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Authors	Lordan, Ronan;Walsh, Aaron M.;Crispie, Fiona;Finnegan, Laura;Cotter, Paul D.;Zabetakis, Ioannis
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University College Cork, Ireland Coláiste na hOllscoile Corcaigh

- **1** 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*}
- ⁴ ^a Department of Biological Sciences, University of Limerick, Co. Limerick, Ireland
- ^b Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland
- ⁶ ^c Alimentary Pharmabiotic Centre, University College Cork, Co. Cork, Ireland
- 7 * Corresponding Author: <u>Ioannis.Zabetakis@ul.ie</u> and <u>Ronan.Lordan@ul.ie</u>
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9 ABSTRACT

The effect of fermentation on the antithrombotic properties of polar lipids in ovine milk has 10 been assessed through the production of yoghurts. The total lipids (TL), total neutral lipids 11 (TNL), and total polar lipids (TPL) were extracted. The fatty acid profiles of all yoghurt polar 12 13 lipids were analysed by GC-MS. The levels of MUFA increased, but there was a reduction in 14 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 15 exhibited potent antithrombotic activities with IC_{50} values ranging from 45–77 µg. Shotgun 16 metagenomics determined the species-level microbial composition and functional potential of 17 the yoghurts. Yoghurts containing L. acidophilus seem to correlate with greater bioactivity. 18 19 Several phospholipid biosynthetic genes have been identified in the most antithrombotic yoghurts. This study has demonstrated that fermentation enhances the antithrombotic 20 properties of yoghurt polar lipids against PAF. 21

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- 28 Keywords: polar lipids; fermentation; yoghurt; inflammation; antithrombotic metagenomics
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30 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
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40 **1. Introduction**

Maladaptive diet and lifestyle play a significant role in the development of chronic diseases 41 42 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, 43 44 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 45 46 Organization, 2017), of which dietary risk factors accounted for 49% of all CVD deaths (Meier 47 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), 48 which can increase cholesterol levels (Lamarche et al., 2016; Lordan & Zabetakis, 2017a). 49 Therefore, low-fat or non-fat dairy products were encouraged by many dietary 50 recommendations in order to reduce to lower cholesterol levels (Lordan, Tsoupras, Mitra, et 51 al., 2018). However, recent research indicates that dairy products may be neutral or even 52 beneficial for cardiovascular health, and may not have significant effects on blood cholesterol 53 levels (Labonté, Couture, Richard, Desroches, & Lamarche, 2013; Lordan, Tsoupras, Mitra, et 54 al., 2018; Lordan & Zabetakis, 2017a; Thorning et al., 2016). Further evidence indicates that 55 56 fermented dairy products may be more beneficial for health than non-fermented dairy products, 57 especially against a number of cardiometabolic risk factors such as hypertension, cholesterol levels, and impaired glucose tolerance (Lordan, Tsoupras, Mitra, et al., 2018). 58

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, & 61 Bes-Rastrollo, 2017; Wu & Sun, 2017). Bovine milk accounts for 85% of the total global milk 62 production (Balthazar et al., 2017), however ovine milk and yoghurts provide a superior 63 nutritional alternative. Ewe's milk and dairy products are not commonly consumed outside of 64 the Mediterranean basin, and are considered a delicacy in many countries (Lordan & Zabetakis, 65 66 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 67 al., 2017; Lordan, Tsoupras, Mitra, et al., 2018). The most predominant fatty acid in ovine milk 68 69 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, 70 whereas high-density lipoprotein (HDL) cholesterol levels are not significantly affected 71 (Lordan & Zabetakis, 2017b; Molkentin, 2000). It has recently be shown that ovine yoghurt 72 consumption does not affect the lipid profile of healthy individuals (Olmedilla-Alonso et al., 73 74 2017). In addition, a recent crossover study has demonstrated a moderate attenuation of several 75 inflammatory markers in participants with a high total cholesterol/HDL cholesterol ratio 76 following ovine yoghurt consumption (Redondo et al., 2018). Evidently, these neutral effects on serum cholesterol levels, putative anti-inflammatory effects, and antithrombotic effects 77 78 (Megalemou et al., 2017; Tsorotioti et al., 2014) indicate that ovine dairy products may be 79 beneficial for human cardiovascular health upon consumption and thus warrant further 80 investigation (Lordan, Tsoupras, Mitra, et al., 2018).

81 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 82 is approximately 9.4-35.5 mg/100g of raw milk. The polar lipid fraction contains 83 phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), which 84 85 are present in abundance with lower quantities of phosphatidylserine (PS) and phosphatidylinositol (PI), which is comparable to other ruminant species (Lordan, Tsoupras, 86 & Zabetakis, 2017; Park, 2009). Although the phospholipid fraction of ovine milk is 87 quantitatively a minor constituent of the overall lipid content, it possesses techno-functional 88 and nutritional properties that are implicated in several physiological processes and are 89 beneficial for health (Lordan et al., 2017). 90

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 94 proinflammatory phospholipid mediator that is implicated in all stages of atherosclerosis that 95 can lead to a major cardiovascular event. PAF and PAF-like molecules act solely through their 96 binding to a unique G-protein coupled seven transmembrane receptor known as the PAF-97 receptor (PAF-R), that subsequently triggers multiple intracellular pathways (Castro Faria 98 Neto, Stafforini, Prescott, & Zimmerman, 2005; Lordan et al., 2017; Tsoupras, Lordan, & 99 100 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 101 102 (Lordan et al., 2017; Palur Ramakrishnan, Varghese, Vanapalli, Nair, & Mingate, 2017). Therapeutic approaches to the proinflammatory effects of PAF focus on disrupting PAF/PAF-103 R interactions through competitive and non-competitive displacement of PAF from the receptor 104 (Lordan, Tsoupras, & Zabetakis, 2018). Dietary PAF inhibitors have been identified in the 105 polar lipids of marine (Lordan et al., 2017; Sioriki, Smith, Demopoulos, & Zabetakis, 2016), 106 meat (Poutzalis, Lordan, Nasopoulou, & Zabetakis, 2018), and dairy sources (Megalemou et 107 al., 2017; Poutzalis et al., 2016). In particular, ovine dairy products possess potent PAF 108 109 inhibitors (Tsorotioti et al., 2014).

110 It has been postulated that fermentation increases the bioactivity of phospholipids against PAF (Antonopoulou, Semidalas, Koussissis, & Demopoulos, 1996; Lordan & 111 Zabetakis, 2017a). However, to date, this has not been definitively established. Thus, the aim 112 of this study was to evaluate the effect of bacterial fermentation on the polar lipid composition 113 and antithrombotic activity of ovine milk and yoghurts via PAF-induced platelet aggregation 114 on human platelets in vitro. Furthermore, shotgun metagenomics was employed to characterise 115 the species-level microbial composition of the yoghurts, and to determine if the detected 116 species contained genes associated with fatty acid and/or lipid metabolism. 117

118

119 2. Materials and methods

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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).

- 131
- 132 2.2. Milk processing & yogurt production

A fresh, commercial, pasteurised, and homogenised ewe's whole milk was obtained from 133 Rockfield Dairy Ltd. (Claremorris, Co Mayo, Ireland). Milk was obtained from a bulk tank 134 containing milk from the Friesland and Lacaune breed of dairy ewe, between March and July 135 2016. The sheep were fed a forage based diet consisting of mainly grass silage or fresh grass, 136 supplemented with cereal at the time of milking, which is typical of the small sheep dairy 137 industry in Ireland; however, atypical in Europe where the diet mainly consists of cereal. The 138 collected milk was pasteurised on site by heating to 91 °C for 15 seconds and then was cooled 139 to 42 °C before being packaged and refrigerated (4 °C \pm 1 °C) for transport to the laboratory. 140 For yoghurt production, all milk samples were heated to 42 °C in pre-sterilised conical flasks 141 in a water bath (Grant JB NV, Cambridgeshire, UK) and held at that temperature throughout 142 the yogurt fermentation process. All yogurts (A-E) were inoculated with specific starter 143 cultures as indicated in Table 1. When inoculated, the milk was mixed thoroughly and the 144 145 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. 146 All yoghurts were made in triplicate. The yogurts were then transferred to glass media bottles 147 148 and stored at -20 °C until required for analysis or a maximum of six weeks.

149

150 *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).

155 *Insert Table 1 Here*

156 2.4. Extraction & isolation

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

163

164 2.5. In vitro human biological assay

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

cuvettes in order induce maximum reversible aggregation (2.6 x 10^{-8} M, final concentration in 188 the cuvette), and 50% PAF-induced aggregation was calculated. Lipid samples were tested and 189 IC₅₀ values were calculated as previously described (Tsoupras, Lordan, Demuru, et al., 2018). 190 All experiments were performed in triplicate using a different donor's blood for each replicate 191 to ensure reproducibility following appropriate control tests of the solvents used on human 192 platelets (saline and BSA-saline solution). The resulting IC_{50} values were expressed as a mean 193 194 value of the mass of lipid (μ g) in the cuvette \pm standard deviation (SD). This procedure was informed by the guidelines for light transmission aggregometry by Cattaneo et al. (2013). 195

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197 *2.6. GC-MS analysis*

Fatty acid methyl esters (FAME) were prepared using 35 mg of the TPL of the milk and 198 yogurt samples in triplicate according to the method of Tsoupras, Lordan, Demuru, et al. (2018) 199 with slight modifications. In brief, FAME were prepared using a solution of 0.5 M KOH in 90 200 % CH₃OH and extracted with *n*-hexane. The GC-MS fatty acid analysis was carried out 201 according the internal standard method as previously described (Tsoupras, Lordan, Demuru, et 202 al., 2018). The equation that described the curve was: y = 0.0041x + 0.12 with an $R^2 = 0.9969$, 203 where the ratio of the area of the analyte peak to that of the internal standard (21:0) represents 204 the y value for the above equation, subsequently the x value represents the analyte 205 concentration of a selected fatty acid in the lipid sample to be tested. Separation of the FAME 206 207 was achieved on an Agilent J&W DB-23 fused silica capillary column (60 m, 0.251 mm, i.d., 208 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 209 210 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, 211 212 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 213 214 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 215 216 library (Gaithersburg, MD, USA).

217

219 2.7. Total DNA extraction from yogurt

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

231

232 2.8. Whole-metagenome shotgun sequencing

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

242

243 2.9. Bioinformatics

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

cess/trimBWAstyle.usingBam.pl). Specifically, reads were trimmed to 200 bp, while all reads 250 with a quality score less than Q30 were discarded. The resulting fastq files were then converted 251 to fasta files using the fq2fa option from IDBA-UD (Peng, Leung, Yiu, & Chin, 2012). Species-252 level analysis was performed using MetaPhlAn2 (Truong et al., 2015), which measures the 253 abundance of species-specific marker genes in metagenomic reads. Microbial pathway analysis 254 was performed using HUMAnN2 (Abubucker et al., 2012), which measures the abundances of 255 UniRef clusters (Suzek et al., 2015) by aligning sequences against the ChocoPhlAn database. 256 257 Bacteriocin genes were quantified by aligning reads against the BAGEL3 (van Heel, de Jong, 258 Montalban-Lopez, Kok, & Kuipers, 2013) bacteriocin database using DIAMOND (Buchfink, Xie, & Huson, 2015). Hits against the BAGEL3 bacteriocin database were counted with 259 SAMtools, after which the results were normalised as copies per million. 260

261

262 2.8. Statistical analysis

All biological experimental analyses were completed in triplicate, and the obtained results were 263 expressed as a mean value ± standard deviation (SD). One-way analysis of variance (ANOVA) 264 was employed in order to find the significant statistical differences (p < 0.05) and Fisher's least 265 significant difference (LSD) test was used to conduct multiple comparisons of the means (SPSS 266 Inc., Chicago, 215 IL, USA). For the bioinformatics, statistical analysis was performed in R-267 3.2.2 (Team R Core, 2013). The vegan package (version 2.3.0) (Oksanen et al., 2007) was used 268 269 for Bray-Curtis based multidimensional scaling (MDS) analysis. The ggplot2 package (version 270 2.2.1) (Wickham, 2016) was used for data visualisation.

271

272 **3. Results**

273 *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).

- 283 *Insert Table 2 Here*
- 284

285 3.2. GC-MS analysis

The fatty acid content of the TPL is shown in Table 3. As a result of fermentation, there seems 286 to be a trend towards the reduction of PUFA levels and an increase of MUFA levels in the fatty 287 288 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, 289 17:1, 18:2 (cis-10, trans-12), 20:4, and 22:5 when milk was fermented to yoghurt. Remarkably, 290 across all yoghurts there was a statistically significant reduction in the levels of ω 3 PUFA in 291 the TPL as the milk was fermented to yoghurts. This data indicates that fermentation directly 292 293 affects the fatty acid composition of milk polar lipids.

294 *Insert Table 3 Here*

295

296 *3.3. Biological assay*

The lipid extracts from the milk and yoghurt samples exhibited potent inhibition against PAF-297 induced platelet aggregation on human PRP in vitro (Table 4). The TNL of various yoghurts 298 exhibited a poor inhibitory effect against platelet aggregation. However, some had moderate 299 300 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 301 the TNL extracts but not as bioactive as the TPL extracts. This indicates that there is a 302 synergistic effect between the TNL and TPL. It is clear from Table 4 that the TPL extract were 303 the most inhibitory against PAF. Ovine milk TPL exhibited the highest IC₅₀ values, indicating 304 that this sample possessed the lowest antithrombotic activity. Yoghurts B and D possessed the 305 306 lowest IC₅₀ values, where the IC₅₀ of yoghurt D was statistically significantly lower than all 307 other samples tested.

308 *Insert Table 4 Here*

310 *3.4. Yoghurt microbial composition*

MetaPhlAn2, which measures the abundance of species-specific marker genes in shotgun 311 metagenomic reads, was used to determine and confirm the composition of the yoghurts made 312 with commercial starter cultures. The genera and species detected in the yoghurts and their 313 relative abundances are depicted in Fig. 1. In all of the yoghurts produced, S. thermophilus was 314 the most dominant species, L. delbrueckii subsp. bulgaricus was the second most abundant 315 species in yoghurts A and B. L. acidophilus was the third most abundant species present in 316 317 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. 318 319 *thermophilus* was the predominant species with *L. paracasei* present in low proportions (<1%) along with E. durans (1.9%). 320

321 *Insert Fig. 1. Here*

322

323 *3.5. Gene composition of the yoghurts*

324 Functional analysis of the shotgun metagenomic data was performed using HUMAnN2 (https://bitbucket.org/biobakery/humann2). The abundances of Gene Ontology (GO) and the 325 abundance of level-4 EC categories of interest are presented in Fig. 2 and 3 respectively. The 326 abundances of genes associated with phospholipid biosynthesis and metabolism were detected 327 in all of the yoghurts. According the data in Fig. 2., the abundance of GO terms associated with 328 polar lipid biosynthesis and metabolism are associated with the presence of *S. thermophilus* 329 and L acidophilus indicating that these microbes have the capacity to alter the polar lipid 330 composition of the milk and yoghurts. Similarly, the data in Fig. 3. indicates that both S. 331 thermophilus and L acidophilus have the greatest capacity to biosynthesise fatty acids and 332 phospholipids according to the abundance of level-4 EC categories of interest. 333

- 334 *Insert Fig. 2. Here*
- 335 *Insert Fig. 3. Here*

336 *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.

345 *Insert Fig. 4. Here*

346

347 4. Discussion

348 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 349 350 by L. delbrueckii subsp. bulgaricus, which was the second most abundant species in yoghurts 351 A and B. L. acidophilus was the third most abundant species present in yoghurt B (4.8%) and 352 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 353 354 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 355 356 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 357 enterococci are generally present in higher amounts in caprine and ovine milk (Del Pozo, Gaya, 358 Medina, Rodríguez-Marín, & Nuñez, 1988) and E. durans in particular is resistant to damage 359 by heat treatment (McAuley, Gobius, Britz, & Craven, 2012). It is unclear what prevented the 360 growth of L. paracasei, as generally this species is grown in the presence of the other lactic 361 acid bacteria present. However, temperature may play a role for its lower abundance as 362 previous research indicates that this species tends to favour growth below 40 °C at an optimum 363 of 37 °C (Collins, Phillips, & Zanoni, 1989), in contrast to the other organisms present, which 364 require 42 °C according to the manufacturers guidelines. Irrespective of these possibilities, 365 366 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 367 368 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. 369

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 372 documented that certain lactic acid bacterial strains have a distinctive phospholipid 373 composition, that may be distinguishable between different genera (Exterkate, Otten, 374 Wassenberg, & Veerkamp, 1971). In particular, based on the abundances of GO terms detected 375 by HUMAnN2, S. thermophilus in yoghurt D seems to have the greatest capacity to synthesise 376 fatty acids and phospholipids and possess the genes for other functions in relation to the 377 metabolic processes of fatty acids and polar lipids including: phosphatidylserine decarboxylase 378 activity (GO:0004609); phosphatidylethanolamine biosynthetic process (GO:0006631); 379 380 glycerophospholipid metabolic process (GO:0006650); phospholipid biosynthetic process; (GO:0008654) cardiolipin synthase activity (GO:0008808); cardiolipin biosynthetic process 381 (GO:0032049); acetyl-CoA carboxylase complex (GO:0009317); glycerol-3-phosphate 382 cytidyltransferase activity (GO:0047348); biotin carboxylase activity (GO:0004075); lipid 383 biosynthetic process (GO:0008610) (Fig. 2.). Several of the genes associated with anabolic 384 385 processes that are crucial for the biosynthesis of various polar lipids and are present in varying amounts in each of the yoghurts. 386

In particular, it seems that L. delbrueckii subsp. bulgaricus has the genetic capacity to 387 express CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase 388 activity (GO:0008444), which is involved in the biosynthesis of phospholipids by catalysing the 389 conversion of CDP-diacylglycerol and glycerol-3-phosphate to CMP and 3-(3-phosphatidyl)-390 glycerol 1-phosphate in the committed step to the synthesis of acidic phospholipids (Gaynor et 391 al., 1991; Vance & Vance, 2008). Furthermore, the detection of GO terms associated with the 392 phosphatidylethanolamine (PE) (GO:0006646) and cardiolipin (CL) (GO:0008808) 393 biosynthesis is not unexpected due to their respective roles in the bacterial and mitochondrial 394 membranes (Vance & Vance, 2008). 395

396 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 397 398 may induce either agonistic or antagonistic effects, but both cardioprotective (Tsoupras, Lordan, & Zabetakis, 2018); structural elucidation of these polar lipid extracts will provide 399 400 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 401 402 including PE and CL in the HUMAnN2 output may explain the levels of bioactivity detected 403 in these yoghurt as these phospholipids have previously been associated with potent

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

406 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 407 fatty acid composition of the yoghurts. Several of the EC categories of interest detected are 408 responsible for the biosynthesis of fatty acids (EC 6.4.1.2: Acetyl-CoA carboxylase), which 409 although expected, are present in abundance. There are also numerous EC categories detected 410 411 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 412 413 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 414 415 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 416 417 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-3-418 phosphate 3-phosphatidyltransferase (EC 2.7.8.5) indicates that the starter cultures in these 419 420 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 421 yoghurts and whether they are incorporated into the dairy matrix or remain bound 422 intracellularly. 423

424 Systemic inflammation is mediated by proinflammatory molecules such as PAF, which leads to the development of chronic conditions such as atherosclerosis and subsequently CVD 425 (Moss et al., 2018; Tsoupras, Lordan, & Zabetakis, 2018). Therefore, considering diet and 426 lifestyle are key modifiable risk factors for the prevention of CVD, the formulation of novel 427 428 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 429 430 development (Balthazar et al., 2017). Previous research indicates that polar lipids in ovine 431 yoghurts possess anti-PAF activity (Megalemou et al., 2017) and that the fermentation of milk 432 may affect the antithrombotic properties of these bioactive lipids (Lordan & Zabetakis, 2017a).

433 As demonstrated in table 2, following milk fermentation and depending on the starter 434 culture used, the IC_{50} decreased when milk was fermented to yoghurt, indicating an 435 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, 436 which is within range of previous polar lipids of animal origin tested on human PRP in vitro 437 (Poutzalis et al., 2018; Tsoupras, Lordan, Demuru, et al., 2018) and dairy derived polar lipids 438 tested on washed rabbit platelets in vitro (Megalemou et al., 2017; Poutzalis et al., 2016; 439 Tsorotioti et al., 2014). In particular, yoghurts B and D possessed the lowest IC₅₀ values, where 440 the IC₅₀ of yoghurt D was statistically significantly (p < 0.05) lower than all other samples 441 tested. These yoghurts in particular contained a higher abundance of L. acidophilus, which 442 443 seems to correlate with greater biological activity against PAF-induced platelet aggregation. 444 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-445 induced inflammation in human colonic NCM460 and Caco-2 cells by reducing nuclear factor 446 kappa B (NF-κB) activation and IL-8 production (Borthakur et al., 2013). L. acidophilus has 447 also demonstrated anti-inflammatory effects in vivo via impairing both the NF-kB and 448 449 mitogen-activated protein kinase (MAPK) signalling pathways (Haihua Li et al., 2016).

Research shows that the fatty acid composition of polar lipids affects their 450 antithrombotic capacity against PAF (Lordan et al., 2017). Following GC-MS analysis (Table 451 452 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 453 antithrombotic capacity of the ovine milk polar lipids. Previous research has shown that as 454 bovine or ovine milk is fermented to yoghurt, cheese, or *kefir*, the fatty acid composition 455 changes due to lipolysis of existing milk lipids and synthesis of lipids by lactic acid bacteria 456 (Florence et al., 2012; Guzel, Yibar, Belenli, Cetin, & Tanriverdi, 2017; Reguła, 2007; Vieira 457 et al., 2015; Yadav, Jain, & Sinha, 2007). In comparison to the milk TPL, there were significant 458 changes in the fatty acid composition of the TPL of all 5 yoghurts following fermentation with 459 460 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 461 462 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 463 and that the metabolism of various bacterial cultures modified the fatty acid profile of the milk. 464

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 469 yoghurts, which may be associated with the enhanced antithrombotic activities of the yoghurts 470 in contrast to the ovine milk. It is noteworthy that in marine products, it has been demonstrated 471 that these ω 3 PUFA are more bioactive when incorporated in a phospholipid structure rather 472 than their free fatty acid forms (Lordan et al., 2017). Remarkably, the fatty acid composition 473 474 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 475 (Demopoulos, Pinckard, & Hanahan, 1979), with acetic acid esterified to the sn-2 position, and 476 477 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 478 et al., 1979). However, further research is required to confirm if there is structural homology 479 480 between these polar lipids and PAF.

Finally, as presented in Fig. 4. it seems that the capacity to produce bacteriocins, which 481 482 are antimicrobial peptides produced by bacterial cultures, may correlate with greater antithrombotic activities. Some bacteriocins such as colicins, which is present in yoghurts D 483 and E are often encoded with a lysis protein, which increases the permeability of the outer 484 485 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 486 into the yoghurt matrix. Several bacteriocins, for example cinnamycin (Machaidze & Seelig, 487 2003), seem to demonstrate specificity for PE (Moll, Konings, & Driessen, 1999), which is the 488 second most abundant phospholipid in most biological membranes (Lordan et al., 2017). 489 Several bacteriocins have demonstrated selective binding towards negatively charged 490 phospholipids on the membranes of cancer cells (Kaur & Kaur, 2015). Some bacteriocins do 491 not seem to bind to neutral choline-containing zwitterionic PC molecules (Chatterjee, Paul, 492 493 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, 494 495 Rink, & Moll, 2011), indicating that the phospholipid charge is a defining feature for 496 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.

508 Overall, the present study has some limitations, and further research is required to 509 reveal the molecular mechanisms by which polar lipids bind to the PAF-R and inhibit the 510 proinflammatory actions of PAF. While the data relating to the bacteriocins is promising, 511 further research is required to confirm these observations. In addition, clinical studies are 512 required to assess the bioavailability of the antithrombotic polar lipids following consumption 513 of the antithrombotic ovine yoghurts.

514

515 **5.** Conclusions

516 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 517 518 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 519 520 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. 521 acidophilus and S. thermophilus plays a key role in improving the antithrombotic properties of 522 these yoghurts. Moreover, functional analysis indicates that the starter cultures present in these 523 yoghurts have the metabolic capacity to synthesise and alter various polar lipids, therefore 524 further research is required to discern whether these polar lipids are bioavailable in human 525 studies. The presence of bacteriocin related genes in some of the most bioactive yoghurts also 526 warrants further investigation to reveal if there are potential interactions between bacteriocins 527 528 and the MFGM. In addition, structural elucidation of these antithrombotic polar lipids and the 529 optimisation of the fermentation process may allow for the enhancement of the antithrombotic 530 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 531 lipid composition and antithrombotic properties. This study highlights that ovine milk and 532

533 yoghurts may have beneficial effects for human cardiovascular health and may lead to the534 future development of functional foods and nutraceuticals.

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548

549 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.

553

- 554 Supplementary data
- 555 <u>https://www.ebi.ac.uk/ena/data/view/PRJEB30083</u>

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834 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.



Abundance of GO terms of interest

Fig. 2. The depicts the abundance of GO terms of interest in each yoghurt according to the 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.

Abundance of level-4 EC categories of interest



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.

Table 1

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

Yogurt	Cultures
Α	0.020 % w/v Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus (YC-380, Chr. Hansen, Denmark).
В	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).
С	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).
Ε	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).

Table 2

Content of total lipids (TL), expressed in grams per 100g of sheep milk and yoghurts (mean \pm 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 \pm 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}	$2.29\pm0.17^{\text{a}}$
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}	$2.25\pm0.10^{\rm a}$
Yoghurt E	9.20 ± 0.55^{bc}	$97.60\pm0.38^{\rm a}$	2.55 ± 0.45^{ab}
^{ab} Different superscripts ind classes when means are con	icate significant differences npared using a Fisher's LSD	among different yoghurt sa multiple comparison test (<i>p</i>	mples within the same $l < 0.05$).

921 **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 \pm 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	Α	В	С	D	Ε	Milk
8:0	ND	$0.22\pm0.14^{\rm a}$	$0.35\pm0.08^{\rm a}$	ND	0.25 ± 0.07^{a}	$0.76\pm0.66^{\rm a}$
9:0	ND	$0.12\pm0.05^{\rm a}$	ND	ND	ND	0.14 ± 0.07^{a}
10:0	3.14 ± 0.32^{b}	$4.90\pm0.89^{\rm c}$	$4.66\pm0.56^{\rm c}$	1.60 ± 0.12^{a}	3.35 ± 0.65^{bc}	$5.58 \pm 1.78^{\rm c}$
10:1	0.17 ± 0.03^{b}	0.22 ± 0.06^{bc}	$0.34\pm0.13^{\rm c}$	0.07 ± 0.01^{a}	0.20 ± 0.04^{bc}	$0.28\pm0.11^{\text{bc}}$
11:0	0.13 ± 0.02^{a}	0.29 ± 0.06^{bc}	0.14 ± 0.12^{abc}	$0.20\pm0.01^{\text{b}}$	0.17 ± 0.03^{ab}	$0.30\pm0.08^{\rm c}$
12:0	3.90 ± 0.50^{a}	5.06 ± 0.82^{ab}	5.51 ± 0.85^{b}	$5.20\pm0.32^{\text{b}}$	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}	$0.20\pm0.01^{\rm c}$	$0.20\pm0.01^{\rm c}$	0.06 ± 0.01^{a}
13:0	0.21 ± 0.02^{a}	0.32 ± 0.08^{bc}	$0.31\pm0.02^{\text{b}}$	$0.36\pm0.02^{\rm c}$	$0.18\pm0.03^{\rm a}$	0.32 ± 0.05^{bc}
14:0	8.20 ± 0.59^{a}	$11.38\pm0.28^{\rm c}$	$13.89 \pm 1.17^{\text{d}}$	$11.49\pm0.78^{\rm c}$	9.83 ± 0.96^{ab}	8.87 ± 1.00^{ab}
14:1ω7 c9	$0.28\ \pm 0.08^{abc}$	$0.34\pm0.01^{\rm c}$	0.32 ± 0.05^{bc}	0.42 ± 0.15^{bc}	$0.31\pm0.01^{\rm b}$	$0.19\pm0.01^{\rm a}$
15:0	1.33 ± 0.08^{b}	$1.93\pm0.22^{\rm a}$	1.31 ± 0.39^{abc}	$1.70\pm0.20^{\rm c}$	1.39 ± 0.07^{b}	$1.56\pm0.10^{\rm c}$
16:0	$17.53\pm0.32^{\rm a}$	$19.87\pm0.48^{\text{b}}$	20.43 ± 0.65^{bc}	21.62 ± 0.60^{c}	19.53 ± 1.56^{bc}	$17.20\pm0.76^{\rm a}$
16:1ω7 c9	$2.00 \pm 0.14^{\circ}$	$2.53\pm0.35^{\text{d}}$	$1.40\pm0.18^{\text{b}}$	2.09 ± 0.31^{cd}	1.71 ± 0.66^{bcd}	$0.88\pm0.06^{\rm a}$
17:0	$0.90 \pm 0.01^{\circ}$	$1.07\pm0.15^{\rm d}$	$0.75\pm0.09^{\text{b}}$	0.96 ± 0.05^{bd}	0.96 ± 0.27^{bcd}	$0.45\pm0.01^{\rm a}$
17:1	$0.61 \pm 0.01^{\circ}$	$0.67\pm0.13^{\rm c}$	$0.27\pm0.17^{\text{b}}$	$0.65\pm0.12^{\rm c}$	0.72 ± 0.30^{bc}	$0.02\pm0.01^{\text{a}}$
18:0	$10.93{\pm}0.68^{\mathrm{b}}$	$11.25\pm1.25^{\rm b}$	$15.76\pm2.64^{\rm c}$	$15.13\pm1.17^{\rm c}$	$8.25\pm0.27^{\rm a}$	$11.32\pm0.72^{\text{b}}$
18:1ω9 c9	$35.56 \pm 1.40^{\circ}$	$27.62 \pm 1.36^{\text{b}}$	23.61 ± 0.31^{ab}	22.97 ± 1.63^{a}	30.53 ± 3.74^{bc}	23.59 ± 3.56^{ab}
18:2 ω6 c9, t12	$6.31 \pm 0.02^{\circ}$	5.67 ± 0.67^{abc}	$4.89\pm0.33^{\rm a}$	5.95 ± 0.06^{b}	5.50 ± 1.70^{abc}	$9.23\pm0.64^{\text{d}}$
18:2ω7 c9, t11	3.51 ± 0.16^{d}	1.94 ± 0.12^{ab}	$1.47\pm0.35^{\rm a}$	$2.00\pm0.06^{\text{b}}$	$3.29\pm0.60^{\text{d}}$	$2.56\pm0.02^{\rm c}$
18:2 ω6 t10, c12	0.46 ± 0.35^{b}	ND	$0.05\pm0.01^{\rm a}$	ND	0.93 ± 0.18^{b}	ND
18:3ω3 c9, c12, c15	2.15 ± 0.01^{d}	$1.44\pm0.16^{\text{b}}$	$0.98\pm0.16^{\rm a}$	$1.83\pm0.05^{\rm c}$	$2.66\pm0.13^{\rm f}$	$2.30\pm0.16^{\text{e}}$
20:0	ND	0.31 ± 0.01^{a}	$0.24\pm0.15^{\rm a}$	0.29 ± 0.11^{a}	ND	ND
20:1 ω 9 c9	$0.65\pm0.04^{\rm a}$	$0.79\pm0.06^{\text{b}}$	0.74 ± 0.14^{ab}	$0.87\pm0.04^{\text{b}}$	0.79 ± 0.44^{abc}	$1.01\pm0.03^{\rm c}$
20:3ω9 c5, c8, c11	ND	$0.24\pm0.01^{\rm a}$	$0.37\pm0.12^{\rm a}$	$0.20\pm0.13^{\rm a}$	$0.32\pm0.18^{\rm a}$	ND
20:4 06 c5, c8, c11, c14	$0.95\pm0.15^{\rm c}$	$0.49\pm0.04^{\rm a}$	$0.61\pm0.10^{\text{b}}$	$0.57\pm0.01^{\text{b}}$	$1.20\pm0.36^{\rm c}$	ND
20:5 03 c5, c8, c11, c14, c17	$0.35\pm0.03^{\circ}$	$0.25\pm0.01^{\rm a}$	0.22 ± 0.08^{ab}	$0.29\pm0.01^{\text{b}}$	$0.42\pm0.05^{\text{d}}$	$3.15\pm0.30^{\rm e}$
22:1 c11	$0.53\pm0.05^{\rm a}$	0.82 ± 0.15^{bc}	0.56 ± 0.22^{ab}	$1.00\pm0.04^{\rm c}$	0.72 ± 0.21^{ab}	$0.89\pm0.11^{\text{b}}$
22:4 ω6 c7, c10, c13, c16	ND	$0.14\pm0.02^{\rm a}$	0.25 ± 0.17^{ab}	ND	$0.44\pm0.04^{\text{b}}$	ND
22:5 ω6 c4, c7, c10, c13, c16	1.62 ± 0.30^{b}	0.97 ± 0.11^{a}	$0.96\pm0.20^{\text{a}}$	1.54 ± 0.07^{b}	1.67 ± 0.14^{b}	ND
22:6w3 c4, c7, c10, c13, c16,	$0.91\pm0.37^{\rm c}$	$0.28\pm0.03^{\rm a}$	0.61 ± 0.36^{abc}	$0.44\pm0.04^{\text{b}}$	0.58 ± 0.10^{b}	$0.97\pm0.04^{\rm c}$
c19						
Total SFA	$46.27\pm1.54^{\mathrm{a}}$	$56.72 \pm 1.33^{\circ}$	63.31 ± 2.62^{d}	$58.56\pm0.89^{\rm c}$	48.44 ± 3.88^{ab}	54.01 ± 4.28^{bc}
Total MUFA	$39.96 \pm 1.37^{\circ}$	$32.90 \pm 1.06^{\text{b}}$	$27.50\pm0.48^{\rm a}$	27.90 ± 0.62^{a}	35.18 ± 3.84^{bc}	$27.35\pm3.35^{\text{a}}$
Total ω7	$5.782\pm0.22^{\rm c}$	4.805 ± 0.49^{b}	$3.196\pm0.48^{\rm a}$	4.519 ± 0.47^{b}	5.309 ± 0.65^{bc}	$3.638\pm0.07^{\text{a}}$
Total ω9	$36.22 \pm 1.39^{\rm c}$	$28.65 \pm 1.42^{\text{b}}$	24.72 ± 0.27^a	24.05 ± 1.73^{a}	31.64 ± 4.03^{bc}	24.60 ± 3.56^{ab}
Total PUFA	$16.37\pm0.60^{\rm c}$	11.46 ± 0.97^{a}	10.42 ± 0.29^{a}	12.83 ± 0.18^{b}	17.01 ± 1.56^{cd}	$18.22\pm1.17^{\rm d}$
Total ω3	3.431 ± 0.38^{c}	$1.970\pm0.16^{\rm a}$	$1.819\pm0.36^{\rm a}$	2.562 ± 0.07^{b}	$3.662\pm0.17^{\text{c}}$	$6.420\pm0.45^{\text{d}}$
Total ω6	9.345 ± 0.38^{c}	$7.270\pm0.68^{\rm a}$	6.763 ± 0.26^{a}	8.057 ± 0.01^{b}	$9.738 \pm 1.17^{\rm c}$	$9.228\pm0.64^{\rm c}$

926 abcdef Mean values (n = 3), \pm standard deviation with different letters in the same row indicating statistical 927 significant differences when means are compared using Fisher's LSD multiple comparison test (p < 0.05). ND: 928 non-detectable

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931 **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 IC₅₀ (μ g) (mean \pm SD, n = 3).

Yogurt	TL	TNL	TPL
Α	306.5 ± 64.1^{ab}	$2938 \ \pm \ 123^{a}$	$77.00 \pm 9.20^{\circ}$
В	331.7 ± 27.3^{b}	$738.6 ~\pm~ 37.7^{b}$	57.41 ± 5.93^{b}
С	253.9 ± 73.1^{ab}	ND	$70.72 \pm 3.95^{\circ}$
D	224.5 ± 21.4^{a}	$640.9 \pm 34.0^{\circ}$	44.84 ± 4.96^{a}
Ε	263.8 ± 55.5^{ab}	ND	68.10 ± 7.55^{bc}
Milk	$378.0 \pm 12.8^{\circ}$	ND	154.4 ± 12.8^{d}

935 abcd Mean values (n = 3), ± standard deviation with different letters in the same column indicating statistical

936 significant differences when means are compared using Fisher's LSD multiple comparison test (p < 0.05). ND: 937 not-detectable

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