CHAPTER V

EFFECT OF SLAUGHTERING METHODS ON THE MICROBIOLOGICAL QUALITY OF BROILER CHICKEN MEAT DURING REFRIGERATED STORAGE

5.1 INTRODUCTION

Effective bleeding is essential in slaughter operation in order to obtain products of high quality (Warriss, 1977). When slaughtering is done properly, 60% of the total blood is drained and the remaining blood is retained in the muscles (10%) and visceral organs (20-25%) (Piske, 1982; Hedrick et al., 1994). There is a positive relationship between residual blood and microbial growth in the muscle during processing and storage. Several factors such as residual blood, meat pH, and storage temperature enhance microbial growth.

Method of slaughter has been implicated in some studies to affect microbial growth (Ali et al., 2011; Nakyinsige et al., 2012; Safa Mustafa et al., 2014; Addeen et al., 2014). Halal slaughter method was reported to record a significantly lower microbial count compared to other slaughter methods during refrigerated storage in chicken and rabbit meat. Bacteria counts ranging between 10^7 and 10^9 cfu/cm^2 during refrigerated storage (Borch et al., 1996) are considered as acceptable threshold for detecting fitness for human consumption. Hence, to avoid bacteria contamination, it has been suggested that carcass of imperfectly bled animal must be consumed immediately because of rapid decomposition of the meat (Thorton, 1949; Hess 1968). Processors and retailers incur major loses annually as a result of raw meat spoilage

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(Nattress et al., 2001). The rate of spoilage is determined by some factors which include; the species of microflora present in the meat, the meat type, processing methods, product composition, and the storage temperature (Borch et al., 1996; Ellis et al., 2002; Nychas et al., 2008).

During refrigerated storage, residual blood left in the carcass of animals as a result of imperfect bleeding undergoes putrefaction (Mucciolo, 1985) thereby, enhancing the growth of pathogenic microbes (*Campylobacter jejuni, Listeria monocytogenes and Pseudomonas* spp.) and psychrotrophic spoilage bacteria (*Pseudomonas spp., Enterobacteriaceae* and lactic acid bacteria). Bacteria count continues to increase as long as nutrient is available to enhance the growth; thus the shelf-life of such meat product is shortened by increasing the rate of deterioration in the meat product. Lactic acid bacteria (LAB) are Gram-positive, non-sporulating, cocci and rod, catalase-negative and facultative anaerobic bacteria with a fermentative metabolism (Axelsson, 1998). Lactic acid bacteria have been reported to be the major spoilage bacteria in vacuum packed meat samples during refrigerated storage (Dainty et al., 1992; Borch et al., 1996; Nychas et al., 1998).

Pre-slaughter and post slaughter factors such as husbandry practices, handling during slaughtering, evisceration and processing, temperature controls during slaughtering, processing and distribution, preservation methods, and packaging type all affect the microbial count. However, halal method of slaughtering has been reported to significantly lower the microbial count of chicken meat (Ali et al., 2011; Safa Mustafa et al., 2014; Addeen et al., 2014) but the mechanism behind it is still unclear. Hence, this study intends to confirm earlier reports that methods of slaughtering affect microbial count and to also explore the spoilage mechanism in chicken meat during refrigerated storage.

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5.2 MATERIALS AND METHODS

Breast meat (200 g) from five carcasses of previously slaughtered birds from both HM and NHM were kept in polythene bags and stored at 4°C. At predetermined days (day 1, 3, 5, 7 and 9), samples were taken for microbial analysis.

5.2.1 Total Aerobic Count

On each sampling day, 5 g of ground breast meat samples was added to 45ml of sterile phosphate buffered saline solution. The mixture was homogenised in the stomacher (Stomacher® 400 Circular Seward). Appropriate dilutions were transferred to already prepared plate count agar PCA (Oxoid CM0361). Plates were incubated at 37°C for 24 hr to enumerate the aerobic plate counts.

5.2.2 Lactic Acid bacteria Count

Ground breast meat (5 g) sample was added to 45 ml of sterile phosphate buffered saline. The mixture was homogenised in the stomacher (Stomacher®400 Circular Seward). Appropriate dilutions were spread on de Man, Rogosa and Sharpe (MRS) agar (Oxoid CM0361) plates containing 0.8% calcium carbonate. Plates were incubated anaerobically in anaerobic jars with AneroGen ™ (Oxoid) at 37°C for 48 h. The colonies obtained were tested for catalase activity by placing a drop of 4% hydrogen peroxide solution on the cells. Bubbles formation indicated the presence of catalase in the cells. Gram staining was done and the morphology of the bacteria was observed using a Nikon microscope (Nikon Eclipse 80i).

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5.2.3 Determination of Proteolytic Activity of LAB

Proteolytic activity of LAB isolates was a carried out by using skim milk agar prepared as follows; 25 g of skim milk (Oxoid LP0031) was reconstituted with 250ml of distilled water. The mixture was thoroughly stirred and autoclaved at 110°C for 10 min. Also a 500 ml of 2.5% agar solution was prepared and sterilized at 121°C for 15 min. Plating was done by thoroughly mixing the skim milk and agar solutions in a water bath at 50°C and then the homogenised mixture was quickly poured into the plates (Pailin et al., 2001).

Protein hydrolysis was determined by inoculating the isolated LAB on the skim milk agar plates and incubated at 37°C for 48 h in an anaerobic jar followed by cooling in a refrigerator (4°C) for 3 days. Protein hydrolysis was observed by the production of clear zones around each colony. The diameter of the clear zones were measured and recorded for each isolate. The determination was carried out in duplicate.

5.2.4 Genotypic Identification of LAB

5.2.4.1 DNA Extraction

Genomic DNA of LAB isolates was extracted using (Genomic DNA Extraction Mini Kit, Yeastern Biotech CO., LTD, Taiwan) the method described by Magnusson et al. (2003) with some modifications. A 1500 µl of overnight culture was harvested by centrifuging at 14,000 x *g* for 1 min using (Eppendorf centrifuge 5804 R) and the supernatant was discarded. The cell pellet was re-suspended in 200 µl of lysozyme and then incubated at room temperature for 10 min with shaking of the tubes every 2-3 min. 200 µl of GB buffer was added to the sample, and mixed by vortexing for 5 sec. The LAB samples were incubated for 20 min until the sample lysate was clear.

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During incubation, the sample tubes were inverted every 3 min. After the sample lysate was clear, elution buffer (200 µl per sample) incubated at 60ºC (for DNA elution) was added. Following that, a volume of 200 µl of the ethanol was added to each sample and mixed immediately by vortexing for 10 sec. The GD column was placed on a 2 ml collection tube. Then all the mixture from the previous step was applied to the GD column, and then centrifuged at 14000 x *g* for 2 min. The flow-through was discarded and the GD column placed in a new 2 ml collection tube. 400 µl of W1 buffer (ethanol added) into the GD column, and then centrifuged at 14000 x *g* for 30 Sec. the flow-through was discarded and the GD column placed back in the 2 ml collection tube, the wash step was repeated with 600 µl of wash buffer (ethanol added) with centrifuge at 14000 x *g* for 30 sec. The flow-through was discarded and the GD column placed back in the 2 ml collection tube, then centrifuged at 14000 x *g* for 3 min to dry the column matrix of any ethanol excess. The dried GD column was transferred into a clean 1.5 ml micro-centrifuge tube, and then 100 µl of the preheated elution buffer was added in the centre of the column matrix. It was left to stand for 3-5 min until elution buffer was absorbed by the matrix. The purified DNA was eluted by centrifuging at 14000 x *g* for 30 sec. Finally, the DNA sample was kept at -20ºC for long term storage.

5.2.4.2 PCR Amplification of 16S rDNA Gene Sequence of LAB Isolates

The extracted DNAs were used directly in PCR reactions to amplify the 16S rDNA gene from LAB. The 16S rDNA region was amplified by using primers (5-AGAGTTTGATCCTGGCTC-3) and 16S reverse: (5-CGGGAACGTATTCAC-CG-3). The PCR reaction mixture contained 5 µl of 1x reaction buffer ($NgCL_2$) (Yeastren

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Biotech CO., LTD. Taiwan), 1 µl of 0.2 mM dNTPs mix (Yeastren Biotech CO., LTD. Taiwan), 1 µl of 10 µM 16S forward and 1 µl of 10 µl 16S reverse primers, 1 µl of template DNA, and 0.5 µl of (1.25 U/ µl) YEAtaq DNA polymerase (Yeastren Biotech CO., LTD. Taiwan), negative control without DNA template was included in parallel. Each sample was toped up with 40.5 µl ddH_2O until 50 µl. The PCR were as follows: (LID 105°C), initial at 95°C for 3 min, denaturation at 95°C for 30s, annealing at 57°C for 15s and extension at 73°C for 1 min, with 34 cycles for each step. Final elongation was at 73°C for 5 min; and then held at 4°C. From each amplification mixture 10 µl were mixed with 2 µl of 6x DNA loading dye (Fermentans), and then subjected to electrophoresis in 1.0% (w/v) agarose gels (Conda S.A, Spain) in 1x TAE buffer (Bio Basic Canada INC, Canada) for 40 min and 80 V. 6 µl of 1Kb DNA ladder (250 to 10000 bp) from (1st Base, Malaysia) was used as standard. After electrophoresis the gel was stained in ethidium bromide (Merck, Germany) for 30 min and then washed with dH_2O for another 30 min. The gel was visualized and photographed with UV transilluminator (BIORAD).

5.2.4.3 PCR Product Clean-up

The PCR products clean up processed using PCR clean up kit from (Yeastern Biotech, Taiwan). 40 µl of the PCR product added into micro-centrifuge tube with 200µl of DF buffer and mixed by vortex. The sample was applied into DF column with collection tube, and then centrifuged at 6000 x *g* for 30s. The flow-through was discarded and the DF column placed back in the collection tube. The centrifugation was repeated at 14000 x *g* for 2 min.The dried DF column was transferred to a new micro-centrifuge tube. 15µl of the elution buffer was added in the centre of the DF column matrix,

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stand for 2 min until the elution buffer absorbed by the matrix. Purified DNA was eluted at 14000 x *g* for 2 min.

5.2.4.4 PCR Product Sequencing

The partial 16S-S DNA and 16 S-R DNA gene sequences of all the isolates were determined by 1st Base, Malaysia and sequences were analysed by the BLAST program on the NCBI website databases (http://www.ncbi.nlm.nih.gov/). (Gen- Bank). Partial sequence of the isolated LABs is listed in Appendix 3.

5.3 RESULTS

5.3.1 Total Aerobic Count

Total aerobic count of both HM and NHM samples generally increased gradually as the storage time increased during the 9 d of refrigerated storage at 4°C (Table 13 and Figure 7). Meat samples from NHM had the highest aerobic microbial count throughout the storage time. HM meat samples recorded counts of 2.26, 3.95 and 4.74 $\log_{10}$ CFU/g, respectively on 1, 3, and 5 d of storage at 4°C. A log increase of 2.60 was observed during 1-5 d of storage in NHM meat samples compared to log increase of 2.48 recorded for HM samples.

However, after the first 5 days of storage at 4°C, the bacteria growth was slow particularly in the HM meat samples. A log increase of 0.52 was recorded for HM meat samples during 5-9 d of storage compared to log increase of 1.25 obtained for NHM samples. The total aerobic count at day 9 of storage reached the highest of 6.28 $\log_{10}$ CFU/g in NHM meat samples compared to 5.26 $\log_{10}$ CFU/g reached in HM meat samples.

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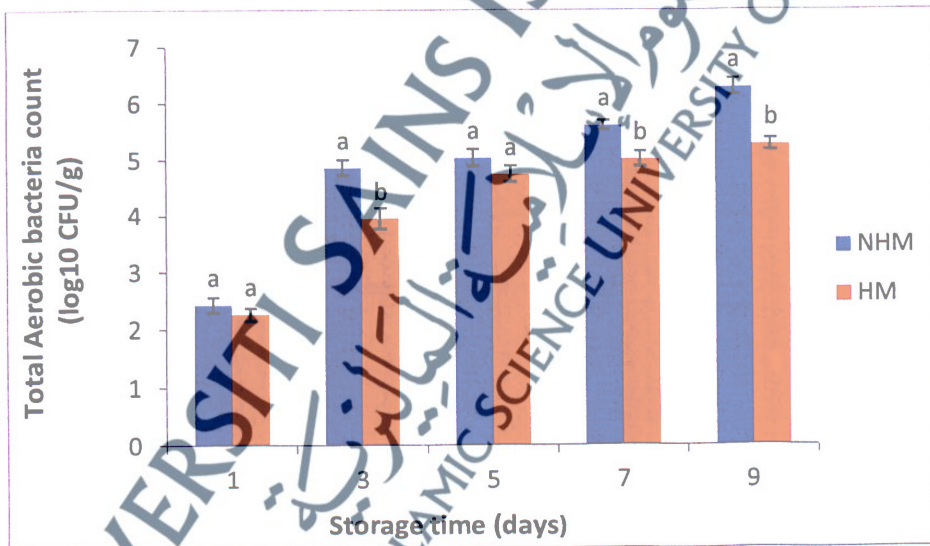
Table 13: Total Aerobic count of broiler chicken meat during refrigerated storage[a].

Storage Time (days)	HM log_{10} CFU/g	NHM log_{10} CFU/g	Statistical Significance
1	2.26±0.11	2.43±0.13	N.S
3	3.95±0.18	4.86±0.14	*
5	4.74±0.14	5.03±0.15	N.S
7	5.00±0.13	5.60±0.08	*
9	5.26±0.10	6.28±0.14	*

[a]HM = Halal method; NHM = Non-Halal method, n = 5 * means significant at P<0.05, NS means not significant at P<0.05.

Figure 7: Total Aerobic count of HM and NHM samples during 9 d of storage at 4°C. HSM= Halal method, NHM= Non Halal method, n = 5. [ab] means with different letters differ significantly at P<0.05.



5.3.2 Lactic acid bacteria Count

Lactic bacteria count increased slightly during 9 d storage at 4°C. No significant (P>0.05) difference was observed in the LAB counts of both HM and NHM samples at 1 and 9 d of storage at 4°C. However, at 3, 5, and 7 d of refrigerated storage, significant differences was observed in the anaerobic counts of both HM and NHM.

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Throughout the storage period, LAB counts of HM samples were lower than values obtained for NHM samples. These isolates showed clear zones on the MRS-$CaCO_3$ agar (Figure 9). They were also catalase negative and Gram positive (Table 14). They were also short rod and cocci in shape observed under microscope (Figure 10). Four isolates from NHM samples showed the good proteolytic activity on skim milk agar (Figure 11).

Figure 8: LAB count (log_{10} CFU/g) of HM and NHM samples during 9 d of refrigerated storage

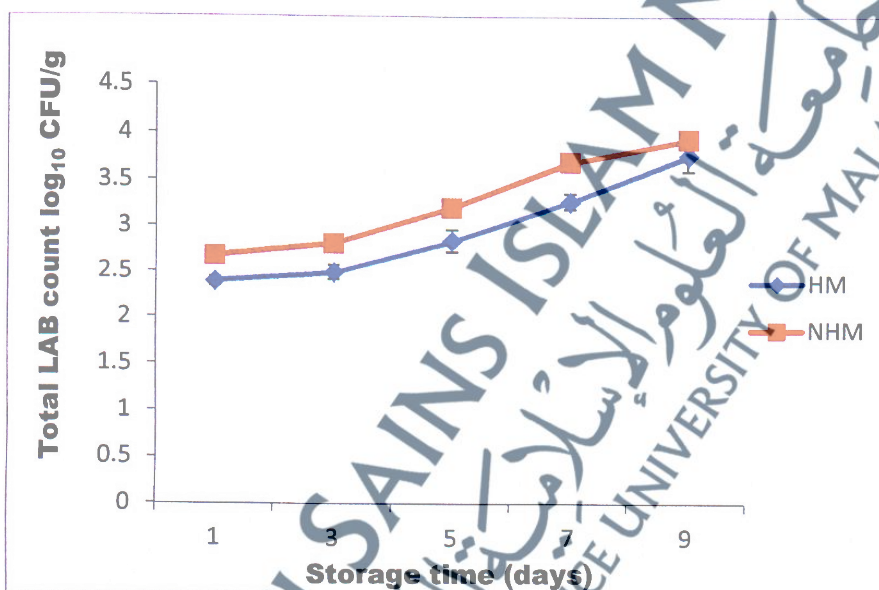

Table 14: Phenotypic characteristics of LAB isolated from breast meat of broiler chicken.

Samples	Catalase reaction	Gram reaction	Morphology	Proteolytic activity zone (mm)
LAB1	-	+	Short rod	8
LAB2	-	+	Cocci	7
LAB3	-	+	Cocci	6
LAB4	-	+	Cocci	6

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Figure 9: Growth of LAB on MRS agar with 0.8% CaCO3 showing clear zone around the colonies

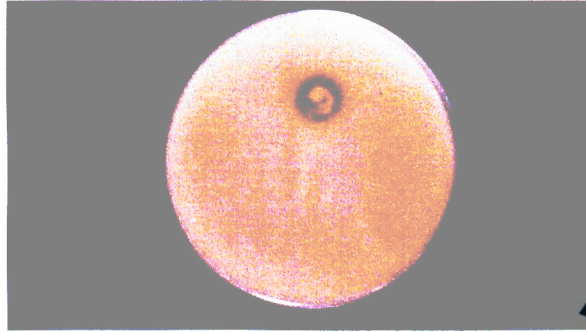
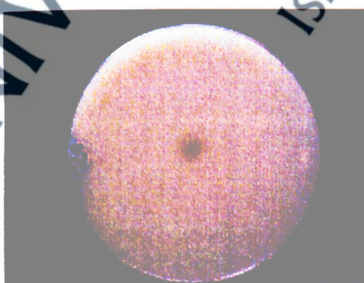
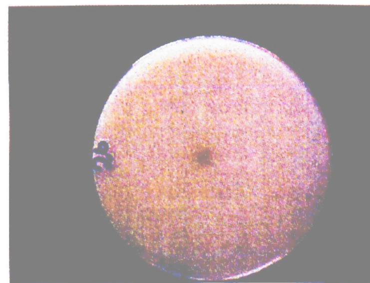


Figure 10: LAB isolated from broiler breast meat under the microscope showing the morphology of the bacteria after Gram reaction.

Figure 11: Clear zones around the colonies indicating proteolytic activity on skim milk agar

LAB 1 LAB 2





LAB 3 LAB 4

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Figure 12: DNA bands of LABs on 1.0% agarose gel. Lane 1: DNA ladder, lane 2: -ve control (water only), lane 3: +ve control (DNA extracted from *E. coli*), lane 4: LAB 1, lane 5: LAB 2, lane 6: LAB 3, lane 7: LAB 4.

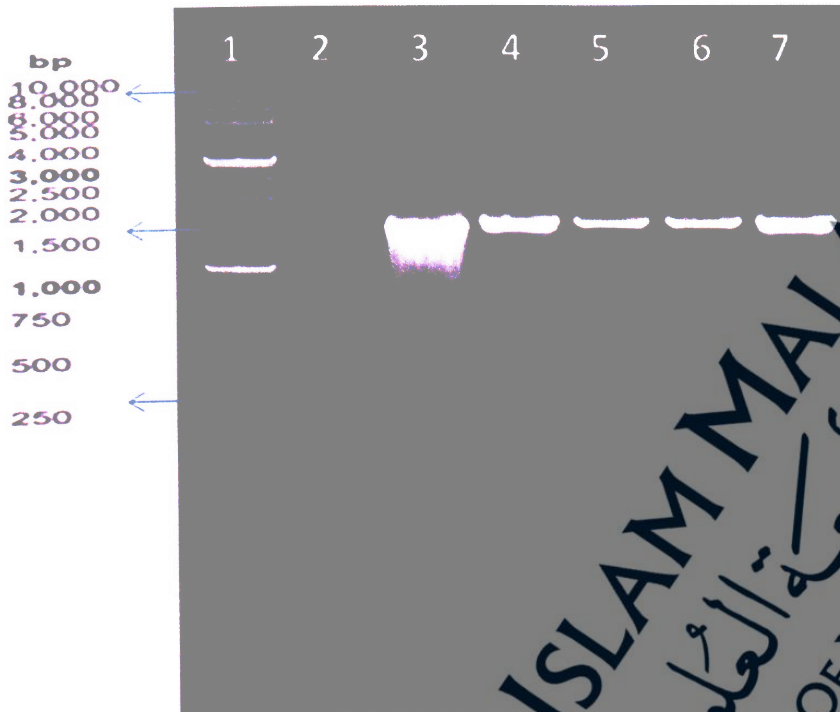

Table 15: Identification of LAB using 16S rDNA sequencing.

LAB Codes	Description	Identification	Accession No.
LAB 1	*Lactobacillus casei*	99%	AB911518.1
LAB 2	*Enterococcus* spp.	99%	EU157916.1
LAB 3	*Enterococcus devriesei*	99%	NR042389.1
LAB 4	*Enterococcus pseudoavium*	100%	NR113907.1

5.4 DISCUSSION

Chicken meat is a perishable product that is easily susceptible to microbial contamination during processing and storage thereby, shorten the shelf life and result in economic loss to producers and health risks to consumers (Jouki & Khazaei, 2011).

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Total viable count is one of the generally accepted measures used to determine the extent of microbial contamination and the hygienic conditions in meat processing plants (Department of Agriculture, Animal Health and Product, 2004). The results obtained from this study indicate a significant increase in total aerobic counts as storage time increased in both HM and NHM birds (Table 13). The total aerobic counts of NHM samples during 9 d refrigerated storage at 4^oC were consistently higher than those samples from HM. Similar observations were reported by Alli et al. (2011); the total viable count of poultry slaughtered by three methods increased with storage time. They reported that the halal slaughtered chicken recorded lower total viable counts during 6 h (3.82 log_{10}CFU/g) and 96 h (8.71 log_{10}CFU/g) of refrigerated storage at 4^oC compared to electrically stunned chickens (3.88 log_{10}CFU/g and 8.79 log_{10}CFU/g). In addition, Alvarado et al. (2007) reported a lower total aerobic plate count for broiler chicken slaughtered by unilateral neck cutting without stunning at day 0 (3.05 log_{10}CFU/g) and day 5 (3.67 log_{10}CFU/g) of refrigerated storage at 4^oC compared to CO_2 stunned bled birds (3.05 log_{10}CFU/g and 3.72 log_{10}CFU/g) and CO_2 stunned un-bled birds (3.85 log_{10}CFU/g and 5.05 log_{10}CFU/g). The method of slaughter used in this study was also without stunning, but the birds slaughtered by the NHM were done by poking of neck without neck cutting. A negative relationship exists between blood loss as a result of slaughter method and microbial count (Nakyinsige et al., 2014). Blood is an excellent medium for bacterial growth due to its high nutritive value which serves as substrate for most bacteria (Alvarado et al., 2007; Alli et al., 2011; Addeen et al., 2014; Nakyinsige et al., 2014). The high microbial counts in NHM meat samples observed during refrigerated storage may be caused by lower blood loss during slaughter. Bacteria count between 10^7 and 10^9 CFU/cm^2 during refrigerated storage is used as the cut-off point for determining fitness for

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human consumption (Insausti et al., 2001; Jeremiah, 2001). The microbial count of both HM and NHM were within the cut-off point limit and, therefore acceptable for human consumption.

Meat ultimate pH (>6.0) significantly affect the growth of spoilage bacteria (Lawrie & Ledward 2006) and meat pH also tends to increase with storage time (Agunbiade et al., 2010; Jouki & Khazaei, 2011; Stanisic et al., 2012). This pH increase during storage may be caused by continuous alkalization initiated by the release of protein degradation product during post-mortem endogenous changes (Florek et al., 2007). The high ultimate pH (6.17) in the NHM meat makes it favourable for high total aerobic count.

Lactic acid bacteria behave as facultative anaerobes and are capable of growing under high CO_2 concentration. The LAB count in this present study continuously increased throughout the 9 d of storage at 4°C with the NHM samples recording higher LAB counts compared to the HM samples. Similar result was reported by Jouki and Khazaei (2011) who also observed an increase in LAB counts of camel meat during 18 d of storage at 4°C. However, recently Sabow et al., (2015) reported that slaughter methods did not significantly affect LAB counts in goat meat (chevon) during 7 d of 4°C. However, Halal slaughter (without stunning) recorded lower LAB counts throughout 7 d storage compared to anaesthesia slaughter (anaesthesia with halothane before exsanguination). They ascribed the higher level of LAB growth in anaesthesia slaughter group to a faster pH decline caused by minimal anaesthesia that caused aging to start earlier in the goat meat. Nortjé and Shaw (1989) suggested that spoilage ensues in meat products when the lactic acid bacteria count reaches 7 log CFU/g. However, the LAB counts in NHM and HM did not reach 7 log CFU/g during the 9 d of refrigerated storage.

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Enzymatic actions are natural process in the muscle cells of slaughtered animals and are the leading cause of meat deterioration (Tauro et al., 1986). The proteolytic enzyme can either be endogenous in nature or produced by microbes. Lactic acid bacteria have the ability to produce proteolytic enzymes that can degrade proteins to produce peptides and amino acids that may contribute to the development of desirable or undesirable flavour (Bonomo et al., 2008). SDS-PAGE revealed protein degradation during 9 days refrigerated storage (Figure 6) in both HM and NHM samples which may be the result from endogenous or microbial enzymes. However, higher total aerobic and LAB counts in NHM samples may be the determining factor responsible for faster rate of protein degradation and meat softening observed in NHM samples. HM meat samples with lower total aerobic and LAB counts caused gradual protein degradation rate.

Genotypic identification of the selected LAB identified the isolates as *Lactobacillus casei, Enterococcus* spp., *E. devriesei*, and *E. pseudoavium* (Figure 12 and Table 15). This result is similar with that of Hayes et al. (2003) who also found *Enterococcus* spp. to be the most dominant bacteria on 971 of the 981 samples (99%) of all meat (chicken, turkey, pork and beef) in the state of Iowa in the United States. *Enterococcus* spp. functions as a facultative anaerobe which can produce ATP by aerobic respiration if oxygen is present and switch to fermentation or anaerobic respiration if oxygen is absent.

5.5 CONCLUSION

The result of this present study showed that slaughter method had an effect on the microbiological quality of both meat samples of HM and NHM. However, the bacteria counts during 9 d of refrigerated storage in both HM and NHM were still within the

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acceptable level for human consumption but bacteria count obtained in HM samples were consistently lower compared to NHM samples. Hence, Halal slaughter ensures better shelf life of meat samples during refrigerated storage.

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