Green Discolouration in Cooked Cured Bologna

BY

Michael David Joseph Peirson

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Food Science
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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I'd like to thank all the little people who made this remarkable achievement possible! But since it might be more politic (and only fair) to give first place to those who contributed directly, perhaps the rest of my adoring public (!) will be satisfied without explicit mention.

First thanks are due, of course, to my advisor Dr. Rick Holley, a man of great knowledge who is very nearly as stubborn as I - but with far better excuse. I have no doubt that his refinements and criticisms have improved the quality of this thesis immeasurably – not to mention that without the funding he arranged my work might never have begun.

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ABSTRACT

Discolourations in cooked cured meat products may have chemical, or physical causes but in practice are usually caused by hydrogen peroxide-producing lactic acid bacteria. Such bacteria grow on the surface of vacuum-packaged cooked cured meat without causing obvious spoilage until the package is opened but once exposed to air they begin generating H_2O_2. The H_2O_2 decolourizes pigments in the meat product. This work was undertaken to identify the organisms responsible for an outbreak of discolouration in bologna produced by a Canadian bologna manufacturing facility, to examine ways of detecting their presence in as-yet unspoiled cooked cured meat products, and to identify methods of killing the relevant organisms or of preventing their growth.

An array of organisms isolated from discoloured bologna and from the manufacturing plant were evaluated, along with laboratory cultures, for their ability to induce green discolourations in irradiation-sterilized bologna. The organisms that were able to cause discolouration were *Weissella viridescens*, tetrad-forming cocci identified by biochemical and genetic tests as *Aerococcus viridans*, and a new species, *Carnobacterium viridans*. Several strains of *Pediococcus*, *Leuconostoc* and *Carnobacterium* were unable to cause discolouration. A meat-agar test system used at the bologna manufacturing plant to detect the presence of organisms capable of causing

greening did not support the growth of *Carnobacterium viridans* and gave false-positive reactions in the presence of pediococci and leuconostocs. It was concluded that meat-agar test systems do not necessarily indicate an organism's potential to cause discolouration in a particular product.

Weissella viridescens, unlike most other lactic acid bacteria, is thought to survive the cooking process used for cured meats. In addition, many bacteria are unable to grow in meat products that contain added sodium lactate or sodium diacetate. Therefore the effects of heat, lactate, and diacetate on the suspect bacteria were examined. Neither *A. viridans* nor *C. viridans* were able to tolerate heat but *W. viridescens* had a thermal death time of 14.7 minutes in bologna batter at 60 °C. *W. viridescens* was hardly affected by sodium lactate and sodium diacetate. *A. viridans* and *C. viridans* were unable to grow at 10 °C in bologna made with 3.0 % (w/w) sodium lactate and 0.30 % (w/w) sodium diacetate, and grew only slowly in meat made with 3.0 % sodium lactate alone. Sodium diacetate alone (0.3 % w/w) had little effect on the growth of these organisms. Unfortunately, however, sodium lactate and sodium diacetate themselves diminished the red colour of bologna. In samples contaminated with *A. viridans* and *C. viridans*, bologna treated with the combination of sodium lactate and sodium diacetate had the worst initial appearance and the best final appearance after 10 weeks at 10°C.

FORWARD

The body of this thesis consists of two papers prepared and submitted for publication. A third paper (Holley *et al.*, 2002) to which the author contributed is also included as an appendix. A literature review, general introduction, and general conclusion comprise the remainder of the work.

The first paper, "**Aerococci and carnobacteria cause discolouration in cooked cured bologna**," was submitted on July 10, 2002 for review and publication in *Food Microbiology*.

The second paper, "**Thermal resistances and lactate and diacetate sensitivities of bacteria causing bologna discolouration**," was submitted on July 10, 2002 for review and publication in the *International Journal of Food Microbiology*.

The appendix, "***Carnobacterium viridans* sp. nov., an alkaliphilic, facultative anaerobe isolated from refrigerated, vacuum-packed bologna sausage**," has been published on line (Holley *et al.*, 2002). It describes MPL-11 and MPL-14, two strains isolated from vacuum-packed discoloured bologna, and gives the reasons for classifying these organisms as a new species, *C. viridans*. The author contributed to this paper by carrying out some of the phenotypic tests (also described in the first paper, above) and

by reviewing the taxonomic literature to demonstrate on phenotypic grounds that MPL-11 and 14 probably represented a new species of *Carnobacterium*. The tables, figures, and references of the appendix are included after the text and only minimal formatting changes have been applied.

1.0 INTRODUCTION

This thesis has its origin in difficulties experienced by a Canadian meat processor in 1999 and 2000 whereby bologna (but not ham) produced at one manufacturing facility tended to become discoloured if exposed to air about 5 weeks after production. Since the normal refrigerated shelf life for this product was 11 weeks, the defect was of great concern to the company and my advisor, Dr. Rick Holley, was able to obtain funding to allow a graduate student, myself, to investigate.

As originally envisioned, the project was to consist of four stages: identification of the organisms responsible for discolouration, determination of the effect of sodium lactate on the organisms, determination of their ability to withstand heat, and design of a simple medium to detect their presence. It became obvious, however, that the project was too large to be completed in the course of a single MSc thesis. Consequently the medium development section was dropped from the thesis and the thermal resistance experiments were designed and (mostly) carried out by Tat Yee Guan.

Discolourations in cured meats can occur for many reasons but are most commonly caused, as here, by hydrogen peroxide-producing lactic acid bacteria. The taxonomy of the lactic acid bacteria is complex and, until recently, very poorly defined. Few investigators have seen fit to define which organisms are responsible for greening in cured meats. Therefore the first and most difficult task of this project was to isolate and identify the organisms that were capable of causing discolourations. That work led to the recognition that *Aerococcus viridans* and a new species, *Carnobacterium viridans*

(Holley *et al.* 2002), grow on refrigerated, vacuum-packaged bologna and cause discolouration.

It is not helpful to know which bacteria cause a particular spoilage problem unless that knowledge aids in preventing the problem. Because other organisms associated with green discolourations have been shown to possess unusual heat tolerance (Niven *et al.* 1954), it was possible that the organisms isolated in this study might be able to survive thermal processing of meats. For this reason we compared the heat tolerances of strains associated with discolouration with those of bacteria known to be unusually heat resistant, to determine whether discolouration might be prevented by raising the cooking temperature used by the manufacturer. In addition, we examined the effects of incorporating sodium lactate and sodium diacetate into the sausages. Both these compounds are naturally found at low levels in cured meats and are being used in these products to prevent growth of the (sometimes fatal) pathogen *Listeria*. If the same agents used to enhance meat safety could also prevent greening, manufacturers would have a double incentive for including those agents in their products.

2.0. LITERATURE REVIEW

2.1. Discolouration in Meat Products

The colour of meat can change in ways that would astonish an average consumer. The decisions people make about meat depend on the colours they expect to see: red for fresh cuts, grey-brown for cooked meat, and pink for cured meat products. Generally, people assume that meat quality and safety are tied to colour. This is only partly true.

The colour of meat depends not only on the age of the meat product or the number and type of microorganisms present but also the chemical state of the meat pigments. The pigments of fresh meat are myoglobin, in which the iron is in the ferrous form and interacts weakly with water, oxymyoglobin, the ferrous form covalently bound to oxygen, and metmyoglobin, the ferric form in an ionic bond with OH^-. Consider a thick piece of meat. If one cuts it in half, one can see that the outer edge is bright red, the neighbouring zone brown, and the interior nearly purple. These colours are those of the oxy-, met-, and deoxy- forms of the pigments, respectively, (Fig. 2.1) and reflect the degree to which oxygen penetrates the meat (Lawrie, 1998). For this reason, meat that is stored without oxygen appears purple and consumers avoid it.

The mechanism by which colour develops in cured meats is uncertain; indeed, there may be several mechanisms. It is known that the colour of meat changes upon addition of curing salts from the red of oxymyoglobin to the brown of metmyoglobin, a change which can be attributed (Fox, 1987) to catalysis by low levels of divalent cations or

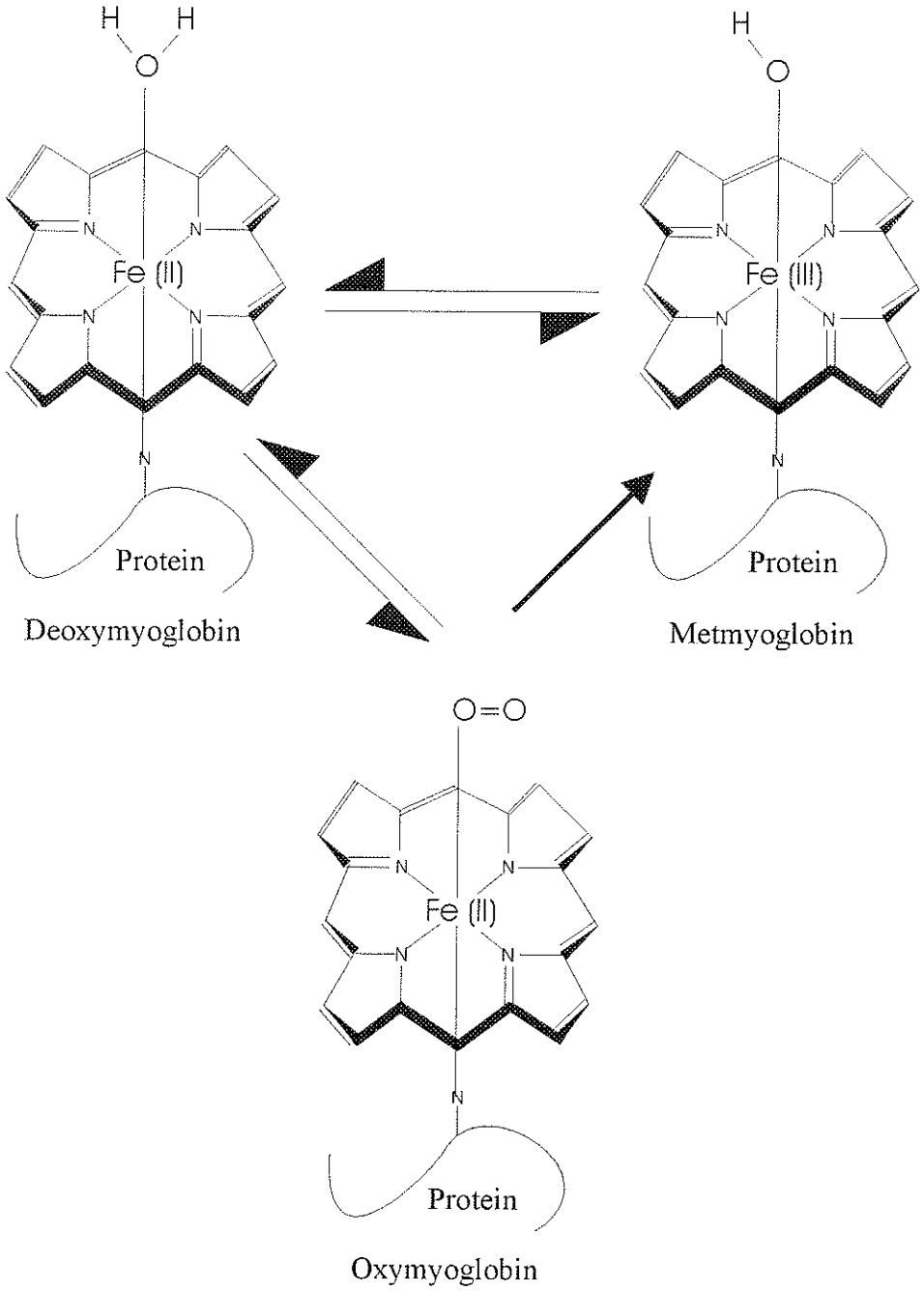


Figure 2.1. Heme pigments of fresh meat (adapted from Pearson and Tauber, 1984).

(Walters and West, 1983) to the action of nitrite. From this point there are at least three ways of reaching nitric oxide myoglobin (Fig. 2.2), the pigment which provides the normal red colour of uncooked cured meat (Walters and West, 1983). The first

possibility is that a complex, metmyoglobin nitrite, forms first and then both components are reduced by ascorbate, NADH, or some other agent. The second is that nitrite becomes reduced to nitric oxide in the presence of reducing agents like ascorbate and erythorbate; the nitric oxide then binds metmyoglobin to form nitric oxide metmyoglobin; this complex autoreduces to nitric oxide myoglobin. The third possibility is that the formation of metmyoglobin is not absolute but rather metmyoglobin is constantly being re-reduced to deoxymyoglobin by various agents. As it forms, deoxymyoglobin spontaneously forms a strong bond with nitric oxide, preventing re-oxidation to metmyoglobin and thus over time nearly all the metmyoglobin is converted to nitric oxide myoglobin.

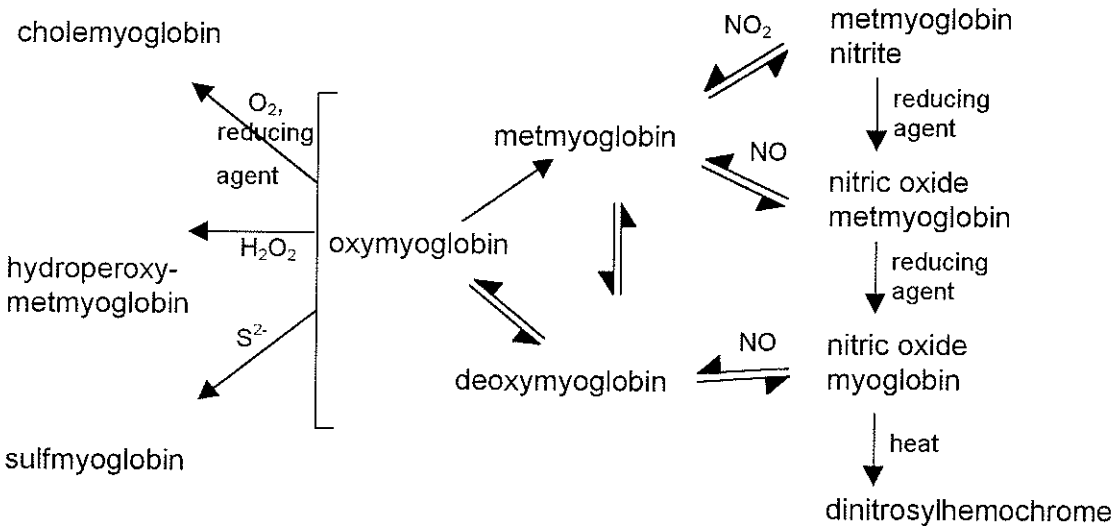


Figure 2.2. Reactions That Affect the Heme Pigments. (Adapted from Fox, 1987)

During cooking of cured meats, myoglobin denatures and dissociates from heme, freeing the iron to bind a second nitric oxide molecule and form a pink pigment,

dinitrosohemochrome. According to Fox (1987) about 60-80% of the heme is converted to dinitrosohemochrome in good quality cooked cured meats, although others claim this number should be 90% (AMSA, 1991). Fox (1987) believed that conversions of more than 80% give the product a bluish-red appearance while conversions less than 50% make it look brown. Other pigments which may be formed in cooked cured meats include nitrihemin, verdoheme, and if the iron is removed, yellow or colourless bile pigments. Formation of these latter pigments requires the presence of large excess amounts of nitrite, denaturing agents, and heat. It may also be possible for dinitrosohemochrome to be oxidized by H_2O_2 or H_2S to produce green pigments analogous to choleomyoglobin and sulfmyoglobin, both of which are associated with discolourations in uncooked meat products. Cooked cured meat becomes discoloured in the presence of H_2O_2 (Niven *et al.* 1949). Judging from current summaries (Lawrie, 1998; Fox, 1987), however, the chemistry of discolouration seems to have been studied almost entirely using the intact, uncooked pigments.

Bacteria induce green discolourations in vacuum-packaged fresh and cured meats, apparently by different mechanisms. In fresh meats, green spoilage is generally attributed to hydrogen sulfide-producing bacteria (Jay, 2000). Of these, some are lactic acid bacteria but many others are *Enterobacteriaceae*. In contrast, all reported cases of greening in cooked cured meats seem to involve H_2O_2 producing lactic acid bacteria (Grant *et al.*, 1988). These bacteria are thought to contaminate the meat prior to vacuum packaging, either as post-cooking contaminants or by surviving cooking. They grow quickly in the cured meat package without causing obvious spoilage until they are

exposed to air. Then they begin generating H_2O_2 which, depending on the product, the temperature, and the type and number of bacteria, discolours the meat over a period of minutes or days.

Few authors have devoted much space to bacterial greening in cured meats, and no new knowledge has been added since Grant *et al.* (1988) reviewed the subject. These are the questions that must be asked: (1) What microorganisms are associated with discolourations in cooked cured meat products? (2) By what mechanism do these microorganisms cause greening? (3) How can spoilage be prevented?

Niven *et al.* (1949) provided the first careful description of the 'greening' bacteria. From six widely separated areas in the eastern United States, they isolated 30 bacteria associated with outbreaks of greening. All were heterofermentative lactobacilli or leuconostocs, psychrotrophic, and able to grow in moderate salt. Sausages incubated anaerobically with these organisms did not spoil but when exposed to air they rapidly took on a green appearance. A concentrated solution of catalase prevented greening. In the same report, a homofermentative *Lactobacillus* and several tetrad-forming cocci were also found to cause greening. Niven *et al.* (1949) concluded that any H_2O_2 producing organism that was capable of growing in refrigerated cured meats might produce the same effect. Nevertheless, their heterofermentative lactobacilli (now called *Weissella viridescens*) have come to be considered the most important cause of bacterial greening in cooked cured meats (Grant *et al.*, 1988).

Other organisms described as causing discolouration include streptococci and atypical streptococci, enterococci, pediococci, leuconostocs, *Lactobacillus* (*Lb.*) *fructivorans*, *Lb. jensenii*, *Lb. leichmannii*, *Lb. helveticus,* and *Lb. lactis* (Grant *et al.*, 1988; Lee and Simard, 1984). These claims have not all been investigated with the same rigour. Shank and Lundquist (1963) reported that an undescribed *Streptococcus* caused greening on frankfurters and in a meat-agar model system. Incze and Delenyi (1984) found an organism with a fermentation pattern similar to *Lb. leichmannii*; but the organism's ability to grow at 3°C makes it unlikely that this identification was correct since *Lb. leichmannii* (now *Lb. delbrueckii* subsp. *lactis*) does not grow at 15°C or lower. Lee and Simard (1984) showed that *Lb. lactis*, *Lb. helveticus*, *Lb. jensennii*, and *Lb. fructivorans* all produced greening on a meat-agar model test system. Of these, only *Lb. fructivorans* is cold-tolerant enough to grow in broth at 15°C (Kandler and Weiss, 1986). Furthermore, the relationship between the model system and real meat products is unclear. Organisms isolated from discoloured meat did not necessarily induce greening in the test system but some strains from other sources did. None of the strains produced detectable amounts of H_2O_2 in MRS broth.

Enterococci and pediococci have not been proved to cause greening, although several reviews (Grant *et al.*, 1988; Borch *et al.*, 1996; Jay, 2000) mention them. Niven *et al.* (1949) and Deibel and Niven (1960) did describe some tetrad-forming cocci, but the former authors (Niven *et al.*, 1949) did not describe the organisms in detail and the latter (Deibel and Niven, 1960) believed that aerococci were a species of the genus

Pediococcus. Genetic evidence, however, shows that aerococci are a distinct genus (Collins *et al.* 1990)

2.1.1. Meat-Agar Test Systems

As mentioned above, several authors have devised meat-agar test systems to study greening phenomena. Shank and Lundquist (1963) used a mixture of 500 g lean beef, 0.15 g sodium nitrite, and one litre brain heart infusion agar to study the effects of O_2 barrier films on discolouration. The *Streptococcus*-like organism they used was able to grow under all films regardless of their O_2 transmission. In the absence of O_2 it produced highly reducing conditions under the barrier film and the colour of the meat-agar system deepened to red. But when the film was removed, a reaction prevented by catalase caused the colour to fade to green. The same reaction affected sausages dipped in H_2O_2 solution. Therefore Shank and Lundquist (1963) concluded that H_2O_2 producing bacteria are responsible for greening in cooked cured meats.

Lee and Simard (1984) replaced BHI with MRS agar and used the medium to screen lactobacilli for their greening potential. *Lb. viridescens* and two unidentified strains from discoloured frankfurters caused greening in the test system but two bacteria from green ham did not. As mentioned above, several of the species they found to cause greening are unlikely to be relevant in practice. The meat-agar test system, in order to obtain visible colonies quickly, uses incubation temperatures much greater than refrigerated meat products normally experience. In addition, the decision to use MRS agar may have affected the results. MRS, because it contains large quantities of glucose,

is a poor medium for detecting H_2O_2 producing bacteria (see "Bacterial hydrogen peroxide production" below).

Grant and McCurdy (1986), finding that colour changes were hard to discern in Lee and Simard's (1984) system, replaced lean beef with pork hearts. Heart muscle contains greater concentrations of myoglobin than beef and thus colour differences become more obvious. On this medium, *Lb. viridescens* was able to grow and cause greening at nitrite levels up to at least 500 ppm and up to 5% (but not 6%) salt. Sodium erythorbate had no effect on *Lb. viridescens* growth or greening.

Grant *et al.* (1988) recommended means of preventing green discolouration in cured meats. They proposed improved sanitation protocols and microbial quality of raw ingredients, prompt refrigeration, and more thorough cooking. Other recommendations included producing nitrite-free cured meats (which do not discolour) or adding antimicrobials to the batter. They suggested nisin.

2.1.2. Colour measurement

Grant *et al.* (1988) have suggested that discoloured spots are actually grey or brown in colour, and appear green through an optical illusion. They incorrectly attributed this idea to Jensen (1945). Jensen, although he referred to optical illusions in other contexts, believed that "pink ferrous nitroso derivatives of hemoglobin are sensitive to oxidations of microbial origin and become emerald-green and grey-green in color when known oxidizers like *Streptococcus viridans* ... or other oxidizing bacteria ... act upon them." Nevertheless, the appearance of a green colour may in fact be an illusion, brought about

by a contrast with the normal pink pigment. Neither instrumental measurements nor human panellists have been used to evaluate the changes in colour associated with bacterial greening (Grant *et al.*, 1988). How big are the changes? Is the meat really green?

There are many instrumental ways of measuring cured meat colour but most experiments use convenient, reflectance-based methods. (AMSA, 1991). Some methods measure the reflectance of specific wavelengths; for example, the ratio of reflectance at 650 nm to that at 570 nm has been used to indicate the intensity of cured colour development. The 650/570 nm reflectance ratio is reported to be the most sensitive and interpretable method of evaluating cured meat pigments. According to the American Meat Science Association (AMSA, 1991) 90% of the total heme pigment in well-cured meat is converted to nitrosohemochrome. Such meat exhibits a reflectance ratio of between 2.2 and 2.6.

Colour can be described in three dimensions: hue, value, and chroma. Colours are named based on their hue (for example, 'red'), the result of differences in wavelengths of light. 'Value' refers to the lightness of a colour and 'chroma' to its intensity. An object's appearance depends on its hue, value, and chroma, but also on its size, shape, gloss, and texture. Colour measuring instruments known as colourimeters use three or four coloured lights to illuminate a sample. Other devices, spectrophotometers, use monochromatic light. Spectrophotometers can provide absolute measurement of colour

but colorimeters, which cannot be made exactly alike, should only be used to measure differences in colour (AMSA, 1991).

Colorimeter measurements can be reported in many ways but one of the most common is the Hunter L a b system (AMSA, 1991). This system describes all colours in terms of the three co-ordinates L, a, and b. 'L' represents the lightness of the colour on a scale of 0 to 100. Positive values of 'a' are red, while negative ones are green; positive values of 'b' are yellow, while negative ones are blue. The L a b system is a uniform colour space, which means that the numerical distance between two colours is consistently related to the visual difference between the colours.

Until now, no report has quantified the colour changes that occur when bacteria discolour cooked cured meat.

2.2. Taxonomy of the Lactic Acid Bacteria

As mentioned above, hardly any of the bacteria associated with discoloured meat have been identified. Lactic acid bacteria are hard to identify; in fact, the whole classification system is currently being revised. The old system, which was based on carbohydrate fermentation profiles along with other key tests, is being replaced with a new structure based on DNA homology and 16S rRNA sequences. The 2nd edition of *Bergey's Manual of Systematic Bacteriology* is based on the new scheme. Unfortunately, only Volume I of the *Manual* is currently available and the organisms it deals with are not associated with food. Volume I does, however, include a listing of current genera according to family, order, class, and phylum (Garrity and Holt, 2001).

The new structure is not immediately useful to food microbiologists. Although the lactic acid bacteria are found mostly in a single order ("Lactobacillales"), many of the genera in this order have no known association with food. Several appear not to be lactic acid bacteria at all. *Alloiococcus*, for example, is considered to be more closely related to *Carnobacterium* than *Carnobacterium* is to *Lactobacillus* or *Aerococcus*, yet it does not ferment sugar and is strictly aerobic (Aguirre and Collins, 1992a). For these reasons and because phenotypic methods were used throughout this work, this section is based mainly on the older classification schemes (Kandler and Weiss, 1986; Schleifer, 1986).

Many people consider the lactobacilli, leuconostocs, and pediococci to be the primary groups of lactic acid bacteria. Streptococci are occasionally included but aerococci seem to have been left out of most discussions, perhaps because of doubt about their importance in food spoilage (Holzapfel, 1992). In recent years this list of genera has been revised and expanded. Stiles and Holzapfel (1997) recognized eleven genera (but not *Aerococcus*) as relevant in food products. There is not space here to describe each genus in detail. This discussion, therefore will focus on the organisms unequivocally proved to cause greening in cooked cured meats. These include first of all *Weissella* (*W.*) *viridescens*, the organism most frequently blamed for discolouration, and secondly the species identified in this study, namely *Aerococcus* (*A.*) *viridans* and *Carnobacterium* (*C.*) *viridans*.

W. viridescens, long known to cause discolouration in meat, was originally described as a heterofermentative *Lactobacillus* (Niven *et al.*, 1949). As a short heterofermentative

rod, it was easy to confuse with leuconostocs, especially because many *W. viridescens* strains produce dextran on sucrose agar, a trait normally ascribed to leuconostocs. It also resembles leuconostocs in failing to produce ammonia from arginine and in producing mainly D(-) lactate, although some L(+) lactate is also made (Niven *et al.*, 1949; Holzapfel and Schillinger, 1992). In the early 1990s, 16S ribosomal RNA sequences indicated that certain heterofermentative lactobacilli were more closely related to *Leuconostoc* (*Lc.*) *paramesenteroides* (but not *Lc. mesenteroides*) than to other lactobacilli. Collins *et al.* (1993b) responded to this discovery by creating the genus *Weissella* with *W. viridescens* as the type species. Table 2.1 lists the species of

Table 2.1. List of Species of *Weissella* and *Leuconostoc*

Weissella viridescens[a]	*Leuconostoc mesenteroides*[c]
W. confusa[a]	*Lc. lactis*[c]
W. halotolerans[a]	*Lc. gelidum*[d]
W. kandleri[a]	*Lc. carnosum*[d]
W. minor[a]	*Lc citreum (L. amelibiosum)*[e]
W. paramesenteroides[a]	*Lc. pseudomesenteroides*[f]
W. hellenica[a]	*Lc. fallax*[g]
W. thailandensis[b]	*Lc. argentinum*[h]
	Lc. kimchii[i]

[a] Collins *et al* (1993b)
[b] Tanasupawat *et al.* (2000)
[c] Garvie (1986a)
[d] Shaw and Harding (1989)
[e] Takahashi *et al.* (1992)
[f] Farrow *et al.* (1989)
[g] Martinez-Murcia and Collins (1991)
[h] Dicks *et al.* (1993)
[i] Kim *et al.* (2000)

Weissella and *Leuconostoc* that were known as of November 2001. The two genera cannot be distinguished reliably using phenotypic characters and new species are described primarily on the basis of their 16S rRNA sequences. Nevertheless, Table 2.2

gives cardinal features of both genera. The main differences are that *Weissella* spp. may produce either DL or D(-) lactate and may hydrolyze arginine.

W. viridescens can be distinguished from leuconostocs because it produces DL lactate and because its cells are usually rod-shaped rather than coccoid. It is distinguished from other weissellas by its failure to produce ammonia from arginine and acid from L-arabinose, cellobiose, galactose, and ribose. The negative arginine reaction also separates it from most (but not all) heterofermentative lactobacilli (Schillinger and Lucke, 1987).

Table 2.2. Key features of *Weissella and Leuconostoc*[a]

Weissella	*Leuconostoc*
Short rods or cocci	Coccoid appearance
Heterofermentative	Heterofermentative
Some species hydrolyze arginine	Do not hydrolyze arginine
D(-) or DL lactate	Only D(-) lactate produced from glucose
Acid tolerant	"less aciduric than the lactobacilli"
Growth at 15°C and not at 45°C	Temperature Minimum: 5°C; Maximum: ND[b]
Cell wall contains alanine or serine and alanine	Cell wall contains alanine or serine and alanine

[a] As described by Collins *et al.* (1993b) and Holzapfel and Schillinger (1992).
[b] Not Described in source article.

2.2.1. Identification of *Aerococcus viridans*

A. viridans can be found just about anywhere. It has been isolated from air, hospitals, sick lobsters, and meat curing brines. It has, in fact, been called part of the normal flora of such brines – although this opinion seems to stem entirely from one report (Deibel and Niven, 1960). Aerococci express a lactate oxidase that generates pyruvate and H_2O_2. If they are streaked on cured meat and incubated at 30°C for 24 hours, the streaked area becomes green (Deibel and Niven, 1960). Despite this, discussions about

cured meat spoilage have ignored the aerococci (Grant *et al.*, 1988) or called into question their role in meat spoilage (Holzapfel, 1992).

Aerococci are Gram-positive, catalase and oxidase negative cocci, which form tetrads, pairs and clumps but not chains. Consequently they may be mistaken for other tetrad-forming genera, especially pediococci. Most pediococci, however, do not grow at pH 9 and do grow at pH 5; the reverse is true of aerococci. In addition, aerococci are sensitive to vancomycin, produce L (+) lactate and leucine aminopeptidase, and do not produce pyrolidonyl arylase (Weiss, 1992). They grow in 10 % salt. None of these features are typical of pediococci.

The second edition of Bergey's Manual of Systematic Bacteriology (Garrity and Holt, 2001) places the following organisms in the "Aerococcaceae": *Aerococcus*, *Abiotrophia*, *Dolosicoccus*, *Eremococcus*, *Facklamia*, *Globicatella*, and *Ignavigranum*. Some characteristics of these and other genera are listed in Table 2.3.

Although for many years *A. viridans* was the only species in its genus, the last decade has seen the number of *Aerococcus* species increase to five. *A. urinaehominis* and *A. urinae* were isolated from human urine, *A. sanguicola* from blood, and *A. christensenii* from the human vagina. *A. viridans* is the only species to have been isolated from non-clinical sources. It is identical with the former species *Gaffkya homari* (later *Pediococcus homari*) and *Pediococcus urinae-equi*. Species of *Aerococcus* may be

identified by their growth temperature range, carbohydrate fermentation profile, and key enzymes (Table 2.4).

Table 2.3. Characteristics of genera related to or phenotypically similar to *Aerococcus*[a]

	Cell morphology	10°C[b]	45°C[b]	6.5% salt[b]	Sugars Fermented	Ammonia from arginine
Aerococcus	spheres in pairs, tetrads, and clusters	+[c]	-	+	extensive	+/-
Abiotrophia	cocci; occasional rods	-	-	-	ND	ND
Dolosicoccus	pairs and short chains	-	-	-	few	-
Eremococcus	pairs and short chains	-	ND[d]	w	glucose only	+
Facklamia	pairs and groups	ND	ND	ND[e]	none	+
Globicatella	pairs or short chains	-	-	+	extensive	-
Ignavigranum	cocci in pairs, groups	ND	+	+	few	+[f]
Tetragenococcus	cocci in pairs and tetrads	-	-	+[g]	extensive	-
Helcococcus	cocci in chains, groups	-	-	+/-	few[h]	-
Dolosigranulum	pairs and groups	-	-	-	extensive	+[i]

[a] Data assembled from the following sources. *Aerococcus*; Evans (1986), *Abiotrophia*; Kawamura *et al.* (1995), *Dolosicoccus*; Collins *et al.* (1999a), *Eremococcus*; Collins *et al.* (1999b), *Facklamia*; Collins *et al.* (1997), *Globicatella*; Collins *et al.* (1992), *Ignavigranum*; Collins *et al.* (1999c), *Helcococcus*; Collins *et al.* (1993a), *Dolosigranulum*; Aguirre *et al.* (1993). The description of *Tetragenococcus* is based on Collins *et al.* (1990) and on the description of the former species *Pediococcus halophilus* (Garvie 1986b).

[b] Growth at 10°C, 45°C, or in 6.5% salt

[c] +, positive reaction; -, negative reaction; w, weak reaction; +/- positive or negative; ND, not described in source article.

[d] Grows at 42°C.

[e] Grows in 5% salt.

[f] Arginine dihydrolase reaction is positive using commercial API systems (BioMerieux) but negative using conventional tests.

[g] Requires salt for growth; amount required depends on species (Collins *et al.*, 1990; Satomi *et al.* 1997).

[h] The range of sugars tested was limited.

[i] Assumes that "arginine dehydrogenase" (Aguirre *et al.*, 1993) should be read as "arginine dihydrolase" (= arginine deiminase). A test for arginine dihydrolase is included in the API 20 Strep (BioMerieux) system, which was used in the study; no enzyme named arginine dehydrogenase is included in the *Index of Enzyme Names* (International Union of Biochemistry and Molecular Biology, 1992).

Table 2.4. Differential Characteristics of the Aerococci[a]

	A. viridans	*A. urinae*	*A. urinaehominis*	*A. sanguicola*	*A. christensenii*
Lactose	+[b]	-[b]	-	-	-
Maltose	+	-	+	+	-
Mannitol	v[b]	+	-	-	-
Ribose	v	v	+	-	-
Sucrose	+	+	+	+	-
Trehalose	+	-	-	+	-
Arginine Dihydrolase	-[c]	-	-	+	-
β-glucuronidase	v	+	+	+	-
Pyroglutamic acid arylamidase	+	-	-	+	-
Growth at 10°C	+	-	ND[b]	ND	ND
Growth at 45°C	-	-	ND	ND	ND

[a] Modified from Lawson *et al.* (2001a), Lawson *et al.* (2001b), Evans (1986), and Aguirre and Collins (1992b).
[b] +, positive reaction; -, negative reaction; v, variable; ND, not described
[c] Occasional strains produce ammonia from arginine (Deibel and Niven 1960).

2.2.2. Identification of *Carnobacterium* spp.

Montel *et al.* (1991) prepared a simplified key that separates carnobacteria from lactobacilli. The main features of a *Carnobacterium* are the presence of *meso*-diamino pimelic acid (m-DAP) in the cell wall, the failure to grow on acetate agar, and the production of L-lactate. Carnobacteria can grow at pHs ≥9 and favour low temperatures (Hammes *et al.*, 1992). They are Gram-positive, non spore-forming rods, both catalase and oxidase negative. They ferment many sugars homofermentatively but may sometimes produce gas by decarboxylating lactate or pyruvate, by the hexose monophosphate pathway, and by unknown pathways involving substrates other than glucose (De Bruyn *et al.*, 1988). Many strains fail to grow on MRS agar.

The genera most likely to be confused with *Carnobacterium* are other non spore-forming rods. Possibilities include *Lactobacillus*, *Paralactobacillus*, *Weissella*, and *Desemzia*. The lactobacilli are aciduric, grow on Rogosa acetate agar, and generally do

not grow above pH 7.2 (Stiles and Holzapfel, 1997). Paralactobacilli are also acid-loving and grow on acetate agar; furthermore, they produce DL, not L(+) lactate. *Weissella* spp. produce significant amounts of gas from glucose, form D or DL lactate, and, like *Lactobacillus*, do not like highly alkaline conditions.

Table 2.5. Genera phylogenetically related to *Carnobacterium*[a]

Genus	Cell Morphology	Oxygen Preferences	Cell Wall Type	Sugar Fermentations	pH preferences
Carnobacterium	rods	facultative fermentative	m-DAP[b]	extensive	Grows at pH 9, not pH 5
Alloiococcus	ovoid in pairs and tetrads	aerobic	ND[c]	None	ND
Desemzia	rods	microaerophilic fermentative	lysine	extensive	apparently alkaline[d]
Dolosigranulum	ovoid	facultative fermentative	lysine	extensive	ND
Lactosphaera	cocci	fermentative	lysine	extensive	5.5-9.0
Trichococcus	cocci in long filaments	facultative	lysine	extensive	5.8-9.0

[a] As listed in Garrity and Holt (2001). Genus *Agitococcus* is not included in the table because there is no record of it in *Biological Abstracts*. Data is compiled from the following sources. *Carnobacterium*; Collins *et al.* (1987), *Alloiococcus*; Aguirre and Collins (1992a), *Desemzia*; Stackebrandt *et al.* (1999), *Dolosigranulum*; Aguirre *et al.* (1993), *Lactosphaera*; Janssen *et al.* (1995).
[b] *meso*-diamino pimelic acid.
[c] ND: Not described in the source article.
[d] pH produced in glucose broth after 48h growth is 6.3 (Stackebrandt *et al.*, 1999).

Desemzia, on the other hand, has several features in common with carnobacteria. Unlike the other genera mentioned so far, *Desemzia* is considered to be phylogenetically related to carnobacteria (Garrity and Holt, 2001). Table 2.5 describes the members of the "Carnobacteriaceae". Although *Desemzia* is similar to *Carnobacterium* in producing L lactate and in favouring alkaline pH it, like the other organisms in Table 5, constructs its cell wall using lysine rather than m-DAP. Within the carnobacteria, species are

differentiated mainly on motility, carbohydrate fermentation profiles and certain enzyme tests. Characteristics that distinguish *C. viridans* from other carnobacteria are found in Table 2.6.

Table 2.6. Species of *Carnobacterium* and their characteristics[a]

	Carnobacterium							
	viridans	*divergens*	*gallinarum*	*mobile*	*piscicola*	*funditum*	*alterfunditum*	*inhibens*
Motility	-[b]	-	-	+	-	+	+	+
Amygdalin	-	+	+	-	+	-	+	+
Inulin	-	-	-	+	+(-)	-	-	w
Lactose	+	-	+	v	+(-)	-	-	w
Mannitol	-	-	-	+	+	+	-	+
Xylose	-	-	+	-	-	-	-	-
Tagatose	+	-	+	-	-	NT	NT	-
Ribose	-	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	-	+
37°C	-	+	+	v	+	-	-	-

[a] Modified from Holley *et al.* (2002).
[b] Symbols: - negative, + positive, +(-) occasional strain negative, w weakly positive, v variable, NT not tested.

2.3. Bacterial Hydrogen Peroxide Production

The lactic acid bacteria that produce H_2O_2 do so using a variety of oxidase enzymes, especially NADH oxidases, lactate oxidases, and pyruvate oxidases (Condon, 1987; Gibello *et al.*, 1999). Other H_2O_2 producing oxidase enzymes include α-glycerophosphate oxidase (Condon, 1987), superoxide dismutase (Condon, 1987), and a fatty acyl-CoA-dependant oxidase (Nunez de Kairuz *et al.*, 1988). Most (perhaps all) require flavin adenine dinucleotide (FAD) as a cofactor. Condon (1987) remarked that all lactic acid bacteria appear to have an NADH oxidase; nevertheless not all produce peroxide. Some possess a water-generating oxidase; others have NADH peroxidases that consume H_2O_2 as fast as it is formed (Condon, 1987). Consequently the tendency to accumulate H_2O_2 depends on an organism's total enzyme set, and is not necessarily tied

to its taxonomy. For example, some streptococci, enterococci, and pediococci possess a lactate oxidase similar to that of *A. viridans* but others do not (Gibello *et al.*, 1999).

The following reactions have been suggested (Condon, 1987; Duncan *et al.*, 1989) for the oxidase enzymes:

$$NADH + H^{+} + O_2 \xrightarrow{NADH:H_2O_2\ Oxidase} NAD^{+} + H_2O_2$$

$$Pyruvate + Phosphate + O_2 \xrightarrow{Pyruvate\ Oxidase} Acetyl\ Phosphate + CO_2 + H_2O_2$$

$$\alpha\text{-}Glycerophosphate + O_2 \xrightarrow{\alpha\text{-}Glycerophosphate\ Oxidase} Dihydroxy\ acetone\ phosphate + H_2O_2$$

$$Lactate + O_2 \xrightarrow{Lactate\ Oxidase} Pyruvate + H_2O_2$$

Each of these reactions influences the energy status of the cell. Pyruvate oxidase, for example, generates acetyl phosphate, which can be used to produce acetic acid and ATP, doubling the amount of ATP that is available from glucose. Lactate oxidase and NADH oxidase allow the accumulation of pyruvate. And α-glycerophosphate oxidase generates dihydroxy acetone phosphate, an intermediate in glycolysis. Therefore, cells that can produce hydrogen peroxide in the presence of oxygen often have a significant energy advantage over related cells that cannot.

It should be noted that there appears to have been some confusion in the literature surrounding lactate oxidase. Villegas and Gilliland (1998) refer to an L-lactate oxidase (E.C.1.1.3.2) in the context of hydrogen peroxide producing enzymes and much of the

work under the name *Lactate oxidase* has been done with this enzyme. However, the enzyme E.C.1.1.3.2 is more properly classified as E.C.1.13.12.4, and ought to be called either *Lactate 2-monooxygenase* or *Lactate oxidative decarboxylase*; the reaction it catalyzes forms acetate, CO_2, and water (International Union of Biochemistry and Molecular Biology, 1992). As Gibello *et al.* (1999) correctly state, the H_2O_2-producing L-lactate oxidase has not been assigned an E.C. number. This true lactate oxidase is thought (Gibello *et al.* 1999) to lead to the production of pyruvate, but to my knowledge this has not been directly demonstrated.

The growth conditions under which H_2O_2 can be detected vary depending on the strain. Indeed, each strain tested by Berthier (1993), who developed a medium (PTM) for the detection of H_2O_2 production, reacted differently to varying culture conditions. Media that have been used for testing H_2O_2 production include PTM, MRS (de Mann *et al.*, 1960), LBS (Difco), APT (Evans and Niven, 1951), Yeast-Peptone-Thiamine (YPT; used by Borch and Molin, 1989); Trypticase-Soy Broth (TSB; used by Brashears *et al.*, 1998), Peptonized Milk Broth and Rogosa Broth (both used by Fontaine and Taylor-Robinson, 1990), Trypticase-Phytone-Yeast Extract (TPY; used by Kot *et al.*, 1996), Benzidine-Erythrocyte-Brain Heart agar (BEB; used by Kraus *et al.*, 1957), and Manganese Dioxide Agar (MDO, used by Whittenbury, 1964). Virtually every medium contains some component known to interfere with accurate detection of H_2O_2. Manganese and other metal ions are used by some lactic acid bacteria to regulate reactive oxygen species (Archibald, 1986). Heme can both stimulate peroxide production and act as a cofactor in its destruction. Yeast extract has catalase activity

(Jaroni and Brashears, 2000); moreover it presumably contains pyruvate, which reacts spontaneously with H_2O_2 (Gunsulas and Umbreit, 1945).

Not only the chemical but also the physical characteristics of the medium affect peroxide production and detection. Some strains of bacteria produce more peroxide when shaken (shaking increases the dissolved oxygen tension), but sometimes the increased peroxide pushes the cells early into stationary phase (Marty-Teysset *et al.*, 2000). One report (Fontaine and Taylor-Robinson, 1990) even claims that peroxide production is stimulated under anaerobic conditions, though this is difficult to understand. Berthier (1993) found that greater peroxide production was detected at pH 6.0 than at pH 7.5 for some strains, but the reverse was true for other strains. Fontaine and Taylor-Robinson (1990) noted that peroxide was less stable in alkaline medium than in acid medium. In addition, many authors have assumed that temperature may affect peroxide production.

Hydrogen peroxide-producing bacteria have been investigated in many contexts because of their potential effect on human health. They play a vital role in women's health by preventing bacterial vaginosis (Naidu *et al.*, 1999). Various authors have tried to use the lactic acid bacteria that produce peroxide to prevent the growth of pathogens or spoilage organisms in food. Some examples include the use of *Lb. lactis* (*Lb. delbrueckii* subsp. *lactis*) to kill *E. coli* O157:H7 in refrigerated raw chicken (Brashears *et al.*, 1998), and the use of lactobacilli to prevent growth of psychrotrophs in milk (Gilliland and Speck, 1975). Hydrogen peroxide may interact in milk and saliva with

thiocyanate and lactoperoxidase to generate hypothiocyanate, which is lethal to many Gram-negative organisms and bacteriostatic to lactic acid bacteria (Condon, 1987).

It may be possible to explain the results of many studies in terms of the type of oxidase enzyme and the growth conditions of the cells. In homolactic organisms, pyruvate is converted to lactate in a reaction that oxidizes NADH to NAD^+. If NADH is being oxidized to produce H_2O_2, it will not be available to generate lactic acid. Therefore, organisms that are classified as homolactic based on their aerobic growth either do not possess an NADH oxidase or their NADH oxidase is repressed in high-sugar media. Lactate oxidase is also repressed by glucose (Gibello *et al.*, 1999). Therefore, one would expect homofermentative organisms to be unable to produce large quantities of peroxide during exponential growth in high-sugar media. Peroxide should accumulate only in stationary phase (when lactate concentrations are high) or when sugar concentrations are low. In contrast, one would expect many heterofermentative organisms to possess NADH oxidase and pyruvate oxidase; these cells would accumulate great concentrations of peroxide while growing in the presence of O_2.

Borch and Molin (1989) evaluated the O_2 consumption and end product formation of a variety of lactic acid bacteria. Homofermentative lactobacilli either did not use O_2 or used it mainly during stationary phase. Heterofermentative lactobacilli, leuconostocs, and carnobacteria used large amounts of O_2 during the exponential phase of growth. Many strains that metabolized oxygen during stationary phase had lower final viable cell numbers, which may indicate that they were less tolerant of H_2O_2.

Berthier (1993) noted that discrepancies exist between various methods of identifying H_2O_2-producing organisms, and developed PTM medium in response. PTM is an agar medium with only 0.2% glucose. Cells are grown anaerobically for 48 hours, then exposed to air for up to 5 h. Peroxide production is detected using horseradish peroxidase and the chromogen tetramethylbenzidine. Berthier reported that most strains produced more H_2O_2 at lower glucose concentrations and suggested that high glucose concentrations found in media like MRS and LBS interfered with H_2O_2 production. The only strain with the opposite tendency was also the only heterofermentative organism in the study, a *Leuconostoc*.

Jaroni and Brashears (2000) considered the effect of growth medium on subsequent H_2O_2 production in buffer for a strain of *Lb. delbrueckii* subsp. *lactis*. Cells grown in MRS or LBS broths both produced H_2O_2 for 5 d when suspended in phosphate buffer without a carbon source, apparently because the cells had stored energy left over from the growth medium. If glucose was added to the phosphate buffer, H_2O_2 production dropped. In contrast, cells grown in PTM broth did not produce peroxide during subsequent storage, regardless of the level of glucose in the broth or buffer. A similar observation was reported by Fontaine and Taylor-Robinson (1990). In that case, cells grown on a chromogenic agar containing horseradish peroxidase produced purple colonies. When these colonies were subcultured onto chromogenic agar they produced white colonies. Broth cultures prepared from the colonies did not produce peroxide, even though earlier broth cultures of the same strains had done so. One possible

explanation for these carry-over effects is that the cells may have taken up peroxidase from the growth medium, peroxidase that would then prevent an accumulation of H_2O_2 in the buffer.

In contrast to the above results, reports by Villegas and Gilliland (1998) and Yap and Gilliland (2000) showed that strains of *Lb. delbrueckii* subsp. *lactis* possess both an NADH oxidase and a D-lactate oxidase activity. Some strains produced more H_2O_2 in the presence of lactate; others in the presence of glucose. In the work by Villegas and Gilliland, starved cells in the presence of glucose accumulated a small amount of D-lactate which later disappeared, presumably because a lactate oxidase system was induced. These reports indicate that bacteria normally classified as homofermentative can possess both NADH oxidase and lactate oxidase activities and that both can be active at moderately high (1%) glucose concentrations in non-growing cells.

2.4. Effects of Lactate and Diacetate on Meat Spoilage Bacteria

Many studies (Qvist *et al.*, 1994; Shelef and Addala, 1994; Samelis *et al.*, 2001) have looked at the effects of organic acids in fresh and cured meat products. Most work, however, has focussed on controlling pathogens, especially *L. monocytogenes*. Consequently, the effect that organic acids have on spoilage microorganisms, and on meat colour is less well known.

Studies in broth (Ouattara *et al.*, 1997) have shown that acetic acid was more effective than lactic or citric acid at inhibiting Gram-positive spoilage bacteria, when effects were measured by weight of acid added. But the order of effectiveness was reversed if effects

were measured based on number of moles of acid. *C. piscicola* was completely inhibited by 0.1% (w/v) acetic or lactic acid in lactobacilli MRS broth (Difco) and *Brocothrix* (*B.*) *thermosphacta* was completely inhibited by 0.1% (w/v) acetic acid or 0.5% (w/v) lactic acid in brain heart infusion (BHI) broth (Difco). *Lb. curvatus* and *Lb. sakei* were both completely inhibited by 0.3% w/v acetic acid or 0.5% lactic acid in MRS broth. Thus, the inhibitory effects of acetic acid and lactic acid in broth have the same order of magnitude.

Reports by Houtsma *et al.* (1993 and 1996) demonstrated that sodium lactate had the same a_w-lowering effect as NaCl but that sodium lactate had a much greater antimicrobial activity at similar molar concentrations. Lactobacilli, carnobacteria, lactococci, listeriae, and staphylococci were all far more sensitive to sodium lactate than to sodium chloride. Indeed, of the Gram-positive bacteria tested, only the bacilli had lactate minimum inhibitory concentrations (MICs) that were close to their NaCl MICs (Houtsma *et al.*, 1993). The MIC is the minimum amount of a compound that can prevent a strain of bacteria from growing. The effect of temperature on the MICs was small but pH had a large effect. In general, the antimicrobial activity of sodium lactate was greater in an acidic environment (Houtsma *et al.*, 1996), but this effect did not increase proportionally with increases in the concentration of undissociated lactate ion. At pH 7, the MIC values for sodium lactate and NaCl were almost the same but at lower pHs sodium lactate was much more effective against many bacteria. Staphylococci, lactococci, and lactobacilli were much less sensitive to the effects of sodium lactate than were carnobacteria, listeriae and *Brochothrix.*

A report by Shelef and Addala (1994) showed that sodium diacetate was more effective than acetic acid at inhibiting *L. monocytogenes* in BHI broth. The MIC of sodium diacetate decreased from 35 mM at 35°C to 28 mM at 5°C. In ground beef and beef slurry sodium diacetate suppressed total aerobic growth but it had little effect on *Enterococcus (E.) faecalis, Lb. fermentis, and Staphylococcus (S.) aureus* in BHI broth.

There are many papers describing the antilisterial effects of organic acids in cured meat products. When sliced pork bologna was dipped in solutions of 2.5% or 5% acetic acid or 5% sodium diacetate, vacuum packaged, and then stored at 4°C, *L. monocytogenes* was unable to grow during ≤120 d of storage (Samelis *et al.*, 2001). Ten percent sodium lactate was less effective at inhibiting *Listeria* than 5% sodium diacetate. In a similar study (Samelis *et al.*, 2002), 1.8% sodium lactate inhibited listerial growth for 35 to 50 days when incorporated in frankfurters. Combinations of 1.8% sodium lactate and 0.25% sodium acetate or sodium diacetate inhibited growth for 120 d. There appears to be an interaction between the effects of organic acids and the effect of storage temperature. In bologna sausages with 2% added sodium lactate, *L. monocytogenes* grew rapidly if stored at 10°C but did not grow for 28 d if stored at 5°C (Qvist *et al.*, 1994). Similarly, a combination of 2.5% sodium lactate and 0.25% sodium acetate inhibited *L. monocytogenes* at 4°C for at least 35 days but at 9°C for only 20 d in sliced cooked ham (Blom *et al.*, 1997). At 4°C in the same study, the lactic acid bacteria were also inhibited for 10 d by the antimicrobial combination; this did not occur at 9°C.

Papers describing the effects that organic acids have on spoilage bacteria in cured meat products are few. Lemay *et al.* (2000) showed that 2% sodium lactate strongly inhibited *B. thermosphacta* in cured chicken meat. The sodium lactate had only a small effect on *Lb. alimentarius*. When 3.3% sodium lactate was present in ham (Stekelenburg and Kant-Muermans, 2001), the time required for *Lb. curvatus* to grow at 4°C from 4.5 $\log_{10}$ CFU/g to 7 $\log_{10}$ CFU/g was 5 weeks; the same growth occurred in control samples in 2.5 weeks or less. Sodium diacetate (0.2%) did not affect *Lactobacillus*.

Sodium lactate and sodium diacetate affect the sensory properties of meat products. In fresh meats, sodium lactate may enhance the red colour of the meat (see, for example, Brewer *et al.* 1995). In cured meats, no effect of sodium lactate on colour has been reported. Blom *et al.* (1997) found no sensory defects in sliced ham made with 2.5% lactate and 0.25% acetate, but servelat sausage made with the same combination of antimicrobials was less desirable than control servelat, based on taste. Qvist *et al.* (1994) avoided using 3% lactate in bologna because experience suggested that it might cause sensory defects. Stekelenburg and Kant-Muermans (2001) found no significant colour defects but reported that ham made with 0.2% sodium diacetate was inferior in odour and taste to untreated ham.

A Japanese article (Sameshima *et al.*, 1997) recently looked at the effect of sodium nitrite and sodium lactate on growth of *W. viridescens*, *Lc. mesenteroides*, and *En. faecalis*. In control samples at 10°C, populations increased from 1 $\log_{10}$ to 4-5 $\log_{10}$ in 9d. Sodium nitrite (200 ppm) extended this period to about 17 days. Sodium lactate

(2%) had little effect on *En. faecalis* but apparently prevented *W. viridescens* and *Lc. mesenteroides* from reaching 4 or 5 $\log_{10}$ CFU/g for 9 and 4 days, respectively. The English abstract, however, did not indicate whether lactate was tested alone or whether it was used in addition to the sodium nitrite (if in addition, the total period of inhibition was presumably 21 d for *Lc. mesenteroides* and 26 d for *W. viridescens*).

2.5. Thermal Tolerances of Lactic Acid Bacteria

Grant *et al.* (1988) recommended thorough cooking to destroy bacteria that might cause discolouration. In Canada, a cooked meat product is (usually) a product that has been heated to an internal temperature of 69°C (R. A. Holley, personal communication). Evidence exists, however, that the thermal resistances of bacteria vary according to the nature of the meat product. In recognition of this, and realising the differences in normal pre-cooking bacterial populations, the US Food and Drug Administration (FDA, 1999) recommends different time-temperature combinations for chicken, pork, and beef when cooked at retail or foodservice outlets. The described temperature requirements for pork products and comminuted meats are 63°C for 3 min, 66°C for 1 min, or 68° for 15 sec but all poultry products must be heated to 74°C for 15 sec. The USDA provides similar guidelines for meat processing plants and stipulates that any processor that deviates from the guidelines must demonstrate that the process being used provides a 6.5 $\log_{10}$ (most products) or 7.0 $\log_{10}$ (poultry products) reduction in *Salmonella* numbers (Orta-Ramirez and Smith, 2002). It is difficult to prove this, however, because pathogens cannot be deliberately taken into a meat processing plant and because thermal death times calculated in a laboratory or pilot plant do not necessarily predict actual death rates in a production facility (Orta-Ramirez and Smith, 2002).

Most laboratory determinations of bacterial heat tolerance rely on the concept of *thermal death time*, D_T, which is the time required to reduce an inital bacterial population by 1 $\log_{10}$. Often, the D_T is supplemented with a second value, Z, which is defined as the increase in temperature required to reduce D to one tenth its value. Both numbers are influenced by the strain of bacterium, the temperature history of that strain, the contents of the heating medium, the size and shape of the heating vessel, and the methods used to recover the organisms (Magnus *et al.*, 1986)

Traditional methods of heating, such as the test tube in water method, have been criticized because small amounts of broth may settle on areas of the tube that are above the water level and therefore be cooler than the rest of the tube (Donnelly *et al.*, 1987). In addition, because test tubes are quite thick, temperature come-up times are long and bacteria near the edge of the tube may be exposed to lethal temperatures considerably earlier than those near the centre. Heating vessels that are thin and fully immersable give a better indication of an organism's intrinsic heat resistance. For this reason, the Immersed Sealed Capillary Tube method (ISCT) has been favoured by some in recent years (Foegeding and Leasor, 1990). It indicates the intrinsic heat resistance of an organism better than most other methods and may produce more linear survivor curves. Schuman *et al.* (1997) and Donnelly *et al.* (1987) observed two-phase survivor curves with the test tube method but not with the ISCT method.

Because meat batters are not easily squeezed into thin glass tubes, and because many bacteria are far more thermally resistant in meat than in laboratory broths, some workers (for example, Magnus *et al.*, 1988) have examined bacterial heat tolerance by a variation on the ISCT idea. Meat batter impregnated with *En. faecalis* or *En. faecium* was placed in sterile plastic bags, formed into a thin layer, and sealed. The total thickness of the meat-containing bag was 4 mm. The bags were then immersed in a hot water bath and removed at intervals to determine the number of surviving bacteria. $D_{66°C}$ values obtained in this way for *En. faecium* were in the range of 14 – 30 min, indicating substantially more heat resistance than the organism exhibits in broth ($D_{60°C}$ = 17 to 35 min; Magnus *et al.*, 1986). *En. faecalis* was less hardy than *En. faecium*.

The thin-layer immersion methods described above have the advantage that all organisms are exposed to nearly the same degree of heat for the same period of time but they suffer from the disadvantage of being very unlike the actual conditions of meat processing. For example, the internal temperature of ground beef patties may vary by up to 18°C when the center temperature is measured at 71°C (Orta-Ramirez and Smith, 2002). Thus for ground beef at least, actual and expected thermal death rates will not coincide, even if the thermal resistance of the organism is known. Furthermore, thin layer methods almost never take into account the effect of heating rate on thermal resistance. A typical ISCT procedure is to store the organisms on ice, suddenly immerse them in water at 60°C or more, and then immediately remove them to an ice-water slurry (Schuman *et al.*, 1997). Such a method tends to minimize calculated thermal death times by giving the organisms no opportunity to prepare for the increased

temperature. The experiments of Borch *et al.* (1988) may illustrate the point. When *W. viridescens* was heated suddenly to 68°C in APT broth, a 3 $\log_{10}$ reduction in viable cell numbers occurred in about 6 minutes and a 6 $\log_{10}$ reduction took 40 min. But when the same strain was heated from 12°C to 70°C over a period of 120 min, only a 1 $\log_{10}$ reduction occurred, even though the organism was at temperatures greater than 60°C for nearly 40 min. Nevertheless, fixed temperature D and Z value calculations are still accepted as a standard way of evaluating thermal tolerance.

Relatively few authors have tried to define the heat resistances of lactic acid bacteria. Franz and von Holy (1996) found that *Lb. sakei*, *Lb. curvatus*, and *Lc. mesenteroides* were not particularly heat-tolerant and had $D_{57°C}$ values < 1 min. Borch et al. (1988) recorded that numbers of an unidentified *Lactobacillus* dropped at least 6 $\log_{10}$ over 4 min at 60°C. Among organisms reputed to be heat-tolerant are A. *viridans*, *W. viridescens*, and *En. faecium*. A. *viridans* is supposed to survive 60°C for 30 min, based on a report by Williams *et al.* (1953) but the report did not indicate the method used. Niven *et al.* (1954) reported that a strain of *W. viridescens* isolated from bologna cores survived 120 min when held at 65.5 °C in 5 ml of tomato juice broth. They also found that heat-sensitive strains became more heat-tolerant after they were exposed to successive heat shocks and that heat-resistant strains rapidly become more heat-sensitive when grown in the laboratory. Later, Milbourne (1983) found that *D* values for this organism in MRS broth were 23.5 min ($D_{65°C}$), 12 min ($D_{75°C}$), and 9.5 min ($D_{80°C}$). But the $D_{65°C}$ values varied greatly when the bacteria were heated in other media. In view of these reports, it seems likely that at least some of the bacteria responsible for

green discolouration are heat-tolerant enough to survive cooking to a final internal temperature of 69°C.

2.6. Summary

In conclusion, H_2O_2 producing lactic acid bacteria are the most common cause of discolouration in cooked cured meats. Despite Niven et al.'s (1949) assertion that "any salt-tolerant, catalase-negative microorganism that is capable of growing at low temperatures, and that oxidizes certain substrates with the accumulation of hydrogen peroxide, might be found associated with ... this type of spoilage," repeated isolation of *W. viridescens* and *A. viridans* from cured meats and meat curing facilities argues that the number of species responsible in practice may be small. Some of the organisms are resistant to heat but many probably are not; for this reason the most effective way to prevent green discolourations is to keep the product as close to sterile as possible between cooking and packaging. Whether antimicrobials such as sodium lactate or sodium diacetate can prevent discolouration is unclear from the literature.

3.0. MANUSCRIPT # 1

AEROCOCCI AND CARNOBACTERIA CAUSE DISCOLOURATION IN COOKED CURED BOLOGNA

3.1. Abstract

Alkalitrophic lactic acid bacteria were isolated from discoloured bologna and from a bologna manufacturing plant. The organisms grew on vacuum-packaged bologna at both 4°C and 9°C and induced green discolouration in ≤ 3 d upon exposure to air. *Weissella viridescens* ATCC 12706 also caused greening but *Enterococcus faecalis* ATCC 7080 and other strains of lactic acid bacteria did not. The organisms that caused greening in meat were *Aerococcus viridans* and *Carnobacterium viridans.* Meat-agar test systems failed to predict an organism's ability to produce green discolouration. The speed and extent of green discolouration varied greatly depending on the organism and the composition of the bologna. Greening in cooked cured meats may often be due to alkalitrophic bacteria and not to the acidiphilic lactobacilli, leuconostocs or pediococci, as is commonly thought.

3.2. Introduction

Occasionally, manufacturers of vacuum-packaged, cooked, cured meat products experience problems with green discolouration caused by bacteria. Unfortunately, studies on greening have been intermittent, since funding for research tends to end when the problem does. Little work has been done since Grant *et al.* (1988) reviewed the subject but the problem remains and a laboratory method is needed to detect the presence of the bacteria responsible.

Though hydrogen sulfide-producing bacteria cause discolouration in vacuum-packaged fresh meats, only hydrogen-peroxide producing bacteria have been shown to produce green discolouration in cooked cured meat products (Incze and Delenyi, 1984; Niven *et al.*, 1949; Lee and Simard, 1984). Two species, *Weissella viridescens* and an organism resembling the former species *Lactobacillus leichmannii*, are able to survive cooking, but others presumably contaminate the meat during the slicing and packaging steps (Grant *et al.*, 1988). The organisms grow in the vacuum-packaged product without causing visible spoilage. After a consumer opens the package the bacteria begin generating hydrogen peroxide which reacts with the cured-meat pigment, nitrosohemochrome (Grant *et al.*, 1988), and turn the meat green within a few days.

Previous studies have attributed greening largely to the acid-loving genera *Lactobacillus, Leuconostoc,* and *Pediococcus* (Grant *et al.*, 1988), even though cooked cured meat products are nearly neutral in pH. In the last 15 years the taxonomy of the lactic acid bacteria has changed. Some strains that would once have been identified as

lactobacilli or leuconostocs, are now better placed in the genus *Weissella* (Collins *et al.*, 1993b) or *Carnobacterium* (Collins *et al.*, 1987). In addition, though they have both been known for some time, the acidiphilic pediococci and the alkalitrophic aerococci are phenotypically similar and are often hard to distinguish from one another.

In this study, several organisms from green vacuum-packaged bologna and from a Canadian bologna manufacturing facility were identified. These organisms cause greening on sterile vacuum-packaged bologna slices. In this report it is shown that some alkalitrophic lactic acid bacteria can cause discolouration in a cooked, cured meat product and that similar bacteria could have been misidentified in earlier research. It is also demonstrated that some model systems, previously used to study greening, do not predict whether discolouration will occur in a particular cured meat product.

Alkaliphilic organisms have been defined as those that have pH optima for growth above pH 8.5 (Prescott *et al.*, 1990). Since the upper pH optima for growth of the spoilage organisms used in this study remain undefined, we have chosen to use the term alkalitrophic to describe their alkali-tolerant character.

3.3. Methods

3.3.1. Type of Bologna

This study used sliced vacuum-packaged bologna from two Canadian meat processors. Regular bologna from plant A was made with pork, mechanically separated chicken, mechanically separated pork, mechanically separated beef, beef, water, salt, sugar, sodium phosphate, spices, sodium erythorbate, dextrose, sodium nitrite, and smoke. It contained 12.5% protein, 21.7% fat, and 0.2% carbohydrate. The composition of all-beef bologna from plant A changed slightly between the two trials. During the first greening trial, all-beef bologna from plant A was similar to regular bologna but lacked chicken, pork, sugar, and dextrose. The all-beef bologna used during the second trial lacked these ingredients and also did not contain mechanically separated beef. Its composition was 13% protein, 20% fat, and 0.3% carbohydrate.

All beef bologna from plant B was made using the following ingredients: beef, water, wheat flour, modified milk ingredients, salt, spice, sugar, sodium erythorbate, garlic powder, sodium nitrite, and smoke. It contained 12% protein, 20% fat, and 6.7% carbohydrate.

3.3.2. Bacteria

Carnobacterium piscicola ATCC 43225, *Carnobacterium divergens* ATCC 35677, *Carnobacterium gallinarum* ATCC 49517, *Carnobacterium mobile* ATCC 49516, *Weissella viridescens* ATCC 12706, *Aerococcus viridans* ATCC 11563, and *Enterococcus faecalis* ATCC 7080 were obtained from the American Type Culture

Collection (Rockville, MD). *Leuconostoc mesenteroides* # 1 was isolated during a previous study (Holley *et al.*, 1996). Strains MPL-A through MPL-F were isolated from equipment swabs by quality control staff at plant A. To isolate strains MPL-1 through MPL-15, a package of regular bologna from plant A was stored unopened at 10 °C until a month after the product expiry date shown on the package. The packages were then aseptically cut open to expose the contents to air, overwrapped, and stored unsealed at 10°C. Bacteria were isolated from a slice that was visibly discoloured after 24 h, as follows. The slice was homogenized in 0.1% peptone using a Stomacher 400 Lab-blender; the resulting bacterial suspension was serially diluted, and the dilutions were plated on a variety of agars, including APT (Difco), MRS (Difco), M5 (Zúñiga *et al.*, 1993), PTM (Berthier, 1993), and the MPL meat-agar system (see below). All strains were maintained in APT broth or agar, and stored at –80°C in APT broth containing 20% (w/v) glycerol.

3.3.3. Identification procedures

Cells were examined by phase-contrast microscopy and tested for catalase and oxidase reactions by standard methods (ASM, 1981). Gram reaction was determined by the 3% KOH test (Gregersen, 1978). Growth at various temperatures and salt concentrations was tested in APT broth. Gas production from glucose was tested using inverted Durham vials in MRS broth with citrate omitted (Schillinger and Luckë, 1987). Ammonia production from arginine was tested by the method of Schillinger and Luckë (1987). Growth at pH 9.6 was tested in the medium of Chesbro and Evans (1959). Carbohydrate fermentation profiles were determined using the API 50CHL system (bioMerieux Vitek Inc., Hazelwood, MO). The API 20Strep system (bioMerieux) was

used to detect the presence of various enzymes. The presence of *meso*-diamino-pimelic acid (*meso*-DAP) in the cell wall was determined by thin layer chromatography of whole cell hydrolysates (Bousefield *et al.*, 1985). The test kit for lactate optical isomer was from Boehringer-Mannheim (FRG). Hydrogen sulfide production was tested using triple sugar iron agar and lysine iron agar (Difco Laboratories Inc., 1984). Nitrate reduction was tested using Bacto-Nitrite test strips (Difco Laboratories, Detroit Michigan). Gelatin hydrolysis was tested in gelatin agar consisting of gelatin, 30 g/l; sodium chloride, 10 g/l; trypticase, 10 g/l; and agar, 15 g/l. Hemolysis was tested on defibrinated sheep blood agar. Dextran production was determined on sucrose agar (Garvie, 1984) and acetate tolerance on Rogosa SL agar (Difco). Pulsed field gel electrophoresis (PFGE) was done by the method of Zhang and Holley (1999). Isolates were ribotyped by Qualicon Inc. using the Riboprinter® Microbial Characterization System following the manufacturer's procedure (Bruce, 1996)

3.3.4. Demonstration of greening potential

Two incubation experiments were carried out. In the first experiment, 375g retail packages of regular and all-beef bologna were obtained from plant A and electron beam-irradiated at Acsion Industries, Pinawa MB (estimated dose 5-8 kGy). Individual slices were aseptically transferred to WinPak Deli*1 bags. Overnight cultures of bacteria were diluted in peptone to about 10^5 CFU/ml and 400µl of this suspension was applied to the upper surface of the slice and spread with a sterile glass rod to yield about 500 CFU/cm^2. The packages were heat-sealed under vacuum using a Bizerba model GM 2002 vacuum packaging machine. Four treatment groups were set up based on bologna type (regular or all-beef) and incubation temperature (4°C or 9°C). Each group

of 44 slices was subdivided into 4 series of 11 slices each with 10 bacterial cultures to a series, plus one control treated with sterile peptone water. Two of the series received no additional treatment and were stored in the dark. A third series was stored under light (0.03 – 1.3 k lux) and for the fourth, sterile pieces (about 1 cm^2) of mild steel, stainless steel T304, and aluminium foil were placed on top of the inoculated bologna, inside the vacuum bag. Samples were incubated at 9°C for 33-41 d or at 4°C for 57-67 d, before being opened. Upon opening, each slice was sampled for bacteria by removing a cross sectional piece (10 cm^2 on each side) from a point half way between the center and edge of the slice, homogenising it for ≤ 2 min in a Stomacher 400 Lab-blender, and then plating serial dilutions on APT agar. After sampling, the remainder of each slice was transferred to a sterile Petri dish and incubated aerobically at 4°C or 9°C, as appropriate.

The protocol was altered for the second experiment. Slices of all-beef bologna from plant A were individually vacuum-packaged in WinPak Deli*1 bags before being irradiated (estimated dose 5-6 kGy). After irradiation, they were frozen until used. Overnight or 48 h cultures were serially diluted in 0.86% (w/v) saline to give a bacterial concentration of $2.5x10^7$ CFU/ml. Thawed vacuum packs were opened on one side and 400 µl of bacterial suspension were added (estimated bacterial concentration: $1x10^5$ CFU/cm^2) to the top side of the slice. The inoculum was spread over the top surface by massaging the bag. Bags were vacuum-sealed again and kept in the dark at 9°C for 25-28 d. Upon opening, slices were sampled and transferred to Petri dishes, as above. Numbers of bacteria/cm^2 were determined by sampling (10 cm^2 per side) from the center of each slice, homogenizing as above, and plating on APT agar. Colour

changes were measured using a HunterLab Miniscan spectrocolourimeter (Reston, Virginia) after 3 d of aerobic incubation. Where discolouration was uneven, measurements were taken from the area that appeared to have the most intense greening. Statistical analyses were carried out using Bonferroni's T test on the SAS computer program version 6.12 (Statistical Analysis Systems Institute, 1999).

The second experiment included two supplementary tests. In the first, samples were treated normally, except that the inoculum was prepared in 10% (w/v) sodium erythorbate plus saline (0.86% NaCl w/v) rather than in saline alone. In the second, inoculated samples were treated with an additional 0.4ml of either 0.86% saline or 0.86% saline plus 3% (w/v) catalase, after the 25-28 d storage period and before the 3 d of aerobic incubation.

3.3.5. Meat-Agar Systems

The meat-agar medium (GMA) designed by Grant and McCurdy (1986) was prepared. Another meat-agar test system (MPL) developed at plant A was also prepared, as follows. Refrigerated pork hearts obtained commercially were prepared by removing connective tissue, weighing out 200 g sections, and freezing. When needed, a 200 g section was thawed, chopped into small pieces, and ground in a blender. The following ingredients were added: 37.5 ml of citrated bovine blood (Oxoid Inc., Nepean, ON Canada), 27.5 g MRS broth, 10.0 g agar, 18.75 g NaCl, 0.075 g $NaNO_2$, 1.0 g sodium erythorbate, and 200 ml water. The mix was pureed and then transferred to a beaker, heated to boiling, with stirring, until the meat was cooked (approx 30 min). It was then transferred back into the blender jar, blended, and sterilised by treatment at 121 °C for

≥15 minutes. After sterilisation the medium was blended again until it had a smooth and consistent texture; it was then poured into Petri dishes and allowed to solidify.

3.3.6. Microbial Analysis of Chilling Brine

Fifty ml aliquots of chilling brine (26.4% sodium chloride) from plant A were passed through hydrophobic grid membrane filters (QA Life Sciences Inc., San Deigo, CA). After being rinsed twice with sterile water, the filters were placed on the following agars: APT (Difco), PTM (Berthier, 1993), GMA (Grant and McCurdy, 1986), CTSI (Wasney *et al.*, 2001), and blood agar containing potassium tellurite plus crystal violet (Evans, 1986). They were examined in air after having been incubated 51 h at 22°C in jars made anaerobic with BBL™ GasPak Plus™ envelopes (Becton Dickinson and Company, Sparks MD). After examination, plates were placed in an anaerobic (20% CO_2: 80% N_2) incubator (22°C) and incubated 14 h. Aliquots of brine were also surface-spread on plates of the various agars. In addition, a swab from the brine tank was massaged in 0.86% saline and the resulting suspension was plated on the above media and incubated at 22°C for 24 h in an anaerobic incubator.

3.4. Results

Table 3.1 provides the carbohydrate fermentation profiles of strains isolated in this study. DNA fingerprint analyses using pulsed field gel electrophoresis (*Sma* I restriction endonuclease) and ribotyping (Riboprinter® Microbial Characterization System using endonuclease *Eco* RI) showed that isolates MPL-4,5,6,7,and 10 were a single strain and that MPL-1 was closely related to these organisms. While MPL-12 was shown to be a different strain, MPL-1, 4,5,6,7,10, and 12 belonged to the same species (data not shown). All strains were Gram-positive, catalase and oxidase negative cocci arranged in tetrads. They formed colonies about 1 mm in diameter on APT agar. In broth they produced uniform turbidity, except for strain 12, which had a more flocculent appearance. The organisms did not contain *meso*-DAP and they produced L (+) lactate but no gas from glucose. They produced ammonia from arginine, but did not produce hydrogen sulfide. They grew at pH 9.6 and at 40°C but not 45°C. In APT broth some strains failed to grow at 8°C. At least one strain (MPL-1) grew on otherwise sterile bologna at 4°C. Nitrate was not reduced and gelatin was not hydrolysed. No dextran was produced from sucrose. The strains grew in the presence of 10% salt but did not grow on Rogosa SL (acetate) agar. A zone of greening could be seen on blood agar and colonies on PTM agar were dark blue, indicating that they produced hydrogen peroxide. Growth on KF Streptococcus agar and CTSI agar was variable. The final pH in MRS broth was between 5.2 and 5.9. These strains were identified as *Aerococcus viridans*. The limited number of profiles of *A. viridans* in the Riboprinter® database did not permit an identification of this group of organisms using this method.

The second group (MPL-11 and MPL-14) represented a previously unidentified species of *Carnobacterium*. Holley *et al.* (2002) described these strains in detail and assigned the name *Carnobacterium viridans* to both. They were Gram-positive, catalase and oxidase negative rods. PFGE, ribotyping, and 16s rDNA sequencing all indicated that the two strains were the same (Holley *et al.*, 2002). They produced L(+) lactate from glucose and possessed *meso*-DAP in their cell walls. They did not grow on acetate agar, PTM agar (at pH 6.0 or pH 8.0), MPL agar, sucrose agar, KF Streptococcus agar, or the pH 9.6 medium of Chesbro and Evans but did grow on APT agar, CTSI agar, and M5 agar. In MRS broth, growth was very poor unless the broth was supplemented with 0.25% cysteine and readjusted to a pH of at least 6.8.

The third group (MPL-3 and MPL-15) were Gram positive, catalase-positive rods. They were identified as *Brochothrix thermosphacta* because of their cellular morphology, colony morphology, and carbohydrate fermentation pattern (API 50CHL), and they were not responsible for green discolouration.

In addition to the bologna isolates, 6 strains of Gram-positive, catalase and oxidase negative cocci (MPL-A to F) were isolated from equipment swabs at the processing plant. Characteristics of the equipment isolates, as well as those isolated from meat, are listed in Tables 3.1 and 3.2. MPL-A was identified as *Leuconostoc* sp. and MPL-C as *Leuconostoc mesenteroides* from phenotypic data. Ribotyping showed MPL-E and F to be two distinct strains of *Pediococcus pentosaceus*. MPL-B and D were identified as *A. viridans*. Since ribotyping yielded a similarity index of 0.99 for MPL-B and D, it was

clear that they were the same strain. They were similar to the aerococci isolated from bologna except in the following characters. They had a flocculent growth style like that of MPL-12 in broth. They formed clusters and pairs but rarely tetrads. Acid production from many carbohydrates was weak and the carbohydrate fermentation pattern differed slightly from the other aerococci (Table 3.1). The presence of meso-DAP in their cell walls was not determined. These strains were not compared to the other aerococci by PFGE and the small number of aerococci in the Riboprinter® database did not permit an identification.

Two experiments were carried out in an attempt to induce greening in bologna. The first experiment examined the effects of light, several metals, and bacteria on irradiation-sterilised bologna stored at either 4°C or 9°C. Regular and all-beef bologna from plant A were used in this experiment. The two types of bologna reacted in essentially the same way. Irradiation at doses ≤8 kGy had no apparent effect on the colour of vacuum-packaged bologna nor did exposure to metals – mild steel, stainless steel T304 and aluminium foil. Exposure to light also had no effect on vacuum-packaged bologna during 9 weeks of storage, but after the packages were opened, all light-exposed slices became visibly discoloured within 2 d. The degree of discolouration associated with bacteria, however, was much greater. Each bologna slice was sampled for bacteria when the package was opened and was then observed daily for greening. Strains MPL-1 (*A. viridans*) and MPL-2 (a strain of *Carnobacterium* that was lost on subsequent transfer) consistently grew and induced greening at either 4°C or 9°C. Visible greening occurred only after the vacuum packages were opened and took 2 d or less at 9°C and ≤3 d at

4°C. The following strains failed to grow or grew poorly on bologna in this trial: MPL-B (*A. viridans)*, MPL-E and F (*P. pentosaceus*). Other strains grew but did not cause greening; these included *Leuconstoc* spp. MPL-A, *Leuconostoc mesenteroides* # 1 and MPL-C, *Enterococcus faecalis* ATCC 7080, and *Brochothrix thermosphacta* MPL-3.

In the second experiment, irradiated vacuum-packed bologna slices were inoculated with approximately 10^5 CFU/cm^2 and growth was considered positive if the number of CFU/cm^2 reached 10^6 before package opening. In addition to bacteria, the effects of added sodium erythorbate and catalase were considered. The level of sodium erythorbate (10 % w/v in 400µl of solution) was chosen to mimic the effect of a sodium erythorbate dip as permitted in the U S Code of Federal Regulations 9 CFR 318.7 (Anonymous, 2001). Erythorbate reduced the extent of discolouration (Table 3.3). Strains that caused greening included MPL-B and D, *W. viridescens* ATCC 12706, *A. viridans* MPL-1, 4, and 12, and *C. viridans* MPL-11 (Table 3.3). The following strains grew but did not induce greening: *C. piscicola* ATCC 43225, *C. divergens* ATCC 35677, *C. gallinarum* ATCC 49517, *C. mobile* ATCC 49516, *A. viridans* ATCC 11563, and *P. pentosaceus* MPL-E. *P. pentosaceus* MPL-F failed to grow.

Three days after opening packages, the colour of each slice was evaluated using the HunterLab colour measurement system. With this system, colour is measured by three parameters: *L* (lightness), *a* (red-green colour scale), and *b* (yellow-blue colour scale). Bacteria affected all three colour parameters ($P< 0.0001$) but the effects on *L* and *b* were small. Selected *a* measurements are listed in Tables 3.3 and 3.4; they were typically

around 9 and never dropped below 1.6. Since positive values of *a* represent the colour red and negative values represent green it appears that the overall colour of bologna did not truly become green, although there may have been small pockets within the measurement area (about 3 cm diameter) that were actually green. The discoloured slices appeared green to the eye when viewed against white or black backgrounds in the presence of sterile (non-discoloured) control slices.

The intensity of greening depended on the bacterial strain, the bologna formulation, and the presence of anti-oxidants. Beef bologna from plant B discoloured more quickly than that from plant A (Table 3.4), although the final bacterial concentrations were similar. The two bologna products differed in composition, because the all-beef bologna from plant B contained added wheat flour, modified milk ingredients, sugar, and dextrose. Consequently the carbohydrate content of all-beef bologna from plant B was approximately 20 x greater than that of the all-beef bologna from plant A, although the fat and protein contents were similar. The age of the bologna may also have been a factor since the bologna from plant B was eleven d past the end of its shelf life when it was sterilized by irradiation; that from plant A had 7 weeks remaining. However, the effects of age and carbohydrate content on greening were not examined in detail.

C. viridans MPL-11 consistently produced more intense greening than either the aerococci or *W. viridescens* (Tables 3.3 and 3.4). Addition of 3 % catalase to the surface of the meat prevented or reduced discolouration in every case. The same volume of

sterile saline had no effect on greening (data not shown). Sodium erythorbate addition had less effect on greening than did catalase addition.

Previous authors (Shank and Lundquist, 1963; Lee and Simard, 1984; Grant and McCurdy, 1986) devised meat-agar systems that might predict an organism's ability to cause the green defect in meat. Table 3.5 shows the reactions of bacteria used in this study on two meat-agar systems and on the medium (PTM) designed by Berthier (1993) to detect hydrogen peroxide production. As shown, none of the systems predicted discolouration of meat reliably.

Since aerococci have been found in meat curing brines (Deibel and Niven, 1960), and since many of our isolates survived several weeks in APT broth that contained 26.4% salt (Table 3.6), we attempted to detect greening organisms in a chilling brine used at plant A. Blood-tellurite-crystal violet agar, which Evans (1986) considered the best selective agar for aerococci, permitted the growth of yeasts and Gram negative bacteria. On the other hand, CTSI agar, which selects for carnobacteria, inhibited most of the organisms found in salt brine. Only a single colony (a catalase-positive rod) grew, although more than a dozen plates of this agar were used. Most isolates from the brine were catalase positive strict aerobes; no aerococci or carnobacteria were found.

3.5. Discussion

Few researchers have investigated the bacteria that produce discolouration in cured meats. Grant *et al.* (1988) reviewed the subject and found that the bacteria most often associated with greening were lactobacilli, leuconostocs, pediococci, and streptococci. Since then the taxonomy of the lactic acid bacteria has changed but no report has considered the effects these changes have had on identities of bacteria responsible for discolouration of cooked cured meats or the effects of imprecision in earlier generic identifications.

We have studied the greening problems experienced in a commercial bologna manufacturing facility. Irradiation at doses up to 8 kGy, light at intensities of up to 1.3 k lux, and bacteria concentrations of 10^7 CFU/cm^2, all failed to induce greening in vacuum-packaged bologna. Both light and certain strains of bacteria consistently caused greening in bologna that was exposed to air. None of the bacteria that caused green discolouration were able to produce H_2S. The enzyme catalase, which destroys hydrogen peroxide, or the antioxidant sodium erythorbate reduced or eliminated greening in every case. Similarly, earlier reports (Grant *et al.*, 1988; Shank and Lundquist, 1963; Watts, 1954) have shown that bacteria or light can react with oxygen to generate hydrogen peroxide at the surface of a cured meat product, and that the hydrogen peroxide in turn reacts with the meat pigments, causing discolouration.

Phenotypic and genotypic tests showed that the organisms that caused discolouration were *A. viridans, C. viridans,* and *W. viridescens* but the ATCC type strain of *A.*

viridans, and representative strains of most known species of *Carnobacterium* failed to cause discolouration. In contrast to previous reports (Grant *et al.*, 1988), three strains of *Leuconostoc*, two of *Pediococcus*, and one of *Enterococcus* also failed to induce greening on bologna, though they did induce greening on meat-agar test systems.

Considering the above results and recent taxonomic changes, it is possible that the acididuric genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* do not cause greening in cooked cured meat. The report by Lee and Simard (1984) that several lactobacilli cause greening is open to question because the strains isolated from meat products were not identified to the level of species and the strains that had been identified to species were not associated with spoiled meat products. Furthermore, the organism best known for producing a green appearance in cured meats, *Lactobacillus viridescens,* is now a member of the genus *Weissella* (Niven *et al.*, 1949; Niven and Evans, 1957; Collins *et al.*, 1993b). Another such organism was identified by Incze and Delenyi (1984) as *Lactobacillus leichmanii,* but its pH tolerances were not determined, and its growth temperature profile disagrees with the summary given by Kandler and Weiss (1986) for *L. delbrueckii*, of which *L. leichmanii* is now a subspecies. The organism might have been a *Carnobacterium*. So far as we can determine, no current member of the genus *Lactobacillus* has been conclusively shown to discolour cooked cured meats.

The claim that leuconostocs and pediococci cause greening seems to be based on work by Niven *et al.* (1949) and Deibel and Niven (1960), who found that leuconostocs and pediococci, in addition to *Lb. viridescens*, induced greening in sausages. However, the

leuconstocs were never identified to the level of species and might, if tested now, be classified in the genus *Weissella* (Collins *et al.*, 1993b). Deibel and Niven (1960) believed the aerococci to be a species within the genus *Pediococcus* and recommended that the genus *Aerococcus* be rejected. Nevertheless, *Aerococcus* is accepted as a legitimate genus (Collins *et al.*, 1990). However, the statement in Bergey's Manual (Evans, 1986) that aerococci do not hydrolyze arginine can be misleading since 3 of 19 strains examined by Deibel and Niven (1960) were able to use arginine, as were all our isolates. Deibel and Niven (1960) also observed that aerococci, but not *Pediococcus cerevisiae* , produced discolouration when incubated overnight on a cured meat product, albeit at much higher temperatures than used in the current study. None of the leuconostocs or true pediococci that we tested were able to cause greening. Consequently, *Weissella* is possibly the only acididuric genus that can cause discolouration in cooked cured meats under normal refrigerated storage conditions.

Some authors have studied greening using meat-agar model systems rather than cured meat products. Lee and Simard (1984), for example, used such a system to screen lactobacilli for greening potential but could not report a consistent connection between greening in a meat product and in the test system. Similarly, we were unable to correlate greening in sliced bologna with greening in two model systems (Table 5). Even two commercial bologna formulations differed remarkably in the speed and extent that discolouration developed. The factors that influence hydrogen peroxide production (and therefore greening) are poorly known. Since in our study a high-carbohydrate bologna was especially vulnerable to discolouration, it is possible that model systems that

incorporate high-sugar media (like MRS agar) may overestimate the potential of a strain to cause greening. On the other hand, the high carbohydrate bologna was from a different manufacturing plant (plant B), was past the end of its shelf life before being sterilized by irradiation, and had additional compositional differences.

The factors that influence hydrogen peroxide production are not fully known. Many lactic acid bacteria possess oxidase enzymes capable of reducing oxygen to hydrogen peroxide while oxidizing NADH, lactate, pyruvate, or other metabolites (Condon, 1987). The oxidation of pyruvate is also a decarboxylation reaction that ultimately leads to production of acetic acid and ATP rather than lactic acid. Borch and Molin (1989) observed that homofermentative lactic acid bacteria tend to consume oxygen mainly during stationary phase while species considered heterofermentative (based on their end products in glucose broth) consumed large amounts of oxygen during exponential phase. This difference may be related to the oxidase enzymes. For example, an organism that uses NADH to generate large amounts of H_2O_2 may not have enough NADH, when growing on glucose, to produce stoichiometric amounts of lactic acid. If that organism also possesses a pyruvate oxidase/decarboxylase enzyme, it may generate more acetic acid than lactic acid, even when fermenting glucose by glycolysis. Interestingly, carnobacteria, which ferment glucose by glycolysis (De Bruyn *et al.*, 1988), produce large amounts of acetate while consuming oxygen (Borch and Molin, 1989). Also, Berthier (1993) found that H_2O_2 production from several homofermentative organisms was greatest at very low glucose concentrations. It is possible to interpret this result as indicating that these strains possessed an oxidase

enzyme (perhaps a lactate oxidase; Gibello *et al.*, 1999) that was repressed in the presence of glucose. The only heterofermentative strain in that study, a *Leuconostoc* spp., apparently produced larger amounts of H_2O_2 when grown on agar that contained 4 g or 20 g of glucose/l than when grown on agar that contained 0.2 g glucose/l.

The bacteria that caused green discolouration in this study shared at least two common features: salt tolerance and pH preferences. All survived several weeks in broth containing 26.4% salt and grew in mildly alkaline broth but failed to grow on acetate agar (pH 5.4). Perhaps these characteristics are typical of the lactic acid bacteria that discolour cooked cured meats.

Our findings have implications for manufacturers of cooked cured meats. It seems clear that simply adding reducing agents like sodium erythorbate to cooked cured meat products will not prevent the loss of red colour that certain lactic acid bacteria induce. The erythorbate levels used in this study are close to the maximum levels permitted in the United States, 9 CFR 318.7 (Anonymous, 2001). Refrigeration also fails to prevent this colour loss. Some hope, however, may lie in the pH sensitivity of the causative organisms, if the bacteria found in this study are indeed typical. Manufacturers must be made aware that reliace upon alkaline cleaners for sanitation is therefore unwise and periodic use of an acid cleaning step to kill alkalitrophic bacteria is advisable. Additionally, a small reduction in the pH of a cured meat product should slow the growth of carnobacteria and aerococci. Carnobacteria in particular are sensitive to organic acids and might be inhibited by them even without a change in pH.

Nevertheless, a more comprehensive survey of the psychrotrophic lactic acid bacteria is needed to identify the full range of organisms that cause green discolouration.

3.6 TABLES

Table 3.1. Carbohydrate fermentation patterns of MPL isolates[a]

Carbohydrate	Strain Designation[b]							
	1, 4, 5, 6, 7, 10, 12	11, 14	3, 15	A	B, D	C	E	F
Glycerol	+/w[c]	-[c]	+	-	w	-	-	w
L-Arabinose	-	-	-	-	-	+	+	+
Ribose	+[d]	-	+	w	w	+	+	+
D-Xylose	-	-	-	-	-	+	+	-
Galactose	+	+	-	-	+	+	+	+
Rhamnose	-	-	-	-	-	-	w	w
Inositol	-[d]	-	+	-	-	-	-	-
Mannitol	+	-	+	w	w	w	-	-
α methyl-D-glucoside	w	-	-	+	w	+	-	-
Amygdalin	+	-	w	-	w	w	-	+
Arbutin	+	-	w	-	w	-	w	+
Esculin	+	+	+	-	+	+	-	+
Salicin	+	-	+	-	w	w	w	+
Cellobiose	+	+	w	-	+	w	+	+
Maltose	+	+	+	w	+	+	-	+
Lactose	+	w	-	-	+	+	-	w
Melibiose	-	-	-	-	-	+	-	+
Saccharose	+	+	+	+	+	+	-	+
Inulin	-	-	-	-	-	-	-	+
D-Raffinose	-[d]	-	-	-	w	+	-	+
Starch	-/w	-	w	-	-	-	-	-
β-Gentiobiose	+	-	+	-	+	w	w	+
D-Turanose	-/w	-	w	+	-	+	-	-
D-Tagatose	-	+	+	-	-	-	-	+
Gluconate	w	-	w	-	w	w	w	w
2-keto-Gluconate	-	-	-	-	-	-	-	w
5-keto-Gluconate	-	-	-	-	-	w	-	-

[a] Profiles were determined using the API 50 CHL system. All strains were positive (+) or weakly positive (w) for glucose, fructose, trehalose, N-acetyl-glucosamine, and mannose. All strains were negative (-) for erythritol, D-arabinose, L-xylose, adonitol, β-methyl-xyloside, sorbose, dulcitol, sorbitol, α methyl-D-mannoside, melezitose, glycogen, xylitol, lyxose, fucose, and arabitol.

[b] Strain identities as follows: MPL-1,4,5,6,7,10, and 12, *A. viridans*; MPL-11, 14, *C. viridans*; MPL-3, 15, *B. thermosphacta*; MPL-A, *Leuconostoc sp.*; MPL-B and D, *A. viridans*; MPL-C, *L. mesenteroides*; MPL-E and F, *Pediococcus pentosaceus*.

[c] Reactions were determined by colour after 72 h at 30 °C. (-), blue (negative reaction); (+) yellow or yellow-green (positive reaction); (w) green (weak reaction).

[d] Except MPL-12, which was positive for raffinose and inositol and negative for ribose.

Table 3.2. Characteristics of cocci from a bologna manufacturing facility

Test	Strain[a]				
	MPL-A	MPL-B, D	MPL-C	MPL-E	MPL-F
Vogues-Proskauer[b]	+[d]	+	+	+	+
Hippurate hydrolysis[b]	-	+	-	+	+
Esculin hydrolysis[b]	-	+	+	w	+
Pyrrolidonyl arylamidase[b]	-	+	-	-	-
α-galactosidase[b]	-	-	+	-	+
β-galactosidase[b]	-	-	+	-	+
Leucine arylamidase[b]	+	-	+	+	+
Gas from glucose	+	-	+	-	-
Ammonia from arginine	-	+	-	+	+
Growth at pH 9.6	-	+	-	-	-
Growth in 6.5 % salt	+	+	+	-	+
Growth in 4.0 % salt	+	+	+	+	+
Produces dextran from sucrose	-	-	+	-	-
Growth at 10 °C	+	+	+	+	+
Growth at 40 °C	-	+	+	+	+
Growth at 45 °C	-	-	-	+	-
H_2S Production	-	-	-	-	-
Growth in sodium azide[c]	-	+	+	+	+
Tetrazolium reduction[c]	n. g.[e]	+	-	-	-

[a] Strain identities: see text or footnote to Table 1.

[b] Tests were carried out using the API 20 Strep system. All strains were positive for Vogues-Proskauer reaction and negative for β-glucuronidase and alkaline phosphatase.

[c] Strains that grew on KF Streptococcus agar were positive for growth in azide. Red colonies were considered positive for tetrazolium reduction; white colonies were negative.

[d] Symbols as in Table 1.

[e] No growth on KF Streptococcus agar.

Table 3.3. Effect of bacterial strain, sodium erythorbate, and catalase on colour of all-beef bologna[a]

Species	Strain	Treatment	n[b]	a[c]
Sterile control			14	9.1 ± 0.3
A. viridans	MPL-B		3	5.8 ± 0.8
A. viridans	MPL-D		3	2.1 ± 0.4
A. viridans	ATCC 11563		3	8.7 ±0.3
A. viridans	MPL-1		3	6.4 ± 1.7
A. viridans	MPL-1	erythorbate	1	7.8
A. viridans	MPL-1	catalase	3	8.4 ± 0.3
A. viridans	MPL-4		2	7.0 ± 1.9
A. viridans	MPL-12		3	4.9 ± 1.2
Carnobacterium sp.	MPL-11		3	1.6 ± 0.0
Carnobacterium sp.	MPL-11	erythorbate	3	3.3 ± 1.8
Carnobacterium sp.	MPL-11	catalase	2	6.7 ± 0.6
W. viridescens	ATCC 12706		3	7.8 ± 0.2
W. viridescens	ATCC 12706	erythorbate	3	7.8 ± 0.2
W. viridescens	ATCC 12706	catalase	3	8.8 ± 0.3

[a] Slices were kept at 9±1°C. Colour measurements were taken 3 d after opening the vacuum packages.
[b] number of slices tested
[c] HunterLab red/green colour scale. The larger the number the more intense the red colour. Negative numbers would have indicated green.

Table 3.4. Greening reactions using two brands of all-beef bologna[a]

Strain	Manufacturer	n[b]	Days before visible discolouration	HunterLab "a" value[c]
Sterile control	Plant A	14	-[d]	9.1 ± 0.3
	Plant B	3	-	8.7 ± 0.1
A. viridans MPL-1	Plant A	3	3	6.4 ± 1.7
	Plant B	1	2	2.7
Carnobacterium MPL-11	Plant A	3	2	1.6 ± 0.0
	Plant B	3	1	2.2 ± 0.1
W. viridescens ATCC 12706	Plant A	3	3	7.8 ± 0.2
	Plant B	3	1	2.5 ± 0.2

[a] Irradiated all-beef bologna slices were inoculated with bacterial cultures and vacuum-packaged before being incubated at 9 ± 1 °C for 24-28 days. After opening, each slice was stored aerobically at 9 ± 1 °C for 3 d.

[b] Number of slices tested.

[c] Red/green colour measurements were taken after 3 d of aerobic storage. The larger the number the more intense the red colour.

[d] No visible discolouration.

Table 3.5. Comparison of laboratory media for predicting greening in meat

Bacterial Strain	Strain	Greening Reaction on Bologna[a]	GM agar[b]	MPL agar[c]	PTM agar[d]
Leuconostoc sp.	MPL-A	-	+	+	++
L. mesenteroides	MPL-C	-	+	-	+
P. pentosaceus	MPL-E	-	+	-	+
P. pentosaceus	MPL-F	n.g.[e]	-	+	++
A. viridans	MPL-B	+	+	+	+++
A. viridans	MPL-D	+	+	+	++
A. viridans	MPL-1	+	+	+	++
A. viridans	MPL-4	+	+	+	++
A. viridans	MPL-12	+	+	+	++
C. viridans	MPL-11	+	+	n.g.	++[f]
B. thermosphacta	MPL-3	-	-	n.g.	-
E. faecalis	ATCC 7080	-	-	-	++
L. mesenteroides	#1[g]	-	+	n.d.[h]	++

[a] Bologna was incubated aerobically at 4°C or 9°C for 3 d.
[b] Meat-agar test system of Grant and McCurdy (1986). A green zone (+) forms around colonies with potential to cause greening.
[c] A modification of Grant and McCurdy's (1986) meat-agar system, used by a major Canadian bologna manufacturer to predict greening. Green zone (+).
[d] Berthier (1993). Colonies capable of producing hydrogen peroxide are light blue (+), dark blue (++) or brown (+++) on this agar, while colonies that do not produce peroxide are white (-).
[e] No growth.
[f] Failed to grow in some trials
[g] Isolated from vacuum-packaged sliced ham (Holley *et al.*, 1996).
[h] Not determined.

Table 3.6. Viability of bacteria responsible for the green defect during storage at high NaCl concentration[a]

		16 Days			37 Days		
		4°C	10°C	25°C	4°C	10°C	25°C
A. viridans	MPL-1	98[b]	91	3.8	100	89	0.0[c]
A. viridans	MPL-4	69	46	0.4	30	24	0.6
A. viridans	MPL-12	92	61	1.3	35	21	0.0[c]
C. viridans	MPL-11	29	13	0.0[c]	11	0.0[c]	0.0[c]
A. viridans	MPL-B	100	100	100	100	31	63

[a] As measured by percent survival following incubation for varying lengths of time in APT broth containing 26.4 % NaCl. Initial $\log_{10}$ CFU were between 3.8 (MPL-1) and 4.5 (MPL-11) except for MPL-B, which started at 2.5.

[b] Percent of cells surviving as detected by hydrophobic grid membrane filtration on APT agar. Counts indicating greater than 100% survival are recorded as 100.

[c] No viable cells detected

4.0. MANUSCRIPT # 2

THERMAL RESISTANCES AND LACTATE AND DIACETATE SENSITIVITIES OF BACTERIA CAUSING BOLOGNA DISCOLOURATION

4.1. Abstract

This report describes the effects of heat, sodium lactate, and sodium diacetate on viability of *Weissella viridescens*, *Aerococcus viridans*, and *Carnobacterium viridans*, all of which produce green discolourations in cooked cured bologna. *W. viridescens* was quite heat resistant in beef bologna ($D_{60°C}$ = 14.7 min) but not in APT broth. *A. viridans* and *C. viridans* were much more sensitive to heat ($D_{60°C}$ in beef bologna ≤ 1.3 min), indicating that these organisms were probably post-pasteurization contaminants. In addition, 3.0 % sodium lactate alone or in combination with 0.3 % sodium diacetate slowed the growth rate and reduced the final cell numbers of *A. viridans* and *C. viridans* in inoculated bologna. *W. viridescens* was only slightly affected by even the combination treatment. The combination of sodium lactate and sodium diacetate prevented *A. viridans* and *C. viridans* from affecting the colour of beef bologna. However, lactate and diacetate themselves reduced red colour, as measured by HunterLab colourimetry. HunterLab 'a' values for fresh beef bologna were 13.4 (no antimicrobial added); 9.6 (3.0 % sodium lactate); 8.0 (0.30 % sodium diacetate); and 7.9 (3.0 % sodium lactate + 0.30 % sodium diacetate).

4.2. Introduction

Many factors influence the colours of meat and meat products by changing the oxidation and oxygenation states of the heme iron in myoglobin (Fox, 1987). In cooked, cured meats the normal pink colour is that of dinitrosohemochrome, a stable complex of ferrous heme iron with two NO_2 groups. Hydrogen peroxide produced by some bacteria can react with dinitrosohemochrome to give the meat a green or grey appearance. It is thought that the H_2O_2 breaks down the dinitrosohemochrome to form various green and brown pigments (Grant *et al.*, 1988).

We recently showed that *Aerococcus* (*A.*) *viridans* and *Carnobacterium* (*C.*) *viridans* can cause discolouration in bologna (Holley et al, 2002; Peirson et al, 2002a). *Weissella* (*W.*) *viridescens* was also able to reduce the intensity of red colour in the product tested, but several pediococci, leuconostocs, and carnobacteria (other than *C. viridans*) had no effect on the colour. Based on these results and incomplete information in some earlier reports about green discolouration, we suggested that typical acidiphilic lactic acid bacteria – the genera *Lactobacillus*, *Leuconostoc*, and *Pediococcus* – have not been proven to cause discolouration in properly refrigerated meat products. Since aerococci and carnobacteria are alkalitrophic, and since even *W. viridescens* grows above pH 8 in APT broth and fails to grow on Rogosa acetate agar, we speculated that cooked cured meat products with a lower pH than usual might be less vulnerable than usual to colour loss (Peirson *et al.*, 2002a).

Many authors have recently discussed the effect that salts of organic acids have on bacterial growth. Sodium lactate and sodium diacetate prevent or slow the growth of *Listeria* (*L.*) *monocytogenes* in many meat products (Blom *et al.*, 1997; Stekelenburg and Kant-Muermans, 2001; Mbandi and Shelef, 2002). The effects that these agents have on food spoilage bacteria have been less well examined but most reports indicate that shelf life increases when salts of organic acids are added to meat batter (Lamkey *et al.*, 1991; Bloukas *et al.*, 1997). In broth, organic acids inhibit many spoilage microorganisms, including carnobacteria (Ouattara *et al.*, 1997). Therefore salts of organic acids might inhibit the bacteria that cause discolouration.

In cooked cured meats there are two types of bacterially induced green spoilage: surface greening caused by post-cooking contaminants and core greening caused by bacteria that are heat-resistant enough to survive cooking (Niven *et al.*, 1954). Among the lactic acid bacteria, *W. viridescens,* and certain enterococci are considered unusually heat resistant. Aerococci also have been reported to survive 30 min at 60°C but the heat resistances of most lactic acid bacteria are largely unknown (Franz and von Holy, 1996). Furthermore, the methods originally used to determine the heat resistances of *W. viridescens* and *A. viridans* are now obsolete.

Here we report the effects of adding 0.3 % (w/w of aqueous phase) sodium diacetate, 3.0 % (w/w of aqueous phase) sodium lactate, or both to an all-beef bologna batter before cooking, on the colour of the product after cooking and on the growth rates of bacteria associated with green discolouration in those products. We also report thermal

death time values for a selection of bacteria associated with greening, as determined in broth and in beef bologna.

4.3. Methods

All strains of bacteria used in this study were obtained from the American Type Culture Collection or were isolated during a previous study (Peirson *et al.*, 2002a).

4.3.1. Preparation of Bologna

Frozen uncooked beef bologna batter was obtained from Maple Leaf Consumer Foods (Mississauga, ON, Canada) and thawed overnight at 0°C. Sausage casings (salted beef bung caps, 5 cm diameter) were obtained from Canada Compound Ltd. (Winnipeg, MB, Canada) and soaked in water overnight at 4°C. Four batches of beef bologna were prepared: regular (no antimicrobial), lactate-treated, diacetate-treated, and a combined treatment (both lactate and diacetate). Where used, sodium lactate (60% w/w in water; purchased from Canada Compound Ltd.) and sodium diacetate (Sigma-Aldrich Co. Canada Ltd; Oakville, ON, Canada..) were added according to the following formulae:

Na-lactate solution (g) = 0.03 x bologna batter (g) x moisture (g/g batter) x (100/60)

Na-diacetate (g) = 0.003 x bologna batter (g) x moisture (g/g batter)

The moisture content was assumed to be 0.6455 g/g batter based on a typical analysis provided by Maple Leaf Consumer Foods. The same batch contained 12.3% protein, 19.0% fat, 2.5% salt, and 1.0-1.5% carbohydrate.

Each batch of bologna was prepared as follows. Meat batter (2.5 kg) was placed in the bowl cutter of a Hobart Model 84142 (The Hobart Manufacturing Co. Ltd, Don Mills

ON Canada) and chopped for 2 minutes before being moved to a 9 L manual sausage stuffer (F. Dick, Germany). Batter was forced into sausage casings, and linked at 0.5 m lengths with a metal clip (Republic Fastener Products Corp. Great Falls, SC, USA). Sausages were cooked in steam-jacketed kettles according to the following schedule: 20 min at 54 ± 2 °C, 20 min at 65 ± 2 °C, and 30 min at 75 ± 1 °C. To reduce the opportunity for antimicrobials to leach out of the sausages during cooking and to provide a means of handling the sausages without touching them, each sausage was cooked in an unsealed plastic bag.

After cooling to room temperature, each sausage was removed from its bag, aseptically placed on a sterile surface in a laminar flow hood, and sliced at 0.5 cm intervals with a sterile knife. Slices were put, using sterile tweezers, into WinPak Deli*1 (low oxygen permeability) bags, heat sealed under vacuum in a Bizerba Model GM2002 vacuum-packaging machine (Bizerba Canada Inc. Mississauga ON Canada), and frozen (-40°C). Two slices of sausage were included in each bag. None of the slices were taken from within 3.5 cm of the end of any sausage. Bags were randomly assigned to groups of 12 bags each according to antimicrobial treatment and test microorganism.

4.3.2. Effect of bacteria on bologna colour

W. viridescens ATCC 12706, *A. viridans* MPL-1, *A. viridans* MPL-B, and *C. viridans* ATCC BAA 336 were subcultured daily in APT broth (Difco; Becton Dickinson and Company, Sparks, MD, USA). Each culture (500µl) was transferred to 50 ml APT broth and grown overnight. The culture was centrifuged, washed in 30 ml 0.86 % sterile

saline solution, and re-suspended in 5 ml 0.86 % saline to a final concentration of approximately 5.6 $\log_{10}$ CFU per ml based on a standard curve of absorbance (500 nm) versus CFU. The packages of thawed bologna were cut open along one side and 100 µl of bacterial suspension were added to the top surface of each slice (that is, 200 µl per package). The estimated concentration of bacteria was $\log_{10}$ 3.5/g of bologna. Then the bags were re-sealed under vacuum and placed at 10 ± 1 °C.

Packages were opened after 0, 1, 5, and 10 weeks of storage at 10 ± 1 °C. From each package, one slice was transferred to a sterile Petri dish, sealed with Parafilm M® (American National Can, Chicago, IL, USA) to prevent drying, and kept at 10 ± 1 °C for approximately 3 d (64-74 h). At the end of this storage period, the colour of the meat was measured using a HunterLab Miniscan spectrocolourimeter (Reston, Virginia).

The remaining slice from each package was used immediately to determine the number of bacteria present. The slice was aseptically placed in a Stomacher 400™ bag, weighed, and homogenized (1-2 min) with 50 ml sterile saline in a Stomacher 400™ LabBlender. The resulting suspension was surface-plated on APT agar or, to detect higher bacterial numbers, serially diluted and plated with an Autoplate 4000 (Spiral Biotech, Norwood, Massachusetts, USA). All plates were inverted and incubated aerobically in plastic bags (to prevent drying) at 30 °C for 48 to 72 h. The only exceptions were the 10 week *A. viridans* MPL-B samples, which were incubated at 22-24°C for 72 h.

4.3.3. Analysis of bologna

Packages which were held frozen for 90 days after cooking were thawed at 4°C, transferred to Petri dishes, sealed with Parafilm M® and placed at 4°C. One slice from each package was used to determine moisture, lactate, and acetate content; the other was used for water activity and pH measurement.

For moisture analysis the sample preparation was modified from AOAC method 983.18 (AOAC, 1996). Slices (which weighed 9 to 17 g) were cut into pieces using a metal spatula and placed into a 50 ml blender jar of a Sorvall Omni Mixer™ (Ivan Sorvall Inc. Norwalk CT, USA). Each sample was chopped, with periodic manual mixing, until it formed a smooth paste; a portion (about 5 g) was then rapidly weighed in a FisherBrand™ aluminium weighing dish (Fisher Scientific, Winnipeg, MB, Canada; catalog # 08-732) and placed at 103°C in a Stabli-Therm® Mechanical Convection Oven (Blue M Electric Co., Blue Island IL, USA) for 17.5 – 18.0 h. The loss in weight was considered to be moisture.

Lactate and acetate contents of the unused portion of the ground slice prepared for moisture analysis were measured as described for meat samples in the Roche/Boehringer Mannheim D-Lactic acid/L-Lactic acid enzymatic test kit (Xygen Diagnostics Inc. Burgessville, ON, Canada). The perchloric acid extract prepared for lactate analysis was also used for acetate analysis, using the Roche/Boehringer Mannheim acetic acid enzymatic test kit (Xygen).

Water activities of intact bologna slices were measured with a Novasina AW SPRINT™ water activity-measuring machine (Axzair Ltd, Pfaffikon, Switzerland). The sample cup itself was used to excise a piece of bologna that just filled the unit.

The pH of a 1:10 dilution of bologna chopped and homogenised in distilled water was measured using an Accumet Basic™ pH meter (Fisher Scientific)™

4.3.4. Thermal Resistances in Broth

For thermal resistance measurement, bacterial cultures were grown as follows: *Listeria monocytogenes* ATCC 19111 in brain heart infusion (BHI) broth (Difco) at 35°C; *A. viridans* ATCC 11563 in BHI broth at 37°C; *A. viridans* MPL-1, 4, and 12 in APT broth (Difco) at 30°C; *A. viridans* MPL-B and *Enterococcus faecalis* ATCC 7080 in Lactobacilli MRS broth (Difco) at 30°C; *W. viridescens* in APT broth at 30°C anaerobically; *C. viridans* ATCC BAA 336 and *C. piscicola* ATCC 43224 in APT broth at 22-25°C anaerobically. Each culture was prepared by inoculating a single colony grown on agar plates (made from the appropriate broth medium + 1.5% Difco agar) into 10 ml of broth. After 24 h, 0.1 ml broth was transferred to 10 ml broth and incubated 48 h, to give viable stationary phase cells. Depending on strain, initial bacterial concentrations were 10^7-10^9 CFU/ml.

Decimal reduction times (D values) were determined using an immersed sealed capillary tube (ISCT) procedure based on the method of Foegeding and Leasor (1990). Using a 12.7 cm 22 gauge disposable spinal needle (Becton Dickinson), culture broth

(50μl) was dispensed into sterilised capillary tubes (0.8-1.1 mm inner diameter, 100 mm long; Kimax-51, Kimble Glass, Vineland NJ) that had been sealed on one end. Filled capillary tubes had 4 cm of headspace and were placed on ice ≤ 20 min before being flame-sealed. After sealing, three tubes were kept on ice (unheated controls) but the remaining tubes were placed upright in an aluminium foil-covered test tube rack. The rack was completely submerged into a preheated circulating water bath at 60 °C or 65 °C. After 4 sec of come-up time, tubes were removed at the end of each of 5 regular intervals and placed in an ice-water slurry for 5 min. They were then immersed in 500 ppm sodium hypochlorite (pH 6.5) for 10 min, rinsed twice with sterile distilled water, and transferred to individual test tubes containing 5 ml of sterile peptone water. Once in the test tubes, the capillary tubes were broken with sterile tweezers and finely ground with a sterile glass rod. The test tubes were kept on ice. Serial tenfold dilutions were carried out and plated on the corresponding agar in duplicate using the Autoplate 4000. Plates were incubated for 5 d as described above and colonies were counted. D values (min) were calculated as the negative reciprocal of the survivor curve slope (log viable CFU/ml versus time) obtained by linear regression analysis.

4.3.5. Thermal resistances in cooked bologna

Heat resistance studies in bologna batter were conducted using an end-point procedure based on the method of Mazzotta and Gombas (2001) with some modifications. Bacterial cultures were grown in APT broth aerobically at 30°C, as described above. Frozen uncooked bologna batter was thawed on ice overnight. Thawed bologna batter (100 g) was placed in the 400 ml stainless steel cup of a Sorvall Omni Mixer, covered with aluminium foil, and autoclaved (121°C, 15 min). The lid and blades for the cup

had been sterilised in boiling water for 10 min. After cooling the meat to room temperature, 10 ml of a 48 h culture was added to the cup and mixed at high speed for 5 to 10 min to ensure thorough distribution of the inoculum. To minimize heating the bologna, the cup was partially immersed in an ice-water slurry during mixing. Inoculated bologna (3g) was placed into each of 19 WinPak Deli*1 bags, which were then placed between two thickness guides and flattened with a rolling pin to a total width (bag plus sample) of 0.5 ± 0.1 mm. The bags were sealed in vacuum and then held at room temperature until testing was begun. One bag was left unsealed so that a thermocouple connected to a digital thermometer (model DP 460-T-DS; Omega Engineering Inc, Stamford, CA, USA) could be inserted.

The heat-resistance tests were carried out using a custom-made thermospacer (Fig. 4.1), to ensure even heating by standardizing the spacing and orientation of the bags. The thermospacer was constructed entirely of acrylic in the following dimensions. The base was 33 cm x 23 cm. Twenty vertical dividers (17.5 cm x 17.5 cm x 0.3 cm thick) stood in parallel upon the base, with a 1.3 cm space on either side of each divider (the *bag space*). Removable combs on either side of the dividers ensured that the bag space was the same size at the top as at the bottom. There were seven round holes (1.3 cm diameter) in each divider to permit water movement throughout the device. There were also 19 holes in the base (each 7.6 cm x 0.6 cm), one for each bag space. A thin copper wire was threaded through the hole in the base and through a hole in the sealed edge of each bag to prevent the bags from floating up during the trial. One bag was placed in each bag space, and the bag containing the thermocouple was placed in the central bag

space. Once all the bags were secured, the thermospacer was placed in a water bath at 60°C and moved back and forth to facilitate temperature equilibration. Because the bag that contained the thermocouple was not sealed, the top centimetre of each bag had to be kept above the water level but the sealed portion of each bag was always fully below the water level. When the temperature of the thermocouple reached 59.5 °C, three bags were removed. Every min thereafter a further three bags were removed. Immediately upon removal, each bag was plunged into an ice-water slurry and held for at least 5 min. Bags were opened aseptically and 27 ml of 0.1% (w/v) peptone (proteose peptone # 3, Difco) was added; the bags were then massaged in the Stomacher 400 LabBlender for 1 min. Serial dilutions were prepared in 0.1% peptone and spread plated manually or spiral plated using the Autoplate 4000, in duplicate. Plates were incubated at 30°C for 5 d before colonies were counted, as described above.

4.4. Results

Table 4.1 displays the effects that sodium lactate and sodium diacetate had on some characteristics of bologna sausage. Both reduced the water activity without affecting the moisture content. Sodium diacetate, but not sodium lactate, slightly reduced the pH of sausages into which it was incorporated. Table 4.1 also shows the lactate and acetate content detected in cooked beef bologna. Surprisingly, only about 75.6% of the added lactate could be detected, even though the added acetate was fully recovered, and even though the extraction procedure used was designed for lactate analysis and not for acetate analysis. We note, however, that Stekelenburg and Kant-Muermans (2001) also reported poor (78%) recovery of lactate from some ham products.

The effects of sodium lactate and sodium diacetate on the growth of bacteria that cause greening are shown in Fig. 4.2. In no case did lactate or diacetate have a bactericidal effect. In every case but one (*W. viridescens*) the combination of 3.0% sodium lactate and 0.3% sodium diacetate limited the organism's growth to less than a $\log_{10}$ CFU increase over 10 weeks. Sodium lactate alone was more effective than sodium diacetate alone and sodium diacetate alone was more effective than the control. The combination treatment was always the most effective but none of the treatments prevented *W. viridescens* from growing. Un-inoculated samples of the untreated (control) bologna occasionally showed high levels of bacterial growth; this effect increased over time and the total number of bacteria on some un-inoculated slices at 10 weeks was too great for the dilutions used. Bacterial growth (4.6 $\log_{10}$ CFU) was also found in one of the un-inoculated, lactate-treated samples but not in the diacetate or combination-treated

samples. In addition, adventitious organisms were observed in four inoculated packages but maximum levels were at ≥ 1 $\log_{10}$ CFU lower than the inoculated organism.

Figure 4.3 illustrates the effect of organic acid salts on the colour of beef bologna at the beginning of the experiment. Figure 4.4 shows how each strain of bacteria influenced the colour of bologna without organic acid salts after 0, 1, 5, and 10 weeks of incubation at 10°C. The untreated bologna was more red than the lactate or diacetate-treated bolognas at the start of the experiment, and un-inoculated slices of control bologna remained more red than un-inoculated slices of lactate or diacetate-treated bologna throughout the experiment. However, all the organisms tested produced visible and measurable discolouration in the untreated bologna after 5 weeks or less of storage. The combination of lactate and diacetate had the worst initial appearance (week 0, all samples) but the best final appearance (week 10) in samples inoculated with aerococci or carnobacteria. Sodium lactate alone or in combination with sodium diacetate prevented *A. viridans* (not shown) and *C. viridans* (Fig. 4.5) from causing substantial colour loss. Sodium diacetate alone was ineffective. None of the antimicrobial treatments prevented colour loss caused by *W. viridescens* (not shown).

As shown in Fig. 4.4, *W. viridescens* ATCC 12706 produced the smallest degree of greening and *C. viridans* the largest. But the strong discolouration produced by *C. viridans* at 1 week of incubation does not necessarily indicate that it is a more powerful producer of greening than *A. viridans* because the initial level of *C. viridans* was 1.5-2.0 log units greater than that of the other organisms (Fig. 4.2).

As Table 4.2 shows, none of the organisms associated with discolouration had great heat resistance in broth. The semi-logarithmic survivor curves (not shown) for most organisms were nearly linear and their slopes indicated that the heat resistances of bacteria associated with greening are low compared to *E. faecalis* ($D_{60°C}$ = 3.3-3.5 min in Sorenson's buffer; Magnus *et al.*, 1986) or *L. monocytogenes* ($D_{60°C}$ = 1.3-1.7 min in liquid whole egg; Foegeding and Leasor, 1990). Much higher thermal resistance values were obtained when the organisms were heated in packages of meat (Table 4.2). In fact, the thermal death time of *W. viridescens* ATCC 12706 was estimated at 14.7 min, which is far longer than the 5 min thermal challenge used.

4.5. Discussion

Many reports (eg. Qvist *et al.*, 1994; Blom *et al.*, 1997; Samelis *et al.*, 2002) have established that sodium lactate and sodium diacetate can prevent *L. monocytogenes* from growing in refrigerated meat products. In addition, a few studies have shown that organic acids might inhibit microorganisms responsible for meat spoilage. For example, Ouattara *et al.* (1997) showed that acetic acid and lactic acid inhibit *C. piscicola*, *Brochothrix thermosphacta*, *Lactobacillus* (*Lb.*) *sakei*, and *Lb. curvatus* in broth. A recent Japanese study (Sameshima *et al.*, 1997) demonstrated that sodium lactate slowed the growth of *W. viridescens* and *Leuconostoc mesenteroides* but not *Enterococcus faecalis* in broth. Similarly, Blom *et al.* (1997) found that a combination of 2.5% (w/w of moisture content) sodium lactate and 0.25% sodium diacetate in ham inhibited both *L. monocytogenes* and background lactic acid bacteria.

The mechanisms by which sodium lactate and sodium diacetate inhibit cells are unclear but may be related to their effect on water activity (Houtsma *et al.*, 1993). Their antimicrobial effects increase with decreasing pH (Houtsma *et al.* 1996), although the increase is not proportional to the concentration of undissociated organic acid. Consequently they should be more effective against organisms such as *Listeria*, *Carnobacterium*, and *Aerococcus*, which grow poorly at low pHs, than against acidiphilic genera like *Lactobacillus*. One would expect that the spoilage microflora of sausages containing lactate or acetate would be mainly acid-loving species. Our results are consistent with these expectations since *W. viridescens* was less affected by sodium lactate than were the aerococci and carnobacteria. If alkalitrophic species are a major

cause of greening, as we have suggested but not proved (Peirson *et al.*, 2002a), then organic acid salts may prevent many cases of discolouration.

There is reason, however, to question this hypothesis. *W. viridescens*, though less acid tolerant than many lactobacilli, was hardly affected by even the combination treatment used in this study. In addition, no comprehensive survey of the organisms responsible for greening has ever been made. Further, the negative effect that both lactate and diacetate had on colour may be a concern, although most reports either have not mentioned the effects that organic acid salts have on colour, or have indicated that their effects are negligible. Colour development in cooked cured meat products is a complex phenomenon involving heme, nitrite, and reducing agents like sodium erythorbate. Notably, Stekelenburg and Kant-Muermans (2001) found lower than expected levels of residual nitrite in ham made with 0.2% sodium diacetate but not in ham made with 3.3% sodium lactate. They attributed the difference to the pH-lowering effect of sodium diacetate but observed no detrimental effect on colour. If lactate and diacetate normally induce colour changes as large as those observed in the present study they might render certain cooked cured meat products unattractive to the consumer. On the other hand, given that many studies have been carried out using sodium lactate and sodium diacetate in cured meats, and given that colour has rarely been mentioned as a significant issue, it seems likely that organic acids will, most of the time, do very little harm to normal colour development.

4.5.1. Thermal tolerances of lactic acid bacteria

Relatively few studies have dealt with the thermal resistances of lactic acid bacteria, presumably because they are not thought to cause disease. One of the few lactic acid bacteria to be examined in detail is *W. viridescens*, which has been studied because of its association with green 'cores' and 'rings' in cooked cured meats (Niven *et al.*, 1954). The study of thermal resistance has improved in recent years with the development of methods that promote rapid temperature equilibration. Consequently we chose to re-evaluate the heat resistances of *W. viridescens* and other species using the Immersed Sealed Capillary Tube Method of Foegeding and Leasor (1990) and the immersed bag method of Mazzotta and Gombas (2001).

So far as we know, this is the first thermal death time report for any species of *Carnobacterium* or *Aerococcus* and the second such report for *W. viridescens*. Thermal death times for typical lactic acid bacteria are around $D_{60°C}$ = 0.25-0.66 min (Franz and von Holy, 1996). Contrary to the claim of Williams *et al.* (1953) that aerococci survive 30 min at 60°C, all aerococci tested in this study had $D_{60°C}$ <1.4 min. The $D_{60°C}$ values of *A. viridans*, *C. viridans*, and *C. piscicola* were similar to those of other non-thermally resistant lactic acid bacteria (Table 4.2). *W. viridescens* also had little heat resistance in broth but its $D_{60°C}$ in meat was 14.7 min. Niven *et al.* (1954), Milbourne (1983), and Borch *et al.* (1988) have likewise reported that *W. viridescens* has extraordinary heat resistance in meat. Our results indicate that carnobacteria and aerococci are very unlikely to survive cooking to an internal temperature of 69°C. The $D_{60°C}$ of the most heat-resistant aerococcus (MPL-4) was only 1.37 min whereas species known to be

thermally resistant had $D_{60°C}$ = 2.53 min (*L. monocytogenes*) and $D_{65°C}$ = 1.07 min (*E. faecalis*). Strangely, *W. viridescens* also was not heat resistant in APT broth, a finding which contradicts all previous reports. Several explanations are possible for this observation. First, the ISCT method used in this study frequently provides shorter D values than the test tube method used in the studies by Niven *et al.* (1954) and Milbourne (1983). In addition, we used spent APT broth rather than the fresh broth used in other studies. Fewer heat-protective factors may have been present in the broth. Also, previous studies have shown that *W. viridescens* exhibits increased heat tolerance in response to successive heat shocks (Niven *et al.* 1954). Strains isolated from the cores of sausages (presumably having survived cooking) were typically more heat resistant than those isolated from the meat surface (presumably post-pasteurization contaminants). *W. viridescens* ATCC 12706, the strain used in the present study, was isolated more than 50 years ago and may have lost any special heat tolerance it once had. On the other hand, strains NCDO 1655 and NCIB 8965 (both of which were originally identical with ATCC 12706 (ATCC, 1996)) were tested by Milbourne about 30 years after their first isolation and yet had retained their thermal resistance. It is also noteworthy that the most recent study (Borch *et al.* 1988) indicated that in APT broth at 68°C the number of *W. viridescens* cells decreased rapidly from 10^7 to 10^3 CFU/ml within 10 min, then remained nearly constant for 20 min, before decreasing rapidly again. After 40 min only 1CFU/ml was present. If each of these declines were treated as being linear, the calculated $D_{68°C}$ values would be 2.5 min for the first 10 min and 5.7 min for the total process. These numbers are much smaller than many of the values obtained at 65°C by Milbourne (1983).

Thus far we have mainly discussed the thermal resistances that bacteria exhibit in broth. But thermal death time values are of practical value only to the extent that they reflect the actual environment of the microorganism during cooking – in this case, cured meat. A $D_{65°C}$ of 71 min has been suggested (Milbourne, 1983) for *W. viridescens* on the surface of sliced ham, but the heating method was not described. In the present study, each bacterial culture was thoroughly mixed in cooked bologna, which was then spread in a thin layer in sealed bags to ensure rapid and uniform heating. The thermal death curve thus obtained should reflect well the conditions to which an organism would normally be exposed. *W. viridescens* ATCC 12706 with a $D_{60°C}$ of 14.7 min would probably survive cooking to 69°C if the initial number of organisms were reasonably high (eg. $\geq 10^5$ CFU/g). A strain made thermally resistant by successive heat shocks would be even more likely to survive.

In conclusion, *C. viridans* and *A. viridans* are probably exclusively post-pasteurization contaminants on cooked cured meats. They may be prevented from causing discolouration by adding sodium lactate alone or in combination with sodium diacetate to the meat batter. The discolouration caused by *W. viridescens*, however, is unlikely to be prevented either by heat or by sodium lactate and sodium diacetate added to the batter.

4.6 Tables

Table 4.1. Characteristics of bologna manufactured with sodium lactate and sodium diacetate.

Treatment	Moisture[a]	A_w	pH	Lactate[b]	Acetate[c]
None	59.9 ± 0.9	0.970 ± 0.001	6.71 ± 0.01	0.53 ± 0.03	0.028 ± 0.014
Lactate[d]	59.8 ± 0.9	0.965 ± 0.001	6.74 ± 0.01	2.27 ± 0.05	0.014 ± 0.001
Diacetate[d]	59.8 ± 0.3	0.967 ± 0.001	6.56 ± 0.01	0.53 ± 0.07	0.303 ± 0.005
Both[d]	59.2 ± 0.5	0.960 ± 0.001	6.57 ± 0.01	2.27 ± 0.37	0.294 ± 0.034

[a] As % Weight

[b] Lactate ion expressed as % (w/w) of the moisture content.

[c] Acetate ion expressed as % (w/w) of the moisture content.

[d] Lactate, bologna with 3.0% (w/w moisture content) sodium lactate added; Diacetate, bologna with 0.30% (w/w moisture content) sodium diacetate added; Both, bologna with both 3.0% sodium lactate and 0.3% sodium diacetate added.

Table 4.2. Decimal reduction times of gram positive bacteria in broth and cooked bologna.

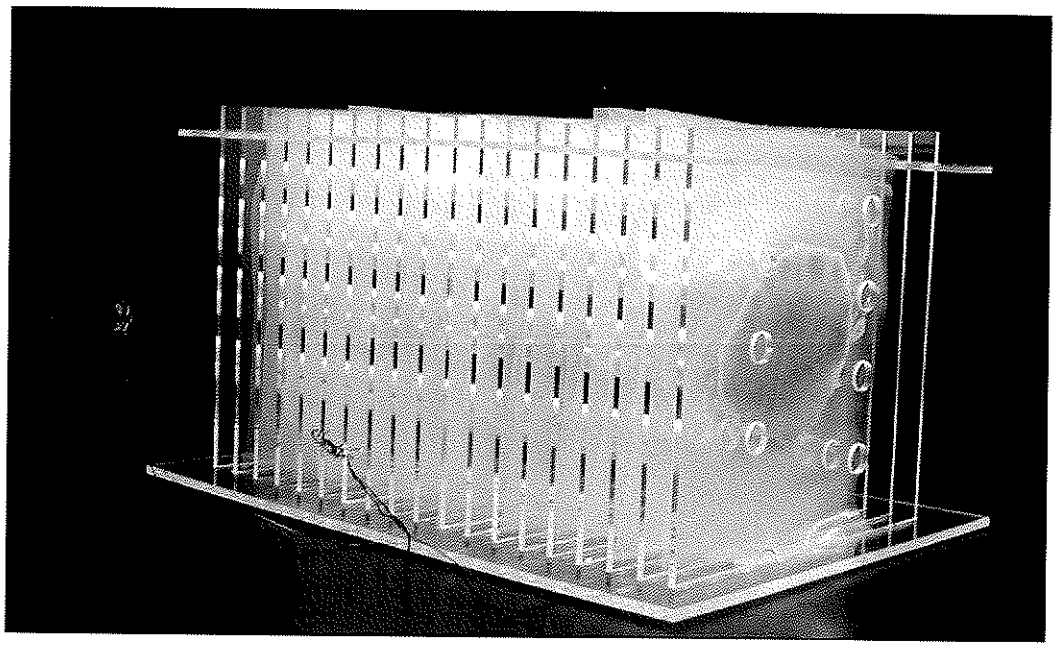
	Broth[a]		Cooked Bologna
	$D_{60°C}$	$D_{65°C}$	$D_{60°C}$
A. viridans MPL-1	0.27[b]	0.09	1.01
A. viridans MPL-4	1.37	0.44	
A. viridans MPL-12	0.86	0.38	
A. viridans MPL-B	NS[c]		1.06
A. viridans ATCC 11563	1.00	0.30	
C. viridans ATCC BAA 336	NS		1.34
C. piscicola ATCC 43224	0.54	0.21	
W. viridescens ATCC 12706	NS		14.7
E. faecalis ATCC 7080	NR[d]	1.07	
L. monocytogenes ATCC 19111	2.53		

[a] *L. monocytogenes* ATCC 19111 and *A. viridans* ATCC 11563 were grown and tested in brain heart infusion (BHI) broth; *E. faecalis* ATCC 7080 and *A. viridans* MPL-B were grown and tested in Lactobacilli MRS broth; carnobacteria and the other aerococci were grown and tested in APT broth.
[b] All times reported in min.
[c] No surviving cells detected at 1 min.
[d] No reduction in viable cell numbers.

4.7 Figures

Figure 4.1. The *Thermospacer*, used to separate inoculated bags of bologna batter during thermal challenge. The thermospacer is shown just before a thermal challenge test, with bags of flattened bologna batter inserted and secured in place at the bottom with copper wire.

thermal 1b.jpg (1173x708x256 jpeg)



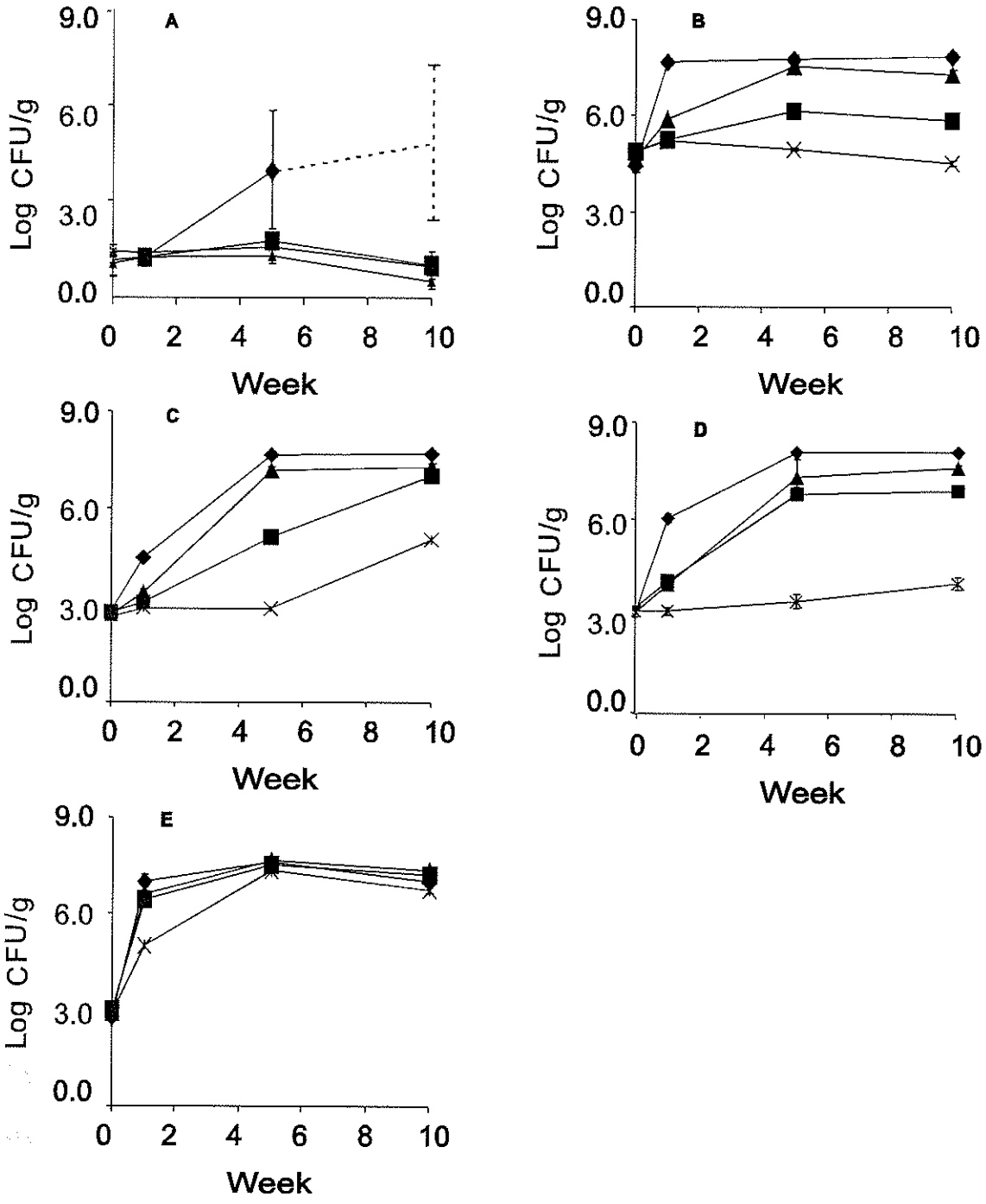


Figure 4.2. Effect of no antimicrobial, ◆; 0.30% sodium diacetate, ▲; 3.0% sodium lactate, ■ ;and 0.30% sodium diacetate + 3.0% sodium lactate, ✕ , on bacterial growth in beef bologna.

(A) Control (no added microorganisms); (B) *C. viridans* MPL-11;
(C) *A. viridans* MPL-1; (D) *A. viridans* MPL-B;
(E) *W. viridescens* ATCC 12706.

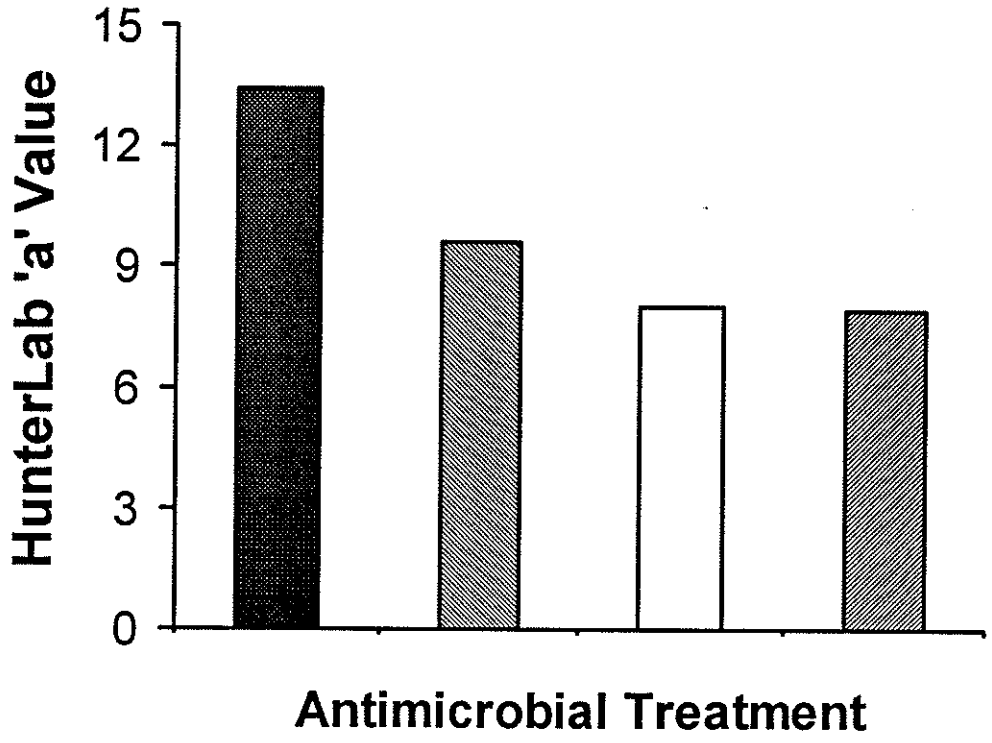


Figure 4.3 Effect of sodium lactate and sodium diacetate on initial (0 d of storage at 10°C) colour of uninoculated beef bologna immediately (<1 h) after package opening.

■ No antimicrobial ▨ 3.0% sodium lactate
□ 0.30% sodium diacetate ▨ 0.30% sodium diacetate + 3.0% sodium lactate

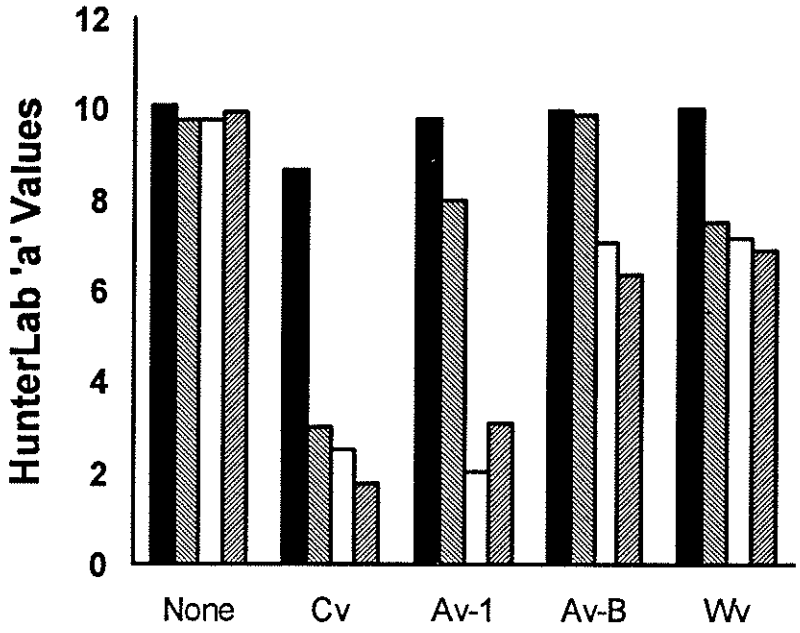


Figure 4.4. Intensity of red colour three days after opening inoculated packages of beef bologna. None, no added microorganism; Cv, *C. viridans* MPL-11; Av-1, *A. viridans* MPL-1; Av-B, *A. viridans* MPL-B; Wv, *W. viridescens* ATCC 12706. Days of storage at 10°C before package opening:

■ 0 days
□ 35 days
▨ 7 days
▨ 70-71 days

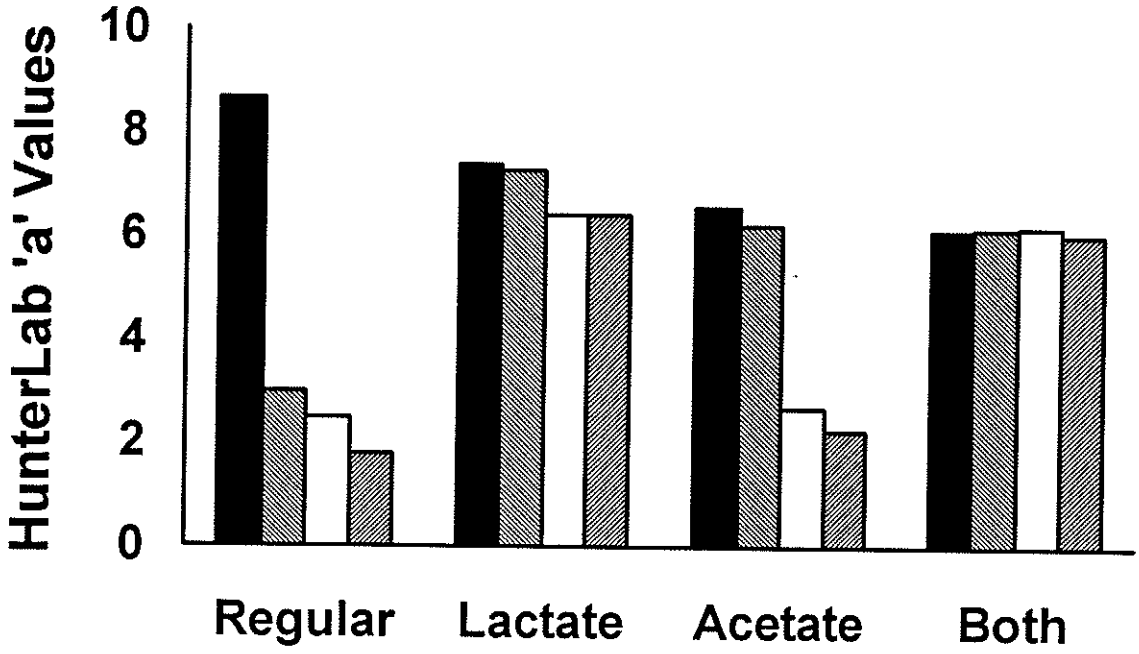


Figure 4.5. Intensity of red colour three days after opening packages of beef bologna contaminated with *C. viridans* MPL-11. Antimicrobial treatments were Regular (no antimicrobial); Lactate (3.0% sodium lactate); Acetate (0.30% sodium diacetate) and Both (0.30% sodium diacetate + 3.0% sodium lactate. Days of storage at 10°C before package opening:

■ 0 days
□ 35 days
▨ 7 days
▨ 70-71 days

5.0 DISCUSSION

Cooked cured meat products like bologna take on a green appearance when exposed to oxidizing agents, the most important of which is H_2O_2. The way in which H_2O_2 reacts with dinitrosohemochrome, the principle pigment of cooked cured meats, is thought to be analogous to that in which it reacts with nitrosomyoglobin, the red pigment of uncooked cured meats. Thus, oxidation of dinitrosohemochrome forms molecules that are similar to choleomyoglobin except that they are not attached to a protein. Hydrogen peroxide may be produced on the surface of aerobically stored cooked cured meats by lactic acid bacteria that either survive thermal processing or that come in contact with the surface of the product after cooking (post-process contamination). In vacuum-packed, refrigerated products, such organisms quickly become the dominant population and often may number 10^7 or more per cm^2 of surface area. Once the package is breached, the organisms are exposed to oxygen; they generate H_2O_2. Dinitrosohemochrome is oxidized, its porphyrin ring broken, and thus converted to pigments that are green, yellow, or brown in colour. Discolourations of this sort are not usually spread evenly over the surface but are interspersed with regions of normal colour, which makes them appear greener than they are, by contrast.

The identities of bacteria that cause discolouration are not well understood. Except for *W. viridescens*, which was described in detail by Niven *et al.* (1949 and 1954), most of the organisms associated with greening have either been named without phenotypic description (Lee and Simard, 1984) or given names which cannot now be reconciled

with their phenotypic descriptions (Incze and Delenyi, 1984). Aerococci produce green discolourations on cured meats at 30°C and are found in meat curing plants (Deibel and Niven, 1960), but have not been considered a factor in meat spoilage (Holzapfel, 1992). Pediococci, at least some of which do not induce greening at 30°C (Deibel and Niven, 1960) have been described (Grant *et al.*, 1988) as potential causes of discolouration, as have enterococci. There was an attempt to screen potential greening organisms using a cured meat-agar system (Lee and Simard, 1984).

This thesis confirms the ability of *W. viridescens* to induce discolourations in bologna, but more importantly it provides a careful and taxonomically up to date analysis of two groups of bacteria that caused an outbreak of greening in bologna produced by a Canadian manufacturer. The first group was composed of tetrad-forming cocci that grew at pH 9.6, produced a final pH around 5.0 in glucose broth, and did not grow on Rogosa acetate agar. They were identified as *A. viridans*. The second group was also alkalitrophic but were rod-shaped and possessed meso-diaminopimelic acid in their cell walls. They were identified as a new species of *Carnobacterium*, *C. viridans*. Organisms identified as pediococci, leuconostocs, and one *Enterococcus* were either unable to grow on refrigerated vacuum-packed bologna or, in most cases, grew without being able to induce any green discolouration. The close phenotypic similarities between *Aerococcus* and *Pediococcus*, between *Leuconostoc* and *Weissella*, and between *Carnobacterium* and *Lactobacillus*, coupled with recent changes in the taxonomy of lactic acid bacteria, suggest that many of the organisms previously found to cause green discolourations may be misclassified. It also became obvious that an

organisms ability to induce greening on a meat-agar test system or to produce hydrogen peroxide in laboratory media bore little relationship to its ability to produce greening in an actual cured meat product.

Reports that *W. viridescens* possesses extraordinary heat resistance have led to the belief that bacteria associated with greening may survive industrial cooking of meat products (Niven *et al.*, 1954). The pH sensitivity of aerococci and carnobacteria suggested that organic acids or their salts might inhibit their growth in meat. Only *Weissella*, of the organisms tested in this study, appeared to be thermally tolerant in meat. Sodium lactate (3.0% w/w), alone or in combination with sodium diacetate (0.3% w/w) was quite effective in inhibiting the growth of *C. viridans* and *A. viridans* but not the growth of *W. viridescens*. As a result, *C. viridans* and *A. viridans* were unable to induce greening in products that contained the combination of sodium lactate and sodium diacetate. Surprisingly, sodium diacetate alone had little effect on any of the bacteria, though it did enhance the growth-limiting effect of sodium lactate. Even more surprising, however, was the discovery that sodium lactate and especially sodium diacetate reduced the initial red colour of beef bologna. Although there are many reports about the effectiveness of both lactate and diacetate for inhibiting *Listeria* in meat products, no significant effect of either organic acid on colour of cooked cured meats seems to have been described before now.

This thesis has described several alkalitrophic bacteria that are capable of affecting the colour of cured meats, and has shown a way in which they may be prevented from

doing so. It remains to be seen whether the organic acids used in this research can also prevent cases of greening caused by other microorganisms. Indeed, although it seems unlikely, it may be that all cases of H_2O_2-induced green discolouration are caused by organisms closely related to those in this study. A broader survey of organisms that may produce hydrogen peroxide, and of the connection between H_2O_2 production in lab media and greening in cured meats would be useful. A more reliable fast method of screening microorganisms for their ability to induce greening could facilitate quality control at cured meat manufacturing facilities. Finally, future research should use carefully identified strains to find ways of producing cured meats that are less vulnerable to discolouration. In addition, the effects of acid and alkaline cleaners on the microbial flora of meat processing plants should be studied to eliminate the possibility that current cleaning protocols selectivly favour the alkalitrophic lactic acid bacteria associated with greening.

6.0 REFERENCES

Aguirre, M., and Collins, M. D. 1992a. Phylogenetic analysis of *Alloiococcus otitis* gen. nov., sp. nov., an organism from human middle ear fluid. Int. J. Syst. Bacteriol. 42:79-83.

Aguirre, M., and Collins, M. D. 1992b. Phylogenetic analysis of some *Aerococcus*-like organisms from urinary tract infections: description of *Aerococcus urinae* sp. nov.. J. Gen. Microbiol. 138:401-405.

Aguirre, M., Morrison, D., Cookson, B. D., Gay, F. W., and Collins, M. D. 1993. Phenotypic and phylogenetic characterization of some *Gemella*-like organisms from human infections: description of *Dolosigranulum pigrum* gen. nov., sp. nov. J. Appl. Bacteriol. 75:608-612.

AMSA. 1991. Guidelines for Meat Color Evaluation. Pages 1-17 in: *Proceedings of the 44th Reciprocal Meat Conference*. National Live Stock and Meat Board, Chicago IL. USA.

Anonymous. 2000. Approval of substances for use in the preparation of products. Pages 246-261 in: *United States Code of Federal Regulations. Title 9, Section 318.7.* General Services Administration, Office of the Federal Register. Washington DC, USA.

AOAC International. 1996. Method 983.18. Page 39-1 in: *Official Methods of Analysis of AOAC International.* Cunniff, P. ed. AOAC International, Gaithersburg, MD, USA.

Archibald, F. 1986. Manganese: its acquisition by and function in the lactic acid bacteria. Crit. Rev. Microbiol. 13:63-109.

ASM. 1981. *Manual of Methods for General Bacteriology*. Gerhardt, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R., and Briggs Phillips, G. eds. American Society for Microbiology, Washington, DC, USA.

ATCC. 1996. *ATCC Bacteria and Bacteriophages 19th edition*. Pietta, P., Tang, J., and Cote, R. eds. American Type Culture Collection, Rockville, MD, USA.

Berthier, F. 1993. On the screening of hydrogen peroxide-generating lactic acid bacteria. Lett. Appl. Microbiol. 16:150-153.

Blom, H., Nerbrink, E., Dainty, R., Hagtvedt, T., Borch, E., Nissen, H., and Nesbakken, T. 1997. Addition of 2.5% lactate and 0.25% acetate controls growth of *Listeria monocytogenes* in vacuum-packed, sensory-acceptable servelat sausage and cooked ham stored at 4°C. Int. J. Food Microbiol. 38:71-76.

Bloukas, J. G., Paneras, E. D., and Fournitzis, G. C. 1997. Sodium lactate and protective culture effects on quality characteristics and shelf-life of low-fat frankfurters produced with olive oil. Meat Sci. 45:223-238.

Borch, E., Kant-Muermans, M. -L., and Blixt, Y. 1996. Bacterial spoilage of meat and cured meat products. Int. J. Food Microbiol. 33:103-120.

Borch, E., and Molin, G. 1989. The aerobic growth and product formation of *Lactobacillus*, *Leuconostoc*, *Brochothrix*, and *Carnobacterium* in batch cultures. Appl. Microbiol. Biotechnol. 30: 81-88.

Borch, E., Nerbrink, E., and Svensson, P. 1988. Identification of major contamination sources during processing of emulsion sausage. Int. J. Food Microbiol. 7:317-330.

Bousefield, I. J., Keddie, R. M., Dando, T. R., and Shaw, S. 1985. Simple rapid methods of cell wall analysis as an aid in the identification of aerobic coryneform bacteria. Pages 222-234 in: *Chemical Methods in Bacterial Systematics*. Goodfellow, M. and Minnikin, D. E. eds. Academic Press. London. UK.

Brashears, M. M. , Reilly, S. S., and Gilliland, S. E. 1998. Antagonistic action of cells of *Lactobacillus lactis* toward *Escherichia coli* O157:H7 on refrigerated raw chicken meat. J. Food Prot. 61:166-170.

Brewer, M. S., Rostogi, B. K., Argoudelis, L., and Sprouls, G. K. 1995. Sodium lactate/sodium chloride effects on aerobic plate counts and color of aerobically packaged ground pork. J. Food Sci. 60:58-62.

Bruce, J. 1996. Automated system rapidly identifies and characterizes microorganisms in food. Food Technol. 50 (1):77-81.

Chesbro, W. R., and Evans, J. B. 1959. Factors affecting the growth of enterococci in highly alkaline media. J. Bacteriol. 78:858-862.

Collins, M. D., Aguirre, M., Facklam, R. R., Shallcross, J., and Williams, A. M. 1992. *Globicatella sanguis* gen. nov., sp. nov., a new Gram-positive catalase-negative bacterium from human sources. J. Appl. Bacteriol. 73:433-437.

Collins, M. D., Facklam, R. R., Rodrigues, U. M., and Ruoff, K. L. 1993a. Phylogenetic analysis of some *Aerococcus*-like organisms from clinical sources: description of *Helcococcus kunzii* gen. nov., sp. nov.. Int. J. Syst. Bacteriol. 43:425-429.

Collins, M. D., Falsen, E., Lemozy, J., Åkervall, E., Sjödén, B., and Lawson, P. A. 1997. Phenotypic and phylogenetic characterization of some *Globicatella*-like organisms from human sources: description of *Facklamia hominis* gen. nov. sp. nov.. Int. J. Syst. Bacteriol. 47:880-882.

Collins, M. D., Farrow, J. A. E., Phillips, B. A., Ferusu, S., and Jones, D. 1987. Classification of *Lactobacillus divergens, Lactobacillus piscicola,* and some catalase-negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium.* Int. J. Syst. Bacteriol. 37:310-316.

Collins, M. D., Jovita, M. R., Hutson, R. A., Falsen, E., Sjödén, B., and Facklam, R. R. 1999a. *Dolosicoccus paucivorans* gen. nov., sp. nov., isolated from human blood. Int. J. Syst. Bacteriol. 49:1439-1442.

Collins, M. D., Jovita, M. R., Lawson, P. A., Falsen, E., and Foster, G. 1999b. Characterization of a novel Gram-positive, catalase-negative coccus from horses: description of *Eremococcus coleocola* gen. nov., sp. nov.. Int. J. Syst. Bacteriol. 49:1381-1385.

Collins, M. D., Lawson, P. A., Monasterio, R., Falsen, E., Sjödén, B., and Facklam, R. R. 1999c. *Ignavigranum ruoffiae* sp. nov., isolated from human clinical specimens. Int. J. Syst. Bacteriol. 49:97-101.

Collins, M. D., Samelis, J., Metaxopoulos, J., and Wallbanks, S. 1993b. Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. J. Appl. Bacteriol. 75:595-603.

Collins, M. D., Williams, A. M., and Wallbanks, S. 1990. The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis: description of *Tetragenococcus* gen. nov. FEMS Microbiol. Lett. 70: 255-262.

Condon, S. 1987. Responses of lactic acid bacteria to oxygen. FEMS Microbiol. Rev. 46:269-280.

De Bruyn, I. N., Holzapfel, W. H., Visser, L., and Louw, A. I. 1988. Glucose metabolism by *Lactobacillus divergens.* J. Gen. Microbiol. 134:2103-2109.

Deibel, R. H., and Niven, C. F. Jr. 1960. Comparative study of *Gaffkya homari, Aerococcus viridans,* tetrad-forming cocci from meat curing brines, and the genus *Pediococcus.* J. Bacteriol. 79:175-180.

De Man, J. C., Rogosa, M., and Sharpe, M. E. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23:130-135.

Dicks, L. M. T., Fantuzzi, L., Gonzalez, F. C., Du Toit, M., and Dellaglio, F. 1993. *Leuconostoc argentinum* sp. nov., isolated from argentine raw milk. Int. J. Syst. Bacteriol. 43:347-351.

Difco Laboratories Inc. 1984. *Difco Manual, Tenth Edition.* Difco Laboratories, Detroit, MI, USA.

Donnelly, C. W., Briggs, E. H., and Donnelly, L. S. 1987. Comparison of heat resistance of *Listeria monocytogenes* in milk as determined by two methods. J. Food Prot. 50:14-17.

Evans, J.B. 1986. Genus *Aerococcus.* Page 1080 in: *Bergey's Manual of Systematic Bacteriology.* Sneath, P. H., Mair, N. S., Sharpe, M. E., and Holt, J. G. eds. Williams and Wilkins, Baltimore, MD. USA.

Evans, J. B., and Niven, C. F. Jr. 1951. Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. J. Bacteriol. 62:599-603.

Farrow, J. A. E., Facklam, R. R., and Collins, M. D. 1989. Nucleic acid homologies of some vancomycin-resistant leuconostocs and description of *Leuconostoc citreum* sp. nov. and *Leuconostoc pseudomesenteroides* sp. nov.. Int. J. Syst. Bacteriol. 39:279-283.

FDA. 1999. *Food Code, Section 3-401.*[Online Document] US Department of Health and Human Services, Public Health Service, Food and Drug Administration, Washington, DC. USA. URL: http://www.cfsan.fda.gov/~dms/fc99-toc.html (17 July, 2002)

Foegeding, P. M., and Leasor, S. B. 1990. Heat resistance and growth of *Listeria monocytogenes* in liquid whole egg. J. Food Prot. 53:9-14.

Fontaine, E. A., and Taylor-Robinson, D. 1990. Comparison of quantitative and qualitative methods of detecting hydrogen peroxide produced by human vaginal strains of lactobacilli. J. Appl. Bacteriol. 69:326-331.

Fox, J. B. Jr. 1987. The pigments of meat. Pages 193-216 in: *The Science of Meat and Meat Products, Third Edition.* Price, J. F., and Schweigert, B. S. eds. Food and Nutrition Press, Inc. Westport CT, USA.

Franz. C. M. A. P., and von Holy, A. 1996. Thermotolerance of meat spoilage lactic acid bacteria and their inactivation in vacuum-packaged Vienna sausages. Int. J. Food Microbiol. 29: 59-73.

Garrity, G. M., and Holt, J. G. 2001. The road map to the manual. Pages 119-166 in: *Bergey's Manual of Systematic Bacteriology, 2nd Edition, Volume 1: The Archaea and the Deeply Branching and Phototrophic Bacteria.* Garrity, G. M. editor in chief: Boone, D. R., and Castenholz, R. W. editors. Springer-Verlag. New York, NY, USA.

Garvie, E. I. 1984. Separation of species of the genus *Leuconostoc* and differentiation of the leuconostocs from other lactic acid bacteria. Pages 147-178 in: *Methods in Microbiology, vol. 16.* Bergen, T. ed. Academic Press. London. UK.

Garvie, E. I. 1986a. Genus *Leuconostoc*. Pages 1071-1075 in: *Bergey's Manual of Systematic Bacteriology.* Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. eds. Williams and Wilkins., Baltimore, MD. USA.

Garvie, E. I., 1986b. Genus *Pediococcus.* Pages 1075-1079 in: *Bergey's Manual of Systematic Bacteriology.* Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. eds. Williams and Wilkins., Baltimore, MD. USA.

Gibello, A., Collins, M. D., Dominguez, L., Fernandez-Garayzabal, J. F., and Richardson, P. T. 1999. Cloning and analysis of the L-lactate utilization genes from *Streptococcus iniae*. Appl. Environ. Microbiol. 65:4346-4350.

Gilliland, S. E., and Speck, M. L. 1975. Inhibition of psychrotrophic bacteria by lactobacilli and pediococci in nonfermented refrigerated foods. J. Food Sci. 40:903-905.

Grant, G. F., and McCurdy, A. R. 1986. Effect of curing agents on the growth of *Lactobacillus viridescens* in model meat systems. Can. Inst. Food Sci. Technol. J. 19:78-81.

Grant, G. F., McCurdy, A. R., and Osborne, A. D. 1988. Bacterial greening in cured meats: a review. Can. Inst. Food Sci. Technol. J. 21:50-56.

Gregersen, T. 1978. Rapid method for distinction of Gram-negative from Gram-positive bacteria. Eur. J. Appl. Microbiol. Biotechnol. 5:123-127.

Gunsulas, I. C., and Umbreit, W. W. 1945. The oxidation of glycerol by *Streptococcus faecalis*. J. Bacteriol. 50:347-357.

Hammes, W. P., Weiss, N., and Holzapfel, W. H. 1992. Chapter 70: the genera *Lactobacillus* and *Carnobacterium.* Pages 1535-1594 in: *The Prokaryotes, 2nd Edition.* Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K. -H. eds. Springer-Verlag. New York, NY. USA.

Holley, R. A., Guan, T. Y., Peirson, M., and Yost, C. 2002. *Carnobacterium viridans* sp. nov., an alkaliphilic, facultative anaerobe isolated from refrigerated, vacuum packed bologna sausage. Int. J. Syst. Evol. Microbiol. 52:1881-1885. DOI: 10.1099/ijs.0.02216-0

Holley, R. A., Lamoureaux, M., and Dussault, F. 1996. Identification of lactic spoilage bacteria from vacuum-packed cooked luncheon meat and induction of repairable injury by mild thermal stress. Lebensm. Wiss. u. -Technol. 29:114-122.

Holzapfel, W. H. 1992. Culture media for non-sporulating Gram-positive food spoilage bacteria. Int. J. Food Microbiol. 17:113-133.

Holzapfel, W. H., and Schillinger, U. 1992. The genus *Leuconostoc*. Pages 1508-1534 in: *The Prokaryotes, 2nd Edition. Vol. 2.* Balows, A., Truper, H. G., Dworkin, M., Harder, W., Schleifer, K. H. eds. Springer. New York, NY. USA.

Houtsma, P. C., de Wit, J. C., and Rombouts, F. M. 1993. Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. Int. J. Food Microbiol. 20:247-257.

Houtsma, P. C., de Wit, J. C., and Rombouts, F. M. 1996. Minimum inhibitory concentration (MIC) of sodium lactate and sodium chloride for spoilage organisms and pathogens at different pH values and temperatures. J. Food Prot. 59:1300-1304.

Incze, K., and Delenyi, M. 1984. Discoloration of cured meat pigment by bacteria. Page 242 in: The 30th European Meeting of Meat Research Workers, Bristol, UK.

International Union of Biochemistry and Molecular Biology. 1992. *Enzyme Nomenclature 1992: Recommendations of the Nomenclature Committee of the Internaional Union of Biochemistry and Molecular Biology on the Nomenclature and Classification of Enzymes.* Prepared for IUBMB by Edwin C. Webb. Academic Press. San Diego, CA. USA.

Janssen, P. H., Evers, S., Rainey, F. A., Weiss, N., Ludwig, W., Harfoot, C. G., and Schink, B. 1995. *Lactosphaera* gen. nov., a new genus of lactic acid bacteria, and transfer of *Ruminococcus pasteurii* Schink 1984 to *Lactosphaera pasteurii* comb. nov. Int. J. Syst. Bacteriol. 45:565-571.

Jaroni, D., and Brashears, M. M. 2000. Production of hydrogen peroxide by *Lactobacillus delbrueckii* subsp. *lactis* as influenced by media used for propagation of cells. J. Food Sci. 65:1033-1036.

Jay, J. M. 2000. *Modern Food Microbiology*. Aspen Pub. Inc. Gaithersburg. MA. USA. 679 pp.

Jensen, L. B. 1945. *Microbiology of Meats*. The Garrard Press, Champaign, IL. USA. 389pp.

Kandler, O., and Weiss, N. 1986. Regular nonsporing Gram positive rods. Pages 1208-1234 in: *Bergey's Manual of Systematic Bacteriology*. Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. eds. Williams and Wilkins., Baltimore, MD. USA.

Kawamura, Y., Hou, X., Sultana, F., Liu, S., Yamamoto, H., and Ezaki, T. 1995. Transfer of *Streptococcus adjacens* and *Streptococcus defectivus* to *Abiotrophia* gen. nov. as *Abiotrophia adiacens* and *Abiotrophia defectiva* comb. nov., respectively. Int. J. Syst. Bacteriol. 45:798-803.

Kim, J., Chun, J., and Han, H. 2000. *Leuconostoc kimchii* sp. nov., a new species from kimchi. Int. J. Syst. Evol. Microbiol. 50:1915-1919.

Kot, E., Furmanov, S., and Bezkorovainy, A. 1996. Hydrogen peroxide production and oxidation of ferrous iron by *Lactobacillus delbrueckii* ssp. *bulgaricus*. J. Dairy Sci. 79:758-766.

Kraus, F. W., Nickerson, J. F., Perry, W. I., and Walker, A. P. 1957. Peroxide and peroxidogenic bacteria in human saliva. J. Bacteriol. 73:727-735.

Lamkey, J. W., Leak, F. W., Tuley, W. B., Johnson, D. D., and West, R. L. 1991. Assessment of sodium lactate addition to fresh pork sausage. J. Food Sci. 56:220-223.

Lawrie, R. A. 1998. *Lawrie's Meat Science, Sixth Edition*. Woodhead Pub. Ltd. Cambridge, UK. 336 pp.

Lawson, P. A., Falsen, E., Truberg-Jensen, K., and Collins, M. D. 2001a. *Aerococcus sanguicola* sp. nov., isolated from a human clinical source. Int. J. Syst. Evol. Microbiol. 51:475-479.

Lawson, P. A., Falsen, E., Ohlén, M., and Collins, M. D. 2001b. *Aerococcus urinaehominis* sp. nov., isolated from human urine. Int. J. Syst. Evol. Microbiol. 51: 683-686.

Lee, B. H., and Simard, R. E. 1984. Evaluation of methods for detecting the production of H_2S, volatile sulfides, and greening by lactobacilli. J. Food Sci. 49:981-983.

Lemay, M. J., Choquette, J., Delaquis, P., Gariepy, C., Rodrigue, N., and Saucier, L., 2000. Efficacy of natural antimicrobial agents in a chicken meat model. Presented at the Canadian Meat Science Association annual meeting, Quebec City, QC. Canada. Feb. 2.

Magnus, C. A., Ingledew, W. M., and McCurdy, A. R. 1986. Thermal resistance of *Streptococci* isolated from pasteurized ham. Can. Inst. Food Sci. Technol. J. 19:62-67.

Magnus, C. A., McCurdy, A. R., and Ingledew, W. M. 1988. Further studies on the thermal resistance of *Streptococcus faecium* and *Streptococcus faecalis* in pasteurized ham. Can. Inst. Food Sci. Technol. J. 21:209-212.

Martinez-Murcia, A. J., and Collins, M. D. 1991. A phylogenetic analysis of an atypical leuconostoc: description of *Leuconostoc fallax* sp. nov. FEMS Microbiol. Lett. 82:55-60.

Marty-Teysset, C., De La Torre, F., and Garel, J., -R. 2000. Increased production of hydrogen peroxide by *Lactobacillus delbrueckii* subsp. *bulgaricus* upon aeration: involvement of an NADH oxidase in oxidative stress. Appl. Environ. Microbiol. 66: 262-267.

Mazzotta, A. S., and Gombas, D. E. 2001. Heat resistance of an outbreak strain of *Listeria monocytogenes* in hot dog batter. J. Food Prot. 64:321-324.

Mbandi, E., and Shelef, L. A. 2002. Enhanced antimicrobial effects of combination of lactate and diacetate on *Listeria monocytogenes* and *Salmonella* spp. in beef bologna. Int. J. Food Microbiol. 76:191-198.

Milbourne, K. 1983. Thermal tolerance of *Lactobacillus viridescens* in ham. Meat Sci. 9:113-119.

Montel, M. –C., Talon, R., Fournaud, J., and Champomier, M. –C. 1991. A simplified key for identifying homofermentative *Lactobacillus* and *Carnobacterium* spp. from meat. J. Appl. Bacteriol. 70:469-472.

Naidu, A. S., Bidlack, W. R., and Clemens, R. A. 1999. Probiotic spectra of lactic acid bacteria (LAB). Critic. Rev. Food Sci. and Nutrit. 38*:13-126.

* Although this article is formally part of volume 38, the page numbers are consecutive with those of volume 39 (1999) and it is bound together with volume 39 in the Science and Technology Library.

Niven, C. F. Jr., Buettner, L. G., and Evans, J. B. 1954. Thermal tolerance studies on the heterofermentative lactobacilli that cause greening of cured meat products. Appl. Microbiol. 2:26-29.

Niven, C. F. Jr., Castellani, A. G., and Allanson, V. 1949. A study of the lactic acid bacteria that cause surface discolorations of sausages. J. Bacteriol. 58:633-641.

Niven, C. F. Jr., and Evans, J. B. 1957. *Lactobacillus viridescens* nov. spec., a heterofermentative species that produces a green discoloration of cured meat pigments. J. Bacteriol. 73:758-759.

Nunez de Kairuz, M. S., Olazabal, M. E., Oliver, G., Pexce de Ruiz Holgado, A. A., Massa, E., and Farias, R. N. 1988. Fatty acid dependent hydrogen peroxide production in *Lactobacillus*. Biochem. Biophys. Res. Comm. 152:113-121.

Orta-Ramirez, A. and Smith, D. M. 2002. Thermal inactivation of pathogens and verification of adequate cooking in meat and poultry products. Pages 147-194 in: *Advances in Food and Nutrition Research, Vol. 44*. Taylor, S. L. ed. Academic Press. San Diego CA. USA.

Ouattara, B., Simard, R. E., Holley, R. A., Piette, G. J.,-P., and Bégin, A. 1997. Inhibitory effect of organic acids upon meat spoilage bacteria. J. Food Prot. 60:246-253.

Pearson, A. M., and Tauber, F. W. 1984. *Processed Meats Second Edition*. AVI Pub. Co. Inc. Westport CT. USA. 427 pp.

Peirson, M. D., Guan, T. Y., and Holley, R. A. 2002a. Aerococci and carnobacteria cause discolouration in cooked cured bologna. Food Microbiology. Submitted for publication July 10, 2002.

Peirson, M. D., Guan, T. Y., and Holley, R. A. 2002b. Thermal resistances and lactate and diacetate sensitivities of bacteria causing bologna discolouration. Int. J. Food Microbiol. Submitted for publication July 10, 2002.

Prescott, L. M., Harley, J. P., and Klein, D. A. 1990. *Microbiology*. Wm. C. Brown. Dubuque, IA. USA. 882pp.

Qvist, S., Sehested, K., and Zeuthen, P. 1994. Growth suppression of *Listeria monocytogenes* in a meat product. Int. J. Food Microbiol. 24:283-293.

Samelis, J., Bedie, G. K., Sofos, J. N., Belk, K. E., Scanga, J. A., and Smith, G. C. 2002. Control of *Listeria monocytogenes* with combined antimicrobials after postprocess contamination and extended storage of frankfurters at 4°C in vacuum packages. J. Food Prot. 65: 299-307.

Samelis, J., Sofos, J. N., Kain, M. L., Scanga, J. A., Belk, K. E., and Smith, G. C. 2001. Organic acids and their salts as dipping solutions to control *Listeria monocytogenes* inoculated following processing of sliced pork bologna stored at 4°C in vacuum packages. J. Food Prot. 64: 1722-1729.

Sameshima, T., Takeshita, K., Miki, T., Arihara, K., Itoh, M., and Kondo, Y. 1997. Effect of sodium nitrite and sodium lactate on the growth rate of lactic acid

spoilage bacteria isolated from cured meat products. Jap. J. Food Microbiol. 13:159-164.

Satomi, M., Kimura, B., Mizoi, M., Sato, T., and Fujii, T. 1997. *Tetragenococcus muriaticus* sp. nov., a new moderately halophilic lactic acid bacterium isolated from fermented squid liver sauce. Int. J. Syst. Bacteriol. 47:832-836.

Schillinger, U., and Lücke, F. –K. 1987. Identification of lactobacilli from meat and meat products. Food Microbiol. 4:199-208.

Schleifer, K. H. 1986. Gram positive cocci. Pages 999-1002 in: *Bergey's Manual of Systematic Bacteriology.* Sneath, P. H. A., Mair, N. S., Sharpe, M. E., and Holt, J. G. eds. Williams and Wilkins., Baltimore, MD.

Schuman, J. D., Sheldon, B. W., and Foegeding, P. M. 1997. Thermal resistance of *Aeromonas hydrophila* in liquid whole egg. J. Food Prot. 60:231-236.

Shank, J. L., and Lundquist, B. R. 1963. The effect of packaging conditions on the bacteriology, color, and flavor of table-ready meats. Food Technol. 17 (9):83-86.

Shaw, B. G., and Harding, C. D. 1989. *Leuconostoc gelidum* sp. nov. and *Leuconostoc carnosum* sp. nov. from chill-stored meats. Int. J. Syst. Bacteriol. 39:217-223.

Shelef, L. A., and Addala, L. 1994. Inhibition of *Listeria monocytogenes* and other bacteria by sodium diacetate. J. Food Safety, 14:103-115.

Stackebrandt, E., Schumann, P., Swiderski, J., and Weiss, N. 1999. Reclassification of *Brevibacterium incertum* (Breed 1953) as *Desemzia incerta* gen. nov., comb. nov. Int. J. Syst. Bacteriol. 49:185-188.

Statistical Analysis Systems Institute (1999) SAS version 6.12. Statistical Analysis Systems Institute Cary NC.

Stekelenburg, F. K., and Kant-Muermans, M. L. T. 2001. Effects of sodium lactate and other additives in a cooked ham product on sensory quality and development of a strain of *Lactobacillus curvatus* and *Listeria monocytogenes*. Int. J. Food Microbiol. 66:197-203.

Stiles, M. E., and Holzapfel, W. H. 1997. Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36:1-29.

Takahashi, M., Okada, S., Uchimura, T., and Kozaki, M. 1992. *Leuconostoc amelibiosum* Schillinger, Holzapfel, and Kandler 1989 is a later subjective synonym of *Leuconostoc citreum* Farrow, Facklam, and Collins 1989. Int. J. Syst. Bacteriol. 42:649-651.

Tanasupawat, S., Shida, O., Okada, S., and Komagata, K. 2000. *Lactobacillus acidipiscis* sp. nov. and *Weissella thailandensis* sp. nov., isolated from fermented fish in Thailand. Int. J. Syst. Evol. Microbiol. 50:1479-1485.

Villegas, E., and Gilliland, S. E. 1998. Hydrogen peroxide production by *Lactobacillus delbrueckii* subsp. *lactis* I at 5°C. J. Food Sci. 63:1070-1074.

Walters, C. L., and West, S. I. 1983. The chemistry of nitrite in curing. Pages 178-191 in: *Recent Advances in the Chemistry of Meat.* Bailey, A. J. ed. The Royal Society of Chemistry. London. UK.

Wasney, M. A., Holley, R. A., and Jayas, D. S. 2001 Cresol red thallium acetate sucrose inulin (CTSI) agar for the selective recovery of *Carnobacterium* spp. Int. J. Food Microbiol. 64:167-174.

Watts, B. M. 1954. Oxidative rancidity and discolorationin meat. Pages 1-52 in: *Advances in Food Research, Volume 5.* Mrak, E. M., and Stewart, G. F. eds. Academic Press. New York, NY. USA.

Weiss, N. 1992. The genera *Pediococcus* and *Aerococcus*. Pages 1502-1507 in: *The Prokaryotes, 2nd Edition. Vol. 2.* Balows, A., Truper, H. G., Dworkin, M., Harder, W., Schleifer, K. H. eds. Springer. New York, NY. USA.

Whittenbury, R. 1964. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. J. Gen. Microbiol. 35:13-26.

Williams, R. E. O., Hirch, A., and Cowan, S. T. 1953. *Aerococcus*, a new bacterial genus. J. Gen. Microbiol. 8:475-480.

Yap, P. S., and Gilliland, S. E. 2000. Comparison of newly isolated strains of *Lactobacillus delbrueckii* subsp. *lactis* for hydrogen peroxide production at 5°C. J. Dairy Sci. 83:628-632.

Zhang, G. and Holley, R. A. 1999. Development and PFGE monitoring of dominance among spoilage lactic acid bacteria from cured meats. Food Microbiol. 16:633-644. DOI:fmic. 1999.0281.

Zúñiga, M., Pardo, I., and Ferrer, S. 1993. An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. Int. J. Food Microbiol. 18:37-42.

7.0 APPENDIX

Carnobacterium viridans sp.nov., an alkaliphilic, facultative anaerobe isolated from refrigerated, vacuum-packed bologna sausage.

by

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The GenBank accession number for *Carnobacterium* "viridans" MPL-11^T 16S rDNA sequence is AF425608

A facultatively anaerobic, non-spore forming, psychrotrophic, Gram-positive, non-aciduric but alkaliphilic, rod-shaped bacterium (MPL-11^T) was found responsible for green discolouration of refrigerated vacuum-packaged bologna upon package opening. Although *Aerococcus viridans*, which had been implicated earlier in causing the same problem was also found, this is the first report of discolouration caused by an organism shown to be a species of *Carnobacterium*. Bacterial discolouration was caused by H_2O_2 production upon meat exposure to air. The organism MPL-11^T is catalase and oxidase negative. It is not motile and does not reduce nitrate to nitrite or produce ammonia from arginine. It does not grow in acetate-containing broth or agar (Rogosa), or produce H_2S. The peptidoglycan is of the *meso*-diaminopimelic acid type and it produces predominantly L-(+) -lactic acid from glucose. It grows from at least 2° C to 30°C over a pH range from 5.5 to 9.1. Ribotyping suggested that MPL-11^T could be either a *Lactobacillus* or *Carnobacterium* species, but analysis using DNA sequences from the 16S rRNA gene conclusively showed the organism belonged to the genus *Carnobacterium*. Since acid is not produced from amygdalin, inulin, mannitol, α-methyl-D-glucoside or D-xylose, the organism differs from the 7 described species of *Carnobacterium*. In addition, MPL-11^T is the first species of the genus found which does not produce acid from ribose. It is capable of acid production / growth on: galactose, glucose, fructose, mannose, N-acetyl glucosamine, esculin, cellobiose, maltose, lactose, sucrose, trehalose, and tagatose. Although extremely salt tolerant it does not grow in ≥ 4% NaCl. On the basis of phenotypic and genotypic data, it is concluded that this isolate represents a separate new species. Accordingly, the name *Carnobacterium viridans* is proposed. The type strain is MPL-11^T (=ATCC BAA-336^T) (=DSM 14451^T).

Green discolouration of vacuum-packed cooked cured meats is a periodic problem that can be caused by bacterial production of H_2S or result from oxidation of di-nitrosyl hemochrome (pink) by bacterial peroxides (H_2O_2) upon opening the meat package and exposure to oxygen. The former yields greenish sulfmyoglobin, while the latter causes the formation of green cholemyoglobin (Lawrie, 1998) or oxidized porphyrins which are also green. Peroxide-based discolouration can arise from the presence of thermally tolerant organisms like *Weissella viridescens*, *Enterococcus (E.) faecium* or *E. faecalis*. These organisms can cause greening in the core or central portions of the cooked cured meat. Just as commonly, if post-heat process contamination occurs (e.g. at slicing or packaging) a variety of Gram-positive, catalase negative, H_2O_2 producers such as the homo- and heterofermentative lactobacilli , or leuconostocs may be involved (Borch *et al.* 1996). Grant *et al.* (1988) suspected *Pediococcus* species could be responsible, but low temperature limits for their growth (8°C) and our recent work (Peirson and Holley, unpublished) has shown that *Aerococcus viridans* (which can be confused with pediococci) was also responsible for this colour defect in cured meat stored at $\geq$ 4°C. Isolation of *Aerococcus viridans* from meat curing brines and its production of similar discolouration reactions in cured meats were reported much earlier by Deibel & Niven (1960). Borch *et al.* (1996) noted that carnobacteria also have the potential to cause H_2O_2- based colour defects in cooked cured meats, but this observation has not been reported in the literature. Carnobacteria have become of increasing interest to food microbiologists because they can represent a significant proportion of the microbiota in perishable foods stored at low temperature for extended periods under reduced oxygen tension (Lai & Manchester, 2000). Some are capable of producing potent antimicrobial compounds which inhibit more noxious organisms (Barakat *et al.* 2000; Jöborn *et al.* 1999). Originally described as non-aciduric, atypical lactobacilli (Collins *et al.* 1987), carnobacteria appear to be regularly present in vacuum or modified atmosphere packaged pork (McMullen & Stiles, 1993; Nadon *et al.*2001;

Wasney *et al.* 2001), poultry (Grant & Patterson, 1991; Barakat *et al.* 2000), and lamb (Nissen *et al.* 1994).

In this study we report the morphologic, phenotypic and phylogenetic characteristics of a *Carnobacterium* strain MPL-11^T (isolated from commercially prepared vacuum packaged bologna sausage) which was responsible for causing green discolouration of the product after package opening. Phenotypic and phylogenetic characteristics were different from all known species of *Carnobacterium*, indicating this organism was a new species. The isolate MPL-11^T has been designated *Carnobacterium viridans.*

The organism MPL-11^T was isolated from sliced bologna in a vacuum-sealed retail package obtained directly from the federally inspected plant where it was manufactured. Two other similar strains (MPL-2 and MPL-14) were isolated from the same package. Strain MPL-2 was lost during subculture. The packages were held at 10°C until product expiry code was exceeded by 30d. A sample of meat (10g) from one aseptically opened pack showing green discolouration was homogenized (Stomacher), diluted in 0.1% peptone and plated on All Purpose Tween (APT, Difco), M5 (Zuniga *et al.* 1993) and MRS (Hammes *et al.* 1991) agars. Plates were incubated anaerobically at 25°C or 30°C for 48h (BBL Gaspak). The isolates were routinely grown in APT broth and preserved in APT broth containing 20% (w/v) glycerol, stored at -80°C. Carnobacteria and aerococci dominated the microbiota of the commercial package of discoloured bologna and were each present at 10^7 CFU bacteria cm^{-2}. Ten of 15 isolates produced a defect typical of that seen in commercial products and all 10 were from these two genera. Two of the three commercial lots of product did not discolour.

Isolates were tested for their ability to grow on Peptonized Agar Medium, PTM, containing $MnSO_4$ and TMBZ (Berthier, 1993), Lactobacillus Selective Agar, LBS (Difco), KF Streptococcus Agar (Difco), Brain Heart Infusion Broth, BHI (Difco), the meat-based medium of Grant *et al.* (1988) and

Cresol Red Thallium Acetate Sucrose Inulin Agar, CTSI (Wasney *et al.* 2001). All agar plates were incubated anaerobically at 30°C for 48h, except for CTSI plates which were incubated 2d at 25°C plus 2d at 8°C (anaerobically).

To determine whether isolates caused green discolouration of meat, they were grown in APT broth for 48h at 30°C, centrifuged and the pellet resuspended in sterile 0.1% peptone to contain about 10^7 CFU bacteria ml^{-1}. Commercial, 500g packages of sliced all beef bologna, were treated with e-beam irradiation (≥3 KGy) to eliminate the background microflora. Individual slices of bologna (10 cm diameter) were transferred to O_2-barrier bags (Winpak, Deli*1) and 0.4 ml of the test bacterial suspension was spread evenly over the meat surface with a sterile glass rod or by massaging the plastic film. This yielded 10^5 CFU bacteria cm^{-2} of bologna. A vacuum was drawn (Bizerba model GM 2002), bags were heat-sealed and stored at 4°C or 9°C for up to 40d. Packages were opened and half were treated with 0.4 ml of an aqueous, sterile 3% (w/v) solution of catalase (Sigma-Aldrich). Unsealed, but still wrapped slices were held at 4 °C for ≤3 d and visually inspected for discolouration.

Morphologic examination of the isolates was by phase contrast microscopy. Cell measurements were made by bright field examination of crystal violet-stained cells. Gram reactions were done using 3% KOH (Gregersen 1978); catalase and oxidase activities were measured by standard methods. Growth over the range 2°C to 45°C and in ≤ 14% NaCl, ammonia production from arginine and gas production from glucose were monitored in modified MRS (acetate omitted) (Hammes *et al.* 1991) at 25°C following inoculation with late logarithmic cells. Survival in APT broth supplemented with 26.4% NaCl was examined for up to 37d at 4°C, 10°C and 25°C. Survivors were plated on APT agar using the hydrophobic grid membrane filtration technique (Entis & Boleszczuk, 1986).

Carbohydrate fermentation tests were done using the API 50CHL (bioMérieux Vitek Inc., Hazelwood, MO) and Biolog AN MicroPlate (Biolog Inc., Hayward, CA) systems. D- and L-lactic acid production were quantified using D(-) and L(+) lactate dehydrogenases (Boehringer-Mannheim,

FRG). Whole cell hydrolysates were used for the detection of *meso*-diaminopimelic acid (*meso*-DAP) in the peptidoglycan by thin layer chromatography (Bousefield *et al.* 1985).

The thermal resistance of MPL-11^T and other isolates was characterized using an immersion sealed capillary tube (ISCT) procedure (Foegeding & Leasor, 1990).

Several isolates were obtained and their identity to MPL-11^T was examined using PFGE (Zhang & Holley, 1999). The isolate MPL-11^T and others were ribotyped using the RiboPrinter Microbial Characterization System following the manufacturer's procedures (Bruce, 1996).The near complete sequence of 16S rDNA for MPL-11^T (1465 bp) and a partial sequence for MPL-14 (>600bp) were obtained using a PCR amplification strategy outlined by Kim *et al.* (2000). PCR products were sequenced at the UC DNA Services facilities (University of Calgary, Calgary, AB). The resultant sequences were aligned to the small-subunit rRNA sequences from the Ribosomal Database Project (Maidak *et al.* 2001). Positions that were unambiguously aligned were used in the construction of a evolutionary distance matrix using the maximum - likelihood method (Bratina *et al.* 1998). An unrooted evolutionary tree was constructed from the distance matrix using a neighbour-joining method (Saitou & Nei, 1987). The phylogenetic analysis was conducted using the PHYLIP software package (Felenstein, 1993).

Strain MPL-11^T grew on bologna inoculated with 10^5 CFU cells cm^{-2} and reached 10^7CFU cm^{-2} within 25d at 10°C. Slices of pasteurized bologna each inoculated with either of two carnobacteria isolates as well as those inoculated with each of the several strains of *Aerococcus viridans*, turned greenish within 3 days of package opening. When slices were treated with catalase immediately after opening the package, discolouration was prevented.

Results from the API 50CHL and Biolog fermentations showed there were differences among the MPL-11^T isolate and other known strains of *Carnobacterium* (Table 1). The isolate was consistent in its ability to produce acid from those substrates normally fermented by carnobacteria except for ribose.

Like other species of carnobacteria, MPL-11^T did not produce acid from arabinose, arabitol, dulcitol, erythritol, fucose, glycogen, inositol, raffinose, L-rhamnose, L-sorbose, xylitol or L-xylose (Hammes *et al.* 1991). The MPL-11^T isolate produced small colonies with light colour (beige to gray) on CTSI agar (Wasney *et al.*2001) with slight yellowing of the medium following anaerobic incubation (25°C for 2d plus 8°C for 2d). The organism produced 97.8% of lactic acid as the L(+) - enantiomer.

The organism was not thermally resistant and did not survive one min heating at 60°C, so a D-value could not be calculated at this temperature. In the same test, *C.piscicola* ATCC 43224 had a D_{60} value of 0.54min with no survivors at 4 min.

Restriction endonuclease digestion of DNA (*Sma*I), followed by PFGE showed that the two viable isolates from bologna (MPL-11^T and MPL-14) were genetically similar, sharing 8 electrophoretic bands. MPL-14 had an additional band at 242.5kb near the top of the gel, but this still indicated significant homology (Tenover *et al.*1995).

Comparison of the RiboPrint patterns obtained from the two isolates revealed a similarity index of 0.99 ± 0.1. Comparison of these profiles against those of six known *Carnobacterium* species (*C. inhibens*, a motile species, Jöborn *et al.*1999, was not included) and all lactobacilli strains held in the DuPont Identification Databank, generated matches with $\leq 85\%$ similarity. Although identification was not possible using this method, the profiles of the unknowns in terms of conservation of intensity and positioning of the bands, were taken to indicate they were members of the genus *Lactobacillus* or *Carnobacterium*.

The 16S rDNA sequence data showed that MPL-11^T is a *Carnobacterium* in a clade separate from the known species of this genus (Fig. 1). MPL-11^T is most closely related to *Carnobacterium* sp. Y6, a clinical strain isolated from a patient with multi-bacterial synergistic gangrene and is clearly different from all previously named species of this genus.

The organism MPL-11^T exhibited major characteristics that define membership in the genus *Carnobacterium* (Lai & Manchester, 2000; Stiles & Holzapfel, 1997; Holt *et al.* 1994; Hammes *et al.* 1991; Montel *et al.* 1991; Collins *et al.* 1987). However, the organism exhibited characteristics that did not allow assignment to any of the currently accepted species of this genus. A principal phenotypic difference from all other *Carnobacterium* spp. was that MPL-11^T did not produce acid from ribose (Table 1). It should be noted that in Jöborn *et al.* (1999), *C.funditum* DSM 5970^T and *C.alterfunditum* DSM 5972^T were incorrectly reported to be ribose negative. Their observation arose from a tabular error by Franzmann *et al.* (1991) which also involved *C.mobile*. The latter showed that both *C.funditum* DSM 5970^T and *C.alterfunditum* DSM 5972^T produced moderate amounts of formic and acetic acids plus ethanol in addition to lactic acid from D(-)-ribose. *C. mobile* NCFB 2765^T produced acid from ribose (Collins *et al.* 1987).

16S rDNA analysis of MPL-11^T showed that it and MPL-14 belonged in a separate phylogenetic group from other species of *Carnobacterium* (Fig.1). Interestingly, its closest relatives are clinical and aquatic environmental isolates while *Carnobacterium* species from meat and meat products are more distantly related. In addition, Ribotyping results indicated no match with known strains of either *Carnobacterium* or *Lactobacillus* in the DuPont Identification Databank.

Perhaps this is not surprising since Lai & Manchester (2000) found in their study of 73 carnobacteria isolates that several strains represented single-membered clusters. They suggested that the genus *Carnobacterium* is under-speciated because of this observation, and the recognition that only a very few phenotypic traits are traditionally used for their speciation. Accordingly, and on the basis of phenotypic results we have named the isolate MPL-11^T *Carnobacterium viridans.*

7.1 Description of *Carnobacterium viridans* sp.nov.

Carnobacterium viridans (vi.rí dans. NL. adj.*viridans* from L.v. *viridare*, to make green, referring to the production of a green colour in cured meat by the organism.)

Carnobacterium viridans is a Gram-positive, non-motile, non-sporeforming facultatively anaerobic organism which occurs as slightly curved rods singly or in pairs, or as straight rods (0.8 x 3.6 ± 0.6μm) in chains sometimes 20μm long. Grows satisfactorily in BHI, APT, M5 and CTSI media, but poorly on a variety of media including MRS. Does not grow on Rogosa agar. It grows over a range of pH from 5.5-9.1, from 2°C to 30°C but the range may be slightly greater. No growth at 37°C. Does not produce catalase or oxidase and is *β*- hemolytic on blood agar base with 0.8% sheep blood. Ammonia is not produced from arginine. No gas is produced from glucose; nitrate is not reduced and H_2S is not produced. The Voges - Proskauer reaction is negative. Acid is not produced from amygdalin, inulin, mannitol, α-methyl-D-glucoside, ribose or D-xylose. Thus the organism differs from the seven described species of *Carnobacterium*. It is capable of acid production / growth on: galactose, glucose, fructose, mannose, N-acetyl glucosamine, esculin, cellobiose, maltose, lactose, sucrose, trehalose, and tagatose (API 50CHL). The organism also metabolized N-acetyl D-mannosamine, arbutin, dextrin, gentibiose, glucose-6-PO_4, maltotriose, 3-methyl-D-glucose, salicin, α -hydroxybutyric acid, α-ketovaleric acid, pyruvic acid and uridine (Biolog AN). Negative for all other substrates used in the API 50CHL and Biolog AN panels. Does not grow in 4% (w/v) NaCl but will tolerate 26.4% (w/v) NaCl (saturated brine) for long periods at 4°C. Does not grow on KF Streptococcus Agar. Produces predominantly L(+)-lactic acid from glucose and the cell wall peptidoglycan contains *meso*-DAP. The type strain is MPL-11 ^{T} (=ATCC BAA-336 ^{T})(=DSM 14451 ^{T}) and the GenBank accession number for its 16S rDNA sequence is AF425608.

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7.3 References

1. Barakat, R.K., Griffiths, M.W & Harris, L.J. (2000). Isolation & characterization of *Carnobacterium*, *Lactococcus*, and *Enterococcus* spp. from cooked modified atmosphere packaged, refrigerated, poultry meat. *Int J Food Microbiol* **62,** 83-94.

2. Berthier, F. (1993). On the screening of hydrogen peroxide-generating lactic acid bacteria. *Lett Appl Microbiol,* **16,** 150-153.

3. Bousefield, I.J., Keddie, R.M., Dando, T.R. & Shaw, S. (1985). Simple rapid methods of cell wall analysis as an aid in the identification of aerobic coryneform bacteria. In *Chemical Methods in Bacterial Systematics*, pp. 222-234. Edited by M. Goodfellow & D.E. Minnikin. London: Academic Press.

4. Bratina, B.J., Stevenson, B.S., Green, W.J. & Schmidt, T.M. (1998). Manganeese reduction from oxic regions of the Lake Vanda (Antartica) water column. *Appl Env Microbiol* **64,** 3791-3797.

5. Bruce, J. (1996). Automated system rapidly identifies and characterizes microorganisms in food. *Food Technol* **50**(1), 77-81.

6. Borch, E., Kant-Muermans, M-L. & Blixt, Y. (1996). Bacterial spoilage of meat and cured meat products. *Int J Food Microbiol* **33,** 103-120.

7. Collins, M.D., Farrow, J.A.E., Phillips, B.A., Ferusu, S. & Jones, D. (1987). Classification of *Lactobacillus divergens, Lactobacillus piscicola*, and some catalase negative, asporogenous, rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. *Int J Sys Bacteriol* **37,** 310-316.

8. Deibel, R. H. & Niven, C.F. (1960). Comparative study of *Gaffkya homari*, *Aerococcus viridans*, tetrad-forming cocci from meat curing brines, and the genus *Pediococcus*. *J Bacteriol* **79,** 175-180.

9. Entis, P. & Boleszczuk, P. (1986). Use of fast green FCF with tryptic soy agar for aerobic plate count by the hydrophobic grid membrane filter. J. Food Protect **49,** 278-279.

10. Felenstein, J. (1993). PHYLIP (phylogenetic inference package) version 3.5c. Seattle: University of Washington.

11. Foegeding, P.M. & Leasor, S.B. (1990). Heat resistance and growth of *Listeria monocytogenes* in liquid whole egg. *J Food Protect* **53**, 9-14.

12. Franzmann, P.D., Höpfl, P., Weiss, N. & Tindall, B.J. (1991). Psychrotrophic, lactic acid - producing bacteria from anoxic waters in Ace Lake, Antartica; *Carnobacterium funditum* sp.nov. and *Carnobacterium alterfunditum* sp.nov. *Arch Microbiol* **156,** 255-262.

13. Grant, G.F., McCurdy, A.R. & Osborne, A.D. (1988). Bacterial greening in cured meats: a review. *Can Inst Food Sci Technol J* **21,** 50-56.

14. Grant, I.R. & Patterson, M.F. (1991). Antibacterial taxonomic study of lactic acid bacteria isolated from irradiated pork and chicken packaged under various gas atmospheres. *J Appl Bacteriol* **70,** 302-307.

15. Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. *Eur J Appl Microbiol Biotechnol* **5,** 123-127.

16. Hammes, W.P., Weiss, N. & Hozapfel, W. H. (1991). The genera *Lactobacillus* and *Carnobacterium*. In *The Procaryotes*, Vol II, 2nd edn., pp. 1535-1594. Edited by A. Ballows, H.G. Trüper, M. Dworkin, W. Harder & K-H. Schleifer. New York: Springer-Verlag.

17. Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley J.J. & Williams, S.T. (1994). Regular, nonsporing Gram-positive rods. In *Bergey's Manual of Determinative Bacteriology*, pp. 565-570. Baltimore: Williams & Wilkins.

18. Jöborn, A., Dorsch, M., Olsson, J.C. Westerdahl, A, & Kjelleberg, S. (1999). *Carnobacterium inhibens* sp.nov., isolated from the intestine of Atlantic salmon (*Salmo salar*). *Int J Sys Bacteriol* **49,** 1891-1898.

19. Kim, J. Chun, J. & Han, H-U. (2000) *Leuconostoc kimchii* sp. nov., a new species from kimshi. *Int J Syst Evol Microbiol* **50,**1915-1919.

20. Lai, S. & Manchester, L.N. (2000). Numerical phenetic study of the genus *Carnobacterium*. *Antonie van Leeuwenhoek* **78,** 73-85.

21. Lawrie., R.A. (1998). *Lawrie's Meat Science*, 6th edn. Woodhead Publishing Ltd., Cambridge, England.

22. Maidak, B.L. Cole, J.R., Lilburn, T.G., Parker, C.T.Jr, Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G. J., Schmidt, T.M. & Tiedje J. M. (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29**,173-174.

23. McMullen, L.M. & Stiles M.E. (1993). Microbiol ecology of fresh pork stored under modified atmosphere at -1, 4.4 and 10°C. *Int J Food Microbiol* **18**, 1-14.

24. Montel, M-C., Talon, R., Fournaud, J. & Champomier, M-C. (1991). A simplified key for identifying homofermentative *Lactobacillus* and *Carnobacterium* spp. from meat. *J Appl Bacteriol* **70**, 469-472.

25. Nadon, C.A., Ismond, M.A.H. & Holley, R. A. (2001). Biogenic amines in vacuum packaged CO_2 controlled atmosphere fresh pork stored at -1.5°C. *J Food Protect* **64**, 220-227.

26. Nissen, H., Holck, A. & Dainty, R.H. (1994). Identification of *Carnobacterium* spp. and *Leuconostoc* spp. in meat by genus specific 16S rRNA probes. *Lett Appl Microbiol* **19,** 165-168.

27. Saitour, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4,**406-425.

28. Stiles, M.E. & Holzapfel, W.H. (1997). Lactic acid bacteria of foods and their current taxonomy. *Int J Food Microbiol* **36,** 1-29.

29. Tenover, F.C., Arbeit, R.D., Goering, R.V. Mickelsen, P.A., Murray, B.E., Persing, D.H. & Swaminathan, B. (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33,** 2233-2239.

30. Wasney, M.A., Holley R.A. & Jayas, D.S. (2001). Cresol red thallium acetate sucrose inulin (CTSI) agar for the selective recovery of *Carnobacterium* spp. *Int J Food Microbiol* **64,** 167-174.

31. Zhang, G. & Holley R.A. (1999). Development and PFGE monitoring of dominance among spoilage lactic acid bacteria from cured meats. *Food Microbiol* **16,** 633-644.

32. Zuniga, M., Pardo, I. & Ferrer, S. (1993). An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. Int. J. Food Microbiol **18**, 37-42.

Table 7.1. Characteristics useful in differentiating *Carnobacterium* species*

Species: 1, *Carnobacterium divergens* NCDO 2763^T; 2, *Carnobacterium gallinarum* NCFB 2766^T; 3, *Carnobacterium mobile* NCFB 2765^T; 4, *Carnobacterium piscicola* NCDO 2762^T; 5, *Carnobacterium funditum* DSM 5970^T; 6, *Carnobacterium alterfunditum* DSM 5972^T; 7, *Carnobacterium inhibens* CCUG 31728^T; 8, *Carnobacterium viridans* MPL-11^T.

	Carnobacterium Species							
Characteristic	1	2	3	4	5	6	7	8
Motility	-	-	+	-	+	+	+	-
Acid from:								
amygdalin	+	+	-	+	-	+	+	-
inulin	-	-	+	+(-)†	-	-	w‡	-
D-lactose	-	+	± v	(-)	-	-	w	+
mannitol	-	-	-	+	+	-	+	-
α-methyl-D-glucoside	-	+	-	+	NT‖	NT	-	-
D-xylose	-	+	-	-	-	-	-	-
D-tagatose	-	+	-(+)¶	-	NT	NT	-	+
D-ribose	+	+	+	+	+	+	+	-
D-trehalose	+	+	+	+	+	-	+	+
Esculin hydrolysis	+	+	+	+	-	±	+	+
Growth at 37°C	+	+	±	+	-	-	-	-
Voges-Proskauer	+	+	-(+)	+	NT	NT	NT	-

* Modified from: Collins *et al.* 1987, Franzmann *et al.* (1991), Holt *et al.* (1994), Jöborn *et al.* (1999), and Lai & Manchester (2000).

† occasional strain negative

‡ weakly positive

v variable

‖ not tested

¶ occasional strain positive

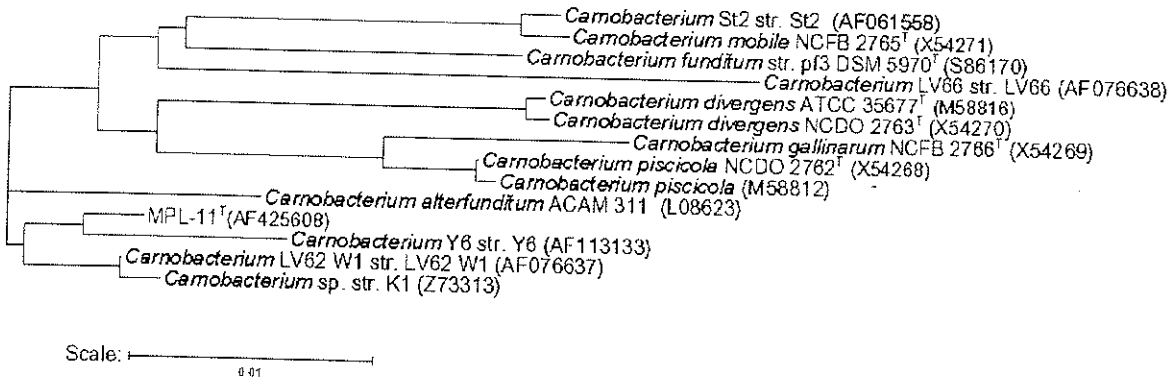

Figure 7.1. Unrooted Neighbour-Joining Tree of the Genus *Carnobacterium*. Based on alignment of 16S rDNA sequences between strain MPL-11^T and members of the genus *Carnobacterium*. The scale indicates 0.01 nucleotide substitutions per nucleotide position. The strain *Carnobacterium* sp. K1 is *C. inhibens* described by Jöborn *et al.* (1999).