Yoghurt fermentation alters the composition and antiplatelet properties of milk polar lipids

Ronan Lordan[a,b,c,*], Natalia P. Vidal[c], Thu Huong Pham[c], Alexandros Tsoupras[a,b], Raymond H. Thomas[c,*], Ioannis Zabetakis[a,b]

[a] Department of Biological Sciences, University of Limerick, Limerick, Ireland
[b] Health Research Institute (HRI), University of Limerick, Limerick, Ireland
[c] School of Science and the Environment/ Boreal Ecosystem Research Initiative, Grenfell Campus, Memorial University of Newfoundland, Corner Brook A2H 5G4, Canada

ABSTRACT

Dairy polar lipids (PL) seem to exhibit antiplatelet effects. However, it is not known what molecular species may be responsible. In this study, we confirmed using C30 reversed-phase (C30RP) ultra-high-performance liquid chromatography (UHPLC) coupled to high resolution accurate mass tandem mass spectrometry (HRAM-MS/MS) that fermentation of yoghurts from ovine milk using specific starter cultures altered the PL composition. These lipid alterations occurred concomitant with increased antithrombotic properties of the yoghurts PL fractions against platelet-activating factor (PAF) and thrombin-induced platelet aggregation. Specifically, elevation in phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylcholine (PC) and their molecular species were observed following yoghurt fermentation. Furthermore, PC(18:0/18:1), PE(18:1/18:2), SM(d18:0/22:0) and several other molecular species were significantly inversely correlated with the inhibition of PAF and thrombin. These molecular species were abundant in the most bioactive yoghurts fermented by *S. thermophilus* and *L. acidophilus*, which suggest that fermentation by these microorganisms increases the antithrombotic properties of ovine milk PL.

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of global mortality. Systemic inflammation is a key biochemical process implicated in the development of chronic diseases such as cancer and CVD (Moss, Williams, & Ramji, 2018), whereby platelets are integral for the protection of the vascular system (Koupenova, Clancy, Corkrey, & Freedman, 2018). However, during unresolved systemic inflammation, activated platelets can induce a myriad of pro-inflammatory events that contribute to the initiation and progression of atherosclerosis (Koupenova, Clancy, Corkrey, & Freedman, 2018). Considering platelet hyperactivity is a risk factor for CVD, it is imperative to develop therapeutic functional foods with antiplatelet properties. Platelet-activating factor (PAF) is a potent phospholipid pro-inflammatory mediator implicated in the development of atherosclerosis (Tsoupras, Lordan, & Zabetakis, 2018). PAF and PAF-like lipids (PAFLL) are structurally homologous species that exhibit similar biological activity by binding to the PAF-receptor (PAF-R), which is a seven transmembrane G-protein-coupled receptor (GPCR). However, PAFLL tend to be less potent than PAF and or they can act as PAF inhibitors (Tsoupras, Lordan, & Zabetakis, 2018).

Fermented dairy products may be cardioprotective with respect to reducing CVD risks (Astrup, Geiker, & Magkos, 2019). Yoghurt consumption is associated with the prevention of obesity, metabolic syndrome, type II diabetes mellitus, and CVD (Lordan, Tsoupras, Mitra, & Zabetakis, 2018; Wu & Sun, 2017). However, not all the cardioprotective mechanisms associated with dairy consumption are fully understood (Da Silva & Rudkowska, 2015). Ovine milk and dairy products are a key component of the diet of populations surrounding the Mediterranean basin, regions around the Black Sea, the Indian subcontinent, and Africa, where they provide essential nutrients for people with low incomes. Ovine milk is a rich source of lipids that are thought to be bioactive by exerting anti-inflammatory and antithrombotic effects (Megalemou et al., 2017), including the bioactive phospholipid and sphingolipid fractions collectively known as polar lipids (PL) (Lordan & Zabetakis, 2017). Milk polar lipids have been associated with several cardiovascular benefits (Anto, Warykas, Torres-Gonzalez, & Blesso, 2020; Millar et al., 2020). In humans, milk PLs have been associated with a reduction of cardiovascular disease risk factors in overweight postmenopausal women (Vors et al., 2019).

* Corresponding authors at: Department of Biological Sciences, University of Limerick, Limerick, Ireland.
  E-mail addresses: Ronan.Lordan@ul.ie (R. Lordan), rthomas@grenfell.mun.ca (R.H. Thomas).

It was recently reported that various bacterial cultures can alter the fatty acid composition of PL in ovine milk when it was fermented to yoghurt, and that these changes led to an increase in the inhibitory effects of the PL against platelet-activating factor (PAF)-induced platelet aggregation (Lordan, Walsh, Crispie, Finnegan, Cotter, & Zabetakis, 2019), which is a measure of the antithrombotic and potentially the anti-inflammatory effects of the PL. However, this study had several limitations as the main PL constituents responsible for the activity were not reported. Similar anti-PAF PL have been reported in cheeses produced from caprine milk fermented with different lactic acid bacteria (LAB) (Lordan, Walsh, Crispie, Finnegan, Demuru, Tsoupras, and Zabetakis et al., 2019). Therefore, PL-rich ovine dairy products are putative candidates for the development of cardioprotective functional foods (Balthazar et al., 2017).

While these previous studies focused on the PAF pathways of platelets, it has recently been shown that PL also affect the thrombin pathways (Tsoupras, Lordan, Demuru et al., 2018). However, little is known about the effect of dairy PL on the activation of the thrombin platelet receptors (PAR-1 and PAR-4) or similar GPCR receptor like that of adenosine diphosphate (ADP) receptors $P2Y_1$ and $P2Y_{12}$. Therefore, the aim of this study is to determine the change of polar lipid composition after the fermentation of ovine milk to yoghurts produced using different starter cultures and to analyse their antiplatelet activity against PAF, ADP, and thrombin. Finally, C30RP-UHPLC-HRAM-MS/MS and multivariate statistical analysis were used to determine the composition of the PL extracts of the ovine dairy products and to assess if there were structure/function relationships between the PL and their antithrombotic properties. Based on previously published research, it is hypothesised that there will be significant differences between the PL compositions of the milk and yoghurts, which will lead to differences in the antithrombotic properties of the PL extracts.

2. Materials and methods

2.1. Materials & chemicals

All consumables and HPLC-grade solvents: chloroform, methanol, and acetonitrile were obtained from Fisher Scientific Ltd. (Ottawa, ON, Canada). LC–MS-grade formic acid, acetic acid, and ammonium acetate were purchased from Sigma-Aldrich (ON, Canada). For solution preparation, deionised water (PURELAB Purification System, ELGA Labwater, ON, Canada) was used. Individual external lipid standards using SPLASH mix of deuterated standards were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA), the composition of which is outlined in the Supplementary material (Table S-2).

The 20G safety needles and evacuated sodium citrate S-monovettes were purchased from Sarstedt Ltd. (Wexford, Ireland). The platelet aggregation bioassay was carried out on a Chronolog-490 two channel turbidimetric platelet aggregometer (Havertown, PA, USA), coupled to the accompanying AGGRO/LINK software package. All platelet aggregation consumables were purchased from Labmedics LLP (Abingdon on Thames, UK). Standard thrombin, saline, and BSA were purchased from Sigma Aldrich (Wicklow, Ireland), while collagen and ADP were obtained from CHRONOLOG, Havertown, PA, USA. Centrifugations were carried out on an Eppendorf 5702R centrifuge (Eppendorf Ltd Stevenage UK). Spectrophotometric analysis was carried out on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). The bacterial cultures used in this study were obtained in freeze dried form and turned into mother culture solutions as outlined in Table S-1 of the Supplementary materials. The cultures were kindly provided by Chr. Hansen (Cork, Ireland).

2.2. Ovine milk and yoghurt polar lipid extracts

The PL extracts used in this study were obtained from a previously published study (Lordan, Walsh, Crispie, Finnegan, Cotter, & Zabetakis, 2019). In brief, whole milk was obtained from a bulk tank containing milk from the Friesland and Lacaune breed of dairy ewe between March and July in 2016. The sheep were fed a forage-based diet consisting of grass silage or fresh grass, supplemented with cereal at the time of milking at Rockfield Dairy Ltd. (Claremorris, Co. Mayo, Ireland). The milk was pasteurised at 91 °C for 15 s and was cooled to 42 °C before being homogenised, packaged, refrigerated at 4 °C, and transferred to the laboratory. The milk was placed in sterilised conical flasks and heated to 42 °C in a water bath (Grant JB NV, Cambridgeshire, UK) before specific concentrations of starter cultures were used to inoculate the milk as per Table S-1 (Supplementary material). The flasks were held at 42 °C throughout the yoghurt fermentation process. When inoculated the milk was stirred thoroughly and the pH monitored. The fermentation was stopped by cooling to 4 °C once the yoghurts reached a pH between 4.4 and 4.6. The yoghurts were transferred to sterile media bottles and stored at − 20 °C until required for analysis. The final microbial composition of the yoghurts as previously determined by Lordan, Walsh, Crispie, Finnegan, Cotter, and Zabetakis (2019) is presented in Table S-1. The milk, the control yoghurt (yoghurt A in this study), and the yoghurt deemed to be most bioactive in the previous study (yoghurt B in this study) were then extracted using the modified Bligh and Dyer method and the counter-current distribution as previously described (Lordan, Walsh, Crispie, Finnegan, Cotter, & Zabetakis, 2019). This method of polar lipid extraction is favoured over other methods as it does not require heat or harsh treatments that may affect the lipid structure or biological activities of the polar lipids. All lipid extracts were stored in the absence of any solvent under a nitrogen atmosphere in sealed vials at − 20 °C. The mean extraction results and PL yields have previously been reported by Lordan, Walsh, Crispie, Finnegan, Cotter, and Zabetakis (2019).

2.3. Analysis of polar lipid extracts using C30-RPLC-HRMS/MS

The milk and yoghurt PL extracts were analysed using C30RP-UHPLC-HRAM-MS/MS in quadruplicate as previously described (Pham et al., 2019). In brief, the C30RP-UHPLC was carried out using an Accucore C30 column (150 × 2 mm I.D., particle size: 2.6 μm, pore diameter 150 Å, (Thermo Fisher Scientific Ltd., ON, Canada). The mobile phase system comprised of solvent A (acetonitrile: water, 60:40 v/v) and solvent B (isopropanol: acetonitrile: water, 90:10:1 v/v/v), which both contained 10 mM ammonium formate and 0.1% formic acid. The column oven temperature was set at 30 °C with a flow rate of 0.2 mL/min, and 5 μL of the lipid extract suspended in chloroform: methanol: isopropyl alcohol (2:2:1 v/v/v) was injected onto the column. The following system gradient was employed to separate the lipid classes and molecular species: 30% solvent B for 3 min, 43% solvent B for 5 min, 50% solvent B for 1 min, 90% solvent B for 9 min, then to 99% B for 8 min, and finally kept at 99% B for 4 min. The column was then re-equilibrated to starting conditions (70% solvent A) for 5 min prior to each new injection.

The lipids were analysed using a Q-Exactive Orbitrap mass spectrometer controlled by X-Calibur software 4.0 (Thermo Fisher Scientific Ltd., MO, USA) with an automated Dionex UltiMate 3000 UHPLC system controlled by Chromeleon software. The Q-Exactive mass spectrometer was operated using the following conditions - sheath gas: 40, auxiliary gas: 2, ion spray voltage: 3.2 kV, capillary temperature: 300 °C; S-lens RF: 30 V; mass range: 200–2000 m/z; full scan mode at a resolution of 70,000 m/z; automatic gain control target: le5. The instrument was externally calibrated to 1 ppm using ESI positive and negative calibration solutions (Thermo Fisher Scientific Ltd., MO, USA). Tuning parameters were optimised using a mixture of lipid standards obtained from Avanti Polar Lipids Inc. (AL, USA). Data was acquired and processed using X-Calibur 4.0. (ThermoScientific, MO, USA) and LipidSearch version 4.1 (Mitsui Knowledge Industry, Tokyo, Japan) software packages. LipidSearch was used for the identification and semi-quantification of the polar lipid classes and their molecular

species. The LipidSearch parameters were as previously reported (Pham et al., 2019). Briefly, there were as follows: Target database: Q-Exactive; precursor tolerance: 5 ppm; product tolerance: 5 ppm; product ion threshold: 5%; m-score threshold: 2; Quan *m/z* tolerance: ± 5 ppm; Quan RT (retention time) range: ± 1 min; use of all isomer filter and ID quality filters A, B, and C; Adduct ions: $[M+H]^+$ and $[M+NH^4]^+$ for positive ion mode, and $[M-H]^-$, $[M + HCOO]^-$, and $[M-2H]^{2-}$ for negative ion mode. Fatty acyl chains position identification present in the molecular species found in the lipid classes of each sample was based on the fragmentation patterns of the MS/MS spectra, and manually confirmed using X-Calibur 4.0. The relative amount of each molecular species identified was calculated from the normalised area counts and the values expressed as nanomole percent.

2.4. Platelet aggregometry assay for ADP, PAF, and thrombin

Blood withdrawal and preparation of human platelet-rich plasma (hPRP) was conducted as previously described (Lordan, Walsh, Crispie, Finnegan, Cotter, & Zabetakis, 2019; Tsoupras, Zabetakis, & Lordan, 2019). The study was approved by the Ethics Committee of the University of Limerick, which was performed in accordance with the Declaration of Helsinki. Healthy donors provided written consent and were free from any antiplatelet therapies. Briefly, blood samples were collected from six donors (n = 6) by a trained phlebotomist using sodium citrate anticoagulant (0.106 mol/L in a 1:10 ratio of citrate to blood; Sarstedt Ltd., Wexford, Ireland) and were centrifuged at $194 \times g$ for 18 min at 24 °C with no brake applied. The supernatant hPRP was then transferred to polypropylene tubes at room temperature for the aggregation bioassays, whereas platelet-poor plasma (PPP) was obtained by further centrifuging the remainder of the blood at $1465 \times g$ for 20 min at 24 °C with no brake applied. hPRP was adjusted to 500,000 platelets/μL if required by addition of the respective volume of PPP according to the absorbance of the hPRP measured using spectrophotometer at 530 nm (Shimadzu UV-1800, Kyoto, Japan). The examined PL extracts were dissolved in BSA (2.5 mg BSA/mL saline) with standard stock solutions of PAF also being dissolved in BSA. Standard stock solutions of active thrombin (0.1 U/μL) and ADP (1 mM) were dissolved in saline prior testing.

The 250 μL of PRP was added to an aggregometer cuvette at 37 °C with stirring at 1000 rpm. The PRP was calibrated using the PPP as a blank. The maximum-reversible platelet aggregation curve induced by ADP, PAF, or thrombin was determined as 100% aggregation. This was used as a baseline (0% inhibition) value in the absence of any lipid-sample. This value was determined by adding the appropriate amount of each platelet agonist to the aggregometer cuvette, in order to reach specific final concentrations. These concentrations were approximately 0.01–0.4 U/mL, for thrombin 2.6×10^{-10} to 2.6×10^{-12} M for PAF, and at 2–10 μM for ADP.

The ADP-, PAF-, and thrombin-induced aggregation of hPRP was calculated first at 0% inhibition of baseline in a cuvette (100% aggregation) in the absence of any lipid sample, whereas after the pre-incubation of hPRP with several amounts (μg) of the lipid samples in a different cuvette for 2 min, the same amount of ADP, PAF or thrombin that induced maximum-reversible platelet aggregation was added and the reduced aggregation was calculated. Thus, a linear curve at the 20–80% range of the percentage of inhibition against ADP-, PAF-, and thrombin-induced aggregation of hPRP was deduced for each sample. From this curve, the concentration (μg) of the lipid sample that led to 50% of agonist-induced aggregation of hPRP was calculated as the 50% inhibitory concentration value also known as the IC_{50} value (half-maximal inhibitory concentration) for each PL sample. The resulting IC_{50} values were expressed as a mean value of the mass of lipid (μg) in the aggregometer cuvette ± standard deviation (SD). All experiments were performed in sextuplicate (n = 6) using a different donors blood sample for each replicate to ensure reproducibility.

2.5. Statistical analysis

The Kruskal-Wallis test was used to determine significant statistical differences between the extracts tested in the aggregometry experiments (XLSTAT, Premium, version 2019.3.1, Addinsoft, New York, USA). Statistical analysis of the lipidomic data was carried out using XLSTAT, whereby principal component analysis (PCA), heatmaps, and bar charts depicting relative abundance of each PL species and the ANOVA were plotted using variable importance of projection (VIP) > 1 following partial least square discriminant analysis (PLS-DA). ANOVA was conducted using Fisher's least significant difference (LSD) posthoc test ($p < 0.05$ was deemed statistically significant) to assess the differences in the lipidomic profiles of the milk and yoghurts. All heatmaps were conducted using a hierarchical clustering based on Euclidian distance at 0.15 interquartile range. PCA and Pearson correlation coefficients were used to discern if the biological activity correlated with the PL species present (XLSTAT). Chemical structures were generated using ChemDraw (Ultra 7.0, PerkinElmer Inc., Waltham, MA, USA), chromatograms by X-Calibur software 4.0 (Thermo Fisher Scientific Ltd., MO, USA), and Fig. 5 was generated using BioRender (Toronto, ON, Canada).

3. Results and discussion

3.1. Composition of the ovine milk and yoghurt polar lipids

We hypothesised that there would be significant changes to the milk phospholipidome following fermentation to yoghurt. Using C30RP-UHPLC-HRAM-MS/MS it is now confirmed that the PL composition of the sheep milk when fermented to yoghurt did significantly alter the overall PL composition and the molecular species of the various PL groups, namely PC, PE, PG, PS, PI, and SM. This is in accordance with recent research concerning bovine milk, yoghurt, and cheese (Furse, Torres, & Koulman, 2019). An example of a chromatogram obtained using C30-RPLC-HRMS/MS in the negative ion mode is presented in Fig. 1A. Fig. 1B-D are representative mass spectra of some of the main PL species identified in the milk and yoghurt extracts. The base peaks of fatty acids were typically generated from glycerophospholipids confirming their fatty acyl composition, e.g. *m/z* 281.24 and 283.26 in Fig. 1B, C representing C18:1 and C18:0 fatty acyl anion produced from PC(18:0/18:1), PE(18:0/18:1), respectively. The base peak of *m/z* 168.04 in Fig. 1D was distinguishable for the phosphocholine polar headgroup and the presence of sphingosine backbone in SM(d16:0/18:1). The analysis determined that PE (46–52%) was the most abundant PL fraction detected in the milk and yoghurts, followed by SM (19–26%), PC (16–17%), PS (10–12%), PI (~1%), and PG (< 1%) (Fig. 1E). While the SM and PS composition of the ovine milk was generally in accordance with the literature, the relative abundance of PE and PC was found to be higher in the milk used in this study in comparison to the literature (Zancada et al., 2013). These differences may be due to multiple reasons such as the time of year the milk was taken, the stage of lactation, the breed of the sheep, and their diets; all of which tend to have a significant impact on the composition of the milk of various ruminant animals (Balthazar et al., 2017). It is clear from Fig. 1E that there are differences in the total relative percentage of the PL groups between the milk and yoghurts and within the yoghurts themselves, indicating that the fermentation of milk to yoghurt and the starter cultures used considerably altered the PL composition. Notably, PE was significantly higher ($p < 0.05$) in the ovine milk than in yoghurt A. Juxtaposed, the percentage of SM tends to significantly increase in the yoghurts, whereas a 1% percent increase in the relative abundance of PC was observed as a function of the different starter cultures used during fermentation.

To determine whether there were differences in the PL subclasses at the fatty acyl chains level, the molecular species of each PL class were also evaluated. As aforementioned, PE was the most abundant PL
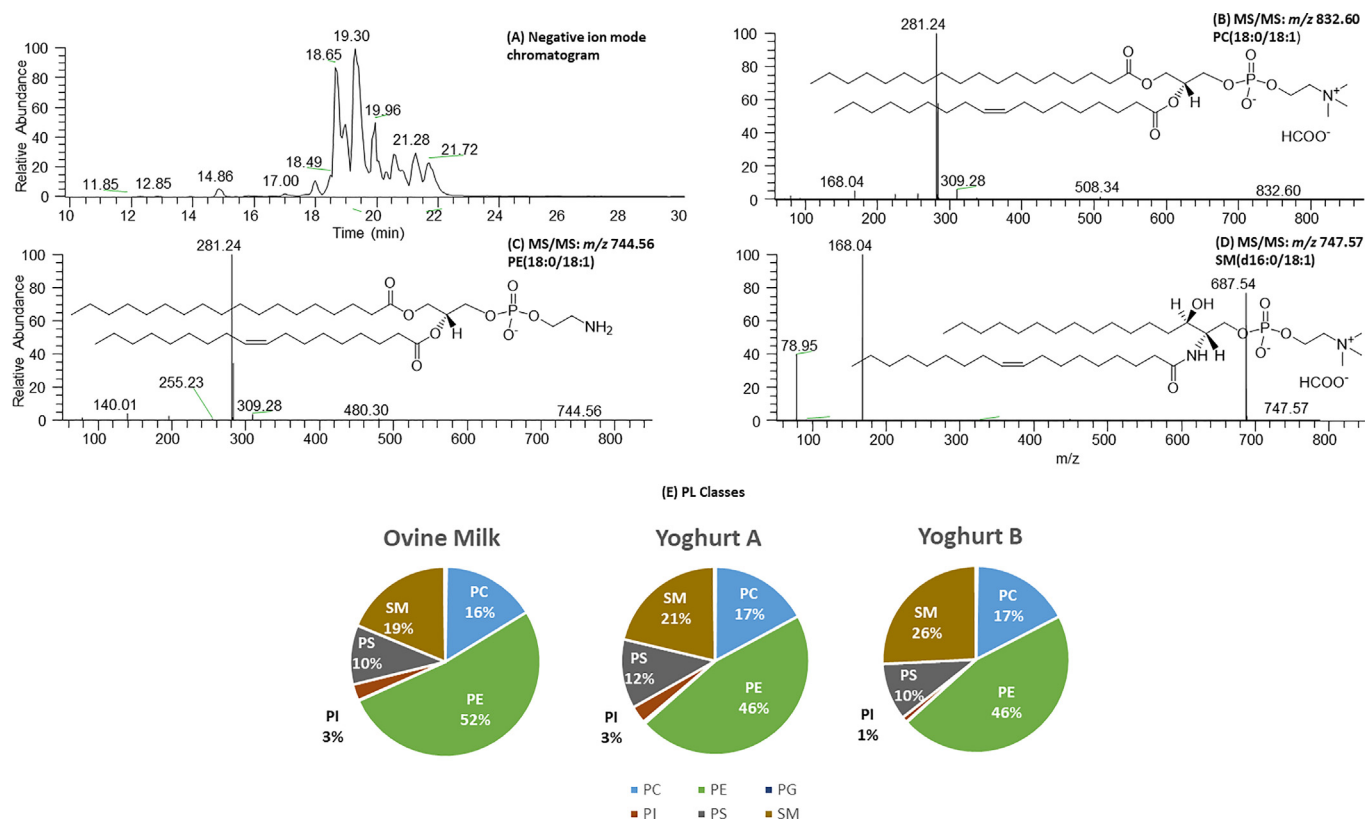
Fig. 1. (A) Chromatogram showing the of the C30RP-UHPLC-HRAM-MS/MS separation of the PL extracts in the negative ion mode. (B, C, and D) Representative LC-MS/MS mass spectra showing the fragmentation patterns of (B) PC(18:0/18:1), (C) PE(18:0/18:1), and (D) SM(16:0/18:1), which are all examples of molecular species that were identified in the three most abundant PL classes present in the milk and yoghurt. (E) The pie charts depict the relative abundance of the ovine milk, yoghurt A, and yoghurt B polar lipid (PL) extracts expressed as a nmol percentage of the total extracts. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PL, polar lipids; SM, sphingomyelin.
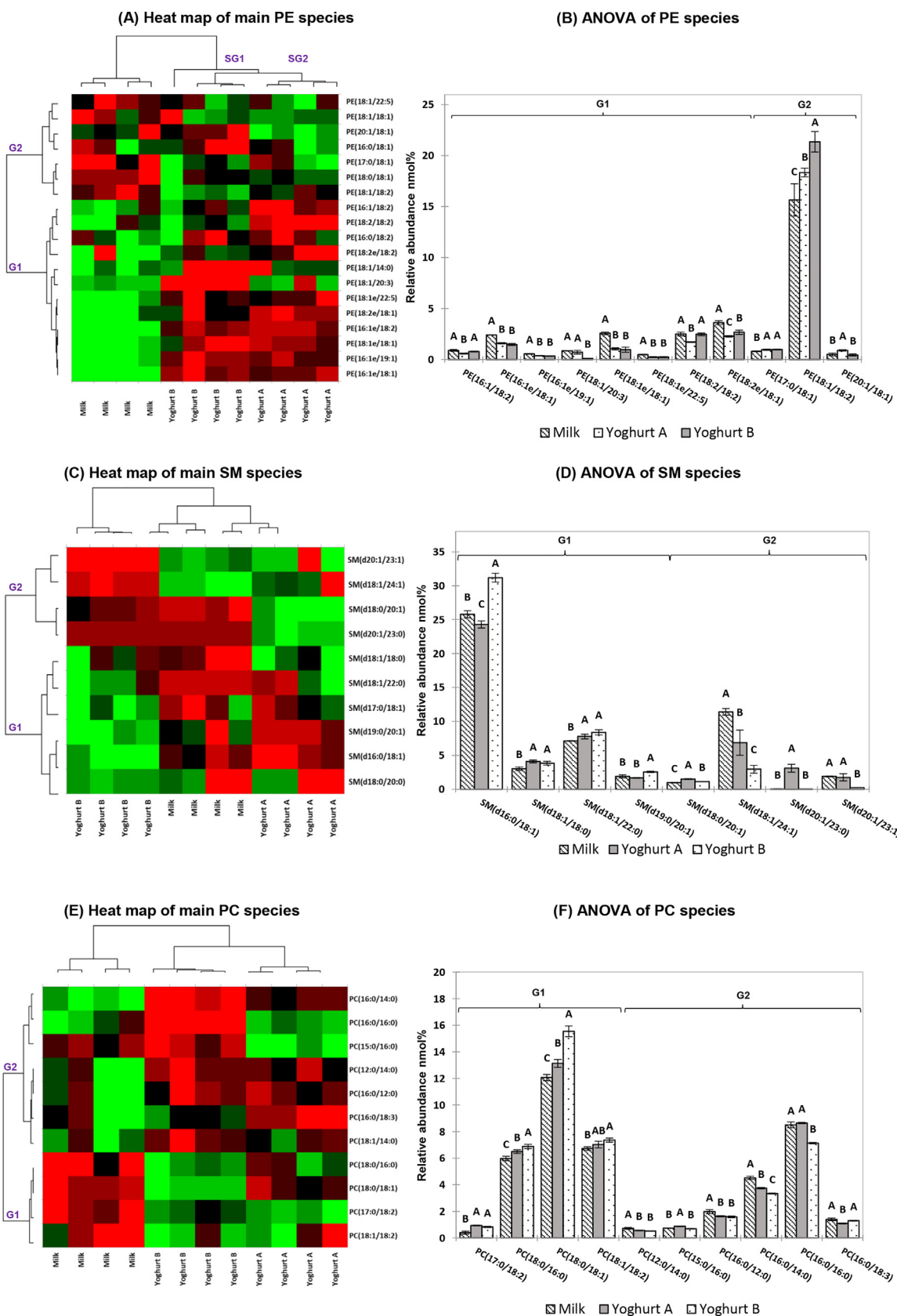
detected in the milk and yoghurts. The heatmap in Fig. 2A., demonstrates that the molecular species of the yoghurt PE cluster together, but separately from the milk PE, indicating that the yoghurt PE species are different from the ovine milk PE species. This demonstrates that fermentation alters the composition of the PE molecular species. The heatmap clusters the PE molecular species into two general groups, G1 and G2, which distinguish the milk from the yoghurt PE molecular species. In particular, there is a statistically significant higher level of ether linked PE molecular species (G1), where the relative abundance (nmol%) of PE(16:1e/18:1), PE(16:1e/19:1), PE(18:1e/18:1), PE(16:1e/22:5), and PE(18:2e/18:1) are significantly higher ($p < 0.05$) in the milk than yoghurts A or B. The heatmap (G2) also shows that there is a higher concentration of unsaturated PE species that lack an ether bond in the yoghurts in contrast to the milk. In particular PE(17:0/18:1) and PE(18:1/18:2) are in higher abundance in the yoghurts. This is corroborated by Fig. 2B., that shows that there is a statistically significant difference in the concentration of these PE species between the milk and yoghurts. There are also subtle differences in the concentration of certain PE molecular species as demonstrated by the clustering of the two yoghurts separately from each other as denoted by subgroup 1 and 2 (SG1 and SG2) in Fig. 2B. Thus, fermentation specifically alters the content of PE molecular species, particularly by significantly reducing the relative abundance of PE species with ether bonds.

SM was the second most abundant PL detected in the milk and yoghurt TPL extracts (19–26%). The heat map in Fig. 2C. demonstrates that yoghurt B segregated from yoghurt A and the milk, which also clusters independently from each as indicated by the hierarchical clustering based on a Euclidian distance set at an interquartile range of 0.15. In particular, yoghurt B is separated from the milk and yoghurt A

by a higher relative abundance of SM species that generally consist of a carbon chain length of 40 carbons or lower and generally contain 1 double bond. Indeed, yoghurt B had a statistically significantly higher concentration of SM(d16:0/18:1) and SM(d19:0/20:1) as confirmed in Fig. 2D. Conversely, the milk and yoghurt A share a statistically significantly ($p < 0.05$) higher abundance of SM(d18:1/24:1). In addition, yoghurt A is separated from the milk and yoghurt B by a statistically significantly higher abundance ($p < 0.05$) of SM(d20:1/23:0) as depicted in Fig. 2C and 2D. Indeed, the yoghurts are also segregated by a lower relative abundance of SM(d20:1/23:1) in yoghurt B in contrast to yoghurt A and the milk.

The relative abundance of PC in the milk and yoghurts was between 16 and 17% of the total PL composition, making it the third most prominent group of PL. The PC heat map (Fig. 2E) indicates that the yoghurts clustered together, but separately from the milk by higher concentration of unsaturated PC molecular species (G1), particularly PC(17:0/18:2), PC(18:0/16:0), PC(18:1/18:2), and PC(18:0/18:1). Conversely, the milk segregates distinctly from the yoghurts by having a significantly ($p < 0.05$) higher abundance of saturated PC species (G2), including PC(12:0/14:0), PC(16:0/12:0), and PC(16:0/14:0). Yoghurt A and B are also mainly segregated from each other due to a significantly higher concentration of saturated PC(16:0/16:0) and PC(15:0/16:0) molecular species in yoghurt A, and a higher concentration of PC(18:0/16:0) in yoghurt B (Fig. 2E and 2F) amongst other species. In contrast to the composition of the PE, there were no ether lipids detected in either the milk or yoghurt PC molecular species.

PS was the fourth most abundant PL detected in the milk and yoghurt TPL extracts (10–12%). The PS heatmap (Fig. 3A.) demonstrates that the milk and yoghurts differ from each other based on two groups of PS species. G1 demonstrates that yoghurt B is significantly different

(A) Heat map of main PE species

(B) ANOVA of PE species

(C) Heat map of main SM species

(D) ANOVA of SM species

(E) Heat map of main PC species

(F) ANOVA of PC species

(caption on next page)

Fig. 2. Heat maps (2A, 2C, and 2E) demonstrating the variation between the most abundant PL lipid (PE, SM, and PC) molecular species present in ovine milk and yoghurts A and B. The various dairy sources and PL classes were clustered independently using ascendant hierarchical clustering based on Euclidian distance at 0.15 interquartile range. The left columns represent the segregation of the PL species, whereas the columns above segregate the milk and yoghurts based on similarities in abundance. Red, black, and green colours denote lower, intermediate, and higher abundance of the PL species. Group 1 and 2 (G1 and G2) and subgroups (SG1 and SG2) are the PL species that were responsible for the resulting clustered patterns in the heat maps that were used to determine statistical significance between the milk and yoghurt PL species in each of the bar charts (2B, 2D, and 2F) coupled to the heat maps. These bar charts report the relative abundance of the various molecular species of each lipid class as a mean nmol% $\pm$ SE (n = 4). Statistically significant differences between the lipid species are denoted by the letter A-C above the error bars as determined by Fisher's LSD multiple comparisons test following ANOVA (α = 0.05). Group 1 and 2 (G1 and G2) and subgroups (SG1 and SG2) are the PL species that were responsible for the resulting clustered patterns in the heat maps that were used to determine statistical significance between the milk and yoghurt PL species as depicted in the bar charts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from the milk by the higher relative abundance of PS(18:0/21:0) and PS(18:1/21:0), where yoghurt A is different from the milk only by the higher relative abundance of PS(18:0/21:0) (Fig. 3A and B). On the other hand, G2 demonstrates that milk and yoghurt A has a statistically significantly higher relative abundance of PS(18:0/18:1), PS(18:0/22:5), PS(18:1/18:1), and PS(18:1/18:2) in contrast to yoghurt B. Therefore, it is clear that yoghurt B has a significantly different PS composition in contrast to the milk or yoghurt A.

The relative abundance of PI in the milk and yoghurts was 1–3%, while this is considerably low, it is clear from the heat map that the milk and yoghurts segregates independently from each other (Fig. 3C). In general, the PI molecular species were broadly distinguished by G1 and G2. The PI species in G2 are present in abundance in the milk, in less abundance in yoghurt A, and in low abundance in yoghurt B. In particular, the relative abundance of PI(18:1/18:1) and PI(16:0/18:1) is statistically significantly higher in the milk and yoghurt A, than yoghurt B (Fig. 3C and D). However, in G1, PI(18:1/18:2) is in significantly higher abundance in yoghurt B in contrast to yoghurt A and the milk.

Overall, it is clear that fermentation significantly alters not only the TPL profile, but the overall composition of PE, SM, PC, PS, and PI in the milk and yoghurts. Furthermore, there were statistically significant differences in the fatty acyl composition in each of the various PL species, which led to two significantly different yoghurt PL profiles following ovine milk fermentation.

3.2. Antiplatelet effects of ovine milk and yoghurt polar lipids

The milk and yoghurt TPL extracts were assessed against ADP, PAF, or thrombin-induced platelet aggregation and the results are presented in Table 1. Results are expressed as IC_{50} values of the mean mass of PL (μg) $\pm$ standard deviation (SD). Previously it was demonstrated that these extracts exhibited anti-PAF activity against human platelet aggregation (Lordan, Walsh, Crispie, Finnegan, Cotter, & Zabetakis, 2019). In this study, these aggregometry tests were replicated in six different human blood samples and the results against PAF were in accordance with the previously published data (Lordan, Walsh, Crispie, Finnegan, Cotter, & Zabetakis, 2019). However, the anti-ADP or anti-thrombin effect of dairy PL extracts were not previously assessed. The results of this study indicate that ovine milk and yoghurts exhibit potent anti-thrombin activity. Yoghurt A (33.7 $\pm$ 6.4) had a lower IC_{50} than the ovine milk (37.5 $\pm$ 5.2). However, this was not significantly different ($p > 0.05$). In contrast, yoghurt B had an IC_{50} (14.7 $\pm$ 6.0) that was significantly ($p < 0.05$) lower than the milk and yoghurt A. These anti-thrombin effects were more potent than those previously published for salmon PL characterised by several diacyl-PC/PE and alkyl-acyl-PC/PE species bearing mostly eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) at the *sn*-2 position of the glycerol backbone (Tsoupras, Lordan, Demuru et al., 2018; Tsoupras, Lordan, Shiels et al., 2019). This is important as these are ω3 fatty acids that are purported to exhibit antithrombotic activity. However, as our study indicates, PL extracts low in ω3 fatty acid content may be just as antithrombotic as those that are characterised as PL with a high ω3 fatty acid content.

Juxtaposed, the anti-ADP effects of these milk and yoghurt PL extracts were not as potent as thrombin or PAF, but still appreciable.

Neither the milk nor yoghurt IC_{50} values were significantly different from each other, indicating that fermentation to yoghurt did not seem to impact the anti-ADP activities of these extracts. Therefore, further research is warranted to discern whether other dairy PL extracts can affect ADP-induced platelet aggregation.

3.3. Milk and yoghurt polar lipid structures affect biological activity

Fig. 4 shows the PCA biplots of the relative abundance of the overall (Fig. 4A) and individual (Fig. 4B-F) PL species with a variable importance of projection (VIP) > 1 following PLS-DA plotted with the antithrombotic data. In accordance with the previous results, the milk and yoghurts are distinct from each other due to their PL composition. It was observed that the SM, PC, and PA classes in yoghurt B clustered together with the thrombin and PAF antiplatelet activity (see Fig. 4A). This suggests a possible relationship between these PL classes and the superior antithrombotic properties detected in yoghurt B PL extracts (Table 1). The milk clustered predominantly into quadrant 1 due to the PE and PI classes and yoghurt A segregated into quadrant 2 due to its PG and PS classes. These groupings accounted for 55.52% of the total variance present in the data. Yoghurt B exhibited the most potent antiplatelet effects against these agonists and the SM class was inversely correlated with both thrombin and PAF-induced platelet aggregation (Fig. 4A and Table S-3). This indicates that the changes in the milk and yoghurt PL profiles can lead to more favourable antithrombotic properties.

Furthermore, the observed PL molecular species that clustered with the thrombin and PAF antiplatelet activity in the milk and yoghurt samples were then evaluated to determine if there were any significant associations between these parameters. The biplot of Fig. 4B presents the PCA of the PE molecular species of the milk and yoghurts and their biological activity. The milk samples are clustered in the positive quadrant and the yoghurts are clustered on the negative quadrants of the first component respectively, whereby these grouping account for 67.03% of the total variance in the data set. This can mainly be attributed to the higher relative abundance of ether lipids in the milk in contrast to their lower abundance in the yoghurts, as observed in the heatmap of the PE molecular species in section 3.1. Moreover, yoghurt A and yoghurt B are divided based on the PE molecular species in component 2 of the PCA. PE molecular species were associated with inhibition of PAF and thrombin. In particular, PE(17:0/18:1), PE(18:0/18:0), and PE(18:0/18:1) were significantly but inversely correlated with anti-PAF effects, whereas PE(18:1/18:2) was inversely correlated with the inhibition of both PAF and thrombin (Table S-3). Notably, PE(18:0/18:0) has previously been identified as a main constituent of potent anti-PAF PL extracts of gilthead sea bream (Sioriki, Smith, Demopoulos, & Zabetakis, 2016). Indeed, as observed in the ovine yoghurts, most of the PE species in these fish PL contained either 18:0 or 18:1 at the *sn*-1 position, which may be a contributory factor in the biological activity of PE species. Previous research indicates that isolated PE extracts of ovine and caprine dairy products inhibited PAF-induced platelet aggregation (Megalemou et al., 2017), thus indicating that the PE fraction of ovine milk has the potential to inhibit PAF-related biological activity.
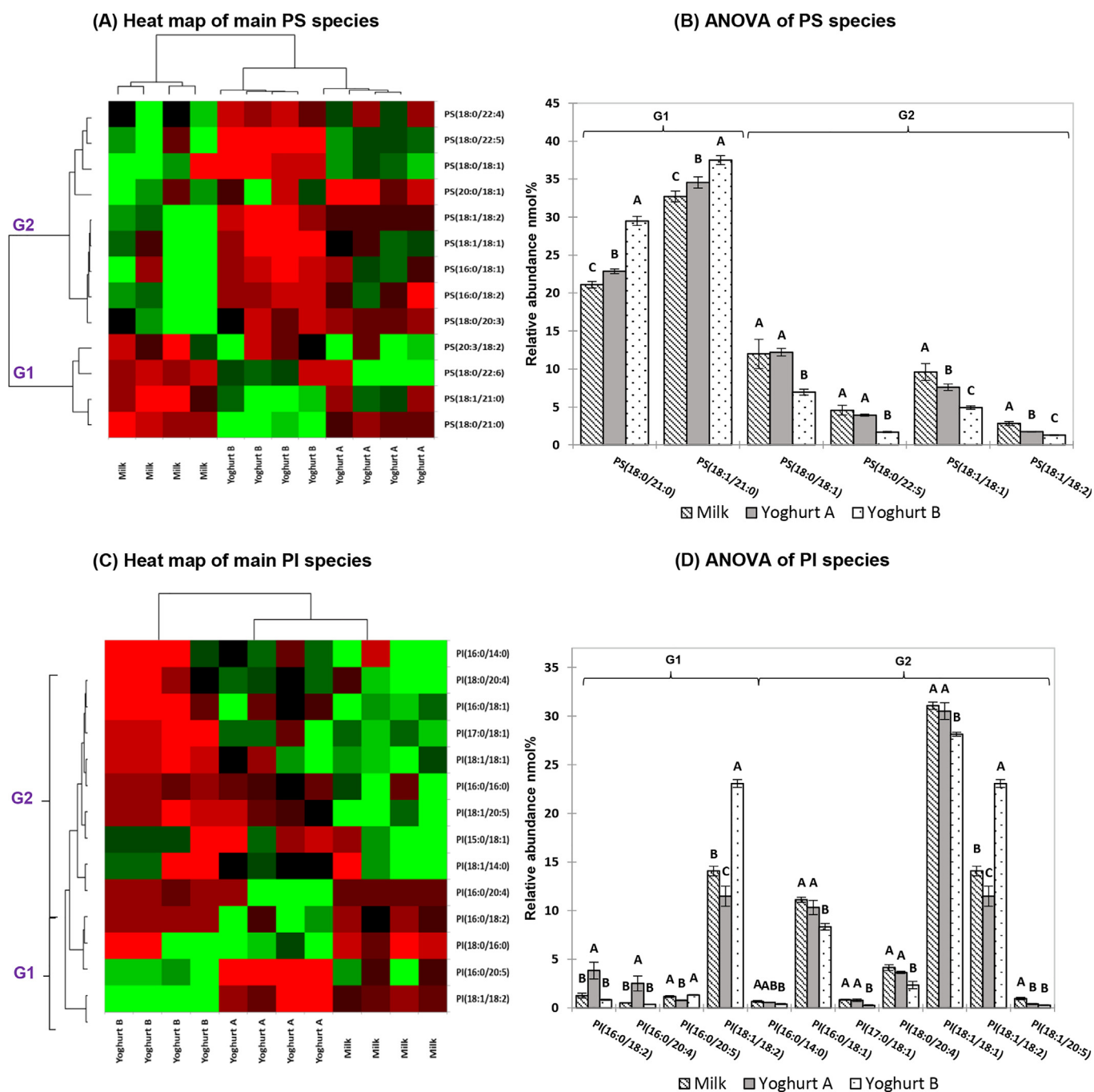
(A) Heat map of main PS species

(B) ANOVA of PS species

(C) Heat map of main PI species

(D) ANOVA of PI species

Fig. 3. Heat maps (3A and 3C) demonstrating the variation between PS and PI molecular species in ovine milk and yoghurts A and B. The various dairy sources and PL classes were clustered independently using ascendant hierarchical clustering based on Euclidian distance at 0.15 interquartile range. The left columns represent the segregation of the PL species, whereas the columns above segregate the milk and yoghurts based on similarities in abundance. Red, black, and green colours denote lower, intermediate, and higher abundance of the PL species. Group 1 and 2 (G1 and G2) are the PL species that were responsible for the resulting clustered patterns in the heat maps that were used to determine statistical significance between the milk and yoghurt PI and PS species in each of the bar charts (3B and 3D) coupled to the heat maps. These bar charts report the relative abundance of the various molecular species of each lipid class as a mean nmol% $\pm$ SE (n = 4). Statistically significant differences between the lipid species are denoted by the letter A-C above the error bars as determined by Fisher's LSD multiple comparisons test following ANOVA (α = 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The SM biplot (Fig. 4C.) clustered the milk and the yoghurt PL in components 1, 3, and 4 of the biplot and this grouping accounted for 73.03% of the total variance present in the data. Yoghurt B mainly clusters into quadrant 1 and was correlated with the PAF and thrombin inhibition. The SM molecular species SM(d18:1/18:1), SM(d16:0/18:1), and SM(d18:0/22:0) were all significantly inversely correlated with thrombin inhibition, whereas the latter SM species was also significantly inversely correlated with PAF inhibition (Table S-3).

Sphingolipids are integral to platelet activation, particularly in relation to thrombin, which causes platelets to release sphingosine-1-phosphate and increase levels of sphingosine, thus potentiating platelet aggregation (Simon & Gear, 1999). As such, sphingolipids are considered bioactive regulators of thrombin generation, whereby sphingosine, sphinganine, glucosylsphingosine, lysosphingomyelin, and similarly structured sphingolipids, but not ceramide or sphingosine-1-phosphate, which down-regulate thrombin generation (Deguchi,

Table 1
Inhibition of adenosine diphosphate (ADP), platelet-activating factor (PAF), and thrombin induced platelet aggregation in human PRP by ovine milk and yoghurts total polar lipid extracts (TPL).

Samples	ADP	PAF	Thrombin
Milk	190.8 ± 72.4[a]	154.9 ± 13.2[c]	37.5 ± 5.2[b]
Yoghurt A	196.0 ± 79.2[a]	80.4 ± 10.2[b]	33.7 ± 6.4[b]
Yoghurt B	198.8 ± 78.0[a]	48.5 ± 5.7[a]	14.7 ± 6.0[a]

[abc] Mean values (n = 6), ± standard deviation with different letters in the same column indicating statistical significant differences when means are compared using the Kruskal Wallis test ($p < 0.05$). The antithrombotic activity is denoted by the IC_{50} value (µg; mean ± SD). Abbreviations: adenosine diphosphate; IC_{50}, 50% inhibitory concentration; PAF, platelet-activating factor; PRP, platelet-rich plasma; TPL, total polar lipids

Yegneswaran, & Griffin, 2004). However, sphingolipids have a complex relationship with human cardiovascular health, whereby some endogenous sphingolipid metabolites are associated with a pro-inflammatory role in atherosclerosis and others an anti-inflammatory role (Edsfeldt et al., 2016; Lordan & Zabetakis, 2017). Previous studies suggest that the SM fraction of bovine, caprine, and ovine dairy products seem to exhibit PAF inhibitory or weakly agonistic effects that are several order of magnitude less potent than PAF (Megalemou et al., 2017; Tsorotioti, Nasopoulou, Detopoulou, Sioriki, Demopoulos, & Zabetakis, 2014). However, the structures involved in these biological effects were never previously elucidated.

In this study, we report that the total sum of SM species, and several individual molecular species of SM, especially SM(d18:0/22:0) and SM (d18:1/22:0), were strongly inversely correlated with the inhibition of both PAF and thrombin (Table S-3). This is in accordance with previous research assessing PL of marine (Tsoupras, Lordan, Demuru et al., 2018; Tsoupras, Lordan, Shiels et al., 2019) and dairy (Megalemou et al., 2017; Tsorotioti et al., 2014) PL extracts. It is essential to further research the SM fraction of dairy products and particularly these ovine milk and yoghurts, as they are a rich natural source of bioactive SM that have the potential to be utilised and altered for the production of functional foods and nutraceuticals capable of affecting platelet function and inflammatory pathways.

While the relative abundance of the PC species did not seem to differ significantly between the milk and yoghurts (Fig. 1E), a clear separation of the milk and yoghurt PC molecular species was evident in this study (Fig. 4D). Furthermore, PAF and thrombin activities were clustered in quadrant 3 with yoghurt B derived PC molecular species (total variance 74.92%). In particular, PC(18:0/16:0) and PC(18:0/18:1) were inversely correlated with PAF and thrombin inhibition, whereas PC(17:0/18:2) was inversely correlated with only PAF inhibition. The PC fraction of various yoghurts and salmon have previously exhibited PAF inhibitory effects (Megalemou et al., 2017; Sioriki et al., 2016; Tsoupras, Lordan, Demuru et al., 2018) and salmon PC has also exhibited anti-thrombin effect (Tsoupras, Lordan, Demuru et al., 2018; Tsoupras, Lordan, Shiels et al., 2019). Notably, PC species in goat and sheep meat fractions and salmon PC fractions appear to be very dissimilar to those of the bioactive yoghurts evaluated in this study (Poutzalis, Lordan, Nasopoulou, & Zabetakis, 2018; Tsoupras, Lordan, Demuru et al., 2018). These fractions are characterised by higher levels of ω3 fatty acids versus the PL of yoghurts. This demonstrates that PC fractions that do not contain PUFA can exhibit antiplatelet activities.

Finally, the antithrombotic activities of PS and PI fractions of dairy products have not previously been assessed due to their low relative abundance, thus it was not known whether these species contribute to the antiplatelet effects of PL extracts. However, in this study, PS accounts for 10–12% and PI accounts for approximately 1% of the relative abundance of the total PL of the milk and yoghurts. Notably, some species of PS and PI were inversely correlated with the observed antiplatelet effects of the milk and yoghurts namely PS(18:0/21:0), PS

(18:1/21:0), and PI(18:1/18:2) (Fig. 4E-F; Table S-3). Mechanistically, many PS and PI species are integral to proper platelet function. In terms of PS, in an activated state, the platelet plasma membrane surface gradually exposes aminophospholipids through translocation via ATP dependent scramblases and flippases to trigger the coagulation cascades. The externalisation of PS and PE is a crucial process necessary for effective inflammation related platelet activation in both acute and resolving stages (Ghoshal & Bhattacharyya, 2014). Similarly, PI species are major signalling molecules in platelet function (O'Donnell, Murphy, & Watson, 2014). It is known that dietary lipids, such as the intake of polyunsaturated fatty acids (PUFA) obtained from food or supplementation, can alter platelet lipid composition and function (Walker et al., 2015). However, it is not known exactly how dietary PL may affect these pathways, thus further research is warranted.

3.4. Potential antiplatelet mechanisms of milk and yoghurt polar lipids

The mechanisms by which dietary PL seem to exert their biological influences against the PAF and thrombin pathways are complex and in the case of thrombin not fully elucidated. Fig. 5 is a schematic illustration of some of the potential modes of action of dietary PL, detailing their possible effect on the PAF and thrombin receptors and the surrounding platelet membrane. For example, Fig. 5A is a heat map of the PL species observed in this study that were highly correlated with antiplatelet activity against PAF and thrombin. Again, it is clear from the heat map that there is a higher concentration of the bioactive PL clustering with yoghurt B (S1). In contrast, the ovine milk and yoghurt A do cluster separately from each other but are more similar to each other than to yoghurt B (S2). The PL molecular species that highly correlated with the PAF activities are potential structural ligands of the PAF-R as they are structurally homologous to PAF (Lordan, Tsoupras, & Zabetakis, 2017), which indicates that there mode of action may be through competitive binding of the PAF-R. Notably, the majority of the PL molecular species that are present in higher relative abundances in yoghurt B tend to be PC, PE, and SM species, which have also previously exhibited antiplatelet activity in studies of marine extracts (Tsoupras, Lordan, Demuru et al., 2018).

As per Table S-3, there seems to be a general trend that indicates that the dairy PL molecular species that are highly correlated to the antiplatelet effects of dairy PL against PAF and thrombin are characterised by either stearic acid (18:0) or oleic (18:1) at the sn-1 position such as PE(18:0/18:1), PE(18:1/18:2), and PC(18:0/18:1). This may be a significant structural feature that dictates whether PL are potent PAF antagonists or not considering that naturally occurring PAF from rabbit basophils are reported to contain either stearic acid (~90%) or palmitic acid (~10%) at the sn-1 position (Hanahan, Demopoulos, Liehr, & Pinckard, 1980). Additionally, oleic acid as a free fatty acid has exhibited inhibition of PAF signal transduction in platelets by decreasing polyphosphoinositide metabolism, which is critical to platelet activation (Nunez et al., 1990). Whether a phospholipid bearing oleic acid can affect these same signalling pathways remains to be determined. Likewise, other fatty acyl groups that are associated with platelet reactivity such as DHA (22:6), arachidonic acid (20:4), and palmitic acid were identified on the sn-2 position of some PL molecular species, the latter bring a structural component of the classic PAF molecule. Further research is warranted to distinguish to what extent these PL molecular species affect PAF and thrombin induced platelet aggregation in humans.

Fig. 5B1 and B2 demonstrates how dairy PL and the structures observed in this study may competitively bind with the PAF-R, thus inhibiting the effects of PAF and PAFLL preventing downstream signalling events. Dairy PL molecular species may inhibit Gq-linked mechanisms whereby phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis is mediated by phospholipase C beta (PLCβ) to produce inositol triphosphate (IP_3) and diacylglycerols (DAG) that leads to a transient elevation of cytosolic Ca^{2+} and activation of protein kinase C (PKC).
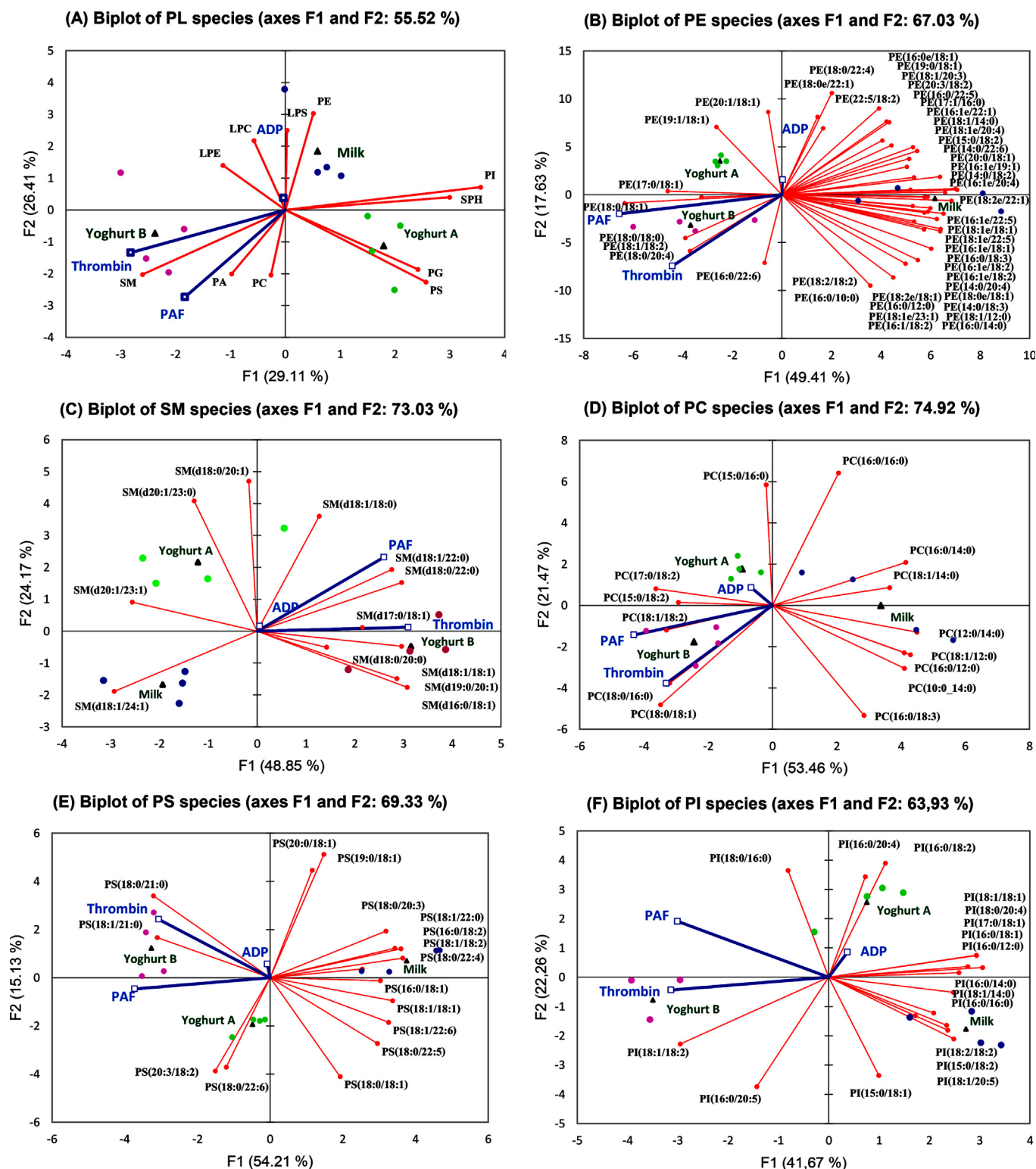
Fig. 4. Biplots showing the ovine milk and yogurt PL and molecular species associated with the IC$_{50}$ values of the ADP, PAF, and thrombin platelet aggregation outputs derived from the PL extracts. A = PL biplot; B = PE biplot; C = SM biplot; D = PC biplot; E = PS biplot; F = PI biplot. The PCA analyses demonstrates that there tends to be a correlation between PAF and thrombin inhibition and the PL molecular species associated with yoghurt B in particular, which exhibited the most potent inhibitory effects against these two platelet agonists. However, no significant deduction can be made for ADP inhibition as none of the PL species tended to significantly cluster with ADP. Abbreviations: ADP, adenosine diphosphate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPS, lysophosphatidylserine; PA, phosphatidic acid; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, polar lipids; PS, phosphatidylserine; SM, sphingomyelin; SPH, sphingosine.

(A) Heat map of main bioactive polar lipids

(B) Potential mechanisms of PAF and thrombin inhibition by dairy polar lipids

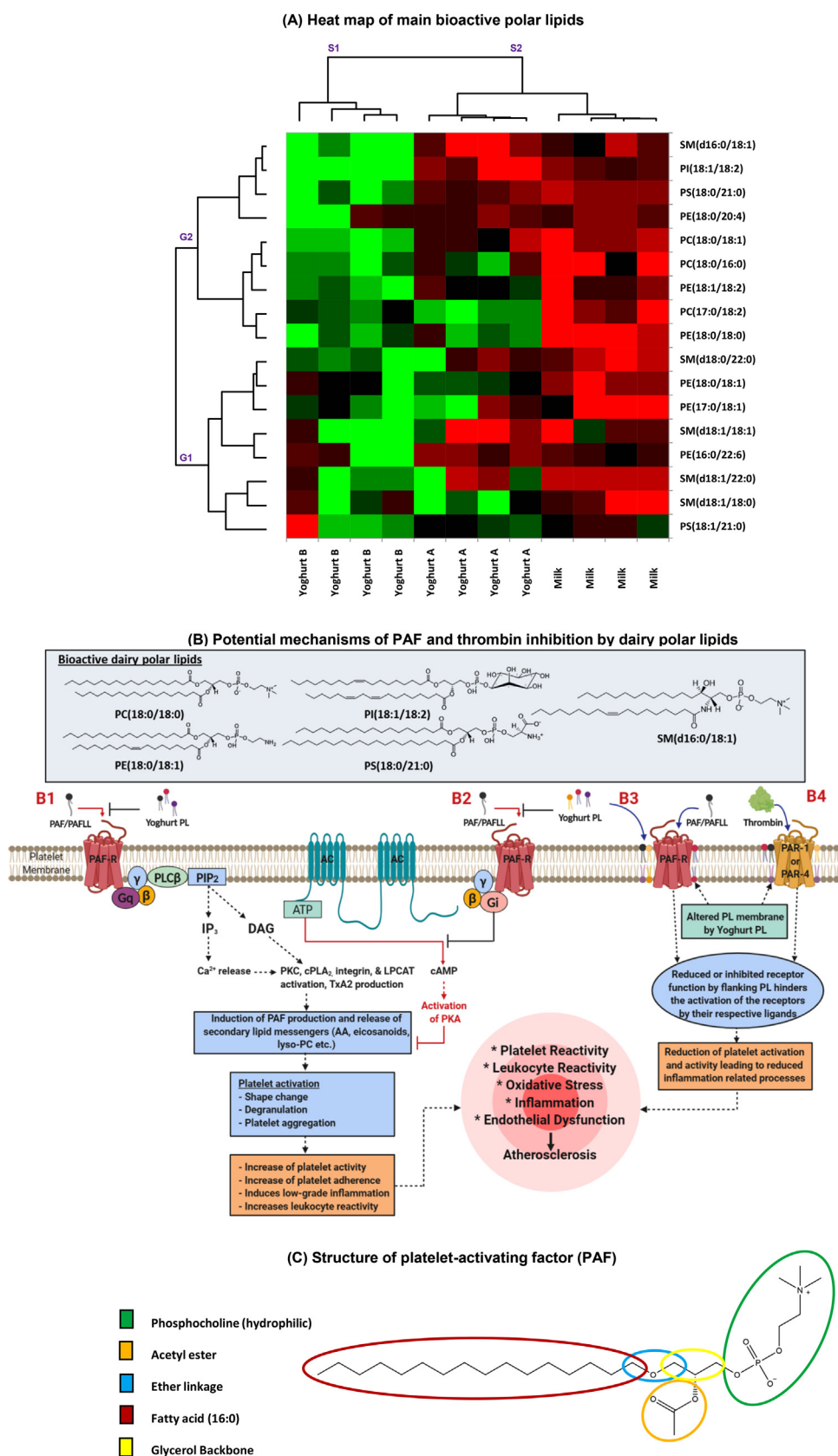**(C) Structure of platelet-activating factor (PAF)**

Fig. 5. Flow diagram showing the proposed pathways associated with the potential mechanisms of PAF and thrombin inhibition by dairy PL. (A) presents the heat map of the PL species of the milk and yoghurts that were significantly inversely correlated with inhibition of PAF and thrombin-induced platelet aggregation. It is clear from the heatmap that the majority of these proposed bioactive PL species were present in abundance in yoghurt B, which was the most bioactive yoghurt, but were present in less amounts in yoghurt A and in very low abundance in the milk. Image (B) is a schematic illustration of the most probable modes of action of these bioactive dairy PL against the PAF and thrombin pathways of platelet activation. In B1 and B2, PAF or PAFLL can bind to the PAF-R inducing platelet aggregation in platelets of inflammation related events in other cells. Structurally similar PL species can competitively bind the PAF-R preventing reducing platelet reactivity due to their lower potency. In B3 and B4, as some of the intact PL species are structurally homologous to the platelet membrane lipids they can be incorporated into the membrane. This leads to structural changes of the membrane surrounding the PAF-R for PAF or PAR-1 and PAR-4 for thrombin, which may impede the function of these receptors. Therefore, the biological activity of these receptors is reduced and their downstream effects are dampened or inhibited. This may be the same mode of action for ADP, however, further research is required. Image (C) is the a structural representation of PAF with its various critical features highlighted. It is postulated that dietary PL exert their effects on the PAF-R as depicted in B1 and B2 due to their structural similarities, i.e. hydrophilic head group, glycerol backbone, and fatty acid at the *sn*-1 position. Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; cAMP, 3′–5′-cyclic adenosine monophosphate; cPLA2, cytosolic phospholipase A2; DAG, diacylglycerol; IP3, Inositol 1,4,5-triphosphate; LPCAT, lyso-phosphatidylcholine acyltransferase; PAF, platelet-activating factor; PAFLL, PAF-like lipids; PAF-R; PAR, protease activated receptor; platelet-activating factor receptor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PL, polar lipids; PLCβ, phospholipase C β; PS, phosphatidylserine; SM, sphingomyelin; TxA2, thromboxane A2.

Elevated Ca^{2+} activates cytosolic phospholipase A2α (cPLA2α) that leads to a release of lysophosphatides and arachidonic acid that act as substrates for the synthesis of eicosanoids and PAF respectively, which amplifies the platelet response. Inhibition of these pathways would prevent platelet aggregation and inflammatory signalling. Juxtaposed, dairy PL may also affect signalling through Gi-linked mechanisms and related anti-inflammatory signalling events (Tsoupras, Lordan, & Zabetakis, 2018). However, further research is required to discern how

these mechanisms are affected by dairy PL.

While there is a wealth of evidence indicating that dietary PL directly act on the PAF-R (Lordan, Tsoupras, & Zabetakis, 2017) due to their structural similarities with PAF, including a hydrophilic head group, a glycerol backbone, and a fatty acyl group at the *sn*-1 position (Fig. 5C), the mechanisms by which dietary PL affect thrombin or even ADP activation is not known. However, it is thought that PL may indirectly affect platelet GPCRs by altering the microenvironment and polarisation of the phospholipid membrane, which potentially alters the affinity of respective platelet agonists to their receptor relating to platelet activation as demonstrated in B3 and B4 (Lordan, Tsoupras, & Zabetakis, 2017; Yoshida et al., 2019). Notably, platelet receptors for ADP, PAF, and thrombin are all GPCRs, indicating that this may be the common feature that explains the antiplatelet effects of the dairy PL. However, while ADP was inhibited by the PL extracts, it seems that fermentation and the subsequent change in the PL composition did not significantly affect ADP inhibition by the PL, in contrast to PAF or thrombin. Further research is required to understand how dairy PL may affect ADP-induced platelet aggregation.

Generally, there is a paucity of literature assessing the antithrombotic effects of milk and dairy consumption including the PL and LAB lipid metabolites in dairy products. In the previous assessment of these ovine milk and yoghurt PL extracts it was determined that yoghurts that contained a higher level of *S. thermophilus* and *L. acidophilus* in the initial starter culture and in the final yoghurt product (yoghurt B in this study) possessed greater bioactivity against PAF. Furthermore, soluble factors released by *L. acidophilus* in particular have exhibited anti-inflammatory effects against PAF by affecting signalling pathways such as the nuclear factor kappa B (NF-κB), and the mitogen activated protein kinase (MAPK) signalling pathways in vitro and in vivo (Borthakur et al., 2013).

This study demonstrates that fermentation of ovine milk by *L. acidophilus* and *S. thermophilus* changes the milk PL composition leading to greater antithrombotic properties. The is due to the metabolic capacity of the starter cultures used (Lordan, Walsh, Crispie, Finnegan, Cotter, & Zabetakis, 2019). However, further research is required to discern the exact metabolic pathways responsible for altering the PL content of milk in order to utilise these pathways for the production of tailored functional cardioprotective foods.

3.5. Study limitations

This current study has its limitations in what can be concluded with regards to potential in vivo platelet function. However, generally light transmission aggregometry is considered the gold standard of platelet function testing as it reflects in vivo platelet aggregation (McEwen, 2014). It is important to test the effect of these PL against other platelet agonists such as arachidonic acid and collagen in future human studies and using alternative methodologies such analysing direct thrombus formation in vivo. Indeed, this study is also limited by its assessment of intact PL. The majority of PL consumed are digested by phospholipase A_2, which cleaves the ester bond at the *sn*-2 position leading to the release of the lyso-form of the PL and free fatty acids. These digestive products are then absorbed by enterocytes in the gastrointestinal tract, which are then transported to the lymphatic system and subsequently the circulatory system as chylomicrons, whereby they are eventually resynthesised into phospholipids or triacylglycerols (Dixon, 2010). Within 5–6 h of ingestion, some PL are transferred directly into high-density lipoproteins (HDL) (Tall, Blum, & Grundy, 1983), which then can transport the PL to their target cells, tissues, and organs (Burri, Hoem, Banni, & Berge, 2012). Approximately 20% of the intestinal and dietary PL are passively absorbed intact and unaffected by digestive lipases, whereby they are incorporated into HDL (Lordan, Tsoupras, & Zabetakis, 2017). Notably, PL can also be integrated into pre-existing HDL particles in the intestine, which can subsequently join the plasma HDL pool (Burri, Hoem, Banni, & Berge, 2012), thereby increasing the

levels of intact PL absorbed into circulation capable of affecting platelet function and reactivity. The incorporation of intact PL into HDL particles has important ramifications for cardiovascular pathology as HDL particles possess antithrombotic properties, which is attributed to their PL membranes (Camont et al., 2013). Further studies investigating the incorporation of intact PL into plasma HDL are warranted. Furthermore, studies are warranted to assess how effective the antithrombotic properties of dairy polar lipids are following human gastrointestinal digestion postprandially and long-term.

4. Conclusion

The totality of the evidence indicates that ovine milk and yoghurt PL possess potent antithrombotic activity against PAF and thrombin and moderate biological activity against ADP-induced human platelet aggregation. C30RP-UHPLC-HRAM-MS/MS was employed to assess the effect of fermentation on the PL fraction of milk following fermentation by specific starter cultures. It was determined using multivariate statistical analysis that there was a significant difference in the PL composition in the yoghurts in contrast to the milk and that there were significant differences between the PL extracts of yoghurts A and B due to their differing starter cultures and the fermentation process. It is evident that these processes affect the antiplatelet properties of these yoghurts and that certain PL compositions are strongly inversely correlated with PAF- and thrombin-induced platelet aggregation. Notably, this study highlights that dietary SM, PE, and PC have the potential to affect platelet aggregation and warrant further intensive research in vivo. This research highlights that further research is required to discern the exact metabolic pathways responsible for altering the PL content of milk in order to utilise these pathways for the production of tailored antithrombotic functional cardioprotective foods.

CRediT authorship contribution statement

Ronan Lordan: Funding acquisition, Project administration, Supervision, Visualization, Writing - review & editing, Data curation, Resources, Investigation, Formal analysis, Validation, Software, Methodology, Conceptualization. **Natalia P. Vidal:** Visualization, Writing - review & editing, Investigation, Formal analysis, Software, Methodology. **Thu Huong Pham:** Visualization, Writing - review & editing, Data curation, Investigation, Formal analysis, Validation, Software, Methodology. **Alexandros Tsoupras:** Data curation, Investigation. **Raymond H. Thomas:** Funding acquisition, Writing - review & editing, Resources, Formal analysis, Validation, Software, Methodology. **Ioannis Zabetakis:** Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Statement

All supplementary data is available on request and will be made available in a *Data in Brief* file that is in preparation for Elsevier.

Funding sources

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127384.

References

Anto, L., Warykas, S. W., Torres-Gonzalez, M., & Blesso, C. N. (2020). Milk polar lipids: Underappreciated lipids with emerging health benefits. *Nutrients, 12*(4), 1001. https://doi.org/10.3390/nu12041001.

Astrup, A., Geiker, N. R. W., & Magkos, F. (2019). Effects of full-fat and fermented dairy products on cardiometabolic disease: Food is more than the sum of its parts. *Advances in Nutrition, 10*(5), 924S–930S. https://doi.org/10.1093/advances/nmz069.

Balthazar, C. F., Pimentel, T. C., Ferrão, L. L., Almada, C. N., Santillo, A., Albenzio, M., ... Cruz, A. G. (2017). Sheep milk: Physicochemical characteristics and relevance for functional food development. *Comprehensive Reviews in Food Science and Food Safety, 16*(2), 247–262. https://doi.org/10.1111/1541-4337.12250.

Borthakur, A., Bhattacharyya, S., Kumar, A., Anbazhagan, A. N., Tobacman, J. K., & Dudeja, P. K. (2013). *Lactobacillus acidophilus* alleviates platelet-activating factor-induced inflammatory responses in human intestinal epithelial cells. *PLoS One, 8*(10), Article e75664. https://doi.org/10.1371/journal.pone.0075664.

Burri, L., Hoem, N., Banni, S., & Berge, K. (2012). Marine omega-3 phospholipids: Metabolism and biological activities. *International Journal of Molecular Sciences, 13*(11), 15401. https://doi.org/10.3390/ijms131115401.

Camont, L., Lhomme, M., Rached, F., Le Goff, W., Nègre-Salvayre, A., Salvayre, R., ... Kontush, A. (2013). Small, dense high-density lipoprotein-3 particles are enriched in negatively charged phospholipids: Relevance to cellular cholesterol efflux, anti-oxidative, antithrombotic, anti-Inflammatory, and antiapoptotic functionalities. *Atherosclerosis, Thrombosis, and Vascular Biology, 33*(12), 2715–2723. https://doi.org/10.1161/atvbaha.113.301468.

Da Silva, M. S., & Rudkowska, I. (2015). Dairy nutrients and their effect on inflammatory profile in molecular studies. *Molecular Nutrition & Food Research, 59*(7), 1249–1263. https://doi.org/10.1002/mnfr.201400569.

Deguchi, H., Yegneswaran, S., & Griffin, J. H. (2004). Sphingolipids as bioactive regulators of thrombin generation. *The Journal of Biological Chemistry, 279*(13), 12036–12042. https://doi.org/10.1074/jbc.M302531200.

Dixon, J. B. (2010). Mechanisms of chylomicron uptake into lacteals. *Annals of the New York Academy of Sciences, 1207*(1), E52–E57. https://doi.org/10.1111/j.1749-6632.2010.05716.x.

Edsfeldt, A., Dunér, P., Ståhlman, M., Mollet, I. G., Asciutto, G., Grufman, H., ... Melander, O. (2016). The pro-inflammatory role of sphingolipids and glycosphingolipids in the human atherosclerotic plaque. *Arteriosclerosis, Thrombosis, and Vascular Biology, 241*(1), E30. https://doi.org/10.1016/j.atherosclerosis.2015.04.113.

Furse, S., Torres, A. G., & Koulman, A. (2019). Fermentation of milk into yoghurt and cheese leads to contrasting lipid and glyceride profiles. *Nutrients, 11*(9), 2178. https://doi.org/10.3390/nu11092178.

Ghoshal, K., & Bhattacharyya, M. (2014). Overview of platelet physiology: Its hemostatic and non-hemostatic role in disease pathogenesis. *The Scientific World Journal, 2014*, Article 781857. https://doi.org/10.1155/2014/781857.

Hanahan, D. J., Demopoulos, C., Liehr, J., & Pinckard, R. (1980). Identification of platelet activating factor isolated from rabbit basophils as acetyl glyceryl ether phosphorylcholine. *The Journal of Biological Chemistry, 255*(12), 5514–5516.

Koupenova, M., Clancy, L., Corkrey, H. A., & Freedman, J. E. (2018). Circulating platelets as mediators of immunity, inflammation, and thrombosis. *Circulation Research, 122*(2), 337–351. https://doi.org/10.1161/CIRCRESAHA.117.310795.

Lordan, R., Tsoupras, A., Mitra, B., & Zabetakis, I. (2018). Dairy fats and cardiovascular disease: Do we really need to be concerned? *Foods, 7*(3), 29. https://doi.org/10.3390/foods7030029.

Lordan, R., Tsoupras, A., & Zabetakis, I. (2017). Phospholipids of animal and marine origin: Structure, function, and anti-inflammatory properties. *Molecules, 22*(11), 1964. https://doi.org/10.3390/molecules22111964.

Lordan, R., Walsh, A. M., Crispie, F., Finnegan, L., Cotter, P. D., & Zabetakis, I. (2019). The effect of ovine milk fermentation on the antithrombotic properties of polar lipids. *Journal of Functional Foods, 54*, 289–300. https://doi.org/10.1016/j.jff.2019.01.029.

Lordan, R., Walsh, A. M., Crispie, F., Finnegan, L., Demuru, M., Tsoupras, A., ... Zabetakis,

I. (2019). Caprine milk fermentation enhances the antithrombotic properties of cheese polar lipids. *Journal of Functional Foods, 61*, Article 103507. https://doi.org/10.1016/j.jff.2019.103507.

Lordan, R., & Zabetakis, I. (2017). Invited review: The anti-inflammatory properties of dairy lipids. *Journal of Dairy Science, 100*(6), 4197–4212. https://doi.org/10.3168/jds.2016-12224.

McEwen, B. J. (2014). The influence of diet and nutrients on platelet function. *Seminars in Thrombosis and Hemostasis, 40*(02), 214–226. https://doi.org/10.1055/s-0034-1365839.

Megalemou, K., Sioriki, E., Lordan, R., Dermiki, M., Nasopoulou, C., & Zabetakis, I. (2017). Evaluation of sensory and *in vitro* anti-thrombotic properties of traditional Greek yogurts derived from different types of milk. *Heliyon, 3*(1), https://doi.org/10.1016/j.heliyon.2016.e00227 Article e00227.

Millar, C. L., Jiang, C., Norris, G. H., Garcia, C., Seibel, S., Anto, L., ... Blesso, C. N. (2020). Cow's milk polar lipids reduce atherogenic lipoprotein cholesterol, modulate gut microbiota and attenuate atherosclerosis development in LDL-receptor knockout mice fed a Western-type diet. *The Journal of Nutritional Biochemistry, 79*, Article 108351. https://doi.org/10.1016/j.jnutbio.2020.108351.

Moss, J. W. E., Williams, J. O., & Ramji, D. P. (2018). Nutraceuticals as therapeutic agents for atherosclerosis. *Biochimica et Biophysica Acta -Molecular Basis of Disease), 1864*(5 Part A), 1562–1572. https://doi.org/10.1016/j.bbadis.2018.02.006.

Nunez, D., Randon, J., Gandhi, C., Siafaka-Kapadai, A., Olson, M. S., & Hanahan, D. (1990). The inhibition of platelet-activating factor-induced platelet activation by oleic acid is associated with a decrease in polyphosphoinositide metabolism. *The Journal of Biological Chemistry, 265*(30), 18330–18338.

O'Donnell, V. B., Murphy, R. C., & Watson, S. P. (2014). Platelet lipidomics: Modern day perspective on lipid discovery and characterization in platelets. *Circulation Research, 114*(7), 1185–1203. https://doi.org/10.1161/CIRCRESAHA.114.301597.

Pham, T. H., Zaeem, M., Fillier, T. A., Nadeem, M., Vidal, N. P., Manful, C., ... Thomas, R. H. (2019). Targeting modified lipids during routine lipidomics analysis using HILIC and C30 reverse phase liquid chromatography coupled to mass spectrometry. *Scientific Reports, 9*(1), 5048. https://doi.org/10.1038/s41598-019-41556-9.

Poutzalis, S., Lordan, R., Nasopoulou, C., & Zabetakis, I. (2018). Phospholipids of goat and sheep origin: Structural and functional studies. *Small Ruminant Research, 167*, 39–47. https://doi.org/10.1016/j.smallrumres.2018.07.015.

Simon, C. G., & Gear, A. R. L. (1999). Sphingolipid metabolism during human platelet activation. *Thrombosis Research, 94*(1), 13–23. https://doi.org/10.1016/S0049-3848(98)00186-8.

Sioriki, E., Smith, T. K., Demopoulos, C. A., & Zabetakis, I. (2016). Structure and cardioprotective activities of polar lipids of olive pomace, olive pomace-enriched fish feed and olive pomace fed gilthead sea bream (*Sparus aurata*). *Food Research International, 83*, 143–151. https://doi.org/10.1016/j.foodres.2016.03.015.

Tall, A. R., Blum, C. B., & Grundy, S. M. (1983). Incorporation of radioactive phospholipid into subclasses of high-density lipoproteins. *American Journal of Physiology Endocrinology and Metabolism, 244*(5), E513–E516. https://doi.org/10.1152/ajpendo.1983.244.5.E513.

Tsorotioti, S. E., Nasopoulou, C., Detopoulou, M., Sioriki, E., Demopoulos, C. A., & Zabetakis, I. (2014). *In vitro* anti-atherogenic properties of traditional Greek cheese lipid fractions. *Dairy Science and Technology, 94*(3), 269–281. https://doi.org/10.1007/s13594-014-0161-x.

Tsoupras, A., Lordan, R., Demuru, M., Shiels, K., Saha, S. K., Nasopoulou, C., & Zabetakis, I. (2018). Structural elucidation of Irish organic farmed salmon (*Salmo salar*) polar lipids with antithrombotic activities. *Marine Drugs, 16*(6), 176. https://doi.org/10.3390/md16060176.

Tsoupras, A., Lordan, R., Shiels, K., Saha, S. K., Nasopoulou, C., & Zabetakis, I. (2019). In vitro antithrombotic properties of salmon (*Salmo salar*) phospholipids in a novel food-grade extract. *Marine Drugs, 17*(1), 62. https://doi.org/10.3390/md17010062.

Tsoupras, A., Lordan, R., & Zabetakis, I. (2018). Inflammation, not cholesterol, is a cause of chronic disease. *Nutrients, 10*(5), 604. https://doi.org/10.3390/nu10050604.

Tsoupras, A., Zabetakis, I., & Lordan, R. (2019). Platelet aggregometry assay for evaluating the effects of platelet agonists and antiplatelet compounds on platelet function *in vitro. MethodsX, 6*, 63–70. https://doi.org/10.1016/j.mex.2018.12.012.

Vors, C., Joumard-Cubizolles, L., Lecomte, M., Combe, E., Ouchchane, L., Drai, J., ... Michalski, M.-C. (2019). Milk polar lipids reduce lipid cardiovascular risk factors in overweight postmenopausal women: towards a gut sphingomyelin-cholesterol interplay. *Gut*. https://doi.org/10.1136/gutjnl-2018-318155.

Walker, C., West, A., Browning, L., Madden, J., Gambell, J., Jebb, S., & Calder, P. (2015). The pattern of fatty acids displaced by EPA and DHA following 12 months supplementation varies between blood cell and plasma fractions. *Nutrients, 7*(8), 5285. https://doi.org/10.3390/nu7085285.

Wu, L., & Sun, D. (2017). Consumption of yogurt and the incident risk of cardiovascular disease: A meta-analysis of nine cohort studies. *Nutrients, 9*(3), 315. https://doi.org/10.3390/nu9030315.

Yoshida, K., Nagatoishi, S., Kuroda, D., Suzuki, N., Murata, T., & Tsumoto, K. (2019). Phospholipid membrane fluidity alters ligand binding activity of a G protein-coupled receptor by shifting the conformational equilibrium. *Biochemistry, 58*(6), 504–508. https://doi.org/10.1021/acs.biochem.8b01194.

Zancada, L., Pérez-Díez, F., Sánchez-Juanes, F., Alonso, J., García-Pardo, L., & Hueso, P. (2013). Phospholipid classes and fatty acid composition of ewe's and goat's milk. *Grasas y Aceites, 64*(3), 304–310. https://doi.org/10.3989/gya.095312.