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Research article

Effect of garlic and onion extract chitosan nanoparticles on selected intestinal bacterial flora in indigenous rainbow rooster chicken in Kenya

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Abstract: Bacterial microbes play a vital role in the nutrition and health of birds. Food passaging through the gastrointestinal tract of poultry favored microflora that have rapid growth and can adhering to the mucosal wall. The caeca are ideal habitats for diverse bacteria with effect on the host nutrition and health. Antibiotics in poultry for therapeutic and as growth promotor can decrease the number of most susceptible bacterial communities and enhance the growth of resistant bacteria. The aim of the study was to determine the effect of garlic and onion extract chitosan nanoparticles on the intestinal microflora of Rainbow Rooster Indigenous Chicken in Kenya in which a total of 18 chickens were used with 2 chickens drawn from each of the 9 groups and both caecum and jejunum content sampled with a total of 36 samples. The chickens were treated with Chitosan and Aqueous extracts of Garlic and Onion (CHIAGO), Chitosan with total Phenol, Ajoene rich extract (CHITPA) nanoparticles, and Chitosan Solution (CHISOLN) all at 5% and 10% and 1 g and 0.5 g Fosbac (Antibiotic) were applied orally to the chickens twice a week for 8 weeks. About 1.5 g of caecum and jejunum contents for micro-organisms *Escherichia coli (E. coli), Salmonella typhi (S. typhi), Campylobacter jejuni (C. jejuni), Lactobacillus acidophilus (L. acidophilus), Bifidobactreum bifidium (B. bifidium) from* caecum and *L. acidophilus* from jejunum were analyzed at 8th week of the treatment using conventional PCR to optimize bacteria 16S rRNA gene specific bands and qPCR for the 16S rRNA gene copy numbers was determined. *Lactobacillus acidophilus, E. coli , S. typhi, C. jejuni from* caecum and *L. acidophilus* from jejunum indicated specific

bands in 1.2% agarose gel. The qPCR revealed primers efficiency in most of the assay with exception of the jejunum *L. acidophilus* assay. There was a significant differences among the treatments for *L. acidophilus* ($p < 0.0001$), *E. coli* ($p < 0.0001$), *S. typhi* ($p < 0.0001$), *C. jejuni* ($p < 0.1059$) in caecum and jejunum *L. acidophilus at* ($p \leq 0.0001$) for 16S rRNA gene copy numbers μg/μl DNA in 1.5 g of caecum and jejunum content. The results indicated normal percentage for caecum *L. acidophilus to* jejunium *L. acidophilus* at 96.65–87.63%, 90.27% to 35% was shown in *L. acidophilus* to *E. coli, L. acidophilus* to *C. jejuni* was 99.97% to 95.94% and low percentage of *L. acidophilus* to *S. typhi* 16S rRNA gene copy numbers after the treatment with CHITPA and CHIAGO. Garlic and onion extract chitosan nanoparticles prepared revealed the presence of selected commensal bacteria and acceptable percentage for caecum *L. acidophilus* to *E. coli* and *C. jejuni* in intestine of Rainbow Rooster Chicken.

Keywords: chitosan; garlic and onion extract; intestinal bacterial flora; indigenous rainbow rooster chicken

1. Introduction

The microbial communities in the gastrointestinal (GI) tract of poultry may be influenced by factors that include stocking density, diet, feeding practices, housing conditions, age of bird and pathogen [1]. The organisms maybe located in lumen of intestine or carried within the mucus [2]. The multidimensional role played by the microflora in the gut of poultry includes digestion, metabolism, pathogen exclusion, immune system stimulation, vitamin synthesis among others [3]. The microflora can metabolize several nutrients and convert them to end products such as short chain, fatty acids that can be utilized by the host as energy and carbon source. The microflora can also act as suppliers of vitamin (especially B vitamin) [4]. Bacterial fermentation in the intestine can occur in most parts of avian gut but primarily takes place in caecum which is densely populated with bacteria [5]. The average time of food passage through the gastro intestinal tract (GI) of poultry such as chicken, turkey and duck is 3.5 hours [6] and the shortest retention time selects bacteria that can adhere to mucosal layer and/or grow fast. The food passage time in caecum is slow [7], which make caecum an ideal habitat for diverse microbes that have considerable effect on the host nutrition and health [7]. Microbes in gut play a positive role in controlling the gut flora and stimulating the development of the gut wall [8]. Studies indicated that the major species of bacteria present in the small intestine and caecum of young chicks are *Lactobacillus* and *Clostridium* while *Bifidobacteria*, *Salmonella*, *Campylobacter*, and *E. coli* species are more dominant in the caeca at older chickens [9]. Lactic acid bacteria (LAB) inhibit harmful micro-organisms through competitive exclusion mechanism based on competition in the binding sites and nutrients. LAB endowed with specific enzymes (amylase, protease) that improve nutrient acquisition as well as stimulation of immune system [10]. *Bifidobacterium* and *Lactobacillus* increases the digestive enzyme activity whilst suppressing the growth of other bacteria such as *Escherichia coli* that can either impair digestive enzyme secretion by damaging villus and microvillus of mucosa or secreting proteolytic enzyme that degrade digestive enzyme [11]. Although *C. jejuni* does not cause disease in chickens through ingestion of *C. jejuni* contaminated feed may lead to severe gastroenteritis in human [12]. Low doses of antibiotics decrease the number of most susceptible bacterial communities

and enhance the growth of resistant bacteria [13]. Bacteria species notwithstanding antibiotics, improves feeding efficiency in bird and maintain health of gut, growth and development [14]. Moreover, additives such as probiotics can be used as nutritional tool in poultry feed to promote growth, modulation of intestinal microflora, pathogen inhibition, immunomodulation and promoting meat quality in poultry [15]. The use of natural additives as alternative to antibiotics for prevention of diseases and performance improvement in poultry production has increased [16]. Herbal feed additive tend to increase both the jejunum and caecum lactic acid bacteria counts as indicated by Giannenas et al. [17]. The study of supplementation of phytogenic feed additive revealed the quantitative increase in the beneficial bacteria by 79% and reduction of pathogenic bacteria population [18]. The gut microbial community is mostly beneficial Gram-positive bacteria at least 85% of total bacteria [19]. The useful microbes in gut play a positive role in controlling the gut flora and stimulate the development of the gut wall [8]. Studies of Selenium nanoparticles (NPs) reported to have improved the gut health by increasing the abundance of beneficial bacteria especially the *Lactobacillus* [20].

2. Methods

2.1. Study location

The experimental chickens were reared at Safari Animal Research Facility at Jomo Kenyatta University of Agriculture and Technology (JKUAT). About 36 samples of caecum and jejunum content were taken from 18 chickens after scarification then followed by DNA extraction and molecular analysis. The nanoparticles preparation was done at Pan African University of Science Technology and Innovations (PAUSTI)-Biotechnology and Molecular Biology Laboratory in (JKUAT)-Kenya.

2.2. Preparation of aqueous onion and garlic, total phenol and ajoene rich extracts

Onion (*Allium cepa* L.) and garlic (*Allium sativum*) were sourced from Juja market in Kiambu County. Onion (red *Creole orallium*) and garlic (softneck) were identified by the Department of Botany at Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya. The aqueous extract of garlic preparation was done according to the method described [21] with modification. About 50 g of Peeled bulbs of garlic was chopped, ground, and mixed with 500 ml distil water then stored for 24 hours, and then filtered the following day using filter paper (Whatman No. 1) and finally stored at 4 ℃ ready for use. The ajoene rich extract was prepared according to [22] with modification in which 50g of peeled garlic bulbs was chopped and homogenously blended with 500 ml of cold distilled water, well stirred, filtered with cotton cloth, then transferred in a new flask containing ethyl acetate then stirred and the mixture allowed to stabilize for separation of Ajoene rich extract. The upper layer was pipetted into a filter paper (Whatman No. 1) to remove all the water leaving only the semi aqueous substance which is ajoene rich extract and the ethyl acetate evaporates and the extracted product stored at 4 ℃ ready for use. Preparation of aqueous extract of onion was done according to [23] with some modification. The onion bulbs together with the outer part were cleaned with ethanol to remove dirt and chopped into smaller and thin slices then air dried for 2 weeks and 50 g weighed mixed with 500 ml of distil water and heated at 72 ℃ for 3 hours and allowed to cool at room temperature and the extract was then filtered with filter paper (Whatman No. 1), then stored at 4 ℃ ready for use. Preparation of total phenol extract was done

according to [24] with some modification in which 50 g of chopped air dried onion bulb soaked in 500 ml of 70% ethanol was left over night and filtered the following day using filter paper (Whatman No. 1) with the ethanol solvent completely removed by rotary evaporator under vacuum at temperature 55 ℃ and the extract stored at 4 ℃ ready for use.

2.3. The preparation of chitosan solution, chitosan nanoparticles, characterization and nanoparticles size determination

Chitosan solution was prepared as described by Rasaee et al. [25] with slight modification. The Low molecular weight chitosan was purchased from Sigma Aldrich and 2 g (w/v) was used with 0.5% (v/v) acetic acid and pH of the solution was elevated to 5 with 1 N NaOH under a magnetic stirring for 24 hours and top up to a volume of 200 ml with distil water, then stored at 4°C ready for application. Preparation of chitosan nanoparticles was done through ionic gelation interaction between positive and negative charged compounds as described by Rasaee et al. [25], with slight modification. The aqueous chitosan was prepared by mixing 40 ml of chitosan solution with 10 ml of garlic and onion aqueous extract (CHIAGO) for treatment 1, and a mixture of 10 ml of total phenol and homogenous ajoene rich extract with 40 ml chitosan solution (CHITPA) for treatment 2. The mixture was then stirred for 10 minutes at 60 ℃, centrifuged at 200 rpm and allowed to rest at room temperature for 30 minutes to form an opalescent solution. The nanoparticles prepared from aqueous of garlic and onion, total phenol and ajoene rich extract were characterized by their sensitivity to pH in confirming the formation of the nanoparticles. The Fourier Transform Infrared Spectroscopy (FTIR) was used to identify the functional groups responsible for the formation of the chitosan nanoparticles of chitosan with the extracts of garlic and onion, and the Field Emission Scanning Electron Microscopy (SEM) analysis was done to confirm the morphology and the size of the nanoparticles prepared. The size of nanoparticles prepared was determined using ImageJ (Figure 1A and 1B).

2.4. Husbandry, experimental design and sampling

A total of 18 chickens were used in this study from the eight groups of treatment and a control with two chickens from each group were sampled for both caecum and jejunum content, an equivalent of 36 samples (18 caecum and 18 Jejunum) were taken for DNA extraction. The chickens used were selected from week 8 of treatment of mixed population of indigenous Rainbow Rooster Chicken. The chickens were sourced from Kukuchic Company Ltd and reared on wooden cages at week 8 of age. Nine chickens were placed in one set of treatment with three chickens per cage with the four treatments at 5% and 10% of (CHISOLN, CHIAGO and CHITPA) and 0.5 g, 1 g of Fosbac and controls were all placed in two opposite blocks. Feed was provided to chickens ad-libitum for both control and the treated groups; the chickens were fed on Kienyeji grower mash produced from the commercial feed company (First Animal Feed). The chickens were acclimatized for two weeks as adaptation period and followed by oral administration of the treatments (twice a week) for a period of 8 weeks. A total of two chickens from each treatment and control were sacrificed using cervical dislocation method according to Laudadio et al. [26]. The weekly sacrifice run for a period of 8 weeks in which, 1.5 g of jejunum and caecum content were sampled at the 8th week of treatment and stored at -20^{o}C for DNA extraction to determine the

effect of garlic and onion extract chitosan nanoparticles on the selected intestinal microflora of Rainbow Rooster Indigenous Chicken of Kenya.

2.5. Primers design for detecting selected intestinal bacterial flora

New species-specific primers (Table 1) targeting the variable region two (V2) of 16S rRNA gene loci were designed for *E. coli, C. jejuni, S. typhi, L. acidophilus* and *B. bifidum and* were done using *Primer3Blast* tool [27]. The universal primers targeting conservative 16S rRNA gene fragments of enteric bacteria were used with slight modification based on alignment of 16S rRNA gene using *FastPCR* tool [28].

Table 1. Species-specific primers for selected intestinal bacterial flora.

Name	Sequence F	Sequence R	Product Length
Bacteria 16S rRNA gene universal primer	CCGAATTCGTCGACA ACAGAGTTTGATCCT GGCTCAG	CCCGGGATCCAAGCTT ACGGCTACCTTGTTAC GACTT	1500 bp
Bifidobacterium bifidum	ATTCGAAAGGTACA CTCACC	CCACATTGGGACTGAG ATAC	169 bp
Campylobacter jejuni	ACGGGTGAGTAAGG TATAGT	ATCATCCTCTCAGACC AGTT	199 bp
Escherichia coli	GGTAACGTCAATGA GCAAAG	GATTAGCTAGTAGGTG GGGT	248 bp
Lactobacillus acidophilus	ACACTTAGCACTCAT CGTTT	TAAGTCTGATGTGAAA GCCC	240 bp
Salmonella Typhi	CCAGATGGGATTAG CTTGTT	AGTACTTTACAACCCG AAGG	204 bp

2.6. Extraction of jejunum and caecum contents

After sampling of each content of 1.5 g jejunum and caecum from the 18 chickens (18 jejunum and 18 caecum); thus a total of 36 samples removed and treated as described by [29] with minor modification. The contents of those segments were inverted into a sterile 15 mL tube containing 9 mL of sterile PBS and then homogenized by vortexing for 3 min, and the Debris removed by centrifugation at 700× *g* for 1min and the supernatant was then collected and centrifuged at 12,000× g for 5 min. The pellet was washed twice with PBS and 200 µl of autoclave distil water was added and DNA was extracted.

2.7. Extraction of bacterial DNA

The bacterial DNA was extracted using qiagen kit protocol in which, twenty microlitres of protease K were added into a tube, 200 µl of the extracted sample pellet from the gut content and 200 µl of lysis buffer was added and incubated at 56 ℃ for 10 minutes. The sample was then centrifuged briefly and 200 µl of 99% ethanol added then pulse vortexed for 15sec and again centrifuged briefly and transferred into mini spin column and centrifuged at 6000 × g for a minute and the supernatant discarded, then Wash buffer one (AW1) 500 µl was added into the column and centrifuged at 6000 × g for a minute, and then Wash buffer two (AW2) 500 µl was added into column and centrifuge at 20000 × g for 3 minutes and flowed. The column was centrifuged again at 20000 x g for a minute eluted into new Eppendorf tube with 50 µl of elution buffer and stored at −20 ℃.

2.8. Preparation of bacterial standards

The pure colonies of *L. acidophilus, E. coli and S. Typhi* (Botany – Microbiology lab-JKUAT) *and C. jejuni* (KEMRI-KENYA) were grown in broth media, and cultured in nutrient agar media and selective media for *Salmonella* and *Campylobacter*. A small portion of 35 bacteria colonies were picked using a sterilized wire loop and placed into 1 ml eppendorf tube containing 200 µl of sterilized distilled water and DNA was extracted using the qiagen DNA extraction kit.

2.9. Quantification of DNA and identification of selected intestinal bacterial flora by conventional PCR

Total bacterial genomic DNA from 1.5 g section of jejunum and caecum from each treatment and a control group from the 18 chickens with 18 samples from each of caecum and jejunum making a total of 36 samples where pooled to 9 each and quantified using Nano-spectrophotometer. The Polymerase Chain Reaction (PCR) amplification of bacteria from jejunum and caecum contents was used as described by Zhu et al. [29] with slight modifications and, 12.5 µl of Dream Tag Green PCR Master Mix (2 ×) (Thermo Scientific), 10.5 µl of nuclease-free water, 0.5 of each primer and 1 µl of DNA extract was used to a total volume of 25 µl. The PCR was conducted with the following amplification conditions; initial denaturation 1cycle of 95 ℃ for 3min, denaturation at 95 ℃ for 30 sec, primer annealing at 35 cycles of 57 ℃ for 30 sec and elongation at 72 ℃ for 7 min. The PCR reaction was optimized at same primer melting temperature (TM) and all run at 35 cycles for all the primers set and the products of PCR was visualized by 1.2% agarose gel electrophoresis.

2.10. qPCR assay and determination of bacterial 16S rRNA gene copy numbers in the jejunum and caecum contents

The qPCR Amplification was carried out on 96 well optical plates on qTower qPCR detector with 5 × HOT FIREPol Evergreen qPCR Mix Plus (Solis BioDyne) in a reaction volume which included 0.5 µl of each primer, 4 µl of the master mix, 14 µl of nuclease free water and 1µl of the DNA template in a final volume of 20 µl per reaction. The qPCR was set at the temperature initial denaturation at 95 ℃ for 3 min, then 35 cycles of denaturation for 20 sec at 95 ℃, annealing plus elongation at 57 ℃ for 30 sec melting curve analysis (60–95 ℃) for 15 sec. Cycle threshold (CT) values were recorded and the melting curve

analysis was performed at the end of the amplification. The bacterial 16S rRNA copy numbers per reaction was determined by qPCR using the primers in Table 1**,** standard curve prepared from the pure culture of the four bacteria with a concentration of 10 fold serial dilution for *E.coli, S. typhi*, caecum *L. acidophilus*, jejunum *L. acidophilus* and *C. jejuni*. The qPCR efficiency was calculated by the formula; Efficiency (E) = $-1+10^{(-1/\text{slope})}$ according to [30]. The slope, R^2 was determined from the standard curve generated from the amplification of the bacteria standard serial dilution and copy numbers of 16S rRNA gene for *E.coli* (0.05754 to 57.54 µg/µl DNA), *S. typhi* (0.089 to 8.9 µg/µl DNA), ceacum *L. acidophilus* (0.00288 to 28.8 µg/µl DNA) and jejunum *L. acidophilus* (0.0120 to 120.226 µg/µl DNA) and *C. jejuni* (0.0138 to 138.03 µg/µl DNA). The unknown copy numbers of 16S rRNA gene of the selected bacteria in the samples was determined from the standard curve. The CT values, the Standard deviation (SD) were recorded and all the sample variation at the lower concentration was determined and claimed to be within the linear interval although it is poorly defined according to MIQM guidelines [31]. The 16S rRNA gene copy numbers in the samples was calculated using the following formula; Concentration (N) = $10^{(n-b/m)}$, where n = CT value, b = intercept and m = Slope [25,30].

2.11. Statistical analysis

Graphpad Statistical Package version 7.1 was used to draw graphs, excel and the data analyzed statistically using one-way ANOVA and Tukey mean differences in Statistical Analysis System (SAS) software version 9.1, and difference considered statistically significant at ($p \leq 0.05$).

2.12. Ethical approval

This research work was approved by Jomo Kenyatta University of Agriculture and Technology (JKUAT) ethical review board vide ethical approval number REF: JKU/2/4/896B.

3. Results

3.1. Determination of nanoparticles size

Imagej software was used to determine the nanoparticles sizes of CHIAGO and CHITPA. The diameter and length of the prepared nanoparticles were obtained with the frequency dstrubution. CHITPA (Figure 1A) yielding particles sizes ranging from (100–600 nm) and CHIAGO (Figure 1B) yielding particles sizes ranging from (200–290 nm) and with the frequency depends on the particles number of the SEM result.

3.2. Gel DNA of bacteria

All selected bacteria isolates with exception of *B. bifidium* were detected by gel electrophoresis indicated the prevalence of caecum *S. typhi* (Figure 2A), *E. coli* (Figure 2B), *C. jejuni* (Figure 2C), *L. acidophilus* (Figure 2D) and *jejunum L. acidophilus* (Figure 2E), in 1.2% agarose gel electrophoresis.

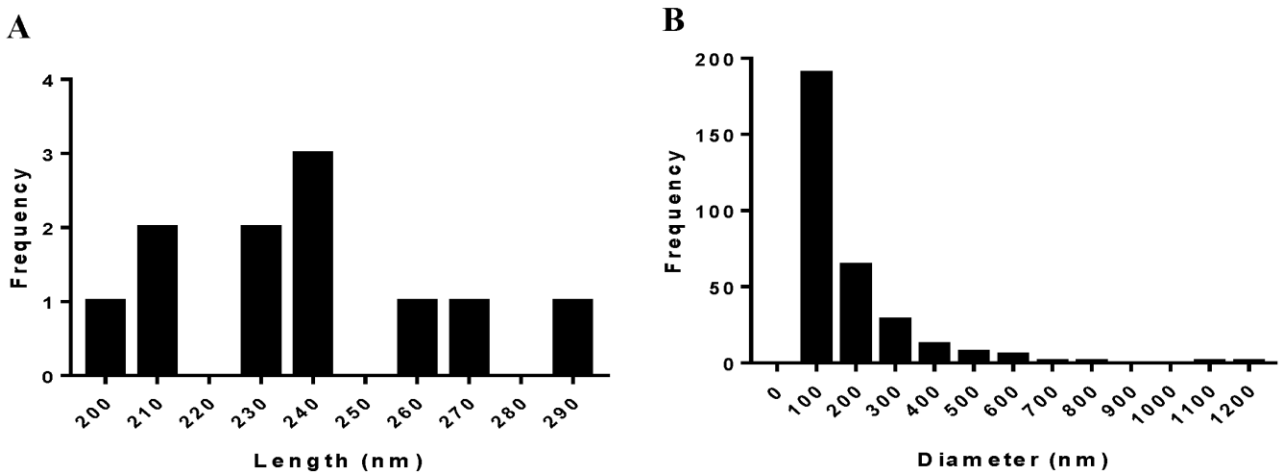

Figure 1. Indicated the CHIAGO and CHITPA nanoparticles size and frequency disturbuttion in SEM result. (A) CHIAGO nanoparticles size in length; (B) CHITPA nanoparticles size in diameter.

3.3. Gel DNA of standard bacteria

The standard bacteria in 1.2% agarose gel electrophoresis (Figure 3) were run to determine specific bands of bacteria to be used in qPCR. The result showed specific bands and was used for the development of qPCR standard curve to determine bacteria 16S rRNA gene copy numbers in the samples.

3.4. qPCR standard curves and limit of detection (LOD) of 16S rRNA gene copy number

The qPCR standard curves for the DNA extracted from pure bacterial culture was determined for caecum *L. acidophilus* 16S rRNA gene copy numbers ranging from 0.00288 to 28.8 gene copies µg/µl DNA (Figure 4A), *E. coli* from 0.05754 to 57.54 gene copies µg/µl DNA (Figure 4B), *S. typhi* from 0.089 to 8.9 gene copies µg/µl DNA (Figure 4C), *C. jejuni* from 0.0138 to 138.03 gene copies µg/µl DNA (Figure 4D), and jejunum *L. acidophilus* from 0.0120 to 120.226 gene copies µg/µl DNA (Figure 4E) which were all calculated from the standard curve of 10 fold serial dilution and utilized in calculating the unknown 16S rRNA gene copy numbers in caecum and jejunum samples of rainbow rooster chicken subjected to the treatments.

3.5. Primer efficiencies

The quantitative PCR efficiencies for individual primer sets were determined using the standard curve slopes achieved from standard bacteria DNA in the below mentioned isolates (Table 2), Regression analysis of the data in different assays obtained indicated efficiencies ranging from (0.798 to 1.124) and linearity of R^2 = (0.954 to 0.999) as shown in the Table 2.

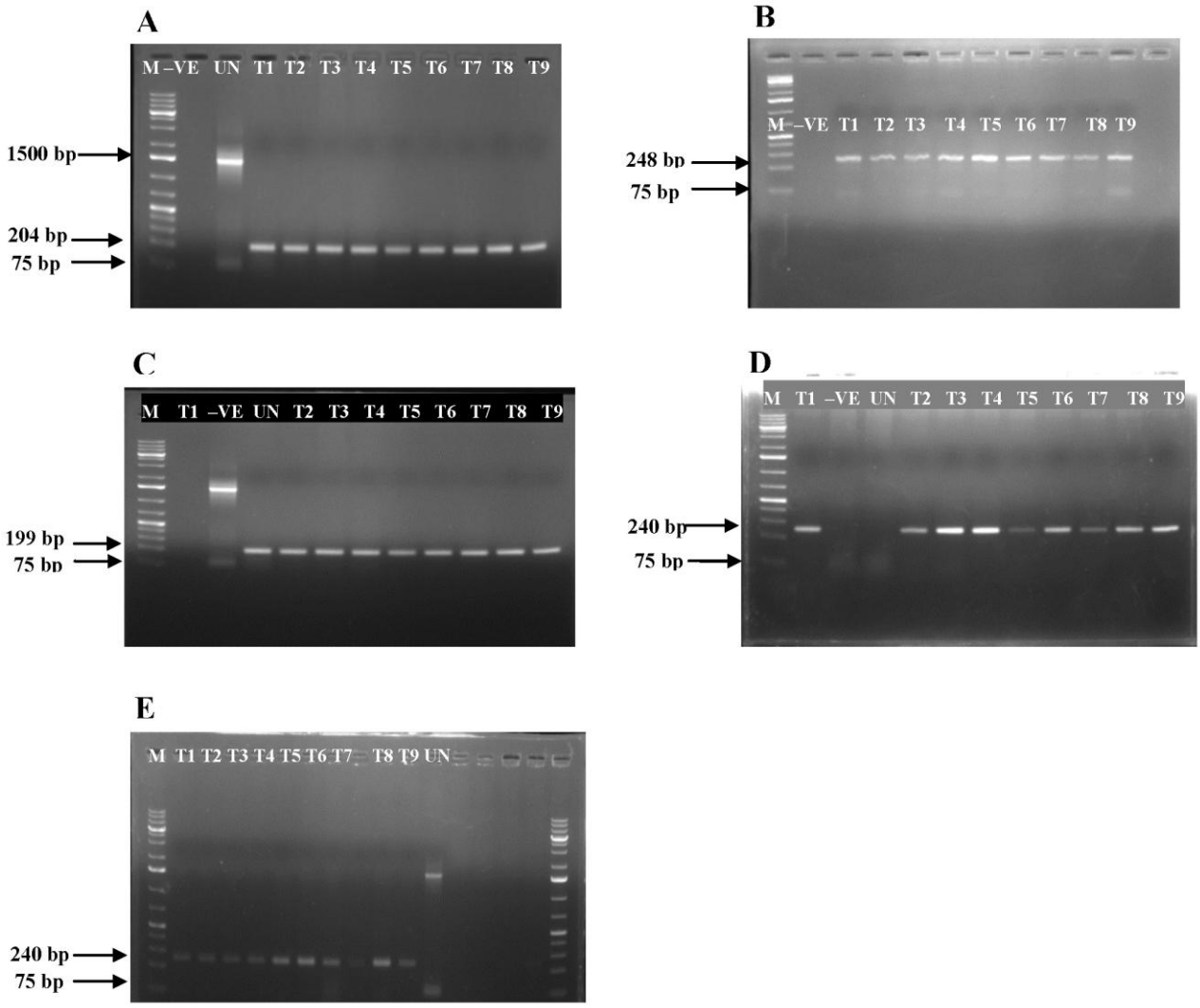

Figure 2. Gel electrophoresis of bacteria isolates with exception of *B. bifidium*. (A) *Caecum S. typhi* in 8th weeks of the treatment; (B) *Caecum E.coli* in 8th weeks of the treatment; (C) Caecum *C. jejuni* in 8th weeks of the treatment; (D) Caecum *L. acidophillus* in 8th weeks of the treatment; (E) Jejunum *L. acidophillus* in 8th weeks of the treatment. T1 (control), T2 (Fosbac 0.5 g), T3 (Fosbac 1 g), T4 (CHITPA 5%), T5 (CHITPA 10%), T6 (CHIAGO 5%), T7 (CHIAGO 10%), T8 (CHLSOLN 5%), and T9 (CHLSOLN 10%), -VE (control negative), M (Marker) and UN (universal primer).

3.6. qPCR melting curves for the bacteria

The melting curve of the three bacteria species showed specificity of one amplicons as indicated in the peaks (Figure 5B, 5C, 5D) and the lengths were confirmed by the gel electrophoresis of standard and the sample. The *L. acidophilus* (Figure 5A, 5E) showed amplicons peak for some of the samples at earlier stage when compared with the standard but the length of standard of *L. acidophilus* bps indicated the same length as that of samples in gel electrophoresis Figure 3.

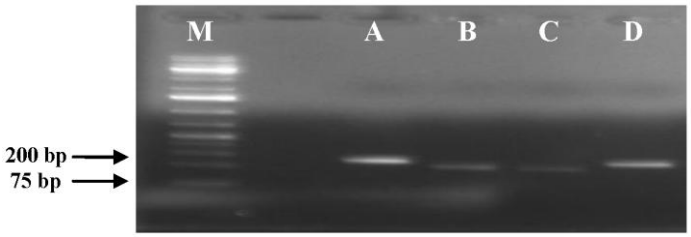

Figure 3. Gel DNA of standard bacteria. M: Marker, A: *E.coli* standard (248 bp); B: *S.typhi* standard (204 bp); C: *C. jejuni* standard (199 bp); and D: *L. acidophillus* standard (240 bp).

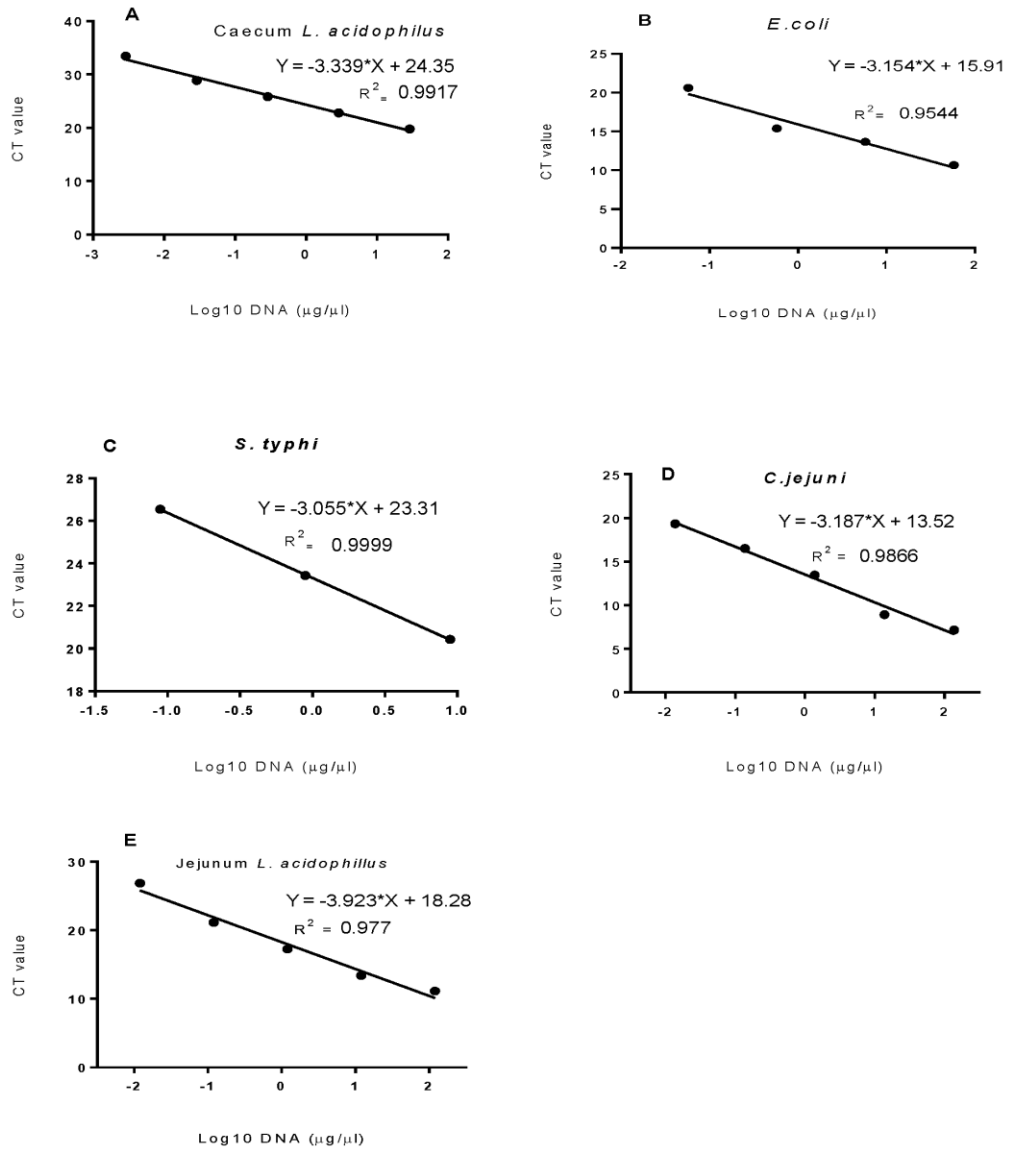

Figure 4. Representative standard curves obtained by plotting the average Ct values against estimated log10 µg/µl DNA of *L. acidophilus* (A), *E. coli* (B), *S. typhi* (C), *C. jejuni* (D) *from caecum and L. acidophilus* (E) from jejunum.

Table 2. Efficiencies of individual primers tested as standards.

S/N	Bacteria Strain	Gene	Efficiency	R^2	Slope	Y-intercept
1	Caecum *Escherichia coli*	16S rRNA	1.075189	0.9544	−3.154	15.91
2	Caecum *Salmonella typhi*	16S rRNA	1.124869	0.9999	−3.055	23.31
3	*Caecum-Campylobacter jejuni*	16S rRNA	1.062839	0.9866	−3.18	13.52
4	Caecum *Lactobacillus acidophilus*	16S rRNA	0.992925	0.9917	−3.339	24.35
5	Jejunum *Lactobacillus acidophilus*	16S rRNA	0.798486	0.977	−3.923	18.28

3.7. Bacterial 16S rRNA gene copy numbers

The 16S rRNA gene copy numbers in Figure 6 calculated from the recorded CT values and the standard curve formula with all the samples variance at the lowest concentration were determined and claimed to be within the linear interval although, is poorly defined according to MIQM guidelines [31]. Caecum *L. acidophilus* (A) gene copy numbers increased significantly in treated chickens for Fosbac 0.5 g, CHITPA 5% and CHISOLN 5% at (p = 0.0001). For *E. coli* in (B) there was significant decrease among the treatments at (p = 0.0001) for CHITPA (5% & 10%), CHIAGO (5% & 10%) and CHISOLN (5% & 10%) in the copy numbers in comparison to the control group. The *S. Typhi* (C) 16S rRNA gene copy numbers was significant at (p = 0.0001) with almost all the treatments indicated reduction in the copy numbers with exception CHIAGO 10% indicated high copy number, and CHISOLN 5% having no expression. The 16S rRNA gene copy numbers of *C. jejuni* (D) was not significant statistically at (p = 0.1059) but with reduction in the copy numbers of the treated groups when compared with the control group. Jejunum *L. acidophilus* (E) copy numbers was significant at (p = 0.0001) among the treatments with Fosbac (0.5 g and 1 g), CHIAGO 5% showed increased.

3.8. Treatment percentage of caecum L. acidophilus to E. coli, C. jejuni, S. typhi from caecum & jejunum L. acidophilus

The result indicated in the Pie Chart below (Figure 7). In Figure 7A with CHIAGO 5% had better percentage of jejunum *L. acidophilus* to caecum *L. acidophilus and* caecum *L. acidophilus* to jejunum *L. acidophilus* for CHITPA (5% & 10%) and CHIAGO 10% had better percentage, Figure 7B had better percentage of caecum *L. acidophilus to E .coli* among the treatments showed in CHIAGO 10% and (CHITPA 5% & 10%), caecum *L. acidophilus* to C. *jejuni* at Figure 7D signifying a good percentage with CHITPA 5% yielding 98.75% and CHIAGO 5% yielding 95.94%, CHIAGO 10% had 99.97 and CHITPA 96.06% and at Figure 7C, caecum *L. acidophilus* to caecum *S. typhi* was poorly represented.

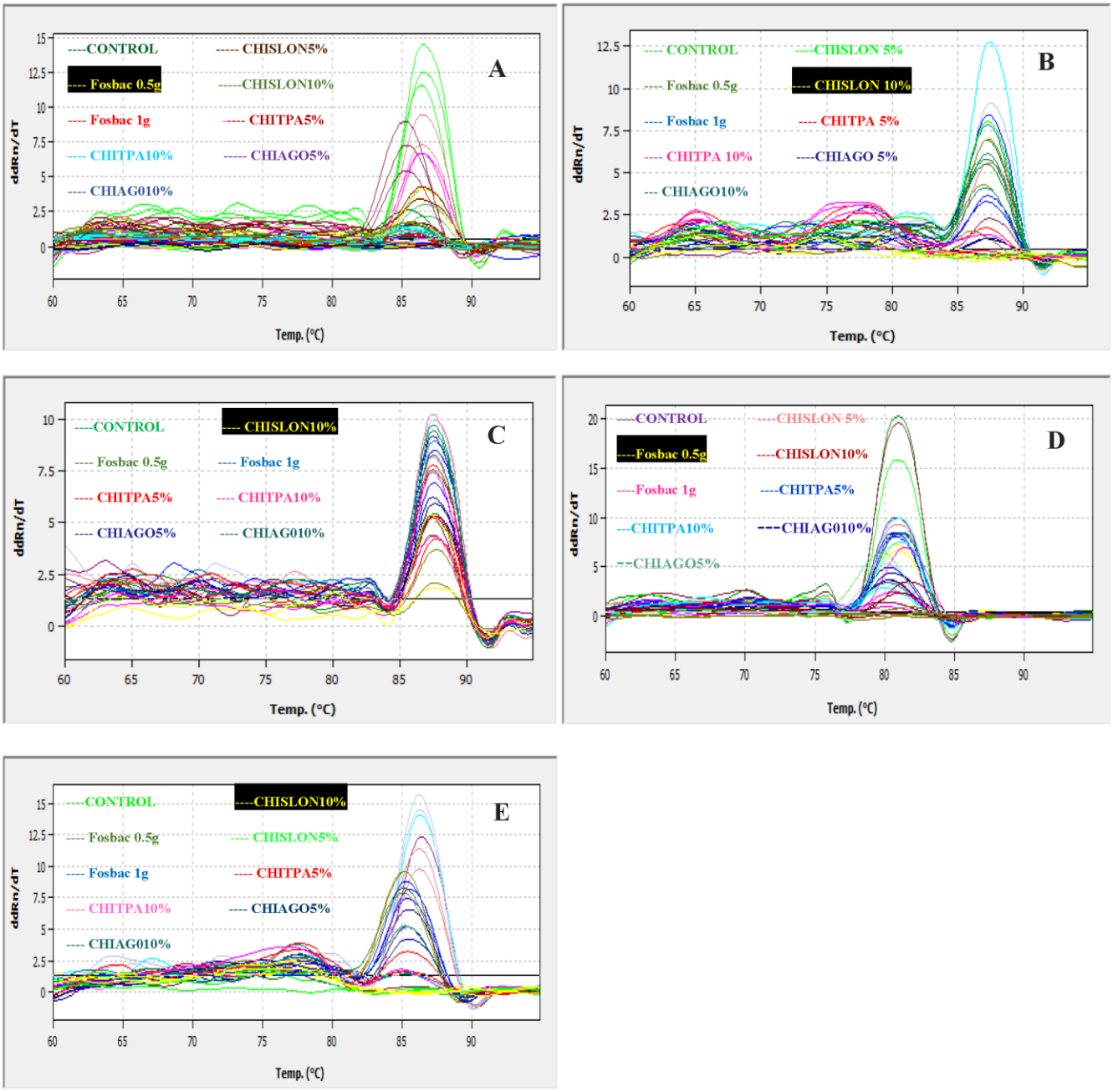

Figure 5. The qPCR melting curves for samples amplifications with the Standard. Caecum *L. acidophilus* (A), *E. coli* (B), *S. typhi* (C), *C. jejuni* (D) & jejunum *L. acidophilus* (E).

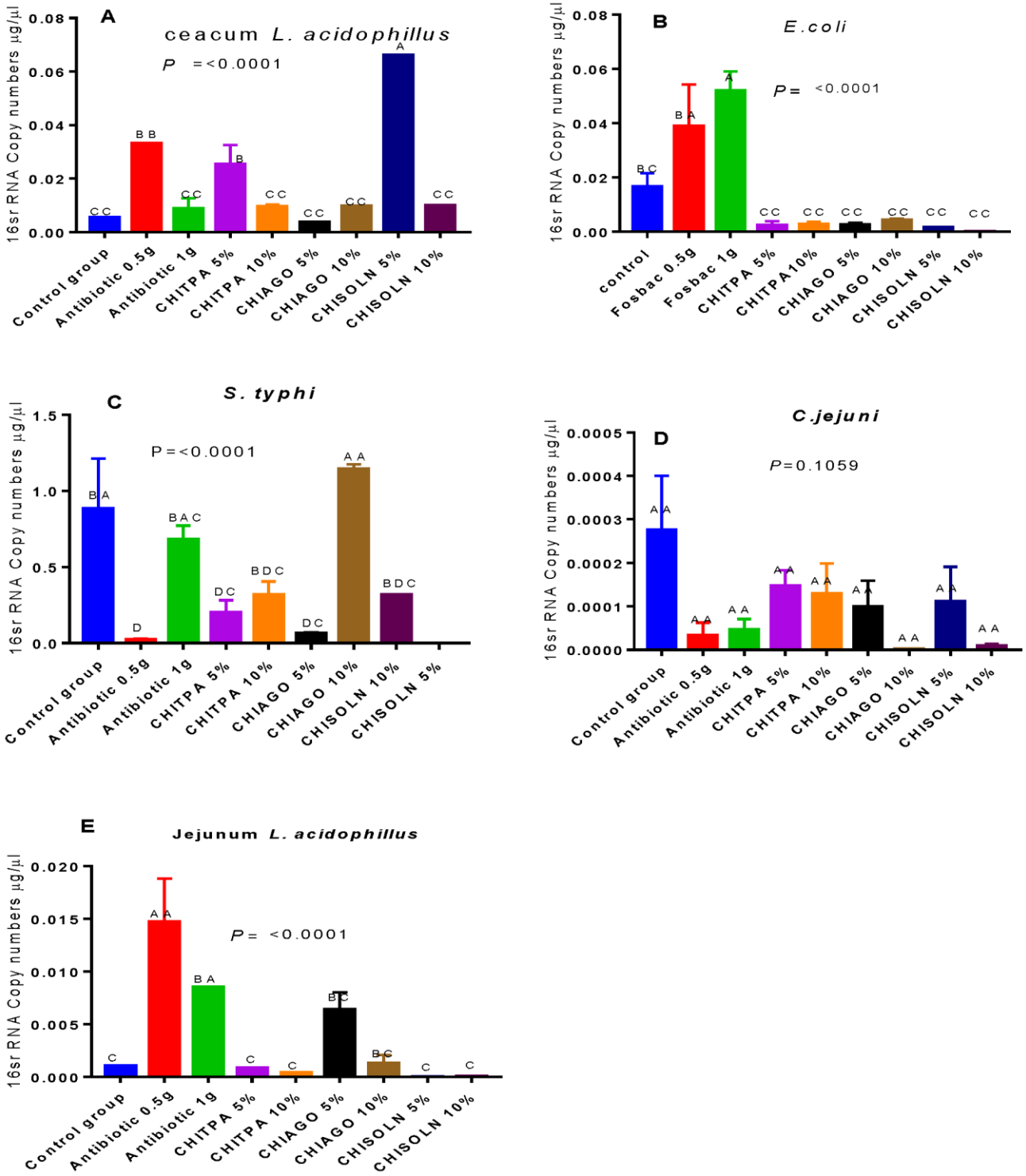

Figure 6. 16S rRNA copy numbers for caecum *L. acidophilus* (A), *E. coli* (B), *S. Typhi* (C), *C. jejuni* (D) & jejunum *L. acidophilus* (E). Control (with only water and feed), Fosbac is antibiotics (control positive); CHISOLN is Chitosan solution; CHIAGO is chitosan with aqueous of garlic and onion; CHITPA is Chitosan with total phenol and ajoene rich extract. Means with different letters are significant difference at ($p \leq 0.05$).

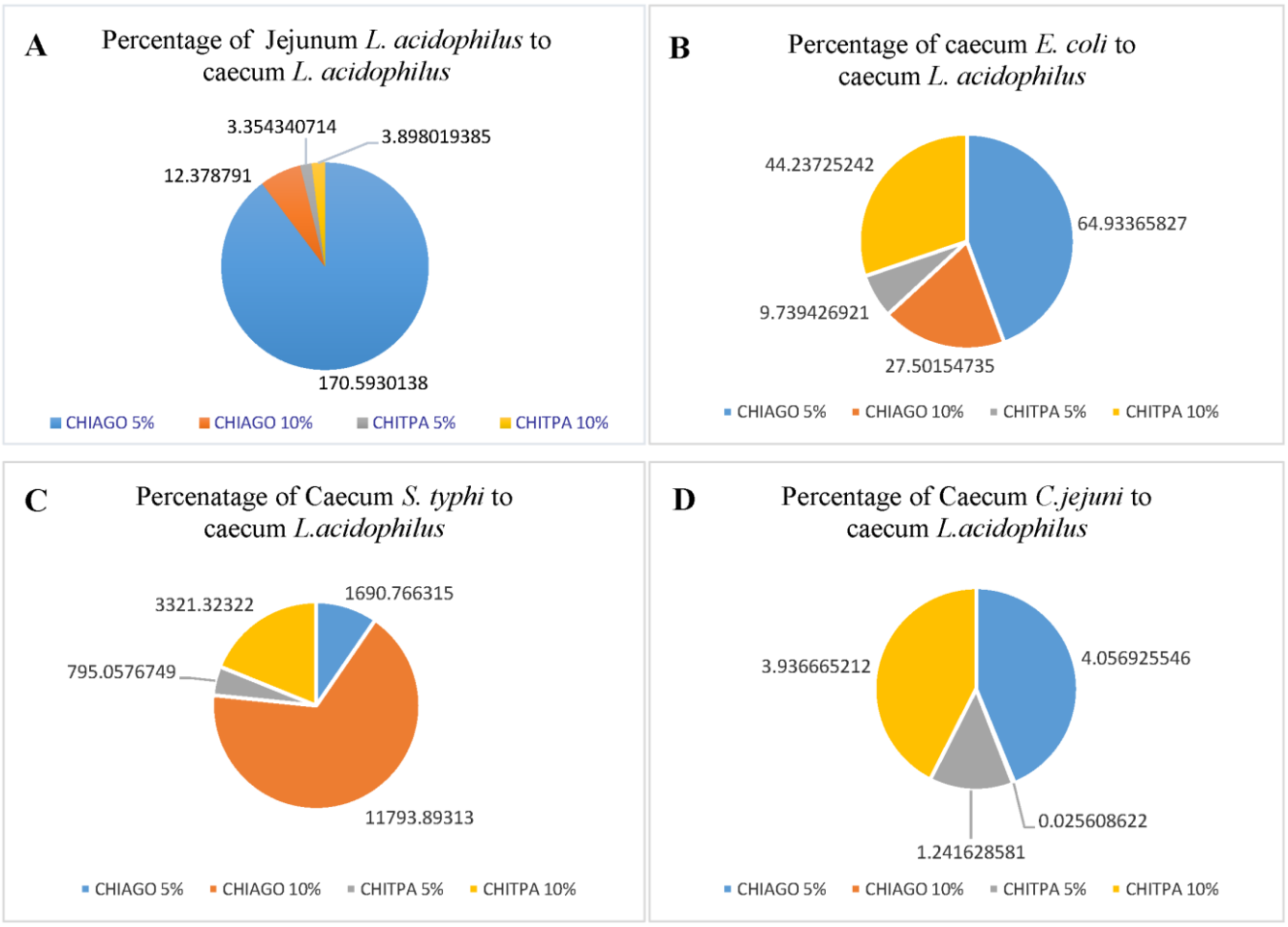

Figure 7. Treatment percentage of the bacteria strain. Percentage of Jejunum *L. acidophilus* to Caecum *L. acidophilus* (A), *E.coli* to *L. acidophilus* (B), *S. typhi to L. acidophilus* (C) and C. *jejuni to L. acidophilus* (D).

4. Discussion

4.1. Nanoparticles sizes

The prepared nanoparticles produced particles sizes ranging from (100–600 nm) and (200 to 290 nm) for CHITPA (Figure 1A) and CHIAGO (Figure 1B) respectively. CHITPA yielded acceptabe frequency with spherical shape and CHIAGO produced a rod shape with frequency depending on the particles number in the SEM result. The nanoparticles sizes fell within the particles size as reported by Rasaee et al. [25] having a result of nanoparticles sizes prepared from chitosan and aqueous leaf extract of *Ocimum basilicum* with a shape nearly spherical and sizes ranging from 135 to 729 nm.

4.2. Primer efficiencies

The primers designed have efficiency to amplify the targeted 16S rRNA gene with the quantitative PCR efficiencies for individual primer sets obtained from the standard curve slopes achieved from standard bacteria isolates DNA. The regression analysis of the data obtained showed efficiencies ranging from (0.798 to 1.124) and high linearity r^2 = (0.954 to 0.999) with exception of jejunum assay indicated 79.8% efficiency. The efficiencies was within the range values (80–110% efficiency), and the slopes between −3.9 and −3.0 was considered acceptable [32].

4.3. qPCR bacteria detection

All melting curves peaks for the three bacteria indicated a single melting curve which was also confirmed by 1.2% agarose gel electrophoresis without dimers but, when *L. acidophilus* compared with the reference standard, the *L. acidophilus* assay was slightly different in some of samples in the melting temperature although the length of standard of *L. acidophilus* bps indicated same length as the samples in gel electrophoresis and the differences might resulted from differences in nucleotides [33], and guanine and cytosine (GC) regions which were more stable and do not melt immediately and other additional sequencing factors such as misalignment [34]. The primer dimers resulted to non-specific products occurring through PCR template-independent primer interactions [35]. The gut microbial community was stable although it can be affected by dietary changes such as feed additives, prebiotics, probiotics, pathogenic infections and antibiotic administration [7]. The greater diversity of microbial community occurs at species level although the age of bird has a significant effect [36]. The report of Amit-Romach et al. [9] indicated *Lactobacillus* in luminal was consistently detected in all intestinal regions compared to *campylobacter*, *E. coli*, *Salmonella* and *B. bifidum* .The major source of *campylobacter* infection in the world is through chicken meat [37]. Although after chickens were treated, the presence of band of *C. jejuni* by qPCR was not an indication that the treatment has no effect. Allicin as one of garlic component showed strong inhibitory properties and degradation products towards *C. jejuni* in vitro as reported [38,39], allicin when given preventively in drinking water, was not able to decrease caecum C. *jejuni* colonization in broilers although there was a development toward lower cecal *C. jejuni* numbers in allicin-treated broilers observed [39].

4.4. Bacteria 16S rRNA copy numbers and L. acidophilus percentage

In this study, all the sample variation at the lowest concentration were determined and claimed to be within the linear interval, although it is poorly defined [31] and as reported, in clinical, veterinary and food microbial detection, there is no specific recommendations for the repeatability standard deviation (SD) value in terms of its proportion with respect to the mean [30].

Caecum *L. acidophilus* 16S rRNA gene copy numbers result, indicated significant differences among the treatments at ($p < 0.0001$) with some of the treatments such as Fosbac 0.5 g, CHITPA 5% and CHISOLN 5% have high copy numbers compared to the other treatments and control. Caecum *L. acidophilus* to *E. coli* and *C. jejuni* 16S rRNA gene copy numbers indicated an improved percentage for the prepared treatments CHITPA and CHIAGO but with *S. typhi* at low percentage. Caecum *L. acidophilus* to *E. coli* of the two treatments (CHIAGO 10%, CHITPA 5% & 10%) with different

concentrations prepared correlated with the findings of [40], reported on the supplementation of onion in broiler diet with significant reduction of *E. coli* and increased significance in *L. acidophilus* and *Streptococcus species.* In healthy chickens, the gut microbial community is mostly beneficial gram-positive bacteria to about 85% of total bacteria [19] and the study of supplementation of phytogenic feed additive revealed the quantitative increase in the beneficial bacteria by 79% and reduced pathogenic bacteria population [18]. Although treatments CHITPA and CHIAGO showed low percentage of 16S rRNA gene copy numbers for *L. acidophilus to S. typhi*, still reduction showed among the treatments in the copy number for *S.typhi* and it correlates with the findings of garlic and onion studied considered as natural bacteriostatic agent that can inhibit the growth of *S. typhi* [41] and the in vivo feedings of chitosan [42] had indicated a reduction of *S.typhi* which might decrease the overall pathogen load in birds, and making them less likely to spread the infection, and with an indication of chitosan 0.2% was able to reduce *S. typhi* both in vitro and in vivo. There were lower set of *L. acidophilus* 16S rRNA gene copy numbers observed among the treatments in jejunum correlated with the reports of Adil et al. [8] indicated the main site of bacteria activity are crop and caecum with lesser extent to small intestine. In this study, Ajoene rich extract which is more stable component of garlic than allicin was used for the preparation of CHITPA treatment, and the encapsulation of the extracts help to avoid inactivation of cysteine groups and other gastrointestinal bacteria that inactivates allicin as reported to caused no effect on the reduction of Campylobacter when allicin is applied in drinking water of broilers [39]. The encapsulation might be the cause of low maintained colonization of caecum *C. jejuni* copy numbers among most of the treatments. Although there is high prevalence of *C. jejuni* in chickens, intestinal disease does not appear to occur after naturally acquired infection of *C. jejuni*, no intestinal inflammation, and no cellular attachment in the intestine of colonized birds [43]. The result of *L. acidophilus* and *E. coli* copy numbers correlates with the reports on herbal feed additive tend to increase both jejunum and caecum lactic acid bacteria counts as indicated [17] In environmental microbiology, the Standard Curve (SC) method used to quantify the concentrations of target genes in diverse samples, e.g., microbial communities of biofilms, the rumen and alpine soils. In most cases, the sample template is different from the standard template used for the preparation of the SC. Standards usually originate from pure cultures, while the sample is composed of a mixture of different species. The SC method assumes a constant efficiency for standard and sample. Although this introduces the possibility of increased quantification errors, it is still the method of choice in environmental microbiology [44].

5. Conclusions

Garlic and onion extract chitosan nanoparticles have a positive effect on the selected intestinal bacteria flora in rainbow rooster chicken. The percentage of *L. acidophilus* to *E. coli* and *C. jejuni* after the application of garlic and onion extract chitosan nanoparticles indicated a good balance in some of the nanoparticles treatment dosages, caecum *L. acidophilus* to C. *jejuni* yielded a good percentage with CHITPA 5% yielded 98.75% and CHIAGO 5% yielded 95.94%, CHIAGO 10% yielded 99.97% and CHITPA 96.06%, although among the treatments including the control group, there were no statistical differences in the copy numbers for *C. jejuni*, the treated groups still showed a reduction in the copy numbers. Caecum *L. acidophilus* to caecum *S. typhi* poorly represented with low percentage but the treated groups in comparison with the control group still observed to be capable to enable the reduction of caecum *S. typhi* 16S rRNA copy numbers which is a good indicator to enable microbial balance in the

gut of chicken for better performances and this will improve gut health, reduction in antibiotic application as growth promoter and improves chicken welfare.

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Conflict of interest

The authors declare no conflict of interest in this manuscript.

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