Small Ruminant Research 167 (2018) 39–47

Contents lists available at ScienceDirect

Small Ruminant Research

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

Phospholipids of goat and sheep origin: Structural and functional studies

Stylianos Poutzalis[a], Ronan Lordan[b], Constantina Nasopoulou[c], Ioannis Zabetakis[b,*]

[a] Laboratory of Food Chemistry, Department of Chemistry, National and Kapodistrian University of Athens, 15771, Athens, Greece
[b] Department of Biological Sciences, University of Limerick, V94 T9PX, Limerick, Ireland
[c] Department of Food Science and Nutrition, School of the Environment, University of the Aegean, Myrina, Lemnos, 81400, Greece

ARTICLE INFO

Keywords:
Atherogenesis
Anti-atherogenic activity
Polar lipids
Phosphatidylcholine
Meat

ABSTRACT

The lipidomic profiles of goat and sheep meat were studied. Polar lipid fractions of raw and baked meat samples were tested for their *in vitro* anti-atherogenic properties. The total lipid (TL) content was extracted using the Bligh-Dyer method and was subsequently separated into total polar lipids (TPL) and total neutral lipids (TNL). The fatty acid profiles of the TPL and TNL of all three samples were determined by GC-FID. The TPL of all samples were further separated by preparative TLC into their constituent phospholipid and sphingolipid fractions. In all samples, polar lipid fraction 3 had a similar R_f value to phosphatidylcholine. These phosphatidylcholine fractions were tested for their *in vitro* capacity to inhibit platelet-activating factor (PAF) induced platelet aggregation (anti-inflammatory activity) using human platelets. The phospholipid content of each fraction 3 was determined using LC–MS. These results provide a novel insight into the structure of phosphatidylcholine derivatives in goat and sheep meat and highlight the nutritional value of these meats in terms of their antithrombotic and cardioprotective properties before and after the baking process.

1. Introduction

Foods and fats of animal origin receive undue criticism from society and scientific communities due to their perceived negative effects on health upon consumption. Recent research trends indicate that these negative perceptions may be unwarranted as numerous studies suggest that meat consumption may be associated with a positive effect on health when eaten in moderation despite their SFA and cholesterol content (Lordan et al., 2017).

Red meat is a highly nutritious and valuable source of proteins, iron, phosphorus, zinc, selenium and B group vitamins. Meat from ruminant animals, such as sheep and goat, contain *trans* fatty acids that are, unlike industrial fatty acids, beneficial for the human health (Bendsen et al., 2011; Mulvihill, 2001). The most common source of *trans* fatty acids in ruminant meat is conjugated linoleic acid (CLA), which has been linked to a protective role against cancer, cardiovascular diseases (CVD) and has been associated with weight loss (Bendsen et al., 2011; Blankson et al., 2000; Schmid et al., 2006).

According to the lipid hypothesis, which stemmed from the Seven Countries Study, saturated fatty acids (SFA), cholesterol and LDL levels are responsible for atherogenesis and consequently their presence in excessive levels can increase the risk of CVD development (Steinberg, 2006). Several studies have implicated red meat in the development of CVD due to their effect on blood lipid parameters but also the effect of additives and preservatives (Pan et al., 2012; Wolk, 2016). However, there are meta-analyses and reviews that refute these claims and they have shown that red meat consumption does not increase the risk for CVD development (Connor et al., 2016; Mcafee et al., 2010).

Recent studies that separated red meat into processed and unprocessed forms, have brought to light the fact that unprocessed red meat has a minor association or no association with increased mortality and CVD (Larsson and Orsini, 2014; Micha et al., 2012, 2010). It has also been suggested that potential bias in many of the studies investigating the consumption of meat and its effect on atherosclerosis may have influenced the conclusions (Larsson and Orsini, 2014; Micha et al., 2012).

Other studies have shown that a diet containing meat and cheese can lead to increased levels of HDL cholesterol and thus, it appears to be less atherogenic than a low-fat, high-carbohydrate diet (National Obesity Forum and Public Health Collaboration, 2016). There is considerable concern that red meat consumption elevates levels of choline and L-carnitine. Phosphatidylcholine (PC) is broken down to choline, which is transformed by the intestinal microbiota to trimethylamine (TMA), which along with L-carnitine is metabolised to trimethylamine N-oxide (TMAO) (Wang et al., 2011). It is proposed that excess dietary PC increases the levels of TMAO resulting in a pro-inflammatory and

* Corresponding author at: Department of Biological Sciences, University of Limerick, Limerick, Ireland.
E-mail address: ioannis.zabetakis@ul.ie (I. Zabetakis).

https://doi.org/10.1016/j.smallrumres.2018.07.015
Received 23 November 2017; Received in revised form 29 June 2018; Accepted 20 July 2018
Available online 30 July 2018
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prothrombotic state that may lead to eventual insulin resistance, type II diabetes mellitus, and CVD (Lordan et al., 2017; Zhu et al., 2016). However, some results indicate that dietary choline may not be to blame, and that the presence of specific gut bacteria promotes the conversion of choline into TMAO (Clouatre and Bell, 2013; Koeth et al., 2013). Other researchers suggested that dietary choline from PC derivatives in dairy and marine sources possess antithrombotic properties, contrary to the effects of TMAO (Lordan et al., 2017; Lordan and Zabetakis, 2017a).

With regards to atherogenesis, platelet-activating factor (PAF) is a crucial potent inflammatory phospholipid mediator (Demopoulos et al., 1979), which is produced by many cells and is involved in the activation of leukocytes and their binding to endothelial cells (Demopoulos et al., 2003; Lordan and Zabetakis, 2017b). CVD such as coronary heart disease and stroke are clinical events as a consequence of atherosclerosis, which is a chronic inflammatory condition mediated by PAF and other molecules (Nasopoulou et al., 2013c)

Our previous work has shown that polar lipids are strong inhibitors of the inflammatory and atherogenic actions of PAF (Megson et al., 2016; Zabetakis, 2013). Recently, polar lipids from sheep and goat dairy products have been evaluated for their inhibitory activities against PAF (Lordan and Zabetakis, 2017b; Megalemou et al., 2017; Poutzalis et al., 2016; Tsorotioti et al., 2014); the most potent inhibitory properties were exhibited by TLC polar lipid fractions with R_f similar to that of PC (Megalemou et al., 2017; Poutzalis et al., 2016). However, little is known about the bioactivity of phospholipids present in sheep or goat meat.

Muscles from mammals are excellent sources of phospholipids (Christie, 1978) and especially of PC which often constitutes to almost 50% of the total phospholipids (Perez-Palacios et al., 2007; Sinanoglou et al., 2013). In lamb, the phospholipid content can constitute 42% of the total lipids, with PC and phosphatidylethanolamine being the predominant lipid species (38–55% and 25–31% respectively) (Lordan et al., 2017). Lipid microconstituents that exert *in vitro* anti-PAF activities could retard and/or regress atherosclerosis and consequently CVD, as several *in vivo* studies have demonstrated (Karantonis et al., 2006; Nasopoulou et al., 2010; Tsantila et al., 2010, 2007).

The aim of our present work was to explore the lipidomic profiles of raw and baked goat and sheep meat and to evaluate the anti-atherogenic properties of PC derivatives obtained from these food sources. Our data suggests that goat and sheep meat possess potential functional cardioprotective lipid microconstituents that warrant further research, which assist in re-assessing the nutritional value of these meats.

2. Materials and methods

2.1. Reagents and instruments

All reagents and solvents were of analytical grade purchased from Merck (Darmstadt, Germany). Fatty acid methyl ester standards were of GC-quality and supplied by Sigma-Aldrich (St. Louis, MO, USA), as well as bovine serum albumin (BSA) and PAF.

The Chromatographic material used for thin-layer chromatography (TLC) was silica gel G-60 supplied by Merck (Darmstadt, Germany) and the polar lipid standards used for TLC were supplied by Sigma-Aldrich as a standard mix of hen egg yolk (St. Louis, MO, USA). Gas chromatographic analysis was carried out on a Shimadzu CLASS-VP (GC-17 A) (Kyoto, Japan) gas chromatograph equipped with a split/splitless injector and flame ionization detector.

Liquid chromatography-mass spectrometry (LC–MS) was carried out using a Thermo Exactive Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK), equipped with a heated electrospray ionization (HESI) probe and coupled to a Thermo Accela 1250 UHPLC system. Platelet aggregation was measured on a Chrono-Log (Havertown, PA, USA) aggregometer (model 400-VS) coupled to a Chrono-Log recorder (Havertown, PA, USA).

2.2. Meat samples

Meat samples were purchased from a local market in Athens. Both the sheep and the goat were male and were 6–10 weeks of age. The sheep originated from Amphilochia region in central Greece, whereas the goat originated from Lakonia region in southern Greece. The meat samples used were untrimmed rib steaks, as typically consumed in Greece. Initially, the bone was removed. The baked meat samples were prepared as follows: the meat was cut into $2 \times 2 \times 1$ cm^3 pieces, wrapped in aluminium foil, and cooked in a preheated oven at 180 °C for 20 min. The raw and baked meat samples used for analysis were further chopped into as small as possible pieces using a knife. The remaining meat juices left after the baking process were discarded. Four groups of meat samples have been generated, namely goat raw (GR), goat baked (GB), sheep raw (SR) and sheep baked (SB) and these meat sample codes are used throughout the manuscript.

2.3. Isolation of lipids

Extraction of the total lipids (TL) from each 100 g sample (kid and lamb, raw or baked) was carried out using the Bligh and Dyer method (Bligh and Dyer, 1959). The TL were weighed and one fourth of the sample was stored in sealed vials under a nitrogen atmosphere at -20 °C, while the rest of the TL was further separated by counter-current distribution (Galanos and Kapoulas, 1962) into total polar lipids (TPL) and total neutral lipids (TNL). In brief, petroleum ether and 87% aqueous ethanol were pre-equilibrated in a separatory funnel. The lower phase containing equilibrated 87% ethanol and the upper phase containing equilibrated petroleum ether were collected separately. Approximately 2 g of TL were dissolved in 9 mL pre-equilibrated petroleum ether and afterwards 3 mL pre-equilibrated 87% ethanol were added and stirred. The lower phase (ethanolic phase) was collected and transferred to a second test tube containing 9 mL of pre-equilibrated petroleum ether and was stirred again. The ethanolic phase in the second test tube was transferred to a round-bottom flask. The procedure was repeated eight times in total. Finally, the ethanolic phase (8×3 mL), containing the TPL, and the petroleum ether phase (2×9 mL), containing TNL, were evaporated to dryness, weighed and stored under nitrogen in sealed vials at −20 °C. Each extraction was carried out in triplicate.

2.4. Fractionation of TPL by preparative TLC

The TPL from all samples were further separated by preparative TLC. The TLC glass plates (20×20 cm, thickness 1.0 mm) were coated with silica gel G-60 and activated by heating at 120 °C for 60 min. Approximately 40 mg of TPL was applied to the TLC plates. The developing system consisted of chloroform/methanol/water 65:35:6 (v/v/v). After development, the plates were stained under iodine vapors. Eight bands appeared after the separation of TPL of the samples. Subsequently, the bands were scraped off, and the lipids from the desirable bands were extracted according to the Bligh-Dyer method (Bligh and Dyer, 1959). The chloroform phase was evaporated to dryness under nitrogen, and lipids were weighed and stored at −20 °C.

2.5. Gas chromatographic analysis

Fatty acid methyl esters (FAME) were prepared from 35 mg of TPL and 35 mg of TNL of all four samples (GR, GB, SR and SB) using a solution of 0.5 N KOH in CH_3OH (KOH-CH_3OH method with reaction time 5 min) and extracted with n-hexane. Analysis was carried out on a Shimadzu CLASS-VP (GC-17 A) (Kyoto, Japan) gas chromatograph equipped with a split/splitless injector and flame ionization detector. Separation of FAMEs was achieved using an Agilent J&W DB-23 fused silica capillary column (60 m × 0.251 mm i.d., 0.25 μm; Agilent, Santa Clara, CA, USA). The oven temperature was initially set at 120 °C for 5 min, raised to 180 °C at 10 °C min^{-1}, then to 220 °C at 20 °C min^{-1},

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