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A methodological approach to screen diverse cheese-related bacteria for their ability to produce aroma compounds

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ABSTRACT

Microorganisms play an important role in the development of cheese flavor. The aim of this study was to develop an approach to facilitate screening of various cheese-related bacteria for their ability to produce aroma compounds. We combined i) curd-based slurry medium incubated under conditions mimicking cheese manufacturing and ripening, ii) powerful method of extraction of volatiles, headspace trap, coupled to gas chromatography-mass spectrometry (HS-trap-GC-MS), and iii) metabolomics-based method of data processing using the XCMS package of R software and multivariate analysis. This approach was applied to eleven species: five lactic acid bacteria (*Leuconostoc lactis, Lactobacillus sakei, Lactobacillus paracasei, Lactobacillus fermentum,* and *Lactobacillus helveticus*), four actinobacteria (*Brachybacterium articum, Brachybacterium tyrofermentans, Brevibacterium aurantiacum,* and *Microbacterium gubbeenense*), *Propionibacterium freudenreichii*, and *Hafnia alvei*. All the strains grew, with maximal populations ranging from 7.4 to 9.2 log (CFU/mL). In total, 52 volatile aroma compounds were identified, of which 49 varied significantly in abundance between bacteria. Principal component analysis of volatile profiles differentiated species by their ability to produce ethyl esters (associated with *Brachybacteria*), sulfur compounds and branched-chain alcohols (*H. alvei*), branched-chain acids (*H. alvei, P. freudenreichii* and *L. paracasei*), diacetyl and related carbonyl compounds (*M. gubbeenense* and *L. paracasei*), among others.

1. Introduction

Characterization of microorganisms for their production of odor-active volatile compounds and evaluation of their utility as ripening cultures in cheese manufacture is an ongoing scientific challenge in dairy microbiology. New strains isolated from dairy or non-dairy environments should be evaluated for their aromatic potential, since flavor is a very important characteristic from the consumer's point of view (Niimi et al., 2014). The formation of flavor compounds in cheese results from numerous metabolic reactions and is largely influenced by microbial diversity and the complex dynamics of growth and metabolism during cheese ripening (Hassan et al., 2013; Steele et al., 2013). The microbiota of traditional Protected Designation of Origin (PDO) raw milk cheeses depends on the microbial community, which naturally arises from raw milk and natural whey culture, and from the environment, and contributes to specific intense flavor of raw milk cheeses (Gatti et al., 2014; Neviani et al., 2013; Ordiales et al., 2013). However, in cheese manufacturing, there is a continual need to modulate cheese flavor via the addition of selected new strains with aroma potential, particularly in the case of cheeses made from pasteurized milk. For example, there is currently a demand to diversify the rather mild flavor of some semi-hard cheeses. Therefore, efficient aroma screening approaches are required to evaluate diverse species of microorganisms. Most studies have targeted a few groups of bacteria, mainly lactic acid bacteria (LAB) such as *Lactobacillus, Lactococcus,* and *Leuconostoc*, or propionibacteria (De Bok et al.,

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2011; Dhaisne et al., 2013; Pedersen et al., 2013; Sgarbi et al., 2013; Zareba et al., 2014). Considering the diversity of the cheese microbiota, we aimed to evaluate the ability of diverse bacterial strains (7 genera, 11 species), to grow and produce volatile aroma compounds in a unique curd-based medium under conditions that mimicked those of semi-hard cheese manufacture.

Several methods can be used to analyze volatile compounds – also referred to as the volatilome (Sgarbi et al., 2013 – (Croissant et al., 2011). Many volatile compounds have been identified in cheese and other fermented dairy products (Maarse et al., 1994; Steele et al., 2013). They include free carboxylic acids, sulfur compounds, carbonyl compounds, alcohols, and esters, among others (Mariaca and Bosset, 1997; Curioni and Bosset, 2002). The analysis of volatiles involves sample preparation, extraction, and concentration, followed by separation, detection, and identification, generally performed by gas chromatography coupled to mass spectrometry (GC-MS). The choice of the sample preparation and extraction method is crucial because it influences both the qualitative and quantitative profiles of the volatiles extracted, as well as the accuracy of the results (Marsili, 2011; Jeleń et al., 2012). Headspace (HS) sampling has become the most frequently used technique in the investigation of food flavor. It is a means of separating volatiles from the sample prior to GC-MS analysis. HS-solid phase micro-extraction (SPME) is the most widely used method to extract volatiles from food products, including cheese (Jeleń et al., 2012). Additionally, other HS-related micro-extraction methods have been developed, for example, the HS-trap technique has recently been successfully applied to extract volatiles from various samples (Schulz et al., 2007; Aberl and Coelhan, 2012).

GCMS data processing and analysis is the final step and can be very time-consuming when there are many samples to compare. A great number of volatile compounds can potentially be extracted and must be identified and quantified in each sample. Data analysis can be facilitated using tools developed for MS-based metabolomic approaches, which have emerged over the past decade in many scientific areas, including food science (Wishart, 2008). To facilitate the handling of large datasets generated from liquid chromatography/MS-based metabolomic approaches, dedicated software has been developed, including the open-source XCMS package (Smith et al., 2006). These tools convert the initial three-dimensional raw data (*m/z*, retention time, ion current) into a two-dimensional data table containing information about the abundance of each metabolite in all samples (Antignac et al., 2011).

Our aim was to develop an efficient approach to screen various cheese-related bacteria species for their ability to produce aroma compounds. Ideally, such an approach should allow assessment of the growth of a variety of targeted species, should be sensitive and sufficiently simple and automated to be useful for large-scale screening. Our strategy was to combine the use of i) a curd-based medium incubated under conditions mimicking cheese manufacture and ripening, ii) the extraction of volatiles using the recently developed headspace trap method coupled to gas chromatography-mass spectrometry (GC-MS), and iii) a metabolomics-based method of data processing using the open-source XCMS package of *R* software, followed by statistical and multivariate analyses.

2. Materials and methods

2.1. Bacterial strains

Twelve bacterial strains (Table 1) of different species of interest for cheese aromatization were used in this experiment, five from the collection of the International Centre for Bacteria of Food Interest - Centre International de Ressources Microbiennes–Bactéries d'Intérêt Alimentaire (CIRM-BIA, UMR1253, INRA Rennes, France) and seven from Laboratoires Standa, Caen, France.

2.2. Preparation of bacterial suspensions used for inoculation

The strains were reactivated from frozen (−80 °C) glycerol stocks in a broth medium, and cultures were then streaked on an agar medium and incubated in the conditions described in Table 1. Cell suspensions were prepared from bacterial colonies collected from agar plates with a sterile loop (about 1 μl) and suspended in 10 mL of a 9 g/L NaCl solution. Preliminary experiments were carried out to determine the optical density and viable counts of these bacterial suspensions, so as to ensure inoculation with an accurate number of viable cells. The day of the experiment, the cell suspensions were used immediately after preparation to inoculate the curd-based medium.

2.3. Preparation of curd-based medium

A curd-based medium was prepared from a fresh curd of semi-hard cheese, provided by an industrial cheesemaker. This cheese was manufactured from pasteurized milk and inoculated only with a commercial lactococci starter, according to the usual cheese manufacture process. Blocks (4 kg) of a non-brined curd (52–53% dry matter, 48–50% fat) were cut and stored wrapped in aluminum foil in plastic bags under vacuum at −20 °C. The curd was left to

Table 1
Bacterial strains used and conditions of revitalization and enumeration.

Strain[a]	Medium[b] (broth or agar)	Growth conditions in broth[c]		Growth conditions in agar[c]	
		T/Atmosphere	Time, h	T/Atmosphere	Time, h
Brachybacterium articum LSBA53	TSB-YE	30 °C/agitation	24	30 °C/AE	72
Brachybacterium tyrofermentans LSBT17	TSB-YE	30 °C/agitation	24	30 °C/AE	48
Brevibacterium aurantiacum LSBA 57	TSB-YE + NaCl	30 °C/agitation	24	30 °C/AE	72
Microbacterium gubbeenense LSMG39	TSB-YE + NaCl	30 °C/agitation	24	30 °C/AE	72
Lactobacillus fermentum LSLF202	MRS	37 °C/AE	24	37 °C/AN	24
Lactobacillus helveticus CIRM-BIA108	MRS	37 °C/AE	24	37 °C/AN	48
Lactobacillus paracasei LSLP248	MRS	30 °C/AE	24	30 °C/AN	24
Lactobacillus sakei LSLS89	MRS	30 °C/AE	24	30 °C/AN	24
Leuconostoc lactis CIRM-BIA1541	MRS	30 °C/AE	24	30 °C/AE	48
Propionibacterium freudenreichii CIRM-BIA1426	YEL	30 °C/AE	24	30 °C/AN	120–144
Hafnia alvei CIRM-BIA1620	BHI-YE	30 °C/AE	48	30 °C/AE	48
Hafnia alvei CIRM-BIA1621	BHI-YE	30 °C/AE	48	30 °C/AE	48

[a] Strain named CIRM-BIA are from CIRM-BIA, INRA, the other strains are from Laboratoires Standa.
[b] Media: MRS: Man Rogosa Sharpe; TSB-YE: tripticase soy broth yeast extract; YEL: yeast extract lactate broth; BHI-YE: brain heart infusion yeast extract, TSA trypticase soy agar.
[c] Agitation at 90 RPM; AN, anaerobic; AE, air atmosphere.