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Research Article

CRUDE EXTRACTS OF FECAL BACTERIA ISOLATED FROM PHILIPPINE NATIVE CHICKEN (*GALLUS GALLUS DOMESTICUS*) SHOW *IN VITRO* ANTIMICROBIAL ACTIVITY AGAINST *ESCHERICHIA COLI*

F.B.R. Matias*, E.V.B. Bachoco, M.B.S. Salinas

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ABSTRACT: The study isolated and prepared crude extracts of fecal bacteria from four known strains of Philippine native chicken. It also evaluated the antimicrobial activity of the crude extracts against *E. coli* as indicator bacteria and compared to selected antimicrobials using modified disc diffusion method. Fecal suspensions were plated on nutrient agar and then incubated at 37 ºC for 24 to 48 h. Colonies were purified and two distinct isolates were characterized morphologically through Gram-staining. A loopful of bacteria was inoculated in 10ml nutrient broth and incubated at 37 ºC at different time intervals (6, 12, 18 and 24 h), then centrifuged and the cell-free supernatant (crude extract) was collected. Filter paper disc was impregnated with a total of 30 µl of crude extract and was used for antimicrobial activity testing along with three selected antimicrobial discs against *E. coli*. A total of 26 bacterial isolates was identified while two distinct colony morphologies were observed, and all isolates have rod shape appearance. The incubation period with the greatest number of isolates with zone of inhibition is 6 h, while the strain with the greatest number of isolates having zone of inhibition was *Joloanon*. All crude extracts showed significantly higher antimicrobial activity compared to these two antimicrobials except gentamicin.

Key words: Philippine native chicken, Fecal bacteria, Antimicrobial activity, *Escherichia coli*.

INTRODUCTION

The Philippine native chicken is a common fowl found in the backyards of most rural households. The Philippine native chicken is under the family Phasianae and the domestic chicken is simply called *Gallus gallus domesticus*. It is believed to be a diverse mixture of different breeds and descended from the domesticated red jungle fowl from Southeast Asia. Indigenous chickens are raised under the free-range system of management (Salces *et al*. 2013). Strains of Philippine native chickens are documented as those of *Banaba* from Batanggas, *Bolinao* from Pangasinan, *Camarines* from Bicol, *Darag* from Iloilo/Panay and the *Paraoakan* from Palawan (Argana 2001). Native chickens are well known for the distinctive taste of their meat (Lambio *et al.* 1997, Cocjin *et al*. 2001), adaptability to local agro-climatic conditions, hardiness, ability to utilize farm-by-products (Lopez 2008) and resistance to diseases. Moreover, they require minimal care, management and inputs.

The chicken microbiome consists of around 1,000 bacterial species, though the composition varies over time, between breeds and lines of birds, between flocks, individuals, and at different sites within the gut (Schokker *et al*. 2015). According to Chen *et al.* (2014), chicken litter contains a large diverse population of microorganisms which can reach up to 10^{10} CFU/g which include *Actinobacillus, Bordetella, Campylobacter, Clostridium, Corynebacterium, Escherichia coli (E. coli), Globicatella, Listeria, Mycobacterium, Salmonella, Staphylococcus*, and *Streptococcus* (Bolan *et al.* 2010). These microbes have properties of either pathogen or commensal depending on the bacterial pathotype, host immune status, diet, and coinfection (Wigley 2015).

College of Veterinary Science and Medicine, Central Luzon State University, Science City of Munoz 3120, Nueva Ecija, Philippines.
**Corresponding Author, e-mail: fbrmatias@clsucvsm.edu.ph*

Identified bacteria include anaerobic Gram-negative cocci, facultative anaerobic cocci, and streptococci. *Peptostreptococcus, Propionibacterium, Eubacterium, Bacteroides,* and *Clostridium* were the major genera (Pan and Yu 2014). Bacteriocins are generally defined as ribosomally synthesized peptides produced by bacteria that have bacteriostatic or bactericidal activity against other related and unrelated microorganisms (Cleveland *et al*. 2001). Bacteriocins are produced by all major lineages (more than 99%) of bacteria, most of which are not identified (Riley and Wertz 2002). Gram-negative bacteria produce a wide variety of bacteriocins, which are specifically named after the genus or species of the producing bacteria (Chavan and Riley 2007).

E. coli infection is one of the serious problems that cause a great threat to the profitability of birds' enterprises all over the world (Ahmad *et al*. 2009). They are common commensal organisms on the distal ileum and colon but presence of colonization factors on pathogenic strains causes diarrheal disease that has a great impact on animals' health (Cassels and Wolf 1995).

Majority of studies on native animals are focused on evaluating their origins or production performance compared to commercial or improved breeds. There is scarce information on why native animals thrive in well even with minimal human intervention, especially in their ability to resist or survive common diseases encountered by commercial breeds. In a previous study, crude extracts of fecal bacteria from Philippine native pigs shows antimicrobial activity to *E.coli* as indicator bacteria (Matias *et al*. 2019). This information generally provides an insight on how these animals may have adapt and survive on environment not suitable for commercial breeds. On the other hand, adaptability traits are also observed on the Philippine native chicken (*G. gallus domesticus*), unfortunately, there are no reported studies about their normal enteric bacteria, especially those with possible antimicrobial potential. Antibiotics are used not only in treating infections but also have been used as promotant and as prophylaxis in farm animals. Thus, discovering and implementing safer alternatives to antibiotics is an emerging concern not only in animal production but also in public health. In the current study, fecal bacteria from Philippine native chicken especially those with antimicrobial characteristics were evaluated.

MATERIALS AND METHODS

Fecal samples collection and bacterial isolation

The fecal samples were collected from a total of 12 chickens (three samples each from four known strains of Philippine native chicken). Freshly voided feces, about one gram, was mixed in a four ml sterile physiologic saline solution. About 100 µl fecal homogenized suspension were cultured on nutrient agar plates and then incubated at 37 ºC in aerobic condition for 24 to 48 h. Colonies producing clearly defined zone with different morphologies were collected and purified using the same medium. Gram staining was used to morphologically characterize the isolated bacteria. At least two distinct isolates were collected and used in the following experiment. Isolates were respectively labelled for proper identification. The letter indicates the strain (B-*Banaba*, D-*Darag*, J-*Joloanon*, and P-*Paraoakan*). The first digit indicates fecal sample while the second digit indicates the bacterial isolate number.

Crude extract production and disc preparation

A loopful of bacteria was inoculated in 10ml nutrient broth in conical tubes and incubated at 37 ºC at different time intervals (6, 12, 18, and 24 h). After each time interval, the broths were centrifuged at 6,000 rpm for 15 min and the cell-free supernatants (crude extract) were collected. The crude extracts were individually transferred to microcentrifuge tubes and stored at -20 ºC until further used. Filter paper discs (7mm diameter) with 20 µl crude extracts from each isolate were prepared by initially impregnating each disc with 10 µl crude extract then dried, followed by an additional 10 µl then dried again.

Antimicrobial activity test

A spread plate culture of the indicator bacteria (*E. coli*) from a stock culture kept at the Molecular Biology laboratory, College of Veterinary Science and Medicine, Central Luzon State University on Mueller-Hinton (MH) agar was prepared. The prepared filter paper discs with 20 µl crude extract were evenly placed on the plate. An additional 10µl crude extract was impregnated on each disc. Different antimicrobial discs: 10 µg ampicillin, 10 µg gentamicin and 2.5 µg trimethoprim/sulfamethoxazole (TMPS) were also placed on the agar plate. The plates were incubated at 37 º C for 18h, and then the zones of inhibition were measured using a Vernier caliper.

Statistical analysis

The data were presented as mean ± standard deviation (SD) of the triplicate and were analyzed using analysis of variance (ANOVA) followed by Turkey's highly significant difference (HSD). The level of significant difference was set at 95 % confidence interval at a p-value of < 0.05.

RESULTS AND DISCUSSION

A total of 12 fecal samples were collected from the four strains of Philippine native chicken (three samples from each strain). Fecal bacteria were isolated and purified. Isolated fecal bacteria were morphologically identified using Gram staining. A total of 26 bacterial isolates were identified. Antimicrobial activity of the samples was evaluated through modified disc diffusion method and then compared with selected antimicrobials.

Bacterial culture and isolation

Two distinct bacterial colony morphologies were observed from fecal samples collected from four strains of Philippine native chicken. First, large thick colonies, grayish in color and with smooth texture were observed in isolate D21, D31 and P31. Second, flat colonies with slightly convex surface and with irregular edges were observed in the rest of the isolates.

Bacterial isolates from *Banaba* strain namely: B11, B12, B21, B22, B31 and B32 were all Gram-positive and appeared to have a rod shape morphology (table 1). Bacterial isolates from *Darag* strain namely: D11, D12, D22, D23, D32 and D33 were all Gram-positive while D21 and D31 were Gram-negative. All isolates appeared to have a rod shape morphology. Bacterial isolates from *Joloanon* strain namely: J11, J12, J21, J22, J31 and J32 were all Gram-positive and appeared to have a rod shape morphology. Lastly, bacterial isolates from *Paraoakan* strain namely P11, P12, P21, P22, and P32 are all Gram-positive, except P31, but all appeared to have a rod shape morphology.

After colony differentiation, bacterial purification and Gram staining, the different isolates were identified and correspondingly labeled thereafter. Two colonies were identified for most of the fecal samples except the fecal sample 2 and 3 of *Darag* strain which have three bacterial isolates each.

Antimicrobial Activity

Highest zone of inhibition was observed in isolate P11, which was statistically significant among 6h crude extracts, except isolates B11, B21, B22, B31, P12, P21, P22 and P32 (table 2). The strain of Philippine native chicken that showed the greatest number of isolates with zones of inhibition were *Paraoakan* and *Banaba* followed by *Joloanon*, with *Darag* having the least number of isolates. Among the 12h crude extracts, the highest zone of inhibition was observed in isolate P12, which was statistically significant to most of the isolates, except isolates P11, P31, B11, B21, and B22. The strain of Philippine native chicken that showed the greatest number of isolates with zone of inhibition was *Paraoakan* followed by *Banaba* then *Joloanon*, with *Darag* having the least number of isolates. While among the 18h crude extracts, the highest zone of inhibition was observed in isolate P12, which was statistically significant to all isolates. The strain of Philippine native chicken that showed the greatest number of isolates with zone of inhibition was *Joloanon* followed by *Paraoakan* and *Banaba*, with *Darag* having the least number of isolates. Lastly, among the 24 h crude extracts, the highest zone of inhibition was observed in isolate P21, which was statistically insignificant to all isolates. The strain of Philippine native chicken that showed the greatest number of isolates with zone of inhibition was *Joloanon* followed by *Banaba* then *Paraoakan*, with *Darag* having the least number of isolates.

Zone of inhibition indicates antimicrobial activity of crude extract in disc diffusion method. According to Arfani *et al.* (2017), the size of the zone of inhibition may indicate the potency or effectivity of interest. The crude extracts of fecal bacteria produced at different time interval resulted to zone of inhibitions on *E. coli* as indicator bacteria. Some bacteria can produce substances with antimicrobial activity that can inhibit the growth of other bacteria. Sure *et al*. (2016) stated that this competitive inhibition arises from the need for survival in over populated environment or culture. Bacteriocin is the general term that refers to the protein produced by bacteria with antimicrobial activity. It is usually produced 18h after the bacteria experienced stressful conditions. The optimum time required in the production of bacteriocin was reported by Arfani *et al.* (2017) where the initial 6h of incubation their bacterial isolates are at log growth phase and produced the highest number of zones of inhibition. At 18h, the bacterial isolates reach its stationary phase, and this hour showed the second highest number of zones of inhibition. Similar observations were reported in a previous study using fecal bacteria from Philippine native pigs (Matias *et al.* 2019).

According to Elayaraja *et al*. (2014), regarding bacteriocin production, cell growth started from late log phase and the maximum production was obtained in early stationary growth phase. Growth beyond stationary phase resulted decrease bacteriocin production, especially at 24h of incubation. The bacteriocin-like material is reduced due to protease produced by bacteria when entering death phase. As bacteriocin is peptide in nature, various studies reported that protease has ability to degrade bacteriocins (Sharma *et al.* 2011). Some of the indicator organisms tested viz. *Bacillus cereus, B. megaterium, subtilis, E. coli, S. aureus, P. aeruginosa*

Table 1. Colony and Gram-stain characteristics of fecal bacteria isolated from four strains of Philippine native chicken on nutrient agar.

Isolates		Colony Characteristic	Gram Stain	Morphology
Banaba				
Sample 1				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 2				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 3				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Darag				
Sample 1				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 2				
	1	Greyish color, and moist and smooth surface	Negative	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
	3	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 3				
	1	Greyish color, and moist and smooth surface	Negative	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
	3	Flat, slightly convex surface with irregular edges	Positive	Rod
Joalanon				
Sample 1				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 2				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 3				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Paraoakan				
Sample 1				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 2				
	1	Flat, slightly convex surface with irregular edges	Positive	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod
Sample 3				
	1	Greyish color, and moist and smooth surface	Negative	Rod
	2	Flat, slightly convex surface with irregular edges	Positive	Rod

Table 2. Zone of inhibition produced by the crude extracts of fecal bacteria isolated from Philippine native chicken on *E. coli* as indicator bacteria.

	Isolates	Zone of Inhibition (mm) of Crude Extract			
		6th	12th	18th	24th
Banaba					
	B11	9.77±2.42^{ab}	9.57±1.62^{ab}	7.10±0.17^{a}	7.03±0.06^{a}
	B12	7.10±0.17^{a}	7.03±0.06^{a}	-	-
	B21	9.23±0.45^{ab}	8.97±0.81^{ab}	9.87±0.65^{b}	7.97±1.67^{a}
	B22	10.00±0.17^{ab}	9.07±0.76^{ab}	9.47±0.60^{ab}	7.97±0.49^{a}
	B31	7.87±1.42^{ab}	-	7.87±1.50^{ab}	7.97±1.12^{a}
	B32	7.63±1.10^{a}	-	-	7.63±1.01^{a}
Darag					
	D11	7.43±075^{a}	7.60±1.04^{a}	7.53±0.29^{ab}	7.60±0.79^{a}
	D12	7.10±0.17^{a}	-	7.40±0.69^{ab}	-
	D21	7.60±1.04^{a}	-	7.03±0.06^{a}	7.10±0.17^{a}
	D22	7.07±0.12^{a}	7.30±0.52^{a}	7.23±0.40^{ab}	-
	D23	-	-	-	-
	D31	7.17±0.29^{a}	-	-	-
	D32	7.43±0.75^{ab}	7.23±0.40^{a}	-	-
	D33	-	-	-	-
Joloanon					
	J11	7.10±0.17^{a}	7.87±0.78^{a}	9.53±0.64^{ab}	8.27±0.32^{a}
	J12	7.07±0.12^{a}	-	9.20±1.15^{ab}	9.13±0.72^{a}
	J21	7.63±1.10^{a}	8.03±0.90^{a}	7.83±1.44^{ab}	7.40±0.36^{a}
	J22	7.47±0.57^{a}	7.10±0.17^{a}	8.87±1.23^{ab}	7.50±0.87^{a}
	J31	7.37±0.64^{a}	8.17±1.46^{ab}	8.50±1.30^{ab}	8.40±0.69^{a}
	J32	-	-	7.93±1.62^{ab}	7.63±1.10^{a}
Paroakan					
	P11	11.08±2.97^{b}	8.20±1.10^{ab}	7.60±1.04^{ab}	7.43±0.75^{a}
	P12	8.80±1.35^{ab}	10.78±2.36^{b}	16.19±0.43^{c}	9.20±0.52^{a}
	P21	9.27±1.30^{ab}	7.73±1.27^{a}	-	9.27±1.19^{a}
	P22	7.92±0.79^{ab}	7.03±0.06^{a}	-	-
	P31	7.50±0.70^{a}	7.17±0.21^{a}	7.63±1.01^{ab}	7.73±1.27^{a}
	P32	8.43±0.81^{ab}	7.50±0.62^{a}	8.60±1.90^{ab}	-

[Legend: The letter indicates the strain (B- *Banaba*, D- *Darag*, J- *Joloanon*, and P- *Paraoakan*). The first digit indicates fecal sample while the second digit indicates the bacterial isolate number. The measurements of the zone of inhibition were presented as the mean of the triplicate ± standard deviation (SD). The values followed by different superscript letters (^{a,b}), in a column are significantly different from each other at $p< 0.05$. "–" sign indicates no zone of inhibition observed.]

and *Alkaligens feacalis* produced potent protease (Anwar and Saleemuddin 1998). Proteases could break down the bacteriocin as per their specificity towards respective bacteriocin. In the present study, different zones of inhibition observed in the crude extract produced at different time interval may indicate their various

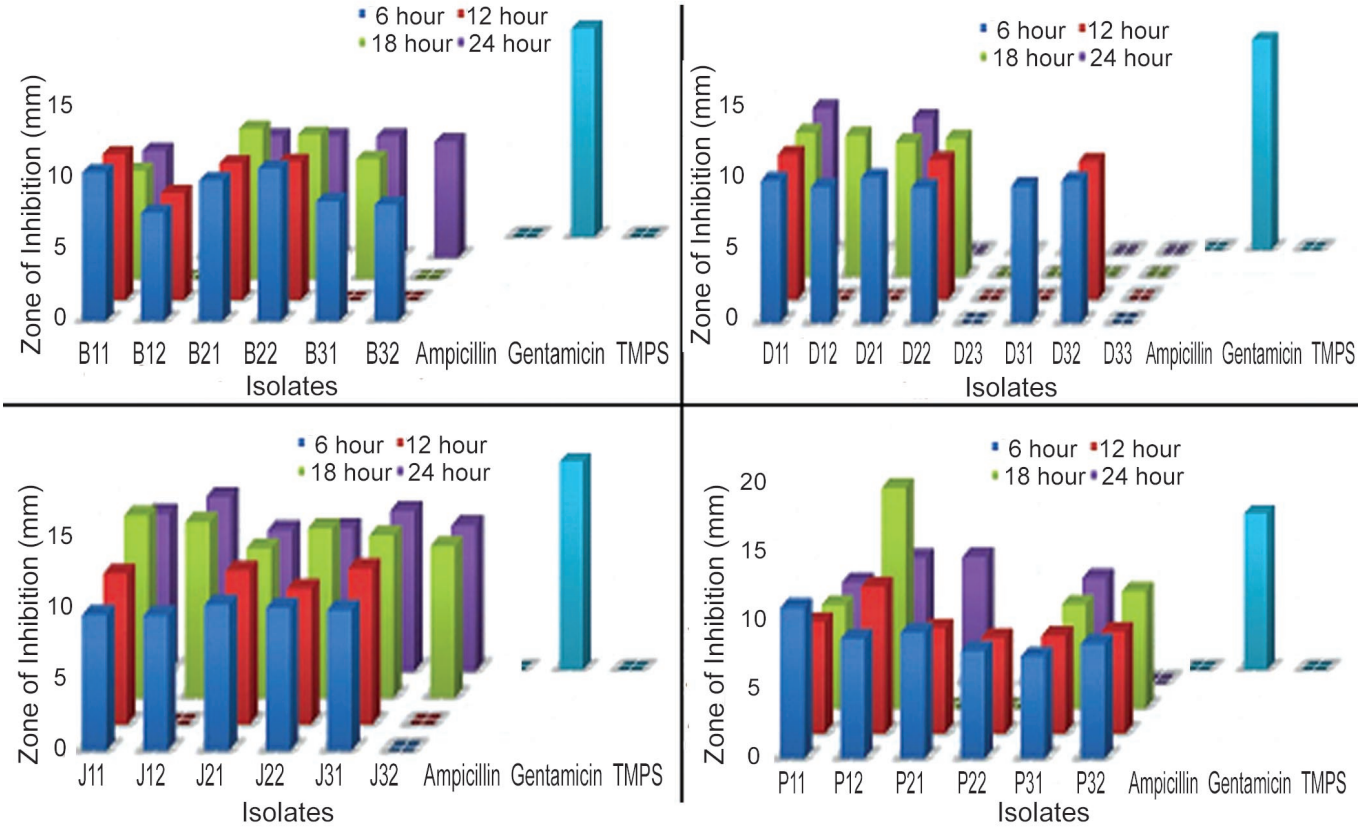

Fig. 1. Zone of inhibition of crude extracts of fecal bacteria isolated from (A) Banaba strain, (B) Darag strain, (C) Joloanon strain, and (D) Paraoakan strain compared to selected antimicrobial agents against *E. coli* as indicator bacteria.

antimicrobial activities. Isolates with no observed antimicrobial activity in earlier time interval but showed antimicrobial activity on later time interval signifies the period when the antimicrobial substances are produced. Also, it can be noted that some crude extracts showed antimicrobial activity in earlier time interval but none in later time. Thus, this may be associated with the production of protease by the isolates that may have degraded the antimicrobial substances produced earlier.

Figure 1 shows the comparison of the antimicrobial activity of the crude extracts of fecal bacteria isolated from four different strains of Philippine native chicken. The *E. coli* used in the study showed resistance to ampicillin and TMPS as indicated by the absence of zone of inhibition. Consequently, all crude extracts that showed antimicrobial activity are statistically significant than these two antimicrobials. On the other hand, gentamicin showed an antimicrobial activity against *E. coli*, which was statistically higher than all the crude extract.

During the last decade, an alarming worldwide increased in the incidence of community acquired infections with pathogens resistant to multiple antibiotics of common use has been observed (Calva *et al*. 1996). *E. coli* antimicrobial resistance has been reported worldwide (Sabate *et al*. 2008; Yusha'u et al. 2010). *E. coli* was reported to be resistant to almost all antimicrobial agent except cephalothin, chloramphenicol and trimethoprim-sulfamethoxazole (Sayah *et al.* 2005). Rasheed *et al.* (2014) reported that *E. coli* has the highest resistance to ampicillin and has the least resistance to gentamicin among the 19 antibiotics used in their study. The current study showed that *E. coli* was resistant to ampicillin and TMPS. As with other antibiotics, TMPS must be given in a sufficient dose at a proper frequency to produce adequate concentrations at the site of infection for successful eradication of the pathogen (Brown 2014). TMPS concentration used in the study was very low thus resulting to absence of zone of inhibition. The crude extract of fecal bacteria from Philippine native chickens showed significantly larger zone of inhibition than ampicillin and TMPS while significantly smaller zone of inhibition than gentamicin.

CONCLUSION

Fecal bacteria isolated from Philippine native chicken showed antimicrobial activity against *E. coli* as manifested by the different zones of inhibitions. This activity provides an insight on one of the factors that allow native chickens to adapt and survive environment even with no or minimal human intervention.

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