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### ► **To cite this version:**

Alison Yee, Marie-Bernadette Maillard, Nathalie Roland, Victoria Chuat, Aurélie Leclerc, et al.. Great interspecies and intraspecies diversity of dairy propionibacteria in the production of cheese aroma compounds. *International Journal of Food Microbiology*, 2014, 191, pp.60-68. <10.1016/j.ijfoodmicro.2014.09.001>. <hal-01209716>

**HAL Id: hal-01209716**

**<https://hal.science/hal-01209716v1>**

Submitted on 25 Dec 2025

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# Great interspecies and intraspecies diversity of dairy propionibacteria in the production of cheese aroma compounds

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Flavor is an important sensory property of fermented food products, including cheese, and largely results from the production of aroma compounds by microorganisms. *Propionibacterium freudenreichii* is the most widely used species of dairy propionibacteria; it has been implicated in the production of a wide variety of aroma compounds through multiple metabolic pathways and is associated with the flavor of Swiss cheese. However, the ability of other dairy propionibacteria to produce aroma compounds has not been characterized. This study sought to elucidate the effect of interspecies and intraspecies diversity of dairy propionibacteria on the production of aroma compounds in a cheese context. A total of 76 strains of *Propionibacterium freudenreichii*, *Propionibacterium jensenii*, *Propionibacterium thoenii*, and *Propionibacterium acidipropionici* were grown for 15 days in pure culture in a rich medium derived from cheese curd. In addition, one strain each of two phylogenetically related non dairy propionibacteria, *Propionibacterium cyclohexanicum* and *Propionibacterium microaerophilum* were included. Aroma compounds were analyzed using headspace trap gas chromatography mass spectrometry (GC-MS). An analysis of variance performed on GC-MS data showed that the abundance of 36 out of the 45 aroma compounds detected showed significant differences between the cultures. A principal component analysis (PCA) was performed for these 36 compounds. The first two axes of the PCA, accounting for 60% of the variability between cultures, separated *P. freudenreichii* strains from *P. acidipropionici* strains and also differentiated *P. freudenreichii* strains from each other. *P. freudenreichii* strains were associated with greater concentrations of a variety of compounds, including free fatty acids from lipolysis, ethyl esters derived from these acids, and branched chain acids and alcohols from amino acid catabolism. *P. acidipropionici* strains produced less of these compounds but more sulfur containing compounds from methionine catabolism. Meanwhile, branched chain aldehydes and benzaldehyde were positively associated with certain strains of *P. jensenii* and *P. thoenii*. Moreover, the production of compounds with a common origin was correlated. Compound abundance varied significantly by strain, with fold changes between strains of the same species as high as in the order of 500 for a single compound. This suggests that the diversity of dairy propionibacteria can be exploited to modulate the flavor of mild cheeses.

## 1. Introduction

Aroma compounds play the major role in the perception of flavor, which is an important property for consumers choosing food products.

*Abbreviations:* ANOVA, analysis of variance; DMS, dimethyl sulphide; DMDS, dimethyl disulfide; DMTS, dimethyl trisulfide; FFAs, free fatty acids; GC-MS, gas chromatography-mass spectrometry; LSD, least significant difference; PCA, Principal Component Analysis; YEL, yeast extract lactate.

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Flavor stems from the five basic tastes perceived at the tongue and, more importantly, from a variety of aroma notes due to the release and retronasal perception of volatile aroma compounds during and after food consumption. Fermented foods in particular contain numerous aroma compounds, due to the activity of microorganisms. For example, in cheese, the conversion of curd components during manufacture and ripening results in aroma compounds that span a range of chemical classes, including acids, esters, diacetyl, and sulfur containing compounds (Urbach, 1997). Lactate fermentation can result in the production of acids, ethanol, diacetyl and some esters (Curioni and Bosset, 2002). The catabolism of branched chain, aromatic amino acids and methionine can produce a variety of alcohols, aldehydes, and acids (Curioni and Bosset, 2002; Yvon and Rijnen, 2001). The hydrolysis of

milk fat results in the formation of free fatty acids (FFAs) that are not only important aroma compounds themselves, but also serve as precursors for other aroma compounds, such as esters and methylketones (Curioni and Bosset, 2002). The cheese microorganisms responsible for such reactions include lactic acid bacteria, used as starter cultures, and, depending on the cheese variety, adjunct cultures, which consist of bacteria such as propionibacteria and smear bacteria, yeasts, and fungi (McSweeney, 2004). Aroma compounds are present in different cheese varieties in varying amounts. The diversity and abundance of the cheese aroma compounds are markedly influenced by the density and richness of the microbiota, which varies according to manufacture conditions. Therefore, microorganisms can be used to modulate or intensify cheese flavor. In particular, there is a demand to diversify the generally mild flavor of some varieties of semi hard cheeses manufactured from pasteurized milk inoculated with only lactic acid bacteria.

Propionibacteria are an example of cheese microorganisms associated with the production of aroma compounds. The most commonly studied dairy *Propionibacterium* species is *Propionibacterium freudenreichii*, widely used as a ripening culture in Swiss cheese, where it contributes to the formation of the typical flavor and eyes (Langsrud and Reinbold, 1973). It can also modulate the flavor of other cheese varieties without eyes, such as Cheddar (Fernandez Espla and Fox, 1998), Raclette and Morbier cheeses (Thierry et al., 2005a; Thierry and Maillard, 2002). It is able to produce a variety of aroma compounds associated with Swiss cheese flavor through the following metabolic pathways: lactate fermentation, lipolysis, and amino acid catabolism (Langsrud and Reinbold, 1973; Thierry et al., 2011). The production of aroma compounds by *P. freudenreichii* is highly strain dependent (Abeijon Mukdsi et al., 2014; Thierry et al., 2011). In addition to *P. freudenreichii*, there are three other dairy related species: *Propionibacterium jensenii*, *Propionibacterium thoenii*, and *Propionibacterium acidipropionici*, which have all been isolated from milk and dairy products, as well as from soil, silage, and dairy plants (Cummins and Johnson, 1992). *P. jensenii*, *P. thoenii*, and *P. acidipropionici* have different tolerances to heat, pH, and salt than *P. freudenreichii* (Darilmaz and Beyatli, 2012; Rossi et al., 2000) and therefore may be useful in cheese manufacture. Their possible contribution to cheese flavor have never been reported.

The goal of this study was to evaluate the interspecies and intraspecies diversity of propionibacteria in terms of their ability to produce aroma compounds in a cheese context. Towards that aim, we screened a collection of strains of the four dairy *Propionibacterium* species according to an approach recently described: culture in a curd based medium, extraction and analysis of volatiles using headspace trap coupled to gas chromatography and mass spectrometry, and a workflow of data processing and analysis (Pogacic et al., 2015). For the sake of comparison, we included a strain each of *Propionibacterium cyclohexanicum* and *Propionibacterium microaerophilum*, two food related *Propionibacterium* species that are phylogenetically close to *P. freudenreichii* and *P. acidipropionici*, respectively (Koussemon et al., 2001; Kusano et al., 1997).

## 2. Materials and methods

### 2.1. Strains and growth conditions

A total of 78 genetically distinct strains (Table 1) of six *Propionibacterium* species were used, 40 from the International Centre for Microbial Resources collection of bacteria (CIRM BIA, UMR1253, INRA, Rennes, France) and 38 from Laboratoires STANDA (Caen, France), a manufacturer of cheese starter cultures. Bacteria were stored at  $-80^{\circ}\text{C}$  in glycerol and pre cultured twice in yeast extract lactate (YEL) medium (Malik et al., 1968) at  $30^{\circ}\text{C}$ . To screen the ability of propionibacteria to produce aroma compounds, the bacteria were incubated for 15 days at  $30^{\circ}\text{C}$  in pure culture in a rich medium derived from cheese curd prepared as described below.

**Table 1**  
List of the 78 *Propionibacterium* strains used in the present study.

Species	Number of strains	Strain name <sup>a</sup>
<i>P. freudenreichii</i>	41	CB6, CB123, CB141, CB1397, CB1406, CB1412, CB1413, CB1414, CB1426, CB1497, CB1499, LSP1F, LSP2, LSP3F, LSP3S, LSP9; LSP10, LSP11S, LSP13, LSP14, LSP18A, LSP19, LSP22, LSP23, LSP24, LSP26, LSP28, LSP31, LSP51F, LSP51S, LSP56, LSP100, LSP101, LSP102, LSP108, LSP110, LSP209, LSP210, LSP214, LSP219, LSP223
<i>P. jensenii</i>	19	CB133, CB1430, CB1432, CB1505, CB1506, CB1507, CB1655, CB1657, CB1658, CB1659, CB1660, CB1661, LSP36B, LSP37, LSP38, LSP39, LSP40B, LSP58, LSP61
<i>P. acidipropionici</i>	8	CB1425, CB1427, CB1428, CB1652, CB1653, CB1656, CB1664, LSP96
<i>P. thoenii</i>	8	CB1329, CB1429, CB1434, CB1508, CB1509, CB1510, CB1662, CB1663
<i>P. microaerophilum</i>	1	CB677
<i>P. cyclohexanicum</i>	1	CB698

<sup>a</sup> CB is for CIRM-BIA, LSP strains are from Laboratoires STANDA.

### 2.2. Preparation and inoculation of the curd medium

The curd based medium was prepared as previously described (Pogacic et al., 2015), with modifications. Briefly, a non brined curd from a semi hard cheese was obtained from a cheese manufacturer. The curd was grated, vacuum packed, and stored at  $-20^{\circ}\text{C}$  until use. Frozen curd was thawed at ambient temperature and 50 g curd was homogenized in 100 g of a sterilized ( $115^{\circ}\text{C}$  for 15 min) solution (1.2 g/L tryptic peptone of casein, 18 g/L NaCl, 1.2 g/L lactose monohydrate, in deionized water). The curd and solution were homogenized in a Waring blender, and 10 mL of the homogenate was immediately transferred to a glass culture tube. Tubes were autoclaved at  $110^{\circ}\text{C}$  for 15 min, vortexed for 5 s, and stored at  $4^{\circ}\text{C}$  until use.

Immediately prior to inoculation of curd with the bacterial strains, each tube was vortexed  $\geq 15$  s to ensure homogeneity of the medium. 100  $\mu\text{L}$  of a sterile solution of a mixture of ethanol (Sigma Aldrich, St. Quentin Fallavier, France) and 3 methyl 1 butanol (Sigma Aldrich) were added to each tube and vortexed, yielding final concentrations of 2.0 and 0.01 mM of ethanol and 3 methylbutanol, respectively. Stationary phase (48 h) bacterial precultures in YEL were added (1% v/v), resulting in inoculation at about  $10^7$  cells/mL. Cultures were incubated for 14 days at  $30^{\circ}\text{C}$ . Each culture was performed in biological duplicate (precultures incubated on different days). For each series of inoculations, a control of curd medium with alcohol solution but no bacteria was included.

### 2.3. pH measurement and microbial enumeration

At the end of incubation, the pH of each culture and control was measured using a pH meter. Culturable populations were determined for one quarter of the strains at the end of incubation, 19 selected strains representing all six species. Serial ten fold dilutions were performed in 0.1% sterile tryptone water. For each dilution, two Petri dishes of YEL agar were incubated anaerobically at  $30^{\circ}\text{C}$  for 6 days.

### 2.4. Analysis of volatile compounds using GC MS

Volatile compounds were analyzed using a Clarus 680 gas chromatograph coupled to Clarus 600 T quadrupole mass spectrometer (Perkin Elmer, Courtaboeuf, France) as previously described (Pogacic et al., 2015). Briefly, the volatiles were separated on an Elite 5MS capillary column ( $60\text{ m} \times 0.25\text{ mm} \times 1\text{ }\mu\text{m}$ ; Perkin Elmer), with helium as the mobile phase. The temperature of the oven was initially  $35^{\circ}\text{C}$ , maintained

for 5 min. The temperature was increased at 7 °C/min to 140 °C and then at 13 °C/min to 280 °C. The mass spectrometer was operated in the scan mode (scan time 0.3 s, interscan delay 0.03 s) within a mass range of m/z 29 to 206. Ionization was done by electronic impact at 70 eV.

A 2.5 mL sample of each culture and control at day 15 post inoculation was placed in a 20 mL Perkin Elmer vial. Vials were stored at -80 °C until analysis. All samples were analyzed in the same GC MS run over a period of 10 consecutive days, with all the samples within a replicate injected in a randomized order over the GC MS run. Standard (a 20 ng/g aqueous solution of a mixture of 11 volatile compounds, prepared as previously described (Pogacic et al., 2015)) and blank samples (boiled deionized water) were regularly injected to control the stability of the GC MS system and detect possible carry over.

### 2.5. Data processing

The GC MS raw data files were converted to netCDF format with Data Bridge (Perkin Elmer, Waltham, Massachusetts, USA) for further analysis. GC MS data were processed by converting the raw data to time and mass aligned chromatographic peaks areas using the open source XCMS package implemented with the R statistical language (Smith et al., 2006). The full width at half maximum was set to 5, the maximum number of peaks per ion to 10, the group bandwidth to 3, and all other parameters set to the default settings. A total of 1812 signals (one m/z at one retention time) were generated by XCMS, corresponding to about 80 different compounds, since multiple signals are generated per compound. Compounds were identified by comparison of mass spectra and retention times with those of authentic standards, or on the basis of their retention index and mass spectral data from the NIST 2008 Mass Spectral Library (Scientific Instrument Services, Ringoes, NJ, USA) and other databases (Flavornet, www.flavornet.org (Kondjoyan and Berdagué, 1996)). Some compounds were tentatively identified on the basis of mass spectral data alone when retention index data were not available. From all the signals generated by XCMS, 72 compounds were identified and quantified from the abundance of one specific signal. Non odorant compounds (alkanes, alkenes, furans, among others), and some contaminants, such as trichloromethane and toluene, were not retained in the dataset.

### 2.6. Statistical and multivariate analysis

An analysis of variance (ANOVA) was performed on bacterial counts, pH values and volatile abundance to determine if they significantly depend on species or strain, using R statistical software. Means were compared using the least significant difference (LSD) test. For species dependence, only the four species with multiple strains represented (*P. freudenreichii*, *P. acidipropionici*, *P. jensenii*, and *P. thoenii*) were compared for the sake of statistical significance. A total of 34 compounds that varied significantly by strain with P value < 0.001 and two compounds (dimethyl disulfide and dimethyl trisulfide) with a tendency to vary by strain (P value < 0.3) and a significant effect of the species, were selected for further analysis. A principal component analysis (PCA) was performed on the mean of replicates for these 36 compounds, after a log(x) transformation and Pareto scaling, using the package FactomineR of the R software.

## 3. Results

### 3.1. Growth and pH change

The average population reached at two weeks was highly strain dependent, with no specific features distinguishing species (Table 2). The unique strain of *P. microaerophilum* tested exhibited the highest viable population at the end of incubation, at 10<sup>9</sup> cfu/mL, while *P. jensenii* CIRM BIA133 showed the lowest viable counts, in the order

**Table 2**

Viable counts, expressed as colony-forming units (CFU)/mL, for 19 selected *Propionibacterium* strains incubated for two weeks at 30 °C in a curd-based medium. Values are means and standard errors of two biological replicates.

Strain	Viable counts, CFU/mL	pH
<i>P. freudenreichii</i> CIMRM-BIA1397	1.98 10 <sup>8</sup> fgh	5.28 ef
<i>P. freudenreichii</i> CIRM-BIA1426	6.82 10 <sup>7</sup> h	5.50 a
<i>P. freudenreichii</i> CIRM-BIA1497	5.49 10 <sup>8</sup> cde	5.51 a
<i>P. freudenreichii</i> LSP1F	2.78 10 <sup>8</sup> efgh	5.48 ab
<i>P. freudenreichii</i> LSP10	3.78 10 <sup>8</sup> def	5.51 a
<i>P. freudenreichii</i> LSP19	2.56 10 <sup>8</sup> fgh	5.49 a
<i>P. freudenreichii</i> LSP28	3.36 10 <sup>8</sup> defgh	5.17 g
<i>P. freudenreichii</i> LSP56	6.94 10 <sup>8</sup> bc	5.22 fg
<i>P. freudenreichii</i> LSP100	5.67 10 <sup>8</sup> cd	5.21 fg
<i>P. jensenii</i> CIRM-BIA133	6.61 10 <sup>7</sup> h	5.23 fg
<i>P. jensenii</i> CIRM-BIA1430	3.49 10 <sup>8</sup> defg	5.21 fg
<i>P. jensenii</i> CIRM-BIA1660	2.61 10 <sup>8</sup> fgh	5.21 fg
<i>P. jensenii</i> LSP36B	9.21 10 <sup>7</sup> gh	5.25 efg
<i>P. acidipropionici</i> CIRM-BIA1425	8.44 10 <sup>8</sup> b	5.36 cd
<i>P. acidipropionici</i> LSP96	2.36 10 <sup>8</sup> fgh	5.41 bc
<i>P. thoenii</i> CIRM-BIA1434	2.89 10 <sup>8</sup> efgh	5.47 ab
<i>P. thoenii</i> CIRM-BIA1509	1.57 10 <sup>8</sup> fgh	5.20 g
<i>P. microaerophilum</i> CIRM-BIA677	1.18 10 <sup>9</sup> a	5.44 ab
<i>P. cyclohexanicum</i> CIRM-BIA698	2.87 10 <sup>8</sup> efgh	5.23 fg

a-h means within a column followed by a common letter do not significantly differ according to the LSD test (P < 0.05).

of the inoculation level (~10<sup>7</sup> cfu/mL). However, viable counts may have decreased during incubation.

The pH of cultures at the end of incubation ranged from 5.17 to 5.51 (Table 2). The pH of the control medium was 5.42. The average culture of seven strains out of 78 showed significantly less acidification than the control medium, with pH ranging from 5.48 to 5.51. Sixty two of the 78 strains had an average pH significantly more acidic than the control, ranging from 5.15 to 5.36. There did not appear to be a significant effect of species on pH.

### 3.2. Aroma compounds produced

A total of 45 volatile aroma compounds were identified, including six esters, seven acids, nine ketones, four alpha dicarbonyl and derived compounds, seven alcohols, eight aldehydes, three sulfur compounds and one of miscellaneous compounds (Table 3). The coefficient of variation of the abundance of volatiles between biological replicates was 0.44, on average.

The results of the ANOVA performed on the abundance of aroma compounds showed significant differences between strains for 34 out of 45 compounds and significant differences between the four dairy species for 39 out of the 45 (Table 3), with marked differences in abundance. For compounds with significant differences by strain, the ratio between the maximum and minimum production ranged from 5.7 for nonanal to >1000 for 2 methylbutanal (data not shown). For compounds with significant differences by species, the ratio between the maximum and minimum production ranged from 2.3 for dimethyl trisulfide to >500 for ethyl propanoate. The five compounds that did not significantly vary in abundance neither by strain nor species were all ketones (acetone, 2 butanone, pentanone, nonanone, and undecanone).

The concentration of most aroma compounds in most strains increased by the end of incubation. However, most aldehydes (straight chain aldehydes, 2 methylpropanal, and benzaldehyde) were present at higher concentrations in the control medium than in all cultures, suggesting that these aldehydes were converted by *Propionibacterium* metabolism. This decreased abundance was also true for the two added alcohols in *P. acidipropionici*.

**Table 3**

Volatile compounds identified, ion (m/z) of quantification, and P-value of the ANOVA showing the effect of the species or of the strain on the amount of the compound.

RI <sup>a</sup>	Compounds (abbreviated code or trivial name)	m/z	Identification <sup>b</sup>	P value, strain <sup>c</sup>	P value, species <sup>c</sup>
471	Ethanol	31	S, RI, DB	***	***
493	Acetone	58	RI, DB	0.85	0.07
517	Dimethyl sulfide (DMS)	62	S, RI, DB	***	**
556	2-Methylpropanal	72	S, RI, DB	***	***
589	2,3-Butanedione (diacetyl)	86	S, RI, DB	***	***
597	2-Butanone	43	S, RI, DB	0.99	0.37
613	Ethyl acetate (ethyl C2)	70	S, RI, DB	0.41	*
622	Acetic acid (C2)	60	S, RI, DB	***	***
629	2-Methylpropanol	33	S, RI, DB	***	***
659	3-Methylbutanal	58	S, RI, DB	***	***
666	2-Methylbutanal	57	S, RI, DB	***	**
688	2-Pentanone	58	S, RI, DB	0.99	0.71
695	2,3-Pentanedione	100	RI, DB	***	***
701	Pentanal	57	RI, DB	***	***
711	Ethyl propanoate (ethyl C3)	102	S, RI, DB	***	***
712	Propanoic acid (C3)	74	S, RI, DB	***	***
712	2-Butanone-3-hydroxy (acetoin)	45	S, RI, DB	***	***
739	3-Methylbutanol	70	S, RI, DB	***	***
745	2-Methylbutanol	59	S, RI, DB	***	***
752	Dimethyl disulfide (DMDS)	94	S, RI, DB	0.14	***
769	2-Methylpropanoic acid	88	S, RI, DB	***	***
781	Butanoic acid (C4)	60	S, RI, DB	***	***
792	2-Hexanone	100	S, RI, DB	0.98	***
800	Ethyl butanoate (ethyl C4)	116	S, RI, DB	***	***
803	Hexanal	72	RI, DB	***	***
813	2-Hydroxy-3-pentanone	59	RI, DB	***	***
836	3-Methylbutanoic acid	87	S, RI, DB	***	***
845	2-Methylbutanoic acid	102	S, RI, DB	***	***
859	Furanmethanol	98	RI, DB	0.37	***
871	1-Hexanol	69	S, RI, DB	***	***
891	2-Heptanone	114	S, RI, DB	1.0	*
900	2-Heptanol	98	RI, DB	***	***
903	Heptanal	81	S, RI, DB	***	***
915	Dimethylpyrazine	108	S, RI, DB	***	***
948	5-Methyl-2-heptanone	95	DB	***	***
952	Hexanoic acid (C6)	87	S, RI, DB	***	***
961	Benzaldehyde	106	S, RI, DB	***	***
969	Ethyl hexanoate (ethyl C6)	88	S, RI, DB	***	***
972	Dimethyl trisulfide (DMTS)	126	RI, DB	0.29	***
1052	2-Nonanone	58	S, RI, DB	1.0	0.45
1104	Nonanal	82	RI, DB	***	***
1116	Phenylethanol	91	RI, DB	***	0.67
1183	Ethyl octanoate (ethyl C8)	101	S, RI, DB	***	***
1282	2-Undecanone	71	RI, DB	0.98	0.21
1390	Ethyl decanoate (ethyl C10)	101	RI, DB	***	***

<sup>a</sup> RI, Kovats retention index;

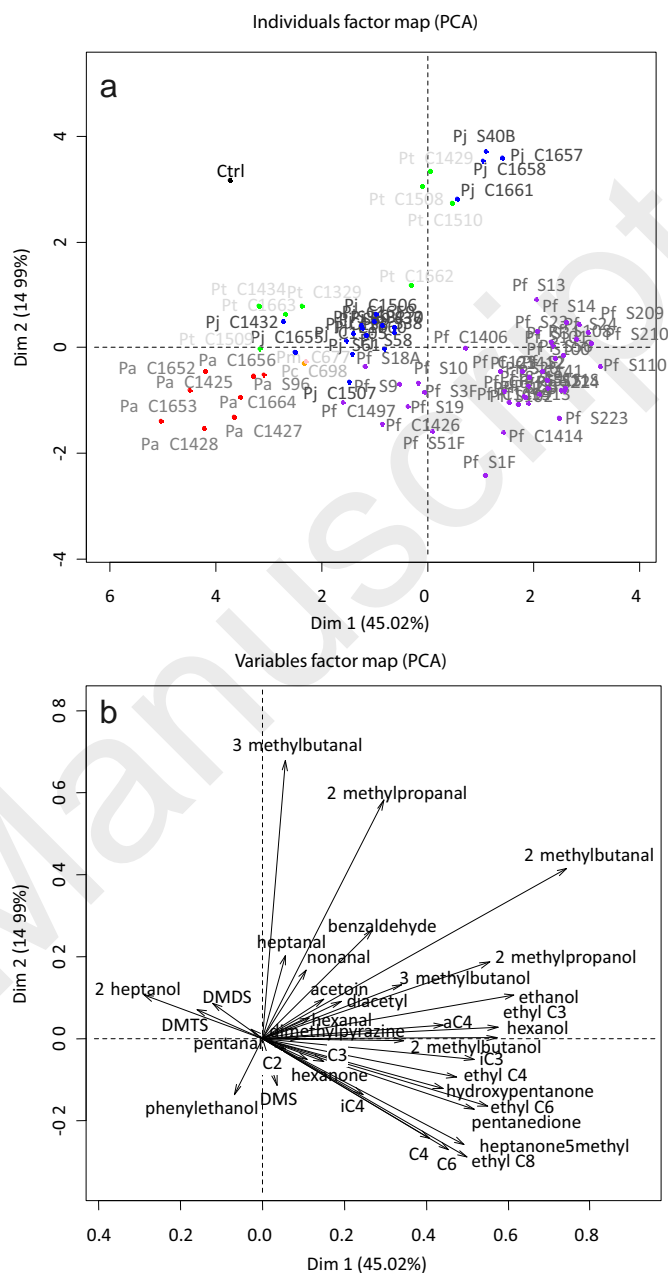
<sup>b</sup> Compounds identified on the basis of: S, retention time and mass spectrum from standard; RI, retention index; DB, mass spectral data Library NIST.

<sup>c</sup> P-value of ANOVA: \*\*\*P < 0.001, \*\*P < 0.01, \*P < 0.05.

### 3.3. PCA analyses of selected aroma compounds

A PCA was performed to summarize the differences in volatile profiles of all cultures and controls (Fig. 1). The first two axes accounted for 60% of the total variability. PC1, describing 45% of the variability, was related to the abundance and diversity of aroma compounds. High concentrations of FFAs, ethyl esters, branched chain acids and alcohols, among others, were positively associated with PC1, while 2 heptanol, DMDS and DMTS were negatively associated with PC1. PC2, accounting for 15% of the variability, was positively related to straight and branched chain aldehydes, and negatively associated with FFAs, and the corresponding ethyl esters.

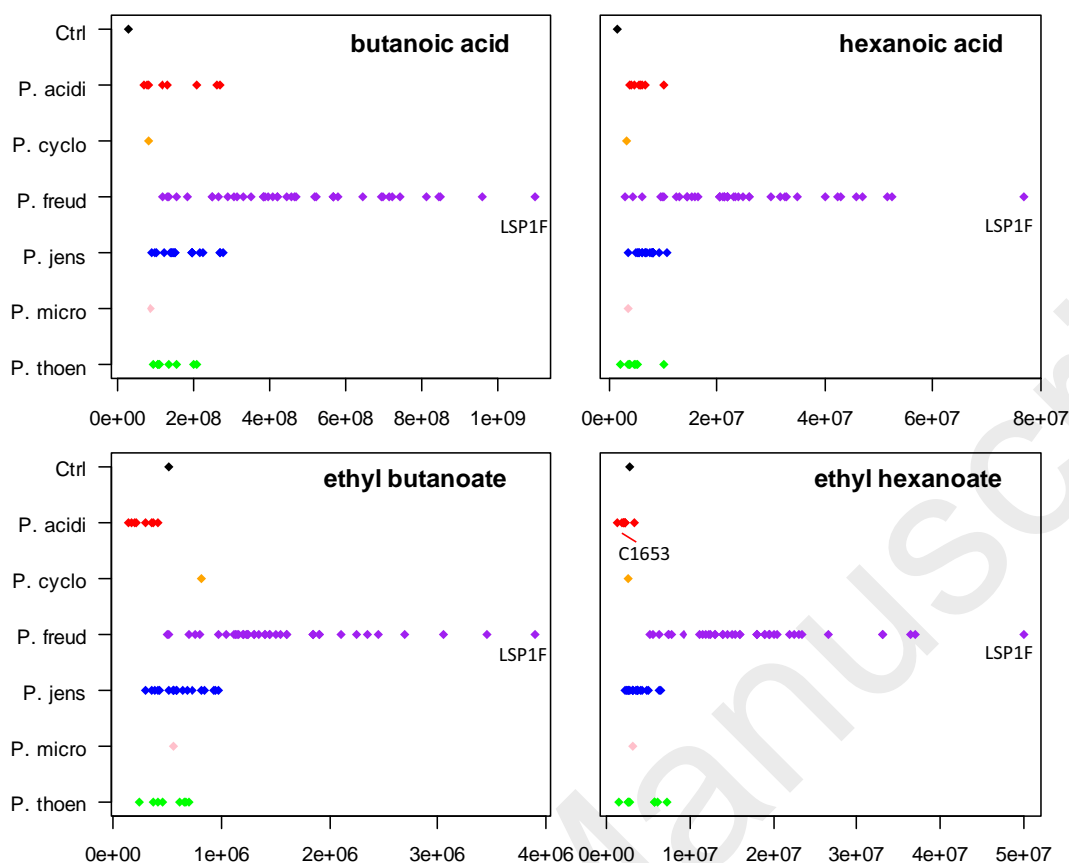
The control appeared in the upper left quadrant, negatively associated with most variables. PC1 was positively associated with *P. freudenreichii* species and a group of strains that included closely related members of *P. jensenii* and *P. thoenii* species. PC1 was also negatively related to *P. acidipropionici* species and some strains of *P. thoenii* and *P. jensenii*. *P. freudenreichii* strains were associated



**Fig. 1.** Individual factor map (a) and variable factor map (b) of principal component analysis on 36 potential aroma compounds analyzed using headspace gas chromatography-mass spectrometry produced in 78 *Propionibacterium* cultures of six species (*P. acidipropionici*, *P. cyclohexanicum*, *P. freudenreichii*, *P. jensenii*, *P. microaerophilum*, and *P. thoenii*, abbreviated as Pa, Pc, Pf, Pj, Pm, and Pt, respectively), grown in a curd-based medium incubated for 14 days at 30 °C. Ctrl is a non-inoculated control medium incubated under the same conditions. Variable names are listed in Table 3.

with greater concentrations of a variety of compounds, including free fatty acids from lipolysis, ethyl esters derived from these acids, and branched chain acids and alcohols from amino acid catabolism, while *P. acidipropionici* strains produced less of these compounds but DMDS, DMTS and 2 heptanol. *P. freudenreichii* was the only species in the lower right quadrant but some strains were placed near to *P. jensenii* and *P. acidipropionici* strains, in the lower left quadrant. PC2 was positively associated with certain strains of *P. jensenii* and *P. thoenii* producing high amounts of branched chain aldehydes.

Ethyl esters and their precursor acids were associated with PC1, as mentioned (Fig. 1). The amounts of ethyl hexanoate differed on average 8 fold between *P. freudenreichii* and *P. acidipropionici*, with differences



**Fig. 2.** Abundance of butanoic and hexanoic acids, and of the ethyl esters corresponding, as area of a specific ion ( $m/z$ ), in arbitrary units, in 78 *Propionibacterium* cultures of six species (*P. acidipropionici*, *P. cyclohexanicum*, *P. freudenreichii*, *P. jensenii*, *P. microaerophilum*, and *P. thoenii*, abbreviated as *P. acidi*, *P. cyclo*, *P. freud*, *P. jens*, *P. micro*, and *P. thoen*, respectively), grown in a curd-based medium incubated for 14 days at 30 °C and in the control non-inoculated medium (Ctrl) incubated under the same conditions.

between strains as high as 39 fold higher in *P. freudenreichii* LSP1F than *P. acidipropionici* CIRM BIA1653. Meanwhile, the amounts of hexanoic acid differed on average 4 fold between *P. freudenreichii* and *P. acidipropionici* species (Fig. 2). The abundance of butanoic and hexanoic acids were highly correlated across strains ( $r = 0.89$ ), as well as the abundance of ethyl esters from fatty acids C4 through C8 ( $r$  ranging from 0.83 to 0.89). Moreover, the abundance of ethyl esters was correlated with the abundance of the corresponding acids ( $r$  ranging from 0.65 to 0.80).

Branched chain acids were also present in higher concentrations in *P. freudenreichii* strains than in *P. acidipropionici*. For example, 2 methylbutanoic acid was, on average, 6 fold higher in *P. freudenreichii* than than *P. acidipropionici* strains, with a maximum difference of 245 fold between *P. freudenreichii* LSP108 and *P. acidipropionici* LSP96 (Fig. 3). Similarly, two branched chain alcohols, 3 methylbutanol and 2 methylbutanol, were 29 and 5 fold higher, respectively, in *P. freudenreichii* LSP108 than *P. acidipropionici* CIRM BIA1653. 3 methylbutanol, added to the medium, was originally present but was found at lower average concentrations in *P. acidipropionici* strains than in the control medium (Fig. 3). Further, 2,3 pentanedione, 2 hydroxy 3 pentanone and 5 methyl 2 heptanone were associated positively with PC1. These three compounds were significantly produced compared to the control medium only in *P. freudenreichii* cultures (half of the strains) (Fig. 4a). The abundance of 2,3 pentanedione was correlated with that of 2 hydroxy 3 pentanone ( $r = 0.68$ ) and 5 methyl 2 heptanone ( $r = 0.79$ ).

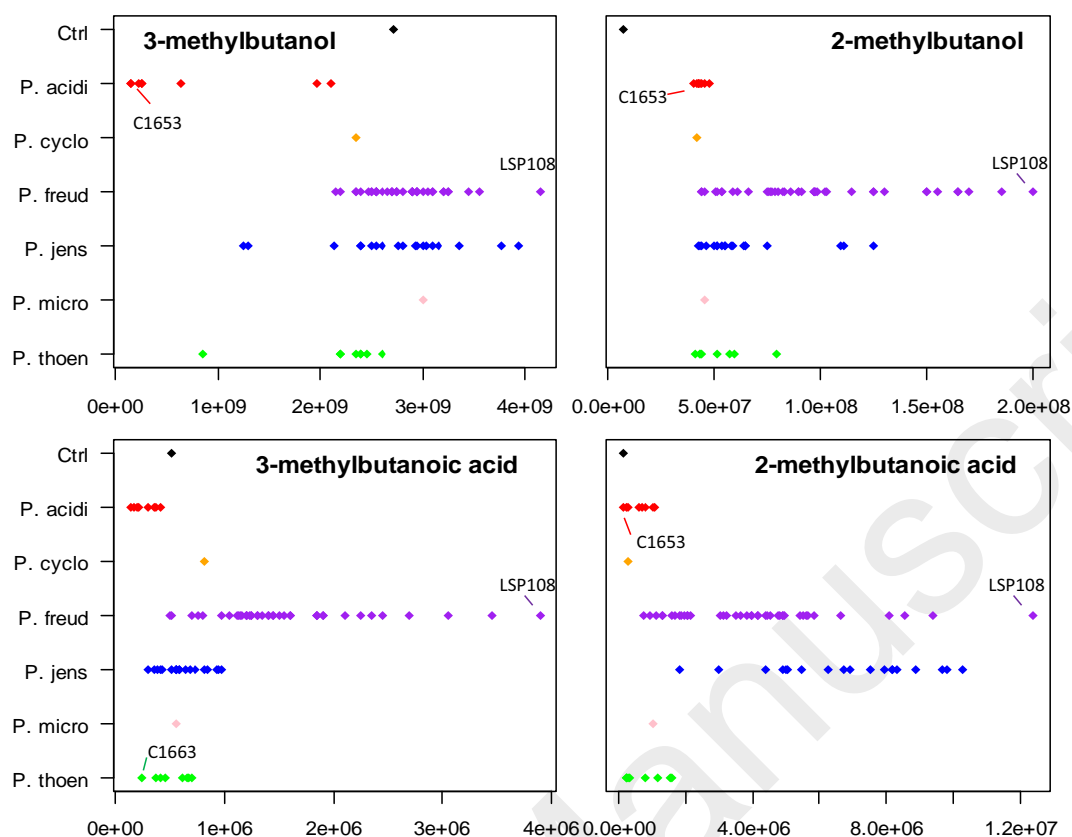
Conversely, the sulfur containing compounds dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) were found in higher concentrations in *P. acidipropionici* strains than in *P. freudenreichii* strains. On average, *P. acidipropionici* cultures produced 2 fold more DMDS than did the mean of *P. freudenreichii* cultures, with a maximum difference

of 7 fold between *P. acidipropionici* CIRM BIA1652 and *P. freudenreichii* CIRM BIA1406 (Fig. 4b). The formation of DMDS and DMTS was correlated ( $r = 0.80$ ). Another sulfur containing compound, dimethyl sulfide (DMS), was also present in six cultures, all strains of *P. freudenreichii*, at amounts significantly higher compared to controls (Fig. 4b). Its abundance was not correlated with that of DMDS and DMTS ( $r = 0.21$  and 0.10, respectively).

PC2 differentiated some *P. thoenii* and *P. jensenii* strains from all other strains on the basis of concentrations of branched chain aldehydes, and to a lesser extent, of other aldehydes. Branched chain aldehyde levels were found at significantly higher levels in some *P. thoenii* and *P. jensenii* strains. In contrast, straight chain aldehydes and benzaldehyde were found at significantly higher levels in the controls than in all strains. For example, the branched chain aldehydes 2 methylbutanal and 3 methylbutanal were about 75 and 19 fold more abundant, respectively, in *P. thoenii* than in *P. acidipropionici* cultures. *P. thoenii* cultures averaged 9 fold more benzaldehyde compared with *P. acidipropionici* cultures (data not shown).

Propionic and acetic acids were produced by strains of all six species in higher concentrations than found in the control medium. Differences in production between strains were observed between the corresponding ethyl esters of these acids. For example, the amounts of ethyl propanoate differed on average 16 fold between *P. freudenreichii* and *P. acidipropionici*, with differences between strains up to 212 fold higher in *P. freudenreichii* LSP13, which produced the highest amounts, and *P. acidipropionici* CIRM BIA1652, which produced the lowest amounts (data not shown).

While the abundance of most compounds was highly dependent on the species, significant variability was also observed between strains of the same species. For example, there was a 405 fold difference in the abundance of 2 methylpropionic acid between *P. freudenreichii* LSP108



**Fig. 3.** Abundance of four branched-chain volatiles, as area of a specific ion ( $m/z$ ), in arbitrary units, in 78 *Propionibacterium* cultures of six species (*P. acidipropionici*, *P. cyclohexanicum*, *P. freudenreichii*, *P. jensenii*, *P. microaerophilum*, and *P. thoenii*, abbreviated as P. acidi, P. cyclo, P. freud, P. jens, P. micro, and P. thoen, respectively), grown in a curd-based medium incubated for 14 days at 30 °C and in the control non-inoculated medium (Ctrl) incubated under the same conditions.

and *P. freudenreichii* LSP19 (data not shown); a 3 fold difference in the abundance of DMTS between *P. acidipropionici* CIRM BIA1652 and *P. acidipropionici* CIRM BIA1653 (Fig. 4b); a 158 fold difference in the abundance of 2 methylpropanal between *P. jensenii* CIRM BIA1657 and *P. jensenii* LSP61; and a 181 fold difference in the abundance of 3 methylbutanol between *P. thoenii* CIRM BIA1429 and *P. thoenii* CIRM BIA1663 (data not shown).

Dimethylpyrazine was found at higher abundances in all species than in the control, with a maximum difference of 8 fold between *P. freudenreichii* LSP13 and *P. freudenreichii* LSP28 (data not shown).

#### 3.4. Aroma profiles of *P. cyclohexanicum* and *P. microaerophilum*

The two strains tested representing the two other *Propionibacterium* species, *P. cyclohexanicum* and *P. microaerophilum*, exhibited markedly different profiles for a few compounds (data not shown). The *P. cyclohexanicum* strain showed significantly greater abundances of diacetyl, acetoin, and 2 heptanol than all other strains and controls, with maximum differences of 70, 37, and 100 fold, respectively, compared to other cultures. The *P. microaerophilum* strain showed a significantly higher level of phenylethanol, with a maximum difference of 1058 fold compared to other cultures (data not shown).

## 4. Discussion

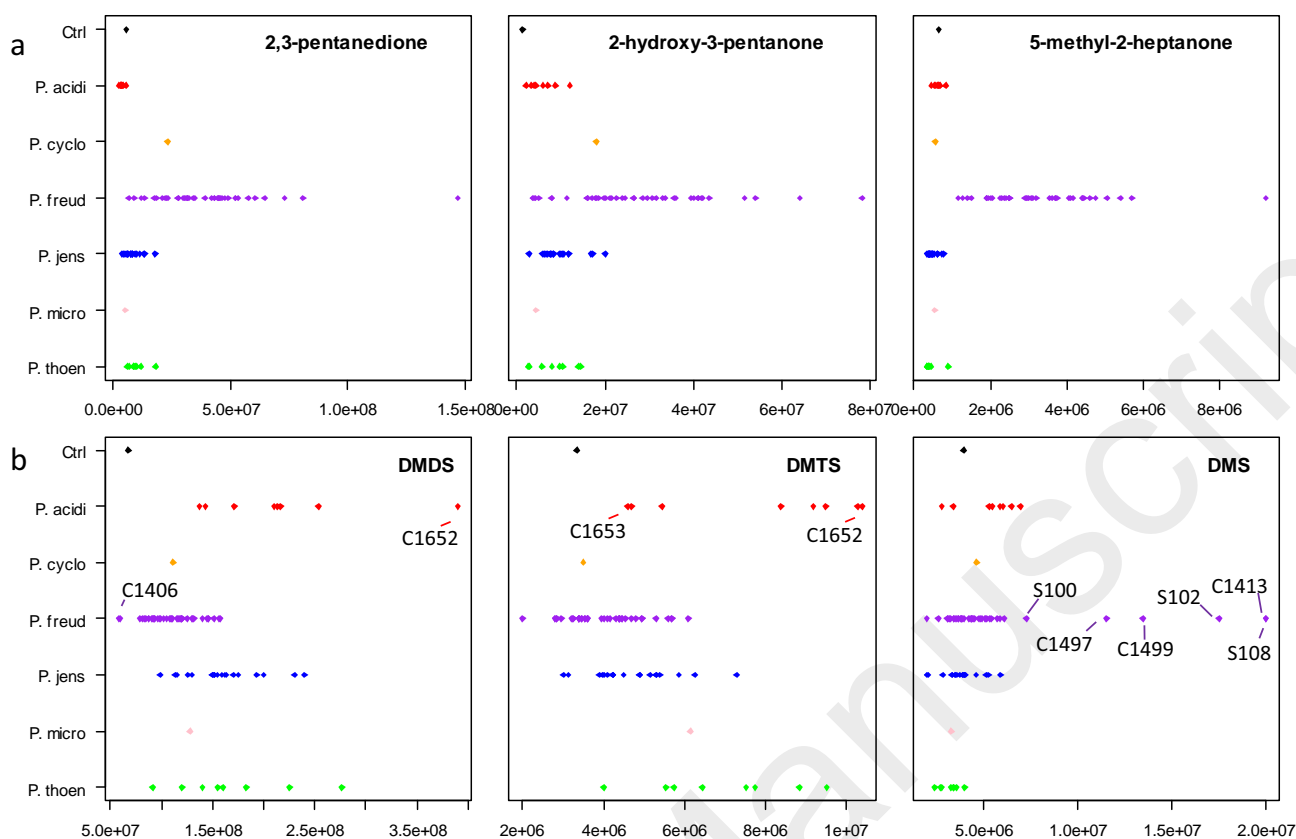
### 4.1. Interest of the screening medium used

We chose the curd medium recently proposed to model a cheese context (Pogacic et al., 2015) because the production of aroma compounds results from the conversion of curd components. Ethanol was added to promote the formation of ethyl esters, since it has been shown that

ethanol is a limiting reagent in ethyl ester synthesis in (semi) hard cheeses (Richoux et al., 2008; Thierry et al., 2006; Urbach, 1997), as well as another alcohol commonly found in cheese, 3 methylbutanol. Lactose was added because small amounts of residual lactose are present in many cheeses at the beginning of the ripening. All dairy propionibacteria can use lactic acid as the sole carbon and energy source, but certain strains can also ferment lactose: *P. acidipropionici*, *P. cyclohexanicum*, most strains of *P. jensenii* and *P. freudenreichii* and some strains of *P. thoenii* (Cummins and Johnson, 1986; Koussemon et al., 2001; Kusano et al., 1997). Growth is faster and a higher cell yield is reached during the fermentation of lactose compared to lactic acid (Piveteau, 1999). Casein peptone was added to promote the growth of propionibacteria, which are non proteolytic bacteria (Dupuis et al., 1995). Propionibacteria grow in cheese during ripening and use for their nitrogen requirements the peptides and free amino acids produced in the curd by lactic acid bacteria proteases, milk plasmin and rennet (Gagnaire et al., 2001). Therefore, the curd medium used was similar to a semi hard cheese in terms of pH, source of carbon and nitrogen, and salt in moisture level. Differences between this *in vitro* model and real cheese included the incubation temperature and the fact that the bacteria were cultured in slurry rather than as immobilized colonies in a solid matrix. Many previous studies comparing *P. freudenreichii* in cheese versus model broth showed that their metabolism was very similar between the conditions (Crow, 1986), including their lipolytic activity (Abejion Mukdsi et al., 2014) and their ability to produce volatile compounds (Thierry et al., 2004b; Thierry et al., 2004a).

### 4.2. Aroma compounds from different origins

Propionibacteria have long been known to produce acetic and propionic acids, along with CO<sub>2</sub>, as the main products of lactate



**Fig. 4.** Abundance of a, three ketones, and b, three volatile sulfur compounds DMS (dimethyl sulphide), DMDS (dimethyl disulfide) and DMTS (dimethyl trisulfide), as area of a specific ion (m/z), in arbitrary units, in 78 *Propionibacterium* cultures of six species (*P. acidipropionici*, *P. cyclohexanicum*, *P. freudenreichii*, *P. jensenii*, *P. microaerophilum*, and *P. thoenii*, abbreviated as P. acidi, P. cyclo, P. freud, P. jens, P. micro, and P. toen, respectively), grown in a curd-based medium incubated for 14 days at 30 °C and in the control non-inoculated medium (Ctrl) incubated under the same conditions.

fermentation (Cummins and Johnson, 1986). These two acids are considered flavor compounds in cheese, although their contribution has not been definitively demonstrated. More recently, the ability of *P. freudenreichii* to produce other aroma compounds via different metabolic pathways, including lipolysis and amino acid catabolism, has been reported (Abeijon Mukdsi et al., 2014; Thierry et al., 2011).

Several compounds produced from fermentation of carbon sources were produced in *Propionibacterium* cultures. Propionic and acetic acids were produced in the six species, as expected. However, the quantification of these two acids may be unreliable since they were likely present at concentrations of several mg/g, exceeding the linearity range determined at 0.5 mg/g using this head space GC MS (Pogacic et al., 2015). The production of diacetyl in milk by *P. freudenreichii* has been reported (Lee et al., 1970), but here the abundance of diacetyl was significantly higher than the control only in the unique strain of *P. cyclohexanicum*, suggesting that most *Propionibacterium* strains tested did not produce much diacetyl compared with the lactococci metabolism in the curd used in the medium.

Lipolysis yields FFAs, including short chain acids with four to ten carbons that are particularly important for aroma (Collins et al., 2003). The FFA products of lipolysis are important aroma compounds in cheese *per se* and can also be further converted into different aroma compounds including esters, methyl ketones and lactones (Collins et al., 2003). The lipolytic activity of *P. freudenreichii* is the main cause of the release of FFAs from milk triglycerides during Swiss cheese ripening and the extracellular lipolytic esterase involved in this activity has been identified (Abeijon Mukdsi et al., 2014; Chamba and Perréard, 2002; Dherbécourt et al., 2010). The lipolytic activity is strain dependent (Abeijon Mukdsi et al., 2014). Here, we identified two short chain fatty acids in *P. freudenreichii* cultures, confirming the

lipolytic activity of about three quarters of the strains of these species. In contrast, none of the 37 non *freudenreichii* strains tested produced significantly more of each FFA than the control.

Regarding the formation of esters, associated with fruity flavors, the presence of *P. freudenreichii* in cheese has been associated with the formation of different esters of propanoate, but not of esters of short chain FFAs such as ethyl butanoate and ethyl hexanoate, which are both widely present in cheese (Thierry et al., 2006; Thierry et al., 2005b; Thierry et al., 2004a). In the present study we demonstrated that most *P. freudenreichii* strains have the ability to produce not only ethyl propanoate, but also ethyl esters of FFA from lipolysis, with the abundance of all these esters correlated with each other and with that of the corresponding acids, suggesting that they could result from the activity of the same esterase. Regarding the production of esters by other *Propionibacterium* species, only a few strains of *P. jensenii* and *P. thoenii* produced significantly more ethyl propanoate than the control. Esters of 3 methylbutanol, which were added in the medium as a precursor, were not detected in any culture, although the presence of some strains of *P. freudenreichii* in cheese has been associated with the presence of increased amounts of methyl butyl esters. This discrepancy could be due to differences in alcohol concentrations.

The catabolism of branched chain, aromatic amino acids and methionine produces various aroma compounds including acids, alcohols and aldehydes, which yield a variety of aroma notes (Yvon and Rijnen, 2001). In pure culture, *P. freudenreichii* produces volatile compounds derived from branched chain amino acids, methionine and phenylalanine (Thierry et al., 2004a; Thierry and Maillard, 2002). It produces mainly branched chain acids from branched chain amino acid catabolism (Thierry et al., 2002). In Swiss and semi hard cheeses, the presence of *P. freudenreichii* has been associated with a higher amount of

branched chain acids, alcohols and/or aldehydes derived from leucine (3 methyl branched) and, particularly, from isoleucine catabolism (2 methyl branched) (Thierry et al., 2005b; Thierry et al., 2004a; Thierry et al., 2005a; Thierry and Maillard, 2002). In the present study, branched chain volatiles were produced by the four dairy species. In nearly all *P. acidipropionici* cultures, the 3 methylbutanol added to the medium was consumed and likely oxidized to the corresponding acid, 3 methylbutanoic acid. The elevated production of 2 methyl branched compounds was confirmed for strains of *P. freudenreichii*, but was also shown to be true for some strains of *P. jensenii*. The particular ability to produce 2 methyl branched (i.e. *anteiso* branched) compounds can be linked to the synthesis of membrane fatty acids, mainly consisting of *anteiso* branched fatty acids in *P. freudenreichii* (Dherbécourt et al., 2008; Moss et al., 1969). All dairy propionibacteria synthesize branched chain membrane fatty acids, but the other dairy *Propionibacterium* species synthesize principally *iso* branched fatty acids (Moss et al., 1969). In the present study, unexpectedly, a high production of 2 methyl branched compounds was also observed in some strains of *P. jensenii*. Three other related compounds were found in about half of the cultures of *P. freudenreichii* but not in other species: 5 methyl 2 heptanone, an *anteiso* branched ketone that may result from cell FA degradation, 2,3 pentanedione, related to isoleucine metabolism (Xu et al., 2005), and its reduced product, 2 hydroxy 3 pentanone. Sulfur containing compounds are derived from the catabolism of methionine or methionine containing peptides, and are important contributors to cheese flavor (Martinez Cuesta et al., 2013). DMS is the main sulfur containing compound in Swiss cheese (Langler et al., 1967). We observed that six *P. freudenreichii* strains produced DMS, in agreement with earlier studies (Keenan and Bills, 1968), while half of the *P. acidipropionici* strains and a quarter of the *P. thoenii* strains produced DMDS and DMTS. DMDS and DMTS were abundant in the same strains, consistent with their common origin (Dias and Weimer, 1999), but the correlation did not hold for DMS, which is not produced by the catabolism of free methionine, but rather from the catabolism of sulfur containing amino acids in peptide bonds (Dykstra et al., 1971).

#### 4.3. Strain and species variations

We confirmed in this study that *P. freudenreichii* produced a variety of aroma compounds via different metabolic pathways, consistent with the literature (Thierry et al., 2011; Thierry and Maillard, 2002). Meanwhile, we demonstrated for the first time that *P. acidipropionici*, *P. jensenii* and *P. thoenii* also produced aroma compounds attributable to different metabolic pathways. In particular, a few strains of *P. jensenii* and *P. thoenii* were high producers of branched chain aldehydes, which are important compounds in cheese flavor that confer malty aroma notes, which may or may not be desirable, depending on the type of cheese (Dunn and Lindsay, 1985). *P. acidipropionici* also displayed distinct features, with about half the strains producing two sulfur compounds, which are considered important aroma compounds in all cheeses (Martinez Cuesta et al., 2013). However, *P. freudenreichii* produced substantially more FFAs, ethyl esters, among other aroma compounds, than any of the other dairy propionibacteria.

In the present study, marked differences in the abundance of aroma compounds were observed between strains. Such strain dependent variation in metabolism has previously been demonstrated in *P. freudenreichii* and other bacteria. For example, in lactic acid bacteria, the level of ethyl ester production varies about four fold between strains of the same species (Abeijon Mukdsi et al., 2009). In *P. freudenreichii*, too, levels of volatile production, particularly of FFAs and branched chain fatty acids, vary up to 14 and 9 fold, respectively, depending on the strain (Abeijon Mukdsi et al., 2014; Dherbécourt et al., 2008). Here, strain dependence as high as in the

order of 500 was found within each species of *Propionibacterium* tested, demonstrating that wild strains represent a remarkable source of biodiversity.

#### 4.4. Perspectives on application in cheese

The results presented in this study allow evaluation of the aromatizing potential of dairy propionibacteria, in comparison to the “reference” species, *P. freudenreichii*, for which the contribution to cheese flavor has been widely documented (Thierry et al., 2011). To evaluate the potential of dairy propionibacteria as ripening cultures in cheese, several properties not tested in this study must be considered, including their safety and their ability to grow in cheese. Regarding safety concerns, the tested species of dairy propionibacteria can be isolated from milk and other food products (Cummins and Johnson, 1992; Fessler et al., 1999). The species *P. freudenreichii* has long been used in human diets and animal feed and is considered Generally Recognized As Safe (GRAS) for use in cheese (Mogensen et al., 2002). Both *P. freudenreichii* and *P. acidipropionici* have been classified using the Qualified Presumption of Safety (QPS) approach (EFSA, 2012). Additionally, *P. thoenii*, *P. jensenii*, and *P. acidipropionici* have also been considered for their probiotic potential (Cousin et al., 2011). To have useful applications in cheese, strains must also be selected based on other relevant criteria depending on the targeted cheese. Such criteria include, but are not limited to, salt concentration and temperatures tolerated, because cheese manufacture may require higher salt concentration than the conditions in the current study, and low ripening temperatures. Swiss cheeses involve a “cooking” step at around 50–54 °C, which *P. freudenreichii* can tolerate. For cheeses manufactured without cooking, the other dairy *Propionibacterium* species may be of interest. Previous studies have shown that a population of approximately  $5 \times 10^7$ – $10^8$  bacteria can be expected to have an impact on cheese flavor (Thierry et al., 2005a). Thus, *Propionibacterium* strains that grow to these levels in conditions similar to manufacture of the desired cheese may be expected to influence cheese flavor. Preliminary indicators of possible safe use in cheese and the different potentials for producing aroma compounds make propionibacteria important candidates to modulate cheese flavor.

## 5. Conclusion

This study highlights the large inter- and intraspecies diversity of propionibacteria in the production of aroma compounds. The production of compounds with a common origin was generally correlated. Fold changes between strains of the same species as high as in the order of 500 for a single compound. Both common and specific abilities were observed for the different species, but *P. freudenreichii* appears to be the species harboring the widest potential to produce cheese aroma compounds. This study suggests that the diversity of dairy propionibacteria can be exploited to modulate the flavor of mild cheeses.

## Acknowledgments

This work was performed in the framework of the PropArom project supported by the Brittany Regional Council, France. Alyson Yee was awarded a Fulbright Advanced Student 2013–2014 Grant for a six month stay at the UMR1253 INRA in Rennes, France.

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