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ARTICLE

Characterization of the Biodiversity of the Spoilage Microbiota in Chicken Meat Using Next Generation Sequencing and Culture Dependent Approach

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Abstract

This study investigated the psychrotrophic bacteria isolated from chicken meat to characterize their microbial composition during refrigerated storage. The bacterial community was identified by the Illumina MiSeq method based on bacterial DNA extracted from spoiled chicken meat. Molecular identification of the isolated psychrotrophic bacteria was carried out using 16S rDNA sequencing and their putrefactive potential was investigated by the growth at low temperature as well as their proteolytic activities in chicken meat. From the Illumina sequencing, a total of 187,671 reads were obtained from 12 chicken samples. Regardless of the type of chicken meat (i.e., whole meat and chicken breast) and storage temperatures (4°C and 10°C), *Pseudomonas weihenstephanensis* and *Pseudomonas congelans* were the most prominent bacterial species. *Serratia* spp. and *Acinetobacter* spp. were prominent in chicken breast and whole chicken meat, respectively. The 118 isolated strains of psychrotrophic bacteria comprised *Pseudomonas* spp. (58.48%), *Serratia* spp. (10.17%), and *Morganella* spp. (6.78%). All isolates grew well at 10°C and they induced different proteolytic activities depending on the species and strains. Parallel analysis of the next generation sequencing and culture dependent approach provides in-depth information on the biodiversity of the spoilage microbiota in chicken meat. Further study is needed to develop better preservation methods against these spoilage bacteria.

Keywords next generation sequencing, psychrotrophic bacteria, spoilage, proteolytic activity, chicken meat

Introduction

Due to improvements in economic drivers for local communities, demand for animal products and particularly meat, are increasing in order to meet dietary demands. Especially, chicken meat is relatively cheap and a good source of animal protein for human health. However, as more and more chicken meat is being consumed, there are concerns about food safety and security. Chicken meat has a short shelf life because psychrotrophic bacteria causes spoilage or off-flavors even at cold storage conditions (Carrizosa *et al.*, 2017). The spoilage of meat depends on pH level, availability of oxygen, biodiversity of bacterial groups, and storage temperature (Ercolini *et al.*, 2009). These factors, in turn, are closely associated with the growth of spoilage bacteria.

In food industries, the increase in contamination of meat is a major issue at slaughter and during processing (Remenant *et al.*, 2015). In addition, increased pH after

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rigor mortis induces bacterial growth of putrefactive bacteria. *Pseudomonas* spp. is a major psychrotrophic bacteria that produces proteinase and its optimal pH is from 6.5 to 8.0. Proteinase hydrolyses chicken protein and causes spoilage (Fairbairn and Law, 1986; Nowak *et al.*, 2012). Availability of oxygen generally affects the microbial populations that will proliferate. When oxygen levels are high, aerobic or facultative anaerobic gram-negative bacteria dominate but at low oxygen levels, facultative anaerobic or anaerobic gram-positive microbiota grow (Doulgeraki *et al.*, 2012).

Storage temperature, however, is the most important factor that affects the growth of bacteria present in chicken meat. Psychrotrophic bacteria can grow at refrigerated conditions, and temperature can affect various microbial growth parameters including maximum growth rate and total bacterial counts (Mataragas *et al.*, 2006). Doulgeraki *et al* (2010) reported that storage temperature can affect the spoilage potential of bacteria and that different strains of the same species do not necessarily grow at the same rates.

Traditionally, microbial populations of meat has been analyzed by culture-dependent plating methods. It is based on the isolation and culturing of bacteria from chicken meat and identification by 16S rDNA sequencing. This method has limitations in that not all bacteria present in chicken meat can be cultivable on the plate media used in the microbial analysis (Grama *et al.*, 2002). However, in recent studies, molecular technologies are being developed with much higher resolving power to advance research (Doulgeraki *et al.*, 2012). Next generation sequencing (NGS) could play an important role in understanding the in-depth biodiversity of spoilage microbiota by analyzing many different samples. Also, NGS can discriminate taxa to specific strains allowing for detailed analysis of bacteria present in meat (Broekaert *et al.*, 2011; Casaburi *et al.*, 2011; Diez *et al.*, 2008).

The objectives of this study were to characterize the putrefactive bacteria isolated from chicken meat during cold storage and to analyze the composition of chicken meat spoilage microbiota using NGS.

Material and Methods

Preparation of chicken meat for spoilage

Chicken meat was purchased commercially as whole chicken (with skin) and chicken breast in local markets. To test for spoilage, two types of chicken meat were refrigerated at 4°C and 10°C. The chicken samples were kept for 5 to 15 d until spoilage. After storage, phosphate buffer saline (PBS) at 2 X the sample weight was added to samples and was homogenized using a stomacher (3M, Korea) at maximum speed for 2 min. The supernatant was then filtered through sterilized gauze to remove floating matter.

Isolation and identification of psychrotrophic bacteria

The filtrate was serially diluted 10-fold with PBS solution, and one hundred microliters was inoculated onto Tryptic Soy Agar (TSA, BD Difco, USA). Inoculated plates were incubated at 25°C for 24 to 48 h. After incubation, colonies were randomly selected from the most diluted plates for the isolation of psychrotrophic bacteria. The isolates were cultured and stored at -80°C in 10% skim milk media.

For this analysis, the isolates were amplified using the PCR process and isolates were identified by 16S rDNA sequencing (Solgent Co., Korea). We used universal primers 27F and 1492R with 2x H-star Taq PCR master mix (Biofact Co., Ltd, Korea). The sequences were confirmed using the BLAST search and EzBioCloud (Yoon *et al.*, 2017).

Analysis of bacterial community in chicken meats

For the analysis of bacterial communities, bacterial DNA was extracted from the chicken meat filtrates. The filtrates were centrifuged at 13,000 rpm for 10 min and re-suspended with PBS. Bacterial DNA was extracted using QIAamp powerfecal DNA isolation kit (Qiagen, Netherlands). DNA concentration and purity was evaluated for subsequent analysis and samples were sequenced by Illumina MiSeq platform and identified using CLcommunity software (Chunlab, Korea).

Evaluation of putrefactive potentials for isolates

Isolated psychrotrophic bacteria were measured for growth at different temperature and by media. The isolates were inoculated on TSA and Plate Count Agar (PCA, BD Difco, USA) and incubated at 10°C, 25°C, and 37°C. The plates were stored for 7 d and bacterial growth was measured at 24 h intervals.

Skim milk agar (SMA, 10% of skim milk solution and 1.5% of Bacto agar [BD Difco, USA]) and chicken juice agar (CJA, 50 g chicken breast meat ground in 250 mL sterilized deionized-water and filtered through two layers

of gauzes; the filtrate were kept in a water bath at 50°C) was used to compare proteolytic activity of the isolates. For making agar plates, 250 mL of 3% Bacto agar solutions were prepared at 50°C and mixed it with the filtrate solution prepared as above (Wang *et al.*, 2017). The isolated psychrotrophic bacteria were inoculated on SMA and CJA plates, and incubated at 25°C for 2 d. After incubation, the plates were examined for clear zones around colonies.

Results and Discussion

Isolation and identification of psychrotrophic bacteria

For the isolation of psychrotrophic bacteria, 12 samples of chicken meat were stored at 4°C and 10°C, and the period of storage was continued until observing similar patterns of spoilage on the surface including microbiological, visible and organoleptic features. During the experimental conditions, the number of bacteria increased gradually with storage time. After 5 d, bacterial growth in chicken meat increased over 8.3 Log CFU/g. In addition, it was confirmed that significantly more bacterial growth was observed on whole chicken than on chicken breast. According to Carrizosa *et al*. (2017), putrefactive bacteria at concentrations more than 8 Log CFU/g can cause spoilage and off-flavor due to the degradation of amino acids.

For the isolation of psychrotrophic bacteria, colonies were randomly selected from TSA plate at the most diluted concentrations. We isolated 118 strains from chicken meat and they were incubated at 25°C for > 12 h before use. The identification of bacterial isolates was carried out using 16S rDNA sequencing. Because bacteria have conserved regions in common, they can be identified after PCR amplification based on universal primers (Nocker *et al.*, 2007).

From the culture-dependent approach, we isolated 118 strains of psychrotrophic bacteria and they comprised *Pseudomonas* spp. (58.48%), *Serratia* spp. (10.17%), and *Morganella* spp. (6.78%) (Fig. 1). In total, 16 different species of *Pseudomonas* were identified with *Pseudomonas weihenstephanensis* and *Pseudomonas psychrophila* the most abundant (Table 1). *Pseudomonas weihenstephanensis* has been recently reported as a novel species from raw cow's milk (von Neubeck *et al.*, 2016). It is similar to *Pseudomonas fragi* and *P. psychrophila* based on phylogenetic analyses, and has a potential to cause spoilage of food. In general, *Pseudomonas* spp. mainly appeared at low temperatures and aerobic conditions, and species of this genus such as *P. fragi*, *P. lundensis*, and *P. fluorescens* have been reported in the putrefaction of meat (Casaburi *et al.*, 2015; Lebert *et al.*, 1998).

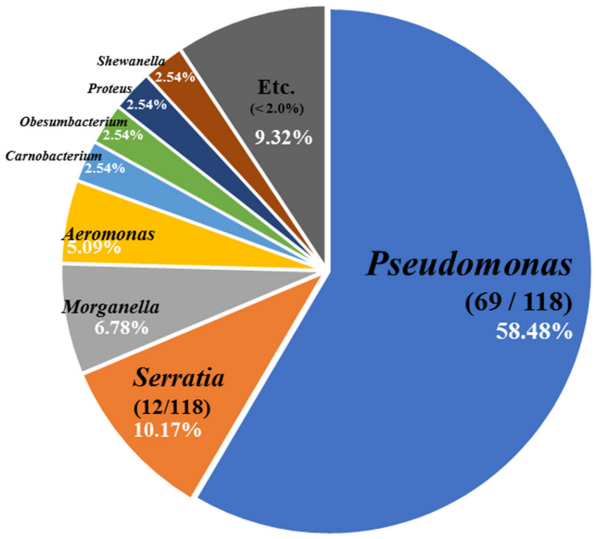

Fig. 1. Composition of the major bacterial genera isolated from the spoiled chicken meat.

Table 1. Composition of the *Pseudomonas* species isolated from chicken meat

Pseudomonas species	Number of strains
Pseudomonas weihenstephanensis	17
Pseudomonas psychrophila	13
Pseudomonas fragi	11
Pseudomonas lundensis	8
Pseudomonas gessardii	4
Pseudomonas congelans	2
Pseudomonas synxantha	2
Pseudomonas tremae	2
Pseudomonas trivialis	2
Pseudomonas yamanorum	2
Pseudomonas caeni	1
Pseudomonas deceptionensis	1
Pseudomonas ficuserectae	1
Pseudomonas koreensis	1
Pseudomonas poae	1
Pseudomonas taetrolens	1
Total	69

All experiments were divided into four treatment groups, which were composed of two different types of chicken meat (chicken breast, CB; whole chicken meat, CS) and two different temperatures (4°C and 10°C). The most frequently observed bacteria in the microbial populations of each treatment group were *Pseudomonas* spp., and *Serra-*

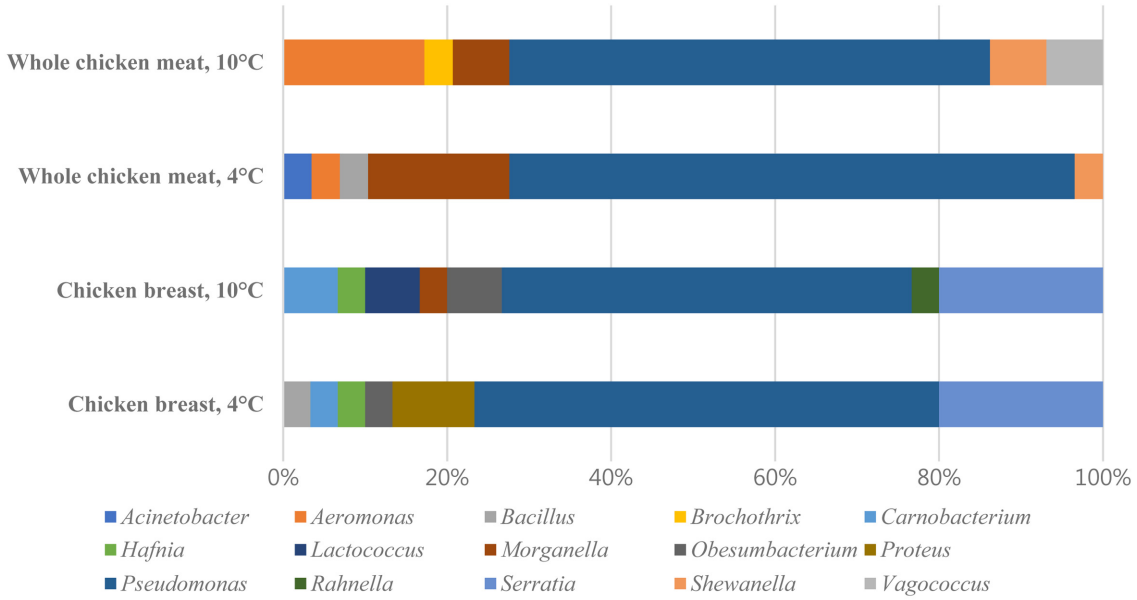

Fig. 2. Composition of isolated psychrotrophic bacteria at different storage conditions. In total 29 strains were isolated from whole chicken meats, and 30 strains were isolated from chicken breast meats, respectively.

tia spp., *Carnobacterium* spp., *Hafnia* spp., and *Obesumbacterium* spp. Especially, *Serratia liquefaciens* and *S. proteamaculans* were the most predominant species in air packed meat (Pennacchia *et al.*, 2011).

In addition, the treatment groups were compared to evaluate differences in bacterial composition. Fig. 2 shows the composition of isolated psychrotrophic bacteria and indicates that CB4 and CB10 exhibited similar patterns than other treatments. Also, bacterial sharedness was measured which is an index that represents different groups sharing common microbiota. It was demonstrated that CB 10-CB4 had the highest score (80%), followed by CS10-CS4 with 72% and implies that the psychrotrophic bacteria was more affected by different meat than the storage temperature.

Analysis of bacterial community in chicken meats

To identify bacterial communities, bacterial DNA was extracted from each treatment group and analyzed by an Illumina MiSeq platform and 187,671 reads were obtained. *Pseudomonas* spp. comprised ~85% followed by *Serratia* spp. (5.95%), *Acinetobacter* spp. (3.45%), and *Morganella* spp. (1.06%) (Fig. 3). In addition, *P. weihenstephanensis* (34.59%) and *P. congelans* (13.74%) were the most prominent species in the NGS analysis (Table 2).

Although the biodiversity of this bacterial community was not highly correlated to the psychrotrophic bacteria,

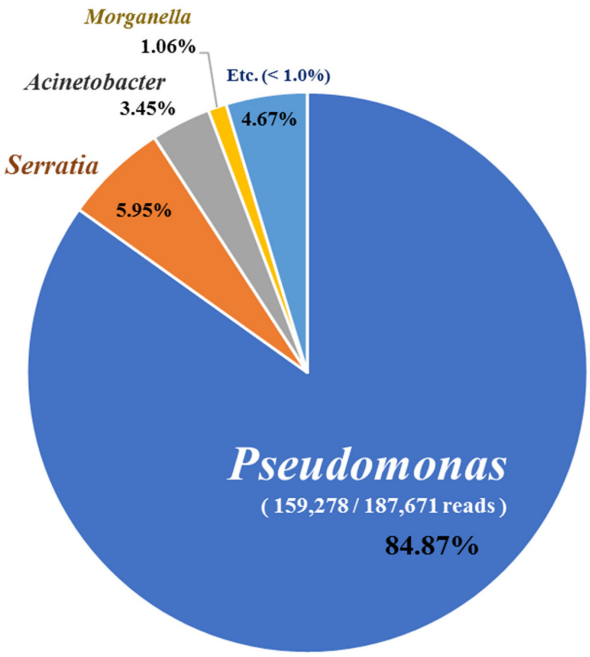

Fig. 3. Bacterial community structure at the genus level during the spoilage of chicken meat. This data was obtained from Illumina MiSeq process.

they shared similar patterns. Predominant genera such as *Pseudomonas*, *Serratia*, and *Morganella* were identified from both experiments. Among these two analyses, the advantage of using NGS was the fact it had higher resolv-

Table 2. Major bacterial species from the community analysis by Illumina MiSeq

Bacterial species	Reads	%
Pseudomonas weihenstephanensis	64,921	34.59
Pseudomonas congelans	25,782	13.74
Pseudomonas syringae	9,602	5.12
Pseudomonas ficuserectae	8,225	4.38
Pseudomonas deceptionensis	6,735	3.59
Pseudomonas asturiensis	5,785	3.08
Pseudomonas fluorescens	5,585	2.98
Serratia proteamaculans	5,482	2.92
Pseudomonas fragi	4,374	2.33
Pseudomonas koreensis	3,690	1.97
Acinetobacter baumannii	3,654	1.95
Pseudomonas helleri	3,447	1.84
Serratia myotis	3,099	1.65
Pseudomonas extremaustralis	2,934	1.56
Pseudomonas caeni	2,576	1.37
Serratia grimesii	2,273	1.21
Pseudomonas tremae	2,036	1.08
Morganella psychrotolerans	1,974	1.05
Etc. (< 1.0%)	25,497	13.59
Total	187,671	100

ing power than the culture-dependent approach. For example, traditional methods of analyzing microbial population are species-specific species but NGS technology can evaluate bacterial communities from environmental samples (Wang *et al.*, 2017). In our study, the culture-dependent approach identified only 16 species of *Pseudomonas* whereas NGS provided 111 species. Consequently, advanced metagenomic technology could play an important role in understanding the biodiversity of spoilage microbiota in chicken meat (Doulgeraki *et al.*, 2012).

To evaluate differences in bacterial communities, MiSeq sequences were analyzed by CLcommunity programs and their relationships were evaluated using sharedness and depicted by UPGMA dendrograms. The bacterial similarity between CB10 and CB4 was much closer than the other populations. The sharedness of each treatment was 80.13% between CB10-CB4 and 80.61% between CS10-CS4. The amount of sharedness was, however, higher than CB4-CS4 (74%) and CB10-CS10 (67%) at the same storage temperature. This indicates that the type of chicken meat was a greater influence than the storage temperatures on bacterial communities. It was assumed that whole chicken (with skin) could be contaminated from feathers, feces, and soil, so that it might harbor different microbial populations than chicken breast (Russell, 2009; Tuncer and Sireli, 2008).

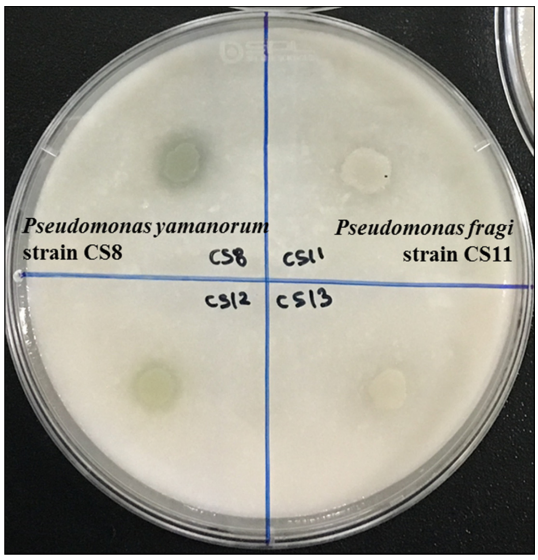

Fig. 4. Proteolytic activity of the isolated psychrotrophic bacteria in chicken juice agar.

Table 3. Proteolytic activity of the psychrotrophic bacteria isolated from chicken meat

Psychrotrophic bacteria	Skim milk agar	Chicken juice agar
Pseudomonas spp.	(**39** / 69)	(**41** / 69)
Serattia spp.	(**12** / 12)	(**11** / 12)
Morganella spp.	(**0** / 8)	(**0** / 8)
Aeromonas spp.	(**6** / 6)	(**5** / 6)
Etc.	(**5** / 23)	(**1** / 23)
Total	(**62** / 118)	(**58** / 118)

Evaluation for the putrefactive potentials of the isolated strains

The isolated psychrotrophic bacteria from chicken meat was composed of many species, and strains of the same species. The isolates were assessed for the growth at different temperatures and media, and also evaluated for proteolytic activity to predict their putrefactive potential.

Almost all of the isolated psychrotrophic bacteria were able to grow at 10°C and 25°C but some of these bacteria did not grow at 37°C even after 5 d. This is probably due to their psychrophilic characteristics.

For the assessment of proteolytic activity, activity was identified by a clear zone around the colonies (Fig. 4). In this experiment, some species were shown to exhibit similar proteolytic activities in both media. However, *Proteus* spp. only able to cause the clear zone in skim milk agar. About two-thirds of *Pseudomonas* spp. showed some degree of proteolytic activity, and the highest activity was

Table 4. Characterization of the *Pseudomonas weihenstephanensis* strains isolated from chicken meat

Isolated psychrotrophic bacteria	Tryptic soy agar & growth at : (No. of days)			Skim milk agar	Chicken juice agar
	10°C	25°C	37°C		
Pseudomonas weihenstephanensis 17 strains					
Pseudomonas weihenstephanensis CB11	1	1	1	+	+
Pseudomonas weihenstephanensis CB12	1	1	2	-	-
Pseudomonas weihenstephanensis CB15	1	1	2	+	+
Pseudomonas weihenstephanensis CB17	1	1	1	-	-
Pseudomonas weihenstephanensis CB18	1	1	2	+	+
Pseudomonas weihenstephanensis CB19	1	1	1	+	+
Pseudomonas weihenstephanensis CB23	1	1	1	+	+
Pseudomonas weihenstephanensis CB28	1	1	2	+	++
Pseudomonas weihenstephanensis CS9	1	1	1	+	+
Pseudomonas weihenstephanensis B5	1	1	1	+	+
Pseudomonas weihenstephanensis B6	1	1	1	+	+
Pseudomonas weihenstephanensis B16	1	1	1	+	+
Pseudomonas weihenstephanensis B24	1	1	1	+	+
Pseudomonas weihenstephanensis S8	1	1	1	+	++
Pseudomonas weihenstephanensis S12	1	1	1	+	+
Pseudomonas weihenstephanensis S14	1	1	1	+	+
Pseudomonas weihenstephanensis S20	1	1	1	+	+

exhibited by *Serratia* spp. and *Aeromonas* spp. that were able to hydrolyze proteins in the plates. However, *Morganella* spp. of all isolates showed no activity (Table 3). These strains were considered non-spoilage bacteria although it is apparent they can grow at refrigerated conditions (Grama *et al.*, 2002).

In addition, we demonstrated mixtures of strain-specific characteristics based on proteolytic activity. For example, among 17 strains of *Pseudomonas weihenstephanensis* tested, two strains had no activity at all, two strains had high activity on chicken juice agar, and the other thirteen strains had moderate proteolytic activity (Table 4). This result was in accordance with other studies that reported different strains exhibited different characteristics (Ercolini *et al.*, 2011; Nieminen *et al.*, 2012).

Conclusion

In this study, we analyzed psychrotrophic bacteria from chicken meat using a culture-dependent approach and next generation sequencing analysis. Though both methods identified similar taxa, the NGS analysis provided much higher resolving power than the cultured method. Therefore, parallel analysis using NGS and culture-dependent approaches provides in-depth information of the biodiversity of the spoilage microbiota in chicken meat.

The predominant genus responsible for chicken meat spoilage was *Pseudomonas*, but other species belonging to the genera *Serratia* and *Aeromonas* were also identified. The combination of psychrotrophic growth of these bacteria in cooled chicken meat with concomitant production of proteolytic enzymes presents a big challenge to the meat industry. Therefore, it is essential to know the dynamics of the microbiota composition as a function of the refrigerated storage time in order to better understand how the specific spoilage bacteria evolves in chicken meat and to develop useful preservation methods.

Consequently, further study is needed to develop better preservation methods against these spoilage bacteria. For example, different packing methods such as modified atmosphere packaging and vacuum packaging should be tested, and bacteriophages targeting specific psychrotrophic bacteria can be applied for this purpose.

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