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

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Beef burgers were stored at 4°C in a vacuum in nisin-activated antimicrobial packaging. Microbial ecology analyses were performed on samples collected between days 0 and 21 of storage to discover the population diversity. Two batches were analyzed using RNA-based denaturing gradient gel electrophoresis (DGGE) and pyrosequencing. The active packaging retarded the growth of the total viable bacteria and 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 operational taxonomic units (OTUs) shared between control and treated samples. Beta diversity analysis of the 16S rRNA sequence 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 nisin, such as *Kocuria rhizophila*, *Staphylococcus xylosus*, *Leuconostoc carnosum*, and *Carnobacterium divergens*, compared to control samples from batch A. However, only from batch B was it possible to find a significant difference between controls and treated samples during 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 spoilage. The present study aimed to assess the viable bacterial communities in beef burgers stored in nisin-based antimicrobial packaging, and it highlights the efficacy of this strategy to prolong beef burger shelf life.

Spoilage of meat or meat products is caused by 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 recent 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, refrigerated storage conditions, addition of natural preservatives, vacuum pack (VP), and modified atmosphere packaging (MAP) could influence the development and activities of the ephemeral spoilage microorganisms 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 suggested the use of treatments with propidium monoazide (PMA) or ethidium monoa-

zide (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 a more informative target than DNA, as rRNA can be proportionally more abundant in microbial cells than DNA copies, and this could lead to a more detailed picture of the matrix analyzed (15). Many studies showed that species from *Lactobacillales*, *Bacillales*, *Enterobacteriales*, *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 to follow the changes in bacterial counts and diversity during storage at 4°C.

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FIG 1 Layout of the experimental plan. A total of 324 burgers were prepared in 12 bags, with 6 bags for each batch. (a.) A total of 27 beef burgers were placed in 3 layers, on top of each other, with 9 burgers in each layer in a 3-by-3 square. Three activated strips were placed 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. At each sampling point, the bags were opened (c.) and 6 burgers were collected (d.) by taking and pooling samples from two burgers from each layer at random positions. For microbiological analysis, 3 pools were then used, while 2 pools were used for the microbial population assessment, to perform DGGE and rRNA-based pyrosequencing. Another series of 324 burgers in 12 bags were packed as described above, using nonactivated bags and strips, and used as a control.

MATERIALS AND METHODS

Preparation of antimicrobial bags and films. A nisin-based antimicrobial solution at 2.5% (nisin; Sigma, Milan, Italy) was prepared as described by Ercolini et al. (17). Four milliliters of antimicrobial solution was used to manually coat the inner layer of 12 bags of linear low-density polyethylene (LLDPE; oxygen transmission, $0.83 \text{ cm}^3 \cdot \text{m}^{-2} \cdot \text{h}^{-1}$ at 23°C , $30 \text{ cm} \times 30 \text{ cm}$). A coating rod able to form a $45\text{-}\mu\text{m}$ -thick coating was used. One milliliter of antimicrobial solution was used to manually coat both sides of 72 LLDPE strips ($30 \text{ cm} \times 10 \text{ cm}$). 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 placed onto the surface of brain heart infusion (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, the antimicrobial activity was evaluated by observing a clear zone of growth inhibition in correspondence of the active film.

Beef burger samples and microbial analysis. Beef burger samples (100 g each in a square shape) were manufactured in a local meat factory in the area of Turin, Italy. The formulation used in the manufacture included minced beef, salt, and pepper. Two independent batches (batches A and B) were analyzed. Both batches were prepared with meat from the same supplier in two different periods of time. Figure 1 presents a schematic representation of the experimental plan. Twenty-seven beef burgers were placed inside the activated bag in three layers, on top of each other, with nine burgers in each layer in a three-by-three square. Three activated strips were placed on top of the first and second layers of burgers in order to let both faces of each burger be in contact with a strip or with the inner face of the activated bag. No space was left between each burger in every layer to avoid oxygen accumulation during successive packaging. A total of 324 burgers were prepared in 12 bags, with 6 bags for each batch. The samples were then vacuum packed before thermal sealing and stored at 4°C . After 1, 3, 5, 7, 14, and 21 days of storage, one bag from each batch was opened, and six samples from each bag were taken for microbio-

logical analysis and microbial population assessment. The burgers were collected by taking two samples from each layer at random positions; the two samples were then pooled, and 10 g of each of the three pools were homogenized with 90 ml of Ringer's solution (Oxoid, Milan, Italy) for 2 min in a stomacher (LAB blender 400; PBI, Italy; stomacher bags, Sto-circul-bag; PBI, Italy) at room temperature. Before packing, at time zero, three burgers for each batch were also analyzed. Another series of 324 burgers in 12 bags were packed as described above using nonactivated bags and strips and were used as a control. From each control (C) and treated (T) sample, 1 ml of the first 10-fold serial dilution was used to determine: (i) total aerobic bacteria, (ii) lactic acid bacteria (LAB), (iii) *Staphylococcaceae*, (iv) *Enterobacteriaceae*, (v) yeasts, and (vi) molds by using selective media and conditions previously described (19). Results were expressed as means of log CFU for three independent determinations on each batch. The pH of each sample was measured by using a digital pH meter (waterproof pH tester; Thermo Scientific, Nijkerk, the Netherlands).

Total DNA and RNA extraction from beef burger samples. At each sampling point, 1 ml of the first 10-fold serial dilution was collected and directly centrifuged at maximum speed for 30 s. Nucleic acid was extracted from two of three biological replicates from each batch. Total DNA was extracted as described by Alessandria et al. (20). DNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Milan, Italy) and standardized at $100 \text{ ng}/\mu\text{l}$. For RNA extraction, $200 \mu\text{l}$ of RNeasy lysis buffer (Qiagen, Milan, Italy) was immediately added to the pellet and stored at -80°C . Total RNA from the samples was extracted using the MasterPure complete DNA and RNA purification kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions. Three microliters of Turbo DNase (Ambion) was added to digest DNA in the RNA samples, with an incubation of 3 h at 37°C . RNA was quantified using the NanoDrop and standardized at $300 \text{ ng}/\mu\text{l}$. Reverse transcription (RT) reactions were performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Milan, Italy). Then, 300 ng of RNA was mixed with $1 \mu\text{l}$ of universal primer 518R (10 mM) and DNase- and RNase-free sterile water (Sigma) to a final volume of $10 \mu\text{l}$ and then incubated at 75°C for 10 min. The mix was placed on ice, and a mixture containing 50 mM Tris-HCl (pH 8.3),

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

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

^a Comparing control and activated packaging data, values with different lowercase letters in the same column and corresponding to the same time of storage differ significantly ($P < 0.05$). Different uppercase letters in the same row indicate significant differences for each medium among times ($P < 0.05$). MRS, de Man, Rogosa, and Sharpe agar; MSA, mannitol salt agar; VRBA, violet red bile agar; MEA, malt extract agar plus tetracycline (0.05 g/liter); GPA, gelatin peptone agar.

75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 2 mM each deoxynucleoside triphosphate, 1 μ l of 200 U $\cdot$ μ l⁻¹ M-MLV, and 0.96 U of RNasin RNase inhibitor (Promega) was transferred to the reaction tube. Reverse transcription was carried out at 42°C for 1 h.

Denaturing gradient gel electrophoresis analysis. A total of 1 μ l of DNA, or cDNA, from two biological replicates of each batch was used as a template in the PCR. The V3 region of the 16S rRNA gene was amplified with the primers 338f-GC/518r, as previously described (21). PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) at 30% to 60% by using a Bio-Rad Dcode, as suggested elsewhere (19).

RNA analysis by pyrosequencing. cDNA was used to study the microbial diversity of the viable populations by pyrosequencing of the amplified V1 to V3 region of the 16S rRNA gene by using primers and a PCR condition previously reported (22). PCR products were purified by the Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and quantified

using the QuantiFluor (Promega, Milan, Italy), and an equimolar pool of the PCR templates was obtained prior to further processing. The amplicon pool was processed by using Titanium chemistry on a GS Junior platform (454 Life Sciences, Roche, Monza, Italy) according to the manufacturer's instructions.

Statistical analysis. Data from microbiological counts were analyzed by one-way analysis of variance (ANOVA) for each individual packaging condition, with time or batch as the main factor, using SPSS 22.0 statistical software package (SPSS, Inc., Cary, NC, USA). When ANOVA revealed significant differences ($P < 0.05$), the Duncan honestly significant difference (HSD) test was applied. A t test was used to assess the differences in microbial loads between C and T samples at the same time of sampling and between C samples of the two batches. A database of fingerprints was created by using the software Bionumerics version 4.6 (Applied Maths, Sint Marten Latem, Belgium). A combined data matrix, including DNA

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

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

^a Comparing control and activated packaging data, values with different lowercase letters in the same column and corresponding to the same time of storage differ significantly ($P < 0.05$). Different uppercase letters in the same row indicate significant differences for each medium among times ($P < 0.05$). MRS, de Man, Rogosa, and Sharpe agar; MSA, mannitol salt agar; VRBA, violet red bile agar; MEA, malt extract agar plus tetracycline (0.05 g/liter); GPA, gelatin peptone agar.

FIG 2 PLS-DA models built on the similarity distance matrix on RNA-DGGE similarity matrix. Plot A is color coded as a function of the nucleic acids: DNA (cyan) and RNA (yellow); plot B is color coded as a function of the batch: A (red) and B (green); plot C is color coded as a function of the packaging condition in batch B: active packaging (blue) and nonactive packaging (red). Comp, component.

and RNA fingerprints, was obtained, and dendrograms of similarity were retrieved by using the Dice coefficient and unweighted pair group method using average linkages (UPGMA) clustering algorithm (23). The similarity distance matrix generated via Bionumerics was used to build partial least-squares discriminant analysis (PLS-DA) by using the R package mixOmics (www.r-project.org).

Bioinformatics and metagenome prediction. Raw reads were first filtered according to the 454 processing pipeline. Sequences were then analyzed and further filtered by QIIME 1.9.0 software (24) using `split_library.py` and `denoiser.py` scripts (25). Then, 99% operational taxonomic units (OTUs) were picked against the Greengenes database 16S rRNA gene (26). The abundance of OTUs from two biological replicates of each sampling time was averaged. Alpha and beta diversities were evaluated through QIIME (4). Weighted UniFrac distance matrices (27) and OTU tables were used to perform Adonis, Anosim, `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 a function of batches (A and B) and packaging (C and T). The Shannon-Wiener diversity index H' was further analyzed using ANOVA, with time being the main factor. When ANOVA revealed significant differences ($P < 0.05$), the Duncan HSD test was applied. A filtered OTU table at 0.5% abun-

dance in at least 2 samples was used to make a heatmap by the R package `heatmap3`. A filtered OTU 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 the abundances of gene families based on 16S rRNA sequence data. OTUs were redetermined by using the `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 imported in the GAGE Bioconductor package (30) to identify biological pathways overrepresented or underrepresented between T and C samples. KEGG Orthology (KO) gene tables, filtered for KO gene presence of ≥ 1 in at least 5 samples, were then used to build a principal component analysis (PCA) as a function of the batch by using the `made4` package of R. Spearman's correlations between OTUs occurring at 5% in at least 2 samples and predicted metabolic pathways related only to amino acid, lipid, energy, and carbohydrate metabolism were taken into account and used to produce a heatmap.

Nucleotide sequence accession number. All of the sequencing data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information (accession number [SRR052241](https://www.ncbi.nlm.nih.gov/sra/SRR052241)).

FIG 3 OTU network summarizing the relationships between taxa and samples. Only OTUs occurring at 5% in at least 2 samples are shown. The abundance of OTUs in the 2 biological replicates for each sampling time was averaged. Sizes of the OTUs are made proportional to weighted degree (i.e., for OTUs, this measures the total occurrence of an OTU in the whole data set) using a power spline. OTUs and samples are connected with a line (i.e., edge) to a sample node, and its thickness is made proportional to the abundance of an OTU in the connected sample. Samples are color coded as a function of the batch: A (red) and B (green).

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 batches A and B are reported in Tables 1 and 2, respectively. For batch A, few differences between C and T samples were observed. The total viable counts, as well as LAB counts, were not affected by the use of the antimicrobial packaging, and they increased in all the samples throughout storage, reaching a final load of about 6 log CFU/g (Table 1). Few differences were observed in the count of yeasts, while no differences were detected for *Staphylococcaceae*, *Enterobacteriaceae*, and molds. For batch B, LAB increased from 4.4 to 6 log CFU/g in C samples during storage, while the load in T samples was kept to about 4.4 log CFU/g during the whole storage period, with a slight increase at day 3. The effect of the antimicrobial packaging was shown, with a significant reduction ($P < 0.05$) of the total viable counts of about 1 log unit at end of storage (Table 2). No differences were observed for *Staphylococcaceae*, *Enterobacteriaceae*, mold, and yeast counts. A significant decrease of pH was observed for both batches ($P < 0.05$) (Table 1). When comparing batches A and B, viable counts at time zero in all of the media appeared to be significantly higher in batch B ($P < 0.05$).

DGGE analysis. Partial least-squares discriminant analysis (PLS-DA), as a function of nucleic acids, showed a certain gradient of separation between DNA and RNA samples (Fig. 2A), while

PLS-DA as a function of the batches (Fig. 2B) presented a clear separation. The distinction was particularly important for samples of batch A, which appeared to group together and separated from samples of batch B. On the other hand, it was possible to observe a certain degree of separation among C and T samples of batch B (Fig. 2C).

Pyrosequencing results. A total of 371,314 raw reads were obtained after 454 processing. A total of 290,245 reads passed the filters applied through QIIME, with an average value of 5,023 reads per sample and an average length of 462 bp. The rarefaction analysis and the estimated sample coverage (ESC) (see Table S1 in the supplemental material) indicated that there was satisfactory coverage for all the samples (ESC, >98%). The richness of the samples varied from a minimum of 44 to a maximum of 194 OTUs. The results, based on storage days, revealed that there was a significant reduction on biological diversity only from C samples of batch B at 3 and 5 days compared to the other samples. The OTU network, presented in Fig. 3, showed that *Photobacterium phosphoreum*, *Lactococcus piscium*, *Lactobacillus sakei*, and *Leuconostoc carnosum* were the major OTUs shared between C and T samples 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* was found in all the samples and was

FIG 4 Incidence of the major taxonomic groups detected by pyrosequencing. Only OTUs with an incidence above 5% in at least 2 samples are shown. Abundance of OTUs in the 2 biological replicates for each sampling time was averaged. Samples are labeled according to time (0, 1, 3, 5, 7, 14, and 21 days), batch (A and B), and treatment (active [T] and nonactive [C]) vacuum packaging.

never lower than 3%. *Lc. 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, *Lc. piscium* was present (about 10%) in all of the samples. Analyzing the microbial diversity, the development of genera and species in both batches could be observed in the heatmap depicted in Fig. 5. It was possible to define a subcluster 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 the 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 of <0.001) through the `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 that they were well separated from batch B on the basis of their microbiota (Fig. 6). The Adonis and Anosim statistical tests run through the `compare_categories.py` script of QIIME confirmed this difference (*P* < 0.001). Comparing T samples from batch A and batch B, no differences in terms of composition were found, while C samples from batches A and B differed significantly (*P* < 0.001). However, according to statistical tests, it was possible to find a significant difference between C and T during the storage only from batch B (*P* < 0.001). ANOVA and *g*-test run through the `group_significance.py` script of QIIME showed that *Kocuria rhizophila*, *Lc. piscium*, *Staphylococcus xylosum*, *Le. carnosum*, and

Carnobacterium divergens were significantly more abundant in C samples of batch B than in C samples of batch A.

Regarding the predicted metagenomes, the weighted nearest-sequenced-taxon index (NSTI) for the samples, expressed as the mean $\pm$ SD, was 0.053 ± 0.010 . This index is the average branch length that separates each OTU from a reference bacterial genome, weighted by the abundance of that OTU in the sample. Thus, an NSTI score of 0.053 indicates a satisfactory accuracy for all of the samples (95%). The pathway enrichment analysis (performed by GAGE) of the predicted metagenomes showed an enrichment of propanoate metabolism (ko00640), butanoate metabolism (ko00650), biosynthesis of unsaturated fatty acids (ko01040), and sulfur metabolism (ko00920) in C samples compared to T samples from batch B only (data not shown). In contrast, from batch A, only pathways involved in cellular processes, biosynthesis of secondary metabolites, and metabolism of amino acids were found to be more abundant in C samples than in T samples. Differences between the two batches were further demonstrated by principal component analysis (PCA) comprising all of the predicted pathways (see Fig. S1 in the supplemental material). The PCA clearly showed that A samples were different from B samples. When plotting the correlation between OTUs and predicted pathways (Fig. 7) of batch B, it appeared that *Le. carnosum* and *Lb. sakei* were positively correlated with the metabolism of volatile fatty acids, such as propanoate and butanoate. *Lc. piscium* was mainly correlated with the biosynthesis of unsaturated fatty acids, while *Le. carnosum* was found linked to sulfur metabolism (Fig. 7). On the other hand, despite the strong Spearman's correlation,

FIG 5 Distribution of OTUs in samples stored in active (T) and nonactive (C) vacuum packaging. The dendrogram of samples (top) was divided into two parts based on the correlation between samples. The categorical annotations (top) were separated into columns, and the samples were labeled by black squares as a function of batches or packaging conditions. The numeric annotation (day of storage) was demonstrated by a scatter plot, and the values were labeled at the right axis. Abundance of OTUs in the two biological replicates for each sampling time was averaged.

FIG 6 Principal coordinate analysis (PCA) based on weighted Unifrac distance matrix. Samples are color coded according to the batch: A (red) and B (green).

the relationship between OTUs and predicted pathways was not statistically significant ($P > 0.05$).

DISCUSSION

The present study aimed at providing a more integrated view on the live viable microbiota development during storage of beef burgers in nisin-based antimicrobial vacuum packaging. For this purpose, an extensive sampling procedure of two different batches with six biological samples replicated each time was used. This may be helpful to limit the intersample variability. The concentration of the antimicrobial agents was chosen because of its effectiveness in retarding growth of spoilage bacteria in beef stored in a vacuum condition (11, 17). Differences in microbial composition during storage were investigated by using classical plate counts, RNA-based DGGE, and rRNA-based pyrosequencing.

Comparing C samples from the two batches, it was observed that the initial counts of the main microbial groups were higher in batch B than in batch A. The microbial load of meat depends on several factors, such as the initial physiological status of the animal, contamination at slaughterhouses and in the equipment used for the meat manipulation, and temperature and storage conditions (3). An effect of the antimicrobial packaging on the reduction of the total viable counts, as well as the LAB counts, was observed only in batch B. However, for both batches, the micro-

biological counts at the end of storage (21 days) showed how the beef burgers stored in active packaging were acceptable in terms of final counts of the main microbial group monitored, as previously reported (31), because the total viable count was lower than 7 log CFU/g.

The OTU network clearly showed that the core of OTUs was dominated by the presence of *P. phosphoreum*, *Lc. piscium*, *Lb. sakei*, *Carnobacterium divergens*, and *Le. carnosum*. *P. phosphoreum* was previously reported as the dominant microbe of spoiled cod under modified atmosphere packaging (MAP) conditions (32) and recently found as the core OTU of the seafood community (1). *Lc. piscium* and *Lb. sakei* have been recently found in a variety of meat products under MAP conditions (33). Their effect on the food matrix appeared to be related to the production of off flavors (34). In accordance with these observations, a positive correlation between *Lc. piscium* and the metabolic pathways of histidine metabolism and fatty acid biosynthesis was found, together with a presumptive abundance of genes related to amino acid metabolism for *Lb. sakei*. On the other hand, the most abundant OTU, *P. phosphoreum*, appeared to be positively correlated with volatile fatty acid metabolism, biosynthesis of unsaturated fatty acid, and nitrogen metabolism. As previously demonstrated (32), this species produces ammonia-like off odors, but it needs to reach

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

a concentration of 10^7 CFU/g in order to have an organoleptic impact on the food product. Unfortunately, in our study, a specific medium for the detection of *P. phosphoreum* was not included. The presence of *Enterobacteriaceae* under VP conditions is reported to be particularly important, both for its high deteriorating potential and for food safety (34). In this study, based on the viable microbiota, the relative abundance of the members of *Enterobacteriaceae* was very low, indicating that only a few taxa could play a role during the spoilage of meat under the VP condition used here.

By PLS-DA analysis based on DNA and RNA DGGE profiles, a

certain degree of separation of the samples based on the nucleic acid analyzed was observed. To evaluate the metabolically active populations, only the RNA data were taken into account. Beta diversity calculation, PLS-DA, and rRNA-based pyrosequencing results confirmed the impact of the antimicrobial packaging only for batch B. By using rRNA-based pyrosequencing, it was possible to find a significant change in the relative abundance of the most abundant OTUs in response to treatment only for batch B. However, this was related not to the initial load but only to the species diversity. Interestingly, C samples from batch B showed a significantly higher abundance of some taxa, such as *Kocuria rhizophila*,

Staphylococcus xylosus, *Le. carnosum*, and *Carnobacterium divergens*, compared to C samples from batch A. These OTUs are those sensitive to the nisin treatment (11, 35–37), explaining the differences between the two batches.

The evidence presented in this study showed that the nisin-based antimicrobial packaging was effective only as a function of the microbiota. The treatment impact was observed when microbiota sensitive to nisin were present in the samples at the beginning and independent of the initial load in the food matrix. In conclusion, our study based on viable microbiota showed that only a few taxa can really play a role during the storage of beef burgers. Further, the use of nisin-based antimicrobial packaging can reduce the abundance of microbes that produce compounds of specific metabolic pathways related to spoilage, with a potential impact on the prolongation of the shelf life. Further studies are needed for verifying this possible prolongation by evaluating the sensory properties of the samples and by metabolomic and meta-transcriptomic studies.

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