Food Chemistry 240 (2018) 67–74

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Polar lipid composition of bioactive dairy co-products buttermilk and butterserum: Emphasis on sphingolipid and ceramide isoforms

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ARTICLE INFO

*Keywords:*
Buttermilk
Butterserum
Polar lipids
Ceramides
Sphingolipids

ABSTRACT

Bioactive lipids of the milk fat globule membrane become concentrated in two co-products of the butter industry, buttermilk and butterserum. Their lipid composition is detailed here with special emphasis on sphingolipid composition of nutritional interest, determined using GC, HPLC and tandem mass spectrometry. Butterserum was 2.5 times more concentrated in total fat than buttermilk, with 7.7 ± 1.5 *vs* 19.5 ± 2.9 wt% and even more concentrated in polar lipids, with 1.4 ± 0.2 *vs* 8.5 ± 1.1 wt%. Both ingredients constitute concentrated sources of sphingomyelin (3.4–21 mg/g dry matter) and contained low amounts of bioactive ceramides in a ratio to sphingomyelin of 1:5 mol% in buttermilk and 1:10 mol% in butterserum. Compared to other natural lecithins, these two co-products are rich in long and saturated fatty acids (C22:0-C24:0), contain cholesterol and could have interesting applications in neonatal nutrition, but also as brain-protective, hepatoprotective and cholesterol lowering ingredients.

## 1. Introduction

During the last decade, the lipid components of the bovine milk fat globule membrane (MFGM) have attracted much attention among nutritionists and food scientists due to their potential health benefits for the general population (*e.g.* cell regulation properties of sphingolipids and metabolites, bactericidal effects, anticholesterolemic effects of SM and PC) or specific nutritional targets (*e.g.* for the elderly with effects on neuronal functions and anti-degenerative effects of sphingolipids and phosphatidylserine; and for infant nutrition with antiviral, bactericidal and potential incidence on microbiota of gangliosides etc.) (Bourlieu & Michalski, 2015; Dewettinck et al., 2008; Kuchta, Kelly, Stanton, & Devery, 2012; Rombaut & Dewettinck, 2006). The milk fat globule membrane ingredient has thus been presented as a potential nutraceutical. MFGM constitutes a unique biological trilayer surrounding and stabilizing the triglyceride core of the milk fat globule from the milk aqueous phase. Several mechanical treatments, among which heating, agitation, homogenization, aeration or churning lead to the release of the MFGM into the aqueous phase. Phase inversion specifically leads to two types of milk co-products concentrated in MFGM: buttermilk and butterserum. Buttermilk (BM) refers to the liquid phase released during churning (destabilization) of cream in the butter making process (Morin, Britten, Jimenez-Flores, & Pouliot, 2007), while butterserum (BS) is the liquid phase obtained when the butter is further transformed by centrifugation into anhydrous milk fat (Dewettinck et al., 2008).

The MFGM is thin (~15 nm) but of a complex organization and composition: it is based on a complex mixture of proteins, polar and apolar lipids which makes up to 90% of its dry weight. The most numerous polar lipids (PL) in the MFGM are (i) glycerophospholipids, *i.e.*

*Abbreviations:* BM, buttermilk; BS, butterserum; DAG, diacylglycerol; CER, ceramide; FA, fatty acid; HLB, hydrophilic lipophilic balance; MAG, monoacylglycerol; MFGM, milk fat globule membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, polar lipids; PS, phosphatidylserine; SM, sphingomyelin

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http://dx.doi.org/10.1016/j.foodchem.2017.07.091
Received 28 November 2016; Received in revised form 20 June 2017; Accepted 18 July 2017
Available online 19 July 2017
0308-8146/ 

phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and (ii) sphingolipids, among which sphingomyelin (SM) is the most abundant. PL are asymmetrically distributed among the MFGM layers. The choline-containing PL, *i.e.* PC and SM, and the glycolipids (cerebrosides and gangliosides) are largely located on the outside of the membrane, with SM being co-localized with cholesterol in the condensed microdomains, so-called lipid rafts (Gallier, Gragson, Jimenez-Flores, & Everett, 2010; Lopez, Madec, & Jimenez-Flores, 2010). In turn, PE, PS and PI are mainly concentrated in the inner surface of the membrane, originally derived from the endoplasmic reticulum of the lactating cell (El-Loly, 2011).

Sphingolipids are highly bioactive molecules of nutritional interest (Vesper et al., 1999). This class of lipids contain a long chain base, the so-called sphingoid base (*i.e.* a 2-aminoalk[ane or ene]1,3-diol with 2S,3R stereochemistry) that differ by chain length, number of double bonds and hydroxyl moities (Zheng et al., 2006). Sphingoid bases vary a lot among living organisms but little within a given species; for instance sphingosine (d18:1) is the principal sphingoid base in human but other species including unsaturations or other chain lengths can be found in other mammalian species. Sphingosine forms a ceramide when its amino group is linked *via* an amide bond with a fatty acid, generally saturated. Ceramide further constitutes the molecular backbone for the synthesis of other sphingolipids, including SM.

SM is a dominant PL class in mammalian milk sphingolipids and it is composed of a phosphorylcholine head group linked to the ceramide. SM is suspected to exert several biological activities when consumed from milk or co-products. SM is a very important structural component in cell membranes and notably in brain cells, which was thus suspected to promote brain health (Wehrmuller, 2008). Bovine milk SM has recently been shown to promote neurobehavioral development in premature babies after eight weeks of supplementation (Tanaka et al., 2013). SM was also described to favour gut maturation during the neonatal period in infants (Motouri et al., 2003) and is suspected to be a promoting factor of colonic mucus secreting cell multiplication after milk PL consumption in high-fat fed mice (Lecomte et al., 2015). During the digestive process, milk SM would also modulate cholesterol micellar solubility and thereby limit cholesterol absorption, possibly explaining the hypocholesterolemic effect of milk PL described in humans (Conway et al., 2013; Conway, Gauthier, & Pouliot, 2010). Milk PL were also shown to stimulate intraluminal digestive lipolysis in mice and *in vitro* (Lecomte et al., 2015). Dietary SM is hydrolyzed in the distal part of the gastro-intestinal tract under the action of alkaline sphingomyelinase or bacterial enzymes into ceramide or other bioactive metabolite (sphingosine, sphingosine-1-phosphate). Buttermilk was shown to present antiproliferative and immunomodulatory properties, which was interpreted as an effect of the products of SM digestion including ceramides (Zanabria, Tellez, Griffiths, Sharif, & Corredig, 2014).

MFGM is certainly the most diverse fraction of milk and thus various proteomic and lipidomic approaches have been applied to MFGM. However, despite numerous reviews or publications of the MFGM chemical composition, none has detailed the entire spectrum of lipid species found in this unique biological ingredient except the publication of Fong, Norris, and MacGibbon (2007). In the latter work however, the identification of bioactive lipids (sphingolipids and ceramides) is only partial. In addition there are few published characterizations of industrial buttermilks and butterserums despite their great interest as a nutritional source of bioactive lipids (Gassi et al., 2016), most works being centered on ingredients obtained at laboratory or pilot-plant scales (Dewettinck et al., 2008; Morin et al., 2007; Rombaut & Dewettinck, 2006).

The objective of the present paper is to analyze the PL species contained in industrial buttermilks and butterserums, with special emphasis on sphingolipid and ceramide molecular isoforms. The difference of bioactive constituents in the two types of co-products is discussed in the light of their manufacturing process and related to their potential application in the field of nutrition for health promotion and disease prevention.

## 2. Materials and methods

### 2.1. Materials

Unless otherwise stated, chemicals were from commercial origin (Sigma-Adrich, Saint-Quentin Fallavier, France).

Liquid industrial buttermilks (BM) and butterserums (BS) were obtained from large milk pools and were collected from several French factories. The co-products were stored at 4 °C and used as liquid within the 24 h after their collection. Sweet BM were produced at the industrial scale in a continuous way according to the NIZO process (Netherlands Dairy Research Institute, 1976). Industrial BS were obtained after melting and centrifugation of butters produced from the NIZO process. For lipid analyses, samples were freeze-dried at −20 °C during 72 h using a lyophilizer CIRP CS 10-0.8 (Serail, La Coudray Saint Germer, France). Freeze drying resulted in a powder (considered as basis for dry matter). The powder was stored at −20 °C under vacuum before analysis.

### 2.2. Fat extraction and total fatty acid characterization

Total lipids were extracted from 2 g aliquots of lyophilized butterserums and buttermilks by Folch extraction which was already used in (Gassi et al., 2016). This Folch extraction (in triplicate) was used as a basis for fat quantification in the two co-products. Extracted total lipids were stored at −20 °C until further analysis. Total fatty acid characterization was done by GC as previously described by Briard-Bion, Juaneda, Richoux, Guichard, and Lopez (2008). Briefly derivatization into methyl esters was achieved using sodium methoxide and Boron trifluoride; methyl esters were analyzed by GC Agilent 7890A (Agilent, Massy, France) equipped with a flame ionization detector and two 70% Cyanopropyl polysilphenylene-siloxane (BPX-70, SGE) capillary columns mounted in series (50 m by 0.32 mm; film thickness 0.25 μm each one). Samples were methylated and injected in duplicate.

### 2.3. Quantification of free cholesterol by GC-MS

Total lipids were extracted twice from the buttermilks and butterserums using ethanol/chloroform (1:2, v/v). The organic phases were dried under nitrogen and the different lipids classes were then separated by TLC using the solvent mixture: hexane/diethylether/acetic acid (80:20:1 v/v/v). Lipids were detected by UV after spraying with 0.2% dichlorofluorescein in ethanol and identified by comparison with standards. Silica gel was scraped off.

Cholesterol was extracted by a mixture of ethanol/chloroform (1:2, v/v). The dry residue was derivatized with BSTFA (*N, O-bis (trimethylsilyl)trifluoroacetamid)*) and then analyzed by gas chromatography coupled with an ion trap mass spectrometry (GC–MS/MS) using electron impact ionization (EI) mode (Thermo Electron, Polaris Q).

### 2.4. Analysis of classes of polar lipids by HPLC

Classes of Polar lipids (glycerophospholipids and sphingomyelin) were determined by HPLC (HP 1100, Agilent, Massy, France) fitted with an evaporative light scattering detector (ELSD) as already described in Gassi et al. (2016).

### 2.5. Sphingomyelin and ceramide profiling by electrospray ionization-tandem mass spectrometry (ESI-MS/MS)

CER an SM were extracted according to the method by Kyrklund in the presence of deuterium-labeled standards (*N*-heptadecanoyl-D-erythro-sphingosine (C17:0-Ceramide); *N*-palmitoyl(d31)-D-erythro-sphingosylphosphorylcholine (C16:0D31SM) from Avanti Polar Lipids,