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Development of a Quantitative Real-time PCR Method to Determine the Total Bacterial Count in Fresh Poultry Meat

Entwicklung einer quantitativen Real-time PCR Methode zur Bestimmung der Gesamtkeimzahl in Geflügelfleisch

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Summary

A real-time PCR approach was developed to determine the total bacterial count in fresh poultry meat comparing the results with standard plate count technique. An initial nucleotide sequence analysis based on 16S rRNA PCR fragment was performed to estimate the bacterial diversity in poultry meat. 71 bacterial strains were identified among which the bacterial family Enterobacteriaceae and the genus *Pseudomonas,* and *Lactobacillus* were found to be predominant. The *rpoB* gene, encoding the ß-subunit of RNA polymerase, which has only one copy in the bacterial genome, was selected as an amplification target. The "total viable count (TVC)" of commercially obtained poultry meat samples was determined using standard plate count method. A DNA isolation process was performed subsequently. The primer set designed to target the highly conserved regions on the *rpoB* gene was applied to detect a wide range of bacteria on poultry meat samples. Using the "SYBR® Green I" system, a melting temperature analysis was performed to confirm the amplification specificity of the primer. The comparison of C_t-values obtained by real-time PCR analysis with the bacterial count resulting from the standard plate count method (Log_{10} CFU/g) showed a good correlation (R^2 = 0.89, y = –2.73x + 47.52) over the range of 10^3 to 10^9 CFU/g of sample tissue. The student's t-test has shown no significant difference between the results of the two methods ($p<0.001$). The real-time PCR assay developed in this study has shown a robust potential to determine the total bacterial count on poultry meat.

Keywords: *rpoB* gene, total viable count (TVC), spoilage bacteria

Zusammenfassung

Es wurde eine Real-Time PCR Methode entwickelt, um die Gesamtbakterienzahl in frischem Geflügelfleisch zu bestimmen. Die erhaltenen Ergebnisse wurden mit denen der klassischen Kulturmethode (DIN EN ISO: 2293: 1988) verglichen. Um das in Geflügelfleisch vorhandene Keimspektrum zu erfassen, wurden 16S rRNA PCR Sequenzen von Einzelkolonien, erhalten aus verschiedenen Fleischproben, analysiert. Hierbei wurden 71 Bakterienstämme identifiziert, wobei die Familie der Enterobacteriaceen und die Genera *Pseudomonas* und *Lactobacillus* vorherrschend waren. Als PCR-Zielsequenz wurde das *rpoB*-Gen, welches die ß-Untereinheit der RNA Polymerase kodiert und im bakteriellen Genom als Einzelkopie vorliegt, ausgewählt. Mittels der klassischen Kulturmethode wurde die Gesamtbakterienzahl in Geflügelfleischproben verschiedener Hersteller bestimmt. Aus diesen Proben wurde die bakterielle DNA isoliert. Ein Primer-Paar zur Detektion der hochkonservierten Regionen des rpoB-Gens wurde entwickelt, um den Großteil der in Geflügelfleisch vorkommenden Keime zu erfassen. Zur Bestätigung der Spezifität der PCR, die ein SYBR® Green I Detektionssystem beinhaltete, wurde eine Schmelzkurzvenanalyse an die PCR angeschlossen. Die mittels Real-Time PCR ermittelten C_t-Werte wurden mit den Ergebnissen der Standardkulturmethode (lg KbE/g) korreliert. Der Nachweisbereich erfasste 10^3 bis 10^9 KbE/g und wies eine gute Korrelation auf (R^2 = 0.89, y = –2.73x + 47.52). Die statistische Überprüfung der Korrelation mittels des Student-t-Tests ergab keine signifikanten Unterschiede hinsichtlich der Ergebnisse der beiden verglichenen Methoden ($p<0.001$). Die in dieser Studie entwickelte Real-Time PCR Methode hat das Potential, zur Bestimmung der Gesamtbakterienzahl in Geflügelfleisch eingesetzt zu werden.

Schlüsselwörter: *rpoB* Gen, Gesamtbakterienzehl, Verderbnis erregende Bakterien

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Introduction

The application of quantitative real-time PCR has provided food microbiologists with a powerful and rapid analysis tool in the field of food safety (Cocolin and Rantsiou, 2012). This advancement is particularly conspicuous in quantifying food pathogens, where specific detection and identification of microorganisms is required (Gutiérrez et al., 1998). However, few studies have reported, using this method for analyzing the spoilage processes especially in perishable food products such as meat (Dolan et al., 2009; Gutiérrez et al., 1998). Considering the need for rapid procedures in the meat production industry to evaluate the microbiological quality of meat products, the sensitive and rapid nature of real-time PCR assays make them a highly attractive alternative to the standard method (Russell, 2005; Hartman et al., 2005).

It is well documented that TVC reflects the hygienic status of food processing and the degree of decomposition (Elliott and Michener, 1960). The "aerobic standard plate count" is recognized as the gold standard method for assessment of microbial numbers in food samples and is routinely applied worldwide (DIN EN ISO: 2293:1988) (Anon., 2003; Harris et al., 1995). Despite being effective and also accepted nationally and internationally, this method has shown some deficiencies in practical use (Russell, 2005). Besides being labor-intensive and costly, the most important disadvantage of such culture-dependent techniques is the long time required for obtaining results (48–72 h) (Swanson et al., 1992). These pitfalls can be avoided by using real-time PCR which has the advantages of high specificity, enhanced assay speed, and absence of time-consuming post-PCR analysis. Another positive aspect of such DNA-based methods is the detection of "viable but non-culturable" bacteria that are difficult to be identified by culture-based methods (Swanson et al., 1992; Rowan, 2004; Oliver, 2005).

To date, most of the PCR-based approaches aiming at the quantification of bacteria have targeted the variable or conserved regions of the 16S *rRNA* gene. However, a disadvantage of this approach is that most bacteria have multiple copies (Klappenbach et al., 2001) suggesting that this gene is rather an improper target for quantifying purposes (Farrelly et al., 1995; Suzuki and Giovannoni, 1996; Větrovskỳ and Baldrian, 2013). The *rpo*B gene is in contrast, a universal single-copy gene, playing an essential and central role in bacterial cellular metabolism (Morse et al., 1996; Mollet et al., 1997; Qi et al., 2001). The highly conserved regions on this gene appear to be an appropriate amplification target for quantification of total bacteria by real-time PCR (Palenik, 1992).

A quantitative real-time PCR method was developed to determine the total bacterial count on fresh poultry meat using newly designed universal primers that target *rpoB* gene for amplification in this study. The results of the real-time PCR analysis (C_t-values: cycle threshold values) were compared with the results of TVC (Log_{10} CFU/g: colony forming unit per gram) obtained by standard plate count method. SYBR® Green I, a fluorescent intercalating dsDNA (double stranded DNA) binding dye, was chosen as the detection system. This has the advantage of performing a low cost assay with an easy design and set up, whereas, the subsequent melting curve analysis helps to confirm the specificity of the primer set for the respective range of bacteria.

Materials and Methods

Isolation of bacteria from poultry meat samples:

All poultry meat samples (stored at +4 °C) were purchased from different local retail markets. In order to promote the growth of a wide spectrum of bacterial species, each sample was divided into different sections and stored at up to three different temperatures (4 °C, 20 °C and 30 °C) overnight. In this way the resulting bacterial colonies could be used as a representative panel for poultry meat. Each sample was cultivated onto a range of different selective media and incubated at the appropriate temperature following the recommended medium cultivation condition (Tab. 1).

After the incubation time, the bacterial colonies were photographed and selected according to their diverse morphological characteristics. In order to pre-differentiate among the bacterial colonies and achieve a rough estimation of the microorganism's identities an initial gram stain process for each colony selected for the DNA extraction was performed. The bacterial colonies, stained on glass slides, were visualized by microscopy and documented photographically.

Identification of microflora:

For the purpose of pre-enrichment, each of the representative bacterial colonies was inoculated into "Tryptic Soy Broth" medium (TSB) for 18 h at 30 °C. For DNA extraction, 1 ml of the enriched culture was transferred into a 1.5 ml sterile microcentrifuge tube and centrifuged at 6082 x g for 10 min at 4 °C (pre-enrichment of microbial load modified according to Dolan et al., 2009). Discarding the supernatant, a DNA extraction process was performed for the enriched bacteria using a universal commercial DNA extraction kit (Fast ID DNA Extraction Kit, Genetic

TABLE 1: *Culturing conditions of bacterial samples on different nutrient media (all by Merck KG. A. -D).*

Nutrient medium	Incubation condition	Target bacteria
MacConkey agar	18–24 h / 35 °C	*E-coli, Shigella, Salmonella*
Violet Red Bile Glucose agar (VRBD agar)	48 h / 35 °C	Enterobacteriaceae
Glutamate Starch Phenol Red agar base (GSP agar) and Penicillin	72 h / 25 °C	*Pseudomonas* and *Aeromonas*
Pseudomonas agar base and Cetrimide-Fucidin-Cephaloridine selective supplement (C-F-C agar)	44 ± / 25 °C	Pseudomonas
Campylobacter selective agar	Pre-enrichment 4 h / 37 ± 1 °C Main enrichment 42–44 h / 41.5 °C	Campylobacter
pre-enrichment by buffered peptone and Modified Semi-Solid Rappaport Vassiliadis agar base (MSRV agar)	Pre-enrichment: 16–20 h / 37 °C Main enrichment: 24 h / 42 °C	*Salmonella* spp.
Brillant green – Phenole red-Lactose-Saccharose agar (BPLS selective agar)	24 h / 35 °C	*Salmonella* spp.
Rambach selective agar	24–48 h / 35–37 °C	*Salmonella* spp.
PALCAM agar and PALCAM selective supplement	48 h / 30–36 °C	*Listeria*
MRS agar (deMan, Rogosa and Sharpe agar)	72 h / 35 °C	Lactobacillaceae

Europe AG, D) which was reported to be proper for extracting various bacteria (gram positive and gram negative) from different biological matrices (Irwin et al., 2014). Briefly: for each extraction 200 µl of the prepared bacterial suspension was added to 1000 µl "Genomic Lyse" buffer premixed with 10 µl "Proteinase K" solution. After mixing thoroughly and obtaining a homogeneous slurry, the samples were incubated at 65 °C for 25 min and spun at 9500 x g for 5 min. 500 µl of the supernatant was transferred into a new vial and an equal amount of "Genomic Bind" buffer was added and mixed thoroughly. The resulting suspension was passed through the "DNA-Binding Column" by centrifugation at 9500 x g for 5 min at 4 °C. After 1 step of washing with "Genomic Wash" buffer and 3 steps of washing with 75 % ethanol (Carl Roth GmbH, D) using centrifugation, 100 µl TE (10 mM Tris-HCl, 1 mM Na/EDTA, pH 8.0) was added to each sample and incubated for 5 min at 65 °C. After spinning at 9500 x g for 30 s at 4 °C the DNA was eluted and stored at 4 °C.

The quality and concentration of DNA isolates were determined using a "Nano drop™ ND 1000" instrument (Thermo Fisher Scientific, USA). The DNA concentration was calculated based on the absorbency at 260 and 280 nm. The 260/280 ratio, which assesses the purity level of the DNA was calculated. 50 different DNA samples were selected according to their DNA concentration and purity level for the real-time PCR amplification.

The real-time PCR thermo cycler used in this study was a "DNA Engine® Peltier Thermocycler Instrument". The device was equipped with the real-time PCR detector "Chromo 4 ™ System" and detecting software "Opticon Monitor™ version 3.1" (all made by Bio-Rad Laboratories GmbH, D). A SYBR® Green I dye based real-time PCR assay was performed using the following universal primer set amplifying a 1462 bp fragment on *16S rRNA* gene: Forward primer 5'-AGTTTGATCCTGGCTCAG-3' and reverse primer 5'-TACCTTGTTACGACTT-3' (Goldenberger et al., 1997; Paster et al., 2002; Kawashara et al., 2005; Dolan et al., 2009). The total reaction volume of 25 µl for amplification contained 1X iQ™ SYBR® Green Supermix (SYBR® Green I dye, 50 U/ml iTaq™ DNA polymerase, 0.4 mM of each dNTP, 6 mM $MgCl_2$, 40 mM Tris-HCl pH 8, 100 mM KCl, 20 nM Fluorescein and stabilizers) (Bio-Rad Laboratories GmbH, D), 0.6 µM of each primer (TIB MOLBIOL Syntheselabor GmbH, D) and DNase, RNase free (0.1 µm filtered) water (Sigma-Aldrich Chemie GmbH, D). 2 µl DNA templates (extracted from bacterial isolates) were added to each PCR reaction tube. The negative control (duplicate) contained 2 µl of purified water as substitute for DNA template.

The real-time PCR amplification started with an initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 25 s, primer annealing at 58 °C for 40 s, DNA extension at 72 °C for 30 s. A final extension step was added at 72 °C for 5 min. The fluorescence was detected automatically at the end of each cycle. A melting temperature analysis of the PCR products was performed following the amplification. The melting curves were obtained by slow heating at 0.5 °C/s from 55 °C to 90 °C with collection of fluorescence after each increase of 0.5 °C. A PCR product purification process was performed to promote the DNA sequencing efficiency using a commercial PCR purification kit (Gene Jet PCR Purification Kit, Fisher Scientific GmbH, D). The principle of the PCR purification process was based on silica membrane technology in the form of a spin column. In this way the primers, dNTP, unincorporated labeled nucleotides, enzymes and salts could be removed effectively from the PCR reaction mixture. The purified PCR products were separated by gel electrophoresis technique and the DNA bands were visualized using an ultra-violate transilluminator.

The purified PCR products were prepared and shipped at ambient temperature to a sequencing lab (Eurofins MWG GmbH, Germany). The obtained sequencing results were analyzed using the chromatograms of the forward and reverse sequencing. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Sequence Tool (BLAST) service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to figure out the bacterial strains identity. The results were also compared with the gram staining and microscopy results.

Choice of target gene and primer design:

In order to quantify a wide-range of bacterial species using real-time PCR, a target gene should be selected which is present in all bacteria identified in poultry meat. In this study the *rpoB* gene was chosen as the amplification target, considering the advantages of this gene (Palenik, 1992; Morse et al., 1996; Mollet et al., 1997; Qi et al., 200) as previously discussed. A BLAST search in the NCBI database was performed in order to find the existing rpoB sequences of all identified bacteria for the purpose of primer design. The resulting sequences were aligned in Clustalx 2.1 (http://clustalx.software.informer.com/2.1/) in order to find consensus sequences. The final primer design was realized with the help of NCBI "Primer-blast" (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The selected primer set (forward: rpoB-15-F 5' TATCCGTTCCGTTGGCGAAA 3' and reverse: rpoB-15-R 5' GAGTTCTTCGGTTCCAGCCA 3') detects a 174 bp fragment of the *rpoB* gene of bacteria. The designed primer set was checked for primer-dimers, hairpin structure, melting temperature (Tm) and GC content using Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The specificity of the primer set has been tested by BLAST search in the NCBI database.

In order to determine the optimal annealing temperature for the newly designed primer set, a gradient real-time PCR assay was performed. The optimal annealing temperature of 57 °C was selected after performing a gel electrophoresis assay and visualizing the DNA bands.

Correlation experiment and statistical analysis:

In order to confirm the suitability of the "*rpoB* gene-targeting" primer set for the quantification of bacteria by real-time PCR in poultry meat a parallel analysis of plate count technique and real-time PCR was performed as follows: poultry meat samples (stored at 4 °C) were obtained commercially from various local retail markets. Each sample (10 g) was homogenized with 90 ml of Sodium Chloride-peptone buffer in a filter containing blender bag using a stomacher 400 (Seward Ltd., UK) at high speed for 1 min. 2 aliquots (each 1.5 ml) of homogenized sample were transferred into 2 vials and centrifuged at 380 x g at 4 °C for 2 min to pellet tissue debris. 1 ml of the supernatant was transferred to the test tube containing 9 ml of the dilution buffer and was decimally diluted. 1 ml of each dilution level was cultivated onto plate count agar using the pour plate technique. The PC plates were incubated at 30 °C for 72 h.

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The TVC of all samples was calculated and given as Log_{10} CFU/g of sample tissue.

1 ml from the supernatant of the second vial was transferred into another microcentrifuge tube and bacterial cells were harvested by spinning at 9500 x g for 10 min at 4 °C (wash step modified according to Lee and Levin, 2007). The pellet was resuspended in 1 ml SMS solution (0.85 % NaCl and 1 mM $MgSO_4$) and was then applied for DNA extraction. 200 µl of the prepared cell suspensions were used for the DNA extraction. The DNA extraction process of all samples was performed using a commercial kit (Fast ID DNA Extraction Kit, Genetic Europe AG, D) according to the manufacturer's instructions as described previously.

All of the real-time PCR assays were performed using the *rpoB* gene amplifying primer set, designed in this study. The real-time PCR total reaction volume was 25 µl and consisted of 1X iQ™ SYBR® Green Supermix, 0.6 mM of each primer and water. 2 µl of DNA templates (extracted from poultry meat samples) were added to each PCR reaction tube. The negative control contained 2 µl of purified water substituting the DNA template. The real-time PCR assay started with an initial denaturation at 95 °C for 3 min following by 40 cycles of denaturation at 95 °C for 25 s, primer annealing at 57 °C for 40 s and DNA extension at 72 °C for 30 s. To promote the complete synthesis of all PCR products a final extension at 72 °C for 5 min was added to the PCR protocol. A melting temperature analysis was performed post PCR. The melting curves were obtained by slow heating at 0.5 °C/s from 55 to 90 °C. The fluorescence was collected after each increase of 0.5 °C. A gel electrophoresis assay was performed to confirm the PCR amplification specificity after purifying the PCR products using a commercial purification kit (Gene Jet PCR Purification Kit, Fisher Scientific GmbH, D).

The results of standard plate count (Log_{10} CFU/g of samples) were compared in a correlation with the results of real-time PCR assay (C_t-values) and the student's t-test for paired value was performed.

Results

Identification of microbial species:

In this study a nucleotide sequence analysis based on 16S rRNA PCR fragment was performed to estimate the spoilage associated bacterial diversity in fresh poultry meat. The primer set selected for the real-time PCR amplified a 1462 bp sequence fragment in the representative colonies. This was confirmed by the gel electrophoresis assay.

Blast analysis was performed using forward and reverse sequences of the bacterial isolates. The results which had the highest "query coverage" (min. 90 %) and "max. identity" (min. 95 %) were included in the analysis. For most of the sequences, several bacterial strains with the similar or sometimes even identical "query coverage" and "max. identity" were given as result. As the aim of this study was to design universal primers that can detect preferably a large range of bacteria in meat samples, up to 3 bacterial strain identities with the same "query coverage" and "max. identity" were taken into account as applicable. Consequently, 71 bacterial strain identities were resulted from 50 PCR products that were analysed by using the Blast search.

These bacterial identities belonged to 16 different taxonomic genera. Among gram negative bacteria the family Enterobacteriaceae and the genus *Pseudomonas* and among gram positive species *Lactobacillus* spp. and Staphylococci were found to be predominant (Tab. 2). The majority of identified bacterial strains were comparable to those mentioned previously in the literature to be associated with fresh poultry meat spoilage (Cunningham, 1987; Cox et al., 1998; Mead, 2004).

TABLE 2: *Bacterial strains identification figured out by 16S rRNA fragment sequencing and using BLAST search analysis tool for DNA samples isolated from 50 Bacterial colonies.*

No.	Taxon	No.	Taxon	No.	Taxon	No.	Taxon
1	*Shigella flexneri*	19	*Pseudomonas pohangensis*	37	*Pantoea agglomerans*	55	*Lactobacillus reuteri*
2	*Shigella dysenteriae*	20	*Pseudomonas vranovensis*	38	*Pantoea ananatis*	56	*Lactobacillus vaginalis*
3	*Escherichia coli*	21	*Pseudomonas fulva*	39	*Pantoea stewartii* subsp. *stewartii*	57	*Aeromonas bestiarum*
4	*Escherichia fergusonii*	22	*Pseudomonas migulae*	40	*Citrobacter youngae*	58	*Aeromonas salmonicida* subsp. *salmonicida*
5	*Escherichia albertii*	23	*Pseudomonas reinekei*	41	*Salmonella enterica* subsp. *salamae*	59	*Aeromonas salmonicida*
6	*Serratia liquefaciens*	24	*Pseudomonas chlororaphis* subsp. *aurantiaca*	42	*Salmonella enterica* subsp. *houtenae*	60	*Aeromonas salmonicida* subsp. *achromogenes*
7	*Serratia proteamaculans*	25	*Pseudomonas caricapapayae*	43	*Hafnia alvei*	61	*Aeromonas molluscorum*
8	*Serratia grimesii*	26	*Pseudomonas plecoglossicida*	44	*Staphylococcus sciuri* subsp. *sciuri*	62	*Aeromonas salmonicida* subsp. *pectinolytica*
9	*Serratia fonticola*	27	*Pseudomonas monteilii*	45	*Staphylococcus sciuri* subsp. *carnaticus*	63	*Aeromonas salmonicida* subsp. *smithia*
10	*Serratia plymuthica*	28	*Pseudomonas cremoricolorata*	46	*Staphylococcus sciuri* subsp. *rodentium*	64	*Shewanella hafniensis*
11	*Pseudomonas lundensis*	29	*Pseudomonas putida*	47	*Staphylococcus lentus*	65	*Shewanella putrefaciens*
12	*Pseudomonas fragi*	30	*Pseudomonas oryzihabitans*	48	*Staphylococcus vitulinus*	66	*Shewanella baltica*
13	*Pseudomonas psychrophila*	31	*Klebsiella pneumoniae*	49	*Staphylococcus fleurettii*	67	*Acinetobacter junii*
14	*Pseudomonas moraviensis*	32	*Klebsiella variicola* subsp. *rhinoscleromatis*	50	*Staphylococcus saprophyticus* subsp. *bovis*	68	*Acinetobacter baumannii*
15	*Pseudomonas koreensis*	33	*Klebsiella pneumoniae* subsp. *rhinoscleromatis*	51	*Lactobacillus salivarius*	69	*Acinetobacter radioresistens*
16	*Pseudomonas taetrolens*	34	*Klebsiella pneumoniae* subsp. *ozaenae*	52	*Lactobacillus sakei* subsp. *carnosus*	70	*Acinetobacter towneri*
17	*Pseudomonas mosselii*	35	*Rahnella aquatilis*	53	*Lactobacillus sakei*	71	*Acinetobacter soli*
18	*Pseudomonas japonica*	36	*Obesumbacterium proteus*	54	*Lactobacillus frumenti*		

Detection sensitivity and effectiveness of real-time PCR analysis:
The primer set was designed to amplify a 174 bp fragment on the conserved sequences of the *rpoB* gene of bacteria associated with poultry meat spoilage. A BLAST search was performed to test the primer sensitivity. This analysis has shown that rpoB primer set could theoretically detect a large number of bacteria. The electrophoresis assay confirmed the PCR product size of 174 bp resulting from rpoB primer amplification (Fig. 1). This indicates a primer specific amplification.

Real-time PCR:
The real-time PCR analysis of 29 DNA samples extracted from poultry meat using rpoB-15 primer set with the PCR conditions described previously led to successful amplification of this gene (Fig. 1). The C_t-values' range was between 20.07, derived from samples with Log_{10} 9.86 CFU/g in the cultural method, and 38.74, derived from samples with Log_{10} 3.59 CFU/g (Fig. 1).

The subsequent melting curve analysis conduced to the PCR products confirmation. The melting temperatures (Tm) of the samples appeared between approximately 83 °C and 87 °C with more accumulation on 85 °C (Fig. 1). However, a few nonspecific PCR products were observed, which were probably due to primer-dimer formation or other PCR artefacts to which the SYBR® Green dye I bound. These PCR artefacts show a different melting temperature than that of the target amplicons (58–75 °C).

The electrophoretically separated DNA bands appeared at the expected location (174 bp) in the agarose gel. However, other PCR artefacts which were observed in melting curve analysis were also observable as bands with different fragment size on the gel (Fig. 1).

Statistical comparison of the real-time PCR analysis and cultural method:
The results of the real-time PCR assay (C_t-values) and the results obtained from cultural method (Log_{10} CFU/g) (Tab. 3) were compared in a correlation diagram illustrating the relationship between the two methods. As depicted in Figure 2 this comparison has shown a linear standard curve over the range of Log_{10} 3.6 (corresponding to C_t-value 38.06) – Log_{10} 9.8 (corresponding to C_t-value 22.44) and a well correlation coefficient of $R^2 = 0.89$ (n = 29; y = –2.73x + 47.52) ($P<0.001$) was achieved. The student's t-test for paired values has shown no significant difference between the results of the two methods.

Discussion

The identification of bacterial species based on 16S rRNA sequence analysis using PCR has previously been used successfully: Takahashi et al. (2006) and Dolan et al. (2009)

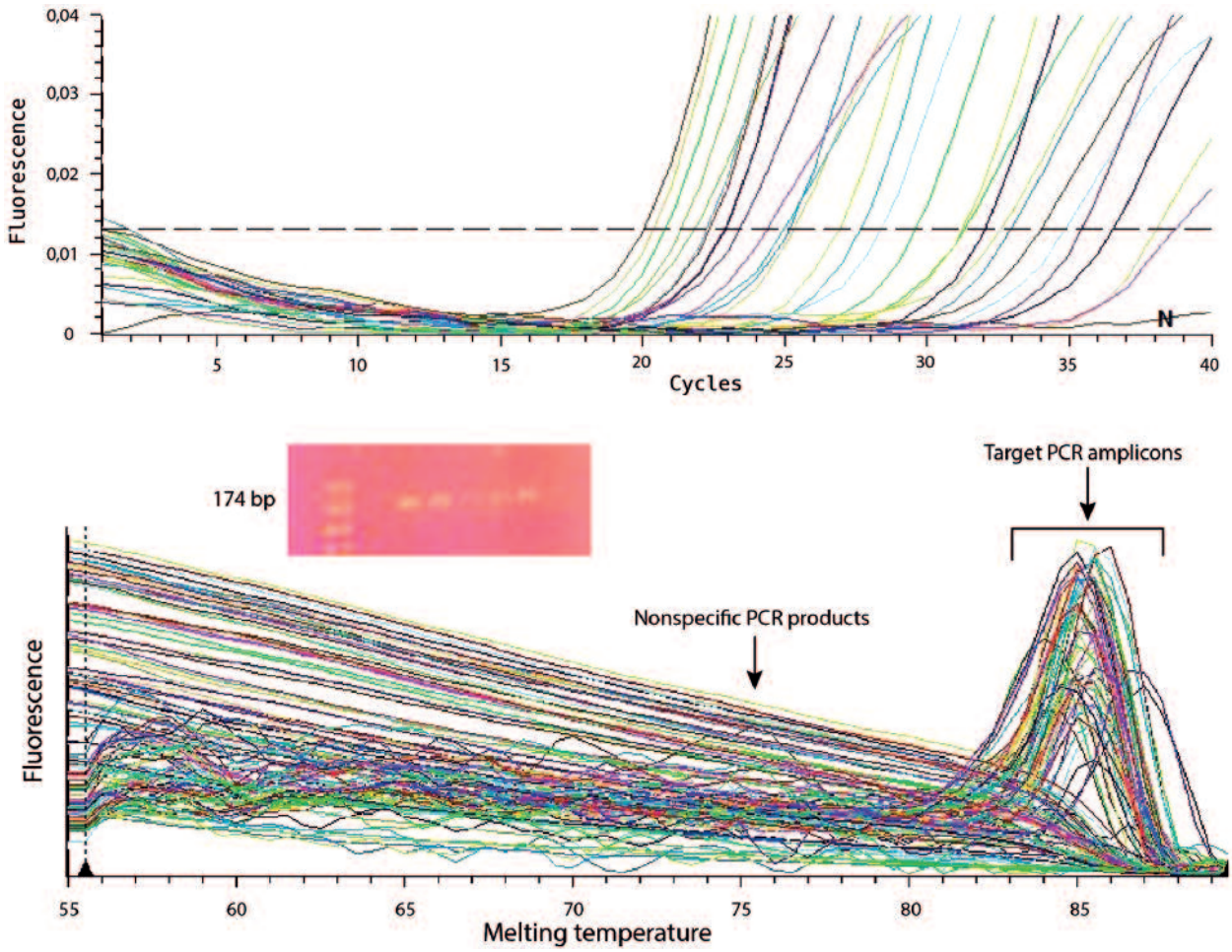

FIGURE 1: *Real-time PCR amplification curves for bacterial DNA templates extracted from poultry meat samples using rpoB gene amplifying primer set, plotted one curve/sample (N = negative control), Melting curve analysis of real-time PCR products for bacterial DNA templates extracted from poultry meat samples using rpoB-15 primer set.*

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TABLE 3: *Results of real-time PCR amplification of samples (Ct-values: average of duplicates), traditional plate count method (Log_{10} CFU/g: weighted average of duplicates) and melting temperatures of the PCR products measured after PCR amplification (average of duplicates; for negative control duplicate is mentioned).*

Samples	No.	bacterial count (Log_{10} CFU/g)	Ct-Values	Melting temperatures of PCR products (°C)
Chicken inner fillet	1	9.86	22.44	85
	2	3.59	38.06	67.5
Chicken breast inner fillet	3	8.37	23.00	85.5
	4	6.81	27.90	85.5
	5	3.83	38.74	83.5
Chicken breast fillet	6	6.50	26.99	85.25
	7	3.93	35.66	85.5
	8	8.80	21.96	85.5
	9	7.75	23.32	85.5
	10	5.18	33.705	85.25
Organic Chicken breast fillet	11	8.86	26.09	85.25
	12	3.89	36.54	85
Chicken breast fillet-parts	13	9.26	24.28	87.25
	14	8.49	25.58	85.5
	15	6.00	32.89	85.5
Chicken breast inner fillet	16	9.15	24.79	85.25
	17	7.73	29.00	84.75
	18	4.88	34.43	85.5
	19	8.61	20.81	85.75
	20	7.46	25.05	86
	21	4.71	35.44	86
	22	9.49	20.92	85.5
	23	9.04	22.35	86
	24	7.16	31.98	86
	25	9.40	20.40	85.5
	26	8.57	22.26	85
Turkey breast inner fillet	27	6.02	29.45	86
Quail breast	28	6.55	28.80	85.25
Duck inner fillet	29	7.94	25.82	85.5
Negative control	N	–	–	58/75

used this method to amplify a 1462 bp fragment of *16S rRNA* sequences (almost as large as the whole bacterial 16S rRNA gene: 1550 bp; Weisburg et al., 1990; Janda et al., 2007) by PCR to identify different bacterial species isolated from food products. The universal primer set used by Dolan et al. (2009) was chosen in this study for the amplification of the 16S rRNA sequence for the purpose of identification of spoilage associated bacteria isolated from fresh poultry meat.

Many of the bacterial species identified in poultry meat in this study using direct 16S rRNA sequencing were comparable with those associated with poultry meat spoilage described previously by other authors: Mead et al. (2004) reported that *Pseudomonas* spp., accompanied by lower numbers of *Acinetobacter, Moraxella,* and *Psychrobacter* spp., including *Acinetobacter johansonii* and *Psychrobacter imnobilis* (not identified in this study) predominate at spoilage. Arnaut-Rollier et al. (1999) found *Pseudomonas fragi, Pseudomonas lundensis,* and *Pseudomonas fluorescence* biovars as three major clusters among bacterial strain isolated from freshly processed and stored poultry meat in a numerical taxonomy study. *Shewanalla putrefaciens* and various cold-tolerant strains of Enterobacteriaceae such as *Enterobacter* and *Serratia* spp. can also be found in spoiled poultry according to Mead (2004). They were also detected in this study. Large varieties of gram negative bacterial strains such as *Pseudomonas* spp., *Aeromonas* spp. along with a wide spectrum of Enterobacteriaceae and gram positive strains such as *Lactobacillus* spp., and *Staphylococcus* spp. were detected in poultry meat samples.

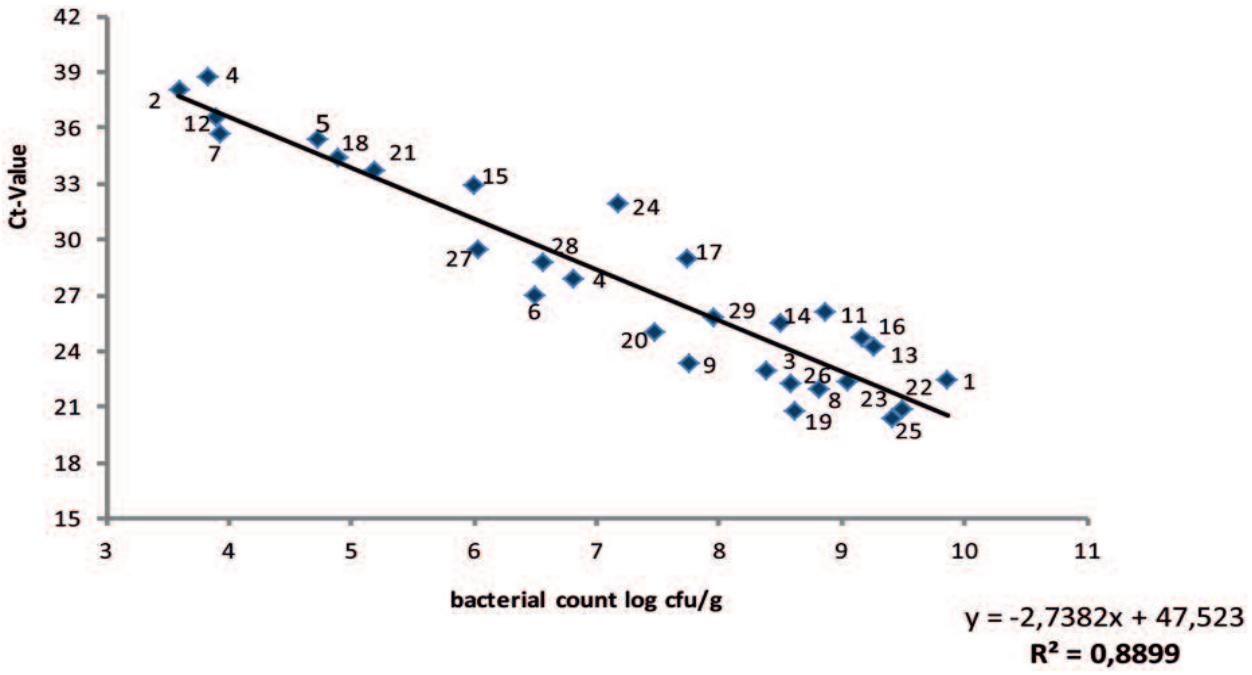

FIGURE 2: *Comparison of real-time PCR results (C_t-values: average of duplicates) and total viable counts (Log_{10} CFU/g: weighted average of duplicates) by standard plate count technique. ($y = –2.73x + 47.52$, $R^2 = 0.89$)*

Conserved single copy genes as an amplification target for quantification of bacteria has been used previously. Dolan et al. (2009) have performed a reverse-transcriptase qPCR to determine the total bacterial count on beef carcasses. However, as RNA extraction and the subsequent reverse transcription complicates the process of real-time PCR assay and raises the risk of components' contamination (Dolan et al., 2009), a DNA-based assay has been preferred in this study. The *rpoB* gene has been used as an amplification target for the enumeration of bacteria in ready-to-eat vegetables and fruits previously by Takahashi et al. (2006). These authors have obtained a high correlation coefficient (R^2 = 0.90) between the results of real-time PCR and plate count method. As the bacterial pattern associated with poultry meat spoilage is rather different than that of fruits and vegetables (Takahashi et al., 2006) a new *rpoB* gene-targeting primer set was designed in this study in order to target the respective range of bacteria. The 174 bp PCR product detected by this primer pair presents an optimal length (approximately 150 bp) for quantitative PCR analysis. The NCBI Blast Primer search has shown that the *rpoB* gene-targeting primer set was able to identify a wide range of bacteria associated with meat spoilage. However, for a perfect primer design targeting *rpoB* gene, a more compassed bacterial rpoB sequence database is required. Although a large part of the rpoB sequence is available in the genetic databases, most of them do not contain the whole gene sequence (Drancourt and Raoult, 2002; Adékambi et al., 2008). This complicates the alignment of sequences and consequently the design of a perfect primer set. Hence, progress in completing the *rpoB* gene sequencing could pave the way for designing more powerful primers leading to successful and time-saving quantification of bacteria in food samples by real-time PCR.

The comparison of C_t-values derived from PCR amplification of poultry meat DNA samples, using the *rpoB* gene targeting primer set, with the Log_{10} CFU/g obtained by cultural method has shown a good correlation (R^2 = 0.89). Nevertheless, some samples have shown deviations from the correlation curve. This might result from the culturability of the bacterial species in the respective culturing conditions. Since the cultural technique is recognized as the standard method for the total bacterial count in food samples, all alternative novel assays should be compared with this method (Dolan et al., 2009). Considering the diversity of bacterial populations in meat and their different growth requirements (Pearson and Dutson, 1986), it is to be expected that the standard nutritional and environmental condition provided by the total plate count technique might not fulfill the optimal growth conditions of all these bacteria (Swanson et al., 1992). Since PCR amplifies the entire DNA existing in a sample, the culturability of the existing bacteria does not play a role in their quantification (Cocolin and Rantsiou, 2012). Nevertheless, the inability of PCR technique to distinguish between target DNA sequences from viable and dead bacterial cells can also lead to an overestimation of the real bacterial load (Nocker and Camper, 2005) that can potentially contribute to meat spoilage. Therefore, RNA-based PCR assays are sometimes preferred for quantification of bacteria. However, the simple handling, rapid performance, and high sensitivity of SYBR® Green dye I system (Levin, 2004) as applied in this study, provides a more suitable PCR method for quantification of total bacteria in food.

The melting curves resulting from SYBR® Green dye I amplification of poultry meat DNA samples are shown in Figure 1. The melting temperatures of most of the samples were between 83 °C and 87 °C which were assumed to be the target amplification products. The melting temperature (Tm) of nucleic acids is affected by length, GC content, and the presences of base mismatches (Ririe et al., 1997). As the target PCR products have the same length (174 bp) they should have almost similar Tm. The slight variation (± 2 °C from the average: 85 °C) of melting temperature might result from the fact that a wide spectrum of bacterial species exists in the poultry meat (Cunningham, 1987). These bacteria might have some variations in their rpoB sequences. This can lead to formation of PCR products having variations in their rpoB sequences (various GC content or base mismatches) and consequently result in slight melting temperature deviations. It is assumed that such variations in the melting temperature of amplicons would not affect the quantification sensitivity significantly as long as the PCR products' length remains constant. The few non-specific melting temperatures might result from the formation of primer dimers or other PCR artefacts (Fig. 1) which normally have a lower melting temperature (70–75 °C) (Ririe et al., 1997).

The good correlation achieved by comparing the results of the two methods has shown that this assay was set well enough to present a potential to be applied for quantification of bacteria in poultry meat and substitute the time- and cost-consuming cultural method in the food industry.

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