

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

2 Ronan Lordan <sup>a\*</sup>, Aaron M. Walsh <sup>b</sup>, Fiona Crispie <sup>bc</sup>, Laura Finnegan <sup>b</sup>, Paul D. Cotter <sup>bc</sup>, and  
3 Ioannis Zabetakis <sup>a\*</sup>

4 <sup>a</sup>Department of Biological Sciences, University of Limerick, Co. Limerick, Ireland

5 <sup>b</sup>Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

6 <sup>c</sup>Alimentary Pharmabiotic Centre, University College Cork, Co. Cork, Ireland

7 \* **Corresponding Author:** [Ioannis.Zabetakis@ul.ie](mailto:Ioannis.Zabetakis@ul.ie) and [Ronan.Lordan@ul.ie](mailto:Ronan.Lordan@ul.ie)

8

9 **ABSTRACT**

10 The effect of fermentation on the antithrombotic properties of polar lipids in ovine milk has  
11 been assessed through the production of yoghurts. The total lipids (TL), total neutral lipids  
12 (TNL), and total polar lipids (TPL) were extracted. The fatty acid profiles of all yoghurt polar  
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  
15 against platelet-activating factor (PAF) induced platelet aggregation. All yoghurt polar lipids  
16 exhibited potent antithrombotic activities with IC<sub>50</sub> values ranging from 45–77 µg. Shotgun  
17 metagenomics determined the species-level microbial composition and functional potential of  
18 the yoghurts. Yoghurts containing *L. acidophilus* seem to correlate with greater bioactivity.  
19 Several phospholipid biosynthetic genes have been identified in the most antithrombotic  
20 yoghurts. This study has demonstrated that fermentation enhances the antithrombotic  
21 properties of yoghurt polar lipids against PAF.

22

23

24

25

26

27

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

29

30 **Highlights:**

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

38

39

40 **1. Introduction**

41 Maladaptive diet and lifestyle play a significant role in the development of chronic diseases  
42 such as cardiovascular disease (CVD), insulin resistance, obesity, and cancer (Mozaffarian,  
43 2016). Diet and lifestyle are key modifiable risk factors for the prevention of CVD (Lordan,  
44 Tsoupras, Mitra, & Zabetakis, 2018). In 2016, CVD was responsible for 17.7 million deaths,  
45 where 80% of CVD events were either a myocardial infarction or stroke (World Health  
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,  
48 scientific, and media circles, as they are energy dense foods rich in saturated fatty acids (SFA),  
49 which can increase cholesterol levels (Lamarche et al., 2016; Lordan & Zabetakis, 2017a).  
50 Therefore, low-fat or non-fat dairy products were encouraged by many dietary  
51 recommendations in order to reduce to lower cholesterol levels (Lordan, Tsoupras, Mitra, et  
52 al., 2018). However, recent research indicates that dairy products may be neutral or even  
53 beneficial for cardiovascular health, and may not have significant effects on blood cholesterol  
54 levels (Labonté, Couture, Richard, Desroches, & Lamarche, 2013; Lordan, Tsoupras, Mitra, et  
55 al., 2018; Lordan & Zabetakis, 2017a; Thorning et al., 2016). Further evidence indicates that  
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  
58 levels, and impaired glucose tolerance (Lordan, Tsoupras, Mitra, et al., 2018).

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

61 2016; Lordan, Tsoupras, Mitra, et al., 2018; Sayon-Orea, Martínez-González, Ruiz-Canela, &  
62 Bes-Rastrollo, 2017; Wu & Sun, 2017). Bovine milk accounts for 85% of the total global milk  
63 production (Balthazar et al., 2017), however ovine milk and yoghurts provide a superior  
64 nutritional alternative. Ewe's milk and dairy products are not commonly consumed outside of  
65 the Mediterranean basin, and are considered a delicacy in many countries (Lordan & Zabetakis,  
66 2017b). Ovine milk owes its nutritional superiority over bovine and caprine milk due to the  
67 higher levels of protein, lipid, minerals, and vitamins essential to human health (Balthazar et  
68 al., 2017; Lordan, Tsoupras, Mitra, et al., 2018). The most predominant fatty acid in ovine milk  
69 and yoghurts are oleic acid (18:1), palmitic acid (16:0), and myristic acid (14:0) (Balthazar et  
70 al., 2017). Diets high in oleic acid decrease low-density lipoprotein (LDL) cholesterol levels,  
71 whereas high-density lipoprotein (HDL) cholesterol levels are not significantly affected  
72 (Lordan & Zabetakis, 2017b; Molkentin, 2000). It has recently been shown that ovine yoghurt  
73 consumption does not affect the lipid profile of healthy individuals (Olmedilla-Alonso et al.,  
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  
77 on serum cholesterol levels, putative anti-inflammatory effects, and antithrombotic effects  
78 (Megalemu 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  
82 to their polar lipid content (Lordan & Zabetakis, 2017a). The polar lipid content of ovine milk  
83 is approximately 9.4-35.5 mg/100g of raw milk. The polar lipid fraction contains  
84 phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SM), which  
85 are present in abundance with lower quantities of phosphatidylserine (PS) and  
86 phosphatidylinositol (PI), which is comparable to other ruminant species (Lordan, Tsoupras,  
87 & Zabetakis, 2017; Park, 2009). Although the phospholipid fraction of ovine milk is  
88 quantitatively a minor constituent of the overall lipid content, it possesses techno-functional  
89 and nutritional properties that are implicated in several physiological processes and are  
90 beneficial for health (Lordan et al., 2017).

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

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

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

118

## 119 **2. Materials and methods**

### 120 *2.1. Chemicals and reagents*

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

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

131

## 132 *2.2. Milk processing & yogurt production*

133 A fresh, commercial, pasteurised, and homogenised ewe's whole milk was obtained from  
134 Rockfield Dairy Ltd. (Claremorris, Co Mayo, Ireland). Milk was obtained from a bulk tank  
135 containing milk from the Friesland and Lacaune breed of dairy ewe, between March and July  
136 2016. The sheep were fed a forage based diet consisting of mainly grass silage or fresh grass,  
137 supplemented with cereal at the time of milking, which is typical of the small sheep dairy  
138 industry in Ireland; however, atypical in Europe where the diet mainly consists of cereal. The  
139 collected milk was pasteurised on site by heating to 91 °C for 15 seconds and then was cooled  
140 to 42 °C before being packaged and refrigerated (4 °C ± 1 °C) for transport to the laboratory.  
141 For yoghurt production, all milk samples were heated to 42 °C in pre-sterilised conical flasks  
142 in a water bath (Grant JB NV, Cambridgeshire, UK) and held at that temperature throughout  
143 the yogurt fermentation process. All yogurts (A-E) were inoculated with specific starter  
144 cultures as indicated in Table 1. When inoculated, the milk was mixed thoroughly and the  
145 temperature was held at 42 °C. The pH was monitored until the yogurt fermentation reached  
146 between 4.4 – 4.6 pH units, then the fermentation was stopped by cooling the yogurts to 4 °C.  
147 All yoghurts were made in triplicate. The yogurts were then transferred to glass media bottles  
148 and stored at -20 °C until required for analysis or a maximum of six weeks.

149

## 150 *2.3. Yogurt cultures*

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

155 **\*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  $-20^{\circ}\text{C}$ , 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  $-20^{\circ}\text{C}$ .  
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  $194 \times g$  for 18  
177 minutes at  $24^{\circ}\text{C}$  with no brake applied in order to obtain the supernatant platelet-rich plasma  
178 (PRP). A second centrifugation at  $1500 \times g$  for 20 minutes at  $24^{\circ}\text{C}$  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  $\mu\text{l}^{-1}$  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  $24^{\circ}\text{C}$  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  $\mu\text{l}$  of PRP was added to an aggregometer cuvette at  $37^{\circ}\text{C}$  with stirring at  
187 1000 rpm, and was calibrated prior to testing using the PPP as a blank. PAF was added to the

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

196

## 197 2.6. GC-MS analysis

198 Fatty acid methyl esters (FAME) were prepared using 35 mg of the TPL of the milk and  
199 yogurt samples in triplicate according to the method of Tsoupras, Lordan, Demuru, et al. (2018)  
200 with slight modifications. In brief, FAME were prepared using a solution of 0.5 M KOH in 90  
201 %  $\text{CH}_3\text{OH}$  and extracted with *n*-hexane. The GC-MS fatty acid analysis was carried out  
202 according the internal standard method as previously described (Tsoupras, Lordan, Demuru, et  
203 al., 2018). The equation that described the curve was:  $y = 0.0041x + 0.12$  with an  $R^2 = 0.9969$ ,  
204 where the ratio of the area of the analyte peak to that of the internal standard (21:0) represents  
205 the *y* value for the above equation, subsequently the *x* value represents the analyte  
206 concentration of a selected fatty acid in the lipid sample to be tested. Separation of the FAME  
207 was achieved on an Agilent J&W DB-23 fused silica capillary column (60 m, 0.251 mm, i.d.,  
208 0.25  $\mu\text{m}$ ; Agilent Technologies, Santa Clara, CA, USA) using a Varian 410-GC coupled to a  
209 Varian 210-MS equipped with a split/splitless injector (Agilent Technologies). The injector  
210 was set at 230 °C with a split ratio of 1:5. The carrier gas was high purity helium with a liner  
211 flow rate of 1 ml/min. The oven temperature was initially programmed to 100 °C for 5 min,  
212 raised to 240 °C at 3 °C/min, and finally held isothermal at 240 °C for 10 mins. FAME were  
213 identified using 37-component FAME standards mix (Sigma Aldrich, Wicklow, Ireland) by  
214 comparison of the retention times and mass spectra of relative peaks with the aid of the Varian  
215 Star Chromatography Workstation Version 6 software (Agilent Technologies) and a NIST  
216 library (Gaithersburg, MD, USA).

217

218

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  $5,444 \times g$  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  $2 \times 300$  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>

250 [cess/trimBWAstyle.usingBam.pl](#)). Specifically, reads were trimmed to 200 bp, while all reads  
251 with a quality score less than Q30 were discarded. The resulting fastq files were then converted  
252 to fasta files using the fq2fa option from IDBA-UD (Peng, Leung, Yiu, & Chin, 2012). Species-  
253 level analysis was performed using MetaPhlan2 (Truong et al., 2015), which measures the  
254 abundance of species-specific marker genes in metagenomic reads. Microbial pathway analysis  
255 was performed using HUMAnN2 (Abubucker et al., 2012), which measures the abundances of  
256 UniRef clusters (Suzek et al., 2015) by aligning sequences against the ChocoPhlAn database.  
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,  
259 Xie, & Huson, 2015). Hits against the BAGEL3 bacteriocin database were counted with  
260 SAMtools, after which the results were normalised as copies per million.

261

## 262 2.8. Statistical analysis

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

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

281 Megale mou et al., 2017), where generally the polar lipid content is reported as relatively low  
282 (Lordan et al., 2017).

283 \*Insert Table 2 Here\*

284

### 285 3.2. GC-MS analysis

286 The fatty acid content of the TPL is shown in Table 3. As a result of fermentation, there seems  
287 to be a trend towards the reduction of PUFA levels and an increase of MUFA levels in the fatty  
288 acids of the polar lipids, whereas the levels of SFA vary depending on the yoghurt produced.  
289 Specifically, there is a statistically significant ( $p < 0.05$ ) increase in the levels of 16:1, 17:0,  
290 17:1, 18:2 (*cis*-10, *trans*-12), 20:4, and 22:5 when milk was fermented to yoghurt. Remarkably,  
291 across all yoghurts there was a statistically significant reduction in the levels of  $\omega$ 3 PUFA in  
292 the TPL as the milk was fermented to yoghurts. This data indicates that fermentation directly  
293 affects the fatty acid composition of milk polar lipids.

294 \*Insert Table 3 Here\*

295

### 296 3.3. Biological assay

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

308 \*Insert Table 4 Here\*

309

310 3.4. Yoghurt microbial composition

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

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

334 \*Insert Fig. 2. Here\*

335 \*Insert Fig. 3. Here\*

336 3.6. Bacteriocins

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

340 mechanisms of action vary considerably due to their structural diversity. As depicted in Fig.  
341 4., it is clear that the microbes present in the yoghurts possess the genetic capacity to produce  
342 a variety of type II and type III bacteriocins. Notably, yoghurt D, C, and E had a greater number  
343 of hits per class of bacteriocin, which may correlate with greater bioactivity against platelet  
344 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.  
349 1A). *S. thermophilus* was the most dominant species in all of the yoghurts produced, followed  
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  
353 species present in yoghurt C was *B. animalis* (6.9%). Although *L. paracasei* was added to  
354 yoghurt E, this species was present in low proportions (< 1%) in the final yoghurt. Furthermore,  
355 *E. durans* was also detected in yoghurt E (1.9%). This is a non-pathogenic bacterial species of  
356 human, animal or environmental origin that is often identified in various dairy products and  
357 may be probiotic (Andrighetto et al., 2001). Its presence may be explained by the fact that  
358 enterococci are generally present in higher amounts in caprine and ovine milk (Del Pozo, Gaya,  
359 Medina, Rodríguez-Marín, & Nuñez, 1988) and *E. durans* in particular is resistant to damage  
360 by heat treatment (McAuley, Gobius, Britz, & Craven, 2012). It is unclear what prevented the  
361 growth of *L. paracasei*, as generally this species is grown in the presence of the other lactic  
362 acid bacteria present. However, temperature may play a role for its lower abundance as  
363 previous research indicates that this species tends to favour growth below 40 °C at an optimum  
364 of 37 °C (Collins, Phillips, & Zannoni, 1989), in contrast to the other organisms present, which  
365 require 42 °C according to the manufacturers guidelines. Irrespective of these possibilities,  
366 yoghurt E possessed a different microbial composition in comparison to the other yoghurts,  
367 which is characterised by a high proportion of *S. thermophilus*. The fact that yoghurt E had an  
368 IC<sub>50</sub> lower than that of milk, indicates that fermentation of milk with *S. thermophilus* plays a  
369 significant role in the bioactivity of polar lipids present in these yoghurts.

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

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

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

396 Interestingly, yoghurt D exhibited the greatest capacity for glycerol ether metabolic  
397 processes. As these lipids exhibit similar structures to PAF, yoghurts that contain these lipids  
398 may induce either agonistic or antagonistic effects, but both cardioprotective (Tsoupras,  
399 Lordan, & Zabetakis, 2018); structural elucidation of these polar lipid extracts will provide  
400 more information about the structure function relationship between these polar lipids and the  
401 PAF-R. Moreover, the detection of genes associated with the synthesis various phospholipid  
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

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

406 The abundance of level-4 EC categories of interest was also assessed (Fig. 3.). It is clear  
407 that the microbes present in each of the yoghurts play a key role in the alteration of the overall  
408 fatty acid composition of the yoghurts. Several of the EC categories of interest detected are  
409 responsible for the biosynthesis of fatty acids (EC 6.4.1.2: Acetyl-CoA carboxylase), which  
410 although expected, are present in abundance. There are also numerous EC categories detected  
411 in abundance that are specific to phospholipid biosynthesis. For example glycerol-3-phosphate  
412 acyltransferase (EC 2.3.1.n3) and phosphate acyltransferase (EC 2.3.1.n2) are the rate limiting  
413 enzymes for phosphatidic acid synthesis, which is critical for the synthesis of phospholipids  
414 (Wendel, Lewin, & Coleman, 2009). Both of these EC terms were detected in abundance in  
415 yoghurt D, which corresponds with the yoghurt that exhibited significant changes in the TPL  
416 lipid composition and the most potent bioactivity. Similarly, the detection of  
417 phosphatidylserine decarboxylase (EC 4.1.1.65), glycerol-3-phosphate cytidyltransferase (EC  
418 2.7.7.39), inositol-3-phosphate synthase (EC 5.5.1.4), and CDP-diacylglycerol-glycerol-3-  
419 phosphate 3-phosphatidyltransferase (EC 2.7.8.5) indicates that the starter cultures in these  
420 yoghurts have the ability to synthesise phospholipids. However, further research is required to  
421 establish to what extent these starter cultures influence the phospholipid content of these  
422 yoghurts and whether they are incorporated into the dairy matrix or remain bound  
423 intracellularly.

424 Systemic inflammation is mediated by proinflammatory molecules such as PAF, which  
425 leads to the development of chronic conditions such as atherosclerosis and subsequently CVD  
426 (Moss et al., 2018; Tsoupras, Lordan, & Zabetakis, 2018). Therefore, considering diet and  
427 lifestyle are key modifiable risk factors for the prevention of CVD, the formulation of novel  
428 nutraceutical and functional foods to combat these inflammatory processes is imperative.  
429 Ovine milk is an underutilised nutritious milk with the potential to be used for functional food  
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

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

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

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

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

481 Finally, as presented in Fig. 4. it seems that the capacity to produce bacteriocins, which  
482 are antimicrobial peptides produced by bacterial cultures, may correlate with greater  
483 antithrombotic activities. Some bacteriocins such as colicins, which is present in yoghurts D  
484 and E are often encoded with a lysis protein, which increases the permeability of the outer  
485 membrane of the producer organism and is lethal to the producing cells (Snijder & Dijkstra,  
486 2000). Consequently, it is possible that bacteriocins may play a role in releasing phospholipids  
487 into the yoghurt matrix. Several bacteriocins, for example cinnamycin (Machaidze & Seelig,  
488 2003), seem to demonstrate specificity for PE (Moll, Konings, & Driessen, 1999), which is the  
489 second most abundant phospholipid in most biological membranes (Lordan et al., 2017).  
490 Several bacteriocins have demonstrated selective binding towards negatively charged  
491 phospholipids on the membranes of cancer cells (Kaur & Kaur, 2015). Some bacteriocins do  
492 not seem to bind to neutral choline-containing zwitterionic PC molecules (Chatterjee, Paul,  
493 Xie, & van der Donk, 2005), and changes to the overall charge of phospholipids due to a change  
494 in the lipid composition is associated with bacteriocin resistance in some bacteria (Kuipers,  
495 Rink, & Moll, 2011), indicating that the phospholipid charge is a defining feature for  
496 bacteriocin specificity.

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

502 the levels of microbial cells it became unavailable to destroy these cells but was bioavailable  
503 and active again when a detergent was added to permeabilise the MFGM (Jung, Bodyfelt, &  
504 Daeschel, 1992). Considering there is a wealth of evidence to suggest that bacteriocins can  
505 bind to various types of membranes, there is speculative evidence to suggest that the  
506 bacteriocins produced by the starter cultures in this study may interact with the MFGM  
507 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  
517 polar lipids during fermentation through the lipolysis and biosynthesis of fatty acids. By  
518 altering the polar lipid composition, the antithrombotic properties of these yoghurts have been  
519 enhanced. Further research is required to discern the exact polar lipid structures responsible for  
520 these bioactivities and how fermentation influences the phospholipid structure of milk polar  
521 lipids. Shotgun metagenomic characterisation of the yoghurts indicates that the use of *L.*  
522 *acidophilus* and *S. thermophilus* plays a key role in improving the antithrombotic properties of  
523 these yoghurts. Moreover, functional analysis indicates that the starter cultures present in these  
524 yoghurts have the metabolic capacity to synthesise and alter various polar lipids, therefore  
525 further research is required to discern whether these polar lipids are bioavailable in human  
526 studies. The presence of bacteriocin related genes in some of the most bioactive yoghurts also  
527 warrants further investigation to reveal if there are potential interactions between bacteriocins  
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  
531 required to assess the use of various milk sources and animal diets that may alter the milk polar  
532 lipid composition and antithrombotic properties. This study highlights that ovine milk and

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

535

### 536 **Acknowledgments**

537 The authors would like to thank the volunteers who donated blood and Elaine Ahern and Breda  
538 Moloney for their phlebotomy support. The authors acknowledge the financial support of both  
539 Enterprise Ireland (grant reference: IP-2016-0488Y) and the Department of Biological  
540 Sciences, University of Limerick, Ireland. The authors would like to extend their sincere  
541 gratitude to Michael and Aisling Flanagan of Rockfield Dairy Ltd. trading as Velvet Cloud,  
542 Claremorris, Co Mayo, Ireland, for donating milk samples for experimentation. The authors  
543 appreciate the support of Chr-Hansen A/S Cork, Ireland for the donation of their cultures and  
544 Michael Heffernan of Orchard Valley Dairy Supplies Worcestershire, UK for the donation of  
545 the Danisco yogurt cultures. The grant providers had no role in the design of the study, in the  
546 collection, analyses or interpretation of the data; in the writing of the manuscript; nor in the  
547 decision to publish the results.

548

### 549 **Author contributions**

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

553

### 554 **Supplementary data**

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

556

557

558

559

560

561 **References**

- 562 Abubucker, S., Segata, N., Goll, J., Schubert, A. M., Izard, J., Cantarel, B. L., . . .  
 563 Huttenhower, C. (2012). Metabolic reconstruction for metagenomic data and its  
 564 application to the human microbiome. *PLoS Computational Biology*, 8(6), e1002358.  
 565 doi: <https://doi.org/10.1371/journal.pcbi.1002358>
- 566 Andrighetto, C., Knijff, E. D. O., Lombardi, A., Torriani, S., Vancanneyt, M., Kersters, K., . . .  
 567 . Dellaglio, F. (2001). Phenotypic and genetic diversity of enterococci isolated from  
 568 Italian cheeses. *Journal of Dairy Research*, 68(2), 303-316. doi:  
 569 <https://doi.org/10.1017/S0022029901004800>
- 570 Antonopoulou, S., Semidalas, C. E., Koussissis, S., & Demopoulos, C. A. (1996). Platelet-  
 571 activating factor (PAF) antagonists in foods: a study of lipids with PAF or anti-PAF-  
 572 like activity in cow's milk and yogurt. *Journal of Agriculture and Food Chemistry*,  
 573 44(10), 3047-3051. doi: <https://doi.org/10.1021/jf950619y>
- 574 Balthazar, C. F., Pimentel, T. C., Ferrão, L. L., Almada, C. N., Santillo, A., Albenzio, M., . . .  
 575 Cruz, A. G. (2017). Sheep milk: Physicochemical characteristics and relevance for  
 576 functional food development. *Comprehensive Reviews in Food Science and Food*  
 577 *Safety*, 16(2), 247-262. doi: <https://doi.org/10.1111/1541-4337.12250>
- 578 Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification.  
 579 *Canadian Journal of Biochemistry and Physiology*, 37(8), 911-917. doi:  
 580 <https://dx.doi.org/10.1139/o59-099>
- 581 Borthakur, A., Bhattacharyya, S., Kumar, A., Anbazhagan, A. N., Tobacman, J. K., &  
 582 Dudeja, P. K. (2013). *Lactobacillus acidophilus* alleviates platelet-activating factor-  
 583 induced inflammatory responses in human intestinal epithelial cells. *PLoS One*, 8(10),  
 584 e75664. doi: <https://doi.org/10.1371/journal.pone.0075664>
- 585 Buchfink, B., Xie, C., & Huson, D. H. (2015). Fast and sensitive protein alignment using  
 586 DIAMOND. *Nature Methods*, 12(1), 59. doi: <https://doi.org/10.1038/nmeth.3176>
- 587 Castro Faria Neto, H. C., Stafforini, D. M., Prescott, S. M., & Zimmerman, G. A. (2005).  
 588 Regulating inflammation through the anti-inflammatory enzyme platelet-activating  
 589 factor-acetylhydrolase. *Memórias do Instituto Oswaldo Cruz*, 100, 83-91. doi:  
 590 <http://dx.doi.org/10.1590/S0074-02762005000900014>
- 591 Cattaneo, M., Cerletti, C., Harrison, P., Hayward, C., Kenny, D., Nugent, D., . . . Watson, S.  
 592 (2013). Recommendations for the standardization of light transmission aggregometry:  
 593 a consensus of the working party from the platelet physiology sub-committee of  
 594 SSC/ISTH. *Journal of Thrombosis and Haemostasis*, 11(6), 1183-1189. doi:  
 595 <https://doi.org/10.1111/jth.12231>
- 596 Chatterjee, C., Paul, M., Xie, L., & van der Donk, W. A. (2005). Biosynthesis and mode of  
 597 action of lantibiotics. *Chemical Reviews*, 105(2), 633-684. doi:  
 598 <https://doi.org/10.1021/cr030105v>
- 599 Collins, M. D., Phillips, B. A., & Zanoni, P. (1989). Deoxyribonucleic acid homology studies  
 600 of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp.  
 601 *tolerans*, and *Lactobacillus rhamnosus* sp. nov., comb. nov. *International Journal of*  
 602 *Systematic and Evolutionary Microbiology*, 39(2), 105-108. doi:  
 603 <https://doi.org/10.1099/00207713-39-2-105>
- 604 Cotter, P. D., Hill, C., & Ross, R. P. (2005). Bacteriocins: developing innate immunity for  
 605 food. [Review Article]. *Nature Reviews Microbiology*, 3, 777. doi:  
 606 <https://doi.org/10.1038/nrmicro1273>
- 607 Da Silva, M. S., & Rudkowska, I. (2015). Dairy nutrients and their effect on inflammatory  
 608 profile in molecular studies. *Molecular Nutrition & Food Research*, 59(7), 1249-  
 609 1263. doi: <http://dx.doi.org/10.1002/mnfr.201400569>

- 610 Del Pozo, B. F., Gaya, P., Medina, M., Rodríguez-Marín, M. A., & Nuñez, M. (1988).  
611 Changes in the microflora of La Serena ewes' milk cheese during ripening. *Journal of*  
612 *Dairy Research*, 55(3), 449-455. doi: <https://doi.org/10.1017/S0022029900028703>
- 613 Demopoulos, C., Pinckard, R., & Hanahan, D. J. (1979). Platelet-activating factor. Evidence  
614 for 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine as the active component (a  
615 new class of lipid chemical mediators). *Journal of Biological Chemistry*, 254(19),  
616 9355-9358.
- 617 Exterkate, F., Otten, B., Wassenberg, H., & Veerkamp, J. (1971). Comparison of the  
618 phospholipid composition of Bifidobacterium and Lactobacillus strains. *Journal of*  
619 *Bacteriology*, 106(3), 824-829.
- 620 Florence, A. C. R., Béal, C., Silva, R. C., Bogsan, C. S. B., Pilleggi, A. L. O. S., Gioielli, L.  
621 A., & Oliveira, M. N. (2012). Fatty acid profile, *trans*-octadecenoic,  $\alpha$ -linolenic and  
622 conjugated linoleic acid contents differing in certified organic and conventional  
623 probiotic fermented milks. *Food Chemistry*, 135(4), 2207-2214. doi:  
624 <https://doi.org/10.1016/j.foodchem.2012.07.026>
- 625 Galanos, D. S., & Kapoulas, V. M. (1962). Isolation of polar lipids from triglyceride  
626 mixtures. *Journal of Lipid Research*, 3(1), 134-136.
- 627 Gaynor, P., Hubbell, S., Schmidt, A., Lina, R., Minskoff, S., & Greenberg, M. (1991).  
628 Regulation of phosphatidylglycerolphosphate synthase in *Saccharomyces cerevisiae*  
629 by factors affecting mitochondrial development. *Journal of Bacteriology*, 173(19),  
630 6124-6131. doi: <https://doi.org/10.1128/jb.173.19.6124-6131.1991>
- 631 Gijsbers, L., Ding, E. L., Malik, V. S., de Goede, J., Geleijnse, J. M., & Soedamah-Muthu, S.  
632 S. (2016). Consumption of dairy foods and diabetes incidence: a dose-response meta-  
633 analysis of observational studies. *The American Journal of Clinical Nutrition*, 103(4),  
634 1111-1124. doi: <https://doi.org/10.3945/ajcn.115.123216>
- 635 Guzel, S., Yibar, A., Belenli, D., Cetin, I., & Tanriverdi, M. (2017). The concentrations of  
636 adipokines in goat milk: relation to plasma levels, inflammatory status, milk quality  
637 and composition. *Journal of Veterinary Medical Science*, 16-0061. doi:  
638 <https://doi.org/10.1292/jvms.16-0061>
- 639 Jung, D.-S., Bodyfelt, F. W., & Daeschel, M. A. (1992). Influence of fat and emulsifiers on  
640 the efficacy of nisin in inhibiting *Listeria monocytogenes* in fluid milk. *Journal of*  
641 *Dairy Science*, 75(2), 387-393. doi: [https://doi.org/10.3168/jds.S0022-](https://doi.org/10.3168/jds.S0022-0302(92)77773-X)  
642 [0302\(92\)77773-X](https://doi.org/10.3168/jds.S0022-0302(92)77773-X)
- 643 Kaur, S., & Kaur, S. (2015). Bacteriocins as potential anticancer agents. [Review]. *Frontiers*  
644 *in Pharmacology*, 6(272). doi: <https://doi.org/10.3389/fphar.2015.00272>
- 645 Kuipers, A., Rink, R., & Moll, G. N. (2011). Genetics, Biosynthesis, Structure, and Mode of  
646 Action of Lantibiotics. In D. Drider & S. Rebuffat (Eds.), *Prokaryotic Antimicrobial*  
647 *Peptides: From Genes to Applications* (pp. 147-169). New York, NY: Springer New  
648 York.
- 649 Labonté, M. E., Couture, P., Richard, C., Desroches, S., & Lamarche, B. (2013). Impact of  
650 dairy products on biomarkers of inflammation: A systematic review of randomized  
651 controlled nutritional intervention studies in overweight and obese adults. *American*  
652 *Journal of Clinical Nutrition*, 97(4), 706-717. doi:  
653 <http://dx.doi.org/10.3945/ajcn.112.052217>
- 654 Lamarche, B., Givens, D. I., Soedamah-Muthu, S., Krauss, R. M., Jakobsen, M. U., Bischoff-  
655 Ferrari, H. A., . . . Després, J.-P. (2016). Does milk consumption contribute to  
656 cardiometabolic health and overall diet quality? *Canadian Journal of Cardiology*,  
657 32(8), 1026-1032. doi: <https://doi.org/10.1016/j.cjca.2015.12.033>
- 658 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., . . . Genome Project  
659 Data Processing Subgroup. (2009). The sequence alignment/map format and

660 SAMtools. *Bioinformatics*, 25(16), 2078-2079. doi:  
661 <https://doi.org/10.1093/bioinformatics/btp352>

662 Li, H., Zhang, L., Chen, L., Zhu, Q., Wang, W., & Qiao, J. (2016). *Lactobacillus acidophilus*  
663 alleviates the inflammatory response to enterotoxigenic *Escherichia coli* K88 via  
664 inhibition of the NF- $\kappa$ B and p38 mitogen-activated protein kinase signaling pathways  
665 in piglets. *BMC Microbiology*, 16(1), 273. doi: <https://doi.org/10.1186/s12866-016-0862-9>

666

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

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

673 Lordan, R., Tsoupras, A., & Zabetakis, I. (2018). The potential role of dietary platelet-  
674 activating factor inhibitors in cancer prevention and treatment. *Advances in Nutrition*,  
675 *In Press*. doi: <https://10.1093/advances/nmy090>

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

679 Lordan, R., & Zabetakis, I. (2017b). Ovine and caprine lipids promoting cardiovascular  
680 health in milk and its derivatives. *Advances in Dairy Research*, 5(176). doi:  
681 <https://doi.org/10.4172/2329-888X.1000176>

682 Machaidze, G., & Seelig, J. (2003). Specific binding of cinnamycin (Ro 09-0198) to  
683 phosphatidylethanolamine. comparison between micellar and membrane  
684 environments. *Biochemistry*, 42(43), 12570-12576. doi:  
685 <https://doi.org/10.1021/bi035225b>

686 McAuley, C. M., Gobius, K. S., Britz, M. L., & Craven, H. M. (2012). Heat resistance of  
687 thermotolerant enterococci isolated from milk. *International Journal of Food*  
688 *Microbiology*, 154(3), 162-168. doi:  
689 <https://doi.org/10.1016/j.ijfoodmicro.2011.12.033>

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

694 Meier, T., Gräfe, K., Senn, F., Sur, P., Stangl, G. I., Dawczynski, C., . . . Lorkowski, S.  
695 (2018). Cardiovascular mortality attributable to dietary risk factors in 51 countries in  
696 the WHO European Region from 1990 to 2016: A systematic analysis of the Global  
697 Burden of Disease Study. [journal article]. *European Journal of Epidemiology*, 34(1),  
698 37-55. doi: <https://doi.org/10.1007/s10654-018-0473-x>

699 Molkentin, J. (2000). Occurrence and biochemical characteristics of natural bioactive  
700 substances in bovine milk lipids. *British Journal of Nutrition*, 84(S1), 47-53. doi:  
701 <https://doi.org/10.1017/S0007114500002245>

702 Moll, G. N., Konings, W. N., & Driessen, A. J. M. (1999). Bacteriocins: mechanism of  
703 membrane insertion and pore formation. In W. N. Konings, O. P. Kuipers & J. H. J.  
704 H. In 't Veld (Eds.), *Lactic Acid Bacteria: Genetics, Metabolism and Applications:*  
705 *Proceedings of the Sixth Symposium on lactic acid bacteria: genetics, metabolism and*  
706 *applications, 19–23 September 1999, Veldhoven, The Netherlands* (pp. 185-198).  
707 Dordrecht: Springer Netherlands.

708 Moss, J. W. E., Williams, J. O., & Ramji, D. P. (2018). Nutraceuticals as therapeutic agents  
709 for atherosclerosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of*

710 *Disease*, 1864(5, Part A), 1562-1572. doi:  
711 <https://doi.org/10.1016/j.bbadis.2018.02.006>

712 Mozaffarian, D. (2016). Dietary and policy priorities for cardiovascular disease, diabetes, and  
713 obesity. *Circulation*, 133(2), 187-225. doi:  
714 <https://doi.org/10.1161/CIRCULATIONAHA.115.018585>

715 O'Shea, E. F., Cotter, P. D., Stanton, C., Ross, R. P., & Hill, C. (2012). Production of  
716 bioactive substances by intestinal bacteria as a basis for explaining probiotic  
717 mechanisms: bacteriocins and conjugated linoleic acid. *International Journal of Food*  
718 *Microbiology*, 152(3), 189-205. doi:  
719 <https://doi.org/10.1016/j.ijfoodmicro.2011.05.025>

720 Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M. H. H., Oksanen, M. J., &  
721 Suggests, M. (2007). The vegan package. *Community Ecology Package*, 10, 631-637.

722 Olmedilla-Alonso, B., Nova-Rebato, E., García-González, N., Martín-Diana, A.-B.,  
723 Fontecha, J., Delgado, D., . . . Asensio-Vegas, C. (2017). Effect of ewe's (semi-  
724 skimmed and whole) and cow's milk yogurt consumption on the lipid profile of  
725 control subjects: a crossover study. *Food and Nutrition Research*, 61(1), 1391669.  
726 doi: <https://doi.org/10.1080/16546628.2017.1391669>

727 Palur Ramakrishnan, A. V. K., Varghese, T. P., Vanapalli, S., Nair, N. K., & Mingate, M. D.  
728 (2017). Platelet activating factor: A potential biomarker in acute coronary syndrome?  
729 *Cardiovascular Therapeutics*, 35(1), 64-70. doi: [https://doi.org/10.1111/1755-  
730 5922.12233](https://doi.org/10.1111/1755-5922.12233)

731 Park, Y. W. (2009). *Bioactive Components in Milk and Dairy Products* (1 ed.). 2121 State  
732 Avenue, Ames, Iowa 50014-8300, USA: Wiley-Blackwell.

733 Peng, Y., Leung, H. C. M., Yiu, S. M., & Chin, F. Y. L. (2012). IDBA-UD: a *de novo*  
734 assembler for single-cell and metagenomic sequencing data with highly uneven depth.  
735 *Bioinformatics*, 28(11), 1420-1428. doi: <https://doi.org/10.1093/bioinformatics/bts174>

736 Poutzalis, S., Anastasiadou, A., Nasopoulou, C., Megale mou, K., Sioriki, E., & Zabetakis, I.  
737 (2016). Evaluation of the in vitro anti-atherogenic activities of goat milk and goat  
738 dairy products. *Dairy Science & Technology*, 96(3), 317-327. Retrieved from  
739 doi:<https://doi.org/10.1007/s13594-015-0266-x>

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

743 Redondo, N., García-González, N., Diaz-Prieto, L. E., Olmedilla-Alonso, B., Martín-Diana,  
744 A. B., Asensio-Vegas, C., & Nova, E. (2018). Effects of ewe's milk yogurt (whole  
745 and semi-skimmed) and cow's milk yogurt on inflammation markers and gut  
746 microbiota of subjects with borderline-high plasma cholesterol levels: A crossover  
747 study. [journal article]. *European Journal of Nutrition*. doi:  
748 <https://doi.org/10.1007/s00394-018-1626-0>

749 Reguła, A. (2007). Free fatty acid profiles of fermented beverages made from ewe's milk. *Le*  
750 *Lait*, 87(1), 71-77. doi: <https://doi.org/10.1051/lait:2006024>

751 Sayon-Orea, C., Martínez-González, M. A., Ruiz-Canela, M., & Bes-Rastrollo, M. (2017).  
752 Associations between yogurt consumption and weight gain and risk of obesity and  
753 metabolic syndrome: A systematic review. *Advances in Nutrition*, 8(1), 146S-154S.  
754 doi: <https://doi.org/10.3945/an.115.011536>

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

- 759 Snijder, H. J., & Dijkstra, B. W. (2000). Bacterial phospholipase A: structure and function of  
760 an integral membrane phospholipase. *Biochimica et Biophysica Acta (BBA) -*  
761 *Molecular and Cell Biology of Lipids*, 1488(1), 91-101. doi:  
762 [https://doi.org/10.1016/S1388-1981\(00\)00113-X](https://doi.org/10.1016/S1388-1981(00)00113-X)
- 763 Suzek, B. E., Wang, Y., Huang, H., McGarvey, P. B., Wu, C. H., & The UniProt Consortium.  
764 (2015). UniRef clusters: a comprehensive and scalable alternative for improving  
765 sequence similarity searches. *Bioinformatics*, 31(6), 926-932. doi:  
766 <https://doi.org/10.1093/bioinformatics/btu739>
- 767 Team R Core. (2013). R: A language and environment for statistical computing.
- 768 Thorning, T. K., Raben, A., Tholstrup, T., Soedamah-Muthu, S. S., Givens, I., & Astrup, A.  
769 (2016). Milk and dairy products: good or bad for human health? An assessment of the  
770 totality of scientific evidence. *Food and Nutrition Research*, 60(1), 32527. doi:  
771 <https://doi.org/10.3402/fnr.v60.32527>
- 772 Truitt, A., McNeill, G., & Vanderhoek, J. Y. (1999). Antiplatelet effects of conjugated  
773 linoleic acid isomers. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell*  
774 *Biology of Lipids*, 1438(2), 239-246. doi: [https://doi.org/10.1016/S1388-](https://doi.org/10.1016/S1388-1981(99)00055-4)  
775 [1981\(99\)00055-4](https://doi.org/10.1016/S1388-1981(99)00055-4)
- 776 Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., . . .  
777 Segata, N. (2015). MetaPhlan2 for enhanced metagenomic taxonomic profiling.  
778 *Nature Methods*, 12, 902. doi: <https://doi.org/10.1038/nmeth.3589>
- 779 Tsorotioti, S. E., Nasopoulou, C., Detopoulou, M., Sioriki, E., Demopoulos, C. A., &  
780 Zabetakis, I. (2014). *In vitro* anti-atherogenic properties of traditional Greek cheese  
781 lipid fractions. [journal article]. *Dairy Science & Technology*, 94(3), 269-281. doi:  
782 <http://dx.doi.org/10.1007/s13594-014-0161-x>
- 783 Tsoupras, A., Lordan, R., Demuru, M., Shiels, K., Saha, S. K., Nasopoulou, C., & Zabetakis,  
784 I. (2018). Structural elucidation of Irish organic farmed salmon (*Salmo salar*) polar  
785 lipids with antithrombotic activities. *Marine Drugs*, 16(6), 176. doi:  
786 <https://doi.org/10.3390/md16060176>
- 787 Tsoupras, A., Lordan, R., & Zabetakis, I. (2018). Inflammation, not cholesterol, is a cause of  
788 chronic disease. *Nutrients*, 10(5), 604. doi: <https://doi.org/10.3390/nu10050604>
- 789 Tsoupras, A., Zabetakis, I., & Lordan, R. (2019). Platelet aggregometry assay for evaluating  
790 the effects of platelet agonists and antiplatelet compounds on platelet function *in*  
791 *vitro*. *MethodsX*, 6, 63-70. doi: <https://doi.org/10.1016/j.mex.2018.12.012>
- 792 van Heel, A. J., de Jong, A., Montalban-Lopez, M., Kok, J., & Kuipers, O. P. (2013).  
793 BAGEL3: automated identification of genes encoding bacteriocins and (non-)  
794 bactericidal posttranslationally modified peptides. *Nucleic Acids Research*, 41(W1),  
795 W448-W453. doi: <https://doi.org/10.1093/nar/gkt391>
- 796 Vance, J. E., & Vance, D. E. (2008). Chapter 8 - Phospholipid biosynthesis in eukaryotes. In  
797 D. E. Vance & J. E. Vance (Eds.), *Biochemistry of Lipids, Lipoproteins and*  
798 *Membranes* (5th ed.). Oxford, UK: Elsevier.
- 799 Vieira, C. P., Álvares, T. S., Gomes, L. S., Torres, A. G., Paschoalin, V. M. F., & Conte-  
800 Junior, C. A. (2015). Kefir grains change fatty acid profile of milk during  
801 fermentation and storage. *PLoS One*, 10(10), e0139910. doi:  
802 <https://doi.org/10.1371/journal.pone.0139910>
- 803 Walsh, A. M., Crispie, F., Kilcawley, K., O'Sullivan, O., O'Sullivan, M. G., Claesson, M. J.,  
804 & Cotter, P. D. (2016). Microbial succession and flavor production in the fermented  
805 dairy beverage kefir. *mSystems*, 1(5), e00052-00016. doi:  
806 <https://doi.org/10.1128/mSystems.00052-1>
- 807 Walsh, A. M., Crispie, F., O'Sullivan, O., Finnegan, L., Claesson, M. J., & Cotter, P. D.  
808 (2018). Species classifier choice is a key consideration when analysing low-

809 complexity food microbiome data. [journal article]. *Microbiome*, 6(1), 50. doi:  
810 <https://doi.org/10.1186/s40168-018-0437-0>  
811 Wendel, A. A., Lewin, T. M., & Coleman, R. A. (2009). Glycerol-3-phosphate  
812 acyltransferases: Rate limiting enzymes of triacylglycerol biosynthesis. *Biochimica et*  
813 *biophysica acta*, 1791(6), 501-506. doi: <https://doi.org/10.1016/j.bbaliip.2008.10.010>  
814 Wickham, H. (2016). *ggplot2: elegant graphics for data analysis*: Springer.  
815 World Health Organization. (2017). Cardiovascular Diseases (CVD) Retrieved 19 June  
816 2018, from <http://www.who.int/mediacentre/factsheets/fs317/en/>  
817 Wu, L., & Sun, D. (2017). Consumption of yogurt and the incident risk of cardiovascular  
818 disease: A meta-analysis of nine cohort studies. *Nutrients*, 9(3), 315. doi:  
819 <https://doi.org/10.3390/nu9030315>  
820 Yadav, H., Jain, S., & Sinha, P. R. (2007). Production of free fatty acids and conjugated  
821 linoleic acid in probiotic dahi containing *Lactobacillus acidophilus* and *Lactobacillus*  
822 *casei* during fermentation and storage. *International Dairy Journal*, 17(8), 1006-1010.  
823 doi: <https://doi.org/10.1016/j.idairyj.2006.12.003>

824

825

826

827

828

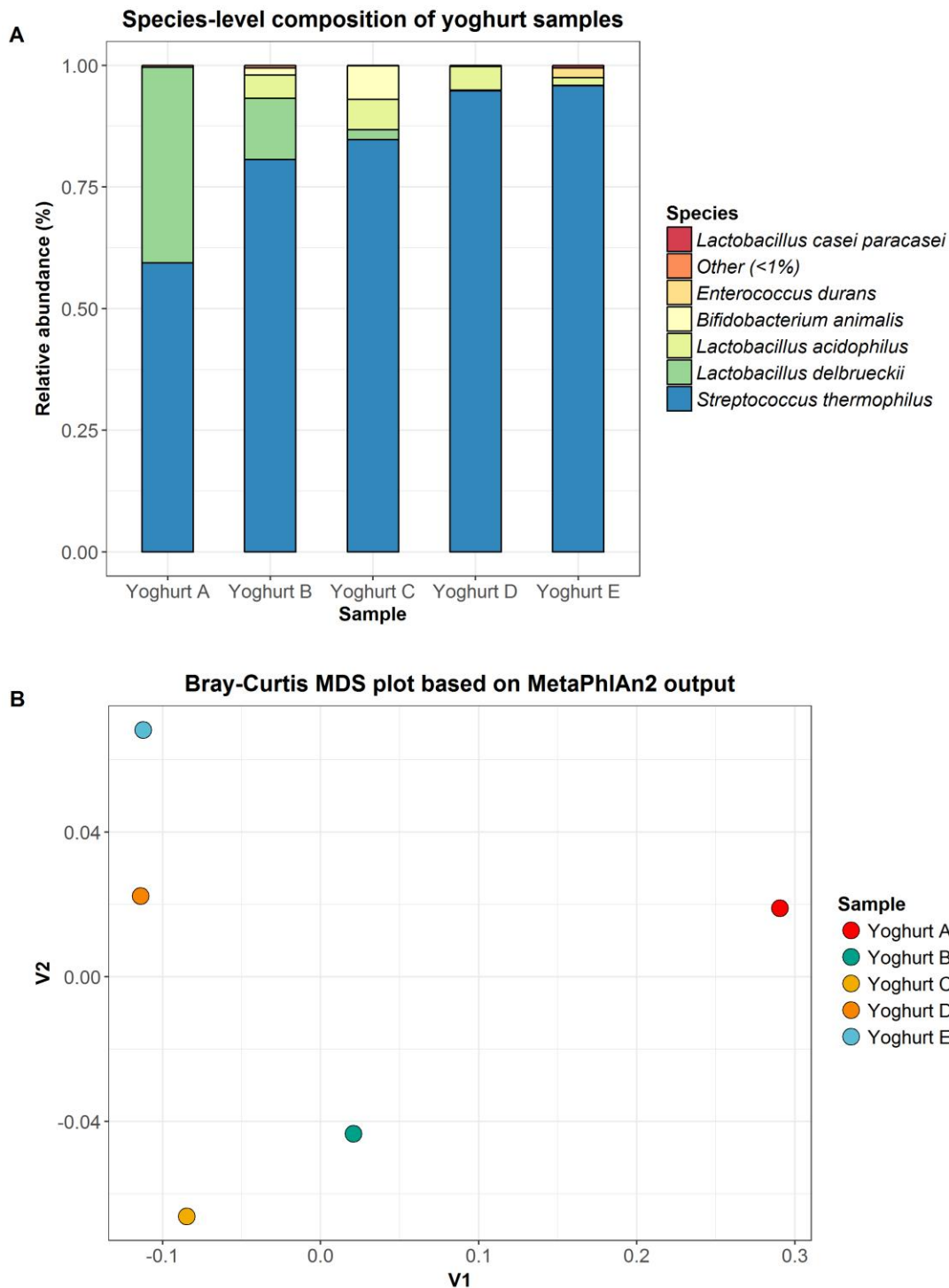
829

830

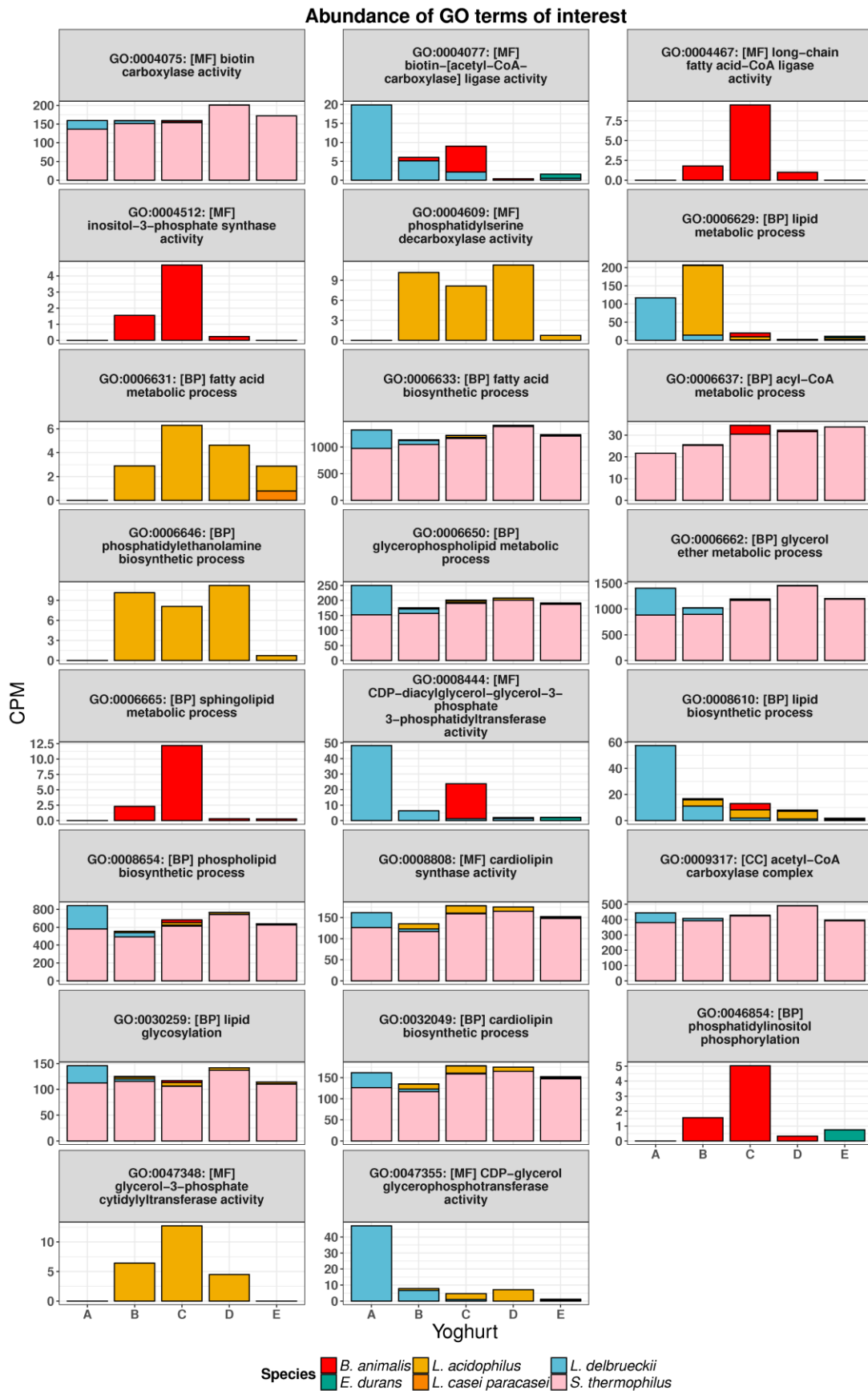
831

832

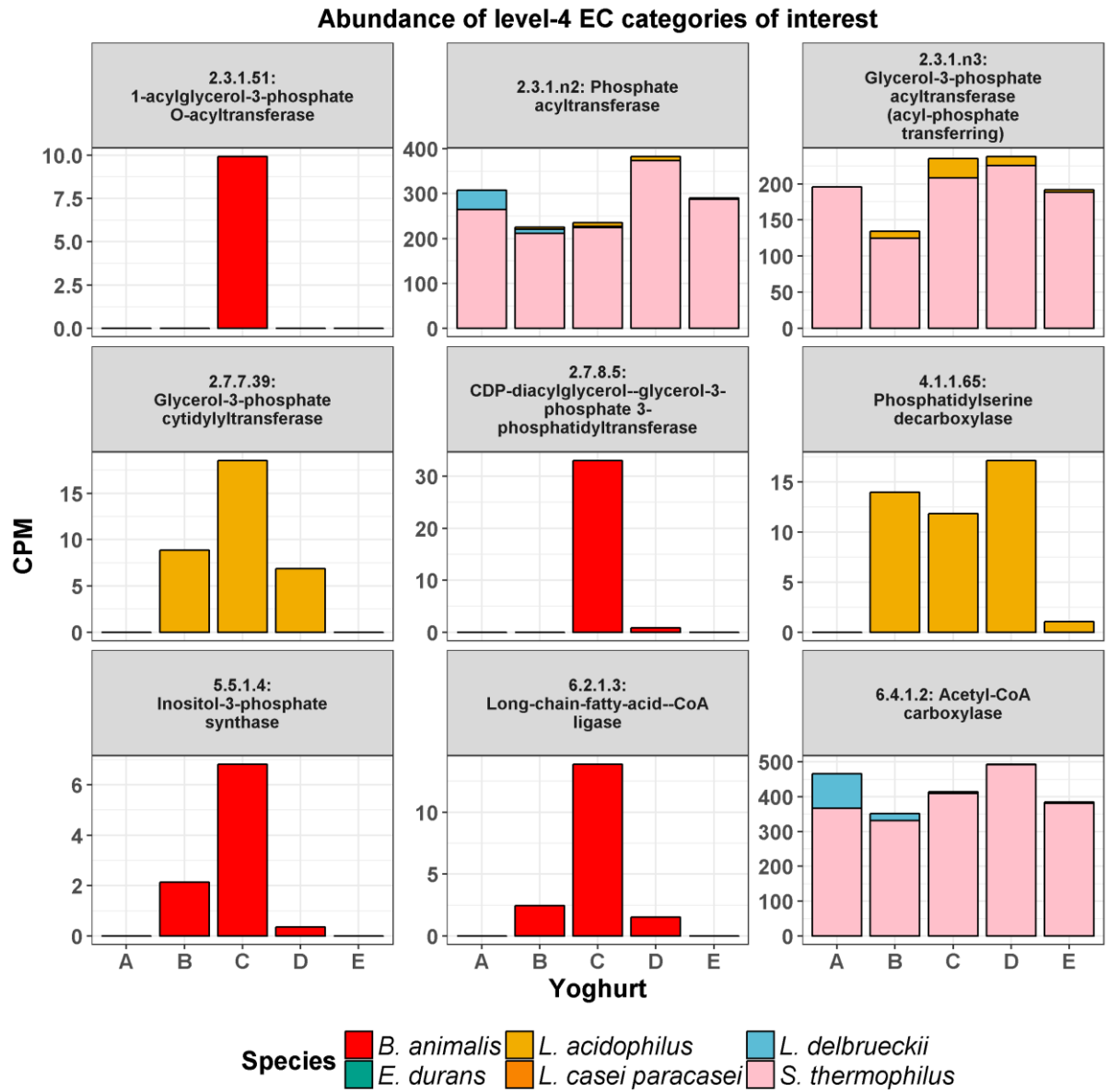
833



855 **Fig. 1.** (A) a stacked bar chart presenting the species-level microbial profile of yoghurts A-E  
 856 as determined by 16s rRNA gene sequencing. (B) A Bray-Curtis MDS plot based on the  
 857 MetaPhlAn2 output on the right that demonstrates that the microbial composition of yoghurts  
 858 D and E are similar, yoghurts B and C are more similar to each other, whereas the microbial  
 859 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.



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

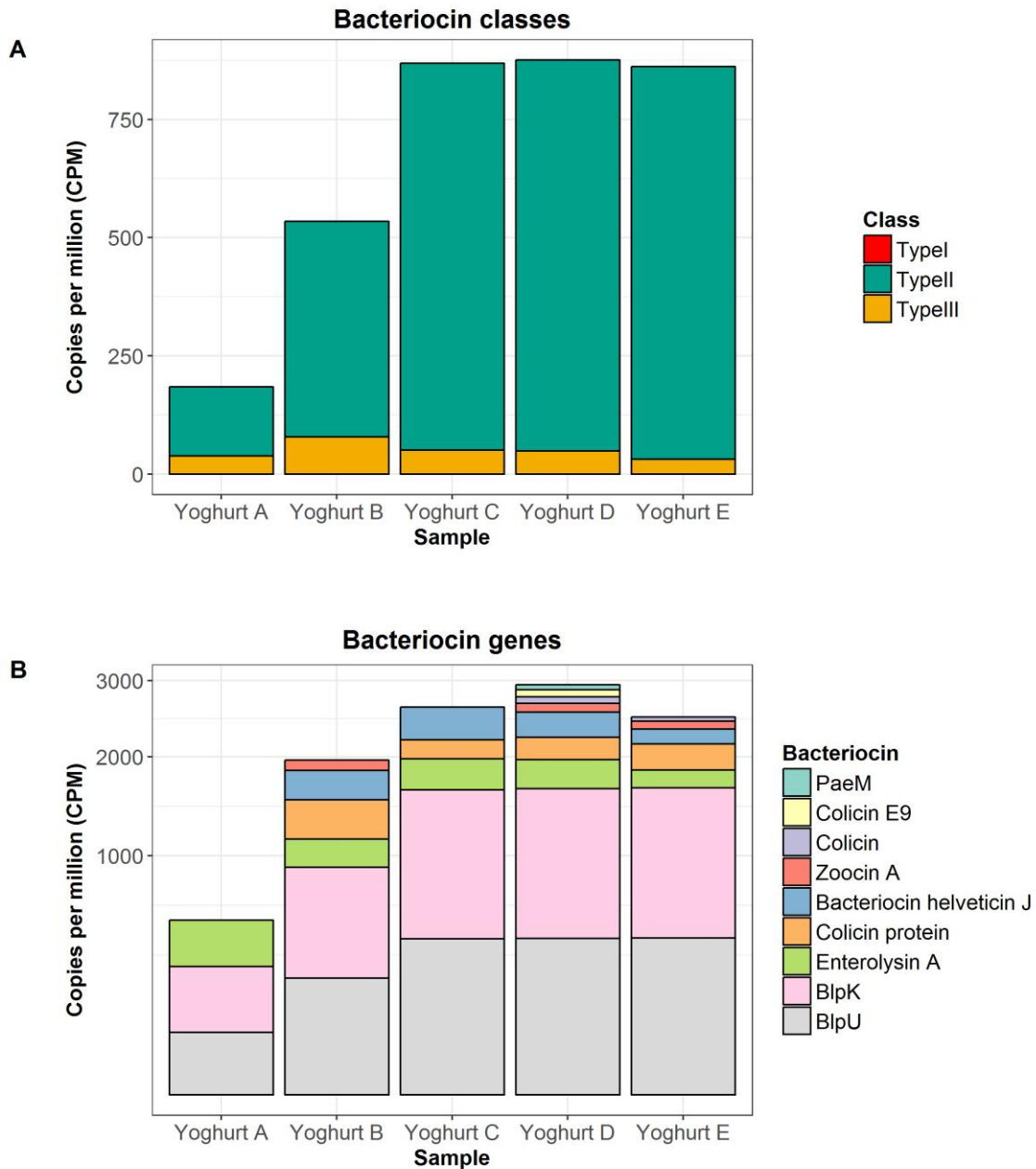
865

866

867

868

869



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

873

874

875

876

877

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

881

882

883

884

885

886

887

888

889

890

891

892

893

894  
895  
896  
897  
898  
899  
900  
901  
902  
903  
904  
905  
906  
907  
908  
909  
910  
911  
912  
913  
914  
915  
916  
917  
918  
919  
920

**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 $\pm$ 0.37 <sup>a</sup>	95.15 $\pm$ 2.30 <sup>a</sup>	3.20 $\pm$ 0.56 <sup>b</sup>
Yoghurt A	8.10 $\pm$ 0.43 <sup>b</sup>	96.46 $\pm$ 1.07 <sup>a</sup>	2.45 $\pm$ 0.20 <sup>ab</sup>
Yoghurt B	8.23 $\pm$ 1.59 <sup>bc</sup>	97.62 $\pm$ 0.22 <sup>a</sup>	2.29 $\pm$ 0.17 <sup>a</sup>
Yoghurt C	7.23 $\pm$ 0.60 <sup>b</sup>	97.47 $\pm$ 0.53 <sup>a</sup>	2.10 $\pm$ 0.37 <sup>a</sup>
Yoghurt D	7.47 $\pm$ 0.36 <sup>b</sup>	97.34 $\pm$ 0.47 <sup>a</sup>	2.25 $\pm$ 0.10 <sup>a</sup>
Yoghurt E	9.20 $\pm$ 0.55 <sup>bc</sup>	97.60 $\pm$ 0.38 <sup>a</sup>	2.55 $\pm$ 0.45 <sup>ab</sup>

<sup>ab</sup>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 ( $p < 0.05$ ).

921 **Table 3**

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

Fatty Acids	A	B	C	D	E	Milk
<b>8:0</b>	ND	0.22 $\pm$ 0.14 <sup>a</sup>	0.35 $\pm$ 0.08 <sup>a</sup>	ND	0.25 $\pm$ 0.07 <sup>a</sup>	0.76 $\pm$ 0.66 <sup>a</sup>
<b>9:0</b>	ND	0.12 $\pm$ 0.05 <sup>a</sup>	ND	ND	ND	0.14 $\pm$ 0.07 <sup>a</sup>
<b>10:0</b>	3.14 $\pm$ 0.32 <sup>b</sup>	4.90 $\pm$ 0.89 <sup>c</sup>	4.66 $\pm$ 0.56 <sup>c</sup>	1.60 $\pm$ 0.12 <sup>a</sup>	3.35 $\pm$ 0.65 <sup>bc</sup>	5.58 $\pm$ 1.78 <sup>c</sup>
<b>10:1</b>	0.17 $\pm$ 0.03 <sup>b</sup>	0.22 $\pm$ 0.06 <sup>bc</sup>	0.34 $\pm$ 0.13 <sup>c</sup>	0.07 $\pm$ 0.01 <sup>a</sup>	0.20 $\pm$ 0.04 <sup>bc</sup>	0.28 $\pm$ 0.11 <sup>bc</sup>
<b>11:0</b>	0.13 $\pm$ 0.02 <sup>a</sup>	0.29 $\pm$ 0.06 <sup>bc</sup>	0.14 $\pm$ 0.12 <sup>abc</sup>	0.20 $\pm$ 0.01 <sup>b</sup>	0.17 $\pm$ 0.03 <sup>ab</sup>	0.30 $\pm$ 0.08 <sup>c</sup>
<b>12:0</b>	3.90 $\pm$ 0.50 <sup>a</sup>	5.06 $\pm$ 0.82 <sup>ab</sup>	5.51 $\pm$ 0.85 <sup>b</sup>	5.20 $\pm$ 0.32 <sup>b</sup>	4.54 $\pm$ 0.54 <sup>ab</sup>	4.93 $\pm$ 0.73 <sup>ab</sup>
<b>12:1</b>	0.15 $\pm$ 0.05 <sup>bc</sup>	0.12 $\pm$ 0.05 <sup>ab</sup>	0.12 $\pm$ 0.07 <sup>abc</sup>	0.20 $\pm$ 0.01 <sup>c</sup>	0.20 $\pm$ 0.01 <sup>c</sup>	0.06 $\pm$ 0.01 <sup>a</sup>
<b>13:0</b>	0.21 $\pm$ 0.02 <sup>a</sup>	0.32 $\pm$ 0.08 <sup>bc</sup>	0.31 $\pm$ 0.02 <sup>b</sup>	0.36 $\pm$ 0.02 <sup>c</sup>	0.18 $\pm$ 0.03 <sup>a</sup>	0.32 $\pm$ 0.05 <sup>bc</sup>
<b>14:0</b>	8.20 $\pm$ 0.59 <sup>a</sup>	11.38 $\pm$ 0.28 <sup>c</sup>	13.89 $\pm$ 1.17 <sup>d</sup>	11.49 $\pm$ 0.78 <sup>c</sup>	9.83 $\pm$ 0.96 <sup>ab</sup>	8.87 $\pm$ 1.00 <sup>ab</sup>
<b>14:1<math>\omega</math>7 c9</b>	0.28 $\pm$ 0.08 <sup>abc</sup>	0.34 $\pm$ 0.01 <sup>c</sup>	0.32 $\pm$ 0.05 <sup>bc</sup>	0.42 $\pm$ 0.15 <sup>bc</sup>	0.31 $\pm$ 0.01 <sup>b</sup>	0.19 $\pm$ 0.01 <sup>a</sup>
<b>15:0</b>	1.33 $\pm$ 0.08 <sup>b</sup>	1.93 $\pm$ 0.22 <sup>a</sup>	1.31 $\pm$ 0.39 <sup>abc</sup>	1.70 $\pm$ 0.20 <sup>c</sup>	1.39 $\pm$ 0.07 <sup>b</sup>	1.56 $\pm$ 0.10 <sup>c</sup>
<b>16:0</b>	17.53 $\pm$ 0.32 <sup>a</sup>	19.87 $\pm$ 0.48 <sup>b</sup>	20.43 $\pm$ 0.65 <sup>bc</sup>	21.62 $\pm$ 0.60 <sup>c</sup>	19.53 $\pm$ 1.56 <sup>bc</sup>	17.20 $\pm$ 0.76 <sup>a</sup>
<b>16:1<math>\omega</math>7 c9</b>	2.00 $\pm$ 0.14 <sup>c</sup>	2.53 $\pm$ 0.35 <sup>d</sup>	1.40 $\pm$ 0.18 <sup>b</sup>	2.09 $\pm$ 0.31 <sup>cd</sup>	1.71 $\pm$ 0.66 <sup>bcd</sup>	0.88 $\pm$ 0.06 <sup>a</sup>
<b>17:0</b>	0.90 $\pm$ 0.01 <sup>c</sup>	1.07 $\pm$ 0.15 <sup>d</sup>	0.75 $\pm$ 0.09 <sup>b</sup>	0.96 $\pm$ 0.05 <sup>bd</sup>	0.96 $\pm$ 0.27 <sup>bcd</sup>	0.45 $\pm$ 0.01 <sup>a</sup>
<b>17:1</b>	0.61 $\pm$ 0.01 <sup>c</sup>	0.67 $\pm$ 0.13 <sup>c</sup>	0.27 $\pm$ 0.17 <sup>b</sup>	0.65 $\pm$ 0.12 <sup>c</sup>	0.72 $\pm$ 0.30 <sup>bc</sup>	0.02 $\pm$ 0.01 <sup>a</sup>
<b>18:0</b>	10.93 $\pm$ 0.68 <sup>b</sup>	11.25 $\pm$ 1.25 <sup>b</sup>	15.76 $\pm$ 2.64 <sup>c</sup>	15.13 $\pm$ 1.17 <sup>c</sup>	8.25 $\pm$ 0.27 <sup>a</sup>	11.32 $\pm$ 0.72 <sup>b</sup>
<b>18:1<math>\omega</math>9 c9</b>	35.56 $\pm$ 1.40 <sup>c</sup>	27.62 $\pm$ 1.36 <sup>b</sup>	23.61 $\pm$ 0.31 <sup>ab</sup>	22.97 $\pm$ 1.63 <sup>a</sup>	30.53 $\pm$ 3.74 <sup>bc</sup>	23.59 $\pm$ 3.56 <sup>ab</sup>
<b>18:2<math>\omega</math>6 c9, t12</b>	6.31 $\pm$ 0.02 <sup>c</sup>	5.67 $\pm$ 0.67 <sup>abc</sup>	4.89 $\pm$ 0.33 <sup>a</sup>	5.95 $\pm$ 0.06 <sup>b</sup>	5.50 $\pm$ 1.70 <sup>abc</sup>	9.23 $\pm$ 0.64 <sup>d</sup>
<b>18:2<math>\omega</math>7 c9, t11</b>	3.51 $\pm$ 0.16 <sup>d</sup>	1.94 $\pm$ 0.12 <sup>ab</sup>	1.47 $\pm$ 0.35 <sup>a</sup>	2.00 $\pm$ 0.06 <sup>b</sup>	3.29 $\pm$ 0.60 <sup>d</sup>	2.56 $\pm$ 0.02 <sup>c</sup>
<b>18:2<math>\omega</math>6 t10, c12</b>	0.46 $\pm$ 0.35 <sup>b</sup>	ND	0.05 $\pm$ 0.01 <sup>a</sup>	ND	0.93 $\pm$ 0.18 <sup>b</sup>	ND
<b>18:3<math>\omega</math>3 c9, c12, c15</b>	2.15 $\pm$ 0.01 <sup>d</sup>	1.44 $\pm$ 0.16 <sup>b</sup>	0.98 $\pm$ 0.16 <sup>a</sup>	1.83 $\pm$ 0.05 <sup>c</sup>	2.66 $\pm$ 0.13 <sup>f</sup>	2.30 $\pm$ 0.16 <sup>e</sup>
<b>20:0</b>	ND	0.31 $\pm$ 0.01 <sup>a</sup>	0.24 $\pm$ 0.15 <sup>a</sup>	0.29 $\pm$ 0.11 <sup>a</sup>	ND	ND
<b>20:1<math>\omega</math>9 c9</b>	0.65 $\pm$ 0.04 <sup>a</sup>	0.79 $\pm$ 0.06 <sup>b</sup>	0.74 $\pm$ 0.14 <sup>ab</sup>	0.87 $\pm$ 0.04 <sup>b</sup>	0.79 $\pm$ 0.44 <sup>abc</sup>	1.01 $\pm$ 0.03 <sup>c</sup>
<b>20:3<math>\omega</math>9 c5, c8, c11</b>	ND	0.24 $\pm$ 0.01 <sup>a</sup>	0.37 $\pm$ 0.12 <sup>a</sup>	0.20 $\pm$ 0.13 <sup>a</sup>	0.32 $\pm$ 0.18 <sup>a</sup>	ND
<b>20:4<math>\omega</math>6 c5, c8, c11, c14</b>	0.95 $\pm$ 0.15 <sup>c</sup>	0.49 $\pm$ 0.04 <sup>a</sup>	0.61 $\pm$ 0.10 <sup>b</sup>	0.57 $\pm$ 0.01 <sup>b</sup>	1.20 $\pm$ 0.36 <sup>c</sup>	ND
<b>20:5<math>\omega</math>3 c5, c8, c11, c14, c17</b>	0.35 $\pm$ 0.03 <sup>c</sup>	0.25 $\pm$ 0.01 <sup>a</sup>	0.22 $\pm$ 0.08 <sup>ab</sup>	0.29 $\pm$ 0.01 <sup>b</sup>	0.42 $\pm$ 0.05 <sup>d</sup>	3.15 $\pm$ 0.30 <sup>e</sup>
<b>22:1 c11</b>	0.53 $\pm$ 0.05 <sup>a</sup>	0.82 $\pm$ 0.15 <sup>bc</sup>	0.56 $\pm$ 0.22 <sup>ab</sup>	1.00 $\pm$ 0.04 <sup>c</sup>	0.72 $\pm$ 0.21 <sup>ab</sup>	0.89 $\pm$ 0.11 <sup>b</sup>
<b>22:4<math>\omega</math>6 c7, c10, c13, c16</b>	ND	0.14 $\pm$ 0.02 <sup>a</sup>	0.25 $\pm$ 0.17 <sup>ab</sup>	ND	0.44 $\pm$ 0.04 <sup>b</sup>	ND
<b>22:5<math>\omega</math>6 c4, c7, c10, c13, c16</b>	1.62 $\pm$ 0.30 <sup>b</sup>	0.97 $\pm$ 0.11 <sup>a</sup>	0.96 $\pm$ 0.20 <sup>a</sup>	1.54 $\pm$ 0.07 <sup>b</sup>	1.67 $\pm$ 0.14 <sup>b</sup>	ND
<b>22:6<math>\omega</math>3 c4, c7, c10, c13, c16, c19</b>	0.91 $\pm$ 0.37 <sup>c</sup>	0.28 $\pm$ 0.03 <sup>a</sup>	0.61 $\pm$ 0.36 <sup>abc</sup>	0.44 $\pm$ 0.04 <sup>b</sup>	0.58 $\pm$ 0.10 <sup>b</sup>	0.97 $\pm$ 0.04 <sup>c</sup>
<b>Total SFA</b>	46.27 $\pm$ 1.54 <sup>a</sup>	56.72 $\pm$ 1.33 <sup>c</sup>	63.31 $\pm$ 2.62 <sup>d</sup>	58.56 $\pm$ 0.89 <sup>c</sup>	48.44 $\pm$ 3.88 <sup>ab</sup>	54.01 $\pm$ 4.28 <sup>bc</sup>
<b>Total MUFA</b>	39.96 $\pm$ 1.37 <sup>c</sup>	32.90 $\pm$ 1.06 <sup>b</sup>	27.50 $\pm$ 0.48 <sup>a</sup>	27.90 $\pm$ 0.62 <sup>a</sup>	35.18 $\pm$ 3.84 <sup>bc</sup>	27.35 $\pm$ 3.35 <sup>a</sup>
<b>Total <math>\omega</math>7</b>	5.782 $\pm$ 0.22 <sup>c</sup>	4.805 $\pm$ 0.49 <sup>b</sup>	3.196 $\pm$ 0.48 <sup>a</sup>	4.519 $\pm$ 0.47 <sup>b</sup>	5.309 $\pm$ 0.65 <sup>bc</sup>	3.638 $\pm$ 0.07 <sup>a</sup>
<b>Total <math>\omega</math>9</b>	36.22 $\pm$ 1.39 <sup>c</sup>	28.65 $\pm$ 1.42 <sup>b</sup>	24.72 $\pm$ 0.27 <sup>a</sup>	24.05 $\pm$ 1.73 <sup>a</sup>	31.64 $\pm$ 4.03 <sup>bc</sup>	24.60 $\pm$ 3.56 <sup>ab</sup>
<b>Total PUFA</b>	16.37 $\pm$ 0.60 <sup>c</sup>	11.46 $\pm$ 0.97 <sup>a</sup>	10.42 $\pm$ 0.29 <sup>a</sup>	12.83 $\pm$ 0.18 <sup>b</sup>	17.01 $\pm$ 1.56 <sup>cd</sup>	18.22 $\pm$ 1.17 <sup>d</sup>
<b>Total <math>\omega</math>3</b>	3.431 $\pm$ 0.38 <sup>c</sup>	1.970 $\pm$ 0.16 <sup>a</sup>	1.819 $\pm$ 0.36 <sup>a</sup>	2.562 $\pm$ 0.07 <sup>b</sup>	3.662 $\pm$ 0.17 <sup>c</sup>	6.420 $\pm$ 0.45 <sup>d</sup>
<b>Total <math>\omega</math>6</b>	9.345 $\pm$ 0.38 <sup>c</sup>	7.270 $\pm$ 0.68 <sup>a</sup>	6.763 $\pm$ 0.26 <sup>a</sup>	8.057 $\pm$ 0.01 <sup>b</sup>	9.738 $\pm$ 1.17 <sup>c</sup>	9.228 $\pm$ 0.64 <sup>c</sup>

926 <sup>abcdef</sup> 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

929

930

931 **Table 4**

932 Inhibition of PAF-induced platelet aggregation in human PRP by sheep milk and yogurts total  
 933 lipids (TL), total polar lipids (TPL), and total neutral lipids (TNL) produced by various starter  
 934 cultures. This activity is represented by their IC<sub>50</sub> (μg) (mean ± SD, n = 3).

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

935 <sup>abcd</sup> 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

938

939