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Microbial Growth Inhibition and Decomposition of Milk Mineral and Sodium Tripolyphosphate Added to Media or Fresh Ground Beef

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MICROBIAL GROWTH INHIBITION AND DECOMPOSITION OF MILK MINERAL AND SODIUM TRIPOLYPHOSPHATE ADDED TO MEDIA OR FRESH GROUND BEEF

by

Rossarin Tansawat

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ABSTRACT

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Utah State University, 2009

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Milk mineral (MM) is a type II antioxidant (metal chelator) that can bind iron and prevent iron catalysis of lipid oxidation. Thus, MM might have microbial growth inhibition effects on iron-dependent bacteria. Objective 1 was to evaluate effects of MM on growth of non-pathogenic iron-dependent bacterial strains (*Listeria innocua*, *Eschericia coli*, *Pseudomonas fluorescens*). MM (1.5 % w/v) did not significantly inhibit growth of *Listeria* and *E. coli*. However, growth of *Pseudomonas fluorescens* was consistently and significantly reduced by ~1 log colony forming units per ml (CFU/ml) with all levels of MM (0.5, 0.75, 1.5 % w/v). All levels of MM also had no growth inhibition effects against the mixed microflora of fresh ground beef during storage for up to 10 days at 2°C. In conclusion, MM had little or no effect to inhibit microbial growth. The strong affinity of MM to ionic iron inhibits lipid oxidation, but does not inhibit

bacterial growth supported by other forms of iron (heme or amino acid + iron complexes).

Several studies report that MM has greater antioxidant effect than sodium tripolyphosphate (STP) in ground meats, especially at longer storage time. Objective 2 was to compare stability of MM and STP in ground beef patties by monitoring the decomposition to soluble orthophosphates (P*i*). Patties (control) and patties with 0.75 % MM or 0.5 % STP were stored at 2 or 22°C for 0, 1, or 2 days. CFU/g and P*i* were measured. As expected, CFU/g at 22°C was much higher than treatment at 2°C. P*i* levels at 2°C were lower ($P < 0.05$) than at 22°C. At day 0, for both temperatures, patties formulated with MM had the highest P*i* levels. However, after 2 days storage, samples with added STP had the highest level of P*i*, followed by MM and control. Thus, decomposition as measured by release of P*i* was significantly higher for STP than for MM added to beef patties. There was a significant positive correlation (0.77) between CFU/g and P*i* during storage of beef patties for 2 days at 22°C. In conclusion, increased P*i* during storage of beef patties was at least partially due to bacterial phosphatases. A third experiment was conducted to examine the stability of 0.75 % MM or 0.5 % STP added to growing cultures of *Pseudomonas fluorescens* at 2°C or 22°C for 0, 1, and 2 days. Neither MM nor STP was stable in autoclaved media (P*i* increased significantly). The factors responsible for decomposition of MM or STP in autoclaved media remain to be determined.

(75 pages)

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Rossarin Tansawat

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LIST OF SYMBOLS, NOTATION, DEFINITIONS

AOAC	Association of Official Analytical Chemists
APC	Aerobic plate count
ATCC	The American Type Culture Collection
BHI	Brain heart infusion
C	Celsius
CFU	Colony forming unit
Coag. Pos. Staph.	Coagulase test for gram-positive Staphylococcus
CPM	Calcium phosphate monobasic
Da	Dalton
Den DF	Denominator degree of freedom
DW	Distilled water
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
Fe^{2+}	Ferrous
Fe^{3+}	Ferric
Feo	Ferrous iron uptake system
Ka	Acid dissociation constant
K_{aff}	Affinity constant
KH_2PO_4	Potassium phosphate
min.	Minute
MM	Milk mineral

NaOH	Sodium hydroxide
Num DF	Numerator degree of freedom
OH^-	Hydroxide
OH·	Hydroxy radical
PO_4^{3-} or P*i*	Orthophosphate
PVC	Polyvinyl Chloride
SH3 domain	Src homology 3 domain
STP or STPP	Sodium tripolyphosphate
TBA	Thiobarbituric acid
TSB	Tryptic soy broth
w/v	Weigh by volume
w/w	Weigh by weigh

CHAPTER 1

INTRODUCTION

Milk mineral (MM), the dried mineral fraction from ultra-filtered whey, is a good calcium source, and has powerful antioxidant properties in cooked ground meats, and also prevents browning in raw ground beef. To receive greater acceptance for MM as a food ingredient, further studies are needed to gain better understanding of MM properties.

Recent studies explain the mechanism of MM as a type II antioxidant (metal ion chelator) that contains negatively charged phosphates that have high affinity for iron cations and other trace minerals. Thus, MM may possibly have microbial growth inhibition effects on iron-dependent bacteria. In Chapter 3 of this study, iron-dependent bacteria species were chosen to determine possible growth inhibition effects of MM added to media or against the mixed microflora of fresh ground beef.

Stability of MM is another property of interest. Previous research has shown that MM retains antioxidant effect for 14 days when added to fresh ground beef, compared to only 7 days for sodium tripolyphosphate (Vissa and Cornforth 2006). It is known that sodium tripolyphosphate (STP) is decomposed to orthophosphate (P*i*) by meat or microbial phosphatases during long term storage of ground beef (Li and others 1993) and P*i* is a less effective iron chelator than STP (Molins 1990). MM is a stronger iron chelator than STP (Allen and Cornforth 2007). Thus, it is the hypothesis of this study (part 1, Chapter 4) that STP decomposes to P*i* more rapidly than MM in ground beef. Stated another way, it is hypothesized that MM is more stable than STP, accounting for the greater antioxidant effect of MM compared to STP in fresh ground beef at longer storage time. Thus, the objective in part 1 (Chapter 4) of this

experiment was to compare STP and MM stability (resistance to decomposition to P*i*), by measuring the P*i* content in ground beef or media with added MM or STP, and stored for 0 - 2 days at 2 or 22°C. Colony forming units per g (CFU/g) beef CFU/ml for media were also measured on all treatment combinations, and correlation coefficients were calculated to determine the possible relationship between bacterial numbers and P*i* formation.

CHAPTER 2

LITERATURE REVIEW

MILK MINERAL

Milk mineral (MM), consisting primarily of colloidal calcium phosphate particles (Allen and Cornforth 2006), is a dried natural calcium phosphate source derived from ultra-filtered whey. The composition and microbial analysis of MM Trucal® D-7(Glanbia®, Twin Falls, ID) is showed in Table 1 and Table 2.

Table 1. Milk mineral composition.

Constituents	Proportion
Non Mineral	23.5 %
Citrate	4.0 %
Lactose	10.0 %
Protein	5.0 %
Free Moisture	4.0 %
Fat	0.5 %
Mineral (ash)	77.5 %
Calcium	23.0 - 25.0 %
Phosphorous	13.0 - 13.5 %
pH	6.4
Typical Particle Size	90% < 7 microns
Bulk Density	0.59 g/ml

Source: Trucal® D-7(Glanbia®),
Accessed on 01/30/09 at - http://glanbiaingredients.com/PDF/TruCalD7.pdf

Table 2. Microbiological analysis of milk mineral.

Microbiological Analysis	
Standard Plate Count	<10,000 /g
Coliform	Negative /1g
Yeast and Mold	<50 /g
Coag. Pos. Staph.	Negative /1g
Listeria	Negative /50g
Salmonella	Negative /50g

Source: Trucal® D-7(Glanbia®),
Accessed on 01/30/09 at - http://glanbiaingredients.com/PDF/TruCalD7.pdf

MM functions as type II antioxidant by chelating iron and preventing iron catalysis of lipid oxidation. There are several studies demonstrating that MM has powerful antioxidant properties. Cornforth and West (2002) conducted the first studies to investigate the possible antioxidant effects of dried MM when added at various levels to ground beef, pork, and turkey. They found that dried MM inhibited lipid oxidation in cooked ground meat crumbles in a dose-dependent manner. They also reported the optimum level of dried MM to inhibit lipid oxidation was 2 % MM to decrease lipid oxidation in raw beef and pork, and only 1 % was required to maintain oxidative stability in ground turkey. They hypothesized that antioxidant properties of MM were due to mechanism of iron-chelation to colloidal phosphate, thus removing iron as a catalyst for lipid oxidation. Jayasingh and Cornforth (2003) reported that MM lowered lipid oxidation in cooked ground pork. They found that the use of 1 %, 1.5 % or 2 % MM were effective at maintaining low thiobarbituric acid (TBA) numbers in cooked ground pork through 6 months of storage at -20°C. They further reported that 1.5 % MM was the minimum level for optimum antioxidant effect at

refrigerator storage (2°C). Vasavada and Cornforth (2005) evaluated the MM antioxidant activity in beef meatballs and nitrite-cured sausage and found that 1.5 % MM was very effective for inhibition of oxidation in cooked meatballs during 15 days of refrigerated storage. They indicated that MM has a potential application as an antioxidant for addition to ground meatballs before cooking. Vissa and Cornforth (2006) compared MM with sodium tripolyphosphate (STP) and vitamin E as antioxidants in ground beef in 80 % oxygen modified atmosphere packaging (MAP). Type II, metal chelating antioxidants (MM and STP) were more effective than vitamin E (type I antioxidant). Also, MM at 0.75 % was more effective than 0.5 % STP for lipid and color stability since MM had lower ($P < 0.05$) TBA values and greater ($P < 0.05$) redness values than samples containing 0.5 % STP. MM at 0.75 % could inhibit lipid oxidation and maintain red color of ground beef in 80 % MAP for 14 day. Allen and Cornforth (2006) studied the effect of nonheme iron on myoglobin oxidation in a model system at pH 5.6 and 7.2 at 23°C to examine the effect of iron removal by iron chelators. They found that MM (at pH 5.6) negated the effect of added iron and slowed oxidation of myoglobin. They reported that MM bound more iron per gram ($P < 0.05$) and was much less soluble ($P < 0.05$) than either sodium tripolyphosphate (STP) or calcium phosphate monobasic (CPM). Mineral localization showed an even distribution of calcium, phosphorus, oxygen, and iron across the MM particle surface and directly demonstrated iron binding to MM particles.

SODIUM TRIPOLYPHOSPHATE

Sodium tripolyphosphate (STP or STPP), with formula $Na_5P_3O_{10}$, is the sodium salt of triose polyphosphate. STP molecular weigh is 367.91; composed of Na 31.25 %, O 43.49 %, and P 25.26 %. STP is prepared by molecular dehydration of

mono- and disodium phosphates. STP is composed of slightly hygroscopic granules. Solubility in water is 20 g/100 ml at 25°C and 86.5 g/100 ml at 100°C (MERCK Index, 1976).

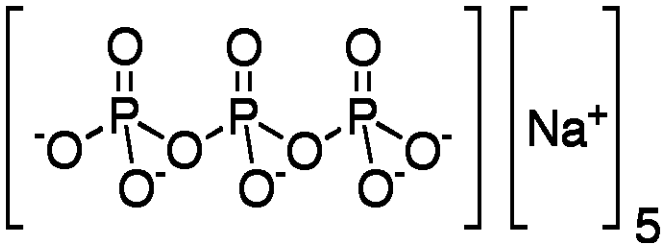

Figure 1. Sodium tripolyphosphate chemical structure.

Source: http://en.wikipedia.org/wiki/Image:Sodium_tripolyphosphate.png, (Accessed on 01/30/09)

In foods, STP is used to preserve meat (MERCK Index, 1976). This substance is generally recognized as safe when used in accordance with good manufacturing practice (FDA 2002). STP solution (0.3 - 0.5 %) is commonly injected into pork chops, fresh pork roasts, fresh beef steaks and beef roasts to enhance water retention, juiciness and cooked yield after cooking (Shults and others 1976; Trout and Schmidt 1984; Molins and others 1987; Anjaneyulu 1989; Li and others 1993; Moiseev and Cornforth 1997; Lee et al. 1998).

STP is also a type II antioxidant used to inhibit lipid oxidation of fresh meat during storage. Smith and others (1984) indicated that phosphate injection reduced warmed-over flavor in reheated pork roasts. Molins and others (1987) reported that addition of phosphates appeared to significantly ($P < 0.05$) reduce the development of oxidative rancidity in frozen beef patties. Craig and others (1991) compared the effects of added sodium tripolyphosphate or sodium ascorbate monophosphate in water solutions (0.3 and 0.5% levels) or water only to ground turkey which was

cooked, vacuum packaged, and stored frozen and found that rancid flavor scores were lower in samples with phosphate salts. Liu and others (1992) reported that STP significantly ($P < 0.01$) reduced lipid oxidation in cooked steaks during refrigerated storage at 4°C for 8 days and in raw steaks stored at -30°C for 8 months.

The antimicrobial effects of STP are uncertain. Tompkin (1984) reported that phosphates have potential value for enhancing the microbial safety and stability of foods. Davidson and Juneja (1990) stated that polyphosphates may suppress the growth of bacteria by complexing metal ions essential for cell division. Vareltzis and others (1997) conducted an experiment to evaluate the effects of STP against bacteria attached to the surface of chicken carcasses. The data showed that the spoilage bacteria were always significantly less in the STP treated samples over the control with storage at 4°C for up to 10 days. However, some other papers reported different results. Molins and others (1985) conducted an experiment to evaluate the effect of polyphosphates on bacterial growth in refrigerated uncooked bratwurst and reported that no significant microbial inhibition by phosphates was found during refrigerated storage. They also stated that enzymatic hydrolysis of phosphates was postulated as a major factor in loss of antimicrobial properties of phosphates in processed meats. In addition, Rhee and others (1997) conducted an experiment to compare the antioxidative and antimicrobial effects of 3 % sodium lactate, 0.05 % ascorbate, 0.5 % tripolyphosphate, and 0.5 % citrate in aerobically refrigerated beef. They reported that STP had no antimicrobial effect, possibly due to hydrolysis of STP by mixing STP with hot (100°C) water to enhance its solubility.

PHOSPHATE HYDROLYSIS

Studies of the hydrolysis of phosphate in foods is very important because it results in loss of functional phosphate properties. Phosphate hydrolysis in foods could occur by many factors; i.e. enzyme activities (bacterial or meat phosphatases), high temperatures, or acidic condition.

In general, linkage between PO_4^- groups in linear polyphosphates tends to be stable in aqueous solution at 25°C. A result of hydrolysis breakdown of polyphosphates is a continuous built up of orthophosphate (PO_4^{3-} or P*i*), which is the end product of the hydrolysis process. Hydrolysis of tripolyphosphate results in the intermediate formation of the pyrophosphate which may further hydrolyze into two orthophosphate ions (Figure 2). One step cleavage of tripolyphosphate into three orthophosphate ions never takes place (Molins 1990).

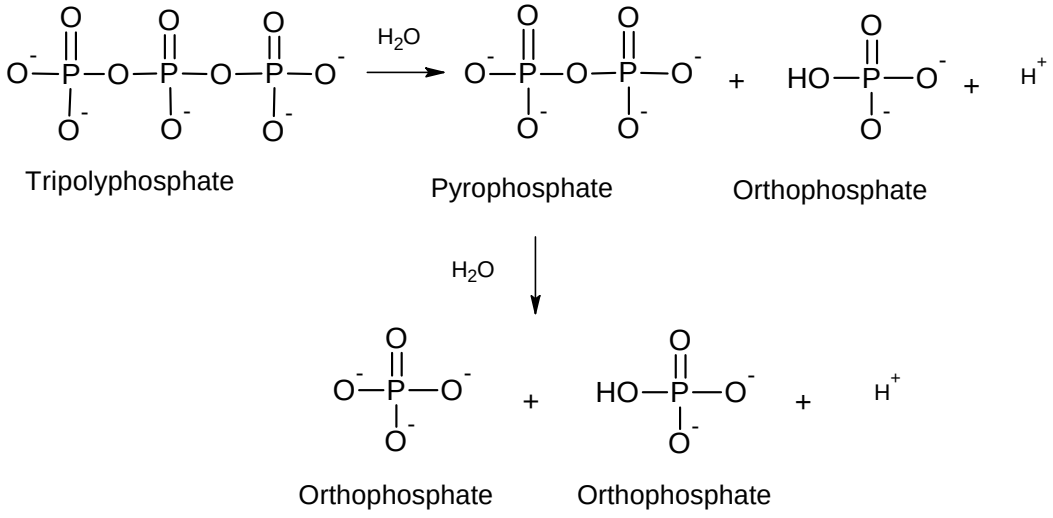

Figure 2. Hydrolysis of tripolyphosphate.

The MERCK Index (1976) states that STP tends to revert to the orthophosphate with prolonged heating of its solutions. Molins and others (1987) studied the effect of inorganic polyphosphates on ground beef characteristics on frozen beef patties and indicated that all phosphates studied increased the pH and the soluble orthophosphate content of beef patties. Hydrolysis of commercial phosphates (Brifisol®) added to ground beef occurred even during frozen storage at -20°C. However, when we look at the data for added STP, P*i* levels did not significantly increase during 60 days frozen storage. For instance, the P*i* of 2 days storage was 1292 ppm and on 60 days was 1250 ppm, so it did not increase from 2 days to 60 days. Thus, STP seems to be stable for 60 days at frozen temperature. Molins and others (1987) also conducted experiments about the extension of the shelf-life of fresh ground pork with polyphosphates. They reported that there was no correlation between the microbial data and soluble orthophosphate content of meat at 2 - 4°C storage for 6 days. Li and others (1993) studied the stability of 0.5% STP in cooked and uncooked ground turkey stored at 5°C for different time periods. They evaluated STP stability by determining soluble orthophosphate. They reported that STP hydrolyzed rapidly in uncooked samples but refrigerated storage time up to 6 days did not affect STP hydrolysis in cooked samples. They also indicated that heating accelerated the rate of STP hydrolysis. Li and others (1993) also cited previous work stating that endogenous meat phosphatase caused STP degradation during refrigerated storage of fresh meat.

BACTERIAL IRON-UPTAKE SYSTEMS

Iron mainly presents in one of two redox states; the reduced ferrous form (Fe^{2+}) or the oxidized ferric form (Fe^{3+}). At physiological pH in an aerobic

environment, Fe^{2+} is the major form but its solubility is very low and it is toxic (Wandersman and Delepelaire 2004). When reduced, the Fe^{2+} activates the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$), where oxygen is partially reduced into hydroxyl radicals that are toxic for most macromolecules. These reasons limit the amount of free iron which bacteria can uptake. Therefore, to extract and use iron from a host or the environment, bacteria have to possess specialized uptake mechanisms. The major steps of these mechanisms consist of; 1) recognition at the cell surface, 2) transport across the outer membrane, 3) transport across periplasm by using periplasmic binding proteins, and 4) transport through the inner membrane. Uptake and transportation mechanisms are regulated by bacterial iron-uptake genes.

There are two main pathways by which bacteria can uptake iron.

1. **Direct Uptake Iron from Iron/Heme Sources:**

   Insoluble ferric iron (Fe^{3+}) can sometimes be reduced to soluble ferrous iron (Fe^{2+}) under anaerobic or reducing conditions. This highly soluble ion can diffuse freely through the outer membrane of gram-negative bacterial strains. Gram negative bacteria can detect environmental iron using outer membrane transporters, and then send a signal across the outer membrane, the periplasmic space, and the inner membrane resulting in regulating certain transport genes to take advantage of a readily available iron source (Buchanan 2005). *Escherichia coli*, gram-negative fecal bacteria, has the ferrous iron uptake system (Feo) which may make an important contribution to the iron supply of the cell under anaerobic conditions (Hantke 1987; Kammler and others 1993). Feo systems are composed of three proteins: FeoA, a small, soluble SH3-domain protein probably located in the cytosol; FeoB, a large protein with a cytosolic N-terminal G-protein domain and a C-terminal integral inner-membrane domain containing two gate motifs

which likely function as the Fe^{2+} permease; and FeoC, a small protein apparently functioning as an [Fe–S]-dependent transcriptional repressor (Cartron and others 2006). This system aids in the ability of *E. coli* to be able to colonize in the gastrointestinal tracts. Other bacteria that also use the Feo system are *Pseudomonas aeruginosa* and *Streptococcus* spp. (Clarke and others 2001).

Most pathogenic bacteria can acquire iron directly from the host iron binding proteins; transferrin and lactoferrin. Transferrin is found in serum while lactoferrin is found in lymph and mucosal secretions. Transferrin functions as iron transporter and protector whereas lactoferrin has only protective function. Yet, both proteins exhibit an extremely high affinity constant for the Fe^{3+} ($Ka \sim 10^{20}\,M^{-1}$) and a much lower constant for Fe^{2+} ($Ka \sim 10^{3}M^{-1}$) (Wandersman and Delepelaire 2004). Examples of bacterial species using both transferrin and lactoferrin as iron sources are *Neisseria* spp. (Hagen and Cornelissen 2006; Perkins-Balding and others 2004), *Helicobacter pylori* and *Haemophilus influenzae* (Clarke and others 2001).

Ferritin is a globular protein complex consisting of 24 protein subunits. It is the main intracellular iron storage protein filled with several thousands of Fe^{3+} ions (Carrondo 2003). Ferritin function is to protect against ferrous iron catalyzed formation of OH· radicals (Wandersman and Delepelaire 2004). Some reports indicate that ferritin is used as an iron source by bacteria. One study reported that this iron acquisition system is used by *Listeria monocytogenes*, a gram positive opportunistic pathogen (Adams and others 1990). In addition, in 2000 there was a study describing a novel intersubunit iron-binding site of the dodecametric ferritin from *Listeria innocua* (Ilari and others 2000) which could be part of the iron uptake mechanism of this bacterial species.

Heme, a prosthetic group of hemoglobin and myoglobin consists of an iron atom in the center of a porphyrin ring. It is an iron source for many bacterial species. An outer membrane receptor can either bind the hemophore, a signaling molecule that acts as an extracellular heme-scavenging protein (Cwerman and others 2006), or heme itself for transfer to the periplasm. Other forms of heme such as hemoglobin, haptoglobin-hemoglobin, and hemopexin could also be used as an iron source. Examples of bacteria using this system are *Escherichia coli* O157, *Neisseria* spp., *Shigella dysenteriae*, *Vibrio cholerae*, *Yersinia* spp. (Clarke and others 2001) and *Haemophilus influenzae* (Andrews and others 2003).

2. Indirect Uptake Iron Sources:

Many bacterial species often secrete and take up chelating compounds or "siderophores" that enable acquisition of iron. Siderophores are normally low molecular mass (< 1000 Da) and highly specific, and with affinity ($K_{aff} > 10^{30}$ M^{-1}) towards ferric iron which can rapidly bind and form complex with Fe^{3+} (Wandersman and Delepelaire 2004).

Siderophores are generally produced by gram-positive and gram-negative bacteria and they are generally synthesized and secreted when the iron sources are limited. Gram-negative bacteria can take up ferri-siderophore complexes by the use of specific outer membrane (OM) receptors which is driven by the cytosolic membrane (CM) potential and mediated by the energy-transducing TonB-ExbB-ExbD system. Then, periplasmic binding proteins transport ferri-siderophores complexes from the OM receptors to CM ATP-binding cassette (ABC) transporters that deliver the ferri-siderophores to the cytosol where the complexes are probably dissociated by reduction (Andrews and others 2003). However, gram-positive bacteria lack an outer membrane. A periplasmic transport protein

and several inner membrane associated proteins then complete the transport of ferri-siderophores complexes to CM by using an ABC transporter (Andrews and others 2003, Clarke and others 2001). The siderophore-mediated iron uptake in gram-negative (A) and gram-positive (B) bacteria is shown in Figure 3.

More than 500 different siderophores have been described (Wandersman and Delepelaire 2004). Well known siderophores, include enterobactin (a catecholate) and ferrichrome (a hydroxamate) (Andrews and others 2003). An example of bacteria employing this iron acquisition system are Pseudomonad species (Poole and McKay 2003, Visca and others 2002), which are gram-negative aerobic bacteria. Pseudomonad species synthesize ferrichrome siderophores called pyoverdines or pseudobactins which are yellow-green, water-soluble, and fluorescent (Clarke and others 2001). These properties aid in the identification of the Pseudomonad genus. *Escherichia coli* can secrete a catecholate siderophore call "enterobactin" (Visca and others 2002).

Other bacterial species using siderophores for iron uptake are *Bacillus subtilis* (Ollinger et al. 2006), *Campylobacter coli*, *Erwinia chrysanthemi* (Franza and others 2005), *Vibrio* spp., and *Yersinia enterocolitica* (Clarke and others 2001).

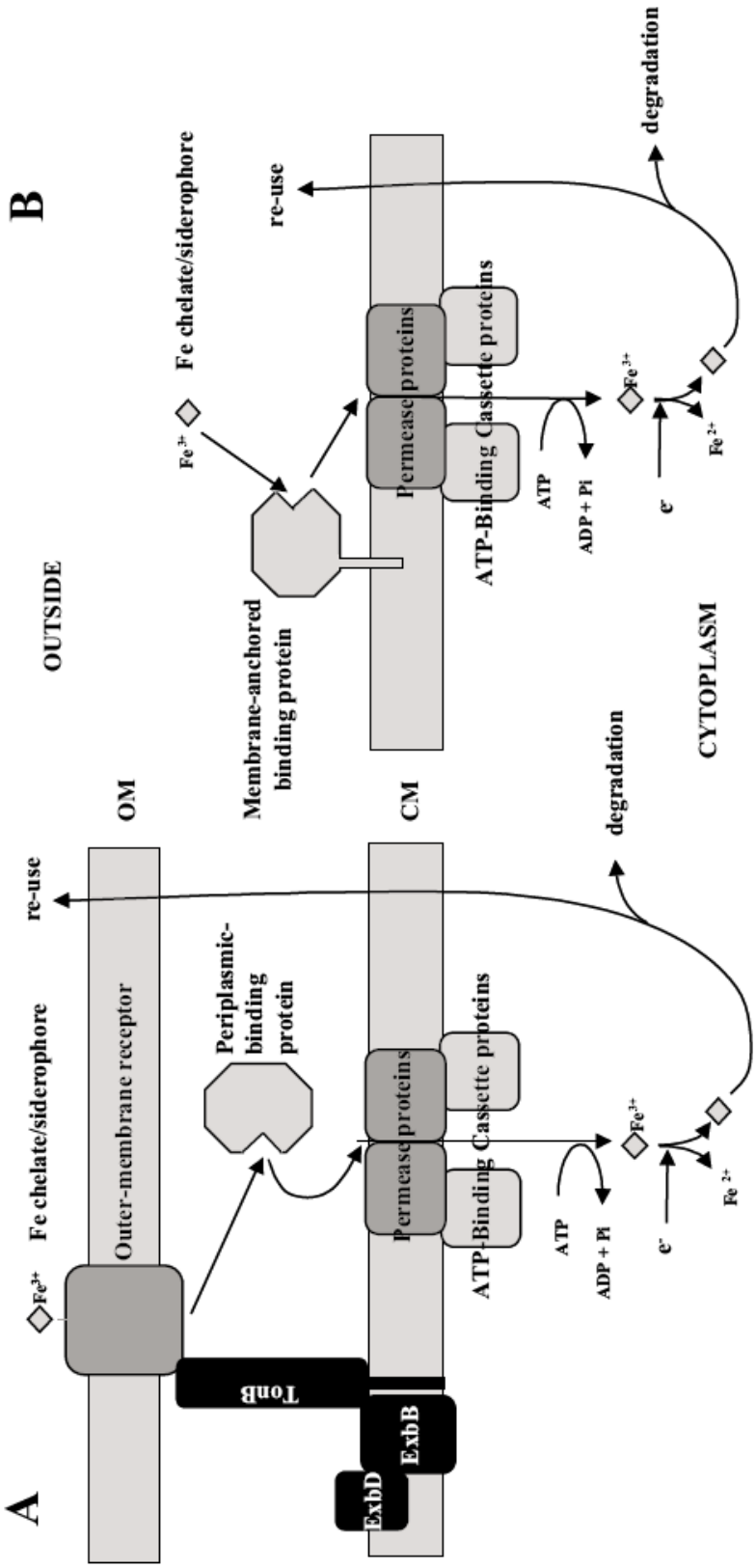

Figure 3. Schematic representation of siderophore-mediated iron uptake in gram-negative (A) and gram-positive (B) bacteria.

Source: Andrews and others (2003)

HYPOTHESES

Hypothesis 1: It is known that negatively charged phosphates of MM have high affinity for iron cations. Thus, I hypothesize that MM has growth inhibition effects on iron-dependent bacteria, and can be used as a growth inhibition agent in fresh ground beef.

Hypothesis 2: It is known that MM has more prolonged antioxidant effects than STP in fresh ground beef. Thus, I hypothesize that the reduced antioxidant effects of STP are due to its degradation by bacterial or endogenous meat phosphatases during storage.

OBJECTIVES

Objectives for hypothesis 1:

1. Evaluate the effects of MM to inhibit the growth of non-pathogenic iron-dependent bacteria strains, including *Listeria innocua* ATCC 33090, *Eschericia coli* DH5-α MCR, and *Pseudomonas fluorescen*s ATCC 948.
2. Determine the possible growth inhibition effects of MM against the mixed microflora of fresh ground beef (hamburger patties).

Objectives for hypothesis 2:

1. Compare the stability of 0.75 % MM and 0.5 % STP in growing cultures of *Pseudomonas fluorescen*s ATCC 948 and in ground beef patties at 2°C and 22°C.
2. Determine the extent of STP degradation due to microbial phosphatases, or endogenous meat phosphatases. Control studies will be done to determine STP degradation in microbial growth media or water after heat sterilization (121°C for 15 min).

CHAPTER 3

IRON-BINDING BY MILK MINERAL – A POSSIBLE GROWTH INHIBITION EFFECT ?

INTRODUCTION

The cleanliness of food products is a major concern for consumers. Many types of microorganisms are involved in food spoilage and foodborne illnesses, resulting in loss of quality and health problems. In order to minimize microbial growth, various methods of preservation are employed to maintain product quality and extend product shelf-life and safety. Anti-microbial additives are one of the methods applied for inhibiting spoilage and pathogenic microorganisms in many kinds of foods.

Numerous bacterial proteins are involved in microbial iron uptake and transport by different bacterial species. The major role of iron is its involvement in enzymatic redox reactions. Iron also plays a structural role in proteins and changes the reactivity of active site residues (Clarke and others 2001). Milk mineral (MM) is dried mineral fraction by-product from whey processing. Preliminary studies showed that MM has potential application to prolong red color stability and inhibit lipid oxidation in raw ground beef (Vissa and Cornforth 2006). Additional studies illustrated that MM is a type II antioxidant, which can chelate iron and prevent iron catalysis of lipid oxidation (Allen and Cornforth 2006). Negatively charged phosphates of MM have high affinity for iron cations (Allen and Cornforth 2007).

Reprinted from Tansawat R, Cornforth DP. 2009. Iron-binding by milk mineral -- A possible growth inhibition effect? J Food Sci (not yet submitted).

Since MM is able to tightly bind trace levels of iron, it may possibly have anti-microbial effects on iron-dependent bacteria.

Raw meats are sensitive to contamination and provide substrates for growth of microorganisms. *Pseudomonas spp.* is a type of bacteria commonly predominating in spoilage of fresh meat aerobically stored in a refrigerator or cooler. It causes putrefactive odors and slime when the numbers of cells exceed 10^7 /cm^2 (Ayres 1960; Gill 1983; Egan 1984; Egan and others 1988). *Pseudomonas spp.* usually employs chelating compounds called "siderophores" for acquisition of iron. Some studies also mentioned that *Pseudomonas aeruginosa* use the ferrous iron uptake system (Feo) to uptake iron (Clarke and others 2001).

Escherichia coli is a gram-negative, facultative, non-spore-forming bacteria. It is sometimes involved in foodborne illness, which causes gastrointestinal diseases in humans. The *E. coli* type of concern in meat product is O157:H7, which can cause hemorrhagic colitis, or bloody diarrhea, and hemolytic uremic syndrome (Doyle and Schoeni 1987). *E. coli* can secrete a catecholate siderophore call "enterobactin" to uptake iron (Visca and others 2002). Some studies also reported that *E. coli* has an iron (II) transport system (Feo) which is an important contribution to the iron supply of the cell under anaerobic conditions (Kammler and others 1993).

Listeria monocytogenes, a gram-positive, catalase-positive, non-spore-forming rod, is considered important to the safety of refrigerated muscle foods. It can grow at temperatures as low as 0°C (Miller and others 1990). Listeriosis is the bacteria infection caused by *Listeria monocytogenes*. Though rare, listeriosis is a fatal disease which is mostly found in pregnancy. *Listeria monocytogenes* can use ferritin as an iron source for its iron acquisition system (Adams and others 1990).

MATERIALS AND METHODS

Bacterial Strains

Non-pathogenic bacterial strains, including *Listeria innocua* ATCC 33090, *Eschericia coli* DH5-α MCR, and *Pseudomonas fluorescen*s ATCC 948 were used in this study. *Listeria innocua* ATCC 33090 and *Eschericia coli* DH5-α MCR strains were obtained from the Utah State University, Food Microbiology Lab, courtesy of Dr. Jeff Broadbent. *Pseudomonas fluorescens* ATCC 948 strain was obtained from the Center for Microbe Detection & Physiology (CMDP) at Utah State University, courtesy of Dr. Bart Weimer. Frozen (-80°C) stock cultures were grown in appropriate broth; using Brain Heart Infusion broth (BBL, Becton Dickinson Co., Sparks, MD) for *Listeria innocua* and Tryptic Soy broth (BBL, Becton Dickinson Co., Sparks, MD) for *E. coli* and *Pseudomonas fluorescens* (see media formula in Appendix A). Each strain was grown by transferring an aliquot (0.1 ml of stock culture) to 10 ml of broth, with incubation at 37°C for *Listeria innocua* and *E. coli*, and at 22°C for *Pseudomonas fluorescens* for 24 hour so that the cultures entered rapid growth (log phase growth). All strains were sub-cultured five times in broth before use in this experiment.

Inoculation Studies

Sterile 1.5 % w/v MM Trucal® D-7 (Glanbia®, Twin Falls, ID) in each broth was prepared by autoclave at 121°C for 20 min. A 9 ml broth was inoculated with 1 ml of log phase bacterial culture containing 10^2 - 10^3 CFU/ml. Serial dilutions were made at 10^{-1} - 10^{-9} cells per ml, with or without MM at 1.5 % w/v. Microbial aerobic plate counts were determined at 0, 6, 12, 24, and 48 hour, using aerobic plate count Petrifilm™ (3M Corporation, St. Paul, MN), in duplicate. The Petrifilm™ plates

were then incubated aerobically at 37°C for *Listeria innocua* and *E. coli*, and at 22°C for *Pseudomonas fluorescens*, for 48 hours. Colonies were counted with counts between 30 and 300 colonies were used to calculate the total colony forming units (CFU) for each treatment. Any strains for which MM exhibited an antimicrobial effect in nutrient broth were examined further at more than one levels of MM (0.75 % w/v and 0.5 % w/v).

Hamburger Pattie Preparation

Fresh ground beef was obtained from the Utah State University Meat Lab, courtesy of lab manager, Dick Whittier. Patties (100 g) of hamburger patties were made from ground beef, with and without MM at 0.5 %, 0.75 %, and 1.5 % w/w. Total plate counts were taken on hamburger patties samples at days 0, 1, 3, 5, 7 and 10 of storage at 2°C, based on AOAC method 990.12 (AOAC 1995). A 10 g of sample was weighed in sterile stomacher bag (17.8 x 30.5 cm, VWR Co., West Chester, PA) and mixed with 90 ml of sterile Butterfield's phosphate diluent. Butterfield's buffer was prepared by adding 34 g of potassium acid phosphate (KH_2PO_4) to ~500 ml distilled water, then adjusted to pH 7.1 with 1 N sodium hydroxide (NaOH), made up to 1 ml with distilled water and sterilized prior to use (FDA 1978). Each sample bag was placed in a stomacher (Seward 400, Golden Valley, MN) to be homogenized for 30 seconds on medium speed (230 rpm). The bag contents were serially diluted with sterile 0.85 % w/v sodium chloride solution and plated, using aerobic plate count Petrifilm™ (3M Co., St. Paul, MN) in duplicate. The Petrifilm™ plates were then incubated at 37°C for 48 hours. Colony counts were taken with counts between 30 and 300 colonies used to calculate the total CFU for each treatment.

Statistical Analysis

Microbiological counts for statistical significance were analyzed by Statistical Analysis Software (SAS) version 9.1 (SAS Institute, Inc., Cary, NC). The repeated measures design, using first order autoregressive covariance structure with the proc mixed function was used for analysis. Analysis of variance was used to identify statistically significant differences at the 95% confidence level. Comparison of the means was made based on p-values ($\alpha = 0.05$) using the Tukey-Kramer adjustment to obtain differences of least means squares.

RESULTS

Effect of 1.5% milk mineral (MM) on bacterial growth in liquid media

MM at 1.5 % w/v was not inhibitory ($P < 0.05$) to growth of *Listeria innocua* or *Eschericia coli* DH5-α during 48 hour incubation at 37°C (P-value = 0.21 and 0.50, respectively; Figure 4 and 5).

Microbial load of *Pseudomonas fluorescens* was significantly inhibited by 1.5 % w/v MM treatment ($P < 0.05$) during 48 hour incubation at 22°C (Figure 6). Significant inhibition occurred at 48 hour of incubation. *Pseudomonas* growth were about 1.0 log less than controls after 48 hours incubation with CFU/ml of 2.79×10^8 for 1.5 % MM treatment and 1.79×10^9 for control (see Appendix Table B4). However, the preservative effects of 1.5 % MM in media were only inhibitory after bacterial numbers were already high ($> 10^7$).

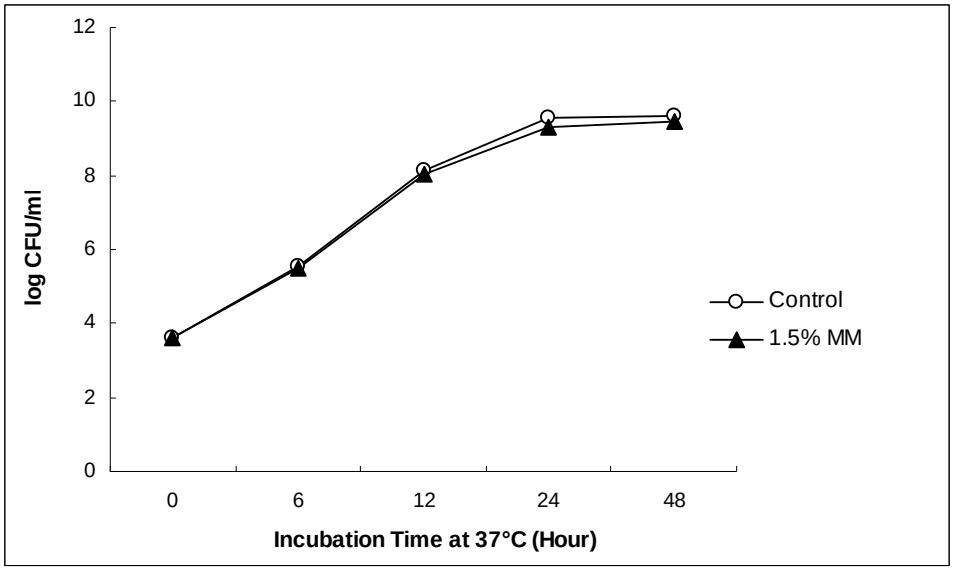

Figure 4. Growth of *Listeria innocua* in brain heart infusion (BHI) media, incubated for 48 hours at 37°C, with and without 1.5 % milk mineral (MM) treatment. (Note that culture tubes were autoclaved after addition of MM to media.)

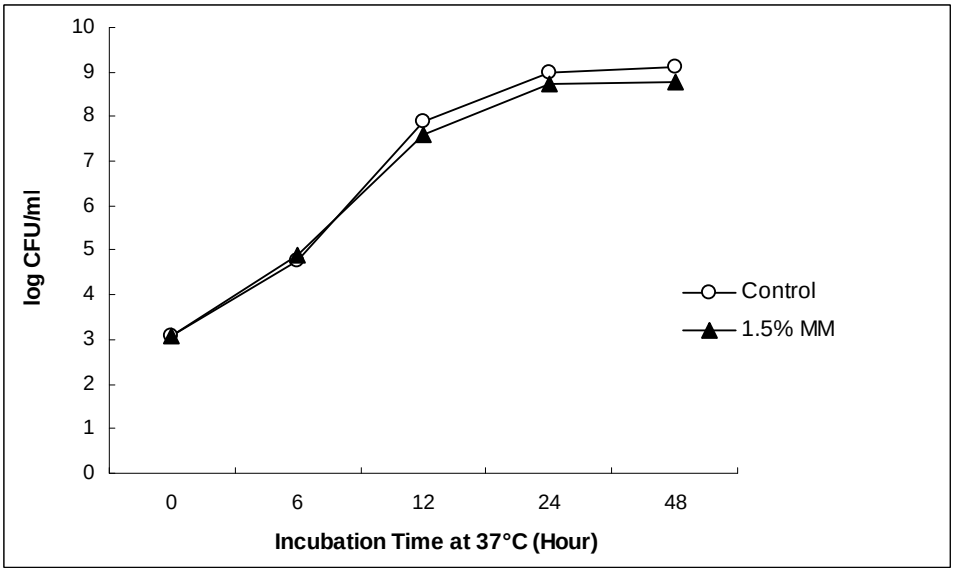

Figure 5. Growth of *Eschericia coli* DH5-α in tryptic soy broth (TSB) media, incubated for 48 hours at 37°C, with and without 1.5 % milk mineral (MM) treatment. (Note that culture tubes were autoclaved after addition of MM in media.

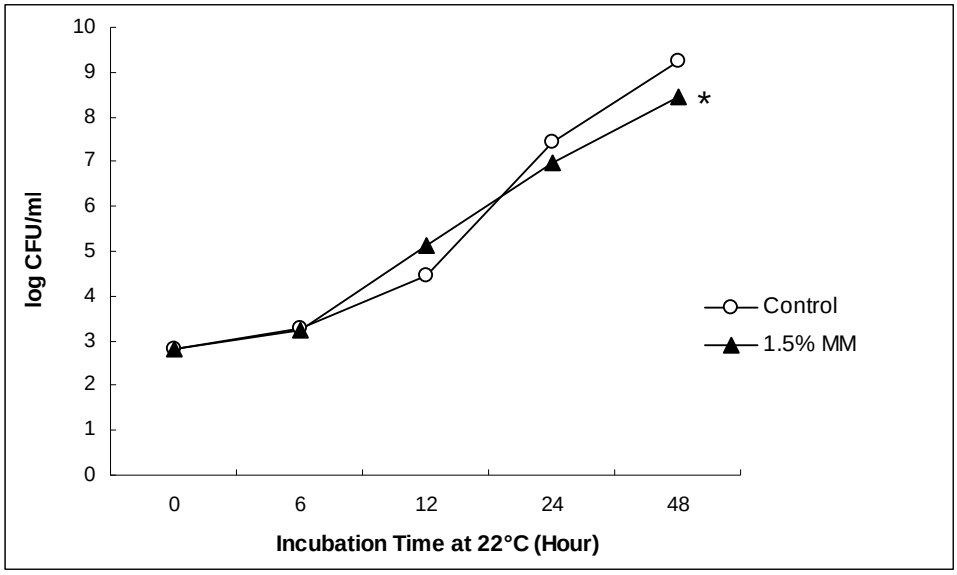

Figure 6. Growth of *Pseudomonas fluorescens* in tryptic soy broth (TSB) media, incubated for 48 hours at 22°C, with and without 1.5 % milk mineral (MM) treatment. * Indicates the first time when growth of 1.5 % MM treatment was significantly lower than control treatment. (Note that culture tubes were autoclaved after addition of MM in media.)

Growth of *Pseudomonas fluorescens* as affected by milk mineral level (0.5%, 0.75%, and 1.5% MM)

Compared to control without MM, all levels of MM treatment significantly ($P = 0.0022$) reduced growth of *Pseudomonas fluorescens* after 48 hours incubation. However, no significant inhibitory effects of MM were observed after 6-12 hours incubation. At 48 hour of incubation, CFU/ml levels were 2.30×10^8, 2.05×10^8, 2.65×10^8, and 2.55×10^9 for 0.5 %, 0.75 %, 1.5 % MM, and control, respectively (Figure 7, see Appendix Table B7).

Total aerobic plate count (APC) of hamburger patties as affected by milk mineral level (0.5%, 0.75%, and 1.5% MM)

Total APC of hamburger patties (20 % fat) was not affected by MM treatment, during storage for up to 10 days at 2°C (Figure 8).

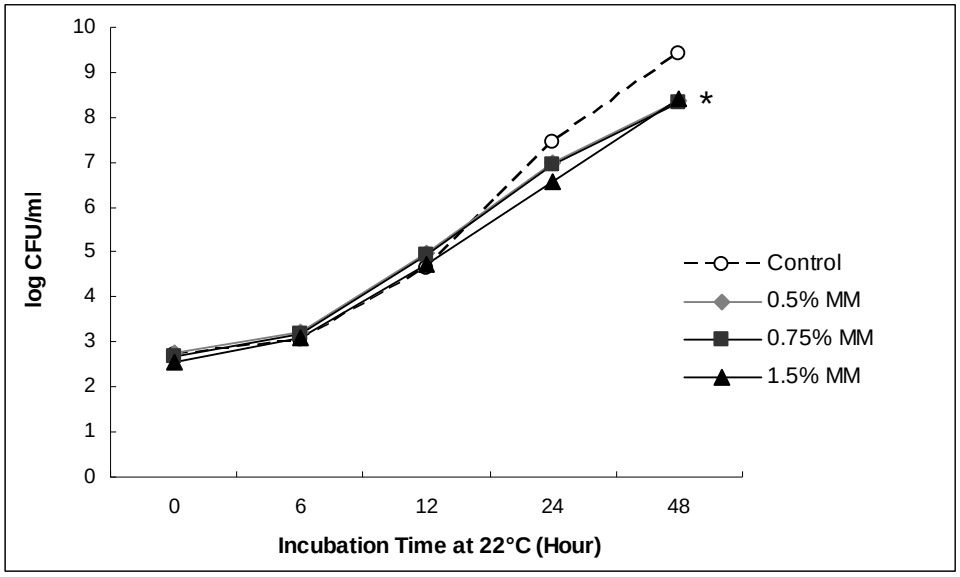

Figure 7. Growth of *Pseudomonas fluorescens* in tryptic soy broth (TSB) media, incubated for 48 hours at 22°C. * Indicates the first time when growth of milk mineral (MM) treatment was significantly lower than control treatment. (Note that culture tubes were autoclaved after addition of MM in media.)

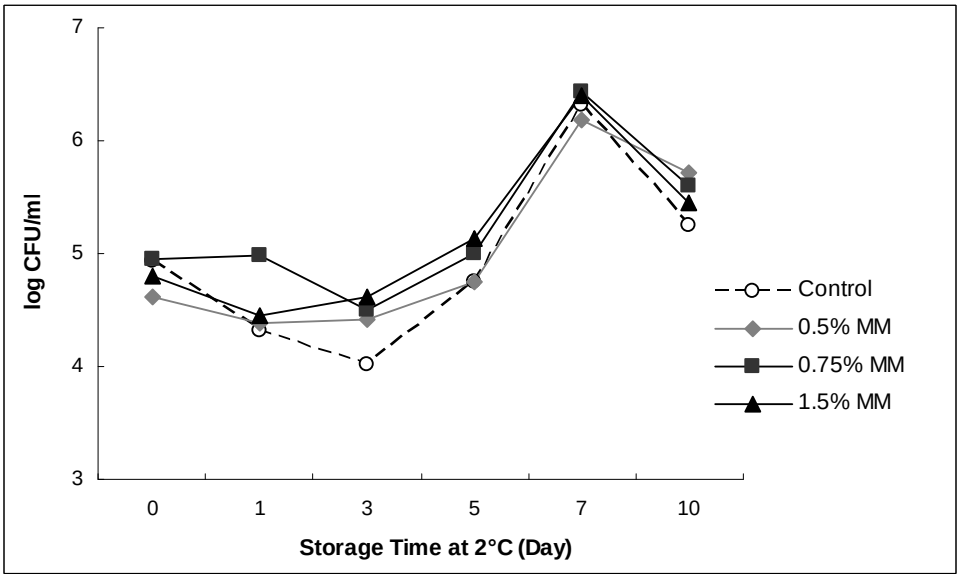

Figure 8. Effect of 0.5 %, 0.75 %, and 1.5 % milk mineral on aerobic plate count of ground beef patties (20 % fat) during storage in PVC film wrap for 10 days.

DISCUSSION

In this study, 1.5 % w/v MM did not significantly inhibit growth of *Listeria innocua* ATCC 33090 in BHI and *Eschericia coli* DH5-α MCR in TSB incubated at 37°C for up to 48 hours. Growth of *Pseudomonas fluorescens* ATCC 948 was not significantly inhibited by MM over the range of 10^3 to 10^7 CFU/ml, but there was a slight (~1 log CFU/ml) reduction of *Pseudomonas fluorescens* at growth over 10^8 CFU/ml in TSB at 22°C incubation for 48 hours. However, there were no statistically significantly different among MM treatment levels (0.5 %, 0.75 %, and 1.5 % w/v) in the reduction of growth of *Pseudomonas fluorescens*. In other words, 0.5 % MM was equally as effective as 1.5 % MM to inhibit growth of *Pseudomonas fluorescens* after 2 days storage. However, all levels of MM (0.5 %, 0.75 %, and 1.5 % w/w) had no anti-microbial effects against the mixed microflora of fresh ground beef (hamburger patties, 20 % fat) during storage for up to 10 days at 2°C.

Bacteria used in this study require iron for growth. An explanation for the lack of inhibition of bacterial growth by MM is that MM binds only soluble ionic iron (ferrous or ferric). Iron bound to soluble compounds such as amino acids, small peptides, or heme may be available to support bacterial growth, but would not be bound to MM. The strong affinity of MM to ionic iron can inhibit lipid oxidation, but not inhibit bacterial growth supported by heme or amino acid + iron complexes. Another possible explanation is that the high heat treatment of autoclave sterilization (121°C for 20 min.) used to sterile the broth with MM before inoculation studies possibly resulted in MM degradation and loss of iron-chelation ability, and thus, the loss of anti-microbial effects.

CONCLUSION

MM had little or no anti-microbial effects. The strong affinity of MM to ionic iron can inhibit lipid oxidation, but not inhibit bacterial growth supported by other forms of iron (heme or amino acid + iron complexes).

CHAPTER 4

DECOMPOSITION OF MILK MINERAL AND SODIUM TRIPOLYPHOSPHATE AS AFFECTED BY BACTERIAL GROWTH IN MEDIA OR GROUND BEEF

INTRODUCTION

Milk mineral (MM), consisting primarily of colloidal calcium phosphate particles, is a dried ultra-filtered from whey. MM has powerful antioxidant properties in cooked ground meats and prevents browning in raw ground beef (also an oxidation process) by the mechanism that MM has negatively charged phosphates which have high affinity for iron cations and other trace minerals. Iron-binding by MM prevents iron catalysis of lipid oxidation.

Sodium tripolyphisphate (STP) or $Na_5P_3O_{10}$, is the sodium salt of triose polyphosphate. STP is approved to use in meat products (Molins 1987). STP solution (0.3 - 0.5 %) is commonly injected into pork chops, fresh pork roasts, fresh beef steaks and beef roasts to enhance water retention, juiciness and cooked yield after cooking, and to inhibit lipid oxidation during storage and retail display (Shults and others 1976; Trout and Schmidt 1984; Molins and others 1987; Anjaneyulu 1989; Li and others. 1993; Moiseev and Cornforth 1997; Lee and others 1998).

Milk mineral (MM) and sodium tripolyphisphate (STP) are both type II metal chelating oxidants. However, the previous studies usually reported that MM has more powerful antioxidant properties greater than STP. Lipid oxidation in ground beef

Reprinted from Tansawat R, Cornforth DP. 2009. Decomposition of milk mineral and sodium tripolyphosphate as affected by bacterial growth in media or ground beef. J Food Sci (not yet submitted).

packing in 80 % oxygen atmosphere and stored at 2°C was inhibited and red color was maintained for 14 day by addition 0.75 % MM which significantly ($P < 0.05$) more effective than 0.5 % STP to lower of thiobarbituric acid (TBA) values and maintain higher of redness values (Vissa and Cornforth 2006). Moreover, the recent studies also indicated that MM can bind more iron per gram than STP (Allen and Cornforth 2007).

Tripolyphosphate can revert to orthophosphates (MERCK Index 1976) by hydrolysis reaction caused by many factors; i.e. enzyme activities, high temperatures, or even a result of bacterial metabolism (Molin 1990). Hydrolysis of polyphosphate finally results in soluble orthophosphate (P*i*) which could use as a parameter to determine the stability of polyphosphates (Li and others 1993).

MATERIALS AND METHODS

Experimental Design and Statistics

Part 1 - Comparison of stability of milk mineral (MM) and sodium tripolyphosphate (STP) in ground beef patties at 2°C and 22°C. This experiment was a factorial design comparing 3 ground beef formulations (control without added polyphosphates, 0.5 % STP, 0.75 % MM) stored at 2 temperatures (2°C and 22°C) with repeated measures for aerobic plate count (APC; in duplicate) and inorganic phosphate (P*i*; in triplicate) at 0, 1, 2 days. Main effect mean values were calculated and compared by ANOVA using Statistical Analysis Software (SAS) version 9.1 (SAS Institute, Inc., Cary, NC). The repeated measures design, using first order autoregressive covariance structure was used for analysis of microbiological counts and soluble orthophosphate values where the measurements were made on each treatment over time. The factorial design was used for orthophosphate analysis

experiment 1). Proc mixed function was used for all designs. Analysis of variance was used to identify statistically significant differences at the 95% confidence level. Comparison of the means was made based on p-values ($\alpha = 0.05$) using the Tukey-Kramer adjustment to obtain differences of least means squares. An analysis of data for correlation between inorganic phosphate levels and microbial plate count was done by using STATISTICA (Statsoft Inc., Tulsa, OK).

Part 2 - Comparison of stability of milk mineral (MM) and sodium tripolyphosphate (STP) in growing cultures of *Pseudomonas fluorescens* incubated at 2°C and 22°C. This experiment was a factorial design comparing 3 media formulations in tryptic soy broth as followed; control TSB without added polyphosphate, TSB + 0.5 % STP, TSB + 0.75 % MM). Samples were incubated at 2 temperatures (2°C and 22°C) with repeated measures for APC (in duplicate) and P*i* (in triplicate) at 0, 1, 2 days. A control experiment was also done to evaluate the effect of autoclave treatment in TSB broth or distilled water (DW) on decomposition of STP or MM before addition of *Pseudomonas fluorescens* cultures. Statistics were done as described in Part 1.

Sample Preparation and Storage

Fresh boneless beef meat was obtained from the Utah State University Meat Lab, courtesy of lab manager, Dick Whittier, and stored at 2°C until the treatments were applied. Beef (≈ 1.5 kg) was passed once through a 3 mm diameter pore size grinder plate (Model 4152, Hobart Mfg Co., Troy, OH). Ground beef was divided into appropriate amounts (≈ 500 g each) for preparation of the three treatments as follows:

1) Fresh ground beef (used as a control).

2) Fresh ground beef with 0.5 %w/w STP added.

3) Fresh ground beef with 0.75 %w/w MM powder (Glanbia®, Twin Falls, ID) added.

For STP treatment, ≈ 500 kg ground beef was manually mixed with 0.5 % w/w STP. An STP solution was prepared by adding 2.5 g STP granular (Fisher Scientific Co., Pittsburgh, PA) into 20 ml distilled water and then heated to about 80°C. For a MM treatment, ground beef was mixed with 0.75 % w/w MM. The MM powder (3.75 g/ 500 g meat) was sprinkled and manually mixed until homogeneous. Twelve 100-g patties of each treatment were formed with a patty mold (12 cm in diameter, 113 g capacity). Each patty was placed in a Styrofoam tray and over-wrapped with polyvinyl chloride (PVC) film. Each treatment was divided into two groups of six patties and stored at 2°C and 22°C. Orthophosphate analysis and aerobic plate count were taken at day 0, 1, and 2.

Bacterial Strain

Non-pathogenic *Pseudomonas fluorescen*s strain ATCC 948 was used in this study, obtained from the Center for Microbe Detection & Physiology (CMDP) at Utah State University, courtesy of Dr. Bart Weimer. Frozen (-80°C) stock cultures were grown in tryptic soy broth (TSB) (BBL, Becton Dickinson Co., Sparks, MD) by transferring 1 % (0.1 ml of stock culture to 10 ml of broth) and then incubated at 22°C. Cultures were transferred (0.1 ml to 10 ml of broth) to fresh broth every 24 hour until the culture was in rapid growth (log) phase before using in this experiment.

Orthophosphate Analysis

Soluble orthophosphate was determined by a modification of EPA method 365.3 (Miller 1996), approved for both drinking water and wastes. Soluble orthophosphate of the three experiments were analyzed including:

- Part 1 - Soluble orthophosphates in hamburger patties, patties with 0.5 % w/w STP, and patties with 0.75 % w/w MM. Each treatment was divided into two groups and stored at either 2°C or 22°C.
- Part 2 - Soluble orthophosphates in sterile TSB, sterile TSB with 0.5 % w/v STP, and sterile TSB with 0.75 % w/v MM. Each treatment was divided into two groups and incubated at either 2°C or 22°C.
- Control experiment (for part 2) - Soluble orthophosphates in TSB or DW, TSB or DW with 0.5 % w/v STP, and TSB or DW 0.75 % w/v MM, before addition of *Pseudomonas fluorescens* cultures, with or without sterilization in autoclave at 121°C for 20 min.

For part 1, ground beef (10 g) was sampled from each patty. A 10 g sample was weighed in a stomacher bag (17.8 x 30.5 cm, VWR Co., West Chester, PA) and then homogenized in 90 ml of distilled water. Each sample bag was placed in a stomacher (Seward 400, Golden Valley, MN) and homogenized for 30 seconds on medium speed (230 rpm). For part 2 and control experiments, 1 ml of each sample was added into 9 ml distilled water and then mixed thoroughly by vortex.

Each mixture then was diluted again to obtain soluble orthophosphate concentration between 0 to 5.0 µg/g sample, which was the concentration range of the standard solutions. A 10 µg/ml of potassium phosphate (KH_2PO_4) in distilled water was used as a stock solution of orthophosphate standard solutions. Phosphate standard solutions at concentration of 0.25, 0.5, 1.0, 2.5, and 5.0 µg/ml were prepared.

An aliquot (1 ml) from each of the diluted mixture and standard solution was transferred into a clean test tube. 0.2 ml of 1.1 N H_2SO_4 was added to each tube, followed by 0.1 ml of color reagent (1.6 % w/v ammonium molybdate combined with 0.04 % w/v potassium antimony tartrate in distilled water). An ascorbic acid solution

(4 ml of 0.2 % w/v) was then added to each tube. Each tube was mixed thoroughly and allowed to sit at least 10 minutes to develop the blue color. The absorbance was read at 650 nm. The concentration of orthophosphate was calculated using the standard curve prepared from KH_2PO_4 and multiplied by the dilution factor (usually 1000). Soluble orthophosphates were reported in mg P*i*/g patty or mg P*i*/ml TSB.

For part 1, soluble orthophosphate was analyzed the day of manufacture (day 0), the first and the second day of storage (day 1 and day 2). Note that on day 0 the same batch of hamburger patties was used for analysis (samples were analyzed prior to storage). For part 2, soluble orthophosphate was analyzed on day 0, 1, and 2. Each treatment was analyzed in triplicate.

Microbial Studies

Aerobic plate count (APC) was determined for two experiments including:

- Part 1 - Hamburger patties, patties with 0.5 % w/w STP, and patties with 0.75 % w/w MM. Each treatment was divided into two groups and stored at two different temperatures; 2°C and 22°C.
- Part 2 - TSB, TSB with 0.5 % w/v STP, and TSB with 0.75% w/v MM. After autoclaving, all the treatments were inoculated with *Pseudomonas fluorescen*s ATCC 948 and then divided into two groups and stored at 2°C and 22°C.

For part 1, a 10 g of sample was weighed in sterile stomacher bag (17.8 x 30.5 cm, VWR Co., West Chester, PA) and mixed with 90 ml of sterile 0.85 % w/v sodium chloride solution. Each sample bag was placed in a stomacher (Seward 400, Golden Valley, MN) and homogenized for 30 seconds on medium speed (230 rpm). The bag contents were serially diluted with sterile 0.85 % w/v sodium chloride solution and plated, using aerobic plate count Petrifilm^{TM} in duplicate. The Petrifilm^{TM} plates were

then incubated at 37°C for 48 hours. Microbial plate counts were determined at the manufacturing day (day 0), day 1, and day 2 of storage.

For part 2, sterile TSB, TSB with 0.5 %w/v STP, and TSB with 0.75 %w/v MM were prepared. After autoclaving, each treatment then was inoculated with *Pseudomonas fluorescen*s ATCC 948. Serial dilutions were made at 10^{-1} - 10^{-9} cells per ml in each treatment. Serial dilutions were plated, using aerobic plate count Petrifilm™ in duplicate. The Petrifilm™ plates were then incubated at 22°C for 48 hours. Microbial plate counts were determined at day 0, 1, and 2 of incubation.

Colony counts were taken with counts between 30 and 300 colonies used to calculate the total colony forming units (CFU) for each treatment.

RESULTS

Part 1

Comparison of stability of milk mineral (MM) and sodium tripolyphosphate (STP) in ground beef patties at 2°C and 22°C

Treatment Effects on Aerobic Plate Count in Ground Beef Patties

Bacterial APC of beef patties increased significantly for all treatments (control, 0.5 % STP, and 0.75 % MM) during 2 day storage at 22°C, but no significant change in APC was observed for patties held at 2°C (Figure 9). The MM treatment was significantly higher than initial after 24 hour (indicated by *).

Treatment Effects on Orthophosphate Levels in Ground Beef Patties

Soluble orthophosphates (P*i*) were all significantly different among control, 0.5 % STP, and 0.75 % MM treatments. For control at 2°C without added phosphate, soluble orthophosphates did not increase during storage. The P*i* level was about 1

µg/ml (bottom line, Figure 10). However, P*i* levels in control increased ($P < 0.05$) to about 2 µg/ml when patties were held at 22°C for 2 days. P*i* levels similarly increased in MM or STP treatments at 22°C, compared to 2°C. P*i* levels were significantly higher in patties with added STP at 22°C, compared to MM patties. P*i* for MM samples were not significantly higher than initial (day 0) until after 48 hours storage at 22°C. Thus, MM was significantly more stable than STP in beef patties held at 22°C.

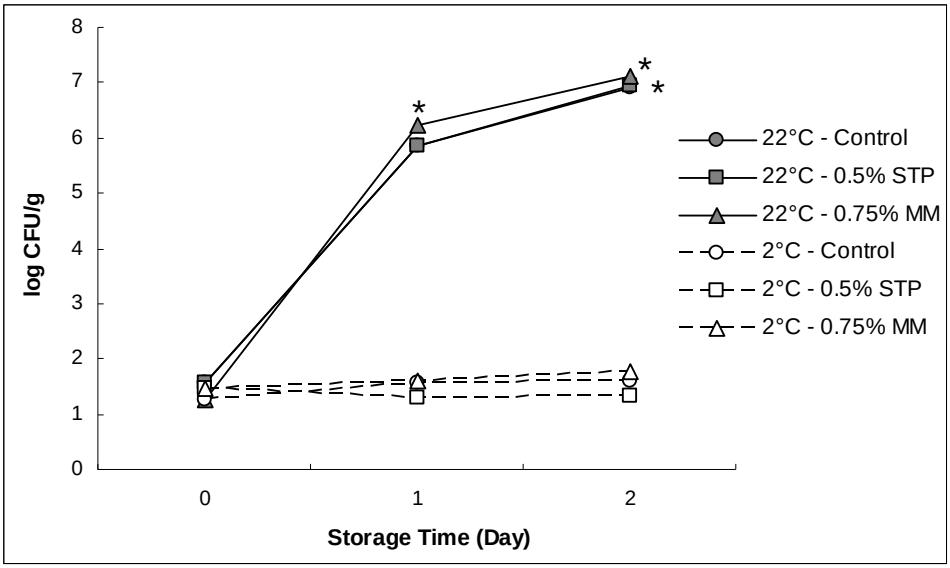

Figure 9. Growth of aerobic bacteria at 2°C and 22°C for up to 2 days in patties formulated without added phosphate (control), 0.5 % sodium tripolyphosphate (STP), or 0.75 % milk mineral (MM). * Indicates that bacterial numbers were significantly higher than original inoculation levels ($P < 0.05$).

Correlation between aerobic plate count and orthophosphates data of ground beef patties

There was a high correlation (r = 0.77) between bacterial APC and P*i* levels of patties held at 22°C for 2 days (Table 4). The correlation coefficient was lower (r = 0.63), but still significant ($P < 0.005$) between APC and P*i*, when data was pooled for

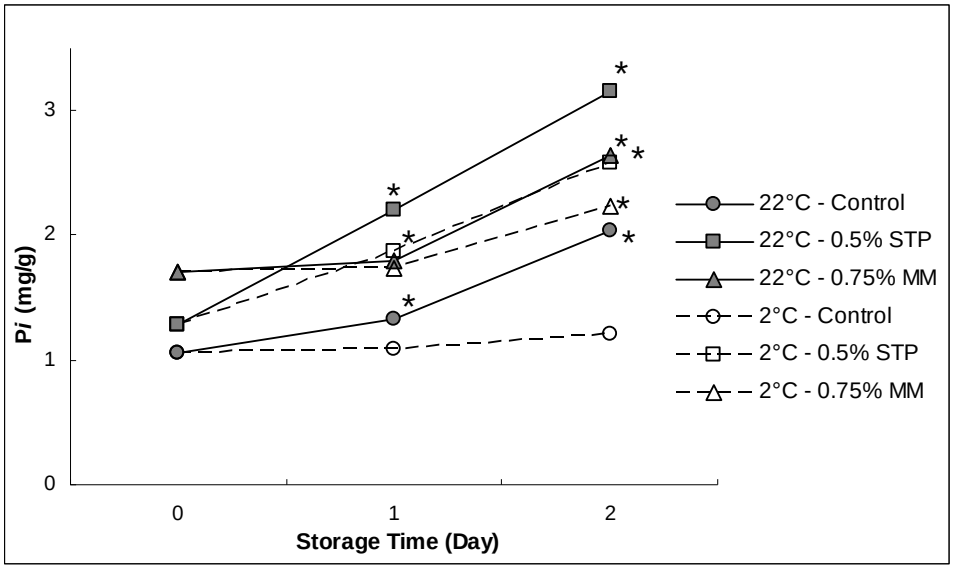

Figure 10. Soluble orthophosphate (P*i*) levels in ground beef patties formulated without added phosphate (control), 0.5 % sodium tripolyphosphate (STP), or 0.75 % milk mineral (MM) and stored at 2°C and 22°C for up to 2 days. * Indicates that orthophosphate levels were significantly different than initial (day 0; $P < 0.05$).

Table 3. Percent conversion of polyphosphates in sodium tripolyphosphate (STP) or milk mineral (MM) to inorganic monophosphate (P*i*) after addition to ground beef patties and storage for 2 days at 22°C or 2°C.

Temperature (°C)	Treatments *	P*i* as % of added polyphosphate **	
		Day 0	Day 2
22°C	STP (0.5 %)	5.9 %	29.0 %
	MM (0.75 %)	21.7 %	20.3 %
2°C	STP (0.5 %)	5.9 %	35.1 %
	MM (0.75 %)	21.7 %	34.0 %

* STP and MM were added at 0.5 % and 0.75 % of meat weight, respectively. [Calculation for STP: (285 g polyphosphates MW / 367.93 total STP MW) x 100 = 77.5 % P*i*, i.e. STP is 77.5 % by weight polyphosphates. Calculation for MM: (13 g phosphorous / 100 g MM) x (100 g P*i* / 32.6 g polyphosphates) x 100 = 39.9 % P*i*, i.e. MM is 39.9 % by weight polyphosphates.]

** % Values are calculated as measured P*i* concentration (mg P*i* /g meat on a given day / total possible P*i* if added polyphosphates are completely hydrolyzed. For 0.5 % STP, 100 % hydrolysis would yield (0.05 g STP / 100 g sample) x 77.5 % P*i* = 3.9 mg P*i*/g sample. For 0.75 % MM: 100 % hydrolysis would similarly yield (0.075 g MM / 100 g sample) x 39.9 % P*i* = 3.0 mg P*i*/g sample.

both 2°C and 22°C (Table 5). However, there was no significant correlation between APC and P*i* for patties held at 2°C, because neither APC nor P*i* increased during 2 days holding time (Table 6).

Table 4. Correlation (r) between aerobic plate count (APC) and orthophosphates (P*i*) of ground beef patties with storage at 22°C for up to 2 days.

Correlation	P*i*	APC
P*i*	1.0000 *P* < 0.000 (N = 9)	0.7704 *P* < 0.015 (N = 9)
APC	0.7704 *P* < 0.015 (N = 9)	1.0000 *P* < 0.000 (N = 9)

Table 5. Correlation (r) between aerobic plate count (APC) and orthophosphates (P*i*) of ground beef patties (data pooled for 2°C and 22°C for up to 2 days storage).

Correlation	P*i*	APC
P*i*	1.0000 *P* < 0.000 (N = 18)	0.6344 *P* < 0.005 (N = 18)
APC	0.6344 *P* < 0.005 (N = 18)	1.0000 *P* < 0.000 (N = 18)

Table 6. Correlation (r) between aerobic plate count (APC) and orthophosphates (Pi) of ground beef patties with storage at 2°C for up to 2 days.

Correlation	P*i*	APC
P*i*	1.0000 *P* < 0.000 (N = 9)	0.1175 *P* < 0.763 (N = 9)
APC	0.1175 *P* < 0.763 (N = 9)	1.0000 *P* < 0.000 (N = 9)

Part 2

Comparison of stability of milk mineral (MM) and sodium tripolyphosphate (STP) in growing cultures of *Pseudomonas fluorescens* incubated at 2°C and 22°C

Treatment Effects on Growth of *Pseudomonas fluorescens*

Compared to initial (day 0), bacterial numbers were significantly higher for control treatment at 22°C at after 1 or 2 day incubation (Figure 11). Bacterial numbers for 0.5 % STP treatment at 22°C were first significantly higher than initial at day 2. Bacterial numbers for 0.75 % MM treatment at 22°C were not significantly higher than initial until day 2. For all treatments, bacterial APC did not increase during 2 days storage at 2°C.

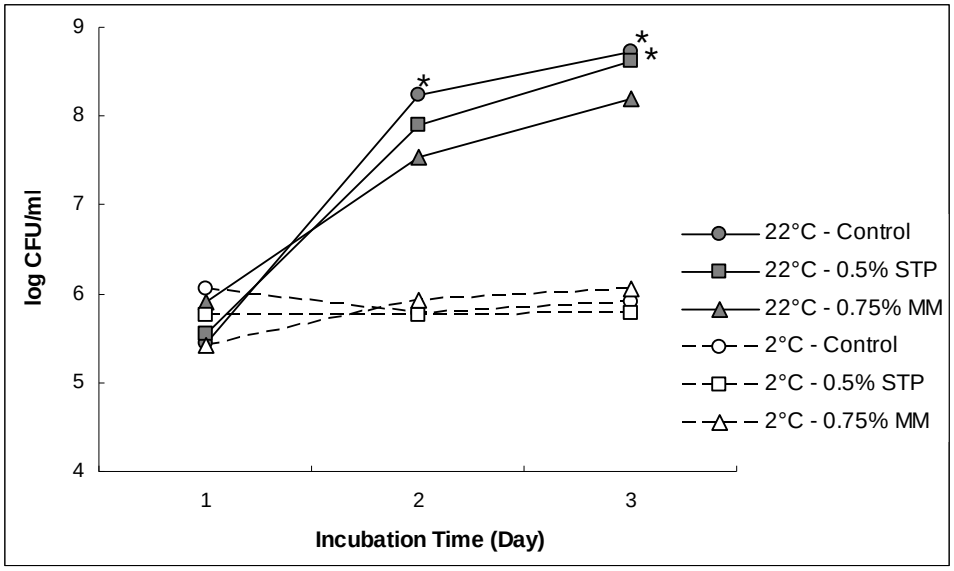

Figure 11. Growth of *Pseudomonas fluorescens* in autoclaved tryptic soy broth (TSB) media without added phosphate (control), 0.5 % sodium tripolyphosphate (STP), or 0.75 % milk mineral (MM) and incubated for up to 2 days at 2°C and 22°C.
* Indicates that bacterial numbers were significantly higher than original inoculation levels ($P < 0.05$).

Treatment Effects on Orthophosphate Levels in TSB Inoculated with *Pseudomonas fluorescens*

Soluble phosphate (Pi) levels at day 0 were significantly different ($P < 0.05$) among control, 0.5 % STP, and 0.75 % MM treatments but neither incubation treatment (2°C or 22°C) or storage time (1 - 2 days) affected Pi levels (Figure 12).

Effect of Autoclave Sterilization of Tryptic Soy Broth (TSB) on Stability of Added Sodium Tripolyphosphate (0.5%) or Milk Mineral (0.75%)

There was no detectable orthophosphate (P*i*) in TSB and TSB with 0.5% STP which was not sterilized by autoclave (121°C for 20 min; Table 8). However, there were some soluble P*i* in MM in TSB (1.43 mg/ml) without heat treatment. After sterilization, there was 0.08 mg/ml P*i* in TSB, but much more P*i* was measured when

either 0.5 % STP or 0.75 % MM were added (3.79 and 4.02 mg/ml, respectively).

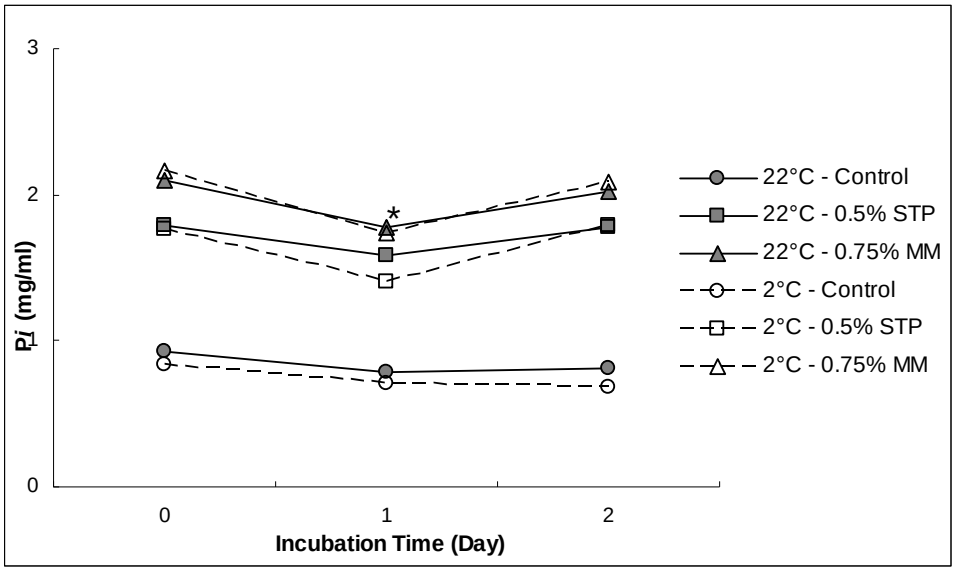

Figure 12. Soluble orthophosphates (P*i*) levels in autoclaved tryptic soy broth (TSB) media without added phosphate (control), 0.5 % sodium tripolyphosphate (STP), or 0.75 % milk mineral (MM), inoculated with *Pseudomonas fluorescens*, and incubated at 2°C and 22°C for 0 - 2 days. * Indicates that orthophosphate levels were significantly different than on initial (day 0) ($P < 0.05$).

Table 7. Percent conversion of polyphosphates in sodium tripolyphosphate (STP) or milk mineral (MM) to inorganic monophosphate (P*i*) after addition to trytic soy broth (TSB), then autoclaved (121°C, 20 min.). The media was then inoculated with *Pseudomonas fluorescens* and incubated at 22°C or 2°C for 2 days.

Temperature (°C)	Treatments *	P*i* as % of added polyphosphate **	
		Day 0	Day 2
22°C	STP (0.5 %)	22.1 %	24.9 %
	MM (0.75 %)	39.3 %	40.3 %
2°C	STP (0.5 %)	23.6 %	28.2 %
	MM (0.75 %)	44.0 %	46.7 %

* , ** Same as Table 3.

There were some soluble P*i* in 0.75 % MM in DW without heat treatment, and it was significant increased ($P < 0.05$) with sterilization by autoclave. Nevertheless, there was no detectable P*i* value for 0.5 % STP in DW in both sterilized and non-sterilized treatments (Table 9). Thus, STP was not degraded by autoclaving in water, but STP was significantly degraded when autoclaved with TSB.

Table 8. Soluble orthophosphates (P*i*) levels in tryptic soy broth (TSB), TSB with 0.5 % sodium tripolyphosphate (STP), and TSB with 0.75 % milk mineral (MM), with and without sterilization by autoclave at 121°C for 20 min. Note that treatments sharing the same superscript letter are not significantly different at $P > 0.05$.

Temperature	Treatments	P*i* (mg/ml)
Room temperature (22°C)	TSB	0.00 a
	TSB + 0.5%STP	0.00 a
	TSB + 0.75%MM	1.43 b
Sterile temperature (121°C, 20 min.)	TSB	0.08 a
	TSB + 0.5%STP	3.79 c
	TSB + 0.75%MM	4.02 d

Table 9. Soluble orthophosphates (P*i*) levels in distilled water (DW), DW with 0.5 % sodium tripolyphosphate (STP), and DW with 0.75 % milk mineral (MM), with and without sterilization by autoclave at 121°C for 20 min. Note that treatments sharing the same superscript letter are not significantly different at $P > 0.05$.

Temperature	Treatments	P*i* (mg/ml)
Room temperature (22°C)	DW	0.00 a
	DW + 0.5%STP	0.00 a
	DW + 0.75%MM	1.65 b
Sterile Temperature (121°C, 20 min.)	DW	0.00 a
	DW + 0.5%STP	0.00 a
	DW + 0.75%MM	2.89 c

Correlation between aerobic plate count and orthophosphates data of *Pseudomonas fluorescens* inculation data

There was no correlation between APC and P*i* for inculation data of *Pseudomonas fluorescens* in autoclaved TSB media without added phosphate (control), TSB with 0.5 % STP or with 0.75 % MM, with r = -0.18 (Table 10) because P*i* levels were not affected by storage time or treatment (MM or STP), or probably because all MM or STP was already degraded by autoclaving in TSB.

Table 10. Correlation (r) between aerobic plate count (APC) and orthophosphates (Pi) of *Pseudomonas fluorescens* inculated into tryptic soy broth (TSB), data pooled for both 2°C and 22°C for up to 2 days storage.

Correlation	P*i*	APC
P*i*	1.0000 *P* < 0.000 (N = 18)	- 0.1784 *P* < 0.0479 (N = 18)
APC	- 0.1784 *P* < 0.0479 (N = 18)	1.0000 *P* < 0.000 (N = 18)

DISCUSSION

As the name implies, STP is a triose polyphosphate. Milk mineral is a colloidal calcium phosphate; i.e., an amorphous, insoluble network of - Ca^{++} and - PO_4^- - (Ca^{++} - PO_4^- -). In this experiment, degradation of STP or MM was measured as an increase in soluble inorganic mono-phosphate; denoted as "P*i*".

The experiment in Part 1 was to compare the stability of STP and MM in ground beef patties, by monitoring the increase of inorganic phosphate (P*i*) during storage at 2 °C or 22°C. After 0 to 48 hr at 22°C, patties formulated with 0.5 % STP

had 1.28 - 3.16 mg P*i*/g (Figure 10; see Appendix Table C3). This corresponds to 5.9 % - 29.0 % of STP polyphosphates being hydrolyzed to monophosphate (P*i*; Table 3). Similar treatments of patties with 0.75 % MM had 1.70 - 2.64 mg P*i*/g which is equivalent to 21.7% - 20.3 % conversion of MM polyphosphates to P*i* (Table 3) after 0 to 48 hr at 22°C. Thus, release of P*i* was significantly higher at 22°C for STP than for MM added to ground beef patties. At 2°C, MM was again more stable (less P*i* release), with P*i* values of 1.28 - 2.58 and 1.70 - 2.23 mg P*i*/g for STP and MM samples, respectively (Figure 10). These correspond to 5.9 % - 35.1 % and 21.7 % - 34.0 % of STP and MM polyphosphates being hydrolyzed to monophosphate, respectively (Table 3). These results indicate that the colloidal calcium phosphate matrix of MM was more stable than STP in ground beef.

The experiment in Part 2 was to examine the stability of MM or STP added to TSB, autoclaved, and inoculated with growing cultures of *Pseudomonas fluorescens.* P*i* values were 1.78 and 2.10 mg P*i*/ml media after addition of 0.5 % STP or 0.75 % MM, respectively, compared to 0.92 mg P*i*/ml in control media, and did not change significantly during 48 hour storage at 2 or 22 °C (see Appendix Table C7). The corresponding % conversions of polyphosphates to P*i* are shown in Table 7.

Effect of autoclave sterilization on decomposition of STP or MM indicated that both polyphosphates were degraded by heating in TSB. The P*i* levels of 0.5 % STP in DW were not changed by heat treatment but P*i* levels of 0.5 % STP in TSB increased after sterilization. MM in DW or TSB had some soluble P*i* which was detectable without any heat treatment. Soluble P*i* levels of 0.75 % MM in both DW and TSB were increased by autoclave sterilization.

Therefore, soluble P*i* levels increased in the media after STP or MM addition and sterilization, but bacterial growth effects did not further increase P*i* levels during

2 days storage. There was also no correlation between APC and P*i* for inculation data of *Pseudomonas fluorescens* in TSB.

In summary, MM or STP were both partially degraded when heated in TSB media. However, STP was less stable than MM in ground beef stored 2 days at 2°C or 22°C. This helps explain the longer lasting antioxidant effects of MM in ground beef samples, compared to STP. Previous work by Allen and Cornforth (2006, 2007) demonstrated that MM and STP antioxidant effects are due to the iron binding by phosphate groups (Free iron stimulates lipid oxidation, and iron chelation to MM or STP decreases lipid oxidation). However, STP is degraded rather rapidly in ground beef, losing its antioxidant effects. Results of the present experiment indicate that the greater stability of MM would result in less lipid oxidation and longer shelf life compared to samples with added STP.

CONCLUSIONS

Higher P*i* was observed during storage of beef patties with added STP compared to patties with added MM. Thus, MM was significantly more stable than STP in beef patties held at either 2°C or 22°C. There was a relatively high correlation ($r = 0.77$) between P*i* levels and APC of beef patties held at 22°C for 2 days, possibly indicating that bacterial phosphatases were contributing to the degradation of STP in these samples.

CHAPTER 5

OVERALL SUMMARY

From an application, standpoint, addition of STP or MM directly to fresh ground beef did not result in inhibition of growth of the mixed microflora of ground beef. MM may be superior to STP as an antioxidant in fresh ground beef, because results of this study demonstrated that STP was more susceptible to degradation than MM in ground beef during 48 hour storage.

Future studies are needed to determine the causes of STP degradation in ground beef. Is it due to action of bacterial phosphatases, endogenous meat phosphatases, or some combination of both type of phosphatases? Or is STP degradation due to non-enzymatic hydrolysis reactions?

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APPENDICES

Appendix A

Formula of Media

Table A1. Approximate formula of Bacto™ Tryptic Soy Broth (soybean-casein digest medium).

Constituents	Proprotion (per liter)
Pancreatic digest of casein	17.0 g
Enzymatic digest of soybean meal	3.0 g
Dextrose	2.5 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Final pH	7.3 ± 0.2

Table A2. Approximate formula of BBL™ Brain Heart Infusion.

Constituents	Proprotion (per liter)
Brain heart, infusion form (solids)	6.0 g
Peptic digest of animal tissue	6.0 g
Sodium chloride	5.0 g
Dextrose	3.0 g
Pancreatic digest of gelatin	14.5 g
Disodium phosphate	2.5 g
Final pH	7.4 ± 0.2

Appendix B
Data for Chapter 3

IRON-BINDING BY MILK MINERAL –

A POSSIBLE GROWTH INHIBITION EFFECT ?

Table B1. Comparison of mean growth of *Listeria inocula* in BHI, incubated for 48 hours at 37°C, with or without 1.5% MM treatment.

Incubation Time at 37°C (Hour)	Mean Growth	
	Control	1.5% MM
0	4.15E+03	3.95E+03
6	3.67E+05	3.24E+05
12	1.32E+08	1.09E+08
24	3.60E+09	1.97E+09
48	4.15E+09	2.79E+09

Table B2. Analysis of variance for growth of *Listeria inocula* in BHI, incubated for 48 hours at 37°C, with or without 1.5% MM treatment.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	1	8.15	0.2145
Hour	4	4	58.63	0.0008
Treatment*Hour	4	4	3.27	0.1386

Table B3. Comparison of mean growth of *Eschericia coli* DH5-α in TSB, incubated for 48 hours at 37°C, with or without 1.5% MM treatment.

Incubation Time at 37°C (Hour)	Mean Growth	
	Control	1.5% MM
0	1.22E+03	1.25E+03
6	5.60E+04	8.05E+04
12	8.10E+07	4.00E+07
24	9.90E+08	5.50E+08
48	1.28E+09	5.70E+08

Table B4. Analysis of variance for growth of *Eschericia coli* DH5-α in TSB, incubated for 48 hours at 37°C, with or without 1.5% MM treatment.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	1	1.00	0.500
Hour	4	4	1.00	0.500
Treatment*Hour	4	4	1.00	0.500

Table B5. Comparison of mean growth of *Pseudomonas fluorescens* in TSB, incubated for 48 hours at 22°C, with or without 1.5% MM treatment.

Incubation Time at 22°C (Hour)	Mean Growth	
	Control	1.5% MM
0	6.65E+02	6.30E+02
6	1.94E+03	1.79E+03
12	2.95E+04	1.33E+05
24	2.85E+07	9.70E+06
48	1.79E+09	2.79E+08*

* Indicates the first time when growth of 1.5% MM treatment was significantly lower than control treatment.

Table B6. Analysis of variance for growth of *Pseudomonas fluorescens* in TSB, incubated for 48 hours at 22°C, with or without 1.5% MM treatment.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	1	3722.36	0.0104
Hour	4	4	13047.9	<.0001
Treatment*Hour	4	4	6101.60	<.0001

Table B7. Comparison of mean growth of *Pseudomonas fluorescens* in TSB, incubated for 48 hours at 22°C, among control, 0.5%, 0.75%, and 1.5% MM treatments.

Incubation Time at 22°C (Hour)	Mean Growth			
	Control	0.5% MM	0.75% MM	1.5% MM
0	5.30E+02	5.85E+02	4.85E+02	3.40E+02
6	1.15E+03	1.62E+03	1.50E+03	1.27E+03
12	4.40E+04	9.80E+04	8.70E+04	5.25E+04
24	2.80E+07	9.70E+06	9.00E+06	3.55E+06
48	2.55E+09 [a]	2.30E+08* [b]	2.05E+08* [b]	2.65E+08* [b]

* Indicates the first time when growth of MM treatment was significantly lower than control treatment. Significant different first occurred at incubation of 48 hour which treatments sharing the same superscript letter are not significantly different at $P > 0.05$.

Table B8. Analysis of variance for growth of *Pseudomonas fluorescens* in TSB, incubated for 48 hours at 22°C, in control and different MM levels (0.5%, 0.75%, and 1.5%) treatments.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	3	83.28	0.0022
Hour	4	4	157.64	0.0001
Treatment*Hour	12	12	80.58	<.0001

Table B9. Mean growth of total aerobic plate count of hamburger patties as affected by milk mineral levels (0.5%, 0.75%, and 1.5% MM) for up to 10 days storage at 2°C.

Storage Time at 2°C (Day)	Mean Growth			
	Control	0.5% MM	0.75% MM	1.5% MM
0	8.55E+04	4.10E+04	8.80E+04	6.20E+04
1	2.08E+04	2.43E+04	9.75E+04	2.77E+04
3	1.06E+04	2.65E+04	3.20E+04	4.20E+04
5	5.55E+04	5.70E+04	1.02E+05	1.35E+05
7	2.06E+06	1.54E+06	2.70E+06	2.51E+06
10	1.80E+05	5.15E+05	4.05E+05	2.85E+05

Table B10. Analysis of variance for total aerobic plate count of hamburger patties as affected by milk mineral levels (0.5%, 0.75%, and 1.5% MM) for up to 10 days storage at 2°C.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	3	3	5.85	0.0904
Day	5	5	273.18	<.0001
Treatment*Day	15	15	4.16	0.0045

Appendix C
Data for Chapter 4

DECOMPOSITION OF MILK MINERAL AND SODIUM TRIPOLYPHOSPHATE AS AFFECTED BY BACTERIAL GROWTH IN MEDIA OR GROUND BEEF

Table C1. Mean growth of aerobic plate count of ground beef patties at storage temperature 2°C and 22°C storage for up to 2 days.

Temperature	Treatment Type	Mean Growth		
		Day 0	Day 1	Day 2
22°C	Control	3.75E+01	7.05E+05	8.45E+06*
	STP 0.5%	3.75E+01	7.30E+05	8.80E+06*
	MM 0.75%	1.85E+01	1.76E+06*	1.26E+07*
2°C	Control	1.85E+01	3.60E+01	4.10E+01
	STP 0.5%	2.80E+01	2.00E+01	2.15E+01
	MM 0.75%	2.80E+01	4.10E+01	5.75E+01

* Indicates that bacterial numbers were significantly higher than original inoculation levels ($P < 0.05$).

Table C2. Analysis of variance for aerobic plate count of ground beef patties at storage temperature 2°C and 22°C storage for up to 2 days.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	8.43	58.01	<.0001
Temp	1	8.43	2548.44	<.0001
Treatment*Temp	2	8.43	58.01	<.0001
Day	2	17.3	2141.81	<.0001
Treatment*Day	4	17.3	32.94	<.0001
Temp*Day	2	17.3	2141.80	<.0001
Treatment*Temp*Day	4	17.3	32.94	<.0001

Table C3. Soluble orthophosphate levels (P*i*) of ground beef patties at storage temperature 2°C and 22°C storage for up to 2 days.

Temperature	Treatment Type	Mean P*i* levels (mg/ml)		
		Day 0	Day 1	Day 2
22°C	Control	1.05	1.33*	2.03*
	STP 0.5%	1.28	2.21*	3.16*
	MM 0.75%	1.70	1.80	2.64*
2°C	Control	1.05	1.08	1.21
	STP 0.5%	1.28	1.87*	2.58*
	MM 0.75%	1.70	1.74	2.23*

* Indicates that orthophosphate levels were significantly different than initial levels on day 0 ($P < 0.05$).

Table C4. Analysis of variance for soluble orthophosphate levels in ground beef patties at storage temperature 2°C and 22°C storage for up to 2 days.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	12	872.75	<.0001
Temp	1	12	278.53	<.0001
Treatment*Temp	2	12	13.97	0.0007
Day	2	24	1464.82	<.0001
Treatment*Day	4	24	165.72	<.0001
Temp*Day	2	24	140.60	<.0001
Treatment*Temp*Day	4	24	7.92	0.0003

Table C5. Mean growth of *Pseudomonas fluorescens* in autoclaved TSB, incubated for up to 2 days at 2°C and 22°C.

Temperature	Treatment Type	Mean Growth		
		Day 0	Day 1	Day 2
22°C	Control	2.80E+05	1.71E+08*	5.35E+08*
	STP 0.5%	3.55E+05	8.05E+07	4.25E+08*
	MM 0.75%	8.05E+05	3.40E+07	1.55E+08
2°C	ctrl	1.15E+06	6.00E+05	8.20E+05
	STP 0.5%	5.65E+05	5.60E+05	6.05E+05
	MM 0.75%	2.64E+05	8.35E+05	1.16E+06

* Indicates that bacterial numbers were significantly higher than original inoculation levels ($P < 0.05$).

Table C6. Analysis of variance for aerobic plate count of growth of *Pseudomonas fluorescens* in autoclaved TSB, incubated for up to 2 days at 2°C and 22°C.

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	7.82	14.35	0.0024
Temp	1	7.82	136.92	<.0001
Treatment*Temp	2	7.82	14.33	0.0024
Day	2	14.1	83.10	<.0001
Treatment*Day	4	14.1	7.60	0.0018
Temp*Day	2	14.1	82.90	<.0001
Treatment*Temp*Day	4	14.1	7.68	0.0017

Table C7. Soluble orthophosphate levels (P*i*) in autoclaved TSB, inoculated with *Pseudomonas fluorescens* and incubated at 2°C and 22°C for up to 2 days.

Temperature	Treatment Type	Mean P*i* levels (mg/ml)		
		Day 0	Day 1	Day 2
22°C	Control	0.92	0.79	0.81
	STP 0.5%	1.78	1.58	1.78
	MM 0.75%	2.10	1.78	2.02
2°C	Ctrl	0.84	0.71	0.69
	STP 0.5%	1.76	1.41	1.79
	MM 0.75%	2.16	1.73	2.09

Table C8. Analysis of variance for soluble phosphates in autoclaved TSB, inoculated with *Pseudomonas fluorescens* and incubated at 2°C and 22°C for up to 2 days

Effect	Num DF	Den DF	F Value	Pr > F
Treatment	2	12	3566.97	<.0001
Temp	1	12	13.16	0.0033
Treatment*Temp	2	12	7.58	0.0074
Day	2	24	111.51	<.0001
Treatment*Day	4	24	16.57	<.0001
Temp*Day	2	24	3.21	0.0582
Treatment*Temp*Day	4	24	1.83	0.1556