Explor Anim Med Res,
Vol.9, Issue - 2, 2019, p. 168-173

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

IN VITRO ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACT OF FECAL BACTERIA FROM PHILIPPINE NATIVE PIG (*SUS SCROFA*) AGAINST *ESCHERICHIA COLI*

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Received 06 August 2019, revised 01 November 2019

ABSTRACT: The study assessed the in vitro antimicrobial activity of crude extract of fecal bacteria isolated from Philippine native pig (*Sus scrofa*) against *Escherichia coli* and compared with three commonly used antimicrobials namely, ampicillin (10 µg), gentamicin (10 µg) and trimethoprim-sulfamethoxazole (TMPS) (25 µg) using modified disc diffusion method. After Gram staining, 23 out of the 25 bacterial isolates were observed to be Gram positive rods. Using different incubation period, the 18h incubation period showed the highest number of zones of inhibition against *E. coli*. While among samples, the isolates collected from the Philippine native pigs raised in San Miguel, Bulacan and Doña Remedios Trinidad, Bulacan showed the highest number of zones of inhibition. The antimicrobial activity of all the crude extracts were significantly higher than ampicillin while significantly lower than gentamicin and TMPS against *E. coli*. This activity may induce the characteristics resistance of Philippine native pig to different conditions, specifically on gastro-intestinal associated bacterial pathogen.

Key words: Fecal bacteria, Philippine native pig, *Sus scrofa*, *Escherichia coli*.

INTRODUCTION

The swine genetic groups present in the Philippines are classified into exotic standard purebreds, synthetic hybrids, Philippine wild pigs, and Philippine native pigs and its upgrades or crosses. The Philippine native pig is characterized by being black or black with a white belly (Oh *et al.* 2014). Indigenous swine breeds are in general much hardy and resistant to the vectors and diseases that are associated with the region they come from, implying that they might have a smaller need for disease intervention than commercial breeds. Much more research is however needed to understand the underlying physiological and genetic mechanisms responsible for native breeds' greater resistance and tolerance to certain diseases (Towers 2016).

Gut microbiota is a significant contributor to the digestion of food substrates that influences overall physiological growth, immunologic responses, and pathogenesis in the host (Dowd *et al.* 2008). Recent studies show that the commensal microbiota can prevent or even cure infection that are caused by pathogenic bacteria through inhibition (Buffie and Pamer 2013). Animals harbor gut microbial communities whose composition and relative proportions of dominant microbial groups vary among species (Pajarillo *et al.* 2015). In the study of Richards *et al.* (2005), the swine fecal microbiota profiles were only limited to microorganisms that can grow and multiply in culture media under laboratory conditions. According to Pajarillo *et al.* (2015), Firmicutes and Bacteroidetes predominated at the phylum level, while Clostridia and Bacteroidia were most abundant at the class level on the bacterial composition of Duroc pigs. Almost all groups of bacteria can inhibit the growth of the other bacteria through the

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production of ribosomally-synthesized cationic peptides known as bacteriocin (Kaur and Kaur 2015). Bacteriocins are protein molecules with antimicrobial properties that can help the producing microorganism to outcompete with other species of microorganism (Alvarez-Sieiro *et al*. 2016). Bacteriocins are produced by all major lineages (more than 99%) of bacteria, most of which are not identified (Riley and Wertz 2002). The production of bacteriocins is often regarded as an important trait not only in the context of bacterial fitness but also in terms of probiotic efficacy and traditional. The production of antimicrobial peptides has been an important criterion in the selection of probiotic strain, albeit that few studies have definitively demonstrated the impact of bacteriocin production on the ability of a strain to compete within the GI tract and/or positively influence the health of the host (Dobson *et al.* 2012).

Escherichia coli is a fast-growing bacterium discovered as early as 1884 (Blount 2015). They are common commensal organisms on the distal ileum and colon but the presence of colonization factors on pathogenic strains can cause diarrheal disease that has a great impact on animals' health (Cassels and Wolf 1995). This bacterium creates a significant problem in the pig industry due to decreased weight gain or even death (Zhao *et al*. 2012). In addition, this bacterium is a common pathogen responsible for both intra-intestinal and extra-intestinal infections in both man and animals (Tenaillon *et al*. 2010). *E. coli* causes diarrheal diseases which are of economic concern particularly in piglets and weaners due to mortality, treatment costs, weight loss, and growth retardation in survivors (Ikwap *et al*. 2016).

With the emergence of antimicrobial resistance among pathogens, especially bacteria, due to the continuous use of antibiotics as growth promotant and prophylaxis against common diseases, regulated use of antimicrobials and finding safer alternatives should be implemented in livestock raising. This study provided information on fecal bacteria with antimicrobial activity from Philippine native pigs that can be a basis in swine production. Specifically, this study evaluated the *in vitro* antimicrobial activity of crude extract of fecal bacteria isolated from Philippine native pig (*S. scrofa*) against *E. coli.*

MATERIALS AND METHODS

Collection of samples

Fecal samples were collected from Philippine native pigs from four different sites (three fecal samples per pig). About one gram of freshly voided feces was diluted with 4 ml sterile physiological saline solution and was thoroughly homogenized.

Bacterial culture and isolation

About 100 µl of fecal suspension was plated on the nutrient agar and then incubated at 37ºC in aerobic condition for 24 to 48 hours. Colonies producing distinct colonies and 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 succeeding experiment. Isolates were respectively labeled for proper identification. The letter indicates the site of collection (A – Science City of Muñoz, Nueva Ecija, B – Science City of Muñoz, Nueva Ecija, C – San Miguel, Bulacan, D – Doña Remedios Trinidad, Bulacan). The first digit indicates fecal sample number while the second digit indicates the bacterial isolate number.

Crude extract production and disc preparation

A loopful of bacteria was inoculated in 10 ml nutrient broth and incubated at 37ºC at different incubation period (6, 12, 18, and 24 h). After incubation, the broth was centrifuged at 6,000 rpm for 15 min and the cell-free supernatant (crude extract) was collected. The crude extracts were transferred to microcentrifuge tubes and stored at -20ºC until further used. Filter paper discs (7mm diameter, Whatman No. 3) were prepared by impregnating the discs with a total volume of 30 µl crude extract.

Antimicrobial activity testing

A spread plate culture of *E. coli* from a stock culture from the Molecular Biology Laboratory, College of Veterinary Science and Medicine, Central Luzon State University on Mueller Hinton agar was prepared. The prepared discs with crude extract were evenly placed on the plate. Selected antimicrobial discs (10 µg ampicillin, 10 µg gentamicin, and 25 µg TMPS) were also placed on the plate. The plates were incubated at 37 ºC for 18-24 h. The zone of inhibition was measured using a vernier caliper.

Statistical analyses

The data on antimicrobial activity were presented as mean ± standard deviation (SD) of the triplicate and were analyzed using analysis of variance (ANOVA) followed by Tukey'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

Bacterial culture and isolation

Isolates A11, B21, B22, C23, C32, D31, and D32 showed large thick colonies with grayish color, moist and

Table 1. Gram-stain and colony characteristics of fecal bacteria isolated from Philippine native pigs from four different collection sites on nutrient agar.

Pig		Isolates	Colony characteristics	Gram stain	Shape
Pig A	Sample 1	1	Grayish color, moist and smooth surface	Positive	Cocci
		2	Flat, slightly convex surface with irregular edges	Positive	Cocci
	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
Pig B	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	Grayish color, moist and smooth surface	Positive	Rod
		2	Grayish color, moist and smooth surface	Positive	Rod
	Sample 3	1	Flat, slightly convex surface with irregular edges	Positive	Rod
		2	Flat, slightly convex surface with irregular edges	Positive	Rod
Pig C	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
		3	Grayish color, moist and smooth surface	Positive	Rod
	Sample 3	1	Flat, slightly convex surface with irregular edges	Positive	Rod
		2	Grayish color, moist and smooth surface	Positive	Rod
Pig D	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	Grayish color, moist and smooth surface	Positive	Rod
		2	Grayish color, moist and smooth surface	Positive	Rod

[Legend: Philippine native pig from (A) Science City of Muñoz, Nueva Ecija, (B) Science City of Muñoz, Nueva Ecija, (C) from San Miguel, Bulacan, and (D) Doña Remedios Trinidad, Bulacan].

smooth surface while the rest of the isolates showed flat colonies that have a slightly convex surface with irregular.

Bacterial isolates from Philippine native pig – A from Science City of Muñoz, Nueva Ecija namely: A21, A22, A31, and A32 were all rods while A11 and A12 were cocci. All isolates were Gram-positive. Bacterial isolates from Philippine native pig – B also from Science City of Muñoz, Nueva Ecija namely: B11, B12, B21, B22, B31 and B32 were all Gram-positive and appeared to have rod shape morphologies. Bacterial isolates from Philippine native pig – C from San Miguel, Bulacan namely: C11, C12, C21, C22, C23, C31, and C32 were all Gram-positive and appeared to have a rod-shape morphology. Lastly, the bacterial isolates from Philippine native pig – D from Doña Remedios Trinidad, Bulacan namely: D11, D12, D21, D22, D31 and D32 were all Gram-positive and appeared to have rod shape morphologies (Table 1).

After colony differentiation, bacterial purification and Gram staining, different isolates were identified and correspondingly named thereafter. Two colonies were identified for most of the samples except Philippine native pig – C from where the fecal sample 2 has three isolates.

Antimicrobial activity

Table 2 shows the zone of inhibition produced by the crude extracts of fecal bacteria isolated from Philippine native pigs on *E. coli*. According to Guzeldag *et al.* (2014), antimicrobial activity from discs containing extracts can be quantitatively evaluated by measuring the zone of

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

Isolates		Zone of inhibition (mm)			
		6h	12h	18h	24h
Pig A					
	A11	8.00±1.39[a]	7.27±0.12[a]	7.50±0.53[a]	-
	A12	-	7.53±0.35[a]	-	-
	A21	8.10±1.10[a]	-	7.10±0.09[a]	7.07±0.03[a]
	A22	8.97±1.86[a]	-	7.07±0.12[a]	7.08±0.03[a]
	A31	-	-	-	7.02±0.03[a]
	A32	-	7.70±1.21[a]	-	-
Pig B					
	B11	-	-	-	-
	B12	-	7.27±0.06[a]	7.28±0.45[a]	7.57±0.50[a]
	B21	-	-	7.08±0.03[a]	7.07±0.03[a]
	B22	-	-	7.07±0.03[a]	7.03±0.06[a]
	B31	-	7.63±0.49[a]	7.12±0.03[a]	-
	B32	-	-		-
Pig C					
	C11	-	7.27±0.46[a]	7.13±0.08[a]	7.73±0.46[a]
	C12	-	7.17±0.29[a]	7.05±0.05[a]	7.03±0.03[a]
	C21	7.20±0.35[a]	7.53±0.46[a]	9.90±3.57[ab]	7.08±0.03[a]
	C22	8.73±1.10[a]	8.07±1.44[a]	12.67±2.02[b]	12.73±3.10[b]
	C23	7.10±0.10[a]	7.13±0.06[a]	7.52±0.48[a]	-
	C31	8.03±0.64[a]	7.53±0.47[a]	10.47±3.51[ab]	7.93±0.64[a]
	C32	7.93±0.68[a]	7.70±0.17[a]	8.38±1.50[a]	8.43±1.24[a]
Pig D					
	D11	7.57±0.49[a]	7.33±0.12[a]	7.97±1.03[a]	8.33±1.15[a]
	D12	-	7.07±0.12[a]	7.40±0.61[a]	7.05±0.00[a]
	D21	-	7.23±0.25[a]	8.03±1.70[a]	8.23±0.49[a]
	D22	7.17±0.29[a]	7.43±0.23[a]	8.35±1.48[a]	9.27±1.27[a]
	D31	-	7.17±0.15[a]	7.12±0.08[a]	7.22±0.14[a]
	D32	7.13±0.12[a]	7.13±0.23[a]	7.40±0.40[a]	7.60±0.52[a]

[Legend: Philippine native pig from (A) Science City of Muñoz, Nueva Ecija, (B) Science City of Muñoz, Nueva Ecija, (C) from San Miguel, Bulacan, and (D) Doña Remedios Trinidad, Bulacan. The data were presented as the mean ± SD of the triplicated. The value followed by the different superscript letters in a column are significantly different with each other at $p<0.05$. —sign indicates no zone of inhibition observed].

inhibition produced. The relative size of the zone of inhibition may also indicate the potency or effectivity with larger zones suggesting greater susceptibility of the bacteria to a particular extract (Brown and Kothari 1975). The zone of inhibitions observed in the different crude extracts of the fecal bacterial isolated from Philippine native pigs can provide evidences of their antimicrobial activity.

Dobson *et al.* (2012) stated that some bacteria can produce peptides with antimicrobial activity to inhibit the growth of other bacteria. Competitive inhibition or exclusion allows the bacteria to proliferate and reduce competition for nutrients (Hibbing *et al*. 2010).

Bacteriocin is the general term that refers to the protein produced by bacteria with antimicrobial activity (Yang *et al*. 2012). This substance is usually produced by bacteria when there is a presence of other bacteria that may act as a stress signal (Gutierrez-Cortes *et al*. 2018).

According to Arfani *et al.* (2017), bacteriocin production peak towards the end of log phase and early stationary phase, however, they can also produce proteases that have the ability to degrade this microbial peptide. Almost all bacteria that produce bacteriocin also produce proteases (Sure *et al.* 2016).

The different zone of inhibitions observed on the crude extracts may indicate their various antimicrobial activity. Isolates with no observed antimicrobial activity in the earlier time interval but with antimicrobial activity on later time interval signifies the period when the antimicrobial substances were produced. In addition, it can be noted that some crude extracts showed antimicrobial activity in the earlier time interval but none in the later time interval. This may be associated with the production of proteases by the isolates to degrade the antimicrobial substances produced earlier.

In the 6 h crude extracts, the highest zone of inhibition was observed on isolate A22, which, however, was statistically insignificant to all isolates. Pig C produced the highest number of crude extracts with zone of inhibition followed by Pig A and Pig D, with Pig B having the least number of isolates with zone of inhibition. In the 12 h crude extracts, the highest zone of inhibition was observed on isolate C22, which however, was statistically insignificant to all isolates. Pig C and Pig D produced the highest number of crude extracts from isolated bacteria with zone of inhibition followed by Pig A, with Pig B having the least number of isolates with zone of inhibition. In the 18 h crude extracts, the highest zone of inhibition was observed on isolate C22 which was statistically significant to all isolates except isolates C21 and C31. Pig C and Pig D produced the highest number of crude extracts from isolated bacteria with zone of inhibition followed by Pig A, with Pig B having the least number of isolates with zone of inhibition. Lastly, in the 24 crude extracts, was observed on isolate C22 which was statistically significant to all isolates. Pig D produced the highest number of crude extracts from isolated bacteria with zone of inhibition followed by Pig A and Pig C, with Pig B having the least number of isolates with zone of inhibition.

Crude extracts vs selected antimicrobials

It was observed that the crude extracts of isolated fecal bacteria from Pig A, Pig B, Pig C and Pig D were significantly lower than gentamicin and TMPS but significantly higher than ampicillin, except isolates C22 and C31 at 18 h of incubation and C22 at 24 h of incubation which have comparable zone of inhibition to gentamicin.

Antimicrobial resistance in *E. coli* has been reported worldwide and treatment for infection associated with these bacteria has been increasingly complicated due to the emergence of resistance to most first-line antimicrobial agents (Sabate *et al.* 2008; Yusha'u *et al.* 2010). Rasheed *et al.* (2014) found out that *E. coli* has the highest resistance to ampicillin and has the least resistance to gentamicin among the 19 antibiotics used in their study. Sayah *et al.* (2005) demonstrated the susceptibility of *E. coli* to trimethoprim-sulfamethoxazole.

CONCLUSION

Crude extracts of fecal bacteria isolated from Philippine native pig showed antimicrobial activity as exemplified by their various zones of inhibition. Some isolates showed antimicrobial activities even better than some antimicrobials. This activity may induce the characteristic resistance of Philippine native pig to different conditions.

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***Cite this article as:** Matias FBR, De Sagun KNG, Salinas MBS (2019) *In vitro* antimicrobial activity of crude extract of fecal bacteria from Philippine native pig (*Sus scrofa*) against *Escherichia coli.* Explor Anim Med Res 9(2): 168-173.