The effect of ovine milk fermentation on the antithrombotic properties of polar lipids

Ronan Lordan [a*], Aaron M. Walsh [b], Fiona Crispie [bc], Laura Finnegan [b], Paul D. Cotter [bc], and Ioannis Zabetakis [a*]

[a] Department of Biological Sciences, University of Limerick, Co. Limerick, Ireland

[b] Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[c] Alimentary Pharmabiotic Centre, University College Cork, Co. Cork, Ireland

[*] **Corresponding Author:** Ioannis.Zabetakis@ul.ie and Ronan.Lordan@ul.ie

ABSTRACT

The effect of fermentation on the antithrombotic properties of polar lipids in ovine milk has been assessed through the production of yoghurts. The total lipids (TL), total neutral lipids (TNL), and total polar lipids (TPL) were extracted. The fatty acid profiles of all yoghurt polar lipids were analysed by GC-MS. The levels of MUFA increased, but there was a reduction in PUFA as milk was fermented to yoghurt. The bioactivity of each lipid extract was assessed against platelet-activating factor (PAF) induced platelet aggregation. All yoghurt polar lipids exhibited potent antithrombotic activities with IC_{50} values ranging from 45–77 µg. Shotgun metagenomics determined the species-level microbial composition and functional potential of the yoghurts. Yoghurts containing *L. acidophilus* seem to correlate with greater bioactivity. Several phospholipid biosynthetic genes have been identified in the most antithrombotic yoghurts. This study has demonstrated that fermentation enhances the antithrombotic properties of yoghurt polar lipids against PAF.

Keywords: polar lipids; fermentation; yoghurt; inflammation; antithrombotic metagenomics

Highlights:

- Fermentation alters the polar lipid fatty acid profile of ovine milk
- Specific starter cultures alter the antithrombotic properties of ovine milk in the production of yoghurts
- *S. thermophilus* and *L. acidophilus* alter the anti-PAF and antithrombotic properties of yoghurts
- Genes associated with phospholipid biosynthesis have been detected in the most bioactive yoghurts

1. Introduction

Maladaptive diet and lifestyle play a significant role in the development of chronic diseases such as cardiovascular disease (CVD), insulin resistance, obesity, and cancer (Mozaffarian, 2016). Diet and lifestyle are key modifiable risk factors for the prevention of CVD (Lordan, Tsoupras, Mitra, & Zabetakis, 2018). In 2016, CVD was responsible for 17.7 million deaths, where 80% of CVD events were either a myocardial infarction or stroke (World Health Organization, 2017), of which dietary risk factors accounted for 49% of all CVD deaths (Meier et al., 2018). Dairy products were long perceived as negative dietary components by public, scientific, and media circles, as they are energy dense foods rich in saturated fatty acids (SFA), which can increase cholesterol levels (Lamarche et al., 2016; Lordan & Zabetakis, 2017a). Therefore, low-fat or non-fat dairy products were encouraged by many dietary recommendations in order to reduce to lower cholesterol levels (Lordan, Tsoupras, Mitra, et al., 2018). However, recent research indicates that dairy products may be neutral or even beneficial for cardiovascular health, and may not have significant effects on blood cholesterol levels (Labonté, Couture, Richard, Desroches, & Lamarche, 2013; Lordan, Tsoupras, Mitra, et al., 2018; Lordan & Zabetakis, 2017a; Thorning et al., 2016). Further evidence indicates that fermented dairy products may be more beneficial for health than non-fermented dairy products, especially against a number of cardiometabolic risk factors such as hypertension, cholesterol levels, and impaired glucose tolerance (Lordan, Tsoupras, Mitra, et al., 2018).

Yoghurt consumption is associated with numerous health benefits including; preventing type II diabetes mellitus, obesity, metabolic syndrome, and CVD (Gijsbers et al.,

2016; Lordan, Tsoupras, Mitra, et al., 2018; Sayon-Orea, Martínez-González, Ruiz-Canela, & Bes-Rastrollo, 2017; Wu & Sun, 2017). Bovine milk accounts for 85% of the total global milk production (Balthazar et al., 2017), however ovine milk and yoghurts provide a superior nutritional alternative. Ewe's milk and dairy products are not commonly consumed outside of the Mediterranean basin, and are considered a delicacy in many countries (Lordan & Zabetakis, 2017b). Ovine milk owes it nutritional superiority over bovine and caprine milk due to the higher levels of protein, lipid, minerals, and vitamins essential to human health (Balthazar et al., 2017; Lordan, Tsoupras, Mitra, et al., 2018). The most predominant fatty acid in ovine milk and yoghurts are oleic acid (18:1), palmitic acid (16:0), and myristic acid (14:0) (Balthazar et al., 2017). Diets high in oleic acid decrease low-density lipoprotein (LDL) cholesterol levels, whereas high-density lipoprotein (HDL) cholesterol levels are not significantly affected (Lordan & Zabetakis, 2017b; Molkentin, 2000). It has recently be shown that ovine yoghurt consumption does not affect the lipid profile of healthy individuals (Olmedilla-Alonso et al., 2017). In addition, a recent crossover study has demonstrated a moderate attenuation of several inflammatory markers in participants with a high total cholesterol/HDL cholesterol ratio following ovine yoghurt consumption (Redondo et al., 2018). Evidently, these neutral effects on serum cholesterol levels, putative anti-inflammatory effects, and antithrombotic effects (Megalemou et al., 2017; Tsorotioti et al., 2014) indicate that ovine dairy products may be beneficial for human cardiovascular health upon consumption and thus warrant further investigation (Lordan, Tsoupras, Mitra, et al., 2018).

Ovine dairy products also possess potent antithrombotic properties that are attributed to their polar lipid content (Lordan & Zabetakis, 2017a). The polar lipid content of ovine milk is approximately 9.4-35.5 mg/100g of raw milk. The polar lipid fraction contains phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), which are present in abundance with lower quantities of phosphatidylserine (PS) and phosphatidylinositol (PI), which is comparable to other ruminant species (Lordan, Tsoupras, & Zabetakis, 2017; Park, 2009). Although the phospholipid fraction of ovine milk is quantitatively a minor constituent of the overall lipid content, it possesses techno-functional and nutritional properties that are implicated in several physiological processes and are beneficial for health (Lordan et al., 2017).

Systemic inflammation is the key biochemical process implicated in the initiation and progression of atherosclerosis (Moss, Williams, & Ramji, 2018). Circulating inflammatory mediators such as PAF actively contribute to vascular and atheromatous change (Da Silva &

Rudkowska, 2015; Lordan et al., 2017; Tsoupras, Lordan, & Zabetakis, 2018). PAF is a potent proinflammatory phospholipid mediator that is implicated in all stages of atherosclerosis that can lead to a major cardiovascular event. PAF and PAF-like molecules act solely through their binding to a unique G-protein coupled seven transmembrane receptor known as the PAF-receptor (PAF-R), that subsequently triggers multiple intracellular pathways (Castro Faria Neto, Stafforini, Prescott, & Zimmerman, 2005; Lordan et al., 2017; Tsoupras, Lordan, & Zabetakis, 2018). PAF plays a key role in various physiological responses such as modulation of normal inflammatory responses and the regulation of blood pressure and coagulation (Lordan et al., 2017; Palur Ramakrishnan, Varghese, Vanapalli, Nair, & Mingate, 2017). Therapeutic approaches to the proinflammatory effects of PAF focus on disrupting PAF/PAF-R interactions through competitive and non-competitive displacement of PAF from the receptor (Lordan, Tsoupras, & Zabetakis, 2018). Dietary PAF inhibitors have been identified in the polar lipids of marine (Lordan et al., 2017; Sioriki, Smith, Demopoulos, & Zabetakis, 2016), meat (Poutzalis, Lordan, Nasopoulou, & Zabetakis, 2018), and dairy sources (Megalemou et al., 2017; Poutzalis et al., 2016). In particular, ovine dairy products possess potent PAF inhibitors (Tsorotioti et al., 2014).

It has been postulated that fermentation increases the bioactivity of phospholipids against PAF (Antonopoulou, Semidalas, Koussissis, & Demopoulos, 1996; Lordan & Zabetakis, 2017a). However, to date, this has not been definitively established. Thus, the aim of this study was to evaluate the effect of bacterial fermentation on the polar lipid composition and antithrombotic activity of ovine milk and yoghurts via PAF-induced platelet aggregation on human platelets *in vitro*. Furthermore, shotgun metagenomics was employed to characterise the species-level microbial composition of the yoghurts, and to determine if the detected species contained genes associated with fatty acid and/or lipid metabolism.

2. Materials and methods

2.1. Chemicals and reagents

All organic solvents and glassware used in the lipid extraction and isolation process were purchased from Fisher Scientific Ireland Ltd. (Dublin, Ireland). All chemical reagents used for platelet aggregometry and lipid standards for GC-MS were purchased from Sigma-Aldrich (Wicklow, Ireland). All platelet aggregometry consumables were purchased from Labmedics LLP (Abingdon on Thames, U.K.). All GC-MS consumables were purchased from Apex

Scientific Ltd. (Kildare, Ireland). The PowerSoil DNA Isolation kit and PowerNad tubes were purchased from Cambio (Cambridge, United Kingdom). Lysozyme, mutanolysin and proteinase K were purchased from Sigma-Aldrich (Wicklow, Ireland). The Qubit High Sensitivity DNA assay was obtained from Life Technologies (ThermoFisher Scientific, Dublin, Ireland).

2.2. Milk processing & yogurt production

A fresh, commercial, pasteurised, and homogenised ewe's whole milk was obtained from Rockfield Dairy Ltd. (Claremorris, Co Mayo, Ireland). Milk was obtained from a bulk tank containing milk from the Friesland and Lacaune breed of dairy ewe, between March and July 2016. The sheep were fed a forage based diet consisting of mainly grass silage or fresh grass, supplemented with cereal at the time of milking, which is typical of the small sheep dairy industry in Ireland; however, atypical in Europe where the diet mainly consists of cereal. The collected milk was pasteurised on site by heating to 91 °C for 15 seconds and then was cooled to 42 °C before being packaged and refrigerated (4 °C ± 1 °C) for transport to the laboratory. For yoghurt production, all milk samples were heated to 42 °C in pre-sterilised conical flasks in a water bath (Grant JB NV, Cambridgeshire, UK) and held at that temperature throughout the yogurt fermentation process. All yogurts (A-E) were inoculated with specific starter cultures as indicated in Table 1. When inoculated, the milk was mixed thoroughly and the temperature was held at 42 °C. The pH was monitored until the yogurt fermentation reached between 4.4 – 4.6 pH units, then the fermentation was stopped by cooling the yogurts to 4 °C. All yoghurts were made in triplicate. The yogurts were then transferred to glass media bottles and stored at -20 °C until required for analysis or a maximum of six weeks.

2.3.Yogurt cultures

The bacterial cultures used to manufacture the yogurts detailed in Table 1 were obtained in freeze dried form. Mother culture solutions were produced from the freeze dried cultures. The cultures used for the production of yogurts were kindly provided by Chr-Hansen (Cork, Ireland) and Orchard Valley Dairy Supplies (Worcestershire, UK).

Insert Table 1 Here

156 *2.4.Extraction & isolation*

157 The total lipids (TL) of all yogurt samples and milk were extracted from 100 g of sample
158 according to the method of Bligh and Dyer (1959). One tenth of the TL was stored in sealed
159 vials at −20 °C, while the TL was then further separated into total neutral lipids (TNL) and
160 total polar lipids (TPL) by counter-current distribution (Galanos & Kapoulas, 1962). All lipid
161 extracts were stored devoid of solvent in sealed vials under a nitrogen atmosphere at -20 °C.
162 All extractions were carried out in triplicate.

163

164 *2.5. In vitro human biological assay*

165 Blood was obtained from healthy human volunteers (n = 12) as previously described
166 (Tsoupras, Lordan, Demuru, et al., 2018; Tsoupras, Zabetakis, & Lordan, 2019). The Ethics
167 Committee of the University of Limerick approved the protocol and it was performed in
168 accordance with the Declaration of Helsinki. Healthy donors were fully aware that their blood
169 samples were used in the study and written consent was provided to the specialised
170 phlebotomist. All fasting blood samples provided were from participants not receiving anti-
171 platelet therapy. A total of 50 ml of blood was drawn from the median cubital vein via
172 venepuncture using a 20 G safety needle into sodium citrate anticoagulant S-monovettes using
173 the aspiration method (0.106 mol/L in a 1:10 ratio of citrate to blood; Sarstedt Ltd., Wexford,
174 Ireland). For plasma isolation, blood was drawn into evacuated sodium citrate Monovettes
175 (0.106 mol/L in a 1:10 ratio of citrate to blood; Sarstedt Ltd., Wexford Ireland) and rested at
176 room temperature for 15 minutes followed by immediate centrifugation at 194 x g for 18
177 minutes at 24 °C with no brake applied in order to obtain the supernatant platelet-rich plasma
178 (PRP). A second centrifugation at 1500 x g for 20 minutes at 24 °C was carried out to obtain
179 the platelet-poor plasma (PPP). All centrifugations were processed using an Eppendorf 5702 R
180 centrifuge (Eppendorf Ltd., Stevenage, UK). The PRP was standardised to 500,000 platelets
181 μl^{-1} using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan), before analysis on a
182 Chronolog-490 two channel turbidimetric platelet aggregometer, coupled to the accompanying
183 AGGRO/LINK software package (Chronolog, Havertown, PA, USA). All analyses were
184 carried out within 2.5 hours of the initial blood draw and PRP was stored at 24 °C before use.
185 PAF and lipids samples were dissolved in a solution of BSA-saline (2.5 mg BSA/ml saline).
186 Prior to testing, 250 µl of PRP was added to an aggregometer cuvette at 37 °C with stirring at
187 1000 rpm, and was calibrated prior to testing using the PPP as a blank. PAF was added to the

cuvettes in order induce maximum reversible aggregation (2.6×10^{-8} M, final concentration in the cuvette), and 50% PAF-induced aggregation was calculated. Lipid samples were tested and IC_{50} values were calculated as previously described (Tsoupras, Lordan, Demuru, et al., 2018). All experiments were performed in triplicate using a different donor's blood for each replicate to ensure reproducibility following appropriate control tests of the solvents used on human platelets (saline and BSA-saline solution). The resulting IC_{50} values were expressed as a mean value of the mass of lipid (μg) in the cuvette ± standard deviation (SD). This procedure was informed by the guidelines for light transmission aggregometry by Cattaneo et al. (2013).

2.6. GC-MS analysis

Fatty acid methyl esters (FAME) were prepared using 35 mg of the TPL of the milk and yogurt samples in triplicate according to the method of Tsoupras, Lordan, Demuru, et al. (2018) with slight modifications. In brief, FAME were prepared using a solution of 0.5 M KOH in 90 % CH_3OH and extracted with *n*-hexane. The GC-MS fatty acid analysis was carried out according the internal standard method as previously described (Tsoupras, Lordan, Demuru, et al., 2018). The equation that described the curve was: $y = 0.0041x + 0.12$ with an $R^2 = 0.9969$, where the ratio of the area of the analyte peak to that of the internal standard (21:0) represents the y value for the above equation, subsequently the x value represents the analyte concentration of a selected fatty acid in the lipid sample to be tested. Separation of the FAME was achieved on an Agilent J&W DB-23 fused silica capillary column (60 m, 0.251 mm, i.d., 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) using a Varian 410-GC coupled to a Varian 210-MS equipped with a split/splitless injector (Agilent Technologies). The injector was set at 230 °C with a split ratio of 1:5. The carrier gas was high purity helium with a liner flow rate of 1 ml/min. The oven temperature was initially programmed to 100 °C for 5 min, raised to 240 °C at 3 °C/min, and finally held isothermal at 240 °C for 10 mins. FAME were identified using 37-component FAME standards mix (Sigma Aldrich, Wicklow, Ireland) by comparison of the retention times and mass spectra of relative peaks with the aid of the Varian Star Chromatography Workstation Version 6 software (Agilent Technologies) and a NIST library (Gaithersburg, MD, USA).

219 *2.7.Total DNA extraction from yogurt*

220 DNA was extracted from 15 ml yoghurt as described by Walsh et al. (2016) with slight
221 modifications. Yoghurt samples were centrifuged at 5,444 × g for 30 min at 4 °C to pellet the
222 microbial cells in the liquid. The cell pellet was resuspended in 200 µl of PowerBead solution
223 from the PowerSoil DNA Isolation kit (Cambio, Cambridge, United Kingdom). The
224 resuspended cells were transferred to a pre-heated (at 60 °C) PowerBead tube (Cambio,
225 Cambridge, United Kingdom). A 90 µl volume of 50 mg/ml lysozyme (Sigma-Aldrich, Dublin,
226 Ireland) and 50 µl of 100 U/ml mutanolysin (Sigma-Aldrich, Dublin, Ireland) were added, and
227 the sample was incubated at 60 °C for 15 min. A 28 µl volume of proteinase K (20 mg/ml;
228 Sigma-Aldrich, Dublin, Ireland) was added, and the sample was incubated at 60 °C for a further
229 15 min. DNA was then purified from the sample by the standard PowerSoil DNA Isolation kit
230 protocol (Cambio, Cambridge, United Kingdom).

231

232 *2.8. Whole-metagenome shotgun sequencing*

233 Whole-metagenome shotgun libraries were fragmented and adaptors and indices added
234 using the Illumina Nextera XT guide in accordance with manufacturer's instructions, except
235 that tagmentation time was increased from 5 min to 7 min. After indexing, the average fragment
236 size was assessed using an Agilent Bioanalyser High Sensitivity Assay (Agilent) and quantified
237 using a Qubit High Sensitivity assay (Life Technologies). Samples were then pooled
238 equimolarly and the final pool was quantified by quantitative PCR using the Kapa Library
239 Quantification Kit for Illumina (Roche). The pool was then sequenced on the Illumina MiSeq
240 sequencing platform in the Teagasc sequencing facility, with a 2 × 300 cycle V3 kit, in
241 accordance with standard Illumina sequencing protocols.

242

243 *2.9. Bioinformatics*

244 Shotgun metagenomic fastq files were processed as described previously (Walsh et al.,
245 2018). Briefly, raw fastq files were converted to unaligned bam files using SAMtools (H. Li et
246 al., 2009). Duplicate reads were subsequently removed using Picard Tools
247 (https://github.com/broadinstitute/picard). Next, low quality reads were removed using the
248 trimBWAstyle.usingBam.pl script from the Bioinformatics Core at UC Davis Genome Center
249 (https://github.com/genome/genome/blob/master/lib/perl/Genome/Site/TGI/Hmp/HmpSraPro

cess/trimBWAstyle.usingBam.pl). Specifically, reads were trimmed to 200 bp, while all reads with a quality score less than Q30 were discarded. The resulting fastq files were then converted to fasta files using the fq2fa option from IDBA-UD (Peng, Leung, Yiu, & Chin, 2012). Species-level analysis was performed using MetaPhlAn2 (Truong et al., 2015), which measures the abundance of species-specific marker genes in metagenomic reads. Microbial pathway analysis was performed using HUMAnN2 (Abubucker et al., 2012), which measures the abundances of UniRef clusters (Suzek et al., 2015) by aligning sequences against the ChocoPhlAn database. Bacteriocin genes were quantified by aligning reads against the BAGEL3 (van Heel, de Jong, Montalban-Lopez, Kok, & Kuipers, 2013) bacteriocin database using DIAMOND (Buchfink, Xie, & Huson, 2015). Hits against the BAGEL3 bacteriocin database were counted with SAMtools, after which the results were normalised as copies per million.

2.8. Statistical analysis

All biological experimental analyses were completed in triplicate, and the obtained results were expressed as a mean value ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed in order to find the significant statistical differences ($p < 0.05$) and Fisher's least significant difference (LSD) test was used to conduct multiple comparisons of the means (SPSS Inc., Chicago, 215 IL, USA). For the bioinformatics, statistical analysis was performed in R-3.2.2 (Team R Core, 2013). The vegan package (version 2.3.0) (Oksanen et al., 2007) was used for Bray-Curtis based multidimensional scaling (MDS) analysis. The ggplot2 package (version 2.2.1) (Wickham, 2016) was used for data visualisation.

3. Results

3.1. Lipid extraction

The resulting TL, TNL, and TPL of all yoghurt samples are presented in Table 2. It is clear from the extraction data that the milk TL is statistically significantly different from all but yoghurt C. The percentage of TNL in all yoghurts were not statistically significantly different from each other. The percentage of TPL of the milk was statistically significantly different from yoghurts A, B, and E, but similar to the other yoghurts. The percentage of TPL of all yoghurts were not statistically significantly different from each other. This agrees with previously published data relating to ovine milk and yoghurts (Balthazar et al., 2017;

Megalemou et al., 2017), where generally the polar lipid content is reported as relatively low (Lordan et al., 2017).

Insert Table 2 Here

3.2. GC-MS analysis

The fatty acid content of the TPL is shown in Table 3. As a result of fermentation, there seems to be a trend towards the reduction of PUFA levels and an increase of MUFA levels in the fatty acids of the polar lipids, whereas the levels of SFA vary depending on the yoghurt produced. Specifically, there is a statistically significant ($p < 0.05$) increase in the levels of 16:1, 17:0, 17:1, 18:2 (*cis*-10, *trans*-12), 20:4, and 22:5 when milk was fermented to yoghurt. Remarkably, across all yoghurts there was a statistically significant reduction in the levels of ω3 PUFA in the TPL as the milk was fermented to yoghurts. This data indicates that fermentation directly affects the fatty acid composition of milk polar lipids.

Insert Table 3 Here

3.3. Biological assay

The lipid extracts from the milk and yoghurt samples exhibited potent inhibition against PAF-induced platelet aggregation on human PRP *in vitro* (Table 4). The TNL of various yoghurts exhibited a poor inhibitory effect against platelet aggregation. However, some had moderate inhibitory effects. Similarly the TL exhibited moderate inhibitory effects against PAF-induced platelet aggregation. Notably, the TL of the yoghurts were considerably more bioactive than the TNL extracts but not as bioactive as the TPL extracts. This indicates that there is a synergistic effect between the TNL and TPL. It is clear from Table 4 that the TPL extract were the most inhibitory against PAF. Ovine milk TPL exhibited the highest IC_{50} values, indicating that this sample possessed the lowest antithrombotic activity. Yoghurts B and D possessed the lowest IC_{50} values, where the IC_{50} of yoghurt D was statistically significantly lower than all other samples tested.

Insert Table 4 Here

3.4. Yoghurt microbial composition

MetaPhlAn2, which measures the abundance of species-specific marker genes in shotgun metagenomic reads, was used to determine and confirm the composition of the yoghurts made with commercial starter cultures. The genera and species detected in the yoghurts and their relative abundances are depicted in Fig. 1. In all of the yoghurts produced, *S. thermophilus* was the most dominant species, *L. delbrueckii* subsp. *bulgaricus* was the second most abundant species in yoghurts A and B. *L. acidophilus* was the third most abundant species present in yoghurts B (4.8%) and C (6.2%) and was the second most abundant in yoghurt D (4.9%). *B. animalis* was the second most abundant species (6.9%) present in yoghurt C. In yoghurt E, *S. thermophilus* was the predominant species with *L. paracasei* present in low proportions (<1%) along with *E. durans* (1.9%).

Insert Fig. 1. Here

3.5. Gene composition of the yoghurts

Functional analysis of the shotgun metagenomic data was performed using HUMAnN2 (https://bitbucket.org/biobakery/humann2). The abundances of Gene Ontology (GO) and the abundance of level-4 EC categories of interest are presented in Fig. 2 and 3 respectively. The abundances of genes associated with phospholipid biosynthesis and metabolism were detected in all of the yoghurts. According the data in Fig. 2., the abundance of GO terms associated with polar lipid biosynthesis and metabolism are associated with the presence of *S. thermophilus* and *L acidophilus* indicating that these microbes have the capacity to alter the polar lipid composition of the milk and yoghurts. Similarly, the data in Fig. 3. indicates that both *S. thermophilus* and *L acidophilus* have the greatest capacity to biosynthesise fatty acids and phospholipids according to the abundance of level-4 EC categories of interest.

Insert Fig. 2. Here

Insert Fig. 3. Here

3.6. Bacteriocins

Bacteriocins are ribosomally synthesised antimicrobial peptides produced by several bacterial species that generally inhibit strains closely related to the producer in order to compete within their specific ecological niche (O'Shea, Cotter, Stanton, Ross, & Hill, 2012). However, their

mechanisms of action vary considerably due to their structural diversity. As depicted in Fig. 4., it is clear that the microbes present in the yoghurts possess the genetic capacity to produce a variety of type II and type III bacteriocins. Notably, yoghurt D, C, and E had a greater number of hits per class of bacteriocin, which may correlate with greater bioactivity against platelet aggregation in these yoghurts.

Insert Fig. 4. Here

4. Discussion

The microbial composition of the yoghurts were assessed following fermentation (Fig. 1A). *S. thermophilus* was the most dominant species in all of the yoghurts produced, followed by *L. delbrueckii* subsp. *bulgaricus*, which was the second most abundant species in yoghurts A and B. *L. acidophilus* was the third most abundant species present in yoghurt B (4.8%) and C (6.2%) and was the second most abundant in yoghurt D (4.9%). The second most abundant species present in yoghurt C was *B. animalis* (6.9%). Although *L. paracasei* was added to yoghurt E, this species was present in low proportions (< 1%) in the final yoghurt. Furthermore, *E. durans* was also detected in yoghurt E (1.9%). This is a non-pathogenic bacterial species of human, animal or environmental origin that is often identified in various dairy products and may be probiotic (Andrighetto et al., 2001). Its presence may be explained by the fact that enterococci are generally present in higher amounts in caprine and ovine milk (Del Pozo, Gaya, Medina, Rodríguez-Marín, & Nuñez, 1988) and *E. durans* in particular is resistant to damage by heat treatment (McAuley, Gobius, Britz, & Craven, 2012). It is unclear what prevented the growth of *L. paracasei*, as generally this species is grown in the presence of the other lactic acid bacteria present. However, temperature may play a role for its lower abundance as previous research indicates that this species tends to favour growth below 40 °C at an optimum of 37 °C (Collins, Phillips, & Zanoni, 1989), in contrast to the other organisms present, which require 42 °C according to the manufacturers guidelines. Irrespective of these possibilities, yoghurt E possessed a different microbial composition in comparison to the other yoghurts, which is characterised by a high proportion of *S. thermophilus*. The fact that yoghurt E had an IC_{50} lower than that of milk, indicates that fermentation of milk with *S. thermophilus* plays a significant role in the bioactivity of polar lipids present in these yoghurts.

Functional analysis of the shotgun metagenomics data was performed using HUMAnN2, which indicated that the microbial species present in all yoghurts had the

metabolic capacity to synthesise polar lipids and various fatty acids (Fig. 2.). It is already well documented that certain lactic acid bacterial strains have a distinctive phospholipid composition, that may be distinguishable between different genera (Exterkate, Otten, Wassenberg, & Veerkamp, 1971). In particular, based on the abundances of GO terms detected by HUMAnN2, *S. thermophilus* in yoghurt D seems to have the greatest capacity to synthesise fatty acids and phospholipids and possess the genes for other functions in relation to the metabolic processes of fatty acids and polar lipids including: phosphatidylserine decarboxylase activity (GO:0004609); phosphatidylethanolamine biosynthetic process (GO:0006631); glycerophospholipid metabolic process (GO:0006650); phospholipid biosynthetic process; (GO:0008654) cardiolipin synthase activity (GO:0008808); cardiolipin biosynthetic process (GO:0032049); acetyl-CoA carboxylase complex (GO:0009317); glycerol-3-phosphate cytidyltransferase activity (GO:0047348); biotin carboxylase activity (GO:0004075); lipid biosynthetic process (GO:0008610) (Fig. 2.). Several of the genes associated with anabolic processes that are crucial for the biosynthesis of various polar lipids and are present in varying amounts in each of the yoghurts.

In particular, it seems that *L. delbrueckii* subsp. *bulgaricus* has the genetic capacity to express CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase activity (GO:0008444), which is involved in the biosynthesis of phospholipids by catalysing the conversion of CDP-diacylglycerol and glycerol-3-phosphate to CMP and 3-(3-phosphatidyl)-glycerol 1-phosphate in the committed step to the synthesis of acidic phospholipids (Gaynor et al., 1991; Vance & Vance, 2008). Furthermore, the detection of GO terms associated with the phosphatidylethanolamine (PE) (GO:0006646) and cardiolipin (CL) (GO:0008808) biosynthesis is not unexpected due to their respective roles in the bacterial and mitochondrial membranes (Vance & Vance, 2008).

Interestingly, yoghurt D exhibited the greatest capacity for glycerol ether metabolic processes. As these lipids exhibit similar structures to PAF, yoghurts that contain these lipids may induce either agonistic or antagonistic effects, but both cardioprotective (Tsoupras, Lordan, & Zabetakis, 2018); structural elucidation of these polar lipid extracts will provide more information about the structure function relationship between these polar lipids and the PAF-R. Moreover, the detection of genes associated with the synthesis various phospholipid including PE and CL in the HUMAnN2 output may explain the levels of bioactivity detected in these yoghurt as these phospholipids have previously been associated with potent

antithrombotic properties in polar lipids of dairy products (Megalemou et al., 2017; Poutzalis et al., 2016).

The abundance of level-4 EC categories of interest was also assessed (Fig. 3.). It is clear that the microbes present in each of the yoghurts play a key role in the alteration of the overall fatty acid composition of the yoghurts. Several of the EC categories of interest detected are responsible for the biosynthesis of fatty acids (EC 6.4.1.2: Acetyl-CoA carboxylase), which although expected, are present in abundance. There are also numerous EC categories detected in abundance that are specific to phospholipid biosynthesis. For example glycerol-3-phosphate acyltransferase (EC 2.3.1.n3) and phosphate acyltransferase (EC 2.3.1.n2) are the rate limiting enzymes for phosphatidic acid synthesis, which is critical for the synthesis of phospholipids (Wendel, Lewin, & Coleman, 2009). Both of these EC terms were detected in abundance in yoghurt D, which corresponds with the yoghurt that exhibited significant changes in the TPL lipid composition and the most potent bioactivity. Similarly, the detection of phosphatidylserine decarboxylase (EC 4.1.1.65), glycerol-3-phosphate cytidyltransferase (EC 2.7.7.39), inositol-3-phosphate synthase (EC 5.5.1.4), and CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (EC 2.7.8.5) indicates that the starter cultures in these yoghurts have the ability to synthesise phospholipids. However, further research is required to establish to what extent these starter cultures influence the phospholipid content of these yoghurts and whether they are incorporated into the dairy matrix or remain bound intracellularly.

Systemic inflammation is mediated by proinflammatory molecules such as PAF, which leads to the development of chronic conditions such as atherosclerosis and subsequently CVD (Moss et al., 2018; Tsoupras, Lordan, & Zabetakis, 2018). Therefore, considering diet and lifestyle are key modifiable risk factors for the prevention of CVD, the formulation of novel nutraceutical and functional foods to combat these inflammatory processes is imperative. Ovine milk is an underutilised nutritious milk with the potential to be used for functional food development (Balthazar et al., 2017). Previous research indicates that polar lipids in ovine yoghurts possess anti-PAF activity (Megalemou et al., 2017) and that the fermentation of milk may affect the antithrombotic properties of these bioactive lipids (Lordan & Zabetakis, 2017a).

As demonstrated in table 2, following milk fermentation and depending on the starter culture used, the IC_{50} decreased when milk was fermented to yoghurt, indicating an enhancement of the antithrombotic activity of the polar lipids. All of the yoghurt TPL had an

IC_{50} value lower than 85 µg, indicating that they all possessed potent antithrombotic properties, which is within range of previous polar lipids of animal origin tested on human PRP *in vitro* (Poutzalis et al., 2018; Tsoupras, Lordan, Demuru, et al., 2018) and dairy derived polar lipids tested on washed rabbit platelets *in vitro* (Megalemou et al., 2017; Poutzalis et al., 2016; Tsorotioti et al., 2014). In particular, yoghurts B and D possessed the lowest IC_{50} values, where the IC_{50} of yoghurt D was statistically significantly ($p < 0.05$) lower than all other samples tested. These yoghurts in particular contained a higher abundance of *L. acidophilus*, which seems to correlate with greater biological activity against PAF-induced platelet aggregation. Notably, *L. acidophilus* has previously been associated with anti-inflammatory activities against PAF; soluble factors released by *L. acidophilus* have been shown to alleviate PAF-induced inflammation in human colonic NCM460 and Caco-2 cells by reducing nuclear factor kappa B (NF-κB) activation and IL-8 production (Borthakur et al., 2013). *L. acidophilus* has also demonstrated anti-inflammatory effects *in vivo* via impairing both the NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways (Haihua Li et al., 2016).

Research shows that the fatty acid composition of polar lipids affects their antithrombotic capacity against PAF (Lordan et al., 2017). Following GC-MS analysis (Table 3), it is evident that the microbial starter cultures play a key role in augmenting the polar lipid composition of ovine milk following bacterial fermentation, which in turn altered the antithrombotic capacity of the ovine milk polar lipids. Previous research has shown that as bovine or ovine milk is fermented to yoghurt, cheese, or *kefir*, the fatty acid composition changes due to lipolysis of existing milk lipids and synthesis of lipids by lactic acid bacteria (Florence et al., 2012; Guzel, Yibar, Belenli, Cetin, & Tanriverdi, 2017; Reguła, 2007; Vieira et al., 2015; Yadav, Jain, & Sinha, 2007). In comparison to the milk TPL, there were significant changes in the fatty acid composition of the TPL of all 5 yoghurts following fermentation with various starter cultures. Fermentation reduced the PUFA and increased the MUFA levels of the fatty acids of the polar lipids, whereas the levels of SFA varied depending on the yoghurt produced. Florence et al. (2012) demonstrated that increases in unsaturated fatty acids during milk fermentation was related to an improvement of *L. delbrueckii* subsp. *bulgaricus* growth and that the metabolism of various bacterial cultures modified the fatty acid profile of the milk.

There was a significant increase in the levels of 18:2 fatty acids in the TPL. Many of these 18:2 fatty acids are classed as conjugated linoleic acids (CLA) that are associated with various health benefits including anti-inflammatory (Lordan & Zabetakis, 2017a) and antithrombotic effects (Truitt, McNeill, & Vanderhoek, 1999). There was also a significant

increase in the levels of 20:4 and 22:5 in the polar lipid fatty acid composition of all the yoghurts, which may be associated with the enhanced antithrombotic activities of the yoghurts in contrast to the ovine milk. It is noteworthy that in marine products, it has been demonstrated that these ω3 PUFA are more bioactive when incorporated in a phospholipid structure rather than their free fatty acid forms (Lordan et al., 2017). Remarkably, the fatty acid composition of the polar lipids in yoghurt D contained several similarities to the classical PAF structure. PAF is generally composed of 16:0 (68 %), 18:0 (27 %), or 18:1 (4 %) at the *sn*-1 position (Demopoulos, Pinckard, & Hanahan, 1979), with acetic acid esterified to the *sn*-2 position, and phosphocholine group at the *sn*-3 position, whereas the three major fatty acids present in the polar lipids of yoghurt D were 16:0 (21.6 %), 18:0 (15.1 %), and 18:1 (23.0 %) (Demopoulos et al., 1979). However, further research is required to confirm if there is structural homology between these polar lipids and PAF.

Finally, as presented in Fig. 4. it seems that the capacity to produce bacteriocins, which are antimicrobial peptides produced by bacterial cultures, may correlate with greater antithrombotic activities. Some bacteriocins such as colicins, which is present in yoghurts D and E are often encoded with a lysis protein, which increases the permeability of the outer membrane of the producer organism and is lethal to the producing cells (Snijder & Dijkstra, 2000). Consequently, it is possible that bacteriocins may play a role in releasing phospholipids into the yoghurt matrix. Several bacteriocins, for example cinnamycin (Machaidze & Seelig, 2003), seem to demonstrate specificity for PE (Moll, Konings, & Driessen, 1999), which is the second most abundant phospholipid in most biological membranes (Lordan et al., 2017). Several bacteriocins have demonstrated selective binding towards negatively charged phospholipids on the membranes of cancer cells (Kaur & Kaur, 2015). Some bacteriocins do not seem to bind to neutral choline-containing zwitterionic PC molecules (Chatterjee, Paul, Xie, & van der Donk, 2005), and changes to the overall charge of phospholipids due to a change in the lipid composition is associated with bacteriocin resistance in some bacteria (Kuipers, Rink, & Moll, 2011), indicating that the phospholipid charge is a defining feature for bacteriocin specificity.

Because bacteriocins can permeabilise the phospholipid bilayer of microbial cells (Cotter, Hill, & Ross, 2005; O'Shea et al., 2012), it is possible that phospholipids from damaged or lysed bacterial cells may be released to the surrounding matrix, thus increasing their bioavailability. However, bacteriocins may also have the capacity to interact with the milk fat globule membrane (MFGM). Research has shown that when nisin was added to milk to reduce

the levels of microbial cells it became unavailable to destroy these cells but was bioavailable and active again when a detergent was added to permeabilise the MFGM (Jung, Bodyfelt, & Daeschel, 1992). Considering there is a wealth of evidence to suggest that bacteriocins can bind to various types of membranes, there is speculative evidence to suggest that the bacteriocins produced by the starter cultures in this study may interact with the MFGM increasing the levels of bioavailable phospholipids, however further research is required.

Overall, the present study has some limitations, and further research is required to reveal the molecular mechanisms by which polar lipids bind to the PAF-R and inhibit the proinflammatory actions of PAF. While the data relating to the bacteriocins is promising, further research is required to confirm these observations. In addition, clinical studies are required to assess the bioavailability of the antithrombotic polar lipids following consumption of the antithrombotic ovine yoghurts.

5. Conclusions

This study confirms that specific starter cultures can alter the fatty acid composition of dairy polar lipids during fermentation through the lipolysis and biosynthesis of fatty acids. By altering the polar lipid composition, the antithrombotic properties of these yoghurts have been enhanced. Further research is required to discern the exact polar lipid structures responsible for these bioactivities and how fermentation influences the phospholipid structure of milk polar lipids. Shotgun metagenomic characterisation of the yoghurts indicates that the use of *L. acidophilus* and *S. thermophilus* plays a key role in improving the antithrombotic properties of these yoghurts. Moreover, functional analysis indicates that the starter cultures present in these yoghurts have the metabolic capacity to synthesise and alter various polar lipids, therefore further research is required to discern whether these polar lipids are bioavailable in human studies. The presence of bacteriocin related genes in some of the most bioactive yoghurts also warrants further investigation to reveal if there are potential interactions between bacteriocins and the MFGM. In addition, structural elucidation of these antithrombotic polar lipids and the optimisation of the fermentation process may allow for the enhancement of the antithrombotic and anti-inflammatory health benefits of these ovine yoghurts. Similarly, further studies are required to assess the use of various milk sources and animal diets that may alter the milk polar lipid composition and antithrombotic properties. This study highlights that ovine milk and

yoghurts may have beneficial effects for human cardiovascular health and may lead to the future development of functional foods and nutraceuticals.

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Author contributions

R.L. and I.Z. conceived and designed the study. R.L. performed the experiments. A.M.W., F.C., L.F., and P.D.C. performed the sequencing. R.L. and A.M.W. analysed the data and wrote the manuscript. All authors approved the manuscript.

Supplementary data

https://www.ebi.ac.uk/ena/data/view/PRJEB30083

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Figure Legend

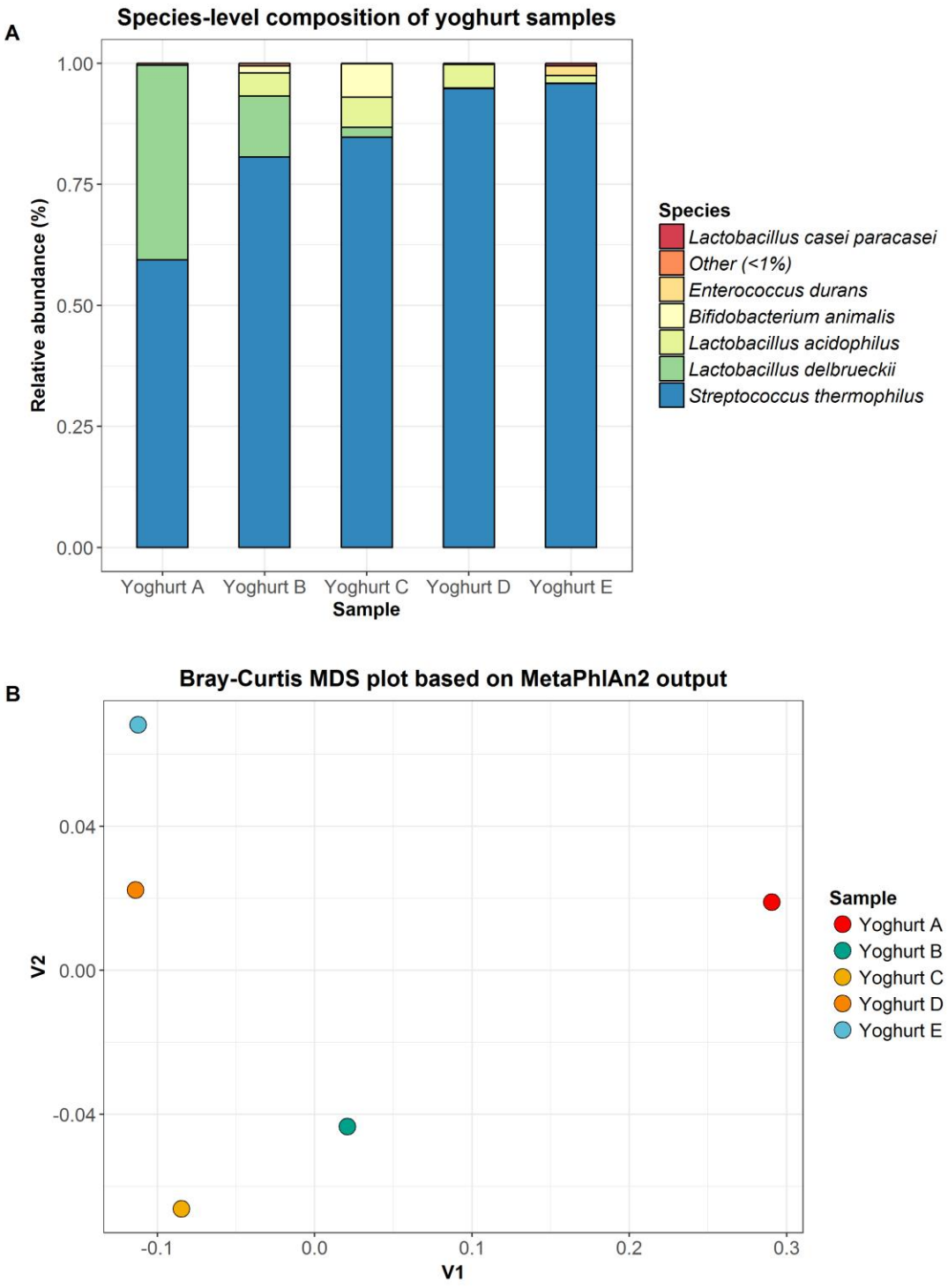
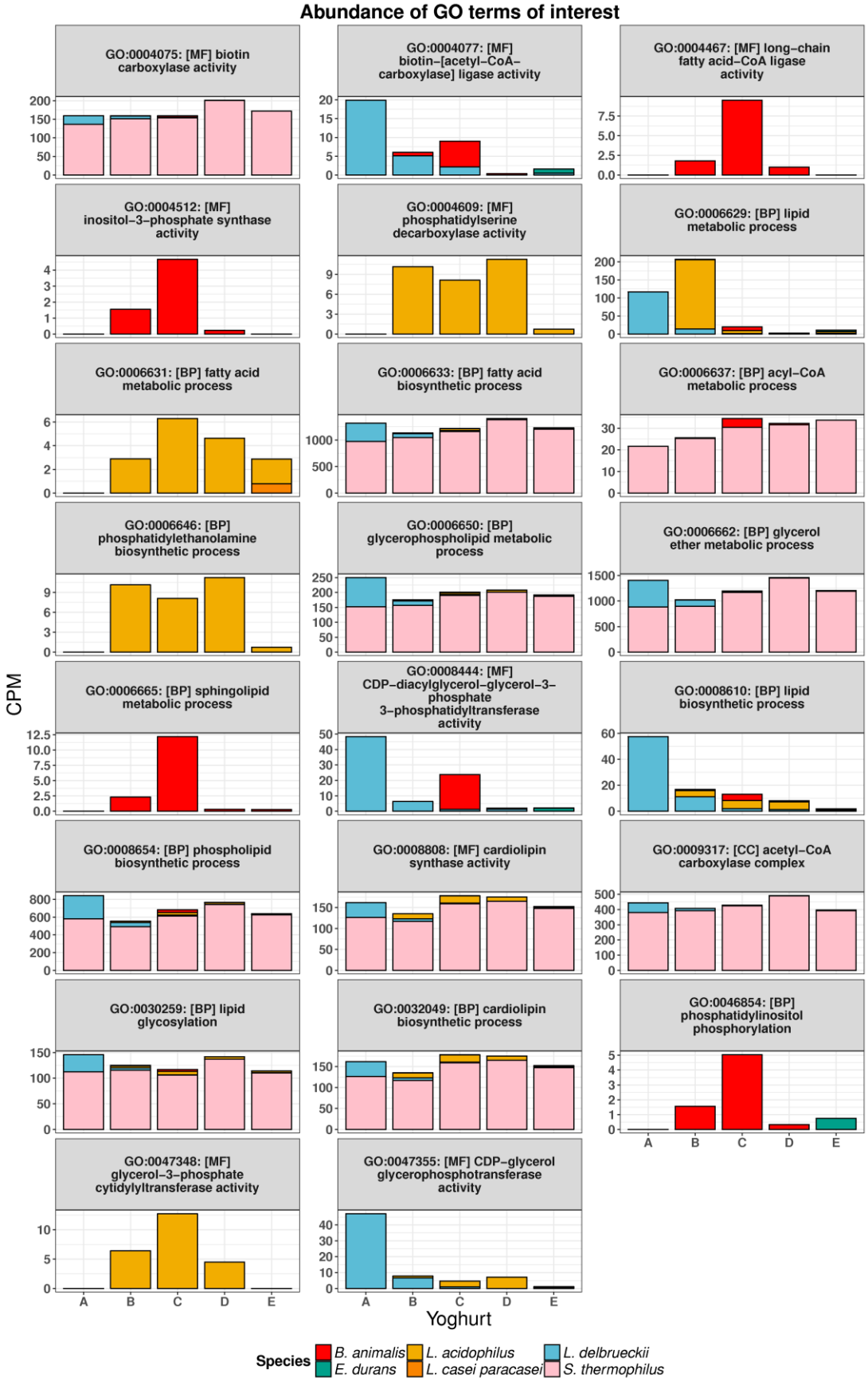


Fig. 1. (A) a stacked bar chart presenting the species-level microbial profile of yoghurts A-E as determined by 16s rRNA gene sequencing. (B) A Bray-Curtis MDS plot based on the MetaPhlAn2 output on the right that demonstrates that the microbial composition of yoghurts D and E are similar, yoghurts B and C are more similar to each other, whereas the microbial composition of yoghurt A is dissimilar to all the other yoghurts.



860 **Fig. 2.** The depicts the abundance of GO terms of interest in each yoghurt according to the
861 corresponding bacterium associated with the GO term using HUMAnN2 output.

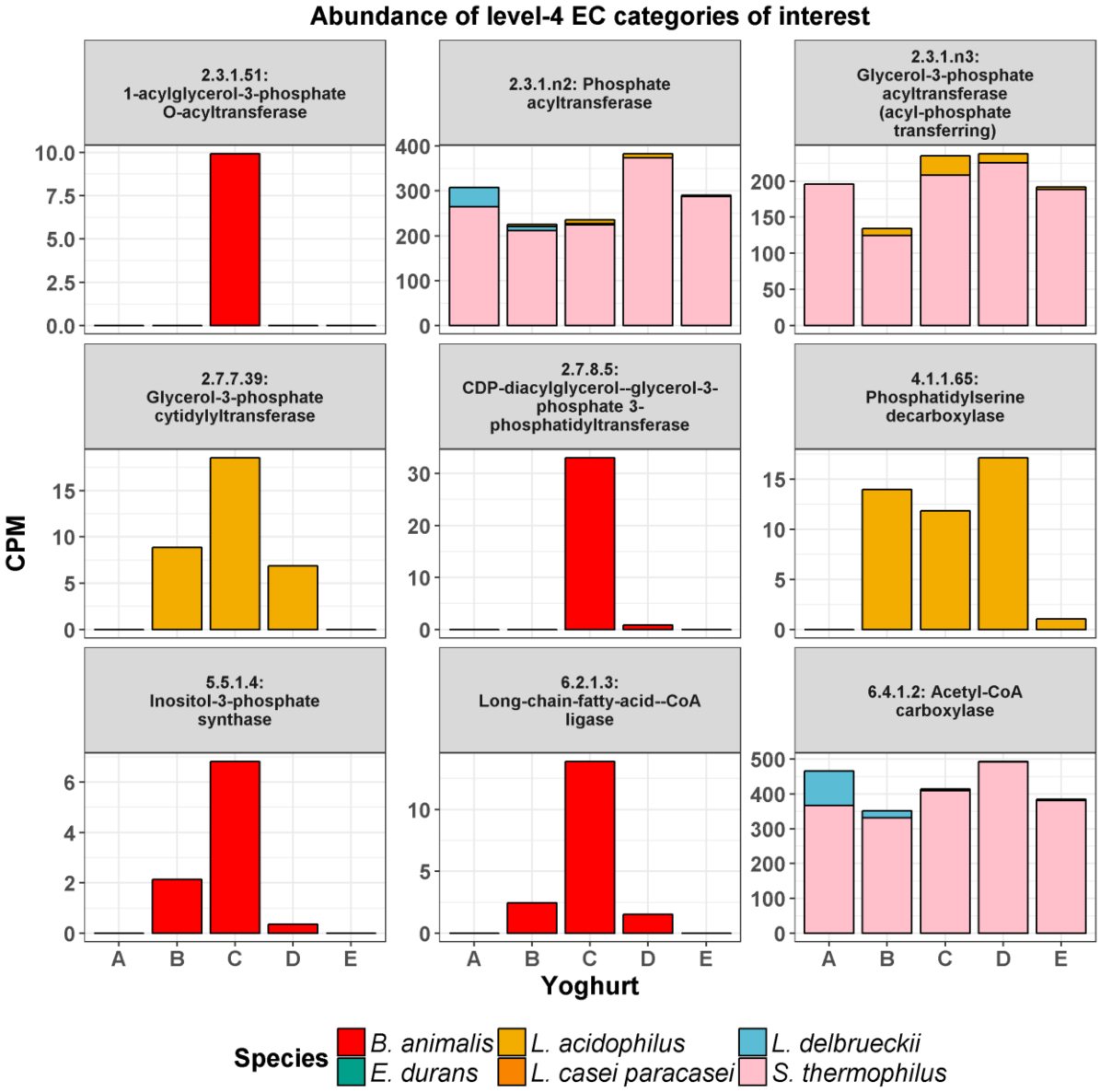


Fig. 3. The abundance of level-4 EC categories of interest in each yoghurt according to the corresponding bacterium associated with the GO term using HUMAnN2 analysis.

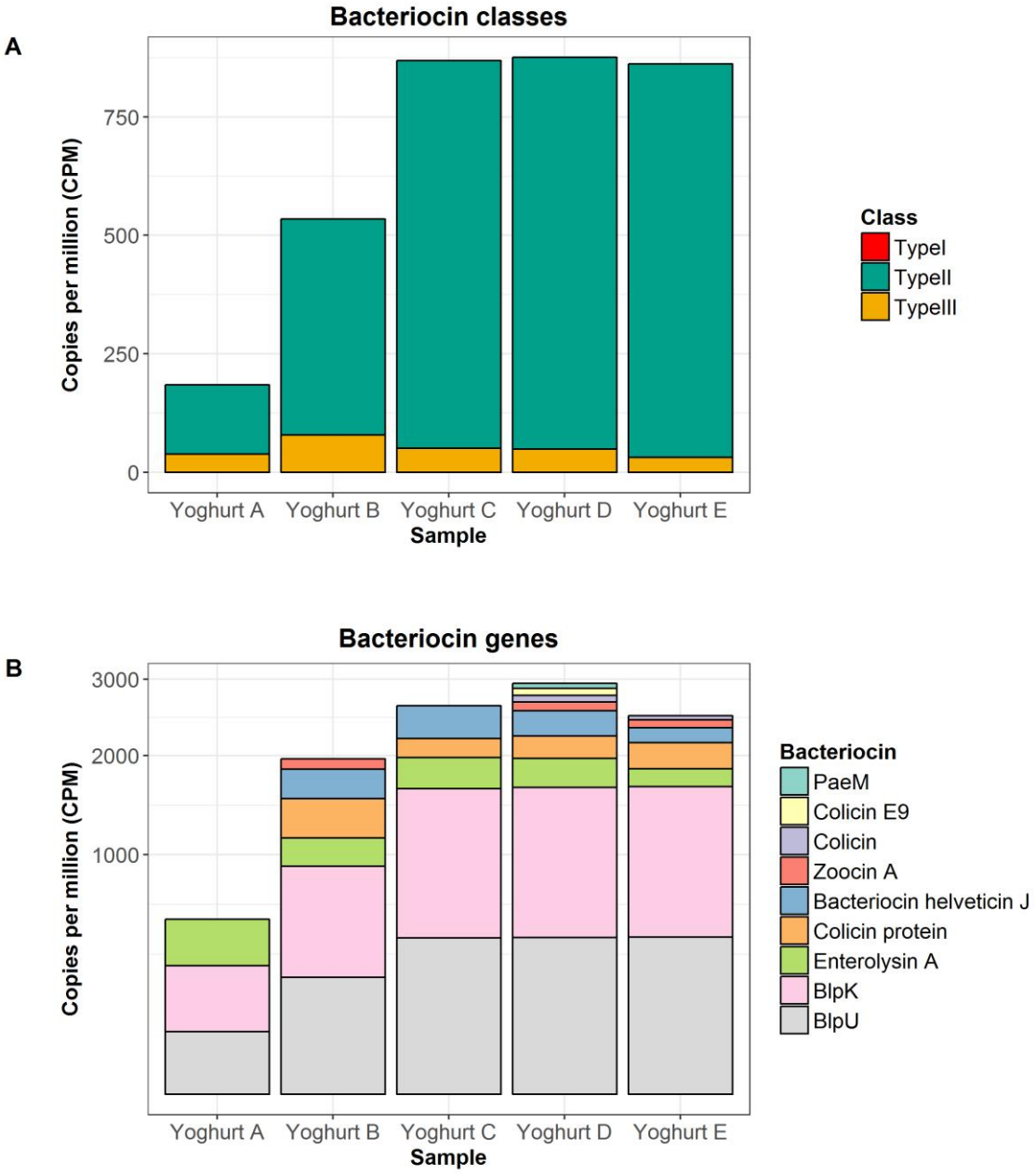


Fig. 4. (A) The total number of hits per class of bacteriocin for yoghurts A-E. Type II bacteriocins are in a greater abundance in all yoghurt samples. (B) A breakdown of the most abundant bacteriocin genes detected in each yoghurt.

878 **Table 1**

879 The composition of the starter cultures used in the inoculation of ovine milk to produce
880 yogurts A-E.

Yogurt	Cultures
A	0.020 % w/v *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (YC-380, Chr. Hansen, Denmark).
B	0.015 % w/v *Streptococcus thermophilus, Lactobacillus delbrueckii, subsp. bulgaricus, Lactobacillus acidophilus,* and *Bifidobacterium animalis* subsp. *lactis* (YOMIX^{TM}-205 LYO 250 DCU, Danisco, Denmark).
C	0.015 % w/v *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus acidophilus,* and *Bifidobacterium animalis* subsp. *lactis* (YOMIX^{TM}-205 LYO 250 DCU, Danisco, Denmark) with an additional 0.020 % w/v *Bifidobacterium animalis* subsp. *lactis* (BB-12, Chr. Hansen, Denmark).
D	0.015 % w/v *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* (YOMIX^{TM}-205 LYO 250 DCU, Danisco, Denmark) with an additional 0.020 % w/v *Lactobacillus acidophilus* (LA-5, Chr. Hansen, Denmark).
E	0.015 % w/v *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* (YOMIX^{TM}-205 LYO 250 DCU, Danisco, Denmark),with an additional 0.020 % w/v *Lactobacillus paracasei* subsp. *paracasei* (L-431, Chr. Hansen, Denmark).

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Table 2

Content of total lipids (TL), expressed in grams per 100g of sheep milk and yoghurts (mean ± SD, n = 3), total polar lipids (TPL), and total neutral lipids (TNL), expressed as percentages of TL in the sheep milk and yoghurt samples (mean ± SD, n = 3).

Sample	TL (g/100g)	TNL (%TL)	TPL (%TL)
Sheep Milk	5.28 ± 0.37^{a}	95.15 ± 2.30^{a}	3.20 ± 0.56^{b}
Yoghurt A	8.10 ± 0.43^{b}	96.46 ± 1.07^{a}	2.45 ± 0.20^{ab}
Yoghurt B	8.23 ± 1.59^{bc}	97.62 ± 0.22^{a}	2.29 ± 0.17^{a}
Yoghurt C	7.23 ± 0.60^{b}	97.47 ± 0.53^{a}	2.10 ± 0.37^{a}
Yoghurt D	7.47 ± 0.36^{b}	97.34 ± 0.47^{a}	2.25 ± 0.10^{a}
Yoghurt E	9.20 ± 0.55^{bc}	97.60 ± 0.38^{a}	2.55 ± 0.45^{ab}

[ab]Different superscripts indicate significant differences among different yoghurt samples within the same lipid classes when means are compared using a Fisher's LSD multiple comparison test ($p < 0.05$).

Table 3

Fatty acid profile of total polar lipids (TPL) of milk and each yoghurt expressed in percentage (%) of total fatty acids of each sample (mean ± SD, n = 3). Total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are shown as a percentage of total lipid.

Fatty Acids	A	B	C	D	E	Milk
8:0	ND	0.22 ± 0.14^{a}	0.35 ± 0.08^{a}	ND	0.25 ± 0.07^{a}	0.76 ± 0.66^{a}
9:0	ND	0.12 ± 0.05^{a}	ND	ND	ND	0.14 ± 0.07^{a}
10:0	3.14 ± 0.32^{b}	4.90 ± 0.89^{c}	4.66 ± 0.56^{c}	1.60 ± 0.12^{a}	3.35 ± 0.65^{bc}	5.58 ± 1.78^{c}
10:1	0.17 ± 0.03^{b}	0.22 ± 0.06^{bc}	0.34 ± 0.13^{c}	0.07 ± 0.01^{a}	0.20 ± 0.04^{bc}	0.28 ± 0.11^{bc}
11:0	0.13 ± 0.02^{a}	0.29 ± 0.06^{bc}	0.14 ± 0.12^{abc}	0.20 ± 0.01^{b}	0.17 ± 0.03^{ab}	0.30 ± 0.08^{c}
12:0	3.90 ± 0.50^{a}	5.06 ± 0.82^{ab}	5.51 ± 0.85^{b}	5.20 ± 0.32^{b}	4.54 ± 0.54^{ab}	4.93 ± 0.73^{ab}
12:1	0.15 ± 0.05^{bc}	0.12 ± 0.05^{ab}	0.12 ± 0.07^{abc}	0.20 ± 0.01^{c}	0.20 ± 0.01^{c}	0.06 ± 0.01^{a}
13:0	0.21 ± 0.02^{a}	0.32 ± 0.08^{bc}	0.31 ± 0.02^{b}	0.36 ± 0.02^{c}	0.18 ± 0.03^{a}	0.32 ± 0.05^{bc}
14:0	8.20 ± 0.59^{a}	11.38 ± 0.28^{c}	13.89 ± 1.17^{d}	11.49 ± 0.78^{c}	9.83 ± 0.96^{ab}	8.87 ± 1.00^{ab}
14:1ω7 c9	0.28 ± 0.08^{abc}	0.34 ± 0.01^{c}	0.32 ± 0.05^{bc}	0.42 ± 0.15^{bc}	0.31 ± 0.01^{b}	0.19 ± 0.01^{a}
15:0	1.33 ± 0.08^{b}	1.93 ± 0.22^{a}	1.31 ± 0.39^{abc}	1.70 ± 0.20^{c}	1.39 ± 0.07^{b}	1.56 ± 0.10^{c}
16:0	17.53 ± 0.32^{a}	19.87 ± 0.48^{b}	20.43 ± 0.65^{bc}	21.62 ± 0.60^{c}	19.53 ± 1.56^{bc}	17.20 ± 0.76^{a}
16:1ω7 c9	2.00 ± 0.14^{c}	2.53 ± 0.35^{d}	1.40 ± 0.18^{b}	2.09 ± 0.31^{cd}	1.71 ± 0.66^{bcd}	0.88 ± 0.06^{a}
17:0	0.90 ± 0.01^{c}	1.07 ± 0.15^{d}	0.75 ± 0.09^{b}	0.96 ± 0.05^{bd}	0.96 ± 0.27^{bcd}	0.45 ± 0.01^{a}
17:1	0.61 ± 0.01^{c}	0.67 ± 0.13^{c}	0.27 ± 0.17^{b}	0.65 ± 0.12^{c}	0.72 ± 0.30^{bc}	0.02 ± 0.01^{a}
18:0	10.93± 0.68^{b}	11.25 ± 1.25^{b}	15.76 ± 2.64^{c}	15.13 ± 1.17^{c}	8.25 ± 0.27^{a}	11.32 ± 0.72^{b}
18:1ω9 c9	35.56 ± 1.40^{c}	27.62 ± 1.36^{b}	23.61 ± 0.31^{ab}	22.97 ± 1.63^{a}	30.53 ± 3.74^{bc}	23.59 ± 3.56^{ab}
18:2ω6 c9, t12	6.31 ± 0.02^{c}	5.67 ± 0.67^{abc}	4.89 ± 0.33^{a}	5.95 ± 0.06^{b}	5.50 ± 1.70^{abc}	9.23 ± 0.64^{d}
18:2ω7 c9, t11	3.51 ± 0.16^{d}	1.94 ± 0.12^{ab}	1.47 ± 0.35^{a}	2.00 ± 0.06^{b}	3.29 ± 0.60^{d}	2.56 ± 0.02^{c}
18:2ω6 t10, c12	0.46 ± 0.35^{b}	ND	0.05 ± 0.01^{a}	ND	0.93 ± 0.18^{b}	ND
18:3ω3 c9, c12, c15	2.15 ± 0.01^{d}	1.44 ± 0.16^{b}	0.98 ± 0.16^{a}	1.83 ± 0.05^{c}	2.66 ± 0.13^{f}	2.30 ± 0.16^{e}
20:0	ND	0.31 ± 0.01^{a}	0.24 ± 0.15^{a}	0.29 ± 0.11^{a}	ND	ND
20:1ω9 c9	0.65 ± 0.04^{a}	0.79 ± 0.06^{b}	0.74 ± 0.14^{ab}	0.87 ± 0.04^{b}	0.79 ± 0.44^{abc}	1.01 ± 0.03^{c}
20:3ω9 c5, c8, c11	ND	0.24 ± 0.01^{a}	0.37 ± 0.12^{a}	0.20 ± 0.13^{a}	0.32 ± 0.18^{a}	ND
20:4ω6 c5, c8, c11, c14	0.95 ± 0.15^{c}	0.49 ± 0.04^{a}	0.61 ± 0.10^{b}	0.57 ± 0.01^{b}	1.20 ± 0.36^{c}	ND
20:5ω3 c5, c8, c11, c14, c17	0.35 ± 0.03^{c}	0.25 ± 0.01^{a}	0.22 ± 0.08^{ab}	0.29 ± 0.01^{b}	0.42 ± 0.05^{d}	3.15 ± 0.30^{e}
22:1 c11	0.53 ± 0.05^{a}	0.82 ± 0.15^{bc}	0.56 ± 0.22^{ab}	1.00 ± 0.04^{c}	0.72 ± 0.21^{ab}	0.89 ± 0.11^{b}
22:4ω6 c7, c10, c13, c16	ND	0.14 ± 0.02^{a}	0.25 ± 0.17^{ab}	ND	0.44 ± 0.04^{b}	ND
22:5ω6 c4, c7, c10, c13, c16	1.62 ± 0.30^{b}	0.97 ± 0.11^{a}	0.96 ± 0.20^{a}	1.54 ± 0.07^{b}	1.67 ± 0.14^{b}	ND
22:6ω3 c4, c7, c10, c13, c16, c19	0.91 ± 0.37^{c}	0.28 ± 0.03^{a}	0.61 ± 0.36^{abc}	0.44 ± 0.04^{b}	0.58 ± 0.10^{b}	0.97 ± 0.04^{c}
Total SFA	46.27 ± 1.54^{a}	56.72 ± 1.33^{c}	63.31 ± 2.62^{d}	58.56 ± 0.89^{c}	48.44 ± 3.88^{ab}	54.01 ± 4.28^{bc}
Total MUFA	39.96 ± 1.37^{c}	32.90 ± 1.06^{b}	27.50 ± 0.48^{a}	27.90 ± 0.62^{a}	35.18 ± 3.84^{bc}	27.35 ± 3.35^{a}
Total ω7	5.782 ± 0.22^{c}	4.805 ± 0.49^{b}	3.196 ± 0.48^{a}	4.519 ± 0.47^{b}	5.309 ± 0.65^{bc}	3.638 ± 0.07^{a}
Total ω9	36.22 ± 1.39^{c}	28.65 ± 1.42^{b}	24.72 ± 0.27^{a}	24.05 ± 1.73^{a}	31.64 ± 4.03^{bc}	24.60 ± 3.56^{ab}
Total PUFA	16.37 ± 0.60^{c}	11.46 ± 0.97^{a}	10.42 ± 0.29^{a}	12.83 ± 0.18^{b}	17.01 ± 1.56^{cd}	18.22 ± 1.17^{d}
Total ω3	3.431 ± 0.38^{c}	1.970 ± 0.16^{a}	1.819 ± 0.36^{a}	2.562 ± 0.07^{b}	3.662 ± 0.17^{c}	6.420 ± 0.45^{d}
Total ω6	9.345 ± 0.38^{c}	7.270 ± 0.68^{a}	6.763 ± 0.26^{a}	8.057 ± 0.01^{b}	9.738 ± 1.17^{c}	9.228 ± 0.64^{c}

^{abcdef} Mean values (n = 3), ± standard deviation with different letters in the same row indicating statistical significant differences when means are compared using Fisher's LSD multiple comparison test ($p < 0.05$). ND: non-detectable

Table 4

Inhibition of PAF-induced platelet aggregation in human PRP by sheep milk and yogurts total lipids (TL), total polar lipids (TPL), and total neutral lipids (TNL) produced by various starter cultures. This activity is represented by their IC_{50} (µg) (mean ± SD, n = 3).

Yogurt	TL	TNL	TPL
A	306.5 ± 64.1^{ab}	2938 ± 123^{a}	77.00 ± 9.20^{c}
B	331.7 ± 27.3^{b}	738.6 ± 37.7^{b}	57.41 ± 5.93^{b}
C	253.9 ± 73.1^{ab}	ND	70.72 ± 3.95^{c}
D	224.5 ± 21.4^{a}	640.9 ± 34.0^{c}	44.84 ± 4.96^{a}
E	263.8 ± 55.5^{ab}	ND	68.10 ± 7.55^{bc}
Milk	378.0 ± 12.8^{c}	ND	154.4 ± 12.8^{d}

^{abcd} Mean values (n = 3), ± standard deviation with different letters in the same column indicating statistical significant differences when means are compared using Fisher's LSD multiple comparison test ($p < 0.05$). ND: not-detectable