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Prevalence of Microorganisms and Suggestion for Potential Contribution of Microorganisms to Volatile Basic Nitrogen Production in Beef at Current Purchase Stages

Abstract

This study investigated the prevalence of microorganisms related to meat quality and analyzed volatile basic nitrogen (VBN) levels in beef samples to suggest potential bacteria that might contribute to VBN production in current purchase stages with metagenomic analysis. Seventy beef samples were analyzed for coliform, *Escherichia coli*, enterohaemorrhagic *E. coli*, *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*, total aerobic bacteria (TAB), Enterobacteriaceae, lactic acid bacteria (LAB), *Pseudomonas* spp., yeast and molds (YM), and psychrotrophic bacteria (PB). VBN levels ranged from 0.69 to 22.51 mg%. Microbiota in three samples with the highest VBN levels and three samples with the lowest VBN levels were analyzed. *S. aureus* was detected in only one sample at 1.2 Log CFU/g. The cell counts for TAB, coliform, Enterobacteriaceae, LAB, *Pseudomonas* spp., YM, and PB were 5.1, 1.7, 2.6, 4.2, 1.9, 2.9, and 5.4 Log CFU/g, respectively. Microbiota analysis revealed that samples with high VBN levels had high relative abundances of *Lactobacillus* and *Leuconostoc*. This study showed that relatively abundant LAB were potential bacteria that might contribute to producing more VBN in beef at current purchase stages. However, the potential bacteria were suggested only by metagenomic analysis with a limited sample size without considering the endogenous meat enzymes. Therefore, further research is necessary to identify and isolate these bacteria with a larger sample size while excluding VBN produced by endogenous enzymes. Additionally, environmental factors not involved due to the limited objective of this study could also be considered in further research with the different objectives from this study.

Keywords: volatile basic nitrogen, Korean beef, meat quality, microbiota

1. Introduction

Meat is vulnerable to degradation from oxidation and microbial contamination, given its nutrient richness and favorable conditions for microorganisms (Lee and Yoon, 2023; Yu et al., 2018). Meat preservation is associated with food safety and meat spoilage. Thus, various research aimed to address the microorganisms responsible for these issues (Devlieghere et al., 2004). Meat spoilage occurs because of various microorganisms, depending on storage temperature, contamination, and packaging conditions. Enzymatic activity in animal muscle cells is a natural occurrence in animal muscle cells after slaughtering and contributes to meat spoilage (Dave and Ghaly, 2011). Key enzymes involved in this process include NADPH oxidase, cyclooxygenase, xanthine oxidase, nitric oxidase, and peroxidases (Bekhit et al., 2021b). Despite the implementation of cold chain systems to mitigate these concerns, vulnerabilities persist in the meat cold chain (Kwon et al., 2022); also, undesirable changes (e.g., protein decomposition, lipid oxidation, discoloration, and the growth of spoilage bacteria) may occur as meat spoilage progresses.

Volatile basic nitrogen (VBN) can be used as an indicator of meat spoilage and to assess the freshness of fish (Jeong et al., 2015; Huang et al., 2015). Thus, the VBN could be a critical consideration at the purchase stage. These compounds are produced through the microbial degradation of protein and non-protein nitrogenous substances, such as amino acids and nucleotide catabolites (Liu et al., 2013).

With recent advancements in gene analysis technology for identifying and characterizing microorganisms, it is now possible to predict meat quality based on microbiota (Gagaoua et

al., 2022). Since meat spoilage is related to microorganisms in the meat, researches have been conducted to identify potential microorganisms for VBN production (Fang et al., 2022; Saenz-Garcia et al., 2020; Wang et al., 2017).

Therefore, the objectives of this study were to investigate the prevalence of microorganisms and VBN in beef and to suggest potential bacteria that might contribute to VBN production in current purchase stages with metagenomic analysis.

2. Materials and Methods

2.1. Analysis of microorganisms and VBN contents in beef

2.1.1. Preparation of beef samples

Seventy beef samples [34 (high grade: 17 and low grade: 17) sirloin (relatively higher fat content) and 36 (high grade: 19 and low grade: 17) top round (relatively lower fat content) samples] were collected from the wholesale stage of distribution (17%), butcher's shops (41%), hypermarkets (17%), and supermarkets (24%) between July and August 2022. These distribution stages and portions for each retail outlet were determined according to the data from the Korea Institute for Animal Product Quality Evaluation (KAPE, 2022), and the distribution stages were also where the consumer purchased beef in current distribution conditions. All collected beef samples were transported in a cooler and analyzed within 3 h of purchase.

2.1.2. Microbiological analysis

According to the analysis method by the Ministry of Food and Drug Safety (MFDS, 2022), for qualitative analysis of *Escherichia coli*, 25 g of beef samples were aseptically placed in a filter bag containing 225 mL sterile 0.1% buffered peptone water (BPW; Becton, Dickinson, and Company, Detroit, MI, USA), and homogenized with a pummeler

(BagMixer®, Interscience, St. Nom, France) for 1 min. The homogenate was diluted with 0.1% BPW, and 1 mL aliquots of the diluents were placed into 9 mL EC medium (Becton, Dickinson, and Company) and incubated at 44℃ for 24 h. A positive sample was identified by turbidity and gas production in the EC medium. Subsequently, a loopful of the positive EC medium was streaked on eosin methylene blue (EMB; Becton, Dickinson, and Company) agar and incubated at 37℃ for 24 h. Colonies displaying a green metallic sheen were subjected to identification through 16S rRNA sequencing. To confirm colony formation of enterohemorrhagic *E. coli* (EHEC) with a method by the MFDS (2022) with the modification, 10 g of the beef sample was aseptically placed in a filter bag containing 90 mL modified tryptic soy broth (mTSB; MBCell, Seoul, Korea). The sample was placed at 37℃ for 24 h for the enrichment of EHEC. A loopful of the culture was streaked onto MacConkey sorbitol agar (Becton, Dickinson, and Company) supplemented with cefixime tellurite (MBCell) (TC-SMAC), and 5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide (BCIG) agar (Oxoid, Basingstoke, Hampshire, UK). The agar plates were incubated at 37℃ for 24 h. After the colony formation of EHEC was confirmed, the following experiment was conducted to detect the DNA of EHEC. To extract DNA from the colonies on the agar plates after incubation, a method by Fratamico et al. (2000) was used with the modification. Two to four red colonies on TC-SMAC and turquoise colonies on the BCIG agar were each suspended in 100 μL of sterile distilled water, incubated at 99℃ for 10 min, and the resulting mixture served as the template DNA for PCR amplification with multiplex PCR. The Powerchek™ diarrheal E. coli 8-plex detection kit (Kogene Biotech, Seoul, Korea) was used to detect *stx1* and *stx2*, which are specific DNA markers for EHEC. PCR amplification was conducted according to the manufacturer's instruction and consisted of initial denaturation at 95℃ for 12 min, followed by 32 cycles of 95℃ for 30 sec, 60℃ for 45 sec, 72℃ for 60 sec, and a

107 final extension at 72℃ for 10 min. The PCR products were electrophoresed on 2% agarose
108 gel, and DNA bands were visualized under UV light.
109 The homogenates previously prepared for qualitative analysis of *E. coli* were diluted in 9
110 mL of 0.1% BPW and used for quantitative analysis of microorganisms. The diluents were
111 plated on Petrifilm™ E. coli/Coliform Count Plates (3M, Saint Paul, MN, USA), Palcam agar
112 (Oxoid), xylose lysine deoxycholate agar (XLD; Becton, Dickinson, and Company), Baird-
113 Parker agar (BPA; MBcell) supplemented with egg yolk tellurite (MBcell), Petrifilm™
114 Aerobic Count plates (3M), Petrifilm™ Enterobacteriaceae Count Plates (3M), de Man,
115 Rogosa, and Sharpe agar (MRS agar; Becton, Dickinson, and Company), cetrimide agar
116 (Becton, Dickinson, and Company), plate count agar (PCA; Becton, Dickinson, and
117 Company), and Petrifilm™ Yeast & Mold Count Plates (3M) for *E. coli* and coliform,
118 *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus*, total aerobic counts,
119 Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp., psychrotrophic bacteria, and
120 yeast and mold, respectively. Petrifilm™ E. coli/Coliform Count Plates, BPA supplemented
121 with egg yolk tellurite, and Petrifilm™ Aerobic Count plates were incubated at 37℃ for 48 h.
122 Palcam agar and cetrimide agar were incubated at 30℃ for 48 h. XLD and MRS agar were
123 incubated at 37℃ for 24 h. Petrifilm™ Enterobacteriaceae Count Plates were incubated at
124 37℃ for 48 h. PCA and Petrifilm™ Yeast & Mold Count Plates were incubated at 7℃ for 10
125 d and at 25℃ for 5 d, respectively. The cell counts of the following bacteria were determined
126 based on the colonies identified through the following methods and 16S rRNA sequencing.
127 For the identification of *E. coli*, blue colonies that produced gas on Petrifilm™ E.
128 coli/Coliform Count Plates were streaked onto EMB agar. Colonies exhibiting a green
129 metallic sheen after 24 h of incubation at 37℃ were isolated. For *L. monocytogenes*, colonies
130 presumptively identified on Palcam agar plates were streaked to CHROMagar™ Listeria
131 (CHROMagar, Paris, France) and incubated at 37℃ for 24 h. Colonies appearing blue with a

diameter of less than 3 mm and displaying a regular white halo on CHROMagar™ Listeria were isolated. Since no colonies indicative of *Salmonella* were observed, no further analysis was conducted for this bacterium. For *S. aureus*, colonies presumptively identified on BPA supplemented with egg yolk tellurite were streaked onto CHROMagar™ Staph aureus (CHROMagar) and incubated at 37℃ for 24 h. Pink to mauve colonies were isolated. For *Pseudomonas* spp., colonies on cetrimide agar were streaked onto CHROMagar™ Pseudomonas (CHROMagar) and incubated at 30℃ for 24-36 h. Blue-green colonies were isolated. All the isolated colonies were then subjected to the 16S rRNA sequencing. The 16S rRNA sequencing was performed by BIONICS (BIONICS Co., Ltd, Seoul, Korea) using universal primers 27F and 1492R. The resulting sequences were analyzed by comparing them with microbial sequences in the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST (Basic Local Alignment Search Tool) to identify the bacteria. In this experiment, the presumptive colonies on the media where the homogenates were plated were streak-plated on the second media with higher selectivity, and isolated colonies on the second media were identified by 16S rRNA sequencing. Based on this identification, only identified colonies of the presumptive colonies were counted.

2.1.3. Analysis of VBN content

VBN contents were also analyzed for samples corresponding to microbiological analysis. VBN content was evaluated with the micro-diffusion method (Conway and O'Mally, 1942; MFDS, 2022). In brief, a sample bag containing 5 g beef and 25 mL distilled water was homogenized with a pummeler for 1 min, and the homogenate was left at room temperature for 30 min. The homogenate was then filtered using Qualitative Filter Papers No.131 (Advantec, Tokyo, Japan). Subsequently, 1 mL of 0.01 N sulfuric acid (H_2SO_4)

(Daejung Chemicals & Metals Co. Ltd., Siheung-si, Gyeonggi-do, Korea) was placed into the inner chamber of the Conway diffusion cell (Daihan Scientific Co., Wonju, Gangwon-do, Korea), and 1 mL of the filtrate and 1 mL of a saturated K_2CO_3 solution (Samchun Chemical Co., Ltd., Seoul, Korea) were placed into the outer chamber. The Conway diffusion cell was sealed with glycerin and incubated at 25°C for 1 h. After incubation, the VBN-captured H_2SO_4 solution was titrated with 0.01 N sodium hydroxide (Daejung Chemicals & Metals Co. Ltd., Siheung-si, Gyeonggi-do, Korea) with the addition of 10 μL indicator solution to the inner chamber. To prepare the indicator solution, 0.1 g of methyl red (Duksan Pure Chemicals, Ansan, Gyeonggi-do, Korea) and 0.1 g of methylene blue (Sigma-Aldrich, St. Louis, MO, USA) were each dissolved in 100 mL of ethanol, filtered, and mixed in a 2:1 ratio (v/v). The following equation was used to calculate the concentration of VBN (MFDS, 2022).

$$\text{Volatile basic nitrogen (mg\%)} = 0.14 \times \frac{(b-a)\times f}{W} \times 100 \times DW$$

W = sample weight, a =blank, b =sample, f = factor of 0.01N NaOH, DW = distilled water volume

2.2. Microbiota analysis in beef

To analyze microbiota in beef corresponding to changes in VBN content, of 70 samples, three samples with the highest VBN content (VBNH) and three samples with the lowest VBN content (VBNL) were selected. To extract DNA from the sample, a method by Li et al. (2020) was used with some modifications. Each sample (25 g) was placed in a sample bag containing 225 mL sterile 0.1% BPW and pummeled for 1 min. Ten milliliters of the homogenate were spun down for 10 min, and the supernatant was transferred to a conical tube. The supernatant was then centrifuged at 5,000×g for 15 min at 4°C, and the pellet was resuspended with 10 mL of phosphate-buffered saline (PBS; pH 7.4; KH_2PO_4 0.2 g,

Na_2HPO_4 1.5 g, NaCl 8.0 g, KCl 0.2 g/distilled water 1 L). The suspension was centrifuged at 5,000×*g* at 4℃ for 15 min. The pellet was then used for genomic DNA extraction. Genomic DNA was extracted from the pellet according to the manufacturer's instructions using a DNeasy PowerSoil Pro Kit (Qiagen, Hilden, NRW, Germany). For sequence library preparation, the Illumina 16S Metagenomic sequence libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols, and the V3 and V4 regions were amplified. Library preparation and paired-end sequencing were performed at Macrogen (Seoul, Korea) with the MiSeq™ platform (Illumina, San Diego, CA, USA). Sequencing results in FASTQ files were subsequently processed and analyzed with the 16S based microbiome taxonomic profiling (MTP) pipeline of the EzBioCloud (CJ Bioscience, Inc., Seoul, Korea) for microbial community and diversity analysis. The PKSSU 4.0 version of the EzBioCloud was used as the reference database for the classification and identification of bacteria with a cut-off percentage set at 0.5% to exclude low-abundance taxa. Microorganisms identified below this threshold were classified into the et cetera (ETC) group. The cut-off was used to avoid the complexity in data analysis caused by including very low-abundance microorganisms, which may often result from sampling errors or other technical variances (Brumfield et al., 2020; Sadurski et al., 2024). Metagenomic analysis for yeast and mold was not conducted because they were analyzed with 16S rRNA, and thus, it was not appropriate to compare the relative abundance with bacteria. Additionally, yeast and mold populations were much lower than those of bacteria in beef samples.

3. Results and discussion

3.1. Prevalence in beef

3.1.1. Microorganisms

206 Qualitative analysis showed no presence of *E. coli* and EHEC in beef (data not shown).
207 Quantitative analysis also showed that *E. coli*, *Salmonella* spp., and *L. monocytogenes* counts
208 were below the detection limit (<1.0 Log CFU/g) (Table 1). Coliform counts were 1.7-2.1
209 Log CFU/g. *S. aureus* was detected at 1.2 Log CFU/g in only one out of 70 samples.
210 Regardless of part, distribution channel, and grade, total aerobic bacteria levels were
211 observed between 4.5 and 5.7 Log CFU/g, while Enterobacteriaceae ranged from 2.0 to 3.3
212 Log CFU/g. Lactic acid bacteria counts varied from 3.8 to 4.4 Log CFU/g. *Pseudomonas* spp.
213 counts were in a range of 1.7 to 2.4 Log CFU/g. Psychrotrophic bacterial counts ranged from
214 4.7 to 6.2 Log CFU/g. Yeast and mold counts were from 2.9 to 3.3 Log CFU/g.
215 The cold chain system is used to delay meat spoilage by maintaining low temperatures
216 during various stages, including post-slaughter carcass storage, cut handling, meat transport
217 to distributors, and storage at retail sites (Ercolini et al., 2009). These practices might be
218 related to the higher levels of psychrotrophic bacteria than those of other microorganisms in
219 meat, as these bacteria could proliferate at refrigeration temperature. In contrast, the growth
220 of microorganisms with higher optimal growth temperatures was inhibited under these
221 conditions (Anas et al., 2019). This reason might cause somewhat higher psychrotrophic
222 bacterial cell counts than the other bacteria. Some lactic acid bacteria in meat are
223 psychrotrophic (Yost and Nattress, 2002; Ercolini et al., 2009), and *Pseudomonas* spp. are
224 also psychrotrophic (Gill and Newton, 1978; Ledenbach and Marshall, 2009; Kim et al.,
225 2013). Thus, their cell counts might contribute to relatively higher cell counts of
226 psychrotrophic bacteria and total aerobic bacteria.
227
228 **3.1.2. VBN**
229 VBN serves as an indicator of protein or amine degradation, and its value was assessed to
230 measure the freshness of beef (Bekhit et al., 2021a). On average, sirloin and top rounds

231 exhibited 8.51±4.44 mg% and 8.15±5.33 mg% of VBN values, respectively (Fig. 1A). In
232 distribution, the VBN values were 8.01±4.75 mg% and 8.39±4.95 mg% for meat packaging
233 plants and retail shops, respectively (Fig. 1B). High-grade samples showed 10.33±5.04 mg%
234 of VBN values, and low-grade samples had 6.26±3.79 mg% (Fig. 1C). In summary, VBN
235 values did not depend on parts, distribution channels, and grades. However, it may vary
236 depending on the sampling season, the number of samples, and the geographic region of
237 sampling. In the other study, raw beef had a VBN content of 8.70±0.40 mg% (An et al., 2020),
238 which is similar to the findings in our study. Based on this result, three VBNH samples and
239 three VBNL samples were selected from 70 samples; their VBN values were 1.06±0.64 mg%
240 and 20.73±2.79 mg% for VBNH and VBNL samples, respectively.
241
242 **3.2. Microbiota in beef**
243 To compare the differences in beef microbiota by alpha diversity, the Chao1 (species
244 richness) and Shannon (species diversity) indices were calculated (Chao et al., 2014) (Fig. 2A
245 and 2B). The Chao1 index did not reveal a significant difference between the VBNH and
246 VBNL groups. However, the Shannon index was higher in the VBNL group than in the
247 VBNH group, indicating higher species diversity in these samples. Beta diversity (the
248 variation in species composition) using the Principal Coordinate analysis (PCoA) was
249 conducted to compare microbial distribution (Legendre et al., 2005) (Fig. 2C). Different
250 microbial community cluster patterns were observed between the VBNH and the VBNL
251 groups. The PCoA analysis showed that more spread was observed in the VBNL group, and
252 it indicates a higher diversity in microbial composition. Specifically, the VBNL samples
253 displayed a wider spread along the principal coordinate (PC), particularly PC1 and PC2.
254 At the phylum level, Firmicutes and Proteobacteria were identified as common phyla in
255 all samples (Fig. 3A). The VBNH group comprised 96.6% Firmicutes, a minor presence of

Proteobacteria, and other phyla of the total composition. Conversely, the VBNL group showed a more diverse microbiota composition: Firmicutes accounted for approximately 68.7%, Actinobacteria accounted for about 19.8%, and smaller proportions of Proteobacteria and other phyla. At the genus level, microbiota was also more varied in the VBNL group than in the VBNH group, and the VBNH group had relative abundance in order of *Lactobacillus* (61.3%) > *Leuconostoc* (17.9%) > *Lactococcus* (11.4%) > *Carnobacterium* (5.7%) > *Pseudomonas* (2.6%), and their relative abundances were higher compared to the VBNL group (Fig. 3B). These genera, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Carnobacterium*, are all lactic acid bacteria (Ringø et al., 1998). Lactic acid bacteria belong to the family of Firmicutes (Liu et al., 2010). Hence, this result may correspond to the comparison for Firmicutes and Proteobacteria, as the VBNH group showed a high relative abundance of Firmicutes with lactic acid bacteria. Among the *Lactobacillus* species, which were found in high proportions in both VBNH (61.3%) and VBNL (39.1%) groups, most of them frequently isolated from meat and meat products are psychrotrophic bacteria (Ercolini et al., 2009; Morishita and Shiromizu, 1986). Similarly, certain *Leuconostoc* species, such as *Leuconostoc gelidum* and *Leuconostoc gasicomitatum* were also psychrotrophic bacteria frequently isolated from meat (Comi et al., 2024; Johansson et al., 2022; Mun et al., 2021; Shaw and Harding, 1984). At the species level, microbiota was more varied in the VBNL group than in the VBNH group (Fig. 3C). *Lactobacillus* spp. in the VBNH group were predominantly represented by *Lactobacillus algidus* and *Lactobacillus sakei* (Fig. 3C). The other notable species were *Leu. gelidum* and *Lactococcus piscium*. *L. algidus* is known as *Dellaglioa algida* (Poirier et al., 2018; Sun et al., 2015; Zheng et al., 2020), and it grows within a temperature range of 0-25℃ (Kato et al., 2000). *L. algidus* has been isolated from various meat and dairy products, including cattle milk, cured seasoned pork, cured ripened sausages, cooked cured or seasoned pork, and bovine meat (Parente et al., 2023; Pothakos et

al., 2014; Sakala et al., 2002; Stoops et al., 2015). *L. sakei* is also a psychrotrophic lactic acid bacteria commonly found in fresh meat and fish (Chaillou et al., 2005). Vihavainen and Björkroth (2007) identified *Leu. gelidum* and *L. sakei* as predominant populations in the lactic acid bacteria in packaged spoiled beef. This result shows that microbiota differs between the samples with high and low VBN, especially for lactic acid bacteria such as *Lactobacillus* and *Leuconostoc* which are the first and second most. These bacteria are known to have proteolytic activity (García-Cano et al., 2019; Kieliszek et al., 2021), which may lead to protein degradation and the production of volatile nitrogenous compounds (Thorn and Greenman, 2012; Bekhit et al., 2021b). Thus, they might be related to producing more VBN contents in the VBNH samples in this study. However, this is just a potential suggestion because this result is based only on metagenomic analysis. The results of the metagenomic analysis show the relative plentifulness of certain bacteria rather than indicating an absolute relation of the higher relative abundance to the higher VBN production. Meat contains calpain, cathepsin, and caspases, which function as proteases, and alanine aminopeptidase, arginine aminopeptidase, and serine aminopeptidases which function as peptidases (Toldrá and Flores, 2000; Sentandreu et al., 2002). Decarboxylases in meat may be involved in the decarboxylation of amino acids, leading to the formation of VBN (Halász et al., 1994; Tosukhowong et al., 2011). These enzymes may act as endogenous factors which contribute to the increase in VBN. Thus, there is a possibility that VBN detected in the beef samples may not be solely caused by the lactic acid bacteria suggested by metagenomic analysis. Because of these reasons, further research is necessary to clarify the results of this study.

4. Conclusion

Total aerobic bacteria, Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp., yeast and molds, and psychrotrophic bacteria were primarily detected in beef samples collected in

current distribution conditions, and their populations varied among the samples. VBN contents also varied among the beef samples. As samples were categorized with VBNH and VBNL, the metagenomic analysis showed that VBNH samples had a high relative abundance of *Lactobacillus* and *Leuconostoc* mostly. Therefore, these results suggest that microorganism populations and VBN varied among beef samples, but specific lactic acid bacteria might be potential bacteria contributing to the production of more VBN in beef. However, the number of samples used was limited and potential VBN production-related bacteria were suggested only by metagenomic analysis, and storage period and storage methods, which may affect the composition of microorganisms, were not examined in this study. Additionally, endogenous enzymes in meat could also contribute to VBN production. Therefore, further research is necessary to identify and isolate these bacteria. This research may involve the inoculation of potential bacteria based on the metagenomic analysis into a larger sample size, followed by the analysis of their VBN production while excluding VBN production by endogenous factors. In further research, an investigation of the bacteria contributing to VBN production under different storage methods and storage periods may also be necessary followed by a comparison of the result with the one in this study. If the design of the research needs to contain additional factors such as temperature, hygiene practices, sanitary conditions in the production process, etc., affecting the compositions of microorganisms in beef that are related to VBN production, the research needs to be conducted with experimental designs of setting levels of these factors with objective standard and with sufficient sample size.

Conflict of interest

The authors declare no potential conflicts of interest.

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Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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Figure Legends

Fig. 1. Volatile basic nitrogen (VBN) contents according to parts (A), distribution channel (B), and grade (C) in beef sample.

Fig. 2. Microbiota diversity with Chao indices (A), Shannon indices (B), and PCoA (C) in beef samples with high volatile basic nitrogen (VBN) contents (VBNH) and beef samples with low VBN contents (VBNL). PCoA: principal coordinate analysis, PC: principal coordinate.

Fig. 3. Comparison of microbiota compositions at phylum (A), genus (B), and species levels (C) in beef samples with high volatile basic nitrogen (VBN) contents (VBNH) and beef samples with low VBN contents (VBNL). ETC group: et cetera group.

Table 1. Prevalence [populations (mean ± standard deviation; log CFU/g) (the number of positive samples/the number of analyzed samples)] of food spoilage microorganisms in beef

	Part		Distribution stages		Grade	
	Sirloin	Top round	Meat packaging plant	Meat retail	High	Low
Escherichia coli	-[1)] (0/34)	- (0/36)	- (0/12)	- (0/58)	- (0/36)	- (0/34)
Coliform	2.1±0.9 (28/34)	1.7±0.7 (21/36)	1.9±0.8 (12/12)	1.9±0.9 (19/58)	1.9±0.7 (0/36)	2.0±0.9 (0/34)
Listeria monocytogenes	- (0/34)	- (0/36)	- (0/12)	- (0/58)	- (0/36)	- (0/34)
Salmonella spp.	- (0/34)	- (0/36)	- (0/12)	- (0/58)	- (0/36)	- (0/34)
Staphylococcus aureus	1.2 (1/34)	- (0/36)	- (0/12)	1.2 (1/58)	- (0/36)	1.2 (1/34)
Total aerobic bacteria	5.7±1.3 (34/34)	4.5±1.1 (36/36)	5.1±1.3 (12/12)	5.1±1.6 (58/58)	5.0±1.2 (36/36)	5.2±1.4 (34/34)
Enterobacteriaceae	3.3±1.1 (30/34)	2.0±0.8 (33/36)	2.6±1.1 (11/12)	2.9±1.3 (52/58)	2.5±0.9 (30/36)	2.8±1.3 (33/34)
Lactic acid bacteria	4.4±1.2 (34/34)	3.9±0.9 (34/36)	4.3±1.0 (12/12)	3.8±1.4 (56/58)	4.0±1.0 (34/36)	4.4±1.1 (34/34)
Pseudomonas spp.	2.4±1.3 (9/34)	1.9±0.9 (16/36)	2.3±1.1 (4/12)	1.7±0.6 (21/58)	2.0±1.0 (14/36)	2.4±1.1 (11/34)
Psychrotrophic bacteria	6.2±1.4 (34/34)	4.7±1.3 (34/36)	5.4±1.5 (12/12)	5.5±1.8 (58/58)	5.3±1.4 (36/36)	5.6±1.6 (34/34)
Yeast and molds	3.1±1.1 (34/34)	2.9±0.8 (33/36)	2.9±0.9 (12/12)	3.3±0.9 (57/58)	2.9±1.0 (35/36)	3.0±0.8 (34/34)

[1)] Below detection limit.

511 **Fig. 1.**

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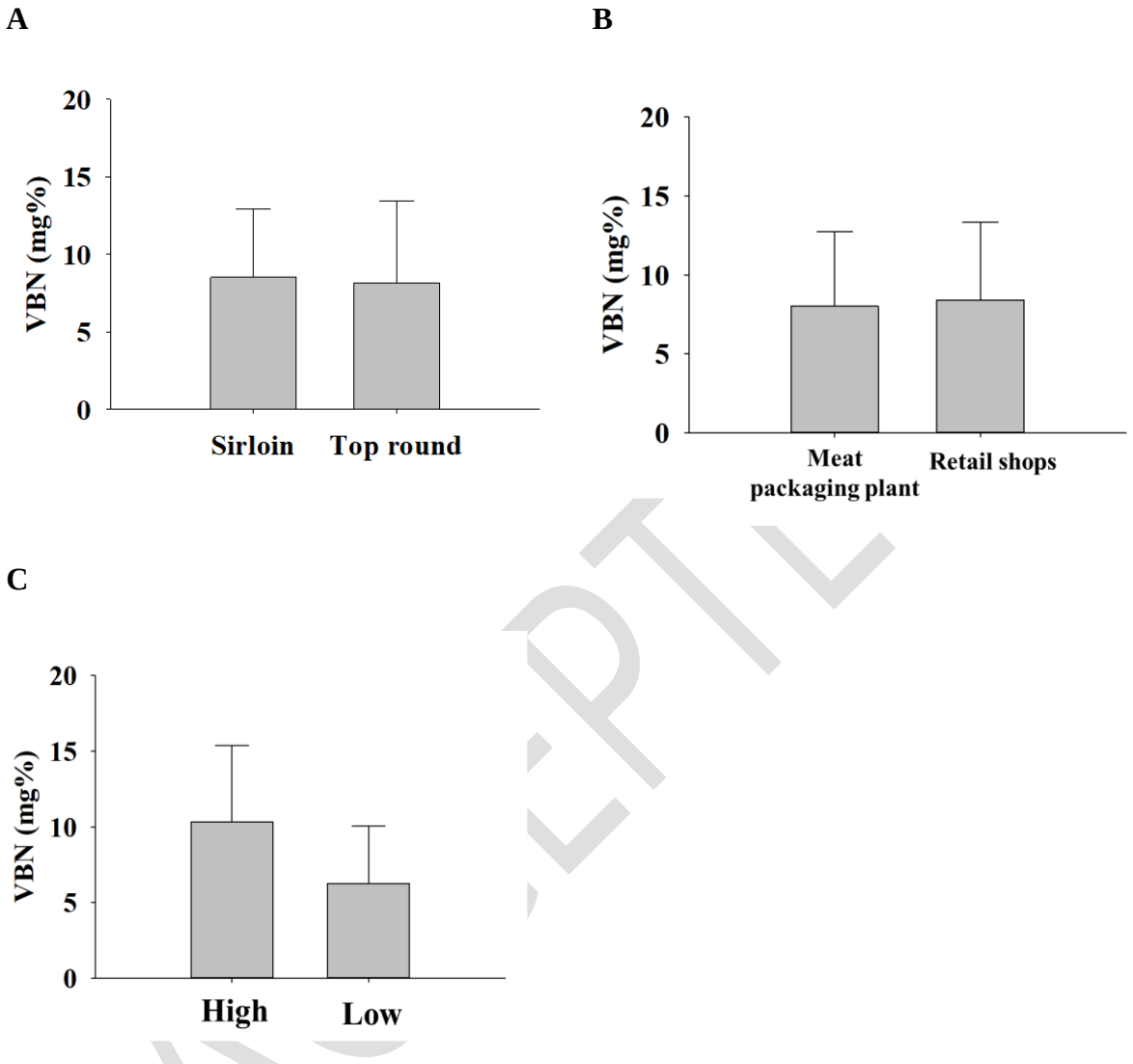

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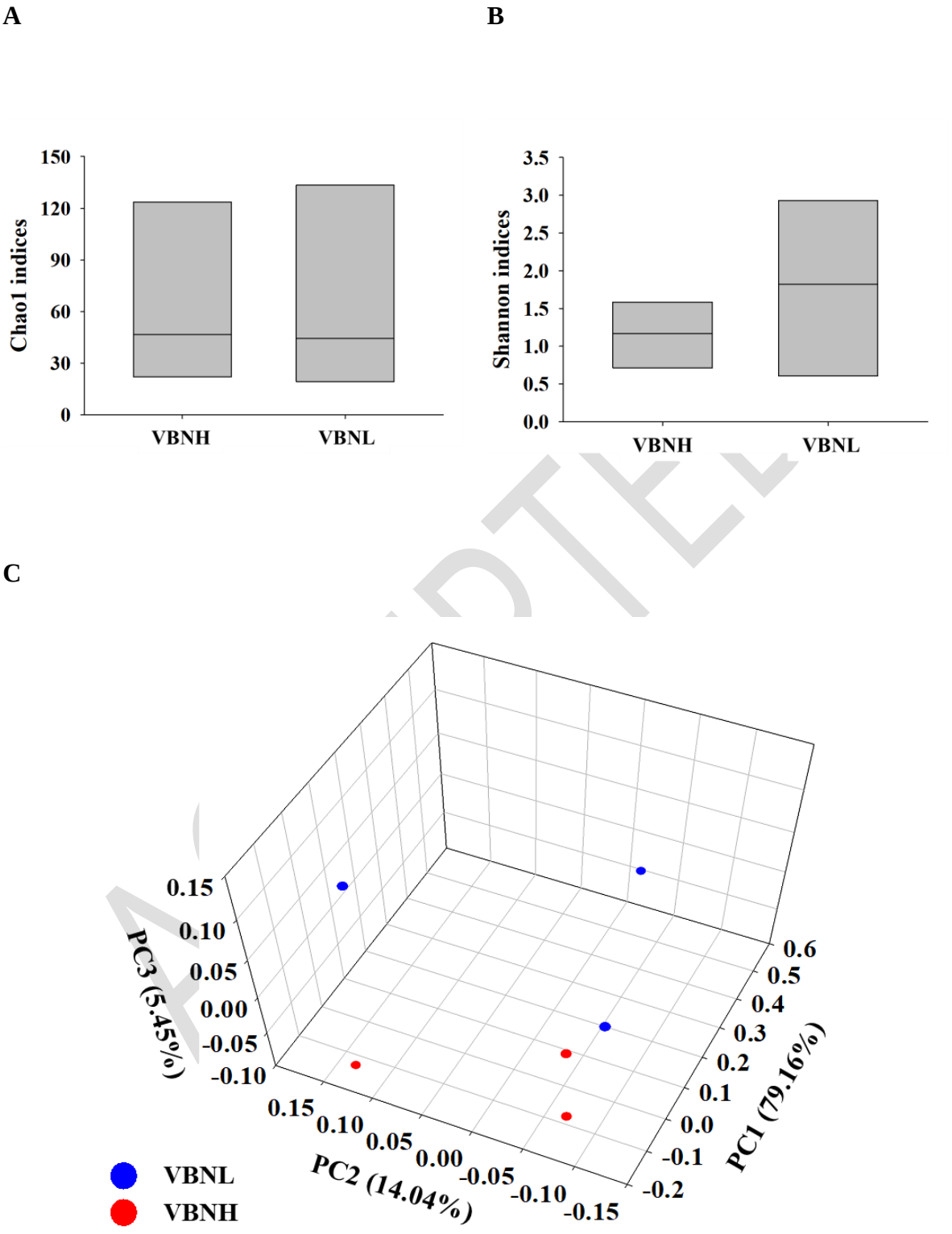
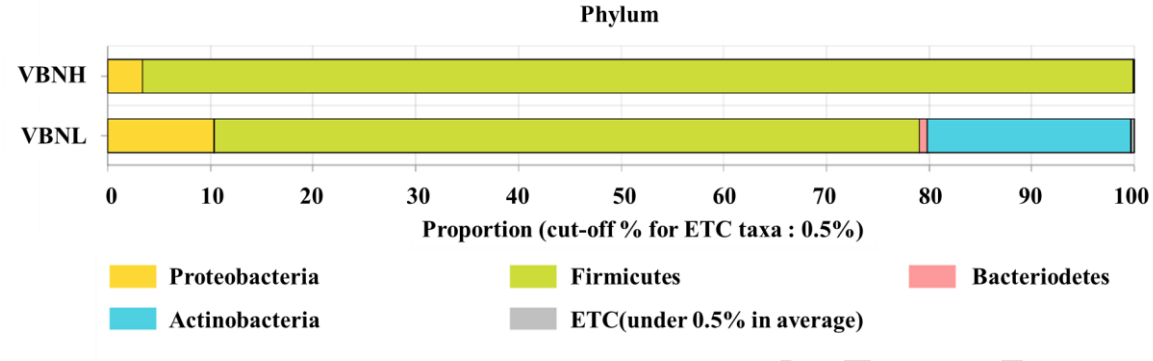
518 **Fig. 2.**


A
B
Chao1 indices
150
120
90
60
30
0
VBNH
VBNL
Shannon indices
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
VBNH
VBNL
C
PC3 (5.45%)
0.15
0.10
0.05
0.00
-0.05
-0.10
PC2 (14.04%)
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
PC1 (79.16%)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
VBNL
VBNH

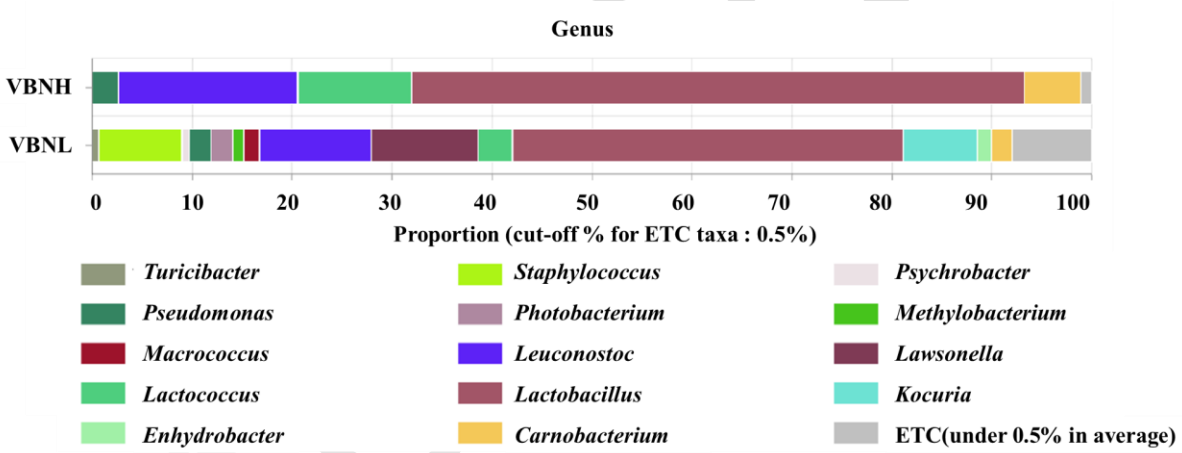
519

Fig. 3.

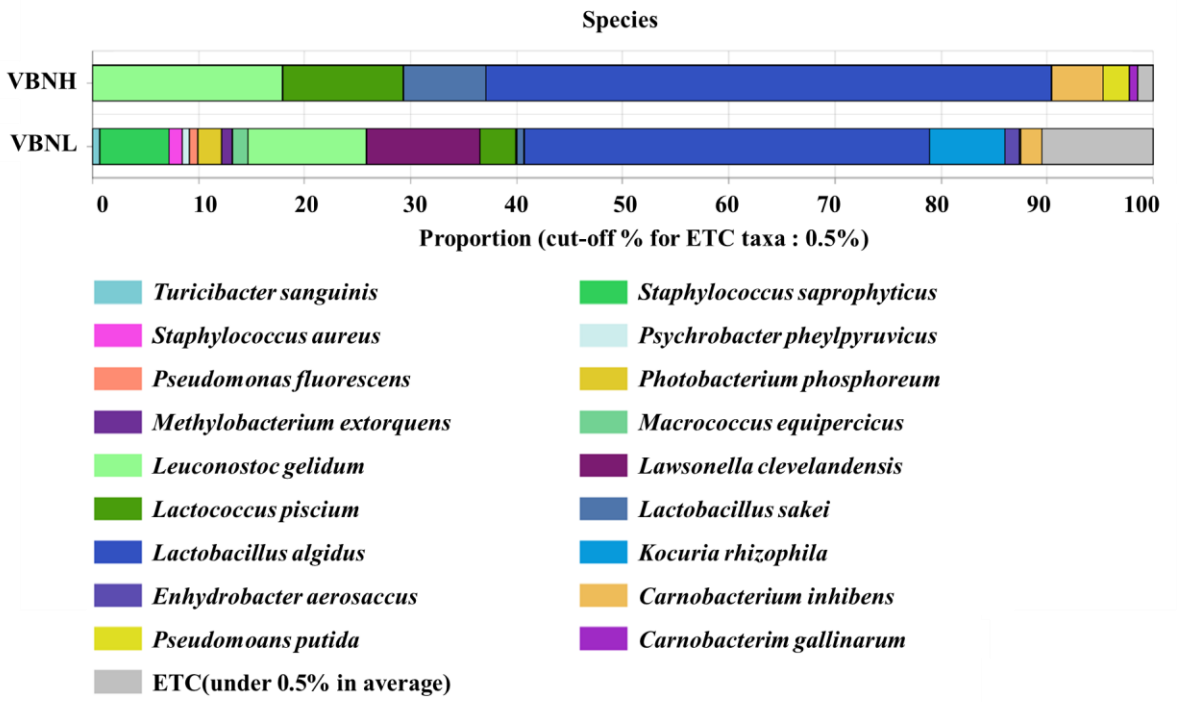
A


Phylum
VBNH
VBNL
0
10
20
30
40
50
60
70
80
90
100
Proportion (cut-off % for ETC taxa : 0.5%)
Proteobacteria
Firmicutes
Bacteriodetes
Actinobacteria
ETC(under 0.5% in average)

B


Genus
VBNH
VBNL
0
10
20
30
40
50
60
70
80
90
100
Proportion (cut-off % for ETC taxa : 0.5%)
Turicibacter
Staphylococcus
Psychrobacter
Pseudomonas
Photobacterium
Methylobacterium
Macrococcus
Leuconostoc
Lawsonella
Lactococcus
Lactobacillus
Kocuria
Enhydrobacter
Carnobacterium
ETC(under 0.5% in average)

C


Species
VBNH
VBNL
0
10
20
30
40
50
60
70
80
90
100
Proportion (cut-off % for ETC taxa : 0.5%)
Turicibacter sanguinis
Staphylococcus saprophyticus
Staphylococcus aureus
Psychrobacter pheylpyruvicus
Pseudomonas fluorescens
Photobacterium phosphoreum
Methylobacterium extorquens
Macrococcus equipercicus
Leuconostoc gelidum
Lawsonella clevelandensis
Lactococcus piscium
Lactobacillus sakei
Lactobacillus algidus
Kocuria rhizophila
Enhydrobacter aerosaccus
Carnobacterium inhibens
Pseudomoans putida
Carnobacterim gallinarum
ETC(under 0.5% in average)