Short Communication: *Carnobacterium maltaromaticum*: The Only *Carnobacterium* Species in French Ripened Soft Cheeses as Revealed by Polymerase Chain Reaction Detection

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ABSTRACT

The PCR technique using Cb1-Cb2R and species-specific primers was applied to various French soft flowered or washed rind cheeses to identify *Carnobacterium* species. Thirty cheeses made from cow's, ewe's, or goat's milk (raw or pasteurized), of which 20 were Appellation d'Origine Contrôlée, were analyzed in the autumn and spring. The results revealed that, irrespective of the season, the *Carnobacterium* genus was initially detected in 5 cheeses and was detected in 5 others after an enrichment period. Polymerase chain reaction results using species-specific primers of *Carnobacterium* showed that these 10 cheeses contained only the species *Carnobacterium maltaromaticum*. Six different patterns of fermentation were found, and 3 of the 10 cheeses contained *C. maltaromaticum* isolates with anti-*Listeria* activity.

Key words: *Carnobacterium*, polymerase chain reaction, French soft cheese

In the manufacture of soft cheeses, lactic acid bacteria are involved in all phases of cheese making. Initially present as natural microbiota or added as starter cultures, they actively participate in the development of flavor and texture in a wide range of cheeses (Giraffa, 2004; Marilley and Casey, 2004). *Carnobacterium* is a lactic acid bacterium that is not used as a starter because of its nonaciduric and psychrotrophic properties. Indeed, it cannot participate actively in the first phase of fermentation, but its growth can be induced during ripening as pH values increase and after a cold-storage period. Studies have shown that the *Carnobacterium* genus is predominant in French surface-mold-ripened soft Brie cheeses (Millière et al., 1994; Millière and Lefebvre, 1994). This genus has frequently been associ-

ated with the spoilage of seafood and meat products (Pilet et al., 1994; Connil et al., 1998; Paludan-Muller et al., 1998; Dalgaard et al., 2003). However, the bacteriocin-production ability of *Carnobacterium* genus may be considered useful in preventing the growth of food-borne pathogens such as *Listeria* (Duffes et al., 1999; Brillet et al., 2005; Laursen et al., 2005).

The aim of this work was to determine whether *Carnobacterium* is a ubiquitous genus present in many traditional soft cheeses and whether a diversity of species exists. Samples were taken from cheeses manufactured from cow's, goat's, and ewe's milk (raw or pasteurized) in different French provinces during 2 periods of the year. A previous study showed that genus-specific primers could be applied for the detection of this genus in cheeses (Cailliez-Grimal et al., 2005). Species-specific primers (Rachman et al., 2004) were then tested and applied to the cheese samples.

Strains were purchased from different collections (Table 1) and were maintained at –20°C in 15% (vol/vol) glycerol. They were subcultured in trypticase-soy medium (TS; Biomerieux, Craponne, France) supplemented with 0.6% (wt/vol) yeast extract (YE; Biokar Diagnostics, Beauvais, France) and incubated at their optimal growth temperature (Table 1).

Thirty cheeses, manufactured in France and purchased from a local supermarket in October 2004 and April 2005 (Table 2), were analyzed and their *Carnobacterium* flora enumerated by plate count and by PCR (see below). Fractions (10 g) of whole cheese (surface and interior) were homogenized in 90 mL of citrate buffer (0.5 M, pH 7.0). The whole suspension was submitted to a serial decimal dilution. Improvements in the detection of *Carnobacterium* spp. from cheese can be obtained on nonselective media (TS-YE agar or broth) with high pH values (pH 9; Holzapfel, 1992) supplemented with 40 mg/L of nalidixic acid (Sigma, St. Louis, MO) and 5 mg/L of amphotericin (Sigma) and incubated under psychrotrophic conditions (at 7°C for 10 d). For the plate count method, each serial decimal dilution was plated using spiral plating (Whitley Auto-

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Table 1. Strains used in this study¹

Bacterial species	Strain designation	Growth temperature, °C
<i>Carnobacterium alterfunditum</i>	CIP 105796	20
<i>Carnobacterium divergens</i>	DSM 20624 ^T	30
<i>Carnobacterium funditum</i>	CIP106503	22
<i>Carnobacterium gallinarum</i>	DSM 4847 ^T	30
<i>Carnobacterium inhibens</i>	CIP 106863	37
<i>Carnobacterium maltaromaticum</i>	DSM 20730 ^T	30
<i>C. maltaromaticum</i>	LMA 28	30
<i>Carnobacterium mobile</i>	DSM 4848 ^T	30
<i>Carnobacterium viridans</i>	CIP 107728	25

¹CIP = Collection de l'Institut Pasteur (Paris, France); DSMZ = Deutsche Sammlung von Mikro-Organismen und Zellkulturen (Braunschweig, Germany); LMA = Laboratoire de Microbiologie Alimentaire, ENSAIA-INPL (Nancy, France); ^T = type strain.

matic Spiral Plater, WASP 2; AES Laboratoire, Bruz, France). For the enrichment method, 10 g of each cheese was mixed with 90 mL of TS-YE broth medium (described above). The interior pH of the cheese was measured with an electrode pHM210 standard pH meter (Radiometer Analytical, Villerbanne, France).

Five colonies from each cheese sample were collected randomly. Isolates were Gram stained and examined for cell morphology and catalase activity. The carbohydrate fermentation patterns were identified with API 50 CHL galleries (API System, Montalieu-Vercieu, France).

Template DNA was obtained by the boiling method (Yost and Nattress, 2000; De Medici et al., 2003). One isolated bacterial colony was picked from the modified TS-YE agar plates with a sterile toothpick and resuspended in 50 µL of sterile ultrapure water (Easypure, RF; Fisher Bioblock, Illkirch, France). From the TS-YE broth culture, a volume of 50 µL was centrifuged in a microcentrifuge at 8,000 × *g* for 3 min and the cell pellet was resuspended in 100 µL of sterile ultrapure water. These suspensions were boiled for 5 min, and the cellular debris was then pelleted by centrifugation at 8,000 × *g* for 3 min. Ten microliters of each supernatant was retained for the PCR reaction.

Fractions of whole cheese (10 g) were homogenized as described above. A 1,000-fold-diluted matrix was used for the PCR assay. For the enrichment method, 10 g of each cheese was mixed with 90 mL of enrichment medium, as described above. After 10 d at 7°C, a 1,000-fold-diluted sample was used for PCR.

Deoxyribonucleic acid sequences of the primers (obtained from Eurogentec, Herstal, Belgium) are indicated in Table 3. The amplification was carried out in a thermal cycler iCycler System (Bio-Rad, Marnes-la-Coquette, France). The reaction mixture (50 µL) contained 25 µL of the ready-to-use master mixes (Eurogentec) and 10 µL of the sample (described below), completed to 50 µL with sterile ultrapure water. The PCR

program was as follows: 5 min at 95°C for primary DNA denaturation, followed by a series of 30 cycles, each of which comprised 30 s at 95°C (denaturation), 30 s at the corresponding annealing temperature (see Table 3), 30 s at 72°C (extension), and a 7-min final extension step at 72°C. Products were stored at 4°C until analysis. Aliquots (20 µL) of the amplified products were subjected to electrophoresis in 1.5% (wt/vol) agarose gels (Agarose DNA grade; Euromedex, Souffelweyersheim, France) in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2). Gels were stained with ethidium bromide (5 µg/mL) and visualized under UV light (312 nm). A 100-bp DNA ladder polymer was used as a molecular mass marker (Eurogentec).

To test the production of antimicrobial compounds, 50 µL of neutralized and pasteurized cell-free culture supernatant fluid of each isolate was tested for the ability to produce antagonistic substances against *Listeria monocytogenes* CIP 82110 by the agar well-diffusion method (Mayr-Hasting et al., 1972).

Among the 30 cheeses tested, only DNA extracted from 5 cheeses (Brique de Jussac, Petit Munster Val de Weiss, Petit Munster fermier, Camembert Réo, Epoisses) gave an amplicon of 340 bp with *Carnobacterium* genus-specific Cb1-Cb2R primers. From these cheeses, after 10 d at 7°C, the enumeration of plates showed population levels of *Carnobacterium* spp. ≥10⁵ cfu/g (Table 2). Colonies on modified TS-YE agar were nonpigmented, convex, circular, and smooth.

After the enrichment period, with the same primers Cb1-Cb2R, DNA extracted from 5 other cheeses [Pérail, Picodon, Petit Munster (Petits amis), Camembert Issigny, Petit Livarot] also gave one band of 340 bp (Table 2). In this case, the amount of *Carnobacterium* was ≤10² cfu/g.

With the species-specific primers, DNA extracted from these 10 cheeses showed a positive response with the *C. maltaromaticum*-specific primers Cpis-23S-7 (Figure 1A). With the 5 other species-specific primers,

Table 2. Detection of *Carnobacterium* spp. in French cheeses by the culture method and PCR, using primers Cb1-Cb2R, directly on the homogenate or after enrichment¹

Cheese (brand name)	Milk type	Ripening time	Department code	pH ($\pm$ 005)	Detection of <i>Carnobacterium</i> spp.		
					Direct PCR	On plate, log cfu/g	PCR after enrichment
Pérail (Lou Pérac)	Pasteurized ewe's	2 wk	Aveyron F 12.225.01 CEE	7.10	–	<2	+
Margalet (Monbrenac)	Pasteurized ewe's	2 to 3 wk	Aveyron F 12.299.20 CEE	7.10	–	<2	–
Brique de Jussac (Jussac)	Raw ewe's	3 wk	Haute-Loire F 43.162.02 CEE	7.47	+	6.2 $\pm$ 0.1	+
Rocamadour (Patrimoine gourmand) ²	Raw goat's	1 to 4 wk	Lot F 46.177.01 CEE	6.80	–	<2	–
Crottin de Chavignol (Cora) ²	Raw goat's	10 d	Cher F 18.194.50 CEE	6.50	–	<2	–
Cavet (Fermier)	Raw goat's	2 wk	Drôme F 26.114.01 CEE	4.76	–	<2	–
Picodon (Rians) ²	Raw goat's	2 to 3 wk	Drôme F 26.011.01 CEE	6.80	–	<2	+
Le Chèvre de caractère (Soignon)	Raw goat's	2 to 3 wk	Vienne F 86.092.01 CEE	6.94	–	<2	–
Sainte Maure (Soignon) ²	Raw goat's	2 to 5 wk	Deux-Sèvres F 79.276.01 CEE	6.50	–	<2	–
Coulommiers (Cœur de Lion)	Pasteurized cow's	>4 wk	Calvados F 14.762.01 CEE	7.40	–	<2	–
Coulommiers (Cora)	Pasteurized cow's	>4 wk	Ille et Vilaine F 35.239.01 CEE	7.67	–	<2	–
Brie (Cora) ²	Pasteurized cow's	>4 wk	Vosges F 88.115.01 CEE	7.55	–	<2	–
Petit Munster (Frech) ²	Pasteurized cow's	6 to 8 wk	Bas-Rhin F 67.216.01 CEE	7.10	–	<2	–
Petit Munster (Fermier) ²	Pasteurized cow's	6 to 8 wk	Haut-Rhin F 68.249.02 CEE	7.05	–	<2	–
Petit Munster (Petits amis) ²	Pasteurized cow's	6 to 8 wk	Haut-Rhin F 68.252.02 CEE	7.10	–	<2	+
Petit Munster (Val de Weiss) ²	Pasteurized cow's	6 to 8 wk	Haut-Rhin F 68.175.01 CEE	7.64	+	6.6 $\pm$ 0.1	+
Petit Munster (Fermier) ²	Raw cow's	6 to 8 wk	Bas-Rhin F 67.216.01 CEE	7.10	+	7.3 $\pm$ 0.1	+
Banon de Chalais (Bourdin)	Raw cow's	3 wk	Isère F 38.559.01 CEE	6.50	–	<2	–
Camembert de Normandie (Lanquetot) ²	Raw cow's	>4 wk	Calvados F 14.478.01 CEE	6.69	–	<2	–
Camembert de Normandie (Lepetit) ²	Raw cow's	>4 wk	Calvados F 14.422.01 CEE	7.26	–	<2	–
Camembert de Normandie (Patrimoine gourmand) ²	Raw cow's	>4 wk	Calvados F 14.342.01 CEE	6.29	–	<2	–
Camembert de Normandie (Isigny Sainte Mère) ²	Raw cow's	>4 wk	Calvados F 14.342.01 CEE	6.40	–	<2	+
Camembert de Normandie (Réo) ²	Raw cow's	>4 wk	Manche F 50.267.01 CEE	7.10	+	7.80 $\pm$ 0.1	+
Epoisses (Lincet) ²	Raw cow's	>4 wk	Haute Marne F 52.092.01 CEE	7.30	+	5.47 $\pm$ 0.1	+
Petit Pont-l'Évêque (Patrimoine gourmand) ²	Raw cow's	>2 wk	Calvados F 14.342.01 CEE	7.40	–	<2	–
Petit Livarot (Lanquetot) ²	Raw cow's	1 to 2 mon	Calvados F 14.478.01 CEE	7.96	–	<2	+
Langres (Germain) ²	Thermic cow's	1 to 2 mon	Haute Marne F 52.092.01 CEE	6.80	–	<2	–
Chaource (Lanquetot) ²	Thermic cow's	>2 wk	Aube F 10.399.01 CEE	7.13	–	<2	–
Camembert biologique (Cora)	Thermic cow's	>4 wk	Morbihan F 56.179.01 CEE	6.96	–	<2	–
Camembert (Président)	Thermic cow's	>4 wk	Calvados F 14.162.01 CEE	7.70	–	<2	–

¹Results as expressed as means of 3 measures ($\pm$ SD) of 3 samples purchased in October 2004 and in April 2005, and the data from October and April are computed together.²AOC (Appellation d'Origine Contrôlée) cheese.

Table 3. Oligonucleotides used for PCR amplification

Primer	Sequence	T _m , °C	Reference
Cb1	5'-CCG TCA GGG GAT GAG CAG TTA C-3'	69	Nissen et al. (1994)
Cb2R	5'-ACA TTC GGA AAC GGA TGC TAA T-3'	69	Yost and Nattress (2000)
16S-2	5'-CTT GTA CAC ACC GCC CGT C-3'	56	Rachman et al. (2004)
23S-7	5'-GGT ACT TAG ATG TTT CAG TTC-3'	56	Rachman et al. (2004)
Cdiv	5'-TTC TAA AAA AAT AGT ACT CTT G-3'	56	Rachman et al. (2004)
Cgal	5'-TAG TTT CAC TCT AAA AAA AAC-3'	56	Rachman et al. (2004)
Cinh	5'-TTT TAT TTG CAC AAA TA-3'	50	Rachman et al. (2004)
Cmob	5'-CTC ATT TAA TTG CAC AAA AAG G-3'	58	Rachman et al. (2004)
Cpis	5'-TTT ATT TTT AAT TAA ATA CCC-3'	53	Rachman et al. (2004)
Cvir	5'-ATA AGT ATA AGA TGT TTT TT-3'	53	Rachman et al. (2004)

no response was obtained (example with the specific primer of *Carnobacterium divergens*; Figure 1B).

Five colonies isolated from these 10 cheeses were picked and stained. They were rods that appeared singly, in pairs, or in short chains, and were gram positive and catalase negative. Cells were nonmotile and non-spore forming. They were tested by species-specific PCR and gave a response only with *C. maltaromaticum*-specific primers (data not shown).

A comparison of their biochemical characteristics is shown in Table 4. Among the 50 isolates from the 10 cheeses (5 colonies isolated from each positive cheese), results from the API 50 CHL showed 6 differences in the fermentation patterns. All isolates were consistent in their ability to produce acid from the following substrates normally fermented by carnobacteria: β -gentiobiose, saccharose, *N*-acetyl glucosamine, amygdalin, arbutin, esculin, salicin, maltose, cellobiose, D-mannose, D-fructose, D-glucose, and ribose (Table 4).

Like other carnobacteria, isolates did not produce acid from the following: erythritol, D-arabinose, L-xylose, adonitol, β -methyl-xyloside, rhamnose, dulcitol, inulin, melezitose, D-raffinose, glycogen, xylitol, D-lyx-

ose, D-fucose, L-fucose, D-arabitol, and L-arabitol (Table 4). Isolates from Camembert Réo (*C. maltaromaticum* reo), Epoisses (*C. maltaromaticum* epoisses), and Petit Munster (Petits amis; *C. maltaromaticum* amis) exhibited the same pattern, whereas Petit Munster (Fermier and Val de Weiss; *C. maltaromaticum* fermier and *C. maltaromaticum* weiss) and Isigny (*C. maltaromaticum* isigny) were identical. The other isolates, Pérail (*C. maltaromaticum* perail), Petit Livarot (*C. maltaromaticum* livarot), Brique de Jussac (*C. maltaromaticum* jussac) exhibited different patterns.

The ability to produce antagonistic substances against *L. monocytogenes* CIP 82110 was found with isolates from 3 different cheese samples, 1 from pasteurized ewe's milk (Pérail) and 2 from raw cow's milk (Réo and Epoisses). The diameter of inhibition against *L. monocytogenes* CIP 82110 for the 3 isolates tested was 19 ± 1.0 mm.

The primers Cb1-Cb2R were used successfully to conduct a rapid screening of the genus in a wide variety of soft French cheeses. Thirty cheeses of 19 types that differed in the methods used in manufacture and ripening were analyzed. The presence and the number of

Figure 1. Electrophoresis in a 1.5% agarose gel of PCR amplification products with primers (A) Cpis-23S-7 and (B) Cdiv-23S-7. Lanes for panels A and B are as follows: 1: 100 to 1,000 bp molecular weight ladder; 2: no template negative control; 3: *Carnobacterium maltaromaticum* LMA 28 (A) or *Carnobacterium divergens* DSM 20624^T (B); 4: Pérail (Lou Pérac); 5: Brique de Jussac (Jussac); 6: Picodon (Rians); 7: Petit Munster (Petits amis); 8: Petit Munster (Val de Weiss); 9: Petit Munster (Fermier); 10: Camembert de Normandie (Isigny) 11: Camembert de Normandie (Réo); 12: Epoisses (Lincet); 13: Petit Livarot (Lanquetot). ^T = type strain.

Table 4. Comparison of the characteristics of the *Carnobacterium* strains isolated from cheeses with those of *Carnobacterium maltaromaticum*¹

Item	DSM 20 730 ^T	LMA 28	Reo epoisses amis	Fermier weiss isigny	Livarot	Pérail	Jussac	Picodon
Glycerol	+	+	w	—	w	+	—	—
L-Arabinose	—	—	—	+	—	—	—	w
D-Xylose	—	—	—	+	—	—	—	+
Galactose	—	w	—	+	+	—	+	w
L-Sorbose	—	—	—	—	—	—	+	—
Inositol	+	+	—	—	—	—	—	—
Mannitol	+	+	w	—	w	+	+	—
Sorbitol	—	—	—	—	w	—	w	—
α-Methyl-D-mannoside	+	+	—	—	—	+	—	—
α-Methyl-D-glucoside	+	+	—	+	—	+	—	—
Lactose	+	+	—	—	+	+	—	w
Melibiose	—	—	—	+	—	—	+	—
Trehalose	+	+	+	+	w	+	+	—
Amidon	—	+	—	—	—	—	—	—
D-Turanose	+	+	—	+	—	w	—	—
D-Tagatose	—	—	—	—	+	—	+	—
Gluconate	+	+	—	—	w	—	—	w

¹Results from 5 isolates of each cheese: Réo (*C. maltaromaticum* reo), Epoisses (*C. maltaromaticum* epoisses), Petit Munster (Petits amis; *C. maltaromaticum* amis), Petit Munster (Fermier; *C. maltaromaticum* fermier), Petit Munster (Val de Weiss, *C. maltaromaticum* weiss), Isigny (*C. maltaromaticum* isigny), Petit Livarot (*C. maltaromaticum* livarot), Pérail (*C. maltaromaticum* perail), Brique de Jussac (*C. maltaromaticum* jussac), Picodon (*C. maltaromaticum* picodon). Isolates produced acid from the following: β-gentiobiose, saccharose, N-acetyl glucosamine, amygdalin, arbutine, esculin, salicin, maltose, cellobiose, D-mannose, D-fructose, D-glucose, and ribose. Isolates did not produce acid from the following: erythritol, D-arabinose, L-xylose, adonitol, β-methyl-xyloside, rhamnose, dulcitol, inulin, melezitose, D-raffinose, glycogen, xylitol, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol. + = positive response; — = negative response; w = weak response.

Carnobacterium in these cheeses, between 1 and 10⁷ bacteria/g, were compatible with the amounts found previously (Millière et al., 1994; Millière and Lefebvre, 1994). This genus has been detected in cheeses manufactured in various departments in the East (Vosges, Bas-Rhin, Haute-Marne), in the West (Manche, Calvados), and in the South (Aveyron, Drôme, Haute-Loire) of France.

Ten positive samples with Cb1-Cb2R primers were also positive with the species-specific primers of *C. maltaromaticum*. The other *Carnobacterium* species (except *C. divergens*) were isolated from others sources, so it was not surprising that they were absent from the cheese (Collins et al., 1987; Franzmann et al., 1991; Joborn et al., 1999; Holley et al., 2002; Pikuta et al., 2005). On the other hand, the detection of *C. divergens*, previously found in cheeses (Millière and Lefebvre, 1994; Morea et al., 1999) was expected.

A previous study showed *Carnobacterium* only in cow's milk cheese (Millière et al., 1994). In this work, ewe's and goat's milk cheeses also contained this genus. The pH values of cheeses tested varied from 5.6 to 8.1, which are in the pH range required for the growth of the genus *Carnobacterium*. No correlations were found among the ripening of cheese, use of raw or pasteurized milk, and *C. maltaromaticum* counts. Cheeses con-

taining *Carnobacterium* did not differ significantly in taste or off-flavors from the same cheeses without *Carnobacterium* (Camembert, Munster). Eight cheeses were AOC cheeses, a designation that guarantees the quality of the product. The role of the *Carnobacterium* in these cheeses was therefore unclear.

Isolates from 3 different cheeses (1 from pasteurized ewe's milk, 2 from raw cow's milk) exhibited anti-*L. monocytogenes* activity. In raw milk soft cheese manufacturing, *L. monocytogenes* could be present at the end of the ripening period, with the rind being the preferential site of colonization. After food processing, this species can potentially grow in refrigerated products stored for extended periods. The anti-*Listeria* property of isolates from industrial cheeses should then be an advantage. Assuming that no product defect is related to the presence of *Carnobacterium*, it could be interesting to investigate their positive influence in the preservation of cheese.

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