Part 3

Applications

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Probiotics as a Source of Aromas in Functional Food: Selected Examples and Analytical Methodology

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ABSTRACT

Probiotics play an important role in functional foods by generating natural aromas that are fundamental for the acceptability of such products by consumers. Selected strains are added to conventional functional foods, not just for health properties but also for their capability to improve flavor and overall taste. Beyond their applications to functional dairy products, the use of microorganisms for the aroma in-situ generation is now expanded to meat and plant-based products. However, aromas produced by probiotics are often complex and all odor compounds are not always suitable to all foodstuff types. Therefore, it is also valuable to overview, in the present chapter, the specific analytical methodology currently used for characterizing and selecting interesting aroma compounds.

***Key words*:** Probiotics, Natural aroma, Functional food, Sampling methods, Gas chromatography

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1. INTRODUCTION

Probiotic foods and beverages are part of an expanding industry with a global current estimated value of $35 billion (Global Market, 2017; Jankovic, 2010). Probiotics are accurately defined as live microorganisms which, when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2002). However, the acceptance of a new probiotic food is not only determined by its health capabilities (Granato *et al.*, 2010).

Although consumers are increasingly demanding natural products with new functional properties, the consumption patterns of new foodstuffs are strongly determined by their sensorial characteristics (Breslin *et al.*, 2001; Cheung *et al.*, 2016). This consumer appeal for probiotic containing foodstuffs has opened the way to investigate probiotic new aspects: their role in flavor generation.

Flavor is the most important aspect of food (Weerawatanakorn *et al.*, 2015). Flavor is defined as a combination of taste, aroma and trigeminal perception (Tournier *et al.*, 2007). Aroma gives a clearly distinguishable character to foodstuffs. It generally consists of hundreds of volatile organic compounds with various chemical structures (acids, alcohols, ketones, esters, etc.) in a well-defined proportion (Schwarb *et al.*, 2008). Individual aroma compounds are found at very low concentrations in food, typically parts-per-million (ppm) or part-per-billion (ppb) level (Mc Gorrin, 2002; Qian *et al.*, 2006). On that point, the relationship between aroma concentration, obtained by analytical methods and their perception is not obvious. The human nose's perception of volatile organic compounds can vary in a large extend depending on the compound, *e.g.*, from 10^{-8} ppm for 1-p-Menthene-8-thiol "citrus" to 10^{2} ppm for ethanol (Schieberle *et al.*, 2009).

Aroma compounds can originate from various sources. They may come from the ingredients itself (*e.g.*, lemon zest), added flavoring agents (natural or not) or technological transformation processes (heating, drying, smoking, frying, baking, etc.). On other side, aroma compounds can also derive from enzymatic reactions, including processing aids for the liberation of flavor compounds from nonvolatile precursors (*e.g.*, cysteine conjugates), and the conversion of a precursor to a desired flavoring (Winterhalter et Skouroumounis, 1997; Sakho *et al.*, 1998) or by fermentation through microbial activities.

Historically, probiotics are non-starter microorganisms but some strains have shown their capability to go along fermentation and generate volatile compounds contributing to food aroma (Salmeron *et al.*, 2009). Therefore, probiotic cultures have been developed to specifically bring out the desired flavors in the products in which they are used (Saarela *et al.*, 2000).

Based on these three categories of foodstuffs (dairy products, meat products and plant-based products), this chapter will provide an overview of probiotic strains which beyond their health benefit, significantly contribute to the food aroma. As analytical methods are clearly the bottleneck of the study of aroma compounds derived from probiotic strains, the second part of the chapter will focus on analytical aspects.

2. FUNCTIONAL FOODS WITH PROBIOTICS AS SOURCE OF AROMAS

2.1 Dairy Products

The substrates play a significant role in probiotic growth and viability. Dairy products such fermented milk, yogurt, and cheese are current food probiotic carriers. The physic-chemical composition of milk-based products, which is rich in carbohydrates, proteins and lipids, acts as a nutriment sources and protective matrix for the fermentation (Vijaya Kumar *et al.*, 2015). The main actions of probiotics in dairy fermentations are preservation of the milk by generating antimicrobial compounds, production of flavor compounds and other metabolites that will provide consumers desired organoleptic properties (Parvez *et al.*, 2006). Particularly, lactic acid bacteria are widely used for the fermentation of cheeses, yogurts and milks. These microorganisms have a variety of enzymes that enable them to turn milk carbohydrates and proteins, firstly into citrate and peptides and, secondary into aroma compounds (Matar *et al.*, 1996). In this case, Table 1 presents the summarized form of key volatile organic compounds (VOCs) from probiotic dairy products.

a. Yogurt aroma compounds derived from probiotic activities

Yogurt is a basic dairy product that has been consumed for centuries as a part of the diet and its beneficial effect has well-known and scientifically proven. Typical yogurt results of the mixture of milk (whole, low-fat, or nonfat) or cream fermented with a culture of lactic acid-producing

Table 1: Key VOCs from probiotic dairy products

Product names	*Main microorganism*	*Character impact compound*	*CAS number*	*Amount (ppm)*	*Descriptors*	*References*
Soft cheese	*L. plantarum* I91	Diacetyl	431-03-8	n.q	Buttery	Milesi *et al.* (2010)
	L. casei I90	Acetoin	513-86-0	n.q	Buttery	
Semi-hard ewe-milk Castellano cheeses	*L. reuteri INIA* P5	3-methyl-1-butanol	123-51-3	n.q	Sweaty[c]	Gómez-Torres *et al.* (2016)
		2-butanol	78-92-2	n.q	Fruity[c]	
		2-propen-1-ol	107-18-6	n.q	Alliaceous[c]	
		2-butanone	78-93-3	n.q	Fruity[c]	
		3-methyl-pyridine[b]	108-99-6	n.q	Green[c]	
		2,3-pentane-dione[b]	600-14-6	n.q	Buttery[c]	
		propionic acid[b]	79-09-4	n.q	Vinegar[c]	
white-brined cheese	*L. acidophilus* LA-5	diacetyl	431-03-8	n.q	Buttery	Ozer *et al.* (2009)
	B. bifidum BB-12 acetaldehyde	75-07-0	7.2 - 27.8	Fresh		
Cheddar cheese	*L. casei* LC2W	heptanoic acid	111-14-8	n.q	Cheesy[c]	Hong-Xin *et al.* (2015)
		nonanoic acid	112-05-0	n.q	Cheesy[c]	
		undecanoic acid	112-37-8	n.q	Creamy[c]	
		myristoleic acid	544-64-9	n.q	Fatty[c]	
	E. faecium PR88	diacetyl	431-03-8	n.q	Buttery	Gardiner *et al.* (1999)
		pentan-2-one	107-87-9	n.q	Protein-aceous	
		3-methyl-butanol	123-51-3	n.q	Sweaty	
		butyric acid	107-92-6	n.q	Buttery	
		3-methyl-butyric acid	503-74-2	n.q	Cheesy	
Yogurt*	*L. bulgaricus,*	Diacetyl[d]	431-03-8	0.2-3.0	Buttery	Tamime et Robinson (2007); Innocente *et al.* (2016)
	S. thermophilus,	acetaldehyde[d]	75-07-0	2.0-41.0	Fruity	
	L. rhamnosus DSA LR1	acetoin[d]	513-86-0	2.2-5.7	Buttery	
		2-butanone	78-93-3	n.q	Fruity	
		2-heptanone	110-43-0	n.q	Fruity	
		2-nonanone	821-55-6	n.q	Fruity	
		Acetone[d]	67-64-1	1.3-4.0	Fruity	

Table 1: (*Contd...*)

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Product names	***Main microorganism***	***Character impact compound***	***CAS number***	***Amount (ppm)***	***Descr iptors***	***Refere nces***
Kefir	*S. cerevisiae* A13	ethanol	64-17-5	4800.0	Alcoholic[c]	Beshkova
	L. helveticus MP1	2-butanone	78–93-3	0.04	Fruity	*et al.* (2003)
	S. thermophilus T15	diacetyl	431-03-8	1.62	Buttery	
	L. delbrueckii subsp. *bulgaricus* HP1	acetaldehyde	75-07-0	18.3	Fruity	
	S. thermophilus T15	Acetone	67-64-1	0.3	Fruity	
	L. lactis subsp. *lactis* C15	ethyl acetate	141-78-6	0.06	Fruity	
Ice-cream	*L. reuteri*, *B. bifidum*	n.i	n.i	n.q	Sour	Salem *et al.* (2005)
Pudding	*L. rhamnosus*	diacetyl	431-03-8	n.q	Buttery	Helland *et al*. 2004

[a]yogurt makes with cow milk, [b]Cheese formulation with specific adjuncts of glycol, [c]Data from *http://www.thegoodscentscompany.com*, [d]Mix culture *L. delbrueckii* subsp. *bulgaricus* and *S. thermophiles* ni: not identify, nq: not quantify

bacteria, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (Routray and Mishra, 2011). There are first potent probiotic strains that have well-researched health benefits (Mater *et al.*, 2005). Whereas, *L. rhamnosus* DSA LR1 is a novel probiotic which has been recently experimented for its possible aroma contribution (Routray and Mishra, 2011). The most aroma compounds usually found in yogurt are acetaldehyde, acetone, acetoin, diacetyl, 2-butanone, 2-heptanone and 2-nonanone (Innocente *et al.*, 2016; Routray and Mishra, 2011).

Indeed, acetaldehyde is the most impact flavor compound of the yogurt which concentration ranges between 2.0 and 41.0 ppm (Tamime *et al.*, 2007). The common level reported is 10–15 ppm (Bottazzi, 1973). Consumer acceptability is generally associated with amount of acetaldehyde, it is responsible of the freshness taste and "fruity" note of the yogurt. The quality of yogurt aroma is quietly depending on its relationship with other volatile compounds; for instance, an aldehyde-to-acetone and aldehyde-to-diacetyl (Accolas, 1980). Overall good flavor of yogurt starts around 8.0 ppm of acetaldehyde, whereas concentration lower than 4.0 ppm give a product which flavor is weak and atypical (Routray and Mishra, 2011). Compounds such as glucose, catechol,

glyceraldehyde, and amino acids such as threonine and glycine, can act as the precursors of acetaldehyde. However, the breakdown of threonine to acetaldehyde and glycine by *L. delbrueckii* subsp. *bulgaricus* threonine aldolase has been identified as a main biosynthesis pathway (Mirjana Hruškar *et al.*, 1995; Routray and Mishra, 2011).

Diacetyl is the second major aroma compound of the yogurt. It is highly exhibited buttery smell, allowed as an impact character of yogurt. Its amount varies from 0.3 to 3.0 ppm, and lowest concentration is observed in mix culture starters. Diacetyl is a secondary metabolite derived from the fermentation of citrate and lactose contained in the milk (Tamime *et al.*, 2007; Nilsson *et al.*, 2008; Cheung *et al.*, 2016; Innocente *et al.*, 2016). Microorganisms involved in its production are *S. thermophilusis, L. bulgaricus* and *L. rhamnosus* (Rasic and Kurmann, 1978; Beshkova *et al.*, 1998; Innocente *et al.*, 2016). Acetoin is derived from enzymatic degradation of diacetyl and imparts buttery notes similar to those of its precursor. The amount (2.2–5.7 ppm) of acetoin is increased during the storage and remains significant at the limit of expired date (Tamime *et al.*, 2007; Innocente *et al.*, 2016; Routray and Mishra, 2011).

Besides aldehydes and diacetyl, several ketones such as 2-butanone, 2-heptanone, 2-nonanone and acetone act in the yogurt aroma. Ketones are characterized by a "fruity" note that makes yogurt very pleasant. Among them, acetone derives from acetaldehyde, whereas 2-heptanone and 2-nonanone derive respectively from β-oxidation of octanoic acid and decarboxylation of decanoic acid. *Lactobacillus rhamnosus* DSA LR1 has a strong activity of these two methyl ketones, while other ketones are common to lactic bacteria (McSweeney *et al.*, 2000; Routray and Mishra, 2011; Innocente *et al.*, 2016).

b. Cheeses aromas derived from probiotic activities

Freshly made curds of various cheeses have in similar bland flavors. Characteristic aroma compounds of each variety of cheese are formed during the ripening period (McSweeney *et al.*, 2000). However, common cheeses produced are not probiotics. Indeed, ripening conditions induce highly decrease of cell viability under 8 log CFU, required for the labelling of products as probiotics. Soft cheese, semi-hard ewe-milk castellano cheese, white-brined cheese and cheddar cheese are some which have been successfully ripening with probiotic strains in order to highlight

aroma character of the final product (Gardiner, 1999; Özer *et al.,* 2009; Milesi *et al.*, 2010; Gomez-Torres *et al.*, 2016; Hong-Xin *et al.*, 2015).

In this context, Milesi *et al.* (2009) have studied production of flavor compounds in soft cheeses and reported that cheeses inoculated with non-starter probiotic strains are impacted by Diacetyl and acetoin. These volatiles are characterized by "buttery to cheesy" note and often produced though various starter Lactobacilli pathway. However, authors assume that cheeses inoculated with non-starters probiotic strains *L. plantarum* I91 or *L. casei* I90 are characterized by a significantly higher concentration of diacetyl and acetoin.

Besides, Gomez-Torres *et al.* (2016) have investigated the effect of reuterin-producing *L. reuteri* coupled with glycerol on the volatile fraction and aroma of *semi-hard ewe milk cheese*. They demonstrated that the addition of probiotic strain *L. reuteri* INIA P5 coupled with 30 mM glycerol enhanced the production 3-methyl-1-butanol "sweaty", 2-butanol "fruity", 2-propen-1-ol "alliaceous", 2-butanone "fruity", 3-methyl pyridine "green", 2,3-pentanedione "buttery" and propionic acid "vinegar" of the cheese. Probiotic semi-hard ewe milk cheese is characterized by "cheesy" aroma due to the individual contribution of those aromatic compounds to the overall flavor.

Another relevant study was carried out in the viability of *B. bifidum* BB-12 and *L. acidophilus* LA-5 in model white-brined cheese by microencapsulation (Özer *et al.*, 2009). The study is also compared the amount of acetaldehyde and diacetyl produced by probiotic cheese and a control. The results show that concentrations of medium and long-chain acids have been increased in experimental cheese. Similarly the acetaldehyde (7.2–27.8 ppm) and diacetyl levels were considerably higher than the control. The model cheese was highly scored in "aroma and flavor" attribute and for overall acceptability.

As regards Cheddar cheese, it has been proven to be a good carrier of probiotics. It contains a complex combination of microorganisms, which change with time, alongside the non-starter lactic acid bacteria (Gardiner *et al.*, 1999; McSweeney *et al.*, 2000; Özer *et al.*, 2009; Phillips *et al.*, 2006; Hong-Xin *et al.*, 2015). In this sense, *L. casei* LC2W was used as an adjunct in the production of probiotic Cheddar cheese ripened. Analysis of aroma compounds showed that probiotic cheese is higher in aroma compounds than control cheese. Heptanoic acid "cheesy", nonanoic acid "cheesy", undecanoic acid "creamy" and myristoleic acid "fatty" were

only detected in probiotic cheese. Acids have a greater contribution to the flavor profile of cheese, and furthermore, they are not only aroma compounds by themselves but also precursors of other compounds, such esters, aldehydes and ketones (Delgado *et al.*, 2011; Hong-Xin *et al.*, 2015). The effect of a probiotic adjunct culture of *E. faecium* on the quality of Cheddar cheese was also reported by Gardiner *et al.* (1999). They found that *E. faecium* PR88 increases proteolysis and higher levels of acid compounds such as butyric acid. This compound has a strong impact on the cheese flavor due to its typical sweaty and cheesy odor.

c. Kefir aromas derived from probiotic activities

Kefir is a fermented milk beverage produced by combined action of bacteria and yeasts that exist in a symbiotic association in kefir grains. The large number of microorganisms present in kefir and their microbial interactions produce active compounds that confer to this beverage a status of natural probiotics (De Oliveira *et al.*, 2013). Starter-kefir grains are the main source of volatile compounds that confers to traditional kefir its unique flavor and aroma. References providing qualitative and quantitative data about flavor production by kefir probiotic lactic acid bacteria and yeasts taking separately are quite rare (Güzel-Seydim *et al.*, 2000; Simova *et al.*, 2002). One of the few studies on this topic has been reported by Beshkova *et al.* (2003) who have followed the production of volatile aroma compounds by single-strain cultures of kefir. The results show that the highest amount of acetaldehyde (18.3 ppm), diacetyl (1.62 ppm), acetone (0.3 ppm), 2-butanone (0.04 ppm) and ethyl acetate (0.06 ppm) and ethanol (4800 ppm) were recorded with *L. delbrueckii* subsp. *bulgaricus* HP1, *S. thermophilus* T15, *L. helveticus* MP12, *L. lactis* subsp. *lactis* C15 and *S. cerevisiae* A13, respectively. The amount of ethanol combined with CO_2 (1.98 ppm) provide to kefir their distinctive alcoholic aroma. Other volatile compounds are yogurt-like aroma commonly found in fermented dairy products.

d. Probiotics contribution in overall aroma of ice cream and pudding

Ice cream and pudding are two dairy products that have been recently investigated as innovative matrices for probiotic growth and viability. Ice cream is a frozen product and pudding is characterized by high thickness and more hydrocolloidal environment than the normal yogurt. Some lactic bacteria strains have displayed their ability to survive in

these restrictive environments (Alamprese *et al.*, 2002; Helland *et al.*, 2004; Moussa Salem *et al.*, 2005; Cruz *et al*., 2009). Alongside the health benefit, additional flavor functionalities of probiotics in such matrices were reported by the same authors. Salem *et al.* (2005) reveal that ice cream containing *L. reuteri* has been judged to be sourer and attained a higher score for probiotic flavor. But their studies do not provide more qualitative and quantitative data regarding volatile organic compound contributions. After study the growth and the metabolism of probiotic strain in milk-based pudding, Helland *et al.* (2004) found that high concentration of diacetyl (18 ppm) were detected in puddings inoculated with *L. rhamnosus* GG. They assume that due to a smaller amount of acetaldehyde produced, diacetyl has a significant impact to the overall pudding aroma.

2.2 Meat Products

The market of probiotics is much expanded in dairy industry, but the concept of probiotic functional food has not obtained similar development in the meat industry (Arihara and Ohata, 2010). Manufacturing of most meat products include thermal treatment, and this process stage is very critical for the survival of probiotic microorganisms. Main probiotic meat products target dry sausages and, to a lesser extent, hams due to their convenient thermal process. Several studies have been recently reported in the topics related with the possibility of functional meat including probiotics (Leroy *et al.*, 2006; Ammor and Mayo, 2007; Arihara and Ohata, 2010). However, most of them are focused in the strain survivability and health benefit. The fewer studies that correlated probiotic adjuncts and aroma improvement are presented in Table 2.

a. Aroma contribution of probiotic strains in fermented sausages

Dry-fermented sausage is a food usually made from meat that is ground, fermented, and then dry cured. During the fermentation period, lactic bacteria add in the sausage formula proliferate and produce volatile organic compounds. Among the compounds produced, some of them have aromatic properties that impart the overall flavor of sausage. Ruiz-Moyano *et al.* (2011) have investigated the volatile compounds change of Iberian dry fermented sausages by adding probiotic *L. reuteri* PL519 during the manufacturing. The products were characterized by significant amount of 2-butanone, 3-methylbutanal and benzene

Table 2: VOCs from probiotic functional meats

Product names	*Main microorganism*	*Character impact compound*	*CAS number*	*Amount (ppm)*	*Descriptors*	*References*
Dry-fermented sausages	*L. reuteri* PL519	2-Butanone	78–93-3	n.q	Aceton-like	Santiago Ruiz-Moyano *et al.* (2011)
		3-Methyl-butanal	590-86-3	n.q	Nutty	
		Benzene acetaldehyde	122-78-1	n.q	Floral	
Heat treated probiotic sausages	*L. casei* ATCC 393	Ethyl butanoate	105-54-4	0.1-1	Fruity[b]	Sidira *et al.* (2015)
		Ethyl decanoate	110-38-3	0.03	Fruity[b]	
		Ethyl lactate	97-64-3	0.01	Fruity, buttery[b]	
Fermented hams	*L. plantarum* L155, *L. plantarum* L167, *P. damnosus* L12	Ethanol[a]	64-17-5	2.08	Alcoholic[b]	Kim *et al.* (2016)
		Ethyl acetate[a]	141-78-6	0.70	Fruity	
		2-Furfural[a]	98-01-1	0.03	Bready	

[a]After 21 days ripening, [b]Data from *http://www.thegoodscentscompany.com*, nq: not quantify

acetaldehyde which concentrations represent 16.3, 0.93 and 2.1 per cent of total volatile compounds, respectively. The authors emphasize that benzene acetaldehyde and 3-Methyl butanal are associated with floral and nutty notes, respectively, and correlated with the flavor of cured meat products, whereas 2-butanone has a moderate effect with an aceton-like flavor. These aroma compounds make dry sausage fermented with *L. reuteri* PL519 fairly acid, and globally more flavored.

b. Aroma contribution of probiotic strains in heat treated sausages

Volatiles of heat treated probiotic sausages were studied with Gas chromatography analytical method by Sidira *et al.*, (2015). These authors reported that immobilized *L. casei* cells resulted in a higher content of ethyl esters and alcohols. Main ethyl esters produced in that case, are ethyl lactate (0.01 ppm), ethyl decanoate (0.03 ppm) and ethyl butanoate (0.1–1 ppm) which are all fruity-like notes. Ethyl-esters contribute to proper sausage aroma (Flores *et al.*, 2004).

c. Aroma contribution of probiotics strains in fermented hams

Functional properties and volatile compounds of fermented hams have

been evaluated by a mixed probiotic starter cultures isolated from Kimchi which is the most popular dish Korea. In the same context, Kim *et al.* (2016) have noticed that hams fermented with Kimchi, which is composed mostly by *L. plantarum* L155*, L. plantarum* L167 and *P. damnosus* L12 (LLP), give similar aroma profile to those obtained from the commercial starter. Ethanol, ethyl acetate and 2-furfural, representing approximately 78 per cent of total VOCs, were very close in experimental and control harms. Thus, they suggested that probiotic (LLP) isolated from kimchi, could be used as starter culture due to their similarity in composition of volatile compounds produced.

2.3 Plant-based Products

Plants are generally rich in carbohydrates and have long been used as fermentation materials. The trend to apply probiotic microbial strains for the fermentation of cereals, fruits and legumes is a rational approach for the development of functional foods (Mishra and Mishra, 2013). Furthermore, the manufacturing of probiotic food with non-dairy food matrices can overcome limitations imposed by milk-based products. Lactose and high content in cholesterol of milk prevent consumption of dairy probiotic foods by the segment of people suffering from lactose intolerance or exposed to hypercholesterolemia related diseases (Granato *et al.*, 2010). Except fruits, the quality of the aroma produced is a permanent challenge in other vegetal materials. Indeed, raw cereals and legumes are often characterized by intense green and beany notes, respectively. Using of suitable probiotic strains can produce volatile compounds that help to mask the negative notes at the same time developing quality aroma compounds. Table 3 presents probiotic strains which have already beneficially used to develop aroma active compounds in plant-based functional foods and their aroma contribution.

a. Aroma compounds derived from probiotics in cereal-based beverages

Cereal-based probiotics are largely dominated by beverages. Salmeron *et al.* (2014) have studied the volatile compounds and flavor characteristics of a malt fermented beverage formulated with *B. breve* NCIMB 702257. Twelve key aroma compounds were produced and most of them (acetic acid, butanoic acid, propanoic acid, acetoin, ethanol 2-butoxy and 2-pentanone 4-hydroxy-4-methyl) were made through the

Table 3: VOCs from probiotic functional foods with plant materials

Product names	*Main micro-organism*	*Character impact compound*	*CAS number*	*Amount (ppm)*	*Descriptors*	*References*
Fermented malt beverage	*B. breve* NCIMB 702257	Acetic acid	64-19-7	n.q	Vinegar[e]	Salmerón *et al.* (2014)
		Butanoic acid	107-92-6	n.q	Sour, rancid	
		Propanoic acid	79-09-4	n.q	Slightly sour	
		Acetoin	513-86-0	n.q	Flowery, wet	
		Ethanol 2-butoxy	111-76-2	n.q	Sweet, fruity	
		2-pentanone 4-hydroxy-4-methyl	68113-55-3	n.q	Butter, caramel	
Probiotic cereal drinks (malt, barley and wheat)	*L. plantarum* NCIMB 8826	Acetic acid[a,b]	64-19-7	n.q	Acidic, vinegary	Salmeron *et al.* (2009)
		Isoamyl alcohol[b]	123-51-3	n.q	Pungent	
		2-butanol[a]	78-92-2	n.q	Leaves, grasses	
		2-methoxyethylbenzene[bc]	3558-60-9	n.q	Floral, chrysanthemum	
Cereal-mix slurry (Pearl millet, red sorghum, white sorghum, wheat)	*P. kudriavzevii* OG32[d]	Tetradecanoic acid	544-63-8	n.q	Creamy, cheesy	Ogunremi *et al.* (2015)
		9, 12-octadecadienoic acid methyl ester	112-63-0	n.q	Bland	
		Benzyl alcohol	100-51-6	n.q	Floral, rose	
Probiotic coconut	*L. acidophilus* L10	Acetic acid	64-19-7	n.q	Vinegar[e]	Lee *et al.* (2013)
		Diacetyl	431-03-8	n.q	Buttery[e]	
		2,3-Heptanedione	96-04-8	n.q	Buttery[e]	
		2-Nonanone	821-55-6	n.q	Fruity[e]	
		Benzaldehyde	100-52-7	n.q	Bitter[e] almond	
		2-Heptanone	110-43-0	n.q	Fruity[e]	
		2-Heptanol	543-49-7	n.q	Citrus[e]	
		2-Nonanol	628-99-9	n.q	Citrus[e]	
		ä-octalactone	698-76-0	n.q	Coconut[e]	
		ä-dodecalactone	705-86-2	n.q	Coconut[e]	
	L. casei L26	Acetic acid	64-19-7	n.q	Vinegar[e]	
		Diacetyl	431-03-8	n.q	Buttery[e]	
		Acetoin	513-86-0	n.q	Buttery[e]	
		ä-Decalactone	705-86-2	n.q	Coconut[e]	
		3-Methyl-3-buten-1-ol	4516-90-9	n.q	Herb[e]	
		1-Octanol	111-87-5	n.q	Orange[e]	
		P-tolualdehyde	104-87-0	n.q	Fruity[e]	
		Ethyl 2-hydroxypropanoate	97-64-3	n.q	Fruity[e]	
		Linalool	78-70-6	n.q	Citrus, floral[e]	

[a]Barley, [b]Malt, [c]Wheat, [d]Probiotic yeast cells, [e]Data from *http://www.thegoodscentscompany.com*, nq: not quantify

metabolic activity of the *B. breve* NCIMB 702257. The sensory evaluation exhibited that the fermented malt beverage had a sour flavor. The authors have concluded that volatile acid compounds identified can be appointed as significant character aroma compounds of the novel fermented cereal beverage.

Salmeron *et al.* (2009) had previously issued a study in the same topic. They have formulated various cereal-based media (oat, barley, wheat and malt) with probiotic strain *L. plantarum* NCIMB 8826 in order to obtain functional drink. About sixty volatile compounds have been identified after Gas chromatography analysis of each fermented drink. Significant differences have been noticed in the amount of acetic acid (vinegary), isoamyl alcohol (pungent), 2-butanol (leaves, grasses) and 2-methoxyethylbenzene (chrysanthemum) produced. Eventually, this study emphasizes the contribution of probiotic strains in the production of flavor compounds and provides useful information to develop probiotic drinks.

b. Aroma compounds derived from probiotics in cereal-mix slurry

The effect of probiotic yeast strains in quality attributes and functional value on traditional fermented cereal-based food has been proved. Cereal-mix slurry (pearl millet, red sorghum, white sorghum, wheat) has inoculated with *P. kudriavzevii* OG32 in selected parameters of the fermentation process. Volatile organic compounds generated during the fermentation have been compared with a control. Forty volatile compounds were identified in the fermented product, while acids (32.21%) and esters (32.37%) accounted for the largest proportions (Ogunremi *et al.*, 2015). In particular, levels of 9, 12-octadecadienoic acid (cheesy), benzyl alcohol (floral) and tetradecanoic acid (creamy) acid were increased after fermentation.

c. Aroma compounds derived from probiotics in fermented coconut water

Another novel probiotic beverage has been assessed with coconut water. Lee *et al.* (2013) have studied the fermentation of coconut water by probiotic strains *L. acidophilus* L10 and *L. casei* L26 and have found significant variations between the two cultures in their ability to produce aroma compounds. *L. acidophilus* L10 generated higher amounts of 2-

heptanone (fruity), 2-nonanone (fruity), benzaldehyde (bitter almond), 2-heptanol (citrus), 2-nonanol (citrus), ™-octalactone (coconut) and ™-dodecalactone (coconut), whereas *L. casei* L26 produced higher amounts of acetic acid (vinegar), diacetyl (buttery), acetoin (buttery), ™-decalactone (coconut), 3-methyl-3-buten-1-ol (herb), linalool (citrus), 1-octanol (orange), p-tolualdehyde (fruity) and ethyl 2-hydroxypropanoate (fruity). Although this notice able qualitative and quantitative differences before and after fermentation, authors emphasize that sensory analysis is required to evaluate the relative contribution of these volatile to the organoleptic characteristics of the fermented coconut water.

To sum up, milk-based substrates are well known and widespread matrices for probiotic foods and beverages. However, fruit juices, vegetables and cereals, constitute an emerging segment of non-dairy matrices for the design of probiotic foods. Several studies presented in this paper have shown the ability of some probiotic strains to produce aroma compounds, and also highlighted their contribution to the overall flavor of functional foods. Nevertheless reported studies are primarily focused in qualitative data of volatile; therefore, the establishment of these substrates will require quantitative data for quality control of aromas and further product development. Sensory activity of the individual components of the probiotic food aromas and the dependence between the odor and the chemical composition of these functional foods will, no doubt, lead to a development of specific gas chromatography methods.

3. PROBIOTIC AROMAS: ANALYTICAL ASPECTS

3.1 Introduction

Flavor perception is one of the most important human factors in consumer's food preference. It is tightly linked to specific chemical senses of smell and taste which are of great importance in everyday life. As concerns smell, the "odor" perception depends upon volatile substances liberated by rough or elaborated food products. Nowadays, the terms "Aroma" and "Off-flavor" are used to characterize positive or negative smell properties respectively. Aroma stability of foods and drinks – like probiotic preparations – as well as the study of what produces aroma changes are overriding concerns for commercial purposes and research. As aroma components are complex, their analysis needs powerful tools

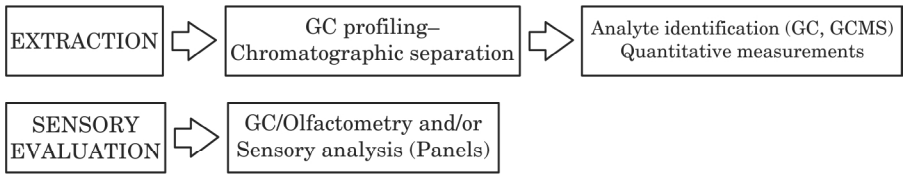
allowing both separation performances and sensitivity. Indeed in many cases some molecules with high odor threshold are present in extremely low amounts. Thanks to gas chromatography (GC and comprehensive GC x GC) and hyphenated techniques (gas chromatography coupled to mass spectrometry GCMS) probiotic aroma characterization is easily achievable for quality assessment. Nevertheless beside instrumental techniques, sensory analysis broadens the performances of the undertaken instrumental investigations. The data supplied by sensory panels is of great help to guide aroma development as well as shelf-life monitoring. This "hybrid" approach (instrumental and sensory) is mandatory (Goodner and Roussef, 2011). In what follows some methodology aspects of aroma analysis are summarized with special attention to probiotic aroma characterization.

3.2 Techniques of Odor Analysis

The main goal of the "odor" analytical chemist is the qualitative and quantitative characterization of aromas. The first challenge he meets is the selection of sampling procedures which must be relevant and of optimal accuracy. Therefore, the choice of extraction method – adapted to the probiotics of interest – is so crucial that it is the keystone for performant determination. Several criteria have to be taken into account when sampling approaches are considered:

a. The "aroma sample" has to be representative; that is, constituents may not be altered during extraction and overheating of the sample should be strictly limited to avoid matrix modification and by-products formation;
b. Reproducible, rapid and possibly automated (whenever possible) methods have to be preferred;
c. When "equilibrium procedures" (see below, § 3.2.1) are used, extraction parameters like temperature, time, pH, ionic strength and salting out of the medium… have to be firstly optimized and systematically applied in routine analyses;
d. While qualitative profiling (comparative studies) is generally the rule in aroma analysis, quantitative measurements of "key molecules" are also required. For that purpose, the establishment of recoveries of the targeted components, as well as the use of internal standard (allowing quantitation by comparison of GC peak areas), are strongly recommended.

Aroma analysis (AA) involves complementary stepwise procedures which are schematically summarized as follows:



The principles of sampling modes for volatile organic compounds (aroma major constituents) studies are exhaustively described by Bazemore (2011) and important criteria are outlined in a white paper: "*The Challenges of Flavour Analysis Comparison and Choices of Extraction Techniques*" by Ridgway K. (*https://www.rssl.com/~/media/rssl/en/files/documents/white-paper/challenges-of-flavour-analysis.pdf*). They can be broadly divided into three main categories: the distillation methods, the equilibrium methods and "purge and trap" (P&T) techniques (both descriptions and some typical examples in the field of probiotics are given below).

a. Sampling of volatile constituents of aromas

Distillation methods

Combined steam distillation-extraction (SDE) allows isolation of volatiles while gentle heating the matrix for a fixed time. This method can lead to some artefacts therefore a modified version named "Solvent Assisted Flavor Evaporation" (SAFE) which combines: vacuum distillation, cold trapping with liquid nitrogen and possibly solvent extraction has been developed by Engel *et al*. (1999). This technique keeps sample at low temperature that minimizes alteration processes and avoid artefacts. In the field of probiotics "classical" SDE has been used to extensively investigate cheese (Hong-Xin *et al.*, 2015), industrial culture medium (Ono *et al.*, 2015) and white salsify (*Tragopogon porrifolius* L.) probiotics (Riu-Aumatell *et al.*, 2011).

Equilibrium methods (head space sampling)

Head space analysis is of special interest for the investigation of volatile compounds liberated by complex matrices and need a reproducible

partition between sample and gaseous phase. Volatile products can be sampled directly (Static Head Space, HS) or after ad(b)sorption on selected polymer phases (Solid Phase MicroExtraction, SPME or Stir Bar Sorptive Extraction, SBSE, See also Goodner and Rouseff, 2011 for general application rules). HS has been applied to the determination of the aroma impact compounds of yogurt flavor (Ott *et al.,* 1997).

SPME has been developed in the early 1990 by Pawliszyn and co-workers. This solvent-free technique is widely used for volatile profiling of food and environmental samples. Due to its flexibility, its broad range of applications and its easy handling, the technique has a widespread acceptance in flavor analytical chemistry. Moreover, in some cases, with SPME, limits of detection approaching the nanogram level can be reached. For long time, the technique has been considerably improved for many and diversified applications see Spietelun *et al.,* 2013) and Ouyang and Jiang, (2016).

SPME design is shown in Fig. 1 whereas the principle of sampling (SPME diagrams) is presented in Fig. 2. The latter is simple and can be briefly presented as follows:

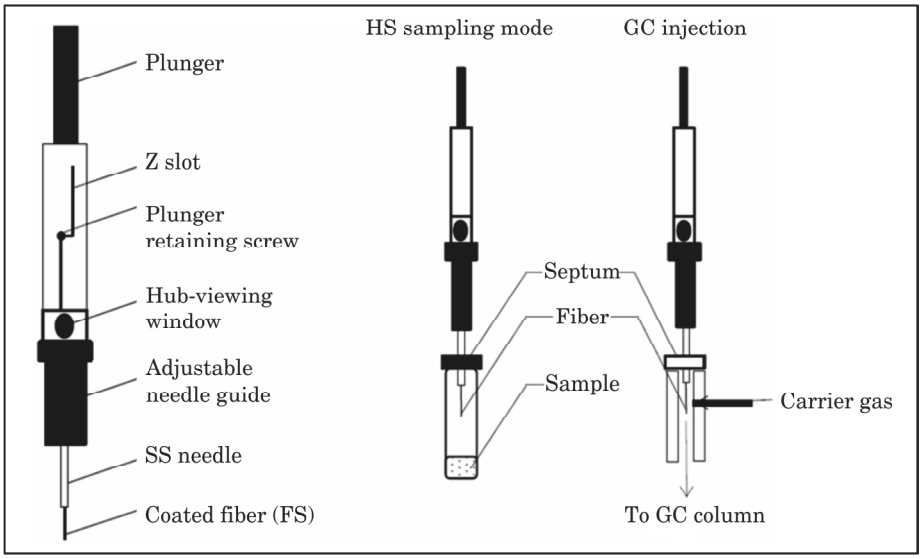


Fig. 1: SPME device

Fig. 2: SPME diagrams

1. The fused silica coated fiber is exposed to the gaseous phase in equilibrium with the probiotic sample in a sealed vial
2. Volatile compound partition between gas phase and the fiber polymer coating is established during optimized sampling time and temperature afterward the fiber is withdrawn and removed into the stainless steel needle
3. Finally the needle is inserted into the GC injection port and the fiber is exposed to rapid thermodesorption leading to the transfer of the analytes to the GC column.

Fiber coating nature and dimensions, as well as intrinsic molecular properties (nature, volatility, etc.), are important factors that influence the interactions. Before (manual or automated) routine analyses, preliminary trials are required to optimize analytical conditions because phase-retention kinetics are strongly influenced by several factors like T°, sampling time, pH, salt effect and so on (Pawliszyn, 1999, 2011; Marsili, 2011, Lin *et al.,* 2007). These particular aspects and developments of SPME have been extensively reviewed by Vas and Vékey, (2004); Merkle *et al.* (2015), Jelen *et al.* (2012); Heaven and Nash (2012); Ouyang and Jiang, (2016). This easy-to-use technique leads to reproducible aroma-profiles nevertheless robust quantification requires careful calibration and method validation (Ouyang and Pawliszyn, 2008; Begnaud and Chaintreau, 2015).

SPME automation is easily undertaken and allows optimal sample throughput. Moreover the use of cooled septum free injection port with narrow –bore inlet liners lead to well resolved chromatographic peaks with good shapes. In order to speed-up the micro-extraction process, the samples can be thoroughly agitated with small magnetic bars or salted at controlled temperature. Head space SPME sampling conditions limit analyte breakdown and therefore warrants sample representativeness.

Purge and Trap (P&T) extraction

P&T is a dynamic process in which volatile organic compounds are continuously displaced from the matrix (purge) or swept from the head space by a continuous flow of inert gas like Helium. The flow passes through a selected trap which adsorbs the molecules of interest which are then desorbed and introduced into a GC column. Cryocooling allows

condensation of very volatile molecules. Typical example of P&T has been described for the study of yogurt flavor by Ott *et al.,* 1997.

b. Chromatographic techniques and molecular identification

Capillary GC is far the most employed technique for aroma analysis. It is easily hyphenated with mass spectrometry (GCMS). The separated molecules are identified on the basis of their typical mass fragmentations in the electron impact mode that are compared with computed data bases (NIST, WILEY, etc.). The identifications are confirmed by comparing their linear retention indices obtained under thermal gradient conditions with those of pure references or from the literature (Goodner and Rouseff, 2011).

c. Gas chromatography/olfactometry (GC/O) and sensory analysis

According to Delahunty *et al.* (2006) *"GC/O refers to the use of human assessors as a sensible and selective detector for odor-active compounds"*. In GC/O this odor-activity of the molecules separated by capillary GC and splitted to a "sniffing-port" are noted and described by human selected and trained "sniffers". Their role is to identify and characterize (with typical sensory vocabulary) potent aroma constituents. GC/O also allows off-flavor detection in food products. Different approaches underpin GC/O analysis: *dilution analysis, detection frequency, time intensity*, etc. They are described in details in Goodner and Rouseff (2011) (see this reference for further details). The method has been reviewed extensively (Brattoli *et al.* 2013; Van Ruth, 2001). Finally, several studies focusing on GC/O are reported within the literature for dairy products (Friederich *et al.,* 1998), fermented sausages (Olivares *et al.,* 2015; Marco *et al.,* 2007) and fruits (Hongley *et al.,* 2014). Beside instrumental techniques a lot of sensory analysis protocols have been described. They are not taken into account in the present overview. Nevertheless, very interesting and systematic descriptions can also be found in Goodner and Rouseff (2011), Borsa *et al.* (2015), Karimi *et al.* (2012), Cruz *et al.* (2010).

d. Literature survey

Among the various available methods and according to the three last decades of scientific literature, SDE and headspace methods have been

used for probiotic flavor analysis. Nevertheless as part of the different head space configuration modes, SPME is the most frequently reported for probiotic aroma profiling. In what follows, typical applications are given.

3.3 Probiotic Aroma Analysis – Some Indicative Examples

To the Authors best knowledge and according to an advanced bibliographic insight, it is confirmed that the majority of the published papers report SPME as sampling procedure for volatile organic compounds. The survey indicates SPME-GCMS as a very powerful method employed for qualifying probiotics. Indeed this could be linked to some observations coming also from the author's experience:

(a) The main drawbacks of simultaneous distillation-extraction (SDE) methods are the potential poor recovery of very volatile components as well as the disappearance of thermolabile molecules associated with possible artefact formation. When solvent evaporation is required, risk of losses of more or less compounds has to be suspected. It seems therefore that these methods are hardly used anymore in recent investigations;

(b) When carefully validated, HS-SPME-GCMS is a fast and reproducible (automated) approach of volatile compound determination even for the detection and quantification of minor compounds in traces.

Typical very indicative applications (studies) related to probiotic aroma are gathered in Table 4. They show the versatility and the flexibility of the HS-SPME-GCMS approaches for the study of complex matrices like dairy products, fermented sausages and other plant products. The method has been applied to the careful sampling of a lot of different compounds of various chemical classes (for some significant examples see related aroma contributing molecules derived from probiotics in Tables 1 to 3)

3.4 Concluding Remark Over Probiotic Characterization

As shown herein, nowadays, the analysis of probiotic assisted production of aroma is mainly guided by the development of head space sampling protocols with GCMS as identification-quantification tool. Nevertheless, other less frequently reported techniques like Proton-Transfer Reaction

Table 4: Typical examples of probiotic aroma analyses by HS-SPME (2007-2017)

Topics	***Authors***
Probiotic dairy products	
• Fermented dairy beverage Kefir	Walsch *et al.*, 2016
• Probiotic viability yogurts produced with wheat bran	Terpou *et al.*, 2017
• Probiotic bacterium in yogurt	Settachaimongkon *et al.*, 2014
• Plant derived lactococci-milk products	Alemayehu *et al.*, 2014
• Caprine Coalho Cheese aromas	Bezzera *et al.*, 2016
• Volatile flavors from yogurt	Cheng, 2010
Sausages & meat products - probiotics	
• Dry fermented sausages produced with probiotics	Sidira *et al.*, 2013
• Effect of L.casei on probiotic fermented sausages	Sidira *et al.*, 2015
• Probiotic cultures, effect on dry fermented sausages	Sidira *et al.*, 2016
• Enterococci- Slightly fermented sausages	Latorre-Moratella *et al.*, 2011
• Effect of mixed probiotics starter on fermented hams	Kim *et al.*, 2016
• Volatiles from slow dry fermented sausage	Cano-Garcia *et al.*, 2013
• Key aromas in Traditional fermented sausages	Corral *et al.*, 2014
• Odor active compounds of fermented sausages	Marco *et al.*, 2007
Other probiotic foods from plant products	
• *Lactobacillus plantarum* in cereal based substrates (aroma)	Salmeron *et al.*, 200
• Volatiles of sourdough breads	Plessas *et al.*, 2008
• Aromas from white salsify (*Tragopogon porrifolius* L.)	Riu-Aumatell *et al.*, 2011
• LAB starters – aroma from tomato juices	Di Cagno *et al.*, 2009

Mass Spectrometry (PTR-MS) and electronic nose technology (which implies odor "perception" by gas sensors) have undergone significant improvements for flavor characterization (Gallardo-Escamilla *et al.,* 2005). Beside these two complementary techniques, and due to its higher peak capacity than mono-dimensional gas chromatography modern "omic" approach like GC x GC (using two columns of different chemistries – Principle of orthogonality) offers very powerful way to deliver comprehensive fingerprints of probiotic food aromas (Cordero *et al.,* 2015; Gasior and Wojtyczak, 2016). Nevertheless the descriptive aroma evaluation requires human nose for a continuous improvement of the reliability of the information. Instrumental and sensory analyses are therefore strictly compatible and complementary.

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