1 **The untargeted lipidomic profile of quarter milk from dairy cows with subclinical**
2 **intramammary infection by *non-aureus staphylococci***
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19 **ABSTRACT**

20 This observational study determined cow milk's lipidome during subclinical
21 intramammary infection (**IMI**) by *non-aureus staphylococci* (**NAS**), also defined as coagulase-
22 negative staphylococci, using an untargeted approach. Among the pathogens causing bovine IMI,
23 NAS have become the most frequently isolated bacteria from milk samples. Although the
24 application of system biology approaches to mastitis has provided pivotal information by
25 investigating the transcriptome, proteome, peptidome and metabolome, the milk lipidome during
26 mammary gland inflammation remains undisclosed. To cover this gap, we determined the milk
27 lipidome of 17 dairy cows with intramammary infection caused by NAS (**NAS-IMI**), and we
28 compared the results with that of the healthy quarter milk from 11 cows. The lipidome was
29 determined following a liquid chromatography–quadrupole time-of-flight mass spectrometry
30 (**LC-QTOF-MS**) approach. Sixteen subclasses of lipids were identified in both groups of animals.
31 From 2556 measured lipids, the abundance of 597 changed more than 10-fold in the quarter milk
32 with NAS-IMI compared to healthy quarters. The results demonstrated the influence of NAS IMI
33 on the milk lipidome, implying significant changes in lipid species belonging to the family of
34 triacylglycerols and sphingomyelins, and contribute to the understanding of inflammatory
35 processes in the bovine udder, highlighting potential novel biomarkers for improving mastitis
36 diagnostics.

37

38 ***Keywords:*** non-aureus staphylococci, dairy cow, lipidomics, mastitis

INTRODUCTION

Mastitis is an inflammation of the mammary gland that negatively impacts the dairy industry by reducing milk yield and quality and increasing the replacement of affected animals (Halasa et al., 2007). Although many pathogens can cause bovine mastitis, non-aureus Staphylococci (**NAS**), also defined as coagulase-negative staphylococci, are among the most frequently isolated bacteria from dairy cows with subclinical mastitis (Vanderhaeghen et al., 2014). The molecular mechanisms regulating the mammary gland inflammatory responses to NAS are unclear (Vanderhaeghen et al., 2014). Many NAS species can cause persistent intramammary infection (**IMI**), as demonstrated by the changes in abundance of numerous proteins, including N-acetyl-β-glucosaminidase (**NAGase**), milk and serum amyloid A (**MAA** and **SAA,** respectively), cathelicidins, and proinflammatory cytokines (Simojoki et al., 2011; Addis et al., 2016a, 2017).

The application of system biology approaches to mastitis has provided pivotal information on the immune defence of the mammary gland in terms of the transcriptome (Loor et al., 2011; Ferreira et al., 2013), proteome (Addis et al., 2016b; Mudaliar et al., 2016; Thomas et al., 2016b), peptidome (Addis et al., 2020), and metabolome (Thomas et al., 2016a).

On the contrary, changes in milk lipidome in mastitis lie virtually undiscovered. During mastitis, the milk lipid profile is likely important since many of the mediators involved in immune defence and inflammation of the mammary gland are derived from arachidonic acid. Previous investigations quantified oxylipid profiles during bovine mastitis caused by *Streptococcus uberis*, demonstrating an imbalance between derivatives of arachidonic acid, such as the milk lipoxin A4 (LXA4)- leukotriene B4(LTB4) ratio (Ryman et al., 2015). Moreover, a specific lipid metabolism pattern has been hypothesized to precede clinical mastitis development in prepartum transition dairy cows in *Streptococcus uberis* mastitis (Zandkarimi et al., 2018). Most of the presently available information is related to the lipid content under the milk quality perspective (Tsiafoulis et al., 2019; Yener and van Valenberg, 2019; Wang et al., 2020), or to identify breed-related differences (Tomassini et al., 2019).

65 The present study aims at covering the knowledge gap by determining the untargeted lipidome of
66 cow milk for the first time during subclinical NAS IMI by applying a liquid chromatography–
67 quadrupole time-of-flight mass spectrometry (**LC-QTOF-MS**) approach.
68
69 **MATERIALS AND METHODS**

70 ***Animal Selection and Collection of Milk Samples***

71 The study was performed on a commercial dairy farm located in Northern Italy. The farm
72 maintained an average of 150 Holstein milking cows housed in free-stall barns in deep-bedded
73 cubicles with chopped straw. The animal selection and collection of samples were carried out as
74 previously described (Addis et al., 2020). Briefly, all the cows were fed with a balanced Total
75 Mixed Ration in feed alleys with headlocks. Lactating cows were milked twice a day in a double-
76 10 herringbone milking parlour. Preliminary milk samples were collected from all individual
77 quarters of 147 lactating cows to assess their udder health status. All the samples were collected
78 before the morning milking time from each quarter, following the National Mastitis Council
79 guidelines (Middleton et al., 2017). Before sampling, all teat ends were carefully cleaned with a
80 pre-dipping foam containing lactic acid and the apex was disinfected with alcohol. First streams
81 of foremilk were discharged, and then approximately 10 mL of milk were collected aseptically
82 from each teat into sterile vials. Samples were stored at 4°C until bacteriological assays, and the
83 somatic cell count (**SCC**) was measured immediately upon arrival using an automated somatic
84 cell counter (Bentley Somacount 150; Bentley Instruments, Chaska, MN).
85
86 ***Bacteriological Analyses of Milk Samples***

87 Bacteriological cultures were performed according to the National Mastitis Council
88 guidelines (Middleton et al., 2017)(NMC, 2017). Ten microliters of milk were spread on blood
89 agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37°C and
90 examined after 24 h. Colonies were provisionally identified based on morphology, hemolysis

91 patterns, and Gram staining. Gram-positive organisms were differentiated in staphylococci and
92 streptococci by the catalase reaction. The coagulase tube test in rabbit plasma was used to
93 differentiate *Staphylococcus aureus* from NAS species.
94
95 ***Sample selection***
96 The following milk samples were included in the study:
97 A: healthy (**H**) quarters that were selected if milk samples had SCC <100.000 cell/mL and were
98 culture negative. Eleven quarter samples met these criteria, of which three were from primiparous
99 and eight from multiparous cows.
100 B: milk from NAS-infected quarters (**NAS-IMI**) quarters (17 samples, of which 11 were from
101 primiparous and 6 from multiparous cows). A threshold of at least five NAS colonies isolated
102 from a 10-µL milk sample was set to classify the samples as NAS-IMI (Dohoo et al., 2011). NAS-
103 IMI quarters were further classified in high SCC quarters (**NAS-IMI-HC**) with SCC > than 200
104 x 10^3/mL and in low SCC quarters (**NAS-IMI-LC**) with SCC < 200 x 10^3/mL. The list of samples
105 included in the study is presented in **Table 1**. Some animals contributed different quarter samples
106 to the healthy or NAS-IMI groups. As detailed in Table 1, two cows contributed multiple samples
107 to the healthy group (cow 815, two quarters; cow 819, three quarters), three cows contributed
108 multiple samples to the NAS-IMI group (cow 620, three quarters; cow 855, two quarters; cow
109 881, two quarters), and two cows contributed multiple samples to both healthy and NAS-IMI
110 groups (cow 618, one healthy quarter and one NAS-IMI quarter; cow 909, one healthy quarter
111 and one NAS-IMI quarter).
112
113 ***Preparation of Milk Samples for Lipidomic Analysis: Lipid extraction***
114 Two aliquots (100 µL) from each sample were added with internal standards and extracted
115 according to the Folch method (Folch et al., 1957). The organic residue was reconstituted with
116 200 µL of 2-propanol: acetonitrile (90:10, v/v), 0.1% formic acid, and 10 mM ammonium acetate.

117 Aliquots of 20 µL were then diluted 1:10 with mobile phase B for lipidomic analysis in positive
118 mode. Aliquots of 5 µL were analyzed in negative ion mode for free fatty acid analysis. Aliquots
119 of 50 µL, after acid saponification, were prepared for total fatty acid quantification in negative
120 mode. Each sample was extracted in duplicate, and two runs were performed for each extraction.
121

122 ***Lipidomic Analysis***

123 Samples have been analyzed at UNITECH platform "OMICs" (Università degli Studi di
124 Milano, Italy) as follows: 2 and 5 µL of sample for the positive and negative ion mode,
125 respectively, were separated by liquid chromatography (**LC**) with a Kinetex EVO C18 - 2.1 x 100
126 mm, 1.7µm (Phenomenex®) column at 45°C connected to an ExionLC™ AD system (ABSciex)
127 maintained at 15°C. Separated metabolites were then ionized through an electrospray ionization
128 (**ESI**) source and analyzed in a TripleTOF 6600 (Quadrupole Time-Of-Flight, QTOF - ABSciex)
129 mass spectrometer. Mobile phases were A) water with 0.1% formic acid and 10 mM ammonium
130 acetate/acetonitrile (60:40); B) 2-propanol with 0.1% formic acid and 10 mM ammonium
131 acetate/acetonitrile (90:10). The following elution gradient was used: 0 min, 55% B; 2 min 55%
132 B; 12 min, 3 % B; 17 min, 3% B; 17.10 min, 55% B; 20 min, 55% B. The flow rate was 0.4
133 mL/min. ESI and mass spectrometer conditions were set as presented in **Supplemental Table S1**.
134

135 ***Data processing***

136 Data are expressed as analyte-to-internal standard area ratio (1-Phenoxy-2-propanol),
137 while fatty acids were expressed as ng/mL of milk. Data processing was carried out using the
138 untargeted data processing program MSDIAL (v3.98) with LipidBlast database v2019. This
139 database contains 143342 tandem mass (**MS/MS**) spectra relating to 110833 analytes belonging
140 to 32 lipid classes.
141

142 ***Univariate and multivariable analyses***

143 The Kruskal-Wallis ANOVA (a nonparametric method) test followed by Dunn's post-hoc multiple
144 comparisons were used to verify differences in lipid species abundance of H and NAS-IMI quarter
145 milk. These analyses were performed by using GraphPad Prims v6.1 software (GraphPad
146 Software, USA).
147
148 Data were checked for integrity, and variables containing more than 20% missing values (i.e.,
149 values lower than the limit of detection, LOD) were not considered for the statistical analysis
150 using the MetaboAnalyst 4.0 webtool (Chong et al., 2019). When present, missing values were
151 imputed by Bayesian Principal Component Analysis (BPCA) by MetaboAnalyst 4.0 (http://
152 www.metaboanalyst.ca). The data were then transformed by generalized log-transformation, and
153 Pareto scaled to correct for heteroscedasticity, reduce the skewness of the data and reduce mask
154 effects (Ghaffari et al., 2019). Partial least squares discriminant analysis (PLS-DA) identified
155 differential lipid metabolites between experimental groups. At the same time, the ranking of lipids
156 was performed by variable importance in projection (VIP) according to metabolite importance in
157 discriminating groups. The permutation test was used with a maximum of 100 permutations with
158 a separation distance test to assess the significance of class discrimination determined by PLS-
159 DA. The classification and cross-validation were performed with a maximum of 5 components
160 and the leave-one-out method. The heatmaps were clustered by Euclidean distance and Ward's
161 minimum variance method (ward.D). Finally, the k-means analysis was used to verify the
162 clustering of the experimental groups, and the number of clusters to test was set as the number of
163 experimental groups. Important milk lipid classes as well the lipid species were identified by the
164 volcano plots based on the size of the biological effect (fold-change) and the x-axis and the t-tests
165 threshold [false discovery rate (FDR) adjusted *P*-value of 0.05] on the y-axis. The principal
166 component analysis (PCA), volcano plots, heatmap, correlation and box and whiskers were
167 generated using MetaboAnalyst 4.0 (http:// www.metaboanalyst.ca) as well as different R (version

168 4.0) Shiny packages (*heatmaply*, *ComplexHeatmap*, and *plotly*, *corrplot* and graph plot *gplots* and

169 *ggplots2*) from R Studio (http://shiny.rstudio.com).

170

171 # RESULTS

172

173 ### *Analytical workflow, animal classification, and disease diagnosis*

174 The analytical workflow followed to investigate H and NAS-IMI quarter milk's lipidome
175 is presented in **Figure 1**. The list of collected samples included in the experimental study is
176 presented in **Table 1**. NAS-IMI quarters were further classified into NAS-IMI-HC, including
177 animals with SCC > 200 x 10^3/mL and NAS-IMI-LC, including animals with SCC < 200 x
178 10^3/mL.

179

180 ### *The lipidome in milk from healthy quarters*

181 In the first part of the study, we characterized the untargeted lipidome of healthy (H) milk,
182 identifying 2556 lipid species. The complete list of analyzed lipids is presented in **Supplemental**
183 **Table S2**. The lipids were classified in 16 classes, namely triacylglycerols (TAG [836 species]),
184 diacylglycerols (DAG [422 species]), monoacylglycerols (MAG [9 species]), phosphatidylserine
185 (PS [38 species]), sphingomyelin (SM [361 species]), phosphatidylinositol (PI [42 species]),
186 phosphatidylglycerol (PG [28 species]), phosphatidylethanolamine (PE [133 species]),
187 phosphatidylcholine (PC [99 species]), lysophosphatidylethanolamine (LPE [69 species]),
188 lysophosphatidylcholine (LPC [28 species]), ceramides (Cer [348 species]), cholesterol esters (CE
189 [47 species]), Bis(monoacylglycerol)phosphate (BMP [6 species]), acylcarnitines (AcCarn [25
190 species]) and fatty acids (FA [50 species]).

191 The abundance of TAG was significantly higher as compared to several lipid categories
192 ($p < 0.0001$, **Figure 2A**). Relevant levels of Cer, PC, and DAG (**Figure 2A**) were also detected.
193 We observed a particularly high abundance of some lipid species, including TAG 38:0:0, 40:1,
194 42:0, 48:1 (**Figure 2B**), PC 30:0, 32:0, 34:1, 36:1 (**Figure 2C**), Cer d37:5, d43.6:8, HexCer d49:7
195 (**Figure 2D**) and DAG 35:7, 39:2e, 34:1, 32:0, 41:1 (**Figure 2E**). Finally, the most abundant fatty

196 acids in healthy cow milk samples were the saturated 16:0, 18:0, 30:0 and the unsaturated 40:5,
197 42:5, 44:5 (**Figure 2F**).
198 Out of the 11 H samples included in the study, three were from cows at their first lactation,
199 and 8 were from multiparous cows. Therefore, we determined the differences between H quarter
200 milk from primiparous and multiparous cows by carrying out a PCA analysis. As shown in
201 **Supplemental Figure S1A**, PCA did not show any separation of H quarter milk samples
202 according to the cow parity (1 vs ≥ 2).
203

204 ***The Lipidome in Milk from infected Quarters***
205 Also in these samples, TAG were significantly more abundant than several lipid species
206 ($p < 0.0001$; **Figure 3A**). We identified higher levels of specific lipid species, including TAG
207 34:0, 36:0, 50:1, 40:0 (**Figure 3B**), PC 32:1, 34:2, 36:3e (**Figure 3C**), Cer 42:5, 45:8, HexCer
208 40:9 (**Figure 3D**), DAG 32:0, 34:1, 35:7e, 39:2e, 41:1 (**Figure 3E**) and FA 16:0, 18:0, 30:0, 40:5,
209 42:5, 44:5 (**Figure 3F**).
210 Given the background that NAS-IMI quarter milk samples were not homogeneous for
211 SCC and cow parity, further analysis was carried out to determine whether somatic cells' content
212 impacted lipid content. The results are presented in **Figure 4**. The IMI quarter milk samples were
213 further divided according to the SCC in NAS-IMI-LC (SCC $< 200 \times 10^3$/ml) and NAS-IMI-HC
214 (SCC $> 200 \times 10^3$/ml). **Figure 4A-C** presents the PCA and the PLS-DA of the lipidomics content,
215 providing evidence of no separation between experimental groups ($p = 0.65$).
216 Out of the 17 NAS-IMI samples included in the study, ten were from cows at their first
217 lactation. According to the cow parity, PCA analysis did not evidence any differences in
218 separation between NAS-IMI quarter milk samples (1 vs ≥ 2) (**Supplemental Figure S1B**). In
219 order to assess whether DIM may affect the lipidome, a PCS analysis was also carried out
220 comparing three groups of animals, divided following their DIM, namely DIM <149, DIM 150-

221 274 and DIM>275. No differences in the milk lipidome between samples clustered in these three
222 groups were found (**Supplemental Figure S2B**).
223
224 ***Comparison of lipid content between healthy and IMI quarters***

225 As a further step of the analysis, we investigated the difference between H and NAS-IMI milk
226 according to the main sixteen lipid classes. Following these criteria, the PCA analysis indicated
227 that H and NAS-IMI quarter milk samples were completely separated (**Figure 5A**). The heatmap
228 and volcano plot identified PG ($Log_2FC = -1.23$; $-log_{10}padj = 11.07$) and PC ($Log_2FC = -1.06$; -
229 $log_{10}padj = 4.41$) as the less abundant classes, whereas PE ($Log_2FC = 1.36$; $-log_{10}padj = 9.82$) and
230 TAG ($Log_2FC = 1.32$; $-log_{10}padj = 5.90$) were the most abundant classes in NAS-IMI milk
231 compared to H milk (**Figure 5B**-**D**), respectively.

232 The lipidome differences between H and NAS-IMI milk were also characterized at the lipid
233 species level (n = 2556), as presented in **Figure 6**. In detail, the PLS-DA plot and k-means
234 clustering (**Figure 6A** and **B)** showed that H milk and NAS-IMI milk were grouped into separate
235 clusters. Results were statistically significant ($p = 0.01$), being the R2 and Q2 of all tested
236 combination of components close to 1 (**Figure 6C** and **D**). PLS-DA and *t*-test (FC > 10; FDR <
237 0.01) identified highly discriminant lipids, providing the evidence that TAG-40:1 (8:0-14:0-18:1;
238 $log_2FC = -16.89$; FDR = $5.06E^{-12}$), TAG-40:0 (10:0-14:0-16:0; $log_2FC = -11.267$; FDR = $8.92E^{-}$
239 ^{09}) are decreased in NAS-IMI milk, whereas HexCer d82:15 (38:3/44:12; $log_2FC = 9.7361$; FDR
240 = $2.19E^{-14}$), Cer-58:2 (16:1/42:1; $log_2FC = 9.215$; FDR = $2.19E^{-14}$) are the most abundant lipids
241 in NAS-IMI compared to H milk (**Figure 6E**, **F**). In total, 597 lipids changed their abundance for
242 more than ten folds (**Supplemental Table S3**). Taken together, 15 lipids, including the decreased
243 TAGs 40:1 (8:0-14:0-18:1; 8:0-16:0-16:1), TAG 40:0, TAG 40:3, Cer d43:6, PC 34:1, TAG 39:0,
244 TAG 41:2, PE 29:1, Cer 44:5 and the increased HexCer d82:15, Cer 58:2, TAG 73:5 and PC 60:8
245 were the most important features identified by PLS-DA (**Figure 6F**). Besides, saturated and
246 unsaturated fatty acids were overall higher in NAS-IMI compared to H milk (**Figure 7A** and **B**).

247 **DISCUSSION**

248 Although milk contains thousands of lipids (Jensen, 2002), only approximately 400 of
249 them have been identified, and many low-represented species remain unknown. This study covers
250 this gap by determining the lipidome of milk from quarters affected by subclinical NAS-IMI
251 compared to healthy milk in an untargeted approach using LC-Q-TOF-M. We identified a total of
252 2556 lipids and provided evidence that NAS-IMI, even when not leading to evident changes from
253 a clinical perspective, can significantly alter the lipid profile of bovine milk. The lipidome content
254 changes involved 16 subclasses, which were identified in both healthy and infected quarters. This
255 study's main findings are that 597 lipids changed their abundance more than tenfold in milk from
256 quarters with a NAS-IMI compared to healthy.

257 This study also provides an advancement in the field of milk lipidomics as compared to
258 previous lipidomics and metabolomics studies that identified 335 (Li et al., 2020), 462 (Li et al.,
259 2017), 362 (Wang et al., 2020), 338 (Brink et al., 2020), 472 (Mitina et al., 2020), and 453 (Li et
260 al., 2017) lipid species, and other studies where the lipid profile was part of more comprehensive
261 studies on the milk metabolome (Zandkarimi et al., 2018; Tsiafoulis et al., 2019).

262 In a first step, we determined the lipidome composition of healthy milk quarters. Bovine
263 milk fats belong mainly to the TAG class (around 98%), while others include DAG, cholesterol,
264 FFA, and polar lipids. The polar lipid fraction contains PC (19.2–57.3%), PE (19.8–42%), SM
265 (18–34%), PI (0.6–13.6%), and PS (1.9–16%) as major constituents, the large variability being
266 related to methodologies used for polar lipid extraction (Bernard et al., 2018). Therefore, as
267 expected, the TAG content in milk from healthy cows was higher than other milk lipid classes and
268 is thus in line with previous reports, the main s'TAG species being 34:0, 36:0, 50:1, and 40:1
269 (Yener and van Valenberg, 2019). Our study included both primiparous and multiparous dairy
270 cows, but the PLS-DA analysis did not reveal statistically significant differences in milk lipidome
271 composition related to parity. These results, which must be regarded as preliminary given the
272 limited number of samples, are consistent with previous reports that found no relationship between

273 parity and overall lipid content, apart from specific lipids (Kgwatalala et al., 2009). Parity's effects
274 on milk's lipid content are still debated, and the conclusions drawn so far are contradictory. Some
275 studies did not find any relationship between lipid composition and parity (Kgwatalala et al.,
276 2009). In contrast, others reported that parity affects fatty acid content (Bilal et al., 2014) and
277 unsaturation degree (Kelsey et al., 2003; Garnsworthy et al., 2006). To adequately address this
278 issue and validate the present results, further studies with a higher number of samples are required.

279 In the second part of the study, we determined the lipidome composition of NAS-IMI
280 milk, and we compared it with the lipidome of H milk. In the first instance, we investigated
281 whether there was any difference between milk with high and low SCC. The information about
282 the impact of somatic cells on milk lipidome is limited to few reports on changes in the total lipid
283 concentration of milk, which was reduced with SCC increase (El-Tahawy and El-Far, 2010;
284 Schwarz et al., 2020). Because the quarters of cows with NAS-IMI were not homogeneous for
285 SCC content, IMI milk was further classified into NAS-IMI-LC (quarters with SCC $< 200 \times$
286 10^3/mL) and NAS-IMI-HC (quarters with SCC $> 200 \times 10^3$/mL). No significant changes in the
287 lipidome composition were found, suggesting that the impact of somatic cell number on the milk
288 lipidome was negligible in our model. It must also be underlined that the number of samples
289 included in the lipidomic analysis was limited, and this hypothesis should be validated on a higher
290 number of samples.

291 The PCA analysis based on lipid classes demonstrated that H and NAS-IMI milk could
292 be separated, identifying PE and TAG as the most abundant, and PG and PC as the less abundant
293 classes in the milk from cows with a subclinical NAS-IMI. The lipid component in milk (3-5%)
294 is present in the form of small droplets defined as milk fat globules (MFG) emulsified in the
295 aqueous phase. The MFG contains a core of non-polar lipids, mainly TAG, that are coated by a
296 tri-layer membrane composed of polar lipids, mainly phospholipids and sphingolipids (Bernard et
297 al., 2018). The changes in the milk lipidome reported in this study suggest that the development
298 of NAS-IMI induces a profound modification of both the non-polar component's content and the

299 MFG membranes. Out of the 15 lipid species found to be most representative for the difference
300 between H and IMI quarter milk, seven belong to the TAG class. The other eight belong to the
301 polar lipid classes, including PC 34:1, PE 29:1, and, remarkably, six SM, of which 4 were Cer and
302 hexo-ceramides. The role of sphingolipids is to maintain the MFG membrane structure
303 (McFadden and Rico, 2019), and changes in polar lipids influence the fluidity of the MFG
304 membrane. Although Cer are minor structural components of membranes, they are present in the
305 MFG membrane at greater levels than the plasma membrane (1.5–5 mol% of the total
306 phospholipids) (Fujino and Fujishima, 1972; Christie et al., 1987). Recent findings demonstrated
307 that changes in ceramide composition might alter the lateral packing of polar lipids, especially the
308 milk SM, increasing membrane thickness and mechanical stability (Murthy et al., 2018), thus
309 changing the biophysical and biological properties of the MFG membrane. Besides this structural
310 role, recent studies also suggested that Cer and SM functions can be extended to a
311 proinflammatory role by activating NFkB expression and upregulating TNFα in macrophages
312 (Boon et al., 2013). A change in the abundance of these molecules playing an immunomodulatory
313 activity may therefore influence the activity of somatic cells in the mammary gland, as suggested
314 by recent reports, that demonstrated that Cer modulate chemotaxis in macrophages (Thomas et
315 al., 1989; Kakazu et al., 2016; Török, 2016), and stimulate phagocytosis (Choi et al., 2011). Recent
316 studies have also demonstrated that changes in milk polar lipids such as sphingolipids affect the
317 gut microbiome (Norris et al., 2019). Based on this evidence, we may not rule out the hypothesis
318 that changes in NAS-IMI, particularly Cer, may impact the milk microbiome.

319 The differential lipidomics analysis between H and NAS-IMI milk also revealed an
320 increase in all the identified fatty acids, including, among the others, several saturated and
321 unsaturated fatty acids involved in the regulation of the immune activity of white blood cells, such
322 as arachidonic acid (20:4) and conjugated linoleic acid (CLA) (18:2). Arachidonic acid has been
323 reported to increase in milk during mastitis (Hayashi et al., 2019), where it plays pivotal functions
324 in regulating inflammation. The increase of CLA in milk from animals with NAS-IMI is equally

325 interesting. *In vitro* studies on a model of mammary gland epithelial cells (BME-UV1) have
326 demonstrated that CLA can modulate inflammation and respiratory burst (Basiricò et al., 2015,
327 2017; Dipasquale et al., 2018). Also, CLA isomers' effects on the activity of immune cells have
328 been investigated in bovine peripheral blood mononuclear cells (PBMC) and monocytes (Ávila et
329 al., 2020). Other studies showed that CLA exerts effects on the *ex vivo* stimulation ability of
330 bovine PBMC (Dänicke et al., 2012), inhibit isolated PBMC mitogen-activated proliferation
331 (Renner et al., 2012, 2013), and increase monocyte respiratory burst (Ávila et al., 2020). We also
332 found that stearic acid abundance is increased in mastitis-affected milk. This result is remarkable,
333 on the background that stearic acid was found to exert anti-inflammatory activity on monocytes
334 by reducing chemotaxis (Ávila et al., 2020).

335 Although this report presents the milk lipidome of dairy cows for the first time, the
336 experimental design has some limitations. The study was carried out on a number of samples that
337 were necessarily limited to the need to focus the collection of samples on the same farm, the
338 microbial positivity to NAS, and be as homogeneous as possible for what concerns the lactation
339 status and parity. The milk fat content and MFG size in cattle could be affected by several factors
340 including physiological characteristics, such as parity, days in milk, pregnancy stage, weight,
341 somatic cell count, and milk production traits, including milk yield, fat yield, protein, and fat
342 content and fat-protein ratio, on the individual animal level. The environmental conditions
343 including, diet, weather, season at herd level (Walter et al., 2019) may affect lipid composition as
344 well, although environmental variables like the proportion of pasture and silage in the diet, have
345 limited effects, at least on MFG, as compared to physiological differences. For what concerns the
346 feeding, the animals included in this study were fed with the same TMR, which did not change
347 during the lactation period, although the individual intake may likely change. The stage of
348 lactation was ranging from DIM 30 to DIM 325. Admittedly, it would have been interesting to
349 test for the stage of lactation as an influencing factor, but from the herd investigated, not all stages
350 could be equally considered.

351 Another limitation of the experimental design is that some of the quarter samples come 352 from the same animal. In the healthy group, 45% of the samples came from 2 cows, and in the 353 NAS-IMI group, 41% of the samples came from 3 cows. Therefore, the sample size is small, and 354 the impact of that clustering of data cannot be ignored: we thus cannot rule out that the cow 355 individual intake and physiological responses may influence the results.

356 Further studies should also investigate the impact of specific NAS strains on milk fat 357 content and lipid composition. In this study, we assessed the impact of NAS on the milk lipidome 358 as a group, which is a limitation of our experimental design. Nevertheless, over 20 different NAS 359 species have been isolated from bovine milk (Vanderhaeghen et al., 2014)) and epidemiological 360 data suggest that some NAS species affect udder health more than others (Supré et al., 2011). 361 Although some research effort is still needed to unravel their relationships with IMI and mastitis, 362 it will be interesting to assess the impact of the different NAS species on the milk lipidome in 363 future studies, given the background that some studies revealed that pathogen-specific clinical 364 mastitis might affect milk yield and milk composition (Kayano et al., 2018).

365 Finally, this study focused on quarter milk. Each quarter has its vascular system, nerve 366 supply, and suspensory apparatus (Berry and Meaney, 2006; Akers and Nickerson, 2011), and 367 thus they were regarded as independent from each other. On the contrary, there is evidence that 368 infection in one udder quarter also influences other neighboring uninfected quarters (Blagitz et 369 al., 2015), and the immune response in the individual mammary gland quarter alters milk 370 composition throughout the udder (Paixão et al., 2017). Still, several studies regard single quarters 371 as independent units (Heimes et al., 2020; Niedziela et al., 2020). In the present experimental 372 design, some animals contributed different quarter samples to the healthy or NAS-IMI groups: 373 two cows contributed multiple samples to the healthy group (cow 815, two quarters; cow 819, 374 three quarters), three cows contributed multiple samples to the NAS-IMI group (cow 620, three 375 quarters; cow 855, two quarters; cow 881, two quarters), and two cows contributed multiple 376 samples to both healthy and NAS-IMI groups (cow 618, one healthy quarter and one NAS-IMI

377 quarter; cow 909, one healthy quarter and one NAS-IMI quarter). Given the limited amount of
378 samples included in this study, the present results may also be partially influenced by the fact that
379 they included multiple quarters from some cows. Given the limited amount of samples included
380 in this study, the present results may be partially influenced by the fact that they included multiple
381 quarters from some cows. Therefore, further studies should be carried out to understand whether
382 the quarter's milk lipidome would react in an interdependent or independent manner to infection
383 with NAS.
384

385 **CONCLUSIONS**

386 By applying the LC-QTOF-MS approach to characterize the quarter milk lipidome with
387 NAS-IMI compared to healthy animals, we measured and identified 2556 lipids belonging to
388 sixteen classes, of which 597 were significantly changed over ten-fold. The lipidomic analysis
389 demonstrated that NAS-IMI changed the abundance of lipid that is physiologically located at both
390 the inner content of MFG and its external membrane. Sphingolipids represent the lipid classes
391 with the most significant changes. Given the recent roles attributed to NAS-IMI in regulating
392 important functions such as apoptosis and macrophages' inflammatory activity, we may speculate
393 that changes in the milk lipidome may also regulate the mammary gland's immune defence. This
394 study identified highly discriminant lipids, particularly those belonging to the ceramide family,
395 that change their abundance by several orders of magnitude in NAS-IMI compared to H quarters.
396 These differently abundant lipids may provide important targets elucidate the role of specific
397 lipids in immune defence. It must be underlined that these results have to be validated on a higher
398 number of samples and also extended to independent cow samples and lactational stage and diet.
399 Therefore, further studies will be required to *i*) validate the reported changes in more extended
400 experimental groups *ii*) identify the impact of different species of NAS on the lipidome and, more
401 in general, investigate the consistency of these results in other intramammary infections to
402 determine how specific these lipidomic changes are.

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406

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615

Table 1: Schematic description of the samples used in lipidomic analysis and their classification.

Cow ID	Quarter ID	Quarter*	Clinical status#	SCC	Group	Delivery	DIM	Parity
784	H1	RR	Healthy	1	Negative	17/09/2018	58	3
795	H2	RR	Healthy	1	Negative	11/1/2018	307	2
819	H3	FL	Healthy	1	Negative	1/9/2018	74	2
819	H4	RR	Healthy	1	Negative	1/9/2018	74	2
819	H5	RL	Healthy	1	Negative	1/9/2018	74	2
905	H6	RR	Healthy	1	Negative	16/08/2018	90	1
918	H7	FR	Healthy	1	Negative	12/8/2018	94	1
618	H8	RL	Healthy	1	Negative	22/07/2018	115	4
815	H9	FR	Healthy	3	Negative	25/08/2018	81	2
815	H10	RL	Healthy	6	Negative	25/08/2018	81	2
898	SM7	FR	NAS-IMI	14	NAS-IMI-LC	15/06/2018	152	1
881	SM8	FL	NAS-IMI	49	NAS-IMI-LC	28/12/2017	321	1
909	H11	RR	Healthy	53	Negative	19/07/2018	118	1
909	SM9	RL	NAS-IMI	53	NAS-IMI-LC	19/07/2018	118	1
932	SM10	FR	NAS-IMI	85	NAS-IMI-LC	15/10/2018	30	1
842	SM11	FR	NAS-IMI	89	NAS-IMI-LC	30/07/2018	107	2
618	SM12	FL	NAS-IMI	109	NAS-IMI-LC	22/07/2018	115	4
620	SM13	RR	NAS-IMI	112	NAS-IMI-LC	25/02/2018	262	4
620	SM14	FR	NAS-IMI	137	NAS-IMI-LC	25/02/2018	262	4
855	SM15	FL	NAS-IMI	152	NAS-IMI-LC	5/3/2018	254	1
894	SM16	FR	NAS-IMI	156	NAS-IMI-LC	29/01/2018	289	1
881	SM17	RL	NAS-IMI	178	NAS-IMI-LC	28/12/2017	321	1
620	SM1	FL	NAS-IMI	224	NAS-IMI-HC	25/02/2018	262	4
855	SM2	RL	NAS-IMI	265	NAS-IMI-HC	5/3/2018	254	1
883	SM3	FL	NAS-IMI	281	NAS-IMI-HC	9/2/2018	278	1
872	SM4	FR	NAS-IMI	602	NAS-IMI-HC	26/03/2018	233	1
703	SM5	RR	NAS-IMI	901	NAS-IMI-HC	20/08/2018	86	3
644	SM6	RR	NAS-IMI	2513	NAS-IMI-HC	24/12/2017	325	3

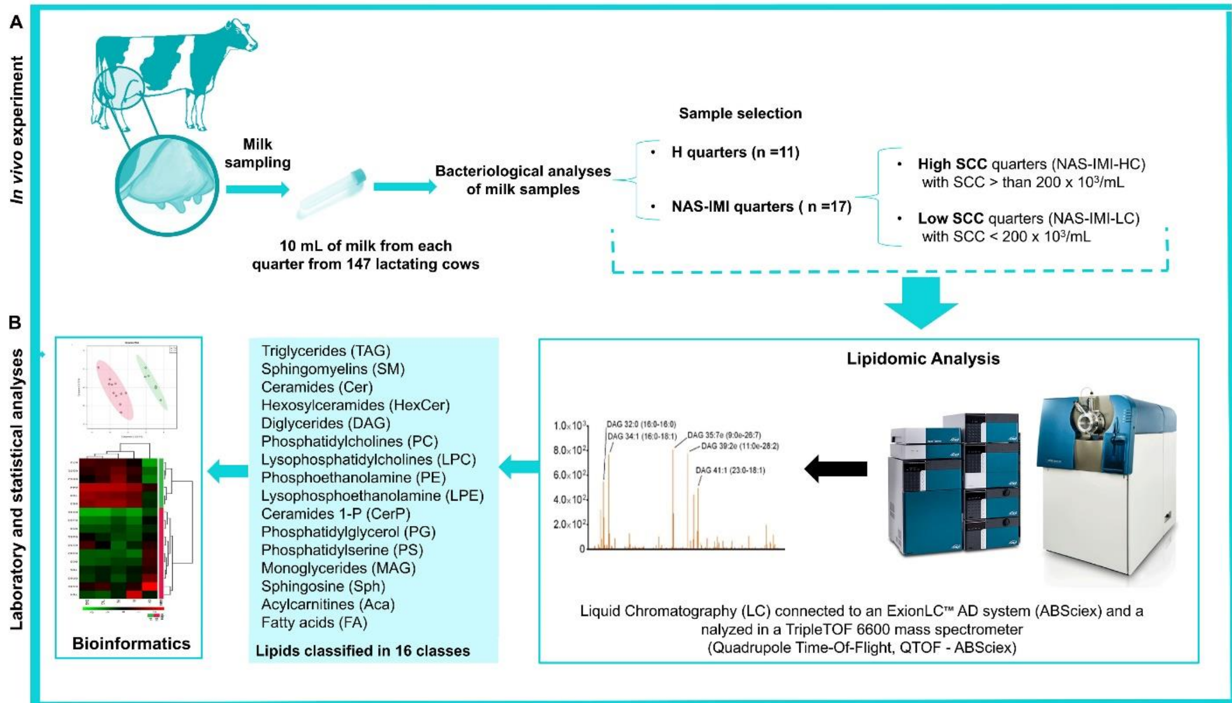
[*] RR, Rear- Right. RL, Rear Left. FR, Front Right. FL, Front Left.
[#] NAS-IMI, subclinical mastitis. H. Healthy. NAS-IMI-LC, Low SCC (<200 x 10^3). NAS-IMI-HC, High SCC (>200 x 10^3).

616

617

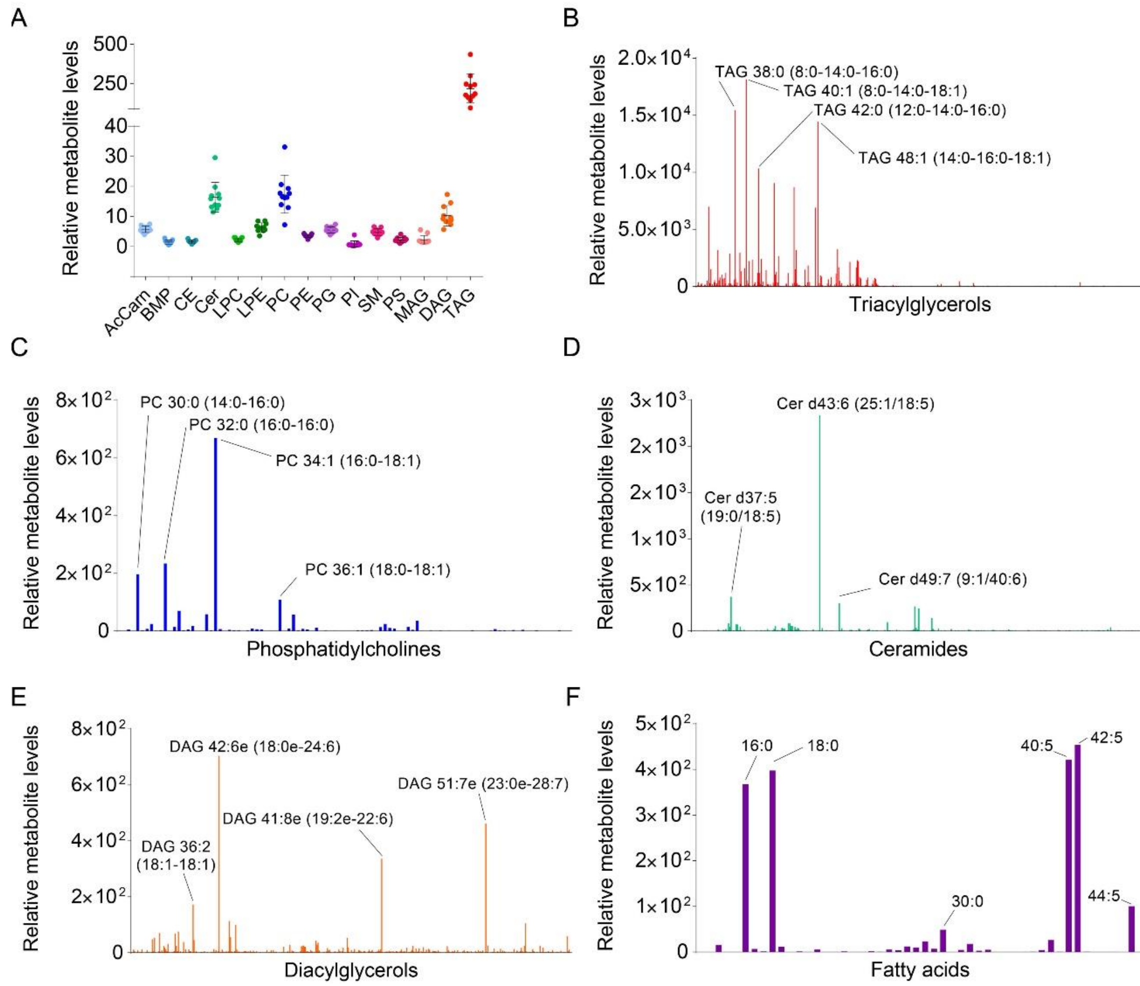
618 **Figure legends**
619 **Figure 1**. Schematic representation of the experimental plan. **A**) Milk was collected from a single
620 individual quarter in 147 lactating cows, and bacteriological analysis was carried out to
621 assess the presence of an IMI. Milk samples from 11 healthy (H) and 17 subclinical
622 intramammary infections by *non-aureus staphylococci* (NAS-IMI) quarters were selected
623 for the evaluation of the lipidome profile, which was performed (**B**) by liquid
624 chromatography quadrupole time-of-flight (LC-Q-TOF). From the mass spectrometry
625 analysis, 16 major lipid classes were identified and subsequently analyzed by
626 bioinformatics tools.
627
628 **Figure 2**. The lipidome of milk from healthy quarters. **A**) Relative levels of lipid classes and
629 several lipid species in healthy quarter milk. **B**) Relative abundance of single
630 triacylglycerols, **C**) phosphatidylcholines, **D**) ceramides, **E**) diacylglycerols, and **F**) fatty
631 acids species. Relative levels of lipids were calculated between detected areas and the
632 area of the internal reference (1-Phenoxy-2-propanol). Abbreviations are reported in the
633 text.
634
635 **Figure 3**. The lipidome of milk from NAS-IMI quarters. **A**) Relative levels of lipid classes and
636 several lipid species in NAS-IMI quarter milk. **B**) Relative abundance of single
637 triacylglycerols, **C**) phosphatidylcholines, **D**) ceramides, **E**) diacylglycerols, and **F**) fatty
638 acids species. Relative levels of lipids were calculated between detected areas and the
639 area of the internal reference (1-Phenoxy-2-propanol). Abbreviations are reported in the
640 text.
641
642 **Figure 4**. The difference in milk lipidome between quarters with high and low somatic cell count
643 (SCC). PLS-DA (**A**), k-means tests (**B**), and t-test (**C**) of quarters with NAS-IMI-LC
644 (quarters with intramammary infection and a SCC $< 200 \times 10^3$/ml – 6 quarters) and NAS-
645 IMI-HC (quarters with intramammary infection and a SCC $>200 \times 10^3$/ml – 11 quarters).
646
647 **Figure 5**. The lipidome differences between healthy (H) and subclinical intramammary infection
648 by *non-aureus staphylococci* (NAS-IMI) affected quarter milk at the lipid class level. **A**)
649 Principal component analysis (PCA), B) heatmap, and **C**) volcano plot of H and NAS-
650 IMI milk lipid classes. **D**) Histogram detailing levels and distribution of total
651 triacylglycerols (TAG) phosphatidylcholine (PC), phosphatidylethanolamine (PE), and
652 phosphatidylglycerol (PG) in H and NAS-IMI quarter milk.
653
654 **Figure 6**. The lipidome differences between healthy (H) and subclinical intramammary infections
655 by *non-aureus staphylococci* (NAS-IMI) affected quarter milk at the lipid species level.
656 **A**) Partial least squares discriminant analysis (PLS-DA), **B**) k-means, and **C**) permutation
657 test to analyze group distribution based on single lipid species. **D**) Cross-validation
658 performed by the leave-one-out method to estimate the predictive ability of the generated
659 models. Asterisk on model N°2 indicates the highest and most consistent model. **E**)
660 Volcano plot showing the biological effect of quarters with NAS-IMI ($\log_2$ fold change,
661 *x*-axis) and statistical significance of differences of lipid species ($-\log_{10}$ *p*-adj). **F**)
662 Variable Importance in Projection (VIP) scores of indicated metabolites listed in model
663 N°2 (highest Q^2).
664
665 **Figure 7**. The lipidome differences between healthy (H) and subclinical intramammary infection
666 by *non-aureus staphylococci* (NAS-IMI) affected quarter milk at the free fatty acid level.
667 **A**) Heatmap and **B**) statistical table of total FAs in H and NAS-IMI quarter milk samples.

668 The heatmap colours reflect the quarter milk FA abundance (mean-cantered and divided
669 by the range of each variable).
670
671 **Supplemental Figure S1**. The lipidome differences between healthy (H) and subclinical
672 intramammary infection by *non-aureus staphylococci* (NAS-IMI) affected quarter milk
673 as related to different parity.
674
675 **Supplemental Figure S2.** The lipidome differences in NAS-IMI as related to different DIM. The
676 NAS-IMI quarters were divided in three groups: DIM <149, DIM 150-274 and
677 DIM>275. No differences in the milk lipidome between samples clustered in these three
678 groups were found.
679

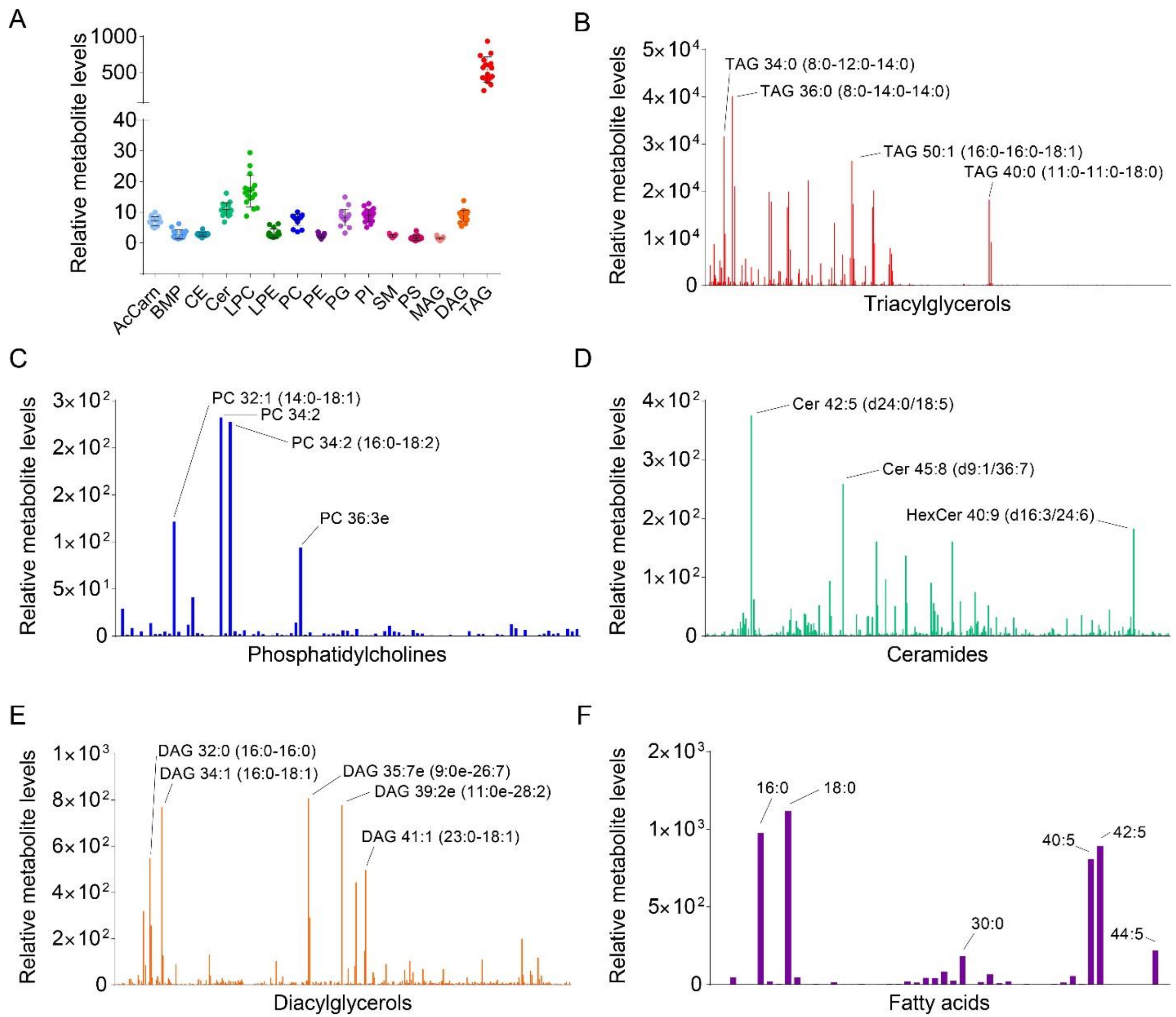


680

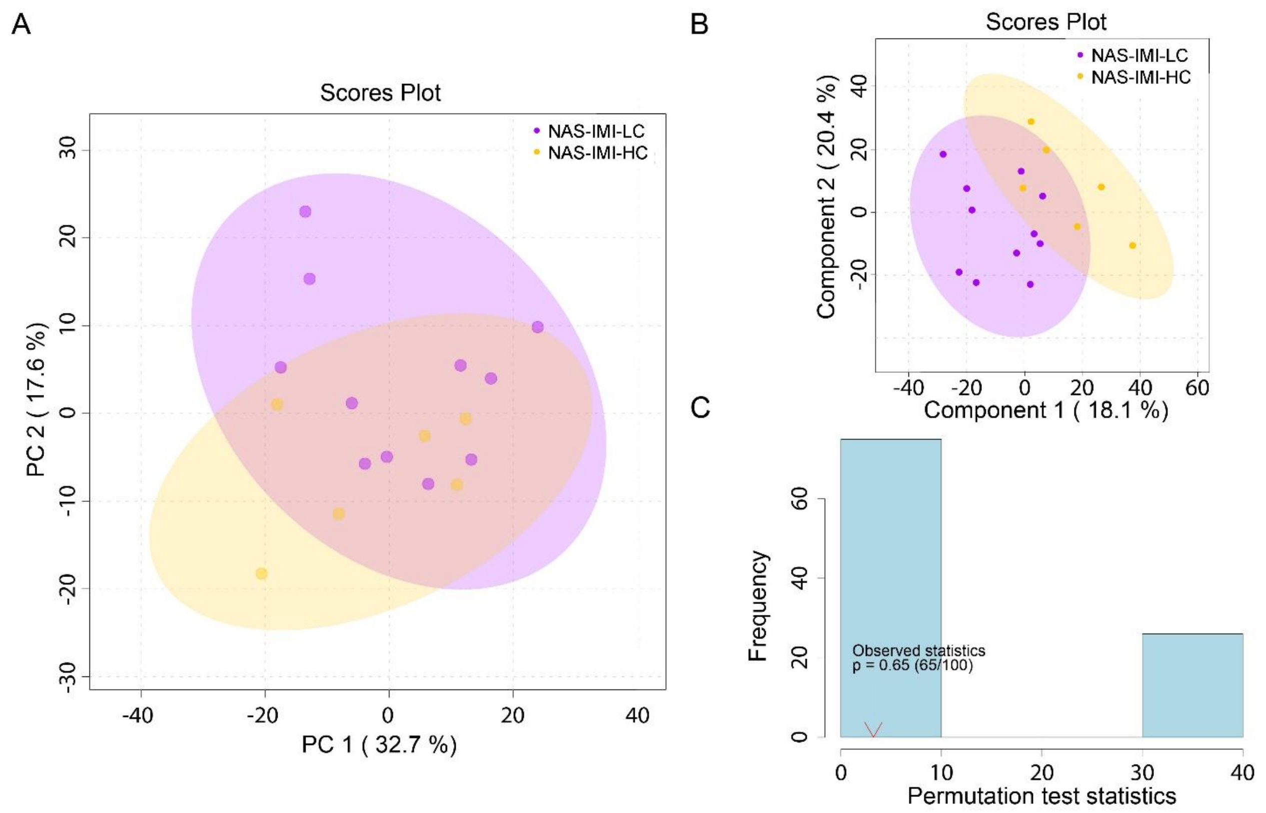
681 Figure 1



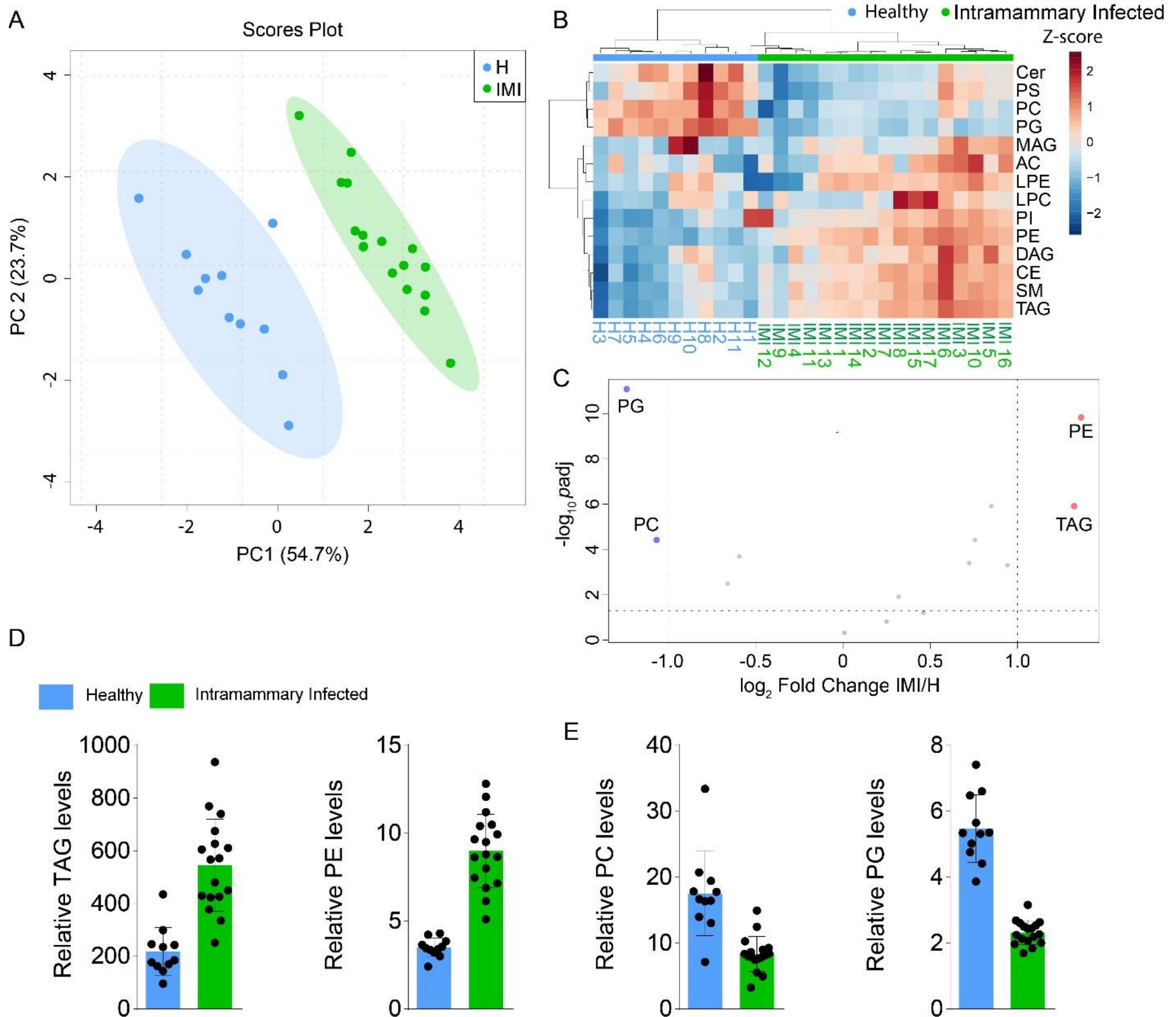
682
683
684 Figure 2
685



686
687 Figure 3
688

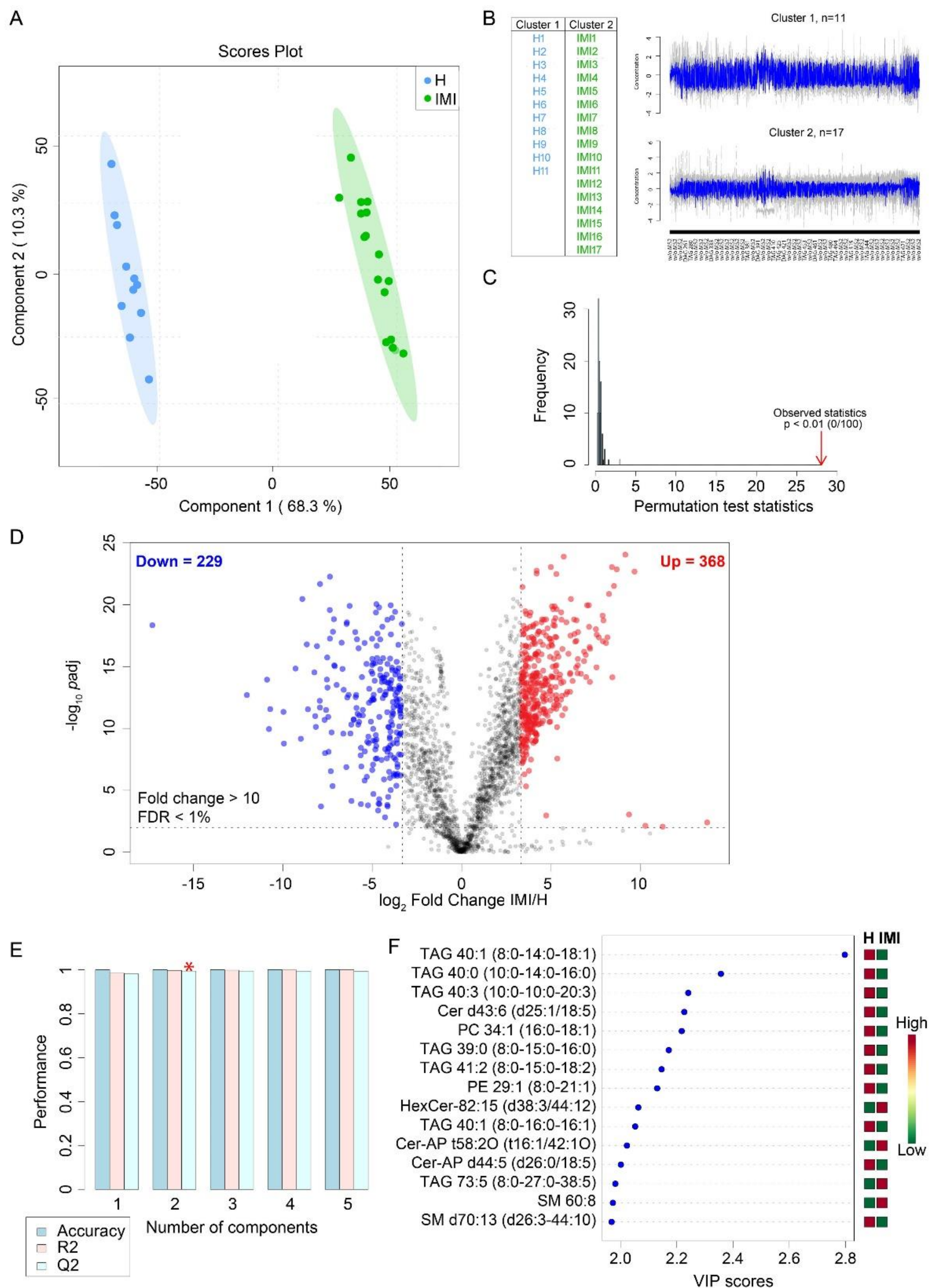


689
690 Figure 4
691



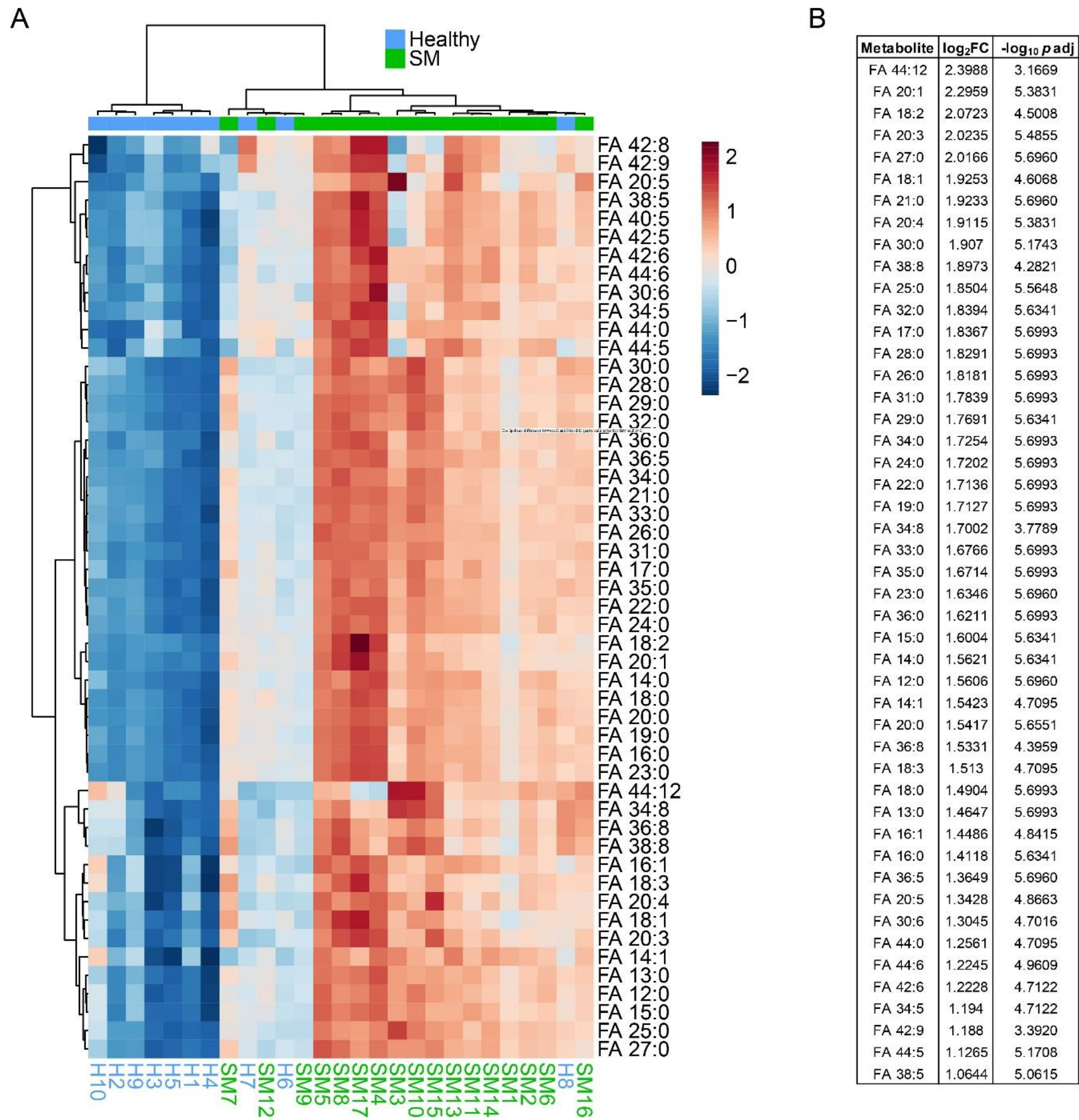
692
693 Figure 5
694
695

The lipidome differences between H (n = 11) and NAS-IMI (n = 17) quarter milk at the lipid species level



696
697 Figure 6
698

The lipidome differences between H and NAS-IMI quarter milk at the free fatty acid level



Metabolite	log_2FC	$-log_{10}$ *p* adj
FA 44:12	2.3988	3.1669
FA 20:1	2.2959	5.3831
FA 18:2	2.0723	4.5008
FA 20:3	2.0235	5.4855
FA 27:0	2.0166	5.6960
FA 18:1	1.9253	4.6068
FA 21:0	1.9233	5.6960
FA 20:4	1.9115	5.3831
FA 30:0	1.907	5.1743
FA 38:8	1.8973	4.2821
FA 25:0	1.8504	5.5648
FA 32:0	1.8394	5.6341
FA 17:0	1.8367	5.6993
FA 28:0	1.8291	5.6993
FA 26:0	1.8181	5.6993
FA 31:0	1.7839	5.6993
FA 29:0	1.7691	5.6341
FA 34:0	1.7254	5.6993
FA 24:0	1.7202	5.6993
FA 22:0	1.7136	5.6993
FA 19:0	1.7127	5.6993
FA 34:8	1.7002	3.7789
FA 33:0	1.6766	5.6993
FA 35:0	1.6714	5.6993
FA 23:0	1.6346	5.6960
FA 36:0	1.6211	5.6993
FA 15:0	1.6004	5.6341
FA 14:0	1.5621	5.6341
FA 12:0	1.5606	5.6960
FA 14:1	1.5423	4.7095
FA 20:0	1.5417	5.6551
FA 36:8	1.5331	4.3959
FA 18:3	1.513	4.7095
FA 18:0	1.4904	5.6993
FA 13:0	1.4647	5.6993
FA 16:1	1.4486	4.8415
FA 16:0	1.4118	5.6341
FA 36:5	1.3649	5.6960
FA 20:5	1.3428	4.8663
FA 30:6	1.3045	4.7016
FA 44:0	1.2561	4.7095
FA 44:6	1.2245	4.9609
FA 42:6	1.2228	4.7122
FA 34:5	1.194	4.7122
FA 42:9	1.188	3.3920
FA 44:5	1.1265	5.1708
FA 38:5	1.0644	5.0615

699

700

701 Figure 7

702

703
704
705 **Supplemental Table S1:** ESI and mass spectrometer parameters.

Item	Lipidomic IDA POS	Lipidomic IDA NEG
Ionization	POS	NEG
Source temperature	350 °C	350 °C
Curtain Gas (CUR)	35	35
GS 1	55	55
GS 2	65	65
Ion Spray Voltage	5500 V	-4500 V
Declustering Potential (DP)	50 V	-50 V
Collision Energy	35V	-40V
Collision Energy Spread	15	20
TOF MS Mass Range	140-2000 Da	150-1100 Da
IDA acquisition Mass Range	50-2000 Da	50-2000 Da
Top N	18	10

706

707 **Supplemental Table S2:** Relative metabolite levels in healthy (H) and NAS-intramammary
708 infected (NAS-IMI) quarter milk.

709 Dataset can be found at https://figshare.com/s/bcdab12c6303949b6648

710

711 **Supplemental Table S3:** Differentially abundant lipids between healthy (H) and NAS-
712 intramammary infected (NAS-IMI) quarter milk (thresholds: log2 fold change > 10; -log10 FDR
713 > 2).

714 Dataset can be found at https://figshare.com/s/bcdab12c6303949b6648