Fine-Tuning of Process Parameters Modulates Specific Metabolic Bacterial Activities and Aroma Compound Production in Semi-Hard Cheese

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Wenfan Cao [1], Julie Aubert [2], Marie-Bernadette Maillard [1], Françoise Boissel [1], Arlette Leduc [1], Jean-Luc Thomas [1], Stéphanie-Marie Deutsch [1], Bénédicte Camier [1], Ali Kerjouh [1], Sandrine Parayre [1], Marielle Harel-Oger [1], Gilles Garric [1], Anne Thierry [1] and Hélène Falentin [1*]

[1] UMR STLO, INRAE, Institut Agro, 35042, Rennes, France

[2] Université Paris-Saclay, AgroParisTech, INRAE, MIA-Paris,75005, Paris, France

*corresponding author, helene.falentin@inrae.fr

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Abstract

The formation of cheese flavor mainly results from the production of volatile compounds by microorganisms. We investigated how fine-tuning cheese-making process parameters changed the cheese volatilome in a semi-hard cheese inoculated with *Lactococcus (L.) lactis, Lactiplantibacillus (L.) plantarum,* and *Propionibacterium (P.) freudenreichii.* A standard (Std) cheese was compared with three variants of technological itineraries: a shorter salting time (7h versus 10h, Salt7), a shorter stirring time (15 min versus 30 min, Stir15min), or a higher ripening temperature (16°C versus 13°C, Rip16°C). Bacterial counts were similar in the four cheese types, except for a 1.4 $\log_{10}$ reduction of *L. lactis* counts in Rip16°C cheeses after 7 weeks of ripening. Compared to Std, Stir15min and Rip16°C increased propionibacteria activity, causing higher concentrations of acetic, succinic and propanoic acids and lower levels of lactic acid. Rip16 °C accelerated secondary proteolysis and volatile production. We thus demonstrated that fine-tuning process parameters could modulate the cheese volatilome by influencing specific bacterial metabolisms.

Figures and tables can be found at the end of the document.

Introduction

The quality of cheese is markedly dependent on the microorganisms used as starter, for both acidification and aromatization purposes. The activity of microorganisms during cheese manufacture and ripening induces modifications to all milk constituents (carbohydrates, proteins and lipids), which in turn leads to the development of cheese flavor. Indeed, flavor development is a dynamic biochemical process that is impacted by (i) milk composition; (ii) curd processing and ripening conditions, and (iii) enzymes naturally present in cow milk and (iv) the indigenous microorganisms or added as starter [1]. Cheese microorganisms are the primary source of the enzymes that influence flavor development. Flavor compounds include sapid compounds (mainly organic acids, peptides and amino acids), alongside added NaCl, and volatile aroma compounds. The microbiological and biochemical processes involved in cheese aromatization have been well deciphered in recent decades. In industrial cheeses, microorganisms developing aroma compounds in cheeses essentially originates from selected strains inoculated as a starter culture in the milk at the beginning of the cheese making. For example, in Cheddar and Emmental cheeses, the production of diacetyl and propionic acid by *Lactococcus (L.) lactis* and *Propionibacterium (P.) freudenreichii*, respectively, has been monitored during cheese making [2,3] and the corresponding metabolic pathways have been established. The ability of microorganisms to produce aroma compound is highly species and strain-dependent, as shown by the results of screening to select the best producers [4]. Many studies show the impact of starters and/or ripening cultures in the differential production of aroma compounds in industrial cheeses: addition of different strains of *P. freudenreichii* in Raclette cheese[5], associations of different strains of *P. freudenreichii* and lactic acid bacteria in Emmental cheese [6,7], diversification of aroma compounds according to the strain of *L. paracasei* in Cheddar cheese [8]. However, little is known regarding the impact of process implemented in the cheese manufacture on the metabolism of bacteria and thus on the production of aroma compounds.

Environmental factors such as pH, salt and temperature are well known to influence the metabolism of bacterial cells in a culture medium *in vitro*. In cheese, process parameters such as the salting step, ripening temperature and duration of different stages can drastically influence the organoleptic quality of cheeses. The effects of salt and temperature have been shown to modulate bacterial growth and, as a consequence, the aroma compounds in cheeses [9–11]. In Cheddar cheeses made with raw milk, a ripening period at 8 °C instead of 1°C increased drastically volatiles compounds and NSLAB growth [9]. In Caciocavallo Silano cheese, a traditional Italian cheese inoculated with an undefined whey culture, an increase in the ripening temperature from 16°C to 20 °C promoted the expression of genes related to proteolysis, lipolysis and amino acid and lipid catabolism and significantly increased the cheese maturation rate and the aroma compounds content [10].

The aim of the present study was to determine how certain variations in process parameters can influence the metabolism of bacteria and therefore whether the choice of process parameters might constitute a lever to modulate the volatilome of semi-hard cheeses.

Our strategy was first of all to develop a reproducible, standard semi-hard model cheese (Std) which was inoculated with three strains: a *L. lactis* ssp *lactis* biovar *diacetylactis* strain for curd acidification and diacetyl production, a *Lactiplantibacillus (L.) plantarum* strain to mimic the role of a non-starter lactic acid bacterium, and a *P. freudenreichii* strain to produce diverse aroma compounds during ripening. The second step was to establish how process parameters modulate the formation of aroma compounds, by modifying three process parameters: reduced stirring time (Stir15min), reduced salting time (Salt7h) or higher ripening temperature (Rip16°C), tested independently. Sampling was performed throughout manufacture and ripening in order to assess the effects of the technological changes versus the Std in terms of biochemistry, bacterial growth and survival and aroma compound content.The relative contributions of time, changes to process parameters were fully discussed.

MATERIALS AND METHODS

2.1 Microorganisms and culture conditions

The consortium used to manufacture the model cheese was composed of three bacterial strains originating from the CIRM-BIA (INRAE, France) collection: *L. lactis* ssp *lactis* biovar *diacetylactis* CIRM-BIA1206, *L. plantarum* CIRM-BIA465 and *P. freudenreichii* CIRM-BIA122.

The strains were stored at -80°C as glycerol stocks (15% v/v). All strains were grown without agitation at 30°C in standard broths: the M17 broth containing 0.5% (w/v) lactose for *L. lactis*, Man, Rogosa and Sharpe broth (MRS, pH 5.4) for *L. plantarum*, and YEL broth [12] for *P. freudenreichii*.

Before cheese making, the frozen strains were revived at 30°C for 2 days in broth media. *L. lactis* and *L. plantarum* were then transferred three times at 1% (v/v) in commercial full-fat UHT cow's milk (Delisse®, France). *L. lactis* was first inoculated in 10 mL milk for 48 h and then in 100 mL milk for 24 h. The final culture of *L. lactis* was performed in 6 L milk and incubated for 41 h to reach a targeted pH of between 5.3 and 5.5. Subcultures of *L. plantarum* were produced in the same manner but in different milk volumes: 30 mL for the second culture and 2 L for the final culture targeting a pH of between 6.3 and 6.4. The revived *P. freudenreichii* was subcultured twice at 1% (v/v) in sterile milk ultrafiltrate supplemented with 10 g/L casein hydrolysate (Organotechnie, La Courneuve, France) and 50 mM sodium L-lactate (Galaflow SL60; Société Arnaud, Paris, France) (medium abbreviated as UF), prepared as previously described [13]. The first subculture of *P. freudenreichii* was performed in 10 mL UF for 72 h. It was then transferred into 0.5 L UF and incubated for 60 h. Before cheese making, the cell concentration in the final culture of *P. freudenreichii* was evaluated by spectrophotometry at 600 nm. An appropriate culture volume, corresponding to a final population of 6.10^6 colony-forming units (CFU) per mL in the milk used for cheese making, was centrifuged for 5 min at 6000 g. The cell pellet was then suspended in 10 mL UHT milk and inoculated into the cheese milk.

Plate counting was also used to control milk quality. The standardized milk was analyzed by plate count agar (PCA) and VRBL agar to check for an absence of contamination. Around 10^3 CFU/mL mesophilic bacteria, ~ 10^2 CFU/mL thermophilic bacteria and <1 CFU/mL coliforms were detected after each pasteurization and each standardization. These levels were judged to be acceptable for cheese making.

2.2 Semi-hard cheese manufacture

Four biological repetitions of cheese production were performed at the pilot scale. The repeatability and reproducibility of the standard cheeses were assessed by making them in two or three different vats, respectively, in order to generate duplicates or triplicates. The standard and three variant itineraries were all performed twice in parallel in three different vats. The choice of vat was randomized to prevent a potential 'vat effect'.

To make each cheese, cow's milk was freshly collected (Entremont, Montauban de Bretagne, France) and treated in batches as follows (**Figure 1**). The cow's milk was pasteurized at 76°C for 20 s, skimmed and standardized to 30 g fat and 36 mg calcium per kg of milk by adding fat and $CaCl_2$ solution, as described by Leyva Salas et al. (2018) [14]. Final cultures of *L. lactis* and *L. plantarum* were inoculated in the standardized milk at ~10^5 CFU/mL. The inoculated milk then underwent prematuration at 14°C for 18 h before cheese manufacture. *P. freudenreichii* was added at a rate of 6.10^6 CFU/mL in matured milk and stirred for 20 min. The milk was then pumped into vats (180 kg/vat) for cheese production.

The standard itinerary for cheese making (**Std**), represented schematically in **Figure 1**, was modified from a previously described method [14]. Briefly, the matured milk was warmed at 33°C for around 30 min. When the pH reached 6.5, 0.25 mL/kg of commercial rennet (520 mg/L chymosin, Carlina 145/80, Dupont Danisco, Dangé, Saint Romain, France) was added to the milk. Gelation time was approximately 20 minutes and firming time was 10 minutes, for a total clotting time of 30 minutes. After cutting the coagulum to the size of corn grains (5x5x5 mm), the curd was stirred for 30 min at 33°C, followed by washing and draining steps. Washing was carried out by replacing 25% of the whey with water at the same temperature (33°C). The washed coagulum was then transferred by gravity into a 56 x 44 cm container and was pre-pressed at 1.96 kPa for 30 min. After pre-pressing, the curd was cut into six pieces (22 x 14 x 12 cm), each weighing around 3.7 kg, which were then molded into cylinders (Ø= 39.6 cm, h = 13.1 cm) and placed on a horizontal press for pressing. This involved three steps: 50 kPa for 30 min, 70 kPa for 1.5 h and then 120 kPa for 2 h. The cheeses were left in their molds overnight at room temperature (the curd temperature falling from 27°C to 23°C during this period) and then demolded on the third day of cheese making, when each cheese weighed around 2.5 kg. The demolded cheeses were then salted by immersion at 12°C for 10 h in saturated brine. After overnight drying at the same temperature, the cheeses were vacuum-packed in plastic bags (La Bovida, France) on the fourth day of cheese making and ripened at 13°C for 7 weeks.

Three variant cheese making itineraries, namely, **Stir15min**, **Salt7h** and **Rip16°C (Figure 1)**, were also followed. These variants differed from the standard itinerary by only one parameter for each variant. Stirring time was reduced from 30 min to 15 min under the **Stir15min** itinerary; salting time was reduced from 10 h to 7 h under the **Salt7h** itinerary and ripening temperature was raised from 13°C to 16°C under the **Rip16°C** itinerary. All other production steps were the same as in the standard itinerary.

2.2 Sample collection

Samples were collected at nine different production stages, ranging from the inoculated milk to cheese ripened for 7 weeks (**Figure 1, Table 1**). Fresh cheese curds were collected at 0, 2 h and 4 h after the start of molding (C_m, C_{m+2h}, C_{m+4h}) and at the demolding stage (C_{dm}). During the ripening period, cheeses were sampled at the start of ripening (C_{0w}) and after 4 and 7 weeks of ripening (C_{4w}, C_{7w}).

The samples were subjected to microbiological, physicochemical and biochemical analyses as detailed in **Table 1**. After pH measurements, the milk samples were aliquoted into sterile jars. Cheese samples were cut with a sterile knife to eliminate the rind (1 cm thick around the cheese surface). Then core samples of around 20 g were cut aseptically for microbial enumeration. The rest of the cheese was mixed using a blender to obtain small cubes with dimensions of less than 1 cm^3 and then aliquoted into sterile jars.

Samples for microbial enumeration were stored at 4°C and analyzed within 24 h. Samples destined for physicochemical (except pH) and biochemical analyses (except volatile compounds) were stored at -20°C. For the analysis of volatile compounds, 2.5 ± 0.1 g of samples were added, in triplicate, to 22 mL PerkinElmer vials and stored at -80°C until analysis.

2.3 Microbiological enumeration

Before enumeration, 10 g of curd or cheese samples were added to the filter stomacher bag (Humeau, Treillière, France), and blended in 90 g 2% (w/v) trisodium citrate (45°C) for 3 min at maximum speed. Ten-fold serial dilutions of milk samples or citrate-cheese solutions were then prepared in peptone salt water. The populations of *L. lactis*, *L. plantarum* and *P. freudenreichii* in the samples were determined using the pour plate technique on M17 agar, MRS agar (pH 5.4) and YEL agar, respectively. The *L. lactis* strain was grown aerobically at 30°C for 24 h, while anaerobic incubation at 30°C was implemented for *L. plantarum* and *P. freudenreichii* for 48 h and 1 week, respectively, as described previously [15].

Counting results were noted as CFU per gram of sample (CFU/g). The bacterial populations at each production stage were expressed as means ± standard deviation for four biological replicates under the standard itinerary, and for two replicates using the three variant itineraries.

2.4 Physicochemical characterization

pH was measured using a pH meter (WTW pH 3100, Weilheim, Germany) equipped with a puncture electrode (LoT406-M6-DXK GmbH, Mettler Toledo, Urdorf, Switzerland) and temperature probe (WTW 325/HC), by direct insertion into the fresh milk and cheese samples. Other analyses were performed on samples after being thawed and equilibrated for 3 h at room temperature. The dry matter content was determined by drying samples for 7 h at 102 ± 2°C [16,17]. Fat content was measured using the Gerber–Van Gulik method with a butyrometer [18]. The calcium content was assessed by atomic absorption spectroscopy [19], results being first expressed as g/100 g of cheese and then converted into the content in dry matter (g/100g DM). Chloride concentrations were determined using a chloridometer based on coulometric titration (Corning 926 Chloride Analyzer, Humeau Laboratoires, La Chapelle-sur-Erdre, France); the results were first expressed as g/100g of cheese and then converted into the content in moisture (g/100g moisture).

2.5 Evaluation of proteolysis

The frozen samples were thawed and equilibrated for 3h at room temperature before nitrogen determinations. Total nitrogen (TN) was determined using the Kjeldahl method [20]; it was then converted to total protein content by multiplying by a factor of 6.38 [21]. The degree of proteolysis was characterized from the non-casein nitrogen content (NCN) (which corresponds to the nitrogen fraction soluble at pH4.6) and from the nitrogen content (NPN), corresponding to the nitrogen fraction soluble in 12% trichloroacetic acid. NCN and NPN were measured according to the method described by Gaucher et al. [22].

2.6 Extraction and quantitation of sugars and organic acids by HPLC

198 Sugars and organic acids in the samples were quantified using high performance liquid
199 chromatography (HPLC). The extraction method was adapted from that described by Leyva Salas et
200 al. [23]. Briefly, frozen samples were first thawed and equilibrated for 3 h at room temperature. Then
201 milk samples were directly diluted 40-fold in the H_2SO_4 solution (0.005 M) and filtered in
202 Vivaspin® 2 (10 kDa MWCO) by centrifugation at 9000 g for 20 min. Curd and cheese samples
203 were first blended in deionized water at 40°C (1:4 w/w) in a filter bag and incubated at 40°C for 1
204 h. The suspensions were then centrifuged (3000 g, 30 min, 4°C) and the supernatants filtered on
205 Whatmann 40 paper. The filtrates were diluted in the H_2SO_4 solution (0.005 M): 6-fold for C_m
206 curds, 4-fold for C_{dm} curds and C_{0w} cheeses, and 2-fold for ripened cheeses (C_{4w} and C_{7w}). The
207 diluted filtrates were then filtered once more (CHROMAFIL® Xtra PVDF-45/13, 0.45 µm pore
208 size, Machery-Nagel GmbH & Co. KG, Germany). HPLC analysis and the identification of
209 metabolites were performed according to the method described by Leyva Salas et al. (2019) [23].

210 **2.7 Analysis of volatile compounds by GC-MS**

211 Volatile compounds were analyzed using headspace (HS) trap extraction coupled to gas
212 chromatography-mass spectrometry (GC-MS). The principle of HS-CGMS has been described
213 elsewhere in detail, including the linearity ranges and limit of detection of 6 of the compounds
214 identified in the present study [24]. The samples were injected in a random order, with standards
215 (mixture of nine volatiles: four esters (ethyl acetate, ethyl propanoate, ethyl butanoate and ethyl
216 hexanoate), two aldehydes (3-methylbutanal and benzaldehyde), two ketones (2-heptanone and 2-
217 nonanone), 2,3-butanedione, dimethyl disulfide and 3-methylbutanol) and blank samples (boiled
218 deionized water) to monitor possible MS drift and carryover. Compounds were eluted on an Elite-
219 WAX ETR (30 m x 0.25 mm ID x 0.25 µm, PerkinElmer USA) column. They were identified by
220 comparing their mass spectral data and linear retention indexes (LRI) calculated on a polar column
221 with that of reference standard compounds, and with data from Library NIST 2008 (Scientific
222 Instrument Services, Ringoes, NJ, USA) and PubChem. The data were processed as described
223 elsewhere [24]. Briefly, raw data files were converted to time- and mass-aligned chromatographic

peaks areas, using the XCMS open source package implemented with the R statistical language [25]. The volatile compounds were semi-quantitied using the abundance of one selected mass fragment (m/z), in arbitrary units. Moreover, previous calibration curves of diacetyl spiked in cream [23] were used to calculate approximate concentrations of diacetyl in milk after prematuration step.

2.8 Statistical analysis

The effect of ripening time, process parameters and their interactions were considered to quantitatively identify, without bias, levers for organoleptic quality. Data were analyzed in a mixed model framework, which explicitly accounted for the correlations between repeated measurements within each type of cheese making, as follows:

$$y_{ijkl} = \mu + I_i + SS_j + (I * SS)_{ij} + CM_k + \epsilon_{ijkl} \qquad (1)$$

where y_{ijkl} is the observed quantity of metabolites l under itinerary i (Std, Stir15min, Salt7h, Rip16°C) at the sampling stage j (M_{LAB}, M_{LAB+P}, C_m, C_{dm}, C_{0w}, C_{4w}, C_{7w}) for cheese making k (1, 2, 3, 4). The symbols I and SS represent the fixed effects due to the itinerary and the sampling stage, respectively. $(I * SS)$ is the interaction effect between the itinerary and the sampling stage. The symbol CM refers to the random effects of cheese making, and accounts for the correlation between repeated measurements within each cheese making cycle. We assumed that CM_k are independent and normally distributed and that ϵ_{ijkl} followed the normal probability distribution with a mean equal to zero. We also assumed the independence between these random effects and ϵ_{ijkl}.

Three models of mixed analysis of variance were performed depending on the question and available data and were fitted by maximizing the log-likelihood using the *nlme* R package.

- *Effect of milk maturation on milk composition*

We first analyzed four variables (two bacterial counts, pH, dry matter and 10 metabolites and volatile compounds, see above) measured at the first two sampling stages under the standard itinerary. Model (1) was simplified into a model with a single fixed effect due to the sampling stage (M_{LAB}, M_{LAB+P}) and the cheese making random effect. From the test of the fixed effect, we

considered metabolites and volatile compounds with p-values lower than 0.05 as being statistically different between the M_{LAB+P} and M_{LAB} sampling stages.

- *Comparison of the four itineraries during ripening*

We focused on the compounds measured during all four itineraries (standard + three variants) at the three last sampling stages (C_{0w}, C_{4w}, C_{7w}). For each compound, we first of all tested the significance of the interaction term in the model (1) using a likelihood ratio test, and only retained it in the model if the p-value was greater than 0.05. In a second step, we computed estimated marginal means for the itinerary fixed effect and performed comparisons between each variant in the itinerary (Stir15min, Salt7h and Rip16°C) and the standard itinerary (Std) using the *emmeans* R package. Raw p-values were adjusted for multiple comparisons using the Tuckey method and the level of significance was fixed at 0.05. For the (I × PS) interaction, the sum of the values for the five production stages was divided by 5, and the results were subsequently referred to as the "global mean" of all production stages. For the (I × RS) interaction, the sum of the values for the three ripening stages was divided by 3 and the results were subsequently referred to as the "global mean" for ripening stages (2). The global means under the variant conditions were then compared to the standard.

Plate counts, pH and dry matter content at each production stage were expressed as means in four biological replicates for the standard itinerary, and two replicates for the variant itineraries. Other concentrations were expressed as means in two biological replicates for both the standard and variant itineraries.

- *Impact of sampling stages*

We analyzed 27 variables (three bacterial counts, pH, salt, dry matter and 21 metabolites and volatile compounds) from the five last sampling stages (C_m, C_{dm}, C_{0w}, C_{4w}, C_{7w}) under the Stir15min and Std itineraries. We followed the same two-step procedure as described in the paragraph above.

All these analyses were implemented using R software version 3.6.1.

- *Principal Component Analysis*

A principal component analysis (PCA) was performed with 31 centered and scaled variables (three bacterial counts, expressed as log-transformed data, pH, dry matter, MFFB, Ca2, Cl, NCN, NPN and 21 metabolites and volatile compounds) for the 32 cheese samples, using XLSstat for Microsoft Excel (Addinsoft 2019, https://www.xlstat.com.).

3 RESULTS

3.1- Evolution of composition during cheese manufacture and ripening

Dry matter (DM) and pH were chosen as physicochemical indicators to assess the repeatability and reproducibility of the **Std** cheese. These parameters were monitored in four biological repetitions of the cheese (**Figure 2**). Before cheese making, the M_{LAB} milk contained 11.65 ± 0.26 g/100 g DM and the pH was 6.59 ± 0.02. At the molding stage, the DM content in cheese was around 40.53 ± 3.21 g/100 g. It then rose progressively with pressing to reach 52.03 ± 1.37 g/100 g at demolding. The DM content remained stable thereafter. The pH in cheese fell from 6.36 ± 0.05 to 5.45 ± 0.07 during molding and remained at around 5.19 ± 0.06 until the end of ripening. For the DM content and pH, the standard deviation (SD) was less than 3% of the mean value at all stages, thus confirming the repeatability and reproducibility of Std cheese manufacture. The only exception was observed regarding DM at molding, where the SD was 8%. This relatively high SD value was due to the non-homogeneity of the fresh cheese sample at that time.

In order to evaluate the influence of variant itineraries on the physicochemical evolution of cheese, six composition parameters (i.e., DM, pH, protein, fat, calcium and chloride) were monitored throughout the production of cheeses under both the standard and variant itineraries.

The physicochemical composition of cheeses at each production stage is summarized in Table 3**.** The evolution of pH in variant cheeses displayed similar behavior and no significant differences

were found compared to the Std cheese. The global mean DM content in Stir15min cheese was 3.9% lower than in the Std cheese, which corresponded to a difference of 1.98 g/100g cheese ($p<0.05$). Salt7h cheese also contained significantly less DM than the Std cheese and the difference was 1.5% ($p<0.05$).

The total protein and fat contents in dry matter did not vary during production regardless of the itinerary. Their contents were around 42.6 g/100g DM for total protein and 47.9 g/100 g DM for fat. However, the moisture content in the fat-free basis (MFFB) of cheese was significantly higher in Stir15min cheese than in the Std cheese ($p<0.001$), with a difference of 2.4%. This was due to a lower DM content in **Stir15min** cheese.

The calcium content in Std cheese increased gradually from 0.61 ± 0.09 g/100g cheese at molding to 0.74 ± 0.04 g/100g at the end of ripening. Compared to **Std**, the **Stir15min** itinerary induced a significantly lower calcium content in cheese (-5.9%, $p<0.05$). However, the calcium content in dry matter did not differ significantly ($p>0.1$). The chloride level in the core of the **Std** cheese were initially 0.35 ± 0.05 g/100g, corresponding to 0.72 ± 0.08 g/100g moisture. This increased over time to reach 0.83 ± 0.03 g/100 g cheese at four weeks of ripening, and was then maintained at around 0.8 g/100g (1.8 g/100g moisture) during the later ripening period. Chloride levels in cheese and in the moisture fraction were both significantly affected by the interaction between the ripening stage and the itinerary (I × RS). **Stir15min** cheese displayed a much more rapid rise in the chloride content during the early ripening period and this was ultimately 10.9% higher than in the cheese ($p<0.05$). Compared to the **Std** cheese, the **Salt7h** cheese contained 8.6% less chloride in the cheese ($p = 0.054$) and 10.9% less chloride in moisture ($p<0.05$).

3.2- Overview of bacterial evolution during cheese manufacture and ripening

During the present study, the kinetic growth of the bacterial consortium was monitored during cheese production according to the standard itinerary (**Std**). This enabled an assessment of the repeatability and reproducibility of the **Std** cheese and the construction of a standard profile for

microbial evolution in a model cheese (**Figure 2**). The standard deviation of population values was less than 0.5 $\log_{10}$ units for all bacterial strains at all stages, thus confirming the repeatability and reproducibility of the **Std** cheese.

The *L. lactis* and *L. plantarum* strains were inoculated in standardized milk at 5.7 and 5.2 $\log_{10}$ CFU/g, respectively, while *P. freudenreichii* was inoculated at 6.1 $\log_{10}$ CFU/g in matured milk. During the prematuration step (14°C for 18 h), the *L. lactis* population increased significantly ($p<0.001$) to reach 7.5 $\log_{10}$ CFU/mL in the mature milk with an average doubling time (DT) estimated at 3.1 h, while *L. plantarum* grew very slowly (DT = 19.7 h). Following molding, the bacteria in milk were concentrated by approximately ten-fold in curd, so that *L. lactis*, *L. plantarum* and *P. freudenreichii* levels in molded cheese (C_m) reached at 8.6, 6.4 and 7.0 $\log_{10}$ CFU/g, respectively. The *L. lactis* population reached the highest level before ripening (9.2 $\log_{10}$ CFU/g) and then fell slightly to reach 8.5 $\log_{10}$ CFU/g at the end of ripening. *L. plantarum* grew rapidly during molding (DT = 5.4 h); the highest population was reached at four weeks of ripening (8.4 $\log_{10}$ CFU/g) and remained stable thereafter. The growth of *P. freudenreichii* occurred during molding (DT = 5.4 h) and the first four weeks of ripening. The final population of *P. freudenreichii* reached 8.5 $\log_{10}$ CFU/g.

Table 2 summarizes the populations of each strain and in the four cheese types. In **Stir15min** cheese, the global mean of the *L. lactis* population was significantly lower than in **Std** cheese ($p<0.05$). However, the difference was less than 0.5 $\log_{10}$ units at each production stage. During the ripening period, a significant (I × RS) interaction effect was detected for *L. lactis* ($p<0.001$), which means that the course of its evolution during ripening stages was dependent on the itinerary. Between 0 to 7 weeks of ripening, the population of *L. lactis* decreased under both **Std** and **Rip16**°C itineraries, but this fall was more marked under **Rip16** °C than **Std** (-1.43 and -0.61 $\log_{10}$ CFU/g, respectively). After seven weeks of ripening, the *L. lactis* population in Rip16°C cheese was significantly lower than in **Std** cheese ($p<0.001$) with a difference of 0.8 $\log_{10}$ units.

3.3- Evolution of nitrogen fractions

Figure 3 shows the evolution of nitrogen fractions under all itineraries. In standard conditions, NCN represented less than 6% of total nitrogen (TN) before ripening, and NPN was lower than 2% TN. At the start of ripening, the nitrogen fractions in cheese at 13°C increased rapidly and reached 14.3 ± 0.6% for NCN and 5.8 ± 0.1 % for NPN at four weeks of ripening. Thereafter, the nitrogen fraction contents continued to increase but at a lower rate. By seven weeks of ripening, the Std cheese contained 16.7 ± 0.1% NCN and 7.2 ± 0.2% NPN.

The nitrogen fractions in **Stir15min** and **Salt7h** cheeses did not differ from those in the **Std**. However, cheese ripened at 16°C (**Rip16**°C) displayed significantly higher levels of proteolysis ($p<0.05$) when compared to the **Std** cheese, with an NCN content of 18.06 ± 0.69% (+ 8.2%) and a NPN content of 8.90 ± 0.08% (+23.2%) after seven weeks of ripening. An interaction between the effects of ripening stage and itinerary was observed for the NPN content, which increased much more rapidly at a higher ripening temperature.

3.4- Evolution of sugar and organic acid levels in milk and cheese during production

The complete metabolome of milk before and after prematuration can be seen in **Table 4.** Lactose and citric acid (48 and 2 g/L, respectively) were the two main carbon sources in milk and did not vary significantly after prematuration. Lactic acid was only detected in matured milk. The acetic acid content increased significantly by 3.4-fold ($p=0.09$) during prematuration. Notable changes to the volatile profile were also observed, with an increase in several compounds in mature milk: hexanoic acid (1.2-fold), octanoic acid (1.7-fold), diacetyl (17.6-fold) ($p<0.05$) and acetoin (6.2-fold, $p = 0.054$).

Overall, 12 molecules were detected, including two sugars (lactose and galactose) and eight organic acids (pyruvic, citric, succinic, lactic, acetic, propanoic, butanoic and phenyllactic acids), as well as two ketones (diacetyl and acetoin). However, the levels of diacetyl and acetoin were too low to be

reliably quantified by HPLC, so instead they were semi-quantified from GC-MS data. According to the previously published calibration curve [26] diacetyl reached between 300 and 400 ng/g of milk during the prematuration step.

Table 5 presents the concentrations of ten compounds in both standard and variant cheeses throughout manufacture and ripening. All molecules displayed significant variations in their concentrations in the cheeses during production ($p<0.001$). Based on the trends in evolution, three groups of molecules were distinguished: **(a)** lactose and citric acid were consumed over time, **(b)** galactose and lactic acid were first produced during cheese manufacture and then consumed, and **(c)** pyruvic, succinic, acetic, propanoic, butanoic, phylactic acids were produced and accumulated over time (**Figure 4**). During the standard cheese manufacture, lactose levels fell rapidly during molding to reach less than 10 g/kg by demolding. Lactose continued to be consumed thereafter and the level was at 0.79 ± 0.06 g/kg by seven weeks of ripening. Citric acid concentrations decreased to 0.15 ± 0.01 g/kg in cheese before ripening and were not detectable in ripened cheeses. Levels of other organic acids rose throughout production, especially during the ripening period.

The **Stir15min** itinerary was compared with the standard itinerary throughout the production cycle (**Table 5**). The interaction effect between itinerary and production stage (I × PS) was significant for citric, succinic, lactic and acetic acids ($p<0.05$). **Stir15min** cheese had a lower citric acid content at the molding stage (-16.5%), and a lower lactic acid content during the middle stages of cheese production (around -10%). At the end of the ripening, it had a higher content in succinic (+ 40.2%) and acetic acids (+18.3%). Other molecules did not display any significant (I × PS) interaction effect. However, the global mean of lactose contents was higher in **Stir15min** cheese (+43.5%), while that of pyruvic and butanoic acids was lower (around -20%). Propanoic acid could only be detected by HPLC during the ripening phase. The itinerary by ripening stage (I × RS) interaction effect was significant for propanoic acid because of its higher content in **Stir15min** cheese at seven weeks of ripening (+13.6%). When all itineraries were compared during ripening, the (I × RS)

interaction effect was significant for all molecules except galactose. The only difference between the **Salt7h** cheese and **Std** cheese was the succinic acid content, which was +57.0% higher in the latter. The **Rip16°C** cheese was the most contrasted sample. The levels of ten molecules differed significantly from those in the **Std** cheese ($p<0.05$). Six organic acids accumulated during ripening (pyruvic, succinic, acetic, propanoic, butanoic and phenyllactic acids) and were produced more rapidly in **Rip16°C** than in Std (**Figure 4 A-F**). The differences in concentration ranged from 34.1% to 158.7%, depending on the molecule ($p<0.001$). The contents of these acids were also the highest among all cheese types. By contrast, the lactic acid content in **Rip16°C** cheese by seven weeks of ripening was 45.3% ($p<0.001$) lower than in the **Std** cheese and the lowest among all cheese types.

3.5-Volatile compound profile in cheese

A total of 14 volatile compounds was detected (**Table 6**), including four ketones (acetone, butan-2-one, diacetyl, acetoin), six acids (acetic, propanoic, butanoic, hexanoic, octanoic, and methylbutanoic acids), three alcohols (propan-1-ol, 2-methylbutan-1-ol and 1-hydroxypropan-2-one), and one sulfur compound (dimethyl disulfide). All compounds were detectable in cheese from the molding stage, but they varied in abundance over manufacture and ripening. Propan-1-ol, 2-methylbutan-1-ol, DMDS and the acids were produced abundantly during ripening and their abundance increased as a function of ripening time. In cheeses ripened for four and seven weeks the high concentrations in acetic, propanoic and butanoic acids induced an overloading of the GC capillary column, thus impairing the accuracy of their quantification. These three acids were therefore quantified using HPLC and only their later concentrations were considered for statistical analyses (**Table 5**).

The comparison between **Std** and **Stir15min** cheeses showed a significant (I × PS) interaction effect for 2-methylbutanol, 2-methylbutanoic acid ($p<0.05$). **Stir15min** cheese always contained higher levels of those two molecules during the ripening period, while the difference *versus* the **Std** cheese diminished with ripening time (**Figure 4G and 4H**). After seven weeks of ripening, the 2-

methylbutanol and 2-methylbutanoic acid contents in **Stir15min** cheese were more than 10% higher than in Std. **DMDS** was significant in both (I × PS) and (I × RS) interaction effects, as the **Stir15min** cheese contained 51.8% ($p<0.001$) less DMDS than **Std** at the end of ripening. As for molecules where the interactions were not significant, the global mean of diacetyl and acetol intensities in ripened cheese was lower in **Stir15min** cheese, the difference being -47.0% ($p<0.1$) and -35.8% ($p=0.056$), respectively.

During ripening, the volatile compound profile of **Salt7h** cheese did not differ from that of **Std**. Concerning the **Rip16°C** cheese, the (RS × I) interaction effect was significant for 2-methylbutanol, 2-methylbutanoic and hexanoic acids ($p<0.001$). Compared to the **Std** cheese, the contents in 2-methylbutanol ($p<0.001$), 2-methylbutanoic ($p<0.05$) and hexanoic acids ($p<0.1$) were higher at four weeks of ripening (**Figure 4G-I**). At the end of ripening, the **Rip16°C** cheese contained +82.7% 2-methylbutanol, +60.4% 2-methylbutanoic and +49.8% hexanoic acids ($p<0.001$).

3.6- Global analysis of the cheese curd profile during cheese production

As shown in **Figure 5**, PCA was performed on 31 variables describing the microbial and biochemical composition of the cheeses. The first two principal components (F1 and F2) accounted for 83.04% of total variability. F1, which described 62.65% of variability, was positively associated with proteolysis parameters, the NaCl content, viable counts of *L. plantarum* and *P. freudenreichii* and most acids and alcohols (**Figure 5A**). By contrast, F1 was negatively associated with viable counts of *L. lactis*, pH, and the contents in sugars and citric acid, two ketones (acetone, butan-2-one) and MFFB. F2, which accounted for 20.38% of variability, was positively correlated with contents in diacetyl, acetoin, lactic acid and acetol, and viable counts of *L. lactis*.

The observation map (**Figure 5B**), showed five cheese groups distinguished as a function of ripening stages. Fresh cheese curds collected at the molding stage (C_m) appeared in the low left quadrant. At the manufacturing steps, the points corresponding to demoulding (D) and after salting (C0w) moved vertically and positively along F2 axis concomitantly to *L. lactis* growth, lactate,

acetol and diacetyl production. Cheeses after demolding (D) and at the start of ripening (C_{0w}) showed similar profiles and were located together in the upper left quadrant. During ripening, points progressively moved positively along F1 axis and negatively along F2 axis concomitantly with *L. plantarum* and *P. freudenreichii* growth, reduction of lactate, acetol, diacetyl and production of esters and proteolysis. Cheeses ripened for four weeks (C_{4w}) and seven weeks (C_{7w}) were mainly in the upper right and lower right quadrants, respectively. Fresh curds were negatively associated with F1 and were therefore characterized by higher pH values, moisture and lactose contents, and viable *L. lactis* counts. Ripened cheeses (C_{4w} and C_{7w}) were mainly associated with higher counts of *L. plantarum* and *P. freudenreichii*, higher proteolysis levels and higher contents in most flavor compounds. Demolded cheeses (C_{dm}) and young-aged cheeses (C_{0w}) were separated from cheeses at molding (C_m) and old-aged cheeses on the F2 and were characterized by high *L. lactis* counts and higher contents in acetoin, diacetyl, lactic acid, and acetol. In order to facilitate comparison of the four cheese types, the cheeses samples were color-coded relative to the itinerary applied (**Figure 5B**). In the groups of cheeses before ripening, the cheeses produced with variant processes were not differentiated from the Std cheese. However, during ripening, cheese ripened at 16°C for four weeks displayed the same profile as the cheese ripened at 13°C for seven weeks. The accelerated ripening at higher temperature was more obvious among cheeses ripened at 16°C for seven weeks. Their profiles contrasted with other cheeses, revealing the highest aroma content and more advanced proteolysis.

4- Discussion

4.1- Standard cheeses as a model cheese

In this work, we developed a model semi-hard cheese with high reproducibility (less than 3% of variation) in terms of moisture (54%), fat/dry matter (48%), protein/dry matter (42%) and growth of starter and ripening bacterial species. For that purpose, we focused in particularly on optimizing the cutting size of curd before stirring, the molding step, the pressing step using a horizontal press and progressive pressing, and the use of plastic vacuum bags to prevent any microbial contamination of

the cheese surface during ripening. We grew our home-made starter cultures using publicly available strains obtained from the CIRM-BIA collection. The ability of *L. lactis* ssp. *lactis* biovar. *diacetylactis* CIRM-BIA1026 to produce diacetyl, and the previous observation of low lysis under carbon source deficiency conditions (data not shown) were features of interest in this work. *L. plantarum* and *P. freudenreichii* were chosen as adjunct bacteria as they contribute to cheese texture and flavor by proteolysis and the production of flavor components[27]. Compliance with the schedule guaranteed a highly reproducible growth of each strain separately during preparation of the inoculum and overall in the milk during the prematuration step and in cheeses. We suggest that this model cheese could be reproduced by other scientists interested in the biochemistry of semi-hard cheeses. Until now, most scientific studies performed on cheese have used Cheddar cheese [2,28,29] which is produced in large quantities throughout the world but which includes a specific step of dry salting (salt is added directly to the grains: cheddarization). The production of our model cheese, which includes a brine salting step, is representative of a broader panel of semi-hard cheeses such as Tommes, Raclette, Edam, Gouda, Manchego, Provolone and Castelmagno cheeses.

4.2 - Microbial and biochemical changes in Std cheese

The activity of microorganisms during cheese making and ripening induces modifications to all curd constituents which in turn leads to the development of cheese flavor. Flavor development is a dynamic biochemical process that is influenced by (i) the type and composition of milk, (ii) processing parameters, and (iii) the microorganisms and enzymes present in the cheese matrix. The cheese microbiota is the primary source of enzymes that influence flavor development through the degradation of carbon sources, proteins and lipids.

4.2.1- Utilization of lactose, production of lactic and acetic acids by LAB

The conversion of lactose to lactic acid is essential for the production of all types of cheeses involving a bacterial acidification step. Lactic acid causes acidification and a refreshing acid taste, which is particularly noticeable in young cheeses. In the **Std** cheese we followed the degradation of

lactose in milk, in curd and in cheeses up to seven-week ripened cheeses. As expected, the utilization of lactose was very rapid during the acidification of milk and curd and was concomitant with *L. lactis* growth. It occurred during the prematuration step and the acidification of curd during pressing (**Figure 1; Table 5**) and 80% of lactose was consumed at the demolding step. The *L. plantarum* strain was also able to use lactose (API gallery results, data not shown) and to produce lactic acid. Lactose levels were very low (nearly zero) as from the fourth week of ripening. Acidification was progressive during ripening because no fungi used the lactic and acetic acids released by lactic acid bacteria. Acetic acid could be produced by all three strains and probably resulted from the successive fermentations by *L. lactis*, *L. plantarum* and then *P. freudenreichii* during ripening, the latter being the main contributor, as suggested by the time-course of production (**Figure 4C**).

4.2.2- Utilization of citric acid, production of diacetyl and acetoin by *L. lactis*

Citric acid is another carbon source for certain lactic acid bacteria strains. The strain of *L. lactis* used during the present study belongs to the biovar *diacetylactis*. As early as the prematuration step in the milk tank at 14°C (M_{LAB+P}), it produced high levels of diacetyl (**Table 4**, fold change × 17.6) and consumed high levels of citric acid (**Table 4**, 90% at the demolding step) in line with the findings of Passerini et al. [30]. The high production of diacetyl during the early stage of cheese production imparts the formation of a buttery flavor. Oxygen is required for this synthesis [31]. Diacetyl production could also be explained by the introduction of oxygen being pumped and stirred in the prematuration tank [32]. The production of diacetyl reached its highest level after four weeks of ripening. The diacetyl content had fallen by the end of the ripening, which probably resulted from its reduction into acetoin by lactic acid bacteria. Acetoin is generally produced in much larger quantities, *i.e.* 10- to 50-fold higher than diacetyl concentrations [33]. It is noteworthy that the partial removal of lactoserum, which was replaced by warm water before molding, led to the loss of one quarter of the diacetyl (and other soluble metabolites) produced before this step.

4.2.3- Primary and secondary proteolysis, a necessary step for volatile production by the bacterial consortium

Proteolysis is essential to flavor formation. The production of small peptides and free amino acids results from the activity of the added coagulant and/or of milk plasmin, in conjunction with cell envelope microbial proteinases and cytoplasmic peptidases [34]. Short peptides and amino acids contribute to the basic flavor of cheeses. Free amino acids are further catabolized into many soluble and volatile compounds. Some of these compounds impact cheese aroma, such as volatile carboxylic acids, aldehydes and alcohols. A range of amino acid-converting enzymes are involved in their formation [35,36].

Primary and secondary proteolysis principally occurred during the four first weeks of ripening (**Figure 3**). According the literature, the primary proteolysis (**Figure 3**, NCN raising from 6 to 16 % of nitrogen fraction) is ensured by surface exposed protease from *L. lactis,* combined with those of *L. plantarum.* The secondary proteolysis (**Figure 3**, NPN) is difficult to attribute. Three aroma compounds derived from the catabolism of amino acids: 2-methylbutan-1-ol and 2-methylbutanoic acid likely resulted from the catabolism of isoleucine by *P. freudenreichi*. [37,38]. All of the corresponding pathways have been described as being expressed in *P. freudenreichii* [39]. DMDS results from the catabolism of methionine[36].

4.2.4-Production of volatiles resulting from *P. freudenreichii* metabolism

P. freudenreichii produced aroma compounds *via* three main pathways: the fermentation of lactic acid, lipolysis, and branched-chain amino acid catabolism.

Lactic acid can be metabolized by a number of pathways to various compounds which may contribute to cheese flavor. *P. freudenreichii* is a ripening culture that is widely used in the manufacture of Swiss-type cheeses, where it uses lactic acid as the main carbon source[40]. Consequently, its growth is reliant on the consumption of lactic acid only. *P. freudenreichii*

converts lactic acid into propanoic acid, acetic acid and carbon dioxide. Unlike acetic acid and carbon dioxide, which are also produced by LAB, propanoic acid was only produced by *P. freudenreichii*. It accumulated gradually in the cheese during ripening.

Milk fat is another essential source for the development of cheese flavor during ripening. Lipolysis, i.e. the hydrolysis of milk fat by lipolytic esterases, results in the formation of free fatty acids (FFAs, namely acids in **Table 6** and **Figure 5**), which even at low concentrations will contribute to cheese flavor, either positively (desirable pungent notes) or negatively (rancid notes), depending on the type of cheese [41,42]. FFAs also act as precursors for certain flavor compounds such as methylketones, secondary alcohols, esters, and lactones [34].

Microorganisms are the principal sources of lipolytic enzymes in cheese. In ripened cheese involving *P. freudenreichii* fermentation, the species is considered to be the main actor in milk fat lipolysis. It has been demonstrated that *P. freudenreichii* is responsible for the formation of up to 96% of FFAs in Emmental cheeses [43], thanks to the activity of an extracellular lipolytic esterase [44]. By contrast, LAB esterases have received much less attention because they are mainly intracellular and have weak lipolytic activity on milk fat [41,42]. In our model cheese, the butanoic and hexanoic acids generated during ripening likely resulted from lipolysis by *P. freudenreichii*.

4.3- Influence of the fine-tuning of process parameters on the profile of aroma compounds

4.3.1- Effect of a reduced salting time on the formation of aroma compounds

The **Sal7h** itinerary differed from **Std** by a 10.9% reduction in the salt concentration of the water phase of cheese. It also resulted in a significant rise in succinic acid levels after seven weeks of ripening (57%, **Table**). This delayed effect on succinic acid could be explained by the slow diffusion of salt (sodium chloride) to the cheese core. Indeed, in pressed and brine-salted cheeses, diffusion rates range from 0.1 to 0.45 cm^2/day [45]. The results of chloride measurements showed that NaCl was only dispersed homogeneously in the core of the cheese after four weeks of ripening,

in line with the size of the model cheeses (see 2.2). We can hypothesize that a lower salt concentration in the core might activate bacterial metabolic activity and cause an accumulation of succinic acid that cannot be degraded by any of the three inoculated species which do not possess a complete tricarboxylic acid pathway capable of metabolizing succinic acid. To the best of our knowledge, succinic acid exerts no influence on cheese flavor, but the lower salt level might directly impact cheese taste (not tested). Therefore, under the conditions of the present study, the reduction in salt did not modify the profile of aroma compounds. It is likely that the salt reduction applied was not sufficiently contrasted; salt levels can affect both bacterial growth and metabolic activities in a strain-dependent manner, as previously illustrated on *L. lactis* [46,47] and *P. freudenreichii* in Swiss-type cheeses [48,49].

4.3.2- Effect of a reduced stirring time on the formation of aroma compounds

A reduction in stirring time selectively reduced the buttery flavor-associated compounds produced by *L. lactis* and increased the amounts of some compounds that are associated with "aged-cheese" and 'Swiss-cheese related flavor' notes, and resulted from the activities of *P. freudenreichii*. At both ripening times (after four and seven weeks), the reduction in stirring time that we applied increased i) the lactose content throughout manufacture and ripening (**Table 4 and 5**), ii) the NaCl content (**Table 3**), iii) the secondary proteolysis during ripening (**Figure 3**), iv) moisture during cheese making (**Table 3**).

Because of the higher contents in lactose (>50 %) and MFFB, we expected that the LAB would produce more lactic acid. However lactic acid content of **Stir15min** was not significantly higher than in the **Std** cheeses. One explanation might be the counter-effect of salt (+10.9%) on the overall equilibrium of the metabolic ecosystem, thus illustrating the complexity of predictive microbiology when one process parameter exerts pleiotropic effects (salt, lactose, MFFB).

603 The slightly higher secondary proteolysis during ripening, currently attributed to higher bacterial
604 metabolic activities -since no lysis was observed- might rather have resulted from the proteolytic
605 activity of *L. plantarum*, as previously demonstrated for some *L. plantarum* strains in Cheddar
606 cheese [50,51]. Moreover, Stir15min decreased DMDS (-52%, $p<0.001$), diacetyl and acetol
607 concentrations (-47% and -36%; $p<0.1$). The higher level of MFFB (+2.4% in ripened cheeses)
608 caused a positive effect on *P. freudenreichii* activity and significantly increased levels of aroma
609 compounds such as propanoic, acetic, butanoic and succinic acids (from +14% up to +159%;
610 $p<0.05$), and 2-methylbutanol and 2- methylbutanoic acid, during the ripening period (+15% and
611 +16% at C7w, $p<0.001$ and $p<0.1$) (**Table 5**). It is noteworthy that the fine-tuning of stirring time
612 on the day of cheese making can lead to a drastic rise (+40.2%) in succinic acid levels, two months
613 after production.

614

615 **4.3.3- Effect of a higher ripening temperature on aroma compound formation**

616 After both periods of ripening, a higher ripening temperature significantly increased secondary
617 proteolysis ($p<0.05$, **Figure 4**), the contents in aroma compounds (2-methylbutanol and carboxylic
618 acids such as acetic and propanoic acids) and lipolysis, as indicated by butanoic and hexanoic acids
619 (**Table**). The rise in acids seen during ripening (from +34% to 159%; $p<0.001$ at 7 weeks of
620 ripening, **Table** , **Figure 4**) arose from *P. freudenreichii* metabolism. A higher lactic acid
621 consumption (-45.3 %, $p<0.05$) was also a sign of more pronounced *P. freudenreichii* activity. The
622 ACP built from the global metabolome of cheeses (**Figure 5**) showed that cheeses ripened at 16°C
623 for four weeks displayed the same profile as the cheeses ripened at 13°C for seven weeks,
624 indicating that a three degree rise in the ripening temperature accelerated the maturing process by
625 three weeks. Similarly, in traditional Italian cheeses, an increase of 4°C in the ripening temperature
626 has been seen to promote the expression of genes related to proteolysis, lipolysis and amino
627 acid/lipid catabolism, and significantly increase the cheese maturation rate [10]. These authors
628 suggested the contribution of non-starter lactic acid bacteria to the aroma profile of cheeses.

629

630 **4.4 Bacterial populations little affected by process parameters**

631 The changes observed in the metabolome resulted from changes to bacterial activity and not from
632 differences in cultivable cell counts.

633 The variant itineraries modified the aromatic profile of cheese by modulating the metabolic activity
634 of the cheese community. This modulation could either be direct, *i.e.*, temperature changing the
635 metabolic activities of bacteria, and/or indirect, *i.e.*, stirring and salting time initially changed the
636 physicochemical composition of the cheese matrix, which in turn influenced bacterial activity.

637 It is generally accepted that process parameters can impact the growth of bacterial species during
638 food fermentation. Surprisingly, during our study, the changes made in variant itineraries did not
639 affect growth kinetics, probably because we only adjusted the process parameters to a minor and
640 insufficient extent. The only effect observed was a significant loss of the cultivability of *L. lactis*
641 during ripening, which might have resulted from both cell lysis and/or the switch from a cultivable
642 state to a viable but non-cultivable (VBNC) state [52,53]. But although *L. lactis* cells are in a VBNC
643 state, their metabolic activity may still influence the aromatic profile of cheeses.

644

645 **4.5 Fine-tuning of process parameters to produce more healthy and hedonic cheeses**

646 Our aim during this work was to evaluate how fine-tuning of process parameters during the
647 production process modulate bacterial aroma compounds of cheese. The conclusions are that, even
648 without impact on microbial growth, fine-tuning of process parameters, like stirring time or
649 ripening temperature influence the final organoleptic quality of cheese by promoting specific
650 bacterial metabolisms. A higher ripening temperature and a shorter stirring time increased the
651 content in *Propionibacterium*-related aroma compounds. It would have been interesting to perform
652 sensory evaluations of the cheeses in order to determine whether the differences in flavor were
653 perceived and appreciated or not. However, a 10.9% reduction in salt content did not influence the

amount of quantified metabolites in the cheeses, excepted for succinic acid. It nevertheless remains noteworthy that this shorter salting step would be beneficial to consumer health. Indeed, there have been calls for decades for less salt in the diet in order to improve public health. The WHO recommends a daily salt intake of less than 5 g/day [54], but in 2017 the global average salt intake remained high and is still estimated at around 15 g/day [55]. A shorter salting time that does not affect aromatic profiles might offer an ideal solution for manufacturers to produce low-salt cheeses with a shorter production time. Similar studies on reducing the salt content in cheese showed that Na-reduced cheeses tasted bitter and were therefore organoleptically unsatisfactory [56]. Further sensory analyses involving less salty cheeses are necessary to confirm their organoleptic quality and conclude as to the positive effects of a shorter salting time.

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Figure 1: Schematic representation of the standard cheese making itinerary and three variant itineraries, and kinetic sampling throughout production.

Figure 2 Kinetic evolutions during cheese manufacture and ripening under the standard itinerary regarding (A) viable counts of the three bacterial species, (B) dry matter and (C) pH. M_{LAB}: milk containing lactic acid bacteria (*L. lactis* and *L. plantarum*); M_{LAB-P}: milk containing lactic acid bacteria and *P. freudenreichii*; C_m: molded cheese; C_{dm}: demolded cheese; C_{0w}: cheese ripened for 0 weeks; C_{4w}: cheese ripened for 4 weeks; C_{7w}: cheese ripened for 7 weeks. Values are the means of four biological replicates of cheese production.

Figure 3: Evolution of (A) the non-casein nitrogen fraction (NPN) and (B) the non-protein nitrogen fraction (NPN) throughout cheese production under the standard and variant itineraries. Std in black, Stir15min in yellow, Salt7h in green, Rip16°C in red. Values are the means of four and two biological replicates of cheese production for Std and others respectively.

Figure 4: Accumulation of pyruvic (A), succinic (B), acetic (C), propanoic (D), butanoic (E) and phenyllactic acids (F), 2-methylbutanol (G), 2-methylbutanoic (H) and hexanoic acids (I) throughout cheese production. ND: not detected. Std in black, Stir15min in yellow, Salt7h in green, Rip16°C in red. Values are the means of four and two biological replicates of cheese production for Std and others respectively.

Figure 5: Principal component analyses of microbial, physicochemical and biochemical components in cheeses throughout production under the standard and variant itineraries. A: variables, B: observations. Std in black, Stir15min in yellow, Salt7h in green, Rip16°C in red. M: molded cheese; D: demolded cheese; C_{0w}: cheese ripened for 0 weeks; C_{4w}: cheese ripened for 4 weeks; C_{7w}: cheese ripened for 7 weeks. Values are the means of four and two biological replicates of cheese production for Std and others respectively. Ellipses were arbitrarily drawn to delimit group of samples to facilitate reading.

Table 1: **Samples collected throughout cheese production and their corresponding analysis**.

Collection time	Sampling stage	Sample label	Microbial analysis	Physicochemical analysis					Biochemical analysis		
			Enumeration	pH	DM [(1)]	Ca^{2+}	Fat	NaCl	Protein (TP, NCN, NPN)[(2)]	Sugars and organic acids	Volatile compounds
0.5 h	Milk inoculated with LAB	M_{LAB}	x	x	x	x	x		x	x	x
18 h	Milk inoculated with *P. freudenreichii*	M_{LAB+P}	x	x	x	x	x		x	x	x
19.5 h	Cheese at molding	C_m	x	x	x	x	x		x	x	x
21.5 h	Cheese molded for 2h	C_{m+2h}		x	x	x					
23.5 h	Cheese molded for 4h	C_{m+4h}		x	x	x					
2nd day	Cheese at demolding	C_{dm}	x	x	x	x	x		x	x	x
3rd day	Cheese before ripening	C_{0w}	x	x	x	x	x	x	x	x	x
4 weeks	Cheese ripened for 4 weeks	C_{4w}	x	x	x	x	x	x	x	x	x
7 weeks	Cheese ripened for 7 weeks	C_{7w}	x	x	x	x	x	x	x	x	x

(1) DM: Dry matter

(2) TP: Total protein; NCN: Non-casein nitrogen; NPN: Non-protein nitrogen.

Table 2. Evolution of the populations of the three bacterial species during the production of model semi-hard cheeses manufactured according to one standard and three variant itineraries [a]. These values are the means of two biological replicates. The last four columns present (I X PS) and (I X RS) interactions, as well as the global mean of the bacterial population under the itinerary analyzed.

		Cheese production					Comparison between Stir15min and Standard at all production stages		Comparison between all variant itineraries and Standard at ripening stages	
		Manufacturing stage		Ripening stage						
Bacteria	**Itinerary**	**C_m**	**C_{dm}**	**C_{0W}**	**C_{4W}**	**C_{7W}**	**I × PS [c]**	**Global mean [d]**	**I × RS [e]**	**Global mean**
L. lactis	Standard	8.78 ± 0.07	9.12 ± 0.05	9.06 ± 0.00	8.95 ± 0.01	8.45 ± 0.02	NS [f]	8.87 ± 0.26		– [h]
CIRM-BIA1206	Stir15min	8.66 ± 0.02	9.03 ± 0.12	9.05 ± 0.04	8.76 ± 0.15	8.39 ± 0.00		8.78 ± 0.27**	*** [g]	–
	Salt7h			9.01 ± 0.08	8.97 ± 0.03	8.52 ± 0.17		–		–
	Rip16°C			9.08 ± 0.02	8.76 ± 0.03	7.65 ± 0.08***		–		–
L. plantarum	Standard	6.49 ± 0.09	7.53 ± 0.03	7.92 ± 0.05	8.47 ± 0.02	8.47 ± 0.02	NS	0.26 ± 7.78		8.29 ± 0.29
CRIM-BIA465	Stir15min	6.47 ± 0.10	7.41 ± 0.05	7.90 ± 0.10	8.42 ± 0.03	8.47 ± 0.13		0.27 ± 7.74	NS	8.27 ± 0.29
	Salt7h			7.96 ± 0.04	8.48 ± 0.07	8.52 ± 0.09		–		8.32 ± 0.28
	Rip16°C			8.00 ± 0.00	8.53 ± 0.01	8.56 ± 0.15		–		8.36 ± 0.29
P. freudenreichii	Standard	7.16 ± 0.09	8.04 ± 0.05	8.11 ± 0.03	8.56 ± 0.07	8.59 ± 0.00	NS	7.78 ± 0.78		8.42 ± 0.24
CIRM-BIA122	Stir15min	7.08 ± 0.05	7.99 ± 0.05	8.20 ± 0.02	8.59 ± 0.07	8.63 ± 0.21		7.74 ± 0.78	NS	8.47 ± 0.23
	Salt7h			8.05 ± 0.11	8.57 ± 0.03	8.65 ± 0.11		–		8.42 ± 0.30
	Rip16°C			8.14 ± 0.00	8.67 ± 0.06	8.62 ± 0.08		–		8.48 ± 0.27

[a] A description of the itineraries is available in Figure 1

[c] I, itinerary; PS, all production stages.

[d] global mean is the estimated marginal mean

[e] RS, ripening stage.

[f] NS, not significant.

[g] *, $p \leqslant 0.1$; **, $p \leqslant 0.05$; ***, $p \leqslant 0.001$.

[h] –, not relevant

Table 3. Physicochemical evolution of cheeses during the production of model semi-hard cheeses manufactured according to one standard *and three variant itineraries* [a]. Reported values are the means of two biological replicates. The last four columns present (I X PS) and (I X RS) interactions, as well as the global means of each composition under the analyzed itinerary.

Parameters	Itinerary	Cheese production					Comparison between Stir15min and Standard at all production stages		Comparison between all variant processes and Standard at ripening stages	
		Manufacturing stages		Ripening stages						
		C_m	C_{dm}	C_{0W}	C_{4W}	C_{7W}	I × PS [b]	Global mean [c]	I × RS [d]	Global mean
pH	Std	6.36 ± 0.08	5.46 ± 0.12	5.32 ± 0.03	5.12 ± 0.05	5.14 ± 0.02	NS [e]	5.48 ± 0.48	NS	5.19 ± 0.11
	Stir15min	6.37 ± 0.01	5.50 ± 0.08	5.33 ± 0.07	5.13 ± 0.05	5.15 ± 0.02		5.50 ± 0.48		5.20 ± 0.11
	Salt7h			5.32 ± 0.05	5.19 ± 0.06	5.14 ± 0.01		–		5.21 ± 0.09
	Rip16°C			5.33 ± 0.09	5.09 ± 0.01	5.18 ± 0.02		–		5.20 ± 0.12
Dry matter	Std	40.53 ± 4.81	51.44 ± 0.15	52.28 ± 1.41	53.41 ± 0.03	53.55 ± 0.26	NS	50.24 ± 5.45	NS	53.08 ± 0.90
in cheese	Stir15min	37.39 ± 4.61	50.36 ± 0.15	50.85 ± 1.99	51.34 ± 1.13	51.39 ± 1.00		48.27 ± 6.01 (-3.9%)** [f]		51.19 ± 1.15 (-3.6%)***
(g/100 g)	Salt7h			51.83 ± 1.48	52.59 ± 0.63	52.39 ± 1.70		–		52.27 ± 1.11 (-1.5%)*
	Rip16°C			52.15 ± 1.93	53.41 ± 0.73	53.23 ± 0.42		–		52.93 ± 1.12
Protein	Std		41.59 ± 1.32	43.34 ± 0.13	42.41 ± 0.66	42.32 ± 0.17	– [g]	–	NS	42.69 ± 0.59
in dry	Stir15min		42.91 ± 0.23	43.60 ± 0.45	41.91 ± 0.95	42.25 ± 0.58		–		42.59 ± 0.96
matter	Salt7h			43.26 ± 0.54	42.43 ± 0.53	42.33 ± 0.32		–		42.67 ± 0.59
(g/100 g)	Rip16°C			42.66 ± 1.41	42.31 ± 0.69	42.84 ± 0.14		–		42.60 ± 0.74
Fat	Std		46.78 ± 1.42	48.15 ± 2.92	48.41 ± 0.03	47.50 ± 1.72	–	–	NS	48.02 ± 1.57
in dry	Stir15min		47.90 ± 1.61	49.29 ± 0.03	47.54 ± 1.80	45.62 ± 0.72		–		47.48 ± 1.86
matter	Salt7h			48.71 ± 0.65	48.48 ± 0.09	46.31 ± 0.46		–		47.84 ± 1.24
(g/100 g)	Rip16°C			48.86 ± 2.26	49.23 ± 1.44	46.50 ± 0.70		–		48.20 ± 1.81
MFFB [h]	Std		63.96 ± 0.48	63.79 ± 0.00	62.84 ± 0.01	62.30 ± 1.02	–	–	NS	62.98 ± 0.82
(g/100 g)	Stir15min		65.43 ± 0.56	65.58 ± 1.81	64.37 ± 1.82	63.50 ± 1.24		–		64.48 ± 1.58 (+2.4%)*
	Salt7h			64.43 ± 1.07	63.63 ± 0.54	62.39 ± 0.74		–		63.48 ± 1.12
	Rip16°C			64.22 ± 0.76	63.21 ± 1.35	62.16 ± 0.70		–		63.20 ± 1.20
Calcium	Std	0.61 ± 0.09	0.70 ± 0.03	0.73 ± 0.01	0.71 ± 0.01	0.74 ± 0.04	NS	0.70 ± 0.06	**	–
in cheese	Stir15min	0.55 ± 0.08	0.68 ± 0.03	0.67 ± 0.02 (-7.8%)**	0.67 ± 0.03	0.72 ± 0.02		0.66 ± 0.07 (-5.9%)**		–
(g/100 g)										
	Salt7h			0.73 ± 0.01	0.70 ± 0.01	0.72 ± 0.00		–		–

	Rip16°C			0.75 ± 0.00	0.70 ± 0.01	0.71 ± 0.02		–		–
Calcium	Std	1.50 ± 0.04	1.36 ± 0.05	1.40 ± 0.02	1.33 ± 0.02	1.38 ± 0.08	NS	1.40 ± 0.07	**	–
in dry	Stir15min	1.46 ± 0.04	1.35 ± 0.07	1.33 ± 0.02	1.31 ± 0.02	1.40 ± 0.07		1.37 ± 0.07		–
matter	Salt7h			1.41 ± 0.03	1.33 ± 0.01	1.37 ± 0.04		–		–
(g/100 g)	Rip16°C			1.44 ± 0.05	1.31 ± 0.01	1.34 ± 0.04		–		–
Chloride	Std			0.35 ± 0.05	0.83 ± 0.03	0.85 ± 0.04	–	–	**	–
in cheese	Stir15min			0.41 ± 0.07	0.96 ± 0.08 (+16.7%)***	0.94 ± 0.03 (+10.9%)**		–		–
(g/100 g)	Salt7h			0.32 ± 0.03	0.76 ± 0.03	0.78 ± 0.07 (-8.6 %)*		–		–
	Rip16°C			0.37 ± 0.10	0.85 ± 0.09	0.85 ± 0.03		–		–
Chloride	Std			0.72 ± 0.08	1.77 ± 0.07	1.83 ± 0.08	–	–	**	–
in water	Stir15min			0.83 ± 0.11	1.98 ± 0.12 (+11.6%)**	1.94 ± 0.02		–		–
(g/100 g)	Salt7h			0.66 ± 0.04	1.61 ± 0.03 (-9.3%)**	1.63 ± 0.08 (-10.9%)**		–		–
	Rip16°C			0.77 ± 0.18	1.82 ± 0.16	1.82 ± 0.05		–		–

[a] A description of the itineraries is available in Figure1

[b] I, itinerary; PS, all production stages.

[c] Global mean is the estimated marginal mean

[d] RS, ripening stage.

[e] NS, not significant.

[f] *, $p \leqslant 0.1$; **, $p \leqslant 0.05$; ***, $p \leqslant 0.001$.

[g] –, not analyzed

[h] MFFB, moisture content in the fat-free basis of cheese.

Table 4, Concentrations of sugars, organic acids and volatiles in milk before and after prematuration at 14°C for 18 h [a]

Compounds	Before prematuration	After prematuration	Comparison	p-value
Lactose (g/kg milk)	48.44 ± 0.27	47.47 ± 0.52	– [b]	p>0.1
Galactose (g/kg milk)	0.22 ± 0.02	0.21 ± 0.04	–	p>0.1
Citric acid (g/kg milk)	2.23 ± 0.04	2.28 ± 0.02	–	p>0.1
Lactic acid (g/kg milk)	ND [c]	0.15 ± 0.01	–	p>0.1
Acetic acid (g/kg milk)	0.01 ± 0.00	0.04 ± 0.00	× 3.4	p = 0.090
propan-2-one (a.u.[d])	5.01E+08	4.97E+08	–	p>0.1
butan-2-one (a.u.[d])	1.42E+09	1.31E+09	–	p>0.1
Butane-2,3-dione (a.u.[d]) (diacetyl)	2.56E+07	4.50E+08	× 17.6	p = 0.049
3-Hydroxybutan-2-one (a.u.[d])	5.39E+07	3.34E+08	× 6.2	p = 0.054
Hexanoic acid (a.u.[d])	3.24E+07	3.72E+07	× 1.2	p = 0.046
Octanoic acid (a.u.[d])	1.67E+04	2.77E+04	× 1.7	p = 0.026

[a] Reported values are the means of two biological replicates.

[b] –, not analyzed

[c] ND, not detected

[d] arbitrary units: volatile compounds were semi-quantified using the abundance of one selected mass fragment (m/z)

Table 5, Concentrations of sugars and organic acids during the production of model semi-hard cheeses manufactured according to one standard and three variant itineraries [a].

Compounds, g/kg	itinerary	Cheese production					Comparison between Stir15min and Standard throughout production		Comparison between all variant processes and Standard throughout ripening	
		Manufacturing stages		**Ripening stages**						
		C_m	**C_{dm}**	**C_{0W}**	**C_{4W}**	**C_{7W}**	**I × PS [b]**	**Global mean [c]**	**I × RS [d]**	**Global mean**
Lactose	Std	22.19 ± 1.17	8.27 ± 0.10	6.24 ± 1.07	1.14 ± 0.09	0.79 ± 0.06	NS [e]	7.73 ± 8.22		– [g]
	Stir15min	25.32 ± 5.72	11.79 ± 0.63	10.23 ± 1.26 (+63.8 %)***	4.62 ± 0.97 (+306.1%)***	3.46 ± 0.44 (+337.7%)***		11.08 ± 8.45 (+43.5%) *** [f]	**	–
	Salt7h			6.98 ± 0.18	1.11 ± 0.27	0.59 ± 0.57		–		–
	Rip16°C			7.28 ± 0.77	0.71 ± 0.53	0.18 ± 0.15		–		–
Galactose	Std	0.77 ± 0.30	0.70 ± 0.07	0.44 ± 0.20	0.21 ± 0.03	0.16 ± 0.01	NS	0.45 ± 0.29		0.27 ± 0.16
	Stir15min	0.59 ± 0.24	0.60 ± 0.04	0.46 ± 0.16	0.18 ± 0.00	0.10 ± 0.01		0.38 ± 0.24	NS	0.24 ± 0.18
	Salt7h			0.44 ± 0.21	0.23 ± 0.03	0.11 ± 0.03		–		0.26 ± 0.18
	Rip16°C			0.46 ± 0.20	0.15 ± 0.02	0.07 ± 0.02		–		0.22 ± 0.20
Pyruvic acid	Std	0.06 ± 0.01	0.24 ± 0.05	0.34 ± 0.06	0.45 ± 0.14	0.64 ± 0.03	NS	0.35 ± 0.21		–
	Stir15min	0.05 ± 0.02	0.19 ± 0.03	0.26 ± 0.06	0.41 ± 0.04	0.53 ± 0.13		0.29 ± 0.19 (-16.5%)*	**	–
	Salt7h			0.32 ± 0.07	0.41 ± 0.09	0.62 ± 0.09		–		–
	Rip16°C			0.33 ± 0.07	0.61 ± 0.02	0.91 ± 0.12 (+40.3%)**		–		–
Citric acid	Std	1.28 ± 0.03	0.25 ± 0.04	0.15 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	***	–		0.48 ± 0.15
	Stir15min	1.50 ± 0.11 (-16.5%)***	0.26 ± 0.04	0.15 ± 0.01	0.00 ± 0.00	0.00 ± 0.00		–	NS	0.40 ± 0.14
	Salt7h			0.11 ± 0.05	0.00 ± 0.00	0.00 ± 0.00		–		0.45 ± 0.15
	Rip16°C			0.16 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		–		0.62 ± 0.27
Succinic acid	Std	0.09 ± 0.01	0.20 ± 0.01	0.17 ± 0.04	0.29 ± 0.04	0.35 ± 0.06	**	–		–
	Stir15min	0.09 ± 0.00	0.17 ± 0.02	0.16 ± 0.01	0.33 ± 0.01	0.49 ± 0.07 (+40.2%)**		–	***	–
	Salt7h			0.12 ± 0.02	0.32 ± 0.01	0.55 ± 0.06 (+57.0%)**		–		–
	Rip16°C			0.15 ± 0.02	0.50 ± 0.01 (+73.0%)***	0.91 ± 0.01 (+158.7%)***		–		–
Lactic acid	Std	4.16 ± 0.16	10.38 ± 0.38	11.65 ± 0.20	13.05 ± 0.20	10.35 ± 0.38	**	–		–
	Stir15min	3.43 ± 0.25	9.50 ± 0.87 (-8.5%)*	10.13 ± 0.10 (-13.0%)**	11.25 ± 1.02 (-13.8%)**	9.98 ± 0.01		–	***	–
	Salt7h			10.89 ± 0.14	13.79 ± 0.03	10.87 ± 0.22		–		–
	Rip16°C			10.78 ± 0.59	10.29 ± 1.16 (-21.1%)***	5.66 ± 0.58 (-45.3%)***		–		–
Acetic acid	Std	0.40 ± 0.05	0.86 ± 0.08	1.01 ± 0.05	1.89 ± 0.16	2.56 ± 0.05	**	–		–
	Stir15min	0.36 ± 0.03	0.91 ± 0.10	0.98 ± 0.05	1.88 ± 0.04	3.02 ± 0.42 (+18.3%)**		–	***	–
	Salt7h			0.98 ± 0.05	1.97 ± 0.09	2.65 ± 0.40		–		–
	Rip16°C			0.94 ± 0.10	2.53 ± 0.05 (+34.2%)**	3.43 ± 0.13 (+34.1%)***		–		–
Propanoic	Std	ND [h]	ND	0.11 ± 0.01	3.13 ± 0.24	4.98 ± 0.02	–	–	***	–

acid										
	Stir15min	ND	ND	0.14 ± 0.01	3.33 ± 0.25	5.65 ± 0.20 (+13.6%)**		–		–
	Salt7h			0.13 ± 0.00	3.57 ± 0.03	5.17 ± 0.57		–		–
	Rip16°C			0.15 ± 0.00	5.14 ± 0.15 (+63.9%)***	7.91 ± 0.23 (+58.9%)***		–		–
Butanoic acid	Std	0.13 ± 0.01	0.21 ± 0.02	0.27 ± 0.03	0.66 ± 0.22	0.68 ± 0.09	NS	0.39 ± 0.26	***	–
	Stir15min	0.12 ± 0.01	0.17 ± 0.01	0.23 ± 0.02	0.40 ± 0.16	0.59 ± 0.00		0.30 ± 0.19 (-23.1%)*		–
	Salt7h			0.24 ± 0.02	0.48 ± 0.03	0.61 ± 0.00		–		–
	Rip16°C			0.22 ± 0.00	0.72 ± 0.14	1.24 ± 0.15 (+82.3%)***		–		–
Phenyllactic	Std	0.01 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.06 ± 0.00	NS	0.04 ± 0.02	***	–
acid	Stir15min	0.01 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.07 ± 0.00		0.04 ± 0.02		–
	Salt7h			0.04 ± 0.00	0.04 ± 0.00	0.06 ± 0.00		–		–
	Rip16°C			0.04 ± 0.00	0.06 ± 0.00 (+34.5%)**	0.10 ± 0.02 (+65.6%)***		–		–

[a] Values are the means of two biological replicates.

[b] I, itinerary; PS, all production stages.

[c] Global mean is the estimated marginal mean

[d] RS, ripening stage.

[e] NS, not significant.

[f] *, $p \leqslant 0.1$; **, $p \leqslant 0.05$; ***, $p \leqslant 0.001$.

[g] –, not analyzed

[h] ND, not detected

Table 6: Volatile compounds detected by GC-MS in cheese during the production of model semi-hard cheeses

Chemical class	CAS Number	Volatile compounds [a] (abbreviated code or trivial name)	Identification [b]	LRI	m/z	Odor descriptor [c]
Ketones	67-64-1	Propan-2-one (acetone)	LRI, DB, S	804	58	Solvent; ethereal; apple; pear
	78-93-3	Butan-2-one	LRI, DB, S	892	72	Acetone-like ethereal fruity camphor
	431-03-8	Butane-2,3-dione (diacetyl)	LRI, DB, S	979	86	Strong butter sweet creamy pungent caramel
	513-86-0	3-Hydroxybutan-2-one (acetoin)	LRI, DB, S	1284	88	Sweet buttery creamy dairy milky fatty
	116-09-6	1-hydroxypropan-2-one (acetol)	LRI, DB	1298	43	Pungent sweet caramellic ethereal
Alcohols	71-23-8	Propan-1-ol	LRI, DB, S	1054	59	Alcoholic fermented fusel musty
	137-32-6	2-methylbutan-1-ol	LRI, DB, S	1231	70	Roasted wine onion fruity fusel alcoholic whiskey
Sulfur compounds	624-92-0	(Methyldisulfanyl)methane (DMDS: dimethyl disulfide)	LRI, DB, S	1068	94	Sulfurous vegetable cabbage onion
Acids	64-19-7	Acetic acid (C2)	LRI, DB, S	1448	60	Sharp pungent sour vinegar
	79-09-4	Propanoic acid (C3)	LRI, DB, S	1533	74	Pungent acidic cheesy vinegar
	107-92-6	Butanoic acid (C4)	LRI, DB, S	1631	60	Sharp acetic cheese butter fruit
	116-53-0	2-Methylbutanoic acid	LRI, DB, S	1674	87	Pungent acid roquefort cheese
	142-62-1	Hexanoic acid (C6)	LRI, DB, S	1829	60	Sour fatty sweat cheese
	124-07-2	Octanoic acid (C8)	LRI, DB, S	1933	60	Fatty waxy rancid oily vegetable cheesy

[a] IUPAC name

[b] Identification based on: LRI, calculated linear retention index; DB, mass spectral data from Library NIST 2008, and S, Standard.

[c] Odor descriptions from thegoodscentscompany.com and foodb. ca (2020).

1 Figures

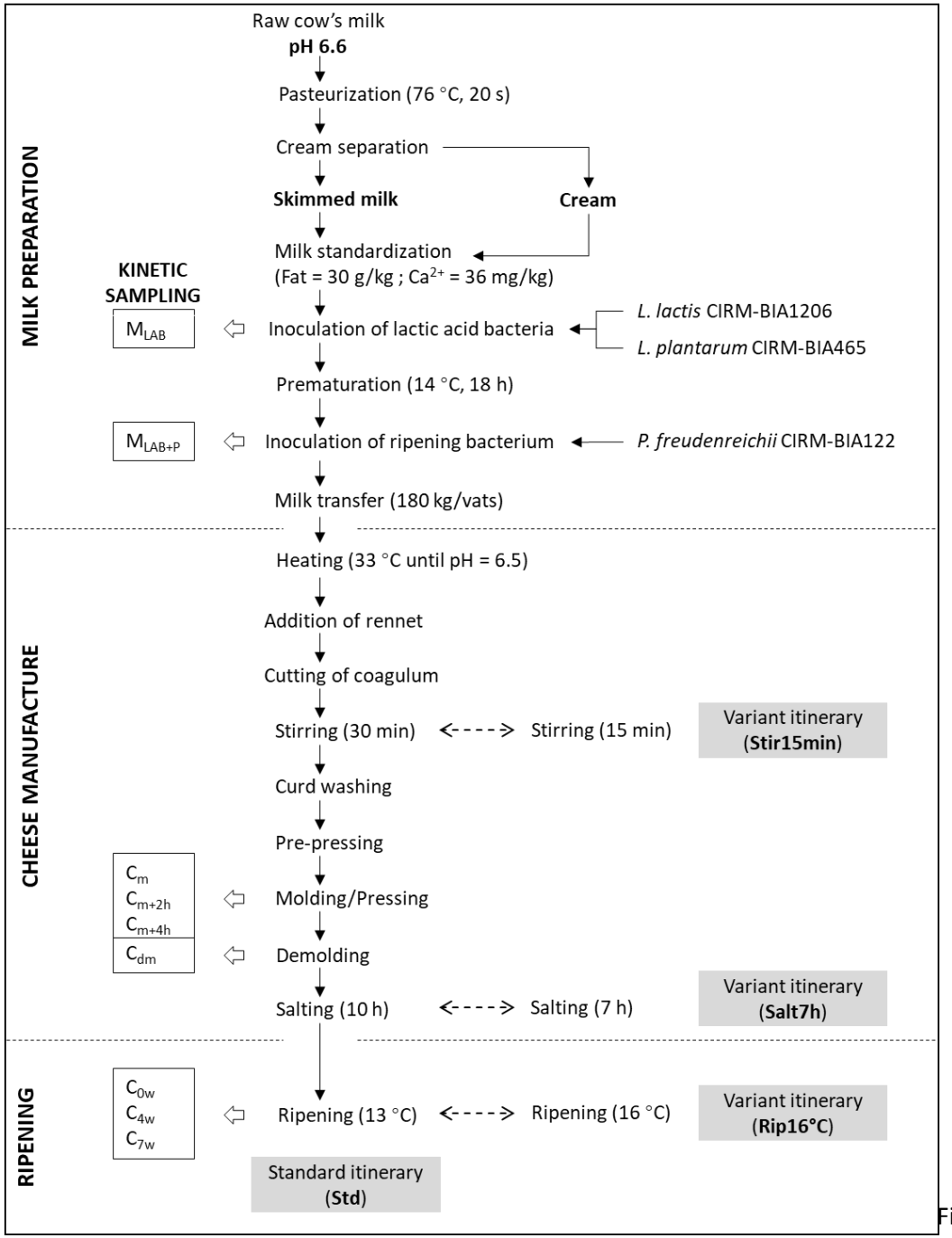

MILK PREPARATION
Raw cow's milk
pH 6.6
Pasteurization (76 °C, 20 s)
Cream separation
Skimmed milk
Cream
Milk standardization
(Fat = 30 g/kg ; Ca2+ = 36 mg/kg)
KINETIC SAMPLING
MLAB
Inoculation of lactic acid bacteria
L. lactis CIRM-BIA1206
L. plantarum CIRM-BIA465
Prematuration (14 °C, 18 h)
MLAB+P
Inoculation of ripening bacterium
P. freudenreichii CIRM-BIA122
Milk transfer (180 kg/vats)
CHEESE MANUFACTURE
Heating (33 °C until pH = 6.5)
Addition of rennet
Cutting of coagulum
Stirring (30 min)
Stirring (15 min)
Variant itinerary (Stir15min)
Curd washing
Pre-pressing
Cm
Cm+2h
Cm+4h
Molding/Pressing
Cdm
Demolding
Salting (10 h)
Salting (7 h)
Variant itinerary (Salt7h)
RIPENING
C0w
C4w
C7w
Ripening (13 °C)
Ripening (16 °C)
Variant itinerary (Rip16°C)
Standard itinerary (Std)

Figure 1

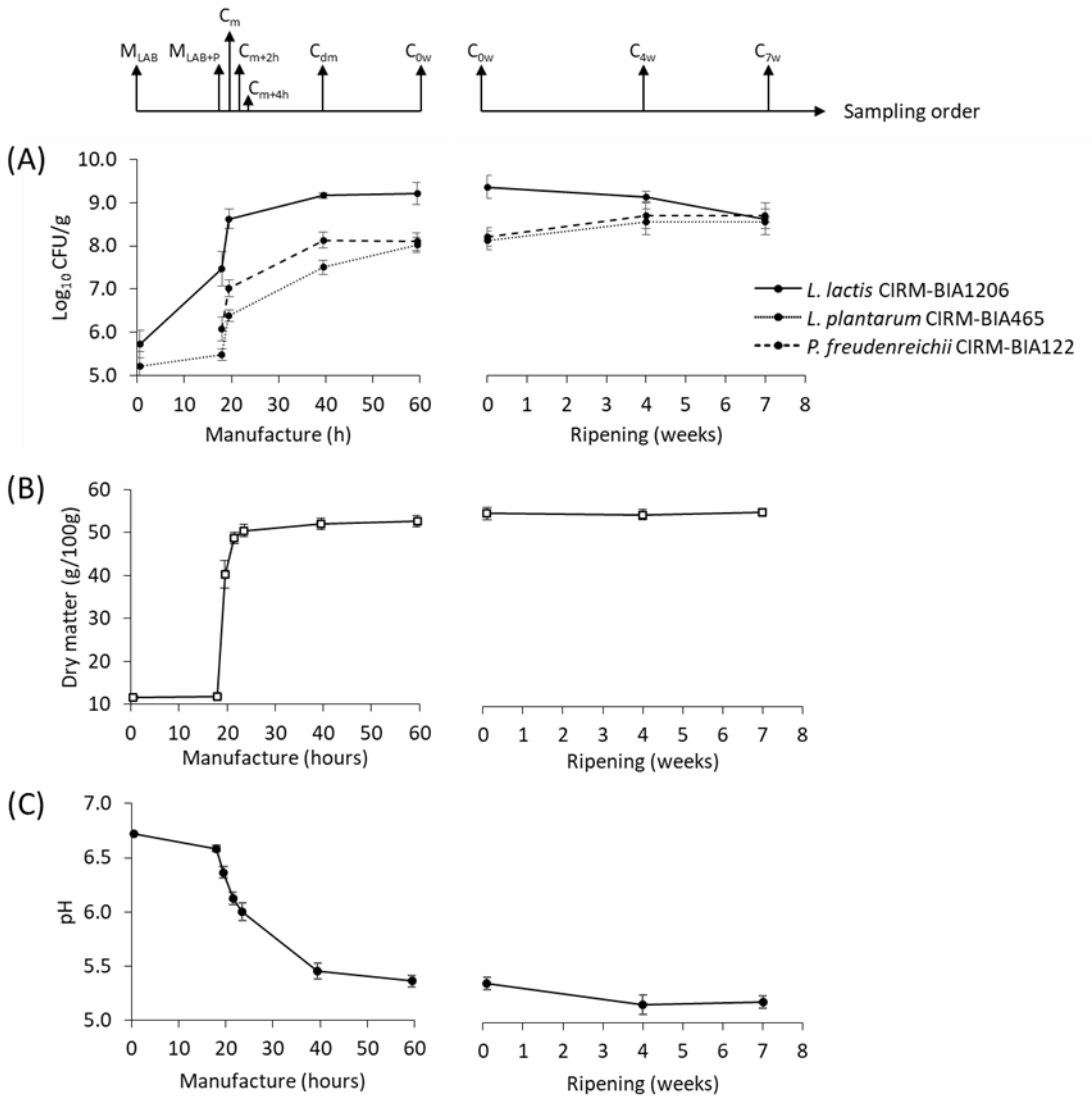

C_m
M_{LAB}
M_{LAB+P}
C_{m+2h}
C_{dm}
C_{0w}
C_{m+4h}
C_{0w}
C_{4w}
C_{7w}
Sampling order
(A)
Log_{10} CFU/g
Manufacture (h)
Ripening (weeks)
L. lactis CIRM-BIA1206
L. plantarum CIRM-BIA465
P. freudenreichii CIRM-BIA122
(B)
Dry matter (g/100g)
Manufacture (hours)
Ripening (weeks)
(C)
pH
Manufacture (hours)
Ripening (weeks)

Figure 2

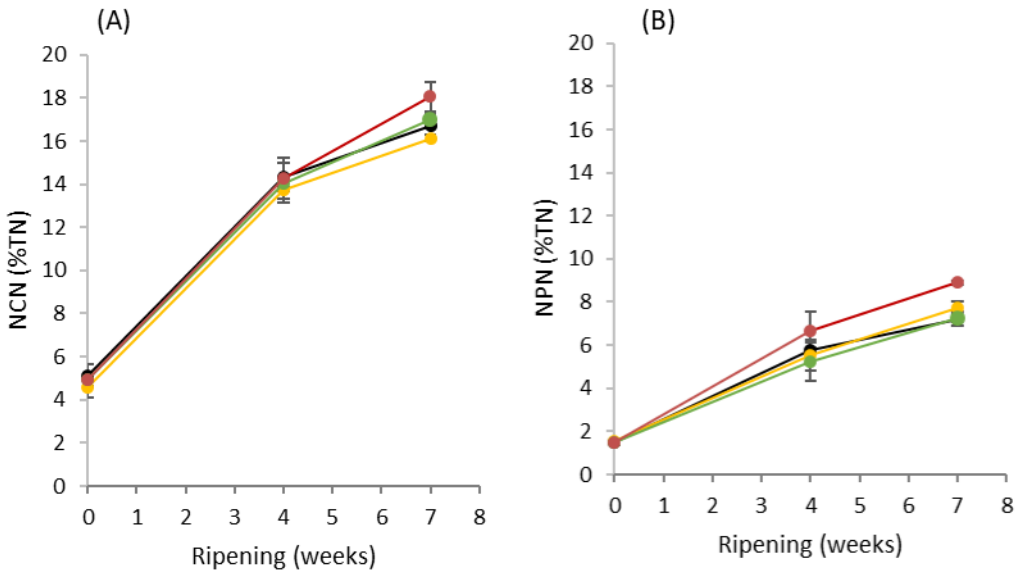


Figure 3

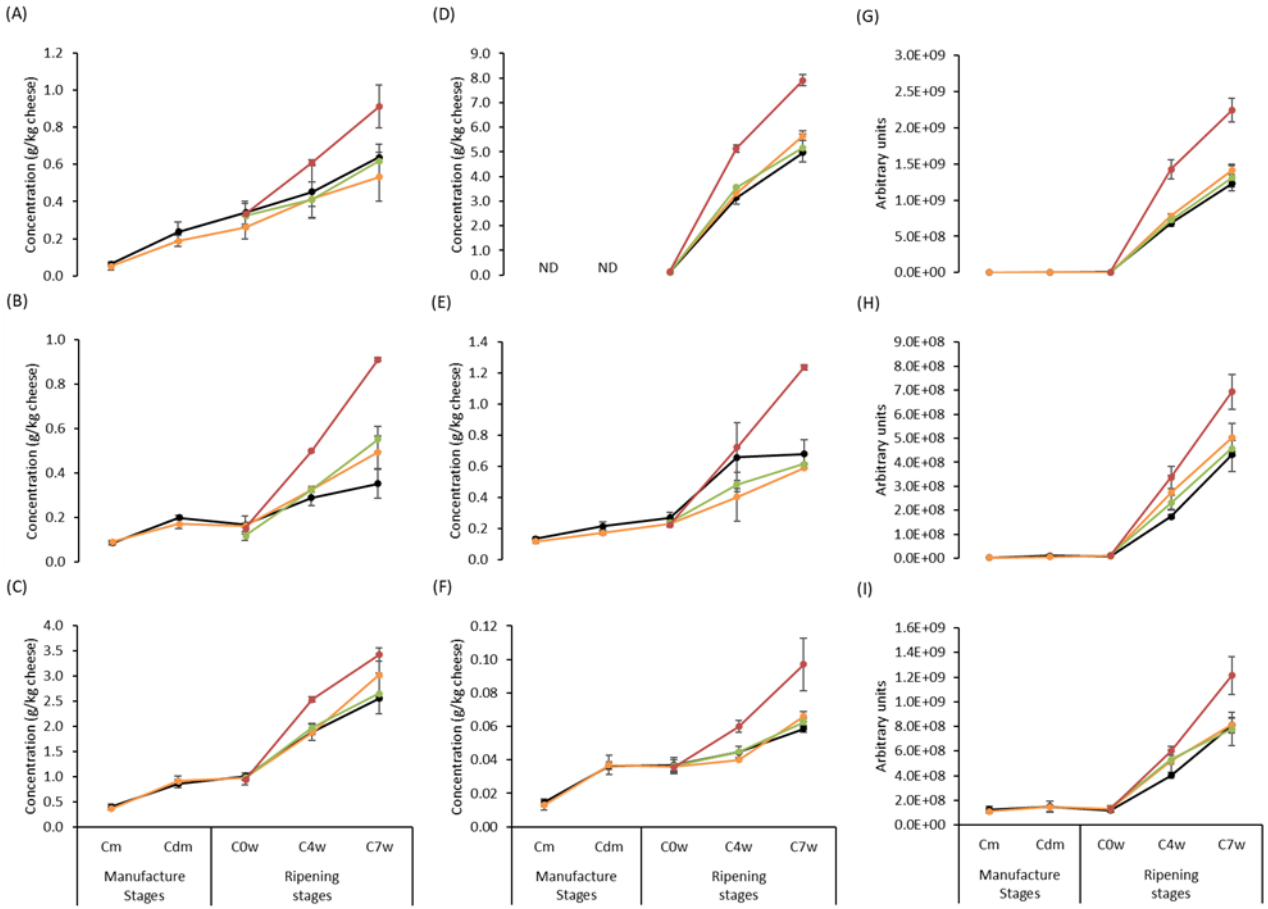

(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
Concentration (g/kg cheese)
Arbitrary units
ND
ND
Cm
Cdm
C0w
C4w
C7w
Manufacture Stages
Ripening stages

Figure 4

(A)

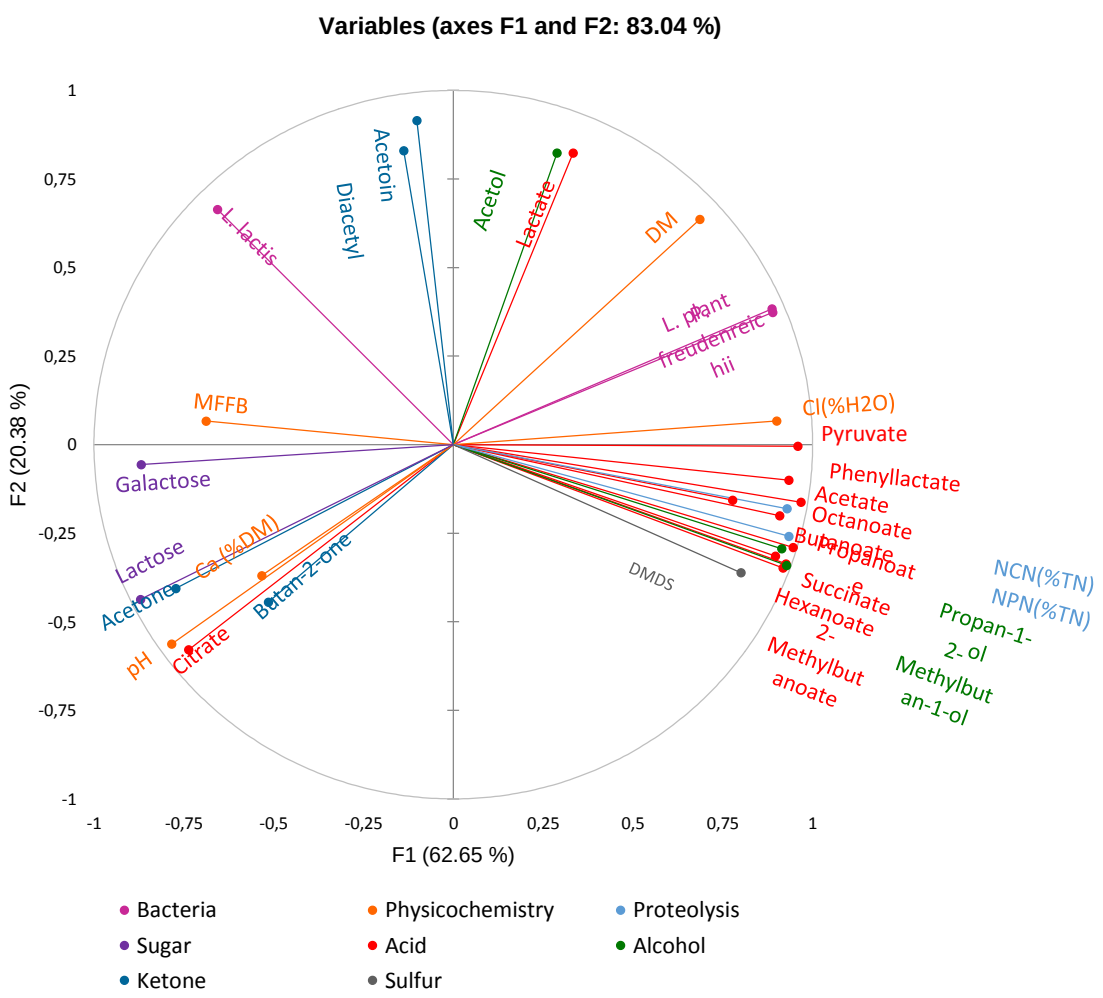
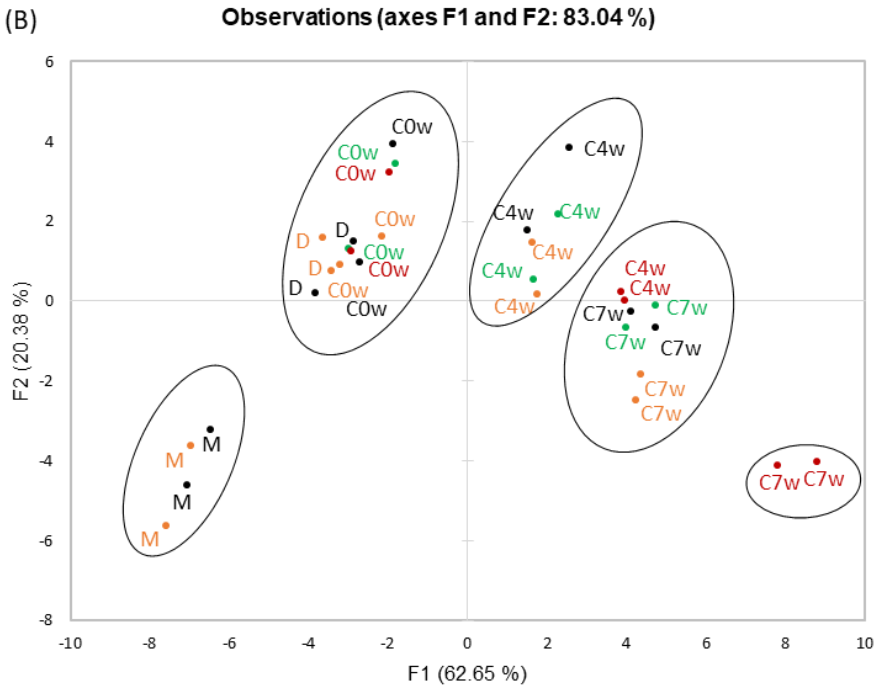

Variables (axes F1 and F2: 83.04 %)
F2 (20.38 %)
F1 (62.65 %)
1
0,75
0,5
0,25
0
-0,25
-0,5
-0,75
-1
-1
-0,75
-0,5
-0,25
0
0,25
0,5
0,75
1
L_lactis
Diacetyl
Acetoin
Acetol
Lactate
DM
L. plant
freudenreic
hii
MFFB
Galactose
Lactose
Acetone
Ca (%DM)
Butan-2-one
pH
Citrate
DMDS
Cl(%H2O)
Pyruvate
Phenyllactate
Acetate
Octanoate
Butanoate
Propanoat
e
Succinate
Hexanoate
2-
Methylbut
anoate
NCN(%TN)
NPN(%TN)
Propan-1-
2- ol
Methylbut
an-1-ol
Bacteria
Physicochemistry
Proteolysis
Sugar
Acid
Alcohol
Ketone
Sulfur

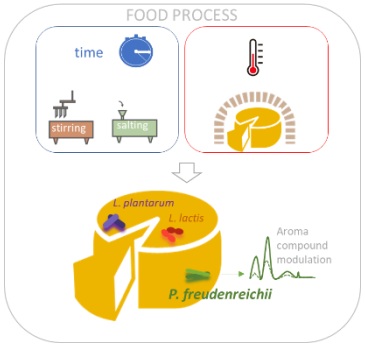
Figure 5A


(B)
Observations (axes F1 and F2: 83.04 %)
F2 (20.38 %)
F1 (62.65 %)
C0w
C4w
C7w
D
M

1

Figure 5B

TOC graphic


FOOD PROCESS
time
stirring
salting
L. plantarum
L. lactis
Aroma
compound
modulation
P. freudenreichii