# Influence of culture conditions on the technological properties of Carnobacterium maltaromaticum CNCM I-3298 starters

Amélie Girardeau, C. Puentes, S. Keravec, P. Peteuil, Ioan-Cristian Trelea, Fernanda Fonseca

# Influence of culture conditions on the technological properties of *Carnobacterium maltaromaticum* CNCM I-3298 starters

A. Girardeau[1,2], C. Puentes[1], S. Keravec[2], P. Peteuil[2], I.C. Trelea[1], F. Fonseca[1*]

[1] UMR GMPA, AgroParisTech, INRA, Université Paris-Saclay, 78850 Thiverval-Grignon, France

[2] CRYOLOG, R&D department, 44261 Nantes, France

*Corresponding author: Fernanda Fonseca, INRA, UMR 782 Génie et Microbiologie des Procédés Alimentaires (GMPA), F-78 850, Thiverval-Grignon, France. E-mail: fernanda.fonseca@inra.fr

**Running title:** Influence of culture conditions on *C. maltaromaticum*

## Abstract

**Aim:** Investigate the effect of a broad spectrum of culture conditions on the acidification activity and viability of *Carnobacterium maltaromaticum* CNCM I-3298, the main technological properties that determine the shelf-life of biological Time-Temperature Integrator labels.

**Methods and results:** Cells were cultivated at different temperatures (20 to 37 °C) and pH (6 to 9.5) according to a modified central composite design and harvested at increasing times up to 10 h of stationary phase. Acidification activity and viability of freeze-thawed concentrates were assessed in medium mimicking the biological label. Acidification activity was influenced by all three culture conditions, but pH and harvest time were the most influential. Viability was not significantly affected by the tested range of culture conditions.

**Conclusions:** *C. maltaromaticum* CNCM I-3298 must be cultivated at 20 °C, pH 6 and harvested at the beginning of stationary phase to exhibit fastest acidification activities. However, if slower acidification activities are pursued, the recommended culture conditions are 30 °C, pH 9.5 and a harvest time between 4 to 6 hours of stationary phase.

**Significance and impact of the study:** Quantifying the impact of fermentation temperature, pH and harvest time has led to a predictive model for the production of biological Time-Temperature Integrators covering a broad range of shelf-lives.

**Keywords:** acidification activity, fermentation, lactic acid bacteria, stress response, biological TTI

## Introduction

Time-Temperature Integrators (TTI) work as 'smart-labels' that relay, by an easily interpreted and irreversible sign, the time-temperature history and quality status of the food they are attached to (Taoukis and Labuza, 1989). Biological TTI are usually based on a pH decline of medium contained within the label, as a result of lactic acid bacteria (LAB) growth and acidification. In the event of a "thermal accident" (a more or less significant break of the cold chain), bacteria contained in the label will grow and acidify their environment, causing a color shift and/or an opacification reaction indicating significant alteration (Varlet-Grancher, 2006; Ellouze *et al.*, 2008; Lee and Jung, 2016).

Bacterial strains used for biological TTI must be carefully chosen and configured to closely match the behavior of micro-organisms endogenous to the food being traced. The only biological TTI currently on the market is TopCryo®, produced by Clock-°T (Cryolog, Nantes, Frances). In TopCryo® labels, the growth of *Carnobacterium maltaromaticum* CNCM I-3298 induces a pH

decline of the label medium leading to a color shift from green to red (EU patent no. EP 1 664 334). *C. maltaromaticum* concentrates used to inoculate TopCryo® TTI labels are produced like other LAB starters by a succession of steps: fermentation, concentration, cryoprotection, freezing or freeze-drying and storage until inoculation. To produce TTI labels, stored concentrates are freeze-thawed, and a certain volume of cell suspension is mixed into the label medium, depending on desired shelf-life (i.e. low concentrations for long shelf-lives). The labels are then frozen and stored at -80 °C until use. For activation, they are freeze-thawed at ambient temperature. The shelf-life covered by the label is thus dependent on both cell concentration and their acidification activity. Today, TopCryo® covers shelf-lives ranging from 30 h to 192 h (8 days) at 4 °C. To further extend the range of the current commercialized biological TTI without changing the bacterial strain that is currently used, two main strategies can be foreseen to modulate acidification activity: (1) modify the label medium, which would lead to several technological constraints and/or (2) modulate the physiological state of *C. maltaromaticum* CNCM I-3298.

It is known that physiological state modulation of LAB mainly depends on fermentation parameters (medium composition, temperature, pH, harvesting time/growth phase) (Rault *et al.*, 2009; Velly *et al.*, 2014; Brillet-Viel *et al.*, 2016; Hansen *et al.*, 2016). One of the main characteristics of *Carnobacteria* is their psychrotrophic nature and their ability to grow at high pH levels (Edima *et al.*, 2006) . The cardinal values of *C. maltaromaticum* CNCM I-3298 were previously assessed by Ellouze *et al*. (2008) in Brain Heart Infusion (BHI) culture medium (**table 1**) and using the experimental protocols developed by Pinon *et al*. (2004) and Membré *et al*. (2004). While exact values may change depending on the culture medium, they illustrate the broad range

of temperature and pH at which *C. maltaromaticum* grows: -5 to 36 °C, pH 5 to 10 (Ellouze et al., 2008).

Few studies have been reported on the fermentation of *Carnobacterium maltaromaticum* (**table 1**) and they mostly concern laboratory fermentations performed in different culture media, at unregulated pH values or restricted range of pH (pH 6 to 6.8). To our knowledge, the impact of fermentation parameters on the physiological state of *C. maltaromaticum* concentrates in conditions close to industrial practices has not yet been reported.

The main objective of this work was therefore to study the effect of three fermentation parameters (temperature, pH and harvest time) on the technological properties, namely viability and acidification activity, of freeze-thawed *C. maltaromaticum* concentrates, in a medium mimicking the TTI label. Mapping the acidification activities of freeze-thawed *C. maltaromaticum* concentrates obtained under a wide range of fermentation conditions would reveal the extent of the possible shelf-life modifications of TopCryo® labels that can be achieved by changing fermentation parameters.

# Materials and Methods

**Frozen starter production process**

An overview of the starter production process is given in **figure 1 A**.

- Strain and inoculum preparation

Stock culture of *Carnobacterium maltaromaticum* CNCM I-3298 was stored at -80 °C in milk (EPI Ingredients, Ancenis, France) supplemented with 15 % (wt/wt) glycerol (VWR, Leuven, Belgium). Inocula were grown in a medium (referred to as MN medium) composed of the following ingredients (g $kg^{-1}$) : trehalose (Treha™; Tokyo, Japan), 40.0; proteose peptone (Oxoid; Waltham, MA, USA), 20.0; yeast extract (Humeau; La-Chapelle-sur-Erdre, France), 5.0; Tween 80 (VWR; Leuven, Belgium), 20.0; $MnSO_4$ (Merck; Darmstadt, Germany), 0.41 and $MgSO_4$ (Merck; Darmstadt, Germany), 0.056. The medium was sterilized at 121°C for 20 min. 10 mL of sterilized medium was inoculated with 100 µL of stock culture and incubated for 13 to 16 h at 30 °C. 1 mL of the resulting culture was transferred into 50 mL of fresh medium before being incubated for 11 h under the same conditions. The resulting culture was used to inoculate the bioreactor.

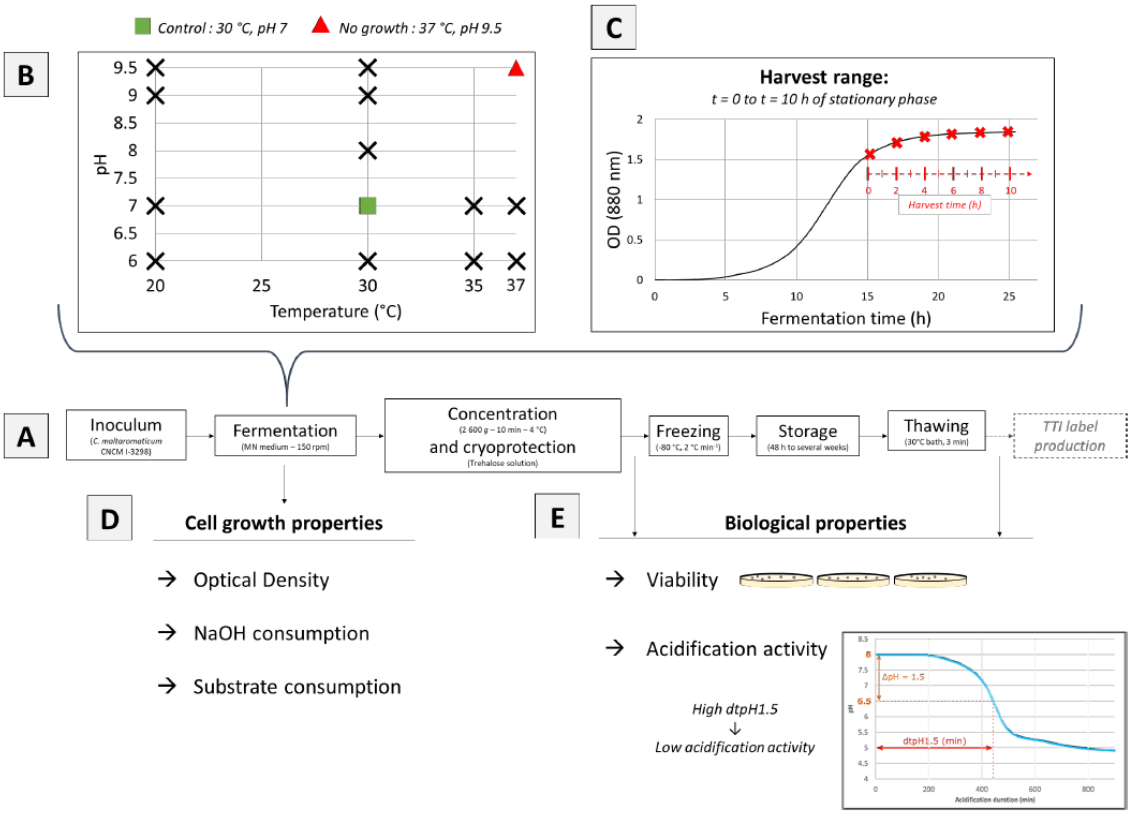


**Figure 1:** Diagram of experimental approach applied to quantify the effect of fermentation temperature, pH and harvest time on viability and acidification activity of *C. maltaromaticum* CNCM I-3298 concentrates: **(A)** Frozen starter production process; **(B)** Range of pH and temperatures tested; **(C)** Harvest time determination; **(D)** Cell growth properties measured throughout fermentation; **(E)** Biological properties of cell concentrates measured before freezing and after freeze-thawing.

- Fermentation

The MN medium was also used as the fermentation culture medium. After adjusting pH to the desired value with NaOH solution (VWR, Pennsylvania, USA) the medium was sterilized at 121 °C for 20 min, cooled down to ambient temperature and introduced into a 3.5 L working volume bioreactor (Minifors, Infors HT, Bottmingen, Switzerland). The pH value of the sterilized medium

was verified and adjusted again when necessary with NaOH or $H_2SO_4$ solution. The inoculation was performed at an initial concentration of approximately $10^7$ CFU $mL^{-1}$. An agitation speed of 150 rpm was applied for culture homogenization. Temperature and pH were set at different values varying between 20 and 37 °C and pH 6 and 9.5, according to the experimental design presented in **figure 1 (B)**. pH was controlled by the automatic addition of 5N NaOH.

- Concentration, cryoprotection and freezing

Cells were harvested at increasing times of fermentation, starting at the beginning of stationary phase (t = 0 h) and up to t = 10 h into stationary phase. Harvested cell suspensions were concentrated by centrifugation (Avanti® J-E centrifuge; Beckman Coulter; Fullerton, CA) at 2 635 *g* for 10 min at 4 °C. Resulting cell pellets were then re-suspended in a cryoprotective solution at a ratio of 1 : 2 (1 g of concentrated cells : 2 g of protective solution) prior to freezing. The protective solution was composed of 200 g $L^{-1}$ of trehalose (Treha™; Tokyo, Japan) and 9 g $L^{-1}$ of NaCl and sterilized at 121 °C for 20 min. Cryoprotected cell suspensions were distributed in 1 mL cryotubes (Sarstedt; Nümbrecht, Germany) prior to freezing at -80 °C. Re-activation was carried out by thawing the frozen cell suspensions in a 30 °C water bath for 3 min.

**Experimental design**

A modified central composite experimental design (**Fig. 1, B and C**) was carried out to investigate the effect of three fermentation parameters (temperature, pH and harvest time) on acidification activity and viability. The experimental ranges chosen for temperature were 20 °C to 37 °C, 6 to

9.5 for pH (**Fig.1, B**) and up to 10 hours after the beginning of stationary phase, for harvest time (**Fig. 1, C**). These ranges were chosen according to the cardinal values of *C. maltaromaticum* determined by Ellouze *et al.* (2008) and preliminary tests. The range of fermentation conditions tested were aimed at creating the largest possible scope of moderately stressful conditions that would change the physiological state of *C. maltaromaticum*, while still allowing enough growth to reach a cell concentration compatible with industrial application (> 1 g $L^{-1}$).

**Kinetic measurements in the bioreactor: cell growth, acidification activity and substrate consumption**

The cell growth and acidification activity were monitored throughout fermentation (**Fig. 1, D**). Cell growth was monitored by an infra-red probe (Excell210, CellD, Roquemaure, France) continuously measuring absorbance at 880 nm (data acquisition every minute). Acidification activity in the bioreactor was determined according to the volume of NaOH solution injected into the bioreactor to maintain a constant pH (data acquisition every minute) with the IRIS NT V5 software (Infors, AG, Bottmingen, Switzerland). NaOH consumption rate (dv/dt, in mL $min^{-1}$) was used to determine the maximum acidification rate. Trehalose concentrations were determined by high-performance liquid chromatography (HPLC, Waters Associates, Millipore; Molsheim, France) in order to verify that carbon source was not depleted (i.e. absence of supplementary stress from carbon source starvation). Before HPLC analysis, each sample was centrifuged at 16 000 *g* for 30 min at 4 °C then filtered through 0.22 µm pores (Sartorius stedim, Biotech; Göttingen, Germany). Analyses were made using a cation exchange column (Aminex Ion Exclusion HPX-87 300x7.8 mm, Biorad,

Richmond, USA) at 35 °C. Mobile phase was 0.005 M $H_2SO_4$ and flow rate was set at 0.6 mL $min^{-1}$ (LC-6A pump, Shimadzu, Courtaboeuf).

**Biological properties of starters**

- Viability

Cell viability was measured using the agar plate count method (**Fig. 1, E**). Thawed cell suspensions were diluted in saline water then plated into Plate Count Agar (Biokar Diagnostics, Paris, France) and aerobically incubated at 30 °C for 48 h. The cell plate counts were expressed in UFC $mL^{-1}$ and results were obtained in triplicate.

- Acidification activity in TTI-like medium

Acidification activity was measured using the CINAC system (Corrieu *et al.*, 1988). The measurements were performed at 30 °C, in a growth medium similar to the one used in TopCryo® labels. The medium contained the following ingredients (g $kg^{-1}$) : glycerol (VWR; Leuven, Belgium), 60.0; trehalose (Treha™; Tokyo, Japan), 15.0; proteose peptone (Oxoid; Waltham, MA, USA), 10.0; yeast extract (Humeau; La-Chapelle-sur-Erdre, France), 5.0; Tween 80 (VWR; Leuven, Belgium), 5.0; $MnSO_4$ (Merck; Darmstadt, Germany), 0.41 and $MgSO_4$ (Merck; Darmstadt, Germany), 0.056. The medium was sterilized at 121 °C for 20 min before being aseptically distributed into 150 mL flasks. Each flask was inoculated with 15 µL of fresh or thawed bacterial suspensions. For each sample, the acidification activity was characterized as the time necessary for a pH drop of 1.5

upH, namely dtpH1.5 (in min) (**Fig.1, E**). Thus, the shorter the value of the dtpH1.5 descriptor was, the higher the acidification activity was. Acidification activities were measured in triplicate.

**Statistical analysis**

Student's paired t-tests were performed using the XLSTAT 2018.3 software (Addinsoft, Paris, France) to evaluate the effect of freezing on viability (in CFU $mL^{-1}$) and on acidification activity (dtpH1.5, in min). Stepwise descending multiple regression analyses were performed to quantify the effect of independent variables (temperature ($X_1$), pH ($X_2$) and harvest time ($X_3$)) on viability and acidification activity (Y) using the MATLAB R2014b software equipped with the Statistics Toolbox (The Mathworks, Inc., Natick, MA). The applied regression model was a second-order polynomial with interactions of the following form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

**(Eq. 1)**

$\beta_0$, $\beta_i$, $\beta_{ii}$ and $\beta_{ij}$ are respectively the intercept, linear, quadratic and interaction coefficients. The adequacy of the model was assessed by its coefficient of determination ($R^2$), a measurement of the percentage of total data variance explained by the model. Regression was initially performed with the complete model. Parameters not significantly different from zero at a 0.05 level were iteratively removed from the model (i.e., set to exactly zero) starting with the one exhibiting the highest coefficient of variation. Response surface plots were generated from the

fitted quadratic polynomial equations obtained in order to visually assess the relationships between variables and responses.

## Results

### Effect of freezing on the viability and acidification activity of *Carnobacterium maltaromaticum* CNCM I-3298

The reported freeze-thaw resistance of *C. maltaromaticum* (Walker *et al*., 2006) was corroborated in this work by measuring the viability (in UFC $mL^{-1}$) and acidification activity (dtpH1.5, in min) of cells produced in reference conditions (pH7, 30°C), before and after freezing. Student's paired t-tests were conducted to compare the viability and acidification activity of cells before and after freezing. Cells produced at pH 7 and 30°C displayed no significant loss of viability or acidification activity after being freeze-thawed ($n = 6$, p-value > 0.05). Moreover, similar freeze-thaw resistance was exhibited by cells produced in two extreme fermentation conditions (pH 9, 30°C and pH 6, 30°C). All the following measurements of viability and acidification activity were thus performed on freeze-thawed concentrates.

### Effect of fermentation conditions (temperature, pH and harvest time) on the acidification activity of TTI label starters.

All residual concentrations of trehalose measured at each harvest time were above 1.3 g $L^{-1}$, thus confirming that biomass had not been subjected to carbon starvation, regardless of applied

fermentation parameters. The effects of fermentation temperature, fermentation pH and harvest time on the acidification activity (dtpH1.5, in min) of the freeze-thawed concentrates were examined in a culture medium close to the TTI label medium. The dtpH1.5 descriptor used in this study for assessing the acidification activity changes, is the main technological property of the produced cell concentrates. It varied between 392 $\pm$ 4 min and 625 $\pm$ 2 min, depending on the culture conditions. Low dtpH1.5 values corresponded to high acidification activities. Results of the stepwise multiple regression analysis are summarized in **table 2**. They enabled quantification of the linear, quadratic and interactive effects of three independent fermentation variables on dtpH1.5, the main technological property of the produced cell concentrates. The model explains more than 87 % of dtpH1.5 variability, according to the coefficient of determination ($R^2$) (**table 2**). Consequently, the model adequately predicts acidification activity of the TTI label starters, as a function of their fermentation temperature and pH as well as the time at which they were harvested. The accuracy of the model and possible outliers can be visualized in **figure 2**, where the 51 predicted versus measured values of dtpH1.5 are plotted.

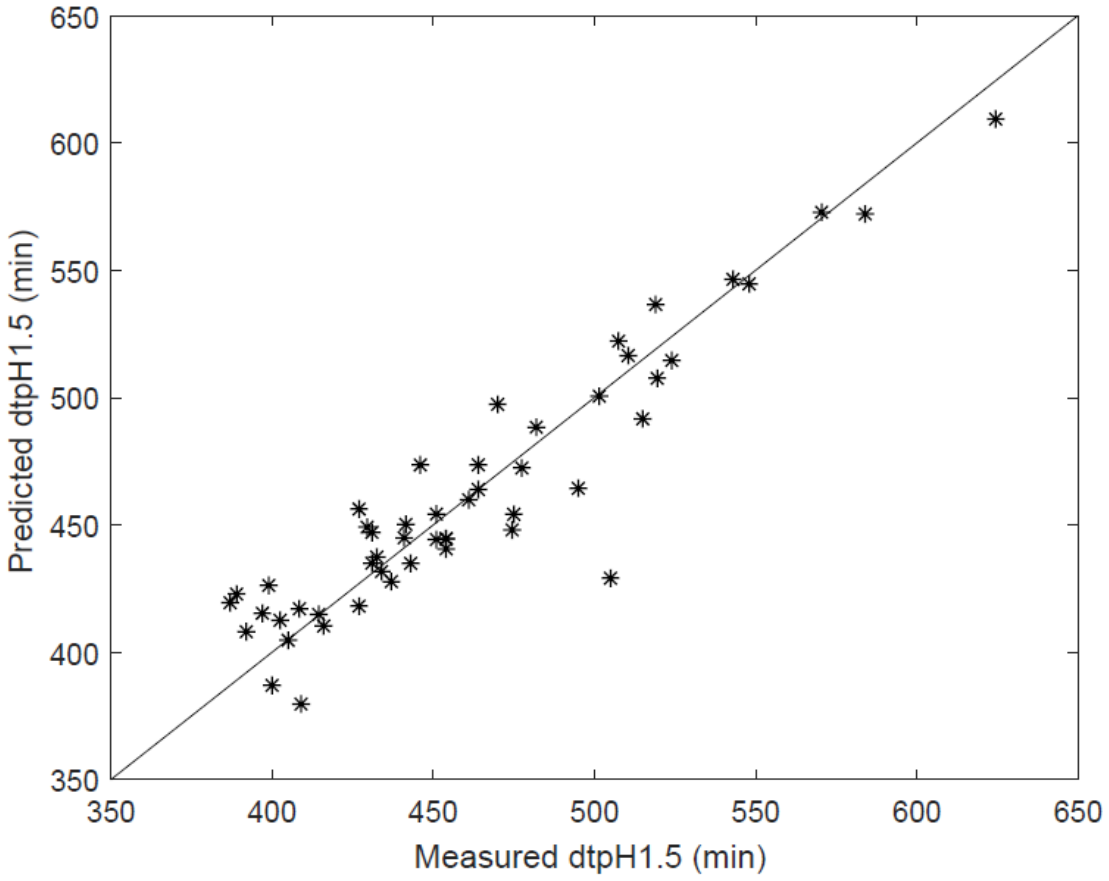


**Figure 2:** dtpH1.5 values predicted by the regression model versus dtpH1.5 values measured. The corresponding coefficient of determination ($R^2$ = 0.8734) indicated adequate prediction of acidification activity in a TTI-like medium.

**Figure 3** shows response surfaces generated with the model for fermentations carried out at 30 °C (**A**), 20 °C (**B**) and 37 °C (**C**), enabling visual assessment of the conjugated effect of fermentation pH and harvest time on dtpH1.5. The limited influence of temperature can be observed through the nearly identical shapes of the response surfaces at both ends of the temperature range (**fig**

**3., B and C**). At all temperatures, dtpH1.5 increased with both pH and harvest time in the considered range. According to the model, for fermentations carried out at 30 °C (**fig. 3, A**), highest acidification activity (lowest dtpH1.5: 407 min) could be achieved with cells cultured at pH 6 and harvested at the beginning of stationary phase (harvest time = 0 h). Lowest acidification activity (highest dtpH1.5: 650 min) could be achieved with cells cultured at pH 9.5, 30 °C (**fig. 3, A**) and harvested after 10 hours of stationary phase (harvest time = 10 h). Cultivating cells at 20 °C instead of 30 °C would lead to higher acidification activities (lower dtpH1.5) (**fig. 3, B**) and inversely when cultivating them at 37 °C (**fig. 3, C**). At pH 9.5 and 37 °C, no growth was observed experimentally. The complete data set used to generate the model is available in supporting information (**table S1**).

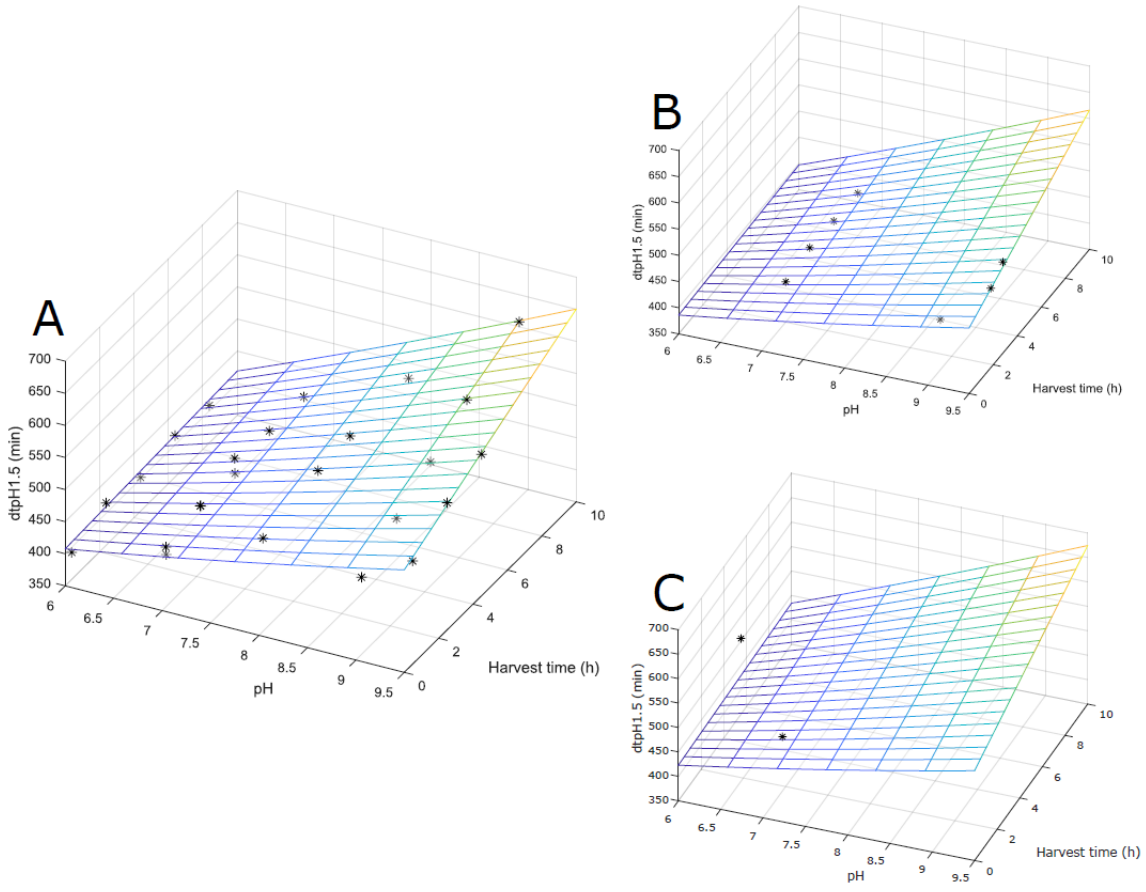


**Figure 3:** Response surface representations of the effect of fermentation pH and harvest time on the dtpH1.5 values of *C. maltaromaticum* CNCM I-3298 concentrates produced at 30 °C **(A)**, 20 °C **(B)** and 37 °C **(C)**. **experimental data points employed in the model at the given temperatures.*

**Effect of fermentation conditions on viability (CFU $mL^{-1}$)**

The viability of freeze-thawed concentrates assessed by the agar plate count method varied between 3.1 $10^{10}$ CFU $mL^{-1}$ and 4.0 $10^{11}$ CFU $mL^{-1}$ for all applied fermentation conditions, similarly

to the range measured in the industrial production of *C. maltaromaticum* CNCM I-3298 concentrates at reference conditions. Stepwise descending multiple regression analyses were performed to investigate the effect of the fermentation conditions on the viability (in CFU $mL^{-1}$) of the freeze-thawed cell concentrates produced according to the experimental design. Results are summarized in **table 3** and the complete data set used to generate the model is available in supporting information (**table S1**).

Only 45 % of viability measurements were explained by the quadratic effect of fermentation pH and the effect of the other design variables were not significant at a 0.05 level. Despite the slight influence of pH on viability, the poor coefficient of determination ($R^2$) level suggests that the range of fermentation parameters applied in the experimental design had a limited influence on the viability of the harvested cells, compared to the variability of the measurement.

## Discussion

Industrial starter production is generally optimized for obtaining high biomass yields and concentrated cells displaying high acidification activity and viability upon long term storage. However, the utilization of *Carnobacterium maltaromaticum* starters for producing biological TTI labels involves different challenges. TTI labels are meant to be used as a tool to measure food shelf-life that can largely vary according to the food type: 3 to 21 days for various meats and 0.5 to 3 days for shellfish, under modified atmosphere packaging, at 4 °C (Dalgaard, 1995). To extend food shelf-life ranges, the physiological state modulation of a strain through fermentation condition adjustment, appears to be a path worth pursuing. For use in TTI labels, physiological

state of *Carnobacterium maltaromaticum* starters can mainly be associated with acidification activity and viable cell concentration. The objective of this work was thus to map the range of acidification activity and viability that can be covered by *Carnobacterium maltaromaticum* CNCM I-3298, by modifying fermentation conditions. It was also aimed at modeling the relationship between fermentation conditions and the physiological state of concentrated cells in order to develop a useful tool for producing TTI labels corresponding to a broadened range of shelf-lives.

All tested fermentation conditions led to biomass yields superior to the threshold for industrial production (1 g $L^{-1}$). All other production steps (concentration, cryoprotection and freezing) were very close to those currently used in industrial production of *C. maltaromaticum* concentrates for biological TTI labels, thus making the direct industrial application of results from this study possible.

As expected, freezing had no significant effect on viability or acidification activity of *C. maltaromaticum* CNCM I-3298 concentrates. This is not surprising, given the psychrotrophic nature of *Carnobacteria* and their distribution in the natural environment (Leisner et al., 2007). In previous work investigating the freeze-thaw tolerance of bacterial soil communities that overwinter, *C. maltaromaticum* showed no significant viability losses after three freeze-thaw cycles and only 1 log CFU $mL^{-1}$ after 12 freeze-thaw cycles. 28 cycles were needed to observe a loss of viability of two to three orders of magnitude (Walker *et al*., 2006). *C. maltaromaticum*'s resistance to freezing could be in part due to their small size (Fonseca *et al*., 2000; Dumont *et al*., 2004). The low sensitivity of small LAB has been related to their resulting high cell surface area to volume (SA/V) ratio, thus facilitating water efflux from the cell during freezing (Fonseca *et al*.,

2000; Dumont *et al*., 2004). Cell sizes of *Carnobacteria* vary between 0.5 and 0.7 µm in diameter and 1.1 and 3.0 µm in length (Schillinger and Holzapfel, 1995), making them relatively small rods with a SA/V ratio of 3.2 to 4.9 $\mu m^{-1}$. They are similar to the size of *Lactobacillus delbrueckii* ssp. *lactis* (0.83 µm in diameter, 3.3 µm in length, SA/V ratio of 5.4 $\mu m^{-1}$), a rod reported to be resistant to freeze-thawing (Fonseca *et al*., 2000). This strain presented similar freeze-thaw resistance to small *Streptococci* and much higher resistance than *Lactobacillus delbrueckii* spp. *bulgaricus* CFL1, respectively exhibiting high (10 to 11 $\mu m^{-1}$) and low (2.9 $\mu m^{-1}$) SA/V ratios. Furthermore, the freeze-thaw resistance of *C. maltaromaticum* CNCM I-3298 concentrates was certainly enhanced in our study by the addition of trehalose before freezing, a sugar known to be an effective cryoprotectant (de Antoni *et al.*, 1989). *C. maltaromaticum*'s resistance to freeze-thawing confirmed previous work (Walker *et al*. , 2006) and supported industrial practices that directly assesses acidification activity and viability on freeze-thawed concentrates, thus inferring that the physiological states of the cells are similar before and after freezing.

Fermentation temperature, pH and harvest time are known factors that affect LAB starter quality (van de Guchte et al., 2002). In this study, *Carnobacterium maltaromaticum* CNCM I-3298 exhibited significant sensitivity to all three factors. Extreme acidification activities of *C. maltaromaticum* concentrates achieved in this study are presented in **figure 4** as a function of harvest time in order to help visually illustrate the effect of each factor and facilitate comparison with control conditions (**fig. 4, fermentation A**). The reference conditions for industrial production of *C. maltaromaticum* concentrates were 30 °C, pH 7 and a harvest time close to the beginning of stationary phase, as indicated by the "TopCryo®" arrow in **figure 4**. Reference

fermentation conditions led to starters exhibiting intermediate acidification activities (dtpH1.5 = 440 min). Highest acidification activity values (i.e. lowest dtpH1.5) were measured in cells produced at pH 6, 30 °C and harvested at the beginning of stationary phase (**fig. 4, fermentation B**). Lowest acidification activity values (i.e. highest dtpH1.5) were measured for those produced at pH 9.5, 30 °C and harvested after 4 hours of stationary phase (**fig. 4, fermentation C**).

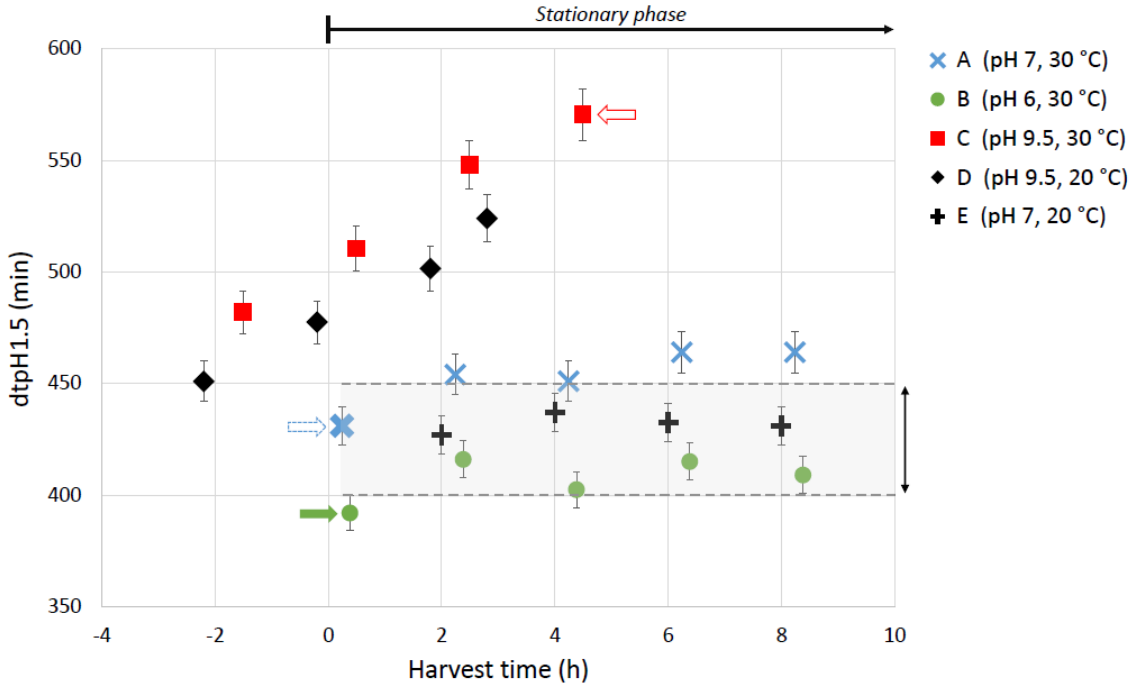


**Figure 4:** dtpH1.5 values of *C. maltaromaticum* CNCM I-3298 concentrates harvested at increasing times of fermentation, carried out at different values of pH and temperature. Fermentation conditions leading to best (full green arrow) and worst (empty red arrow) acidification activities are indicated, as well as the reference conditions corresponding to the TopCryo® commercial TTI (dashed, empty blue arrow). The range of acidification activities leading to technologically similar shelf-lives to the control is signaled by dotted lines. Harvests done before stationary phase are of no industrial interest.

The mean-centered coefficients of the model describing the effect temperature, pH and harvest time on acidification activity are presented in **table 4**. Measurement units are different between the three culture parameters (temperature in °C, pH in upH and harvest time in h) and the response variable (dtpH1.5 in min). The mean-centered analysis makes it possible to reduce coefficients to the same scale in order to rank the influence of the culture variables on dtpH1.5. The variable that is most influent on dtpH1.5 is thus the interactive variable between pH and harvest time (2.218) and the least influent is the interactive variable between pH and temperature (0.289) (**table 4**).

Although the effect of temperature evidenced in **table 4** only appears through the interaction variable with pH, its moderate effect can still be observed in **figure 4**. There is indeed a positive influence of low temperature on acidification activity (**fig. 4, fermentations A *vs* E and C *vs* D**). Low temperature was also reported to induce high acidification activity in both *Lactobacillus sakei* (Hüfner and Hertel, 2008), another psychrotrophic LAB, and *Lactococcus lactis* (Wouters et al., 2000). The adaptive response of LAB to temperature downshift has been linked to the production of cold induced proteins (Panoff *et al.*, 1994; Wouters *et al.*, 1999). During cold acclimation of *Lactococcus piscium* CNCM I-4031 at 5 °C, Garnier *et al.* (2010) detected an upregulation of the histidyl phosphor-carrier protein (HPr), a protein that has been linked to glycolysis regulation (Deutscher *et al*., 2006). The cold induction of the HPr protein has likewise been observed in *Lactococcus lactis* (Wouters *et al.*, 2000), *Bacillus subtilis* (Graumann *et al.*, 1997) and *Lactobacillus sakei* (Marceau *et al.*, 2004). When lowering temperatures, enzyme-catalyzed reactions slow down. As suggested by Hüfner and Hertel (2008), it can thus be expected that cold

induces higher production of glycolytic enzymes, to compensate for slower metabolism (glycolytic capacity) at low temperatures. In their study on *Lc. lactis*, Wouters *et al.* (2000) observed an increase of the maximal glycolytic activity by 2.5 fold following a temperature downshift. We could consequently speculate that an increase in the production of certain proteins (such as HPr) in *C. maltaromaticum* CNCM I-3298 during growth at sub-optimal temperatures (i.e. 20 °C) would lead to an increased acidification activity of the harvested cells at higher temperatures (i.e. 30 °C), such as those used for the industrial quality control in TTI-like medium.

A strong effect of pH on the acidification activity of *C. maltaromaticum* can be observed in **figure 4**. During this study, highest acidification activity values were measured in cells produced at pH 6 (**fig. 4, fermentation B**) and lowest in those produced at pH 9.5 (**fig. 4, fermentations C and D**). This is corroborated by the mean-centered coefficient value attached to the quadratic effect of pH presented in **table 4**. It is generally known that acid adaptation systems in LAB promote synthesis of stress proteins associated with improved stress resistance (Lorca and de Valdez, 2001). The positive effect of low pH on acidification activity observed in this work supports a study aimed at optimizing cell growth and bacteriocin activity of *Carnobacterium divergens* V41 where low pH (i.e. pH 6.5 compared to pH 8) was also shown to improve metabolic activity (Brillet-Viel *et al.*, 2016). Low pH may thus favor metabolic activity in all *Carnobacteria*.

Remarkably, in this study the negative effect of high pH on acidification activity is greatly enhanced by increasing harvest times, as can be observed for pH 9.5 (**fig. 4, fermentations C and D**). The interaction between pH and harvest time displays the highest mean-centered coefficient

value (2.218, **table 4**), thus appearing to be the most influent variable on the dtpH1.5 descriptor. In the studied parameter range, it notably outweighs the seemingly positive effect of increased harvest time on dtpH1.5 brought about by the negative value of its coefficient: -1.805 (**table 4**). The interaction between pH and harvest time on acidification activity was reported to have a similar effect on *Lactococcus lactis* concentrates produced during batch fermentation (Hansen *et al.*, 2016). Highest acidification activities were achieved at low pH (pH 5.5) and the negative effect of increasing harvest times during stationary phase was only seen at high pH (pH 6.5 and 7.5). In their study, Hansen *et al.* (2016) correlated low *Lc. lactis* acidification activities with decreasing cell sizes observed upon entry into stationary phase during fermentations carried out at pH 6.5 and 7.5, but not at pH 5.5. Shrinkage has been generally linked to nutrient starvation (Hochman, 1997), the most frequent type of stress provoking entry into stationary phase (van de Guchte *et al.*, 2002). Interestingly, a mechanism reported to favor cell survival after nutrient depletion is the production of extra ATP through the arginine deiminase (ADI) pathway (Arena *et al*., 1999; Stuart *et al*., 1999), that happens to partly be induced by acid stress (de Angelis and Gobbetti, 2004). The production of ATP by the ADI pathway is due to arginine catabolism and the production of $NH_3$. This helps restore optimal intracellular pH during acid stress (Marquis *et al.*, 1987). The ADI pathway is present in both *Lc. Lactis* (Budin-Verneuil *et al.*, 2003) and *C. maltaromaticum* (Leisner, *et al.*, 1994) and in *Lactobacillus bulgaricus*, a strain that also exhibited more stable acidification activities during stationary phase at pH 5 compared to pH 6 (Rault *et al*., 2009). It is known that the availability of certain essential substrates is linked to cell environmental conditions (Konings *et al.*, 1997). The negative, but still moderate effect of stationary phase when pH is low could therefore also be explained by a decreased availability of certain substrates, rather than complete

depletion. Consequently, at low pH the acidification activity increase (or its maintenance) of *C. maltaromaticum*, *Lc. lactis* and *Lb. bulgaricus* during stationary phase could be explained by a less critical nutritional deprivation, in addition to the induction of the ADI pathway.

All TopCryo® labels are currently inoculated with *C. maltaromaticum* starters produced by fermentations carried out in control conditions and always harvested at the same culture time (**figure 4**). Maximum range of shelf-life covered by these labels at 4 °C is 30 h to 192 h. Concentrates produced during fermentations carried out at 30 °C, pH 6 (**fig. 4, fermentation B**) and harvested at beginning of stationary phase, could potentially reduce the lower limit of TopCryo®'s range by 2 h (-7 % of the current lower limit). On the other hand, concentrates produced at 30 °C, pH 9.5 (**fig 4, fermentation C**) and harvested 4 h into stationary phase could increase the upper limit of the range by 68 h (+35% of the current upper limit). The shelf-life range of TopCryo® is presently achieved by inoculating the labels with varying cell concentrations (i.e. lower cell concentrations for longer shelf-lives and higher cell concentrations for shorter shelf-lives). The difference in acidification activities observed today in TopCryo® labels is thus directly correlated to viable cell concentration. It was therefore important to confirm that the acidification activity modulation presented in this study was not due to gain or loss of starter cell viability. To this end, the influence of the three studied fermentation parameters on viability was analyzed using the same stepwise multiple regression analysis method as the one used for the acidification activity descriptor (dtpH1.5). As expected for psychrotrophic micro-organisms, the range of tested temperatures did not show any significant effect on viability, and neither did harvest time. The only parameter to have an effect on viability was pH, but it only explained 45 % of data (**table**

**3**). It is important to note that due to the intrinsic variability of the plate count agar method, it is common practice to only consider viability variations above 0.3 log CFU $mL^{-1}$ as significant. The variability of this method was particularly critical here as cultures of *Carnobacteria* cells can be present in single, pair or short chains (Schillinger and Holzapfel, 1995). Furthermore, cell chain length is dependent on culture conditions and can significantly affect CFU counts. However, given that plate count variability in this study was under 0.3 log, it can safely be suggested that cell viability was not significantly affected by the applied fermentation conditions. This is substantiated by a study aimed at developing a medium for the selective enumeration of *C. maltaromaticum*, where viability did not vary significantly at pH values ranging from 5 to 9 (Edima *et al.*, 2006). Edima *et al.* (2008) reported that although growth stops under pH 5.0, *C. maltaromaticum* is able to sustain its viability at an even lower pH (pH 4.0 in coculture with *Lc. lactis*). It is therefore not surprising that the viability of *C. maltaromaticum* CNCM I-3298 would not be significantly impacted by the range of pH tested in this experimental design. Furthermore, the results of this work are in agreement with a more recent study investigating the impact of the same three fermentation conditions (pH, temperature and harvest time) on the physiological state of *Lc. lactis* at different steps of starter production (Velly *et al.*, 2014). The authors also observed no significant change in *Lc. lactis* viability, regardless of applied fermentation conditions.

To further explore the possible link between acidification activity and viability, dtpH1.5 measurements of concentrates produced in the experimental design were plotted against their viability (**Figure 5**). A downward trend can be observed, indicating as expected, that as viable cell

concentration increases, dtpH1.5 values decrease (i.e. acidification activity increases). However, this trend explains less than 47 % of variability ($R^2$ = 0.4654). For example, for a dtpH1.5 value of 480 min, corresponding cell viabilities ranged between 10.6 and 11.5 log CFU $mL^{-1}$. Likewise, for a cell viability of 11.4 log CFU $mL^{-1}$, corresponding dtpH1.5 values ranged between 380 and 510 min. Consequently, the changes in acidification activity can be mainly attributed to the physiological state modulation induced by the application of sub-lethal fermentation conditions, and not simply by gain or loss of cell viability.

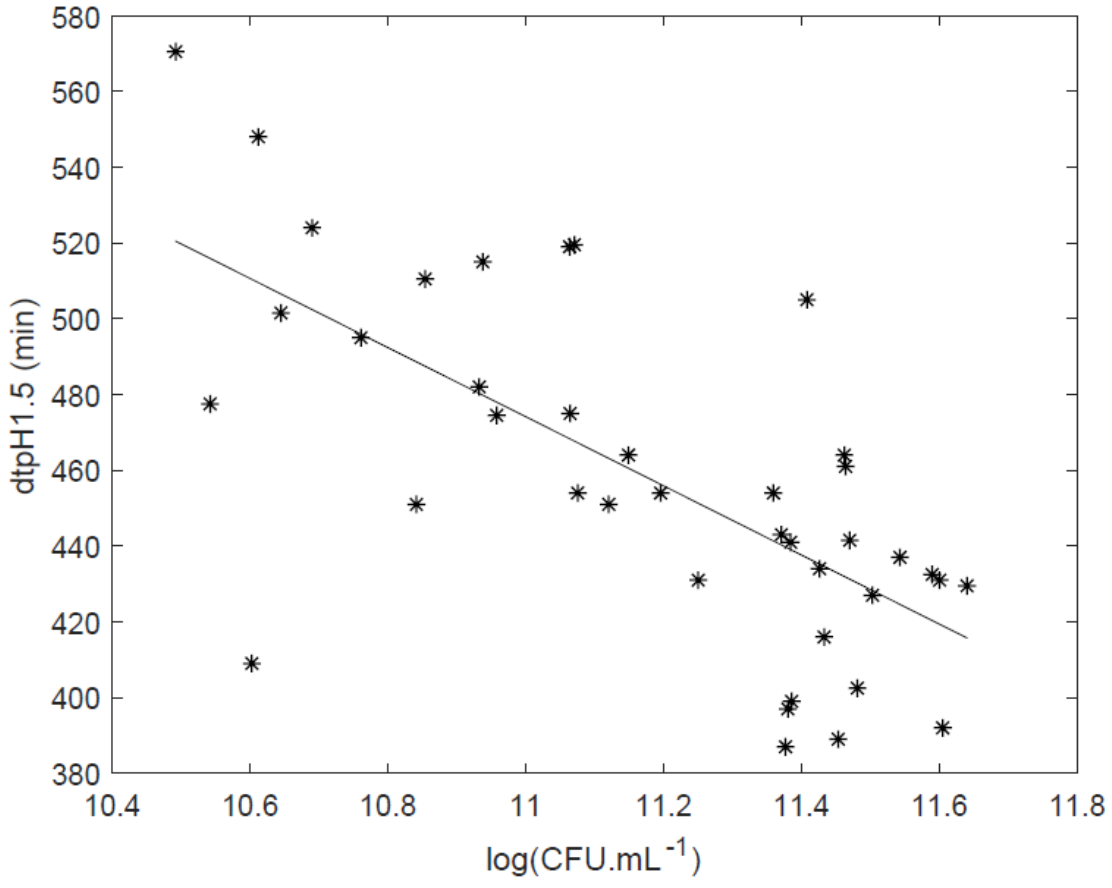


**Figure 5:** Experimental dtpH1.5 (in min) values of all *C. maltaromaticum* CNCM I-3298 concentrates produced following the experimental design, as a function of their viability (in logCFU $mL^{-1}$). $R^2$ = 0.4654

This study has evidenced that the acidification activity of *Carnobacterium maltaromaticum* CNCM I-3298 concentrates can be modulated by applying sub-lethal stress conditions during the fermentation step of production. Although fermentation temperature, pH and harvest time all influenced acidification activity, pH and harvest time were identified to be the most efficient modulation parameters for the production of concentrates exhibiting extreme acidification activities. This work has led to the development of a simple tool in form of a response surface model that can quantify the acidification activity exhibited by *C. maltaromaticum* CNCM I-3298 concentrates according to the applied values of fermentation parameters.

In future work, intracellular pH measurements as well as an integrative approach combining cell membrane characterization (fluidity and lipids composition) and proteomics should offer a better understanding of the mechanisms responsible for the physiological changes induced by sub-lethal stress conditions during fermentation of *Carnobacterium maltaromaticum*.

## Acknowledgements

This work was supported by Clock-°T (Cryolog, Nantes, France). This project has also received funding from the European Union's Horizon 2020 research and innovation program under grant agreement N° 777657. The authors thank Dr Marie-Nöelle Leclercq-Perlat for the HPLC analyses.

## Conflict of Interest

No conflict of interest declared.

## References


Afzal, M.I., Delaunay, S., Paris, C., Borges, F., Revol-Junelles, A.-M., and Cailliez-Grimal, C. (2012). Identification of metabolic pathways involved in the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism by *Carnobacterium maltaromaticum* LMA 28. *Int J Food Microbiol* **157**, 332–339.

Afzal, M.I., Boulahya, K.-A., Paris, C., Delaunay, S., and Cailliez-Grimal, C. (2013). Effect of oxygen on the biosynthesis of flavor compound 3-methylbutanal from leucine catabolism during batch culture in *Carnobacterium maltaromaticum* LMA 28. *J Dairy Sci* **96**, 352–359.

Arena, M.E., Saguir, F.M., and Manca de Nadra, M.C. (1999). Arginine, citrulline and ornithine metabolism by lactic acid bacteria from wine. *Int J Food Microbiol* **52**, 155–161.

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

Brillet-Viel, A., Pilet, M.-F., Courcoux, P., Prévost, H., and Leroi, F. (2016). Optimization of growth and bacteriocin activity of the food bioprotective *Carnobacterium divergens* V41 in an animal origin protein free medium. *Front Mar Sci* **3**, 168.

Budin-Verneuil, A., Maguin, E., Auffray, Y., Ehrlich, S.D., and Pichereau, V. (2003). An essential role for arginine catabolism in the acid tolerance of *Lactococcus lactis* MG1363. *Lait* **84**, 61–68.

Cailliez-Grimal, C., Edima, H.C., Revol-Junelles, A.-M., and Millière, J.-B. (2007). Short communication: *Carnobacterium maltaromaticum*: The only *Carnobacterium* species in French ripened soft cheeses as revealed by polymerase chain reaction detection. *J Dairy Sci* **90**, 1133–1138.

Corrieu, G., Spinnler, H. E., Picq, D. and Jomier, Y. (1988). Automated system to follow up and control the acidification activity of lactic acid starters. FR88 04456

Dalgaard, P. (1995). Quality and quality changes in fresh fish. Rome: Food and Agriculture Organization of the United Nations.

de Angelis, M., and Gobbetti, M. (2004). Environmental stress responses in *Lactobacillus*: A review. *Proteomics* **4**, 106–122.

de Antoni, G.L., Pérez, P., Abraham, A., and Añón, M.C. (1989). Trehalose, a cryoprotectant for *Lactobacillus bulgaricus*. *Cryobiology* **26**, 149–153.

Deutscher, J., Francke, C., and Postma, P.W. (2006). How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev* **70**, 939–1031.

dos Reis, F.B., de Souza, V.M., Thomaz, M.R.S., Fernandes, L.P., de Oliveira, W.P., and de Martinis, E.C.P. (2011). Use of *Carnobacterium maltaromaticum* cultures and hydroalcoholic extract of Lippia sidoides Cham. against *Listeria monocytogenes* in fish model systems. *Int J Food Microbiol* **146**, 228–234.

Dumont, F., Marechal, P.-A., and Gervais, P. (2004). Cell Size and Water Permeability as Determining Factors for Cell Viability after Freezing at Different Cooling Rates. *Appl Environ Microbiol* **70**, 268–272.

Edima, H.C., Cailliez-Grimal, C., Revol-Junelles, A.-M., Tonti, L., Linder, M., and Millière, J.-B. (2006). A selective enumeration medium for *Carnobacterium maltaromaticum*. *J Microbiol Methods* **68**, 516–521.

Edima, H.C., Cailliez-Grimal, C., Revol-Junelles, A.-M., Rondags, E., and Millière, J.-B. (2008). Short communication: impact of pH and temperature on the acidifying activity of *Carnobacterium maltaromaticum*. *J Dairy Sci* **91**, 3806–3813.

Ellouze, M., Pichaud, M., Bonaiti, C., Coroller, L., Couvert, O., Thuault, D., and Vaillant, R. (2008). Modelling pH evolution and lactic acid production in the growth medium of a lactic acid bacterium: Application to set a biological TTI. *Int J Food Microbiol* **128**, 101–107.

Fonseca, F., Béal, C., and Corrieu, G. (2000). Method of quantifying the loss of acidification activity of lactic acid starters during freezing and frozen storage. *J of Dairy Res* **67**, 83–90.

Garnier, M., Matamoros, S., Chevret, D., Pilet, M.-F., Leroi, F., and Tresse, O. (2010). Adaptation to cold and proteomic responses of the psychrotrophic biopreservative *Lactococcus piscium* strain CNCM I-4031. *Appl Environ Microbiol* **76**, 8011–8018.

Graumann, P., Wendrich, T.M., Weber, M.H., Schröder, K., and Marahiel, M.A. (1997). A family of cold shock proteins in *Bacillus subtilis* is essential for cellular growth and for efficient protein synthesis at optimal and low temperatures. *Mol Microbiol* **25**, 741–756.

Hansen, G., Johansen, C.L., Marten, G., Wilmes, J., Jespersen, L., and Arneborg, N. (2016). Influence of extracellular pH on growth, viability, cell size, acidification activity, and intracellular pH of *Lactococcus lactis* in batch fermentations. *Appl Microbiol Biotechnol* **100**, 5965–5976.

Hochman, A. (1997). Programmed cell death in prokaryotes. *Crit Rev Microbiol* **23**, 207–214.

Hüfner, E., and Hertel, C. (2008). Improvement of raw sausage fermentation by stress-conditioning of the starter organism *Lactobacillus sakei*. *Curr Microbiol* **57**, 490–496.

Konings, W.N., Lolkema, J.S., Bolhuis, H., van Veen, H.W., Poolman, B., and Driessen, A.J.M. (1997). The role of transport processes in survival of lactic acid bacteria, energy transduction and multidrug resistance. *Antonie Van Leeuwenhoek* **71**, 117–128.

Lee, S.J., and Jung, S.W. (2016). Time-temperature indicator, method for manufacturing the time-temperature indicator, quality guarantee system using the time-temperature indicator, and quality guarantee method using the quality guarantee system. US9476083B2

Leisner, J.J., Tidemand, J., and Larsen, L.M. (1994). Catabolism of arginine by *Carnobacterium* spp. isolated from vacuum-packed sugar-salted fish. *Curr Microbiol* **29**, 95–99.

Leisner, J.J., Laursen, B.G., Prévost, H., Drider, D., and Dalgaard, P. (2007). *Carnobacterium*: positive and negative effects in the environment and in foods. *FEMS Microbiol Rev* **31**, 592–613.

Lorca, G.L., and de Valdez, G.F. (2001). A low-pH-inducible, stationary-phase acid tolerance response in *Lactobacillus acidophilus* CRL 639. *Curr Microbiol* **42**, 21–25.

Marceau, A., Zagorec, M., Chaillou, S., Mera, T., and Champomier-Verges, M.-C. (2004). Evidence for involvement of at least six proteins in adaptation of *Lactobacillus sakei* to cold temperatures and addition of NaCl. *Appl Environ Microbiol* ***70***, 7260–7268.

Marquis, R.E., Bender, G.R., Murray, D.R., and Wong, A. (1987). Arginine deiminase system and bacterial adaptation to acid environments. *Appl Environ Microbiol* **53**, 198–200.

Membre, J., Leporq, B., Vialette, M., Mettler, E., Perrier, L., Thuault, D., and Zwietering, M. (2004). Temperature effect on bacterial growth rate: quantitative microbiology approach including cardinal values and variability estimates to perform growth simulations on/in food. *Int J Food Microbiol* **100**, 179–186.

Panoff, J.-M., Legrand, S., Thammavongs, B., and Boutibonnes, P. (1994). The cold shock response in *Lactococcus lactis* subsp. *lactis*. *Curr Microbiol* **29**, 213–216.

Pinon, A., Zwietering, M., Perrier, L., Membre, J.-M., Leporq, B., Mettler, E., Thuault, D., Coroller, L., Stahl, V., and Vialette, M. (2003). Development and validation of experimental protocols for use of cardinal models for prediction of microorganism growth in food products. *Appl Environ Microbiol* **70**, 1081–1087.

Rahman, A., Gleinser, M., Lanhers, M.-C., Riedel, C.U., Foligné, B., Hanse, M., Yen, F.T., Klouj, A., Afzal, M.I., Back, A., et al. (2014). Adaptation of the lactic acid bacterium *Carnobacterium maltaromaticum* LMA 28 to the mammalian gastrointestinal tract: From survival in mice to interaction with human cells. *Int Dairy J* **34**, 93–99.

Rahman, A., El Kheir, S.M., Back, A., Mangavel, C., Revol-Junelles, A.-M., and Borges, F. (2016). Repeat-based bequence typing of *Carnobacterium maltaromaticum*. *Int J Food Microbiol* **226**, 1–4.

Rault, A., Bouix, M., and Beal, C. (2009). Fermentation pH influences the physiological state dynamics of *Lactobacillus bulgaricus* CFL1 during pH-controlled culture. *Appl Environ Microbiol* **75**, 4374–4381.

Schillinger, U., and Holzapfel, W.H. (1995). The genus *Carnobacterium*. In The Genera of Lactic Acid Bacteria, B.J.B. Wood, and W.H. Holzapfel, pp. 307–326. Boston, MA: Springer US.

Stuart, M.R., Chou, L.S., and Weimer, B.C. (1999). Influence of carbohydrate starvation and arginine on culturability and amino acid utilization of *Lactococcus lactis* subsp. *lactis*. *Appl Environ Microbiol* **65**, 9.

Taoukis, P.S., and Labuza, T.P. (1989). Applicability of time-temperature indicators as shelf life monitors of food products. J *Food Sci* **54**, 783–788.

van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D., and Maguin, E. (2002). Stress responses in lactic acid bacteria. In Lactic Acid Bacteria: Genetics, Metabolism and Applications, R.J. Siezen, J. Kok, T. Abee, and G. Schasfsma, pp. 187–216. Dordrecht: Springer Netherlands.

Varlet-Grancher, X. (2006). Time temperature indicator (tti) system. EP20050722847

Velly, H., Fonseca, F., Passot, S., Delacroix-Buchet, A., and Bouix, M. (2014). Cell growth and resistance of *Lactococcus lactis* subsp. *lactis* TOMSC161 following freezing, drying and freeze-dried storage are differentially affected by fermentation conditions. *J Appl Microbiol* **117**, 729–740.

Walker, V.K., Palmer, G.R., and Voordouw, G. (2006). Freeze-thaw tolerance and clues to the winter survival of a soil community. *Appl Environ Microbiol* **72**, 1784–1792.

Wouters, J.A., Rombouts, F.M., Vos, W.M. de, Kuipers, O.P., and Abee, T. (1999). Cold shock proteins and low-temperature response of *Streptococcus thermophilus* CNRZ302. *Appl Environ Microbiol* **65**, 4436–4442.

Wouters, J.A., Kamphuis, H.H., Hugenholtz, J., Kuipers, O.P., de Vos, W.M., and Abee, T. (2000). Changes in glycolytic activity of *Lactococcus lactis* induced by low temperature. *Appl Environ Microbiol* **66**, 3686–3691.

Zhang, P., Badoni, M., Gänzle, M., and Yang, X. (2018). Growth of *Carnobacterium* spp. isolated from chilled vacuum-packaged meat under relevant acidic conditions. *Int J Food Microbiol* **286**, 120–127.

# Tables

**Table 1:** Culture conditions applied in published research involving *Carnobacterium maltaromaticum*

| Strain | Fermentation conditions | | | | | References |
|---|---|---|---|---|---|---|
| | Medium | Atmosphere | **T (°C)** | **pH** | **Harvest time** | |
| *C. maltaromaticum* 3 | Yeast extract, proteose peptone, thiamine, glucose | aerobic | 25 | 6.0 | NA | Borch and Molin (1989) |
| *C. maltaromaticum* LMA 28, LMA 29, LMA 30, DSMZ 20730 | TSB-YE broth | aerobic | 30 | unregulated | NA | Cailliez-Grimal *et al.* (2007); Edima *et al.* (2008); Rahman *et al.* (2014 and 2016) |
| *C. maltaromaticum* CNCM I-3298 | BHI broth | aerobic | 1.75 to 35 | 5.1 to 10.4 | NA | Ellouze *et al.* (2008) |
| *C. maltaromaticum C2, A9b$^{+}$, A9b$^{-}$* | BHI broth modified with fish peptone | aerobic | 25 | unregulated | 24 h | dos Reis *et al.* (2011) |
| *C. maltaromaticum* LMA 28 | TSB-YE broth | aerobic | 30 | unregulated | End of exponential phase | Afzal *et al.* (2012) |
| *C. maltaromaticum* LMA 28 | MCGC broth (synthetic minimal medium) | varied DOC 0 % to 90 % | 30 | 6.8 | NA | Afzal *et al.* (2013) |
| *C. maltaromaticum* (10 strains isolated from vacuum packaged meat) | BHI, lactic acid, sodium acetate | aerobic | 30 | unregulated *(Initial pH: 5.4, 6.2, 7.4)* | NA | (Zhang *et al.* (2018) |

*NA : Not Applicable*
*TSB-YE : Trypton Soy Broth and Yeast Extract*
*BHI : Breath Heart Infusion*

**Table 2:** Regression analysis of the acidification activity (dtpH1.5, in min) of *C. maltaromaticum* CNCM I-3298 cells harvested at increasing times during fermentations carried out at different temperatures and pH. ($X_1$: fermentation temperature; $X_2$: fermentation pH; $X_3$: harvest time) (Eq. 1)

| **Term** | **Estimated coefficient (β)** | ***Min*** | ***Max*** |
|---|---|---|---|
| Intercept | 299.99 | *265.21* | *334.77* |
| $X_3$ *(harvest time)* | -21.05 | *-28.39* | *-13.72* |
| $X_1$ $X_2$ *(temperature x pH)* | 0.3623 | *0.2091* | *0.5154* |
| $X_2$ $X_3$ *(pH x harvest time)* | 3.697 | *2.669* | *4.726* |
| $X_2^2$*(pH x pH)* | 1.178 | *0.8414* | *1.516* |
| $R^2$ = 0.8734 | | *95% confidence interval* | |

**Table 3:** Regression analysis of viability (in CFU $mL^{-1}$) of *C. maltaromaticum* CNCM I-3298 cells harvested at increasing times during fermentations carried out at different temperatures and pH. ($X_2$: fermentation pH) (Eq. 1)

| **Term** | **Estimated coefficient (.$10^{11}$) (β)** | ***Min (.$10^{11}$)*** | ***Max (.$10^{11}$)*** |
|---|---|---|---|
| Intercept | 4.25 | *3.37* | *5.13* |
| $X_2^2$ *(pH x pH)* | -0.0414 | *-0.0564* | *-0.0265* |
| $R^2$ = 0.454 | | 95 % confidence interval | |

**Table 4:** Mean-centered regression analysis of the acidification activity (dtpH1.5) of *C. maltaromaticum* CNCM I-3298 cells harvested at increasing times during fermentations carried out at different temperatures and pH.

| **Variables** | **Mean-centered coefficients** | ***Min*** | ***Max*** |
|---|---|---|---|
| Harvest time | **-1.8048** | -2.4338 | -1.1758 |
| Temperature x pH | **0.2891** | 0.1669 | 0.4114 |
| pH x harvest time | **2.2180** | 1.6012 | 2.8349 |
| $pH^2$ | **0.4440** | 0.3169 | 0.5712 |

# Supporting information

**Table S1:** dtpH1.5 (in min) and viability (in CFU $mL^{-1}$) measurements of freeze thawed *Carnobacterium maltaromaticum* CNCM I-3298 concentrates produced according to the culture conditions of the experimental design presented in **figure 1 (B and C).**

| Fermentation | *Culture conditions* | | | *Biological properties* | |
|---|---|---|---|---|---|
| | pH | T (°C) | Harvest time (h) | dtpH1.5 (min) | viability (CFU $mL^{-1}$) |
| 1 | 7 | 30 | 0.22 | 443 | 2.35E+11 |
| | 7 | 30 | 2.22 | 454 | 1.57E+11 |
| | 7 | 30 | 4.22 | 475 | 1.16E+11 |
| 2 | 7 | 30 | 0.24 | 431 | 1.78E+11 |
| | 7 | 30 | 2.24 | 454 | 1.19E+11 |
| | 7 | 30 | 4.24 | 451 | 1.32E+11 |
| | 7 | 30 | 6.24 | 464 | 1.41E+11 |
| | 7 | 30 | 8.24 | 464 | 2.90E+11 |
| 3 | 6 | 30 | 0.38 | 392 | 4.03E+11 |
| | 6 | 30 | 2.38 | 416 | 2.71E+11 |
| | 6 | 30 | 4.38 | 403 | 3.03E+11 |
| | 6 | 30 | 6.38 | 415 | - |
| | 6 | 30 | 8.38 | 409 | - |
| 4 | 9.5 | 30 | -1.51 | 482 | 8.56E+10 |
| | 9.5 | 30 | 0.49 | 511 | 7.15E+10 |
| | 9.5 | 30 | 2.49 | 548 | 4.09E+10 |
| | 9.5 | 30 | 4.49 | 571 | 3.11E+10 |
| 5 | 7 | 20 | 2.00 | 427 | 3.19E+11 |
| | 7 | 20 | 4.00 | 437 | 3.49E+11 |
| | 7 | 20 | 6.00 | 433 | 3.89E+11 |
| | 7 | 20 | 8.00 | 431 | 3.99E+11 |
| 6 | 7 | 35 | -3.07 | 434 | 2.67E+11 |
| | 7 | 35 | 0.55 | 430 | 4.37E+11 |
| 7 | 7 | 37 | -2.28 | 454 | 2.29E+11 |
| | 7 | 37 | -0.28 | 442 | 2.95E+11 |
| | 7 | 37 | 1.72 | 461 | 2.91E+11 |
| 8 | 9.5 | 20 | -2.20 | 451 | 6.94E+10 |
| | 9.5 | 20 | -0.20 | 478 | 3.48E+10 |
| | 9.5 | 20 | 1.80 | 502 | 4.41E+10 |
| | 9.5 | 20 | 2.80 | 524 | 4.90E+10 |
| 9 | 5.7 | 20 | 5.26 | 409 | 4.01E+10 |
| 10 | 6 | 37 | 5.53 | 505 | 2.56E+11 |
| 11 | 9 | 30 | 0.33 | 470 | - |
| | 9 | 30 | 2.37 | 508 | - |
| | 9 | 30 | 4.35 | 543 | - |

| | | | | | |
|---|---|---|---|---|---|
| | 9 | 30 | 6.45 | 584 | - |
| | 9 | 30 | 9.50 | 625 | - |
| 12 | 9 | 20 | -6.03 | 400 | - |
| | 9 | 20 | -4.58 | 405 | - |
| | 9 | 20 | -0.363 | 427 | - |
| | 9 | 20 | 1.054 | 446 | - |
| 13 | 8 | 30 | -1.68 | 475 | 9.08E+10 |
| | 8 | 30 | 0.237 | 495 | 5.77E+10 |
| | 8 | 30 | 3.437 | 515 | 8.67E+10 |
| | 8 | 30 | 5.304 | 520 | 1.18E+11 |
| | 8 | 30 | 8.704 | 519 | 1.16E+11 |
| 14 | 6 | 35 | -2.83 | 397 | 2.40E+11 |
| | 6 | 35 | 0.87 | 387 | 2.38E+11 |
| | 6 | 35 | 3.887 | 389 | 2.84E+11 |
| | 6 | 35 | 6.854 | 399 | 2.43E+11 |
| | 6 | 35 | 23.437 | 441 | 2.42E+11 |