Impact of Nisin-Activated Packaging on Microbiota of Beef Burgers during Storage

Impact of nisin-activated packaging on microbiota of beef burgers during storage

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Running title: Microbiota of beef burgers in nisin active packaging

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ABSTRACT

Beef burgers were stored at 4°C under vacuum in nisin-activated antimicrobial packaging. Microbial ecology analyses were performed from samples collected between days 0 to 21 of the storage to discover the population diversity. Two batches were analyzed using RNA-based DGGE and pyrosequencing. The active packaging retarded the growth of the total viable counts as well as lactic acid bacteria. Culture-independent analysis by pyrosequencing of RNA extracted directly from meat showed that *Photobacterium phosphoreum*, *Lactococcus piscium, Lactobacillus sakei* and *Leuconostoc carnosum* were the major OTUs shared between control and treated samples. Beta diversity analysis of the 16S rRNA sequences data and RNA-DGGE showed a clear separation between two batches based on the microbiota. Control samples from batch B showed a significant high abundance of some taxa sensitive to the nisin such as *Kocuria rhizophila*, *Staphylococcus xylosus*, *Leuconostoc carnosum* and *Carnobacterium divergens* compared to control samples from batch A. However, only from batch B it was possible to find a significant difference between controls and treated samples during the storage due to the active packaging. Predicted metagenomes confirmed differences between the two batches and indicated that the use of nisin-based antimicrobial packaging can determine a reduction in the abundance of specific metabolic pathways related to the spoilage. The present study aimed to study the viable bacterial communities in beef burgers stored in nisin-based antimicrobial packaging and highlights the efficacy of this strategy to prolong beef burgers shelf life.

Keywords: antimicrobial vacuum packaging/meat/nisin/RT-PCR-DGGE/rRNA-based pyrosequencing

INTRODUCTION

Spoilage of meat or meat products is caused by an uncontrolled growth and various metabolic activities of the dominant microbiota found on these foods (1, 2). It is commonly assumed that microbiota of meat can originate from the processed animal's skin or intestines and that contamination may occur during the successive steps of processing and distribution (3–6). Undesired microbial development in meat could also appear during storage. In particular, different storage conditions of meat products can influence the development of different microbial groups and their ability to produce spoilage-associated compounds such as esters, ketones, aldehydes, sulfur compounds, amines and volatile fatty acids (2, 7). In the last decades numerous methods had been applied for storage of meat to control the spoilage process extending the shelf life of raw meat and meat products. Among them, refrigerate storage conditions, addition of natural preservatives, vacuum pack (VP) and modified atmosphere packaging (MAP) could influence the development and the activities of the "ephemeral spoilage microorganisms (ESO)" and consequently the spoilage process (3). Antimicrobial nisin-based active packaging has also been recently developed as a powerful tool for meat storage (8–11). Depending on the storage conditions and other extrinsic factors, only a few species are able to sufficiently develop in the food matrix to significantly affect the food quality.

The development and application of powerful molecular techniques have contributed to produce reliable data on the microbial species and strains occurring during meat storage. High-throughput sequencing (HTS) is becoming an increasingly popular tool in food microbiology offering numerous chances of food microbiota assessment (12, 13). Although DNA-based procedures provide a picture of the global community, they do not necessarily reflect the live population, as the DNA may not originate from living cells. To avoid this bias several authors suggest the use of treatments with PMA or EMA prior to DNA extraction to detect or quantify viable microorganisms. However, in some cases EMA and PMA could also diffuse into living bacterial cells with intact membranes (14). On the other hand, RNA is considered a more useful

target for viable bacteria even though rRNA molecules remain available for detection after bacterial death for some generally not predictable time (14). Moreover, RNA should be considered as a more informative target than DNA as rRNA can be proportionally more abundant in microbial cells than DNA copies, and this could lead to more detailed picture of the matrix analyzed (15). Many studies showed that species from Lactobacillales, Bacillales, Enterobacterales, Pseudomonadales and Vibrionales, are the predominant spoilage microorganisms on meat/meat products (16). Indeed, it is poorly understood whether members of these microbial communities are active during storage of raw meat.

The aim of this work was to study the viable bacterial communities in beef burgers stored in nisin-based antimicrobial VP and at following the changes in bacterial counts and diversity during storage at 4°C.

MATERIALS AND METHODS

Preparation of antimicrobial bags and films. A nisin-based antimicrobial solution (NS) at 2.5% (Nisin, Sigma, Milan, Italy) was prepared as described by Ercolini et al. (17). Four milliliter of antimicrobial solution was used to manually coat the inner layer of 12 bags of Linear Low Density Polyethylene (LLPDE, oxygen transmission 0.83 cm^3 m^{-2} h^{-1} at 23°C, 30 X 30 cm^2). A coating rod able to form a 45 μm thick coating was used. One mL of antimicrobial solution was used to manually coat both sides of 72 LLDPE strips (30 cm x 10 cm). Both bags and films were then air dried at 50 °C and used for the packaging of beef burger samples as described below. The antimicrobial activity of pieces of plastic films was checked in agar assays as previously described (18), using *Listeria monocytogenes* EGDE as the indicator strain. Briefly, the treated films were located onto the surface of a BHI (Oxoid, Milan, Italy) soft (0.75%) agar plates seeded with 2.5% of an overnight culture of *L. monocytogenes* EGDE as the indicator strain. The treated face of the film was in contact with the agar. Untreated films were assayed as negative controls. After incubation

97 the antimicrobial activity was evaluated by observing a clear zone of growth inhibition in
98 correspondence of the active film.

99 **Beef burger samples and microbial analysis.** Beef burger samples (100 g each in a square shape)
100 were manufactured in a local meat factory in the area of Torino, Italy. The formulation used in the
101 manufacture included minced beef, salt and pepper. Two independent batches were analyzed
102 (namely A and B). Both batches were prepared with meat from the same supplier in two different
103 periods of time. Figure 1 presents a schematic representation of the experimental plan. Twenty-
104 seven beef burgers were placed inside the activated bag, on three layers one on the top of each
105 other, nine burgers on each layer in a three by three square. Three activated strips were placed on
106 top of the first and second layers of burgers, in order to let both faces of each burger be in contact
107 with a strip or with the inner face of the activated bag. No space was left between each burger on
108 every layer to avoid oxygen accumulation during successive packaging. A total of 324 burgers were
109 prepared in twelve bags, six bags for each batch. The samples were then vacuum packed before
110 thermal sealing and stored at 4 °C. After 1, 3, 5, 7, 14 and 21 days of storage one bag from each
111 batch was opened and six samples from each bag were taken for microbiological analysis and
112 microbial population assessment. The burgers were collected by taking two samples from each
113 layer at random positions, the two samples were then pooled together and 10 g of each of the three
114 pools were homogenized with 90 mL of Ringer's solution (Oxoid, Milano, Italy) for 2 min in a
115 stomacher (LAB Blender 400, PBI, Italy; stomacher bags: Sto-circul-bag, PBI, Italy) at room
116 temperature. Before packing, at time 0, three burgers for each batch were also analyzed. An equal
117 series of 324 burgers in twelve bags were packed as described above using non activated bags and
118 strips, and used as a control. From each control (C) and treated (T) sample, 1 ml of the first decimal
119 dilution was used to determine: i) total aerobic bacteria, ii) Lactic Acid Bactiera (LAB), iii)
120 *Staphylococcaceae*, iv) *Enterobacteriaceae*, v) yeasts and vi) moulds, by using selective media and
121 conditions previously described (19). Results were expressed as means of Log colony forming units
122 (CFU) for three independent determinations on each batch.

123 The pH of each sample was measured by using a digital pH meter (Waterproof pH Tester, Thermo
124 Scientific, Nijkerk, The Netherlands).
125 **Total DNA and RNA extraction from beef burger samples**. At each sampling point, 1 ml of the
126 first decimal dilution was collected and directly centrifuged at maximum speed for 30 s. Nucleic
127 acid were extracted from two out of three biological replicates from each batch. Total DNA was
128 extracted as described by Alessandria et al. (20). DNA was quantified using a NanoDrop 1000
129 spectrophotometer (Thermo Scientific, Milano, Italy) and standardized at 100 ng/µl. For RNA
130 extraction, 200 µl of RNA-later (Ambion, Applied Biosystems, Milan, Italy) was immediately
131 added to the pellet and stored at -80 °C. Total RNA from the samples was extracted using the
132 MasterPureTM Complete DNA and RNA Purification kit (Epicentre, Madison, WI, USA)
133 following the manufacturer's instructions. Three microliters of TURBO-DN*ase* (Ambion) were
134 added to digest DNA in the RNA samples, with an incubation of 3 h at 37 °C. RNA was quantified
135 using the Nanodrop and standardized at 300 ng/µL. Reverse transcription (RT) reactions were
136 performed using M-MLV reverse transcriptase (Promega, Milan, Italy). Three hundred ng of RNA
137 was mixed with 1 µ l of 10 µM by using the universal primer 518R and DNase- and RNase-free
138 sterile water (Sigma) to a final volume of 10 µl and then incubated at 75 °C for 10 min. The mix
139 was placed on ice and a mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$,
140 10 mM DTT, 2 mM of each dNTP, 1 µl of 200 Uμ l^{-1} M-MLV and 0.96 U of RNasin ribonuclease
141 inhibitor (Promega) was transferred to the reaction tube. Reverse transcription was carried out at
142 42°C for 1 h.
143 **DGGE analysis.** One µl of DNA, or cDNA, from two biological replicates of each batch was used
144 as a template in the PCR reaction. The V3 region of the 16S rRNA gene was amplified with the
145 primers 338f-GC/518r, as previously described (21). PCR products were analyzed by denaturing
146 gradient gel electrophoresis (DGGE) at 30-60% by using a Bio-Rad Dcode as suggested elsewhere
147 (19).

148 **RNA analysis by pyrosequencing**. cDNA was used to study the microbial diversity of the viable
149 populations by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene by using
150 primers and PCR condition previously reported (22). PCR products were purified by Agencourt
151 AMPure kit (Beckman Coulter, Milano, Italy), quantified using the QuantiFluor™ (Promega,
152 Milano, Italy) and an equimolar pool of the PCR templates was obtained prior to further processing.
153 The amplicon pool was processed by using Titanium chemistry on a GS Junior platform (454 Life
154 Sciences, Roche, Monza, Italy) according to the manufacturer's instructions.
155 **Statistical analysis.** Data from microbiological counts were analyzed by one-way analysis of
156 variance (ANOVA) for each individual packaging condition, with time or batch as the main factor,
157 using SPSS 22.0 statistical software package (SPSS Inc., Cary, NC, USA). When ANOVA revealed
158 significant differences ($P < 0.05$), Duncan HSD test was applied. t test was used to assess the
159 differences in microbial loads between C and T samples at the same time of sampling and between
160 C samples of the two batches. A database of fingerprints was created by using the software
161 Bionumerics version 4.6 (Applied Maths, Sint Marten Latem, Belgium). A combined data matrix
162 including DNA and RNA fingerprints was obtained and dendrograms of similarity were retrieved
163 by using the Dice coefficient and Unweighted Pair Group Method using Arithmetic average
164 (UPGMA) clustering algorithm (23). The similarity distance matrix generated via Bionumerics was
165 used to build Partial Least Squares Discriminant Analysis (PLS-DA) by using R package
166 "mixOmics" (www.r-project.org).
167 **Bioinformatics and Metagenome prediction.** Raw reads were first filtered according to the 454
168 processing pipeline. Sequences were then analyzed and further filtered by QIIME 1.9.0 software
169 (24) using split_library.py and denoiser.py scripts (25). 99 % OTUs were picked against the
170 Greengenes database 16S rRNA gene (26). Abundance of OTUs from two biological replicates of
171 each sampling time was averaged. Alpha and beta diversity were evaluated through QIIME (4).
172 Weighted UniFrac distance matrices (27) and OTU tables were used to perform Adonis, Anosim,
173 g_test, ANOVA and distance comparison statistical tests through compare_category.py,

make_distance_comparison_plots.py and group_significance.py scripts of QIIME, in order to verify the difference between the samples as function of batches (A-B) and packaging (C-T). The Shannon-Wiener diversity index *H'* was further analyzed using ANOVA, with time being the main factor. When ANOVA revealed significant differences ($P < 0.05$), Duncan HSD test was applied. Filtered OTUs table at 0.5 % abundance in at least two samples was used to make a heatmap by R package "heatmap3". Filtered OTUs table at 5 % abundance was used to produce nodes and edge tables obtained through make_otu_network.py scripts of QIIME. The tables were then imported in Gephi software (28) and an OTU network was built. PICRUSt (29) was used to predict abundances of gene families based on 16S rRNA sequences data. OTUs were re-determined by using pick_closed_reference_otus.py script of QIIME 1.9.0 with default parameters at 97 % similarity against the Greengenes database. KEGG orthologs were then collapsed at level 3 of hierarchy, and the table was import in "gage" Bioconductor package (30), to identify biological pathways over or under represented between T and C samples. KO genes table, filtered for KO gene presence ≥ 1 in at least 5 samples were then used to build a PCA as a function of the batch by using "made4" package of R. Spearman's correlations between OTUs occurring at 5 % in at least two samples and predicted metabolic pathways related to amino acid, lipid, energy and carbohydrate metabolism were only taken into account and used to produce a heatplot.

Nucleotide sequence accession number. All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (accession number SRP052241).

RESULTS

Microbiological analysis. The antimicrobial activity of the plastic films tested in agar plates proved that the antimicrobial solution was homogeneously distributed on the surface of the plastic film (data not shown). The results of microbial counts of beef burgers in T and C packaging for batch A and B are reported in Tables 1 and 2, respectively. For batch A, few differences between C

200 and T samples were observed. The total viable counts, as well as LAB counts, were not affected by
201 the use of the antimicrobial packaging and they increased in all the samples throughout the storage,
202 reaching a final load of about 6 Log CFU/g (Tab. 1). Few differences were observed in the count of
203 yeasts, while no differences were detected for *Staphylococcaceae, Enterobacteriaceae* and moulds.
204 For batch B, LAB increased from 4.4 to 6 Log CFU/g in C samples during storage, while in the
205 active packaging the load was kept to about 4.4 CFU/g during the whole storage period, with a
206 slight increase at day 3. The effect of the antimicrobial packaging was shown, with a significant
207 reduction ($P < 0.05$) of the total viable counts of about 1 Log at the end of the storage (Tab. 2). No
208 differences were observed for *Staphylococcaceae, Enterobacteriaceae,* mould and yeast counts. A
209 significant decrease of pH was observed for both batches ($P < 0.05$) (Tab. 1). By comparing batches
210 A and B, viable counts at time 0 in all the media appeared to be significantly higher in batch B ($P <$
211 0.05).
212 **DGGE analysis.** Partial Least Squares Discriminate Analysis (PLS-DA), as a function of nucleic
213 acids, showed a certain gradient of separation between DNA and RNA samples (Fig. 2A), while
214 those as a function of the batches (Fig. 2B) presented a clear separation. The distinction was
215 particularly important for samples of batch A, which appear to group together, and separated from
216 samples B. On the other hand it was possible to observe a certain degree of separation among C and
217 T samples of batch B (Fig. 2C).
218 **Pyrosequencing results.** A total of 371,314 raw reads were obtained after 454 processing. 290,245
219 reads passed the filters applied through QIIME, with an average value of 5,023 reads/sample and an
220 average length of 462 bp. The rarefaction analysis and the estimated sample coverage (Table S1)
221 indicated that there was satisfactory coverage for all the samples (ESC > 98%). The richness of the
222 samples varied from a minimum of 44 to a maximum of 194 OTUs. The results, based on storage
223 days, revealed that only from C samples of batch B at 3 and 5 days there was a significant reduction
224 on biological diversity compared to the other samples. The OTU network, presented in Figure 3,
225 showed that *Photobacterium phosphoreum*, *Lactococcus piscium, Lactobacillus sakei* and

Leuconostoc carnosum were the major OTUs shared between C and T in both batches. From the size of the edges, it was possible to see how the relative abundance of the above OTUs increased, as affected by the VP time compared to the samples at day 0. In particular, regarding the most abundant OTUs, *P. phosphoreum* increased from about 15 to 50% of the relative abundance in both batches (Fig. 4), while *Lb. sakei* increased from 10 to 30%. In general, *Leuc. carnosum* were found in all the samples never lower than 3%. *L. piscium* was most abundant at the beginning of the storage reaching 74% of the relative abundance around 5 days of storage (for C of batch A) and reaching 70% in C samples of batch B (day 1). At the end *L. piscium* was present (about 10%) in all the samples. Analyzing the microbial diversity, the development of genera and species in both batches could be observed in the heatmap depicted in Figure 5. It was possible to define a sub-cluster between treated samples at day 7 from batch A (T_7_A) with control samples at day 0 from both batch A and B (C_0_A and C_0_B). Three subclusters of samples were found grouping most of treated and control samples at days 1, 3 and 5. After 14 days the majority of samples clustered together and no differences were found between C and T samples in both batches. Further, the OTUs less represented at the beginning disappeared with time. These results were confirmed (Bonferroni corrected P-value < 0.001), through make_distance_comparison_plots.py script of QIIME (data not shown). Through principal-coordinate analysis (PCoA) with a weighted UniFrac distance matrix it was possible to show that samples from batch A grouped together and they were well separated from batch B on the basis of their microbiota (Fig. 6). The Adonis and Anosim statistical tests run through compare_categories.py script of QIIME confirmed this difference ($P <$ 0.001). Comparing T samples from batch A to B, no differences in terms of composition were found, whilst C samples from batch A to B differed significantly ($P < 0.001$). However, according to statistical tests, only from batch B it was possible to find a significant difference between C and T during the storage ($P < 0.001$). ANOVA and g_test run through group_significance.py script of QIIME showed that *Kocuria rhizophila*, *L. piscium*, *Staphylococcus xylosus*, *Leuc. carnosum* and

251 *Carnobacterium divergens* were significantly more abundant in C of batch B compared to C of
252 batch A.
253 Regarding the predicted metagenomes, NSTI index for the samples was 0.053 ± 0.010. This index
254 is the average branch length that separates each OTU from a reference bacterial genome, weighted
255 by the abundance of that OTU in the sample. Thus a NSTI score of 0.053 indicates a satisfactory
256 accuracy for all the samples (95%). The pathway enrichment analysis performed by "gage" of the
257 predicted metagenomes showed an enrichment of propanoate metabolism (ko00640), butanoate
258 metabolism (ko00650) biosynthesis of unsaturated fatty acids (ko01040) and sulfur metabolism
259 (ko00920) in C samples compared to T from batch B, only (data not shown). In contrast, from batch
260 A, only pathways involved in cellular processes, biosynthesis of secondary metabolites and
261 metabolism of amino acids were found to be more abundant in C compared to T samples.
262 Differences between the two batches were further demonstrated by PCA analysis comprising all the
263 predicted pathways (Fig. S1). The PCA clearly showed that samples from A were different from B
264 samples. Plotting the correlation between OTUs and predicted pathways (Fig. 7) of batch B it
265 appeared that *Leuc. carnosum* and *Lb. sakei* were positively correlated with the metabolism of
266 volatile fatty acids such as propanoate and butanoate. *L. piscium* was mainly correlated with the
267 biosynthesis of unsaturated fatty acids while *Leuc. carnosum* was found linked to sulfur metabolism
268 (Fig. 7). On the other hand, despite the strong Spearman's correlation, the relationship between
269 OTUs and predicted pathways was not statistically significant ($P > 0.05$).
270
271 **DISCUSSION**
272 The present study aimed at providing a more integrated view on the live viable microbiota
273 development during storage of beef burgers in nisin-based antimicrobial vacuum packaging. For
274 this purpose, an extensive sampling procedure of two different batches with six biological samples
275 replicated each time was used. This may be helpful to limit the inter-sample variability. The
276 concentration of the antimicrobial agents was chosen because of its effectiveness to retard the

277 growth of spoilage bacteria in beef stored in vacuum condition (11, 17). Differences in microbial
278 composition during storage were investigated by using classical plate counts, RNA-based DGGE
279 and rRNA-based pyrosequencing.
280 Comparing C samples from the two batches, it was observed that the initial counts of the main
281 microbial groups were higher in batch B, compared to A. The microbial load of meat depends on
282 several factors such as the initial physiological status of the animal, the contamination at
283 slaughterhouses and in the equipment used for the meat manipulation, as well as temperature and
284 storage conditions (3). An effect of the antimicrobial packaging on the reduction of the total viable
285 counts as well as LAB counts was observed only in batch B. However, for both batches the
286 microbiological counts at the end of storage (21 days) showed how the beef burgers stored in active
287 packaging were acceptable in terms of final counts of the main microbial group monitored, as
288 previously reported (31), because the total viable count was lower than 7 Log CFU/g.
289 The OTU network clearly showed that the core of OTUs was dominated by the presence of *P.*
290 *phosphoreum, L. piscium, Lb. sakei*, *C. divergens* and *Leuc. carnosum*. *P. phosphoreum* was
291 previously reported as dominant of spoiled cod under MAP conditions (32) and recently found as
292 core OTUs of seafood community (1). *L. piscium* and *Lb. sakei* have been recently found in a
293 variety of meat products under MAP conditions (33). Their effect on the food matrix appeared to be
294 related to the production of off-flavors (34). In accordance to these evidences, a positive correlation
295 between *L. piscium* and the metabolic pathways of histidine metabolism and fatty acid biosynthesis
296 was found, together with a presumptive abundance of genes related to amino acid metabolism for
297 *Lb. sakei*. On the other hand, the most abundant OTU, *P. phosphoreum,* appeared to be positively
298 correlated with volatile fatty-acid metabolisms, biosynthesis of unsaturated fatty acid and nitrogen
299 metabolism. As previously demonstrated (32), this species produces ammonia-like off-odours, but it
300 needs to reach a concentration of 10^7 CFU/g in order to have an organoleptic impact on the food
301 product. Unfortunately in our study a specific medium for the detection of *P. phosphoreum* was not
302 included. The presence of *Enterobacteriaceae* under VP conditions is reported to be particularly

303 important, both for its high deteriorating potential and for food safety (34). In this study, based on
304 the viable microbiota, the relative abundance of the members of *Enterobacteriaceae* was very low,
305 indicating that only few taxa could play a role during the spoilage of meat under VP condition used
306 here.
307 By PLS-DA analysis based on DNA and RNA DGGE profiles, a certain degree of separation of the
308 samples based on the nucleic acid analyzed was observed. To evaluate the metabolically active
309 populations only the RNA data were taken into account. Beta diversity calculation, PLS-DA and
310 rRNA-based pyrosequencing results, confirmed the impact of the antimicrobial packaging only for
311 batch B. By using rRNA-based pyrosequencing it was possible to find a significant change in the
312 relative abundance of the most abundant OTUs in response to the treatment only for batch B.
313 However, this was not related to the initial load, but only to the species diversity. Interestingly, C
314 samples from batch B showed a significantly higher abundance of some taxa such as *Kocuria*
315 *rhizophila*, *Staphylococcus xylosus*, *Leuc. carnosum* and *C. divergens* compared to C samples from
316 batch A. These OTUs are those sensitive to the nisin treatment (11, 35–37), explaining the
317 differences between the two batches.
318 The evidences presented in this study showed that the nisin-based antimicrobial packaging
319 was effective only as a function of the microbiota. The treatment impact was observed when
320 microbiota sensitive to nisin were present in the samples at the beginning, and independently of the
321 initial load in the food matrix. In conclusion, our study based on viable microbiota showed that only
322 a few taxa can really play a role during the storage of beef burgers. Further, the use of nisin-based
323 antimicrobial packaging can determine a reduction of the abundance of specific metabolic pathways
324 related to the spoilage, with a potential impact on the prolongation of the shelf life. Further studies
325 are needed for verifying this possible prolongation by evaluating the sensory properties of the
326 samples and by metabolomics and metatranscriptomic studies.
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328

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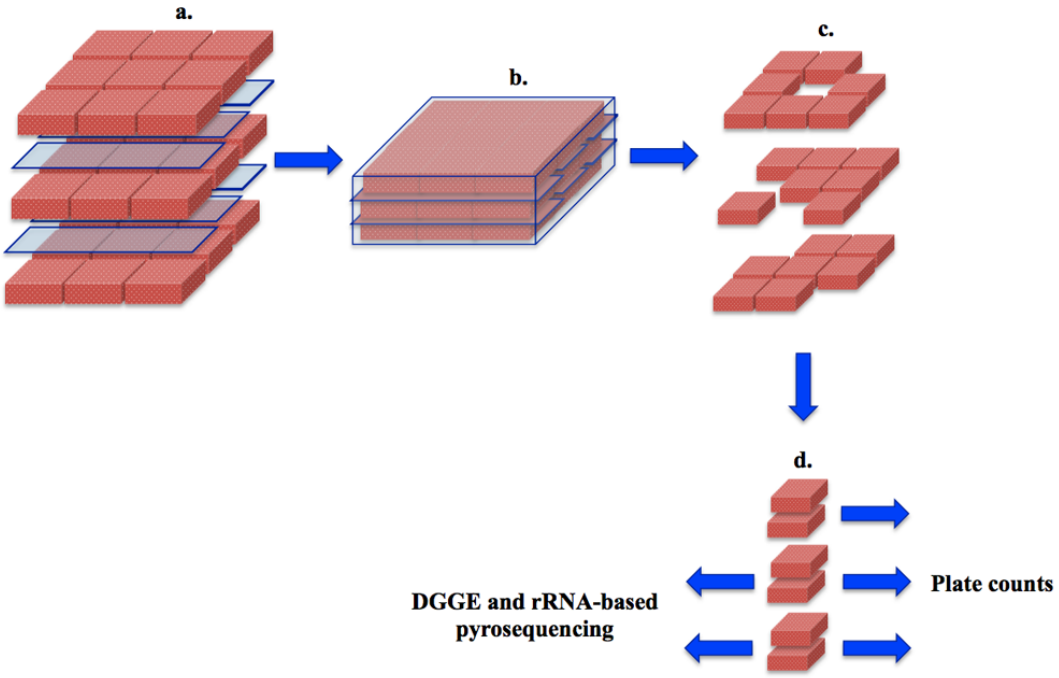
Legends to figures

FIG 1 Layout of the experimental plan. A total of 324 burgers were prepared in twelve bags, six bags for each batch. (a.) Twenty-seven beef burgers were disposed on three layers one on the top of each other, nine burgers on each layer in a three by three square. Three activated strips were disposed on top of the first and second layers of burgers. (b.) Beef-burgers were then vacuum packed in the activated bag in order to let both faces of each burger be in contact with a strip or with the inner face of the activated bag. (c.) At each sampling point the bags were opened and (d.) six burgers were collected by taking and pooled together two burgers samples from each layer at random positions. For microbiological analysis three pools were than used, while for microbial population assessment two pools were used to perform DGGE and rRNA-based pyrosequencing.

489 An equal series of 324 burgers in twelve bags were packed as described above using non-activated
490 bags and strips, and used as a control.
491 **FIG 2** PLS-DA models built on the similarity distance matrix on RNA-DGGE similarity matrix.
492 Plot A is color coded as a function of the nucleic acids: DNA (cyan) and RNA (yellow); Plot B is as
493 a function of the batch: A (red) and B (green), while Plot C is as a function of the packaging
494 condition in batch B: active packaging (blue) non-active packaging (red).
495 **FIG 3** OTU network summarizing the relationships between taxa and samples. Only OTUs
496 occurring at 5% in at least 2 samples are shown. Abundance of OTUs in the 2 biological replicates
497 for each sampling time was averaged. Sizes of the OTUs are made proportional to weighted degree
498 (i.e. for OTUs this measures the total occurrence of an OTU in the whole dataset) using a power
499 spline. OTUs and samples are connected with a line ("edge") to sample node and its thickness is
500 made proportional to the abundance of an OTU in the connected sample. Samples are color coded
501 as a function of the batch: A (red) and B (green).
502 **FIG 4** Incidence of the major taxonomic groups detected by pyrosequencing. Only OTUs with an
503 incidence above 5% in at least two samples are shown. Abundance of OTUs in the 2 biological
504 replicates for each sampling time was averaged. Samples are labelled according to time (0, 1, 3, 5,
505 7, 14 and 21 days), batch (A and B) and treatment: active (T) and non-active (C) vacuum
506 packaging.
507 **FIG 5** Distribution of OTUs in samples stored in active (T) and non-active (C) vacuum packaging.
508 The dendrogram of samples (top) was divided into two parts based on the correlation between
509 samples. The categorical annotations (top) were separated into columns, and the samples were
510 labelled by black squares as a function of batches or packaging conditions. The numeric annotation
511 (day of storage) was demonstrated by a scatter plot, and the values were labelled at the right axis.
512 Abundance of OTUs in the 2 biological replicates for each sampling time was averaged.
513 **FIG 6** Principal Co-ordinate Analysis (PCoA) based on weighted Unifrac distance matrix. Samples
514 are color-coded according of the batch: A (red) and B (green).

515 **FIG 7** Heatplot showing Spearman's correlations between OTUs occurring at 5% in at least 2
516 samples and predicted metabolic pathways, filtered for KO gene samples presence ≥ 1 in at least 5
517 samples, related to amino acid (red squares), lipid (green squares), energy (brown squares) and
518 carbohydrate metabolism (blue squares). Rows and columns are clustered by Ward linkage
519 hierarchical clustering. The intensity of the colors represents the degree of correlation between the
520 OTUs and KO as measured by the Spearman's correlations.
521

522 **FIG 1**



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525 **FIG 2**

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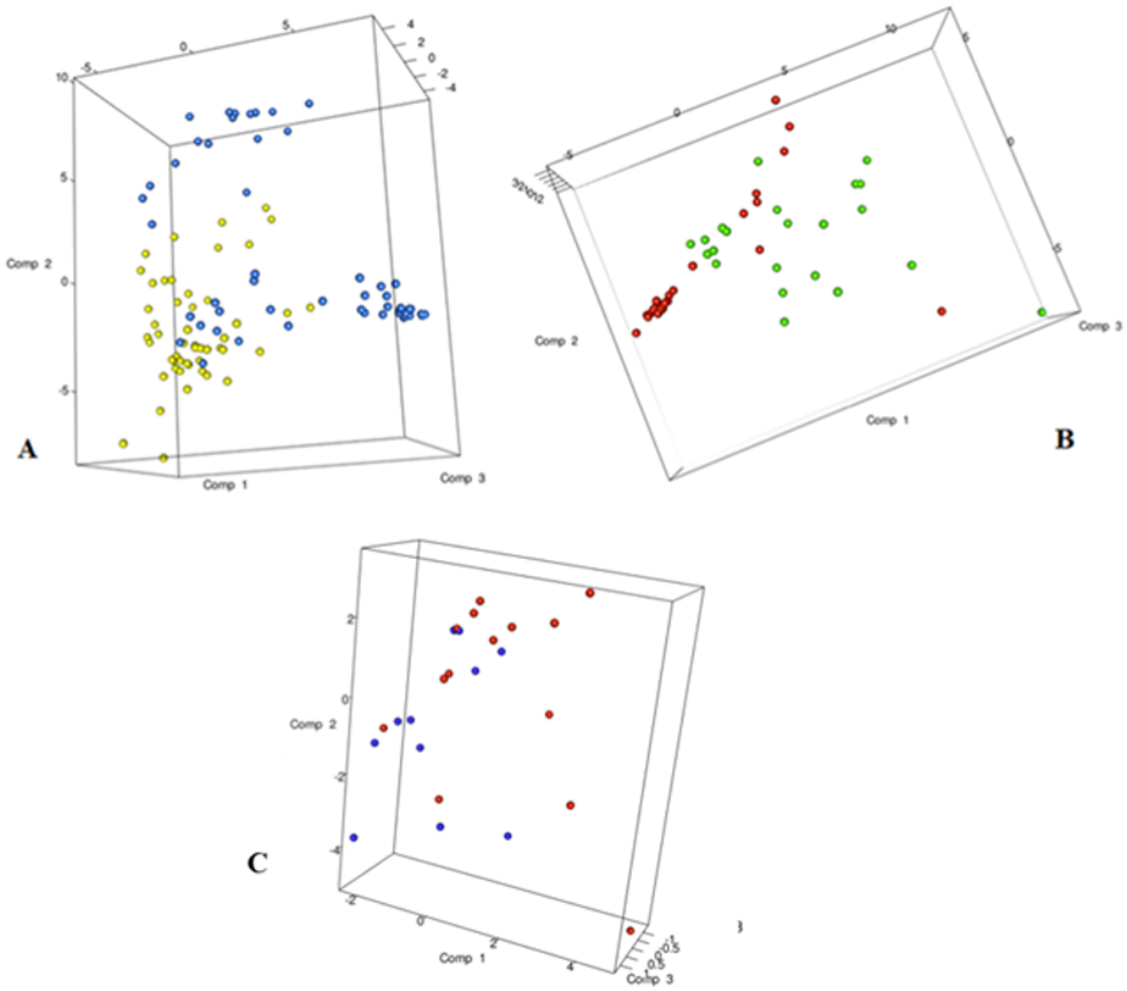

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FIG 3

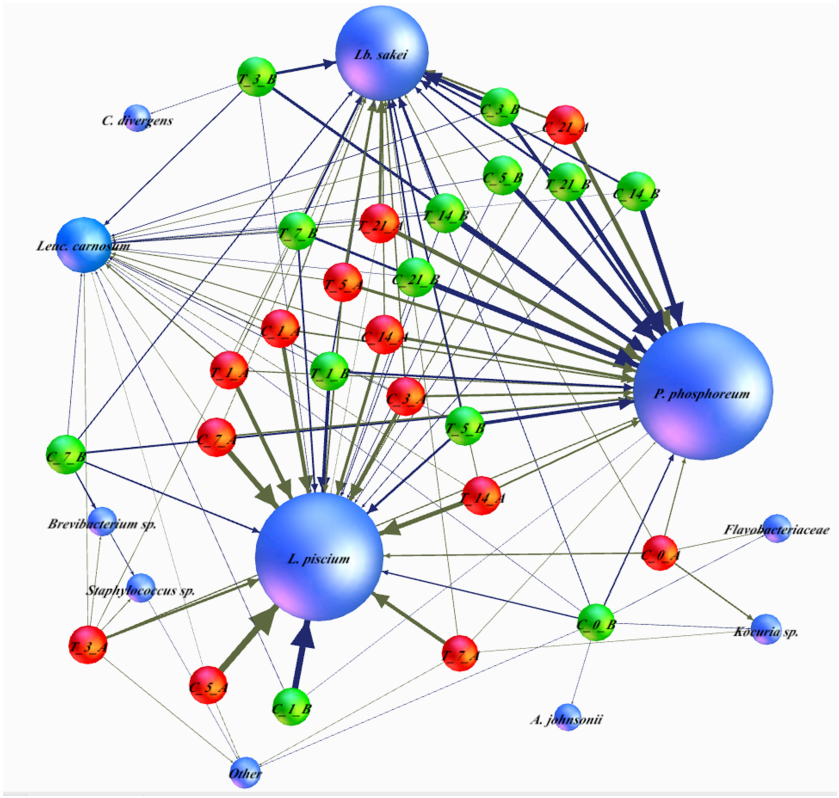
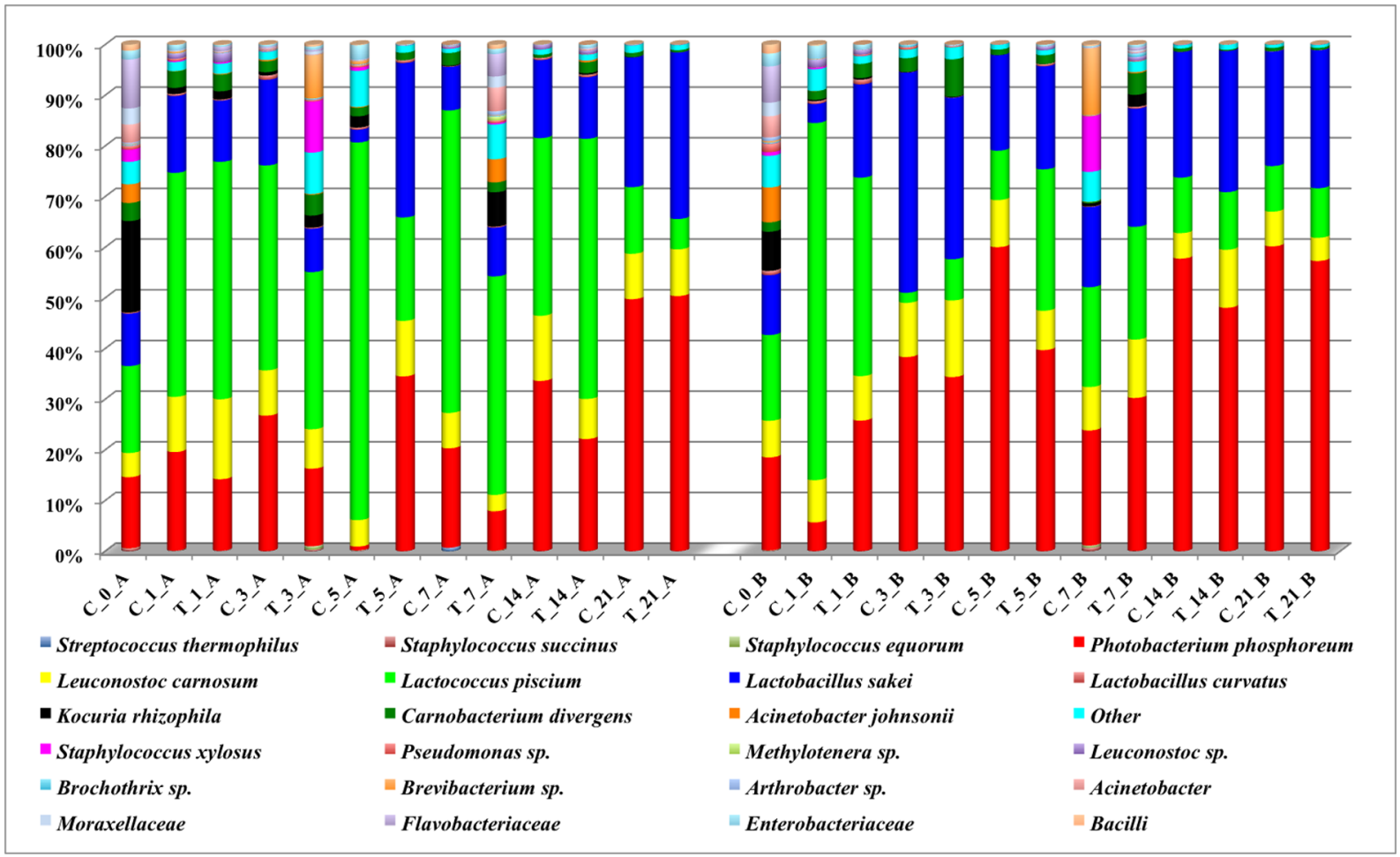

Lb. sakei
C. divergens
Leuc. carnosum
P. phosphoreum
L. piscium
Brevibacterium sp.
Staphylococcus sp.
Flavobacteriaceae
Kocuria sp.
A. johnsonii
Other

FIG 4


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C_0_A
C_1_A
T_1_A
C_3_A
T_3_A
C_5_A
T_5_A
C_7_A
T_7_A
C_14_A
T_14_A
C_21_A
T_21_A
C_0_B
C_1_B
T_1_B
C_3_B
T_3_B
C_5_B
T_5_B
C_7_B
T_7_B
C_14_B
T_14_B
C_21_B
T_21_B
Streptococcus thermophilus
Staphylococcus succinus
Staphylococcus equorum
Photobacterium phosphoreum
Leuconostoc carnosum
Lactococcus piscium
Lactobacillus sakei
Lactobacillus curvatus
Kocuria rhizophila
Carnobacterium divergens
Acinetobacter johnsonii
Other
Staphylococcus xylosus
Pseudomonas sp.
Methylotenera sp.
Leuconostoc sp.
Brochothrix sp.
Brevibacterium sp.
Arthrobacter sp.
Acinetobacter
Moraxellaceae
Flavobacteriaceae
Enterobacteriaceae
Bacilli

539 **FIG 5**

540

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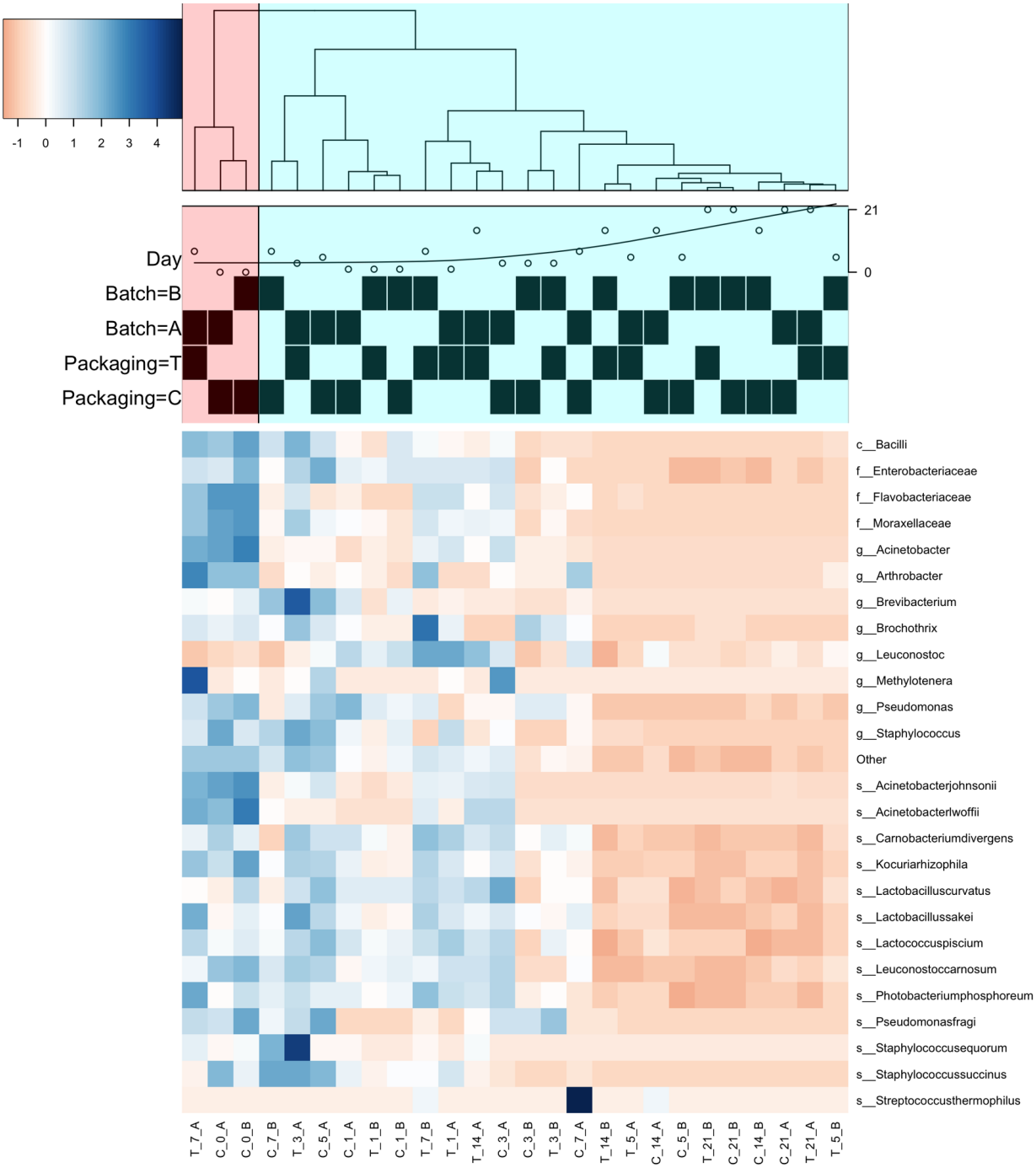

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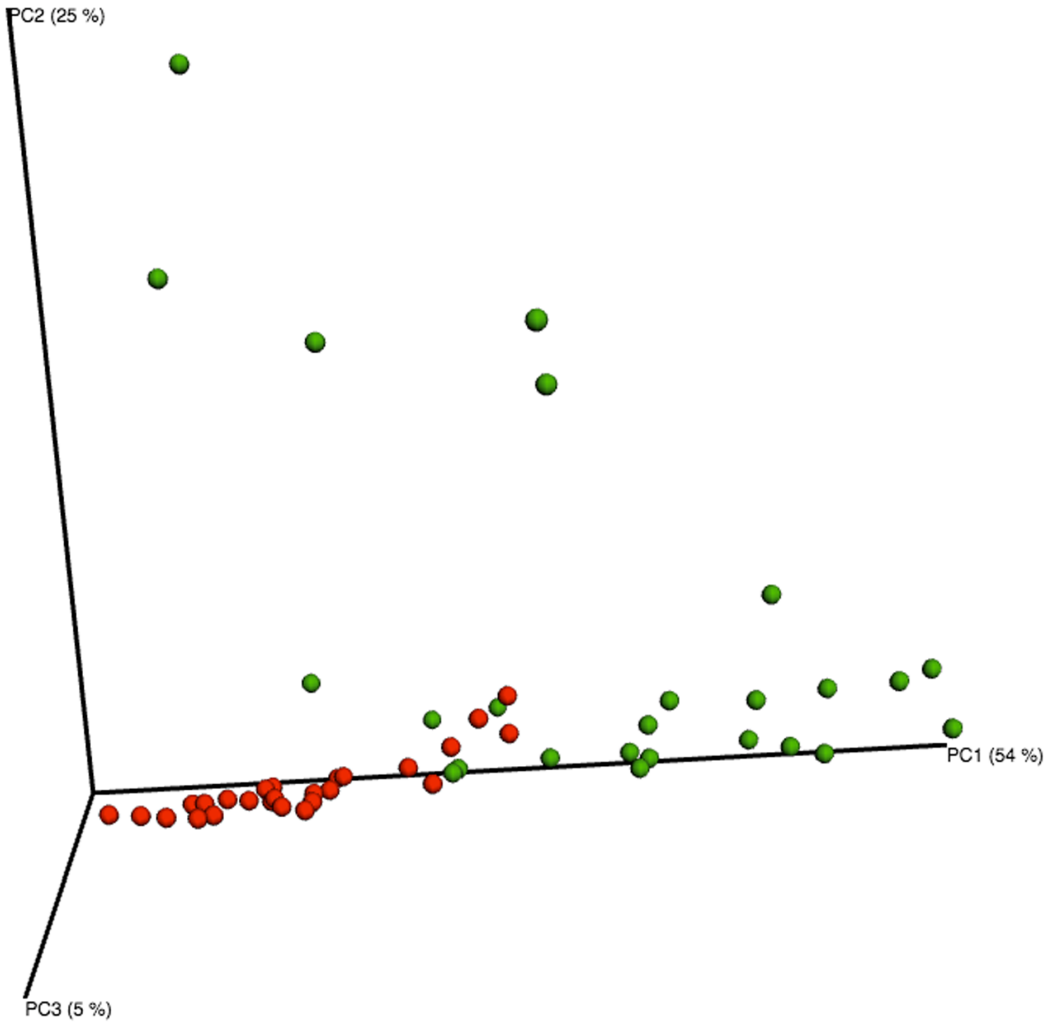
FIG 6



550 **FIG 7**

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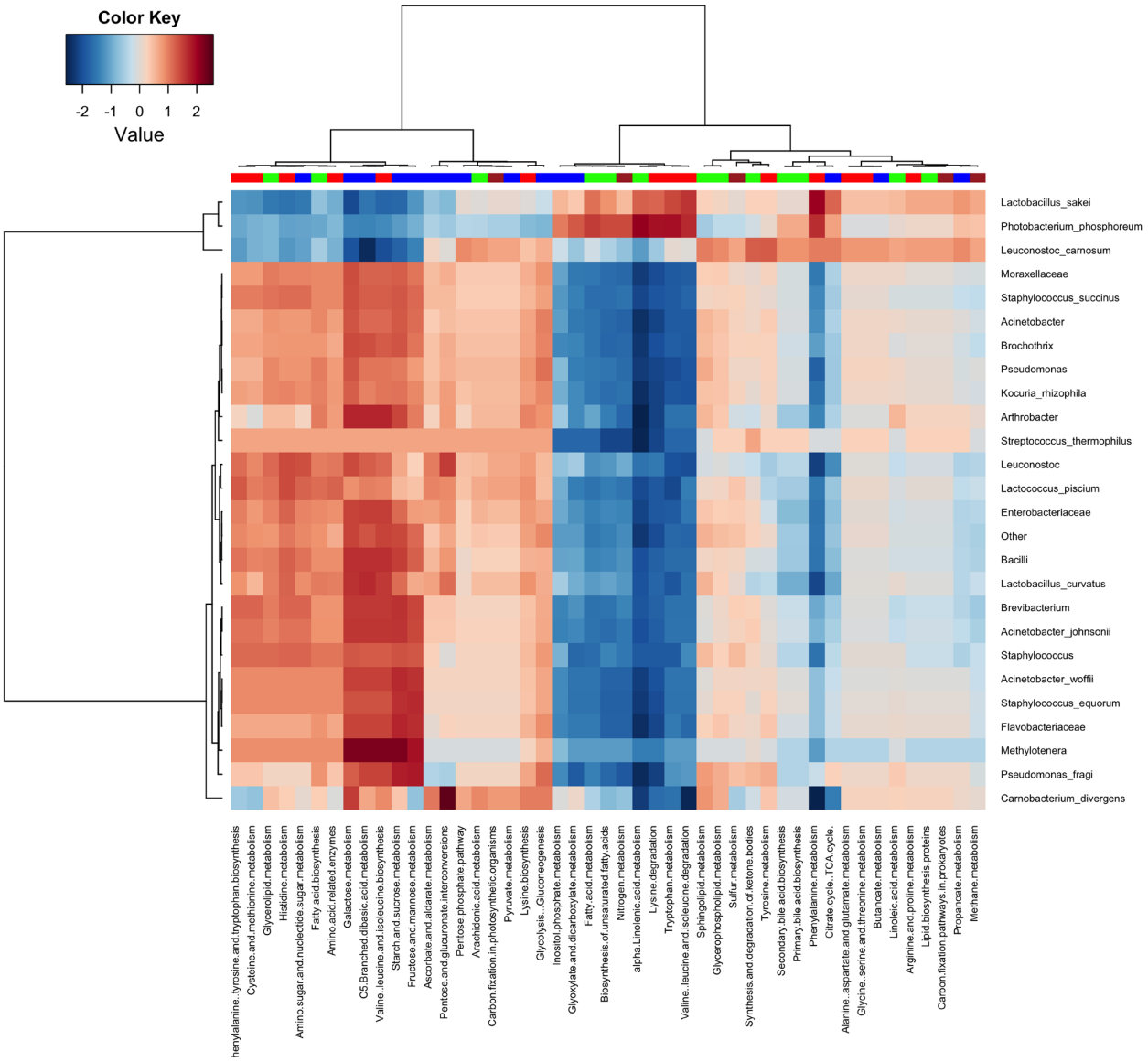

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TABLE 1 Viable counts of different meat spoilage microbial groups in hamburgers vacuum-packed in non active (C) and active (T) packaging from batch A during storage at 4°C for 21 days

Packaging	Storage time (days)		†Log CFU g^{-1} ± SD					
			LAB	*Staphylococcaceae*	*Enterobacteriaceae*	Moulds	Yeast	Total counts
		pH	(MRS agar)	(MSA)	(VRBA)	(AMT)	(AMT)	(GPA)
Non-active (C)	0	6.03 ± 0.02^{Da}	4.09 ± 0.05^{A}	3.57 ± 0.06^{A}	2.59 ± 0.10^{A}	1.59 ± 0.15^{A}	2.55 ± 0.22^{A}	5.02 ± 0.34^{A}
	1	6.03 ± 0.04^{Da}	$4.61 \pm 0,13^{Ba}$	3.85 ± 0.06^{Ba}	2.85 ± 0.02^{DEa}	1.70 ± 0.15^{ABa}	3.07 ± 0.15^{Ba}	5.04 ± 0.02^{Aa}
	3	5.68 ± 0.01^{Ca}	4.89 ± 0.11^{Ca}	4.02 ± 0.08^{BCa}	3.16 ± 0.15^{Ca}	1.85 ± 0.00^{ABCa}	3.12 ± 0.07^{Ba}	5.49 ± 0.04^{Ba}
	5	5.65 ± 0.01^{BCa}	5.19 ± 0.08^{Da}	4.07 ± 0.11^{BCa}	3.29 ± 0.22^{CDa}	1.85 ± 0.59^{ABCa}	3.39 ± 0.06^{Ca}	5.74 ± 0.07^{BCa}
	7	5.63 ± 0.01^{Ba}	4.57 ± 0.36^{Ba}	4.20 ± 0.21^{Ca}	3.36 ± 0.18^{CDEa}	2.09 ± 0.3^{BCa}	3.52 ± 0.12^{Ca}	5.93 ± 0.18^{BCDa}
	14	5.33 ± 0.01^{Aa}	4.68 ± 0.28^{BCa}	4.49 ± 0.45^{Da}	3.52 ± 0.11^{DEa}	2.18 ± 0.20^{Ca}	3.54 ± 0.20^{Ca}	6.18 ± 0.53^{CDAa}
	21	5.31 ± 0.00^{Aa}	6.00 ± 0.76^{Ea}	5.76 ± 0.53^{Ea}	3.60 ± 0.42^{Ea}	3.33 ± 0.09^{Da}	3.60 ± 0.20^{Ca}	6.28 ± 0.90^{Da}
Active (T)	0	6.03 ± 0.02^{Da}	4.09 ± 0.05^{A}	3.57 ± 0.06^{A}	2.59 ± 0.10^{A}	1.59 ± 0.15^{A}	2.55 ± 0.22^{A}	5.02 ± 0.34^{A}
	1	6.03 ± 0.01^{Da}	4.50 ± 0.13^{Ba}	3.72 ± 0.06^{ABa}	3.16 ± 0.27^{Ba}	1.70 ± 0.00^{Aa}	2.25 ± 0.05^{ABb}	4.61 ± 0.05^{Bb}
	3	5.58 ± 0.01^{Bb}	4.75 ± 0.24^{Ca}	4.00 ± 0.02^{BCa}	3.27 ± 0.06^{BCa}	1.60 ± 0.00^{Aa}	2.59 ± 0.05^{ABCb}	4.77 ± 0.20^{Bb}
	5	5.59 ± 0.01^{BCb}	4.86 ± 0.07^{Cb}	4.09 ± 0.16^{Ca}	3.29 ± 0.09^{BCa}	2.20 ± 0.20^{Bb}	2.85 ± 0.27^{CDb}	5.48 ± 0.13^{Cb}
	7	5.61 ± 0.01^{BCb}	4.94 ± 0.15^{Ca}	4.12 ± 0.15^{Da}	3.31 ± 0.15^{BCa}	2.85 ± 0.15^{Cb}	2.97 ± 0.15^{DEb}	5.95 ± 0.07^{Db}
	14	5.61 ± 0.01^{Cb}	5.47 ± 0.07^{Db}	4.55 ± 0.02^{ABa}	3.33 ± 0.07^{BCa}	1.70 ± 0.00^{Aa}	3.52 ± 0.59^{EFa}	5.97 ± 0.30^{Da}
	21	5.53 ± 0.03^{Ab}	6.58 ± 0.15^{Ea}	5.76 ± 0.43^{BCa}	3.47 ± 0.11^{Ca}	3.33 ± 0.00^{Da}	4.05 ± 0.65^{Fb}	6.18 ± 0.19^{Da}

†Comparing control and activated packaging data, values with different superscripts in the same column (lowercase letters) and corresponding to the same time of storage, differ significantl($P<0.05$).

Different letters in the same row (uppercase letters) indicate significant differences for each media among times ($P< 0.05$)

TABLE 2 Viable counts of different meat spoilage microbial groups in hamburgers vacuum-packed in non active (C) and active (T) packaging from batch B during storage at 4°C for 21 days

Packaging	Storage time (days)		†Log CFU g^{-1} ± SD					
			LAB	*Staphylococcaceae*	*Enterobacteriaceae*	Moulds	Yeast	Total counts
		pH	(MRS agar)	(MSA)	(VRBA)	(AMT)	(AMT)	(GPA)
Non-active (C)	0	5.67 ± 0.04^{Ea}	4.46 ± 0.19^{A}	4.27 ± 0.17^{C}	2.79 ± 0.09^{C}	2.59 ± 0.29^{C}	2.56 ± 0.56^{A}	5.44 ± 0.26^{A}
	1	5.67 ± 0.01^{Ea}	5.07 ± 0.09^{Ba}	3.89 ± 0.11^{Ba}	3.47 ± 0.07^{Da}	1.70 ± 0.00^{Aa}	2.85 ± 0.15^{Aa}	6.11 ± 0.09^{Ba}
	3	5.64 ± 0.00^{Da}	5.57 ± 0.09^{Ca}	3.85 ± 0.15^{Ba}	2.72 ± 0.06^{Ca}	1.70 ± 0.00^{Aa}	4.20 ± 0.05^{BCa}	5.27 ± 0.03^{Aa}
	5	5.50 ± 0.00^{Ca}	5.44 ± 0.04^{Ca}	3.36 ± 0.18^{Aa}	2.54 ± 0.06^{Ba}	1.70 ± 0.00^{Aa}	3.86 ± 0.32^{Ba}	5.28 ± 0.02^{Aa}
	7	5.37 ± 0.01^{Ba}	5.89 ± 0.02^{Da}	3.41 ± 0.11^{Aa}	2.87 ± 0.17^{Ca}	1.85 ± 0.15^{ABa}	4.31 ± 0.08^{BCa}	6.13 ± 0.35^{Ba}
	14	5.38 ± 0.01^{Ba}	6.02 ± 0.02^{Da}	3.89 ± 0.19^{Ba}	3.33 ± 0.03^{Da}	2.20 ± 0.50^{BCa}	4.40 ± 0.14^{Ca}	6.39 ± 0.29^{Ba}
	21	5.34 ± 0.00^{Aa}	5.98 ± 0.00^{Da}	3.98 ± 0.20^{Ba}	2.27 ± 0.13^{Aa}	1.70 ± 0.00^{Aa}	4.16 ± 0.23^{BCa}	7.19 ± 0.01^{Ca}
Active (T)	0	5.67 ± 0.04^{B}	4.46 ± 0.19^{A}	4.27 ± 0.17^{B}	2.79 ± 0.09^{CD}	2.59 ± 0.29^{B}	2.56 ± 0.56^{A}	5.44 ± 0.26^{B}
	1	5.68 ± 0.00^{Ba}	$4.64 \pm 0.04A^{Bb}$	3.35 ± 0.05^{Ab}	3.33 ± 0.07^{Ea}	2.70 ± 0.00^{Ba}	2.94 ± 0.06^{ABa}	4.96 ± 0.11^{ABb}
	3	5.63 ± 0.01^{Ba}	5.45 ± 0.07^{Ca}	3.21 ± 0.03^{Ab}	2.62 ± 0.01^{ABCa}	1.70 ± 0.00^{Aa}	3.90 ± 0.00^{BCb}	5.44 ± 0.14^{Ba}
	5	4.92 ± 0.49^{Aa}	4.60 ± 0.05^{ABb}	3.42 ± 0.02^{Aa}	2.41 ± 0.11^{Aa}	1.70 ± 0.00^{Aa}	4.09 ± 0.09^{Ca}	5.35 ± 0.05^{ABa}
	7	5.35 ± 0.00^{Bb}	4.81 ± 0.12^{ABb}	3.29 ± 0.11^{Aa}	3.01 ± 0.17^{Da}	1.70 ± 0.00^{Aa}	4.30 ± 0.10^{Ca}	5.35 ± 0.05^{ABb}
	14	5.34 ± 0.00^{Bb}	5.08 ± 0.54^{BCb}	4.07 ± 0.20^{Ba}	$2.71 \pm 0.07B^{Cb}$	2.10 ± 0.45^{Aa}	3.77 ± 1.05^{BCa}	5.00 ± 0.39^{ABb}
	21	5.33 ± 0.00^{Ba}	4.74 ± 0.49^{ABb}	4.12 ± 0.14^{Ba}	2.52 ± 0.25^{ABa}	2.00 ± 0.51^{Aa}	3.52 ± 0.89^{ABCa}	4.88 ± 0.49^{Ab}

†Comparing control and activated packaging data, values with different superscripts in the same column (lowercase letters) and corresponding to the same time of storage, differ significantly (*P*<0.05).

Different letters in the same row (uppercase letters) indicate significant differences for each media among times (P< 0.05)

Supplementary information:

FIG S1 Principal component analysis (PCA) of the abundance of KO genes. Samples are color-coded according to batch: A (red) and B (green).

TABLE S1 Number of sequences analyzed, observed diversity and estimated sample coverage (Good's coverage) for 16S RNA (cDNA) amplicons analyzed. Samples are labelled according to treatment: active (T) and non-active (C) vacuum packaging; time (0, 1, 3, 5, 7, 14 and 21 days), and batch (A and B).