Article

Evaluation of the Spoilage-Related Bacterial Profiles of Vacuum-Packaged Chilled Ostrich Meat by Next-Generation DNA Sequencing Approach

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Abstract: Monitoring the development of the bacterial community in packaged raw meat refrigerated until two weeks is important for identifying the spoilage-related bacteria, preventing meat putrefaction, and prolong the shelf life. This study aimed to evaluate the influence of vacuum-packaging (VP) on the development of spoilage-related bacterial profiles in chilled ostrich meat among three manufacturing batches produced in different periods by using culture-dependent and 16S rDNA amplicon sequencing. Similar to the culture-dependent method, 16S rDNA sequencing showed that *Photobacterium* was the most prevalent genus detected in VP ostrich meat after 14 days of cold storage. The second-largest group was the population of lactic acid bacteria (LAB), mainly dominated by *Carnobacteriaceae* including *Carnobacterium* spp. and *Lactobacillaceae* with *Lactobacillus* spp. Our results suggest that these taxa could contribute to spoilage of VP ostrich meat and shorten its shelf life, especially *Photobacterium* spp., which is considered as a potential meat spoiler.

Keywords: ostrich meat; vacuum packaging; 16S rDNA sequencing; bacterial community

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1. Introduction

In Poland, ostrich (*Struthio camelus*) breeding was started in 2000, and currently, ostrich meat production is one of the important segments of the food industry, mostly focused on export to the EU. Ostrich meat is one of the most popular choices in western societies because it is frequently described as a healthy alternative to beef and pork products, due to its favourable nutritional properties, such as low cholesterol and intramuscular fat contents and a high percentage of polyunsaturated fatty acids [1–3]. Moreover, ostrich meat has the characteristic of lower sodium and higher iron contents than other red meat such as beef; therefore, it is recommended for people with arterial hypertension and anaemia problems [4]. Currently, ostrich meat is considered ideal game meat for consumers who prefer a healthy eating style [2,3,5]. The relatively high pH (6.0–6.7) of ostrich meat creates an ideal environment for rapid microbial spoilage under some packaging conditions [6–8]. Therefore, extending the shelf life of ostrich meat products is a major concern for the ostrich industry [8]. To allow competitive marketing of ostrich meat, both for internal consumption and for export, it is necessary to know its microbial characteristics under different conditions to prolong its shelf life.

Modern meat packaging techniques are intended to maintain the microbial and sensory quality of the foodstuffs and extending their shelf life by inhibition or regarding the growth of undesirable microbiota. Vacuum packaging (VP) and storage under chilled conditions have proved to be very effective for extending the shelf life of various perishable foods, including meat [9]. This method of packaging can prevent the growth of some

food-borne pathogens and spoilage bacteria commonly present on meat and therefore is widely used for packaging primal cuts for distribution to the retail market [10,11]. Recently, several studies have focused on describing the diversity of the spoilage microbial population in response to different meat storage conditions [12,13]. According to the literature, the predominant genera associated with freshly beef, pork, and poultry spoilage are *Brochothrium thermosphacta*, *Enterobacteriaceae*, *Pseudomonas* spp., and lactic acid bacteria (LAB) [8,13,14], defined as ephemeral spoilage organisms (ESOs) [15]. Several studies have clearly shown that storage temperature combined with packaging conditions induces a selection of spoilage microbiota, such as ESOs, consequently limiting the spoilage process and determining the commercial shelf life of meat products [12,13,15,16].

The basic knowledge of microbial populations that develops during the storage of raw ostrich meat under different refrigerated conditions and packaging techniques has been vested in the past by using culture-dependent studies [6,8,9,14,17]. According to mentioned trials, different ways of packaging as well as storage temperature and time, affect the microbiological abundance of ostrich meat. In several studies, it has been shown a decline in the number of aerobic bacteria under the conditions of VP, modified atmosphere (MAP), and MAP + CO during the first 4 days of storage, and a significant increase in the subsequent stages of storage [6,8,9,17]. Fernandez-Lopez et al. [17] reported that ostrich meat stored under aerobic conditions would have a shelf life of 8 days, while under vacuum, or modified atmospheres (MAP or MAP + CO) it would be 12 days. On the other hand, Otremba et al. [18] showed that the previously frozen (−40 °C), VP ostrich meat stored under refrigerated conditions for 14 days should be used within 10 days; although microbial growth remained below 7 logs CFU/g for up to 21 days, the unacceptable aroma and colour of meat occurred after 14 days of refrigerated storage. Moreover, in a culture-dependent study, Alonso-Calleja et al. [6] reported that *Pseudomonas* spp., *Enterobacteriaceae* and LABs are initial microbiota in fresh ostrich steaks, and VP treatment is an effective method of inhibiting *Pseudomonas* spp. growth. Similarly, Bingol and Ergun [7] reported that packaging of ostrich meat under a MAP effectively inhibited the growth of *Pseudomonas* sp., but a significant increase in the number of LAB was observed during the storage period which was consistent with the previously reported studies [6,8,9]. It should be emphasised that feeding, slaughter environment, and contamination of carcass are important factors shaping the structure of the initial microflora of raw meat and affect the final quality of meat products and their shelf life. Therefore, it is necessary to analyse the microflora of raw meat from the regional commercial farm, especially ostrich meat as a new, less popular product on the retail market.

In recent years, the development and application of molecular techniques such as 16S rDNA amplicon sequencing using the next-generation sequencing (NGS) approach have become a useful tool for the identification and monitoring of bacterial community changes in packaged and chilled-stored meat products. Many studies using the 16S rDNA sequencing to characterise the spoilage-related microflora of beef [15], pork [19,20], and poultry meat [21–23] stored under aerobic conditions or in vacuum packs have been reported. For example, Xiao, Dong, Zhu, and Cui [24], using 454 pyrosequencing, found that bacterial diversity of vacuum-packaged pork meat increased during refrigerated storage. Using 16S rDNA amplicon sequencing, Li et al. [25] determined the profile of potential spoilage-related bacteria as well as the dynamics of their changes to predict the shelf life of pork meat during storage under refrigerated conditions. Although there are a lot of studies focused on the composition of the bacterial community in meat products [14], little is known about the bacterial diversity on the level of the meat production plant, manufacturing batch variation, and microbial changes that occur during and after storage [13,26].

However, to date, there is no metagenomics report on the microbial community present in vacuum-packed ostrich meat stored in refrigerated conditions for two weeks, which is offered in the retail market. The objective of this study was to determine the microbial community composition and diversity of vacuum-packaged chilled ostrich meat among three manufacturing batches produced in different periods and stored 14 days at

4–5 °C using culture-independent 16S rDNA sequencing. Moreover, a culture-dependent method for enumeration of common spoilage bacteria in ostrich meat was also used. In our study, we especially were interested in the volatility of community composition among different batches of ostrich meat sampled at three manufacturing periods (August, October, March) to investigate spoilage-related bacteria profiles and provide a more comprehensive evaluation of the microbial quality of ostrich meat products.

2. Material and Methods

2.1. Meat Samples

Three batches of ostrich meat (steak and fillet) originating from three different manufacturing periods: August 2019 (batch I), October 2019 (batch II), and March 2020 (batch III) were analysed. Meat samples were obtained from the meat processing company Strusia Kraina & MOBAX Sp.j. (Katowice, Poland) from ostrich with a preslaughter weight of 90 to 130 kg. After overnight chilling at a temperature of −2 °C to 0 °C, approximately 300 g of fillet and steaks were collected from three randomly selected ostrich carcasses. During cutting and packing (8 h), the meat was at a temperature of from 2 °C to 4 °C. The ostrich meat samples were packed in 40 µm multilayer barrier bags with a PET/PA/EVOH/PE structure (OTR: O2 23 cc/m²/24 h; Bag supplier: Mercur Group, Poland) on a vacuum machine (Tepro PP12; Tepro S.A. Poland) and were placed into sterile containers and transported out in a compressor refrigerator (CFX3 75 Dometic) at 0 °C to the laboratory within 5 h. The meat delivered to the laboratory was at a temperature of 3 °C to 4 °C. Then, samples of steak and fillet were stored in the refrigerator for 14 days at 4–5 °C.

To investigate the effect of microbial differences between production batches, 24 packages were prepared for each batch. A total of 12 packages (six fillets and six steaks) were used for the initial microbial enumeration (two biological replicates). For VP treatment, 12 packages were prepared; two packages of both fillet and steak for each batch (two biological replicates) were used for 16S rDNA analysis.

2.2. Microbiological Analysis

Microbiological analysis was carried out immediately after delivery (for initial microbial load evaluation) of meat samples and after 14 days of refrigerated storage (final microbial load evaluation). Briefly, 20 g of ostrich fillet and separately steak from each batch were blended (Stomacher Colworth 400) for 2 min in 180 mL of tryptone salt broth (0.85% (*w/v*) NaCl, 0.1% (*w/v*) tryptone, Biokar Diagnostics, Beauvais, France) according to ISO 6887-1 and 4:2017 to prepare an initial suspension. Decimal dilutions were carried out using the same diluent. Culture media and incubation parameters used are shown in Table 1. Duplicate plates were incubated under aerobic conditions. Confirmation of *Enterobacteriaceae*, *Pseudomonas*, and LABs was carried out according to ISO standards (glucose fermentation test, oxidase reactions, and catalase). The results were expressed as log CFU/g. The assays were performed using two independent samples of the fillet (*n* = 6) and two independent samples of the steak (*n* = 6) for each batch.

Table 1. Culture media, incubation times, temperatures, and references for microbiological analysis.

Microbial Group	Culture Medium	Incubation		References
		Temp. (°C)	Time (h)	
Presumptive <i>Photobacterium</i> sp. ^a	Marine Agar (Difco Becton Dickinson Sparks MD, USA)	15	72	[27]
Lactic acid bacteria (LAB) ^a	Man. Rogosa. Sharpe (MRS) agar (Merck, Darmstadt, Germany)	30	72	ISO 15214:1998
<i>Brochothrix</i> spp. ^b	Streptomycin, sulphate thallos acetate agar (STAA) (Oxoid Basingstoke, UK)	25	48	ISO 13722:2017
<i>Pseudomonas</i> spp. ^b	<i>Pseudomonas</i> agar supplemented with ceftriaxone, fucidin, cephalosporin (CFC) (Merck, Darmstadt, Germany)	25	72	ISO 13720:2010
<i>Enterobacteriaceae</i> ^c	Violet red bile glucose agar (VRBGA) (Graso Biotech., Poland)	37	24	ISO 21528-2:2017

^a Pour-plate technique; ^b Spread-plate technique; ^c Pour-plate technique with overlay added before incubation.

2.3. 16S rDNA Amplicon Sequencing

2.3.1. DNA Extraction

DNA was extracted from ostrich meat ($n = 12$) after 14 days of storage in a VP according to the method described by Rouger et al. [28]. Briefly, about 200 g of steak and fillet samples were placed in sterile filter bags, and 200 mL sterile TS buffer (8.5 g/L NaCl, 1 g/L tryptone, 1% Tween 80) was added, followed by 5 min of hand agitation to separate the microflora from the meat surface. The filtrates from the bags were centrifuged in sterile Falcon containers ($4000 \times g$, 20 min at 4 °C), and the pellet was resuspended in 100 mL TS buffer. Total DNA was extracted using the DNeasy PowerFood Microbial Kit (Qiagen, GmbH, Hilden, Germany) according to the protocol. DNA purity was measured by the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Watertown, MA, USA), and DNA concentration was quantified by a Qubit 4.0 Fluorometer using the Qubit dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). DNA samples were stored at −20 °C until further processing for 16S rDNA amplicon sequencing analysis.

2.3.2. PCR Amplification of 16S rDNA Genes

For each sample, 5 ng of total DNA, and one pair of primers (341F: CCTACGGGNGGC WGCAG-3'; 805R: GACTACHVGGGTATCTAATCC-3') were used to amplify the V3-V4 region of the 16S rDNA gene. PCR was conducted using KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) and 10 µM of each forward and reverse primer. PCR assay consisted of 95 °C/3 min, 25 cycles of 95 °C/30 s, 56 °C/30 s, and 72 °C/30 s, followed by 72 °C/5 min. A negative control without template DNA was also included in the PCR assay. Amplicons were purified using MagSi-NGS^{PREP} PLUS (Steinbrenner Laborsysteme, GmbH, Wiesenbach, Germany) and quantified using the Qubit DNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA) in conjunction with a Qubit 4.0.

2.3.3. Library Preparation and Sequencing

The 16S rDNA fragments were indexed using the Nextera XT Kit (Illumina, San Diego, CA, USA) according to the Nextera DNA Sample Preparation Guide (protocol #15044223). Each index PCR reaction contained 5 µL of the i7 and i5 adapter, 10 µL of KAPA HiFi

HotStart ReadyMix, 20 µL of template DNA (7.0 ng/µL), and 10 µL of H₂O for a total reaction volume of 50 µL. The indexed PCR was cycled according to the Nextera DNA Sample Prep Guide, and the libraries were cleaned up using MagSi-NGS^{PREP} PLUS (Steinbrenner Laborsysteme, GmbH, Wiesenbach, Germany). The DNA libraries were quantified using the Qubit 4.0 along with the Qubit DNA HS Assay Kit, and the quality was assessed on a TapeStation 4200 using the High Sensitivity D1000 SreenTape Assay Kit (Agilent, Santa Clara, CA, USA). Indexed libraries were normalised to 4 nM and pooled. The normalised, pooled 4 nM library was denatured using 0.2 N NaOH and diluted to 10 pM using prechilled HT1 buffer supplied in Nextera XT Kit (Illumina). The 10% of denatured PhiX library (Illumina, San Diego, CA, USA) was spiked into the denatured and indexed library, which was loading into Illumina Miseq v3 reagent cartridge, and 16S rDNA gene amplicons were sequenced on an Illumina MiSeq platform using the 600 cycles (2 × 300 bp) v3 chemistry.

2.3.4. Sequencing Data Analysis

Bioinformatic analysis was performed using the CLC genomic workbench v.8.5.1 with Microbial Genomics Module (Qiagen) [29]. Briefly, for microbial community analysis, obtained raw paired-end reads were assembled and filtered for quality (max error rate 1%) and length (minimum 300 bp of merged reads). Low-quality scores and chimeric reads were deleted using the USEARCH (<http://www.drive5.com/usearch> (accessed on 2 May 2021)). The merged sequences were trimmed of barcodes and primers and next the high-quality sequences were clustered into operational taxonomic units (OTUs) (Table S1). A sensitive BLASTN search against the GreenGenes 16S sequence database, v.13.5 (<http://greengenes.lbl.gov> (accessed on 1 May 2019)) to obtain taxonomy assignment [30] was utilised.

2.4. Statistical Analysis

The software TIBCO StatisticaTM, version 13.5 (TIBCO Software Inc, Palo Alto, CA 94303, USA) was used to perform the statistical analysis. Analysis of variance (ANOVA) and Tukey's multiple range test was used to test the significance of differences ($p < 0.05$) between the mean log values of different manufacturing batches and time of storage. Statistically significant differences for each batch depending on family/genus and individually for each genus depending on the batch were determined using one-way ANOVA. If significant differences were found, the Fisher's NIR tests were performed. The occurrence of significant differences for interactions (family/genus × batch) was performed using a two-way ANOVA. To find out which interactions there were statistically significant, the NIR test was also performed. Data were presented as means ± SD (standard deviation), and a value of $p < 0.05$ was used to indicate statistical significance.

3. Results

3.1. Bacterial Enumeration of Initial and VP Meat

Microbial counting was performed to determine the number of culturable bacteria present. The changes in microbial populations in ostrich meat separately for ostrich fillet and steak are given in Table S2. A culture-dependent method was carried out to enumerate *Brochotrix* spp., LABs, *Enterobacteriaceae*, and *Pseudomonas* spp. as common spoilers in raw meat. Moreover, enumeration of presumptive *Photobacterium* spp. was also determined. The average of bacteria enumeration in ostrich meat (mean values from fillet and steak) considered as spoilage is presented in Table 2. Initial *Photobacterium* spp. count was not significantly different in all tested batches, but after 14 days of VP storage, a significant increase from 7.40 log CFU/g to 7.85 log CFU/g was observed ($p < 0.05$). Similarly, a significant increase ($p < 0.05$) of LAB counts after 14 days of storage in VP was determined in batch I (August; 5.70 log CFU/g) and batch II (October; 6.15 log CFU/g), respectively. *Brochotrix* spp. did not show a significant development within all batches ($p > 0.05$), remaining constant after 14 days of packaging. Nonetheless, the significantly highest count of *Brochotrix* spp. ($p < 0.05$) was observed in batch III (4.73 log CFU/g) in

comparison with batch I (2.66 log CFU/g) and batch II (3.27 log CFU/g). Similarly, the initial count of *Pseudomonas* spp. was very low (<1.00–1.87 log CFU/g) and remained at this level after 14 days of storage. The level of initial contamination of ostrich meat with *Enterobacteriaceae* was similar between all batches and the count of these bacteria was increased significantly to 5.02–5.62 log CFU/g ($p < 0.05$) at 14 days of storage; no significant differences in *Enterobacteriaceae* count related to the manufacturing batches were observed.

Table 2. Microbial analysis of initial and VP bacterial populations in refrigerated ostrich meat.

Meat	Colony Count (log CFU/g)									
	Photobacterium spp.		LAB		Brochotrix spp.		Pseudomonas spp.		Enterobacteriaceae	
	Storage Days									
	Initial	14 Days	Initial	14 Days	Initial	14 Days	Initial	14 Days	Initial	14 Days
	Batch I	4.95 ± 0.13 ^{aA}	7.40 ± 0.08 ^{bA}	3.10 ± 0.30 ^{aA}	6.15 ± 0.00 ^{bB}	1.80 ± 0.45 ^{aA}	2.66 ± 0.24 ^{aA}	<1.00 ^{aA}	1.48 ± 0.67 ^{aA}	2.47 ± 0.51 ^{aA}
Batch II	5.12 ± 0.05 ^{aA}	7.65 ± 0.05 ^{bAB}	2.75 ± 0.15 ^{aA}	5.70 ± 0.48 ^{bAB}	1.82 ± 0.47 ^{aA}	3.27 ± 0.43 ^{aAB}	1.87 ± 0.12 ^{aB}	1.73 ± 0.18 ^{aA}	2.91 ± 0.41 ^{aA}	5.62 ± 0.30 ^{bA}
Batch III	4.15 ± 1.05 ^{aA}	7.85 ± 0.08 ^{bB}	3.56 ± 0.68 ^{aA}	4.78 ± 0.10 ^{aA}	2.02 ± 0.08 ^{aA}	4.73 ± 0.45 ^{bB}	0.54 ± 0.66 ^{aAB}	1.65 ± 0.07 ^{aA}	1.70 ± 0.06 ^{aA}	5.56 ± 0.07 ^{bA}

^{a-b} Mean values in rows with different lowercase letters are significantly different at $p < 0.05$, separately for the microbial counts of each group and each batch. ^{A-B} Mean values in columns with different capital letters are significantly different at $p < 0.05$, separately for the microbial counts of each group and day of storage. Data are mean ± standard deviation.

3.2. 16S rDNA Sequencing Data of VP Meat

16S rDNA amplicon sequencing was used to gain insight into the composition of the bacterial community in VP ostrich meat after 14 days of storage at 4–5°C and was performed separately for ostrich steak and fillet (Table 3).

Table 3. Comparison of bacterial community composition at the family/genus level (%) in ostrich steak and fillet between three manufacturing batches after 14 days of refrigerated storage.

Family/Genus	Steak I		Steak II		Steak III		Fillet I		Fillet II		Fillet III	
	N	%	N	%	N	%	N	%	N	%	N	%
<i>Vibrionaceae/Photobacterium</i>	60,540.0	85.63	52,126.0	87.71	52,997.0	47.22	65,651.0	83.47	41,658.0	91.77	62,198.0	65.31
<i>Carnobacteriaceae/Carnobacterium</i>	494.0	0.70	1,550.0	2.61	17,533.0	15.62	1013.0	1.29	1520.0	3.35	8749.0	9.19
<i>Carnobacteriaceae/Other</i>	-	-	-	-	22,224.0	19.80	-	-	-	-	9852.0	10.35
<i>Lactobacillaceae/Lactobacillus</i>	3320.0	4.70	4,078.0	6.86	2474.0	2.20	2125.0	2.70	1051.0	2.32	1924.0	2.02
<i>Enterobacteriaceae/Serratia</i>	-	-	-	-	-	-	657.0	0.84	-	-	-	-
<i>Enterobacteriaceae/Other</i>	-	-	494.0	0.83	5564.0	4.96	-	-	594.0	1.31	3061.0	3.21
<i>Streptococcaceae/Lactococcus</i>	-	-	-	-	3023.0	2.69	-	-	-	-	3785.0	3.97
<i>Enterococcaceae/Vagococcus</i>	-	-	-	-	-	-	-	-	101.0	0.22	-	-
<i>Leuconostocaceae/Weissella</i>	530.0	0.75	650.0	1.09	-	-	720.0	0.92	67.0	0.15	-	-
<i>Shewanellaceae/Shewanella</i>	1241.0	1.76	-	-	-	-	859.0	1.09	-	-	-	-
<i>Bacteroidaceae/Bacteroides</i>	492.0	0.70	-	-	-	-	-	-	-	-	-	-
Unidentified	2607.0	3.69	250.0	0.42	7546.0	6.72	4745.0	6.03	-	-	5050.0	5.30

I—August; II—October; III—March; N—the average number of reads (read counts); %—the average total reads.

Results showed that the majority of bacterial sequences (>0.5%) identified in samples of ostrich meat after 14 days of storage belonged to *Photobacterium*, *Lactobacillus*, *Carnobacterium*, *Serratia*, *Shewanella*, *Weissella*, *Vagococcus*, *Bacteroides*, and *Lactococcus* (Table 4). Similar to culture-dependent analysis, *Photobacterium* spp. constituted the most predominant microflora in all ostrich meat batches, but the highest relative abundance in batch I (84.55%) and batch II (89.74%) was found ($p < 0.05$) (Table 5). The microbial community of batch III was also dominated by *Photobacterium* spp. with a relative abundance of more than 65% in the fillet and 47% in the steak, respectively. The results of our study also revealed a dominance of the LAB (28%) population in ostrich meat after 14 days of storage in a vacuum package under chilled conditions. The LAB population consisted of *Carnobacteriaceae* (20.5%), *Lactobacillaceae* (3.5%), *Streptococcaceae* (3.3%) *Leuconostocaceae* (0.7%) and was mainly dominated by *Carnobacterium* (5.39%) and *Lactobacillus* (3.5%) genus (Table 4). A relatively high abundance of *Carnobacterium* spp. was obtained in the manufacturing batch III, compared with batches I and II ($p < 0.05$) (Table 5). Moreover, both samples from batch III showed a relatively high abundance of psychrotrophic *Lactococcus* spp. (3.97%

and 2.69%) and a relatively low proportion of *Lactobacillus* spp. in the steak (2.2%) in comparison with the steak from batch I (4.7%) and II (6.86%) (Table 3). The rest of the LAB population, i.e., *Weissella* spp. remained at a low level and was detected in the fillet and steak from batch I (0.92%/0.75%) and batch II (0.15%/1.09%). In our study, after 14 days of storage, *Enterobacteriaceae* were identified with relatively low abundance in the fillet from batch I (0.84%) and were more abundant in batch III in both the fillet (3.21%) and the steak (4.96%) (Table 3). *Serratia* spp. belonging to *Enterobacteriaceae* was found in this study but was presented only in the fillet from batch I with a detection limit of 0.84%. Relative abundances of *Brochotrix* spp. and *Pseudomonas* spp. were below to detection limit and were not found in VP ostrich meat after 14 days of storage.

Table 4. Average share (%) of OTU assigned to bacterial taxonomic units between batches of ostrich meat from different manufacturing periods after 14 days of refrigerated storage.

Family/Genus	Batch I (August)	Batch II (October)	Batch III (March)	Relative Abundances (I + II + III) *
	(%)	(%)	(%)	(%)
<i>Vibrionaceae/Photobacterium</i>	84.55	89.74	56.27	76.85
<i>Carnobacteriaceae/other</i>	-	-	15.08	15.08
<i>Carnobacteriaceae/Carnobacterium</i>	1.00	2.98	12.21	5.39
<i>Enterobacteriaceae/other</i>	-	-	4.09	4.09
<i>Lactobacillaceae/Lactobacillus</i>	3.7	4.7	2.11	3.50
<i>Streptococcaceae/Lactococcus</i>	-	-	3.33	3.33
<i>Shewanellaceae/Shewanella</i>	1.43	-	-	1.43
<i>Enterococcaceae/Vagococcus</i>	-	1.35	-	1.35
<i>Leuconostocaceae/Weissella</i>	0.84	0.62	-	0.73
<i>Enterobacteriaceae/Serratia</i>	0.84	-	-	0.84
<i>Bacteroidaceae/Bacteroides</i>	0.7	-	-	0.70
Unclassified	4.86	0.475	6.01	3.78

* mean of (%) abundances from three manufacturing batches.

Table 5. Mean of relative abundances (%) of bacterial community and significant differences between batches and family/genus of ostrich meat after vacuumed, refrigerated storage.

Family/Genus	Batch I (August)	Batch II (October)	Batch III (March)
<i>Vibrionaceae/Photobacterium</i>	84.55 ± 1.53 ^{Cb}	89.74 ± 2.87 ^{Cb}	56.09 ± 12.81 ^{Da}
<i>Bacteroidaceae/Bacteroides</i>	0.35 ± 0.49 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}
<i>Carnobacteriaceae/Carnobacterium</i>	1.00 ± 0.42 ^{Aa}	2.98 ± 0.52 ^{ABa}	12.21 ± 4.6 ^{BCb}
<i>Lactobacillaceae/Lactobacillus</i>	3.70 ± 1.41 ^{Ba}	4.61 ± 3.18 ^{Ba}	2.11 ± 0.13 ^{Aa}
<i>Carnobacteriaceae/Other</i>	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	15.08 ± 6.68 ^{Cb}
<i>Enterobacteriaceae/Other</i>	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	4.09 ± 1.24 ^{ABb}
<i>Enterobacteriaceae/Serratia</i>	0.84 ± 0.59 ^{Aa}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}
<i>Enterococcaceae/Vagococcus</i>	0 ± 0 ^{Aa}	1.35 ± 1.9 ^{Aa}	0 ± 0 ^{Aa}
<i>Streptococcaceae/Lactococcus</i>	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}	3.33 ± 0.91 ^{ABb}
<i>Leuconostocaceae/Weissella</i>	0.84 ± 0.12 ^{Aa}	0.62 ± 0.66 ^{Aa}	0.10 ± 0.01 ^{Aa}
<i>Shewanellaceae/Shewanella</i>	1.43 ± 0.47 ^{Ab}	0 ± 0 ^{Aa}	0 ± 0 ^{Aa}
Unidentified	4.86 ± 1.65 ^{Bb}	0.21 ± 0.3 ^{Aa}	6.01 ± 1.00 ^{ABCb}

Values are mean ± standard errors; ^{a-b} Means with different letters under different batches in the same row are significantly different ($p < 0.05$); ^{A-D} Means with different letters under different family/genus in the same column are significantly different ($p < 0.05$). Data are mean ± standard deviation.

4. Discussion

Even though the nutritional value of ostrich meat is well documented, not enough information is available on the microbial quality and shelf life, especially of the refrigerated vacuum-packaged ostrich fillet and steak form, which are most frequently purchased in development countries. Thus, improvement of the microbiological quality and extension of

shelf life of retail ostrich meat is crucial, both for the local markets and for export [4,5,7]. It is known that the growth of microorganisms and their metabolic activity can significantly affect the quality of packaged raw meat stored under refrigerated conditions and its shelf life. Regarding ostrich meat, the relatively high pH is a supporting factor for rapid microbial spoilage under certain packaging conditions [6,8,9,17]. Therefore, the analysis of microbial counts and changes in the community composition related to the packaging methods, period, and temperature of raw meat storage may contribute to the development of effective methods of inhibiting specific spoilage and extending the shelf life of retail ostrich meat products.

In this study, utilising 16S rDNA sequencing we investigated the microbial community composition and determined the predominant microflora occurring in three different batches of retail vacuum-packed ostrich meat acquired over several months (August, October, March). The culture-dependent method was also used for the enumeration of total initial numbers of common spoilage bacteria of raw ostrich meat among tested manufacturing batches, and their differences after 14 days of VP storage were also compared.

Using the enumeration method, we showed that the initial population of *Photobacterium* spp., LAB, *Brochotrix* spp., *Pseudomonas* spp., and *Enterobacteriaceae* in ostrich meat was relatively similar between the production batches. In line with many reports, the initial population of the indigenous microflora on raw poultry meat has consisted of *Brochotrix* spp., *Pseudomonas* spp., LABs, *Enterobacteriaceae* [13,14,31,32] and to be similar to that red meat [33]. Surprisingly, *Photobacterium* spp. was the predominant genus in both initial and after 14 days of meat storage in VP and the total count of its was significantly higher in batch I (August) and batch II (October). At the end of the storage, the count of *Photobacterium* spp. reached above 7 log CFU/g, which is commonly reported as the microbial spoilage threshold causing sensorial deterioration such as the off-odours and slime [34,35]. Members of this genus are not often identified using the culture-dependent method; therefore, the mechanism underlying meat spoilage by *Photobacterium* has not been clarified. Moreover, the results of the enumeration counts revealed also the dominance of the LAB population at the beginning and after 14 days of meat storage, which is in agreement with previous studies on poultry meat [36–38], beef [11,15], and pork [19,39]. In our study, the initial counts of LAB (2.75–3.56 log CFU/g) were similar to those found in fresh ostrich steak (2–4 log CFU/g and 2.9 log CFU/g) by Otremba et al. [18] and Fernandez-Lopez, [17], respectively. These results were also consistent with those of Jouki and Yazdi [39], who reported a 3.8 log CFU/g of initial LABs count in fresh ostrich meat. On the other hand, a significantly higher initial LABs count of ostrich steak was reported by Alonso-Calleja et al. [6] with a range of 6.5–7.8 log CFU/g in retail ostrich steaks in Spain. In our study, the LAB population showed a significant increase in all packages above 7 log CFU/g at the end of storage time (14 days), which can be associated with initial meat spoilage, especially with sensory deterioration [34]. Interestingly, Alonso-Calleja et al. [6] reported that samples of retail ostrich meat with a higher level of LABs than 7 log CFU/g showed a non-repulsive and slight acid odour after 7 days of packaging. Moreover, none of the samples with <7 log CFU/g of LABs count had any off-odour in VP meat, even when higher than 8 log CFU/g.

In our study, the initial count of *Enterobacteriaceae* as the indicator of meat contamination was relatively low in all batches in comparison to ostrich steaks (5.4–6.7 log CFU/g) from retail outlets in Spain [6]. It is known that the initial contamination of meat can influence the spoilage dynamics during storage. Similar results were also presented by Angulo et al. [40], who found an average of 6.78 log CFU/g for *Enterobacteriaceae* in refrigerated VP ostrich steaks in Spain. Additionally, Fernandez-Lopez et al. [17] showed that *Enterobacteriaceae* counts in ostrich meat stored under aerobic conditions, VP, or MAP and MAP + CO reached an average value of 7.0 ± 0.2 log CFU/g at the end of the storage period (18 day). Under aerobic conditions, the predominant spoilage microorganism in meat and poultry is *Pseudomonas* spp. [8,26,38]. Alonso-Calleja et al. [6] reported a range of 5.0–7.2 log CFU/g for *Pseudomonas* spp. in fresh ostrich steaks, which is much higher than the initial range of <1.00–1.85 log CFU/g found in our study between individual batches. The higher

initial count of *Pseudomonas* spp. (3.12 log CFU/g and 3.0 log CFU/g) in ostrich meat was also reported by Jouki and Yazdi [39], and Bingol and Ergun [7]. Our results showed that VP limited the growth of *Pseudomonas* spp., which ranged from 1.48 log CFU/g to 1.73 log CFU/g in batch I and batch II, respectively. A significantly higher *Pseudomonas* count of VP ostrich steaks was reported by Alonso-Calleja et al. [6] after 7 days of storage as well as by Jouki and Yazdi [39] at the end of storage (day 21). The initial number of *Brochothrix* spp. ranged from 1.8 to 2.3 log CFU/g and fell within the range of values reported by other authors for unpackaged ostrich meat after slaughter [8,9,17,18] and a slight growth was observed in VP meat after 14 days of storage. As mentioned by Pennacchia et al. [15], the growth of both *Pseudomonas* spp. and *B. thermosphacta* was effectively limited in VP chilled beef, as compared to the MAP [11,13,23,33].

In the aforementioned studies, the microbiological characteristics of ostrich meat under different package methods or storage conditions were determined using a culture-dependent technique, assessing the counts and viability of bacteria in the sample. It should be noted that culturable microorganisms represent only a small fraction of the entire microbial diversity and the culture-dependent method is not suitable to investigate changes in the bacterial communities during storage of meat products under different conditions. Currently, the rapid development of a high-throughput sequencing (HTS) approach is becoming more widely used to adequately characterise the composition of bacterial communities and their diversity during the storage of raw meat under different conditions [25,38].

In our study, the amplicon sequencing of the V3/V4 region of the 16S rDNA gene was applied to investigate the bacterial community composition and diversity of VP meat (fillet and steak portion) after 14 days of storage. Nine families were found in the microbiota in VP ostrich meat and the vast majority of detected sequences belonged to the genus of *Photobacterium*, *Carnobacterium*, *Lactobacillus*, and *Enterobacteriaceae* family (Table 4). A relatively taxonomically similar composition was obtained regardless of the manufacturing batches of both fillet and steak, but relative differences in the abundance of several bacterial genera between the tested batches were observed. Our results showed that *Photobacterium* genus was occurred in all samples from meat with a relative abundance from 56% to 89% dependent on the manufacturing batches, indicating a potential contribution to the spoilage of ostrich meat. The members of this genus are considered dominant spoilage-related bacteria of refrigerated fish and seafood products [41,42] due to their ability to produce biogenic amines such as putrescine, tyramine, and cadaverine that can lead to fish poisoning [43]. Recently, based on culture-independent approaches, the presence of *Photobacterium* spp. was reported on the vacuum and air-stored beef [15] and modified-atmosphere-packaged poultry [27,35], pork [21,44], and minced beef [26]. Together with this, our results showed that *Photobacterium* spp. was also present in raw ostrich meat, which could indicate that this genus is not characteristic only for marine species but is rather a widespread issue linked with the meat and poultry industry [27]. Several reports indicate that the source of *Photobacterium* in all kinds of meat could be highly associated with contamination via air or water during slaughtering and subsequent meat processing [27,34]. On the other hand, Hilgarth et al. [35] suggested that the presence of *Photobacterium* could be linked with livestock before the slaughtering process because this bacteria genus is an indigenous member of the animal gut microbiome. Moreover, *Photobacterium* may grow independent of the packaging method, and according to the study of Höll et al. [45], the modified atmosphere composition and vacuum packages, as well as marinating used to extend the shelf-life of meat and fish, are inadequate to restrict spoilage-associated *Photobacterium* in meat. Thus far, the mechanism underlying spoilage by this genus has not been clarified. Thus, further studies should be conducted to discover the source of the initial bacterial contamination of the meat and to know the mechanisms of *Photobacterium* adaptation to the respective storage conditions. To the best of our knowledge, this is the first report that detected the presence of *Photobacterium* in chilled ostrich meat packaged in a vacuum using a culture-independent approach.

Psychrotrophic LABs are the prevailing spoilage organisms in packaged cold-stored meat products. [11,15,36]. Particularly, *Leuconostoc*, *Carnobacterium*, *Lactobacillus*, *Lactococcus*, and *Weissella* are the most abundant LAB genus associated with the spoilage of refrigerated and/or chilled vacuum-packaged beef [10,11,15,33], pork [19,20,46], and poultry meat [8,28]. LAB are recognised as important competitors in raw meat under VP and MAP conditions [10,20,23] and have the potential to cause spoilage of raw meat by discolouration or production of off-odour compounds [6,8,16,33]. It has been shown that their relative abundance is dependent on the type of meat and preservation technology as well as inter-species interactions [26,47]. In our study, 16S rDNA sequencing results showed that the composition of LAB was more diverse between manufacturing batches. A relatively high abundance of the *Carnobacteriaceae* family including *Carnobacterium* genus was detected in both the fillet and the steak of batch II (October) and III (March), whereas *Lactobacillus* became a common genus in all samples of ostrich meat after 14 days of storage. Previous reports showed that *Lactobacillus* spp., *Carnobacterium* spp., and *Weissella* spp. were the dominant bacterial communities during storage in vacuum-packed chilled pork [20,46], lamb [47–49], and beef [10,11,33,50]. Our study confirmed the prevalence of these genera in ostrich meat after 14 days of storage. Wang et al. [51] demonstrated that *Lactococcus* spp. were dominant bacteria at the end of the shelf life of poultry meat under modified-air packaging (MAP). Similar results were reported by Bingol and Ergun [7] regarding ostrich meat packaged under a modified atmosphere (MAP).

In our study, *Carnobacterium* spp. was the dominant genus of the LAB population in all tested manufacturing batches after 14 days of storage. *Carnobacterium* similar to other members of the LAB population is facultative anaerobic bacteria that can adapt to the environment of vacuum [22,31,52]. The significantly high abundance of *Carnobacteriaceae* observed in batch III (March) may be related to the initiation of the meat sensory deterioration process. On the other hand, relative higher *Carnobacterium* spp. in batch III can be associated with an initial higher count of this genus in fresh ostrich meat. In lamb steaks packaged in a vacuum pouch (VAC) with a VP machine and storage under 4 °C for 21 days, the significant growth of *C. divergens* and *C. maltaromaticus* outcompeted the other microbial population and became the dominant bacteria in the latter period of storage was reported by Wang et al. [48]. The two aforementioned species are recognised as predominant species on raw meat regardless of the packaging conditions [14,53–55]. They have been found in air and VP, and are associated with the spoilage of fresh and chilled meat [11,48,56]. The presence of *Carnobacteriaceae* abundance as the predominant population at day 14 (14.54%) and day 21 (3.4%) was also observed in VP chilled pork [20]. In beef steak stored under vacuum, *Carnobacterium* spp. was detected most frequently in the last days of storage with an abundance of 100% after 20 days [15]. According to the sensory evaluation, Jääskeläinen et al. [54] reported that high oxygen MAP beef was spoiled over 10 days earlier than the VP beef. Moreover, LAB species are considered to be important for preventing the growth of other spoilage-related bacteria [56,57] due to their ability to produce antibacterial substances such as hydrogen peroxide, lactic acid, and bacteriocins [58]. *Lactobacillus* spp. acts as competitive microflora in meat and meat products, extending their shelf life by competing with pathogens such as *L. monocytogenes*, inhibiting their excessive growth [58,59]. The presence of *Lactobacillus* spp. in ostrich meat portions may be associated with the production of lactic acid, which inhibits the growth of spoilage-related bacteria under vacuum-packed storage [13,16,26]. A relatively high abundance of *Carnobacterium* spp. and other members of the *Carnobacteriaceae* family in batch III (March) and the simultaneous decrease of *Photobacterium* spp. abundance might be due to the competitive growth of *Carnobacterium* spp. or its inhibitory activity towards *Photobacterium* during the late phase of meat storage.

Höll et al. [31] reported that in high O₂ MAP at 4 °C *Brochothrix thermosphacta* were identified in the chicken breast as typical representative meat spoiling bacteria. *B. thermosphacta* is one of the main spoilers in raw meat packaged in a vacuum or modified atmosphere [60]. It has been reported that *B. thermosphacta* can grow in meat both under

aerobic and anaerobic conditions [6,8,36,38,60]. Jääskeläinen et al. [54] showed that the abundance of *B. thermosphacta* was higher in high O₂ MAP at 6 °C than in VP beef meat. Recently, Chen et al. [36] reported that *Brochothrix* was the dominant genus in fresh roast duck after 7 days of storage under overwrapped packaging (OWP). In our study, the initial cell count of *Brochothrix* spp. in ostrich meat was relatively low (<2 log CTU/g), and similar results were also observed in VP meat after 14 days of storage. The 16S rDNA data showed that the relative abundance of *Brochothrix* spp. was found to be below the detection limit, which indicates that this genus did not belong to ESO in our experiment. Thus, our results confirm the previous finding [6,8,17] that *Brochothrix* spp. are not an important contributor to the spoilage of VP ostrich meat. Our study shows how sequencing and the commonly used culture-dependent method for detection of metagenome result in different outcomes concerning the abundance of bacterial composition. This may be due to DNA extraction or PCR bias in the meat matrix in which different species are present at high levels and compete for 16S rDNA amplification [11,15]. These results indicate the need to use both techniques simultaneously to study bacterial populations in meat and meat products comprehensively.

It is known that the presence of *Enterobacteriaceae* in raw meat is related to the initial contamination of animal carcasses during the slaughtering process and carcass handling [61,62]. A high number of this family is considered to be a hygiene indicator and has a high correlation with the sensory odour of the poultry [22,24,38,51]. Most of the studies have found many members of *Enterobacteriaceae* on raw beef [10,11,14,26,50,55], pork [19–21,25], lamb [47–49], poultry [22,24,31,36], ostrich meat [6,8,9,11] as well as on offal meat [19,63]. Identification of *Enterobacteriaceae* in refrigerated raw meat and meat products is of particular importance both for its high spoilage potential and for food safety concerns because some species are pathogens [12]. *Serratia* is the most commonly found genus in meat stored in air, VP, and MAP and plays an important role in the spoilage of meat, especially in the late phase of chilled meat storage [14,15]. Although *Enterobacteriaceae* has been found as one of the major bacteria in VP chilled meat [6,33,39,48,53,64], using enumeration method and 16S rDNA sequencing, the present study showed that its content was relatively lower in comparison to *Photobacterium* spp. and LAB population. The presence of the unclassified *Enterobacteriaceae* abundance (4.09%) in batch III (March) can be associated with contamination of carcass during handling, but it was not consistent with results obtained from the culture-dependent analysis; the initial count of *Enterobacteriaceae* in batch III was significantly lower than in batch I and batch II. This may be due to DNA extraction or PCR amplification bias [15]. Previous studies reported different counts of *Enterobacteriaceae* chilled beef [54], pork [25,56], lamb [47,48,62], ostrich [6–9], and poultry meat [32,51,60] under different packaging methods and refrigerated storage. In our study, the relative abundance of this family in meat was relatively low (<5%), and the cell count was below the limit of 6 logs CFU/g after 14 days of storage. Several previous studies have revealed that counts of >7 logs CFU/g are considered as the maximum value in a raw meat product at the end of its shelf life [7,26,54]. Regarding ostrich meat, the shelf life of meat storage under VP can be more than 10 days [7,18,26,54]. Although *Enterobacteriaceae* has been shown to be one of the major bacteria in VP chilled meat [15,61], in our experiment this family did not belong to the dominant ESO in ostrich meat. The role of this bacterial group as ESO becomes more significant if the storage temperature of the meat is abused [54].

Moreover, several low-abundance (i.e., *Shewanella* spp., *Bacteroides* spp.) animal-origin bacteria observed in the present study could be associated with contamination via faecal, air, and/or water during slaughtering and subsequent handling. For example, *Bacteroides* spp. detected in the steak (0.7%) from batch I (August) was reported to be intestinal bacteria in livestock animals [62,65]. Similarly, *Shewanella* spp., which was isolated from the intestinal content of fish [61] in our study, was detected at a low level in both fillet (1.09%) and steak (1.76%) from batch I (August). In the food industry, the genus *Shewanella* is closely related to the *Pseudomonas* spp. and is considered the primary cause of meat spoilage during cold storage under vacuum [13,17,65,66] or modified atmosphere

packaging (MAP) [62]. *Pseudomonas* spp. was found as the dominant spoilage-related flora of raw poultry meat [34,44,57,63,66] and MAP and VP meat products [6,8,11,14,26,48]; but its growth occurred more frequently in meat stored in the air than under vacuum, in line with its aerobic ability [18,24,39,55]. In our study, the relative abundance of *Pseudomonas* spp. in all tested manufacturing batches at the end of storage was found to be below to detected limit. Similar results were also found in the culture-dependent method (Table 2); the initial total number of *Pseudomonas* spp. was below to detection limit in both batch I (August) and batch II (October) ($<1.0 \log \text{CTU/g}$), and insignificant low bacterial growth in VP ostrich meat after 14 days of storage at 4–5 °C was noticed. Ntchimani et al. [67] found inhibition of microbial growth, including *Pseudomonas* spp., on turkey fillets stored under air packaging (AP), VP, and vacuum skin pack (VSP) at 4 °C. According to them, VSP is the most effective, followed by VP. In a culture-dependent study, Bingol and Ergun [7] reported that packaging of ostrich meat under a modified atmosphere (MAP) effectively inhibited the growth of *Pseudomonas* spp. with a simultaneous increase in LAB counts during the storage period. Thus, our results confirm the previous finding that *Pseudomonas* spp. is not an important contributor to the spoilage of VP ostrich meat.

5. Conclusions

The identification and monitoring of bacterial community dynamics during meat storage is important for identifying the predominant spoilage bacteria and optimising shelf-life strategies for raw ostrich meat products. In this study, 16S rDNA amplicon sequencing by employing a next-generation sequencing (NGS) approach was applied to determine the microbial community of VP chilled ostrich meat in three manufacturing batches produced in different periods to establish the range and diversity of this community. Nine main genera ($>0.5\%$) of bacteria were detected in refrigerated meat after 14 days of storage at 4–5 °C. *Photobacterium* was the most prevalent genus detected in all samples of ostrich meat at the end of storage, which suggests that this genus may be contributing to meat deterioration and shortening of the commercial shelf life of ostrich meat under VP storage. To the best of our knowledge, this is the first report that detected the presence of *Photobacterium* spp. in chilled ostrich meat packaged under vacuum using a culture-independent approach. Future research to confirm the potential link between the presence of *Photobacterium* spp. and the initiation of the VP ostrich meat deterioration is required.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/pr9050803/s1>. Table S1: Raw bioinformatics data of ostrich meat samples. Table S2: Microbial analysis of initial and VP bacterial population in refrigerated ostrich meat, separately for fillet and steak.

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