# TRANSFORMING RAW MILK INTO SAFE MILK USING ELECTRON BEAM PROCESSING

A Thesis

by

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## ABSTRACT

We hypothesized that at low doses (<2.0 kGy) electron beam processing, a non-thermal food pasteurization technology, would be effective at inactivating the microbial pathogens potentially present in raw milk without compromising or degrading its composition, nutritional value, and aromatic profiles. The $log_{10}$ reductions of background microbial populations and inoculated pathogens (*Coxiella burnetii*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus)* in raw milk was determined. The possible reduction in infection risks associated with these pathogens was also quantified using β-Poisson and exponential risk assessment models. After eBeam processing, milk was analyzed to determine potential losses in the concentrations of lactose, vitamin $B_2$, vitamin $B_{12}$, and calcium. Casein and whey proteins were analyzed for signs of breakdown with SDS-PAGE. Lipid oxidation was measured using the TBARS method, and GC-MS olfactory analysis was used to determine changes in the aromatic compound profile. When exposed to 2.0 kGy, the numbers of aerobic and anaerobic microbial populations (8.1 x $10^4$ and 2.9 x $10^3$ CFU/mL respectively) in raw milk were reduced to below detectable limits representing >3.5 and ~2.5 $log_{10}$-reductions for aerobic and anaerobic microorganisms, respectively. At 2.0 kGy eBeam dose, significant reductions in predicted lethality of raw milk associated pathogens can be observed (between 13-logs and 28-logs). QMRA illustrated the significant reductions in infection risks eBeam pasteurization of raw milk can achieve through pathogen elimination. Without eBeam

pasteurization, ingestion of raw milk containing ~40 CFU/mL *L. monocytogenes* or ~$10^3$ CFU/mL *C. jejuni* or *E. coli* O157:H7 would result in ~8/10, ~8/10, or ~10/10 infections for these pathogens, respectively. However, if raw milk is eBeam pasteurized at 2.0 kGy, the infection risks from consumption would be reduced to ~<1/9.735 million persons. Except for vitamin $B_2$ (which showed a 31.57% loss), none of the other targeted nutrients were affected at 2.0 kGy. There was no indication of lipid oxidation after eBeam processing. However, by day 7 of refrigerated storage, there was a 350% increase in lipid oxidation in the 2.0 kGy samples as compared to the non-irradiated samples. There were only minimal changes in the aromatic compound profiles after eBeam processing.

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## CONTRIBUTORS AND FUNDING SOURCES

### Contributors

This work was supervised by a thesis committee consisting of Professor Suresh D. Pillai (advisor), Assistant Professor Susanne Talcott of the Department of Nutrition and Food Science, and Associate Professor T. Matthew Taylor of the Department of Animal Science.

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## NOMENCLATURE

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| CDC | Centers for Disease Control and Prevention |
| CFU | Colony Forming Unit |
| FDA | U. S. Food and Drug Administration |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| HTST | High-Temperature, Short-Time |
| IRB | Institutional Review Board |
| MDA | Malondialdehyde |
| MPN | Most Probable Number |
| QMRA | Quantitative Microbial Risk Assessment |
| TBARS | Thiobarbituric Acid Reactive Substances |
| USDA | U. S. Department of Agriculture |

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# CHAPTER I

# INTRODUCTION

Raw milk is a high-risk food item to consume due to the potential presence of pathogenic microorganisms such as *Campylobacter jejuni*, shiga-toxin producing *Escherichia coli* spp., and *Listeria monocytogenes* (1). Pathogens such as these are typically introduced into the milk due to either mammary or other infections/inflammation (mastitis) affecting the dairy cow, or from sanitary factors associated with the milking environment, such as cross-contamination from fecal matter or from the human workers (2). Raw milk and food products made with raw milk (such as soft cheeses) have been implicated in numerous foodborne outbreaks that have led to several hospitalizations and deaths (3). Thermal pasteurization of raw milk, a standard practice in the milk industry, is designed to inactivate pathogenic microorganisms present in milk, thus making milk safe for human consumption. Milk is considered to be a good source of potassium and magnesium, and an excellent source of vitamin $B_2$ (riboflavin), vitamin $B_{12}$, and calcium, among other nutrients (4). Thermal pasteurization of milk is a low-heat processing treatment and does not decrease milk's nutritional value, particularly its vitamin and mineral content. Electron beam irradiation is a proven non-thermal (cold) pasteurization technology, and is approved by the FDA for the processing of certain foods such as fresh produce (5-7). There are potential benefits to exploring the use of non-thermal processing technologies for the pasteurization of raw

milk, including an improved creamier (non-cooked) flavor and applications in further processed dairy products, particularly those that would normally use raw milk.

The purpose of the studies presented in this thesis was to determine if eBeam processing technology is a suitable, non-thermal pasteurization alternative to traditional thermal pasteurization of raw milk. The first study focused on determining if eBeam pasteurization of raw milk was effective from a microbiological and public health perspective, while the second study focused on determining its effectiveness from a nutritive value and aroma sensory perspective.

The objective of the microbiological study was to define achievable $\log_{10}$ reductions of raw milk's indigenous microflora, define D-10 reduction values for select raw milk pathogens, and to determine the predicted reduction of public health risks (based upon the defined D-10 values) when eBeam processed milk is consumed as opposed to raw (non-eBeam processed) milk. The objective of the nutrient and aroma sensory study was to determine the changes, if any, of lactose, calcium, vitamin $B_2$, and vitamin $B_{12}$ concentrations before and after eBeam processing. Additional objectives included measuring the amount of lipid oxidation occurring due to eBeam pasteurization, determining breakdown of casein and whey proteins based upon their molecular weights, and determining changes in aroma profile due to eBeam pasteurization. It was hypothesized that eBeam processing at 2.0 kGy is effective for non-thermal milk pasteurization while not affecting the nutritional value of such milk. Additionally, it was

hypothesized a 2.0 kGy dose would not affect the aroma profile of eBeam processed milk.

# CHAPTER II

# LITERATURE REVIEW

## Raw Milk

Unless the dairy cow has a mammary gland infection, such as mastitis, or a systemic disease, raw milk from the udder is considered sterile, free of microorganisms (8). However, as milk leaves the udder, bacterial and other microbial contaminants are introduced into the milk via cross-contamination from sources such as the teat skin or human workers assisting with milking. Microorganisms are able to survive and proliferate in raw milk due to factors such as milk having an approximately neutral pH (~6.8), a high water activity (~0.99), and an abundance of nutrients (9-10). Though the majority of microorganisms present in raw milk are not pathogenic and will not cause illness to humans if consumed, raw milk is known to harbor pathogens known to cause severe illness, and sometimes death, if consumed. Although anyone can be affected, the illness symptoms and other ailments caused by these pathogens are particularly severe when consumed by children, pregnant women, the elderly, or other immune-compromised persons.

Consumption of Raw Milk and Associated Products in the United States

Consuming raw cow's milk is becoming an increasingly popular replacement to heat-pasteurized milk (1, 11). This change of consumer preference is due to many reasons, including a "better nutrient" profile as perceived by the consumer and perceived "increased probiotic health benefits from the bacteria present in raw milk. Some advocates even suggest raw milk helps treat, prevent, and cure many health related ailments, including acne, heart and kidney disease, food allergies, cancer, and lactose intolerance (12-13). However, there is very little scientific research to support these claims. There is not a significant difference in nutritional content between raw and pasteurized milk, and the known health risks from consuming raw milk far exceed any possible health benefits (1, 14).

The CDC's Foodborne Diseases Active Surveillance Network Population Survey (2006-2007) estimated approximately 3% of the U.S. population drank raw milk within the past seven days, a slight decrease from the 3.5% estimated in their 2002 survey (3). Additionally, it was estimated ~1.6% of the U.S. population consumed cheese made from unpasteurized milk, another raw-milk related food vehicle implicated in numerous foodborne outbreaks. The frequency of raw milk consumption among farm families and their employees is even greater, with approximately 35% to 60% of such persons estimated to consume raw milk (14-15).

Sale of Raw Milk Within the United States

Since 1987, the FDA has required all milk packaged and sold for human consumption through interstate commerce to be pasteurized to protect consumers from these pathogen risks. However, as of January 2016, the sale of raw cow's milk in intrastate commerce remains legal in some capacity in 29 U.S. states, including California, Pennsylvania, and Texas (16). The sale of raw milk in these states is allowed either through retail stores or purchase directly on the farm. The other states do not allow for the sale of raw cow's milk for human consumption, though some allow the sale for non-human consumption purposes. In states in which the sale of raw milk is illegal, some still allow for citizens to lawfully obtain raw milk through a cow-share program. In cow-share programs, an individual or group of individuals pay the farmer for boarding, feeding, and milking the cow(s) owned. The milk produced by the cow is then given to the group and is then typically consumption. It should be noted, the incidence of foodborne outbreaks related to raw milk are significantly higher in states which allow for the sale of raw milk, further supporting the argument raw milk is a high-risk food item to consume (16).

Rationale of Thermal Milk Pasteurization

Due to a variety of factors, there is always a certain level of uncertainty pertaining to the safety of raw milk. Even when carefully produced and harvested from disease-free cattle, the production environment cannot consistently be completely aseptic. Heat

pasteurization, defined as a process to kill pathogenic and other microorganisms present, is commonly used in the milk industry to decrease the microbial load of milk and extend shelf life. Heat pasteurization kills the pathogenic bacteria responsible for diseases such as listeriosis, campylobacteriosis, typhoid fever, and brucellosis, as well as other vegetative pathogens. This is most commonly accomplished through batch pasteurization at 145$^{o}$F (63$^{o}$C) for 30 minutes, or through the high temperature, short time (HTST) method at 161$^{o}$F (72$^{o}$C) for 15 seconds. These time and temperature combinations are implemented in order to achieve a 6.0 $\log_{10}$-cycle reduction of *Coxiella burnetii*, a bacterium implicated as the most heat-resistant, non-sporulating pathogen present in milk, known to cause Q-fever in humans (8, 17). A variety of organizations, such as the FDA, CDC, the American Academy of Pediatrics, the National Association of State Departments of Agriculture, the Association of Food and Drug Officials, and other organizations have endorsed the pasteurization of milk to increase consumer safety (18-22).

**Pathogens in Raw Milk and Raw Milk-Made Dairy Products**

Foodborne bacterial pathogens found in raw milk include *Salmonella*, pathogenic *Listeria* and *Campylobacter* spp., Shiga toxin-producing *Escherichia coli*, *Staphylococcus aureus*, and *Brucella abortus*. Raw milk has been implicated in numerous foodborne illnesses and deaths due to consumption of these and other pathogens. According to the CDC (23), from 1993-2006, outbreaks linked to raw milk

consumption were 150 times more likely to occur than from pasteurized milk. Numerous literature reports state outbreaks associated with raw milk have also led to chronic illness, deaths, and stillbirths, as reviewed by Oliver and others (1). The average number of outbreaks caused by raw milk quadrupled in 2007-2012 from 1993-2006 (24). This can be attributed to the fact that more states are legalizing the sale of raw milk, and this increase in legalization positively correlates to the number of raw milk outbreaks.

Incidence of Foodborne Outbreaks Related to Raw Milk in the U. S.: 1990 – 2016

The CDC states that from 2007-2012, there were 81 outbreaks attributed to the consumption of raw milk reported in 26 states. These outbreaks caused 979 illnesses and 73 hospitalizations (23). The three pathogens most frequently implicated in these cases were *Campylobacter* spp. (81%), shiga toxin-producing *E. coli* spp. (17%) and *Salmonella* spp. (3%). Additionally, the CDC (23) noted the average yearly outbreaks count was 4 times higher from 2007-2012 (5 years) than 1993-2006 (13 years). Interestingly, ~81% of these reported outbreaks occurred in states in which the sale of raw milk was legal. Table A-1 shows data for raw milk-implicated foodborne outbreaks from 1992-2016. Data were collected from the CDC Foodborne Disease Outbreak Surveillance System, published literature, and other outbreak reporting online sites. Fifty outbreaks were identified in which the outbreak location, contaminating pathogens, and number of illnesses were identified leading to 897 illnesses, 204 hospitalizations, and 2 deaths. *Campylobacter* spp. was the most frequently-implicated pathogen in these

outbreaks (52%), followed by shiga toxin-producing *E. coli* (24%), *Salmonella* spp. (20%) and *L. monocytogenes* (4%).

*Campylobacter jejuni*

*C. jejuni* is a helical-shaped, Gram-negative bacterium known to cause campylobacteriosis (a form of gastroenteritis). Worldwide, *C. jejuni* causes more cases of gastroenteritis than *Salmonella* spp. or *E. coli* O157:H7 (25). Those who are sickened from *C. jejuni* show acute symptoms such as diarrhea (sometimes bloody), nausea, fever, and abdominal cramps, and typically recover within 2 to 10 days (26). Deaths related to *C. jejuni* are not common (~0.005%) (25). Long-term sequelae are uncommon with *C. jejuni* infection, Guillain-Barré and Reiter syndromes are recognized to occur in ~0.01% and ~1.0% of infections (27). Though most infections arise from handling and consuming poultry, consumption of *C. jejuni*-contaminated raw milk is the most reported cause of outbreak infections (25, 28). Person-to-person transmission is highly unusual, particularly because the infectious dose of *C. jejuni* is relatively high (~800-$10^6$ ingested organisms needed to produce illness) (25). Heat, such as cooking chicken or thermally pasteurizing milk, kills *C. jejuni cells*.

*Coxiella burnetii*

*C. burnetii* is a Gram-negative bacterium that is the causative agent of Q-fever. The infective dose of *C. burnetii* is estimated to be less than 10 bacterial cells (29). In 2007, 167 cases of Q-fever were reported in the U.S. (30). Q-fever is characterized by influenza-like symptoms, such as high fevers, severe headaches, chills, nausea, and vomiting (30). Although typically an acute illness that is significantly shortened in duration with antibiotics, Q-fever can develop into a chronic illness with complications such as pneumonia and granulomatous hepatitis. Pregnant and other immunosuppressed people and those with a pre-existing heart valve defect are most at risk for developing chronic Q-fever. Those who develop chronic Q-fever need long term antibiotic treatment, typically lasting at least 18 months. While the fatality rate of acute Q-fever is less than 2% of hospitalized patients, the fatality rate for chronic Q-fever can be as high as 60% (30). Cattle, sheep, and goats are the primary reservoirs of *C. burnetii*, with the organism excreted in the milk, urine, and feces of infected animals. Although the most common infection route of *C. burnetii* is inhalation, other infection routes include ingestion of raw milk and dairy products made from raw milk. *C. burnetii* is extremely hardy to harsh physical conditions such as heating and drying. It is considered to be the most heat-resistant, non-spore-forming pathogen found raw milk, making it the pathogen of concern for conventional milk pasteurization (29). Current heat pasteurization standards standard for milk are based upon the 6-$\log_{10}$ destruction of *C. burnetii* in raw milk (17, 31, 32).

## Shiga Toxin-Producing *E. coli* (STEC)

*E. coli* is a Gram-negative bacterium commonly found throughout the environment. Though most are non-pathogenic and will not cause harm if consumed by humans, some, such as Shiga toxin-producing *E. coli* (STEC), will cause severe gastrointestinal illness if consumed (33). After ingestion of STEC, illness typically follows 3 to 4 days after, though incubation times can be as long as 10 days after ingestion before illness is evident (33). Common symptoms of illness include bloody diarrhea, severe abdominal cramps, and vomiting. Though most symptoms subside within seven days, the severity of illness can range from mild to extremely severe and even life-threatening (34). It is estimated 265,000 STEC foodborne infections occur each year in the U.S. (33). The most commonly identified STEC in the U.S. regarding foodborne outbreaks is *E. coli* O157:H7, causing more than 63,000 illnesses, 2,100 hospitalizations, and 20 deaths (35). Those who become ill with STEC (particularly O157:H7) can develop hemolytic uremic syndrome (HUS), a type of kidney failure, hemorrhagic colitis, or thrombotic thrombocytopenic purpura, all of which are considered to be life-threatening (36). Approximately 25% other persons infected with *E. coli* O157:H7 are though to develop long-term renal sequelae (37). Foods produced from cattle, such as raw ground beef, raw milk, and cheeses made from raw milk, remain the primary vehicles for STEC foodborne illnesses, though infection through cross-contamination (such as from not properly washing hands) also is a source of concern for STEC (38).

*Listeria monocytogenes*

*L. monocytogenes* is a Gram-positive bacterium commonly implicated in foodborne outbreaks of ready to eat (RTE) or non-processed raw food products. Listeriosis is characterized by fever, muscle aches, and sometimes gastrointestinal distress, similar to symptoms of the influenza virus (39). Listeriosis primarily affects those with a weakened immune system, such as newborns and children, pregnant women, the elderly, among other immune-compromised individuals (40). Infection during pregnancy can be particularly dangerous, as it can lead to miscarriage, stillbirth, or premature delivery (39). Though listeriosis only causes ~0.02% of foodborne related illnesses, it is a significant cause (>25%) of foodborne illness-related deaths (41). Raw meats, vegetables, raw milk and cheeses made with raw milk, and ready to each foods (such as deli meat) have been known to cause *L. monocytogenes* foodborne outbreaks (39). Additionally, newborns can be born with listeriosis if their mother consumes contaminated food during pregnancy (39).

*Salmonella* spp.

Salmonella is a Gram-negative bacterium estimated to cause over one million foodborne illnesses in the U. S. each year, with 19,000 hospitalizations (~1.9%) and 380 deaths (0.038%) associated with these illnesses (42). Approximately 25% of illnesses in the U.S. reported to the CDC are caused by serotype Typhimurium (43). Illness caused by

*Salmonella* consumption, known as salmonellosis (a form of gastroenteritis), is characterized by diarrhea (often bloody), abdominal cramps, fever, nausea, vomiting, and headaches (42). These symptoms typically show up 12 hours to 3 days after consumption/exposure and typically last no longer than 7 days (42). Long-term sequelae associated with salmonellosis infection include reactive arthritis, which can last for a few months up to many years after illness (42). This organism has been implicated in numerous foodborne outbreaks involving beef, poultry, pork, raw or undercooked eggs, and raw milk (44-45).

## Ionizing Irradiation as a Food Processing Technology

### Gamma, X-Ray, and eBeam Technologies

Irradiation of food products as a non-thermal processing aid has become more prevalent in the food industry. Ionizing irradiation of foods can be achieved primarily through gamma rays (such as $^{60}Co$), x-rays, or electron beam (eBeam) irradiation. Ionizing radiation imparts sufficient energy to produce ionized atoms by removing electrons from their orbitals, subsequently damaging microbial DNA by created multiple single and double-strand breaks (46-47). The microbial cells unable to repair their damaged DNA die, while other sub-lethally injured microbial cells are unable to replicate. It is important to mention while the desired results of all three methods are the same (namely microbial inactivation), there are fundamental differences between them in terms of dose

rate, dose penetration, and how the ionizing irradiation is generated, among others (48). Gamma irradiation is continuously produced from a radioactive source (cobalt-60 or cesium-137), and is often subject to waste as it continuously decays regardless of whether it's treating product. Additionally, the safety requirements and logistical factors (such as facility location) of working with gamma sources, such as dealing with isotope procurement and transportation issues, are other things to consider when working with this form of irradiation. However, gamma rays are highly penetrative compared to x-rays and eBeam, making it more ideal for irradiating denser products, especially at high doses. Electron beam irradiation is generated through the use of commercial electricity. X-rays are generated through eBeam technology by converting the high energy electrons into photons. This generation of photons is accomplished by "shooting" the electrons through a metal sheet, such as tungsten or tantalum, with an electron beam accelerator. Both eBeam and x-ray are considered "on-off" technologies. Unlike gamma, these technologies can be turned off when not in use, saving companies both money and energy, and minimizing waste. Furthermore, eBeam continues to be a prominent method for food irradiation, with low equipment costs and relatively short processing times being the primary benefits (49).

Food Irradiation Regulations in the United States

The USDA-Animal and Plant Health Inspection Service (USDA-APHIS) approves the use of food irradiation for phytosanitary applications for foods imported into the U.S.

(≤1.0 kGy).[1] Phytosanitary applications of imported foods are necessary to mitigate pests that may otherwise pose a risk to U.S. agriculture. Such pests are dangerous as they can feed on and introduce disease to crops, thereby potentially destroying the viability of such crops in the U.S. The use of irradiation technology is beneficial to other phytosanitary applications (such as heat or fumigation treatment) as dose treatments (≤1.0 kGy) universally affect pests regardless of the commodity being irradiated (50-51). Additionally, irradiation at phytosanitary doses does not affect the quality of such produce (48, 52-53). Phytosanitary irradiation treatment impacts include mortality, inability to fly, or sterilization of the pests. Some produce, such as guavas and sweet limes from Mexico, must be processed solely by irradiation before U.S. entry (54). The U.S. FDA has approved irradiation for the use of pathogen control in fresh and frozen raw poultry and meats (3.0-7.0 kGy) (55). Irradiation is also approved for the treatment of all fresh produce commodities for the extension of shelf life (≤1.0 kGy) (55). The inactivation of microorganisms present in produce is another benefit of irradiation, increasing the safety of such foods.

Consumer Acceptability of Irradiated Food Products

Irradiation of foods as a processing technology to increase the shelf life and safety of foods is endorsed by a variety of national and international organizations, including the

[1] 1 kGy = 100,000 rd

FDA, USDA, the International Atomic Energy Agency (IAEA), the Food and Agriculture Organization (FAO), and the World Health Organization (WHO), among others. The widespread use of this technology has been hindered by a variety of reasons including a lack of consumer acceptance (48). However, consumers are often not informed on the topics of irradiation and the benefits it provides to the foods they consume, particularly foods that are minimally processed at home (such as fruits and vegetables). Many consumers erroneously believe the foods become radioactive after irradiation or that the technology is used to compensate for unscrupulous food safety practices. Studies have found that once educated, consumers are more likely to accept food irradiation as a safe and beneficial processing technology (56). Other consumer acceptance studies regarding food irradiation have found that societal opinions and attitudes are likely to heavily influence consumer acceptance, but that education helps eliminate consumer misconceptions based on societal opinion (57-58). Nayga and others (59) found that not only did consumer acceptance of irradiated foods increase after education, but consumer willingness to purchase irradiated foods also increased. The food industry and perhaps key policy makers would likely benefit from better understanding the influences of society on consumer opinion to better educate the population on possible misconceptions that may arise. Bruhn (60) suggests consumer education messages include information such as microorganisms (harmless and pathogenic) are present everywhere, comprehensive yet understandable descriptions of foodborne illness, and strategies for preventing such illness. Acceptance of food irradiation technologies is critical for increasing its utilization in the food industry as a

processing technology, and it is equally important to educate consumers so they can make informed and rational decisions regarding buying and eating irradiated foods.

Irradiation of Dairy Products

It is well-accepted irradiation is an effective technology for increasing public health by minimizing (and even eliminating) the presence of pathogens in foods (61). However, other factors of food quality may be affected by this form of processing, such as food appearance and sensory qualities. It is important to consider both safety and quality factors when evaluating the effectiveness of such processing. Though some claim there is no need for irradiation of dairy products (and therefore are slow to adopt due to the already established effectiveness of thermal pasteurization), there is merit to researching the potential of irradiation of dairy products as a cold-pasteurization (non-thermal) technology (62). Many consumers prefer the taste and aroma of raw milk (soft) cheeses compared to these cheeses made with pasteurized milk (63). While the dangers of consuming raw milk, and other raw milk-made dairy products are evidenced, many consumer still desire the organoleptic properties offered by raw milk dairy products (1, 64). Much of current dairy irradiation research revolves determining not only the microbiological safety of such foods, but also in determining whether these foods have acceptable sensory and other qualities. Many studies have also looked into utilizing irradiation on dairy products made with pasteurized milk as an additional processing aid to increase food safety, particular for consumers who are immunocompromised (65-66).

Irradiation of dairy products (particularly gamma irradiation) has been well researched, but further studies, especially in regards to the electron beam irradiation of milk and other dairy foods, would still be beneficial to determine whether such products could be marketed as value-added due to its cold-pasteurization processing. Studies on the irradiation of raw milk, particularly with electron beam, are difficult to find. However, numerous studies conducted on dairy products, such as cheese, and dehydrated infant formula show promising results that electron beam could be an effective technology for the pasteurization of raw milk (67-69). These studies (outlined below) found irradiation effectively reduced pathogen content and increased shelf life of foods without significantly affecting sensory properties at pasteurization doses.

*Raw Milk*

There is minimal literature documenting studies of raw milk irradiation, and none are found that discuss it in the context of eBeam irradiation. Kung and others (70) found gamma pasteurization of raw milk at 240,000 roentgens (~2.0 kGy) significantly decreased both vitamin A and riboflavin (vitamin $B_2$) content by ~45 and ~25%, respectively. Although milk is not a primary source of vitamin A for the typical diet of a consumer from the United States, many consumers receive a significant amount of vitamin $B_2$ in their diet from milk (4). Although specific concentrations of riboflavin were not stated in the literature, one could assume, based upon typical levels of pasteurized whole milk and considering a 25% destruction of such riboflavin, irradiated

milk still remained an excellent source of vitamin $B_2$ and provided more than 20% of the current (as of November 2016) recommended daily value (71). De Oliveira Silva and others (68) studied the effects of gamma irradiation on raw milk's microbiological and sensory properties at 0, 1.0, 2.0, and 3.0 kGy. This study found raw milk had significantly less natural microflora when irradiated at 2.0 and 3.0 kGy compared to the non-irradiated control (~2.50 and 2.80 $\log_{10}$ CFU/mL reductions observed respectively; $p < 0.05$). Though all members of the sensory panel could differentiate between non-irradiated and irradiated milk samples, the investigators found that positive attributes, such as better taste, were still used to describe irradiated samples up to 2.0 kGy. Rancid odors and flavors were perceived by ~22% of panelists at 3.0 kGy. The authors attributed the formation of rancid odors in the 3.0 kGy irradiated milk due to the formation of free radicals affecting the lipid portion of the milk (particularly its fatty acids).

*Cheese*

Literature documents studies of irradiated cheeses made from both raw and thermally pasteurized milk. Kim and others (69) found the natural aerobic microflora of sliced and pizza cheeses (made with thermally pasteurized milk) were reduced to non-detectable levels after 3.0 kGy for both gamma and eBeam irradiation. The study also found significant reductions of *L. monocytogenes* and *S. aureus* (>99.9%) could be achieved at a dose of 3.0 kGy, and inoculated pathogens were reduced to non-detectable levels

(>99.999% reduction) after 5.0 kGy processing. Seisa and others (72) did not find significant differences in organoleptic properties of thermally pasteurized cheddar cheeses after 4.0 kGy gamma irradiation, although they did observe color changes from orange (control) to light yellow (irradiated sample), suggesting the annatto coloring in cheddar cheese is sensitive to gamma irradiation. Other studies have shown gamma, x-ray, and eBeam irradiation technologies improve the safety of raw milk made cheeses by significantly decreasing indigenous microflora without significantly affecting flavor and other sensory qualities of such products at doses up to 5.0 kGy (67, 73-74).

*Other Further-Processed and Related Dairy Products*

Significant pathogen reduction in ice creams (inoculated with pathogens such as *L. monocytogenes*, *B. abortus*, and *B. melitensis* ) can be achieved with as little as 2.0 kGy (75-76). Badr (77) studied how gamma irradiation at 0, 1.0, 2.0, 3.0, and 4.0 kGy affected the microbiological and sensory properties of ice cream. This study concluded that 3.0 kGy significantly reduces inoculated pathogens (*S. aureus, L. monocytogenes, Salmonella* Typhimirium) without adversely affecting sensory attributes such as appearance, color, odor, taste, and texture. Kim and others (78) analyzed gamma irradiated ice cream for flavor and other sensory changes at 0, 1.0, 3.0, and 5.0 kGy. Their study did not find significant decreases in color, moisture, fat, or flavor quality after irradiation. Some ice cream flavors even showed an increased in acceptability after irradiation.

Current methods of preparation for powdered infant formula does not assure product sterility (79). The primary bacterial pathogen of concern for powdered infant formula is *Cronobacter sakazakii* (formally known as Enterobacter sakazakii) (79). This pathogen is highly resistant to desiccation stress and therefore can survive for long periods of time in powdered infant formula once introduced (80). Many studies have shown that ionizing irradiation is an effective processing aid in the inactivation of *C. sakazakii* in dehydrated infant formula. Powdered infant formula inoculated with $10^6$CFU/g *C. sakazakii* saw approximately a 3-log reduction (D10-value 0.76 ± 0.08 kGy) when gamma irradiated at 3.0kGy, and below detection levels were reached at 5.0kGy (81). Similar D10-values for *C. sakazakii* in dehydrated infant formula were also determined by Osaili and others (82) and Osaili and others (83) when treatment was applied shortly after manufacture of the powdered infant formula. One key quality of concern when producing infant formula is assuring that the nutritional quality remains high, even after processing. A study using eBeam processing up to 25.0kGy showed no significant losses of key nutrients nor detrimental effects in dehydrated infant formula, including amino acid, fatty acid, and mineral profiles, protein degradation, and lipid oxidation (84). These studies show promise for the application of irradiation technologies for dehydrated dairy products, such as powdered milk.

## Quantitative Microbial Risk Assessment

Microbial foodborne hazards pose a risk to all populations as they are prevalent throughout all parts of the world. QMRA is a valuable tool in identifying the risks associated with microbial foodborne hazards (85). QMRA is especially useful as it can systematically relate the performance of one or more intervention methods to determine the level of intervention needed to manage food safety risks (86). Risks can be minimized with QMRA analysis to better protect the health of consumers, as the capabilities of intervention methods can be quantitatively articulated in terms of the risk management necessary to protect public health. The four primary steps involved in QMRA are hazard identification, exposure assessment, risk characterization, and risk management.

### Primary Steps of QMRA

A problem scenario must first be developed during hazard identification, in which situations and problems to be addressed in the hazard analysis are identified. The microbial hazards associated with the chosen scenario are identified and described. This includes providing general information regarding the microbial agent, incubation times, and symptoms associated with illness.

Exposure assessment involves determining the dose of the microbial hazard of interest that people come into contact with. This aspect of QMRA can be quite complex, as many factors involved in microbial exposure need to be taken into account. The information needed to thoroughly conduct the exposure assessment can sometimes be difficult (if not impossible) to determine; thus, assumptions often need to be made. However, it is important to be extremely thorough and consider every possibility that may affect the pathogen exposure. Even a small change in exposure can have a significant impact in the risk characterization (87-88). A dose response model is also identified, in which the risk of response (infection, illness, death, etc.) is estimated given a known pathogen dose.[2] The information gathered from the exposure assessment is utilized into the dose response model to predict the risk of response for the chosen scenario.

In risk characterization, the information gathered from the exposure assessment is incorporated into the dose response model to predict the risk of response for the chosen scenario. Risk characterization is typically accomplished through either the use of modeling software, such as R (Wien, Austria), Palisade @Risk (Ithaca, NY), or Oracle® Crystal Ball (Redwood City, CA), or through calculating point estimates. The primary difference between the two methods is modeling software can determine a range of risk

[2] Susceptibility of the consumer affects response risk after exposure. Many studies (such as the one presented in this thesis) assume each person (iteration) is equally susceptible to the defined risk.

values (including the most likely risk of response in a given distribution, with a defined minimum or maximum), while point estimates only determine a single numeric value for risk (per calculation).

Based upon the risk quantified from risk characterization, risk management determines whether the intervention methods used to control risk are acceptable (determine whether the risk assessment goals were met). Communication of new policies and practices arising from QMRA analysis to the food industry and other end-users, key policy makers, and the community is an important aspect of risk management.

QMRA Studies on Raw Milk and Raw Milk Cheeses

Numerous risk assessments have been conducted to determine the risk of illness such as campylobacteriosis, salmonellosis, and listeriosis from consuming raw milk and raw milk-made cheeses. Some studies simply analyzed illness risks without considering possible risk reductions with intervention methods and generated critical data for communicating to the public of the health risks due to consuming raw milk and raw milk made products (89-92). Latorre and others (93) determined the risk of illness from listeriosis due to raw milk consumption was about 57 – 77 times greater when purchased from on-farm and retail stores than when purchased directly from bulk tanks. The authors hypothesized this difference in risk was likely due to time-temperature fluctuations when transporting milk, leading to a growth of *L. monocytogenes* in such

products. Their study suggests handling practices of raw milk greatly affect the microbial growth in such products, particularly pertaining to pathogens, similar to what would be expected with other temperature-sensitive products. Other studies, similar to the QMRA study presented in this thesis, determined illness risks from consuming contaminated raw milk cheeses were significantly less with an intervention treatment than without any intervention treatment (94). Perrin and others (95) explored the effects of varying intervention treatments on the risks of contracting HUS from consuming raw milk cheeses contaminated with STEC, and found ~76.2 - 98.4% reduction in illness risks as a result of these intervention methods. Studies such as these are beneficial in not only communicating the risks of consuming raw milk and related products in a more comprehensible manner, but these studies also quantify the benefits of intervention methods of raw milk to make is safer in terms of public health.

# CHAPTER III

# ELECTRON BEAM PASTEURIZATION OF RAW MILK TO REDUCE POTENTIAL HEALTH RISKS

## Introduction

Raw milk has been implicated in numerous foodborne illnesses and deaths due to pathogens in raw milk. Raw milk related outbreaks are estimated to be 150 times more likely to occur than from pasteurized milk (23). The average annual number of outbreaks caused by raw milk quadrupled between the years 2007-2012 compared to outbreak data from 1993-2006 (24). This can be attributed to the fact that more states are legalizing the sale of raw milk, and this increase in legalization positively correlates to the number of raw milk outbreaks. Heat pasteurization of raw milk by the high temperature short time (HTST) method is routinely used by the milk industry to inactivate the microbial pathogens that may be present in raw milk. Today, the HTST method assures at least a 6.0 $\log_{10}$-reduction of *Coxiella burnetii,* one of the most heat-resistant bacteria potentially present in raw milk (2). The HTST milk pasteurization technology has been endorsed by a variety of national and international government and non-governmental organizations to assure the microbiological safety of milk (19-20, 96). However, in recent years, raw cow's milk has become increasingly popular for a variety of reasons including taste, improved nutrient profile, and increased "health benefits" from the bacteria present in milk (4, 11). However, there is no significant difference in

nutritional content between raw and heat pasteurized milk, and the known health risks from consuming raw milk far exceed any possible health benefits (1, 14). Electron beam (eBeam) processing is a non-thermal food processing technology that is proven as an effective food pasteurizing technology (46, 97-100). This FDA-approved technology is finding increasing applications for fruits and vegetables, a commodity that is especially sensitive to heat treatments (7, 48). The highly energetic electrons in eBeam processing are used to inactivate microorganisms by creating multiple double-strand breaks in the microbial DNA, preventing the cell from multiplying (47). Quantitative microbial risk assessment (QMRA) is a valuable tool for quantifying the risk reduction that can be achieved by adopting pathogen intervention technologies. We have previously used QMRA methods to quantify the reduction in infection risks associated with the adoption of eBeam technology for processing raw oysters, lettuce and spinach and fresh strawberries (5-7). QMRA is especially useful as it can systematically relate the performance of one or more intervention methods to determine the level of intervention needed to manage food safety risks (86). The QMRA approach at quantifying the reduction in infection risks is a powerful risk management and risk communication tool (85). The underlying hypothesis of this study was that eBeam dose at 2.0 kGy is effective for non-thermal raw milk pasteurization. The 2.0 kGy dose was chosen based upon the pathogen reduction study discussed in this thesis chapter. The objectives of this study focused on determining the reduction of the background microbial populations found in raw milk as well as determining the reduction of specific raw milk-associated pathogens *C. jejuni*, *L. monocytogenes*, *S. aureus,* and *E. coli* O157:H7. We also

evaluated the reduction of *Coxiella burnetii* which is currently used as the benchmark organism for heat pasteurization. Based on empirical pathogen reduction data, we quantified the reduction of infection risks associated with *C. jejuni*, *E .coli* O157:H7, and *L. monocytogenes* if raw milk was exposed to eBeam at a 2.0 kGy dose.

## Materials and Methods

### Milk Samples

Raw milk samples were purchased (between ~1.90-3.80 liters) from six different dairy farms within the state of Texas. We chose multiple raw milk sources to obtain a wide diversity of samples having different levels of background microbial populations. The samples were refrigerated ($\leq 7.0^{o}$C) until analysis, which was completed within 48 hr of purchase. A composite raw milk sample was created by combining raw milk samples purchased from three separate dairy farms in equal amounts into a sterile 500 mL bottle. Two and thirty mL of the composite raw milk sample (for D-10 and bioburden studies, respectively) was transferred aseptically into Whirl-Pak bags (Nasco, Fort Atkinson, WI). These samples were then exposed to defined eBeam doses and analyzed for their microbiological profiles.

Electron Beam Irradiation

The eBeam processing was conducted at the National Center for Electron Beam Research at Texas A&M University (College Station, TX). Samples were irradiated using a 10 MeV, 18 kW Electron Beam Linear Accelerator (LINAC). Alanine dosimeters were used to verify the delivered eBeam dose. Dosimetry was performed using alanine dosimeters calibrated to international standards (ISO/ASTM 52628). The dosimeters were read using the Bruker e-scan spectrometer (Bruker, Billerica, MA) to measure the delivered irradiation dose. Preliminary dose-mapping studies were performed to ensure that the experimental samples received uniform doses with Dose Uniformity Ratio (DUR) ($Dose_{max}/Dose_{min}$) values of the experimental samples to as close to 1.0 as possible (5-6). Irradiated samples were packaged thinly (less than 1.0 cm thickness) to ensure a uniform dose so that the DUR was approximately 1.0. A DUR of ~1.0 indicates the dose received was uniform throughout the sample. The delivery of uniform eBeam doses is an important factor when performing irradiation experiments and is especially critical when determining the D-10 value to ensure every aliquot of the milk sample (and accompanying indigenous and inoculated pathogens) receives the same irradiation exposure dose.

TABLE 1. Strains used in the pathogen reduction (D-10) study, along with the media used and growth conditions for each pathogen before milk inoculation.

| | **Strains used** | **Media(um)** | **Incubation Time and Temperature** | **Atmospheric condition** |
|---|---|---|---|---|
| ***C. jejuni*** | ATCC 33560, PR1-1[1], PR1-12[1] | Brain, Heart Infusion Broth with Preston Campylobacter Selective Supplement | 42°C, 7 days | 6-16% $O_2$, 2-10% $CO_2$ |
| ***C. burnetii*** | RSA439 Nine Mile phase II[2] | ACCM-2 (101) | 37°C, 7 days | 5% $CO_2$, 2.5% $O_2$ |
| ***E. coli* O157:H7** | ATCC 43889, ATCC 43895, 8624 | Tryptic Soy Broth (TSB) | 35°C, 24 hours | Aerobic |
| ***L. monocytogenes*** | ATCC 19115, ATCC 15313, ATCC 43256 | TSB with 0.6% yeast extract | 30°C, 24 hours | Aerobic |
| ***S. aureus*** | ATCC 25923, ATCC 29737, ATCC 33862, two strains isolated from raw milk[3] | Tryptic Soy Broth | 35°C, 24 hours | Aerobic |

[1]Laboratory collection
[2]Provided by J. Samuels laboratory, Texas A&M Health Science Center, Texas A&M University, MS 1359, College Station, Texas, USA, 77843-1359
[3]Verified *S. aureus* using a VITEK Gram-positive identification card (GP card, BioMérieux, Marcy-I'Étoile, France)

D-10 Estimations of Specific Pathogens

The D-10 value (the eBeam dose required to achieve 90% reduction (1.0 $\log_{10}$) was determined for *C. jejuni, L. monocytogenes, E. coli* O157:H7*, S. aureus, and C. burnetii*. Multiple strains of these pathogens were grown up in the laboratory under defined conditions (Table 1). The cells were washed and re-suspended in 0.1% peptone. The final titer of these pathogens was estimated be around $10^9$ CFU/ml, verified by an $OD_{600}$ ≈ 1.0 (BioPhotometer, Eppendorf, Hamburg, Germany). The strains from the different bacterial genera were mixed together individually in equal amounts and inoculated into 45 ml raw milk samples contained in conical tubes. Each sample was thoroughly mixed and 2 mL aliquots of the inoculated milk samples were placed in sterile Whirlpak bags. Only 2 ml of test samples were used to ensure dose uniformity which is critical for D-10 estimations. The D-10 value was estimated by subjecting pathogen-inoculated raw milk samples to varying eBeam doses between 0.1 and 1.5 kGy. After eBeam processing, the samples were serially diluted in 0.1% peptone water and aliquots plated on specific selective and differential media (Table 2) and the numbers of surviving populations were estimated. The numbers ($\log_{10}$ CFU/ml) of the surviving pathogens after exposure to the defined eBeam doses were plotted as a function of the measured dose (kGy). The slope of the curve was determined using regression analysis and the negative reciprocal of the slope (i.e. D-10 value) was determined for each of the different bacterial pathogens (5-7). For *C. burnetii* analysis, two independent eBeam irradiation trials were performed. For *C. jejuni* and *E. coli* O157:H7, three independent eBeam irradiation trials were

performed. For *L. monocytogenes* and *S. aureus*, four independent eBeam irradiation trials were performed. Three technical replications were completed for each eBeam irradiation dose for every trial.

TABLE 2. Enumeration conditions of specific pathogens in inoculated raw milk after eBeam processing.

| | **Plating Media** | **Incubation Time and Temperature** | **Atmospheric condition** |
|---|---|---|---|
| ***C. jejuni*** | Campylobacter Blood Free Agar with CCDA supplement | 42°C, 5 days | Microaerophilic (6-16% $O_2$, 2-10% $CO_2$) |
| ***C. burnetii*** | Agarose overlay plates of ACCM-2 (*36*) | 37°C, 14 days | 5% $CO_2$, 2.5% $O_2$ |
| ***E. coli* O157:H7** | MacConkey Agar with Sorbitol | 35°C, 36 hours | Aerobic |
| ***L. monocytogenes*** | Modified Oxford's Agar with antimicrobic supplement | 35°C, 48 hours | Aerobic |
| ***S. aureus*** | Baird Parker Agar with 10% Egg Yolk Tellurite | 35°C, 48 hours | Aerobic |

Raw Milk Background Bioburden Reduction Studies

Aliquots (30 ml) of the raw milk sample were placed in heat-sealed Whirlpak bags and subjected to 1.0 and 2.0 kGy eBeam doses. Non-irradiated control samples were also included in the study. Growth conditions for aerobic and anaerobic vegetative organisms, aerobic and anaerobic sporeformers, and *S. aureus*, all isolated from raw and irradiated milk samples, varied (Table 3). Before enumerating sporeformers, samples were heated to 80$^{o}$C for 12 minutes using a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, Calif.) thermocycler to inactivate any vegetative cells present. All platings were performed in triplicate. After incubation, the colonies were enumerated. Coliforms were enumerated using the IDEXX Colilert®-18 assay per the manufacturer's instructions with a slight modification (a 1:100 dilution was made using 1 mL milk sample in 99 mL 0.1% peptone water for all processed samples) (IDEXX, Westbrook, Maine). After incubation, the yellow and fluorescent wells were counted. Fluorescent wells were counted using a hand-held UV light. The manufacturer-supplied MPN table was then used to estimate the MPN/mL of coliforms in each sample. For bioburden reduction studies, two independent eBeam irradiation trials (three replicates each) were performed.

TABLE 3. Enumeration conditions for select microorganisms isolated from raw milk after eBeam processing.

| | **Media** | **Incubation Time and Temperature** | **Atmospheric condition** |
|---|---|---|---|
| **Aerobic Vegetative Organisms** | Plate Count Agar (PCA) | 35°C, 48 hours | Aerobic |
| **Anaerobic Vegetative Organisms** | Brucella Blood agar with 5% horse blood | 35°C, 48 hours | Anaerobic (≥13% $CO_2$) |
| **Aerobic Sporeformers** | PCA + 0.1% soluble starch | 35°C, 48 hours | Aerobic |
| **Anaerobic Sporeformers** | Brucella Blood agar with 5% horse blood | 35°C, 48 hours | Anaerobic (≥13% $CO_2$) |
| ***S. aureus*** | Baird Parker Agar with 10% Egg Yolk Tellurite | 35°C, 48 hours | Aerobic |

Quantitative Risk Assessment of Infection Risks from Raw and eBeam Pasteurized Milk Samples

The infection risks that would arise from exposure to raw milk contaminated with either *C. jejuni*, *E. coli* O157:H7, or *L. monocytogenes* and the reduction in infection risks

achieved after exposure to eBeam pasteurization (2.0 kGy) were estimated. Possible pathogen titers for *L. monocytogenes* were based on published literature and assumed to follow the Poisson distribution (102-106). Due to lack of published data, the initial pathogen titers for *C. jejuni* and *E. coli* O157:H7 were assumed to be $10^3$ CFU/mL and also assumed to follow the Poisson distribution. We assumed a triangular distribution of the raw milk serving size between 0 and 711 mL, with 237 mL being the most likely serving size (107). The pathogen reduction (D-10) values determined in this study were used in determining the expected log reduction of such pathogens when raw milk exposed to a 2.0 kGy eBeam dose is consumed. The infection risks for *C. jejuni* and *L. monocytogenes* were estimated using the β-Poisson model (equation 1), where *Pi* is the probability of infection, and *N* is the number of pathogenic bacteria ingested. Alpha ($\alpha$) is a slope parameter reflecting the dose-response curve, and $N_{50}$ represents the dose in which half of the population is expected to be infected.

Equation 1: $$Pi = 1 - (1 + N\left(\frac{2^{\frac{1}{\alpha}} - 1}{N_{50}}\right)^{-\alpha}$$

For *C. jejuni*, $\alpha$ (1.44E-01) and $N_{50}$ (8.9E+02) were based on the dose-response analysis published by Medema and others (108). For *L. monocytogenes*, $\alpha$ (2.53E-01) and $N_{50}$ (2.77E+02) were based on the dose-response analysis published by Haas and others (109). The infection risks for *E. coli* O157:H7 was estimated using the exponential distribution model (equation 2), where *Pi* is the probability of infection, *N* is the number

of pathogenic bacteria ingested. *K* represents the probability of the pathogenic bacteria surviving to reach and infect the subject, assuming each pathogen has equal probability.

Equation 2: $Pi = 1 - e^{-k*N}$

For *E. coli* O157:H7, *K* (2.18E-04) was based upon dose-response studies conducted by Cornick and Helgerson (110), and further dose-response analysis from the Center for Advancing Microbial Risk Assessment (111). The infection risk model for all pathogens was simulated with Monte Carlo techniques (10,000 iterations) using Oracle Crystal Ball software (V. 11.1.2.4.600, Redwood City, Calif.). Since the log-reductions for *C. jejuni* and *E. coli* O157:H7 were too large for risk reduction to be quantified using Crystal Ball simulations, a point estimate was calculated to determine the infection risks after eBeam processing using the same dose-response formulas identified above. The pathogen titers in raw milk per serving used in the point-estimate calculations (pre-eBeam processing) were the likely maximum, mean, and median doses consumed before eBeam processing, determined from the risk model generated by Crystal Ball. It was assumed all pathogens in the raw milk were infectious and all exposed individuals were susceptible to infection from single exposure. The infection risks were based on illnesses associated with a single exposure.

**Results**

Figure 1 shows the reduction in the background aerobic and anaerobic microbial populations in the raw milk samples after exposure to 1.0 kGy and 2.0 kGy eBeam dose. There were no detectable organisms after eBeam exposure ($\geq$ 3.5 $\log_{10}$-reduction). The background levels of coliforms were reduced to non-detectable levels after even after 1.0 kGy.

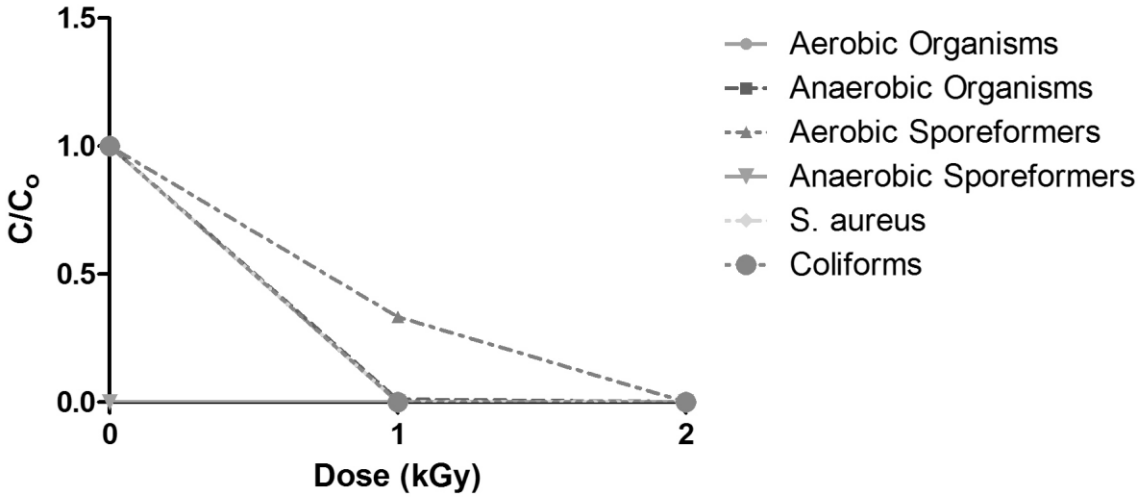


FIG 1. Inactivation of background microbial populations in raw milk after exposure to 1.0 and 2.0 kGy eBeam doses. $C_o$ represents the starting microbial concentration, and C represents the concentration after eBeam treatment.

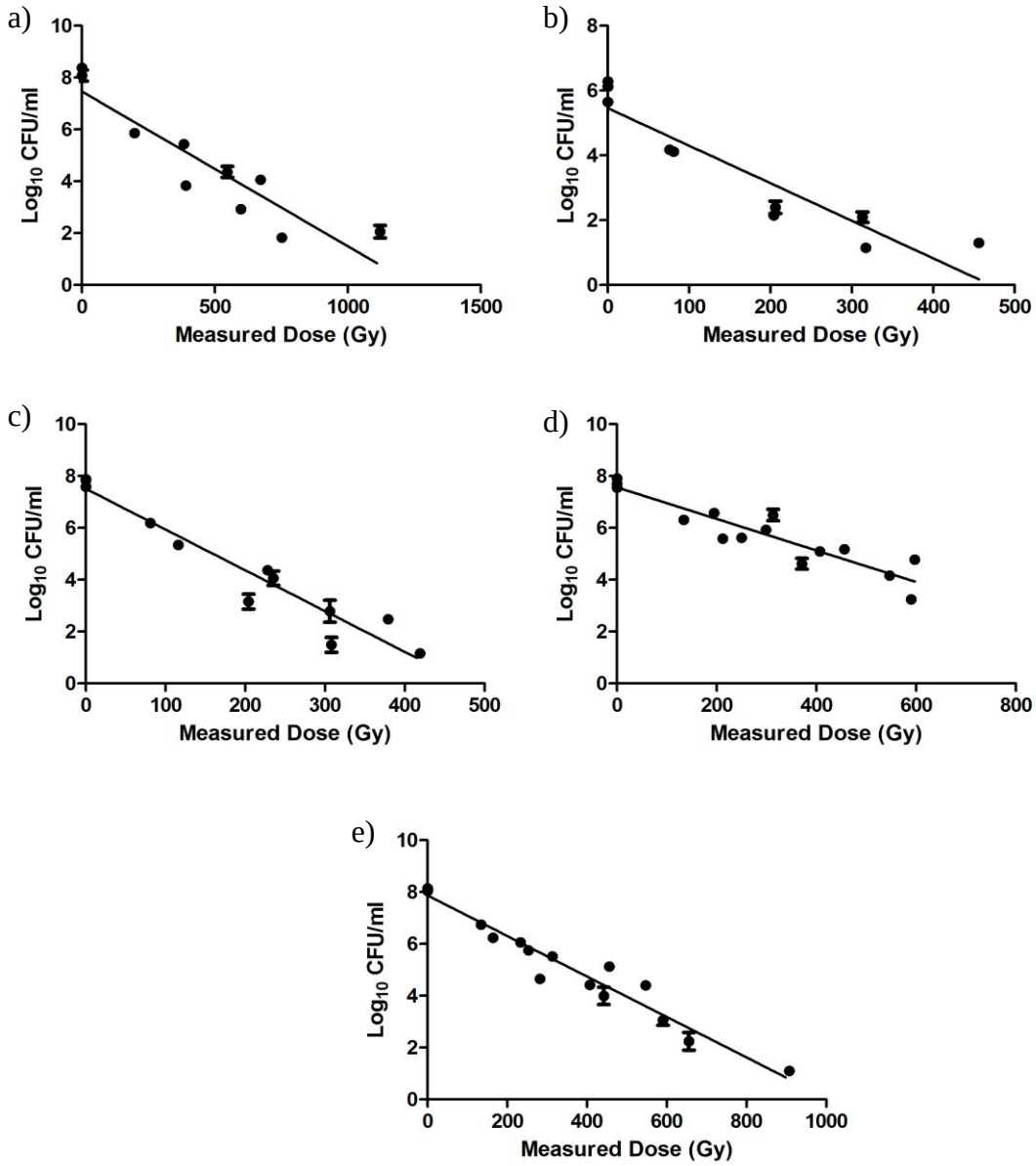


FIG 2. Inactivation of a) *C. burnetii* (n=2), b) *C. jejuni* (n=3), c) *E. coli* O157:H7 (n=3), d) *L. monocytogenes* (n=4), and e) *S.* aureus (n=4) in raw milk after exposure to eBeam processing. "n" represents the number of independent experimental replications performed. Each dose point represents three technical replications. Error bars represent standard deviation.

TABLE 4. Pathogen reduction (D-10) values of key raw milk pathogens in raw milk after eBeam processing.

| | **D-10 Value (kGy)** |
|---|---|
| ***C. jejuni*** | 0.071 ±0.009 |
| ***C. burnetii*** | 0.151 ±0.028 |
| ***E. coli* O157:H7** | 0.062 ±0.008 |
| ***L. monocytogenes*** | 0.156 ±0.017 |
| ***S. aureus*** | 0.129 ±0.008 |

Figure 2 represents the inactivation of the specific pathogens as a function of eBeam dose. Table 4 shows the D-10 values of the selected pathogens investigated. Table 5 shows the estimated risk of infection from consuming raw milk contaminated with C. *jejuni, E. coli* O157:H7, or *L. monocytogenes* as compared to the infection risks if raw milk is eBeam pasteurized with 2.0kGy dose. *C. jejuni* infection risks without eBeam pasteurization ranged between 6 out of 10 persons to as high as 10 out of 10 persons. With the use of 2.0 kGy eBeam pasteurization, these risks were reduced to less than 8 x $10^{-18}$ persons. *E. coli* O157:H7 infection risks without eBeam pasteurization ranged between 9 out of 100 persons to as high as 10 out of 10 persons. With the use of 2.0 kGy eBeam pasteurization, these risks were reduced to less than 2 x $10^{-24}$ persons. *L. monocytogenes* infection risks without eBeam pasteurization ranged between 4 out of 10 persons to as high as 10 out of 10 persons. With the use of 2.0 kGy eBeam

pasteurization, these risks were reduced to 1 out of 1 trillion persons to 2 out of 1,000 persons. To put the risks of infection after eBeam processing into perspective for *C. jejuni* and *E. coli* O157:H7, it was assumed ~3.0% of the U.S. population consumes raw milk on a regular basis (CDC 2007). Therefore, the average infection risks after milk consumption for theses pathogens after eBeam processing was approximately less than 1 out of 9.735 million persons. The average infection risk of illness after consuming milk contaminated with *L. monocytogenes* after eBeam processing was approximately 1 out of 10 million persons.

**Discussion**

The dairy industry is growing rapidly in the United States with the demand for dairy products such as cheese and butter projected to increase at a faster rate than the US population (112). Milk is a popular dairy product among U.S. consumers, and the and Dietary Guidelines for Americans 2015-2020 recommends people consume 2-3 servings of dairy products each day (107). The major finding from this study show that eBeam pasteurization of raw milk even at 2.0 kGy dose can significantly reduce most bacterial infections associated with raw milk. Based on the determined pathogen reduction values, it can be surmised up to a 12-log reduction of vegetative microorganisms can be achieved with 2.0kGy pasteurization. Thermal pasteurization guarantees a 6.0 $\log_{10}$-reduction of *Coxiella burnetii*, the non-sporulating microorganism identified as the most resistant to processing methods in raw milk (17). *C. burnetii* and *L. monocytogenes* were

found to be the most resistant pathogens to eBeam processing, with D-10 values at approximately 0.151 and 0.156 kGy, respectively. Based upon the D-10 values determined in this study, more than a 12.0 $\log_{10}$-reduction of *C. burnetii* is achievable with 2.0 kGy. This reduction translates to more than 2X the reduction achieved with HTST, the cornerstone pasteurization technology of the milk industry today. Based on the measured D-10 values, a 2.0 kGy eBeam dose can achieve at least a 32.0 $\log_{10}$-reduction of *E. coli* O157:H7, a 28.0 $\log_{10}$-reduction of *C. jejuni*, a 15.0 $\log_{10}$-reduction of *S. aureus*, and more than a 12.0 $\log_{10}$-reduction of *C. burnetii* and *L. monocytogenes*. Other investigators have also reported on the total aerobic plate counts of microorganisms in raw milk, with results ranging from $10^4$-$10^6$ CFU/ml (113-115). These results are similar to what was determined in this study. This suggests that at 2.0 kGy, significant inactivation of the background microbial populations is achievable. The USDA Economic Research Service estimates that disease-related infections resulting from *Campylobacter* spp. leads to a loss of $1.93 billion 2013 U.S. dollars, disease-related infections resulting from *E. coli* O157:H7 leads to a loss of $271 million 2013 U.S. dollars, and disease-related infections resulting from *L. monocytogenes* leads to a loss of $2.83 billion 2013 U.S. dollars (116). This includes costs incurred from time lost due to recovery, physician visits and hospitalizations, and mortality. The QMRA performed in this study demonstrates that the infection risks can be significantly reduced for the microbial pathogens *C. jejuni*, *E. coli* O157:H7, and *L. monocytogenes* after eBeam processing at 2.0 kGy in raw milk. These reductions in infection risks should

TABLE 5. Potential infection risks from specific pathogens arising from consumption of raw milk with and without eBeam pasteurization.

| Pathogen | Pathogen Concentration in raw milk (CFU/serving[1]) | Infection risks without eBeam pasteurization | Pathogen Concentration in eBeam pasteurized milk[2,3] (CFU/serving) | Infection risks after eBeam pasteurization | Mean Risk reduction |
|---|---|---|---|---|---|
| ***C. jejuni*** | Mean: $3.16 \times 10^{8}$<br>Median: $2.98 \times 10^{5}$ | Mean: 7.80 / 10 persons<br>Median: 7.83 / 10 persons | < 1 | Mean: 4.34E-21<br>Median: 4.09E-21 | >99.99% |
| ***E. coli* O157:H7** | Mean: $1.13 \times 10^{8}$<br>Median: $2.98 \times 10^{5}$ | Mean: 9.90 / 10 persons<br>Median: 9.90 / 10 persons | < 1 | Mean: 2.46E-28<br>Median: 6.49E-31 | >99.99% |
| ***L. monocytogenes*** | Mean: $1.15 \times 10^{7}$<br>Median: $1.13 \times 10^{4}$ | Mean: 7.94 / 10 persons<br>Median: 8.01 / 10 persons | < 1 | Mean: 1.52E-07<br>Median: 1.50E-10 | >99.99% |

Assumptions:

[1]Serving size: triangular distribution between 0mL – 711mL, with 237mL the most likely

[2]Pasteurization dose: 2.0kGy

[3]*C. jejuni* 28-log reduction; *E.coli* O157:H7 32-log reduction; *L. monocytogenes* 12-log reduction

result in significant improvement in public health since the likelihood of consumers being exposed to pathogens being negligible after eBeam pasteurization. Though less than 1% milk sold to U.S. consumers is raw milk, the reduction in infection risks can still be particularly relevant to foods made with raw milk, such as raw milk made cheeses (64). Raw milk-based cheeses are presently available to consumers in most grocery stores in the U.S. Cheeses made from raw milk were made an exception by the FDA in 1987 and are allowed to be shipped and sold in interstate commerce so long as the cheese has been aged at least 60 days and is clearly labeled as containing raw milk (117). Although there is a requirement for raw-milk made cheeses to be aged, the risk of consuming pathogens from these cheeses is still present, as evidenced by multiple outbreaks in which raw-milk made cheeses were implicated to be the cause (35, 40, 43-44, 118). Previous studies have shown that eBeam processing is a viable technology for inactivating pathogens such as *L. monocytogenes* in a cheese matrix (69, 74, 119). The FDA's Pasteurized Milk Ordinance (PMO) describes ultra-pasteurization (commercial sterilization) as the thermal processing of milk at or above 138$^{o}$C (280$^{o}$F) for at least two seconds (20). Milks processed under these conditions have an extended shelf life under refrigerated conditions. A common problem associated with commercially sterile (UP) milk is an undesirable taste (120-121). Since eBeam technology is a non-thermal process, there is the potential for this technology as a substitute for UP milk. Studies in our laboratory indicate that less than 12% of the volatile compounds are different between raw milk and eBeam pasteurized milk (data included in following chapter). Major advances are occurring in the development of in-line eBeam technology (122).

There are also ASTM standards for the dosimetry associated with flowing liquid streams. This study provides experimental evidence that eBeam processing can be used as a substitute for heat based pasteurization method for raw milk. However, use of ionizing radiation such as eBeam (or other irradiation technologies) for milk pasteurization is not presently permitted by the FDA (123). There are also, unfortunately, a number of regulatory and marketing hurdles preventing the quick adoption of this technology by the milk industry (124-125). Therefore, there should be a concerted effort by the raw milk industry stakeholders to seriously explore the use of eBeam pasteurization of raw milk.

# CHAPTER IV

# AROMA AND NUTRIENT PROFILES OF RAW MILK AFTER ELECTRON BEAM PASTEURIZATION

## Raw Milk and GC-MS Olfactometry Overview

Milk is an excellent dietary source for many nutrients such as calcium, riboflavin, vitamin $B_{12}$, and others (4). The nutrients found in milk are particularly important in the growth and healthy development of children. Raw milk, though sterile before leaving the udder, is host to a vast microflora of bacteria (14, 115). Bacterial populations are able to thrive in raw milk because of its neutral pH, abundance of nutrients, and high water activity (0.99) (9-10). Though some bacterial populations are non-pathogenic, raw milk could potentially contain high numbers of infectious organisms such as *Salmonella* spp.,, *Listeria* spp., and *Campylobacter* spp., which can lead to illness and possibly death (15). Raw milk has been implicated in numerous foodborne illnesses and deaths due to consumption of these and other pathogens (118). Most milk sold to consumers in the U.S. is thermally pasteurized to eliminate pathogens present in the milk, making it safe for consumption (19). Although low heat treatment minimally affects serum proteins and some vitamins (such as vitamins $B_{12}$ and E), it does not significantly contribute to nutrient deficiencies based upon U.S. dietary values (126-127). However, many consumers continue to drink raw milk perceiving it to be more "natural" (1). While there may not be an abundance of evidence suggesting the benefits of raw milk, there is

overwhelming scientific support regarding the dangers of its consumption (11, 14, 23). Electron beam (eBeam) processing is a non-thermal food processing irradiation technology proven to be an effective pasteurization technology for foods (5-7, 46, 128). The highly energetic electrons, generated through commercial electricity, are used to inactivate microorganisms by created single and double-stranded breaks in the microbial DNA (47-48). Studies have shown that at low doses of eBeam processing, foods retain their original sensory and visual characteristics (52-53, 129-130). Studies have also shown that eBeam processing at varying doses (depending on the food matrix) does not significantly affect nutrients present in foods (84, 131-132). The underlying hypothesis for this study was that an eBeam dose at 2.0 kGy is not only effective for non-thermal raw milk pasteurization but does not significantly breakdown key nutrients found in milk, especially when compared to the effects of conventionally pasteurized milk. This study focused on determining concentrations of lactose, calcium, vitamin $B_2$, and vitamin $B_{12}$ before and after eBeam processing. The amount of lipid oxidation occurring due to eBeam pasteurization was also measured. The molecular weights of casein and whey proteins were also analyzed before and after eBeam processing to detect the breakdown of these proteins. Additionally, it was hypothesized that eBeam pasteurization will not affect the aroma profile of raw milk.

## Experimental Design

### Sample Acquisition and Preparation

Raw milk samples were purchased from six different dairy farms within the state of Texas between October 2015 and August 2016. Between one-half gallon and one gallon of milk was purchased from each farm. Thermally pasteurized milk samples were purchased from local grocery stores in College Station, TX, between October 2015 and March 2016. The samples were stored on ice in coolers during transport to Texas A&M University and until analysis. A composite raw milk sample was created by combining raw milk samples purchased from three separate dairy farms in equal amounts into a sterile 500 mL bottle. Thirty and one-hundred milliliters of the composite raw milk sample was transferred aseptically into Whirl-Pak bags (Nasco, Fort Atkinson, Wis.). These samples were then exposed to defined uniform eBeam doses and analyzed for specific parameters as described below. The raw milk and eBeam pasteurized milk samples were compared to determine whether eBeam pasteurization affected any of the raw milk properties.

### eBeam Irradiation Processing

The eBeam processing was conducted at the National Center for Electron Beam Research at Texas A&M University (College Station, TX). Samples were irradiated

using a 10 MeV, 18 kW Electron Beam Linear Accelerator (LINAC). Alanine dosimeters were used to verify the delivered eBeam dose. Dosimetry was performed using alanine dosimeters calibrated to international standards. The dosimeters were read using the Bruker e-scan spectrometer (Bruker, Billerica, MA) to measure the delivered irradiation dose. Preliminary dose-mapping studies were performed to ensure that the experimental samples received uniform doses with Dose Uniformity Ratio (DUR) ($Dose_{max}/Dose_{min}$) values of the experimental samples to be as close to 1.0 as possible (5-6). The samples to be irradiated samples were packaged within thin bags (<1.0 cm thickness) and laid flat to ensure a uniform dose so that the DUR was approximately 1.0. A DUR of ~1.0 signifies the dose received was uniform throughout the sample. The delivery of uniform eBeam doses is an important factor when performing irradiation experiments to ensure every aliquot of the milk sample receives the same irradiation exposure dose.

Nutritional Profile Analysis

*Lactose Concentrations*

The lactose concentrations present in the raw and eBeam processed milk were determined using the Lactose Colorimetric/Fluorometric Assay Kit (#K624-100, BioVision, Milpitas, CA). Sterile Sensoplate™ 96-well, flat, glass bottom plates were used (Greiner bio-one, Stonehouse, UK). To create a standard curve, the Lactose

Standard solution (#K624-100-7) was diluted with assay buffer (#K624-100-1) to 1 nmol/μL, and 0, 2, 4, 6, 8, and 10 μL of this solution was added into individual wells of the 96-well plate. The volume was adjusted to 50 μL in each well with assay buffer to generate 0, 2, 4, 6, 8, and 10 nmol/well of Lactose standard. The eBeam and control raw milk samples were diluted by pipetting 50 μL milk into 950 μL dd$H_2O$ and mixing well. Then, 0.05 μL milk was added to each well, and the volume of each well was adjusted to 50 μL with lactose assay buffer. Lactase (2 μL) was added to each standard and sample well. A reaction mix was created per manufacturer's instructions, and 50 μL reaction mix was pipetted into each standard and sample well. The 96-well plated was covered with aluminum foil to protect from light and incubated at 37$^{o}$C for 60 minutes. The plate was measured at 580 nm using a Wallac Victor$^{2™}$ 1420 Multilable Counter (Perkin Elmer, Waltham, MA). Lactose concentrations present were calculated using Microsoft® Excel per the manufacturer's instructions.

*Casein and Whey Protein Degradation*

Casein proteins were extracted as described by Recio and Olieman (133) with some modifications to the protocol. Control and eBeam processed milk samples were brought to room temperature. The pH was adjusted to 4.6 using 6 M HCl, stirring constantly. Samples were incubated at room temperature for 20 min, and were then centrifuged at 1800 x g for 20 min. The supernatant (whey fraction) was aseptically pipetted into a 50 mL conical tube (VWR, West Chester, PA) and set aside for further purification steps.

The remaining pellet (casein) was washed in 20 mL acetate buffer (0.4 M sodium acetate, pH 4.6) and centrifuged at 1800 x g for 20 min twice. The casein pellet was then washed with 20 mL dichloromethane to remove lipids and centrifuged at 1800 x g for 20 min. Then, the casein pellet was washed in 20 mL acetate buffer and centrifuged at 1800 x g for 20 min. The casein pellet was resuspended in 15 mL acetate buffer. Then, 150 µL of the casein suspension and 450 µL methanol was aseptically pipetted into microcentrifuge tubes (VWR, West Chester, PA), vortexed, and then centrifuged at 13,800 x g for 3 min. The supernatant was discarded, and the casein pellet was resuspended in 200 µL methanol. The mixture was vortexed and then centrifuged at 13,800 x g for 2 min. The supernatant was discarded, and the pellet was allowed to air dry for 5 min. The pellet was then resuspended in 200 µL 10 mM Tris-HCl bufferd (pH 8.0). For whey extraction, the supernatant set aside during casein extraction was filtered using Durapore® Membrane filter (0.45µM) (Millipore Corporation, Billerica, MA). Then, 150 µL of the whey fraction was pipetted into microcentrifuge tubes (VWR, West Chester, PA), and 600 µL methanol, 150 µL chloroform, and 450 µL dd$H_2O$ was added to the whey faction in that order, vortexing in between each addition. The mixture was centrifuged at 13,800 x g for 2 min. The top layer (above the white disk) was discarded, and 450 µL methanol was aseptically pipetted into the mixture, vortexed, and then centrifuged at 13,800 x g for 3 min. The supernatant was discarded, and the whey pellet was resuspended in 200 µL methanol. The whey mixture was vortexed and then centrifuged at 13,800 x g for 2 min. The supernatant was discarded, and the pellet was allowed to air dry for 5 min. The pellet was then resuspended in 50 µL 10 mM Tris-HCl

bufferd (pH 8.0). After extractions, 10 µL of the casein samples and 20 µL of the whey samples were pipetted into microcentrifuge tubes (VWR, West Chester, PA). A 1:1 dilution was made by adding 2X Laemmli sample buffer (Sigma-Aldrich, St. Louis, MO) to each protein sample. The samples were heated at 95-100$^{o}$C for 5 min, and then allowed to cool. The BenchMark™ Prestained Protein Ladder (#10748-010, Life Technologies, Carlsbad, CA) and protein samples were pipetted into the lanes of Precise™ Protein Gels 4-20% (#25244, ThermoScientific, Rockford, IL). The gels were run in Tris-HEPES-SDS buffer at 100 to 110 V according to the manufacturer's instructions (ThermoScientific, Rockford, IL). Once the ladder reached the bottom of the gels, the gels were carefully removed from the cassettes and placed in a 0.25% Coomassie® Brilliant Blue R-250 (ThermoFisher Scientific, Waltham, MA) staining solution for approximately one hour. After staining, the gels were placed in a destaining solution until protein bands were clearly visible (approximately 24 hours). Protein molecular weights were measured using an Odyssey® Imaging System (LI-COR Inc., Lincoln, NE) and Odyssey® 2.1 imaging software.

*Calcium, and Vitamins $B_2$ & $B_{12}$ Concentrations*

Ebeam processed milk samples were shipped on ice overnight to Eurofins Nutrition Analysis Center (Des Moines, IA) for analysis on calcium (AOAC 965.17 / 985.01), vitamin $B_2$ (AOAC 970.65), and vitamin $B_{12}$ (AOAC 952.20). All analyses were performed in a commercial laboratory.

Lipid Oxidation

The extent of lipid oxidation in the eBeam processed milk samples as compared to the raw untreated samples were determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) formed as described by Nagababu and others (134) with slight modifications. For modifications, a 1:1 dilution of the milk sample and butanol was made, and 1mL of the dilution mixture was added to 1 mL of 0.67% thiobarbituric acid (TBA) in 90% Glacial Acetic Acid. The sample mixture was placed in a 95-100$^{o}$C water bath for 10 min. After, the sample mixture was read at 526 nm using a Helios spectrophotometer (ThermoScientific, Waltham, MA).

Volatile Odorous Compounds

Milk volatiles were evaluated using the Aroma Trax gas chromatograph/mass spectrophotometer system with dual sniff ports for characterization of aromatics (MicroAnalytics-Aromatrax, Round Rock, Tx). The milk volatiles in HTST milk samples, the raw milk samples and the eBeam treated (1.0 and 2.0 kGy) samples were compared. The samples were placed in glass jars (473 mL) with a Teflon lid under the metal screw-top to avoid off-aromas. Then the headspace was collected with a Solid-Phase Micro-Extraction (SPME) Portable Field Sampler (Supelco 504831, 75 μm Carboxen/ polydimethylsiloxane, Sigma-Aldrich, St. Louis, Mo). The headspace above each milk sample in the glass jar was collected for 2 hr for each sample. Upon

completion of collection, the SPME was injected in the injection port of the GC where the sample was desorbed at 280°C. The sample was then loaded onto the multi-dimensional gas chromatograph into the first column (30 m X 0.53 mm ID/ BPX5 (5% Phenyl Polysilphenylene-siloxane) X 0.5 μm, SGE Analytical Sciences, Austin, TX) that separated compounds based on boiling point. Through the first column, the temperature started at 40°C and increased at a rate of 7°C/min until reaching 260°C. Upon passing through the first column, compounds were sent to the second column {(30 m X 0.53 mm ID)(BP20- Polyethylene Glycol) X 0.50 μm, SGE Analytical Sciences, Austin, TX}, in which compounds were separated based upon polarity. The gas chromatography column then spilt into three different columns at a three-way valve with one going to the mass spectrometer (Agilient Technologies 5975 Series MSD, Santa Clara, CA) and two going to the two humidified sniff ports with glass nose pieces heated to 115°C. The sniff ports and software for determining flavor and aroma were part of the AromaTrax program (MicroAnalytics-Aromatrax, Round Rock, Tx). Only those compounds determined to have an aroma were used for analysis. Any compounds that were not present during an aroma event were discarded.

Data Analysis

For lactose, casein, whey, vitamin $B_2$, and lipid oxidation (TBARS) analysis, two independent eBeam irradiation trials (with three replicates each) were performed for eBeam pasteurized milk samples, and one eBeam trial was performed for calcium and

vitamin $B_{12}$. For allergen and GC-MS Olfactory (aroma) analysis, one eBeam irradiation trial (with two replicates each) was performed. Analysis of variance (ANOVA) and was used to analyze the remaining nutritional data ($p<0.05$). Newman-Keuls multiple comparison test ($p<0.05$) was used to compare the eBeam processing treatments against each other when ANOVA determined nutritional concentrations were significantly different (0.0 kGy vs. 1.0 kGy, 1.0 kGy vs. 2.0 kGy, and 0.0 kGy vs. 2.0 kGy).

## Results and Discussion

### Nutritional Profile Analysis

#### *Lactose*

Figure 3 shows lactose concentrations in raw milk before and after eBeam processing at 0.0, 1.0 and 2.0 kGy. Mean lactose concentrations for the 0.0 kGy (control), 1.0 kGy, and 2.0 kGy doses were 0.74, 0.70, and 0.74 g/fl. oz., respectively. Mean lactose concentrations did not significantly differ between the three eBeam processing treatments ($p>0.05$).

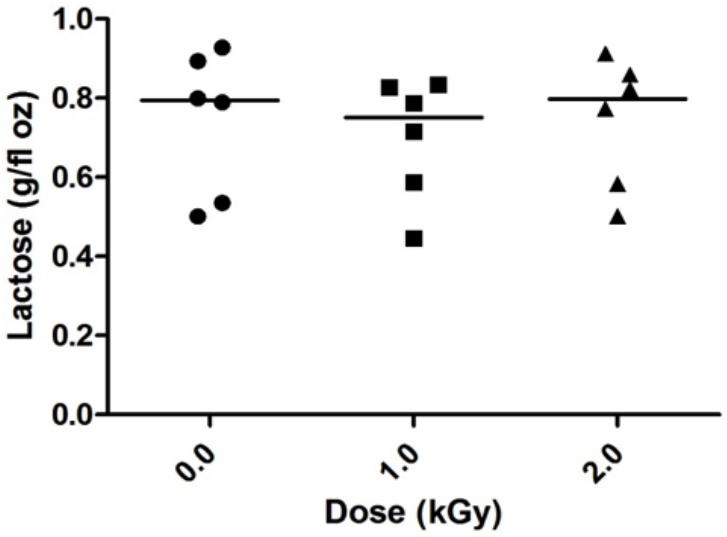


FIG 3. Scatter plot of lactose concentrations remaining in raw milk (0 kGy) and after eBeam processing at 1.0 and 2.0 kGy. The horizontal line represents the median value (n=6).

Our findings correspond to that of previous studies which have reported ionizing irradiation does not significantly break down reducing sugars, such as lactose, in foods (135-136). Direct heating of milk can lead to lactose degradation due to the Maillard reaction, as lactose (a reducing sugar) will react with lysine residues in milk, present mainly in the casein proteins (137). However, in the case of milk pasteurization (a low heat treatment), the Maillard reaction only reaches the early stages and does not negatively affect milk's nutritional value nor does it affect its color or flavor.

*Casein and Whey Proteins*

The molecular weights of two casein and five whey proteins were analyzed after eBeam processing of raw milk at doses 0.0, 1.0, and 2.0 kGy (Figures 4-5). The mean molecular weights between the three eBeam processing treatments did not significantly differ for any of the analyzed proteins ($p>0.05$) (Table 1). Studies have shown thermal pasteurization according to U.S. standards can reduce whey proteins by up to ~7-9%, while casein proteins remain largely unaffected as the micelles are very stable (126, 138-140). Literature largely agrees that proteins in foods remain unaffected when exposed to ionizing irradiation at low doses. Previous studies have reported that eBeam irradiation ($\leq$ 4.0 kGy) did not breakdown proteins in shell eggs and liquid eggs yolks (141-142). Al-Kahtani and others (143) found that gamma irradiation up to 3.0 kGy minimally affected essential and non-essential amino acids found in tilapia and Spanish mackerel. They found some amino acids concentrations to remain the same or increase, while some (~35% measured amino acids) significantly decreased in concentration. Matloubi and others (144) found that gamma irradiation of baby foods did not significantly affect its amino acid profile, an important factor in the nutritional value of proteins in foods. It is not surprising that there was no breakdown in proteins at these doses used in this study. Previous studies in our laboratory have shown that the metabolic activity within bacterial cells persists even after exposure to 7.0 kGy eBeam dose (145-146). This indicates that the enzymatic proteins are not only structurally intact but also functional even after eBeam exposure. We also noticed that the milk allergens (whey and casein) were not

detectable (and presumably unaffected) when exposed to 25.0 kGy using commercial detection kits (Reveal 3-D for Total Milk Allergen prod. # 8479, Neogen Corp., Lansing, MI).

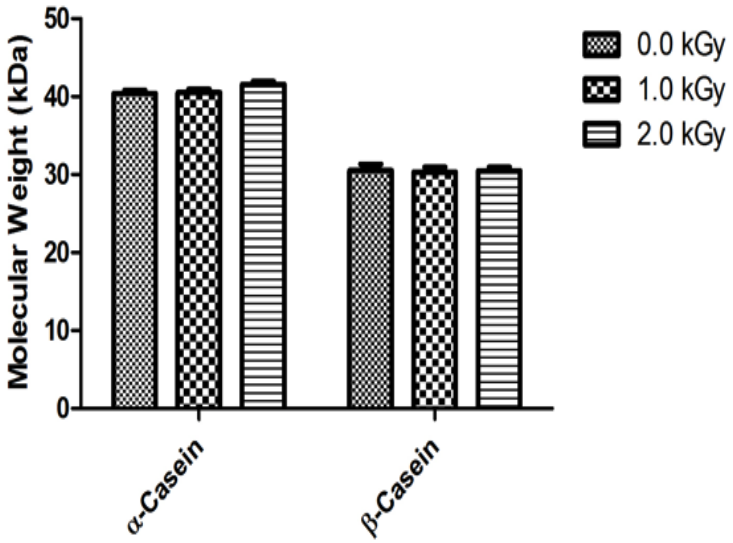


FIG 4. Mean molecular weights of α-Casein and β-Casein in raw milk (0 kGy) and after eBeam processing at 1.0 and 2.0 kGy (n=6).

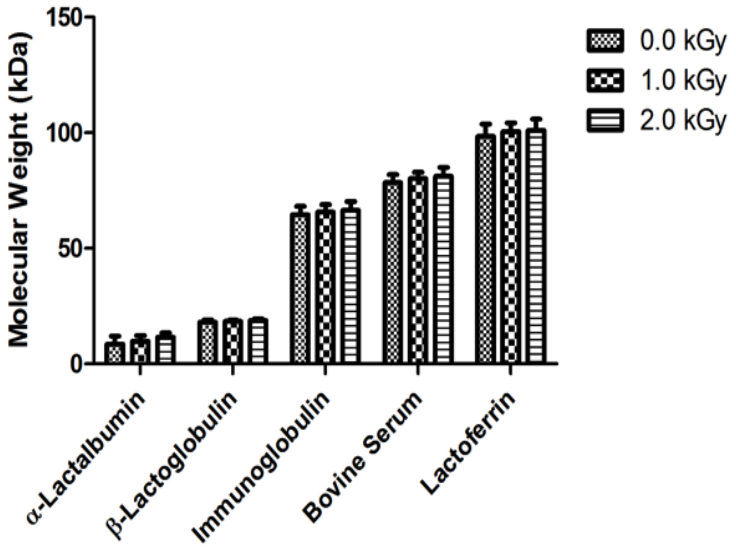


FIG 5. Mean molecular weights of whey proteins in raw milk (0 kGy) and after eBeam processing at 1.0 and 2.0 kGy (n=6).

*Calcium, Vitamin $B_2$, and Vitamin $B_{12}$*

Figure 6 shows calcium concentrations present in raw milk before and after eBeam processing at 0.0, 1.0 and 2.0 kGy. Mean calcium concentrations for each treatment were 1.31 , 1.30, and 1.27 mg/g, respectively. Mean calcium concentrations did not significantly differ between the three eBeam processing treatments ($p>0.05$). Vitamin $B_2$ (riboflavin) concentrations remaining in raw milk after eBeam processing is shown in figure 7. Mean vitamin $B_2$ concentrations for eBeam doses 0.0, 1.0, and 2.0 kGy were 2.08, 1.61, and 1.42 mg/kg, respectively. Mean vitamin $B_2$ concentrations did

significantly differ between the three eBeam processing treatments ($p<0.0001$). Further statistical analysis between groups comparing 0.0 vs. 1.0 kGy, 1.0 vs. 2.0 kGy, and 0.0 vs. 2.0 kGy revealed that all three doses were significantly different ($p<0.05$). Figure 8 shows vitamin $B_{12}$ concentrations present in raw milk before and after eBeam processing at 0.0, 1.0 and 2.0 kGy. Mean vitamin $B_{12}$ concentrations for each treatment were 3.734, 4.009, and 3.848 μg/kg, respectively. Mean vitamin $B_{12}$ concentrations did not significantly differ between the three eBeam processing treatments ($p>0.05$). Studies have shown that calcium, along with other minerals, are not degraded at irradiation doses commonly employed in food pasteurization (ie., <10.0 kGy) (84, 147-148). Care must be taken when reviewing the literature, as many studies state vitamins are sensitive to

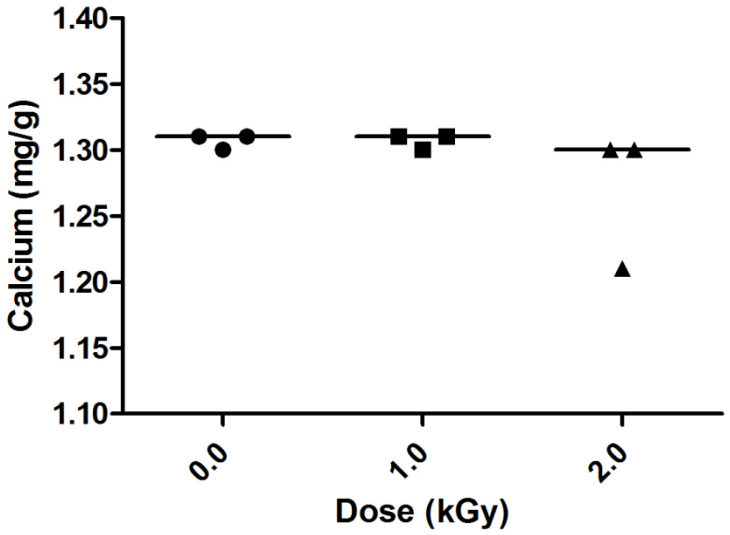


FIG 6. Scatter plot of calcium concentrations remaining in raw milk (0 kGy) and after eBeam processing at 1.0 and 2.0 kGy. The horizontal line represents the median value (n=3).

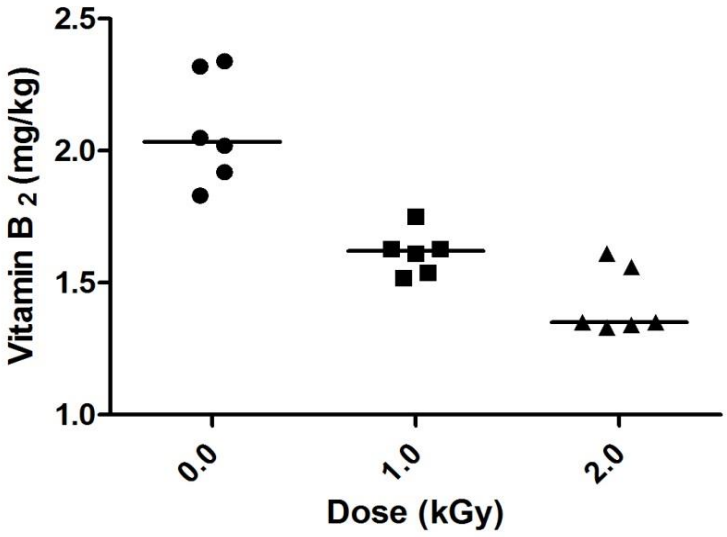


FIG 7. Scatter plot of Vitamin $B_2$ (riboflavin) concentrations remaining in raw milk (0 kGy) and after eBeam processing at 1.0 and 2.0 kGy. The horizontal line represents the median value (n=6).

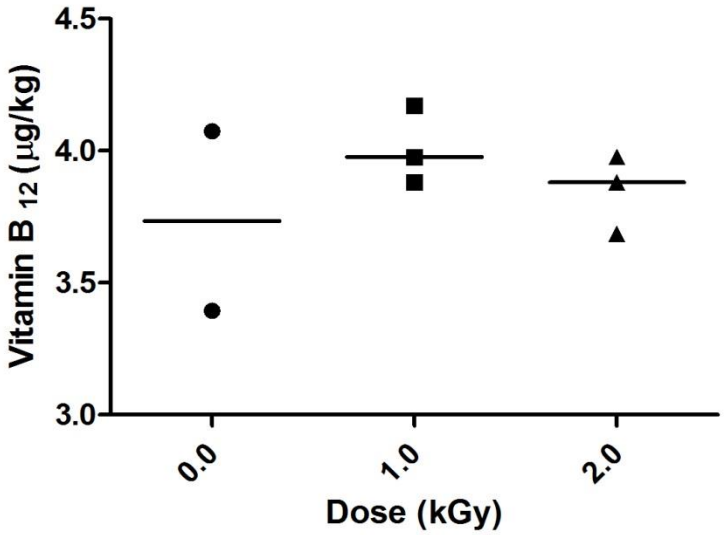


FIG 8. Scatter plot of Vitamin $B_{12}$ concentrations remaining in raw milk (0 kGy) and after eBeam processing at 1.0 and 2.0 kGy. The horizontal line represents the median value (n=3). The data for 0 kGy was based on 2 samples (n=2).

irradiation (and thus, were degraded) using doses much higher than that needed to pasteurize foods (149). Studies have shown that gamma irradiation (≤3.0 kGy) does not significantly affect vitamins B2 and B12 content in fish (150-151). Hau and Liew (152) found that vitamin $B_{12}$ was unaffected by gamma irradiation (≤7.0 kGy) of grass prawns. Even at sterilization doses for gamma irradiation (28.9-48.7 kGy), no changes in vitamins $B_2$ or $B_{12}$ were observed in animal foods (153). Our results from this study were similar to what's found in literature, with the exception of the degradation of riboflavin when exposed to eBeam processing in milk. It must be noted though that the vitamin concentrations (particularly vitamin $B_2$) cited from the literature were present in a solid food matrix when exposed to irradiation. It may be the case that a solid food matrix is better suited for protecting the integrity of vitamin $B_2$ as opposed to an aqueous food matrix, such as that of milk. Water is thought to be able to mobilize catalysts and other compounds in foods that may lead to the formation of free radicals, inducing lipid oxidation (154-155). Similar to the findings in our study, Kung and others (70) found approximately a 25% loss of vitamin $B_2$ when raw milk was exposed to approximately 2.0 kGy gamma irradiation. Furthermore, it must be noted that many food processes, including pasteurization of milk, may result in minor losses of vitamins which are negligible in terms of necessary dietary intake and do not necessarily contribute to vitamin deficiencies in the diet (156-158). This fact must be kept in mind in regards to the potential loss of vitamins and other nutrients in irradiated milks. Ziegler and Keevil (159) found vitamin $B_2$ concentrations in milk to decrease from 9-16% due to thermal pasteurization, while Andersson and Öste (160) found vitamin $B_{12}$ in milk to be

unaffected by thermal pasteurization. After conducting a literature review and performing meta-analysis on the compiled literature, MacDonald and others (127) determined vitamin $B_2$ concentrations significantly decreased in milk ($p<0.05$) after thermal pasteurization. Although this study found riboflavin concentrations were lowered by 31.57% in raw milk as a consequence of eBeam pasteurization, the residual concentration after eBeam processing remains similar to concentrations determined by the USDA (71). Based upon our study, eBeam pasteurized raw milk would still be considered an excellent source of vitamin $B_2$ (≥20% of daily value) at a processing dose of 2.0 kGy.

Lipid Oxidation

Malondialdehyde (MDA) is a secondary byproduct of lipid oxidation, and its concentrations were measured to determine lipid oxidation in raw and eBeam pasteurized milk. Concentrations were measured on day 1 (same day as eBeam processing) and day 7 to determine concentrations of oxidation in raw milk processed with eBeam at 0.0, 1.0, and 2.0 kGy (figures 9-10). MDA concentrations did not significantly differ between the untreated raw milk and the eBeam treated raw milk samples on Day 1 ($p>0.05$).

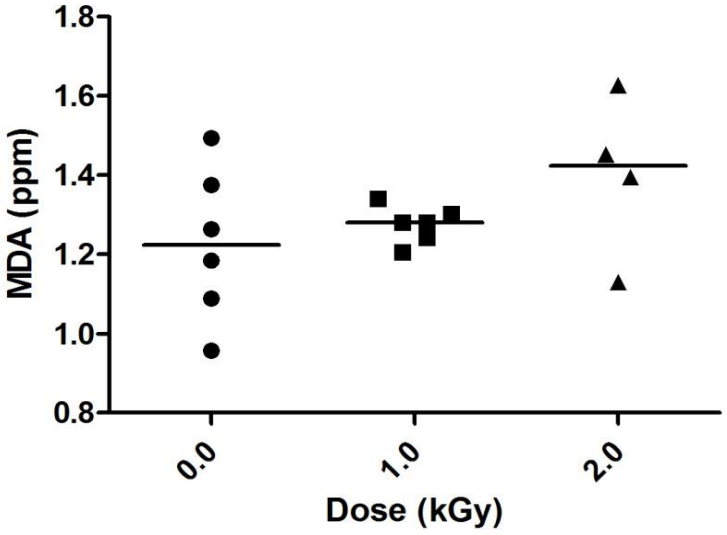


FIG 9. Scatterplot of day 1 MDA concentrations remaining in raw milk (0 kGy) and after eBeam processing 1.0 and 2.0 kGy. The horizontal line represents the median value (n=6). The data for 2.0 kGy was based on 4 samples (n=4).

However, MDA concentrations did significantly differ between eBeam treatments on Day 7 ($p < 0.0001$). Further statistical analysis showed the 0.0 kGy vs. 1.0 kGy treatments did not differ significantly, with ~13% increase in MDA concentrations ($p > 0.05$). All other treatment pairs (0.0 kGy vs. 2.0 kGy, 1.0 kGy vs. 2.0 kGy) differed significantly in MDA concentrations, showing ~58% and ~39% increases in MDA concentrations, respectively ($p < 0.05$).

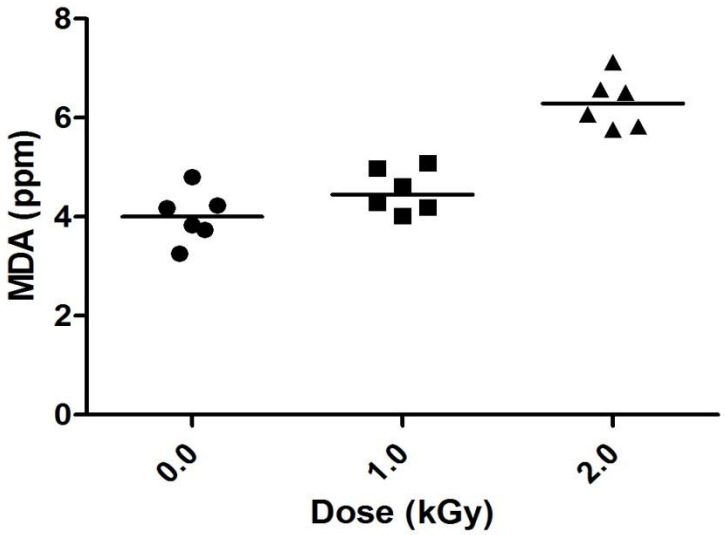


FIG 10. Scatterplot of day 7 MDA concentrations remaining in raw milk (0 kGy) and after eBeam processing 1.0 and 2.0 kGy. The horizontal line represents the median value (n=6).

Although eBeam processed at low doses, it is likely MDA concentrations were significantly different between the three processing treatments (with the exception of the 0.0kGy and 1.0kGy treatments) on day 7 due to the formation free radicals. As eBeam processing is a reducing process and electrons are being introduced in the foods, the high fat environment of milk could easily allow for the formation of free radicals (particularly, reactions with unsaturated fatty acids), which would in turn, lead to secondary oxidative products (161-162). The carbon-carbon double bonds present on

unsaturated fatty acids are likely to be particularly susceptible (163). Similar results were reported in which secondary lipid oxidation products in foods significantly increased as a result of electron beam processing, with significant changes observed as early as day 0 (164-167). Additionally, in the case of polymer sterilization, Costa and others (168) found hydroperoxides being formed up to 15 days after electron beam irradiation. These studies suggest the formation of such compounds post irradiation is to be expected.

Volatile Odorous Compounds

GC-MS olfactory analysis is beneficial in analyzing the odorous (volatile) compounds present in foods by trapping the volatiles in a solid phase microextraction fiber (SPME) before further analysis. This technology has the benefit of combining sensory data with the identification of the aromatic compound (169). Fifty-three odorous compounds were identified in HTST milk and raw milk (untreated) and eBeam treated (1.0 and 2.0 kGy) (table 6). Of these compounds, six (<12%) had at least one treatment significantly differ ($p<0.05$). Most of the significant odorous compounds (~67%) have an aroma associated with their presence, with the exception of 1-octene, which was undetermined, and cycloheptane, a non-aromatic compound. None of the compound classifications stood out as more prevalent amongst the significant compounds. All significant odorous compounds were only detected in the eBeam processed milk samples (1.0 and 2.0 kGy), suggesting eBeam processing slightly alters the volatile compound profile of raw milk.

Linoleic acid is a fatty acid that comprises approximately 60% of milk's polyunsaturated fatty acids, while oleic acid comprises of approximately 100% of milk's monounsaturated fatty acids (71). Alkenes are typical products of lipid oxidation (170). Hexanal, one of the significant aldehydes identified, is a major product of linoleic acid oxidation and is assumed to be a reliable indicator of lipid oxidation in food systems (171-172). The presence of significant hexanal in only the eBeam processed samples (1.0kGy and 2.0kGy) suggests the possibility of greater oxidation occurring as a result of these treatments than compared to raw (0.0 kGy) and store-bought (HTST) milk.

TABLE 6. Odorous compounds present in raw milk after exposure to 1.0 and 2.0 kGy eBeam doses as compared to un-irradiated (0 kGy) raw milk and heat pasteurized (HTST) milk sample. Each data point reflects the mean from 3 different milk sources replicated twice[1].

| Compound | 0.0kGy | 1.0kGy | 2.0kGy | HTST[2] | Classification | Aroma |
|---|---|---|---|---|---|---|
| **Nonanal** | 3.845 | 2.305 | 2.335 | 2.39 | | |
| **p-Xylene** | 2.215 | 2.505 | 0 | 0 | | |
| **Benzene, 1,3-dimethyl-** | 2.135 | 0 | 0 | 0 | | |
| **1-Decene** | 0 | 1.76 | 1.985 | 0 | | |
| **1-Heptene** | 0 | 3.975 | 2.075 | 0 | | |
| **2-methyl-2-Propen-1-ol** | 0[a] | 4.2[b] | 0[a] | 0[a] | Allyl alcohol | Pungent, alcohol |
| **Benzene** | 0 | 2.1 | 4.355 | 0 | | |
| **Benzene, ethyl-** | 0 | 2.21 | 0 | 0 | | |
| **Butanal** | 0 | 4.83 | 2.39 | 0 | | |
| **Butanal, 3-methyl-** | 0 | 3.95 | 1.925 | 0 | | |
| **Butanoic acid, methyl ester** | 0 | 3.625 | 2.015 | 0 | | |
| **Cycloheptane** | 0[a] | 3.05[b] | 3.94[c] | 0[a] | Cycloalkane | Non-odorous |
| **Heptane** | 0 | 4.34 | 2.34 | 0 | | |
| **Hexanal** | 0[a] | 5.29[b] | 5.345[b] | 0[a] | Aldehyde | Green, grassy |

TABLE 6 Continued

| Compound | 0.0kGy | 1.0kGy | 2.0kGy | HTST[2] | Classification | Aroma |
|---|---|---|---|---|---|---|
| **Hexane, 3-ethyl-** | 0 | 1.935 | 0 | 0 | | |
| **Pentasiloxane, dodecamethyl-** | 0 | 2.09 | 0 | 0 | | |
| **Toluene** | 0 | 2.79 | 2.625 | 0 | | |
| **Xylene** | 0 | 1.985 | 4.345 | 0 | | |
| **1-Nonene** | 0 | 1.835 | 0 | 0 | | |
| **3-Tetradecene, (Z)-** | 0 | 2.12 | 2.24 | 0 | | |
| **Cyclobutanone, 2-ethyl-** | 0 | 2.015 | 4.01 | 0 | | |
| **Decanal** | 0 | 1.785 | 2.025 | 0 | | |
| **Ethanol, 2-(hexyloxy)-** | 0 | 1.915 | 0 | 0 | | |
| **Heptenal** | 0 | 1.785 | 2.025 | 0 | | |
| **o-Xylene** | 0 | 2.255 | 0 | 0 | | |
| **Octanal** | 0 | 2.33 | 4.81 | 1.81 | | |
| **Octanoic acid, ethyl ester** | 0 | 1.82 | 2.505 | 4.91 | | |
| **Trans-1-Butyl-2-methylcyclopropane** | 0 | 2.28 | 0 | 0 | | |
| **1-Octene** | 0[a] | 0[a] | 4.695[b] | 0[a] | Alkene | Undetermined |

TABLE 6 Continued

| Compound | 0.0kGy | 1.0kGy | 2.0kGy | HTST[2] | Classification | Aroma |
|---|---|---|---|---|---|---|
| **3-Dodecen-1-al** | 0 | 0 | 1.79 | 0 | | |
| **Butanoic acid, 2-propenyl ester** | 0 | 0 | 2.085 | 0 | | |
| **Dimethyldisulfide** | 0[a] | 0[a] | 4.165[b] | 0[a] | Disulfide | Pungent, garlic |
| **Hexanoic acid, methyl ester** | 0 | 0 | 1.545 | 0 | | |
| **N-Heptanal** | 0 | 0 | 2.44 | 0 | | |
| **Nonane** | 0[a] | 0[a] | 4.62[b] | 0[a] | Alkane | Sharp, pungent |
| **Octane** | 0 | 0 | 2.24 | 0 | | |
| **Propanoic acid, 2-methyl-,2-ethyl-3-hydroxyhexyl ester** | 0 | 0 | 1.65 | 0 | | |
| **Synephrine** | 0 | 0 | 1.835 | 0 | | |
| **Acetic Acid** | 0 | 0 | 1.785 | 0 | | |
| **Benzaldehyde** | 0 | 0 | 1.575 | 0 | | |
| **Benzene, 1-methyl-4-(1-methylethyl)** | 0 | 0 | 2.285 | 0 | | |
| **Benzene, 1,4-dimethyl-2,5-bis(1-methylethyl)-** | 0 | 0 | 1.925 | 0 | | |

TABLE 6 Continued

| Compound | 0.0kGy | 1.0kGy | 2.0kGy | HTST[2] | Classification | Aroma |
|---|---|---|---|---|---|---|
| **Benzene, methyl-** | 0 | 0 | 2.68 | 0 | | |
| **Cyclobutanemethanol** | 0 | 0 | 1.77 | 0 | | |
| **Compound** | **0.0kGy** | **1.0kGy** | **2.0kGy** | **HTST[2]** | **Classification** | **Aroma** |
| **Decanoic acid, ethyl ester** | 0 | 0 | 2.22 | 0 | | |
| **dl-Limonene** | 0 | 0 | 2.355 | 0 | | |
| **Eicosanoic acid, methyl ester** | 0 | 0 | 1.545 | 0 | | |
| **Hydroxylamine, O-decyl-** | 0 | 0 | 1.595 | 0 | | |
| **Naphthalene** | 0 | 0 | 1.815 | 0 | | |
| **Hexanoic acid, ethyl ester** | 0 | 0 | 0 | 1.925 | | |
| **2-Undecanone** | 0 | 0 | 0 | 1.625 | | |
| **3-Oxabicyclononane** | 0 | 0 | 0 | 1.87 | | |
| **Dodecanal** | 0 | 0 | 0 | 1.89 | | |

[1]Values in rows that have different superscripts are statistically ($p<0.05$) different.
[2]HTST: High temperature, short time pasteurization method

## Conclusions

The purpose of this study was to determine the extent to which eBeam pasteurization of raw milk affected the nutrients and aroma profile of raw milk. All nutrients analyzed, with the exception of vitamin $B_2$, were not significantly decreased by eBeam processing. Vitamin $B_2$ content was impacted both at the 1.0 and 2.0 kGy doses, with ~22% and ~32% decreases observed, respectively. However, milk irradiated at 2.0 kGy still remains an excellent source of vitamin $B_2$ according the U.S. dietary recommendations. Vitamin $B_2$ fortification can be an option to replace the trace amounts of vitamin $B_2$ lost. TBARS and GC-MS olfactory analysis studies both suggest lipid oxidation occurs in milk as a result of eBeam pasteurization. MDA concentrations for milk eBeam processed at 2.0 kGy were significantly higher (~58% increase) than the control (0.0 kGy) 7 days after eBeam processing. Aldehydes, compounds often formed due to oxidation of lipids, were significantly greater in eBeam processed milk samples than the control and HTST milk. Less than 12% of odorous compounds identified in milk were determined to significantly differ between at least one treatment. Further sensory analysis would need to be conducted to determine whether humans can detect the subtle changes in odorous compounds.

# CHAPTER V

# CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

The purpose of the studies presented in this thesis was to determine if key pathogens and other indigenous microflora in raw milk could be eliminated with eBeam pasteurization, and to determine the extent to which eBeam pasteurization of raw milk affected the nutrients and aroma profile of raw milk.

Microbial analysis showed eBeam pasteurization achieves up to a 32.0 $\log_{10}$ theoretical reduction of key microbial pathogens at a 2.0 kGy dose, and reduces coliform and indigenous microflora counts to below detectable limits. Significant reductions in infection risks from raw milk-associated pathogens is achievable if raw milk is pasteurized with eBeam. This major finding translates to the availability of a non-thermal milk pasteurization technology that can assure public health and wellness for consumers, particularly to those who desire to consume raw milk and raw milk based products.

None of the nutrients with the exception of vitamin $B_2$, were significantly decreased by eBeam processing. Vitamin $B_2$ content was impacted both at the 1.0 kGy and 2.0 kGy doses, with ~22% and ~32% decreases observed, respectively. However, milk irradiated at 2.0 kGy still retains enough vitamin B2 per US dietary guidelines to be considered an excellent source of vitamin $B_2$. Vitamin $B_2$ fortification can be an option to replace the

trace amounts of vitamin $B_2$ lost. Evidence of lipid oxidation was found in both TBARS and GC-MS olfactory analysis studies, suggesting that lipid oxidation does occur in milk as a result of eBeam pasteurization, similar to findings in other studies (164-167). Malondialdehyde concentrations for milk eBeam processed at 2.0 kGy were significantly higher (~58% increase) than the control (0.0 kGy) 7 days after eBeam processing. Aldehydes, compounds often formed due to oxidation of lipids, were significantly greater in eBeam processed milk samples than the control and HTST milk. Less than 12% of aromatic compounds identified in milk were determined to significantly differ between at least one treatment. These minor changes in aroma compounds due to irradiation suggest that there are minor flavor profile changes occurring. However, sensory analysis would to need to be conducted to determine if humans are able to detect these subtle changes and whether these changes negatively affect the overall acceptability.

Building on these studies, an important next step would be to conduct sensory panel studies to determine consumer acceptability of eBeam pasteurized raw milk, though it is highly unlikely IRB approval would be given for human sensory of raw milk. Studies like this would simply need to determine acceptability of irradiated milk without a control, which could prove challenging as there is nothing for panelists to compare the product to. Additionally, a study to determine how eBeam pasteurized milk affects the quality and other sensory attributes in further processed dairy products (such as soft cheeses, yogurt, ice cream, etc.) would be beneficial in determining its usability.

Sensory panel studies of these further processed dairy products would also be beneficial. Studies like these would allow us to draw conclusions of eBeam pasteurized milk's practicality in the dairy food industry. Raw milk advocates are unlikely to change their opinion of processed milk and drink milk that is not raw. This fact, along with the reality that raw milk represents approximately 1% of all milk sold in the U.S., supports the notion that simply selling eBeam pasteurized milk to the public would not be financially viable. However, if eBeam pasteurized milk is able to maintain (and perhaps improve) the quality of further processed dairy products, then it would be more likely to be widely used in the dairy industry and consumed by its customers.

It would be interesting for a study to focus on developing an in-line eBeam processing method for raw milk. Predicted challenges for this study include temperature control of the raw milk, ensuring uniform dose distribution throughout the product, and the development of suitable packaging. Another aspect to consider when processing raw milk is it is not homogenized. Fat will settle at the top of the product when not agitated, affecting dose distribution in the product. In-line eBeam pasteurization studies would need to consider solutions for dealing with non-homogenized milk, such as agitation before irradiation. It would also be interesting to study the possibility of homogenizing milk before eBeam irradiation.

Finally, an important step for the eBeam pasteurization of raw milk would be developing a legal petition to the US FDA to allow for its legal commercial processing use in the

food industry. Studies such as the ones presented in this thesis, along with the suggested future studies, would all include critical information to validate the equivalency of eBeam pasteurization to conventional thermal pasteurization.

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# APPENDIX

TABLE A-1. Foodborne outbreaks associated with raw milk or raw milk-made products in the United States: 1992-2016.

| Year | State(s) | Contaminating Pathogen | No. Illnesses | No. Hospitalizations | No. deaths | Food Vehicle | Reference(s) |
|---|---|---|---|---|---|---|---|
| **2016** | CO | *Campylobacter* spp. | 20 | 0 | 0 | raw milk | Marler blog |
| **2016** | CA | *E. coli* O157:H7 | 8 | NR[1,2] | 0 | raw milk | Marler blog |
| **2014** | UT | *Salmonella* Newport | 2 | 0 | 0 | raw milk | CDC 2016 |
| **2014** | PA | *Campylobacter* spp. | 2 | 0 | 0 | raw milk | CDC 2016 |
| **2014** | MN | *C. jejuni* | 9 | 2 | 0 | raw milk | CDC 2016 |
| **2014** | MI | *E. coli* O157:H7 | 2 | 0 | 0 | raw milk | CDC 2016 |
| **2014** | NY | *C. jejuni* | 8 | 0 | 0 | raw milk | CDC 2016 |
| **2014** | CA, FL | *L. monocytogenes* | 2 | 2 | 1 | raw milk | CDC 2016 |
| **2014** | UT | *C. jejuni* | 99 | 10 | 1 | raw milk | Davis and others 2016 |
| **2012** | OR | *E. coli* O157:H7 | 16 | 4 | 0 | raw milk | CDC 2016 |
| **2012** | CA | *C. jejuni* | 33 | 2 | 0 | raw milk | CDC 2016 |
| **2012** | CO | *E. coli* 0111 | 2 | 0 | 0 | raw milk | CDC 2016 |
| **2011** | SC | *C. jejuni* | 23 | 1 | 0 | raw milk | CDC 2016 |
| **2011** | MI | *Campylobacter* spp. | 2 | 0 | 0 | raw milk | CDC 2016 |
| **2011** | NY | *C. jejuni* | 4 | 0 | 0 | raw milk | CDC 2016 |
| **2011** | NY | *C. jejuni* | 3 | 0 | 0 | raw milk | CDC 2016 |
| **2011** | NY | *C. jejuni* | 13 | 0 | 0 | raw milk | CDC 2016 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **2011** | CA | *E. coli* O157:H7 | 5 | 3 | 0 | raw milk | CDC 2016 |
| **2010** | CA, NV, AZ, CO, NM | *E. coli* O157:H7 | 41 | 18 | NR | gouda cheese[3] | McCollum and others 2012 |
| **2010** | NY | *C. jejuni* | 20 | 1 | 0 | raw milk | CDC 2016 |
| **2010** | IL | *C. jejuni* | 2 | 0 | 0 | raw milk | CDC 2016 |
| **2008** | CT | *E. coli* O157:H7 | 14 | 5[4] | NR | raw milk | Guh and others 2010 |
| **2008** | TN | *C. jejuni* | 4 | 0 | 0 | raw milk | CDC 2016 |
| **2008** | CT | *E. coli* O157:NM | 14 | 5 | 0 | raw milk | CDC 2016 |
| **2008** | MA | *C. jejuni* | 8 | 0 | 0 | raw milk | CDC 2016 |
| **2008** | MN | *Campylobacter* spp. | 2 | 0 | 0 | raw milk | CDC 2016 |
| **2008** | PA | *C. jejuni* | 65 | 1 | 0 | raw milk | CDC 2016 |
| **2008** | VT | *E. coli* O157:H7 | 6 | 3 | 0 | raw milk | CDC 2016 |
| **2008** | UT | *C. jejuni* | 4 | 0 | 0 | raw milk | CDC 2016 |
| **2008** | CA | *C. jejuni* | 16 | NR | NR | raw milk | CDC 2016 |
| **2008** | ND | *C. jejuni* | 3 | 0 | 0 | raw milk | CDC 2016 |
| **2007** | PA | *Salmonella* Typhimurium | 29 | 2 | NR | raw milk, soft cheese | Lind and others, 2007 |
| **2007** | SC | *C. jejuni, S. enterica* | 11 | 4 | 0 | raw milk | CDC 2016 |
| **2007** | KS | *C. jejuni* | 16 | 0 | 0 | cheddar cheese | CDC 2016 |
| **2007** | WA | *C. jejuni* | 18 | 0 | 0 | raw milk | CDC 2016 |
| **2007** | PA | *Salmonella* Typhimurium | 4 | 0 | 0 | raw milk made cheese | CDC 2016 |
| **2007** | CA | *Campylobacter* spp. | 11 | 0 | 0 | raw milk | CDC 2016 |
| **2006** | VA | *C. jejuni* | 9 | 0 | 0 | raw milk | CDC 2016 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **2006** | PA | *Salmonella* Typhimurium | 20 | 2 | 0 | queso fresco | CDC 2016 |
| **2006** | CA | *E. coli* O157:H7 | 6 | 3 | 0 | raw milk | CDC 2016 |
| **2005** | OR, WA | *E. coli* O157:H7 | 18 | 5[5] | NR | raw milk | Denny and others 2008 |
| **2002** | IL, IN, OH, TN | *Salmonella* Typhimurium | 62 | 2 | NR | raw milk | CDC 2003, Mazurek and others 2004 |
| **2000** | PA | *C. jejuni* | 3 | 1 | 0 | raw milk | CDC 2016 |
| **2000** | NC | *L. monocytogenes* | 13 | 10 | NR[6] | Mexican Style Cheese | MacDonald and others 2005 |
| **1998** | MA | *Salmonella* Typhimurium | 47 | 2 | 0 | raw milk | CDC 2016 |
| **1997** | WA | *Salmonella* Typhimurium | 54 | 5 | NR | Mexican Style Cheese | Villar and others 1999 |
| **1997** | CA | *Salmonella* Typhimurium | 31 | 4 | NR | Mexican Style Cheese | Cody and others 1999 |
| **1997** | CA | *Salmonella* Typhimurium | 79 | 10 | NR | Mexican Style Cheese | Cody and others 1999 |
| **1992** | OR | *E. coli* O157:H7 | 14 | 2 | NR | Raw Milk | Keene and others 1997 |

[1]none reported
[2]2 HUS cases reported
[3] all reported cheese made from raw milk
[4]3 HUS cases reported
[5]4 HUS cases reported
[6]5 stillbirths reported