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Characterization of *Carnobacterium maltaromaticum* LMA 28 for its positive technological role in soft cheese making

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ABSTRACT

Carnobacterium maltaromaticum is a lactic acid bacterium isolated from soft cheese. The objective of this work was to study its potential positive impact when used in cheese technology. Phenotypic and genotypic characterization of six strains of *C. maltaromaticum* showed that they belong to different phylogenetic groups. Although these strains lacked the ability to coagulate milk quickly, they were acidotolerant. They did not affect the coagulation capacity of starter lactic acid bacteria, *Lactococcus lactis* and *Streptococcus thermophilus*, used in dairy industry. The impact of *C. maltaromaticum* LMA 28 on bacterial flora of cheese revealed a significant decrease of *Psychrobacter* sp. concentration, which might be responsible for cheese aging phenomena. An experimental plan was carried out to unravel the mechanism of inhibition of *Psychrobacter* sp. and *Listeria monocytogenes* and possible interaction between various factors (cell concentration, NaCl, pH and incubation time). Cellular concentration of *C. maltaromaticum* LMA 28 was found to be the main factor involved in the inhibition of *Psychrobacter* sp. and *L. monocytogenes*.

1. Introduction

Cheeses host a complex ecosystem characterized by a succession of different microbial groups emerging during milk fermentation, ripening or storage. Lactic acid bacteria (LAB) are usually involved throughout numerous processes occurring during milk transformation into cheese. Microorganisms generally play a positive role during cheese maturation processes, as they provide specific cheese flavors (Irlinger and Mounier, 2009). They generally come from commercial starter cultures but are also found in raw milk, in the dairy environment and are thus called opportunistic LAB (Fleet, 1999). The presence of an opportunistic psychrotrophic LAB named *Carnobacterium maltaromaticum* has been demonstrated in numerous French cheeses (Appellation d'Origine Protégée, AOP = Protected Designation of Origin, PDO) at the end of ripening and cold storage, without affecting the final product quality (Cailliez-Grimal et al., 2007; Millière et al., 1994). These species frequently predominate in a large range of foods, including non-dairy foods such as fish or meat and are commonly associated with the spoilage of modified-atmosphere-packaged products (Vihavainen et al., 2007).

Many *Carnobacterium* strains have been used previously as protective cultures against pathogenic bacteria (for instance *Listeria monocytogenes*) in fish and meat products (Brillet et al., 2005; Duffes et al., 1999; Matamoros et al., 2009; Vescovo et al., 2006). The bacteriocin-producing ability of *Carnobacterium* strains was found to play a key role in controlling spoilage and pathogenic bacteria in food and in *in vitro* model systems (Dos Reis et al., 2011; Martin-Visscher et al., 2011; Bernardi et al., 2011), while external factors, including pH, salt and storage temperatures, were also shown to have a significant impact (Hwang, 2009; Leroi et al., 2012).

The identification, isolation, ecology and technological aspects of *C. maltaromaticum* in cheese were recently documented (Afzal et al., 2010). The objective of this study was to characterize several *C. maltaromaticum* strains isolated from soft cheese by comparing their technological abilities, acid tolerance and

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inhibitory activities. One selected strain was tested during ripening of soft cheese. An experimental plan was then done to evaluate the inhibition potential of this strain against *L. monocytogenes* and *Psychrobacter* sp.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Strains used in this study, along with culture media and growth conditions, are presented in Table 1. Strains were grown in trypticase soy broth (Biokar, Beauvais, France) supplemented with yeast extract (TSB-YE), except for *Escherichia coli*, which was grown in Lysogeny broth (LB) (Biokar, Beauvais, France) and *Psychrobacter* sp. grown in TSB-YE containing 8% NaCl (Table 1). For selective enumeration, PALCAM agar (Biokar, Beauvais, France) was used for *L. monocytogenes* CIP 82110, MCM (Edima et al., 2007) was used for *C. maltaromaticum* LMA 28 and TSB-YE with NaCl (8%) for *Psychrobacter* sp.

2.2. Identification

2.2.1. Determination of carbon assimilation profiles

Carbohydrate fermentation patterns were determined using API 50 CH test strips (BioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions.

2.2.2. Molecular typing using pulsed-field gel electrophoresis (PFGE)

Carnobacterium strains were inoculated in 10 mL of TSB-YE broth and grown at 30 °C overnight. The culture (1 mL) was inoculated in 20 mL of TSB-YE broth and incubated at 30 °C until the bacterial suspension absorbance reached 0.4–0.8 (exponential phase). Pulsed-Field Gel Electrophoresis (PFGE) of all *Carnobacterium* strains was performed with the enzyme *SmaI* as described by Haenni et al. (2010).

Table 1
Bacterial strains used in this study.

Bacterial species	Strain designation[a]	Growth medium	Incubation temperature (°C)
Carnobacterium maltaromaticum	LMA 5[b]	TSB-YE	30
C. maltaromaticum	LMA 7[b]	TSB-YE	30
C. maltaromaticum	LMA 14[b]	TSB-YE	30
C. maltaromaticum	LMA 23[b]	TSB-YE	30
C. maltaromaticum	LMA 28[b]	TSB-YE	30
C. maltaromaticum	LMA 32[b]	TSB-YE	30
C. divergens	DSM 20623^T	TSB-YE	30
C. gallinarum	DSM 4847^T	TSB-YE	25
C. mobile	DSL 4848^T	TSB-YE	30
C. viridans	CIP 107728	TSB-YE	25
Listeria grayi	CIP 6818^T	TSB-YE	30
L. innocua	CIP 12511	TSB-YE	30
L. innocua	CIP 107775	TSB-YE	30
L. ivanovii	CIP 12510	TSB-YE	30
L. ivanovii	CIP 7842	TSB-YE	30
L. monocytogenes	CIP 82110^T	TSB-YE	37
L. monocytogenes	CIP 7831	TSB-YE	37
Enterococcus faecalis	ATCC 19433	TSB-YE	37
E. faecalis	CIP 78117^T	TSB-YE	37
E. faecium	CIP 106742	TSB-YE	37
Escherichia coli	XL-1Blue	LB	37
Lactococcus lactis	DSM 20481	TSB-YE	30
Streptococcus thermophilus	INRA 302	TSB-YE	37
Psychrobacter sp.	LMA 1	TSB-YE + NaCl (8%)	30

[a] ATCC: American Type Culture Collection, Mannassas, USA; CIP: Collection de l'Institut Pasteur, Paris, France; DSM: Deutsche Sammlung von Mikro-Organismen und Zellkulturen, Göttingen, Germany; INRA: Institut National de la Recherche Agronomique; LMA: Laboratoire de Microbiologie Alimentaire, ENSAIA-INPL, Nancy, France; SLCC: Special *Listeria* Culture Collection, University of Wurzburg, Germany; T: Type.
[b] Strains isolated by Millière et al. (1994).

2.2.3. Antibacterial assays and primers used for the detection of genes encoding bacteriocins

Antibacterial activity of six strains of *C. maltaromaticum* was determined against *Carnobacterium*, *Listeria* and *Enterococcus* species by the agar well diffusion method (Mathieu et al., 1993) (Table 1). Primers (Eurogenetec, Herstal, Belgium) (Table 2) were used for the detection of genes encoding different bacteriocins in DNA of *C. maltaromaticum* strains. DNA extraction and PCR were conducted as described above (in 2.4. Nucleic acid extraction).

2.2.4. Isolation of Psychrobacter sp.

Cheese sample (5 g) was suspended in 50 mL of citrate buffer (trisodium citrate, 2% w/v and NaCl, 8.5 g/L) and homogenized in stomacher (Interscience, St. Nom-la-gatehouse, France). A volume of 1.5 mL was then inoculated into three different selective media: brilliant green bile broth, lactose broth (BLBVB Biokar, Beauvais, France) and TSB-YE + NaCl (8%). These media were incubated at 4 °C and 30 °C during 48 and 24 h respectively. After enrichment, bacteria were isolated on TSA-YE. Gram staining, oxidase and catalase tests were performed on each isolated colony.

2.3. Technological aptitude

2.3.1. Milk acidification

C. maltaromaticum strains (LMA 5-7-14-23-28-32), *Streptococcus thermophilus* INRA 302 and *Lactococcus lactis* DSM 20481 were inoculated in 150 mL of semi-skimmed milk supplemented with 1 g/L of yeast extract at the initial population of 10^8 cfu/mL. The cultures were incubated in a thermostatic water bath at 30 °C to monitor acidification using an automatic multimeter (Consort D230, Neuilly-sur-Seine, France). The kinetic parameters, maximum acidification rate (V_m) and time corresponding to V_m (T_m) were determined by the method described by Spinnler and Corrieu (1989).

2.3.2. Milk coagulation

The bacterial strains were subcultured twice in TSB-YE at their optimum growth temperatures during 16 h. The cell pellets were obtained as described above. Milk coagulation times were obtained by the use of a rheometer (Stress Tech, Rheologica Instruments AB, Sweden). The cell pellets were used to inoculate 18 mL (placed in a C25 cup) of semi-skimmed UHT milk supplemented with yeast extract (1 g/L) in a tank thermostatically controlled by a water bath at 30 °C. The geometry used to monitor milk coagulation is a paddle system (four blades placed at right angles to each other) specially designed by Rheologica to follow milk coagulation. The shear rate was fixed at 100 s^{-1} and remained constant throughout the analysis. Data are collected automatically every 20 s and all runs are carried out at least in duplicate.

2.3.3. Acid tolerance

The strains were subcultured twice in TSB-YE at 30 °C for 16 h. These precultures were used to inoculate TSB-YE and semi-skimmed UHT milk at 1:10 ratio and incubated at 30 °C. Cultures were stopped in exponential growth phase ($OD_{660nm} = 0.4$). Volume of 1 mL of the suspension was used to inoculate 9 mL of TSB-YE or milk to a specified value of pH. The pH range selected varied from 6.5 to 3, adjusted using HCl or lactic acid in aseptic conditions. After a contact time of 4 h at 30 °C, spreadings and numerations were