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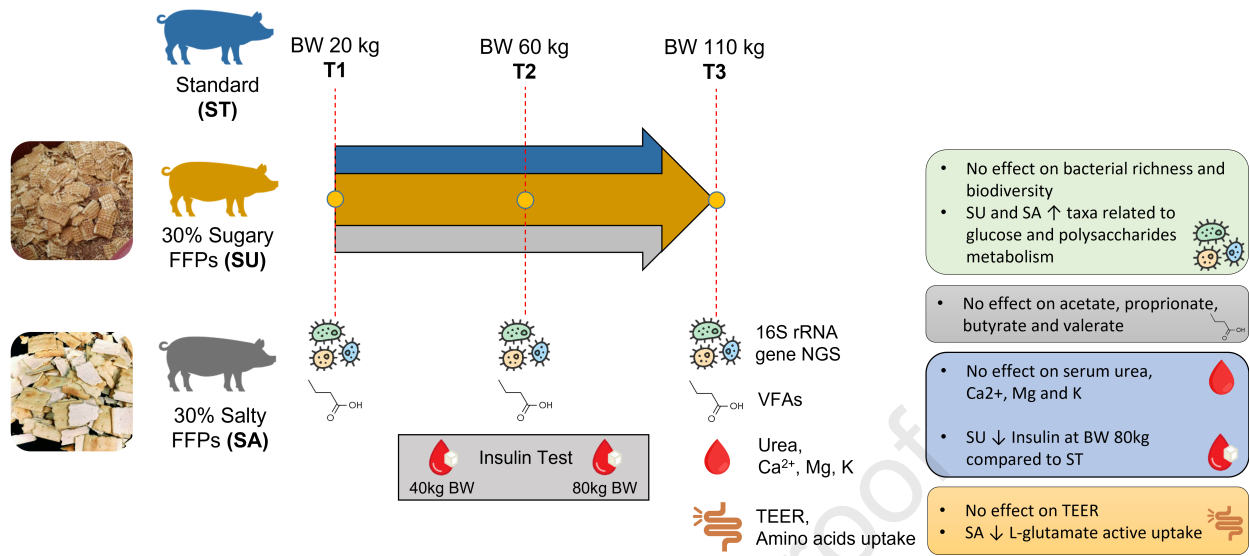
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Journal Pre-proof

Replacing cereal with ultra-processed foods in pig diets does not adverse gut microbiota, L-glutamate uptake, or serum insulin

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Abbreviations:

ASV: Amplicon Sequence Variant
F: Finishing
FFPs: Former Food Products
G: Growing
GI: Glycemic Index
SA: Salty diet
ST: Standard diet
SU: Sugary diet
TEER: Trans-epithelial electrical resistance
TJs: Tight junctions
UPF: Ultra processed food
VFAs: Volatile fatty acids
 Δ Isc: delta short circuit current

1 Abstract

2 Background: Using ultra-processed food (UPF) to replace traditional feed ingredients offers a
3 promising strategy for enhancing food production sustainability.

4 Objective: analyze the impact of salty and sugary UPF on gut microbiota, amino acids uptake,
5 and serum analytes in growing and finishing pig.

6 Methods: Thirty-six Swiss Large White male castrated pigs were assigned to three
7 experimental diets: (1) standard (ST), 0% UPF; (2) 30% conventional ingredients replaced by
8 sugary UPF (SU); and (3) 30% conventional ingredients replaced by salty UPF (SA). The
9 Next Generation Sequencing was used to characterise the fecal microbiota. Trans-epithelial
10 electrical resistance (TEER) and the active uptake of selected amino acids in pig jejunum were
11 also evaluated. Data were enriched with measurements of fecal volatile fatty acids and serum
12 urea, minerals and insulin. All data analyses were run in R v4.0.3. The packages phyloseq,
13 vegan, microbiome and microbiomeutilities were used for microbiota data analysis. The
14 remaining data were analyzed by ANOVA using linear mixed-effects regression models.

15 Results: The UPF did not affect fecal microbiota abundance or biodiversity. The Firmicutes to
16 Bacteroidetes ratio remained unaffected. SU-induced increase in the Anaerostipes genus
17 suggested altered glucose metabolism, while SA increased the abundance of CAG-352 and p-
18 2534-18B. No effects on fecal volatile fatty acids were observed. Assumptions of UPF
19 negatively affecting small intestinal physiology were not supported by the measurements of
20 TEER in pigs. Active amino acids uptake tests showed potential decrease in L-glutamate
21 absorption in the SA compared to the SU diet. Blood serum analysis indicated no adverse
22 effects on urea, calcium, magnesium or potassium concentration but the SU group resulted in
23 a lower blood serum insulin level at the time of blood collection.

24 Conclusions: When incorporated at 30% into a standard growing-finishing diet for pigs, UPF
25 does not have detrimental effects on gut microbiota, intestinal integrity and blood mineral
26 homeostasis.

27 **Keywords:** Former food products, Next Generation Sequencing, Ussing chamber,
28 Sustainability, Dietary intervention

29
30

31 **Introduction**

32 Food security is currently addressing the shortage of land, water and energy and the need to
33 produce more food using fewer natural resources (1). Quality and quantity and therefore food
34 security depend on the industrial processing of food. In recent years, the society raised the
35 awareness about the impact that processing generates on the nutritional value of food.
36 According to the NOVA classification, food products can be classified into: i) unprocessed or
37 minimally processed food, ii) processed culinary ingredients; iii) processed food and iv) ultra-
38 processed food (UPF) (2). Almost all the food produced is processed to some extent but the
39 present study only focuses on UPF, defined as “formulations of ingredients typically created
40 by series of industrial techniques and processes”, such as sweet or savoury packaged snacks,
41 mass-produced packaged bakery products (bread, cakes, etc.), margarines and other spreads,
42 biscuits, breakfast cereals, and many other products (3). Usually, UPF contains high levels of
43 refined carbohydrates and fats (3), specifically sugars, starches, oils, and then also proteins.
44 Some of these nutrients are modified by hydrolysis, hydrogenation, or other
45 physical/chemical/thermal processes. Examples are extrusion, moulding and pre-frying,
46 through which unmodified and modified food substances are assembled with little or no food.
47 Furthermore, the use of high temperature leads to the non-enzymatic production of high levels
48 of advanced glycation end products from proteins and glycated lipids from fats. Preservatives
49 are also used in processed and ultra-processed food to elongate the biological duration, the

50 marketability of the product and to reduce the potential proliferation of micro-organisms (3).
51 Food additives such as colouring and flavouring additives, emulsifiers, sweeteners, thickeners
52 and anti-foaming, bulking, carbonating, and glazing agents are used only for UPF to make
53 them more palatable (3).

54 The human consumption of UPF is positively associated with high glycaemic responses and a
55 low satiety potential (4), and also creates an environment in the gut that selects specific
56 microbes that can potentially activate inflammatory processes at local level (5). The main
57 outcomes are increased obesity (6), hypertension (7), coronary (8) and cerebrovascular
58 diseases (9, 10), dyslipidaemia (11), metabolic syndrome (12), and gastrointestinal disorders
59 (13). The pathological conditions reported above have been mainly related to the high levels
60 of sugar and sweeteners, partially through the gut microbiota (14, 15). Indeed, an increased
61 consumption of sugars and sweeteners influences the composition of the carbohydrate pool
62 available to the gut microbial community. This can lead to the creation of distinct microbial
63 populations in the gut, which are characterized by the presence of endogenous or exogenous
64 microbes, of which some can be pathogenic (16). When consumed at high doses, glucose is
65 known to enhance the absorption in the intestinal epithelium (17) by increasing the
66 permeability of the tight junctions (TJs) and changing the distribution of the main proteins in
67 the TJs, as reported only in the Caco-2 cell line, thus suggesting intercellular leakage (18). It
68 is known that salt in high concentration alters the osmolarity. As like glucose, salt increases
69 the permeability of the intestinal epithelium modulating the action of the TJs (19). Regarding
70 the effect of salt on microbiota, few data are available. It was observed that high salt
71 concentration increased the abundance of *Lachnospiraceae* and *Ruminococcus* genus, while
72 decreasing the abundance of *Lactobacillus* genus (20). Also, high salt concentration increased
73 the *Firmicutes/Bacteroidetes* ratio (20), a known marker of intestinal homeostasis that is
74 related to dysbiosis (21). Other food additives, such as surfactant agents, have been related to

75 increased intestinal permeability and P-glycoprotein inhibition, possibly by decreasing the
76 hydrophobicity of the mucus layer (22). In light of this, processed and UPF also contain
77 compounds and nutrients such as glutamine and polyphenols which are known to potentially
78 protect the integrity of the intestinal barrier (22). The overall effect of UPF on human gut
79 microbiota was confirmed by Atzeni et al. (23), who observed that the high consumption of
80 UPF by senior subjects was positively associated with the abundance of specific taxa, such as
81 *Alloprevotella*, *Negativibacillus*, *Prevotella* and *Sutterella*, associated to inflammatory gastro-
82 intestinal diseases occurrence.

83 In recent years, the use of UPF as feed ingredients for farm animals is considered an
84 innovative solution for a more sustainable livestock food production. This is because of the
85 high amounts of UPF which is lost and/or wasted by the food industry due to logistical or
86 technical reasons (24). Given that pigs use of a lot of feed ingredients which could be directly
87 be consumed by humans, the replacement of human-edible unprocessed grains with UPF (in
88 such context also called former food products, FFPs) in the diet of farm animals could reduce
89 the competition between feed and food and hence reduce the use of natural resources (24).
90 The hypothesis of this study was that the inclusion of UPF to replace the 30% of traditional
91 ingredients in the pigs' diet would affect the gut microbial community both qualitatively and
92 quantitatively. In addition, the high content of simple sugars and salt could promote a leaky
93 gut condition and an insulin resistance. Thus, the present study aimed to clarify if the long-
94 term replacement of slight processed ingredients by sugary or salty UPF in pigs' diet could
95 lead to detrimental effects on gut microbiota, small intestinal physiology, selected serum
96 metabolites of the animals and insulin secretion.

97 **Methods**

98 *Animals, diets, and slaughtering procedure*

99 This study was a continuation of Mazzoleni et al., (25) and details about rearing conditions,
100 diets and slaughter procedure are reported there. Briefly, 36 Swiss Large White male castrated
101 piglets were reared in a single-group pen equipped with three single-space computerized
102 feeders (Mastleistungsprüfung MLP-RAP; Schauer Agrotronic AG, Sursee, Switzerland),
103 which allowed for recording individual feed intake. The BW of all animals was monitored
104 weekly. Three dietary treatments were fed to the pigs when they reached ~20 kg body weight
105 [BW] (start of the grower period), including: standard (ST), salty (SA), and sugary (SU).
106 The SA and SU diets were formulated including products such as savory packaged snacks,
107 pasta, bread or candies, chocolate, breakfast cereals, cookies, for salty and sugary diets,
108 respectively. The three experimental diets underwent identical processing procedures and both
109 SA and SU diets were sourced from the same foodstuff processing company. The chemical
110 composition of the pure SA and SU FFPs used to formulate the experimental diets was similar
111 to the two pure FFPs used for the diets in post-weaned piglets by Luciano et al. (26). The
112 grower and finisher diets were formulated following the Swiss feeding recommendations for
113 pigs (27) (**Table 1**). The standard grower diet (ST-G) and the standard finisher diet (ST-F)
114 were formulated considering a reference BW of 40 kg and 80 kg, respectively. For the SA and
115 SU grower (SA-G and SU-G, respectively) and finisher (SA-F and SU-F, respectively) diets,
116 a portion of conventional ingredients such as cereals and fats included in the ST-G and ST-F
117 diets were replaced by 30% salty and sugary FFPs. During the entire trial and samples
118 collection, the names of the diets were blinded. The pigs had *ad libitum* access to fresh water
119 and to the grower and finisher diets from 20 kg to 60 kg BW and from 60 kg BW to slaughter,
120 respectively. The grower and finisher diets were formulated to be isoenergetic and
121 isonitrogenous.

122 Pigs were slaughtered at the Agroscope research slaughterhouse after fasting for 16 h (28)
123 when they reached ~110 kg BW. The animals were stunned with CO₂, after which they were
124 exsanguinated, scalded, mechanically dehaired, and eviscerated.

125

126 *Sample collection, DNA extraction, and sequencing*

127 The collection of fecal samples from the rectal ampulla occurred at three different time points:
128 before starting feeding the experimental diets (T1); one day before the end of the growing
129 period (T2, 47.4 ± 0.6 days on feed) and one day before the slaughter (T3, 94.5 ± 1.2 days on
130 feed). Samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until
131 analysis. Starting with 200 mg of faeces, the DNA was extracted with the QIAamp Fast DNA
132 Stool Mini Kit (QIAGEN, Germantown, USA) following the manufacturer's instructions and
133 quantified with Nanodrop ND2000. The universal primers for prokaryotic
134 (341F/802R:CCTACGGGNGGCWGCAG/GACTACHVGGGTATCTAATCC, respectively)
135 were used to amplify by PCR the V3 and V4 regions of the 16S rRNA gene. The amplicons
136 were sequenced by BMR Genomics (Pavia, Italy) through the Illumina MiSeq platform and a
137 v2 500 cycle kit (San Diego, CA, USA). The paired-end reads obtained were tested for
138 chastity and subjected to demultiplexing and trimming by Illumina real-time analysis software
139 v2.6. The read quality was checked by FastQC v0.11.8. USEARCH v11.0.667 was used to
140 trim forward and reverse reads of the paired-end reads.

141 *Tissue recovery for ex-vivo analysis*

142 At the slaughterhouse, intestinal segments from the third-metre distal to the pylorus were
143 removed within 15 min after exsanguination. A cold (4°C) saline solution (Phosphate
144 Buffered Saline pH 7.4, Bioconcept Ltd, Allschwil, Switzerland) was used to remove the
145 intestinal content, then tissues were stored in a serosal buffer solution (see the following).

146 Before mounting in the Ussing chamber device (Physiologic Instruments) equipped with eight
147 chambers, the outer muscle layers have been removed from the tissues. Each experiment
148 started within 30 min from the tissue recovery. A minimum of six independent Ussing
149 chamber experiments per each group were performed. Each experiment was carried out using
150 intestinal tissues from two pigs mounted in four different chambers per pig. Thus, a minimum
151 of six biological and 24 technical replicates per experimental group were used.

152 *Ussing chamber experimental procedure*

153 The jejunum tissue (exposed area of 1 cm²) was mounted on an Ussing chamber for the
154 evaluation of D-glucose and amino acids (AA) transport across intestinal epithelial cells. The
155 chambers were filled with 4ml Krebs–Ringer mucosal buffer (115 mmol/l NaCl, 2.4 mmol/l
156 K₂HPO₄, 0.4 mmol/l KH₂PO₄, 1.2 mmol/l CaCl₂, 1.2 mmol/l MgCl₂ and 25 mmol/l
157 NaHCO₃⁻). The serosal buffer (pH 7.4) also contained 10 mmol/l glucose as an energy
158 source which was osmotically balanced with 10 mmol/l mannitol in the mucosal buffer (pH
159 7.4). Indomethacin was added in both the mucosal and serosal buffers at a final concentration
160 of 0.01 mmol/l. Buffers were continuously perfused with a 95 % O₂ and 5 % CO₂ gas
161 mixture. The temperature was kept constant at 37°C by a circulating water bath. After a 30–
162 40 min equilibration period, baseline I_{sc} (in mV) values were measured. The trans-epithelial
163 resistance (TEER) was also measured at 2-min intervals under current clamped conditions.
164 The TEER was determined at an applied current of 100 mA, and the short-circuit current (I_{sc})
165 was calculated using Ohm's law ($R = V/I$). Furthermore, D-Gluc and AA uptake was
166 performed according to the following protocol: after the stabilisation period (10–15 min), 10
167 mmol/l D-glucose (D-Gluc) was added to the mucosal buffer, followed by the addition of the
168 same concentration of L-Arg, L-Meth and L-Glut. The substrates were added in the
169 aforementioned order at intervals of 15 min. D-Gluc or each AA addition was kept in an

170 equilibrated osmotic condition by the addition of equimolar (10 mmol/l) mannitol on the
171 serosal side. Forskolin (10 μ mol/l) was added to the serosal compartment at the end of the
172 experiment to test tissue viability. Active uptake was evaluated according to electrical
173 changes in the short circuit. The total active transport through the tissue was verified by
174 monitoring the change in short-circuit current (ΔI_{sc}), which was representative of ion flux,
175 and thus active transport within the jejunal tissues. Only tissues showing a change in the I_{sc}
176 generated by the addition of forskolin were considered for the data analysis.

177 *Blood collection and serum urea, calcium, magnesium and potassium analysis*

178 Blood was sampled directly during bleeding after CO₂ stunning using blood collection tubes
179 with serum clot activator (Vacuette [®]; Greiner Bio-One GmbH, Kremsmuenster, Austria),
180 which were stored upside down at room temperature for 1 h prior to processing. The Vacuette
181 [®] serum tubes were then centrifuged for 15 min at 3000 g and subsequently for 2 min at 4000
182 g. Two aliquots of serum were stored at -20°C in Eppendorf tubes. Levels of blood urea (UV
183 Urease-GLDH), calcium (Calcium O-Cresolftalein Complexone) and magnesium
184 (Magnesium Xylidyl Blue) were measured in the serum using commercial kits provided by
185 Biotecnica Instruments Spa (Rome, Italy) following manufacturers' procedure using an
186 autoanalyser BT 1500 (Biotecnica instruments Ltd, Roma, Italy), while potassium was
187 quantified by using the Stat Profile PrimeVet ES electrolyte analyzer (Nova Biomedical,
188 Waltham, MA USA).

189 *Insulin secretion test*

190 Eight pigs were randomly selected from each treatment at the beginning of the experiment to
191 undergo the insulin secretion test. Once these pigs reached a body weight of 40 kg (n=4) and
192 80 kg (n=4), they were transferred to clean individual pens for a 2-hour period after fasting

193 overnight. Then, 1 kg of feed (SA, SU or ST growing and finishing diets at 40 and 80 kg BW,
194 respectively) was offered to each pig and 1 h later a blood sample was collected by the jugular
195 vein. This specific time point was selected to ensure that all the pigs could consume the entire
196 kilogram of feed and to standardize blood sampling. To minimize stress for the animals, only
197 one blood sample was taken per pig.

198 Plasma samples were further obtained as described above. The commercial Porcine Insulin
199 ELISA kit (10-1200-01, Mercodia AB, Uppsala, Sweden) was used to quantify insulin
200 concentration according to manufacturer's protocol. The detection limit was 1.15 mU/L as
201 determined with the methodology described in the manufacturer's manual.

202 *Intestinal volatile fatty acids quantification*

203 The VFA profile in feces was determined by HPLC Briefly, feces samples previously
204 weighed and frozen at -20°C with 1 mL of phosphoric acid (25%, w/v) were thawed.
205 Following defrosting, 1 mL of internal standard (pivalic acid at 1%, w/v) and 18 mL of
206 distilled water were added into the tube. This preparation was shaken for 3 hours at room
207 temperature before being centrifuged for 5 minutes at 4000 g. The supernatants were filtered
208 and analyzed for VFA using a liquid chromatography (Ultimate 3000, Thermo Fisher
209 Scientific, Reinach, Switzerland) with an exchange ion column (Nucleogel ION 300 OA 300
210 x 7.8 mm) and equipped with a refractive index detector (RefractoMax 521, Thermo Fisher
211 Scientific, Reinach, Switzerland).

212 **Statistical analysis**

213 All microbiota data analyses were run in R v4.0.3 (Boston, MA, USA). The R packages used
214 were phyloseq v1.26.1, vegan v2.5–5, microbiome v1.12.0, and microbiomeutilities.
215 v1.00.14. The alpha diversity indexes used were the number of ASVs s and Chao1, Simpson,

216 and Shannon indexes (microbiome package, v.1.12.0). Both the weighted and unweighted
217 Unifrac distances were calculated on rarefied ASVs. Both the variance (PERMANOVA) and
218 similarities (ANOSIM) of the tested groups were also calculated. The linear discriminant
219 analysis effect size (LEfSe) between groups was calculated using the following conditions:
220 alpha value <0.05 for the Kruskal–Wallis sum-rank test among the classes; threshold >3.0 on
221 the logarithmic linear discriminant analysis score (29). To estimate the common core
222 microbiota, the “microbiome” library was used (detection threshold: 0.001, prevalence:
223 80/100).

224 Multivariate analysis was conducted using MaAsLin to investigate associations between
225 microbial abundances (from the domain to genus taxonomic level) and fecal VFAs and blood
226 serum measurements. Default settings were used for this analysis, specifically: maximum
227 false discovery rate (significance threshold) = 0.05. Minimum for feature relative abundance
228 filtering = 0.0001. Minimum for feature prevalence filtering = 0.01.

229 Data about fecal volatile fatty acids were analyzed by repeated measures ANOVA using
230 linear mixed-effects regression models (Lmer) (30) implemented in R (version 4.0.5). The
231 model contained the treatment and the time point as fixed effects, while the pig was
232 considered as random effect. The model for Ussing chamber, blood serum and measurement,
233 speed of food consumption and insulin secretion test did not include the time effect. For
234 pairwise comparisons, a modified Tukey test for multiple comparisons of means, the Sidak
235 function was used. Statistical means and standard error of the means (SEM) were calculated
236 with the lsmeans function from the package emmeans (31). Residuals of Lmer models were
237 checked for normality and homoscedasticity. Differences were considered significant for $P <$
238 0.05.

239 **Results**

240 *Performance*

241 Detailed information about the effect of UPF inclusion on growth performance and feeding
242 behaviour can be found in (25). Briefly, UPF did not influenced the average daily gain,
243 average daily feed intake, feed conversion ratio or BW of the pigs at slaughter. The average
244 daily fat intake was higher ($P < 0.05$) in pigs fed the SU diet, even though both categories of
245 UPF had no effects on the parameters related to the pigs' body composition (e.g., average
246 daily fat weight gain).

247 *Microbiota analysis*

248 Fecal samples were obtained from 36 pigs at T1, T2 and T3. At T1, from one SA and one ST
249 piglets, it was not possible to obtain fecal samples. Therefore, a total of 106 samples have
250 been analysed. Because of the low number of sequences obtained in two samples (one from
251 ST treatment at T1 and one from SU treatment at T3), they have been removed from the
252 dataset. The rarefaction curve showing the sequencing depth is reported in the Supplementary
253 figure 1.

254 *Non-phylogenetic diversities and composition*

255 Considering the overall period, the diets did not influence the observed amplicon sequence
256 variant (ASV), the Chao1 and the Shannon indexes (data not showed). Similarly, over time no
257 effect of UPF inclusion on the bacterial abundance nor biodiversity was found (**Figure 1**).

258 As expected, statistically significant differences were found when considering the effect of the
259 pig's age, with increasing abundance and biodiversity with increasing age (**Figure 2**).

260 The composition plots at family level of the fecal microbiota of pigs at the three different time
261 points are reported in **Figure 3**. No differences can be observed between the three dietary
262 treatments in each time point.

263 An effect of the time can be observed at family level, in particular regarding the abundance of
264 the Prevotellaceae family that linearly decreased ($P < 0.05$) with time (**Figure 4**). The
265 Firmicutes/Bacteroidetes ratio was similar between the treatments in each time-point (data
266 now showed).

267 *Beta diversities and core microbiota*

268 The diet did not affect the Unweighted or the Weighted beta-diversity. Specifically, for the
269 Unweighted beta diversity, the PERMANOVA showed no differences between the treatment
270 groups at T1 ($P = 0.141$, $R_2 = 0.06$), at T2 ($P = 0.202$, $R_2 = 0.06$) and at T3 ($P = 0.068$, $R_2 = 0.06$).
271 Similarly, the Weighted beta diversity was similar among treatment groups at T1 ($P = 0.612$,
272 $R_2 = 0.05$), T2 ($P = 0.775$, $R_2 = 0.04$) and T3 ($P = 0.178$, $R_2 = 0.06$). As expected, the time point
273 strongly influenced the beta-diversity. Both the unweighted (**Figure 5A**) and the weighted
274 (**Figure 5B**) Unifrac beta-diversity showed a clear cluster ($P < 0.001$) of the fecal microbial
275 community between T1, T2 and T3. Specifically, the unweighted beta-diversity determined at
276 T1 differed from T2 ($P = 0.048$) and tended to differ from T3 ($P = 0.058$). No differences were
277 observed between T2 and T3 ($P = 0.684$). The weighted beta-diversity differed between T1 and
278 T3 ($P = 0.032$), but not from T2 ($P = 0.838$). The weighted beta-diversity tended to differ
279 between T2 and T3 ($P = 0.055$).

280 The core microbiota composition at T2 and T3 was similar between the three dietary groups.
281 The core microbiota of ST and SU pigs exhibited greater similarity, with 9 and 8 ASVs,
282 respectively. In contrast, the core microbiota of the SA group at T3 consisted of 12 ASVs (see
283 **Figure 6**).

284 *Linear Discriminant analysis of effect size*

285 We conducted a Linear Discriminant Analysis of Effect Size (LEfSe) to identify potential
286 biomarkers among the three dietary groups. At T1, no significant differences in taxa were
287 observed between the groups (data not shown). However, at the genus level, biomarkers were
288 detected between the ST, SU, and SA groups at T2 and T3 (as shown in **Figure 7A** and **7B**,
289 respectively). Both SU and SA diets had a higher number of biomarkers compared to ST at
290 both T2 and T3. For both the time points, the main biomarker of the SU group was the
291 *Anaerostipes* genus, while for ST group was an unclassified genus of the Ruminococcacea
292 family at T2 and an unclassified genus of the Lachnospiraceae family at T3. The main
293 biomarkers for the SA group were the genera CAG-352 and p-2534-18B5 gut group at T2 and
294 T3 respectively (Figure 6A, B).

295 *Fecal volatile fatty acids*

296 Volatile fatty acids (VFAs) were quantified in the feces at T1, T2 and T3 (**Table 2**). The diet
297 did not affect none of the VFA analysed during the overall period. Propionate and valerate
298 were affected by the time point, but not acetate and butyrate. Also, propionate and butyrate
299 level was lower in T1 than T2 and T3 in ST.

300 Values of VFAs in feces were also combined with NGS data to investigate correlations
301 between bacterial taxa and VFAs level in feces though the MaAsLin analysis. Several positive
302 and negative associations between specific bacterial taxa and VFAs level were found (Figure
303 8). Only the top 50 associations with a p-value < 0.05 are reported. Among all the correlations
304 found between the microbiota data and VFAs, only few taxa correlated with both VFAs and a
305 specific dietary treatment. Specifically, the *Anaerostipes* genus was a biomarker of the SU
306 group and at the same time positively correlated with propionate and negatively correlated
307 with butyrate. Similarly, unclassified ASV301, mycoplasma genus, an uncultured
308 Ruminococcaceae family, clostridium sensu stricto and an uncultured prokaryote specie

309 belonging to the Christensenellaceae family negatively correlated with SU but positively
310 correlated with propionate and negatively with butyrate, valerate and, with the exception of
311 the unclassified ASV301, also with acetate. Only the unclassified ASV301 negatively
312 correlated with SA group (**Figure 8**).

313 *Jejunum nutrients active uptake and transepithelial integrity*

314 The ex-vivo trial was performed to further investigate the effect of SU and SA diets on the
315 small intestinal physiology. The use of UPF in pigs' diets did not affect the active D-glucose
316 uptake in the jejunum nor the active uptake of the amino acids L-arginine and L-methionine
317 (Table 3). However, the active uptake of the L-glutamate was lower in the SA group
318 compared to the SU group. The intestinal integrity, represented by the TEER, was also similar
319 between the three experimental groups (**Table 3**).

320 *Effect of salty and sugary processed food on serum urea, minerals and insulin*

321 The SA and SU diets had no effect ($P > 0.05$) on serum urea, calcium, magnesium and
322 potassium concentrations compared to pigs fed the ST diet (**Table 4**).

323 All the pigs completely consumed the kilogram of feed during the insulin test. The average
324 speed of feed consumption was 36.4 ± 2.23 and 39.1 ± 1.85 g/min at BW40 and BW80 pigs,
325 respectively. In each time point, the speed of feed consumption was similar between groups
326 (Supplementary Table 1).

327 Despite the distinct characteristics of SA and SU products compared to the conventional feed
328 ingredients utilized in the ST diet, the dietary treatment did not impact the release of serum
329 insulin at a body weight of 40 kg. However, it significantly ($P = 0.011$) reduced the insulin
330 concentration in SU (20.7 ± 10.1 milliunit/L) compared to the ST (144.5 ± 25.2 milliunit/L)

331 diet after 1 hour of feeding at a body weight of 80 kg (see **Figure 9**). No significant
332 relationship between the microbiota and the serum parameters analysed has been found.

333 **Discussion**

334 *Ultra-processed food and fecal microbiota*

335 To our knowledge there are very few studies that used the pig as model to investigate the
336 effects of UPF on gut microbiota and physiology. One study examined the effects of a
337 maternal Western diet during gestation and lactation (32). The authors demonstrated that the
338 western diet modified offspring's microbiota activity in Yucatan pigs (32). However, the
339 ingredients used in the western diets were not ultra-processed and diets differed mainly in
340 sugar and fat content and not for their processing levels. It is therefore difficult to attribute the
341 effects observed to the processing of the ingredients used. In the present study, the standard
342 and experimental diets were similar in energy, protein and fibre content and the observed
343 effects can be related to the 30% replacement of traditional ingredients by UPF. The gut
344 microbiota refers to the complex community of microorganisms, including bacteria, fungi,
345 and viruses that reside in the digestive tract. In this study, by microbiota we refer only to the
346 bacterial community characterised in the pigs' feces. Surprisingly, the use of UPF did not
347 affect the abundance or the biodiversity indexes in the fecal microbiota of pigs, independently
348 of the age of the pigs and the sugar and salt content of the diets. This is in contrast to what has
349 been observed in human studies where a Western diet is normally associated with lower
350 bacterial richness and biodiversity (9). A reason could be that while human studies often
351 associate the UPF consumption to a lower consumption of fiber and complex carbohydrates,
352 in our study the three experimental diets were similar in fiber and also energy content (9).
353 Even if a comparison between human and pig is not possible due to the different physiology,
354 exposure time to UPF and different chemical composition of the diets, our study suggests that

355 in pigs, the food processing alone do not impair the abundance and the biodiversity of the
356 fecal microbiota when UPF replace 30% of the standard ingredients. The Firmicutes to
357 Bacteroidetes ratio was also unaffected by the UPF. The literature reports that a higher
358 Firmicutes to Bacteroidetes ratio is related to a decrease in diarrhoea in pigs, with a strong
359 negative correlation between Firmicutes and pathogenic bacterial population in the intestine
360 (33).

361 The absence of adverse impacts on the Firmicutes to Bacteroidetes ratio is promising for the
362 potential use of UPF without affecting the gut health in pigs.

363 As expected, both the abundance and the diversity of the bacterial community increased with
364 the age of the pigs, in accordance with the literature (34). The core microbiota was moderately
365 influenced by the presence of UPF in the pig diets. Slight effects could be observed in the
366 finishing period, in particular in the core microbiota of pigs fed the SA diets compared to the
367 ST and SU diets. Given that the primary distinction between the SU and SA UPF lies in their
368 salt and sugar content, one might hypothesize that the salt exerts a more significant influence
369 than refined sugar in modulating the core microbiota, as reported by (35) and (36). The core
370 microbiota of the ST and SU groups were characterized by 9 and 8 ASVs respectively, while
371 the one of the SA group was composed by 12 ASVs. The intestinal core microbiota is defined
372 as the number and the identity of bacteria that are shared among different individuals. The
373 core microbiota focuses therefore only on the stable and permanent members of the bacterial
374 community (37).

375 It is hypothesized that these shared taxa represent the most ecologically and functionally
376 significant microbial associates of the host or environment under the sampled conditions.
377 Indeed, it has been suggested that identifying core microbiome components may aid in
378 addressing various topics, including the maintenance of gut health (38). In the present study,
379 the ASVs that constitute the core microbiota of the pigs fed the ST diet are present also in the

380 core microbiota of the SU and SA groups, and given that the experimental diets did not
381 reduce the size of the core microbiota, we can conclude that the UPF did not lead to any
382 detrimental effect on the pig gut core microbiota.

383 The LefSe analysis performed at the genus level showed that the dietary treatment only
384 influenced a few taxa during the trial. In fact, while at the beginning of the dietary treatment no
385 taxa abundance was found to be significantly different between the three treatment groups,
386 differences were found at T2 and T3. At T2, the SU diet was the one influencing the highest
387 number of bacteria, with 6 ASVs being more abundant compared to the ST and SA diets.
388 Contrastingly, in the finishing period (T3) the SA diet showed the highest number of significant
389 differences, with 6 ASVs being more abundant compared to the other groups. The SU diet
390 increased the abundance of the *Anaerostipes* genus both in the T2 and T3, compared to the other
391 groups. Members of the *Anaerostipes* genus, within the phylum Firmicutes, are strictly
392 anaerobic microorganisms with a strong glucose fermentation metabolism, resulting in the
393 production of mainly butyrate, acetate and lactate (39). In fact, sugar is the main source of
394 carbon and energy for such bacteria (39, 40). This genus represents more than 2% of total
395 colonic microbiota in the healthy human colon (41). This finding suggests a higher presence of
396 rapidly fermentable carbohydrates, such as sugar residues, in the large intestine of SU diet-fed
397 pigs. It is known that the glucose uptake from the intestinal lumen to the systemic circulation
398 takes place mainly in the small intestine and it is mediated by active (Sodium-Glucose
399 Transporter, SGLT1) and passive transporters (Glucose transporters, GLUTs) (42). However,
400 SGLT1 expression in the large intestine remains controversial, because SGLT1 mRNA in the
401 proximal colon has been detected by in situ hybridization but not by PCR (43). Therefore,
402 taking into account the similar fibre content between the three experimental diets, the higher
403 abundance of sugar-utilizing bacteria in the feces of SU-fed pigs suggests a higher amount of
404 unabsorbed sugars reaching the large intestine in both the growing and finishing periods,

405 compared to the ST and SA pigs. Such hypothesis is encouraged by the higher abundance of
406 the *gauvreauii* group belonging to the *Ruminococcus* genus in the SU group at T2 and T3.
407 Similarly to the *Anaerostipes* genus, also *Ruminococcus gauvreauii* produces acetic acid as
408 major end-product of glucose metabolism and mainly utilizes D-glucose, D-galactose, D-
409 fructose, D-ribose, D-sorbitol, D-mannitol, inositol and sucrose as substrate (44). Readily
410 fermentable carbohydrates such as starch, sugar residues, mucus and soluble non-starch
411 polysaccharides have been considered substrate for bacterial growth in caecum and proximal
412 colon of pigs also by Knudsen and colleagues (45). Several of these substrates are also part of
413 soluble fibre (e.g. non-starch polysaccharides), that can probably reach the large intestine and
414 induce microbiota changes in this group. However, starch polysaccharides were not quantified
415 in the large intestine content and the hypothesis cannot be confirmed by the present study.

416 Among the most affected taxa by the dietary treatment, the genus *bacteroidales* p2534-18B5
417 and members of the *Muribaculaceae* family were increased by the SA diet. No information
418 was found about the p2534-18B5 genus, but the literature reports that *Muribaculaceae* family
419 regulates the community composition and metabolites of the gut microbial population and that
420 participates in the degradation of polysaccharides, leading to the production of succinate,
421 acetate, and propionate (46). The increase in the *Prevotellaceae* UCG-003, belonging to the
422 *Prevotella* genus, was already observed by our research about the use of UPF as replacement
423 of traditional ingredients in post-weaning piglets' diets and it is probably correlated to the
424 fermentation of non-structural carbohydrates (47).

425 *Fecal volatile fatty acids and microbial community*

426 The gut microbiota plays a crucial role in the production of VFAs in the intestine. Acetate,
427 propionate, butyrate and valerate are the main VFAs produced by the microbial fermentation
428 of dietary fibres and complex carbohydrates that escape digestion in the small intestine and

429 serve as substrate for microbial growth (48). The UPF undergoes processing procedures aimed
430 to increase mainly their digestibility. Given this characteristic, and because of our previous
431 studies where we observed a high in-vitro digestibility of the UPF-based diets for pigs (49), our
432 hypothesis was that different amount or type of feed material would have escaped the digestion
433 process in pigs fed the SU and SA diets, resulting in different amount of substrate for the large
434 intestine bacterial fermentation and subsequent different VFAs production. By contrast, we did
435 not find differences between treatments in the fecal VFAs production. The majority of the
436 bacterial taxa that correlated (positively or negatively) with specific VFAs were equally
437 expressed in SU and SA groups compared to the ST. Only the *Anaerostipes* taxa, positively
438 correlated to the propionate production and negatively correlated to the butyrate production,
439 was more abundant in the SU group. Among the top 50 taxa that correlated with the VFAs
440 production, only five were less abundant in SU and only 1 in the SA group, compared to the
441 ST. This confirms the lack of detrimental effects of UPF on VFAs production in pigs.

442 *Jejunum physiology and blood serum measurements*

443 In this study we assumed that the long-term consumption of the UPF by pigs could lead to
444 detrimental effects on the small intestinal physiology. Specifically, our hypothesis was that
445 UPF could have impaired the integrity of the intestinal barrier function and lead to a lower
446 trans-epithelial electric resistance, an indicator of a condition known as “leaky gut”,
447 characterized by an increased intestinal permeability (50). In this environment, toxins,
448 bacteria and other unwanted molecules are allowed to enter the systemic circulation triggering
449 inflammation and other health issues (50). However, our theory was not confirmed by the
450 Ussing chamber measurements. In addition, no differences were found in performance traits
451 and health status, as better described in (25). The TEER was in fact similar between pigs fed

452 the UPF-based diets compared to the ones fed the standard diet, indicating that the UPF did
453 not promote a leaky gut condition in pigs.

454 The high content of saturated fatty acids, added sugars and sodium in UPF may interfere with
455 nutrient absorption, including amino acids. In our experiment on jejunum tissues, we
456 considered the L-glutamate, L-arginine and L-methionine to test the activity of different
457 classes of amino acids transporters, specifically anionic, cationic and neutral amino acids
458 transporters, respectively. The jejunum of pigs fed the SA diet showed a lower ability to
459 actively absorb L-glutamate, compared to the SU diet, and tended to absorb less L-glutamate
460 compared to the ST pigs. It has been observed in mice that a high salt content diet created a
461 high local concentration of sodium in the colon, despite the fact that sodium levels from food
462 are rapidly normalized in the small intestine (51). Therefore, we believe that also in our study,
463 the SA diets could have created a high luminal salt concentration at the jejunum level. How
464 such sodium chloride concentration could modulate the physiology of the L-glutamate uptake
465 is unclear. What is known is that the intestinal L-glutamate uptake is mainly mediated by the
466 sodium-dependent excitatory amino acid transporter-3 (EAAT3) (52). Therefore, further
467 studies should focus on the effects of UPF on the activation status of the EAAT3 transporter
468 and related L-glutamate uptake.

469 The consumption of UPF has been associated also with an excessive sodium chloride intake
470 that could disrupt the balance of certain minerals in the body such as potassium, calcium and
471 magnesium, essential for the animal health (53). Blood analysis performed on blood serum
472 showed that in our study, no effect of the SU or SA diets was observed on urea, calcium,
473 magnesium and potassium concentration. This suggests that when used to partially replace
474 traditional ingredients in a balanced diet, UPF did not lead to severe deficiency in pig.

475 At a BW of 80kg, pigs fed a SU diet exhibited significantly lower blood insulin
476 concentrations one-hour post-meal compared to the ST and SA groups. This could be due to

477 the higher simple sugar content in UPF compared to standard feed ingredients (49), leading to
478 a quicker decline in insulin secretion. However, since this study measured insulin at only one
479 time point, this hypothesis cannot be confirmed. Also, when translating the results of UPF
480 studies on insulin secretion from pigs to humans, it is essential to consider significant
481 differences in glucose and insulin metabolism. Pigs are known to be resistant to the
482 spontaneous development of type 2 diabetes mellitus, even after intervention with high-fat,
483 high-fructose, and high-carbohydrate diets (54, 55). The resistance of pigs to type 2 diabetes
484 is likely attributed to variations in the composition of their bile acid pool, particularly the high
485 concentration of hyocholic acid (HCA) and its derivatives. These HCAs play a crucial role in
486 improving glucose homeostasis by modulating the activity of the cell membrane G-protein-
487 coupled BA receptor TGR5 and the nuclear farnesoid X receptor (FXR) signaling mechanism,
488 as described by Zheng and co-authors (56).

489 In conclusion, the partial replacement of traditional feed ingredients with UPF have no
490 detrimental effects on gut microbiota, intestinal integrity and mineral homeostasis when
491 included in a balanced diet for pigs. More targeted studies should be performed to better
492 investigate the effect of sodium chloride intestinal accumulation and its effect on specific
493 intestinal transporter's activity, in particular the EAAT transporters and the related L-
494 glutamate intestinal uptake.

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499
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501 MT conducted research, performed statistical analysis and wrote paper; SM conducted
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505 All authors have read and approved the final manuscript.

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507 Data described in the manuscript will be made available upon request.

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Table 1. Dietary ingredients used for experimental diets in the growing (G) and finishing (F) periods.

Ingredient ² , %	Dietary treatments ¹						
	SA-G	Grower			Finisher		
		ST-G	SU-G	SA-F	ST-F	SU-F	
Barley	39.7	41.1	38.0	41.3	46.4	41.8	
Wheat	-	30.0	-	-	30.0	-	
Salty FFPs ³	30.0	-	-	30.0	-	-	
Sugary FFPs ⁴	-	-	30.0	-	-	30.0	
Fat	-	2.69	0.79	-	2.22	0.68	
Potato protein	5.00	5.00	5.00	5.00	5.00	5.00	
Soybean meal	6.16	6.59	7.36	2.77	3.55	4.03	
Wheat bran	9.06	4.34	8.76	12.3	3.93	9.87	
Dried beet pulp	5.15	5.15	5.15	4.50	4.50	4.50	
L-Lysin-HCl	0.26	0.26	0.23	0.12	0.10	0.09	
DL-Methionine	0.01	0.02	0.02	-	-	-	
L-Threonine	0.03	0.02	0.02	-	-	-	
L-Tryptophan	-	-	0.002	-	-	-	
MCP	0.45	0.45	0.47	0.11	0.13	0.16	
Lime, carbonic acid	1.51	1.53	1.48	1.17	1.20	1.19	
Sodium chloride	-	0.16	-	-	0.27	-	
Pellan ⁵	0.30	0.30	0.30	0.30	0.30	0.30	
Celite 545	2.00	2.00	2.00	2.00	2.00	2.00	
ALP-S 467 Mast ⁶	0.40	0.40	0.40	0.40	0.40	0.40	
Natuphos 5000 G	0.01	0.01	0.01	0.01	0.01	0.01	
Analyzed nutrient composition (g/kg DM)							
Total ash	74.1	68.4	72.1	65.4	61.7	64.1	
Crude fat	53.2	52.2	61.3	53.4	45.3	58.9	
Crude protein	174	173	176	151	152	153	
Crude fiber	39.5	41.6	38.6	38.9	42.2	39.6	
Sodium	3.74	1.25	1.51	3.21	1.66	1.73	
SFA	11.7	18.4	17.7	11.7	16.1	20.3	
MUFA	26.2	18.5	20.2	29.4	14.5	25.4	
PUFA	15.8	17.1	14.1	17.6	16.1	17.9	
Calculated							
Digestible P (g/kg DM)	2.87	2.87	2.87	2.19	2.19	2.24	
Digestible lysine (g/kg DM)	8.29	8.29	8.29	6.24	6.24	6.24	
DE (MJ/kg DM)	13.7	13.7	13.7	13.7	13.7	13.7	
ME (MJ/kg DM)	13.2	13.2	13.2	13.3	13.3	13.3	

¹ All grower diets were formulated for pigs with a body weight (BW) of 40 kg; all finisher diets were formulated for a BW of 80 kg. ST-G, ST-F = standard diet without former foodstuff product (FFP) inclusion for growing (G) and finishing (F) pigs, respectively. SA-G and SA-F = grower and finisher diets where a part of the cereals and fats were replaced with 30% salty FFPs. SU-G and SU-F = diets

where a part of cereals and fats were replaced with 30% sugary FFPs for growing (G) and finishing (F) pigs, respectively.

² MCP, monocalcium phosphate; SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DE, digestible energy; ME, metabolizable energy; DM, dry matter

³ Pure salty former foodstuff products

⁴ Pure sugary former foodstuff products

⁵ Binder that aids in pellet formation

⁶ Mineral-vitamin premix that supplied the following nutrients per kg of diet: 20,000 IU vitamin A, 200 IU vitamin D₃, 39 IU vitamin E, 2.9 mg riboflavin, 2.4 mg vitamin B₆, 0.010 mg vitamin B₁₂, 0.2 mg vitamin K₃, 10 mg pantothenic acid, 1.4 mg niacin, 0.48 mg folic acid, 199 g choline, 0.052 mg biotin, 52 mg Fe as FeSO₄, 0.16 mg I as Ca(IO)₃, 0.15 mg Se as Na₂Se, 5.5 mg Cu as CuSO₄, 81 mg Zn as ZnO₂, and 15 mg Mn as MnO₂

Table 2. Volatile fatty acids (VFAs, g/kg) quantified in pig feces (n=12 per each group) at T1, T2 and T3.

	SA			ST			SU			SEM	P-values		
	T1	T2	T3	T1	T2	T3	T1	T2	T3		Diet	Time	D x T
Acetate	1.35	1.45	1.46	1.29	1.51	1.47	1.35	1.4	1.33	0.057	0.730	0.205	0.251
Propionate	1.75 ^{ab}	2.17 ^{bc}	2.39 ^{bc}	1.54 ^a	2.53 ^c	2.36 ^c	1.91 ^{abc}	2.27 ^{bc}	2.21 ^{bc}	0.164	0.143	0.001	0.035
Butyrate	1.37 ^{ab}	1.58 ^{ab}	1.49 ^{ab}	1.13 ^a	1.92 ^b	1.58 ^{ab}	1.53 ^{ab}	1.59 ^{ab}	1.33 ^{ab}	0.154	0.133	0.525	0.042
Valerate	0.23	0.37	0.35	0.24	0.45	0.31	0.26	0.41	0.32	0.036	0.757	0.001	0.602

Abbreviations: SA = salty UPF-based diet; ST = standard diet; SU = sugary UPF-based diet; Values are least square means with the standard error of the means (SEM). P-values for the diet (D), time point (T) and their interaction (DxT) were calculated by using repeated measures ANOVA using linear mixed-effects regression models (Lmer). For pairwise comparisons, a modified Tukey test for multiple comparisons of means, the Sidak function was used.

Table 3. D-glucose and amino acid-induced change in short-circuit current (ΔI_{sc} , μA) in mid-jejunum of pigs (n=6 in ST and n=7 in SA and SU groups).

	SA	ST	SU	SEM	P value
D-Glucose	2.54	4.49	4.46	1.235	0.327
L-Glutamate	0.33 ^a	0.81 ^{ab}	1.54 ^b	0.251	0.006
L-Arginine	4.25	3.58	4.89	1.431	0.769
L-Methionine	3.52	4.54	4.84	0.951	0.504
TEER	34.2	31.6	34.1	2.902	0.666

Abbreviations: SA = salty UPF-based diet; ST = standard diet; SU = sugary UPF-based diet; TEER =

transepithelial electrical resistance. P-values were obtained by one-way ANOVA statistical analysis.

For pairwise comparisons, a modified Tukey test for multiple comparisons of means, the Sidak function was used.

Table 4. Serum concentration (mmol/l) of urea, calcium, magnesium and potassium of pigs (n=8 per each group) fed a standard growing finishing diet (ST) or a growing finishing diet supplemented with 30% sugary (SU) or salty (SA) ultra-processed food.

	SA	ST	SU	SEM	P value
Urea	6.31	5.79	6.35	0.151	0.231
Calcium	2.55	2.51	2.51	0.010	0.390
Magnesium	0.98	0.99	1.01	0.022	0.872
Potassium	3.38	3.39	3.26	0.051	0.481

Abbreviations: SA = salty UPF-based diet; ST = standard diet; SU = sugary UPF-based diet. P-values

were obtained by one-way ANOVA statistical analysis.

Figures Legend

Figure 1: Non-phylogenetic diversities at the A) T1 (20 kg BW), B) T2 (60 kg BW) and C) T3 (100 kg BW) of fecal microbiota from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF.

Figure 2: Independent of the diet, non-phylogenetic diversities at T1 (20 kg BW), T2 (60 kg BW) and T3 (100 kg BW). **** = P-value < 0.001.

Figure 3: Relative abundance of fecal microbiota families at A) T1 (20 kg BW), B) T2 (60 kg BW) and C) T3 (100 kg BW) from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF

Figure 4: Composition plots of pigs' fecal microbiota families at the T1 (20 kg BW), T2 (60 kg BW) and T3 (100 kg BW).

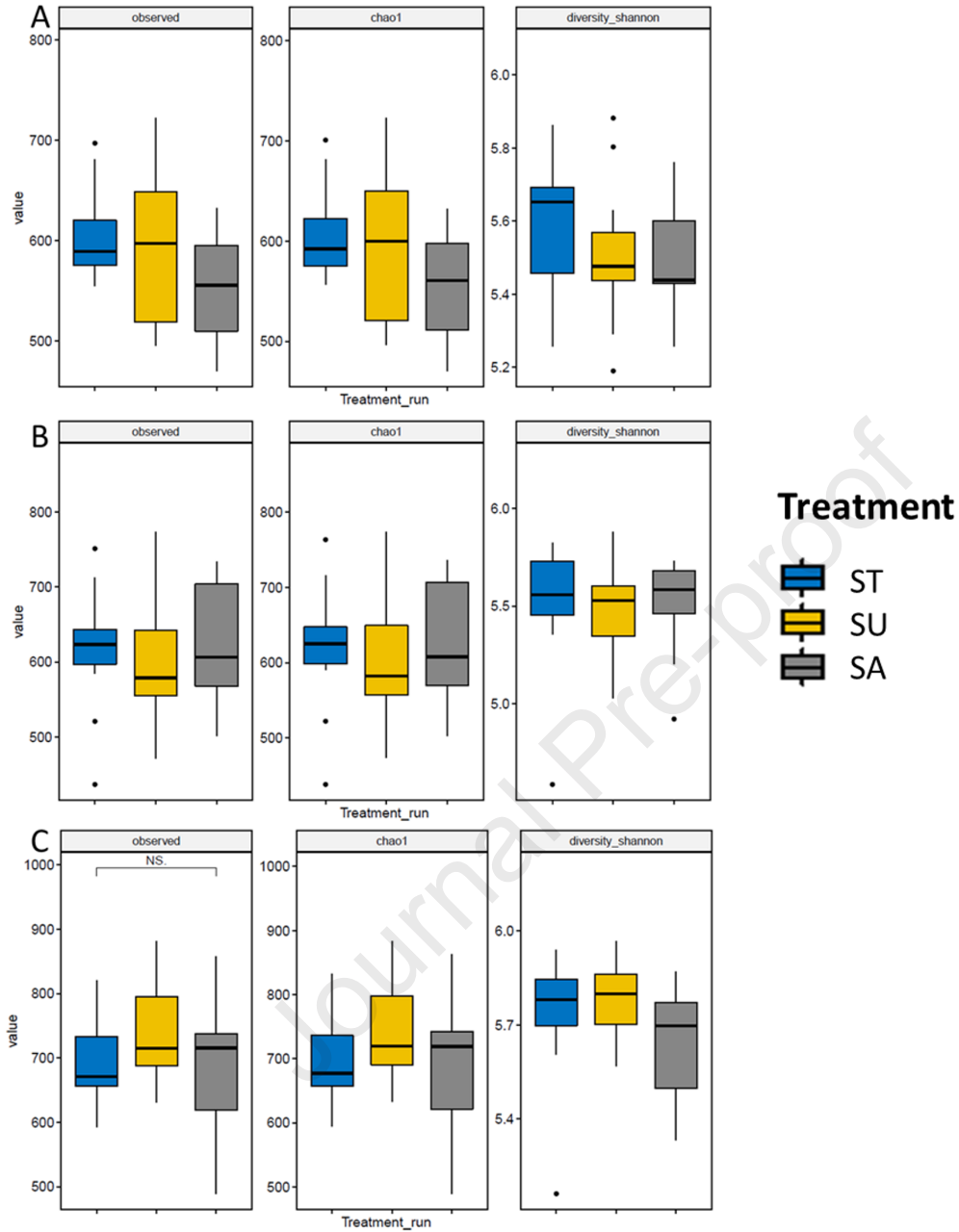
Figure 5: A: Unweighted and **B:** weighted UniFrac beta-diversity distances of the pigs' fecal microbiota at the T1 (20 kg BW), T2 (60 kg BW) and T3 (100 kg BW).

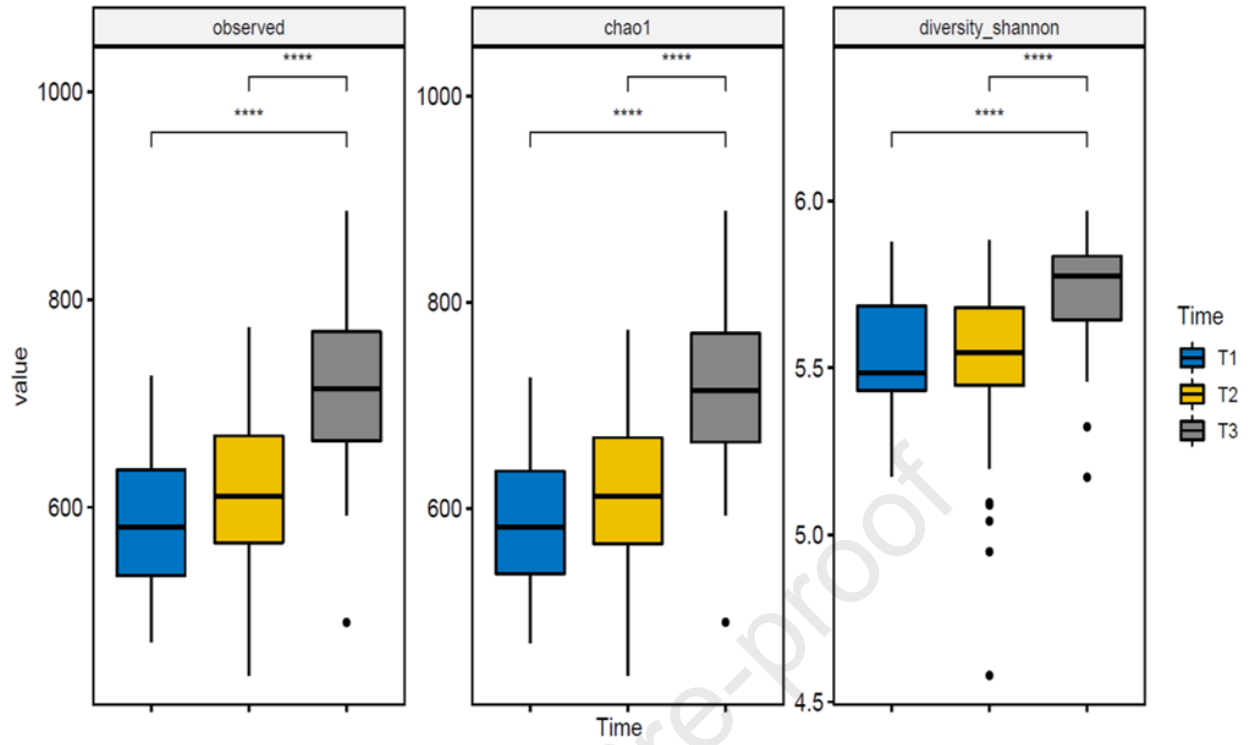
Figure 6: Heatmaps of the fecal core microbiota at T2 (60 kg BW) and T3 (100 kg BW) from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF.

Figure 7: Biomarker taxa analysis conducted on the fecal microbiome at the genus level at **A:** T2 (60 kg BW) and **B:** T3 (100 kg BW) from pigs fed either a basal grower-finisher diet or the basal diet with 30% salty (SA) or sugary (SU) UPF. The outcomes were derived from Linear Discriminant Analysis of Effect Size (LEfSe).

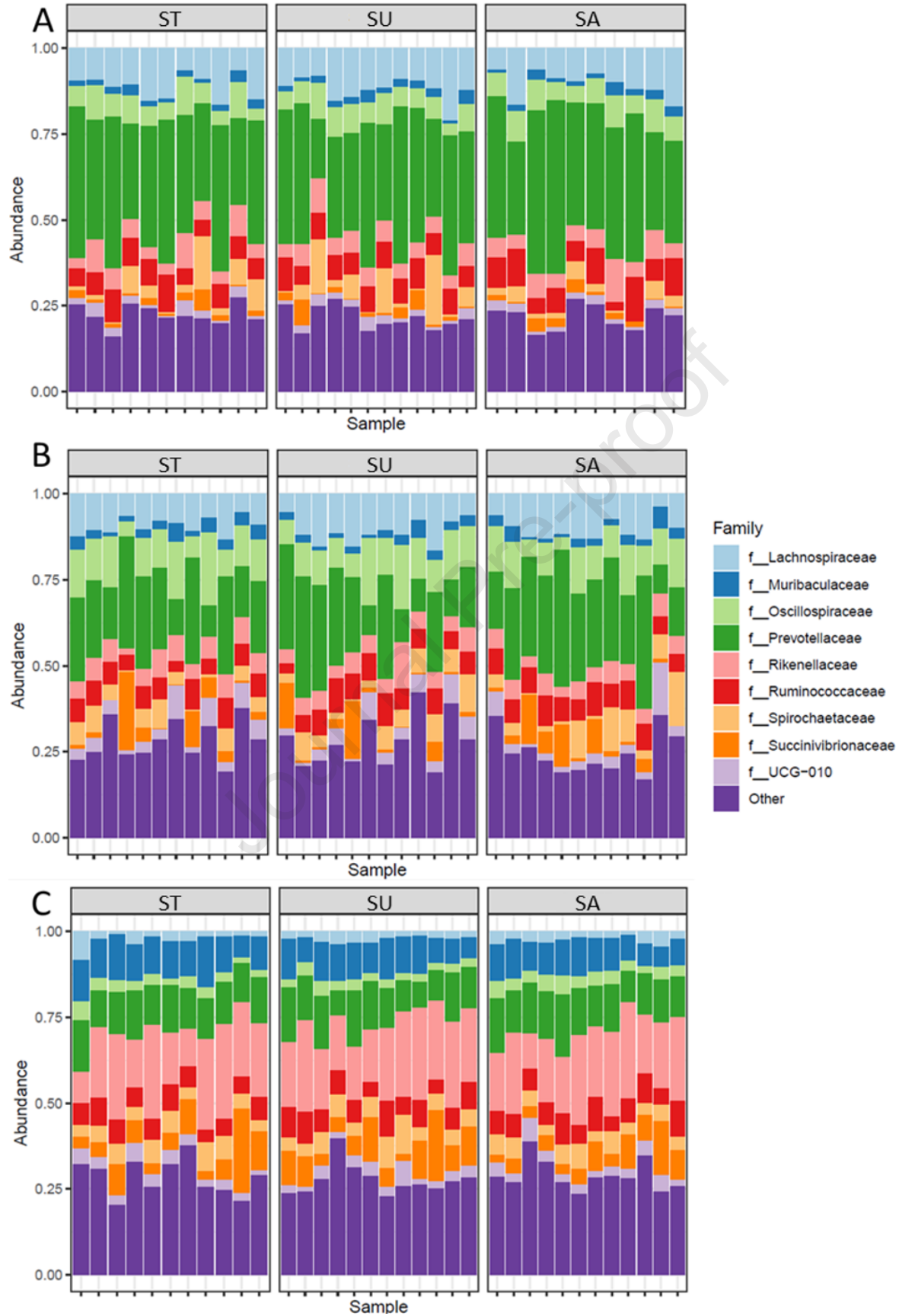
Figure 8: Top 50 significant associations between volatile fatty acids (acetate, valerate, butyrate, and propionate) and gut microbiota composition in pigs, irrespective of time point of fecal collection, fed the growing finishing diets supplemented with sugary or salty ultra-processed food. The MaAsLin2 analysis incorporated dietary treatment effects as fixed factors. The color scale-bar represents positive relationships (red) and negative ones (blue) between taxa and factors derived from normalized significant results. Presented are the top 50 correlations, all exhibiting a P-value < 0.05.

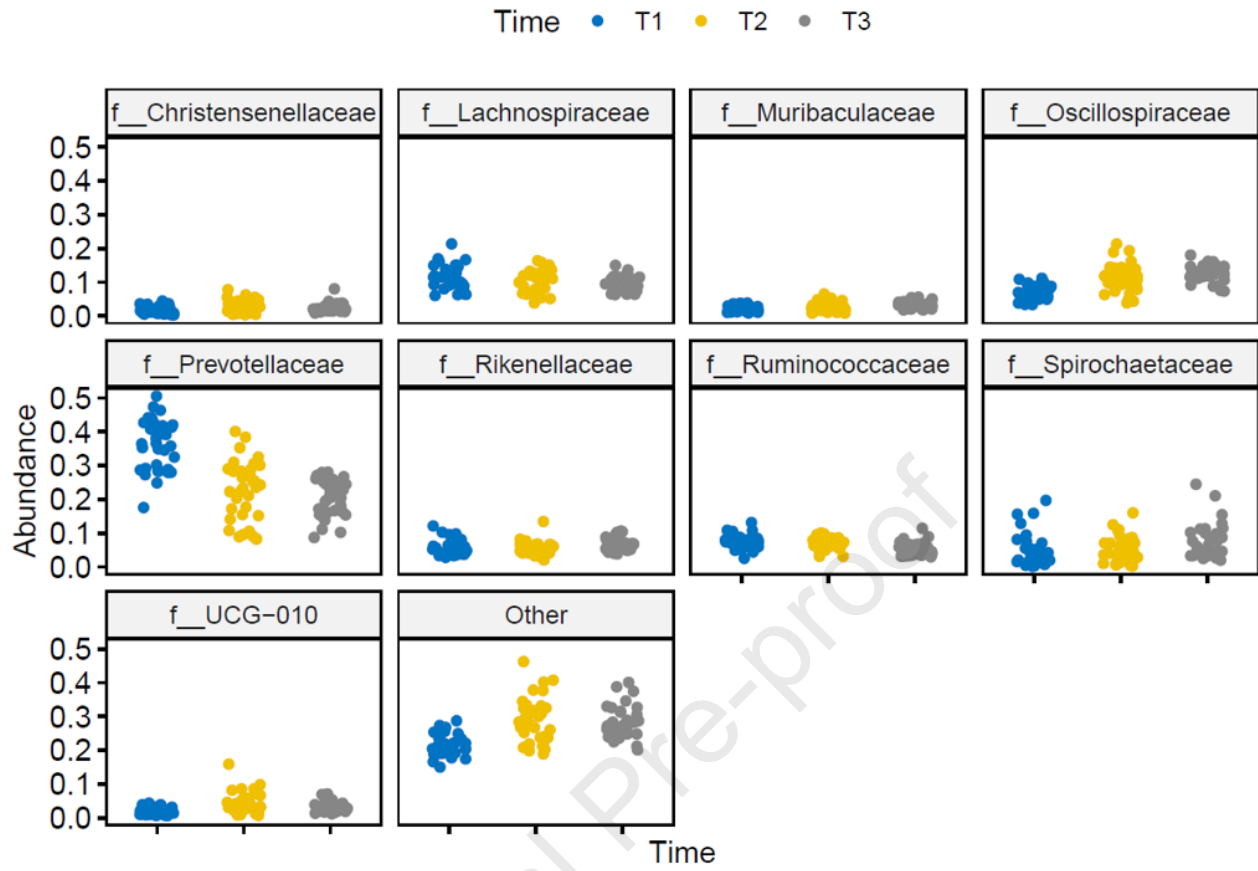
Figure 9: Comparison of insulin concentration (milliunit/l) between pigs fed a standard growing finishing diet (ST) or a growing finishing diet supplemented with 30% sugary (SU) or salty (SA) ultra-processed food at body weight (BW) of 40 or 80 kg. Boxplots with Standard Deviations.

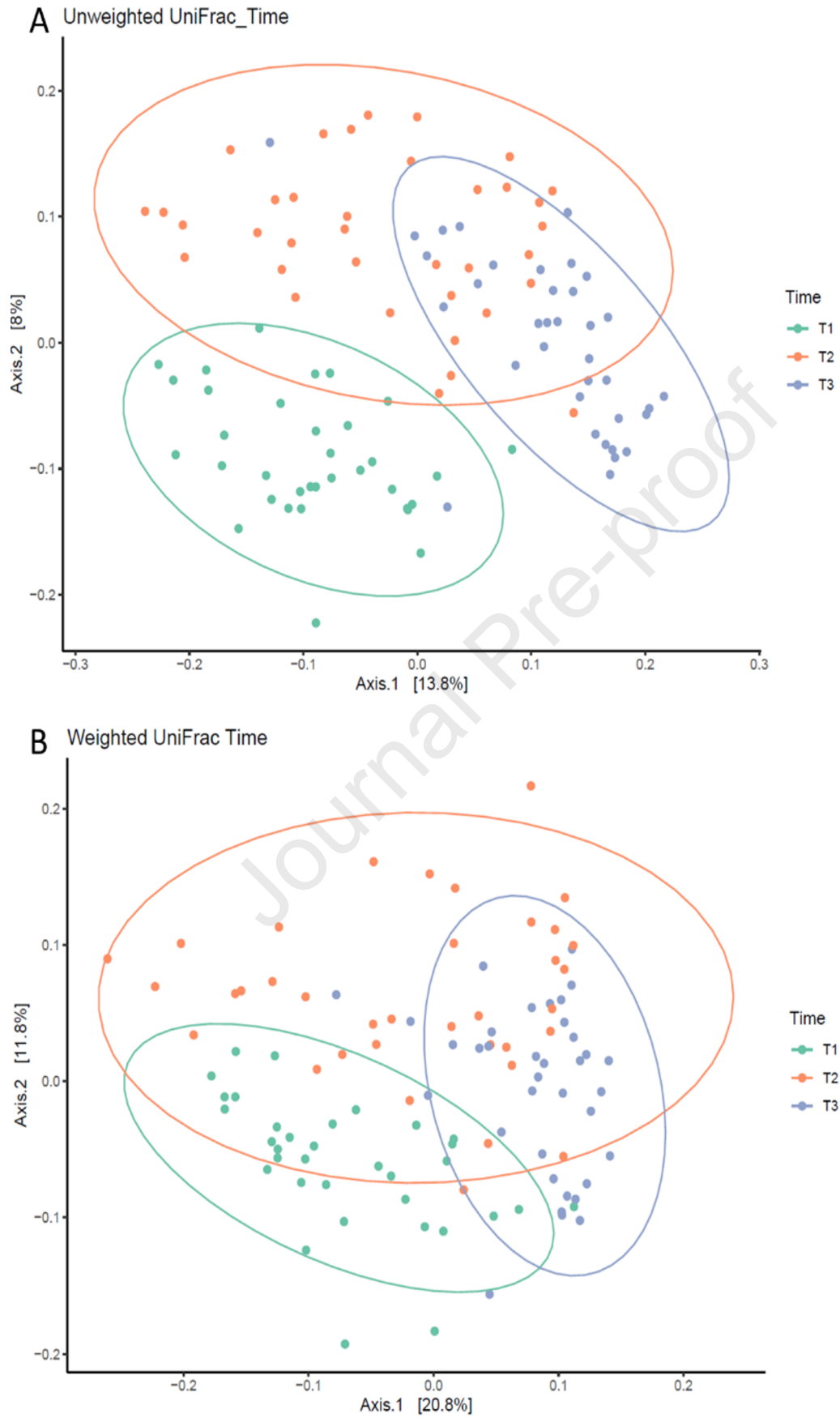


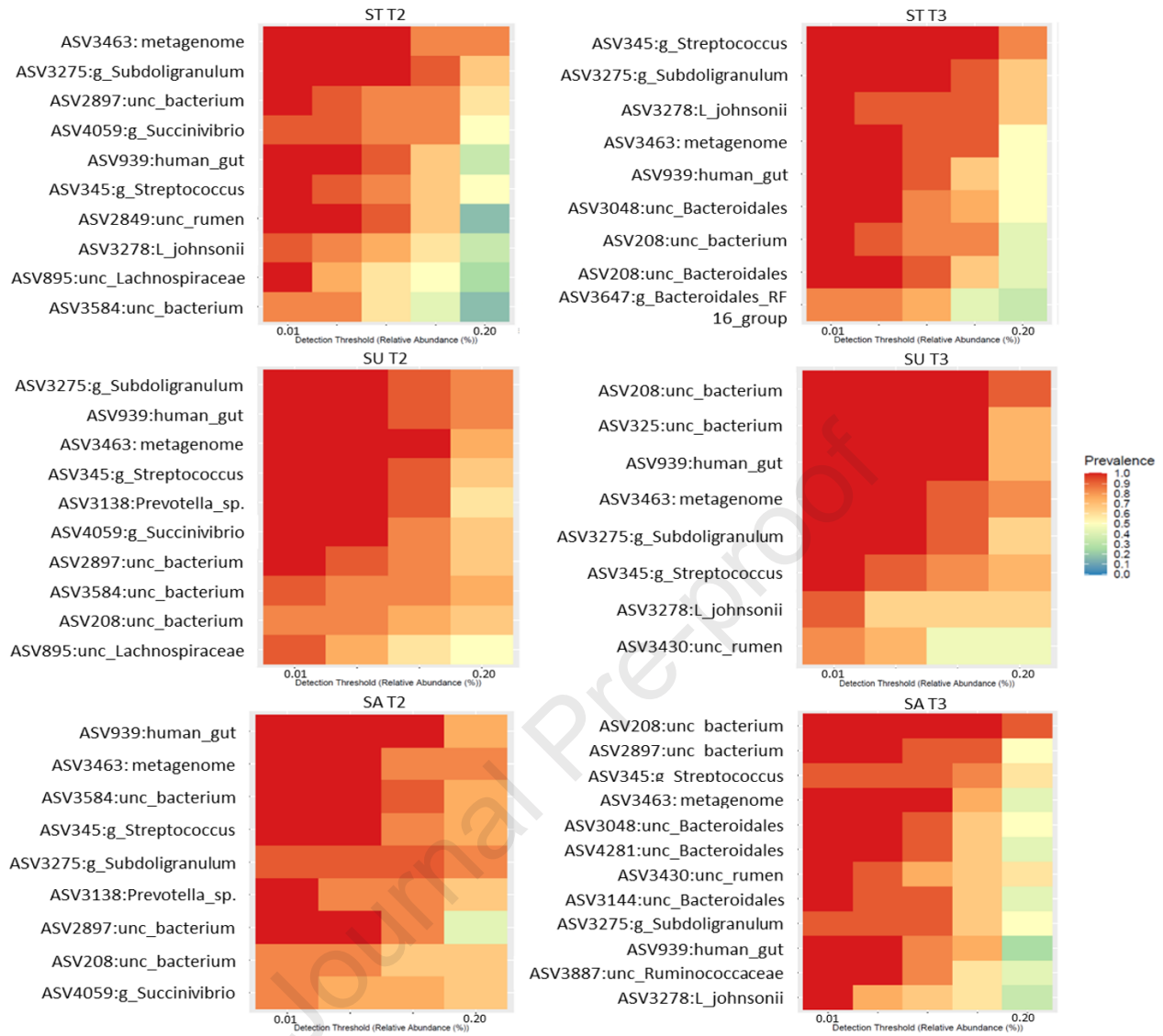


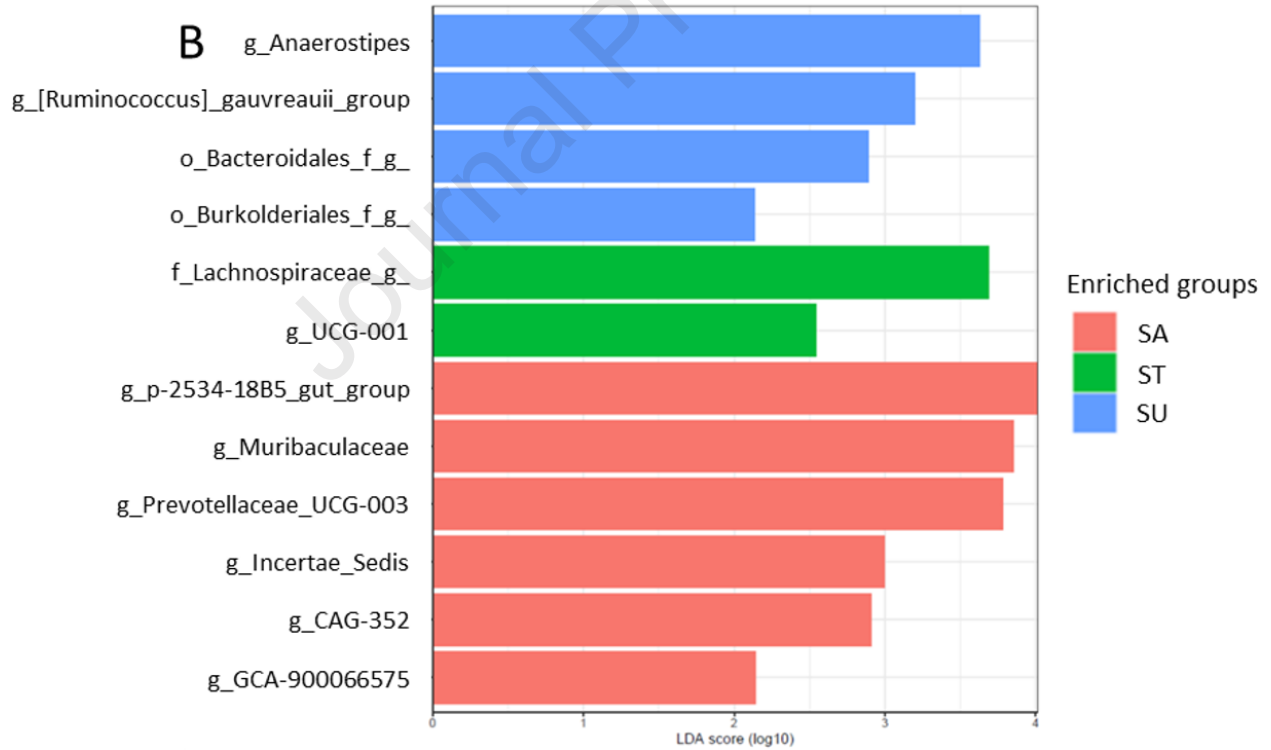
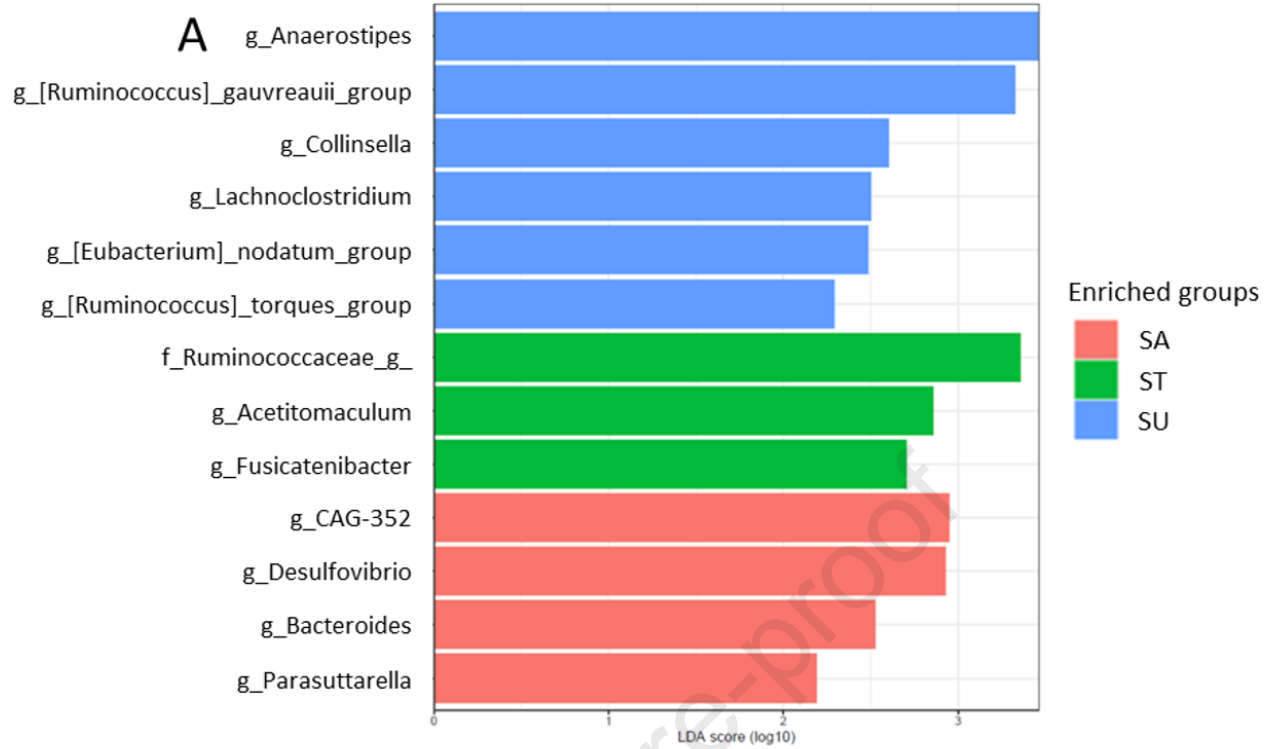
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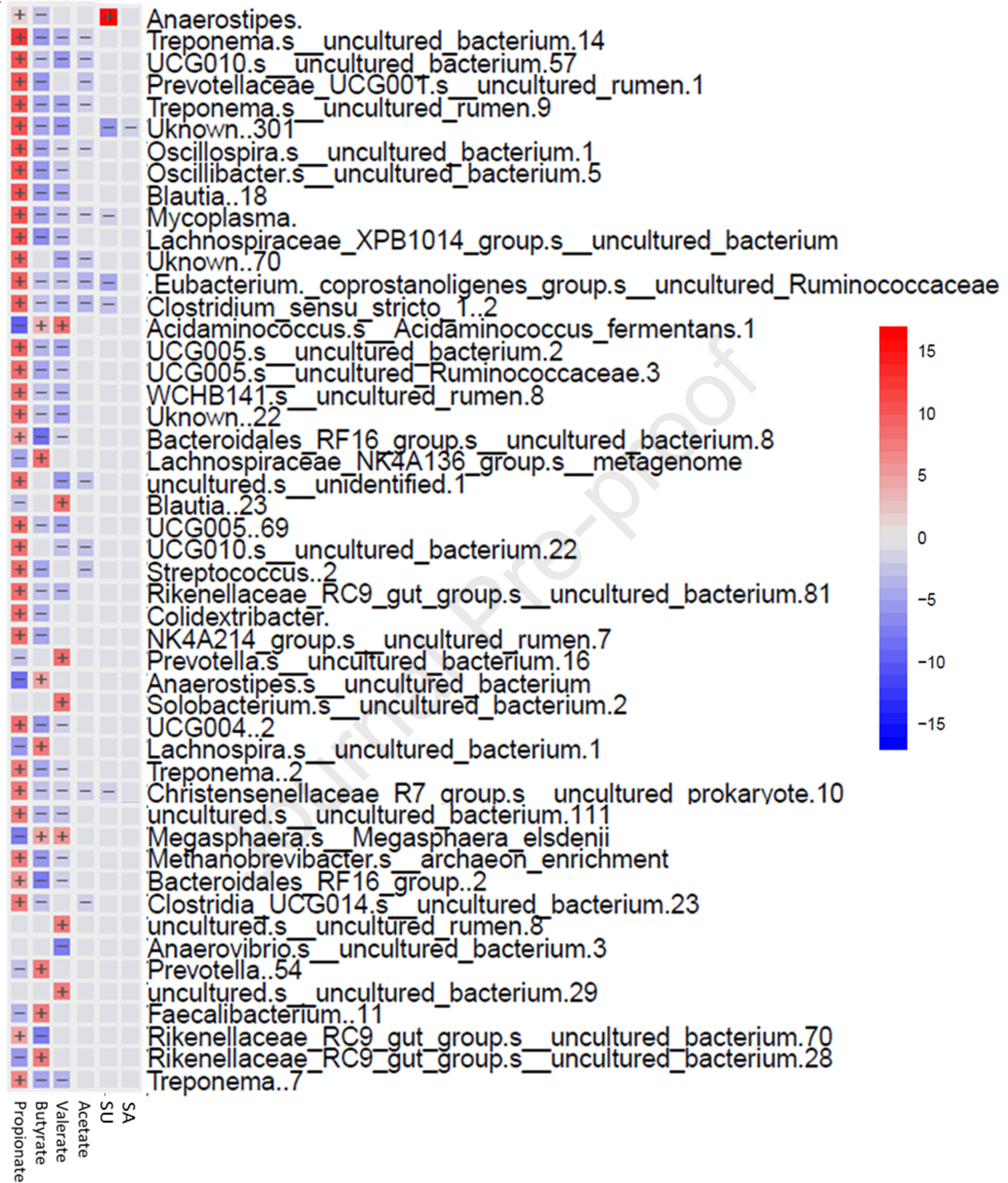


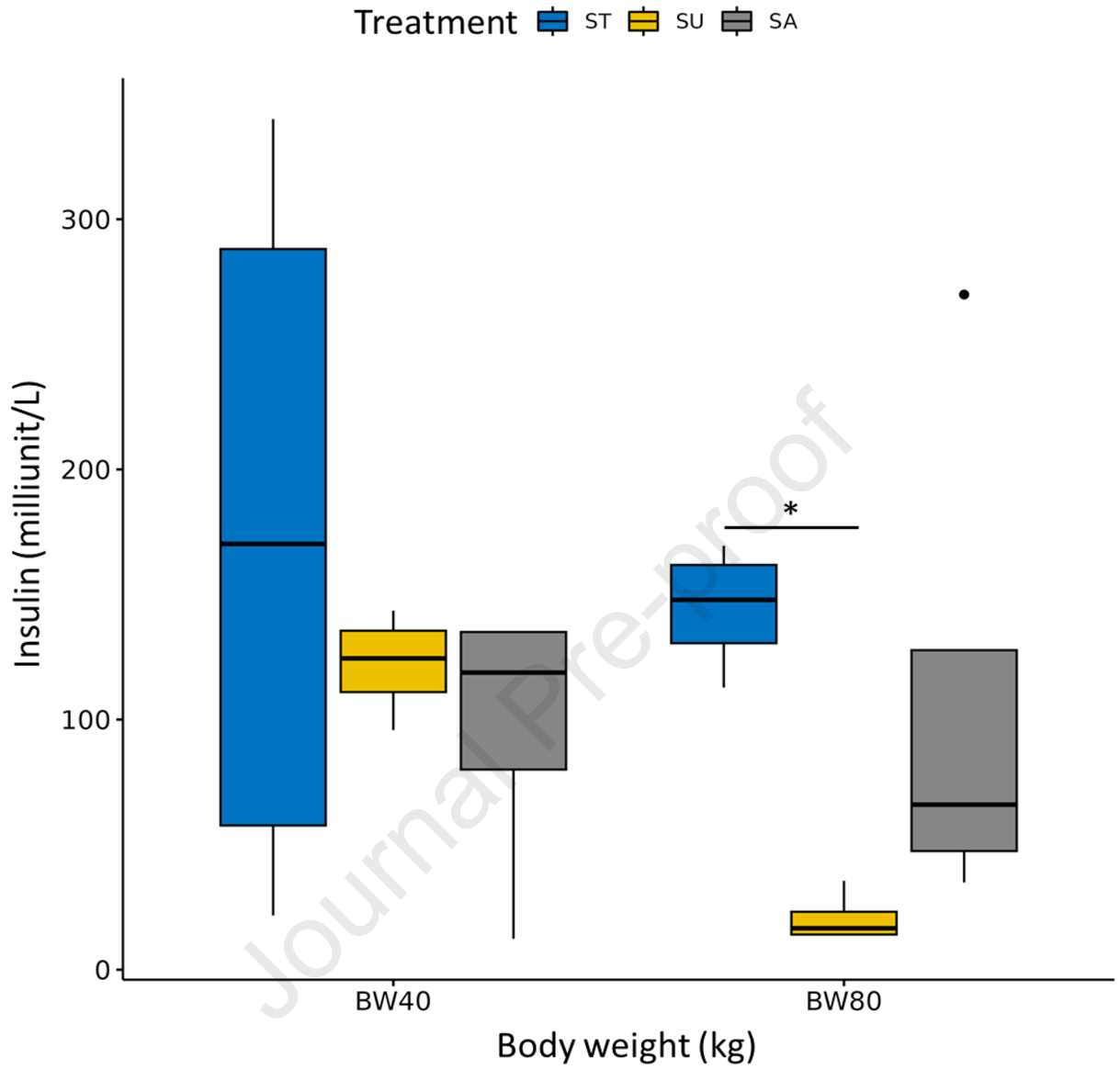












Declaration of interests

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

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Prof. Luciano Pinotti reports financial support was provided by Lombardy Region. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.