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

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ABSTRACT

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. 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

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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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 °C in glycerol and pre-cultured twice in yeast extract lactate (YEL) medium (Malik et al., 1968) at 30 °C. To screen the ability of propionibacteria to produce aroma compounds, the bacteria were incubated for 15 days at 30 °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[a]
P. freudenreichii	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
P. jensenii	19	CB133, CB1430, CB1432, CB1505, CB1506, CB1507, CB1655, CB1657, CB1658, CB1659, CB1660, CB1661, LSP36B, LSP37, LSP38, LSP39, LSP40B, LSP58, LSP61
P. acidipropionici	8	CB1425, CB1427, CB1428, CB1652, CB1653, CB1656, CB1664, LSP96
P. thoenii	8	CB1329, CB1429, CB1434, CB1508, CB1509, CB1510, CB1662, CB1663
P. microaerophilum	1	CB677
P. cyclohexanicum	1	CB698

[a] 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 °C until use. Frozen curd was thawed at ambient temperature and 50 g curd was homogenized in 100 g of a sterilized (115 °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 °C for 15 min, vortexed for 5 s, and stored at 4 °C until use.

Immediately prior to inoculation of curd with the bacterial strains, each tube was vortexed ≥15 s to ensure homogeneity of the medium. 100 μL 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 °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 °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 m × 0.25 mm × 1 μm; Perkin-Elmer), with helium as the mobile phase. The temperature of the oven was initially 35 °C, maintained