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Research Paper

Changes in the Microbiota from Fresh to Spoiled Meat, Determined by Culture and 16S rRNA Analysis

Samart Dorn-In [1,*], Sirkka Mang [2], Raúl O. Cosentino [3,4], Karin Schwaiger [1]

[1] Unit of Food Hygiene and Technology, Institute of Food Safety, Food Technology and Veterinary Public Health, University of Veterinary Medicine, Vienna Veterinaerplatz 1, 1210 Vienna, Austria
[2] Chair of Food Safety and Analytics, Faculty of Veterinary Medicine, Ludwig Maximilian University of Munich, Schoenleutnerstr. 8, 85764 Oberschleissheim, Germany
[3] Department of Veterinary Sciences, Experimental Parasitology Ludwig Maximilian University of Munich, Lena-Christ-Str. 48, 82152 Planegg-Martinsried, Germany
[4] Biomedical Center Munich, Department of Physiological Chemistry, Ludwig Maximilian University of Munich, Großhaderner Str. 9, 82152 Planegg-Martinsried, Germany

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ABSTRACT

Growth of meat microbiota usually results in spoilage of meat that can be perceived by consumers due to sensory changes. However, a high bacterial load does not necessarily result in sensory deviation of meat; nevertheless, this meat is considered unfit for human consumption. Therefore, the aims of this study were to investigate changes in the microbiota from fresh to spoiled meat and whether the proportions of certain bacteria can probably be used to indicate the hygiene status of meat. For this purpose, 12 fresh pork samples were divided into two groups, and simultaneously aerobically stored at 4°C and 22°C. At each time-temperature point (fresh meat, days 6, 13, and 20 at 4°C, and days 1, 2, 3, and 6 at 22°C), 12 meat subsamples were investigated. Sequences obtained from next-generation sequencing (NGS) were further analyzed down to species level. Plate counting of six bacterial groups and NGS results showed that *Pseudomonas* spp. and lactic acid bacteria (LAB) were found in a high proportion in all stored meat samples and can therefore be considered as important "spoilage indicator bacteria". On the contrary, sequences belonging to *Staphylococcus epidermidis* were found in a relatively high proportion in almost all fresh meat samples but were less common in stored meat. In this context, they can be considered as "hygiene indicator bacteria" of meat. Based on these findings, the proportion of the "hygiene indicator bacteria" in relation to the "spoilage indicator bacteria" was calculated to determine a "hygiene index" of meat. This index has a moderate to strong correlation to bacterial loads obtained from culture ($p < 0.05$), specifically to *Pseudomonas* spp., LAB and total viable counts (TVCs). Knowledge of the proportions of hygiene and spoilage indicator bacteria obtained by NGS could help to determine the hygiene status even of (heat-) processed composite meat products for the first time, thus enhancing food quality assurance and consumer protection.

Contamination of meat with bacteria usually occurs during the slaughtering and cutting process. During transport and storage, meat can be microbially spoiled when temperatures are too high or when it is stored at chilled temperatures for too long. Meat spoilage is defined as a change in color and the production of off-flavors, mucus, and exudates that result in unacceptable sensory and organoleptic properties (Zhu et al., 2022). These changes may not be perceived by customers, when meat is only "lightly" spoiled, or spoiled meat was used to produce spiced meat products (such as cooked sausages). Changes in sensory usually strongly correlate with loads of spoilage microorganisms in meat (e.g., *Pseudomonas* spp., and LAB) (Papadopoulou et al., 2013). However, according to the EU Regulation (EC) No 178/2002, Article 14, meat is considered unfit for human consumption and shall not be placed on the market if it is contaminated with high a load of bacteria, even if it does not show any sensory change.

Food authorities in the European Union (EU) and in many other countries had established critical values for microbial contamination in various meat products. In terms of process hygiene criteria in the EU, improvements in production hygiene and in the selection and/or origin of raw materials are required if contamination in the product (at production site) is higher than the critical values, i.e., $>6.5 \log_{10}$ cfu/g for TVC, >5.0 for *Enterobacteriaceae,* and >2.5 for *E. coli* in minced meat (European Commission Regulation (EC) No 2073/2005). For example, in Germany, the critical values mentioned are set for minced meat and fresh pork sold at retail, and in addition,

* Corresponding author.
E-mail address: Samart.Dorn-In@vetmeduni.ac.at (S. Dorn-In).

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the critical values for further bacteria are set for these products, i.e., 6.0 $\log_{10}$ cfu/g for *Pseudomonas* spp. and 3.5 for coagulase-positive *Staphylococcus* spp. (DGHM, 2018).

To verify these values, plate counting is usually applied as a standard method. In the meantime, culture-independent methods (PCR, NGS-based methods) are also increasingly used in the food sector. While qPCR can be used to quantify meat microbiota (Bahlinger et al., 2021), next-generation sequencing (NGS) is commonly used to determine the microbial diversity and their proportion in food products, such as in chicken meat stored under refrigerated temperatures (Dourou et al., 2021), in chilled, vacuum-packaged ostrich meat (Juszczuk-Kubiak et al., 2021), or in chilled, vacuum-packed ham (Piotrowska-Cyplik et al., 2017).

To analyze the microbial diversity in fresh and spoiled meat, almost all NGS-based studies investigated the samples at two time points, i.e., fresh meat and after being stored for a certain duration. The most common spoilage microorganisms found in meat stored at chilled temperatures determined by NGS are *Pseudomonas* spp., LAB, *Enterobacteriaceae*, *Brochothrix thermosphacta,* and *Photobacterium* spp. (Dourou et al., 2021; Juszczuk-Kubiak et al., 2021). Growth of meat microbiota is a dynamic process. Spoilage of meat can be a result of the contiguous growth of different bacteria; thus, quantification of only single bacterial groups may not be sufficient to judge the hygiene status of meat. Therefore, the present study aimed to apply culture methods, NGS, and 16S rRNA gene analysis in parallel to determine the dynamic changes in amounts/proportions of meat microbiota in fresh meat and after storing the same meat for 6, 13, and 20 days (at 4°C) and for 1, 2, 3, and 6 days (at 22°C). Knowledge obtained from this study can be used to become acquainted with the product-specific proportion (not only the absolute amount) of certain bacterial species that may be used to assess the hygiene status of meat.

Material and methods

Meat samples and storage conditions. Fresh meat samples (n = 12 biological replications, approximately 2 kg each) from pork shoulder were collected (within 24 h after slaughtering) on different days from five butchers in Southern Bavaria, Germany (Bahlinger et al., 2021). All materials used for cutting meat and the plastic boxes used to pack meat were disinfected with 70% ethanol and left dried under UV light in a laminar flow cabinet for 20 min. Meat cutting was performed in the same laminar flow. Each pork sample was cut into small pieces (approximately 5 by 5 by 5 cm) under this sterile condition, manually mixed together using gloved hands to assure that indigenous microbiota thoroughly distributed on surface of cut meat. Then, they were equally separated into two subsamples, placed in two sterile plastic boxes (L-W-H, 18 by 11 by 8 cm) and loosely covered with their tops to assure an exchange of the air between inside and outside of the boxes. These two boxes were simultaneously stored at two temperatures, to exemplarily demonstrate the growth (determined by culture) and the proportion (determined by NGS) of meat microbiota in fresh meat and in meat stored at the maximum recommended storage temperature (4°C, but for a long period), and at an extreme storage temperature (at room temperature, 22°C) to imitate temperature abuses.

Sampling and investigation of fresh meat were carried out on the day of purchase (day 0) and of stored meat on days 6, 13, and 20 (4°C) and on days 1, 2, 3, and 6 (22°C). Twelve subsamples were investigated at each time-temperature point, altogether 96 subsamples. Stored meat samples were taken from the surface as well as from the lower part that did not have direct contact to the atmosphere. Ten grams of the meat sample and 90 mL peptone water (Merck) were homogenized for 90 s using a laboratory stomacher. Subsequently, the homogenates (dilution 10^{-1}) were proceeded to plate counting and DNA extraction. In addition, of all meat subsamples (n = 8) from the first repetition, one each was picked up at day 0 (fresh meat), days 6, 11, and 20 (4°C), and days 1, 2, 3, and 6 (22°C), were exemplarily investigated for the pH changes, and by two persons for the changes in odor, color, consistency, gas (bubble formation on meat surface or drip), and drip loss. While the pH of meat was determined using Laboratory pH Meter (Type 766, Knick), the other sensory parameters were scored or described as stated in Table 6. Since the assessment of the sensory deviations was not the focus of the present work, but rather the development of a culture-independent method, e.g., for composite or heat-processed meat products in which the sensory technology reaches its limits, the remaining meat samples were not further investigated for these characteristics.

Plate counting. Each meat homogenate (dilution 10^{-1}) was serially diluted up to 10^{-8} (for stored meat samples). After that, 100 μL of three appropriate dilutions were spread on six different selective agars. In detail, Plate Count Agar (PCA, Merck) was used for mesophilic aerobic bacteria (total viable counts, TVC), DeMan Rogosa Sharpe Agar (MRS, Sifin) for LAB, Violet-Red-Bile-Dextrose Agar (VRBD; Merck) for *Enterobacteriaceae*, Cetrimid-Fucidin-Cephalothin Agar (CFC; Oxoid) for *Pseudomonas* spp., Streptomycin-Thallium-Acetate Agar (STA; Oxoid) for *B. thermosphacta,* and Baird-Parker Agar (BP; VWR International and Merck) for *Staphylococcus* spp. Each selective agar was incubated under the following conditions: aerobically at 30°C for 72 h for PCA and MRS, anaerobically at 30°C for 48 h for VRBD, aerobically at 25°C for 48 h for CFC and STA, and aerobically at 37°C for 48 h for BP. Then, colony counting of all agar plates was performed.

DNA extraction. The DNA extraction of meat homogenates (dilution 10^{-1}) was performed using the Isolate II Genomic DNA Kit (Bioline). A total of 200 μL of sample suspension was first mixed with 10 μL of lysozyme (20 mg/mL in 10 mM Tris-HCl, pH: 8.0; final concentration = 1 mg/mL) and incubated for 30 min at 37°C. The following steps from prelysis with buffer GL, protein digestion with enzyme proteinase K to the extraction of DNA were performed according to the instruction manual of the DNA extraction kit. Each meat subsample was extracted in duplicate (two technical replicates); both DNA extracts were pooled and then proceeded to Amplicon PCR and NGS.

Amplicon primer selection. Three Amplicon primer pairs (see Table 1) were tested, No. 1 and 2 were developed in this study, while No. 3 was recommended by Illumina for the Illumina MiSeq system (Illumina, 2013). All these primer pairs target V3–V4 region of the 16S rRNA gene. Fragment sizes of PCR products of primer pair No. 1, 2, and 3 (including primer and adapter) were approximately 541

Table 1
Sequences of the tested primer pairs for Amplicon PCR (Adapter - **Primer**)

No.	Primer pair	Sequence (direction 5' - 3')	Reference for primer
1	335F 789R	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – **CADACTCCTACGGGAGGCAG** GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – **ATCCTGTTTGCTMCCCACGC**	Dorn In. et al., 2015 (modified from 335F and 769R)
2	335F 803R	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – **CADACTCCTACGGGAGGCAG** GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG - **CTACCAGGGTATCTAATCCTGT**	Dorn-In et al., 2015 (modified from 335F and 783R)
3	341F 805R	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – **CCTACGGGNGGCWGCAG** GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – **GACTACHVGGGTATCTAATCC**	Illumina, 2013; Klindworth et al., 2013

bp, 557 bp, and 552 bp, respectively. All three primer pairs (with adapter) were tested for their efficiencies to amplify DNA in meat samples ($n = 16$) containing different bacterial loads (i.e., low in fresh meat, moderate, and high in stored meat). The type of meat samples and their bacterial loads, determined using culture methods, are described in the legend of Figure 2. The concentration of PCR compositions and the Amplicon PCR protocol were modified from that described by Illumina (2013).

Each Amplicon PCR reaction contained 0.25 μL of each 20 μM primer, 12.5 μL of 2x KAPA HiFi HotStart Ready Mix (Roche) and 2.5 μL of DNA template. The volume was filled up to 25 μL with 9.5 μL H_2O. The amount of DNA template in each extract used for Amplicon PCR was variable between very low (or not detectable in some fresh meat samples) and 4.5 ng/μL (in stored meat), measured using a NanoPhotometer (type NP80 mobile, Implen GmbH). The Amplicon PCR runs were carried out in the Biorad CFX96 Touch™ (Biorad).

Different PCR protocols were primarily tested (e.g., with different annealing temperatures and numbers of PCR cycles) until a good amplification result was obtained from at least one primer pair. Based on the results, the chosen PCR protocol started with initial denaturation at a temperature of 95°C for 3 min, followed by 35 cycles in a series of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, and final elongation at 72°C for 5 min. Fragment sizes of the PCR products were checked by gel electrophoresis using 2.0% Certified Molecular Biology Agarose (Biorad), running at 140 V for 60 min in 0.5% TBE buffer. The Hyper Ladder 50 bp (Bioline) was used as a fragment size marker. The gel was stained with ethidium bromide, and DNA fragments were visualized by ultraviolet transillumination.

Next-generation sequencing. The Amplicon PCR with the selected primer pair was applied for all 95 DNA extracts of fresh and stored meat samples (due to technical disruption, one DNA extract could not have proceeded to this step). The subsequent PCR product purification using AMPure XP beads (Illumina) followed the instructions of the producer. After that, the amounts of purified DNA in samples were determined using a NanoPhotometer.

For the following Index PCR, a 10 ng/μL concentration of purified PCR product of each sample was required. To perform Index PCR, the Nextera XT Index Kit (Illumina) was used, and 95 individual index-primer pairs were required. The concentration of the components and the Index PCR protocol followed the manual instruction of the producer. The purification of Index PCR products using AMPure XP beads was carried out as described for the purification of Amplicon PCR products.

The quality control of the purified Index PCR products was performed using a Bioanalyzer (Agilent), followed by the fluorometric quantification and the pooling of the Index PCR products. The subsequent sequencing was performed in a HiSeq1500 from Ilumina. The pooled sample was dropped in a single Rapid Flow Cell, in which 150 million reads were produced. Since the target DNA fragment was approximately 450 bp (without adapter and primer), paired-end sequencing with a read fragment of 250 bp was performed. All these steps were conducted in the Laboratory of Functional Genome Analysis (LAFUGA) of the Gene Center Munich.

16S rRNA amplicon analysis. The obtained reads were preprocessed with Cutadapt (Martin, 2011), removing primer sequences and discarding reads that were smaller than 200 bases after primer removal or did not have the primer sequence at all. Reads were then processed in R with DADA2 (Callahan et al., 2016). Forward and reverse reads were merged to reconstruct the full target amplicons, Amplicon Sequence Variants (ASVs) were inferred, and occurrences in each sample were counted. To assign taxonomy to the ASVs, the package DECIPHER (Murali et al., 2018) was used with the SILVA SSU (release 138) as reference database (Yilmaz et al., 2014).

The ASVs were then sorted to order level according to their microbial taxonomy. Inspired by Whittaker (1972), gamma, alpha, and beta diversity of the bacterial orders in fresh and stored pork samples were analyzed. Gamma diversity is the total number of bacterial orders in all samples. Alpha diversity is the total number of bacterial orders at each time-temperature point, and beta diversity is the differentiation of bacterial orders between two time-temperature points, meaning the total number of species that are unique to each of the time-temperature points being compared.

In the next step, sequences belonging to the same bacterial order were further sorted according to their family or genus. The most frequently occurring sequences in the order/family/genus (with an incidence of ≥1.0% of total sequence within the group) were subjected to phylogenetic analysis using an online program (http://www.phylogeny.fr/simple_phylogeny.cgi, France) to obtain information about their similarity. After that, individual sequences were aligned with the sequences available in the GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi, NCBI, the United States) to identify/confirm its species or genus.

Hygiene index and statistical analysis. In this study, the hygiene index refers to the ratio value of loads of certain bacterial species or groups that are relevant to the hygiene status and/or the spoilage of meat. A hygiene index is used to judge whether meat is acceptable for human consumption. Data generated by NGS were used to calculate a hygiene index as follows:

$$\text{Hygiene index}(\%) = \frac{\text{Number of reads of hygiene indicator bacteria}}{\text{Number of reads of hygiene and spoilage indicator bacteria}} \times 100$$

A complex bacterial community generally contributes to the spoilage of meat, the hygiene and spoilage indicator bacteria could be a group of bacteria that includes different species, genera, families, and orders. The requirement for candidates for hygiene indicators was that they had to be found in all fresh meat samples but less or not detected in stored meat, while spoilage indicators had to be found in a high proportion in all stored meat samples. No limitation of abundance (minimum or maximum) of both indicators was set for this purpose.

Afterward, the correlation between hygiene index and the amount of spoilage microorganisms obtained from the culture was analyzed using the Pearson correlation coefficient (r, Microsoft Excel, 2016). The strength of the correlation was interpreted as described by Evans (1996), $r = 0–0.19$ is regarded as very weak, 0.20–0.39 as weak, 0.40–0.59 as moderate, 0.60–0.79 as strong, and 0.8–1.0 as a very strong correlation. Additionally, the p-value as described by Dorn-In et al. (2023) was calculated based on two-tailed t test analysis to evaluate if the correlation was statistically significant ($p < 0.05$).

To analyze whether bacterial loads determined by culture and whether proportions of detected bacterial groups determined by NGS in fresh meat (day 0) and in stored meat at different time-temperature points are statistically significantly different, a two-tailed t test analysis (Microsoft Excel, 2020) was applied.

Results and discussion

Plate counting. Figure 1 shows the growth of six microorganisms on fresh and stored meat samples ($n = 12$ at each time-temperature point) determined by culture and results of statistical analysis (t test) comparing between bacterial loads in fresh meat (day 0) and stored meat at different time-temperature points (see also Table S1, supplementary data). Initial contamination of TVC was between 2.0 and 5.8 $\log_{10}$ cfu/g. The TVC, *Pseudomonas* spp., LAB, and *B. thermosphacta* statistically significantly increased ($p < 0.05$) at day 6 (4°C), and day 1 (22°C), while *Enterobacteriaceae* and *Staphylococcus* spp. at day 13 (4°C), and day 1 (22°C). TVC, *Pseudomonas* spp., and LAB grew consistently, reaching their maximum population on day 13 at 4°C and already on days 1–2 at 22°C, depending on the initial contamination

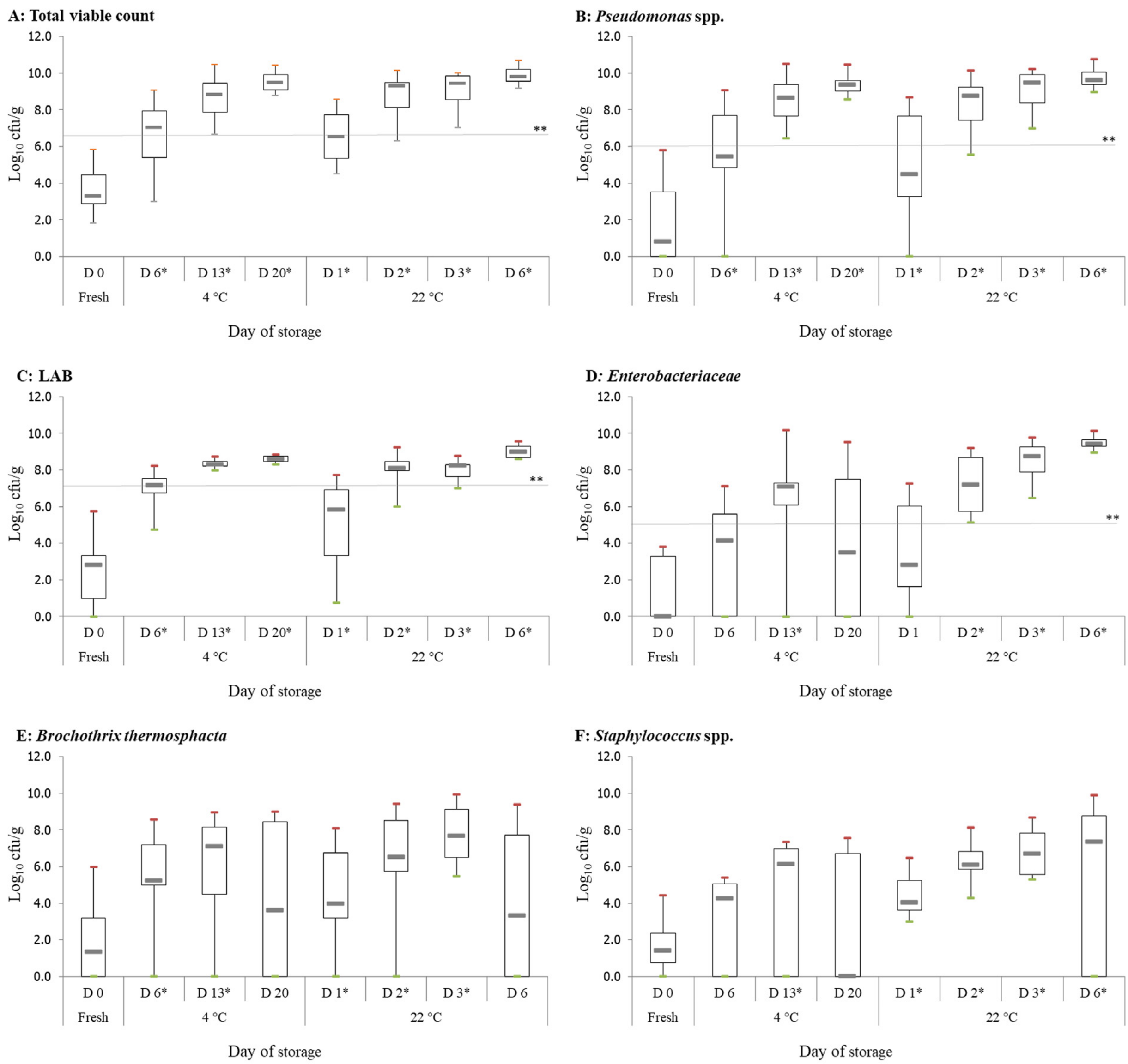

Figure 1. Box whisker plots (Boxplots) showing growth behavior of six microbial groups (A – F) determined by plate counting ($\log_{10}$ cfu/g) on meat (n = 12) stored at 4°C for 20 days and 22°C for six days. Lines above and below the box plots are the maximal and minimal values of the dataset. The boxes indicate ranges between the median of the lower and upper quartiles of the dataset. The bars within the boxes are the median values of all samples. * Bacterial loads at time-temperature points are statistically significantly different (p < 0.5) to loads in fresh meat (day 0). ** Horizontal lines mark the microbial critical values in fresh pork sold at retail: 6.5, 6.0, and 5.0 $\log_{10}$ cfu/g for TVC, Pseudomonas spp., and Enterobacteriaceae, respectively (European Commission Regulation (EC) No 2073/2005; DGHM, 2018), and 7.0 $\log_{10}$ cfu/cm^2 for LAB (EFSA, 2016).

level. Enterobacterales and *Staphylococcus* spp. grew better at 22°C than at 4°C and reached the maximum population on day 3. For *B. thermosphacta*, after reaching the maximum population on day 13 at 4°C, and on day 3 at 22°C, they could not be isolated in some samples using the same three dilutions as on the previous investigation day. Similar findings were also observed in *Staphylococcus* spp. and Enterobacterales. This indicates that after growth stagnation, these bacterial groups were less active or probably died. Based on the microbiological results and the critical values shown in Figure 1, pork samples were considered "unfit for human consumption" if bacterial loads exceeded the critical values set by authority in Germany (see legend of Fig. 1), in almost all cases when the microbiota started to reach their maximum population, namely not later than day 13 and day 2, when they were aerobically stored at 4°C and 22°C, respectively. This corresponds to the description in §2 and §3 of the German Food Hygiene Regulation (Lebensmittelhygiene-Verordnung – LMHV, 2007), and in Article 14 (Food safety requirements) of the EU Regulation (EC) No 178/2002 indicating that meat is unfit for human consumption for reasons of contamination, whether by extraneous matter or otherwise, or through putrefaction, deterioration or decay and thus shall not be placed on the market.

Amplicon primer selection. Nine of ten fresh meat samples contained a low quantity of DNA. This was a criterion for why the comparison of the efficiency of amplicon primer pairs was performed. In case the primer pair has a low efficiency to amplify DNA from sample with low bacterial loads, biased results may occur. Two annealing temperatures (55 vs. 57°C) were tested, and PCR cycles were increased from 25 to 35 cycles (data not shown). All these steps resulted in the decision of using the PCR protocol as described in the section material and methods.

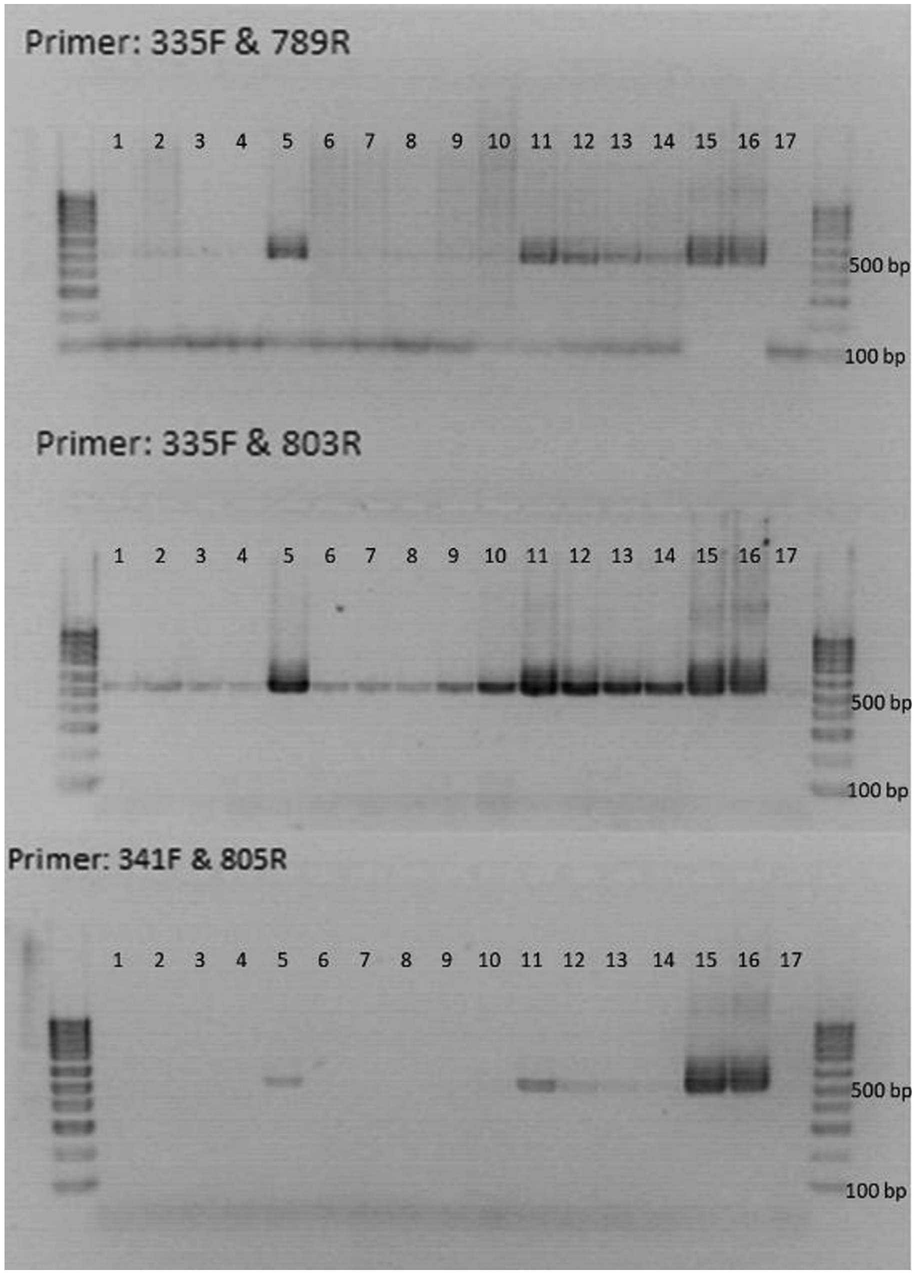

Figure 2. Agarose gel of PCR products of Amplicon primer pair No. 1 – 3: DNA-ladder 100 bp, **1 -12** = meat samples from day 0 (Approaches 1 - 12), **13 - 14** = meat from day 1, incubated at 22°C (Approaches 1 & 4), **15 - 16** = meat from day 6, incubated at 22°C (Approaches 1 & 4), 17 = No Template Control (H_2O). Bacterial loads in meat samples 1 - 16 are 3.0, 2.9, 3.5, 1.8, 5.6, 2.9, 2.1, 3.5, 4.1, 2.8, 5.7, 5.8, 4.7, 6.3, 3.0, and 7.1 $\log_{10}$ cfu/g, respectively.

Figure 2 shows the DNA bands in the agarose gels resulting from the PCR amplification of three amplicon primer pairs, targeting the 16S rRNA gene. PCR products of primer pair No. 1 (335F & 789R) showed the high intensity of a primer-dimer (fragment size approximately 100 bp), especially for samples with low bacterial loads. Primer pair No. 2 (335F & 803R) could amplify sufficient DNA from all fresh meat samples (day 0). Primer No. 3 (341F & 805R) turned out to be not sensitive enough for samples containing low DNA concentrations, thus probably did not produce sufficient PCR products for further steps of NGS. Based on these results, primer pair No. 2 was selected for the amplicon PCR of all 95 DNA extracts of meat samples.

16S rRNA amplicon analysis. A total of 4,378 Amplicon Sequence Variants (ASVs) comprising 49,009,791 amplicon reads were determined. These ASVs belong to 57 bacterial orders, where only 16 orders contain ≥50,000 amplicon reads (corresponding to ≥0.1% of total reads). Table 2 presents the proportions of the bacterial reads after storage at 4°C and 20°C. The bacterial orders were sorted according to the proportion (in %) of their corresponding reads in fresh meat. Table 3 demonstrates alpha and beta diversities of bacterial orders in fresh and in stored meat samples and Table S2 (see supplementary data) shows the number of reads from all bacterial orders (n = 57, gamma-diversity) in fresh or in stored meat samples. It clearly demonstrates the development of meat microbiota and their proportions during storage. In fresh meat (day 0), 51 bacterial orders (alpha-diversity) were found, then the number of orders continually decreased after meat samples were stored at both temperatures (Table 3). Minor reads belonging to seven bacterial orders were found in stored meat samples

Table 2
Percentage of amplicon reads belonging to bacterial orders that were found in fresh meat (day 0), meat stored at 4°C (for 6, 13, and 20 days) and 22°C (for 1, 2, 3, and 6 days); $n = 12$ at each time-temperature point (exception: storage at 4°C for 13 days, $n = 11$, due to technical disruption)

Bacterial order	Fresh	4°C			22°C				Total
	D0	D6	D13	D20	D1	D2	D3	D6	
Pseudomonadales	45.39	26.98	35.48	49.68	32.48	47.65	39.23	27.96	37.93
Enterobacterales	12.90	1.02	1.32	2.39	5.50	11.73	26.32	37.83	23.13
Lactobacillales	10.70	24.78	27.59	21.51	33.92	22.43	21.01	21.97	12.23
Vibrionales (*Photobacterium*)	7.61	34.80	25.75	17.00	0.32	0.02	0	0	10.78
Staphylococcales	4.30	0.24	0	0.01	15.38	5.47	2.82	2.15	3.89
Bacillales (*Brochothrix*)	4.03	10.74	9.10	8.30	8.65	5.22	1.06	0.46	6.02
Propionibacteriales	2.12	0.04	0	0	0.12	0	0	0	0.28
Bacillales (*Kurthia*)	1.57	0.39	0	0	2.56	5.40	5.81	2.14	2.20
Clostridiales	1.25	0.06	0	0.09	0.32	1.26	0.15	1.55	0.58
Burkholderiales	1.10	0.04	0.02	0	0.04	0.01	0.03	0.50	0.21
Xanthomonadales	0.98	0.01	0	0	0.04	0.01	0.01	0.02	0.13
Micrococcales	0.95	0.02	0	0	0.07	0	0	0	0.13
Rhizobiales	0.85	0	0	0	0.02	0	0	0	0.11
Flavobacteriales	0.60	0.05	0.31	0.77	0.03	0.41	2.27	1.37	0.71
Peptostreptococcales	0.20	0.02	0	0	0	0.13	0.16	3.93	0.56
Aeromonadales	0.03	0	0.14	0.01	0.02	0.20	1.08	0.04	0.18
Other orders	5.44	0.81	0.29	0.23	0.52	0.08	0.06	0.09	0.93

Table 3
Diversity of bacterial orders in fresh and stored pork samples ($n = 12$ samples for each time-temperature point)

Diversity of bacterial	Fresh	22°C				4°C		
order	D0	D1	D2	D3	D6	D6	D13	D20
Gamma diversity	– 57 –							
Alpha diversity	51	34	22	24	19	29	17	15
Beta diversity								
D0 (fresh meat)		23	31	27	31	28	34	36
D1, 22°C			16	12	17	17	17	19
D2, 22°C				6	9	11	9	7
D3, 22°C					9	7	9	9
D6, 22°C						14	4	4
D6, 4°C							16	14
D13, 4°C								4

from day 1 (22°C) and day 6 (4°C), but not in fresh meat. Similar to the results obtained from Dourou et al. (2021), the diversity of bacterial groups in fresh meat (chicken) was higher than in spoiled meat. It has to be considered that the proportion of reads resulting from NGS analysis referred to the relative abundance of bacterial sequences determined at different time-temperature points of fresh and stored meat. Thus, this relative abundance is not directly linked to the growth of bacteria, when the proportion of reads of certain bacterial groups (species, genus, or order) increased. In fresh meat, reads belonging to Pseudomonadales were found in a relatively high proportion, followed at a great distance by Enterobacterales, Lactobacillales, Vibriolales (*Photobacterium* spp.), Staphylococcales, and Bacillales (Table 2). Altogether, reads belonging to Pseudomonadales remained in a high proportion in all samples, proportions of Lactobacillales and Bacillales increased considerably in meat stored at both temperatures. While Vibrionales were detected in a high proportion only in meat stored at 4°C, proportions of Enterobacterales and Staphylococcales increased only in meat stored at 22°C. It was observed that proportions of Staphylococcales continually decreased in meat stored at 4°C for 2, 3, and 6 days, respectively. This result contradicted the results obtained from culture, since *Staphylococcus* spp. and Enterobacterales increased continually in some samples that were stored at 4°C (see Fig. 1).

Based on the results presented in Table 2, the ASVs that share ≥0.1% of the reads belonging to the same bacterial order were further analyzed down to the genus level. The boxplots shown in Figure 3 demonstrate the share of sequences of the most frequently found bacterial orders or genera (in % compared to the total of generated ASVs) and results of *t* test analysis of bacterial proportions that were detected in fresh meat and in stored meat (see also Table S3, supplementary data). The numbers of reads belonging to the same bacterial group are very variable between samples. Altogether, proportions of *Pseudomonas* spp. in meat stored at 22°C are statistically significantly lower than the proportion found in fresh meat ($p < 0.5$) Similar results are observed for Bacillales and Enterobacterales in meat stored at 4°C. In all stored meat samples, read proportions of *Staphylococcus* spp. are statistically significantly lower than in fresh meat. For the other bacterial groups, read proportions detected in stored meat on some days were statistically significantly higher than in fresh meat samples ($p < 0.5$).

Almost all NGS studies classify the detected microorganisms down to genus level, except when the sequence is distinguishable to species level such as *B. thermosphacta* (Juszczuk-Kubiak et al., 2021; Piotrowska-Cyplik et al., 2017). This is sufficient to obtain an overview of the microbial diversity in samples. However, different species within the same genus may show different growth rates in meat samples, which can be attributed to different susceptibilities to environmental changes and to inhibition factors produced by other microorganisms. Since this study attempted to find the exact bacterial species that can be used as candidates to generate hygiene index, further tools, namely phylogenetic analysis and sequence alignment with the sequences provided in GenBank, were additionally applied (see supplementary documents, Fig. S1 and Table S4). The bar graphics in Figure 4 demonstrate the identified species of the ASVs and their proportion (in %) within the same microbial group as shown in Figure 3. Similar to the conclusion of Fadeev et al. (2021), the region V3 - V4 of the 16S rRNA can definitely be used to differentiate bacteria

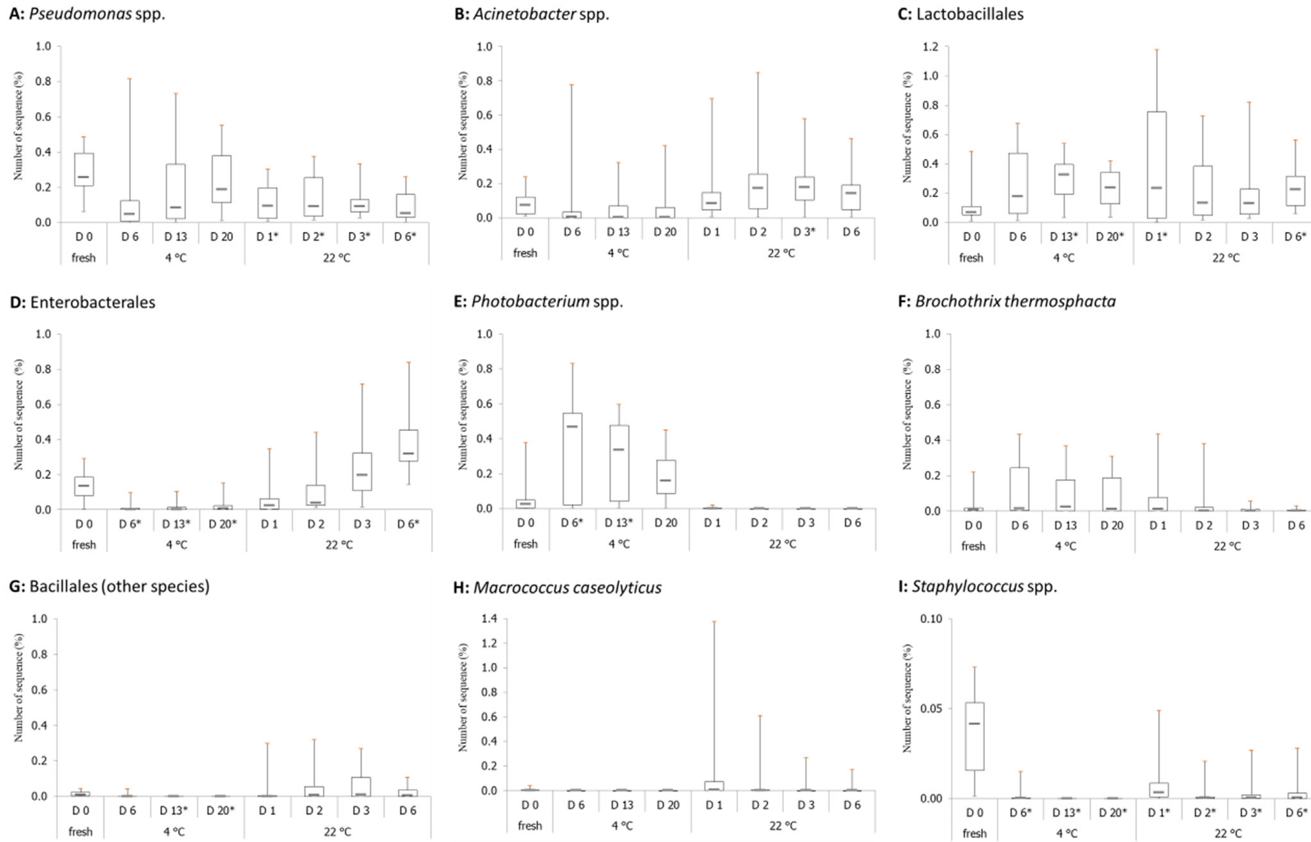

Figure 3. % of reads belonging to eight microbial groups (A to I), sorted according to the day of storage (D) and to the temperature of incubation, $n = 12$ at each time-temperature point (exception: at 4°C for 13 days, $n = 11$). * Bacterial proportions at time-temperature points are statistically significantly different ($p < 0.5$) to proportions in fresh meat (day 0)

down to genus level. In this study, bacteria within five genera, i.e., *Acinetobacter* spp., *Brochothrix* spp., *Macrococcus* spp., *Staphylococcus* spp., and *Escherichia* spp., were relatively different from each other, allowing an identification down to species level (Fig. 4, B, F, H, I, and Fig. S1). However, this region cannot differentiate species of the genera *Pseudomonas* spp., *Lactococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp., *Carnobacterium* spp., *Serratia* spp., *Yersinia* spp., *Photobacterium* spp., and *Kurthia* spp. (see Fig. 3, A, C, D, E, G, and Fig. S1).

As shown in Figure 4, the most important bacteria that contaminated fresh pork were *Pseudomonas* spp. and *Acinetobacter* spp. (order Pseudomonadales), *Lactococcus* spp., *Carnobacterium* spp., *Lactobacillus* spp. (order Lactobacillales), *Serratia* spp., *Hafnia* spp., *Proteus* spp. (order Enterobacterales), *B. thermosphacta* (order Bacillales), and *Photobacterium* spp. (order Vibrionales). These bacteria were again found in high proportions in stored meat, thus indicating their importance as spoilage microorganisms (e.g., Dourou et al., 2021; Juszczuk-Kubiak et al., 2021). The species *Escherichia coli*, *Staphylococcus epidermidis,* and *S. capitis/S. caprae* were also found in a high proportion in almost all fresh meat samples, but continually decreased until not detectable in some stored meat samples.

In summary, *Pseudomonas* spp. and LAB were found in a high amount/proportion both in fresh and in stored pork. For Enterobacterales, *B. thermosphacta,* and *Staphylococcus* spp., growth in some samples was detected by culture but very low read proportions of these bacterial groups were found by NGS. In this context, using NGS with a universal primer pair may lead to an underestimation of certain microorganisms, when classical spoilage bacteria are overrepresented. As shown in the present study, the ostensible absence of *E. coli*, *S. epidermidis,* and *S. capitis/S. caprae* in stored meat investigated by NGS does not necessarily mean that these bacteria are not present. To verify these results, further methods such as culture and probably species-specific qPCR are still required. On the other hand, a relatively high proportion of *E. coli*, *S. epidermidis,* and *S. capitis/S. caprae* was found in all fresh meat samples, but not or very less in stored meat (determined by NGS), revealing that these bacterial species could be further investigated for being used as markers for the hygiene of meat and meat products. However, their absolute amount must not exceed the critical contamination level set by relevant authorities.

For the other bacterial groups, reads belonging to *Acinetobacter* spp. and *Macrococcus* spp. were found in highly varying levels both in fresh and in stored meat. Due to this high variability, they are not considered as reliable spoilage indicators for meat and meat products. Similarly, not all meat samples were contaminated with *Photobacterium* spp. In this study, three of twelve pork samples were negative for these bacteria throughout the incubation period, determined by NGS. Therefore, using *Photobacterium* spp. as an indicator for spoiled meat, if at all, is only suitable to a limited extent.

Hygiene index and correlation to culture results. Since *Pseudomonas* spp. and LAB showed a constant proportion in almost all fresh and stored meat samples determined by NGS, they were chosen to be considered as spoilage indicator bacteria for the hygiene index analysis. The species *E. coli* and *S. epidermidis* were used as hygiene indicators, since they were detected in a high proportion in almost all fresh meat samples but less or not detected in stored meat. However, it has to be mentioned that due to the role of *E. coli* as an indicator of fecal contamination, its suitability as a hygiene marker (for this purpose) seems questionable. The hygiene index was categorized in three levels

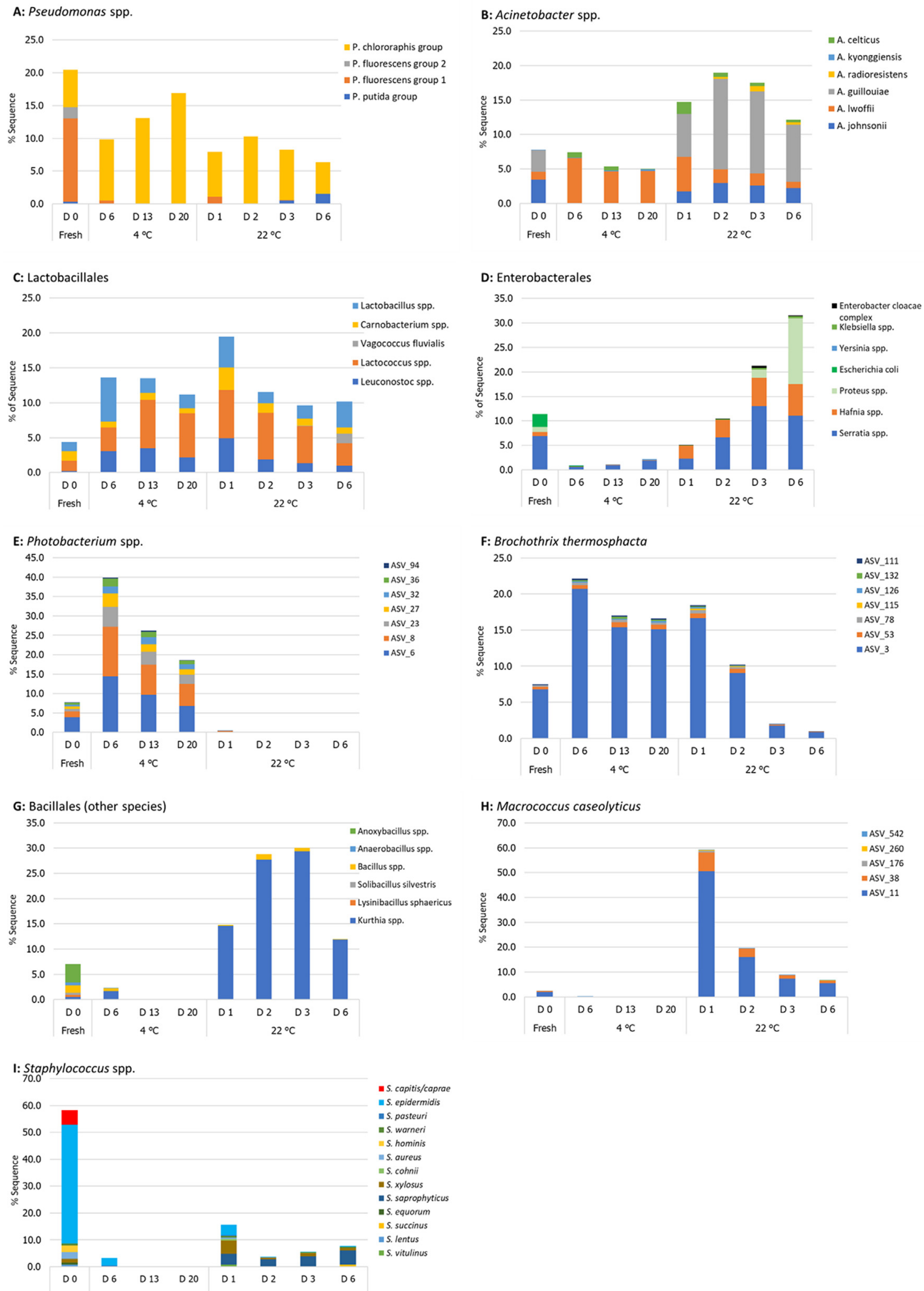

Figure 4. Species identification of the ASVs and % of their corresponding sequences (compared to the same microbial group as shown in Fig 3), $n = 12$ at each time-temperature point (exception: at 4°C for 13 days, $n = 11$)

(Table 4). Table S5 (see supplementary data) shows the $\log_{10}$ cfu/g obtained from culture, and the hygiene index value obtained from NGS in individual meat samples ($n = 96$).

Out of twelve fresh meat samples, ten showed a hygiene index value ≥1.0, indicating high hygienic status. Two fresh meat samples considered as suspicious (index values 0.1–0.9) contained a relatively

Table 4
Number of meat samples at each sampling time-temperature point (n = 12, except at 4°C for 13 days, n = 11) that are categorized according to their hygiene index value, i.e., ≥1 (acceptable), 0.1 – 0.9 (suspicious), and ≤0.1 (unacceptable)

Storage condition & sampling day		*E. coli* vs.						*S. epidermidis* vs.					
		LAB			*Pseudomonas*			LAB			*Pseudomonas*		
		≥1.0	0.1–0.9	< 0.1	≥1.0	0.1–0.9	< 0.1	≥1.0	0.1–0.9	< 0.1	≥1.0	0.1–0.9	< 0.1
Fresh	D0	10	2		10	2		10	2		10	2	
4°C	D6	2		10	5	1	6	2		10	5	1	6
	D13			11		1	10			11			11
	D20			12			12			12			12
22°C	D1	3	4	5	7	1	4	3	1	8	8		4
	D2	2	2	9	2	4	6		2	10		2	10
	D3	1	3	7	1	2	8		1	11		1	11
	D6	1	1	10	2	1	9		2	10		2	10

Table 5
Pearson correlation coefficient (r) between hygiene index and absolute amount of spoilage indicator bacteria (LAB, *Pseudomonas* spp. and total viable counts)

		Cultural results					
		LAB		*Pseudomonas* spp.		TVC	
		r	p value	r	p value	r	p value
NGS results (Index value)	*E. coli* vs.LAB	−0.567	2.1E−09	−0.513	1.0E−07	−0 .684	2.2E−14
	E. coli vs. *Pseudomonas*	−0.541	1.5E−08	−0.536	2.1E−08	−0.693	7.4E−15
	S. epidermidis vs. LAB	−0.521	6.2E−08	−0.526	4.3E−08	−0.704	1.7E−15
	S. epidermidis vs. *Pseudomonas*	−0.587	4.0E−10	−0.570	1.6E−09	−0.763	2.6E−19

high initial amount of TVC (>5.6 $\log_{10}$ cfu/g), although *Pseudomonas* spp. and LAB could not be cultured. Similarly, these bacterial groups were detected in low numbers using NGS. Table 4 shows the number of samples categorized in all index values at each sampling time point, and Table 5 shows Pearson correlation values of hygiene indices and values obtained by culture (LAB, *Pseudomonas* spp., and TVC). As shown in Table 5, there are moderate to strong (negative) correlations between the hygiene index generated using *E. coli* and *S. epidermidis* and the culture results of spoilage microorganisms (TVC, *Pseudomonas* spp., and LAB). These correlations were evaluated as statistically significant ($p < 0.05$). However, as mentioned in the previous paragraph, *E. coli* is probably not suitable to be used for this purpose, since it is an indicator for fecal contamination and for this reason, it should not be detected in fresh meat at all. Additionally, this bacterial species could still be detected in relatively high numbers in some samples (e.g., at days 3 and 6, 22°C, index value >1.0, see supplementary data Table S2). Therefore, it is more reasonable to use *S. epidermidis* as a hygiene indicator to generate a hygiene index.

Sensory changes are important spoilage characteristics and the most important indicators for consumers to assess the freshness of meat. In this context, data in Table 6 exemplarily show the changes of pH, odor, color, consistency, and drip loss and the load of cultured bacteria in pork sample No. 1. The initial bacterial contamination (fresh meat, day 0) was very low and no sensory abnormalities could be observed. After storage for 13 days at 4°C, bacterial loads exceeded the critical values given by European Commission Regulation (EC) No 2073/2005 and DGHM (2018). Additionally, this meat showed sensory deviations like slight odor change, moderate drip loss, and change in meat color. The microbial and sensory changes corresponded to the hygiene index value of this meat sample (<0.1, unacceptable). On the contrary, bacterial loads in meat stored at 22°C for 2 days were in acceptable ranges, but according to deviations in pH, odor, color, and drip loss, it would be considered as spoiled which corresponded to the hygiene index (<0.1) of this meat. However, sensory analysis such as applied for this sample cannot be applied in seasoned/heated meat products, and in these cases, the developed method can be helpful.

Conclusion

Changes in amount/proportion of meat microbiota in fresh pork samples (biological replicates, n = 12) on the day of purchasing (fresh) and after storing them for 6, 13, and 20 days (at 4°C) and for 1, 2, 3, and 6 days (at 22°C) were analyzed using culture and NGS methods. Independent of sensory deviations, meat can be defined as unfit for human consumption if amounts of certain bacterial groups (TVC, LAB, *Pseudomonas* spp., and *Enterobacteriaceae*) exceed the legal critical values (see Fig. 1). In this context and under investigated conditions in this study, all pork samples stored at 4°C for 13 days and at 22°C for 2 days were judged as unfit for human consumption. However, some meat samples fell into this category earlier if they had a high level of initial contamination. Using the NGS method with a newly developed primer pair targeting the region V3 – V4 of the 16S rRNA gene, 4,378 Amplicon Sequence Variants (ASVs) comprising 49,009,791 amplicon reads of 57 bacterial orders were generated. The diversity of meat microbiota continually decreased after the pork meat was stored. According to culture and NGS results, we defined lactic acid bacteria (LAB) and *Pseudomonas* spp. as "spoilage indicator bacteria". Based on NGS data, *Staphylococcus epidermidis* was defined as "hygiene indicator bacteria", since it was detected in a relatively high proportion in all fresh meat samples but to a lesser extent or even not in stored/spoiled meat.

The sequence proportion between hygiene and spoilage indicator bacteria was evaluated to generate a "hygiene index". The index values were categorized in three levels, i.e., ≥1.0 (acceptable), 0.1–0.9 (suspicious), and ≤0.1 (unacceptable). This index shows a moderate to strong correlation to loads of *Pseudomonas* spp., LAB and TVC obtained from culture ($p < 0.05$). It has to be addressed that the study was conducted under laboratory conditions with only two tested storage temperatures and the bacterial composition on pork can depend on the region that the pork samples were obtained (in this study in the Munich area, Germany). For other meat types, storage temperatures and additional factors, the results may be variable. Nevertheless, the generated hygiene index might help to analyze if relevant meat products (e.g., seasoned and/or heated products) contain meat (in this

Table 6
Pork sample No. 1, meat characteristics, culturing results, and calculated hygiene index

Storage condition & sampling day		Meat characteristics						Culture (cfu/g)[5]						Hygiene index *E. coli* vs..		Hygiene index *S. epidermidis* vs	
		pH	Firmness[1]	Odor[2]	Drip[3]	Gas[4]	Color	TVC	Pseu	LAB	EB	Bro	Stap	LAB	Pseu	LAB	Pseu
Fresh	D 0	5.33	1	1	1	1	dark red	3.0	< 2.0	3.2	< 2.0	< 2.0	< 2.0	26.93	7.22	40.68	12.65
4°C	D 6	5.33	1	2	1	1	bright red	3.0	4.7	6.6	4.7	5.0	4.0	0.01	0.60	0.01	0.53
	D 13	5.34	1	2	3	1	bright red, gray and white spot	9.0	9.0	8.3	6.1	7.1	6.2	0.00	0.00	0.00	0.00
	D 20	5.35	2	2	3	1	gray-bright red, green for some part	9.8	9.9	8.7	7.2	7.2	6.6	0.00	0.00	0.00	0.00
22°C	D 1	5.13	1	2	2	1	pale red, some parts were pale gray	4.7	< 2.0	5.4	2.3	3.0	3.2	0.79	10.16	0.24	3.33
	D 2	5.08	2	2	3	1	pale red, some parts were pale gray	6.3	5.6	6.0	5.6	5.3	5.3	0.00	0.23	0.00	0.00
	D 3	5.18	2	3	4	2	pale red - gray, some points were green	7.0	7.0	7.0	7.0	6.1	5.6	0.00	0.00	0.00	0.02
	D 6	5.67	3	4	4	3	dark red to green	9.5	9.4	8.6	9.4	6.6	5.3	0.00	0.00	0.00	0.05

[1] **Firmness and Texture**
1: firm-elastic (standard)
2: slightly soft
3: loose, loss of texture

[2] **Odor**
1: fresh, almost odorless
2: slight deviation
3: distinct deviation
4: spoilage odor

[3] **Drip loss**
1: no drip loss
2: small amount
3: moderately
4: large amount

[4] **Gas (bubble formation)**
1: none
2: 1 - 5 gas bubbles
3: 6 - 20 gas bubbles

[5] **Culture**
TVC: Total viable counts
Pseu: *Pseudomonas*
LAB. Lactic acid bacteria
EB: *Enterobacteriaceae*
Bro: *B. thermosphacta*
Stap: *Staphylococcus* spp.

study, pork) that was unfit for human consumption regarding high loads of bacterial contamination, where the sensory deviation of products may be masked by spices and therefore not be perceived by consumers. Thus, it can be used as a supporting tool to uncover food fraud, and to enhance the security status of food quality, food safety, and consumer protection.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfp.2023.100212.

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