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Monitoring of lactic fermentation driven by different starter cultures via direct injection mass spectrometric analysis of flavour-related volatile compounds

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ABSTRACT

In this work, we used Proton Transfer Reaction-Mass Spectrometry (PTR-ToF-MS), coupled with an automated sampling system, to monitor lactic fermentation driven by different yogurt commercial starter cultures via direct injection mass spectrometric analysis of flavour-related volatile compounds. The aim is the identification of markers for real-time and non-invasive bioprocess control and optimisation as an industrial driver of innovation in food technology and biotechnology. We detected more than 300 mass peaks, tentatively identifying all major yogurt aroma volatiles. Thirteen mass peaks showed statistically significant differences among the four commercial starters. Among these are acetaldehyde, methanethiol, butanoic acid, 2-butanone, diacetyl, acetoin, 2-hydroxy-3-pentanone/pentanoic acid, heptanoic acid and benzaldehyde which play a key role in yogurt flavour. These volatile described the diverse flavour properties claimed by food biotechnological companies and, considering the possible contribution to yogurt flavour, are potential markers for the rapid screening of starter cultures and for the quality design in this fermentation-driven production. The strength of our approach lies in the identification, for the first time, of specific depletion kinetics of four sulphur containing compounds occurring during fermentation (hydrogen sulphide, methanethiol, S-methyl thioacetate/S-ethyl thioformate, pentane-thiol), which suggest a new possible protechnological feature of yogurt starter cultures.

1. Introduction

Lactic acid fermentation (LAF) of milk is a dynamic biochemical process of particular importance for the dairy industry, as it is involved in the production of a large group of products, such as yogurt, fresh cheeses, acidified dairy beverages and desserts (Perina et al., 2015). Yogurt is a product of the lactic acid fermentation of milk, generally obtained upon the addition of a symbiotic culture of the homofermentative lactic acid bacteria (LAB) *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Morell, Hernando, Llorca, & Fiszman, 2015). During fermentation, these bacteria perform three major biochemical conversions of milk components: (i) conversion of lactose into lactic acid (fermentation), (ii) hydrolysis of caseins into peptides and free amino acids (proteolysis) and (iii) breakdown of milk fat into free fatty acids (lipolysis). These reactions lead to the production of various metabolites resulting in the decrease of pH and the formation of semi-solid texture and aroma (Settachaimongkon et al., 2014a). Even though *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are able to grow individually in milk, they can have a symbiotic interaction called "proto-cooperation" in mixed cultures. This interaction is based on the exchange of several metabolites, which provide mutual growth stimulating effects (Settachaimongkon, Nout, Antunes Fernandes, Hettinga, et al., 2014a). Transcriptome analysis has recently revealed the

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molecular basis of this specific mixed-culture growth (Sieuwerts et al., 2010). Mixed cultures show significant higher populations of the two species, faster milk acidification, significant abundance of aroma volatiles and non-volatile metabolites. Therefore, proto-cooperation is one of the key factors which determines the fermentation process and the final quality of yogurt and is hence used regularly by the dairy industry (de Bok et al., 2011). Consumer preference for food is driven by many criteria, including odour, aroma and flavour. The distinct aroma of plain yogurt is formed by a complex mixture of compounds which include volatiles already present in milk and compounds produced from milk fermentation through bacterial metabolism (Cheng, 2010). Although a long list of volatile organic compounds (VOCs) has been identified in yogurt (Cheng, 2010; Routray & Mishra, 2011), the main part of studies focused on the most abundant ones, such as lactic acid and carbonyl compounds (e.g. acetaldehyde and diacetyl). The influences of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* on the formation of aroma volatiles in yogurt are also well documented (Imhof, Glättli, & Bosset, 1995; Ott, Fay, & Ott, Fay, & Chaintreau, 1997; Tamime & Robinson, 2007; Routray & Mishra, 2011; Settachaimongkon, Nout, Antunes Fernandes, Hettinga, et al., 2014a). Recently, metabolite formation in yogurt produced by selected strains of probiotic bacteria in co-fermentation with traditional starters has also been investigated (Settachaimongkon et al., 2014b; Arena et al., 2015). However, the general impact of different commercial starter formulations on volatile organic compounds in yogurt, as well as on the monitoring of VOC during the fermentation process is under-investigated.

Proton transfer reaction mass spectrometry (PTR-MS) is an established method for the rapid, direct and non-invasive on-line monitoring of volatile organic compounds in food, basically based on the hydronium ion protonation of neutral compounds (M) according to the following reaction:

$$H_3O^+ + M \rightarrow H_2O + MH^+$$

PTR-MS is characterized by short response times and high sensitivity with limits of detection in the pptv (parts per trillion by volume) range (Soukoulis et al., 2010). The coupling of proton transfer ionization with Time-of-Flight (ToF) mass spectrometers offers several advantages related to high time and mass resolution (Romano, Capozzi, Spano, & Biasioli, 2015). For example, exact masses are determined with a mass resolution better than 0.001 Th in average (Blake, Monks, & Ellis, 2009). In most cases, this allows for a non-ambiguous determination of the chemical formula. However, unlike gas chromatography (GC), PTR-ToF-MS is unable to separate isomers. In previous works, PTR-ToF-MS has been employed to monitor VOC kinetics during LAF in milk (Soukoulis et al., 2010), or in correlation with textural properties, transglutaminase and heat treatments of yogurt (Soukoulis et al., 2011; Tsevdou et al., 2013). Moreover, PTR-ToF-MS has been recently used to monitor and differentiate starter cultures employed in the bakery sector (Makhoul et al., 2014).

In the present work, PTR-ToF-MS was used to monitor lactic fermentation driven by four different yogurt starter cultures, as a model to demonstrate the interest of non-invasive process monitoring via direct injection mass spectrometric analysis of flavour-related volatile compounds in the control and design of industrial food fermentations (processes).

2. Materials and methods

2.1. Starter cultures preparation

Four different freeze-dried direct-vat-set (DVS) yogurt cultures containing *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were used at the rate of 1 U/l (units per litre). According to the manufacturer's specifications, the four starters were supposed to give products with different characteristics in terms of overall flavour (A, FD-DVS YF-L812 Yo-Flex, Chr. Hansen; B, FD-DVS YC-380 Yo-Flex, Chr. Hansen; C, FD-DVS YC-X11 Yo-Flex, Chr. Hansen; D, YO-MIX 883, Danisco; Table 1).

2.2. Samples preparation and PTR-ToF-MS analysis

Ultra-high-temperature (UHT) low-fat milk (1.5% milk fat, 8.5% solids non-fat, Mila Spa., Bolzano, Italy) was purchased from the local market and used for the preparation of samples. Sixty vials were filled each with 5 ml of milk and heat-treated at 80 °C for 30 min in a water bath. After being rapidly cooled down to 45 °C in a cooler set at 4 °C, the samples were transferred to a tray, thermostated at 45 °C for starter addition. From this point on, all operations were carried out in an automated fashion by means of a GC multifunctional autosampler (Gerstel, Mülheim an der Ruhr, Germany). The robotic arm of the autosampler carried out the inoculation of all vials (except blanks) at a final starter concentration of 0.1% (v/v). The inoculation of all vials required approximately 20 min. The incubation time was set to 240 min, according to preliminary studies on the fermentation process (Routray & Mishra, 2011). The autosampler has been used in this step as well, to monitor VOC concentrations over time. For each experimental mode (the four starters additions and non-inoculated blanks as well), twelve vials were employed; these correspond to different time points, placed at 20-min intervals. The resulting LAFs were monitored for four hours. The whole experiment was performed in three replicates, on separate days, randomizing the starter addition order in each of them.

Headspace measurements of the fermented milks were performed with a commercial PTR-ToF-MS 8000 apparatus from Ionicon Analytik GmbH (Innsbruck, Austria), in its standard configuration (V mode). Headspace air is directly injected in the PTR-MS drift tube without any treatment. The ionization conditions in the drift tube were the following: 110 °C drift tube temperature, 2.30 mbar drift pressure, 550 V drift voltage. This led to an E/N ratio of about 140 Td (1 Td = 10^{-17} cm^2 V^{-1} s^{-1}). The inlet line consisted of a PEEK capillary tube (internal diameter 0.04 in.) heated at 110 °C. The inlet flow was set at 40 sccm. A single analysis corresponded to the average of 30 mass spectra with an acquisition rate of one spectrum per second.

2.3. Data analysis

Dead time correction, internal calibration of mass spectral data and peak extraction were performed according to the procedure described by Cappellin et al. (2010;2011). Peak intensity in ppbv was estimated using the formula described by Lindinger, Hansel, & Jordan (1998), using a constant value for the reaction rate coefficient (k = 2.10^{-9} cm^3 s^{-1}). This introduces a systematic error for the absolute concentration for each compound that is in most cases below 30% and could be accounted for if the actual rate constant coefficient is available (Cappellin et al., 2011). All data detected and recorded by the PTR-ToF-MS were processed and analyzed using MATLAB (MathWorks, Natick, MA) and R (R Foundation for Statistical Computing, Vienna, Austria). Principal Component Analysis, Analysis of Variance, and Tukey's post-hoc test have been performed to spot the differences in the volatile aroma compounds emitted by the four lactic acid cultures used in this study.

3. Results and discussion

3.1. Automated monitoring of lactic acid fermentation by different starter cultures

In fermented foods, such as yogurt, a relevant field of study deals with the contribution of microbiological resources to the organoleptic and sensory properties of the final product (Casarotti, Monteiro, Moretti, & Penna, 2014; Cadena et al., 2014). In the case of yogurt, some of the most characteristic flavour components are already present in cow milk and others are synthesized by homofermentative lactic acid