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Exploring the Bacterial Diversity of Belgian Steak Tartare Using Metagenetics and Quantitative Real-Time PCR Analysis

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ABSTRACT

Steak tartare is a popular meat dish in Belgium. It is prepared with raw minced beef and is eaten with sauce, vegetables, and spices. Because it contains raw meat, steak tartare is highly prone to bacterial spoilage. The objective of this study was to explore the diversity of bacterial flora in steak tartare in Belgium according to the source and to determine which bacteria are able to grow during shelf life. A total of 58 samples from butchers' shops, restaurants, sandwich shops, and supermarkets were collected. These samples were analyzed using 16S rDNA metagenetics, a classical microbiological technique, and quantitative real-time PCR (qPCR) targeting the *Lactobacillus* genus. Samples were analyzed at the beginning and at the end of their shelf life, except for those from restaurants and sandwich shops, which were analyzed only on the purchase date. Metagenetic analysis identified up to 180 bacterial species and 90 genera in some samples. But only seven bacterial species were predominant in the samples, depending on the source: *Brochothrix thermosphacta, Lactobacillus algidus, Lactococcus piscium, Leuconostoc gelidum, Photobacterium kishitani, Pseudomonas* spp., and *Xanthomonas oryzae*. With this work, an alternative method is proposed to evaluate the total flora in food samples based on the number of reads from metagenetic analysis and the results of qPCR. The degree of underestimation of aerobic plate counts at 30°C estimated with the classical microbiology method was demonstrated in comparison with the proposed culture-independent method. Compared with culture-based methods, metagenetic analysis combined with qPCR targeting *Lactobacillus* provides valuable information for characterizing the bacterial flora of raw meat.

Key words: Metagenetics; Microbiota; Quantitative real-time PCR; Spoilage; Steak ~~tartare; Food~~

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Food spoilage is the damage of the nutritional value, texture, and/or flavor of a food *(27)*. Spoiled foods are unpleasant and unsuitable for consumption. In meat, spoilage is primarily microbial in nature and is affected by the initial load and environmental conditions (intrinsic and extrinsic factors). Raw meat, such as steak tartare, is recognized to be a sensitive perishable food with a short shelf life. Steak tartare is prepared with minced beef meat and is eaten with a variety of sauces, vegetables, and spices. Steak tartare is eaten raw and, hence, represents a potential hazard to the consumer due to the growth of bacteria to unacceptable levels, which contributes significantly to lack of safety or to spoilage *(22, 55)*.

In Europe, since the introduction of Regulation (EC) no178/2002, a food is declared unsafe when it is harmful to human health or unfit for human consumption *(16)*. Food may be declared unfit for human consumption for a number of reasons, including putrefaction, decay, and contamination by foreign bodies. Surveillance of spoilage during food storage is based primarily on the enumeration of aerobic plate counts (APCs) at 30°C or of some specific flora growing on selective media *(17)*. However, microbial analysis alone might not be sufficient to understand modifications of the microbial ecology in response to different storage or environmental conditions *(12)*.

Molecular technologies can elucidate the microbial community, including the identification and quantification of culturable and nonculturable organisms, and can do so at a much higher resolution than was previously possible with culture-based methods *(1)*. Among the culture-independent techniques, 16S rDNA metagenetics (also called metagenomic analysis targeting 16S ribosomal DNA) has emerged as a powerful tool for studying the bacterial composition of various ecosystems *(15)*. Food microbiologists are becoming increasingly interested in pyrosequencing analysis to explore the microbial flora of fermented food, marinated poultry, sausages, cheese, tea, and bottled water *(5, 11, 25, 30, 33, 35, 37)*. By using such analysis, scientists are now elucidating the microbial flora distribution at a much higher resolution than was previously possible *(24)*.

The objective of this study was to explore the diversity of bacterial flora present in steak tartare in Belgium according to the source and to determine which bacteria are able to grow during the meat's shelf life. A quantitative

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real-time PCR (qPCR) assay was used in combination with 16S rDNA metagenetics to evaluate the bacterial concentration in the samples. Results are discussed with regard to the potential for using metagenetics, qPCR, and classical microbiological analysis as complementary methods to evaluate bacterial flora of perishable foodstuffs.

MATERIALS AND METHODS

Food samples. In Belgium, beef is sold in supermarkets (49.3%), butchers' shops (29%), and other establishments (21.7 %) *(53)*. Samples of steak tartare were collected from five different sources to represent the respective proportions of the beef meat market, and a minimum of six samples were taken per group to ensure an adequate representation of each group *(2)*. In total, 58 samples of steak tartare were collected between March and April 2014: 16 samples from eight supermarkets selling prepacked steak tartare provided by other establishments (SM1), 16 samples from eight supermarkets preparing their own steak tartare (SM2), 14 samples from seven butchers' shops, six samples from restaurants, and six samples from sandwich shops. Minced beef samples with additional ingredients were provided by the companies without details of their composition, except for the SM1 samples, for which the labels with ingredients are available.

The labels of samples 1 to 3 of SM1 stated that the samples contained minced beef and acidity regulators (lactic acid, sodium acetate, calcium lactate); products 4 to 8 from SM1 contained minced beef (60%) and sauce (40%). Samples 4, 5, 6, and 8 of SM1 originated from the same producer but were sold in different supermarkets. The sauce was composed of oil (canola and olive), egg yolk, vinegar, malt extract, onions, garlic, mustard, anchovy, sugar, salt, acidity regulators (lactic acid, sodium acetate, calcium lactate, citric acid), preservatives (potassium sorbate, sodium benzoate), antioxidants (ascorbic acid, sodium ascorbate), stabilizers (guar gum, carob gum, xanthan gum), and coloring (paprika extract).

Following the Federal Food Agency guide for self-monitoring and food safety in butcheries, minced meat cannot be marketed for more than 2 days *(19)*. Steak tartare samples were analyzed at the beginning (day 0) and at the end of shelf life (day 2). The samples were stored at 4°C on the first day and at 8°C on the second day, following the guidelines for implementing microbiological durability tests of chilled perishable and highly perishable foodstuffs *(4)*. In restaurants and sandwich shops, the samples were analyzed on the day of production only (in these cases, the meat is eaten directly after preparation). All the food samples were packed in food wrap.

Classical microbiological analysis. Microbiological analyses were performed by a laboratory licensed by the Belgian Ministry of Public Health and were accredited in accordance with the requirements of the International Organization for Standardization (ISO) 17.025 standard *(29)*. Samples (25 g) were placed in a Tempo bag with an 80-μm-pore-size mesh screen liner (ref 80015, bioMérieux, Basingstoke, England) and were homogenized for 1 min with sterile physiological water using a Mix 2 Stomacher apparatus with a speed of 230 rpm (AES Chemunex, Bruz, France). When appropriate, the suspension was diluted with sterile physiological water.

Following the requirements of the ISO 4833 method, APCs were obtained using plate count agar (Bio-Rad, Marnes La Coquette, France) incubated for 72 ± 3 h at 30°C *(28)*.

Bacterial 16S rRNA gene amplification and barcoded pyrosequencing. An aliquot (1.5 ml) of the suspension without repetition was then used for total bacterial genomic DNA extraction using the DNeasy Blood & Tissue kit (Qiagen, Crawley, UK), following the manufacturer's recommendations. The resulting DNA extracts were eluted into DNase- and RNase-free water, and their concentration and purity were assessed by means of optical density using a NanoDrop ND-1000 spectrophotometer (Isogen, St-Pieters-Leeuw, Belgium). DNA samples were stored at −20°C until they were used for 16S rDNA amplicon pyrosequencing analysis.

16S rDNA PCR libraries targeting the V1 to V3 hypervariable region (amplicon size of minimum 460 bp) were generated. Primers E9-29 and E514-430 *(6)*, specific for bacteria, were selected for their theoretical ability to generate the lowest amplification bias relative to amplification among the various bacterial phyla *(56)*. Amplification fusion primers contain a directional titanium primer A or B sequence (which includes a 454 library "key" sequence) at the 5-prime end of the oligonucleotide in addition to the template-specific sequence at the 3-prime end. A multiplex identifier sequence is added in between the titanium primer and template-specific sequences to allow for automated software identification of samples after pooling or multiplexing and sequencing (also referred to as "barcoding"). The amplification mix contained 5 U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 1× enzyme reaction buffer, 200 μM deoxynucleoside triphosphates (Eurogentec, Liège, Belgium), 0.2 μM each primer, and 100 ng of genomic DNA in a final volume of 100 μl.

Thermocycling conditions consisted of a denaturation step of 15 min at 94°C, followed by 25 cycles of 40 s at 94°C, 40 s at 56°C, and 1 min at 72°C, and a final elongation step of 7 min at 72°C. These amplifications were performed on a Mastercycler EP gradient system (Eppendorf, Hamburg, Germany). The PCR products were run on 1% agarose gels, and the DNA fragments were purified using a Wizard SV PCR purification kit (Promega Benelux, Leiden, The Netherlands). The quality and quantity of the products were assessed by the Picogreen dsDNA quantitation assay (Isogen). Equal amounts of each PCR product (maximum 20 products) were mixed and subsequently amplified by emulsion PCR. Pyrosequencing was performed with the Roche 454 GS Junior Sequencer (Roche, Basel, Switzerland) using the pyrosequencing protocol *(34)*. The sequences generated in this study have been submitted to the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) (bioproject PRJNA281696, biosamples SUB921066 and SRA experiment SRX1009712).

Bioinformatic analysis. Image and data processing for amplicon sequencing were performed using the Genome Sequencer FLX system software package 2.3 (Roche). The 16S rDNA sequence reads were processed using the mothur software package *(54)*. The quality of all sequence reads was denoised using the PyroNoise algorithm implemented in mothur and was filtered according to the following criteria: minimal length of 425 bp, an exact match to the barcode, and one mismatch allowed to the proximal primer. Sequences from multiplexed samples were assigned based on the presence of the multiplex identifiers assigned to each sample. The sequences that passed the quality check were aligned to the SILVA alignment database *(46)*. Chimeric sequences were detected using the chimera-slayer command included in the mothur package, and the potential chimeras were removed *(23)*. A distance matrix was prepared (distance = 0.03), and the sequences were clustered to operational taxonomic units (OTUs) using the average neighbor algorithm. OTUs representing less than 0.01% of the total number of reads were considered to be artifacts and were discarded.

In the second phase of the experiment, the representative sequences of each OTU were compared with the SILVA microbial

TABLE 1. *Results of TVCs with classical microbiology in steak tartare samples from different sampling locations*[a]

Sample location	Day 0 TVCs		Day 2 TVCs		Wilcoxon test (comparison between day 0 and day 2)
	n	P50 (minimum–maximum)	*n*	P50 (minimum–maximum)	*W* value (significance)
Butchers	7	7.5×10^4 (8.0×10^3–2.8×10^7)	7	3.4×10^5 (8.8×10^3–5.5×10^6)	23 (NS)
SM1	8	3.0×10^4 (9.8×10^3–3.8×10^4)	8	3.3×10^4 (1.4×10^4–3.8×10^5)	9.5 (NS)
SM2	8	3.8×10^4 (7.6×10^3–8.1×10^5)	8	3.8×10^4 (1.5×10^4–3.6×10^5)	19.5 (NS)
Restaurants	6	4.1×10^4 (8.6×10^3–1.7×10^7)		—	
Sandwich shops	6	1.0×10^5 (8.2×10^3–1.9×10^5)		—	

[a] Values given as CFU per gram. TVC, total viable count; NS, not significant; SM1, prepacked steak tartare sold in supermarkets; SM2, steak tartare produced in supermarkets.

database using the Basic Local Alignment Search Tool (BLAST) *(3, 20)*. For each OTU, a standard detailed taxonomic identification was given based upon the identity (less than 1% mismatch with the aligned sequence) and the metadata associated with the best hit (validated bacterial species or not). The genus assignations obtained using mothur and BLAST were compared and were noted as unclassified in the case of a mismatch. Each bacterial population identified by metagenetics was analyzed as a proportion of reads, to deduce the bacterial flora proportion. The sequences corresponding to mitochondria or chloroplasts were removed prior to analysis.

qPCR protocol. Based on the results of the metagenetic analysis, *Lactobacillus* genus was identified in all the samples included in this study. A specific qPCR was developed for the quantification of *Lactobacillus* genus in the steak tartare samples. qPCR assays were carried out in 96-well plates and were performed in triplicate using the LightCycler 480 system (Roche). Each 12-µl PCR amplification contained 6 µl of LC480 probe master mix (Roche), 2 µl of template DNA (at 5 ng/µl), 200 nM each forward and reverse primers (forward primer: 5′-GCYCACGTWGAATAYGAAAC-3′; reverse primer: 5′-CGDACTTCCATTTCAACYAAGTC-3′, designed by the author S.B.), and 100 nM fluorogenic probe (5′-GTGGCATWGGRCCATCAGTTGC-3′). The amplification consisted of a 5-min denaturation step, followed by 40 two-step cycles of 15 s for 95°C and 30 s at 60°C. The real-time system was supplied with Lightcycler 480 software version 1.5, using unique Roche algorithms for highly accurate and robust automated data analysis. Serial dilutions (10^6 to 10 genomic copy numbers) of *Lactobacillus acidophilus* DNA were used for the calibration curve. The mean of the cycle threshold on the three repetitions was used to estimate the load of the *Lactobacillus* population present in the samples.

To estimate the total flora in the samples, the concentration of *Lactobacillus* genus obtained by qPCR was weighted by the results obtained with the metagenetic analysis (equation 1).

$$C_{\text{total flora}} = C_{\text{qPCR } Lactobacillus} \times \left(\frac{N_{\text{reads total}}}{N_{\text{reads } Lactobacillus}} \right) \qquad (1)$$

where $C_{\text{total flora}}$ is the estimated total flora concentration in the sample, $C_{\text{qPCR } Lactobacillus}$ is the concentration of *Lactobacillus* genus obtained by qPCR, $N_{\text{reads total}}$ is the total number of reads per sample in the metagenetic analysis, and $N_{\text{reads } Lactobacillus}$ is the number of reads for *Lactobacillus* genus per sample in the metagenetic analysis. This result was then compared with the result of APC at 30°C obtained using the classical microbiology method.

Statistical analysis. For classical microbiology results, nonparametric statistical tests were used to compare the APC results of different product types and the results obtained from samples taken on the day of production (day 0) and at the end of shelf life (day 2). The Kruskal-Wallis test was performed to make a comparison between the types of establishments on a certain day (i.e., day 0 or day 2). Meanwhile, the Wilcoxon test was used to compare the APC results from the same type of establishment. Spearman's rank correlation coefficient was used to establish an association between values obtained with qPCR and those obtained via the classical microbiology method. Analysis of covariance (ANCOVA) was used to evaluate whether bacterial concentrations were equal between qPCR results and those obtained via the classical microbiology method. All tests were considered significant for *P* values of less than 0.05 *(2)*.

For the metagenetic results, the datasets were subsampled using mothur to obtain the same number of reads per sample and to determine rarefaction curves with the coverage estimator *(10)*. The microbial biodiversity and richness were assessed using, respectively, the nonparametric Shannon diversity index *(8)* and the Chao index *(7)*. The distribution of read percentages for the major bacterial species was characterized by calculating the 25th, 50th, and 75th percentiles.

Bacterial population dissimilarity was assessed using the Bray-Curtis index with the Vegan package in the R program *(41, 48)*. STAMP software *(42)* was used to highlight the statistical differences between samples in their proportions of bacterial populations, using the two-sided Fisher's exact test *(49)*, including the confidence intervals from the Newcombe-Wilson method *(36)*. The differences were considered significant for corrected *P* values of less than 0.05.

RESULTS

Classical microbiology analysis. The median, minimum, and maximum APCs (CFU/g) in relation to sampling location are shown in Table 1. The lowest APC values were observed for SM1, whereas steak tartare sampled from butchers' shops showed the highest APC results.

At day 0, APCs ranged between 3.0×10^4 CFU/g (for SM1) and 1.0×10^5 CFU/g (for samples obtained from sandwich shops). At day 2, APCs ranged between 3.3×10^4 CFU/g (for SM1) and 3.4×10^5 CFU/g (for samples obtained from butchers' shops). No significant differences were observed using a Kruskal-Wallis test between samples at day 0 (H = 3.39; alpha value = 49.5%) and at day 2 (H = 5.21; alpha value = 7.39%). This was also the case for the

Author: Is it common to say "at day" rather than "on day"? Is on day more accurate: something was performed on day 0, or the results on day 0 were...?

TABLE 2. *Number of OTUs, coverage of the bacterial flora, Shannon index, Chao index, and number of identified species and genera from metagenetic analysis according to sampling location*[a]

	Butchers		SM1		SM2		Restaurants	Sandwich shops
	Day 0	Day 2	Day 0	Day 2	Day 0	Day 2	Day 0	Day 0
No. of OTUs	165	124	245	166	198	166	238	320
Coverage (%)	96.4	97.8	96.3	96.9	97.0	97.7	95.2	95.0
Shannon index	2.2	1.7	2.1	1.6	1.9	1.6	3.3	2.5
Chao index	365.7	291.1	566.0	374.8	523.1	414.4	399.0	664.3
No. of identified species	121	124	180	151	155	154	147	145
No. of identified genera	72	70	90	85	81	83	80	79

[a] OTU, operational taxonomic unit; Shannon index, bacterial diversity; Chao index, bacterial richness; SM1, prepacked steak tartare sold in supermarkets; SM2, steak tartare produced in supermarkets.

Wilcoxon test to compare the results at day 0 and day 2 for each product.

Identification of bacterial communities by pyrosequencing. For metagenetic results, the number of reads per sample ranged from a minimum of 741 to a maximum of 5,549, with a median of 1,977 (data not shown). The number of OTUs, the bacterial diversity, richness estimators, coverage, and the number of identified species and genera according to sampling location are presented in Table 2. The highest number of identified species was encountered in SM1 at day 0. SM1 also showed a reduction in identified species between day 0 and day 2. The highest values of OTUs and bacterial richness were found in the samples from sandwich shops. The lowest values were found in the samples from butcher shops at the end of the product's shelf life. The highest bacterial diversity was found in samples from restaurants.

The metagenetic results showed seven predominant major bacterial species: *Brochothrix thermosphacta, Lactobacillus algidus, Lactococcus piscium, Leuconostoc gelidum, Photobacterium kishitani, Pseudomonas* spp. (a genus encompassing different species), and *Xanthomonas oryzae*. The distribution of read percentages for the seven major bacteria across the different sampling locations is shown in Table 3. These data reveal a bacterial profile for SM1. Percentages of reads for samples obtained from this location were lower in comparison with others, especially for *L. algidus, L. piscium, L. gelidum,* and *P. kishitanii*. *X. oryzae* was mainly encountered in SM1, whereas *L. algidus* appeared to increase in samples from butchers' shops and SM2 during the product's shelf life. In restaurants and sandwich shops, there was a more diverse microbiota, as attested by a higher number of identified bacteria. The percentage of reads for the genus *Pseudomonas* was generally very low (although higher in the samples from restaurants, sandwich shops, and butchers' shops at day 0).

Dissimilarity. The dissimilarity of results obtained by metagenetics is shown in Figure 1 and reveals nine groups (GI to GIX) of samples with the same pattern of bacterial communities. The most dominant bacteria in each group (proportion of reads >10%) were *X. oryzae* for GI (11 samples, mainly SM1); *Streptococcus thermophilus* for GII; *Clostridium haemolyticum* for GIII; *L. algidus* and *P. kishitanii* for GV; *P. kishitanii, L. algidus,* and *L. piscium* for GVI; *L. piscium* for GVII; *L. gelidum* for GVIII; and *Pseudomonas antarctica, B. thermosphacta,* and *P. kishitanii* for GIX. By contrast, GIV was not characterized by any specific dominant bacterial species or genera: in this case, 13% of the bacteria identified consisted of *X. oryzae* and *L. gelidum*. Greater bacterial species diversity was observed for this group in comparison with the others.

Comparison of total flora estimated by classical microbiology method and by culture-independent technique. The relationship between the APC incubated for 72 ± 3 h at 30°C and total flora estimation obtained with qPCR on the genus *Lactobacillus* combined with metagenetic analysis (see equation 1) is depicted in Figure 2 (values expressed in log CFU/g). The Spearman correlation (based on 57 paired samples) was $r_s = 0.9931$, which represents a high correlation. On average, there was a lower concentration of total flora obtained with the conventional microbiology method, especially in the SM1 samples. Of 57 samples, 46 presented a higher total flora estimation using qPCR than that obtained from APCs at 30°C. On average, the underestimation was equal to 1.32 log for APCs, with a maximum of 4.33 and a minimum of –0.85 (data not shown). Significant differences of total flora estimated with both methods were evaluated with the ANCOVA analysis.

DISCUSSION

The objective of this study was to explore the diversity of bacterial flora present in steak tartare in Belgium according to the source and to determine changes in bacterial populations during shelf life. Although the differences in APC results between samples were not statistically significant at day 0, contamination levels were found to be lowest in SM1 (3.0×10^4 CFU/g) and highest in the samples from the sandwich shops (1.0×10^5 CFU/g). Based on the information given on food labels from SM1, producers had added acidity regulators and preservatives to the product, which are allowed for use in prepacked minced meat by the European regulation dealing with preservative agents added to food *(18)*. The presence of those acidity regulators and preservatives may explain the lower bacterial

TABLE 3. *Distribution of read percentages for the seven major bacterial species for each source of steak tartare sampled*[a]

Bacterial species	Butchers		SM1		SM2		Restaurants ($n = 6$)	Sandwich shops ($n = 6$)
	Day 0 ($n = 7$)	Day 2 ($n = 7$)	Day 0 ($n = 8$)	Day 2 ($n = 8$)	Day 0 ($n = 8$)	Day 2 ($n = 8$)		
Brochothrix thermosphacta	0.9 (0.2–4.4)	0.3 (0.0–2.2)	0.6 (0.3–1.5)	0.4 (0.2–1.0)	0.4 (0.0–1.6)	0.4 (0.2–0.4)	0.1 (0.1–1.0)	0.2 (0.0–0.3)
Lactobacillus algidus	8.8 (0.7–24.6)	50.0 (17.1–64.3)	0.0 (0.0–0.1)	0.0 (0.0–7.7)	21.0 (2.7–36.7)	68.0 (14.9–76.9)	1.6 (0.2–15.0)	36.7 (18.3–58.7)
Lactococcus piscium	0.7 (0.4–1.0)	0.4 (0.2–10.1)	0.0 (0.0–0.2)	0.0 (0.0–0.1)	3.6 (0.5–14.0)	1.0 (0.4–4.7)	0.2 (0.1–23.6)	3.6 (2.3–4.9)
Leuconostoc gelidum	1.3 (0.9–3.4)	2.1 (1.2–10.8)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	1.4 (0.6–2.3)	2.8 (1.3–4.0)	4.5 (2.0–7.4)	1.0 (0.8–1.2)
Photobacterium kishitanii	15.0 (1.4–31.1)	6.0 (2.8–14.1)	0.0 (0.0–0.1)	0.0 (0.0–0.0)	2.9 (1.2–24.7)	1.5 (0.3–8.8)	0.0 (0.0–26.2)	12.4 (3.9–20.4)
Pseudomonas spp.	11.1 (3.6–22.9)	4.4 (0.5–12.0)	3.0 (1.9–5.5)	1.3 (1.0–2.9)	3.4 (0.5–5.1)	1.6 (0.4–4.8)	5.1 (1.5–5.8)	2.1 (1.6 –6.3)
Xanthomonas oryzae	0.0 (0.0–0.1)	0.1 (0.0–0.2)	81.9 (5.8–85.1)	78.5 (3.6–83.3)	0.2 (0.0–0.4)	0.1 (0.0–0.4)	0.9 (0.3–3.7)	0.2 (0.1–0.7)
Others	62.1	36.8	14.4	19.8	67.2	24.7	87.7	43.9

[a] 50th percentile of results (25th and 75th percentiles of results in parentheses). SM1, prepacked steak tartare sold in supermarkets; SM2, steak tartare produced in supermarkets.

concentration in prepacked steak tartare during the shelf life *(32)*.

In this study, minced beef preparations without any heat treatment step were analyzed, and all the microorganisms identified in the samples were considered to be alive. The metagenetic results show the presence of *Lactobacillus* genus in all the analyzed samples, and this genus was chosen to develop a qPCR. A lower concentration of total flora obtained from conventional microbiological methods (APCs at 30°C) was noted in comparison with the total flora estimated by combining the qPCR results on *Lactobacillus* and the percentage of reads from metagenetic analysis. The difference between the two estimation methods is clearer in the SM1 samples than in other samples. Previous studies have highlighted the potential fallacy of the total aerobic mesophilic count as a reference shelf-life parameter for chilled food products, because it can often underestimate the contamination levels at the end of shelf life *(45)*. Some bacteria such as *L. gelidum* and *L. algidus* either grow very slowly or cannot grow on culture media incubated at temperatures above 30°C, which is the incubation temperature of plate count agar, following the recommendations of the ISO 4833 standards *(51)*.

Metagenetic analysis sheds light on the bacterial diversity in the meat and highlights the presence of lesser-known or as-yet-undescribed bacteria. This has been shown for marinated poultry *(37)* and also for vacuum-packed beef *(14)*. Metagenetic analysis also allows for the assessment of the dynamics of bacterial species within a food matrix (e.g., during the shelf life of a product) *(13)*. However, one drawback is that metagenetics is not yet considered a ~~gold~~ standard, as is the classical microbiology method, owing to the recent rapid development of genetic methods *(9)*. It has, therefore, been necessary to compare the metagenetic results with those obtained via conventional methods, despite the fact that the former can identify a much greater range of bacteria than the latter.

In our study, up to 180 bacterial species and 90 genera were identified using metagenetic analysis, depending on the sampling location (Table 2). In agreement with those of other authors, these results show great diversity of the microbial flora in steak tartare, which was not revealed by plating on conventional media *(9, 13)*. The diversity and the richness appeared to be slightly lower in steak tartare by the end of shelf life (day 2) compared with that on the day of production (day 0) (Table 2). These results are in accordance with the study of Ercolini *(13)*, who observed rarefaction curves in fermentation (e.g., mozzarella) or spoilage (e.g., pork meat) processes.

Seven predominant bacterial species were identified in the steak tartare samples: *B. thermosphacta, L. algidus, L. piscium, L. gelidum, P. kishitanii, Pseudomonas* spp., and *X. oryzae*. Following dissimilarity analysis (using the Bray-Curtis formula), nine main groups of samples were distinguished, denominated GI to GIX (Fig. 1).

The first group (GI) was constituted of samples of prepacked steak tartare obtained from supermarkets at day 0 and day 2. The bacterial flora was very similar between the 2 days of analysis, suggesting that preservatives added to the steak tartare stabilize the flora of the product. In this group, a

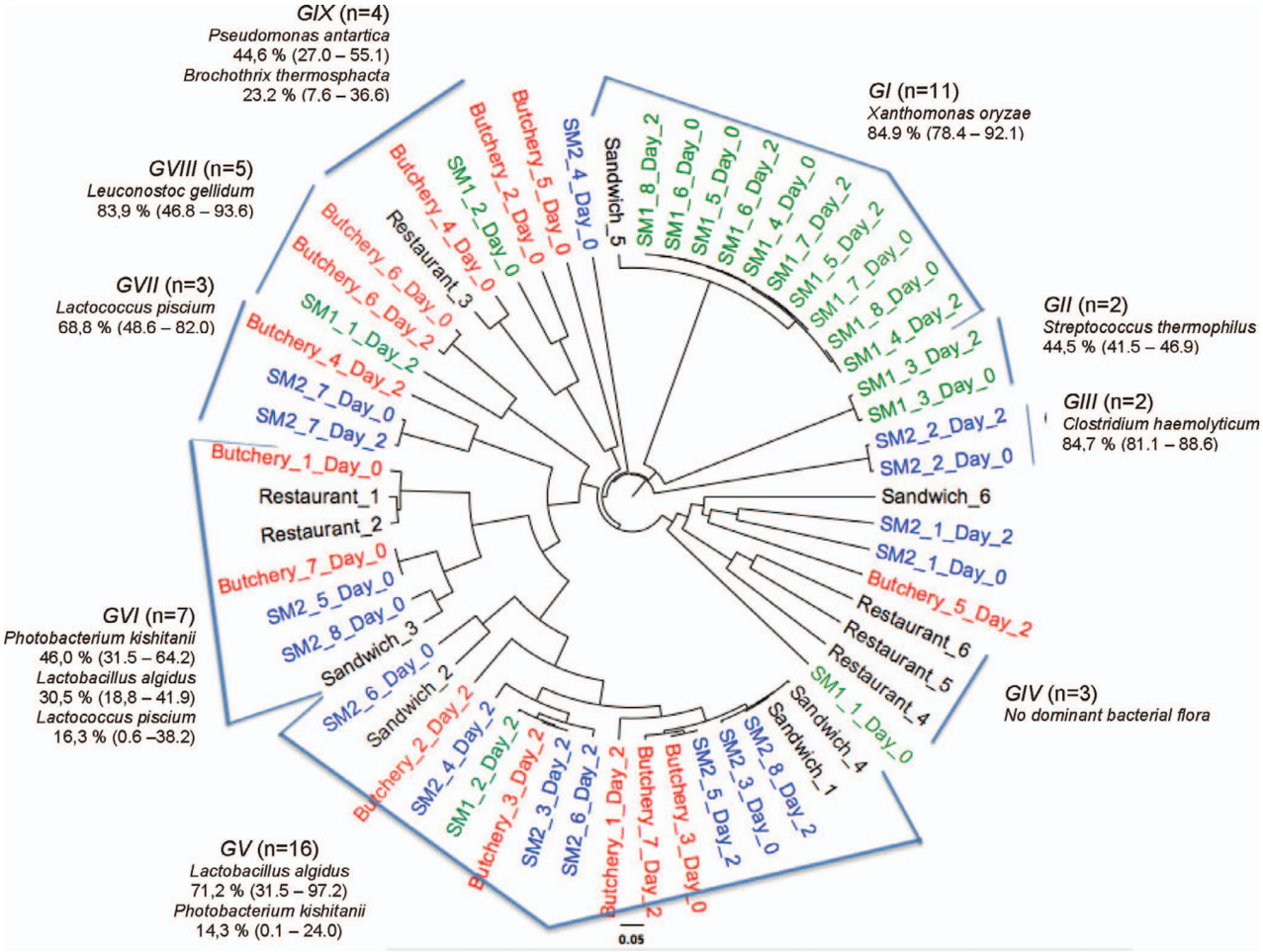

FIGURE 1. *Visual representation of the similarity of samples based on their bacterial community composition using Bray-Curtis index. Dominant bacteria (proportion of reads >10%) are described for each group with the average proportions of reads (minimum and maximum proportion of reads are presented in parentheses). SM1, prepacked steak tartare sold in supermarkets; SM2, steak tartare produced in supermarkets. (Note: Nine groups [GI to GIX] are annotated on the graph. Due to high dissimilarity, seven samples are not included in one of the groups.)*

high proportion of *X. oryzae* reads were observed. This bacterium is a well-known plant pathogen found in rice *(57)*, in which it causes a condition called "bacterial blight" and "bacterial leaf streak" *(39)*. This bacterium causes a similar disease in onions and garlic *(50)*. According to the labels, the sauce added to the product contains vegetables, onions, and garlic. One reason for the homogeneity of the SM1 group is the fact that samples 4, 5, 6, and 8 originated from the same producer but were sold in different supermarkets. In this respect, one may assume that the products were relatively constant between batches and that the presence of the specific ingredients or spices contributed to the pattern of the bacterial flora in the final product.

The second group (GII) encompassed only two samples of prepacked steak tartare obtained from supermarket 3. The dominant bacterial flora found in these two samples was *S. thermophilus*. *S. thermophilus* is usually encountered in fermented food such as yogurt and other fermented milk products *(21)*. So, in this case, the presence of this bacterium suggests that the producer used particular ingredients containing this bacterium, such as fermented milk products, or that cross-contamination occurred during production.

In the case of GIII (one sample of steak tartare produced in a supermarket at day 0 and day 2), the dominant flora was *C. haemolyticum*. This bacterium is responsible for a disease called "bacillary hemoglobinuria" (or "redwater disease") in cattle, and it may be excreted after an acute fluke infection *(26, 52)*. In this case, the presence of this bacterium in the meat product indicates that the cattle from which the meat was obtained might have been slaughtered during a bacteremial phase.

Group GIV was composed only of samples from restaurants and was characterized by a widespread distribution of bacteria. *X. oryzae* and *L. gelidum* were the most abundant taxa, representing 13% of the total population of the group. The high diversity of bacteria might have been related to contamination events during preparation in the restaurant kitchens and to ingredients used in the preparation of steak tartare in these restaurants.

For groups V, VI, and VII, the dominance of three bacterial species was observed, although not in equal

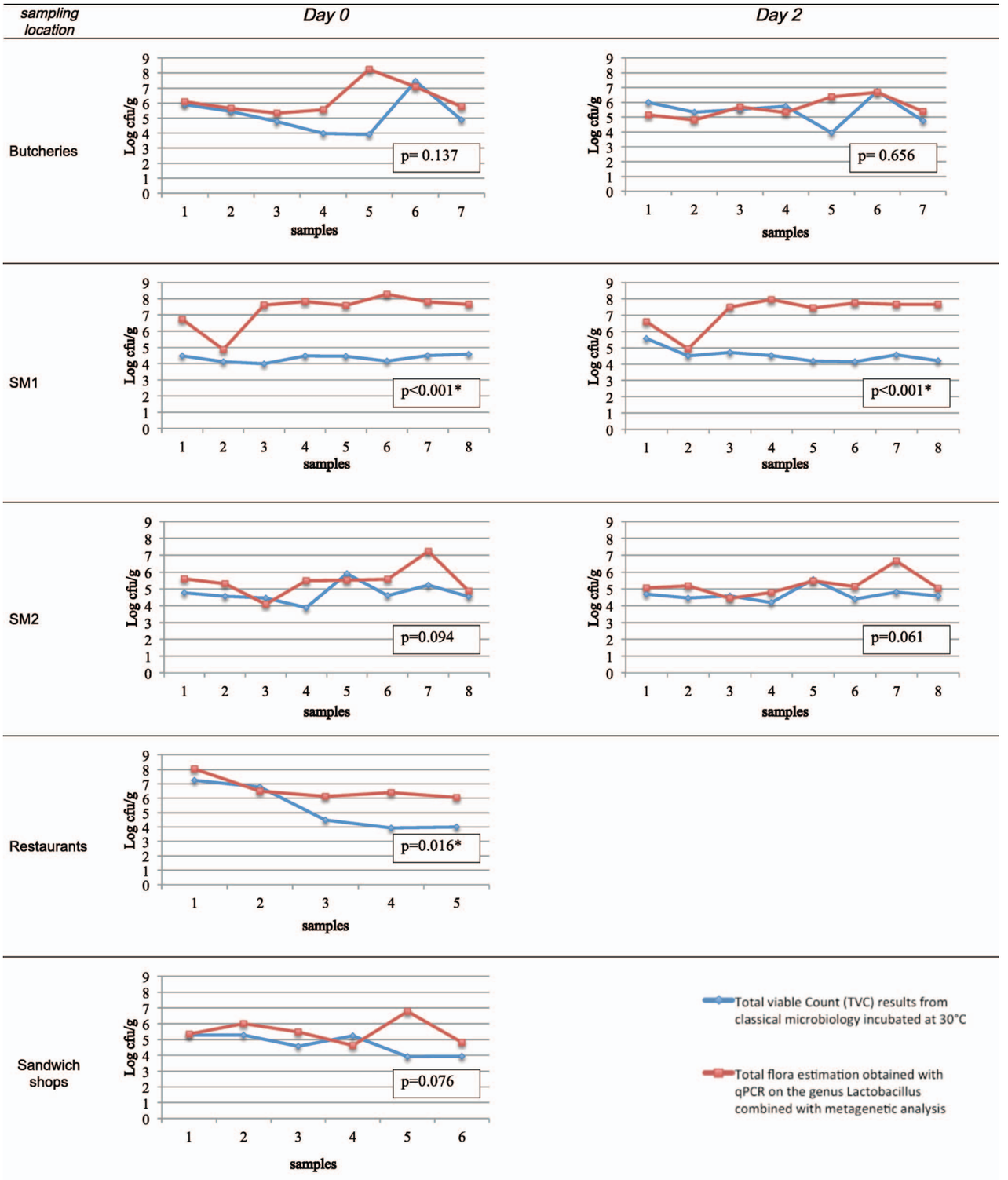

FIGURE 2. *Comparison of total flora estimated by classical microbiology method (in blue) (total viable count [TVC] incubated for 72 h ± 3 h at 30°C) and by culture-independent method (in red) (total flora estimation obtained with qPCR on the genus* Lactobacillus *combined with metagenetic analysis) (log CFU per gram). Analysis of covariance (ANCOVA) was used to evaluate whether bacterial concentrations were equal between classical microbiology and culture-independent method. The test was considered as significant (*) for a* P *value of less than 0.05. SM1, prepacked steak tartare sold in supermarkets; SM2, steak tartare produced in supermarkets.*

distribution throughout the groups: *L. algidus, L. piscium,* and *P. kishitanii*. Group V was mainly constituted of samples taken at day 2, with a high proportion of *L. algidus*; and group VI was constituted exclusively of samples taken at day 0. In group VII, there was a high proportion of *L. piscium*. *L. algidus* was identified a decade ago as a natural contaminant in chilled vacuum-packed beef. This bacterium is a facultative anaerobic gram-positive rod that produces lactic acid from glucose *(31)*. In the present study, a statistically significant increase in the percentage of reads between the day of production (day 0) and the end of shelf life (day 2) was observed for *L. algidus* (data not shown). This is consistent with the observations of Pennacchia et al. *(44),* who showed that *L. algidus* developed mainly in the late stage of storage of vacuum-packed beef, whereas in contrast, *B. thermosphacta* was more prevalent in the early stage of storage. No implication in meat spoilage has been established for *L. algidus*. According to Nieminen et al. *(38),* this organism could be beneficial in meat by hampering the development of spoilage organisms due to its homofermentative ability. *L. piscium* is a gram-positive bacterium that is well adapted for growth in a variety of meats stored in modified atmosphere packaging, owing to its ability to develop in low-temperature conditions *(40, 47)*. Little competition seems to occur between *Leuconostoc* species and other bacterial species, which explains why they are able to coexist in meat until the end of the product's shelf life, taking on the role, among others, of a spoilage agent *(47)*. According to other researchers, *Photobacterium* is mainly involved in the spoilage of fish and is also responsible for the spoilage of beef, in which it has been shown to develop after 7 days of storage *(44)*. The development of this bacterium relies on its ability to grow at low temperatures, within the range of chilled goods, and in anaerobic conditions.

In the present study, group VIII (samples from one butchery and one sample of prepacked steak tartare at day 2) was mainly composed of *L. gelidum,* which is also a well-known spoilage-causing agent encountered in meat stored in modified atmosphere packaging or under vacuum packaging *(12)*. This bacterium is closely related to *Leuconostoc gasicomitatum,* which also belongs to the lactic acid bacteria group and shares its heterofermentative ability *(37)*.

P. antarctica and *B. thermosphacta* were found to represent 68% of group IX. *Pseudomonas* spp. are well-known spoilage agents for meat stored in aerobic conditions. One may thus assume that the meat used for the preparation of steak tartare in these samples was not as fresh as that used in other groups. However, the use of preservatives probably contributed to the stabilization of the bacterial flora during the shelf life of group IX by favoring the development of *L. algidus* over spoilage agents such as *Pseudomonas* or *Brochothrix*. Modified atmosphere packaging and the vacuum packaging of meat are known to lower the contamination levels of those two taxonomic groups *(12, 14)*. To date, three major species of *Pseudomonas, fluorescens, ludensis,* and *fragi,* have been identified as significant spoilage agents, with the last of these being well adapted to the meat "niche" *(12)*. The present study also revealed the presence, at different levels, of *B. thermosphacta*. It was demonstrated that *B. thermosphacta* dominates the microbial flora in meat during the early stage of storage but that it decreases later on *(14)*. These results also confirm another experiment in which a dominance of *B. thermosphacta* during the shelf life of meat products was observed, although the storage mode was different from that of the present study *(44)*. *B. thermosphacta* is responsible for off-odors and off-flavors, due to the conversion of tryptophan into 3-methylindole *(14, 43)*.

This study has highlighted that the microbiota of steak tartare is much more diverse than the cultivable group of bacteria studied by culture media. Depending on the sample location, the predominant bacterial species were *B. thermosphacta, L. algidus, L. piscium, L. gelidum, P. kishitanii, Pseudomonas* spp., and *X. oryzae*. In comparison with culture-based methods on selective media and previous culture-independent techniques, metagenetic analysis combined with the enumeration of *Lactobacillus* by qPCR provides valuable information for helping producers to market safer and more stable products. These technologies are not yet available for routine analysis. But, thanks to the fast evolution of this field, the simplification of protocols, and the decreasing costs of next-generation sequencers, these techniques could be available in the future for industries and public authorities as a complementary technique for characterizing the bacterial flora of raw meat products.

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