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Antimicrobial activity and meat colour stabilizing properties of *Carnobacterium maltaromaticum*

by

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Abstract

Listeria monocytogenes is a foodborne pathogen of concern in meat products. Also, many fresh meat products packaged for retail sale with oxygen permeable films are prone to browning, leading to a decrease in sales. The first objective of this study was to investigate the inhibitory effect of *C. maltaromaticum* UAL307 against *L. monocytogenes* in fresh beef sausage with and without the supernatant extract of *Enterococcus faecalis* 710C or *C. maltaromaticum* UAL307. *C. maltaromaticum* significantly reduced counts of *Listeria* spp. in aerobic and modified atmosphere packaged products. The addition of cultures had no effect on pH or colour in sausages stored in a modified atmosphere; sausages stored aerobically with *C. maltaromaticum* UAL307 maintained a red colour during 10 d of storage. The mechanism behind this colour stabilization was investigated, particularly the effect of *C. maltaromaticum* on the state of myoglobin. Fe^{3+} exists in brown metmyoglobin and Fe^{2+} in red oxymyoglobin and purple deoxymyoglobin. *C. maltaromaticum* UAL307 and several other lactic acid bacteria were added to mMRS containing 0.3% myoglobin stored under aerobic and anaerobic conditions. Under aerobic conditions, only *C. maltaromaticum* promoted oxymyoglobin formation for a sustained length of time, which increased when samples were stored at 4°C. Under anaerobic conditions, metmyoglobin was converted to deoxymyoglobin by several organisms, but only *C. maltaromaticum* maintained the deoxymyoglobin at almost 100% for over 48 h. To evaluate iron reduction, cultures were inoculated into a medium containing Fe^{3+}, and Fe^{2+} levels were determined using

a modified ferrozine assay. *C. maltaromaticum* UAL307 was the only organism to significantly convert Fe^{3+} to Fe^{2+}. The investigation of iron binding capacity did not detect the production of any siderophores. The effect of heme on *C. maltaromaticum* was investigated, as the metabolic activity of some lactic acid bacteria can change from fermentation to respiration in the presence of heme. The heme-binding ability of *C. maltaromaticum* was investigated, as well as the effect of both heme and myoglobin on growth efficiency, NADH oxidase and LDH activity and its ability to produce cytochrome oxidases through expression of cytochrome *bd* genes. Heme and myoglobin had no effect on any of these properties.

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List of Symbols and Abbreviations

A	absorbance
ABC	ATP-binding cassette
APT	all purpose tween
ATP	adenosine triphosphate
°C	degrees Celsius
CAA	casamino acids
CAS	chrome azural S
CFU	colony-forming units
CO	carbon monoxide
CO_2	carbon dioxide
CTSI	cresol red thallium sucrose inulin
Deo	Deoxymyoglobin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
g	gram
x g	times gravity
GRAS	Generally Recognized as Safe

h	hour
IFN	iron-free nutrient
L	litre
LAB	lactic acid bacteria
LDH	lactate dehydrogenase
M	moles per litre
Mb	myoglobin
MetMb	metmyoglobin
MAP	modified atmosphere packaging
min	minute
mL	millilitre
mM	millimoles per litre
mMRS	modified deMan, Rogosa and Sharpe
mol	mole
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
nm	nanometre
nM	nanomoles per litre
O_2	oxygen
OD	optical density
OxyMb	oxymyoglobin
PALCAM	polymyxin-acriflavine-lithium chloride-ceftazidime-esculin-mannitol

PCR	polymerase chain reaction
PMF	proton motive force
rpm	revolutions per minute
RNA	ribonucleic acid
s	seconds
v	volume
w	weight
μL	microlitre
μm	micrometre
μM	micromoles per litre
μmol	micromoles

Chapter One: Introduction and Literature Review

1.1 MICROBIAL FLORA OF FRESH MEAT

Fresh meat products are considered highly perishable foods, as they have a high water activity and plenty of available nutrients for microbial growth (Ercolini et al, 2009). Also, the source of many of the organisms found in raw meat is the animal slaughtered to produce the meat, making these organisms inherent in the product. Although intact meat from healthy animals is virtually sterile, bacteria from the hide or gastrointestinal tract may contaminate the meat during slaughter (Gill et al, 1998). Enteric pathogens for example, may contaminate meat during post-slaughter processing of the animal such as skinning and evisceration, due to the presence of faecal matter in the intestines and on the hide (Gill, 2005). Although the microorganisms already present in or on animal carcasses form a significant portion of the organisms found in resulting fresh meat products, contamination can also occur within food processing plants. This is illustrated by a study by Vihavainen et al (2007), which compared the psychrotrophic lactic acid bacteria (LAB) of incoming broiler chicken carcasses, the resulting modified atmosphere packaged chicken products and the air of the processing plant to determine any links among the three. They found that virtually none of the LAB that were found in modified atmosphere packaged chicken products were also found on chicken carcasses entering the plant. However, they found that 45% of the bacterial isolates in the air were also found on the

processed chicken products, indicating that the broiler chickens were contaminated within the processing plant. Contamination at processing plants is not only a spoilage issue, but also one of safety. Processing plants may harbour pathogenic organisms that can contaminate food products after they have undergone processing and are considered ready-to-eat (RTE). Consumers may then consume the food believing it to be safe to eat without further processing and become ill. Lundén et al (2003) investigated the presence of persistent, meaning they can survive on surfaces within a food processing plant for a period of time, and non-persistent *Listeria monocytogenes* in processing plants that produce RTE beef, pork and poultry products. After taking samples from the processing equipment (which included slicers, packaging machines, spiral freezers and conveyors), and walls and floors of the plant as well as the raw and processed meat products, they found not only that the levels of persistent *L. monocytogenes* were eight times higher in the processed product than the raw, but that processing equipment was frequently contaminated with persistent *L. monocytogenes*. Bacteria were being transferred from the equipment to the food product during processing. Proper sanitation practices are necessary to prevent the presence of organisms and biofilms on equipment.

There are a variety of organisms that can grow and survive on fresh meat products to cause spoilage. The numbers and species of organisms present can differ in the product depending on the storage conditions, including temperature and packaging. Furthermore, the microflora of the product can change during storage due to intrinsic and extrinsic factors such as other organisms that will act

as competitors, available oxygen, pH of the meat and the meat surface morphology (Ercolini et al, 2009).

Due to the high potential for spoilage of fresh meat products, to extend shelf life they are stored under refrigeration temperatures, which selects for psychrotrophic bacteria. For this reason, the primary organisms that grow on refrigerated, aerobically stored meats are Aeromonads (McMullen and Stiles, 1993), *Pseudomonas* spp., *Enterobacteriaceae* and LAB (Ercolini et al, 2009). Typically, spoilage of refrigerated fresh meat and fresh meat products is caused by *Pseudomonas* spp. The organisms metabolize the glucose in the meat, but the low glucose concentration and slow diffusion gradient of glucose from the internal layers of the meat to the surface mean that the organisms begin to metabolize amino acids and proteins. This proteolytic action produces ammonia, amines and sulfides, which leads to the formation of characteristic off-odours in the product (Koutsoumanis et al, 2008).

In addition to temperature, packaging can also select for the type of bacteria present on the product. In modified atmosphere packaging (MAP), the shelf life of meats is extended by the elimination or reduction of oxygen and/or incorporation of CO_2 or CO in the packaging and the use of an oxygen impermeable film. By changing the atmosphere within the package, the dominant microflora is changed, preferably to those organisms with less spoilage potential (Koutsoumanis et al, 2008). For example, in vacuum packaged and MAP refrigerated meat, the dominant microflora is no longer *Pseudomonas* spp., but psychrotrophic LAB (Ercolini et al, 2009). These LAB are able to grow at

refrigerated temperatures as well as tolerate the elevated levels of CO_2 that exist in the MAP, so they multiply to become a large portion of the microbial population of MAP meat products. The growth of aerobic spoilage bacteria in contrast, is restricted (Vihavainen et al, 2007). Although this shift in organisms can lead to an extension of shelf life, spoilage eventually occurs. However, due to the difference in metabolic activity among organisms, the characteristics of spoilage may differ. In meat spoilage caused by LAB or *Brochothrix thermosphacta,* sour, acid or cheesy odours are produced due to production of organic acids from glucose metabolism (Koutsoumanis et al, 2008), as well as the formation of slime and discolouration of the meat (Vihavainen, et al, 2007), which are very different from the odours of the sulphides and ammonia produced by *Pseudomonas* spp. in packaging where oxygen is not excluded. The addition of CO_2 can further prolong shelf life by slowing the growth rate of LAB and thereby the rate of spoilage in the product, as well as inhibiting the growth of other spoilage organisms (Samelis et al, 2000). There are several organisms that can survive and grow in more than one type of atmosphere. However, when the type of packaging changes, the metabolism of the organism may shift leading to different by-products, with different characteristics of spoilage. In the case of *B. thermosphacta* for example, under aerobic conditions acetic acid and acetoin are produced. Under anaerobic conditions such as vacuum packaging or MAP, volatile fatty acids and lactic acid are produced (Pin et al, 2002). Therefore, even if *B. thermosphacta* was the dominant organism in the meat product under aerobic

conditions, the spoilage profile of the product would still be different than under anaerobic conditions.

In addition to spoilage organisms, there are several pathogenic bacteria that can be found in fresh meat products. *Salmonella* spp., *Campylobacter jejuni*, *Campylobacter coli* and verotoxigenic *E. coli* are foodborne pathogens that may be found in the gastrointestinal tract of animals used for meat production (Nørrung, et al, 2009). These organisms can either be natural gut commensals, as is the case with *E. coli* in cattle, or due to the repeated exposure of the animals to the environment (Humphrey and Jørgensen, 2006). Cattle, sheep, and pigs may pick up *Campylobacter* from contaminated water or from their outdoor environment (Manser and Dalziel, 1985; Nielsen, 2002; Humphrey and Jørgensen, 2006). Since fresh meat products are not thermally treated before being distributed to the consumer, the most efficient way for processors to reduce the numbers of pathogenic organisms is in primary production and through proper procedures and hygiene during slaughter (Nørrung, et al, 2009). There are also pathogens that are found in meat as a result of contamination. These organisms such as *L. monocytogenes*, *Clostridium* spp. and *Staphylococcus aureus* are found in the environment and so are best controlled by proper sanitation and hygiene in the processing plant (Nørrung, et al, 2009). In many cases, the numbers of these organisms are actually higher in the processing plant than in the slaughter plant. *L. monocytogenes* is often found throughout pork processing, but the incidence increases in the cutting room compared to the slaughterhouse (Thévenot et al, 2005). In the case of *L. monocytogenes*, improper sanitation can lead to the

presence of persistent organisms on processing equipment, which can then be transferred to the food product.

Although several pathogenic bacteria are unable to grow to high numbers on meat, e.g. *C. jejuni* and *C. coli* cannot grow under aerobic conditions or below 30°C, those with a low infectious dose will still be able to cause foodborne illness upon consumption. Other organisms, such as *L. monocytogenes*, have a high infective dose but are able to grow on meat at refrigeration temperatures (Humphrey and Jørgensen, 2006).

1.2 FRESH SAUSAGES

Fresh sausages contain coarsely ground pork, beef, veal, or chicken and spices and binders. They have not undergone cooking, fermentation steps or cooking steps and are sold fresh or frozen. They are very popular in the United Kingdom (Essien, 2003).

1.2.1 Production and manufacture of fresh sausages

There are several ingredients included in a fresh sausage formulation. The purpose of the addition of binders to the product is to increase water absorption and contribute texture and stability to the mixture (Essien, 2003). Figure 1-1 shows the steps involved in sausage production. Of particular note in the production of fresh sausages is the absence of any cooking or fermentation steps. In fermentation, curing agents and starter cultures of LAB are added. Traditionally, the naturally present bacteria are allowed to grow causing

fermentation, or an old batch that is already fermented is introduced into the new batch of product. The organisms are introduced at the chopping stage of production before filling into casings. After fermentation the sausages are dried. The low pH due to the production of lactic acid and the low a_w of the sausages leads to a much longer shelf-life than that of fresh sausages (Essien, 2003). Cooked sausages also have a longer shelf-life than fresh sausages, although not as long as fermented sausages. The cooking step kills many of the organisms that would cause spoilage of the product, but there are no antimicrobials (such as the lactic acid in fermented sausages) to prevent the growth of any organisms that survived the cooking step or organisms that were introduced in post-process contamination.

Ice is added at the chopping and mixing steps to reduce temperature, which in turn prevents fat melting and the breakdown of the emulsion that would occur as the mixture heats during mixing (Essien, 2003). The increased water can also help increase protein solubilization.

Sausage mixtures are put into casings using vacuum fillers. The mixture is fed from the chamber of the machine by a piston pump through a nozzle into the casings. There are several types of casings that can be used in sausage making. Natural casings consist of the intestines of pigs, sheep or cattle. Artificial casings can be made with collagen, cellulose or plastic (Essien, 2003).

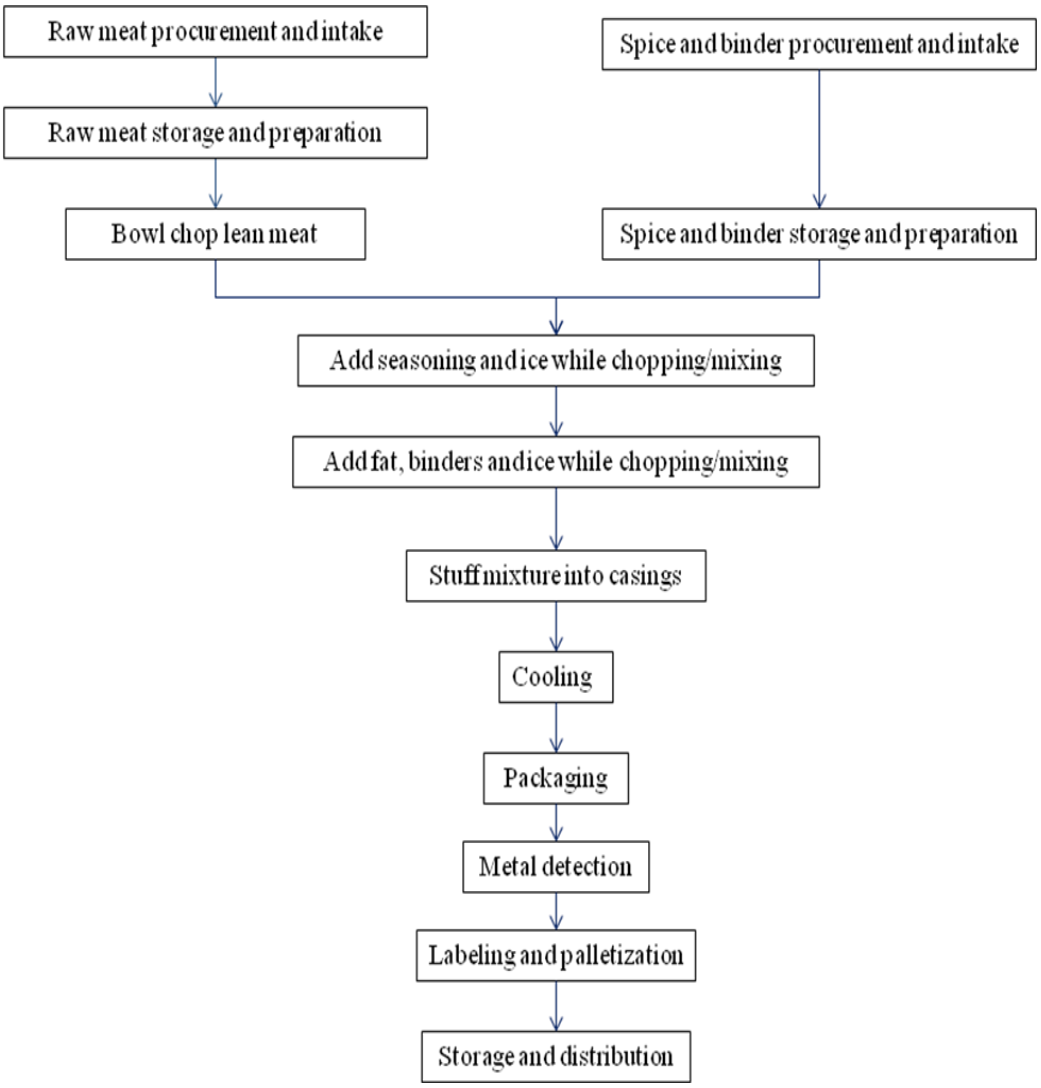

Figure 1-1: Commercial production process for fresh sausages (adapted from Essien, 2003).

1.2.2 Microbial flora of fresh sausages

There are several sources of microorganisms in sausage. The first is the meat used. There are several organisms that are associated with raw meat, whether from the animal carcass or from subsequent contamination. There may also be contamination of the spices and seasoning received from suppliers. Spices can be imported as cleaned, uncleaned or raw uncleaned. Uncleaned spices can

contain stones, rodent droppings, insects, nails, dead rodents, or wood pieces. Sometimes, a seller will package unclean spices as cleaned and sell them at a lower price than reputable spice sellers, illustrating the importance of utilizing trustworthy suppliers (Coggins, 2001). For example, a study of frankfurters made in a processing plant in Turkey showed that the contamination found in their product was due primarily to the spice mix and raw materials. The total bacteria count of their spice mix was 7.61 ± 0.17 log CFU/g, with *S. aureus* counts of 3.41 ± 0.16 log CFU/g (Güngör and Gökoğlu, 2010). It is also possible for contamination to occur within the sausage processing plant itself; from improperly sanitized equipment, drains and sinks. Contamination can also occur during storage and distribution of the product.

Since it is a fresh meat product, the types of organisms found in fresh sausages are often the same as those found in fresh meat. A study by Khalafalla and El-Sharif (1993) evaluated the types of organisms found in fresh beef sausages from retail stores in Beni Suef, Egypt. They found that psychrotrophic *Enterobacteriaceae* were present in 100% of their samples, with mean counts of $2.5 \times 10^5 \pm 10^3$ CFU/g. These organisms consisted of *Citrobacter freundii*, *Enterobacter aerogenes, E. coli, Klebsiella pneumonia, Serratia marcescens, Proteus rettgeri* and *Yersinia enterocolitica*. They also found *Pseudomonas* spp., *Aeromonas* spp. and *B. thermosphacta* in several of the sausages. A more recent study by Cocolin et al (2004) investigated the microbial population of fresh pork sausages made in a local plant in Milan, Italy. They found that the dominant organisms throughout storage of the sausages at 4 °C were *B. thermosphacta* and

Lactobacillus sakei. In particular, *B. thermosphacta* was also found throughout the sausage making process. This organism is often the principal organism in sausages after low temperature storage, especially in products that have been stored in gas-impermeable bags, but have not undergone gas flushing. This is due to the microaerobic atmosphere, which along with the low temperature selects for the growth of *B. thermosphacta* (Khalafalla and El-Sharif, 1993).

1.3 METHODS OF PRESERVATION OF MEAT PRODUCTS

1.3.1 Low-temperature storage

Fresh meat products by definition do not undergo thermal processing. Therefore, a variety of other methods must be used to extend the shelf life of these products and reduce the risk of foodborne illness to consumers. Hurdle technology is often used, the principle of which is that the use of several inhibitory factors together yields a greater inhibition of microbial growth, even if one inhibitory factor is unable to inhibit organisms by itself (Lambert et al, 1991). The first hurdle to bacterial growth applied by processors is temperature. Fresh meat products are stored at 0 to 4 °C to inhibit growth of mesophilic bacteria.

1.3.2 Packaging of fresh meat products

Modified atmosphere packaging is another method that is commonly used as a step to inhibit bacterial growth. MAP is defined as "the packaging of a perishable product in an atmosphere which has been modified so that its composition is other than that of air'' (Brody, 1989). This method has been

utilized in the meat industry for decades, with beef being shipped in containers with carbon dioxide in Australia and New Zealand as early as the 1930's (Laury and Sebranek, 2007). The gases used in MAP of meat are O_2, CO_2, N_2 and CO. The gas that imparts antimicrobial activity is CO_2 (Devlieghere, and Debevere, 2003). It penetrates the food and is dissolved in both the fat and water phases. Although the exact mechanism of the antimicrobial activity of CO_2 is not known, several have been proposed. CO_2 is rapidly absorbed by bacterial cells compared to O_2, and causes a decrease in the internal pH of the cell (Devlieghere, and Debevere, 2003). It has also been proposed that there is an effect on the induction or repression of enzyme synthesis of the cell, particularly carboxylation and decarboxylation. The other possible target of CO_2 in a cell is the lipids in the cell membrane. This interaction could lead to changes in the properties of the cell membrane and inhibition of the uptake of certain ions, causing a change in membrane fluidity (Devlieghere, and Debevere, 2003). Gram-negative organisms such as *Pseudomonas* spp. are very susceptible to CO_2, while Gram- positive organisms such as LAB are resistant (Devlieghere, and Debevere, 2003). A study by Enfors et al (1979) showed that when the head space of packages of pork stored at 4ºC were filled with CO_2, it took 35 days for counts of total aerobic bacteria to reach ca. 6 log CFU/cm^2 as opposed to 5 days in pork stored aerobically. The predominant microflora at the end of storage shifted from *Pseudomonas* spp. to *Lactobacillus plantarum* and heterofermentative LAB with the addition of CO_2 to the packaging. Although the growth of psychrotrophic LAB is not a safety issue, there are some pathogens that can also grow in

atmospheres with elevated CO_2 levels. *L. monocytogenes* is one, although there have been different degrees of inhibition reported depending on the food product that has been MAP and the storage temperature (Devlieghere, and Debevere, 2003). Hudson et al (1994) found that at 3ºC, *L. monocytogenes* growth was slowed on roast beef stored under 100% CO_2 compared to vacuum packaged product, reaching 6 log CFU/g after 10 d and 40 d respectively. However, when the storage temperature was lowered to -1.5ºC, the inhibitory effect of CO_2 increased, with counts of *L. monocytogenes* decreasing to < 2 log CFU/g after 100 d of storage, whereas roast beef reached counts > 8 log CFU/g. In contrast, Zeitoun and Debevere (1991) found that packaging chicken legs in a MAP that contained 90% CO_2 and 10% O_2 had no significant effect on the growth of *L. monocytogenes* at 6ºC compared to aerobically stored chicken legs.

While N_2 acts solely as a filler gas in MAP, O_2 is sometimes added to fresh meat products for the purpose of colour. The purpose of this is not to inhibit microbial growth, but to retard the browning of fresh red meats. Consumers associate browning with a low quality or unsafe product (Zanardi et al, 1999). Myoglobin is the principle compound responsible for fresh red meat colour. When it is in the oxygenated oxymyoglobin form, a red pigment is formed. In the absence of oxygen, it takes the form of deoxymyoglobin, which is purple. Both of these forms can be oxidized into metmyoglobin under a low partial pressure of oxygen, but the process is slower for deoxymyoglobin. For this reason, flushing the MAP with O_2 above atmospheric levels will retard the formation of metmyoglobin, thereby increasing the time that the product remains visually

acceptable to the consumer (Gill, 2003). The drawback is that aerobic spoilage organisms such as *Pseudomonas* are not inhibited under these conditions, and there is only retardation not prevention of the formation of metmyoglobin, as shown in Table 1-1. Also, other types of deterioration can occur in products stored under elevated levels of O_2, such as lipid oxidation (Veberg et al, 2006)

The addition of CO to MAP of meat products is an alternative to elevated O_2 levels when trying to prevent browning of fresh red meats. When low levels (ca 0.4%) of CO are added to a MAP, it combines with myoglobin in the muscle tissue of meat to form carboxymyoglobin, which is a cherry red pigment. This pigment is more stable than oxymyoglobin and therefore oxidizes much more slowly to metmyoglobin (Gill, 2003). However, addition of CO has been a controversial issue. In the U.S., CO has been approved for use in MAP of foods at levels of 0.4% by the Food and Drug Administration; however, Canada and the European Union have not approved its use (Venturini et al, 2010). The concern is that due to the high stability of carboxymyoglobin, meat will still appear red even at the end of the product shelf life, masking microbiological spoilage of the meat (Venturini et al, 2010). Advocates for the technology argue that there are other factors that indicate microbiological spoilage of meat, such as slime formation and odour, therefore negating the risk of consumers consuming meat that is of poor microbiological quality.

Table 1-1: Effect of storage conditions on metmyoglobin levels (%) in fresh beef steak stored at -1.5°C (followed by 1 h display in air).[1]

Storage time (days)	100% N_2	100% CO_2	67% O_2 + 33% CO_2
1	7	60	4
2	25	25	7
4	23	14	0
6	0	2	3
8	8	6	9
12	0	0	17
16	0	6	10
20	7	6	23
24	8	0	42

[1] adapted from Gill, 2003

In contrast to MAP, vacuum packaging is the removal of all gases from the package so there is, in essence, no atmosphere. Although vacuum packaging does prevent the growth of several organisms, there are some pathogens that are not inhibited. *Yersinia enterocolitica* is able to grow under vacuum conditions and has been reported in vacuum packaged lamb, chicken and pork. Its growth is retarded by high levels of CO_2 (Devlieghere, and Debevere, 2003), illustrating the advantage of MAP over vacuum packaging.

1.3.3 Antimicrobial additives in meat

Another hurdle utilized to improve the microbiological quality of meat products is the addition of antimicrobial compounds. Traditionally, there are a limited number of antimicrobial additives used in fresh meat products. Chemical antimicrobials such as nitrite and sodium lactate are utilized to preserve processed meats. For whole cuts of fresh meat, the application of antimicrobials primarily occurs as part of the decontamination of the carcass; for this reason they are not considered ingredients of the product (Simpson and Sofos, 2009). Primarily, organic acids are used in addition to hot water and steam for carcass decontamination, but chemicals can be used as well. Ideally, any antimicrobial used should not alter the sensory properties of the product, and should be environmentally friendly. In addition, the use of the antimicrobial should pose no concerns for consumers, as well as regulators and legislators. According to the U.S. Food Safety and Inspection Service (FSIS) antimicrobial interventions are approved for use in carcass decontamination if they are generally recognized as safe (GRAS), do not lead to adulteration of the product, do not have to be labeled as added ingredients, do not pose any health issues for workers in the plant or consumers who purchase the product, and have efficacy that is scientifically proven (Simpson and Sofos, 2009). The organic acids commonly used in carcass washing are lactic, acetic and citric acid. The antimicrobial activity of lactic and acetic acid lies in the ability of the weak acids to enter the cell, whereupon they dissociate. This causes the bacterial cell to expend energy as it expels the excess protons. Organic acids also inhibit organisms by lowering the intracellular pH of

the cell, which can affect components of the cell membrane, nucleic acids and cellular proteins (Simpson and Sofos, 2009). Table 1-2 shows a number of antimicrobial compounds that are approved for use in the U.S. as part of fresh meat and poultry decontamination.

Table 1-2: Antimicrobial compounds approved in the U.S. for application in fresh meat and poultry carcass decontamination.[1]

Antimicrobial agent	**Maximum use level**
Organic acids (citric, lactic and acetic)	2.0–5.0% aqueous acetic acid at ambient temperature or 55°C
Chlorine compounds	3–50 ppm free chlorine
Acidified sodium chloride	50–1,200 ppm at a pH 2.3–3.2 and 45–55°C
Trisodium phosphate	8–12% in an aqueous solution containing 20 ppm chlorine
Ozone	N/A

[1] adapted from Simpson and Sofos, 2009

Although the efficacy of these compounds against bacterial pathogens such *as E. coli*, *Salmonella* and *Listeria* are documented, that efficacy can be reduced by several factors. These include temperature (organic acids are more effective at higher temperatures such as 55°C while ozone is more effective at low temperatures), pH (most have higher efficacy below pH 7) and the presence of minerals, for example, citric acid and chlorine gas are less effective in hard water (Simpson and Sofos, 2009). The presence of organic matter also negatively affects the antimicrobial efficacy of most antimicrobial agents, which is why their

application is preceded by a hot water wash, which removes excess debris and fecal matter from the carcass.

Processed fresh meat products, such as fresh sausages, may also have antimicrobials added as ingredients. These include bacteriocins, which will be discussed in detail in Chapter 2.4 and herbs and spices. In the case of herbs and spices, their antimicrobial activity *in vitro* is well established and is due to the presence of phenolic compounds, glycosides, essential oils, coumarins and tannins (Lai and Roy, 2004; Mandal et al, 2011; Weerakkody et al, 2010), many of which act by damaging cell membranes. An herb or spice can contain several phenolic compounds, for example rosemary, contains carnosic acid, but also contains carnosol, borneol, isorosmanol, rosmanol, and rosmarinic acid (Simpson and Sofos, 2009). Other examples of antimicrobial herbs and spices used in meat products are oregano, basil, sage and cloves (Nychas and Skandamis, 2003). However, there is variability in their efficacy in food due to the low concentrations that are used in foods, as well as the components and structure of the food matrix and the way the product is stored. Low water activity (a_w), the presence of organic acids and high salt levels tend to enhance the efficacy of plant extracts, while high fat and protein content can lower their antimicrobial activity (Simpson and Sofos, 2009). The diffusivity of essential oils is also greater in a liquid medium such as a nutrient broth than the solid matrix of a food, leading to the increased antimicrobial activity observed against target organisms *in vitro* compared to *in vivo* (Nychas and Skandamis, 2003). Stecchini et al (1993) found

that under vacuum packaging conditions at 10°C the lethality of clove oil against *A. hydrophilia* increased compared to that observed in pork stored under aerobic conditions. Efficacy can also depend on the target organism. In most cases, fungi are more inhibited by plant extracts, while Gram-positive are more affected than Gram-negative bacteria (Zaika, 1988). Rosemary extract, which contains 16-24% carnosic acid, is an example of an herb extract available for commercial use in food (Simpson and Sofos, 2009). It acts as an antioxidant as well as an antimicrobial. Grohs and Kunz (2000) further illustrated the use of spices as antimicrobials in meat products. They formulated a series of mixtures of commercial spices based on the taste preferences of the European palate and applied them to minced pork. They found that the samples with spice mix had *ca*. 2 log less total bacteria after 5 days of storage at 8°C compared to a control with no spices added.

Although the antimicrobial activity of many essential oil extracts of herbs and spices is known, many are not suitable for use in certain food products. This is because the levels necessary for inhibition of bacteria often negatively affect the organoleptic properties of the food (Nychas and Skandamis, 2003).

1.4 COLOUR OF FRESH MEAT PRODUCTS

The colour of fresh meat from mammals is characteristically red, which is a function of the presence of myoglobin. Myoglobin is a globular protein that contains a porphyrin group known as heme (Young and West, 2001). Figure 1-2 shows the structure of myoglobin and heme. Within the heme group lies iron,

which has six binding sites. Four of the sites are bound to the nitrogen atoms of the porphyrin's pyrrole groups. The fifth coordination site is a histidine that is part of the globular protein (Faustman and Cassens, 1990). The sixth binding group of the iron and the oxidation state of the iron play key roles in the chemical state of myoglobin (Figure 1-3). The sixth binding site is available to bind to a number of select molecules. When oxygen is bound to the iron, and the iron is in the ferrous (Fe^{2+}) state, myoglobin exists in the form oxymyoglobin, which is a red pigment (Robach and Costilow, 1961). Metmyoglobin is brown and exists when the iron has undergone oxidation to the ferric state (Fe^{3+}) and water is at the sixth binding site. The reduced form of myoglobin is the purple pigment deoxymyoglobin, where iron is in the Fe^{2+} state in the absence of oxygen (Robach and Costilow, 1961). The oxidation from deoxymyoglobin or oxymyoglobin to metmyoglobin occurs readily through a process called autoxidation. This process occurs most readily at a partial pressure of oxygen (pO_2) of 1.5 mm Hg. The partial pressure of oxygen in air is 760 mm Hg. During storage, the pO_2 of meat decreases because the mitochondria remain active in intact post-rigor muscle for as long as 6 days at 4°C, allowing for the consumption of oxygen in the meat (Faustman and Cassens, 1990). In living muscle, autoxidation occurs as a side reaction of the mediation of oxygen transfer between hemoglobin and mitochondria. To keep the levels of metmyoglobin low in muscle, the enzyme metmyoglobin reductase converts Fe^{3+} to Fe^{2+} so that the myoglobin is able to bind oxygen again (Young and West, 2001). This enzyme requires NADH as a reductant and cytochrome *b5* as a mediator for electron transfer and remains active in stored meat, as long as NADH

is present (Young and West, 2001). However, metmyoglobin formation is still an issue when fresh meat is stored, as NADH becomes depleted in meat, leading to metmyoglobin formation over time. In addition, free radicals are formed, and since the superoxide dismutase and catalase that convert superoxide to molecular oxygen in the living cell are not active, cellular components are damaged, which leads to the acceleration of metmyoglobin production (Young and West, 2001).

Molecules other than oxygen can attach to the sixth binding site of iron within myoglobin. In meat, CO can attach to create the cherry red pigment carboxymyoglobin and the binding of nitric oxide creates nitrosomyoglobin, which gives the pink/red colour seen in cured meats (Young and West, 2001).

The concentration of myoglobin differs depending on the species of animal. The muscles of cattle, sheep and pigs have a higher myoglobin content than the muscles of chicken and other poultry, hence the more intense red colour of meat from those animals (Young and West, 2001). The function of myoglobin in live animals is to act as an oxygen store between the oxygen carried in the blood by hemoglobin, and the reduced oxygen produced by respiration (Young and West, 2001). For this reason, different muscle types within an animal have varying concentrations of myoglobin; e.g. the diaphragm would have a high level of myoglobin because it is used in respiration (Young and West, 2001).

In fresh meat products, meat colour is an important factor. Consumers prefer meat that has a red colour, and believe that browning is an indication that the product is of low quality or unsafe. To give an understanding of how colour compares to other factors utilized by consumers to assess the quality of meat in

the retail store, Glitsch (2000) conducted a study that encompassed six European countries and surveyed consumer perceptions of fresh meat quality in regards to beef, pork and chicken. They found that the most important intrinsic factor that consumers used to evaluate "quality in shop" was colour in beef and pork, while place of purchase was the most important extrinsic factor. Results were more varied for chicken, although colour did play a major role in quality assessment in most countries. A study conducted by Carpenter et al (2001) evaluated the effect of beef colour on the likelihood of consumers to purchase the product as well as on their perception of the taste of the product. This involved packaging raw beef steaks and patties in vacuum packaging, aerobic packaging and MAP with carbon monoxide to obtain meat that was purple, brown and red respectively. To evaluate the effect on taste, the individuals in the panel were served cooked meat that they were told had come from the samples that they evaluated for appearance, while the samples were actually from a single steak or patty that had been untreated. This allowed for the evaluation of the panelists' expectation of taste based on the colour that they observed. Although the consumers were most likely to purchase meat that was red, the scores that the consumers gave for taste were not affected by colour. This illustrated that although the colour appearance of the meat did not affect the consumers' perception of taste, it does have an effect on their initial perceptions of quality, which in turn affects their purchasing decisions. For this reason, browning can reduce the shelf-life of retail meat products leading to a loss in sales for retailers (Renerre, 1990).

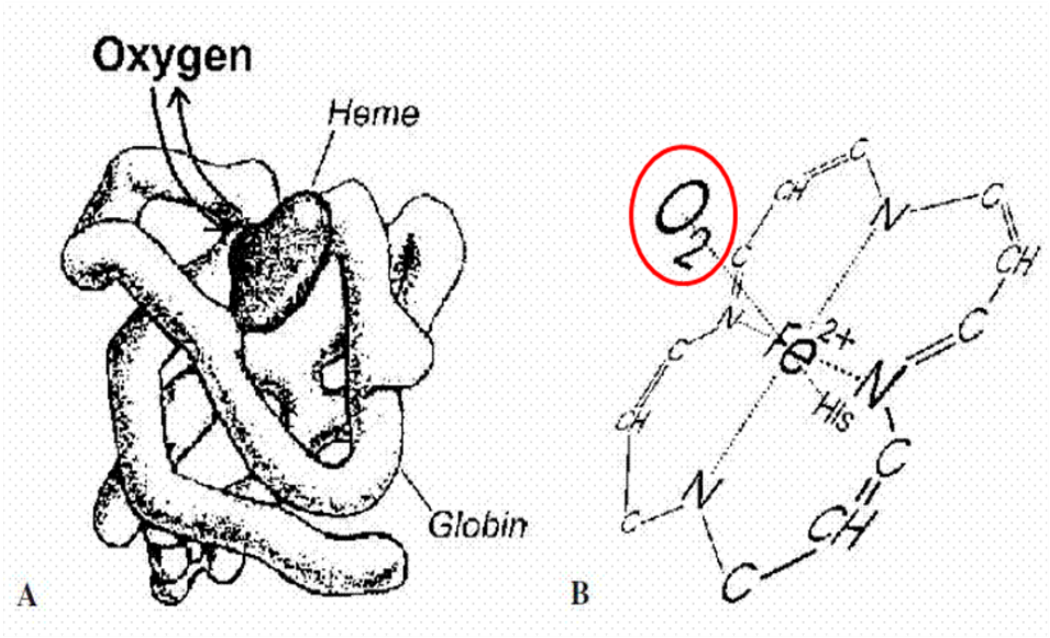

Figure 1-2: Structure of myoglobin (A) and the portion of its porphyrin ring relevant to myoglobin state (B). [From: Young and West, 2001 (Copyright 2001. Reproduced with permission of Taylor & Francis Group LLC-Books)].

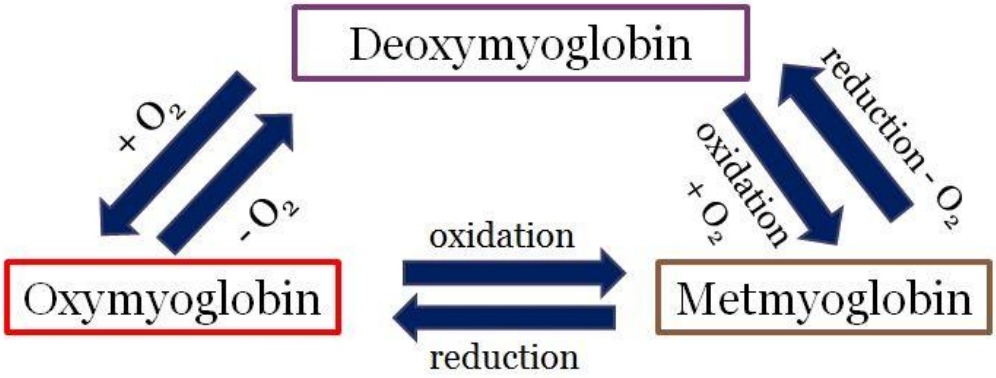

Figure 1-3: Interchangeable states of myoglobin.

The colour of meat can also be affected by bacterial growth. Aerobic bacteria such as *Pseudomonas*, *Achromobacter* and *Flavobacterium* increase the metmyoglobin content on the surface of fresh meat (Renerre, 1990). This is due to

their consumption of oxygen, leading to reduced partial pressure of oxygen on the meat surface (Robach and Costilow, 1961). Bacteria may also increase metmyoglobin through oxidation of the iron in myoglobin (Renerre, 1990). Other forms of discolouration of fresh meat may occur from bacterial growth due to their production of hydrogen sulfide (H_2S) and hydrogen peroxide (H_2O_2). These compounds bind with myoglobin to produce sulphmyogobin and choleglobin, respectively (Renerre, 1990). Both of these compounds cause the greening of meat. *Shewanella putrefaciens* (formerly *Alteromonas putrefaciens*) and *Proteus vulgaris* both produce H_2S (McMeekin and Patterson,, 1979) and have been previously reported to cause greening of dolphin fillets (Faustman and Cassens, 1975).

Another intrinsic factor that can play a role in meat colour is pH. As meat pH increases, the colour of meat becomes darker. As pH increases and the pH moves further from the isoelectric point, the proteins within it bind more water on their negatively charged side chains. This increase in bound water means there is less water on the surface to reflect light, leading to a darker appearance in colour (Abril et al, 2001). The oxidation state of myoglobin can be affected by pH. As pH decreases, myoglobin is more readily oxidized to metmyoglobin. Also, at a higher pH, myoglobin does not degrade as rapidly with heat. That means there may be a persistent red colour in the meat even if an adequate internal temperature has been reached during cooking (Young and West, 2001).

There are several extrinsic factors which also play a role in meat colour. At higher temperatures, meat colour is negatively affected, as bacterial growth

increases and lower O_2 levels prevail due to oxygen consumption from processes such as greater oxygen scavenging activity of residual enzymes in the muscle (Renerre, 1990). Freezing can also have negative effects on the colour of fresh meat. Meat that is frozen at a slower rate appears very dark, due to large ice crystal formation which leads to less scattering of light (Renerre, 1990). Stabilization of meat colour is best achieved when the retail storage/ display temperature is at 0°C (Young and West, 2001). Light can also play a role in meat colour in two ways. The first is by affecting the colour that the consumer perceives. Natural, incandescent and fluorescent lighting vary in their spectral profiles, so that the colour of the meat appears differently to the consumer, thus retailers can choose the right lighting profile so that the appearance of the product is optimized (Young and West, 2001). Light can also affect the state of myoglobin. Photochemical autoxidation of myoglobin occurs when it is exposed to light, particularly that in the ultraviolet range, leading to the formation of metmyoglobin. Frozen meat is more susceptible to this form of autoxidation than chilled meat, and fluorescent lighting should be avoided (Young and West, 2001). As mentioned in Section 1.3.2 of this thesis**,** packaging can play a role in meat colour. Vacuum packaged meat has a purple colour as there is no oxygen present, while high O_2 MAP can prolong the red colour of fresh meat by meeting the oxygen demand caused by the residual mitochondrial activity in the muscle, preventing the lowering of the pO_2 of the meat (Faustman and Cassens, 1990). MAP that utilizes CO will result in meat with a bright cherry red colour.

Myoglobin has a higher binding affinity for CO than for O_2, so carboxymyoglobin is quickly formed (Young and West, 2001).

1.5 *LISTERIA MONOCYTOGENES*

L. monocytogenes is a pathogen that can be found in fresh meat products and there are several reasons why it is a pathogen of concern in food. It is able to grow at temperatures as low as -0.4°C, so it grows at refrigeration temperatures and it is ubiquitous in the environment. It is usually found in water, soil, sewage, silage and has been isolated from milk from healthy and mastitic cows (Farber and Peterkin, 1991). The organism is also able to grow at a wide pH range of 4.5 – 9.6 (Zhu et al, 2005) and is not affected by changes in atmospheric conditions; its growth is the same under aerobic, anaerobic and microaerophilic conditions and it is not inhibited by elevated CO_2 levels (ICMSF, 1996). *L. monocytogenes* is also not inhibited by the NaCl content of several products such as sausage (Hefnawy et al, 1993).

There are 13 serovars of *L. monocytogenes*, three of which are most often implicated in human listeriosis: 4b primarily, 1/2a and 1/2b (ICMSF, 1996). The majority of human cases of listeriosis occur in individuals who are immune compromised e.g. AIDS and cancer patients, the elderly, pregnant women, and infants. In these individuals as few as 100 cells can cause illness (Farber and Peterkin, 1991). Listeriosis infection in pregnant women is particularly of concern as it can cause miscarriages. Often, the mother only develops flu-like symptoms, but the organism is able to pass through the transplacental barrier and infects the

fetus. This infection of both mother and unborn child is termed perinatal listeriosis (ICMSF, 1996). Miscarriages, stillbirth or neonatal listeriosis (infection in a newborn) can then occur. Symptoms of the infection in a fetus can include septicaemia affecting a number of organs and it causes the formation of granulaomatous lesions. If infection occurs before the first trimester, intrauterine death often occurs. Infection later in pregnancy can cause stillbirth or the birth of an infant that is seriously ill (ICMSF, 1996). Symptoms of neonatal listeriosis include rash, respiratory distress (usually the cause of death), shortness of breath, shock, conjunctivitis, pneumonia, hyperexcitability, vomiting, cramps, septicaemia, meningitis, hematologic irregularities, and hyper- or hypothermia (Farber and Peterkin, 1991). The infant may also be infected at birth, which is called late-onset listeriosis, and usually causes meningitis (ICMSF, 1996). In non-neonatal listeriosis, the symptoms are gastroenteritis, meningitis and endocarditis (Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt, 2007; Roed et al, 2012).

Since *L. monocytogenes* is sensitive to heat, cooking is one of the main intervention steps for this organism. In the case of ready-to-eat (RTE) meats such as deli meats that do not undergo any cooking steps by the consumer, *L. monocytogenes* outbreaks have been caused by post-process contamination of the products. There are several intervention steps used in the food industry to control *L. monocytogenes*. With in-package thermal pasteurization where the product is already packaged during the thermal inactivation of the organism, there is no opportunity for post-process contamination at the processor level. The use of

chemical preservatives, such as sodium lactate and diacetate can reduce the growth of *L. monocytogenes*. The use of LAB and their bacteriocins is being studied for the prevention of the growth and survival of *L. monocytogenes* in RTE meats (Zhu et al, 2005). A few of these have been approved for commercial use. Micocin®, a powder that contains the organism *Carnobacterium maltaromaticum* CB1, has been approved for use in Canada in vacuum-packed products such as wieners, sliced roast beef, cooked ham and sliced cooked turkey (Griffith Laboratories, 2011). It also has GRAS approval in the United States (Harrington, 2011).

1.6 BIOPRESERVATION OF FOOD USING LACTIC ACID BACTERIA

LAB are Gram-positive, non-motile, non-sporeforming, rod and coccus-shaped bacteria that primarily produce lactic acid during metabolism of carbohydrates, and have a low (<55%) G+C content in their DNA (Ananou et al, 2007). The genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus* and *Carnobacterium* are included in this group (Deegan et al, 2006). Biopreservation is the term used to describe the "extended storage life and enhanced safety of foods using the natural microflora and / or their antibacterial products" (Stiles, 1996). Several lactic acid bacteria are suited for this purpose as they produce compounds that have antimicrobial activity against some of the pathogens of concern in foods, and several are already considered safe as they have been used in food fermentation (Stiles, 1996). Biopreservation would be an ideal alternative for producers to ensure food safety and increase microbial shelf life of their

product, as consumers are demanding more minimally processed foods with natural food additives rather than the chemical preservatives commonly used (Ananou et al, 2007). Historically, biopreservation has taken the form of fermentation, in which organisms, often LAB, that are naturally present in the food or that have been added are allowed to grow and produce organic acids and other compounds that alter flavour and / or texture of the food and have antimicrobial properties that increase shelf life (Ananou et al, 2007). One type of antimicrobial compound that can be produced by these organisms is bacteriocins. These are small, ribosomally-synthesized antimicrobial peptides. They differ from antibiotics in that they are not produced by enzymes, usually have a narrow spectrum of activity against organisms closely related to themselves and are effective as antimicrobials in nanomolar concentrations rather than the larger concentrations needed for antibiotic efficacy (Nes, 2011). The bacteriocins produced by LAB have several properties that make them ideal for use in food preservation. Many of the producer organisms are GRAS, they do not have a toxic effect on eukaryotic cells and so are safe for human consumption, and they are inactivated by proteases of the digestive tract and have little influence on the gut microflora. They are also usually pH- and heat-tolerant (important properties in food processing). Other properties that make them suitable for use as antimicrobials in food are their lack of cross resistance with antibiotics, and the fact that genes for their production are usually plasmid-encoded, which allows greater ease of genetic manipulation (Galvéz et al, 2007).

In Canada, if a live bacterial culture is added to a food it is considered an ingredient under the Food and Drug Regulations. If the particular culture has been added to foods previously and has a history of safe use, it can be added to other foods. If it does not have a history of safe use in foods or has undergone genetic modification, it is considered a "novel food" under Food and Drug Regulations and must pass a safety assessment, the guidelines of which are set by Health Canada (Health Canada, 2009). These assessments are conducted by the Food Directorate Division of Health Canada (Health Canada, 2006).

For the purposes of biopreservation of food, there are three types of preparation that can be added. The first is a culture of the bacteriocin-producing organism itself, which produces the bacteriocin in the food during storage. The second is to add a concentrated or partially purified fermentate; the producer organism is grown in a substrate such as milk or whey and the fermentate is concentrated. It contains the bacteriocin as well as other compounds produced during growth, such as organic acids. The final option is to use a purified bacteriocin. This option is often not cost effective for producers (Galvéz et al, 2007). In addition to Micocin®, the only other bacteriocins currently used commercially in food are nisin produced by *Lactococcus lactis* and marketed as Nisaplin™, and Pediocin PA-1, which is produced by *Pediococcus acidilactici* and marketed as ALTA™ 2431 (Deegan et al, 2006). Nisin is used in several countries and differs from many other bacteriocins in that it has a wide range of activity against several Gram-positive organisms, such as *L. monocytogenes*, *Bacillus* spp. and *Clostridium* spp. The latter two are important as they are heat-

resistant spore-formers, so nisin is used in the production of processed cheese and cheese spreads to protect against the the outgrowth of these organisms. It is also used in canned foods and dairy products (Deegan et al, 2006).

There are several factors that can affect the efficacy of a bacteriocin in a food system. The first is interaction with components of the food. For example, nisin is not an effective antimicrobial in fresh meat products, due to its formation of an adduct with the compound glutathione, rendering it inactive (Rose et al, 1999). The pH of a food may also be a factor, as nisin has poor solubility at pH > 6.0. Other contributing factors include, storage temperature of the food, inactivation by enzymes within the food, uneven distribution throughout the food and the processing conditions applied to the food (Galvéz et al, 2007).

1.7 BACTERIOCIN CLASSIFICATION, STRUCTURE AND MODE OF ACTION

The classification system of bacteriocins has changed several times in the past. At one time, as many as five classes were proposed. Currently, there are three classes of bacteriocins with most falling into the first two classes (Deegan et al, 2006). They are described in Table 1-3.

Class I bacteriocins are modified extensively after translation, resulting in the formation of their characteristic amino acids lanthionine and methyllanthionine. This process consists of two steps, in which gene-encoded serine and threonine undergo enzymatic dehydration to create dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively.

Table 1-3: Classes of bacteriocins.[1]

Category	Characteristics	Subclass	Example
Class I	Lantibiotics. Contain lanthionine, β-methyllanthionines and dehydrated amino acid residues. <5kDa	Type A (linear, positively charged)	Nisin
		Type B (globular, negative or no charge)	Mutacin II
Class II	Non-modified, heat stable peptides <10kDa	IIa (anti-listerial, pediocin-like, positive charge)	Sakacin P
		IIb (two-peptide)	Plantaricin EF
		IIc (circular bacteriocins)	Enterocin AS48
		IId (non-pediocin like)	Lactococcin A
Class III	Heat labile proteins >30kDa		Helveticin

[1]adapted from Rea et al, 2011

The double bond of Dha or Dhb is attacked by thiol groups from neighboring cysteines, forming lanthionine or methyllanthionine. This process forms covalently closed rings in the peptide, and affects both its structure and functionality (Deegan et al, 2006). These lantibiotics are believed to target bacteria by using lipid II, which is a main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, as a docking molecule. Nisin is a Class Ia bacteriocin and has a dual mode of action. It can use lipid II as a docking molecule to initiate membrane insertion and pore formation leading to rapid cell death and can also bind to lipid II, preventing correct cell wall synthesis which

leads to cell death (Hasper et al, 2006). There are some mutants of nisin that are unable to form pores, as they are composed of a shorter chain of amino acids. They still have antimicrobial activity, however, which is achieved by blocking cell wall synthesis through the removal of the lipid II molecule (Hasper et al, 2006). Class Ib bacteriocins interfere with the enzymatic functions of bacteria, causing their death (Deegan et al, 2006).

Class II bacteriocins do not undergo post-translational modification, and are subdivided into IIa, IIb, IIc and IId. Class IIa bacteriocins have antimicrobial activity against *L. monocytogenes* and the amino acid sequence YGNGVXCXXXXVXV at their N terminus. This group is termed "pediocin-like". These bacteriocins have been the major focus of research into bacteriocins produced by LAB for use in biopreservation. There is much less similarity among Class IIa bacteriocins in their C-terminus, but it has been discovered that some similarities exist that may allow for their division into subgroups. These similarities exist among piscicolin 126, bavaricin A, mundticin, and sakacin P; enterocin A, bavaricin MN, and divercin V41; and carnobacteriocin BM1, enterocin P, and sakacin A (Ennahar et. al, 2000). The mannose permease EII_t^{man} of the phosphotransferase system in bacteria, which is a sugar uptake/phosphorylation system, has been suggested as a putative receptor for Class IIa bacteriocins (Deegan et al, 2006). Recently, Kjos et al (2010) demonstrated that Class IIa bacteriocins specifically target the 40 amino acid structure IIC, an extracellular loop of the mannose phosphotransferase (MPTase) component of *L. monocytog*enes (Kjos et al, 2010). MPTase is a sugar uptake

system that allows for the uptake of glucose into the cell, and consists of the subunits IIa, IIB, IIC and IID. Class IIb bacteriocins consist of two peptides that operate synergistically and neither of the peptides has any antimicrobial activity when used as a single peptide (Deegan et al, 2006). Class IIc is comprised of bacteriocins that are covalently linked at their C and N-terminus, leading to their cyclic structures. Relatively few of these bacteriocins have been identified, but it is known that carnocyclin A, which is produced by *Carnobacterium maltaromaticum* UAL307, forms anion-selective pores in its target organisms, while enterocin AS-48 forms non-selective pores that allow for the dissipation of potassium ions as well as other small solutes out of the cell (Gong et al, 2009). Class II bacteriocins operate as antimicrobials primarily by forming pores which cause an ionic imbalance. This dissipation of the proton motive force (PMF) involves a total dissipation of the pH gradient, but only a partial dissipation of the transmembrane potential. For example, sakacin has an amphiphilic helical structure, allowing it to insert into the membrane of the target cell, leading to cell depolarization and death (Ennahar et al, 2000).

1.8 HEME UTILIZATION IN BACTERIA

1.8.1 The function and acquisition of heme

Heme is produced by several Gram-negative bacteria including *E. coli* and *P. aeruginosa*. It serves as a prosthetic group of several proteins that have roles in electron transport, oxygen sensing and transport, and enzymatic reactions within the cell (Cavallaro et al, 2008). Heme levels in these organisms vary depending

on the growth conditions; low levels are usually present in fermentation conditions when no heme-containing respiratory enzymes are required, and higher levels are found under aerobic growth conditions. It appears that oxygen, nitrate and the carbon source serve as signals for the regulation for heme biosynthesis (Schobert and Jahn, 2002). Several Gram-negative and Gram-positive bacteria have systems for heme acquisition that are used in cases where either no free iron is present so heme is used as a source of iron, or the organism lacks the pathways necessary for heme biosynthesis (Cavallaro et al, 2008). Gram-positive bacteria utilize proteins anchored in the cell wall to shuttle heme from the host compound (e.g. myoglobin) into the cytoplasm through a dedicated ABC transporter (Cavallaro et al, 2008). For example, in *S. aureus*, iron is acquired through the binding of heme using the proteins IsdA, IsdB, IsdC, and IsdH to transport heme across the cell-envelope. They all contain one or more copies of a heme-binding domain called NEAr iron Transporter (NEAT), which is named in this manner because it is part of proteins that are expressed by genes near genes predicted to encode Fe^{3+} transporters (Vermeiren et al, 2006). Once through the cell envelope, heme is imported through the IsdDEF complex into the cytoplasm, where it is degraded by the monooxygenases IsdG and IsdI. Gram-negative bacteria use two different systems for heme acquisition. The first is through hemophores, extracellular heme-chelating proteins which cleave heme from the heme-containing compound, and allow for its uptake through the outer membrane receptors and into the bacterial periplasm. It is also possible for whole heme-containing proteins to be unloaded at the cell surface for subsequent

transportation to the periplasm. Specific outer membrane receptors for a wide range of heme-containing molecules are involved in recognition. Once the heme is in the periplasm, it is transported through the inner membrane to the cytoplasm through ABC transporters similar to those used in Gram-positive bacteria (Cavallaro et al, 2008).

1.8.2 Heme and lactic acid bacteria

LAB lack the ability to synthesize heme. However, in some LAB including *L. lactis*, *E. faecalis*, *Leuconostoc mesenteroides* and several *Streptococcus* species, the presence of heme in a growth medium under aerobic conditions can induce respiration. The difference between this and the typical type of metabolism in LAB, which is fermentation, is the form of oxidation of organic compounds (Brooijmans et al, 2009). Their growth typically takes the form of fermentation; endogenous electron acceptors are used that are usually catabolic intermediates of the same organic compounds. In respiration, extracellular electron acceptors are used through an electron transport chain (ETC) that generates a PMF (Brooijmans, 2008). This ability has been studied in *L. lactis*, and the reason for this switch to respiration is the synthesis of cytochromes and menaquinone using the heme present. Menaquinones act as redox reagents in electron transport and oxidative phosphorylation systems and comprise part of the bacterial plasma membrane. Cytochromes are membrane-bound heme-proteins that are used as terminal oxidases in the ETC of several bacteria. The *bd*-type is an effective oxygen scavenger due to its high affinity for oxygen (Brooijmans, 2008). This cytochrome consists of 2 subunits. Subunit one (also called CydA) is

the site of quinol oxidation in the compound and binds a *b*-type heme. Subunit II (also called CydB) binds two hemes one of which is a chlorin-type heme of cytochrome (Cook et al, 2002). In *E. coli*, it is expressed under low oxygen tension conditions. The genes necessary for menaquinone synthesis and a heme-dependent *bd*-type cytochrome that is encoded by the *cydABCD* operon have also been characterized in *L. lactis* (Brooijmans et al, 2009). These findings indicate a change in the ETC. Figure 1-4 shows the ETC of *L. lactis* during respiration. Respiration is actually energetically favoured by *L. lactis*, leading to an increased biomass, a less acidic environment due to a decrease in the accumulation of fermentation compounds such as lactic acid and an increase in acetoin, and an extension of long-term survival from the few days that are typical to months. This extension is attributed to a lack of oxidative stress due to the decrease in cytoplasmic oxygen from its conversion to water by cytochrome oxidase, and the less acidic environment. The less acidic environment may also contribute to a decrease in toxic oxygen radicals (Pederson et al, 2008). Although there is a metabolic shift in the presence of heme, it is not constant throughout the entire growth of *L. lactis*. Growth in the lag phase remains fermentative, even in the presence of heme and oxygen. The shift begins when the pH decreases to 5.3 due the production of lactic acid. The pH then increases as the genes for respiration are induced (Gaudu et al, 2002).

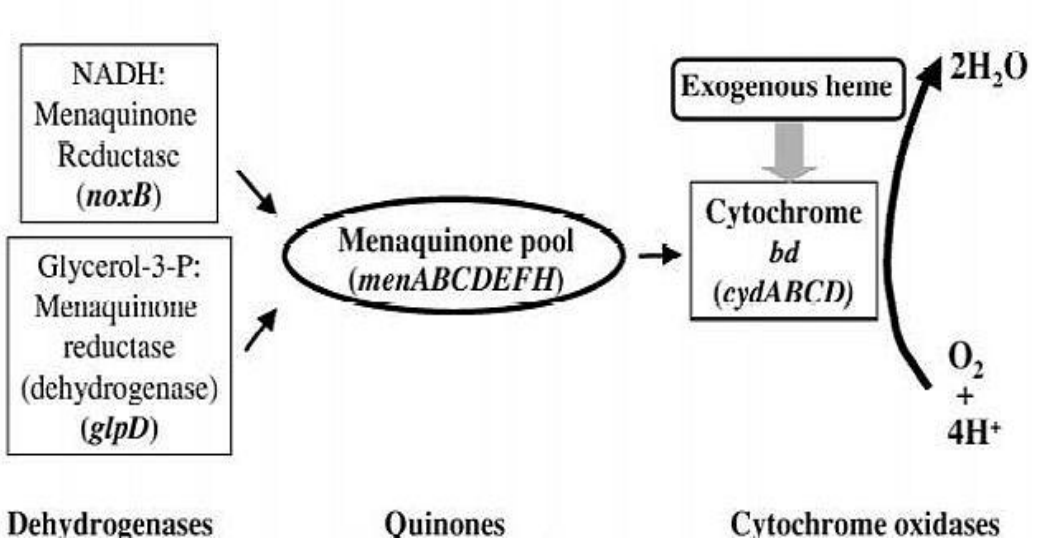

Figure 1-4: Electron transport chain of *L. lactis* in the presence of oxygen and heme (From Gaudu et al, 2002. With permission from Springer Science + Business Media).

1.9 UTILIZATION OF IRON BY BACTERIA

1.9.1 Function of iron in bacteria

Iron is necessary for the growth and survival of many bacteria, especially pathogens. It plays a role in redox reactions as the catalytic center of enzymes. These enzymes play a variety of roles in cellular processes such as amino acid and nucleoside synthesis, DNA synthesis, and electron transport (Wandersman and Deleplaire, 2004). Iron itself may also play a role in electron transport as an electron acceptor (Lovely, 2006). Both Fe^{2+} and Fe^{3+} are used in catalytic reactions that generate hydroxyl radicals, which are used as oxidizing agents (Guerinot, 1994). In many pathogens, including *P. aeruginosa* and *Vibrio cholera*, the production of compounds for iron acquisition has been associated

with virulence (Guerinot, 1994). Several studies have investigated the iron requirement of LAB; however, LAB such as *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Carnobacterium* are not affected by iron starvation conditions, so do not require iron for growth (Bruyneel et al, 1989; Elli et al, 2000; Pandey et al, 1994).

In many environments iron is biologically unavailable. In host organisms, iron is often sequestered in carrier proteins such as transferrin, lactoferrin, and ferritins, or as part of the protoporphyrin ring in hemoproteins, such as myoglobin and hemoglobin. In soil and aquatic environments, and under aerobic conditions and a neutral pH, iron is mostly in the Fe^{3+} state, which is insoluble and therefore not directly utilizable by bacteria. Due to this shortage of biologically available iron, many bacteria have adapted mechanisms to acquire iron (Wandersman and Deleplaire, 2004).

1.9.2 Acquisition of iron by bacteria

There are several ways in which iron is taken up into cells. Heme may be used as a source of iron; however, Gram-negative bacteria can bind other iron containing compounds such as lactoferrin and transferrin to receptors in the outer membrane that are specific to that substrate (Wandersman and Deleplaire, 2004). In situations of iron limitation, pathogens e.g. *V. cholera*, may even release hemolysin / cytolysin and utilize the heme compounds that are released from the lysed epithelial cells of the host (Guerinot, 1994). Both Gram-positive and Gram-negative bacteria may also produce siderophores, which are low molecular weight iron-chelating molecules (Brown and Holden, 2002). These compounds have a

high affinity for iron and bind preferentially to Fe^{3+}. There are several types of siderophores; more than 500 have been identified and are grouped into three types: hydroxamates such as ferrichrome; catechols such as enterobactin, and hydroxyacids such as pyochelin (Wandersman and Deleplaire, 2004). In Gram-negative bacteria, after binding of the siderophore to the outer membrane receptor, it is transported into the periplasmic space, where the iron moiety binds to a specific lipoprotein receptor of an ABC transporter in the inner membrane. Iron is then transported into the cytoplasm through an ABC transporter. The same process occurs for iron-containing proteins. Each iron transporter system is substrate specific, which is determined according to the outer-membrane receptor and the inner-membrane ABC transporter. *Neisseria meningitidis* has separate receptors for the binding of transferrin, lactoferrin, hemin / hemoglobin and hemoglobin / haptoglobulin. The iron transport systems of Gram-positive organisms are less well studied, but there has been some investigation into Gram-positive pathogens. For example, *S. aureus* has five ABC transporters for iron as well as a transferrin receptor and it produces the siderophores staphyloferrin a, staphyloferrin b and aurochelin. *Streptococcus pyogenes*, *Clostridium diptheriae*, *Streptococcus pneumoniae*, and *L. monocytogenes* are other Gram-positive pathogens that possess iron-uptake systems (Brown and Holden, 2002).

Because too much iron is toxic for bacterial cells, its assimilation is strictly regulated and the genes responsible for iron assimilation are expressed only under conditions of iron deficiency and are repressed when the intracellular iron concentration is high (Wandersman and Deleplaire, 2004).

1.9.3 Iron reduction by bacteria

Some bacteria preferentially or exclusively use Fe^{2+} as an iron source. For this reason, they require ways to reduce Fe^{3+} when Fe^{2+} is not available (Guerinot, 1994). This can be carried out by extracellular iron reductases. Dissimilatory iron reduction may also take place in cases where the organism does not require iron, but uses it as an extracellular electron acceptor. This is a form of anaerobic respiration and is mediated by outer membrane cytochromes in Gram-negative bacteria. Members of the family *Geobacteraceae* are known to carry out dissimilatory iron reduction (McKinlay and Zeikus, 2004). Finally, iron reduction may occur as part of an iron-uptake system that utilizes siderophores. *P. aeruginosa*, for example, secretes an enzyme that reduces iron when it has formed a complex with the siderophore pyochelin. This releases the iron from the complex, making it available for the synthesis of necessary iron-containing compounds (Cox, 1980).

1.10 RESEARCH OBJECTIVES

Bacteriocins have potential use as antimicrobial additives that are perceived as natural by consumers. For this reason, they would be very useful to food producers to increase product safety. Before bacteriocins or their producing cultures are used in commercial products, however, it is important to verify their efficacy against pathogens in specific food products.

In addition to benefits to the product safety, a bacteriocin-producing organism that prolongs the red colour of meat would also lengthen the shelf-life of fresh meat products, due to its effect on consumer perception of quality and

safety. Understanding the mechanism behind this colour stabilizing effect would aid researchers in finding additional organisms with this ability and may also allow for the manipulation of the organism to optimize colour stabilization of meat products.

The objectives of this study were to:

1. determine the efficacy of bacteriocin producing cultures and/or bacteriocins at inhibiting the growth of *L. monocytogenes* in fresh sausage;
2. evaluate the efficacy of *C. maltaromaticum* to maintain the red colour of fresh meat products; and
3. determine the mechanism of myoglobin stabilization in fresh meat by *C. maltaromaticum.*

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Chapter Two: The effect of *Carnobacterium maltaromaticum* on the growth of *Listeria monocytogenes* and colour of aerobically stored and modified atmosphere packaged fresh beef sausage

2.1 INTRODUCTION

Listeria monocytogenes is an organism that is increasingly found to be the cause of widespread foodborne illness outbreaks. The infective dose is approximately 0.1-10 million CFU in immunocompromised individuals, children and pregnant women compared to 10-100 million CFU in healthy individuals (Bortolussi, 2008). The elimination of this pathogen would increase food safety and has been of continued concern in the food industry for several reasons. *L. monocytogenes* is ubiquitous in the environment and is carried by several animal species intended for human consumption (Donnelly, 2001). The organism is capable of growing at refrigeration temperatures and is able to withstand conditions that may inhibit the growth of other bacteria, such as salt concentrations greater than 10% and low pH (Farber, 2000). *L. monocytogenes* is prevalent in raw meat and can be found in both cooked and uncooked meat products (Wang and Muriana, 1994). Although the primary concern is post-process contamination of RTE (ready-to-eat) products that will not be cooked by the consumer, *L. monocytogenes* contamination of raw meat products is also of concern because consumers do not always cook their food properly, or engage in cross contamination between raw and processed foods. Fresh or frozen meats can be contaminated from their origins or during processing (Bortolussi, 2008).

The search for intervention strategies to prevent survival and growth of *L. monocytogenes* in food has focused on natural compounds that are generally recognized as safe (GRAS) and cost effective (Barmpalia et al, 2004). Bacteriocins are well suited for this purpose. They are small antimicrobial peptides that are produced by several bacteria. Lactic acid bacteria (LAB) that produce Class II bacteriocins are particularly of interest. This is the largest group of bacteriocins that are heat stable and the group is subdivided into Class IIa, Class IIb and Class IIc bacteriocins (Chen and Hoover, 2003). Class IIa exhibit antilisterial activity *in vitro* and have the ability to retain their activity at the pH present in meat (Ennahar et al, 1999). Lactic acid bacteria that produce Class IIa bacteriocins have been isolated from foods and many produce more than one bacteriocin (Ennahar et al, 1999). The organisms that produce these compounds include species belonging to the genera *Lactococcus, Bacillus, Brochothrix, Carnobacterium* and *Enterococcus* (Ennahar et al, 1999). Of the genus *Carnobacterium*, *Carnobacterium divergens* and *Carnobacterium maltaromaticum* are the only two species frequently isolated from food products and the environment (Leisner et al, 2007). In this study, the effects of two particular organisms were investigated; *Carnobacterium maltaromaticum* UAL307 and *Enterococcus faecalis* 710C. *C. maltaromaticum* UAL307 is a homofermentative lactic acid bacterium; although it produces formate, acetate and ethanol, it utilizes glycolysis during metabolism of glucose (Gänzle, unpublished data). *Carnobacterium* spp. have strong antilisterial activity (Schöbitz et al, 1999) and the ability to grow at temperatures as low as -1.5ºC to 2ºC in a meat juice

medium (Yang et al, 2009). *C. maltaromaticum* UAL307 produces two Class IIa bacteriocins, piscicolin 126 and carnobacteriocin BM1, and a cyclic bacteriocin, carnocyclin A, has been isolated (Martin-Visscher, 2008). These characteristics make it an ideal candidate for use in an intervention strategy against *L. monocytogenes.*

E. faecalis is a bacteriocin-producing LAB that exhibits broad spectrum antimicrobial activity against Gram-positive bacteria and has antilisterial activity. It also has several virulence factors, including cytolisin, gelatinase and β-haemolysin (Sabia et al, 2008). In this study, only the supernatant extract of the organism was used, as the growth of *E. faecalis* is inhibited at temperatures below 10ºC (Riemann and Cliver, 2006). The supernatant fractions obtained after the removal of bacterial cells from cultures of *C. maltaromaticum*, *E. faecalis* and other bacteriocin-producers have been shown to contain bacteriocins produced by these organisms, and have antimicrobial activity *in vitro* (Schöbitz et al, 1999; Villani et al, 1993; Casla et al, 1996;) These supernatant fractions can be concentrated to produce purified bacteriocins using methods such as ammonium sulfate precipitation, and anion exchange and high-performance liquid chromatography (Herbin et al, 1997).

In addition to food safety, quality is also an issue in food production, in which product appearance plays a large role. Consumers often make decisions on food purchases based on the appearance of the product and colour plays a key role in meat products. The absence of the red colour associated with a fresh meat product can have a negative effect on the consumers' perception of product

quality and can also cause them to question the safety of the product (Zanardi et al, 1999).

The objectives of this study were to evaluate the effect of *C. maltaromaticum* UAL307 on the growth of a cocktail of strains of *L. monocytogenes* with and without supernatant extracts of either *C. maltaromaticum* UAL307 or *E. faecalis* 710C, and to evaluate the effects on the colour of fresh beef sausage. The effect of packaging atmosphere (most notably the addition of carbon dioxide and carbon monoxide) on the growth of *C. maltaromaticum* UAL307 in fresh beef sausage was also determined.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains

All bacterial strains were stored at -80°C in 20% glycerol (Fisher Scientific, Fair Lawn, NJ, USA) and subcultured twice in All Purpose Tween Broth (APT; Difco, Becton, Dickinson and Company Sparks, MD, USA). *L. monocytogenes* ATCC 15313 (serotype 1/2a), FS-15 (serotype 1/2b) and CDC 7762 (serotype 4b) and *C. maltaromaticum* UAL307 were grown at 22°C for 18 h. Cultures were centrifuged (10,000 x *g* for 10 min at 4°C) and OD_{600} was adjusted with 0.1% peptone (Difco) to obtain ca. 10^8 CFU/mL. To create a *L. monocytogenes* cocktail, 0.17 mL of each strain was added to 4.5 mL 0.1% peptone to obtain a final concentration of ca. 10^7 CFU/mL *Listeria monocytogenes*. *E. faecalis* 710C was grown at 35°C for 18 h and another culture of *C. maltaromaticum* UAL307 was grown at 8°C for 24 h to obtain culture

supernatants. The cultures were centrifuged and the supernatant was removed by pipetting. The pH was adjusted to 7.0 with 1 N NaOH.

2.2.2 Sausage formulation

Ground beef with 85% lean, 15% fat was obtained from a federally inspected processing facility and hand mixed with the following formulation: 83% ground beef, 10% 0.1% peptone or culture supernatant and 7% fresh beef sausage seasoning and binder (Griffith Laboratories, Toronto, ON). The seasoning and binder mix contained 21.6% salt, bringing the final salt content of the sausage mixture to 1.5%. The final product weight was 4 kg. Six treatments were applied to the sausage mixture:

1. a control with no inoculum added;

2. a treatment with a cocktail of 3 strains of *L. monocytogenes;*

3. a treatment with a cocktail of 3 strains of *L. monocytogenes* and *C. maltaromaticum* UAL307;

4. a treatment with a cocktail of 3 strains of *L. monocytogenes*, *C. maltaromaticum* UAL307 culture and *C. maltaromaticum* UAL307 supernatant;

5. a treatment with a cocktail of 3 strains of *L. monocytogenes* and *E. faecalis* 710C supernatant; and

6. a treatment with a cocktail of 5 strains of *L. monocytogenes*, *C. maltaromaticum* UAL307 culture and *E. faecalis* 710C supernatant.

The three strain cocktail of *L. monocytogenes* was inoculated at approximately 10^4 CFU/g while *C. maltaromaticum* UAL307 inoculated at 10^5 CFU/g by adding 4 mL of cultures, which had been adjusted using OD, to the

0.1% peptone prior to addition to ground beef. In treatments containing the culture supernatants, 0.1% peptone was replaced by the supernatant during formulation.

Sausage mixtures were stuffed into collagen casings (Nippi, Tokyo, Japan) using a stuffer (model SC-100, Koch Equipment, Kansas City, MO, USA). The sausage was twisted into links and packaged two links in each polypropylene tray (Winpak, Chicago Hts., IL, USA) using a traysealer (model T200, Multivac, Kansas City, MO, USA). Modified atmosphere packaged (MAP) trays were flushed with a gas mixture of 30% CO_2, 0.4% CO and the balance nitrogen, and sealed with a 0.5 mil polyester/PVDC/2 mil EVA copolymer film with an oxygen transmission rate of 7.8 cc/m^2 (Winpak Portion Packaging, Newton, PA, USA). Aerobic packages were sealed with a 0.75 mil oriented polypropylene/2 EVA copolymer film with an oxygen transmission rate of 1400 cc/m^2 (Winpak Prtion Packaging). Aerobic packages were stored at 4°C for 10 d and MAP packages were stored at 4ºC for 15 d. Aerobically packaged samples were analyzed every 2 days and MAP samples were analyzed every 3 days.

2.2.3 Microbiological analysis

Samples of 10 g that included meat and casing were cut from sausages and stomached with 90 mL sterile 0.1% peptone water for 2 min in filter stomacher bags (Fisher Scientific). Aliquots of 10 mL were removed and the appropriate dilutions were made in 0.1% peptone water. Samples (0.1 mL) were spread onto the surface of PALCAM agar (Oxoid Ltd., Basingstoke, Hampshire, England) containing selective supplement (Oxoid) to enumerate *Listeria* spp., APT agar

(Difco) to determine the number of LAB present and Cresol red thallium acetate sucrose inulin (CTSI) agar formulated according to Wasney et al (2001) was used for the enumeration of *Carnobacterium* spp. PALCAM agar plates were incubated at 30°C for 48 h. Colonies that were circular and grayish-green with black centres and black halos against the red medium were counted as *Listeria* spp. (van Netten et al, 1989). CTSI plates were incubated at 22°C for 48 h followed by incubation at 8°C for an additional 48 h. *Carnobacterium* spp. appeared as light pink and circular colonies with a red centre and a yellow halo against the dark purple medium (Wasney et al, 2001). APT agar plates were incubated in anaerobic jars with anaerobic GasPak™ (BD BBL, Franklin Lakes, NJ, USA) at 22°C for 48 h.

To further enumerate *L. monocytogenes* when plate counts on PALCAM were below the original detection limit of 2 log CFU/g and to increase the recovery of injured cells, 1 mL of the stomached sample was added to 10 mL University of Vermont Medium (UVM; Oxoid) and incubated at 35ºC for 24 h. A 0.1 mL aliquot of the UVM broth was spread onto the surface of PALCAM agar and incubated at 30ºC for 48 h. If no growth was exhibited on these plates then *Listeria* spp. counts were < 1 log CFU/g. If colonies were present on the PALCAM agar after enrichment, counts were recorded as < 2 log CFU/g.

2.2.4 Colour

The status of the red colour of each sausage sample was evaluated using a Chroma Meter CR-410 (Konica Minolta, Osaka, Japan). Sausage casings were excised using a scalpel and sterile forceps. Sausage meat was spread in a small

Petri dish for colour analysis after 5 min and 3 readings were taken of each sample, turning the Petri dish between readings to better account for variability in colour between physical areas of the sausage meat. The Hunter Lab "a" values of each sample on each day (D_n) were compared to the Hunter Lab "a" value of the first day of evaluation of the control (D_0), to give a percentage:

$(D_n/D_0) \times 100$= red colour value

2.2.5 pH

The pH of each sample was recorded using the samples prepared for colour analysis. A flat surface electrode (Thermo Electron Corporation., Beverly, MA, USA) was used for analysis.

2.2.6 Statistical analysis

The experiment was replicated 4 times. Bacterial counts were transformed to log values and the data was subjected to analysis of variance (ANOVA) using the PROC GLM procedure in SAS version 9.1 software (SAS Institute, Inc., Cary, NC, USA) and Student-Newman-Keuls (SNK) test was used for comparison of treatments. An α value of 0.05 was used as the level of significance. Results of colour analysis were statistically evaluated using PROC GLM, after percentages were transformed to a normal distribution.

2.3 RESULTS

2.3.1 Microbiological analysis

Results for APT counts (Figure 2-1) showed that there was some background microflora in the sausage mixture in control samples at the beginning

of the experiment (3 log CFU/g). The inocula of *L. monocytogenes* and *C. maltaromaticum* UAL307 were evident on the APT agar, resulting in a higher count on Day 0. By the end of 10 days of storage, the counts on APT agar for the control treatment reached approximately 7 log CFU/g. This took 15 d in the MAP sausages (Figure 2-2) as compared to 10 d in the aerobically stored sausages.

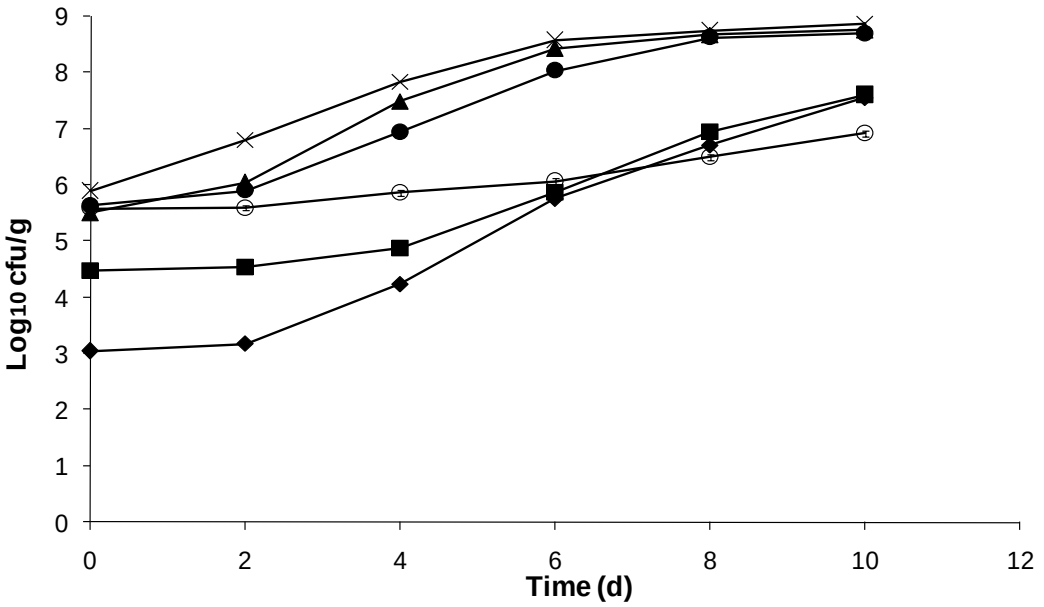

Figure 2-1: Mean log counts of presumptive lactic acid bacteria determined on APT agar for aerobically packaged fresh beef sausage stored at 4ºC. Treatments included a Control with no inocula (♦), *Listeria* spp. inoculum only (■), *Carnobacterium* UAL307 inoculum (▲), *C. maltaromaticum* UAL307 culture and *C. maltaromaticum* UAL307 supernatant (×), *E. faecalis* 710C supernatant (○) and *C. maltaromaticum* UAL307 culture and *E. faecalis* 710C supernatant (●). n=4; error bars are standard deviations and the variation around the means was < 0.25.

Counts on PALCAM agar (Tables 2-1 and 2-2) showed that the addition of *C. maltaromaticum* UAL307 resulted in significantly ($P < 0.05$) lower numbers

of *L. monocytogenes* in fresh beef sausage after 4 days of aerobic storage at 4ºC. When the supernatant of *C. maltaromaticum* UAL307 was added along with the *C. maltaromaticum* UAL307 culture, counts of *L. monocytogenes* were significantly lower than those of other treatments after 2 days of storage. The addition of the *E. faecalis* 710C supernatant did not significantly lower the counts of *L. monocytogenes* as compared to counts of samples were the culture of *C. maltaromaticum* UAL307 was added. Under MAP conditions

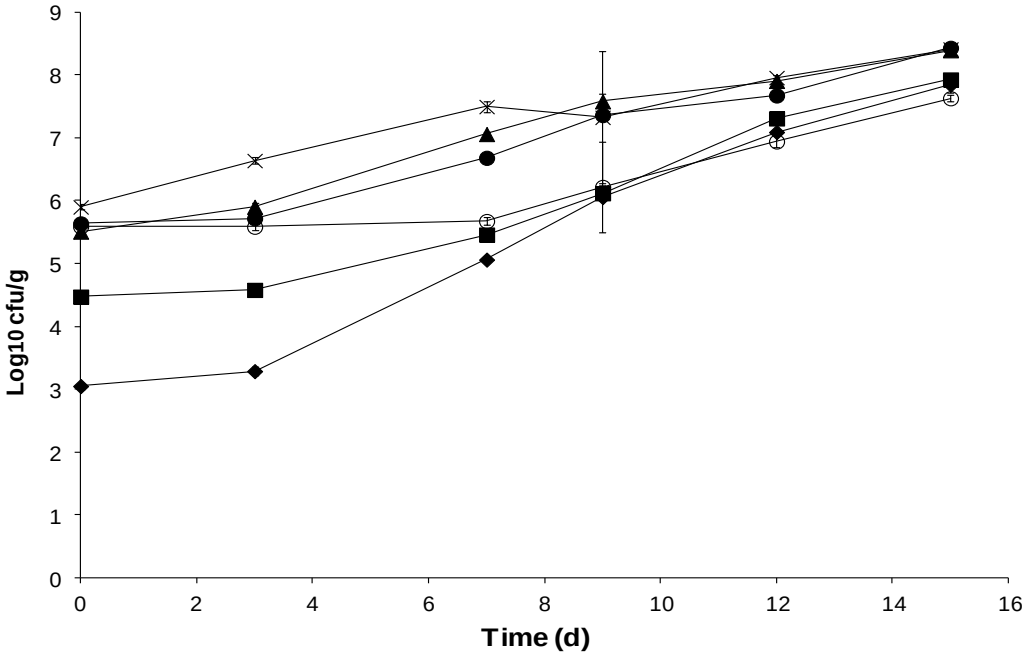

Figure 2-2: Mean log counts of presumptive lactic acid bacteria determined on APT agar for MAP fresh beef sausage stored at 4ºC. Treatments included a Control with no inocula (♦), *Listeria* spp. inoculum only (■), *Carnobacterium* UAL307 inoculum (▲), *C. maltaromaticum* UAL307 culture and *C. maltaromaticum* UAL307 supernatant (×), *E. faecalis* 710C supernatant (○) and *C. maltaromaticum* UAL307 culture and *E. faecalis* 710C supernatant (●). n=4. Error bars are standard deviations.

(Table 2-2), the antilisterial effect was detected in samples with the *C. maltaromaticum* UAL307 culture and supernatant after 3 days of storage. When added to sausages, the *E. faecalis* 710C supernatant, also reduced counts of *L. monocytogenes* compared to the control; however, it was not able to reduce numbers to below detection limit.

Counts on CTSI agar (Tables 2-3 and 2-4) showed that *Carnobacterium* spp. were present in sausage samples to which no *C. maltaromaticum* UAL307 inoculum was added. Although counts of *Carnobacterium* spp. were below detection limit at the beginning of storage in samples were no *Carnobacterium* culture was added, by the end of the experiment, counts of approximately 4 log CFU/g were present in the control samples and in samples inoculated with *L. monocytogenes*. *C. maltaromaticum* UAL307 was able to grow in aerobically packaged sausages (Table 2-3), increasing by 2 logs after 4 d of storage. In aerobically packaged samples that had the *E. faecalis* 710C supernatant added, the growth of *Carnobacterium* spp. was inhibited and counts never increased above 4 log CFU/g. In MAP sausages (Table 2-4), *C. maltaromaticum* UAL307 was able to grow and increased by 2 log CFU/g after 7 d of storage. *Carnobacterium* spp. were present in samples with no *C. maltaromaticum* UAL307 culture but counts were below 4.5 log CFU after 15 d of storage. Samples with the *E. faecalis* 710C supernatant was added had the lowest counts of *Carnobacterium* spp. up to 9 days of storage but after 12 and 15 d counts were similar to those for the control and the sausages that had only *L. monocytogenes* added.

Table 2-1: Mean log counts[*] of *Listeria* spp. (log CFU/g) determined on PALCAM agar of aerobically packaged fresh beef sausage stored at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum*	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
0	<1.00 [d]	4.03±0.09 [**c]	3.98±0.16 [a]	3.22±0.31 [b]	3.45±0.16 [b]	3.42±0.22 [b]
2	<1.00 [d]	4.30±0.18 [a]	4.31±0.19 [a]	2.61±0.16 [c]	3.41±0.18 [b]	3.41±0.18 [b]
4	<1.00 [d]	4.77±0.09 [a]	2.77±0.62 [b, c]	<2.00 [† c]	3.09±0.42 [b]	2.60±0.61 [b, c]
6	<1.00 [d]	5.48±0.02 [a]	<2.00 [c]	<2.00 [c]	2.61±0.51 [b]	<2.00 [c]
8	<1.00 [d]	5.97±0.06 [a]	<2.00 [c]	<1.00 [†† d]	2.93±0.57 [b]	<2.00 [c]
10	<1.00 [d]	6.32±0.43 [a]	<2.00 [c]	<1.00 [d]	2.99±0.90 [b]	<1.00 [d]

[*] n = 4

[a, b, c, d] numbers with different letters in the same row are significantly different at $P < 0.05$.

[**] mean ± standard deviation

[†] Below the detection limit, but colonies are present on PALCAM following enrichment in UVM

[††] No colonies present on PALCAM following enrichment in UVM

Table 2-2: Mean log counts[*] of *Listeria* spp. (log CFU/g) determined on PALCALM agar of MAP fresh beef sausage stored at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum* culture	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
0	<1.00 ^{d}	4.03±0.09 $^{**\,a}$	3.98±0.16 ^{a}	3.11±0.14 ^{c}	3.50±0.12 ^{b}	3.42±0.22 ^{b}
3	<1.00 ^{d}	4.30±0.26 ^{a}	3.96±0.24 ^{a}	2.43±0.18 ^{c}	3.04±0.69 ^{b}	3.20±0.21 ^{b}
7	<1.00 ^{e}	4.76±0.11 ^{a}	4.16±0.06 ^{b}	<2.00 $^{\dagger\,d}$	2.83±0.70 ^{c}	2.81±0.47 ^{c}
9	<1.00 ^{d}	5.15±0.34 ^{a}	3.20±0.29 ^{b}	<2.00 ^{c}	2.91±1.06 $^{b,\,c}$	2.54±0.63 $^{b,\,c}$
12	<1.00 ^{d}	5.64±0.19 ^{a}	2.10±0.17 ^{c}	<1.00 $^{\dagger\dagger\,d}$	3.10±1.28 ^{b}	<2.00 ^{c}
15	<1.00 ^{c}	5.72±0.81 ^{a}	2.08±0.11 ^{c}	<1.00 ^{c}	3.39±1.60 ^{b}	<2.00 ^{c}

[*] n = 4

$^{a,\,b,\,c,\,d}$ numbers with different letters in the same row are significantly different at $P < 0.05$.

[**] mean ± standard deviation

† Below the detection limit, but colonies are present on PALCAM following enrichment in UVM

†† No colonies present on PALCAM following enrichment in UVM

2.3.2 Colour

Packaging atmosphere influenced the colour of the sausages. Under aerobic conditions, control samples and samples inoculated with *Listeria* had 59% of the red colour remaining after 10 d of aerobic storage (Table 2-5). Sausages containing *C. maltaromaticum* UAL307 (with or without the supernatant of a culture of *C. maltaromaticum* UAL307) had the highest Hunter Lab "a" value compared to the control at the end of the storage life, with 83% of the red colour value in sausages at the end of the experiment compared to the red colour value of the control sausages at the beginning of the experiment. When *E. faecalis* supernatant was added to the sausages along with the *C. maltaromaticum* UAL307, the colour was not significantly different from those that contained just the *C. maltaromaticum* UAL307 or the *C. maltaromaticum* UAL307 and its supernatant. Samples containing just the supernatant of *E. faecalis* 710C had the greatest deterioration of meat colour, with only 50% of the red colour value at the end of storage compared to sausages at the beginning of storage. Under MAP, the results were very different (Table 2-6). The addition of carbon monoxide in the gas mixture, maintained the red colour of the sausages throughout storage. There was a slight increase in the red colour over time, most likely due to the development of carboxymyoglobin, which has a more cherry red colour than the oxymyoglobin originally present in the meat. There was no significant difference in the colour among treatments when sausages were stored under MAP.

Table 2-3: Mean log counts[*] of *Carnobacterium* spp. (log CFU/g) determined on CTSI agar for aerobically packaged fresh beef sausage stored at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum* culture	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
0	<2.00 [†, c]	<2.00 [c]	5.27±0.22 [**, b]	5.92±0.07 [a]	<2.00 [c]	5.22±0.19 [b]
2	<2.00 [d]	<2.00 [d]	6.22±0.25 [b]	6.72±0.03 [a]	<2.00 [d]	5.83±0.02 [c]
4	<2.00 [c]	<2.00 [c]	7.58±0.25 [a]	7.63±0.29 [a]	<2.00 [c]	6.80±0.14 [b]
6	3.66±0.24 [c]	3.20±0.32 [c, d]	8.15±0.06 [a]	8.57±0.46 [a]	2.82±0.46 [d]	7.61±0.39 [b]
8	3.39±0.32 [b]	3.69±0.18 [b]	8.58±0.17 [a]	7.86±0.86 [a]	2.67±0.80 [c]	8.04±0.61 [a]
10	4.78±0.21 [b]	4.02±0.67 [b, c]	8.17±0.99 [a]	7.74±1.20 [a]	2.90±0.35 [c]	7.98±0.90 [a]

[*] n = 4

[a, b, c, d] numbers with different letters in the same row are significantly different at $p < 0.05$.

[†] Below the detection limit

[**] mean ± standard deviation

Table 2-4: Mean log counts[*] of *Carnobacterium* spp. (log CFU/g) determined on CTSI agar for MAP fresh beef sausage stored at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum* culture	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
0	<2.00 [†] [c]	<2.00 [c]	5.27±0.22 [**] [b]	5.92±0.07 [a]	<2.00 [c]	5.22±0.19 [b]
3	<2.00 [d]	<2.00 [d]	6.20±0.22 [b]	6.66±0.08 [a]	<2.00 [d]	5.64±0.21 [c]
7	2.93±0.76 [c]	2.72±0.45 [c]	7.27±0.15 [a]	7.10±0.47 [a]	<2.00 [d]	6.47±0.23 [b]
9	3.82±0.16 [c]	3.40±0.36 [c]	7.68±0.06 [a]	6.89±1.07 [a, b]	2.32±0.41 [d]	6.55±0.53 [b]
12	3.05±0.55 [b]	3.32±0.44 [b]	7.71±0.44 [a]	7.77±0.28 [a]	4.39±1.45 [b]	6.59±1.23 [a]
15	4.20±0.72 [b]	4.07±0.90 [b]	7.61±0.90 [a]	6.86±1.24 [a]	4.30±0.22 [b]	6.71±1.40 [a]

[*] n = 4

[a, b, c, d,] numbers with different letters in the same row are significantly different at $p < 0.05$.

[†] Below the detection limit

[**] mean ± standard deviation

Table 2-5: Red colour development[*] of aerobically packaged fresh beef sausage[**] stored at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum* culture	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
0	100.00±3.84 [†] [a]					
2	79.49±16.44 [a,]	95.51±2.64 [b]	94.97±3.38 [ba]	83.67±4.1 [c]	83.33±2.06 [c]	85.21±0.87 [c]
4	70.30±13.07 [b, c]	86.04±4.77 [a,]	92.30±7.51 [a]	75.01±10.42 [a]	67.01±4.34 [a]	72.88±10.87 [a]
6	67.15±2.19 [b, c]	77.35±10.83 [a, b, c]	85.06±2.67 [a, b]	88.47±10.00 [a]	59.27±4.43 [c]	70.27±10.67 [b, c]
8	58.72±3.25 [b]	61.48±5.28 [b, c]	93.17±3.21 [a]	80.02±16.22 [a, b]	54.99±2.20 [c]	77.61±19.05 [a, b]
10		58.71±0.80 [b]	83.50±11.01 [a]	83.53±17.14 [a]	50.09±0.80 [b]	79.75±13.55 [a]

[*]Hunter Lab "a" value as a percentage of the Control sample on Day 0

[**] n = 4

[a, b, c] numbers with different letters in the same row are significantly different from each other at $P < 0.05$

[†]mean ± standard deviation

Table 2-6: Red colour development[*] of MAP fresh beef sausage[**] stored at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum* culture	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
3	100.00±1.71[† a]	95.18±0.02 [b]	106.64±4.24 [a]	107.90±4.01 [a]	102.99±4.18 [a]	105.23±7.62 [a]
7	109.29±8.01 [a]	106.21±10.82 [a]	114.72±6.32 [a]	114.72±7.35 [a]	115.15±0.84 [a]	115.66±4.00 [a]
9	109.87±9.26 [a]	115.70±5.43 [a]	120.02±4.49 [a]	119.31±5.48 [a]	117.05±2.09 [a]	118.05±1.64 [a]
12	114.87±5.78 [a]	122.08±3.19 [a]	117.77±5.49 [a]	111.83±7.54 [a]	114.28±3.25 [a]	119.95±5.40 [a]
15	117.55±5.02 [a]	117.01±6.05 [a]	121.44±5.28 [a]	107.79±12.64 [a]	109.90±13.09 [a]	113.01±2.39 [a]

[*]Hunter Lab "a" value as a percentage of the Control sample on Day 0

[**] n = 4

[a, b] numbers with different letters in the same row are significantly different from each other at $P < 0.05$

[†]mean ± standard deviation

2.3.3 pH

Treatment had no significant effect on the pH of either the aerobic or MAP sausages ($P > 0.05$) and there were few significant differences among treatments (Tables 2-7 and 2-8).

2.4 DISCUSSION

The background microflora detected during storage was to be expected as contamination occurs during meat preparation and manufacturing, and the meat provides an environment highly suited to microbial growth (Khalafalla and El-Dherif, 1993). *C. maltaromaticum* UAL307 was able to inhibit the growth of *L. monocytogenes* in fresh beef sausage. The addition of its supernatant increased the effect as it caused an initial drop in numbers of *L. monocytogenes* and resulted in < 1.0 log CFU/g of the pathogen after 8 days of refrigerated aerobic storage. A reduction of this magnitude would help to reduce the incidence of foodborne *L. monocytogenes* infection in consumers. This reduction was delayed but not lost in an atmosphere of 30% CO_2 and 0.4% CO, with < 1.0 log CFU/g of *L. monocytogenes* after 12 days of storage. The *C. maltaromaticum* UAL307 inocula grew in both aerobic and MAP conditions, with a 2 to 3 log CFU/g increase by the end of storage. This is a positive aspect for the meat industry, as some processors in the U.S. use this type of MAP to increase the shelf life of their meat products. The ratio of gases is ideal, as the carbon dioxide levels reduces both

Table 2-7: Mean [*] pH of fresh beef sausage stored aerobically at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum* culture	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
0	5.81±0.02 [†] [a, b]	5.80±0.05 [b]	5.83±0.00 [a, b]	5.87±0.02 [a, b]	5.86±0.02 [a, b]	5.86±0.01 [a]
2	5.85±0.03 [a]	5.86±0.06 [a]	5.89±0.05 [a]	5.90±0.06 [a]	5.90±0.02 [a]	5.89±0.03 [a]
4	5.87±0.05 [b]	5.90±0.01 [a, b]	5.90±0.02 [a, b]	5.90±0.02 [a, b]	5.94±0.02 [a]	5.93±0.02 [a]
6	5.85±0.04 [a]	5.86±0.01 [a]	5.84±0.04 [a]	5.64±0.26 [b]	5.81±0.01 [a]	5.82±0.07 [a]
8	5.78±0.07 [a]	5.74±0.19 [a]	5.73±0.15 [a]	5.55±0.34 [a]	5.69±0.41 [a]	5.69±0.26 [a]
10	5.71±0.22 [a]	5.75±0.25 [a]	5.63±0.08 [a]	5.49±0.27 [a]	5.15±0.40 [a]	5.59±0.24 [a]

[*] n = 4

[a ,b,] numbers with different letters in the same row are significantly different from each other at $P < 0.05$

[†] mean ± standard deviation

Table 2-8: Mean [*] pH of MAP fresh beef sausage stored at 4ºC after intervention with cultures and supernatant extracts of bacteriocin producers.

Day	Control	*L. monocytogenes* inoculum only	*C. maltaromaticum* culture	*C. maltaromaticum* culture + supernatant	*E. faecalis* 710C supernatant	*E. faecalis* 710C supernatant + *C. maltaromaticum* culture
0	5.81±0.02 [† a, b]	5.80±0.05 [b]	5.83±0.00 [a, b]	5.87±0.02 [a]	5.86±0.02 [a, b]	5.86±0.01 [a, b]
3	5.85±0.00 [c]	5.88±0.02 [b]	5.94±0.01 [a]	5.93±0.00 [a]	5.92±0.00 [a]	5.91±0.01 [a]
7	5.88±0.01 [a]	5.90±0.04 [a]	5.88±0.03 [a]	5.70±0.34 [a]	5.87±0.08 [a]	5.90±0.05 [a]
9	5.89±0.04 [a]	5.94±0.07 [a]	5.84±0.11 [a]	5.55±0.43 [a]	5.73±0.36 [a]	5.79±0.18 [a]
12	5.63±0.28 [a]	5.77±0.13 [a]	5.69±0.19 [a]	5.53±0.48 [a]	5.67±0.39 [a]	5.69±0.35 [a]
15	5.55±0.32 [a]	5.58±0.30 [a]	5.58±0.23 [a]	5.41±0.42 [a]	5.58±0.48 [a]	5.55±0.44 [a]

[*] n = 4

[a,b] numbers with different letters in the same row are significantly different from each other at $P < 0.05$

[†] mean ± standard deviation

microbial growth and lipid oxidation in the product, while carbon monoxide maintains the red colour of the meat (Laury and Sebranek, 2007). In this way, shelf life is extended due to the delay of spoilage and enhancement of consumer perception of freshness. This reduction of overall microbial growth is likely the reason for the slower growth rate of *C. maltaromaticum* UAL307 in the MAP samples, leading to a less rapid decrease in cell counts of *L. monocytogenes*.

Although the addition of the supernatant *C. maltaromaticum* UAL307 increased antilisterial activity, the addition of *E. faecalis* 710C supernatant did not increase the activity to such an extent and had the added detriment of decreasing the red colour of the sausages. *E. faecalis* 710C is known to produce virulence factors (Liu et al, 2011). Although some enterococci have been used in the production and ripening of several European cheeses, the addition of *Enterococcus* species to food is of concern, as several (including some *E. faecalis* strains) have been involved in hospital acquired infections in humans (Cariolato et al, 2008). For this reason, it would be necessary to ensure that the strain that is being considered for potential use in food passes the safety requirements and assessments required set in place by the food regulatory body of the country of production. For example, in Canada it would be necessary to pass a safety assessment for novel foods conducted by the Food Directorate division of Health Canada (Health Canada, 2006). This may be an undesirable barrier for processors to add it to their sausage formulation.

In addition to an antilisterial effect, *C. maltaromaticum* UAL307 had a significant and beneficial effect in maintaining the red colour of fresh beef

sausages under aerobic conditions. In the MAP sausages, it is expected that CO combined with myoglobin to produce carboxymyoglobin, which is a cherry red pigment (Martínez et al, 2005). For this reason, the colour stabilizing effect of *C. maltaromaticum* UAL307 was not apparent in the MAP sausages, as the cherry red colour of the carboxymyoglobin produced likely surpassed any result produced by the organism. Thus, in aerobically packaged products, producers would benefit from the maintenance of red colour as well as antilisterial properties if *C. maltaromaticum* UAL307 was included in their formulation. With use of a MAP with an atmosphere containing carbon monoxide, red colour is maintained, thus *C. maltaromaticum* UAL307 would only be required for its antilisterial activity.

Although no sensory analysis was conducted in this study, the effect of the inocula on pH was measured. This was of concern because some species of *Carnobacterium* have been known to contribute to meat spoilage (Ercolini et al, 2009). Results showed that the addition of *C. maltaromaticum* UAL307 with or without its supernatant had no effect on the pH of the sausages under either packaging condition.

This study demonstrated that *C. maltaromaticum* UAL307 can be used to inhibit the growth of *L. monocytogenes* in fresh beef sausage, and that inhibition of *L. monocytogenes* is increases when combined with a culture supernatant. Combined with its GRAS status, colour stabilizing properties and negligible effect on meat pH, *C. maltaromaticum* UAL307 would provide the meat industry

with an effective intervention that would extend the overall retail storage life of fresh sausage.

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Chapter 3: The production of siderophores by *Carnobacterium maltaromaticum* and its effect on the states of myoglobin and iron

3.1 INTRODUCTION

In the retail market, many fresh meat products such as ground beef and sausages are packaged in trays covered with oxygen permeable films, which allows the formation of metmyoglobin (Greene et al, 1971). The browning that results can cause consumers to mistrust both the safety and quality of the product (Zanardi et al, 1999). When the meat surface pigment is more than 20% metmyoglobin consumers will perceive that the product is not as fresh and, correspondingly, sales will decrease by a factor of two (Renerre, 1990).

Myoglobin is the main contributor of colour to fresh, red meat (Tang et al, 2004). It is a heme protein that exists in 3 different states. Deoxymyoglobin is present in meat stored in an atmosphere without oxygen and results in meat with a purple hue. Oxymyoglobin is the oxygenated form of myoglobin and imparts a red colour. Finally, brown meat colour is caused by metmyoglobin when there is a low oxygen atmosphere (0.5-1%) or meat has been exposed to an oxygenated atmosphere (e.g. air) for an extended period of time (Robertson, 1993). In addition to the presence of oxygen in the package of meat, the state of myoglobin is also dependent on state (oxidized or reduced) of the iron in the porphyrin ring of the molecule. In metmyoglobin, ferric iron (Fe^{3+}) is present and ferrous iron (Fe^{2+}) is present in oxymyoglobin (Robertson, 1993).

Carnobacterium maltaromaticum UAL307 has a strong antilisterial activity as it produces several bacteriocins (Martin-Visscher et al, 2008). *Carnobacterium* spp. have the ability to grow at temperatures of -1.5ºC to 2ºC in meat products (Leisner et al, 2007). These characteristics have lead to the investigation of their use as an antimicrobial in food. *C. maltaromaticum* UAL307 inhibits the growth of *Listeria monocytogenes* in meat products (Chapter 2, this thesis). In addition to its antimicrobial activity, *C. maltaromaticum* h prolongs the red colour of aerobically stored fresh beef sausages (Chapter 2, this thesis). It is likely that the effect on myoglobin is due to the oxidation state of iron. Several bacteria have the ability to reduce ferric iron to ferrous iron, including *Shewanella putrefaciens* and several LAB (Yun et al, 2007). This iron reduction may be assimilatory or dissimilatory. In assimilatory iron reduction, the ferrous iron is taken up into the cell while in dissimilatory reduction the iron is only used as an electron acceptor and reduced iron accumulates outside of the cell (Yun et al, 2007). To sequester iron from their surrounding environment before uptake into the cell, several microorganisms produce iron chelators known as siderophores (Cox, 1980). This allows them to access and bind the iron before transport into the cell.

In this study, the effect of *C. maltaromaticum* on the state of myoglobin and iron was determined, to assess the ability to reduce ferrous iron to ferric iron. The ability of *C. maltaromaticum* to produce siderophores was also investigated. Results were compared to those of other LAB and *Carnobacterium* spp., as well as to a known iron reducer (*Pseudomonas aeruginosa*). *P. aeruginosa* produces

both iron reductases and siderophores (Cox, 1980) and was used as a positive control in assays for iron reduction and siderophore production in this study.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains

All bacterial strains were obtained from the University of Alberta Food Microbiology culture collections and were stored at -80°C in 20% glycerol (Fisher Scientific, Fair Lawn, NJ, USA) and subcultured twice in modified de Man, Rogosa and Sharpe (mMRS) broth. Modified MRS broth (900 mL) contained: 10 g peptone, 5 g beef extract, 5 g yeast extract, 10 g malt extract, 4 g $K_2HPO_4 \cdot 3H_2O$, 2.6 g KH_2PO_4, 3 g NH_4Cl, 0.1 g $MgSO_4 \cdot 4H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$ and 0.5 g L-cysteine HCl. After autoclaving, an autoclaved solution of 5 g dextrose, 5 g fructose and 10 g maltose in 100 mL H_2O was added.

C. maltaromaticum UAL307, *C. maltaromaticum* UAL26, *Carnobacterium mobile* ATCC 49516 and *Listeria innocua* ATCC 33090 were grown at 22°C for 18 h. *Enterococcus faecalis* 710C and *P. aeruginosa* ATCC 27853 were grown at 35°C for 18 h. *Lactobacillus sanfranciscensis* ATCC 20451 was grown at 30°C for 18 h.

3.2.2 Evaluation of the state of myoglobin

Myoglobin from equine skeletal muscle (Sigma Aldrich, St. Louis, MD, USA) was added to mMRS broth at a concentration of 3 mg/mL, and the solution was filter sterilized (0.22 μm pore size, Millipore Corp., Billierica, MA, USA). This mixture was divided into 30 mL portions to which 0.3 mL of 18 h microbial

cultures were added. Control samples contained no inoculum. Samples were incubated at the optimum growth temperature for the organism or at 4ºC under aerobic conditions. The optical density (600 nm) was measured, and samples were centrifuged (5,000 x *g*, 5 min) to remove bacterial pellets before measuring the absorbance of the supernatant at 4 different wavelengths (503, 525, 557, 582 nm). This was done to prevent interference of bacterial cells with absorbance readings.

The effect of bacterial cells on the state of myoglobin under anaerobic conditions (22°C) was also determined. The medium for anaerobic samples was stored in an anaerobic hood (model 1025/1029 Forma Anaerobic System, Thermo Fisher Scientific, Fair Lawn, NJ, USA) for 7 d prior to inoculation with aerobically grown bacterial cultures in the anaerobic hood. Inoculated media were dispensed into microcentrifuge tubes for each sampling. At each sampling time, the microcentrifuge tubes were removed from the anaerobic hood and centrifuged to remove bacterial cells. Samples were put back into the anaerobic hood, the supernatant was transferred to cuvettes and covered with mineral oil as a barrier to oxygen. This was done as it was necessary to move them outside of the anaerobic hood to measure absorbance in a spectrophotometer (model DU 730; Beckman Coulter, Mississauga, Canada).

The percentages of oxymyoglobin (OxyMb), metmyoglobin (MetMb) and deoxymyoglobin (DeoMb) were calculated according to the formulae of Tang et al (2004) (Eq. 1-3):

$$[\text{OxyMb}]\% = (0.722R_1 - 1.432R_2 - 1.659R_3 + 2.599) \times 100 \quad (1)$$

$$[\text{MetMb}]\% = (-0.159R_1 - 0.085R_2 + 1.262R_3 - 0.520) \times 100 \quad (2)$$

$$[\text{DeoMb}]\% = (-0.543R_1 + 1.594R_2 + 0.552R_3 - 1.329) \times 100 \quad (3)$$

Where $R_1 = A_{582}/A_{525}$, $R_2 = A_{557}/A_{525}$, $R_3 = A_{503}/A_{525}$ and where A is the absorbance value recorded at 582, 525, 557, 503 nm, respectively.

3.2.3 Evaluation of iron reduction

A modified ferrozine assay (Viollier et al, 2000) was used to determine the conversion of ferric iron to ferrous iron by bacterial cultures. The following reagents were used:

Reagent A: 0.01 M ferrozine (Sigma Aldrich) in 0.1 M ammonium acetate.

Reagent B: 1.4 M hydroxylamine hydrochloride in 2 M HCl

Reagent C: 10 M ammonium acetate buffer

After 18 h incubation, 10 mL aliquots of cell cultures were centrifuged (6,000 x *g*, 10 min) and washed with 0.1% peptone (Difco, Sparks, MD, USA). Pellets were dispersed in 10 mL 0.1% peptone and 0.2 mL was added to 20 mL of a modified Casamino acids (CAA) broth containing: 3 g/L casamino acids (Difco), 2 g/L glucose (Fisher) and 10,000 µg/L Fe^{3+} (in the form of $FeCl_3$). Measurements were taken at 0, 3, 6, 24, 48, 72, 96, 120, 146, 192 and 218 h by removing a 1.5 mL aliquot of this mixture and centrifuging (8,000 x *g* for 4 min). The supernatant was added to 100 µl of reagent A and the absorbance was measured at 562 nm (A_1). A reduction step followed in which 800 µl of the solution was combined with 150 µl of reagent B and allowed to react for 10 min before the addition of 50 µL of reagent C. The absorbance was read again at 562 nm (A_2).

Ferrous iron concentration was calculated using standard curves created using $FeCl_3$ and $FeSO_4$ and deriving the equation:

$$[Fe^{2+}] = [(A_1 \times 0.3073) - (A_2 \times 0.0079)]/0.115614$$

3.2.4 Production of siderophores

All glassware was washed with 6M HCl to remove residual iron. Iron free nutrient (IFN) broth was made with 3g/L beef extract, 5g/L peptone and 3 g/L glucose in milli-Q water. To remove iron, Chelex 100 (5 g) was added to 100 mL of the broth and the mixture was stirred at 22°C for 2 h (Bio-Rad Laboratories). The resin was removed by filtration and the broth was autoclaved.

Frozen cultures were subcultured in APT broth and incubated for 18 h. A 0.1 mL aliquot was transferred to IFN broth. After cultures were incubated for 48 h at the optimum growth temperature for each culture, 50 µL was spotted onto IFN agar (IFN broth with 15 g/L agar) and incubated for 48 h. A modified version of the Overlaid Chrom azural S (O-CAS) method of Pérez-Miranda et al (2007) was used to determine siderophore production. Plates were over layered with Chrome azural S (CAS) agar and incubated for 48 h at 22°C. The CAS agar was prepared as follows: 36.45 mg of hexadecytrimethylammonium bromide (Sigma Aldrich) was dissolved in 20 mL of milli-Q water and 30.25 mg of CAS; (Sigma Aldrich) was dissolved in 25 mL of milli-Q water and a 5 mL aliquot of a solution with 1mM $FeCl_3 \cdot 6H_2O$ in 10 mM HCl was added. The two mixtures were combined to form a dye and autoclaved. A 15.12 g portion of 1,4-piperazinediethanesulfonic acid (PIPES) (Sigma Aldrich) was dissolved in 440 mL of milli-Q water and 6 g (50% w/w) NaOH was added. If needed, the pH was

adjusted to 6.8 and 7.5 g of agar was added. After autoclaving, all solutions were cooled to 50°C and the dye was slowly added to the PIPES solution while stirring. Plates were assessed visually for colour change indicating production of siderophores. A change from the original blue colour of the agar to orange indicated a hydroxamate type of siderophore, while a purplish hue indicated the presence of catechol (Pérez-Miranda et al, 2007).

3.2.5 Statistical analysis

Percentage values for myoglobin content were transformed so that the data followed a normal distribution, and the data was subjected to analysis of variance using the PROC GLM procedure in SAS version 9.1 software (SAS Institute, Inc., Cary, NC, USA). Student-Newman-Keuls (SNK) test was used for comparison of treatment means. An α value of 0.05 was used as the level of significance. Results of the ferrozine assay were analyzed using PROC GLM.

3.3 RESULTS

3.3.1 Evaluation of the state of myoglobin

To determine the ability of microorganisms to change the state of myoglobin organisms were grown in mMRS containing myoglobin. When *C. maltaromaticum* UAL307 was added to mMRS with 0.3% myoglobin and incubated at 22°C, the level of OxyMb increased within 30 min. After 2 h of incubation the proportion of OxyMb reached a maximum of 80% before slowly decreasing to almost zero during 48 h of storage (Figure 3-1). When the

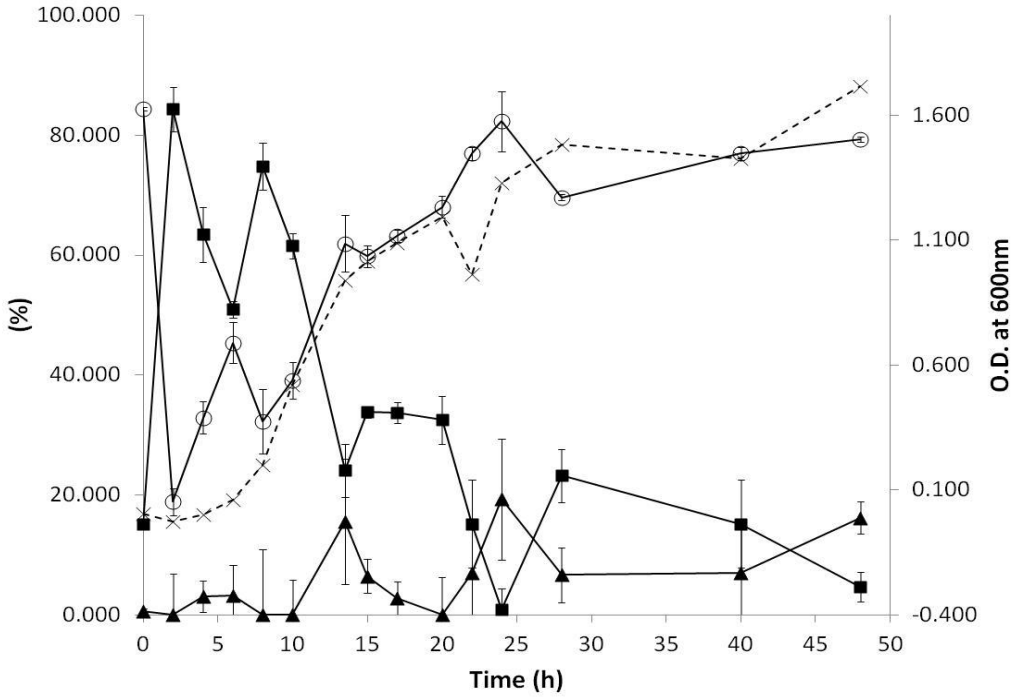

Figure 3-1: Growth (OD_{600}) and concentration of different forms of myoglobin (expressed as a percentage of total myoglobin present in the medium) in mMRS broth containing 0.3% myoglobin inoculated with *C. maltaromaticum* UAL307 and stored aerobically at 25°C. Deoxymyoglobin (Δ), Oxymyoglobin (■) and Metmyoglobin (○). Growth was measured in O.D. (-x-). n = 3; error bars are standard deviations. (Moquin, unpublished data)

concentration of OxyMb reached its maximum, the corresponding OD indicated that *C. maltaromaticum* UAL307 was still in its lag phase. The concentration of oxymyoglobin decreased when *C. maltaromaticum* UAL307 reached the stationary phase of growth. When OxyMb concentration decreased, MetMb increased, while deoxymyoglobin content remained below 20% during the entire storage time.

When the myoglobin content of other bacterial strains was investigated, it was demonstrated that although *P. aeruginosa* ATCC 27853 and *E. faecalis* 710C were able to temporarily increase levels of oxymyoglobin to over 30%, those levels dropped after less than 8 h, while both *C. maltaromaticum* strains were able to maintain oxymyoglobin levels above 50% for 48 h (Figure 3-2). In samples containing *P. aeruginosa* and *E. faecalis*, following centrifugation, the colour in the medium remained with the pellet rather than in solution. Since myoglobin was no longer present in the solution, myoglobin content was not measured after 12 h and 24 h for *E. faecalis* and *P. aeruginosa*, respectively.

When grown at refrigeration temperature (Figure 3-3), samples containing *C. maltaromaticum* UAL307 reached a maximum OxyMb concentration of almost 90%, but it took 36 h for this maximum to be achieved as compared to 4 h when samples were incubated at 22ºC. The OxyMb concentration was also maintained for a longer time at 4ºC, remaining above 70% for 125 h of storage. OxyMb concentrations in samples containing *C. maltaromaticum* UAL26 were not significantly different from those in samples containing *C. maltaromaticum* UAL307. *C. mobile* did not cause a significant increase in OxyMb concentration until 100 h of storage, reaching ca. 30%. All other cultures did not significantly increase the proportion of OxyMb during 125 h storage.

Figure 3-4 illustrates the colour difference observed visually among treatments with different concentrations of oxymyoglobin. There was an increase in red colour when *C. maltaromaticum* was stored at 4°C for 48 h. After 12 h of storage at 35°C, the supernatant samples containing *E. faecalis* reverted to the

original yellow colour of mMRS without added myoglobin, as a brown precipitate was removed with the cell pellet.

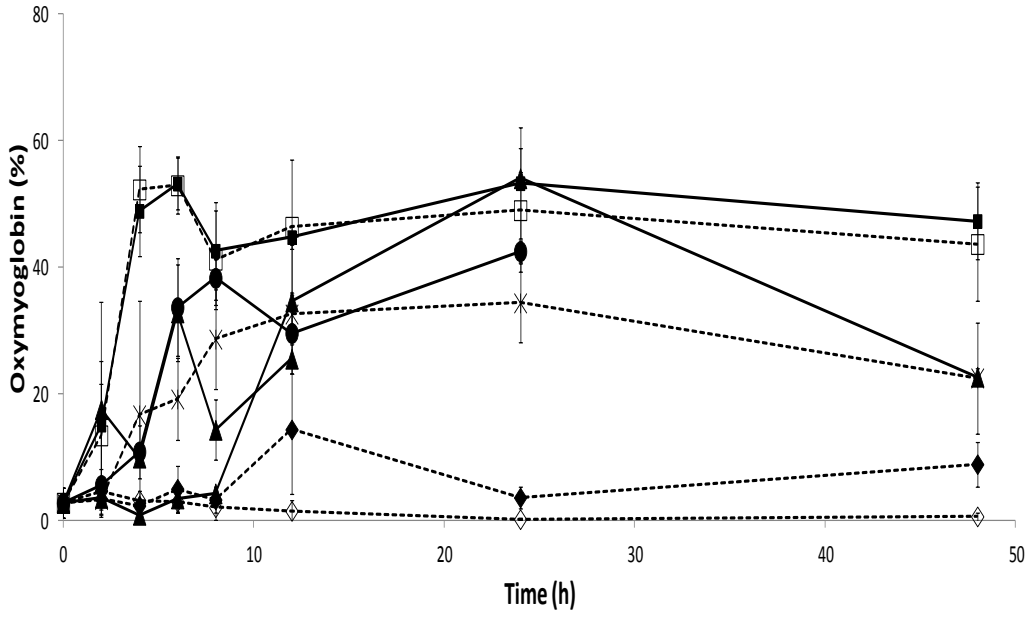

Figure 3-2: Mean concentration of oxymyoglobin (expressed as a percentage of total myoglobin present in the medium) in mMRS broth containing 0.3% myoglobin stored aerobically at the optimum growth temperature for *C. maltaromaticum* UAL307 (■) and UAL26 (-□-), *C. mobile* ATCC 49516 (▲), *L. innocua* ATCC 33090 (-x-), *E. faecalis* 710C (○), *P. aeruginosa* ATCC 27853 (●) and *L. sanfranciscensis* ATCC 20451 (-♦-). A control contained no inoculum (-◊-). n = 4 and error bars are standard deviations.

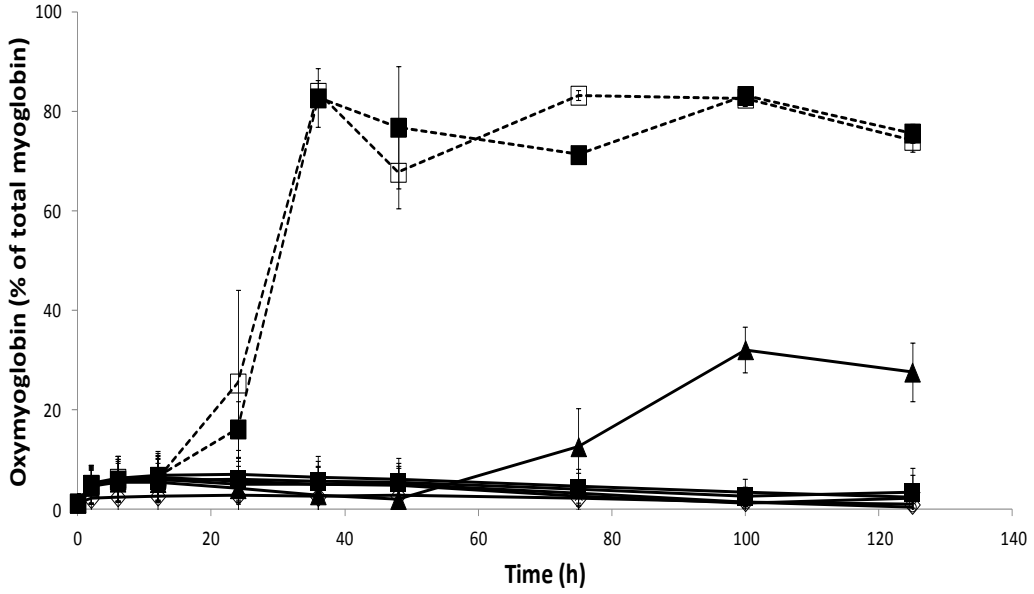

Figure 3-3: Mean concentration of oxymyoglobin (expressed as a percentage of total myoglobin present in the medium) in mMRS broth containing 0.3% myoglobin stored aerobically at 4^{o}C. Samples were inoculated with *C. maltaromaticum* UAL307 (-■-) and UAL26 (-□-), *C. mobile* ATCC 49516 (▲), *L. innocua* ATCC 33090 (-x-), *E. faecalis* 710C (○), *P. aeruginosa* ATCC 27853 (●) and *L. sanfranciscensis* ATCC 20451 (♦). A control contained no inoculum (-◊-). n = 6 and error bars are standard deviations.

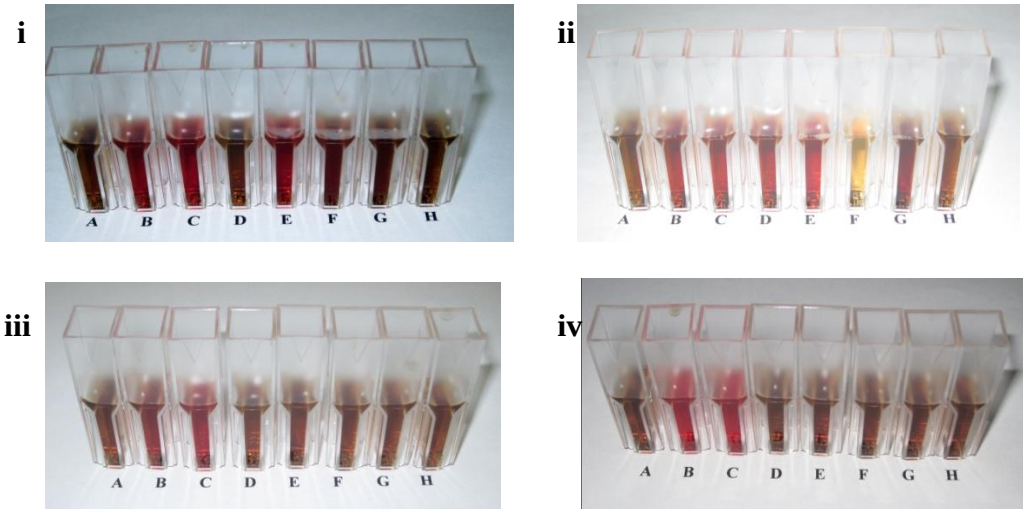

Figure 3-4: Colour development in mMRS broth containing 0.3% myoglobin inoculated with bacteria and stored at the optimum growth temperature of the respective culture for 4 h (i) and 12 h (ii), and stored at 4°C for 24 h (iii) and 48 h (iv). A) Control, B) *C. maltaromaticum* UAL307, C) *C. maltaromaticum* UAL26, D) *C. mobile*, E) *L. innocua*, F) *E. faecalis* 710C, G) *P. aeruginosa* ATCC 27853, H) *L. sanfranciscensis*

Under anaerobic storage (Figure 3-5), no oxymyoglobin was present, only metmyoglobin and deoxymyoglobin. When deoxymyoglobin concentrations increased in the samples, there was a corresponding increase in the red colour of the samples. *C. maltaromaticum* UAL307 and UAL26 increased deoxymyoglobin levels to almost 100% after 6 h, and were able to maintain deoxymyoglobin levels for 72 h. Similar to what was observed when *E. faecalis* 710C was stored under aerobic conditions at optimum growth temperature, it temporarily increased the red colour of the samples. However, after 24 h the concentration of deoxymyoglobin began to decrease and was below 20% by the end of storage.

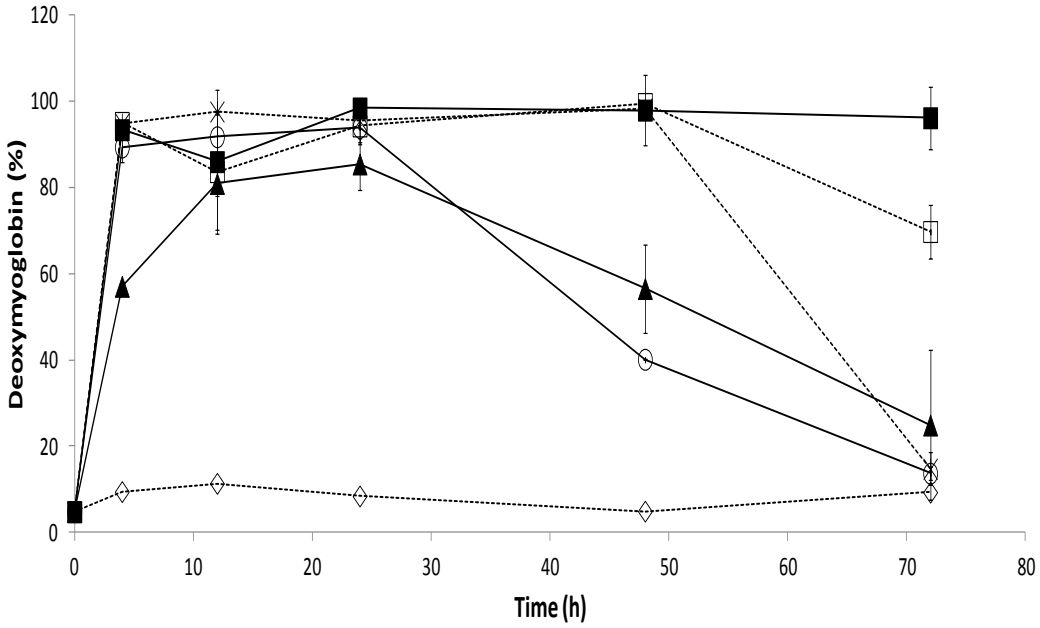

Figure 3-5: Mean concentration of deoxymyoglobin (expressed as a percentage of total myoglobin present in the medium) in mMRS broth containing 0.3% myoglobin stored anaerobically at 22°C. Samples were inoculated with *C. maltaromaticum* UAL307 (■) and UAL26 (□), *C. mobile* ATCC 49516 (▲), *L. innocua* ATCC 33090 (-x-) and *E. faecalis* 710C (○). A control contained no inoculum (◊). n = 3. Error bars are standard deviations.

3.3.2 Iron reduction

There was evidence of iron reduction by *C. maltaromaticum* UAL307, with 200 µg/L ferrous iron being converted from ferric iron to ferrous iron (Figure 3-6). This was not as high as that produced by the positive control *P. aeruginosa* (ca. 500 µg/L), but was higher than that of all other organisms, which stayed below 100 µg/L. Although the peak concentration in samples containing *P. aeruginosa* was more than double compared to that in samples containing *C.*

maltaromaticum UAL307, the concentration dropped after 48 h of incubation and after 75 h of incubation it had fallen below that of the *C. maltaromaticum* samples. The samples containing *C. maltaromaticum* UAL307 maintained a fairly consistent concentration of ferrous iron once it had reached its peak, staying between ca. 130 and 200 µg/L for 216 h. No other organisms evaluated in this study exhibited iron reduction.

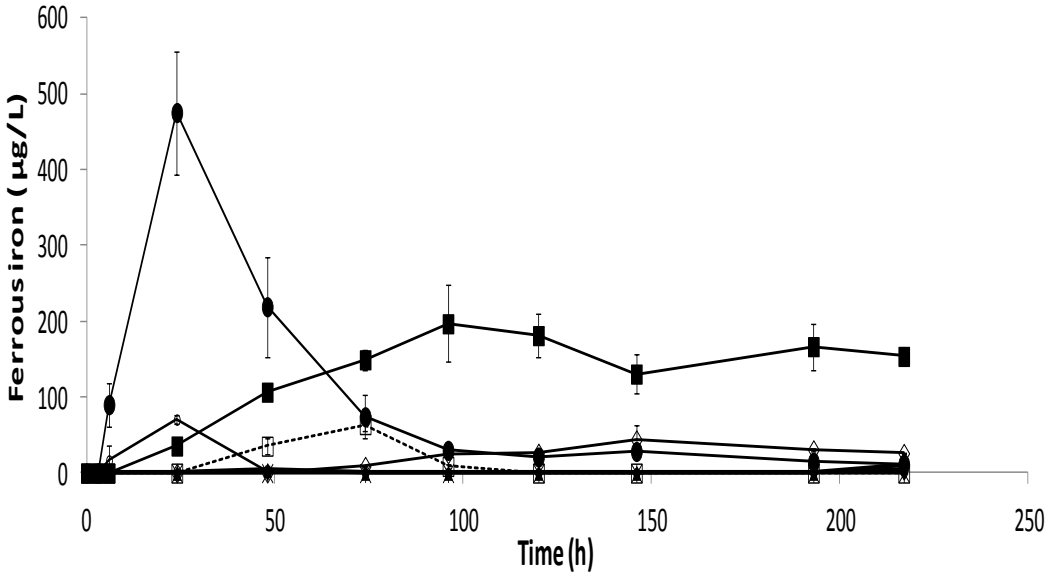

Figure 3-6: Concentration of ferrous iron in a modified CAA medium inoculated with *C. maltaromaticum* UAL307 (■) and UAL26 (-□-), *C. mobile* ATCC 49516 (▲), *L. innocua* ATCC 33090 (x), *E. faecalis* 710C (○), *P. aeruginosa* ATCC 27853 (●) and *L. sanfranciscensis* ATCC 20451 (◊) and incubated at 22°C. n = 4 and error bars are standard deviations.

3.3.3 Production of siderophores

The results of the CAS assay indicated that *C. maltaromaticum* UAL307 and UAL26 did not produce siderophores. In addition, *L. innocua* and *C. mobile* did not produce siderophores (results not shown). The positive control *P. aeruginosa* did cause a colour change in the CAS media from blue to orange and purple (Figure 3-7) indicating siderophore production.

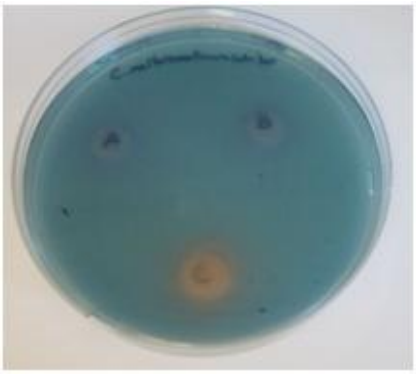

Figure 3-7: CAS agar plate inoculated with *C. maltaromaticum* UAL307 (A, B) and *P. aeruginosa* (C) and incubated for 48 h at 22°C.

3.3 DISCUSSION

Results indicate that the addition of *C. maltaromaticum* to broth containing myoglobin resulted in the conversion of metmyoglobin to oxymyoglobin at both 22°C and 4°C. Maximum concentrations of oxymyoglobin correlated with an OD that corresponded to the lag phase of growth. During the exponential growth phase, concentrations of oxymyoglobin decreased and those

of metmyoglobin increased. This suggests that there is a link between the metabolism of *C. maltaromaticum* and its ability to convert oxymyoglobin to metmyoglobin.

C. maltaromaticum was able to maintain the red colour of the mMRS broth containing myoglobin under anaerobic conditions, resulting from the conversion of metmyoglobin to deoxymyoglobin. Under aerobic conditions, it converted metmyoglobin to oxymyoglobin. This indicates that the organism affects the state of iron in the myoglobin, since the only difference between deoxymyoglobin and oxymyoglobin is oxygenation and both compounds have iron in the ferrous state. This supposition is supported by the results of the iron reduction assay. In the case of *P. aeruginosa* and *E. faecalis*, the removal of the colour in the media with the pellet after centrifugation indicated that the myoglobin was being removed with the bacterial pellet. This may be due to heme binding by the two bacteria (Nygaard et al, 2006).

C. maltaromaticum UAL307 was the only organism used in this study that was able to reduce ferric iron to ferrous iron other than the positive control. However, unlike the positive control (*P. aeruginosa) C. maltaromaticum* was able to maintain the iron in this state, whereas ferrous iron levels in broth inoculated with *P. aeruginosa* decreased during incubation. This was likely due to uptake of the iron by bacterial cells, which were removed during centrifugation. *P. aeruginosa* produces several siderophores and is able to take iron into the cell (Ankenbauer et al, 1988). The CAS assay showed that *C. maltaromaticum* does not produce siderophores to facilitate iron uptake. These results are supported by

the results of Pandey et al (1994) who investigated the production of siderophores by lactic acid bacteria and none of the organisms, including *C. mobile* and *Carnobacterium piscicola*, produced siderophores. Therefore, iron reduction by *C. maltaromaticum* is dissimilatory, allowing reduced iron to accumulate in the medium.

These results suggest that the ability of *C. maltaromaticum* UAL307 to stabilize the red colour of fresh sausage (Chapter 2, this thesis) is the result of a conversion of metmyoglobin to oxymyoglobin. Furthermore, this conversion may be due to the organism's ability to reduce ferric iron to ferrous iron within the heme group of the myoglobin molecule.

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Chapter Four: The utilization of heme and myoglobin by *Carnobacterium maltaromaticum* and its effect on metabolism

4.1 INTRODUCTION

The previous research has shown that in addition to its antimicrobial activity, *C. maltaromaticum* has the ability to prolong the red colour of aerobically stored fresh beef sausages. Subsequent investigation demonstrated that this ability is due to the conversion of metmyoglobin to oxymyoglobin and deoxymyoglobin under aerobic and anaerobic conditions respectively, and that *C. maltaromaticum* has the ability to maintain myoglobin in these states during prolonged storage. Further investigation showed that *C. maltaromaticum* has the ability to reduce ferric iron to ferrous iron, which could explain its colour stabilizing properties as the oxidation state of iron is an important factor in the state of myoglobin; oxymyoglobin contains iron in the ferrous state while metmyoglobin contains ferric iron (Robertson, 1993). Although this ability has not yet been explained, it is known that the metabolic activity of some lactic acid bacteria can change from fermentation to respiration due to the presence of heme. Although these organisms are lacking some of the biosynthetic enzymes necessary for the production of cytochrome oxidases, unlike organisms such as *Bacillus subtilis* and *Escherichia coli*, they are able produce cytochrome oxidases with the addition of exogenous heme, which acts as a cofactor of cytochrome complexes (Brooijmans et al, 2007). *Lactococcus lactis* is able to utilize heme to

produce a *bd*-type cytochrome (Gaudu et al, 2002). Cytochrome *bd* is a terminal oxidase that is present in several bacteria. This enzyme has been well studied in *E. coli* and several other Gram-negative bacteria, but has also been found in some Gram-positive bacteria, namely *B. subtilis* and *L. lactis*. There are four genes encoding the *bd*-type cytochrome. The *cydA* and *cydB* genes form one operon, encoding the two subunits of the complex, while *cydC* and *cydD* encode an ATP-binding cassette (ABC) transporter that is required for the assembly of the cytochrome oxidase (Winstedt et al, 1998). These genes are also present in *C. maltaromaticum* ATCC 35586 (Leisner, personal communication).

The production of cytochrome oxidase causes a change in the metabolism of *L. lactis* under aerobic conditions, from fermentative where pyruvate is converted to lactate by NAD-dependent lactate dehydrogenase (LDH), to respiration where an ETC generates energy through NADH oxidation (Nagayasu et al, 2007). This leads to increased growth efficiency and oxygen consumption (Brooijmans et al, 2007). Such changes or lack thereof in the ETC of *C. maltaromaticum* may help explain its ability to stabilize oxymyoglobin in meat while other LAB do not.

In this study, the utilization of heme by *C. maltaromaticum* was investigated by evaluating its ability to bind heme as a method of accessing the compound. The effect of heme and myoglobin on growth efficiency and metabolism was determined by assessment of NADH oxidase and LDH activity. The ability of *C. maltaromaticum* UAL307 to produce cytochrome oxidases through expression of cytochrome *bd* genes was also determined. Evaluations

were carried out under the same conditions as those used to determine the organisms effect on the state of myoglobin (Chapter 3, this thesis).

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

All bacterial strains were obtained from the University of Alberta Food Microbiology culture collection and were stored at -80°C in 20% glycerol (Fisher Scientific, Fair Lawn, NJ, USA) and subcultured twice in modified de Man, Rogosa and Sharpe (mMRS) broth. mMRS broth (900 mL) contained: 10 g peptone, 5 g beef extract, 5 g yeast extract, 10 g malt extract, 4 g $K_2HPO_4 \cdot 3H_2O$, 2.6 g KH_2PO_4, 3 g NH_4Cl, 0.1 g $MgSO_4 \cdot 4H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$ and 0.5 g L-cysteine HCl. After autoclaving, an autoclaved solution of 5 g dextrose, 5 g fructose and 10 g maltose in 100 mL H_2O was added.

C. maltaromaticum UAL307, *C. maltaromaticum* UAL26, *Carnobacterium mobile* ATCC 49516 and *Listeria innocua* ATCC 33090 were grown at 22°C for 18 h. *Enterococcus faecalis* 710C and *P. aeruginosa* ATCC 27853 were grown at 35°C for 18 h. *Lactococcus lactis* ATCC 11454 was grown at 30°C for 18 h.

4.2.2 Preparation of media

Myoglobin from equine skeletal muscle (Sigma Aldrich, St. Louis, MO, USA) was added to mMRS broth at a concentration of 3 mg/mL, and the mixture was filter sterilized (0.22 µm pore size, Millipore Corp., Billierica, MA, USA). To prepare a solution of 10 mg/L hematin in mMRS, a stock solution of hematin was

made by dissolving 0.05g of hematin (Sigma Aldrich) in 1 mL 1N NaOH and adding it to 99 mL H_2O. After autoclaving, the 0.5g/L stock solution was added to autoclaved mMRS to a final concentration of 1 mg/L.

4.2.3 Effect of hematin on bacterial growth efficiency

Volumes of 25 mL of 0.3% myoglobin in mMRS and 1 mg/L hematin in mMRS were distributed into sterile 50 mL culture tubes. Bacterial cultures (250 µL) were added to each tube and samples were incubated at 22°C with shaking at 150 rpm in a shaking incubator (Infors HT, Bottmingen, Switzerland). At 2, 6, 18 and 24 h, 1 mL samples were taken from each tube and growth was determined by measuring OD at 600 nm using a spectrophotometer (Beckman Coulter, Mississauga, Canada). Triplicate trials were conducted.

4.2.4 Hematin binding activity

Hematin binding activity was determined using a modified version of the method of Tai et al (1997). A standard curve was constructed using concentrations of hematin from 0-15 mg/L in a modified Casamino acids (CAA) medium containing 5g/L casamino acids (Difco, Becton Dickinson, Sparks, MD, USA) and 2 g/L glucose (Fisher Scientific, Fair Lawn, NJ, USA). Absorbance was measured at 405 nm using a spectrophotometer and results were plotted against hematin concentration to obtain a standard curve. Four trials were conducted.

Volumes of 24 mL of modified CAA medium with 10 mg/L hematin were added to sterile 50 mL culture tubes and inoculated with 1 mL of an 18 h bacterial culture. An uninoculated sample served as a negative control and *P. aeruginosa* was used as a positive control. Samples were incubated at 22°C with shaking at 150 rpm. At 1, 2, 5 and 24 h, 1 mL samples were taken and centrifuged at 10,000 x *g* for 3 min. The absorbance of the supernatant was measured at 405 nm and the concentration of hematin was determined using the equation obtained from the standard curve:

$$[\text{Hematin}] = \text{Abs}_{405}/0.063$$

The amount of hematin absorbed by the bacterial cells was determined by subtracting the concentration of hematin in the supernatant from the original hematin concentration of the medium.

4.2.5 Preparation of cell free extracts

Bacterial cultures (5 mL) that had been grown under static conditions for 18 h at 22ºC in mMRS, mMRS with 1 mg/L hematin or mMRS with 0.3% myoglobin were centrifuged at 5,000 x *g* for 10 min at 4°C and washed once in 50 mM potassium phosphate (PPB) buffer, pH 7.0. The washed cell suspensions were centrifuged and cell pellets were re-suspended in 1 mL of PPB buffer. Cells were disrupted using 0.1 mm glass beads in a mini-bead beater (BioSpec Products Inc., Bartlesville, OK, USA). Cell suspensions were beaten for 1.5 min followed by holding 1 min on ice and another 1.5 min of beating. The beads and cell debris were removed from the solution by centrifugation at 12,000 x *g* for 10 min at 4°C.

4.2.6 NADH oxidase activity

The method of De Felipe et al (1998) was used to determine NADH oxidase activity. A standard curve of NADH was created by measuring the absorbance at 340 nm at 25°C of NADH at concentration of 0-300 µg/mL in 50 mM potassium phosphate buffer (PPB) using a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA). A final reaction volume of 1 mL containing 50 mM PPB, 0.29 mM NADH (Sigma Aldrich) and 0.3 mM ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich) was used for enzyme analysis. Cell free extract (10 µL) was added to the mixture to initiate the reaction. The absorbance at 340 nm was monitored over 10 min. Using the standard curve, the NADH concentration was determined with the following equation:

$$[NADH] = A_{340}/0.003322$$

The amount of NADH converted to NAD over time was found by monitoring the decrease in absorbance over time. A unit of NADH oxidase enzyme was defined as the amount required to catalyze the conversion of 1 µmol of NADH to NAD per min. Triplicate trials were conducted and each sample was measured in duplicate.

4.2.7 LDH activity

Enzyme activity was determined using the method of Zhou et al (2003). To initiate the reaction, 10 µl of cell free extract was added to a mixture containing 30 µl of 1 M sodium pyruvate (Sigma Aldrich), 192 µL of 1mM NADH, 400 µl of 50 mM morpholinepropanesulfonic acid buffer (MOPS, Sigma

Aldrich), pH 7.0 and 368 µL of milliQ H_2O. The amount of NADH consumed over 10 min was monitored using a Varioskan to measure absorbance at 340 nm and the NADH standard curve previously constructed to determine NADH oxidase activity. A unit of LDH enzyme was defined as the amount required to catalyze the conversion of 1 µmol of NADH to NAD per min. Triplicate trials were conducted and each sample was measured in duplicate.

4.2.8 Cytochrome oxidase gene expression

Relative gene expression analysis was used to determine the effect of hematin and myoglobin on expression of the genes responsible for cytochrome oxidase activity in *C. maltaromaticum* UAL26 and UAL307. Bacterial cultures (100 µl) were added to 10 mL of one of 3 solutions: mMRS, mMRS with 1 mg/L hematin or mMRS with 0.3% myoglobin and solutions were incubated for 8 h. Solutions were incubated at 22°C under static or, shaking conditions, and under anaerobic conditions in an anaerobic hood (model 1025/1029 Forma Anaerobic System; Thermo Fisher Scientific, Bath, UK). Relative gene expression was determined after cells had been incubated for 8 h. This ensured that the cells were in the exponential phase of growth, an important factor because in *L. lactis cydAB* genes are involved in respiration late in the exponential phase of growth; the metabolism of the cells is still fermentative during the lag phase (Gaudu et al, 2002). To verify that the cells were in the exponential phase of growth, OD was measured at 0, 3, 6 and 8 h of incubation. After 8 h, aliquots of 500 µL were removed from the samples and RNAprotect (Qiagen, Hilden, Germany) was used to stabilize the RNA of the samples. Protected cell pellets were stored at -80°C

until RNA isolation. RNA was isolated using Trizol® and a PureLink® RNA spin column system (Ambion®, Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Isolated RNA was treated with DNase I (Invitrogen®, Life Technologies Corporation, Carlsbad, CA, USA) to remove any remaining DNA. A 10 µL sample was added to 1.25 µL of DNase I and 1.25 µL of 10 x DNase Buffer (Invitrogen®). The mixtures were incubated at 37°C for 30 min in a Mastercycler Thermal Cycler (Eppendorf, Hamburg, Germany). The reaction was terminated by the addition of 0.65 µL of EDTA and incubation at 70°C for 10 min and tubes were immediately put on ice.

Reverse transcription of 2 µL of DNase treated RNA was carried out using a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Following reverse transcription cDNA samples were stored at -20°C until quantitative PCR analysis.

A QuantiTect SYBR green kit (Qiagen) was used to carry out qPCR in an Mx4000™ Multiplex Quantitave PCR System (Stratagene, La Jolla, CA, USA). The cycling protocol is shown in Table 4-1. A reaction volume of 25 µL was used and contained a final concentration of 3 µM each of forward and reverse primers, 12.5 µL of 2 x SYBR green PCR Master Mix (which contained HotStarTaq DNA Polymerase, SYBR Green PCR Buffer, dNTP mix including dUTP, SYBR Green I, ROX passive reference dye and 5 mM $MgCl_2$) and 2 µL of cDNA template. The primers for *cydA*, *cydC* and *cydD* (Table 4-2) were designed using PrimerQuest^{SM} (IDT, http://www.idtdna.com/Scitools/Applications/Primerquest/Default.aspx) based on the genome sequence of *C. maltaromaticum* ATCC 35586 provided by

Dr. Jørgen Leisner at the University of Copenhagen. Validation experiments were carried out to ensure that the efficiencies of the target genes and the normalization reference gene were approximately equal.

Table 4-1: qPCR cycling protocol used to investigate the expression of cytochrome oxidase genes.

Step	**Time**	**Temp (°C)**	**Cycles**
Polymerase activation	15 min	95	1
Denaturation	15 s	94	
Annealing	20 s	55	40
Extension and data collection	30 s	72	

Table 4-2: Oligonucleotide primers used to investigate expression of cytochrome oxidase genes

Name	**Sequence (5' – 3')**
cydA forward	GTCGCAGTGGTTGCGTTATGGTTT
cydA reverse	CCAACCGGCTGTTGTCCCAATAAA
cydC forward	AGCCGCTAACTGGATAGGTGCTTT
cydC reverse	ATTCAGAAACCGCAGTTGGAACCG
cydD forward	ACGAACCAACGGCTCACTTAGACA
cydD reverse	CCAATGAAGTCGATGCGTGAA

The relative expression ratio of each of the three target genes was determined using the following equation:

$$R = 2^{-(\Delta Ct\ sample - \Delta Ct\ calibrator)}$$

Where R is the relative expression ratio of the target gene, Ct is the amplification cycle at which fluorescence exceeds the threshold level for quantification and ΔCt is the Ct of the normalization reference subtracted from the Ct of the sample. The calibrator used was the respective gene (*cydA*, *cydB* or *cydD*) when the bacterial culture was incubated in mMRS without hematin or myoglobin, and 16S rRNA was used as a normalization reference. A relative expression ratio over 1 indicated upregulation of the gene. Negative controls for qPCR consisted of the reaction mixture without cDNA and water or RNA that had not undergone reverse transcription. Isolated chromosomal DNA was used as a positive control. All samples were measured in duplicate. Triplicate trials were conducted.

4.2.9 Statistical analysis

Data obtained in all experiments except relative gene expression was subjected to analysis of variance using the PROC GLM procedure in SAS version 9.1 software (SAS Institute, Inc., Cary, NC, USA) and Student-Newman-Keuls (SNK) test was used to compare individual treatment means overall as well as to determine if there was a significant difference among individual treatment means for each organism. An α value of 0.05 was used as the level of significance for the ANOVA.

4.3 RESULTS

4.3.1 Effect of hematin on bacterial growth efficiency

The effect of hematin on the growth of *L. lactis* was greater than the effect on the other organisms evaluated (Figure 4-1). At the final sample point of 24 h, the absorbance of the *L. lactis* grown with hematin was 0.3 units higher than without added hematin. This difference in growth began to occur at 6 h of incubation during the exponential phase of growth, where the OD of the culture grown in the presence of hematin began to increase more rapidly than that grown without hematin. The growth of all other organisms was not significantly affected by the presence of hematin.

4.3.2 Hematin binding activity

A hematin binding assay was done to determine the ability of the organisms to bind heme in bacterial media. The positive control *P. aeruginosa* was able to bind almost all of the 10 mg/L of hematin in the medium after 5 h (Figure 4-2). The LAB evaluated did not exhibit hematin binding to that extent, although all were significantly higher than the negative control. *L. lactis* had the highest binding activity of the LAB, binding 1.68 mg/L after 5 h of incubation, but increasing to 7.06 mg/L after 24 h. *E. faecalis* bound 1.93 mg/L after 24 and *C. maltaromaticum* had the lowest binding activity, absorbing only 1.39 mg/L after 24 h.

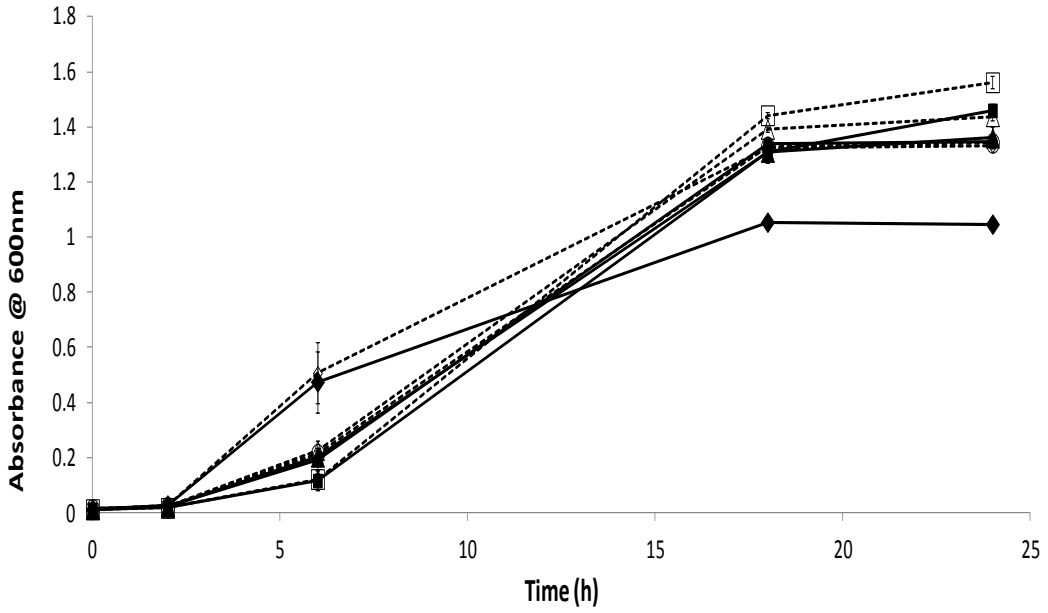

Figure 4-1: The effect of hematin on bacterial growth. *C. maltaromaticum* UAL307 with (-□-) and without (■) hematin, *E. faecalis* 710C with (-Δ-) and without (▲) hematin, *P. aeruginosa* ATCC 27853 with (-○-) and without (●) hematin and *L. lactis* ATCC 11454 with (-◊-) and without (♦) hematin grown at 22°C under aerated conditions in mMRS medium. n = 4 and error bars are standard deviations.

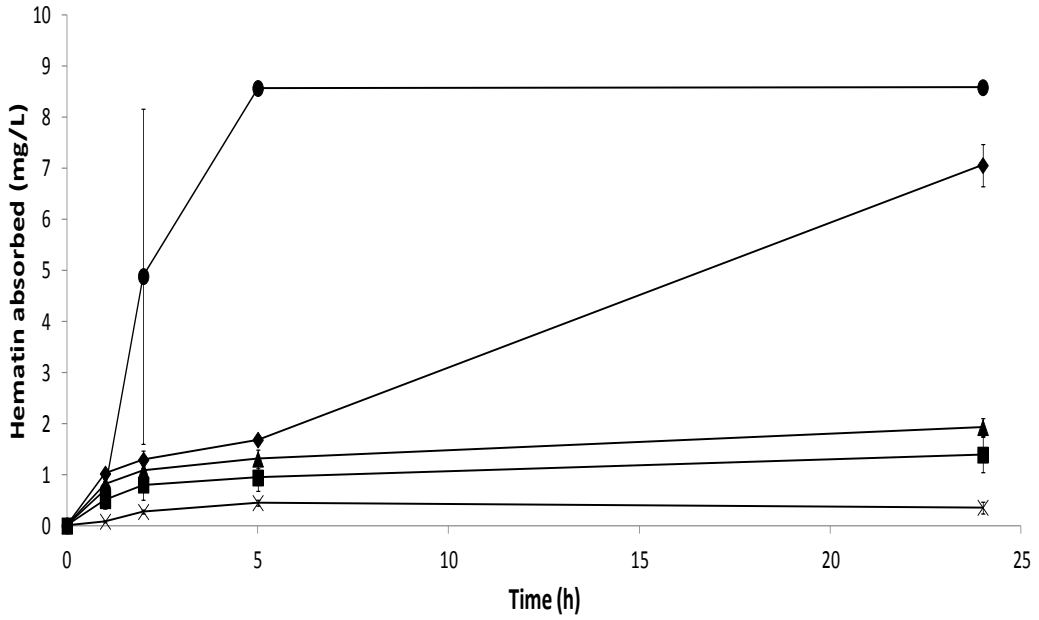

Figure 4-2: Hematin binding activity of several bacteria. *C. maltaromaticum* UAL307 (■), *Enterococcus faecalis* 710C (▲), *P. aeruginosa* ATCC27853 (●) and *L. lactis* ATCC 11454 (♦) grown in CAA medium and incubated at 22°C with shaking. A control contained no inoculum (×). n = 4 and error bars are standard deviations.

4.3.3 NADH oxidase activity

NADH oxidase activity decreased when *C. maltaromaticum* and *L. lactis* were grown in microbiological media containing myoglobin (Figure 4-3). *C. maltaromaticum* also had decreased NADH oxidase activity when grown in the presence of hematin, but there was no significant difference in NADH oxidase activity when *L. lactis* was grown in the presence of hematin compared to mMRS without the addition of hematin or myoglobin. The presence of hematin or myoglobin during growth had no significant effect on the NADH oxidase activity of *C. mobile*.

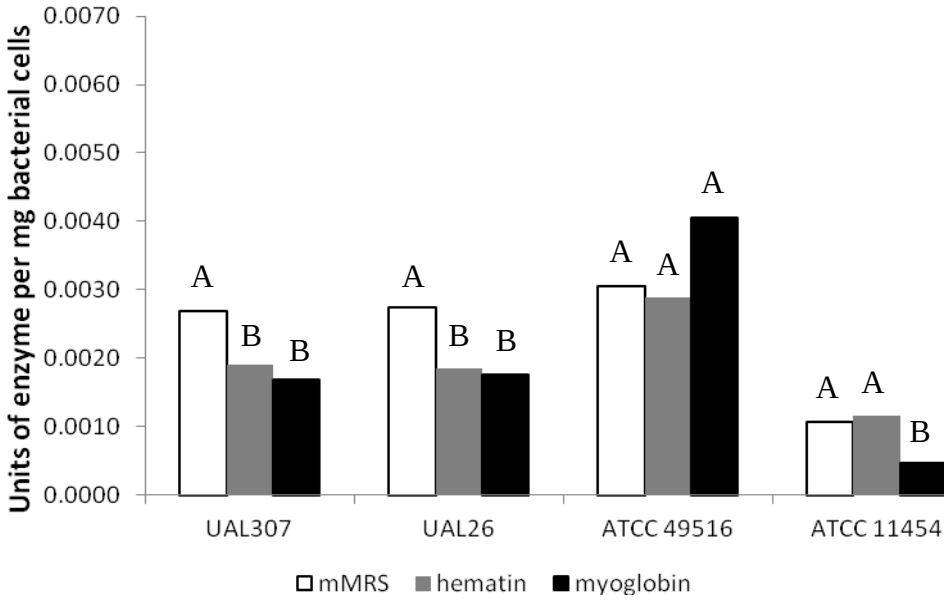

Figure 4-3: Effect of the presence of hematin and myoglobin during bacterial growth at 22°C on the NADH oxidase activity of *C. maltaromaticum* UAL307 and UAL26, *C. mobile* ATCC 49516 and *L. lactis* ATCC 11454 grown at 22°C in mMRS broth, mMRS broth with hematin added or mMRS broth with myoglobin added. n = 3. Within each organism, bars with the same letters are not significantly different ($P > 0.05$).

4.3.4 LDH Activity

The presence of hematin and myoglobin had no significant effect on the LDH activity of *C. maltaromaticum*, *C. mobile* or *L. lactis* (Figure 4-4). However, *C. maltaromaticum* samples showed a slight decrease in LDH activity when the culture was grown in the presence of myoglobin.

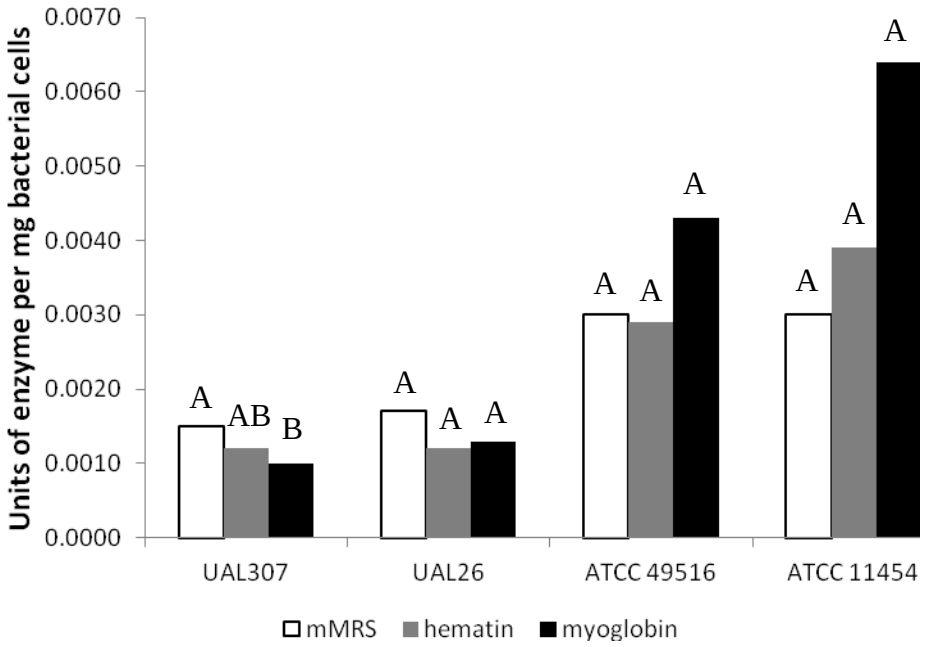

Figure 4-4: Effect of the presence of hematin and myoglobin during bacterial growth at 22°C on the LDH oxidase activity of *C. maltaromaticum* UAL307 and UAL26, *C. mobile* ATCC 49516 and *L. lactis* ATCC 11454 grown at 22°C in mMRS broth, mMRS broth with hematin added or mMRS broth with myoglobin added. n = 3. Within each organism, bars with the same letters are not significantly different ($P > 0.05$).

4.3.5 Cytochrome oxidase gene expression

The presence of hematin or myoglobin during growth did not cause a significant change in the expression of genes related to cytochrome oxidase expression in either strain of *C. maltaromaticum* tested (Tables 4-3 and 4-4). No amplification products were detected in the negative controls.

Table 4-3: Relative expression ratios of *cydA*, *cydC* and *cydD* cytochrome oxidase genes in *C. maltaromaticum* UAL26 after growth under static, aerobic and anaerobic conditions in the presence of hematin or myoglobin. Samples were incubated at 22°C. Results are averages of three replicates ± standard deviations.

Growth Condition	Relative Expression Ratios					
	cydA		***cydC***		***cydD***	
	hematin	**myoglobin**	**hematin**	**myoglobin**	**hematin**	**myoglobin**
Static	0.81±0.08	2.21±3.22	0.23±0.17	0.46±0.45	0.3±0.20	0.75±0.56
Aerobic	2.11±2.59	3.08±3.98	2.85±3.35	0.4±0.51	0.09±0.05	1.99±1.36
Anaerobic	1.56±0.19	1.9±1.92	3.93±2.17	9.66±2.69	4.33±4.0	3.89±3.66

Table 4-4: Relative expression ratios of *cydA*, *cydC* and *cydD* cytochrome oxidase genes in *C. maltaromaticum* UAL307 after growth under static, aerobic and anaerobic conditions in the presence of hematin or myoglobin. Samples were incubated at 22°C. Results are averages of three replicates ± standard deviations.

Growth condition	Relative Expression Ratios					
	cydA		***cydC***		***cydD***	
	hematin	**myoglobin**	**hematin**	**myoglobin**	**hematin**	**myoglobin**
Static	0.39±0.40	0.91±1.10	2.59±3.40	0.44±0.53	0.9±0.36	1.33±1.70
Aerobic	0.40±0.01	1.01±0.14	2.99±0.57	0.49±0.06	0.63±0.38	1.51±0.26
Anaerobic	0.69±0.91	0.72±0.32	1.63±1.45	1.12±1.51	1.08±0.07	1.01±0.18

4.4 DISCUSSION

The experiments in this study were conducted under conditions that would be found in retail meat, microaerobic in the case of aerobically packaged meat and anaerobic conditions similar to vacuum packaged products, to determine whether the heme present in meat caused any changes in the growth and metabolism of *C. maltaromaticum* that could affect the state of myoglobin.

The presence of hematin had no effect on the growth of *C. maltaromaticum* under microaerobic conditions, nor was the organism able to bind significant amounts of the heme compound. These results indicate that *C. maltaromaticum* does not have a mechanism for binding heme to allow the cell access the molecule, and it is not utilizing or taking the compound into the cell. In comparison, *L. lactis* was able to bind heme and increased growth occurred in the presence of heme. These results are in agreement with previous research, which showed that under aerobic conditions and in the presence of heme *L. lactis* undergoes respiration, which leads to an increased biomass (Gaudu et al, 2002). The increase in biomass in *L. lactis* observed in this study was not as significant as that observed by other researchers (Duwat et al, 2001), but this may be due to a lower levels of oxygen present under the growth conditions used in this study; a smaller headspace in the growth medium and a shaking at a lower rpm. The difference in growth between *L. lactis* cultures incubated in the presence of heme and those incubated without occurred during the exponential phase of growth. This corresponds to the findings of researchers, where the switch from fermentation to the more efficient respiration occurs in the log phase (Gaudu et al, 2002). The switch from fermentation to respiration would also be indicated by an increase in NADH oxidase activity, as NADH plays an important role in the electron transport chain during respiration, allowing for increased oxygen consumption in *L. lactis* cells in the presence of heme (Brooijmans et al, 2007). In this study, there was no increase in NADH oxidase activity in *C. maltaromaticum* or *L. lactis* with the addition of heme or myoglobin. The difference in the

behaviour of *L. lactis* from that reported in the literature may be due to the growth conditions used. In this study, the bacterial cultures were grown statically, without aeration, to keep growth conditions closer to the atmosphere present in meat, while those in literature (Duwat et al, 2001; Gaudu et al, 2002; Brooijmans et al 2007; aerated cultures to create an aerobic atmosphere. in the present study, there was no change in LDH in the presence of heme or myoglobin. A decrease in LDH would be expected as NADH oxidase increased (Nagayasu et al, 2007).

The lack of effect with the addition of heme or myoglobin in *C. maltaromaticum* was further illustrated in the lack of upregulation of 3 genes involved in the formation of cytochrome oxidase both under microaerobic and anaerobic conditions.

Previous investigation showed that *L. lactis* does not have the myoglobin stabilizing ability that *C. maltaromaticum* exhibits (Moquin, personal communication). From the results of this study, it can be concluded that *L. lactis* has the ability to bind the heme group in myoglobin. This may prevent the organism from stabilizing the colour of meat by consuming the heme in the compound rather than keeping myoglobin in the oxymyoglobin state. In addition, the increased oxygen consumption could play a role, since it is necessary for oxygen to be bound to the iron within the heme group for myoglobin to be in the oxymyoglobin state (Robach and Costilow, 1961). In contrast, *C. maltaromaticum* did not significantly bind heme, nor did it utilize the compound to produce cytochrome oxidase. Its growth also was unaffected by the presence of heme. This allows for the dissimilatory reduction of the compound without deplenishing

heme or otherwise altering the myoglobin compound. In meat, this would allow for the conversion of oxymyoglobin from metmyoglobin without diminishing the myoglobin content in the meat. The shelf-life of the product would thereby be prolonged due to the delaying of browning of the meat.

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Chapter 5: General discussion and conclusion

Lactic acid bacteria have been used traditionally for many years in fermentation and have delayed microbial spoilage and enhanced food safety, through competition with spoilage and pathogenic organisms (Stiles, 1996). They are known to produce several antimicrobial compounds that inhibit the growth of spoilage and pathogenic bacteria. Bacteriocins are one group of these antimicrobial compounds and their use in food preservation would be helpful in meeting the consumer demand for more "natural" food ingredients to replace the chemical additives currently used, as well as a hurdle for minimally processed products that are more susceptible to microbial growth. Although several of these bacteriocin-producing organisms have exhibited antimicrobial activity in *in vitro* studies, it is important to evaluate their efficacy in food systems, as they can be reduced or inactivated by food components, processing or storage conditions.

In addition to microbial spoilage and microbial safety, there are other quality factors of importance to both retailers and consumers. Browning of fresh meat products is an issue that can cause significant loss of sales for retailers. Many fresh meat products such as ground beef and sausages are packaged for retail sale in trays covered with oxygen permeable films, which allows for the formation of metmyoglobin. The resulting browning can cause consumers to mistrust both the safety and quality of the product, to the extent that when the meat surface pigment content is more than 20% metmyoglobin consumers will perceive the product is not as fresh and, correspondingly, sales will decrease by a

factor of two (Renerre, 1990). Most microorganisms cause, rather than prevent, discoloration of meat either by increasing the metmyoglobin content on the surface of fresh meat through consumption of oxygen or the oxidation of the iron within myoglobin, or by causing greening from the formation H_2S and H_2O_2 (Renerre, 1990).

The first objective of this study was to evaluate the antimicrobial activity of bacteriocin-producing *Carnobacterium maltaromaticum* UAL307 against *Listeria monocytogenes* in fresh beef sausages, as well as to evaluate its effect on the colour of the sausages. It was found that the addition of *C. maltaromaticum* UAL307 with a bacteriocin-containing culture supernatant decreased counts of *Listeria* spp. to < 1.0 log CFU/g under aerobic and MAP storage and maintained the red colour in aerobically stored sausages for 10 days under refrigeration. Combined with its GRAS status, the demonstrated colour stabilizing properties, antimicrobial activity *in vivo* and negligible effect on meat pH, would make *C. maltaromaticum* UAL307 an effective intervention for use by the meat industry to extend the overall retail storage life and increase the safety of fresh sausage.

The ability of *C. maltaromaticum* to stabilize the red colour of meat is unique. The second objective of this study was to determine the mechanism behind this ability. The first experiments evaluated the effect of bacterial growth on the oxidation state of myoglobin. Myoglobin is the main contributor of colour to fresh, red meat (Tang et al, 2004), and is a heme protein that exists in 3 different states. Deoxymyoglobin is present in meat stored in an atmosphere without oxygen and results in meat with a purple hue. Oxymyoglobin is the

oxygenated form of myoglobin and imparts a red colour. The browning observed during retail storage is caused by metmyoglobin when there is a low oxygen atmosphere (0.5-1%) or meat has been exposed to air for an extended period of time (Robertson, 1993). The state of iron plays a very important role in the state of myoglobin. In metmyoglobin, the ferric iron (Fe^{3+}) is present and ferrous iron (Fe^{2+}) is present in oxymyoglobin (Robertson, 1993). Results of the current study showed that *C. maltaromaticum* promotes the conversion of metmyoglobin to oxymyoglobin *in vitro* under aerobic conditions and can maintain the myoglobin in this state. The other LAB studied did not have this ability. Under anaerobic conditions, metmyoglobin was converted to deoxymyoglobin by several organisms, but only *C. maltaromaticum* maintained the deoxymyoglobin at almost 100% throughout storage. This ability indicated that *C. maltaromaticum* was able to change the state of the iron in myoglobin to Fe^{2+}, since iron in this state is common to both oxymyoglobin and deoxymyoglobin, with the only difference between the two states being the presence or absence of oxygen. To confirm this, the ability of *C. maltaromaticum* to reduce Fe^{3+} to Fe^{2+} was evaluated, and compared to *P. aeruginosa*, a known iron reducer. Using a modified ferrozine assay it was found that although its iron reducing capability was not as great as the positive control, *C. maltaromaticum* UAL307 was the only LAB evaluated to significantly convert Fe^{3+} to Fe^{2+}. Many organisms that reduce iron do so before taking it into the cell for uses such as a cofactor for biosynthetic enzymes (Brown and Holden, 2002). To do this, bacteria utilize several mechanisms to bind iron for uptake into the cell. Since initial results showed

myoglobin in the oxymyoglobin state in the presence of *C. maltaromaticum* UAL307, one would expect that although the iron was reduced, it was not being removed from the compound, as this would alter the heme group to the extent that colour would have been affected. This would also have been evident in the spectrophotometric method utilized to evaluate the states of myoglobin, in which the absorbance of the medium is measured at several wavelengths and the state of myoglobin can be determined with calculations based on the known absorbance spectra of the different states of myoglobin. The metmyoglobin absorption peak is at 503 nm, while deoxymyoglobin absorption peaks at 557 nm and oxymyoglobin absorption peak is at 582 nm (Tang et al, 2004). Myoglobin without iron attached to the heme group has a peak absorbance at around 400 nm (Albani and Alpert, 1987). One of the mechanisms that organisms use to bind iron is the production of compounds known as siderophores, so the production of siderophores by *C. maltaromaticum* was determined using a CAS assay (Pérez-Miranda et al, 2007). No siderophores were detected, which supports the conclusion that iron reduction by *C. maltaromaticum* UAL307 was dissimilatory, meaning the iron is only being used as an electron acceptor.

Further investigation examined the mechanisms behind the iron reduction ability of *C. maltaromaticum* UAL307, particularly by comparison to other LAB that do not exhibit the same colour stabilizing properties. Several LAB are affected by the presence of heme during growth and under aerobic conditions demonstrate a metabolic switch from fermentation to respiration. *L. lactis* is one of these organisms. This switch to respiration is facilitated by the synthesis of

cytochromes and menaquinone using heme. These compounds are necessary parts of the ETC utilized during respiration (Brooijmans, 2008). To determine if this also occurs in *C. maltaromaticum* UAL307, its ability to bind heme was investigated, as well as the effect of both heme and myoglobin on growth efficiency. NADH oxidase and LDH activity and its ability to produce cytochrome oxidases through expression of cytochrome *bd* genes were also investigated. Heme and myoglobin had no effect on any of these properties in *C. maltaromaticum* UAL307, under microaerobic and anaerobic conditions. This lack of respiration would promote the reduction of metmyoglobin to oxymyoglobin. A switch to respiration would cause the consumption of oxygen, which would reduce the oxygen tension in the meat, increasing metmyoglobin levels. An increase in NADH oxidase activity may also negatively affect meat colour. NADH plays a key role in the reduction of metmyoglobin in animal muscle. In live muscle, autoxidation of myoglobin occurs as a side reaction of the mediation of oxygen transfer between hemoglobin and mitochondria, so the enzyme metmyoglobin reductase converts Fe^{3+} back to Fe^{2+} so that the myoglobin is able to bind oxygen again (Young and West, 2001). NADH is required as a reductant by this enzyme. As the NADH becomes depleted in post-rigor meat metmyoglobin formation is increased over time. Since *C. maltaromaticum* does not produce more NADH oxidase, it does not contribute to that depletion. The fact that cytochromes are not produced by *C. maltaromaticum* UAL307 would also account for the stabilization of oxymyoglobin in meat. Cytochrome formation by LAB requires the utilization of exogenous heme. If the heme group

or iron were removed from the myoglobin it would have an effect on the colour of the medium. These differences would explain why *C. maltaromaticum* UAL307 exhibits colour stabilizing capabilities and *L. lactis* does not.

Overall, the first and second objectives of this study has been fulfilled; the antimicrobial activity of *C. maltaromaticum* UAL307 against *L. monocytogenes* in a fresh meat product was confirmed, as well as the fresh meat colour stabilizing properties of *C. maltaromaticum* UAL307. Although significant insights have been achieved, the mechanism behind the colour stabilizing properties of *C. maltaromaticum* UAL307 has not been fully explained. Its ability to keep myoglobin in the oxymyoglobin form as well as its iron reducing activity have been confirmed; however, the mechanism behind the iron reduction was not determined.

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