Protective Effect of *Carnobacterium* spp. against *Listeria monocytogenes* during Host Cell Invasion Using *In vitro* HT29 Model

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The pathogenesis of listeriosis results mainly from the ability of *Listeria monocytogenes* to attach, invade, replicate and survive within various cell types in mammalian tissues. In this work, the effect of two bacteriocin-producing *Carnobacterium* (*C. divergens* V41 and *C. maltaromaticum* V1) and three non-bacteriocinogenic strains: (*C. divergens* V41C9, *C. divergens* 2763, and *C. maltaromaticum* 2762) was investigated on the reduction of *L. monocytogenes* Scott A plaque-forming during human infection using the HT-29 *in vitro* model. All *Carnobacteria* tested resulted in a reduction in the epithelial cell invasion caused by *L. monocytogenes* Scott A. To understand better the mechanism underlying the level of *L. monocytogenes* infection inhibition by *Carnobacteria*, infection assays from various pretreatments of *Carnobacteria* were assessed. The results revealed the influence of bacteriocin production combined with a passive mechanism of mammalian cell monolayers protection by *Carnobacteria*. These initial results showing a reduction in *L. monocytogenes* virulence on epithelial cells by *Carnobacteria* would be worthwhile analyzing further as a promising probiotic tool for human health.

Keywords: foodborne pathogens, *Carnobacterium divergens*, *Carnobacterium maltaromaticum*, bacteriocin, HT29, mucus layer

INTRODUCTION

*Listeria monocytogenes* is one of the major foodborne pathogens responsible for listeriosis. The infections in high-risk individuals, such as pregnant women, newborn infants and immunocompromised people may cause meningitis, encephalitis, septicemia or spontaneous late-term abortions with a mortality rate as high as 30% (Farber and Peterkin, 1991; Rocourt et al., 2000; Vázquez-Boland et al., 2001; Swaminathan and Gerner-Smidt, 2007). In 2013, 1763 human cases of listeriosis were reported in Europe by the European Food Safety Authority and the European Center for Disease Prevention and Control (EFSA and ECDC, 2015). The notification rate was 0.44 cases per 100,000 population which represented an 8.6% increase compared with 2012 (EFSA and ECDC, 2015). There was a statistically significant increase in the incidence of listeriosis over the period 2009–2013. In total, 191 deaths due to listeriosis were reported in 2013, with the highest number (64 cases) occurring in France. *L. monocytogenes* is therefore of public concern in terms...
of food safety and regulations to control this microorganism. Currently, antibiotics are the most accepted treatment option for listeriosis infections. As vaccination is unavailable and the use of antibiotics is declining due to an increase in resistance and allergies, there is a need for innovative alternative ways of reducing L. monocytogenes infections in humans.

L. monocytogenes is widespread in the environment and has been isolated from various sources such as dairy products, fresh vegetables, and meats (Beresford et al., 2001; Guerrieri et al., 2009). Isolations performed in the food processing environment reveal that L. monocytogenes can adhere to inert surfaces and grow as biofilms in diverse areas, such as dead ends, crevices and corner cracks (Kim and Frank, 1995; Tresse et al., 2006, 2007; Shi and Zhu, 2009; Pilchová et al., 2014; Guilbaud et al., 2015). Due to the ability of this pathogenic bacterium to grow in foodstuffs at refrigerated temperatures, efficient control methods are required to limit the risk in ready-to-eat food products with a long shelf life. As increasing numbers of consumers prefer foods without chemical preservatives (Cleveland et al., 2001; Devlieghere et al., 2004), there is an opportunity for methods focusing on the use of a protective culture such as lactic acid bacteria (LAB). The protective LAB could produce antimicrobial metabolites such as lactic acid, diacetyl, hydrogen peroxide and bacteriocin or bacteriocin-like compounds (Lindgren and Dodrogoz, 1990).

Some bacteriocin-producing LAB have demonstrated their efficiency in limiting the growth of L. monocytogenes (Harris et al., 1989; Rodriguez et al., 1997; Laukova et al., 1999; Ennahar et al., 2000; Sabia et al., 2002; Trypponen et al., 2003; Todorov et al., 2011; Amado et al., 2012). Carnobacterium spp. are among the bacteriocin-producing bacteria recently studied as protective cultures in foodstuffs. The Carnobacterium genus belongs to the family Carnobacteriaceae within the order of Lactobacillales and currently consists of 12 species (Euzéby, 1997). Two of the Carnobacterium species, C. divergens and C. maltaromaticum, are mainly isolated from the environment and foods (Leisner et al., 2007). Both have been shown to exhibit a wide spectrum of activity against L. monocytogenes which has been attributed in some cases to the production of bacteriocins (Pilet et al., 1995; Buchanan and Bagi, 1997; Duffes et al., 1999; Nilsson et al., 1999; Schöbitz et al., 1999; Yamazaki et al., 2003; Jözelfiak et al., 2010). Attempts to apply extracted bacteriocins against L. monocytogenes have been limited due to (i) the loss of activity following bacteriocin purification steps, (ii) variations in bacteriocin production depending on Carnobacterium spp and (iii) variations in susceptibility among L. monocytogenes strains (Richard et al., 2003; Brillet et al., 2004). Non-bacteriocinogenic strains have also demonstrated inhibitory activity toward L. monocytogenes (Nilsson et al., 2005). Some in vitro experiments on the antagonism of various LAB against pathogenic bacteria have been reported (Alemka et al., 2010; Garnier et al., 2010; Messaoudi et al., 2012). Although, the active ingredients were mainly attributed to the production of bacteriocins, the inhibitory effect of Carnobacterium on L. monocytogenes virulence has not yet been explored.

In this study, we examined the in vitro potential of protective Carnobacterium strains to assess their effect on the virulence of L. monocytogenes. Bacteriocin- and non-bacteriocin-producing Carnobacterium strains were tested on cell line models using HT29 and its mucin-producing counterpart HT29-MTX.

**MATERIALS AND METHODS**

**Bacterial Strains**

Two clinical strains of L. monocytogenes and five strains of Carnobacterium spp. were used in this study. L. monocytogenes Scott A (serotype 4b) and L. monocytogenes LO28 (serotype 1/2c) were previously isolated from listeriosis outbreaks, C. divergens V41, and C. maltaromaticum V1 (formerly C. piscicola) were isolated from salmon and trout intestine and characterized by Pilet et al. (1995), C. divergens V41C9 is a C. divergens V41 mutant deficient in divercin production (Richard et al., 2003), C. divergens NCDO 2763 and C. maltaromaticum (formerly C. piscicola) NCDO 2762 (type strain) were obtained from the National Collection of Dairy Organisms (Reading, UK). All strains were stored in cryotubes at −80°C in brain heart infusion broth (BHI) for L. monocytogenes strains and in Elliker broth for Carnobacterium strains supplemented with 20% glycerol as a cryoprotectant.

**Antimicrobial Activity Determination**

The antimicrobial activity of Carnobacterium strains was tested on two L. monocytogenes strains using the agar diffusion assay (Pilet et al., 1995). Briefly, the L. monocytogenes subculture was grown in BHI broth for 8 h at 37°C and culture was incubated overnight at 37°C. A concentration of 3.5 × 10^7 cfu ml⁻¹ was mixed to the BHI agar. Carnobacterium strains were subcultured in Elliker broth for 24 h at 20°C. Cultures were then incubated overnight at 20°C and 30°C. Similar concentrations of bacteria were obtained after cultivation at 20°C and 30°C (2.6 × 10^9 cfu ml⁻¹ and 1.8 × 10^9 cfu ml⁻¹). The cell-free supernatant of each Carnobacterium strain was obtained by centrifugation (8200 g, 10 min at 4°C) and 10 µl of the filtered supernatant (untreated supernatant and treated culture supernatant adjusted to pH 6.5) was then spotted onto indicator plates of BHI agar (1%) seeded with 10^6 cfu ml⁻¹ of the target strain. After overnight incubation at 37°C, the presence of a translucent halo corresponding to the absence of L. monocytogenes growth was observed.

**Cell Line Cultures**

The human adenocarcinoma cell line HT29 and the mucus-secreting HT29 cells selected by adaptation to methotrexate (HT29-MTX) were used. HT29-MTX cells were obtained from Dr Thécla Lesuffleur (INSERM UMR S 938, Paris France) (Lesuffleur et al., 1993). Cells were routinely grown in 25 cm² plastic tissue culture flasks (Nunc, Life Technologies) in 5 ml of culture medium (Dulbecco's modified Eagle's medium; DMEM; Eurobio, Courtaboeuf, France) supplemented with 10% (v/v) fetal calf serum (Eurobio, Courtaboeuf, France), 2 mM L-glutamine (Eurobio, Courtaboeuf, France) and antibiotics—penicillin 100 IU ml⁻¹ and streptomycin 100 µg ml⁻¹ (Sigma, France). Antibiotics were routinely added to the culture medium except for virulence assays. Cells were maintained in a humidified incubator (at least 90% RH) at 37°C under 5% (v/v) CO₂.
PFA as previously described by Roche et al. (2001). Briefly, cell monolayers were grown until they reached 90% confluence in DMEM supplemented with antibiotics and then without antibiotics for another 24 h. The overnight L. monocytogenes cultures, grown in BHI broth, were appropriately diluted in DMEM without antibiotics. The 96-wells were inoculated with 2 to 8 log CFU L. monocytogenes per well in triplicate and incubated for 2 h at 37°C in a humidified incubator and treated with 100 µg ml⁻¹ gentamicin (Sigma, France). After 1.5 h of incubation, cell monolayers were overlaid with an agarose gel containing 0.48% indubiose (Bio-Rad Laboratories, France) in DMEM supplemented with 10 µg ml⁻¹ of gentamicin. The number of plaques was counted with a microscope after 48 h of incubation at 37°C in a humidified incubator. Each experiment was repeated three times from independent cultures for each strain and the results were expressed as the number of plaques obtained for 7 or 8 log L. monocytogenes loaded per well.

**Ability of C. divergens to Adhere to Epithelial Cells HT29**

After cultivation, C. divergens V41 and V41C9 were harvested by centrifugation, resuspended in DMEM without antibiotics and serum at a concentration of 10⁸ cfu ml⁻¹ and loaded on confluent HT29 and HT29-MTX cell line monolayers. After 1 h or 4 h of incubation (37°C, 5% CO₂), monolayers were washed three times with PBS to remove nonadherent bacteria and lysed with 0.1% Triton X100 for 15 min. The lysate was then diluted and plated on Elliker agar plates to determine the number of adherent bacteria. Each experiment was performed in duplicate.

**PFA in the Presence of Carnobacterium**

Carnobacterium strains were grown as described above. Cultures were pelleted by centrifugation at 8200 g for 10 min and resuspended in DMEM medium without antibiotics. A total of 100 µl of Carnobacterium culture in tissue culture medium was used to coat the cells with 10⁹ cfu ml⁻¹ for 1 h or 4 h at 37°C. After the initial incubation period, 100 µl of L. monocytogenes culture at 10⁹ or 10⁸ cfu ml⁻¹ was added on the cell monolayers and incubated for 2 h at 37°C. Then, the medium was replaced with 100 µl of fresh sterile culture medium containing 100 µg ml⁻¹ gentamicin and incubated for another 1.5 h at 37°C. Next, the same steps as mentioned above were performed to count the number of lysis plaques. As controls, L. monocytogenes and Carnobacterium strains were also tested separately on HT29 and HT29-MTX cell lines. Each experiment was repeated three times from independent cultures for each strain.

**Pretreatment of Carnobacterium before Infection Assays**

Cell monolayers were originally incubated with probiotic cultures at 10⁹ cfu ml⁻¹ in antibiotic-free medium. To cover wide range of active compounds that could potentially affect the listerial invasion, six different pretreatments were applied to the Carnobacterium culture: (a) 1 ml of an overnight culture of Carnobacteria strains was centrifuged (8200 g for 10 min) and the cells obtained in the pellet were resuspended in 1 ml of DMEM (namely—resuspended cells—RS); (b) 1 ml of an overnight culture of Carnobacterium was centrifuged (8200 g for 10 min) and the cells obtained in the pellet were washed in 1 ml of DMEM, centrifuged once more and subsequently resuspended in 1 ml of DMEM (namely—washed cells—WS); (c) 1 ml of an overnight culture was heated at 100°C for 5 min, centrifuged (8200 g for 10 min) and the cells in the pellet resuspended in 1 ml of DMEM (namely—resuspended heated cells—RHC); (d) 1 ml of an overnight culture was heated at 100°C for 5 min, centrifuged (8200 g for 10 min) and the cells were washed in 1 ml of DMEM, centrifuged once more time and then resuspended in 1 ml of DMEM (namely—washed heated cells—WHC); (e) overnight cultures were centrifuged (8200 g for 10 min) and cell-free supernatant was adjusted to pH 6.5 and then either untreated (namely—non-filtered supernatant—NFS), or filtered (0.2 µm filter; namely—filtered supernatant—FS).

**Statistical Analyses**

The data were analyzed using Statgraphics Centurion XVI software (StatPoint Inc., Herndon, Virginia, USA). With the confirmation of a normal distribution for each data set, significant differences were determined using two-sided Student’s t-test comparisons at a 5% significance level.

**RESULTS**

**Effect of Bacteriocin-Producing C. divergens V41 on L. monocytogenes Using In vitro Virulence Models**

First, the antimicrobial activity of the dиверс—in-producing C. divergens V41 (div+) and its non-bacteriocinogenic mutant C. divergens V41C9 (div−) against L. monocytogenes was confirmed (Table 1). As expected, the growth of Scott A and LO28 was inhibited by supernatants of the div+ strain cultivated at 20° and 30°C while no inhibition zone was observed for the div− strain in all conditions. Identical results observed with pH-neutralized supernatants confirmed the dиверсion activity of V41 against L. monocytogenes (Table 1). In the following experiments, the effect of Carnobacteria against L. monocytogenes virulence was assessed using Scott A as it is more virulent than LO28. Plaque-forming on confluent monolayer epithelial cells by L. monocytogenes Scott A was evaluated after 1 h of C. divergens V41 inoculation (Figure 1). No plaque was observed when C. divergens V41 was loaded alone indicating the absence of a cytotoxic effect by C. divergens on HT29 and HT29 MTX cell lines. In contrast, L. monocytogenes Scott A was able to form plaques on both cell lines with a higher level on HT29 cells (log
Table 1: Growth inhibition of *L. monocytogenes* Scott A and LO28 by *C. divergens*.

| Untreated supernatant | Scott A | LO28 |
|------------------------|---------|------|
| V41 (20°C)             | +       | +    |
| V41 (30°C)             | +       | +    |
| V41C9 (20°C)           | −       | −    |
| V41C9 (30°C)           | −       | −    |

Supernatant adjusted to pH 6.5

|           | Scott A | LO28 |
|-----------|---------|------|
| V41 (20°C) | +       | +    |
| V41 (30°C) | +       | +    |
| V41C9 (20°C)| −       | −    |
| V41C9 (30°C) | −       | −    |

Supernatants of cultures at 20° and 30°C of the bacteriocin-producing strain *C. divergens* V41 and its non-bacteriocinogenic variant V41C9 were tested. Growth inhibition was measured using the agar diffusion test as shown in the picture for Scott A with the cell-free culture supernatant of V41 at 20°C (lane I) and at 30°C (lane II). Neutralized supernatants were obtained by adjusting the pH to 6.5.

3.27 ± 0.12 plaques than on their mucus-secreting counterparts (log 2.80 ± 0.08). The significant difference between the two cell lines indicates a role of mucus in the prevention of *L. monocytogenes* plaque-forming. When *Scott A* was inoculated on epithelial cells previously coated with *C. divergens* V41, the plaque-forming ability of *Scott A* was reduced dramatically on both cell lines. At 10^8 cfu ml^-1, plaque-forming by *Scott A* decreased to log 0.44 on HT29 and to log 0.29 on HT29 MTX while at 10^7 cfu ml^-1, the plaque-forming of *Scott A* was reduced to an undetectable level on both lines. Furthermore, when the supernatant (filtered-FS and not filtered-NFS) of V41 culture was used, a similar inhibitory effect on *Scott A* plaque-forming was obtained on both cell lines.

Comparative Effect of Bacteriocinogenic and Non-bacteriocinogenic *C. divergens* Strains on *L. monocytogenes* Scott A In vitro Virulence

In order to determine the contribution of the bacteriocin (divercin) secreted by *C. divergens* V41 to the inhibition of *L. monocytogenes* plaque-forming, the div− mutant, defective in bacteriocin synthesis, was tested. When the HT29 cells were precoated with the div− strain, the inhibitory level of *L. monocytogenes* plaque-forming only decreased to 91.5% indicating a major contribution by another factor besides that of bacteriocin (Table 2). From now on, the antimicrobial effects will be named “the bacteriocin effect” for those due to bacteriocins and “protective effect” for those independent of bacteriocins. The inhibition of plaque-forming by the div− strain was significantly lower than that of div+ on both cell lines confirming a slight but significant contribution of the bacteriocin effect in the div+ strain (Table 2). In addition, a lower plaque-forming inhibition was observed on HT29 MTX for *Scott A* at 10^8 cfu/mL which confirms the role of the mucus observed in Figure 1 in preventing *L. monocytogenes* plaque-forming (Table 2).
**Influence of Precoating Time** *C. divergens* **on the Inhibition of** *L. monocytogenes* **Scott A Infection Using HT29 and HT29-MTX Cell Lines**

The ability of strains div+ and div− to adhere to host cells was assessed by incubating the culture for 1 h and 4 h on confluent monolayers of HT29 and HT29-MTX (Figure 2). The initial *C. divergens* concentration loaded onto cell monolayers was $10^9$ cfu ml$^{-1}$. Overall, approximately $5 \times 10^7$ cfu ml$^{-1}$ of viable *C. divergens* cells were recovered from cell lysates. A significantly greater number of adherent cells was observed on HT29-MTX than on HT29 indicating a positive effect of mucus on *C. divergens* adhesion (Figure 2). In addition, a decrease, although moderate, was observed in the adhesion of div+ after 4 h of contact on both cell lines compared to 1 h while adhesion tended to increase for div− on HT29-MTX. When assessed in the presence of *L. monocytogenes* Scott A, there was no significant difference between 1 and 4 h of contact except for div− after 4 h of contact on HT29 MTX which showed less inhibition of Scott A plaque-forming compared to 1 h (Table 3). Taken together, these results indicate that *C. divergens* V41 remains efficient against Scott A after 4 h of contact with both cell lines by maintaining both its protective and bacteriocin effects.

**Effect of Various Pretreatments of** *C. divergens* **V41 on the Inhibition of** *L. monocytogenes* **Scott A Plaque-Forming on HT29**

To understand further the protective mode of action of *C. divergens* V41, additional infection assays with various pretreatments were carried out (Figure 3). The results showed no significant difference in plaque-forming by *L. monocytogenes* when cells were washed and/or heated before inoculation on HT29, indicating that no extracellular divercin remained after the preparation of the div+ culture and that dead cells were able to achieve the same efficient protective effect.

**Effect of Carnobacterium spp. on the Inhibition of** *L. monocytogenes* **Plaque-Forming on HT29**

In order to determine if the protective effect of *Carnobacterium* against *L. monocytogenes* is specific to *C. divergens* V41, additional strains or species were tested (Figure 4). The anti-listerial activity of cell-free neutralized supernatants of *C. maltaromaticum* V1 was confirmed (inhibition zone $= 10$ mm $\pm 0$; $n = 3$ at 20°C and 9 mm $\pm 1$; $n = 3$ at 30°C) while the supernatants of non-bacteriocinogenic strains *C. divergens* 2763 and *C. maltaromaticum* 2762 did not exhibit any growth inhibition of *L. monocytogenes* Scott A (inhibition zone $< 3$ mm; $n = 3$ at 20°C and 30°C; Figure 4A). All five *Carnobacterium* strains tested, bacteriocinogenic or not, significantly inhibited the number of plaques formed by *L. monocytogenes* Scott A (Figure 4B). However, the decrease in plaque-forming was lower for all strains compared to that of div+ indicating a better potential of *C. divergens* V41 to prevent *L. monocytogenes* infection. In line with what was observed with V41 and its div− mutant, the bacteriocin-producing *C. maltaromaticum* V1 was significantly more efficient at limiting the number of plaques formed by *L. monocytogenes* than the non-bacteriocin-producing strain *C. maltaromaticum* 2762. Similar results obtained with washed or preheated cells confirmed that inhibition of Scott A plaque-forming by *Carnobacteria* can utilize both bacteriocin and protective effects.

**DISCUSSION**

The inhibitory effect of two bacteriocin-producing *Carnobacteria* (C. divergens V41 and C. maltaromaticum V1) and three non-bacteriocin-producing *Carnobacteria* (C. divergens V41C9, C. divergens 2763 and C. maltaromaticum 2762) against

**TABLE 3 | Effect of precoating contact time of Carnobacterium strains on the inhibition of L. monocytogenes plaque-forming on HT29 and HT29 MTX.**

| Contact time of C. divergens (h) | HT29 | HT29 MTX |
|----------------------------------|------|----------|
| 1                                | 100.0 ± 0.0 | 97.0 ± 3.1 |
| 4                                | 100.0 ± 0.0 | 100.0 ± 0.0 |
| V41 (div+)/Scott A               | 100.0 ± 0.0 | 97.0 ± 3.1 |
| V41C9 (div−)/Scott A             | 91.5 ± 6.6  | 82.8 ± 9.9 |
|                                  | 98.7 ± 2.7  | 82.4 ± 2.6* |

*L. monocytogenes* cells were inoculated at $10^9$ cfu ml$^{-1}$ 1 h or 4 h after the inoculation of *C. divergens* V41 (div+) or V41C9 (div−) at $10^9$ cfu ml$^{-1}$. Results were normalized to the plagues obtained with *L. monocytogenes* Scott A ($log_{2} 3.29 \pm 0.13$, $n = 4$ on HT29 and log 2.79 $\pm 0.08$, $n = 4$ on HT29 MTX). The means and standard deviations (SD) were calculated from at least three independent experiments. Asterisks indicate significant difference ($P < 0.05$) between 1 and 4 h of precoating.
Pathogenic *L. monocytogenes* strains has previously been investigated in food (Pilet et al., 1995; Richard et al., 2003; Brillet et al., 2004, 2005). The use of *C. divergens* V41 and *C. maltaromaticum* V1 could represent an alternative strategy to control the growth of *L. monocytogenes* in cold-smoked salmon (Brillet et al., 2004). Overall, LAB isolated from salmon intestine resulted in growth inhibition of *Aeromonas salmonicida* with no reduction in the mortality rate of the fish (Gildberg et al., 1995).

Several methods have been proposed to assess the virulence of *L. monocytogenes*. Mouse infection models have demonstrated their efficiency in differentiating virulent from non-virulent strains (Roche et al., 2001); nonetheless their use is limited due to ethical considerations. Expression of the main virulence genes *hlyA*, *actA*, *inlA*, and *prfA* using RT-qPCR has also been investigated to show the effect of environmental conditions on virulence factor transcript levels of *L. monocytogenes* (Duodu et al., 2010). However, the plaque-forming assay using HT29 cells is usually proposed as the best alternative to animal models as it takes into account the epithelial cell invasion capability of the pathogen (Roche et al., 2001). In this work, HT29 cell assays were completed by mucus-secreting HT29 MTX cells in order to include the potential role of mucus in bacterial invasion capability.

A significant reduction in *L. monocytogenes* virulence on epithelial cells was observed when the cell monolayers were precoated with *C. divergens* V41 cultures during 1 or 4 h. The capability to limit foodborne pathogen virulence has previously been tested for probiotic LAB and found to be strain-specific. For instance, Garriga et al. (2015) reported that only bacteriocinogenic *Lactobacillus sakei* of 5 other LAB tested significantly reduced the adhesion of *L. monocytogenes*. *Lactobacillus* and *Bifidobacterium* were also shown to inhibit significantly the subsequent listerial infection using the *in vitro* C2Bbe1 epithelial cell model (Corr et al., 2007). Pretreatment of intestinal cells T 84 with LAB prevents injury of *Escherichia coli* O157:H7 and *E. coli* O157:H6 induced by attaching-effacing-*Lactobacillus* species (Sherman et al., 2005). In the case of *Campylobacter jejuni*, the leading cause of bacterial foodborne diseases (EFSA and ECDC, 2015; Turonova et al., 2015), adhesion, internalization and translocation of HT29 cells were attenuated by strains of *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, and *Lactobacillus salivarius* (Aleimka et al., 2010). The authors reported that live LAB and prolonged precolonization of mammalian cells with probiotics is a prerequisite for probiotic action against *Campylobacter* virulence. More recently, Srividya et al. (2015) demonstrated an *in vitro* inhibition of 70% of *Shigella dysenteriae* by a probiotic lactic acid bacterial lyase.

In this study, to investigate the mechanism that could be involved in the efficiency of counteracting *L. monocytogenes* Scott A by *Carnobacterium*, comparisons were made between the divercin-producing *C. divergens* V41 (div+) and *C. divergens* V41C9 (div−), a mutant defective in divercin production (Richard et al., 2003). As expected, div− did not show any inhibitory effect on *L. monocytogenes* cultured on plates in accordance with previous studies (Pilet et al., 1995; Richard et al., 2003). In addition, div+ supernatants resulted in a similar inhibitory effect on plaque-forming by *L. monocytogenes* indicating the efficiency of divercin on *L. monocytogenes* during invasion assays. Nonetheless, both div+ and div− were able to reduce dramatically *L. monocytogenes* plaque-forming on HT29 and HT29 MTX cell lines. The effect of the div+ strain was slightly but significantly higher than that obtained with div− indicating the contribution of bacteriocin activity by the div+ strain since the adhesion rate of both protective cultures was similar on both cell line models. Another bacteriocin-producing *C. maltaromaticum* V1 was also more efficient at limiting the number of plaques formed by *L. monocytogenes* than the non-bacteriocinogenic strain counterpart (*C. maltaromaticum* 2762). This work indicates that non-bacteriocinogenic *Carnobacteria* strains are also effective candidates for limiting the pathogenicity of *L. monocytogenes* using the combined effects of bacteriocin activity and mammalian cell protection. The preheated cell treatment suggests that the protective effect of *Carnobacteria* could be attributed to a passive mechanism. A significant inhibition of *L. monocytogenes* adhesion, invasion and transepithelial translocation was obtained using *Lactobacillus paracasei* but only if this strain was recombined to obtain the expression of *Listeria* adhesion protein (LAP, Lmo1634) in order to interact specifically with the host cell receptor Hsp60 (Koo et al., 2012). In our study, an inhibitory effect of *L. monocytogenes* virulence by *Carnobacterium* was obtained without genetically engineered strains. This could be explained by an increase in epithelial barrier functions due to an interaction with secreted components (Shen et al., 2005). Inhibition mechanisms could also involve specific proteins that accumulate on the cell-surface.
In conclusion, this work demonstrates the potential probiotic effect of *Carnobacterium* strains to attenuate the pathogenesis of *L. monocytogenes* Scott A. The probiotic mechanism results from a bacteriocin effect combined to a protective effect of mammalian cells. Probiotics have positive effects on human health and safety risk assessment of microorganisms intentionally added to food and feed (EFSA, 2014). In the USA, the Food and Drug Administration (FDA) provides a notice inventory of substances that have been approved in terms of safety namely substances generally recognized as safe (GRAS) based on specific usage and dosage for each substance. This inventory lists viable bacteria including *C. maltaromaticum* (FDA, 2015).

Previous studies have shown that they do not present an imminence for human illnesses, nor for nosocomial infections in hospitals (Leisner et al., 2007). The genome sequence of the type strain of *C. maltaromaticum* NCDO2762 (ATCC35586) has shown potential virulence factors but none of the specific virulence factors that are present in *L. monocytogenes* strains and it was thus concluded that there are no human safety concerns for this species (Leisner et al., 2012). The whole genome sequence of *C. divergens* V41 is now being annotated and analyzed in our laboratory for further research on its harmlessness. In addition, *C. maltaromaticum* and *C. divergens* are considered microorganisms with technological beneficial use according to Bourdichon et al. (2012). Recently, *C. divergens* was added to the authoritative list of microorganisms with a QPS status (qualified presumption of safety) validated by EFSA for the safety risk assessment of microorganisms intentionally added to food and feed (EFSA, 2014). In the USA, the Food and Drug Administration (FDA) provides a notice inventory of substances that have been approved in terms of safety namely substances generally recognized as safe (GRAS) based on specific usage and dosage for each substance. This inventory lists viable bacteria including *C. maltaromaticum* (FDA, 2015).

In conclusion, this work demonstrates the potential probiotic effect of *Carnobacterium* strains to attenuate the pathogenesis of *L. monocytogenes* Scott A. The probiotic mechanism results from a bacteriocin effect combined to a protective effect of mammalian cells. Probiotics have positive effects on human health and

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**As described for Streptococcus pneumoniae** (Guiral et al., 2005). However, further analyses are required to unravel the protective effect of mammalian cells by *Carnobacteria*.

We also observed that the mucus layer enhanced the impact of the protective effect of *Carnobacteria*. This was correlated to a significantly higher number of adherent cells. Similar results were obtained by Alemka et al. (2010) who reported the contribution of the mucus layer to the potential efficacy of probiotic treatment for the attenuation of *C. jejuni* pathogenicity. Mucus constitutes a physical and chemical protective barrier of epithelial cells. Its complex composition includes electrolytes, plasma proteins, lipids, nucleic acids and a large variety of high molecular weight glycoproteins called mucins that contribute to the viscoelasticity of mucus (Johansson et al., 2011). The mucus is a sheltering interspace for bacteria protecting them from shearing motions due to intestinal peristalsis. In addition, commensal bacteria trapped in the mucus are less motile and could be organized into biofilms reinforcing epithelial cell protection (Zoetendal et al., 2002). Weak interactions between mucus and bacterial cell surfaces such as hydrophilic/hydrophobic bonds or cell appendages such as pili could also contribute to maintaining cells in sheltering interspace (Douillard et al., 2013).

*Carnobacteria* did not alter HT29 cells indicating the absence of cytotoxicity. For their potential use as protective cultures in food, the question of human the safety of these LAB arises. *Carnobacteria* are not known as members of the human gastrointestinal microbial community like several other LAB.
general well-being. They have been historically associated with cultured milk and dairy products. More recently, they have been analyzed for their potential to inhibit pathogenic and spoilage microorganisms (Klaenhammer, 2000; Gill and Guarner, 2004; Grover et al., 2012).

AUTHOR CONTRIBUTIONS

OT conceived the work; MP, JC and OT designed the work; TP performed the experimental work; TP and OT analyzed and interpreted the data; TP drafted the manuscript; OT, MP and JC contributed to the final manuscript.

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