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Evolution of Cloacal Bacteria and Fungi in Brahma Chickens (*Gallus gallus domesticus*) Fed with *Chromolaena odorata* Supplement

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A B S T R A C T

A study was conducted to evaluate the evolution of cloacal bacteria and fungi in Brahma (*Gallus gallus domesticus*) chickens fed with a *Chromolaena odorata* supplement. A total of 180 cloacal samples were collected over a 10 weeks period from August to October 2020. Three feed rations (R_0, R_1 and R_2) containing 0, 1 and 2% dried leaves of *Chromolaena odorata* respectively were tested. Studied microorganisms isolated were total coliforms, *Escherichia coli*, *Salmonella sp.*, *klebsiella sp.*, yeasts and molds. Bacterial load varied from 10 $x10^4$ to 5236 $x10^4$ CFU/g for R_0, from 64 $x10^4$ to 12317 $x10^4$ CFU/g for R_1 and from 1 $x10^4$ to 4292 $x10^4$ CFU/g for R_2 Whereas fungal load fluctuated between 34 $x10^4$ and 3786 $x10^4$CFU/g for R_0, between 153 $x10^4$ and 8988 $x10^4$ CFU/g for R_1 and between 8 $x10^4$ and 18154 $x10^4$ CFU/g for R_2. This flora was subject to wide temporal fluctuations. The analysis of these results considered the interaction of the different variables (gender, weight and time). Apart from the time factor (weeks) which significantly ($p<0.05$) influenced the abundance of *Escherichia coli*, yeast and mold, no other factor had a significant effect on the evolution of the cloacal microflora of these birds. However, linear relationships between the factors ration, gender, time and weight evolution of microbial cells revealed a significant effect ($p<0.05$). This action resulted in an improvement of the feed conversion ratio and an increase in live weight of the studied birds which was most marked for the R_1 ration and in males.

Introduction

Sustainable production of poultry meat and eggs is important to provide safe and quality protein sources in human diets worldwide (Kogut and Oakley, 2016). The gastrointestinal tract (GIT) of chickens contains a diverse and complex flora that plays an essential role in digestion and nutrient absorption, immune system

development and pathogen exclusion (Shang *et al.,* 2018). The digestive flora includes single-celled organisms namely bacteria, fungi and protozoa. Regarding bacterial populations, which are the predominant microorganisms, they represent a wide range of metabolic and morphological types. Their total number is greater than the number of eukaryotic cells constituting the host body. A distinction is made between dominant ($>10^6$ Colony Forming Units (CFU)/g content), subdominant (10^5-10^3 CFU/g content), and residual ($<10^3$ CFU/g content) bacteria (Oakley *et al.,* 2014; Pan and Yu, 2014). In chickens, the main sites of bacterial activity are the crop, caeca and, to a lesser extent, the small intestine.

In the crop, facultative anaerobic bacteria (lactobacilli, streptococci and coliforms) are mainly found, but also yeast. In the caeca, strict anaerobes such as *Eubacterium*, Bifidobacteria or Clostridia are in the majority, but facultative anaerobic bacteria are also present. Thus, in caeca, 10^{11} and 10^9 bacteria are found per g of content respectively (Gabriel *et al.,* 2005). The composition and function of these communities vary according to bird age, gender and diet (Shang *et al.,* 2018).

Several studies have been carried out comparing animals fed with and without antibiotics and the influence of these chemicals on the floral walls of animals (Djellout *et al.,* 2018). With the abolition of Growth Promoting Antibiotics (GPAs), research is now focusing on the microflora of farmed animals, including poultry, and the changes it may undergo (Afssa, 2007). Plant-based products, already used for their effects on zootechnical performance in poultry since the 1990s, have thus seen their use increase significantly (Brenes and Roura, 2010).

Chromolaena odorata (L.) R. M. King and Robinson belongs to the family *Asteraceae* (Ngono Ngane *et al.*,2006; IUCN/PACO, 2013). In traditional medicine, it has been cited for several uses. It has anti-parasitic and insecticidal, antioxidant, anti-diabetic, immunostimulant, antibacterial, anti-inflammatory, antiviral, anti-allergic and antifungal properties (Bouda *et al.,* 2001; Pamo *et al.,* 2004; Ngono Ngane *et al.,* 2006; Mbajiuka *et al.,* 2014; Adedapo *et al.,* 2016). In poultry nutrition, an inclusion level of up to 5% of *C. odorata* leaves seems to support a desirable health status in haematological and biochemical reports (Fasuyi *et al.,* 2005).

These authors also noted that egg production and feed intake increased at up to 5% inclusion of *C. odorata* powder, an a slight decrease beyond that threshold. It is in this context that this work whose aim was to evaluate the evolution of cloacal bacteria and fungi in Brahma chickens (*Gallus gallus domesticus*) fed with a *C. odorata* supplement was carried out. The hypothesis was that addition of *C. odorata* to the birds' diet would favor the development of cloacal microorganisms.

Materials and Methods

Plant material

The plant material for this study was dried leaves of *Chromolaena odorata* (L.) R. M. King and Robinson (*Asteraceae*). Fresh leaves were collected during the period of April-May 2019 corresponding to the rainy season (when biomass is abundant), in the campus of the Institute of Agricultural Research for Development (IRAD) Nkolbisson located in Yaoundé, Cameroon (Central Africa). The choice of this plant as function of its different uses, its availability, its invasiveness and the ethno-medicinal information obtained from traditional healers in certain localities. The identification of this plant was carried out at the National Herbarium (IRAD).

Preparation of Chromolaena odorata powder

The powder was obtained according to the method used in traditional method (Adedapo *et al.,* 2016). The harvested leaves were weighed, washed, drained and dried in the laboratory at room temperature (22±2°C) to avoid any degradation of the active ingredients. During drying, the leaves were regularly aerated to avoid contamination by fungi. Once dried, the leaves were ground using an electric grinder (brand of blender) and the resulting powder was stored in plastic bags at room temperature, in a dry place and protected from humidity and light until further use.

Animal material and experimental set-up

In this study, forty-five parent stocks (*Gallus gallus domesticus*) aged 4-5 months with mean weights of 1kg for females and 1.5kg for males were randomly divided into 3 groups of 15 chickens. Each group was then subdivided into 3 replicates of 5 chickens each (1 male and 4 females) for a total of 9 experimental units. They were reared on deep litter at a density of 5 chickens/m^2. At this stage, a control ration (R_0) was made (Table 1) and 2 other rations containing 1% (R_1) and 2% (R_2) of *C. odorata* powder. Each of the 3 experimental rations was randomly allocated to the experimental units in a completely randomized design. The chemical composition of the proposed rations in the different groups is presented in Table 1. The animals were fed according a ration plan and water given *ad libitum*. The study lasted 10 weeks (W_1 to W_{10}) and sampling and weighing of birds were carried out every week.

Cloacal sampling and microbiological analysis

In each experimental unit, feces were collected from one male and one female directly from the cloaca of each bird, using sterile swabs.

A total of 9 samples were taken on each visit. Each sample was weighed and then introduced into a 2.5-5ml of sterile physiological water depending on the consistency.

The suspension was then homogenized using a grinder to obtain a 1/10 stock suspension. A series of dilutions down to 10^{-5} was made from the stock solution by taking 1 ml each time to add to 9 ml of distilled water contained in a test tube. 100 µl of the final sample was taken with a micropipette and spread on cast agar in Petri dishes around the sterility diameter of the Bunsen burner flame (Rodier *et al.,* 2016).

The main selective media used for isolation and enumeration of microbiological colonies are described in Table 2. The reading of germs (colonies) on the colony counter for the enumeration of considerable colonies was performed according to the ISO-AFNOR standard (NF V08-010 1986).

All colonies present in the solid culture media were identified according to standard biochemical criteria (Holt *et al.,* 2000). The results were expressed as CFU/g (colony forming units per gram).

Statistical analyses

Statistical analyses of the data were carried out using the International Business Machine Statistical Package of Social Science version 20 ((SPSS 20). All histograms were generated using Microsoft Excel 2010. Relationships between cell abundances were assessed by Pearson correlation tests. Comparisons of the means of cell abundances of the data by ration, time and gender were performed using the analysis of variance (ANOVA) test. The significance level considered was 5%.

Results and Discussion

Variation in microbial abundance in males

Total coliform (TC) concentration in males were fed with R_0 control ration that ranged from $0x10^4$ to $10696x10^4$ CFU/g, $10587x10^4$ to $11x10^4$ CFU/g for the 1% *C. odorata* powder ration (R_1), $33589x10^4$ to $11x10^4$ CFU/g for the 2% *C. odorata* powder ration (R_2) (Figure 1A). Maximum concentrations were recorded at W_9 and W_3 for R_0 and R_1 respectively and at W_2 for R_2. The mean bacterial abundances of the three rations were $2035x10^4$, $2465x10^4$ and $5830\ x10^4$ CFU/g respectively.

The concentration of *E. coli* fluctuated from $0x10^4$ to $5323x10^4$ CFU/g for R_0 at W_6 and W_9 respectively, $0x10^4$ to $3327x10^4$ CFU/g for R_1 at W_2 and W_9 respectively, $14319x10^4$ to 2x104 CFU/g for R_2 at W_8 and W_2 respectively (Figure 1B). The mean values of the three rations were $1071x10^4$, $520x10^4$ and $1985x10^4$ CFU/g respectively.

Klebsiella sp. counts ranged from $0x10^4$ to $4041x10^4$ CFU/g for R_0 at W_6 and W_9 respectively, $0x10^4$ to $1599x10^4$ CFU/g for R_1 at W_3 and W_9 respectively and $0x10^4$ to $28822x10^4$ CFU/g for R_2 at W_1 and W_4 respectively (Figure 1C). The means of the three rations were $672x10^4$, $368x10^4$ and $3978x10^4$ CFU/g respectively.

For *Salmonella sp.* concentrations ranged from $167x10^4$ to $0x10^4$ CFU/g for R_0 and $0.05x10^4$ to $0x10^4$ CFU/g for R_1. Maximum concentrations were recorded at W_5 and W_1 for R_0 and R_1 respectively. The R_2 treatment was free of *Salmonella* (Figure 1D).

Yeast concentration ranged from $1475x10^4$ to $0x10^4$ CFU/g for R_0, $0x10^4$ to $252x10^4$ CFU/g for R_1, and $16202x10^4$ to $0x10^4$ CFU/g for R_2. The mean values of the three rations were $234x10^4$, $64x10^4$ and $2643x10^4$ CFU/g respectively. Maximum concentrations were recorded in weeks W_2, W_7, and W_4 for R_0, R_1 and R_2 respectively (Figure 1E).

Mold concentration fluctuated from $0x10^4$ to $96x10^4$ CFU/g for R_0, $0x10^4$ to $40x10^4$ CFU/g for R_1 and $0x10^4$to $55x10^4$ CFU/g for R_2 (Figure 1F). The mean values of the three rations were $15x10^4$, $11x10^4$ and $12x10^4$ CFU/g respectively.

Variation in microbial abundance in females

The total coliform concentration varied from $6x10^4$ to $11754x10^4$ CFU/g for ration R_0 at weeks W_8 and W_9 respectively, $229x10^4$ to $200000x10^4$ CFU/g for R_1 at W_1 and W_2 respectively, $11574x10^4$ to $178x10^4$ CFU/g for R_2 at W_3 and W_{10} respectively (Figure 2A). The mean of the three rations were $2109x10^4$, $22169x10^4$ and $2846x10^4$ CFU/g respectively.

The concentration of *E. coli* fluctuated from $0x10^4$ to $6513x10^4$ CFU/g for R_0 at W_6 and W_9 respectively, $0x10^4$ to $20200x10^4$ CFU/g for R_1 at W_1 and W_2 respectively, $0x10^4$ to $2293x10^4$ CFU/g for R_2 at W_3 and W_7 respectively (Figure 2B). The means of the three rations were $838x10^4$, $2758x10^4$ and $625x10^4$ CFU/g respectively.

Klebsiella sp. counts showed concentrations ranging from $90063x10^4$ to $0x10^4$ CFU/g for R_0 at W_2 and W_6 respectively, $51494x10^4$ to $6x10^4$ CFU/g for R_1 at W_2 and W_8 respectively and $21840x10^4$ to $2x10^4$ CFU/g for R_2 at W_3 and W_{10} respectively (Figure 2C). The mean of the three rations were $9801x10^4$, $9158x10^4$and $3000x10^4$ CFU/g respectively. For *Salmonella sp.* concentrations ranged from $9x10^4$ to $0x10^4$ CFU/g for R_0 and $1067x10^4$ to $0x10^4$ CFU/g for R_1. Maximum concentrations were recorded at W_1 for both rations. The R_2 ration was free of *Salmonella* throughout the study (Figure 2D).

Table.1 Composition of experimental ration

	Rations		
Ingredients (kg)	**R_0**	**R_1**	**R_2**
Maize	51.8	51.8	51.8
Wheat Bran	7	7	7
Soya meal	15	15	15
Cotton seed cake	7	7	7
Fish meal	5	5	5
oyster shell	8	8	8
Concentrate 5%	5	5	5
Bone meal	2	2	2
Elitox	0.1	0.1	0.1
Bicalphos	0.1	0.1	0.1
***Chromonla Odorata* Powder**	0	1	2
Total	100	101	102
Calculated chemical Composition			
ME(Kcal/Kg)	2673	2700	2726.5
Crude Protein %	21.49	21.48	21.15
Fat	4.11	4.12	4.13
Calcium %	4.37	4.33	4.39
Phosphorus %	0.78	0.78	0.77
Total Lysine%	1.13	1.13	1.12
Total Methionine	0.47	0.47	0.47

R_0=0% de la poudre de C. odorata, R_1= 1% de la poudre de C. odorata, R_2=2% de la poudre de C. odorata, ME : metabolic energy

Table.2 Main selective media used for colony isolation and enumeration

Germs	Agar	Temperature	Incubation time
Total coliforms (TC)	Endo agar	37 ° C	18 to 24h
Eschericia coli (E. coli)	EMB agar	44 ° C	48 to 72h
Salmonella sp.	Salmonella-Shigella agar (SS)	37 ° C	18 to 24h
klebsiella sp.	Levine EMB Blue Agar (EMB)	37 ° C	18 to 24h
Yeasts and molds	Potatoes dextrose agar (PDA) + chloramphenicol	22 ° C	48 to 72h

Table.3 Correlation matrix (Pearson) between cell abundances

	***E. coli* (UFC/g)**	**Total coliforms (UFC/g)**	***Salmonella sp.* (UFC/g)**	***Klebsiella sp.* (UFC/g)**	**Yeasts (UFC/g)**	**Molds (UFC/g)**
***E.coli* (UFC/g)**	1					
Total coliforms (UFC/g)	**0.654****	1				
***Salmonella sp.* (UFC/g)**	**0.534****	**0.958****	1			
***Klebsiella sp.* (UFC/g)**	**0.186***	0.072	-0.008	1		
Yeasts (UFC/g)	**0.244****	**0.277****	**0.252****	**0.288****	1	
Molds (UFC/g)	**0.611****	**0.574****	**0.490****	**0.360****	**0.298****	1

*correlation significant at 0.05, ** correlation significant at 0.01

Table.4 Analysis of variance and regression (ANOVA)

		***E. coli* (UFC/g)**	**Total coliformes (UFC/g)**	***Salmonella sp.* (UFC/g)**	***Klebsiella sp.* (UFC/g)**	**Yeasts (UFC/g)**	**Molds UFC/g)**
R^2		0,200	0,099	0,067	0,104	0,152	0,147
F		3.470	1.524	1.005	1.608	2.497	2.395
***p* > F**		**0.000**	0.120	0.447	0.093	**0.005**	**0.007**
Gender	F	0.105	0.946	0.987	2.455	2.756	174.726
	p	0.746	0.332	0.322	0.119	0.099	< 0.0001
Time	F	4.529	1.665	0.963	1.853	2.899	4.237
	p	**< 0.0001**	0.101	0.472	0.062	**0.003**	**< 0.0001**
Ratios	F	0.388	1.182	1.201	0.084	0.557	5.837
	p	0.679	0.309	0.304	0.920	0.574	0.004

Fig.1 Variation in total coliforms (A), *E. coli* (B), *Klebsiella sp.* (C), *Salmonella sp.* (D), yeasts (E) and molds (F) in males fed R_0, R_1 and R_2 rations.

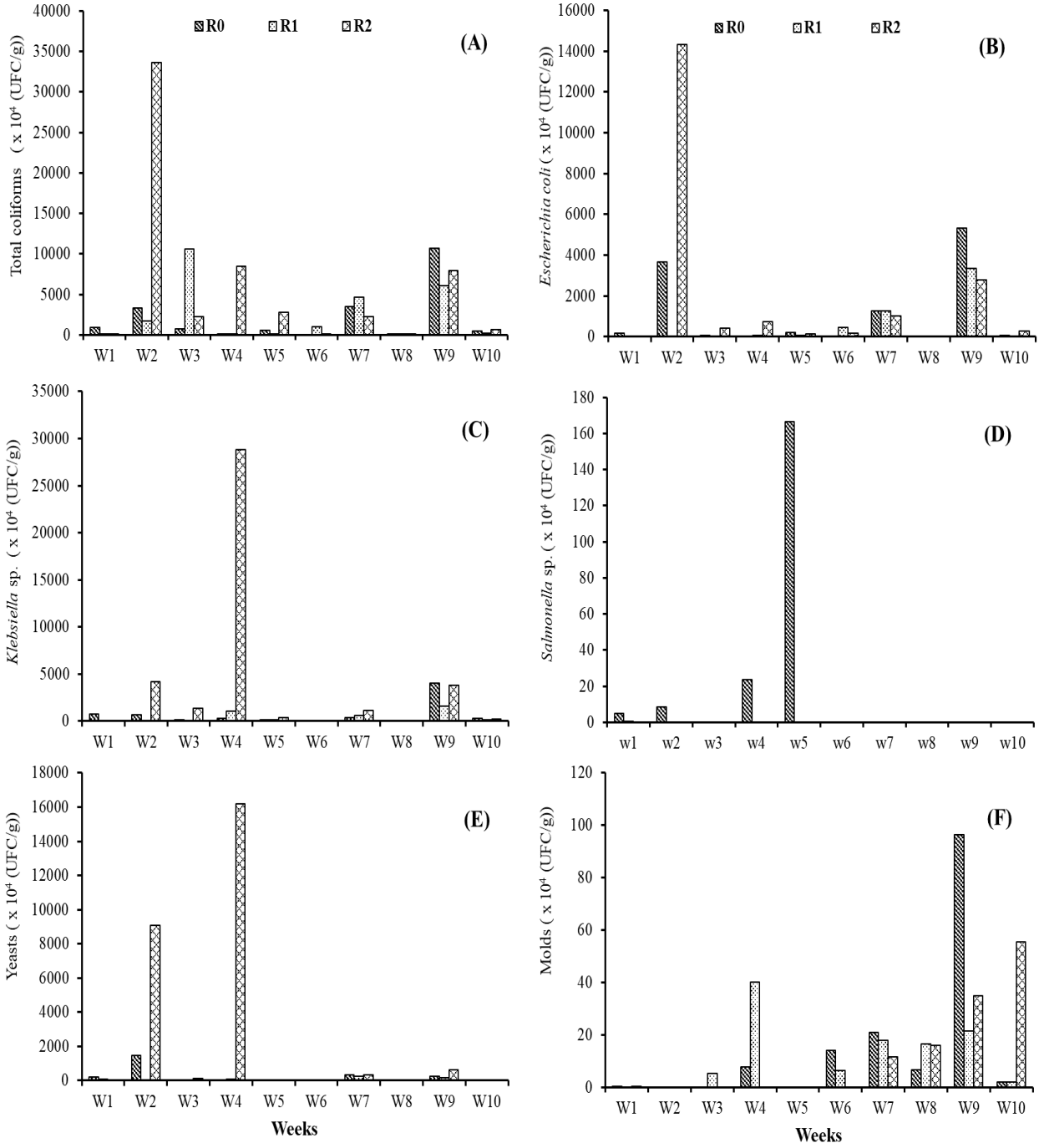


Fig.2 Variation in total coliforms (A), *E. coli* (B), *Klebsiella sp.* (C), *Salmonella sp.* (D), yeasts (E) and molds (F) in females fed R_0, R_1 and R_2.

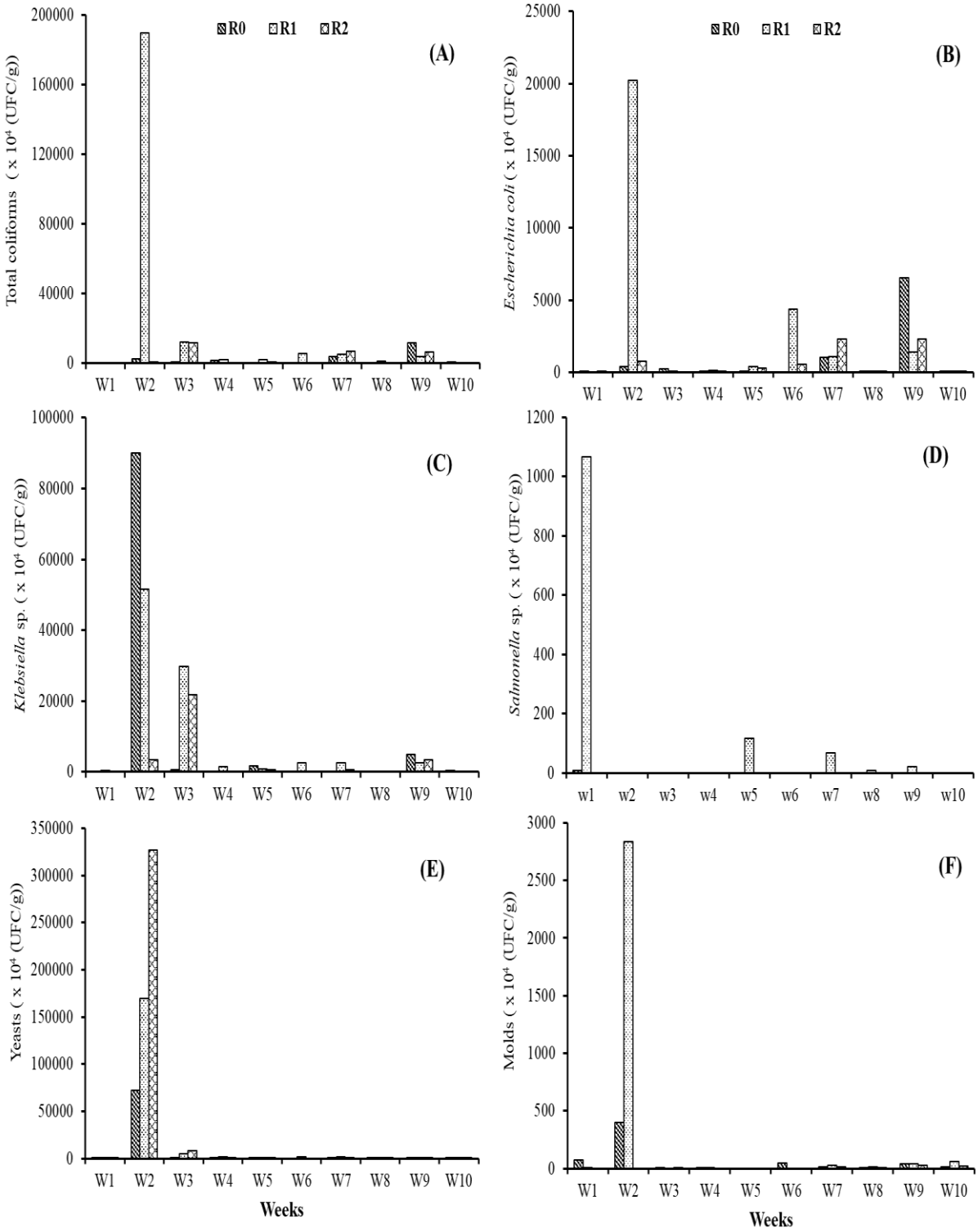
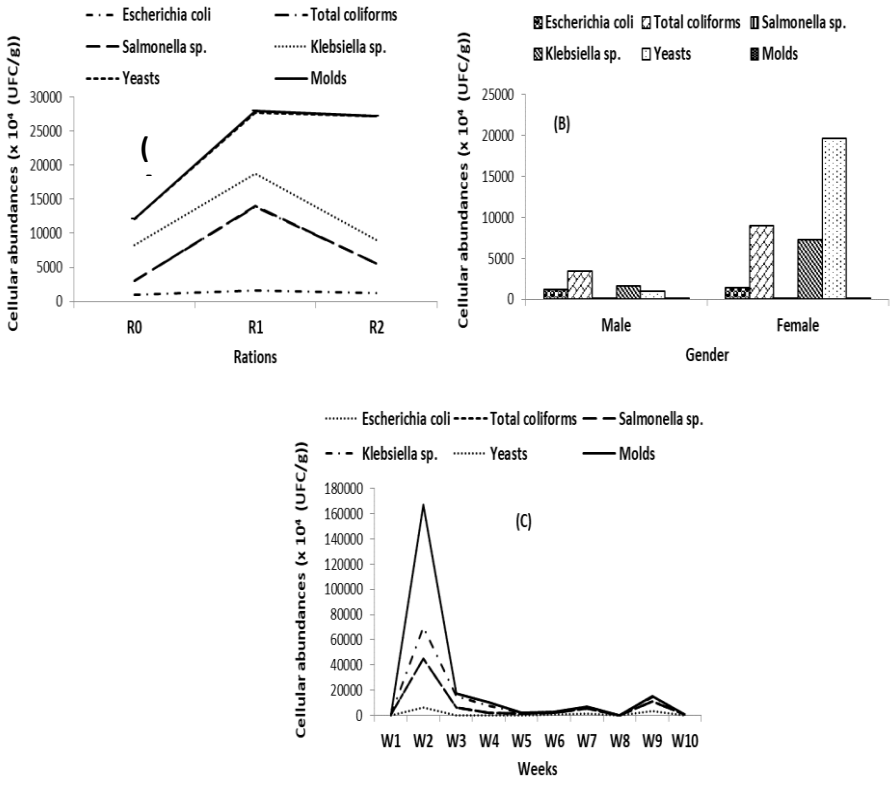


Table.5 Live weight (Kg) of chickens according to ration, gender and time

Sources of variation	Weight (Kg)
Rations	**
R_0	2.072± 0.028b
R_1	2.197± 0.028a
R_2	2.094± 0.028b
Gender	***
Male	2.332± 0.023a
Female	1.910± 0.023b
Time	***
Week 1	2.048 ± 0.051bc
Week 2	2.096 ± 0.051bc
Week 3	2.013 ± 0.051c
Week 4	2.094 ± 0.051bc
Week 5	2.094 ± 0.051bc
Week 6	2.097 ± 0.051bc
Week 7	2.084 ± 0.051bc
Week 8	2.207 ± 0.051b
Week 9	2.382 ± 0.051a
Week 10	2.096 ± 0.051bc

** p<0.01, *** p<0.001, a, b, c: numbers with the same letter are statistically comparable

Fig.3 Concentration of microbial populations as a function of ration (A), gender (B) and time (weeks) (C)



Yeast concentrations ranged from $72467x10^4$ to $0x10^4$ CFU/g for R_0, from $200000x10^4$ to $8x10^4$ CFU/g for R_1, and $300000x10^4$ to $0x10^4$ CFU/g for R_2. The mean of the three rations were $7396x10^4$, $18006x10^4$ and $33666x10^4$ CFU/g respectively. Maximum concentrations were recorded at W_2 for all three rations and minimum concentrations at W_6, W_{10} and W_6 for R_0, R_1 and R_2 respectively (Figure 2E).

Molds concentrations fluctuated from $400x10^4$ to $0x10^4$ CFU/g for R_0, $2833x10^4$ to $0x10^4$ CFU/g for R_1 and $31x10^4$ to $0x10^4$ CFU/g for R_2 (Figure 2F). The means of the three rations were $61x10^4$, $300x10^4$ and $8x10^4$ CFU/g respectively.

Correlation between the parameters studied

Pearson's correlation test for the data from the 180 samples indicated a degree of association between cell abundances (p<0.01) throughout the study (Table 3).

The analysis of variance and regression (Table 4) shows that 20%; 9.9%; 6.7%; 10.4; 15.2% and 14.7% of the variability observed in *E. coli*, total coliforms, *Salmonella sp.*, *Klebsiella sp.*, yeasts and molds, respectively, is explained by ration, time and gender. The rest of the variability was explained by effects that were not identified during the study. The contribution to the total variability observed within these different bacteria was statistically significant (p<0.05) *in E. coli*, yeasts and molds.

Apart from the time factor (weeks) which significantly (p<0.05) influenced *E. coli*, yeasts and molds, no other factor had a significant effect on the evolution of the microbiological flora of chicken feces. However, the linear relationships between the factors (ration, gender and time) and weight evolution reveal a significant effect (p<0.05). Indeed, there is evidence of an increase in microbial flora for all three rations with higher abundances at ration R_1 (Figure 3A). On the other hand, higher abundances were observed in females (Figure 3B), and decrease with time (Figure 3C).

The average weight according to ration, gender and time is summarized in Table 5. This action results in an increase in live weight of the animals, which was more pronounced for the R_1 ration and in males.

The cloacal harbours a very diverse, rich and stable microbial community (Videnska *et al.*, 2013; Juan *et al.*, 2019). The microbial flora of the cloaca analyzed was subject to temporal fluctuations. It varied from 10^4 to 10^7 CFU/g for the R_0 control ration, from 10^5 to 10^8 CFU/g for the 1% *C. odorata* powder ration (R_1) and from 10^4 to 10^8 CFU/g for the 2% *C. odorata* powder ration (R_2). According to Gabriel *et al.*, (2005), in this region, microbial concentrations vary between 10^9 and 10^{11} CFU per g of content. The fluctuations observed would be linked to the ration administered. Indeed, the concentrations of total coliforms, *E. coli* and *klebsiella sp.* increased throughout the study and this increase was more pronounced in subjects fed 1% *C. odorata* powder ration (R_1) supplemented diets. Feeding approaches were monitored to clarify the impact of feed intake on caeca, physiological and functional characteristics in broilers with different residual feed as a measure of feed efficiency (Stanley *et al.*, 2016; Metzler-Zebeli *et al.*, 2019). They described that predominant families such as *Enterobacteriaceae* was more affected by the diet. The composition and function of these communities have been shown to vary with dietary components (Pan and Yu, 2014; Oakley BB, 2014; Kogut MH and Oakley BB, 2016). The digestive flora is directly dependent on the diet since the diet determines the type of substrate available for the growth of microorganisms.

Microbial flora analysis of cloaca differed according to the sex of the animals: higher populations were observed in females. Indeed, everyone has its own microbial community (Zhu *et al.,* 2002) and implies that host-specific factors are involved in the establishment of the gut flora.

As with regards to the relation between fed supplementation, growth and evolution of microbial flora, birds fed with diets supplanted at R_1 had the highest live weight gain. This showed that addition of 1% *C. odorata* to the chickens' diet had an impact on the digestive physiology of the subjects, increasing the digestibility of the feed by the microorganisms. The microorganisms compete with the host for use of feed in the digestive tract and are due to the fact that. They have a very large number of enzymes compared to their host, and those in the intestinal lumen can utilize certain food constituents. Feed that are not easily digestible by the host are the most affected. Thus, depending on the diet, the flora is involved in the digestion of food (Langhout *et al.,* 2000), (Maisonnier *et al.,* 2003). These microorganisms would have a positive effect by releasing nutrients that can be absorbed by the host in the cecum, which also have carbohydrate and amino acid transport capacities (Gabriel *et al.,* 2005). Also, cloacal bacteria can convert uric acid into ammonia, which is then absorbed by the bird and used to produce amino acids such as glutamine (Vispo and Karasov, 1997).

In addition, some of the nitrogen from the diet is incorporated into the cellular proteins of the bacteria and therefore the bacteria themselves can be a source of protein/amino acids (Metges, 2000).

Furthermore, this flora may have a protective effect against harmful microorganisms and be partly responsible for the development of the bird's intestinal immune system. Overall, the presence of harmful flora negatively affects their growth. It can also affect the quality of animal products (meat, eggs). (Shang *et al.,* 2018).

The addition of *Chromolaena odorata* to the birds' diet favored the growth cloacal microorganisms than the ration containing 1% *Chromolaena odorata* powder (R_1). This flora underwent fluctuations which would be related to the gender of the subjects and the incorporation time. The studied supplemented feeds affected not only the intestinal flora but also growth of birds and this was most pronounced at R_1 and in males.

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