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ORIGINAL ARTICLE

Lipidomics as an important key for the identification of beer-spoilage bacteria

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Significance and Impact of the Study: Electrospray ionization-tandem mass spectrometry (ESI-MS/MS) using collision-induced dissociation was used to characterize intact plasmalogen phospholipid molecules in beer-spoilage anaerobic bacteria *Megasphaera* and *Pectinatus*. Using selected ion monitoring (SIM), this method has a detection limit of 1 pg for the standard 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and is linear within four orders of magnitude (2 pg to 20 ng). The limit of detection was about 830 cells of bacteria containing natural cyclopropane plasmalogen (c-p-19:0/15:0). SIM ESI-MS method is useful for analyzing low concentrations of plasmalogens in biological samples contaminated with anaerobic bacteria, e.g. beer or juice.

Keywords
beer, contamination, electrospray tandem mass spectrometry, *Megasphaera*, *Pectinatus*, plasmalogens.

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Abstract

Electrospray ionization-tandem mass spectrometry (ESI–MS/MS) was used for characterizing intact plasmalogen phospholipid molecules in beer-spoilage bacteria. Identification of intact plasmalogens was carried out using collision-induced dissociation and the presence of suitable marker molecular species, both qualitative and quantitative, was determined in samples containing the anaerobic bacteria *Megasphaera* and *Pectinatus*. Using selected ion monitoring (SIM), this method had a limit of detection at 1 pg for the standard, i.e. 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and be linear in the range of four orders of magnitude from 2 pg to 20 ng. This technique was applied to intact plasmalogen extracts from the samples of contaminated and uncontaminated beer without derivatization and resulted in the identification of contamination of beer by *Megasphaera* and *Pectinatus* bacteria. The limit of detection was about 830 cells of anaerobic bacteria, i.e. bacteria containing natural cyclopropane plasmalogenes (c-p-19:0/15:0), which is the majority plasmalogen located in both *Megasphaera* and *Pectinatus*. The SIM ESI-MS method has been shown to be useful for the analysis of low concentration of plasmalogens in all biological samples, which were contaminated with anaerobic bacteria, e.g. juice, not only in beer.

Introduction

Microbial contamination that can occur during the brewing process can negatively affect the process of beer production and the quality of final product and its durability. The contaminants can include, beside the so-called wild yeasts and molds, a number of bacterial genera (Jespersen and Jakobsen 1996; Sakamoto and Konings 2003; Matoulková *et al.* 2012a). Improvements in beer handling and bottling technology have resulted in significant reduction in the oxygen content during the process and in packaged beer, which has permitted the growth of anaerobic beer-spoilage bacteria such as *Megasphaera* and *Pectinatus* (Helander and Haikara 1995; Satokari *et al.* 1998).

These anaerobic beer-spoilage bacteria belong to the class Clostridia and the family Veillonellaceae; despite their phylogenetic affiliation to Gram-positive bacteria, the cells stain as Gram-negative or Gram-variable (Schleifer *et al.* 1990; Juvonen *et al.* 2008). Three *Pectinatus* species, *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*

and *Pectinatus haikarae*, are found only in brewery environments and may be associated with biofilms (Juvonen *et al.* 2008; Matoulková *et al.* 2012b). *Pectinatus brassicae* has recently been isolated from salty pickle wastewater (Zhang *et al.* 2012). *Pectinatus sottacetonis* sp. nov. was isolated from a pickle spoilage tank and described recently (Caldwell *et al.* 2013). The genus *Megasphaera* currently contains five species; *Megasphaera cerevisiae*, *Megasphaera paucivorans* and *Megasphaera sueciensis* were isolated from spoilt beer or contaminated brewer's yeast (Juvonen and Suihko 2006). The species *Megasphaera elsdenii* and *Megasphaera micronuciformis* have been isolated from nonbrewery environments—*M. elsdenii* resides in the sheep and cattle rumen and in the intestinal tract and stool of humans, *M. micronuciformis* was isolated from clinical material (Marchandin *et al.* 2009).

The anaerobic beer-spoilage bacteria *Megasphaera* and *Pectinatus* have become a serious problem in brewing industry because they can cause strong turbidity and sediment in beer and produce unpleasant volatile compounds, mostly acids, e.g. acetic, propionic, butyric, valeric and caproic acid and also acetoin and hydrogen sulphide (Jespersen and Jakobsen 1996; Sakamoto and Konings 2003). As a result, bacterial spoilage activity can completely damage beer taste and quality, and thus cause serious financial problems to the brewery.

Detection of these spoilage bacteria in breweries is still based on traditional incubation on special diagnostic/selective media (Sakamoto and Konings 2003; Matoulková *et al.* 2012a), which is very time-consuming. For example, development of turbidity in beer forcing tests takes 4–6 weeks (Satokari *et al.* 1998; Juvonen *et al.* 2008). In SMMP-medium (a selective medium for *Megasphaera* and *Pectinatus*), the incubation takes up to 14 days (Satokari *et al.* 1998). Further alternatives are analysed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) (Vávrová *et al.* 2014), epifluorescence microscopy, immunofluorescence (Juvonen *et al.* 2008) or modern genetic methods such as PCR (Satokari *et al.* 1998), fluorescence *in situ* hybridization (Juvonen *et al.* 2008) or oligonucleotide microarrays (Weber *et al.* 2008), see also Table 1.

Phospholipids are major constituents of bacterial cell membranes as mixtures of various molecular species depending on the type of polar head groups, the degree of unsaturation and the length of the acyl chains (Christie and Han 2010).

Lipids with ether bonds of long-chain alkyl moieties, i.e. fatty acids, instead of ester bonds are very common in anaerobic bacteria, especially as membrane constituents. Usually the ether bond is to position *sn-1* of a glycerol moiety, which may be a part of phospholipids in membranes. One of two main types of bonds of glycerolethers is alkenyl ether bond, also called plasmenyl or vinyl ether bond. Alkenyl ethers are unstable to acidic hydrolysis and open readily to form aldehydes.

Plasmalogens belong to very specific types of lipids, as they are present only in anaerobic bacteria and in animals from protozoans to mammals (Goldfine 2010). Their presence in fungi including yeast and in plants has not been documented (Feld and Spiteller 1994). We used this

Table 1 Comparison of different methods for the determination of bacterial contamination by the genus *Megasphaera* and *Pectinatus*

Detection method	Sensitivity	Time	Reference
Matrix assisted laser desorption/ionization time-of-flight mass spectrometry	1×10^3–7×10^4 ml^{-1}	1–2 days	Šedo *et al.* (2011); Vávrová *et al.* (2014)
Polymerase chain reaction	1×10^3–5×10^3 CFU 100 ml^{-1} of beer	1–3 days*	Satokari *et al.* 1998; Juvonen *et al.* (2008); Juvonen and Haikara (2009)
Fluorescence *in situ* hybridization	1×10^3 cells 100 ml^{-1} of beer	5 h	Yasuhara *et al.* (2001)
Membrane filter-based fluoroimmunoassay	20–40 cells 100 ml^{-1} of beer	3 h	Gares *et al.* (1993)
Direct epifluorescent filter technique	10–100 cells on membrane	3–5 days*	Haikara (1985a,b)
Hybridization protection assay	5×10^2–1×10^3 CFU ml^{-1}	2–3 days*	Paradh *et al.* (2014)
Selective medium for *Megasphaera* and *Pectinatus*	1 cell 130 ml^{-1} of beer	14 days	Lee (1994)
De Man–Rogosa–Sharpe with tetrahydroiso-α-acids	1 cell from swab sample	1–2 days	Matoulková *et al.* (2012a)
Nachweismedium für Bierschädliche Bacterien –Concentrate	1 cell 150 ml^{-1} of beer	5 days	User Manual (Döhler, Darmstadt, Germany)
Nachweismedium für Bierschädliche Bacterien—Bouillon-Anreicherungsmedium	1 cell from swab sample	3 days	User Manual (Döhler, Darmstadt, Germany)
Electrospray ionization-tandem mass spectrometry selected ion monitoring	8×10^2–9×10^2 cells of sample	20 min	This paper

*PCR identification takes 6–7 h for end-point PCR and 2–3 h for Real-Time PCR, but a cultivation step (so-called enrichment) is required before the PCR.

selective occurrence of plasmalogens for identifying possible contamination of beer by anaerobic bacteria as neither *Saccharomyces* yeast nor the raw materials used for beer production (barley, malt and hops) contain plasmalogens. If present in beer, plasmalogens can thus arrive only from the beer-spoilage bacteria *Megasphaera* or *Pectinatus*.

We therefore developed a fast and sensitive method for the analysis of contaminated beer samples. The crude lipid extract of beer is analysed on a short column by HPLC connected to high resolution selected ion monitoring (SIM). This method identifies appropriate molecular species of the plasmalogen 1-(methylene-nonadecyl)-2-pentadecanoyl-*sn*-glycero-3-phosphoethanolamine (c-p-19:0/15:0 PE), lithium salt, and thus also the *Megasphaera* or *Pectinatus* bacteria. The SIM (electrospray ionization-tandem mass spectrometry) ESI-MS analysis was used in eight samples including those from mixed cultivations, and the presence of highly abundant molecular species (c-p-19:0/15:0 PE) led to the identification of beer contamination. The limit of detection of the appropriate plasmalogen was 1 pg, i.e. about 830 cells.

Results and discussion

Plasmalogens in the crude lipid extract of beer and in lipid extracts from three bacteria and yeast (*M. cerevisiae*, *P. frisingensis*, *Lactobacillus brevis* and *Saccharomyces pastorianus*) were identified by HPLC analysis connected to high resolution SIM ESI-MS. These micro-organisms were selected as the beer can contain residual yeast cells. Among the lactic acid bacteria with beer-spoiling ability, the species *Lact. brevis* is considered the most deleterious. Therefore, the analysis of samples collected as pure cultures concerned the anaerobic bacteria (*Megasphaera* and *Pectinatus*), aerotolerant anaerobic bacterium (*Lactobacillus*), yeast (*Saccharomyces*) and also beers (blank).

As mentioned above, phosphatidylethanolamine (PE) molecular species give rise to a number of pseudomolecular ions of type $[M+H]^+$, $[M+Na]^+$, etc. This previously reported phenomenon (Feld and Spiteller 1994) complicates the analysis of PEs and generally phospholipids in biological samples. As described by Hsu and Turk (2000), addition of lithium salts on the other hand causes preferential formation of ion types $[M+Li]^+$ or $[M-H + 2Li]^+$, their abundance being dependent on the concentration of the lithium salt added. The monolithiated adduct ion was the major ion species when 1 μmol ml^{-1} AcOLi was present in the solution. The dilithiated ion became the exclusive adduct ion species when Li^+ (AcOLi in methanol) was >2 μmol ml^{-1}.

Collision-induced dissociation (CID) of positive ions of phospholipids yields fragment ions that are related to the polar head group, fatty acyl and plasmenyl substituent bonded to the glycerol backbone. In the MS^2 mode, PE species showed an intense fragment $[M+Li-43]^+$ and this fragment was used for SIM detection.

Tandem MS of the *m/z* 736 ion of 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, lithium salt (Fig. S1), i.e. commercially obtained standard (p-18:0/9-18:1 PE) exhibits abundant fragment ions at *m/z* 693 $[M+Li-43]^+$, 613 $[M+Li-123]^+$, 607 $[M+Li-129]^+$, 595 $[M+Li-141]^+$ and 589 $[M+Li-147]^+$, arising from neutral losses of CH_2CH_2NH (aziridine), $(HO)P(O)(OCH_2CH_2NH)$, $(LiO)P(O)(OCH_2CH_2NH)$, $(HO)_2P(O)OCH_2CH_2NH_2$ and $(LiO)(HO)P(O)(OCH_2CH_2NH_2)$, respectively. The spectrum also contains two abundant ions, one of which, at *m/z* 425, arises by a combined loss of aziridine and alk-1′-enyl as alcohol from position *sn*-1. The other, at *m/z* 307 (see the structure $C_{16}H_{33}CH{=}CHOH$) arises by a combined loss of $(LiO)(HO)P(O)(OCH_2CH_2NH_2)$ and fatty acid (structure $C_{17}H_{33}COOH$) from position *sn*-2. The type of fatty acids in position *sn*-2 can be further confirmed by the presence of acylium ion $[C_{17}H_{33}CO]^+$ at *m/z* 265. It is thus possible to determine the type of the substituents in positions *sn*-1 and *sn*-2 and also the length of the acyl chains and the degree of unsaturation of the substituents on glycerol backbone. For the tandem MS, see Fig. S2.

The tandem MS of the $[M+Li]^+$ ion from natural source, i.e. c-p-19:0/15:0 PE, gives rise, in addition to the pseudomolecular ion at *m/z* 708, to another three most abundant ions in the tandem spectra of the lithiated precursors at *m/z* 665 $[M+Li-43]^+$, 567 $[M+Li-141]^+$ and 561 $[M+Li-147]^+$, which represent a neutral loss of aziridine, $(HO)_2P(O)(OCH_2CH_2NH_2)$, and $(LiO)(HO)P(O)(OCH_2CH_2NH_2)$, respectively. These ions are characteristic for PE and its head group, i.e. phosphoethanolamine. The spectrum also contains fragment ion at *m/z* 319, resulting from a neutral loss of fatty acid (pentadecanoic acid). Present is also ion at *m/z* 383 $[M+Li-43-R'_1CH{=}CHOH]^+$ which arises by combined losses of aziridine and alcohol moiety, from position *sn*-1. The spectrum further contains ion at *m/z* 225 (15:0), which represents the acylium ion of fatty acid, see Fig. S3. The proportion of c-p-19:0/15:0 PE molecular species from *Megasphaera* would reach some 10% of total pPE.

Determination of the level of plasmalogen molecular species requires the addition of internal standards because the ionization efficiency significantly differs between different phospholipid classes and also between homologues of ESI-MS. p-18:0/18:1 PE was used as an internal standard in the analyses of pPE, as this molecular species is among the few commercially available ones. Because the chain lengths and unsaturation degree in our samples from bacteria vary in a very narrow interval (our sample, i.e. c-p-19:0/15:0 PE, differs from the standard, i.e. p-18:0/18:1 PE, by only two methylenes), the

standard and bacterial plasmalogens can be taken to be nearly identical. To determine the range of linear response of Orbitrap, we injected the standard of p-18:0/18:1 PE in triplicate in a range of 1 pg μl^{-1} to 2 μg μl^{-1} in chloroform/methanol mixture (1 : 1). The detection limit for a specific pPE, i.e. p-18:0/18:1 PE, was 1 pg injected sample.

In the construction of the calibration curve, p-18:0/18:1 PE concentrations above 20 μg μl^{-1} resulted in a relative decrease in the MS signal response, which was nonlinear and yielding a cubic polynomial curve, where $y = -2950x^3+64\ 979x^2+527\ 009x-143\ 842$, with $R^2 = 0{\cdot}9\ 996$ and/or a quadratic polynomial curve $y = -27\ 952x^2 + 1\ 360\ 441x\text{-}2\ 064\ 425$, with $R^2 = 0{\cdot}9936$. From 2 pg μl^{-1} to 20 ng μl^{-1}, the calibration curve showed a high degree of linearity ($R^2 = 0{\cdot}9983$) with a linear regression curve, see $y = 930\ 000x-794\ 615$ over four orders of magnitude, see Figs S4 and 1 (calibration curve and SIM of ion at *m/z* 693·5408, i.e. ion $[M+Li\text{-}43]^+$).

Lipid extracts obtained from different samples, see above and also Fig. 2, were analysed by ESI MS SIM. Minimal content of the selected, the most abundant, pPE was 1 pg/830 cells, which agrees with literature data calculated from the biomass composition of different *Pectinatus* strains and knowledge of cell size, see an electron microscope (Fig. 3).

Product ion spectrum of adduct ions of monolithiated plasmalogen-PE contains ion fragments that can be readily distinguished from diacyl-PE (Hsu *et al.* 2003), whose base peak in the spectrum of PE plasmalogens shows ions of type $[M+Li\text{-}43]^+$ due to their aziridine loss (Řezanka *et al.* 2011) from the adduct of molecular ion, e.g. $[M+Li]^+$.

When quantifying different molecular species, one should be aware that the ionization efficiency significantly differs between different phospholipid classes and also between homologues of ESI-MS. Precautions were taken in this quantitative study as different homologues are not detected with equal efficiency because of the different length and unsaturation degree of the carbon chains (Berdeaux *et al.* 2010). As shown previously, the standard, i.e. p-18:0/18:1 PE, and a real natural sample, i.e. c-p-19:0/15:0 PE, differ by only 2 CH_2 groups. Despite this, we could not fully quantify the effect of plasmalogens on the instrument response (Hsu *et al.* 2003).

Based on our previous results (Řezanka *et al.* 2011), the c-p-19:0/15:0 PE molecular species was selected as one of the major ions present in *Megasphaera* and also in *Pectinatus*. The *Megasphaera* bacteria feature highly abundant 15:0 fatty acid and also c-p-19:0 alk-1-enyl chain (Helander and Haikara 1995); it was thus assumed, and later confirmed that the major molecular species, i.e. c-p-19:0/15:0 PE, accounts for more than 10% of total plasmalogen PE.

Based on all these data, one can argue that the presence of anaerobic bacteria *Megasphaera* and *Pectinatus* can be demonstrated from concentrations of about 830 cells, which is slightly less than the previously described methods for MALDI-TOF MS (Šedo *et al.* 2011; Vávrová *et al.* 2014). But what is much more important is the unrivalled 20-min time during which one can determine whether the beer is contaminated or not. This value is at least 10-fold lower than any value shown in Table 1.

The method that we used can be employed not only for identifying anaerobic beer-spoiling bacteria in beer; it is much more versatile, as it makes it possible to determine any metabolites in beer provided one can find a suitable biomarker. LC–ESI-LTQ-Orbitrap-MS can thus be used as a multipurpose technique, which was employed, for instance, to identify nearly 50 completely new phenolic compounds in beer including hexosides, dihexosides, pentosides and quinic conjugates (Quifer-Rada 2015), to distinguish

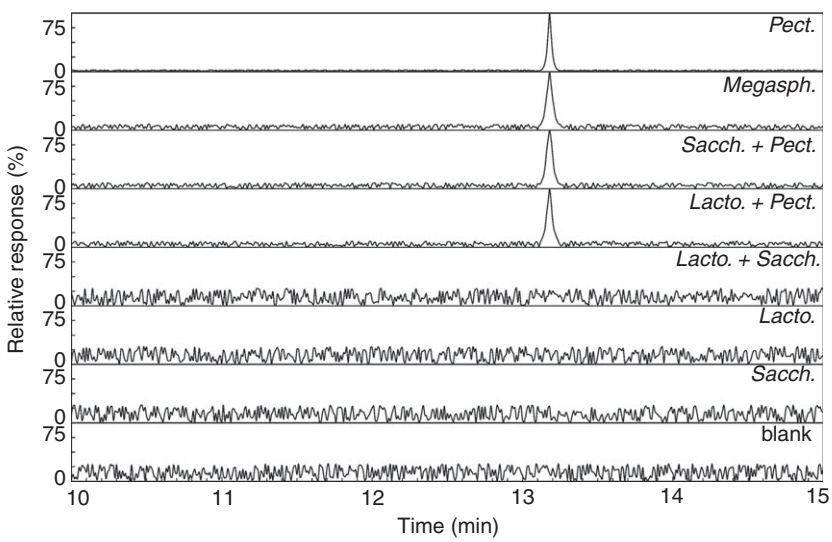

Figure 1 Selected ion monitoring of natural c-p-19:0/15:0 PE from contaminated and uncontaminated samples at *m/z* 665·5092.

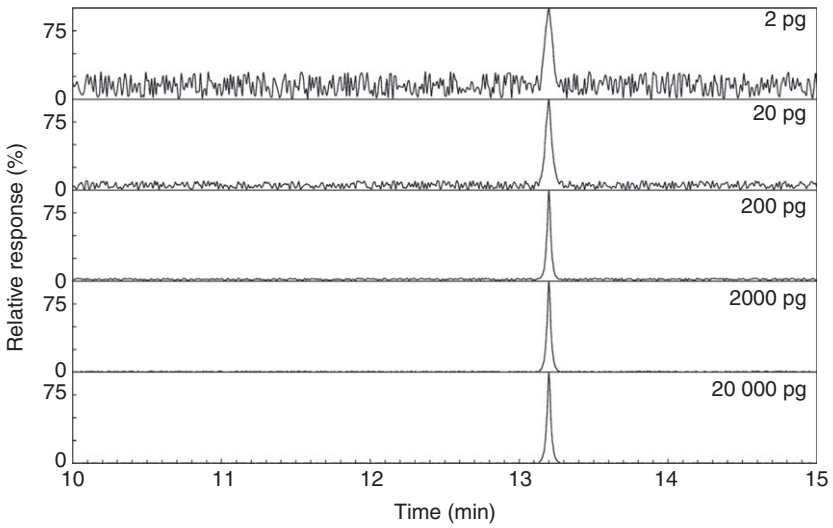

Figure 2 Selected ion monitoring of the standard 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, lithium salt at *m/z* 693·5405.

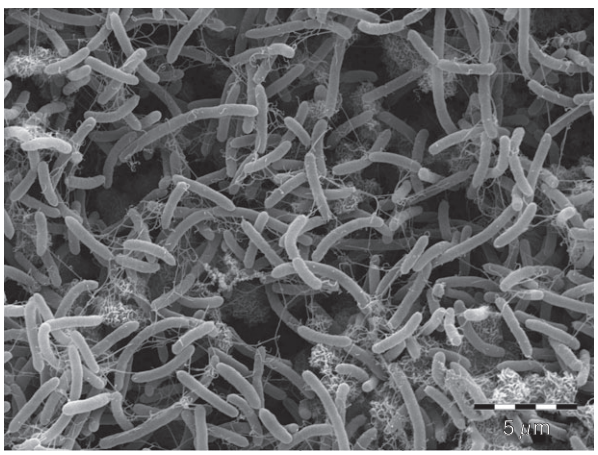

Figure 3 Scanning electron microscopy of *Pectinatus frisingensis* cells adhering onto SPI filter. The image shows bacilliform cells of very variable length ranging from 2 μm to several μm and of uniform thickness of about 0·5 μm (mean ± SD = 0·53 ± 0·04; n = 35). Numerous tubular structures of unknown origin attached to the individual cells or connecting them can be seen.

differential metabolites between regular and nonalcohol beers (Andres-Iglesias *et al.* 2014) or to perform beer fingerprinting (Catharino *et al.* 2009). As such, it can be expected to have numerous applications in future.

Materials and methods

Cultivation and lipid extraction of bacterial strains

Pectinatus frisingensis DSMZ 20465 and *Megasphaera cerevisiae* DSMZ 20461 were purchased from the DSMZ Collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *Lactobacillus brevis* RIBM 2-4 and *S. pastorianus* RIBM 95 were obtained from the RIBM Collection (Research Institute of Brewing and Malting, Prague, Czech Republic). All strains originated from brewery environments, e.g. spoilt beer, brewery equipment and beer production.

Pectinatus and *Megasphaera* were grown in a modified MRS broth (de Man, Rogosa and Sharpe medium; Merck, Darmstadt, Germany) supplemented with cysteine hydrochloride (0·5 g l^{-1}) and sodium thioglycollate (0·5 g l^{-1}), pH 5·6–5·7, for 48 h at 28°C. Cultivation was performed under anaerobic conditions (10 ml of modified MRS broth in 20-ml tubes, the surface of the medium was covered with sterile mineral oil to ensure anaerobiosis). *Lactobacillus brevis* was grown in liquid MRS broth, pH 5·6–5·8, for 48 h at 28°C. Yeast *S. pastorianus* was grown in yeast-peptone-dextrose (YPD) broth (peptone 20 g l^{-1}, yeast extract 10 g l^{-1}, glucose 20 g l^{-1}) for 48 h at 26°C. After cultivation and centrifugation (10 min/6000 rev min^{-1}/4°C), the inoculum of each micro-organism was transferred aseptically to beer (pasteurized 4·3% vol/vol alcohol beer, pH 4·5, containing an average of 25·0 bitterness units) to form a suspension containing 10^3 cells ml^{-1}.

The method of Bligh and Dyer (1959) was used for lipid extraction. Briefly, chloroform/methanol solution (1/2, v/v; 1 ml) was added to the bacterial suspension (10^3 CFU ml^{-1}, 1 ml) and vortexed for 10–15 min. Then chloroform (1 ml) was added and the suspension of cells was shaken for 1 min, and 1 mol l^{-1} NaCl solution (1 ml) was added. The sample was mixed for 1 min and centrifuged. The lower phase was collected, the solvents were evaporated under nitrogen and further redissolved in chloroform/methanol solution (2/1, v/v; 10 μl) prior to analysis. The yeast lipids are extracted in the same way after a previous grinding by glass beads in liquid nitrogen. All reagents were obtained from Sigma (Prague, Czech Republic), except for the phospholipid standard (1-(1*Z*-

octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), which was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). To record mass spectra, 1 μl of the solution was injected to liquid chromatography–mass spectrometry (LC-MS).

Analysis of natural samples, i.e. extracts from *Megasphaera, Pectinatus*, *Lactobacillus*, *Saccharomyces* and their mixed cultures of an uncontaminated beer was carried out under the same conditions as the analysis of the standard (p-18:0/18:1 PE).

Liquid chromatography-mass spectrometry

The HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series; Hewlett Packard, Prague, Czech Republic), and Ascentis Express HILIC HPLC column (2·7 μm particle size, L × I.D. 3 cm × 2·1 mm) (Supelco, Prague, Czech republic). LC was performed at a flow rate of 200 μl min^{-1} for the molecular species pPE (p-18:0/18:1 PE), with a linear gradient from mobile phase containing methanol/acetonitrile/aqueous 1 mmol l^{-1} ammonium acetate (70 : 20 : 10, v/v/v) to methanol/acetonitrile/aqueous 1 mmol l^{-1} ammonium acetate (5 : 75 : 20, v/v/v) with lithium acetate in methanol (1 μmol l^{-1} ml^{-1}) for 20 min. The whole HPLC flow was introduced into the ESI source without any splitting.

LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany), a high resolution hybrid mass spectrometer equipped with a heated electrospray interface was used. ESI–MS analysis was performed in the FT positive ion mode. MS spectra were acquired with target mass resolution of R = 105 000 at *m/z* 800. The ion spray voltage was set at 3·5 kV and the scan range of the instruments was set at *m/z* 150–1000. Nitrogen was used as a nebulizer gas—set at 18 arbitrary units (sheath gas) and seven arbitrary units (auxiliary gas). Helium was used as a collision gas for CID experiments. The CID normalization energy of 35% was used for the fragmentation of parent ions. The MS/MS product ions were detected by the high-resolution FT mode. Further source conditions in the positive mode: vaporizer temperature, 250°C; capillary temperature, 230°C; capillary voltage, 50 V; tube lens voltage, 170 V.

The SIM was used at *m/z* 693·5405, i.e. the appropriate ion $[M+Li-43]^+$ of standard p-18:0/18:1 PE molecular species and at *m/z* 665·5092, i.e. the appropriate ion $[M+Li-43]^+$ of c-p-19:0/15:0 PE natural molecular species.

Scanning electron microscopy

About, 1·5 ml bacteria prefixed with 1·5% glutaraldehyde in culturing medium for 1 h were washed with cacodylate buffer pH 7·4, fixed overnight with 3% glutaraldehyde at 4°C, washed with the buffer and sedimented overnight onto poly-L-lysine-treated SPI Pore Filters (SPI Supplies/Structure Probe, Inc., West Chester, PA, USA) (pore size 0·2 μm) at 4°C. Filters were washed with water and dehydrated (alcohol series, absolute acetone), critical point dried in a Balzers CPD 010 unit, sputter-coated with gold in a Polaron Sputter-Coater (E5100) (Quorum Technologies Ltd, Ringmer, UK) and examined in a Tescan Vega LSU scanning electron microscope (Tescan, Brno, Czech Republic) at 20 kV in secondary electron mode.

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Conflict of Interest

The authors declare no competing financial interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The structure of plasmalogen standard 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, lithium salt.

Figure S2 Tandem mass spectrum of the standard 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine, lithium salt.

Figure S3 Tandem mass spectrum of natural plasmalogen 1-(methylene-nonadecyl)-2-pentadecanoyl-*sn*-glycero-3-phosphoethanolamine (c-p-19:0/15:0), lithium salt.

Figure S4 Test of linearity, detection limit and reproducibility (LC-MS/ESI) for a commercially obtained standard molecular species (p18:0/18:1 PE).