(54) Title: LACTIC ACID BACTERIA FOR THE TREATMENT OF FOOD

Growth of *Listeria monocytogenes* cocktail in the presence of *C. piscicola* CB1 on Palcam in inoculated pork samples stored at 5 C for 20 days

(57) Abrégé/Abstract:
The methods and compositions of the present invention involve the use of *Carnobacterium maltaromaticum* strains (previously known as *C. piscicola*) or associated fermentate or bacteriocin compositions to treat foods, such as fresh or processed meats, against bacterial contamination.
Growth of *Listeria monocytogenes* cocktail in the presence of *C. piscicola* CB1 on Palcam in inoculated pork samples stored at 5°C for 20 days

(57) Abstract: The methods and compositions of the present invention involve the use of *Carnobacterium maltaromaticum* strains (previously known as *C. piscicola*) or associated fermentate or bacteriocin compositions to treat foods, such as fresh or processed meats, against bacterial contamination.
Lactic Acid Bacteria for the Treatment of Food

I. Field of the Invention

This invention relates to novel strains of *Carnobacterium maltaromaticum* that produce bacteriocin molecules having antimicrobial activity. The bacteria of the present invention, and the bacteriocin(s) produced by the bacteria or other bacteria, may be used to treat food and as a food preservative. In a particular application of the invention, the bacteriocin and the bacterial strain that produces the bacteriocin are used to control pathogenic bacteria, including but not limited to, *Listeria monocytogenes* ("L. monocytogenes") in meat products, without jeopardizing the storage life of the meats.

II. Background of the Invention

*Carnobacterium maltaromaticum* is one species of a diverse group of bacteria that are classified as Lactic Acid Bacteria (LAB). LAB have been utilized for centuries in the food and dairy industries in the production of fermented foods. Important in this capacity is their ability to produce aromatic and flavor-enhancing compounds (Stiles and Holzapfel, 1997; Carr et al., 2002). LAB have been characterized by their ability to produce a variety of
isomers of lactic acid from the fermentation of carbohydrates. Atypical Carnobacteria are distinct due their inability to grow on acetate agar at pH 5.6, while being able to produce virtually pure L(+-)-lactic acid from glucose and, their ability to ferment both glycerol and mannitol, properties that are unusual in lactobacilli (Holzapfel and Gerber, 1983; Shaw and Harding, 1984).

One of the methods that *C. maltaromaticum* may inhibit potentially pathogenic bacteria is through the production of bacteriocins. Bacteriocins are ribosomally synthesized, low molecular weight antibacterial proteinaceous materials that are able to kill closely related bacteria (Klaenhammer, 1993). Bacteriocins have been isolated from beef, spoiled ham, as well as from French mold-ripened soft cheese (Jack *et al*., 1996; Herbin *et al*., 1997). Because bacteriocins are isolated from foods such as meat and dairy products, which normally contain LAB, both LAB and bacteriocins have been consumed for centuries. Bacteriocins produced from *C. maltaromaticum* have been shown to be susceptible to proteolytic enzymes. The bacteriocin from *C. maltaromaticum* LV17 is stable during heat treatment at 62°C, boiling for 30 min and, after autoclaving at 121°C for 15 minutes. Trypsin, protease types I, IV, VIII, XIV, α - chymotrypsin, β -chymotrypsin and papain inactivated the bacteriocin, while non-proteolytic enzymes did not (Ahn and Stiles, 1990b). Piscicolin 126, a bacteriocin produced by *C. maltaromaticum* JG126 was inactivated by α - and β -chymotrypsin, proteases (types I, XIV, XXIII and trypsin), but catalase, lipase or lysozyme had no effect (Jack *et al*., 1996). Similarly, the bacteriocin produced by *C. maltaromaticum* LV61 is resistant to
heat (100°C for 20 minutes), while being inactivated by α -chymotrypsin, trypsin, pepsin, papain and proteinase K.

Treatment with catalase, α -amylase, lipase, phospholipase C, DNase I and lysozyme did not affect the antibacterial activity (Schillinger et al., 1993). This evidence has indicated that ingestion of bacteriocins would not have an effect on the beneficial gut microorganisms. Trypsin has been shown to inactivate the bacteriocin, nisin (Hara et al., 1962).

There is a continual need for new food preservatives bearing new and useful properties. Further, there is growing interest in replacing traditional "chemical" food preservatives with effective "natural" preservatives, especially those that inhibit pathogenic microorganisms. In this regard, considerable research has been conducted on bacterial proteins, known as bacteriocins, which are often heat stable and have antimicrobial activity.

Recent years have seen major advances in the development of microbial metabolites with antagonistic activities towards spoilage and pathogenic microorganisms associated with food. There now exists many bacteriocins, but only a few have been fully characterized and evaluated for food use. Additionally, consumer emphasis is now on minimally processed foods that are natural and preservative free. Because of this, there is considerable resistance to the use of chemical additives as food preservatives. Other biological inhibitors produced by microorganisms are currently being investigated for use in foods. Of particular interest are those
antibacterial substances such as bacteriocins that are produced by Lactic Acid Bacteria ("LAB").

Bacteriocins, which are anti-bacterial peptides and proteins produced by LAB as normal by-products of their metabolism, are potentially very attractive natural preservatives. Many LAB are well-established, industrially important bacteria that include the genera *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus* and *Carnobacterium*. They have been used for the production of fermented foods that have been consumed safely for thousands of years. Because they have achieved a status as "safe" microorganisms, they are a particularly suitable source of natural antimicrobials, such as bacteriocins, and for use in foods. Bacteriocins can have a broad or narrow spectrum of antibacterial activity, and are not lethal to the cells that produce them. Bacteria protect themselves from the lethal effects of their own bacteriocins by the production of immunity proteins.

*C. maltaromaticum* is a Gram-positive, non-motile, non-sporeforming, rod-shaped bacterium, recently redefined from the genus *Lactobacillus* to *Carnobacterium*. *C. maltaromaticum* has been indicated as one of a large, diverse group of lactic acid-producing bacteria, which metabolize glucose to produce lactic acid and other acids that inhibit the growth of several pathogenic bacteria. *C. maltaromaticum* was initially found in salmonid fish, but has since been found on various food products, from meats and fish to fruits and vegetables, produced and stored by current agricultural practices, at levels exceeding $1 \times 10^7$ cfu/g. Lactic acid bacteria
have been used for centuries in the fermentation and preservation of food products (e.g., yogurts, sausages, vegetables, breads, wine, cheeses and milk). *C. maltaromaticum* has already been used as part of a starter bacterial culture in sausage fermentation in France.

Notwithstanding the usefulness of the above described natural preservatives, a need still exists for lactic acid bacteria and their bacteriocins that are capable of controlling pathogenic and spoilage bacteria in specific food products.

**III. Summary of the Invention**

This invention relates to novel strains of bacteriocin-producing *Carnobacterium maltaromaticum* ("C. maltaromaticum"), previously known as *Carnobacterium piscicola* ("C. piscicola"), having exceptional antimicrobial activities. The novel strains of the present invention, CB1, CB2, and CB3 produce multiple bacteriocins, including camobacteriocin BM1 and piscicolin 126. These bacteriocins have broad spectrum anti-Listerial activity, and the producer strains grow at refrigeration temperatures and do not cause food spoilage relative to other similarly related spoilage microorganisms or within the typical shelf-life of the food.

An embodiment of the invention includes *Carnobacterium maltaromaticum* strains CB1, CB2, CB3, LV17, UAL26, ATCC 35586 and ATCC 43225 for use as a preservative in ready-to-eat (RTE) and fresh comminuted, processed meat products, preferably at a maximum inoculation concentration of $1 \times 10^4$ colony forming units (cfu)/g.
An embodiment of the present invention includes a method of treating fresh food by applying *C. maltaromaticum*, its pasteurized or unpasteurized fermentate, or combinations thereof to the food. In these embodiments of the invention, the bacteria and its pasteurized or unpasteurized fermentate produce a predictable or controlled storage life.

In preferred embodiments of the invention, the food is treated with the combination of natural bacteria and its pasteurized or unpasteurized fermentate, or one or more bacteriocin fermentates produced by a different bacterium. In the most preferred embodiment of the invention, the food is treated with the combination of selected natural bacteria and a pasteurized or unpasteurized fermentate of a selected natural bacterial culture.

An embodiment of the present invention includes using a composition of the present invention to further protect a food product from the growth of gram positive pathogenic bacteria including, but not limited to, *Listeria monocytogenes*. The compositions of the present invention are effective against strains of *L. monocytogenes* serotypes 1/2a, 1/2b, 3a and 4b.

The method of the present invention includes the use of one or more natural bacterial cultures, homologous pasteurized or unpasteurized fermentate, heterologous pasteurized or unpasteurized fermentate, or combinations thereof. The natural bacterial cultures of the present invention are described above. A homologous fermentate refers to the culture supernatant of a single bacterial culture, typically prepared according to standard preparation techniques. A heterologous fermentate refers to the
culture supernatant derived from a different bacterial culture typically prepared according to standard preparation techniques. The homologous or heterologous fermentate may be i) pasteurized or unpasteurized; ii) lyophilized; or iii) otherwise dried. Two or more bacterial cultures may be mixed or added separately. Two or more fermentates may be mixed or added separately. A bacterial culture combined with one or more fermentates may be mixed or added sequentially.

In another exemplary embodiment, the present invention comprises a culture of bacterial strain CB1. CB1 was deposited in the American Type Culture Collection (10801 University Boulevard, Manassas, Virginia USA 20118) on 9 July 2003, and received Accession No. PTA-5313.

In another exemplary embodiment, the present invention comprises a culture of bacterial strain CB2. CB2 was deposited in the American Type Culture Collection (10801 University Boulevard, Manassas, Virginia USA 20118) on 9 July 2003, and received Accession No. PTA-5314.

In another exemplary embodiment, the present invention comprises a culture of bacterial strain CB3. CB3 was deposited in the American Type Culture Collection (10801 University Boulevard, Manassas, Virginia USA 20118) on 9 July 2003, and received Accession No. PTA-5315.

In another exemplary embodiment, the present invention comprises the use CB1, CB2, and/or CB3, or combinations thereof, for the treatment of food, for the treatment of spoilage bacteria on food, for the treatment of pathogenic bacteria on food, and/or establishes a predictable storage life for a food or food product. Strains CB1, CB2, and/or CB3 may be
used alone or in combination; may be used with or without their respective bacteriocins; may be used with or without a fermentate comprising their respective bacteriocins; may be used in combination with one or more bacteriocin-producing bacteria, including but not limited to a lactic acid bacterium; and/or may be used with one or more bacteriocins produced from a different bacterium; and/or may be used with or without a fermentate comprising one or more bacteriocins produced from a different bacteriocin.

In another exemplary embodiment, the present invention comprises a method of preserving foods or beverages, the method comprising adding to the food or beverage an effective amount of a bacterial culture of the present invention, alone or in combination with a fermentate. The inventors have found that an amount of $10^5$, or less, colony forming units ("cfu") per gram or per cm$^2$ is typically not sufficient to compete with the existing adventitious microbial population. The inventor has found that 10-fold greater than the initial background microflora, typically about $10^3$ cfu per gram or per cm$^2$ or greater, is sufficient to overcome the growth of the existing adventitious bacterial (e.g., background microflora) population. One skilled in the art will recognize that the amount of adventitious bacteria in a food product is variable. In accordance with the present invention, the amount of the composition should be about ten times or more higher than the amount of adventitious spoilage bacteria.

In preferred embodiments of the invention, the method includes treating fresh meat. In the most preferred embodiments of the invention, the
method includes treating or preserving fresh sausage or vacuum-packaged wieners.

The present invention also relates to the use of the bacterial composition and/or bacteriocin produced by the composition in the treatment of Listeria spp., to inhibit the growth of Listeria spp. in meats.

The invention also relates to a fermentate comprising one or more bacteriocins produced by strains CB1, CB2, and/or CB3. In preferred embodiments of the invention, the fermentate comprises piscicolin 126, carnobacteriocin BM1, and an identifiable but yet uncharacterized proteinaceous compound(s) having antibacterial activity.

In the embodiments of the invention that include a bacteriocin, the bacteriocin may be isolated from natural sources, may be produced by one or more strains of the present invention, may be produced by another bacterial strain, or may be produced by genetic modification e.g., the use of a recombinant expression vector).

An advantage of the invention is unprecedented anti-listerial activity. Such a broad anti-listerial spectrum is exceptional. Another advantage of the invention is that there is both bactericidal and bacteriostatic potential. Yet another advantage of the invention is that these bacteria grow at temperatures as low as 0°C, which indicates that they grow and are effective under refrigeration temperatures that are essential for the preservation of meats. Yet a further advantage of the invention is that these strains do not cause significant spoilage of the meats in and of themselves.
The accompanying drawings show illustrative embodiments of the invention from which these and other of the objectives, novel features and advantages will be readily apparent.

IV. Brief Description of the Drawings

FIGURE 1 is a graph of the anti-listerial activity of a composition of the present invention illustrating the reduction of bacterial numbers and the inhibition of a cocktail of four strains of *L. monocytogenes* in the presence of $10^3$ and $10^4$ cfu of *C. maltaromaticum* CB1 inoculated per gram of pork sausage samples stored at 5°C for greater than the proposed 15-day refrigerated storage life of the sausages.

FIGURE 2 is a graph of the first of three replicate trials illustrating the reduction of bacterial numbers and the inhibition of a cocktail of four strains of *L. monocytogenes* inoculated at $10^2$ to $10^3$ cfu per cm$^2$ in the presence of $10^4$ cfu of *C. maltaromaticum* CB1 or CB3 per cm$^2$ on the surface of vacuum-packaged wieners stored at 5°C over the 12-week refrigerated storage life of the product.

FIGURE 3 is a graph of the second of three replicate trials illustrating the reduction of bacterial numbers and the inhibition of a cocktail of four strains of *L. monocytogenes* inoculated at $10^2$ to $10^3$ cfu per cm$^2$ in the presence of $10^4$ cfu of *C. maltaromaticum* CB1 or CB3 per cm$^2$ on the surface of vacuum-packaged wieners stored at 5°C over the 12-week refrigerated storage life of the product.

FIGURE 4 is a graph of the third of three replicate trials illustrating the reduction of bacterial numbers and the inhibition of a cocktail of four
strains of *L. monocytogenes* inoculated at $10^2$ to $10^3$ cfu per cm$^2$ in the presence of $10^4$ cfu of *C. maltaromaticum* CB1 or CB3 per cm$^2$ on the surface of vacuum-packaged Wieners stored at 5°C over the 12-week refrigerated storage life of the product.

V. **Specific Description of the Invention**

A composition of the present invention includes strains of *Carnobacterium maltaromaticum*, and each producing at least one, and typically three, bacteriocins. *C. maltaromaticum* CB1 produces bacteriocins piscicolin 126, carnobacteriocin BM1, and another uncharacterized bacteriocin that exhibits antibacterial activity. *C. maltaromaticum* CB2 produces piscicolin 126, carnobacteriocin BM1, and may produce one or more additional uncharacterized bacteriocins. *C. maltaromaticum* CB3 produces piscicolin 126, carnobacteriocin BM1, and may produce one or more additional uncharacterized bacteriocins.

The compositions and methods of the present invention include the use of one or more natural bacterial cultures, homologous pasteurized or unpasteurized fermentate, heterologous pasteurized or unpasteurized fermentate or combinations thereof. The natural bacterial cultures of the present invention are described above. A homologous fermentate refers to the culture supernatant of a single bacterial culture prepared according to standard preparation techniques. A heterologous fermentate refers to the culture supernatant derived from a different bacterial culture prepared according to standard preparation techniques. The homologous or heterologous fermentate may be i) pasteurized or unpasteurized; ii)
lyophilized; or iii) otherwise dried. Two or more bacterial cultures may be mixed or added separately. Two or more fermentates may be mixed or added separately. A bacterial culture combined with one or more fermentates may be mixed, or added sequentially.

An important aspect of the present invention comprises the use of the bacterial fermentate in the preservation and treatment of fresh meats. In accordance with the teachings of the present invention, the bacteriocins produced by strains CB1, CB2, or CB3 appear to act synergistically to provide greater protection and effectiveness than use of the individual bacteriocins alone.

As used herein, fresh meat products refer to raw or uncooked meat (stored under refrigerated conditions) that may or may not contain additional spice mixtures, and includes integral or ground meat. Processed meat products refer to meats that have been i) formulated and cooked; ii) cured; or iii) uncured to produce a marketable product. “Fresh” and “processed” are intended to be used in their ordinary meaning as known to those skilled in the art. Typical meats include, but are not limited to, wieners, sausage, fish, and poultry.

The compositions and methods of the present invention may also be used to treat other food products including, but not limited to, modified atmosphere packaged vegetables, vacuum-packed pasta and fresh pasta products.

As used herein, predicted storage life refers to the capability of controlling spoilage for a discrete period, at which point spoilage becomes
evident. For example, bacteria can be applied to a food product to attain a storage life of about 10 weeks or greater, at which point spoilage may be detectable. Within the 10-week storage period, the composition of the present invention controls spoilage by one or more of the following ways: i) by applying bacteria having a known time to spoilage; ii) by applying bacteria that produce one or more proteins or bacteriocins that kill or control spoilage bacteria; or iii) by combinations thereof.

As used herein, enhanced safety refers to the inhibition of growth and/or the reduction of numbers of potentially pathogenic bacteria, ranging from bactericidal to bacteriostatic.

As used herein preservation of color refers to the extension of the time that the food product retains its desirable coloration. This concept is well known to those skilled in the art.

EXEMPLARY

Example 1.

Collins et al. (1987) reported that L. piscicola, L. divergens and L. carnis synthesize the major C18:1 isomer as oleic acid (Δ 9,10), indicative of a different unsaturated fatty acid synthase pathway. Genetic homology classifications and chemical as well as physical characteristics also placed L. piscicola, L. carnis and L. divergens in the same DNA homology group. In addition, biochemical and chemical data indicated that L. piscicola and L. carnis should be (and were) reduced to the same species, L. piscicola. L. piscicola, along with L. ivergens, were then re-classified into a new genus,
Carnobacterium (L. gen. N. carnis, of flesh; Gr. dim. n. bakterion, a small rod; M.L. neut. N. Carnobacterium, flesh rodlet) by Collins et al. (1987). This was further substantiated when a 16S rRNA sequence analysis demonstrated that the Carnobacterium genus forms a distinct phylogenetic clade within the lactic acid bacteria and included C. funditum, C. alterfunditum, C. gallinarum and C. mobile (Table 1), with Lactobacillus maltaaromaticus further defined as an objective synonym of Carnobacterium piscicola (Miller et al., 1974; Collins et al., 1991; Lai and Manchester, 2000; Lai et al., 2004). In addition, although the Carnobacterium spp. were originally classified with the lactobacilli, phylogenetically the genus is more closely related to the genera Enterococcus and Vagococcus (Hiu et al., 1984).

Subsequent phenotypic and genetic characterizations of Lactobacillus maltaaromaticus strains DSM 20342T, DSM 20344 and JCM1154 determined that these strains also belonged in the genus Carnobacterium. Further comparison with C. piscicola resulted in the decision that these two species should be considered synonymous. As a result, C. piscicola was reclassified as Carnobacterium maltaaromaticum comb. nov. (Collins et al., 1991; Mora et al., 2003). Therefore, the common name of Carnobacterium maltaaromaticum will be used in reference to the species of the present invention.
Table 1. *Carnobacterium* species, their relationship to previously described bacteria and their habitat (Collins et al., 1987; Collins et al., 1991; Mora et al., 2003).

<table>
<thead>
<tr>
<th>Current nomenclature</th>
<th>Previous nomenclature</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. divergens</td>
<td><em>L. divergens</em></td>
<td>Meat, poultry, surface of ripened mold cheeses</td>
</tr>
<tr>
<td>C. gallinarum</td>
<td></td>
<td>Poultry</td>
</tr>
<tr>
<td>C. mobile</td>
<td></td>
<td>Poultry</td>
</tr>
<tr>
<td>C. maltaromaticum*</td>
<td><em>L. piscicola</em></td>
<td>Meat, poultry or salmonid fish</td>
</tr>
<tr>
<td></td>
<td><em>L. camis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>L. maltaromaticus</em></td>
<td></td>
</tr>
<tr>
<td>C. funditum</td>
<td></td>
<td>Antarctic lake</td>
</tr>
<tr>
<td>C. alterfunditum</td>
<td></td>
<td>Antarctic lake</td>
</tr>
</tbody>
</table>

*Proposed as C. maltaromaticus*(Collins et al., 1991) and C. maltaromaticum*(Mora et al., 2003). C. = *Carnobacterium*; L. = *Lactobacillus*.

Example 2.

Naturally-occurring *C. maltaromaticum* historically belongs to a group of LAB that metabolize glucose heterofermentatively to produce equimolar amounts of lactic acid, carbon dioxide and ethanol or acetic acid from sugars and was previously included in the genus *Lactobacillus* (Stanier et al., 1957; Hiu et al., 1984). Although some research has indicated that *Carnobacterium* spp. are homofermentative for L-lactate [with acetate, formate and CO₂ being produced as end-products of some secondary decarboxylation/dissimilation reactions of pyruvate (Hiu et al., 1984; De Bruyn et al., 1988)], the most recent description and characterization of *C. maltaromaticum* states that L(+)-lactic acid, ethanol and acetate are produced heterofermentatively (Mora et al., 2003). Therefore, for this example, *C maltaromaticum* has been characterized as having heterofermentative properties. *C. maltaromaticum*
was found frequently in fish that had suffered some form of stress, such as that which occurs at spawning or with handling (Hii et al., 1984; Baya et al., 1991). *C. maltaromaticum* has also been found by Ringo et al. (2000) to be associated with the digestive tract of the Atlantic salmon (*Salmo salar* L.). Carnobacteria have been isolated from refrigerated, vacuum-packaged fish and unprocessed beef and lamb, where it was among the predominant LAB on the meats (Ahn and Stiles, 1990a; Baya et al., 1991; Barakat et al., 2000; Carr et al., 2002; Paludan-Muller et al., 1998; Sakala et al., 2002; Yamazaki et al., 2003). The methods used in these studies did not enrich or select for any specific bacterial class or species.

A biochemical and physiological comparison between *C. divergens* and *C. maltaromaticum* is given in (Table 2). *C. maltaromaticum* strain B270T was described as having the following characteristics (Hii et al., 1984; Collins et al., 1987):

- Gram-positive, non-motile, non-sporeforming rods that occur singly and in short chains;
- Grows well on many standard laboratory media, including TSA (Trypticase Soy Agar) and Brain Heart Infusion agar and in deMan, Rogosa and Sharpe (MRS) broth and thioglycolate broth;
- Colonies are pinpoint, convex, white, circular and nonpigmented when grown at 25°C for 24h on TSA;
- Temperature range for growth is 6°C to 40°C; optimum temperature is approximately 30°C;
- Optimum pH range is from 6.0 to 7.0;
- Facultatively anaerobic. D, L-lactate is produced homofermentatively, but the species may exhibit heterofermentative properties under certain conditions; lactic acid production is enhanced under anaerobic growth conditions;

- Folic acid, riboflavin, pantothenate and niacin are required for growth; vitamin B12, biotin, thiamine and pyridoxal are not required;

- Catalase and oxidase are not produced;

- Nitrate is not reduced to nitrite;

- Gas production is variable (depending on substrate) and frequently negative; gas production from glucose in arginine-MRS broth;

- Acid is produced from glycerol, ribose, galactose, gluconate, glucose, fructose, mannose, mannitol, N-acetyl glucosamine, amygdalin, arbutine, salicin, cellobiose, sucrose and trehalose; acid is not produced from arabinose, xylose, sorbose, rhamnose, dulcitol, inositol, methyl-D-mannoside, inulin or melezitose;

- Arginine and esculin are hydrolyzed;

- H₂S is not detected in TSI Triple Sugar Iron Agar) slants;

- Resistant to 0.4 and 0.6% Teepol;

- Cell wall peptidoglycan contains diaminopimelic acid;

- DNA G+C content is 33.7-36.4 mol%;

- Major cellular fatty acids are of the straight-chain saturated and mono-unsaturated types with myristic, palmitic, palmitoleic and Δ 9, 10-oleic acids predominating;
The type strain is B270T (ATCC 35586), isolated in 1970 from a stressed adult cutthroat trout reared at Bandon Trout Hatchery in Coos County, Oregon.

Table 2. Biochemical and physiological comparison of the *Carnobacterium* species.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>C. divergens</em></th>
<th><em>C. maltaromaticum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid producted from³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amidon</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>β-Gentiobiose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gluconate</td>
<td>+(-)⁴</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Melezitose</td>
<td>+(-)</td>
<td>+(-)</td>
</tr>
<tr>
<td>α-Methyl-D-glucoside</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>α-Methyl-D-mannoside</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D-Tagatose</td>
<td>-</td>
<td>+(-)</td>
</tr>
<tr>
<td>D-Turanose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer⁵</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Δ9,10-Methyleneoctadecanoic acid⁶</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Adapted from (Collins et al., 1987). ²Previously designated as *Lactobacillus piscicola* and *Carnobacterium piscicola*. ³Reading performed at seven days. ⁴+(-) = Occasional strain negative; ⁵Glucose metabolism test performed on API 10E system; both strains produced arginine dihydrolase and β-galactosidase; both strains were negative for lysine decarboxylase, tryptophan desaminase, urease, ornithine decarboxylase, indole and H2S; ⁶Greater than 15% of total cellular fatty acids.
Alkaline pH (up to pH 9.5) promotes the growth of Carnobacterium colonies, while inhibiting other Lactobacillus species. Differentiation of C. maltaromaticum from other bacteria may be accomplished by modification of growth substrates. Differentiation of C. maltaromaticum from the enterococci includes microscopic distinction of rods vs. coccii and growing on Cresol Red Thallous Acetate Sucrose (CTAS) medium containing 2% inulin instead of sucrose. Enterococci are not able to ferment inulin, while C. maltaromaticum ferments inulin, forming yellowish to pinkish colonies with a metallic bronze sheen, a yellow color change of the medium and a clearance of precipitate. C. maltaromaticum forms an umbolate or beta-type colony when inosine is substituted for sucrose in CTAS Agar. The enterococci also produce a yellowing of the medium and a clearing of the precipitate, but do not have a metallic sheen (Carr et al., 2002). Different strains of C. maltaromaticum have been shown to produce bacteriocins (Ribosomally synthesized, low molecular weight, antibacterial, proteinaceous materials that are able to inhibit the growth or kill closely related bacteria) that inhibit the growth of Lactobacillus, Listeria and other Carnobacterium species (McMullen and Stiles, 1996; Duffes et al., 1999c; Schillinger et al., 1993).

Example 3.

The strains specified (e.g., Carnobacterium maltaromaticum strains CB1, CB2, CB3, LV17, UAL26, ATCC 35586 AND ATCC43225) have been tested for their resistance to 27 antibiotics (Table 3; Griffiths Labs, 2004). Overall, the C. maltaromaticum strains tested were
sensitive to amoxicillin + clavulanic acid, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, imipenem, netilmicin, rifampin, tetracycline and tobramycin. In viewing the antibiotic resistance profiles (Table 3), the *Carnobacterium* strains are sensitive to those major antibiotics that are commonly associated with transferable genetic elements in grampositive commensal bacteria; specifically, erythromycin, chloramphenicol and tetracycline. Borriello *et al.* (2003) suggested that when used as probiotics, selected strains should be susceptible to greater than two major antibiotics. A comparison with the antibiotics used by Baya *et al.* (1991), Duffes *et al.* (1999b) or Euzéby (2004) indicate that the sensitivity of the C. *malta*romaticum strains (CB1, CB2, CB3, LV17, UAL26, ATCC 35586 and ATCC43225) to various antibiotics correlate well with antibiotic resistance found in *C. malta*romaticum strains isolated from natural fish sources, as noted in Table 3. The antibiotic resistance profile for the *C. malta*romaticum strains specified in this GRAS dossier correlate well with the antibiotic resistance profiles of *Lactobacillus* species already being added to food or found in food naturally. This indicates that the addition of these strains of *C. malta*romaticum to foods would not be adding any new or significant antibiotic resistance determinants that are not normally found in commensals or probiotic lactobacilli.
### TABLE 3.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>CB1</th>
<th>CB2</th>
<th>CB3</th>
<th>LV17</th>
<th>UAL26</th>
<th>ATCC 35586</th>
<th>ATCC 43225</th>
<th>Baye et al., 1991</th>
<th>Euzby, 2004</th>
<th>Duffes et al., 1999b</th>
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<tr>
<td>Amikacin</td>
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<td>R</td>
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<td>R</td>
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<td>R</td>
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<tr>
<td>Amoxicillin + clavulanic acid</td>
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<td>S</td>
<td>S</td>
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<td>R</td>
<td>S</td>
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<td>I</td>
<td>S</td>
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<td>P</td>
<td>P</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>Netilmicin</td>
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<td>S</td>
<td>S</td>
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</table>

**Example 4. Occurrence and use of Lactic Acid Bacteria in Food**

Using direct-plating methods to identify bacteriocin-producing LAB isolates from meat and meat products, milk and dairy products, vegetables, fruit and seafoods, a total of 663,533 colonies from 72 food samples (32 milk and dairy, 40 meat) were examined for bacteriocin production (Coventry et al., 2001).
1997). Many of these food samples were judged to have exceeded an acceptable shelf life. A total of 15% of the meat and meat products yielded bacteriocin producing *Carnobacterium* spp. Of the 72 food samples investigated, 44% yielded bacteriocin producing bacteria. From the total 663,533 colonies tested, 80,992 colonies (12.2%) were found to be *Carnobacterium* spp., with 0.15% of those producing bacteriocins. The antibacterial activities of filter-sterilized culture supernatant fluids from select strains of the bacteriocin producers were not affected by catalase, lipase or lysozyme, but were either completely or partially inactivated by at least one of the proteolytic enzymes, indicating that antibacterial activity was associated with proteinaceous substances. This study also shows that humans are already being exposed to *Carnobacterium* spp. and other food-borne bacteria that produce bacteriocins.

Amezquita and Brashears (2002) report the isolation of 49 strains of LAB from commercially available ready-to-eat (RTE) meat products. These were screened for their ability to inhibit the growth of *Listeria monocytogenes* at 5°C on agar spot tests. *Pediococcus acidilactici, Lactobacillus casei* and *L. paracasei* were identified as the three species with the greatest inhibitory activity. There was significant inhibition (P<0.05) of the growth of *L. monocytogenes* in all of the RTE meat products evaluated (five commercial samples of cooked ham and five commercial samples of frankfurters), when three selected strains of *Pediococcus acidilactici, Lactobacillus casei* and *L. paracasei* were added to the RTE meat products. This study showed that select strains of LAB can be isolated from RTE meat products and these...
strains effectively inhibit the growth of *L. monocytogenes* in frankfurters and cooked ham at 5°C over 28 days of storage. During the time of storage, the numbers of LAB increased by only approximately 1 log cycle and no visible signs of spoilage were evident (*e.g.*, detrimental effect on some organoleptic properties related to external appearance such as color changes, undesirable aromas and stickiness or texture changes) on the surface of the products. A study conducted by Sakala *et al.* (2002) to investigate the psychrotrophic (Bacteria which are able to grow at refrigeration temperatures, but grow optimally at temperatures above 20°C.) spoilage microflora on refrigerator-stored, vacuum-packaged beef. This study utilized a less selective glucose-blood-liver agar and Trypticase Soy Agar plating method (allowing for the widest range of bacterial growth) at an incubation temperature of 7°C.

Various psychrotrophic species on vacuum packaged beef stored at refrigeration temperatures were identified and quantified over a six-week period to determine alterations in the bacterial species or quantities of the bacteria. Five fresh beef cut samples (acquired and vacuum-packaged approximately 48 hours after slaughter) were utilized to determine the types and quantities of the various bacteria found in vacuum-packaged beef. A total of 1493 bacterial strains were identified as: *Brochothrix thermosphacta* (64), *Carnobacterium malcolmaticum* (27), *C. divergens* (79), *Lactobacillus algidus* (637), *Lactobacillus* spp. (4), *Lactococcus piscium* (270), *Leuconostoc gelidum* (375), *Acinetobacter* (3), *Aeromonas* (1), *Bacillus* (10), *Corynebacterium* (3), *Enterobacteriaceae* (1), *Pseudomonas* (13) and *Psychrobacter* (6). *L. gelidum, L. piscium* and *L. algidus* increased during the
first three weeks of storage from approximately $5 \times 10^3$ cfu/g to approximately
$1 \times 10^6$ cfu/g, and remained stable for the rest of the six-week study. *C.
maltaromaticum* was inconsistently detected, but when present increased to
approximately $5 \times 10^7$ cfu/g during the first three weeks of storage and
remained at that level for the last three weeks of the study. Vacuum or
modified atmosphere (CO$_2$) packaging (CO$_2$-MAP) influences the bacterial
species isolated from meat (Labadie, 1999). At low temperatures and with a
limited amount of oxygen, LAB comprise the predominant bacterial population
of CO$_2$-MAP packaged meat, at approximately $1 \times 10^7$ cfu/cm$^2$ (Gill and
Newton, 1978). There have been no studies directly comparing the specific
quantities of different species of *Lactobacillus*, *Leuconostoc* and
*Carnobacterium* on freshly packaged meat under CO$_2$-MAP conditions.
Nilsson *et al.* (1999) isolated $2 \times 10^4$ and $5 \times 10^7$ cfu/g LAB from cold-smoked
salmon at the time of purchase and after thirty-two days of incubation,
respectively.

**Example 5. Natural Occurrence of *Carnobacterium maltaromaticum* on
Meat, Fish and Cheese Products**

*Carnobacterium* species have been isolated from vacuum-packaged
meat, fish and French soft cheese (Ahn and Stiles, 1990a; Buchanan and
Klawitter, 1992b; Stoffels *et al.*, 1992; Pilet *et al.*, 1995; Milliere and Lefebvre,
1994a; Milliere *et al.*, 1994b), as summarized in Table 4. A study by Lewus *et
al.* (1991) identified two bacteriocin-producing strains of *C. maltaromaticum*
from different parts of meat from retail meat products. Other *C.
*maltaromaticum* strains have been isolated from fish, meat and cheese (Milliere *et al*., 1994b; Nissen *et al*., 1994; Pilet *et al*., 1995; Schillinger *et al*., 1993; Shaw and Harding, 1984). Leisner *et al*. (1994) found that eighteen of the 80 strains of bacteria originally isolated from vacuum-packed halibut, salmon or mackerel were lactic acid bacteria. Of these, 28% were identified as *C. maltaromaticum*. Sakala *et al*. (2002) conducted a study to investigate the psychrotrophic spoilage microflora on chill-stored vacuum-packaged beef and determined that out of a total of 1493 strains isolated from five fresh beef cut samples (each from a different meat shop), twenty-seven were identified as *C. maltaromaticum*. This bacterium was detected at 0, 1, 3, 5 and 6 weeks of storage at mean numbers of 2x10^3, 2x10^4, 2.5x10^6, 1x10^7 and 2.5x10^7 cfu/g, respectively, for two samples positive for *C. maltaromaticum* and, persisted at the level of approximately 5x10^7 cfu/g during the last three weeks of the six-week storage period.

The growth of *C. maltaromaticum* in fermented meat products has been noted by Montel (1999), who noted “At the end of the fermentation period, lactic acid bacteria are generally the dominant bacterial flora. The species *Lactobacillus curvatus*, *L. sakei*, *L. plantarum*, *L. viridescens*, *Carnobacterium divergens*, *C. maltaromaticum* and *Leuconostoc* are present naturally, but *Pediococcus* is only found when inoculated as a starter culture. Their count generally exceeds 10^6 cfu/g and remains at this level during the whole ripening period. *Carnobacterium* is present during the fermentation period, but disappears afterwards.”
Table 4. Isolation of *C. maltaromaticum* from food products.

<table>
<thead>
<tr>
<th>Food Category</th>
<th>Food Product</th>
<th><em>C. maltaromaticum</em> strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>Cultured striped bass, channel catfish and bullhead catfish</td>
<td></td>
<td>(Baya <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td></td>
<td>Cold-smoked freshwater fish</td>
<td></td>
<td>(Gonzalez-Rodriguez <em>et al.</em>, 2002)</td>
</tr>
<tr>
<td></td>
<td>Salmonid fish</td>
<td></td>
<td>(Hsu <em>et al.</em>, 1984)</td>
</tr>
<tr>
<td></td>
<td>Cold-smoked salmon</td>
<td></td>
<td>(Leroy <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td></td>
<td>Vacuum-packaged halibut, salmon or mackerel</td>
<td></td>
<td>(Lasner <em>et al.</em>, 1994)</td>
</tr>
<tr>
<td></td>
<td>Cold-smoked salmon</td>
<td>A9a, A9b, A9c, A9J, A10a, A10b, A10f, A10j, S1, S2, S3, S4 V1</td>
<td>(Paludan-Muller <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td></td>
<td>Fish</td>
<td>V1</td>
<td>(Pilet <em>et al.</em>, 1995)</td>
</tr>
<tr>
<td>Beef</td>
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<td>(Ahn and Stiles, 1990a)</td>
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<tr>
<td></td>
<td>Raw ground beef</td>
<td></td>
<td>(Buchanan and Kiawitter, 1992A)</td>
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<tr>
<td></td>
<td>Meat</td>
<td>GN, DX</td>
<td>(Lewus <em>et al.</em>, 1991)</td>
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<td></td>
<td>Vacuum-packaged beef</td>
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<td>(Sakala <em>et al.</em>, 2002)</td>
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<tr>
<td></td>
<td>Meat</td>
<td>LV 61</td>
<td>(Shaw and Harding, 1984)</td>
</tr>
<tr>
<td>Lamb</td>
<td>Modified atmosphere-packaged lamb</td>
<td></td>
<td>(Nissen <em>et al.</em>, 1994)</td>
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<tr>
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<td>(Bakarhat <em>et al.</em>, 2000)</td>
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<td></td>
<td>Poultry</td>
<td></td>
<td>(Collins <em>et al.</em>, 1987)</td>
</tr>
<tr>
<td>Dairy</td>
<td>Soft cheeses</td>
<td>CP5</td>
<td>(Milliere <em>et al.</em>, 1994B)</td>
</tr>
</tbody>
</table>

* = Strain given, if known.
Cold-smoked salmon (CSS) is an extremely perishable food product and is highly susceptible to contamination with *Listeria monocytogenes*. CSS spoilage is primarily due to microbial activity during refrigerated storage (Duffes, 1999a). For CSS, it has been estimated that immediately after packing, bacterial counts range from $1 \times 10^3$ to $1 \times 10^4$ cfu/g with a predominance of Gram-negative bacteria (64%) such as *Shewanella putrefaciens* and *Aeromonas* spp. LAB were found to be present (32%), with the majority being *Carnobacterium* spp. (Donald and Gibson, 1992; Huss et al., 1995). At 8°C, the level of bacterial flora increased to $1 \times 10^7$ – $1 \times 10^8$ cfu/g over three weeks, with a shift in relative bacterial populations such that LAB predominate (60%), mainly as *Carnobacterium* spp. (47%) and *Lactobacillus* spp. (13%). Paludan-Muller et al. (1998) reported a series of studies that evaluated the role of *C. maltaromaticum* in spoilage of vacuum- and modified-atmosphere-packed cold-smoked salmon stored at 5°C. A mixture of LAB and Gram-negative bacteria are usually found on spoiled CSS.

Initial numbers of bacteria were low with total psychrotrophic counts less than $5 \times 10^3$ cfu/g and, specifically, LAB counts of $10 - 1 \times 10^2$ cfu/g. In addition, it was determined (by sensory evaluation) that the shelf life of vacuum-packed cold-smoked salmon peaked at four weeks at 5°C. The microflora at four weeks was composed of LAB ($1 \times 10^6 - 1 \times 10^7$ cfu/g) with Gram negative microflora at varying levels ($1 \times 10^5 - 1 \times 10^7$ cfu/g).

Modified-atmosphere packaging reduced the growth of Gram-negative bacteria and selected specifically for LAB, although growth of LAB was below $3 \times 10^5$ cfu/g during five weeks of storage (Paludan-Muller et al., 1998). The
LAB microflora was dominated by *C. maltaromaticum*, accounting for 87% of the 255 LAB isolates characterized. The spoilage potential of *C. maltaromaticum* was further studied by inoculation of approximately $1 \times 10^6$ cfu *C. maltaromaticum* per gram in CSS stored at 5°C (Paludan-Muller et al., 1998). In vacuum packed salmon inoculated with *C. maltaromaticum* strains, LAB counts reached $1 \times 10^7$ cfu/g after only one week of storage and the level was above $1 \times 10^8$ cfu/g for the rest of the storage period. However, after four weeks of storage, the salmon was not rejected by a sensory taste panel, while the vacuum-packed control was rejected after four to five weeks. In inoculated modified atmosphere-packed salmon, the LAB counts reached final levels of $1 \times 10^6 - 1 \times 10^7$ cfu/g after two weeks, but the salmon was not sensory-rejected until four to five weeks of storage. It was concluded that the growth of *C. maltaromaticum* even at high numbers ($1 \times 10^7 - 1 \times 10^8$ cfu/g) for several weeks did not accelerate the spoilage process of packed cold-smoked salmon.

A bacterial study on the composition of the psychrotrophic and mesophilic microflora of French surface-mold-ripened soft cheeses made from raw cow’s milk found that *C. maltaromaticum* was the dominant bacteria at the end of ripening on five samples of Brie cheese (Milliere and Lefebvre, 1994a). *C. maltaromaticum* bacteria was also isolated from Coulommiers, Camember, Pon-î’Eveue and Munster cheeses. The number of *Camobacterium* colonies isolated from these cheeses ranged from $5 \times 10^5$ to $8 \times 10^8$ cfu/g in the various cheese samples. Milliere *et al.* (1994b) went on to characterize *C. maltaromaticum* strains isolated from five samples of Brie cheese. The pH
values of the cheeses were between 6.8 and 7.6 and no off-odors or organoleptic defects were noted. The *Carnobacterium* species were dominant in the cheese samples, at between $1 \times 10^8$ and $1 \times 10^9$ cfu/g. The results of DNA-DNA hybridizations indicated that 33 of the 36 isolates were of the *C. maltaromaticum* species, while the remaining three (all picked from the same sample) were *C. divergens*.

To summarize, *Carnobacterium* spp. are common components of the microflora on vacuum-packaged meat, poultry, fish and cheese products and in some cases, they can represent a predominant constituent population, reaching levels of $1\times10^8$ cfu/g or higher, on products such as smoked fish, chicken, beef and cheese, without causing detectable spoilage.

**Example 6. Production of *Carnobacterium maltaromaticum* Culture**

*C. maltaromaticum* strains are maintained in lyophilized form under vacuum at 4°C, or as frozen cultures in 20% (v/v) glycerol at −80°C. API® strip analysis (a kit for identification of bacteria to the species level) is conducted to ensure viability and strain purity will be confirmed by the absence of bacteriological contamination and/or by random amplified polymorphic DNA (RAPD) and microbiological analysis. Seven lyophilized vials are prepared for each strain (master seed). From a single vial of the master seed, 15 lyophilized vials are prepared under vacuum and stored at 4°C (secondary seed). From each vial of the secondary seed, enough frozen vials are prepared for one year's production requirements and stored at –
80°C. One out of every 10 vials undergoes microbiological testing to confirm strain purity and the absence of bacteriological contamination.

Seed and Mother Culture

The seed culture is prepared by transferring a loop full of frozen master seed or a vial of lyophilized culture to 10 ml APT (All Purpose Tween). The seed is then grown overnight. The mother culture is prepared from the seed culture by transferring the seed culture (grown overnight) into 6L of APT medium and incubated again overnight.

Fermentation and Concentration

The mother culture is aseptically transferred to the production fermentor, which contains a growth medium and was maintained at 25°C. The fermentation is monitored spectrophotometrically (650 nm) and by plating onto APT agar until a cell density of approximately $10^9$ cfu is reached. The fermented growth medium (containing *C. maltaromaticum*) is then harvested and freeze-dried.

Lyophilization

The lyophilized material is scraped from the trays, ground and milled, and placed into polyethylene bags and double-bagged prior to refrigeration (4-8°C).
Example 7. Microbiological Analysis

The lyophilized material is analyzed microbiologically for total lactic acid bacteria, non lactic acid bacteria, yeast, molds, total coliforms, 

\textit{Staphylococcus aureus, Escherichia coli and Salmonella spp.} (Table 5).

<table>
<thead>
<tr>
<th>Active Ingredients</th>
<th>Carnobacterium maltaromaticum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excipients</td>
<td>Maltodextrin</td>
</tr>
<tr>
<td>Shelf Life</td>
<td>&gt; one year</td>
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<tr>
<td>Storage Conditions</td>
<td>Room Temperature (22°C)</td>
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<table>
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<tr>
<th>Physical Aspects</th>
<th>Specifications</th>
<th>Method</th>
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<td>Pass</td>
<td>Visual inspection APT plate and comparison to standard plate photograph and description</td>
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<tr>
<td>Microbiological Specifications</td>
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<td>Lactic Acid Bacteria</td>
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<td>Non Lactic acid bacteria</td>
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<td>Yeasts</td>
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<td>Molds</td>
<td>&lt;100/g</td>
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<td>Anaerobic Spore Forming Bacteria</td>
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<td>A.P.H.A./USP</td>
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<td>Absent per 50g</td>
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<td>Total coli forms</td>
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<tr>
<td>\textit{Salmonella spp.}</td>
<td>Absent per g</td>
<td>A.P.H.A./USP</td>
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</table>

A.P.H.A. = American Public Health Association; USP = U.S. Pharmacopoeia
Example 8. Reconstitution of Starter Culture

The standardized viable cell blend will be packaged into plastic foil film packages, flushed with nitrogen and package weight will be customized to the finished product application such that between $10^3$ and $10^4$ viable *C. maltaaromaticum* cells per gram of finished product will be achieved. The packages will then be stored at ambient temperature.

The packaged standardized viable cell blend will be analyzed microbiologically for identity, total lactic acid bacteria, non lactic acid bacteria, yeast, molds, total coliforms, *Staphylococcus aureus, Escherichia coli* and *Salmonella* spp. (Table 5).

A dose of reconstituted *C. maltaaromaticum*, containing one or more of strains CB1, CB2, CB3, LV17, UAL26, ATCC 35586 or ATCC 43225 (approximately $1 \times 10^3$ to $1 \times 10^4$ cfu/g of finished product) may also be added directly to ground meat prior to ingredient mixing and further grinding and stuffing into casings. The sausages would then be rapid frozen at $-50^\circ$C until frozen in the center. These sausages would then be wrapped airtight in plastic wrap and kept frozen until thawed for retail sale.

Example 9. Growth Characteristics of *Carnobacterium maltaaromaticum* on Vacuum-Packaged Wieners

A laboratory-scale study was designed to investigate the growth characteristics of *C. maltaaromaticum* on vacuum-packaged wiener inoculated with *C. maltaaromaticum* under conditions comparable to commercial production. In addition, the effects on sensory properties, such as aroma and
flavor profile, were investigated. The methods and results of this study were as follows:

Two strains of *C. maltaromaticum* were chosen for study: LV17 (a synonym for UAL 8) was originally isolated from vacuum-packaged, refrigerated, fresh pork and described by Shaw and Harding (1984) and, strain UAL 26, which was isolated from vacuum-packaged beef (Stiles and Holzapfel, 1997). The inoculum was prepared by adding washed bacterial cells to sterile 0.85% saline to provide an inoculum level of 2.5x10⁶ cfu/ml. Individual wiener was dipped into the inoculum suspension for one minute, drain dried and vacuum-packaged in groups of five wiener per bag (high barrier, low O₂ transmission, VP bags). As a control, wiener was dipped in 0.85% sterile saline without bacterial inoculum. Treated and control samples were then placed into refrigerated (4°C) storage for up to 12 weeks. Sampling of the wiener for microbiological analyses and sensory evaluation was performed on day zero and after 2, 4, 6, 7, 8, 10 and 12 weeks of storage.

Samples were prepared for microbial analysis by cutting 1.8 cm length piece of wiener (equivalent to a surface area of 10 cm²), placing it in a sterile tissue homogenizer bag and homogenizing. Bacterial counts were conducted by standard dilution and plating techniques and included: 1) Total aerobic plate count on Plate Count Agar incubated aerobically at 25°C, 48 hours; 2) Lactic acid bacteria on APT agar incubated anaerobically at 25°C, 48 hours; 3) *Enterobacteriaceae* on Violet Red Bile Agar with one percent added glucose incubated at 35°C, 18 hours. Concentrations of bacteria were reported as cfu per cm² of product (cfu/cm²).
The wiener's to be evaluated for sensory characteristics were cooked in "just boiled" water and allowed to stand for five minutes (internal wiener temperature approximately 83°C). Wieners were cut into pieces, placed in coded foil-covered jars and heated for 15 min in a 94°C oven just prior to evaluation. Sensory evaluation was conducted by a group of nine panelists trained over a three-month period. Samples were evaluated for overall aroma intensity, meat flavor intensity, seasoned flavor, smoke intensity, sourness/acidity, off-flavor and overall acceptability using a 15 cm unstructured line scale with 0 = very bland and 15 = very strong. Between samples, palates were cleansed with crackers and a 1:1 dilution of 7-Up®.

This study reported that sample wiener's that had been inoculated with C. maltaromaticum strains LV17 or UAL 26 reached maximum anaerobic lactic acid bacteria (LAB) counts of 2.75x10⁶ and 1.2x10⁶ cfu/cm² after seven or eight weeks of cold storage, respectively. C. maltaromaticum grew at a slow rate on vacuum-packaged wiener's and growth was accompanied by a relatively small decrease in surface pH during storage. LV17 varied from pH 6.2 at Week 0 to pH 6.1 at Week 10, while UAL 26 varied from an initial pH 6.2 to approximately 5.9 during Weeks 6-8 and Week 12).

It was concluded that in comparison with other lactic acid bacteria, such as L. gelidum, C. maltaromaticum is a slow-growing species when inoculated onto refrigerated (4°C), vacuum packaged wiener's. The levels of C. maltaromaticum reached a maximum of 5x10⁷ cfu/cm² after 12 weeks of cold storage. Based on sensory evaluations using a trained nine-member panel over the 12-week storage period, there were no significant adverse
effects on aroma, off-flavors, sour intensity, or overall acceptability resulting from inoculation with *C. maltaromaticum*.

**Example 10. Growth Characteristics of *Carnobacterium maltaromaticum***

When Inoculated into Sausages

*C. maltaromaticum* CB1 was added to pork in three trials as an inoculant during the production of sausages. Odor intensity and freshness attributes for the sausages (evaluated as both raw and cooked) were treated on a 21-point line scale. Inoculum levels ranged from $1 \times 10^3$ to $1 \times 10^5$ cfu/g of meat. Bacteriological analyses were conducted on Days 0, 5, 10, 15 and 20 to assess the growth of *C. maltaromaticum* and bacteriocin production.

Chilled pork shoulder and pork fat were weighed, coarse ground and divided into four batches to which 2.76% water and 1.8% of seasoning were added. Test product was inoculated with *C. maltaromaticum* up to $10^5$ cfu/g. The ground meat and ingredients were mixed, ground fine and stuffed into collagen casing (UniPac, Edmonton). The stuffed casing was cut into links 3.5-3.75 inches, to give sausages that were approximately 20.4 g/sausage. The individual sausage links were rapid frozen at $-50^\circ$C for approximately 35 minutes. The frozen sausages were packaged on Styrofoam trays (approximately 10 oz per pack) and airtight wrapped and sealed in plastic wrap. The samples were thawed and stored at 4°C prior to bacterial sampling.

Usual manufacturing procedures for meats include the procedure of “flash freezing” meat for shipment, with subsequent thawing for sale or further processing.
Bacteriological sampling was done on samples that had been thawed and stored at 4°C for 0, 5, 10, 15 and 20 days. Duplicate 10 g samples were placed into sterile stomacher bags (VWR International) and blended with 90 ml of sterile 0.1% peptone water. Appropriate serial dilutions in 0.1% peptone water were streaked onto prepoured APT agar and MRS agar plates and incubated at 30°C for 48 h. Duplicate counts (cfu/g of meat) were recorded for each sample after the incubation period.

Over a 20 day period, total anaerobic bacterial counts on MRS agar increased from $10^3 - 10^5$ cfu/g, up to $10^9$ cfu/g of product. The growth of background microflora in the test product did not differ from that associated with un-inoculated samples, as evidenced by the growth on APT agar. This indicates that the inoculation of the sausage meat with C. maltaromaticum did not increase the overall incidence of bacterial growth in the sausage. The microbiology assays indicated that the total number of bacteria growing on APT and MRS agar was similar on the uninoculated control and the test products. Therefore, added C. maltaromaticum cultures did not increase the number of bacteria found on the test products, nor cause the meat to spoil faster than the control.

Bacteriocin production in the sausage samples was tested at Days 0, 5, 10, 15 and 20 by direct and indirect assays and was detected, indicative of bacteriocin production by the added C. maltaromaticum. Suppression of the indicator organism, L. monocytogenes, was noted by Day 10 via the indirect assay [portions of the sausage were heat-treated (to kill the producer organism) and directly embedded into APT agar inoculated with L.}
monocytogenes CDC 7762 (serotype 4b)], with this suppression maintained through Day 20 of the assay. The direct assay for bacteriocin production (heat-treated supernatant of a homogenized sausage was directly added to APT agar plates overlaid with the L. monocytogenes indicator organism) in the sausages inoculated with C. maltaromaticum indicated that bacteriocin production occurred by Day 15 of sausage storage at 4°C and continued through Day 20 of storage.

Example 11. Use of the Addition of Carnobacterium maltaromaticum in Ready-To-Eat (RTE) and Fresh Comminuted, Processed Meat Products

RTE meat and fresh comminuted, processed meat products require preservation techniques that inhibit the growth of potentially pathogenic bacteria. A deadly Listeria monocytogenes outbreak recently spread across the northeast US, resulting in the U.S. Food and Drug Administration and the USDA's Food Safety Inspection Service (FSIS) issuing a health advisory in September, 2003 (Morbidity and Mortality Weekly Report, 2003).

It is proposed that C. maltaromaticum will be added to vacuum- or modified atmosphere packaged ready-to-eat (RTE) meat products and fresh comminuted, processed meat products as a means of mitigating the effects of contamination caused by human pathogenic bacteria, such as L. monocytogenes. During packaging of RTE meat products, such as wiener, it is proposed that a dose (approximately 1.5 ml, or 5x10^6 cfu) of reconstituted C. maltaromaticum will be applied to each 454 g (1 pound) package.
An aliquot of reconstituted *C. maltaromaticum* (to deliver approximately $1 \times 10^3$ to $1 \times 10^4$ cfu/g) would also be added to fresh comminuted, processed meat products prior to ingredient mixing and further grinding and stuffing into casings to produce fresh comminuted, processed meat products. The fresh comminuted, processed meat products would be rapid frozen at $-50^\circ$C until frozen in the center, then wrapped airtight in plastic wrap and stored frozen.

The inoculation ranges for RTE meat products and fresh comminuted, processed meat products would be approximately $1 \times 10^3$ to $1 \times 10^4$ viable *C. maltaromaticum* cells (cfu) per gram of product.

**Example 12.**

Examination of the potential of a bacteriocin-producing strain of *C. maltaromaticum* (strain SF668) isolated from commercial, vacuum-packaged cold-smoked salmon (CSS) to inhibit *L. monocytogenes* growth on CSS by Duffes *et al.* (2000), found that *C. maltaromaticum* SF668 was able to grow from $1 \times 10^5$ to $3 \times 10^7$ cfu/ml in 21 days on vacuum-packaged, cold-smoked salmon stored at 4°C (Table 8). *L. monocytogenes* co-cultured with *C. maltaromaticum* grew from $1 \times 10^3$ cfu/ml to $3.5 \times 10^3$ cfu/ml after three weeks at 4°C. This co-culturing of *C. maltaromaticum* with *L. monocytogenes* resulted in a significant bacteriostatic effect on *L. monocytogenes* growth on cold-smoked salmon (*L. monocytogenes* growth in the absence of *C. maltaromaticum* reached approximately $5 \times 10^4$ cfu/ml).

When screened against 21 strains of *Listeria* spp., distinct zones of inhibition formed by *C. maltaromaticum* LK5 were evident with seventeen of
the strains (Buchanan and Klawitter, 1992a). C. *maltaromaticum* LK5 was found to lack hydrogen peroxide formation, while producing a bacteriocin. The ability of *C. maltaromaticum* LK5 to inhibit *Listeria* spp. was temperature-dependent (determined at both 5°C and 19°C), with a substantially greater suppression of *L. monocytogenes* co-cultured with *C. maltaromaticum* LK5 occurring at 5°C than at 19°C. *C. maltaromaticum* LK5 was shown to be capable of substantially faster growth than *L. monocytogenes* at refrigeration temperatures, whereas the growth rates were approximately the same at 19°C. At 19°C, suppression of *L. monocytogenes* (the inoculation concentration was kept constant at 1×10^3 cfu/ml) was dependent on inoculum ratio, with only LK5:*L. monocytogenes* ratios of ≥ 1:1 producing a substantial degree of inhibition. At 5°C, an increased level of anti-listerial activity was observed with the higher inoculum ratios during the early stages of the incubation, but by approximately 300 h of incubation, LK5 inoculum size had no effect on inhibitory activity; the degree of suppression was equivalent for ratios ranging from 0.01:1 to 1000:1 (10:1×10^3 and 1×10^6:1×10^3 cfu/ml, respectively).

The *Carnobacterium* isolate was extremely competitive at refrigeration temperatures, indicating that even small inocula could be used to control *L. monocytogenes* in refrigerated foods. This study confirms the report by Schillinger and Holzapfel (1990), who reported that out of thirteen strains of *C. maltaromaticum*, ten significantly inhibited the growth of *L. monocytogenes* DSM 20600, as determined by the agar spot test.
Example 13. 7.2. Background Exposure to *Carnobacterium maltaromaticum*

Studies have shown that lactic acid bacteria in general and, specifically *C. maltaromaticum*, are found in retail food products (modified-atmosphere packaging and refrigeration preferentially selects for anaerobic *Carnobacterium* spp.) within the expiration dates (Milliere and Lefebvre, 1994a; Kelly *et al.*, 1996; Schobitz *et al.*, 1999; Amequita and Brashears, 2002; Sakala *et al.*, 2002). Therefore, for an accurate assessment of the numbers of *C. maltaromaticum* that could maximally be consumed, one must take into account any theoretical amount of *C. maltaromaticum* that may already be present on the proposed foods.

An extensive search of the literature resulted in two references specifically analyzing the quantity of *C. maltaromaticum* found on commercially available foods. Sakala *et al.* (2002) determined that two beef samples contained *C. maltaromaticum*. It was detected at 0, 1, 3, 5 and 6 weeks of storage (vacuum-packaged and stored at 2°C) at mean numbers of 2x10^3, 2x10^4, 2.5x10^6, 1x10^7 and 2.5x10^7 cfu/g of meat, respectively. Montel (2000) found that at the end of the fermentation period of sausages, lactic acid bacteria were generally the dominant bacterial flora, with *C. maltaromaticum* naturally present during the fermentation period at levels of approximately 5x10^7 cfu/g of sausage, but disappeared afterwards. Sterile cold smoked salmon inoculated with *C. maltaromaticum* at 10^4-10^5 cfu/g of salmon was found to have final counts ranging between 5x10^7 to 10^9 cfu/g, after two to three weeks of storage at 6°C (Stohr *et al.*, 2001). Nadon *et al*
(2001) showed that LAB (which included carnobacteria) increased from an initial 100 cfu/cm² to an average level of 1×10⁸ cfu/cm² for the first six weeks of storage in vacuum-packaged or carbon dioxide-controlled atmosphere-packaged (CO₂-CAP) treated pork and maintained that level of LAB for the remainder of the thirteen-week study. In the CO₂-CAP pork samples, there was no significant increase in LAB until the 11th week of storage, with a maximum level of LAB at 3.2×10⁵ cfu/cm². Nadon et al. (2001) demonstrated that carnobacteria dominate the LAB microflora during storage at −1.5°C in the absence of oxygen.

Example 14.

Strains of C. maltaromaticum produce several different carnobacteriocins (Quadri et al., 1994), which have been identified as heat-resistant peptides, stable over a wide pH range and capable of acting as bactericides (Jack et al., 1996). A bacteriocin from C. maltaromaticum L103 was recently tested in a study to determine the ability of this bacteriocin to control the growth of Listeria monocytogenes in vacuum-packaged meat (Schobitz et al., 1999). Steaks from beef semitendinosus muscle were inoculated with the partially purified bacteriocin at a concentration of 100 AU/ml (AU/ml = Arbitrary units of activity). L. monocytogenes was added to the meat as an indicator strain at a final concentration of 1×10³ cfu/cm². After assuring good contact with the meat, the steaks were vacuum-packaged and stored at 4°C for 21 days. Non-inoculated controls and meat containing only the indicator strain were included for each sampling date. Duplicate steaks
were sampled at time 0 and every seven days for growth of *L.*
monocytogenes and LAB growth. After seven days of storage at 4°C, a
significant decrease in the *L.* monocytogenes counts were observed, from an
initial count of $2 \times 10^3$ cfu/cm$^2$ to 4 cfu/cm$^2$, with complete inhibition of the
pathogen (<1 cfu/cm$^2$) on day 14 of storage. The LAB multiplied on the
vacuum-packaged meats, reaching counts of $1 \times 10^7$ cfu/cm$^2$ after 14 days,
with a starting level of $1.6 \times 10^2$ cfu/cm$^2$. Color and odor of the meat remained
acceptable during the 14 days of storage. The results of this study indicate
that the bacteriocin from *C. maltaromaticum* was able to inhibit *L.*
monocytogenes on vacuum-packaged meat, while maintaining edible
characteristics of the meat for up to 14 days (Schobitz et al., 1999). *C.*
maltaromaticum LV61 produces a bacteriocin that was active against *C.*
maltaromaticum 2762 and *L.* monocytogenes (strains R2, Lud 1033, Br124 6,
Lud 905 and T), but was inactivated by pronase E, proteinase K and trypsin
(Pilet et al., 1995). Other research has indicated that the purified bacteriocin
from *C. maltaromaticum* LV61 inhibits several strains of *Carnobacterium* and
Enterococcus, but does not inhibit several strains of *Listeria* (Holck et al.,
1994). It was therefore concluded that *C. maltaromaticum* LV61 produces, in
addition to piscicolin 61, another factor involved in the anti-listerial activity.

**Example 15.**

*Carnobacterium* spp. are psychrotrophic, grow at elevated pH values of
8 to 9 and ferment inulin. In culture conditions in the presence of inulin, *C.*
maltaromaticum forms yellowish to pinkish colonies with a metallic bronze
sheen, a yellow color change of the medium and a clearance of precipitate.

Various *C. maltaromaticum* strains have been shown to produce bacteriocins, proteinaceous compounds with the ability to inhibit the growth of other *Carnobacterium, Lactobacillus and Listeria* species.

It is proposed that *C. maltaromaticum* will be inoculated into various ready-to-eat and fresh comminuted, processed meat products at a range of $1 \times 10^3$ to $1 \times 10^4$ cfu/g for enhanced preservation and decreased pathogenic bacterial growth. Based on these inoculation ranges, as well as the theoretical assumption that the bacteria will grow over an extended period of time in storage, the mean *per capita* consumption estimate of *C. maltaromaticum* as an addition to the selected RTE foods would be $4.3 \times 10^9$ cfu/day or $7.2 \times 10^7$ cfu/kg/day for a 60 kg person.

The suppression of the pathogen, *L. monocytogenes*, by *C. maltaromaticum* occurs when assessed in salmon, chicken, pork, beef and other commercial meat products. Co-culturing of *C. maltaromaticum* with *L. monocytogenes* results in log reductions in *L. monocytogenes* growth. Compared with *L. monocytogenes* growth at low temperatures, the suppressive effect of *C. maltaromaticum* on *L. monocytogenes* growth is enhanced at low temperatures. *L. monocytogenes* suppression may be mediated through production of lactic acid, competition for nutrients, as well as the production of bacteriocins. Bacteriocin production is correlated with increased suppression of *L. monocytogenes* growth. The activity of bacteriocins produced by *C. maltaromaticum* is rapidly degraded when
subjected to simulated gastric acid or proteolytic enzymes, an indication of a non-toxic and non-allergenic protein.

*C. maltaromaticum* increases the storage time of RTE and vacuum-packaged meat products, while decreasing the growth of pathogenic bacteria. *C. maltaromaticum* growth has been found to be self-limiting, with levels of *C. maltaromaticum* on RTE meat products and vacuum-packaged, cold-smoked salmon stabilizing at approximately $1 \times 10^9$ cfu/g. Addition of *C. maltaromaticum* to the proposed RTE and fresh comminuted, processed meat products at a level between $1 \times 10^3$ and $1 \times 10^4$ cfu/g, would not significantly increase the overall human consumption of LAB from these food products (theoretical natural consumption determined at $4.3 \times 10^9$ cfu/day). *C. maltaromaticum* has been shown to be self-limiting in its growth; the growth of *C. maltaromaticum* will plateau between approximately $1 \times 10^6$ and $1 \times 10^9$ cfu/g of meat. This has been speculated to be due to the release of specific bacteriocins that limit a higher bacterial density.

**Example 16. Isolation and screening procedures for lactic acid bacteria (LAB) from meat products**

Samples of refrigerated or frozen, raw and ready-to-eat processed meats were:

A) Samples purchased in the retail marketplace; taken to the laboratory for microbiological analysis

B) Frozen samples from a pilot-plant manufacture of raw pork sausages, thawed and subjected to microbiological analysis
C) Samples of ready-to-eat processed meats purchased in the retail marketplace and stored at 4°C in the laboratory until their "best before date" and subjected to microbiological analysis.

Triplicate 10 g samples were aseptically excised from each package, diluted in 90 mL of sterile 0.1% peptone water and homogenized in a Stomacher Lab-Blender 400 (Seward, England) for 2 minutes. Serial dilutions of the homogenate were prepared in 0.1% peptone water and plated onto prepoured plates of APT (All Purpose Tween; Difco) agar (1.5%). Plates were incubated anaerobically (A, B) and aerobically (C) for 48 hours at 25°C (A, B) or 15°C (C). Randomly selected, single colonies were picked with a sterile toothpick from the APT plates and streaked onto onto the required number of sets (one set for each indicator strain used for screening) of prepoured APT plates. The plates were incubated anaerobically (A) and aerobically (B, C) for 24 hours at 25°C. Each set of plates was overlayered with a lawn of L. monocytogenes indicator strain or a universal indicator strain Carnobacterium divergens LV13, seeded at 1% in soft APT agar (0.75%). The overlayered plates were incubated at 37°C for 24 hours. Zones of inhibition, observed as areas of clearing in the overlayer were recorded as organisms producing antibacterial substances. The organisms exhibiting this activity were screened for susceptibility to pronase E (Sigma) and for heat sensitivity. Those that were sensitive to pronase and stable at 60°C for 30 minutes were selected for further characterization.
Example 17.

Many bacteria produce antibacterial peptides or proteins (e.g., bacteriocins) that are generally active against other bacteria, typically closely related. An exemplary list of bacteria and their bacteriocins are shown in Table 6.

Table 6

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacteriocin</th>
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<td>Our lab collection of LAB</td>
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<tr>
<td>1. <em>Carnobacterium maltaromaticum</em> CB1</td>
<td>carnobacteriocin BM1, piscicolin 126 +</td>
</tr>
<tr>
<td>2. <em>C. maltaromaticum</em> CB2</td>
<td>carnobacteriocin BM1, piscicolin 126</td>
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<td>3. <em>C. maltaromaticum</em> CB3</td>
<td>carnobacteriocin BM1, piscicolin 126</td>
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<td>4. <em>C. maltaromaticum</em> UAL26</td>
<td>piscicolin 126</td>
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<tr>
<td>5. <em>C. maltaromaticum</em> LV17</td>
<td>carnobacteriocin A, BM1 and B2</td>
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<td>6. <em>C. maltaromaticum</em> UAL26/8A</td>
<td>piscicolin 126, carnobacteriocin A</td>
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<td>7. <em>Carnobacterium diversgens</em> LV13</td>
<td>divergicin A</td>
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<td>8. <em>Leuconostoc gelidum</em> UAL187</td>
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<td>9. <em>Lactobacillus sakei</em> UAL185</td>
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<td>10. <em>Leuconostoc</em> spp. UAL280</td>
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<td>Non-LAB inhibiting <em>Listeria</em> spp.</td>
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<tr>
<td>11. <em>Brochothrix campestris</em> ATCC43754</td>
<td>brochocin C</td>
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<td>12. <em>Staphylococcus aureus</em> A53</td>
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<td>13. <em>Brevibacterium linens</em> ATCC9175</td>
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<td>14. <em>B. linens</em> OC2</td>
<td>linenscin OC2</td>
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<td>15. <em>Bifidobacterium bifidum</em> NCFB1454</td>
<td>bifidocin B</td>
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<td>Meat applied LAB inhibiting Listeria</td>
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<td>16. <em>C. maltaromaticum</em> LV61</td>
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<td>17. <em>C. maltaromaticum</em> V1</td>
<td>carnobacteriocin BM1, piscicolin 126</td>
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<td>18. <em>C. maltaromaticum</em> CP5</td>
<td>carnobacteriocin BM1 and B2</td>
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<td>19. <em>C. maltaromaticum</em> JG126</td>
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<td>20. <em>Carnobacterium</em> spp. 377</td>
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<td>21. <em>C. maltaromaticum</em> UI49</td>
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<td>22. <em>C. diversgens</em> 750</td>
<td>divergicin 750</td>
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<td>23. <em>Pediococcus acidilactici</em> PAC1.0</td>
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</tr>
<tr>
<td>28. <em>P. acidilactici</em> M</td>
<td>pediocin PA-1</td>
</tr>
<tr>
<td>29. <em>P. pentosaceus</em> Z102</td>
<td>pediocin PA-1</td>
</tr>
</tbody>
</table>
30. *Lactobacillus plantarum* WHE92  pediocin PA-1
31. *L. plantarum* ALC01  pediocin PA-1
32. *Lactobacillus sakei* Lb706  sakacin A
33. *Lb. sakei* CTC494  sakacin A
34. *Lactobacillus curvatus* LTH1174  sakacin A
35. *Lb. sakei* LTH673  sakacin P
36. *Lb. sakei* 874  sakacin P
37. *Lactobacillus bavaricus* Mi401  sakacin P
38. *Lb. sakei* MN  bavarcin MN
39. *Enterococcus faecium* CTC492  enterocin A and B
40. *E. faecium* T136  enterocin A and B
41. *E. faecium* WHE81  enterocin A and B
42. *E. faecium* BFE900  enterocin A and B
43. *E. faecium* L50  enterocin L50A and L50B, P, Q
44. *E. faecium* DPC1146  enterocin A
45. *E. faecium* EK13  enterocin A and P
46. *E. faecium* P13  enterocin P
47. *E. faecium* AA13  enterocin P
48. *E. faecium* G16  enterocin P
49. *E. faecium* JCM5804T  enterocin A, B, P
50. *Enterococcus casseliflavus* M416K1  enterocin 416K1
51. *Leuconostoc carnosum* 4010  leucocin A and C
52. *Lb. plantarum* UG1  plantaricin UG1
53. *E. faecium* CRL35  enterocin CRL35
54. *Lactobacillus casei* CRL705  lactocin CRL705
55. *Lb. sakei* CTC494  sakacin K
56. *L. carnosum*  leucocin F10
57. *L. carnosum*  leucocin B-Ta11a
58. *Lactobacillus brevis* VB286  brevicin 286
59. *Lb. plantarum* CTC305  unknown
60. *Lb. plantarum* CTC306  unknown
61. *Lb. sakei* CTC372  unknown

**LAB inhibiting Listeria**

62. *C. maltaromaticum* CS526  unknown
63. *Streptococcus thermophilus* Sf13  thermophilin 13
64. *E. faecalis* EJ97  enterocin EJ97
65. *E. faecalis* BFE1071  enterocin 1071
66. *E. faecalis* FAIR-E309  enterocin 1071
67. *E. faecalis* Y1717  bacteriocin 31
68. *E. faecalis* LMG2333  enterolysin A
69. *E. faecalis* DPC5280  enterolysin A
70. *E. faecalis* S-48  enterocin AS-48
71. *E. faecalis* INIA4  enterocin AS-48
72. *Lb. plantarum* ALC01  pediocin PA-1
73. *Lb. sake* 2512  sakacin G
74. *Lb. plantarum* 423  plantaricin 423
75. *Enterococcus mundtii* ATO6  mundticin
76. *E. mundtii* NFR17393  mundticin KS
77. *Lactobacillus buchneri*  buchnericin-LB
78. *L. lactis* MMFII  lactococcin MMFII
79. *L. lactis* UL720  diacetin B
80. *Enterococcus gallinarum* 012  enterocin 012

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81. Lb. plantarum  
82. Leuconostoc mesenteroides FR52  
83. L. mesenteroides Y105  

Lantibiotics inhibiting Listeria  
84. L. lactis  
85. L. lactis  
86. L. lactis 61-14  
87. L. lactis DPC3147  

Other bacteriocin producing bacteria  
88. L. lactis  
89. L. lactis LMG280  
90. L. lactis IPLA972  
91. L. lactis DPC5552  
92. Lactobacillus BGMN1-5  
93. Lactobacillus johnsonii/VPI11088  
94. Lactobacillus acidophilus M46  
95. Lb. acidophilus N2  
96. Lactobacillus gasseri LA39  
97. Lactobacillus salivarius UCC118  
98. L. plantarum C11  
99. L. plantarum NC8  
100. Propionibacterium jensenii DF1  
101. Escherichia coli  
102. E. coli  
103. E. coli  
104. Staphylococcus epidermis  
105. Bacillus subtilis 168  
106. Lb. gasseri  
107. Klebsiella pneumoniae  
108. Clostridium tyrobutyricum ADRIAT932  
109. Clostridium beijerinckii ATCC25752  
110. Lactobacillus amylovorus DCE471  
111. Lb. plantarum SA6  
112. Lb. sakei L45  

The following bacteriocins are called microcins produced by gram-negative bacteria:

1. Klebsiella pneumoniae RYC492  

2. E. coli  

3. E. coli  

4. E. coli  

5. E. coli  

6. E. coli  

The following bacteriocins are called microcins produced by gram-negative bacteria:

1. Microcin E492 (same as 107)  
2. Microcin V (same as 101, colicin is "old" name)  
3. Microcin Y101 (same as 102)  
4. Microcin H47  
5. Microcin L  
6. Microcin 24
11. References


American Type Culture Collection (ATCC): Collections and Repository (2004a) - Biosafety Levels.


Biochemical and serological characterization of *Carnobacterium* spp. isolated from farmed and natural populations of striped bass and catfish. *Applied and Environmental Microbiology* 57:3114-3120.


Numbers and types of microorganisms in vacuum-packed cold-smoked freshwater fish at the retail level. International Journal of Food Microbiology 77:161-168.


U. S. Food and Drug Administration (site visited on 11/10/2003). Processing Parameters Needed to Control Pathogens in Cold-Smoked Fish. Potential
Hazards in Cold-Smoked Fish: \textit{Listeria monocytogenes}.


IN THE CLAIMS:

We claim:

1. A method for treating food to inhibit the growth of at least one deleterious microorganism comprising preparing a composition comprising at least one beneficial microorganism, said microorganism is at least one Carnobacterium selected from the group consisting of *Carnobacterium maltaromaticum* strain CB1 (ATCC Accession No. PTA-5313), *Carnobacterium maltaromaticum* strain CB2 (ATCC Accession No. 5314), and *Carnobacterium maltaromaticum* strain CB3 (ATCC Accession No. 5315); or at least one bacteriocin produced by one or more bacteria selected from the group consisting of *Carnobacterium maltaromaticum* strain CB1 (ATCC Accession No. PTA-5313), *Carnobacterium maltaromaticum* strain CB2 (ATCC Accession No. 5314), and *Carnobacterium maltaromaticum* strain CB3 (ATCC Accession No. 5315); or combinations thereof; and treating the food with the composition.

2. The method of claim 1 wherein the at least one deleterious microorganisms comprises a spoilage or pathogenic bacteria.

3. The method of claim 2 wherein the pathogenic bacteria are one or more bacteria selected from the group consisting of Listeria species.

4. The method of claim 2 wherein the spoilage bacteria are susceptible to one or more bacteriocins produced by lactic acid bacteria.

5. The method of claim 4 wherein the spoilage bacteria has a known spoilage rate.

6. The method of claim 5 wherein the known spoilage rate is used to predict the shelf-life of the food product.

7. The method of claim 1 further comprising treating the food with a fermentate comprising a bacteriocin, said fermentate being obtained from one or more of said Carnobacteria.

8. A method of treating a food product against Listeria comprising contacting the food product with a composition comprising one or more bacteria cultures selected from the group consisting of *Carnobacterium maltaromaticum* strain CB1 (ATCC Accession No. PTA-5313), *Carnobacterium maltaromaticum* strain CB2 (ATCC
Accession No. PTA-5314), and Carnobacterium maltaromaticum strain CB3 (ATCC Accession No. PTA-5315).

9. A method of preparing processed meats comprising contacting the meat with an effective amount of a Carnobacterium maltaromaticum strain selected from the group consisting of Carnobacterium maltaromaticum strain CBI (ATCC Accession No. PTA-5313), Carnobacterium maltaromaticum strain CB2 (ATCC Accession No. PTA-5314), and Carnobacterium maltaromaticum strain CB3 (ATCC Accession No. PTA-5315).

10. A method of preserving foods or beverages comprising adding to the food or beverage an effective amount of a bacteriocin composition produced by Carnobacterium maltaromaticum strain CBI (ATCC Accession No. PTA-5313).

11. A method of preserving foods or beverages comprising adding to the food or beverage an effective amount of a bacteriocin composition produced by Carnobacterium maltaromaticum strain CB2 (ATCC Accession No. PTA-5314).

12. A method of preserving foods or beverages comprising adding to the food or beverage an effective amount of a bacteriocin composition produced by Carnobacterium maltaromaticum strain CB3 (ATCC Accession No. PTA-5315).

13. The method of claim 1 or claim 10 further comprising treating the food by adding to the food the strain in combination with a fermentate.


15. Carnobacterium maltaromaticum strain CB2 (ATCC Accession No. PTA-5314).

Growth of *Listeria monocytogenes* cocktail in the presence of *C. piscicola* CB1 on Palcam in inoculated pork samples stored at 5 C for 20 days

**FIGURE 1**

- Listeria Control
- Listeria with CB1 (10^3/g)
- Listeria with CB1 (10^4/g)

Log (CFU/g) vs Time (Days)
FIGURE 3

Growth of Listeria monocytogenes spp. on wiener in the presence of Carnobacterium piscicola CB1 and CB3

log(cfu/cm²)

Days

5 4 3 2 1 0

0 7 14 21 28 35 42 49 56 63 70 77 84
FIGURE 4

Rep 3: Growth of Listeria monocytogenes spp. on wiener in the presence of Carnobacterium piscicola CB1 & CB3
Growth of *Listeria monocytogenes* cocktail in the presence of *C. piscicola* CB1 on Palcam in inoculated pork samples stored at 5°C for 20 days.