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EVALUATION OF AMPICILLIN, VANCOMYCIN, AND GENTAMYCIN ANTIBIOTIC RESISTANT AMONG LACTOBACILLUS ISOLATES

Rozila Alias[1,2*], Thivyah Karunageran[2], Maimunah Mustakim[4], Siti Nurfatehah Kamal[2,3], Siti Hasmah Mohtar[3], Siti Rahayu Hassan[1], Murni Salina[1], Siti Amalina Ahmad Khairuddin[1], Hasliza Muhammad Ali[1], Norazah Mohammad Nawawi[1,2], Mohd Nizam Lani[5] and Abdul Latif Ibrahim[2]

[1]*Centre for Foundation and General Studies, Universiti Selangor, Bestari Jaya Campus, Kuala Selangor, Malaysia*
[2]*Institute of Bio-IT Selangor, Universiti Selangor, Shah Alam Campus, Selangor, Malaysia*
[3]*Faculty of Engineering and Life Sciences, Department of Biotechnology, Universiti Selangor, Bestari Jaya Campus, Kuala Selangor, Malaysia*
[4]*Faculty of Health Sciences, Universiti Teknologi MARA (UiTM), Selangor Branch, Puncak Alam Campus, Selangor, Malaysia*
[5]*Faculty of Fishery and Food Science, Universiti Malaysia Terengganu (UMT), 21030 Kuala Nerus, Terengganu, Malayisa*

[*]*Corresponding author: rozila@unisel.edu.my*

Abstract

Antibiotic resistance is an ever-increasing in worldwide problem nowadays. It is happened when the extensive use of antibiotics which will creates the selective pressure resulting from mutation of normal genes and spreading of a variety of an antimicrobial resistance. Uses of *Lactobacillus* as probiotics or in starter cultures may serve as hosts of antibiotic resistance genes, which could be transfer or resistance to multiple antibiotics. Therefore, it is important to screen the susceptible or resistance towards antibiotics which will not transferable resistance genes. In the recent study, a total of 37 strains of *Lactobacillus* species isolated from various milk and dairy products such as goat's milk, cattle's milk, human's milk, homemade yogurt and commercial yogurt were examined for the antibiotic profile using Kirby-Bauer method. The results of this study show that human's milk has a high number of *Lactobacillus* isolates resistant to ampicillin (75%), vancomycin (62.5%) and gentamycin (62.5%). Whereas *Lactobacillus* isolated from cattle's milk have resistant to ampicillin (42.1%), vancomycin (42.1%) and susceptible to gentamycin (0%). But however, goat's milk still has lower percentage number of resistant to ampicillin (28.0%), vancomycin (14.3%) and are susceptible to gentamycin (0%). These results indicate that ampicillin and vancomycin resistant seems to be very common among *Lactobacillus* isolates but gentamycin are still susceptible used. Vancomycin resistant are more concerned because of the emerging problem in hospital and often described as one of the last resorts against the infection caused by multidrug-resistant pathogens. Therefore, lactobacilli as probiotics would be more monitoring when applied in food industry and clinical especially for immune-compromised patients or during anti-biotherapy. Acquisition and retransfer of resistance genes should be addressed in the new safety aspects of probiotics uses.

Keywords: Lactobacillus, vancomycin, ampicillin, gentamycin, antibiotic resistance

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Introduction

Antibiotics are a lifesaving technology widely used in human and veterinary medicine to prevent, control and treat diseases. However, overprescribing and misuse of antibiotics in medicine, animal feed, aquaculture, and agriculture have led to the emerging and spreading of antibiotic resistant bacteria, which constitutes a serious problem for the health of both humans and animals. The use of antibiotic drugs would create a selective evolutionary pressure to the bacteria and genes resistant to that antibiotics. It's could be able to resist to one or more antibiotics. When resistance has developed to one antibiotic in a class, it's also would be resistance to some or other antibiotics within the same class and across different antibiotic classes. Antimicrobial resistance has now become an important global human health concern, with widespread public and private initiatives aimed at managing resistance (John et al., 2019). Livestock agriculture is a major consumer of antibiotics and contributes to antibiotic resistance in ASEAN, US, EU and other countries

(Lucy et al., 2019; Mami et al., 2019; Stacy et al., 2019). Lactobacilli are important microorganisms as being consumed daily and it is a well-preserved starter culture strains by industrial producers where less investigate on dairy products whether the starter culture strains harbor resistance gene(s) or not. There is a reported that antibiotic residues found in milk (Sabbya et al., 2019). There is a worried that Lactobacillus seems to be resistant to antibiotics and it is important characteristic if *Lactobacillus* consumed as probiotic. Therefore, an investigation for the three common antibiotics tested were screened among *Lactobacillus spp.* locally isolates from milks in order to determine antibiotic profile.

Methods

Collection of samples

Goat milks, cattle milk, homemade yogurt, commercial yogurt and human milk samples were randomly sampling from local farms, markets at Shah Alam and Klang, and 4 volunteer mothers respectively. The samples were collected and kept in ice box until brought to Food Safety and Biosecurity Research Laboratory, Institute of Bio-IT Selangor, Universiti Selangor, Shah Alam, Selangor, Malaysia.

Isolation of *Lactobacillus*

Approximately 1.0 g or 1.0 mL of samples were weighted and done the serial dilution with 9.0 mL sterile MRS broth. 100 mL of suspension was mixed with 20 mL of warm MRS agar medium before pour into a sterile petri dish plate. The plates were incubated for 48 h at 37°C in anaerobic condition. The characteristic of lactic acid bacteria colonies was grown and randomly picked up and streaked on the MRS agar medium for further to obtain a pure culture. Colonies were further identified its morphology through gram stained. A few colonies of pure culture were transferred to MRS agar slants (working culture) and stock bead culture (Protect Microorganism Preservation System) for a long-term storage at -80^{o}C for further study.

Presumptive of *Lactobacillus*

The isolates were subjected to oxidase and catalase biochemical test and morphological studies such as colony morphology, gram staining in order to presumptive the *Lactobacillus* isolates.

Screening for Antibiotic Profile

Antibiotic profile category as susceptible, intermediate and resistant was tested by Kirby-Bauer method according to the CLSI (National Committee for Clinical Laboratory Standards, NCCLS). All isolates were tested for Vancomycin 30ug (V30), Ampicillin 10ug (AM10) and Gentamycin 10ug (G10), (Oxoid). Inoculate were grown in MRS broth (Oxoid, UK) for overnight at 37^{o}C. Then, the suspension was adjusted the cell density to a standard 0.5 McFarland with 10^{8} CFU/mL before swabbed onto the MRS agar plate. The antibiotics disc was placed onto the MRS agar and incubated at 37^{o}C in anaerobic condition. Inhibition of clear zone was measured in milimeter (mm) after 24 hours.

Result and Discussion

A total of 37 strains of *Lactobacillus* were isolated from raw milks and yogurts (goat, cattle, human, homemade yogurt and commercial yogurt). The isolates were presumptive as *Lactobacillus* based on morphology rod shape and purple colour with the magnification 100x view under the compound microscope. Oxidase test assays was done to screen the presence of enzyme cytochrome oxidase or also known as indophenol oxidase. If the isolates contained the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized colored product, purple color. If this enzyme does not present in the isolates, it will react negative result, no color changes. Then the catalase test also was done, if it shows negative results, it indicates that there is no emergence of bubbles and vice versa. Catalase helps to fasten breakdown of hydrogen peroxide (H_2O_2) into water and oxygen and cause the emergence of bubbles. All the 37 isolates show negative result for catalase and oxidase test. *Lactobacillus* were known as lack of catalase. Then, the isolates were proceeded to antibiotic test and the zone of inhibition is interpreted followed the criteria from the Clinical and Laboratory Standard Institute (CLSI) in Table 1. All the *Lactobacillus* isolates were analyzed the antibiotic profile data as presented as in Table 2 and represented in three categories as resistance (R), intermediate (I) and susceptible (S).

Tables 1: Zone Interpreted Standard diameter according to CLSI, NCLSS

	Resistant (R)	Intermediate (I)	Susceptible (S)
Ampicillin (10ug)	<13	14-16	>17
Vancomycin (30ug)	< 9	10-11	>12
Gentamycin (10ug)	<12	13-14	>15

Table 2: Antibiotics profile of *Lactobacillus* isolates

No.	Code ID	Source	Inhibition Zone (mm)		
			AM10	V30	G10
1	ULC001	goat	32 (S)	23 (S)	24 (S)
2	ULC002	goat	10 (R)	9 (R)	30 (S)
3	ULC003	goat	40 (S)	22 (S)	30 (S)
4	ULC004	goat	20 (S)	17 (S)	35 (S)
5	ULC005	goat	34 (S)	10 (I)	32 (S)
6	ULC006	goat	29 (S)	21 (S)	30 (S)
7	ULC007	goat	11 (R)	17 (S)	25 (S)
8	ULC008	cattle	10 (R)	9 (R)	29 (S)
9	ULC009	cattle	20 (S)	9 (R)	33 (S)
10	ULC010	cattle	10 (R)	20 (S)	27 (S)
11	ULC011	cattle	11(R)	10 (I)	20 (S)
12	ULC012	cattle	13 (R)	9 (R)	32 (S)
13	ULC013	cattle	13 (R)	9 (R)	20 (S)
14	ULC014	cattle	12 (R)	27 (S)	25 (S)
15	ULC015	cattle	36 (S)	24 (S)	25 (S)
16	ULC016	cattle	38 (S)	24 (S)	26 (S)
17	ULC017	cattle	30 (S)	18 (S)	18 (S)
18	ULC018	Cattle	29 (S)	22 (S)	25 (S)
19	ULC019	Homemade yogurt	27 (S)	20 (S)	19 (S)
20	ULC020	Homemade yogurt	34 (S)	24 (S)	29 (S)
21	ULC021	Homemade yogurt	17 (S)	9 (R)	25 (S)
22	ULC022	Homemade yogurt	13 (R)	10 (I)	25 (S)
23	ULC023	Homemade yogurt	37 (S)	24 (S)	28 (S)
24	ULC024	Homemade yogurt	20 (S)	9 (R)	27 (S)
25	ULC025	Homemade yogurt	20 (S)	9 (R)	27 (S)
26	ULC026	Homemade yogurt	13 (R)	8 (R)	30 (S)
27	ULC027	Commercial yogurt	30 (S)	9 (R)	30 (S)
28	ULC028	Commercial yogurt	25 (S)	9 (R)	25 (S)
29	ULC029	Commercial yogurt	30 (S)	10 (I)	25 (S)

30	ULC030	Human	23 (S)	22 (S)	29 (S)
31	ULC031	Human	32 (S)	25 (S)	26 (S)
32	ULC032	Human	11 (R)	10 (I)	18 (S)
33	ULC033	Human	12 (R)	9 (R)	11 (R)
34	ULC034	Human	11 (R)	9 (R)	12 (R)
35	ULC035	Human	10 (R)	8 (R)	12 (R)
36	ULC036	Human	10 (R)	9 (R)	11 (R)
37	ULC037	Human	11 (R)	9 (R)	12 (R)

Notes:
ULC = Unisel's *Lactobacillus* Culture

From Table 3 and 4, it shows that human's milk has a high number of *Lactobacillus* isolates resistant to ampicillin (75%), vancomycin (62.5%) and gentamycin (62.5%). It's mean that these *Lactobacillus* were resistant to three antibiotics. These results are similar found as Mao et al., 2019 and Chetan et al., 2017 mentioned *Lactobacillus* in human milk already resistant to multi-antibiotic resistant (MAR). *Lactobacillus* isolated from cattle's milk shown resistant to ampicillin (42.1%), vancomycin (42.1%) and susceptible to gentamycin (0%). Previous research was done by Reem et al., 2019 said that one of the best antibiotics in treatment of bovine mastitis caused by bacteria was using antibiotic gentamicin. This is also similar results to antibiotic resistant studied by Vanniyasingam, Kapilan and Vasantharuba, 2019 and Huiling et al., 2019. But however, goat's milk still has lower percentage number of resistant to ampicillin (28.0%), vancomycin (14.3%) and are still susceptible to gentamycin (0%). Table 3 also shows that most of the commercial yogurt are resistant to vancomycin is one of the characteristics to choose *Lactobacillus* as probiotics. Antibiotic resistant bacteria are now spreading in worldwide such as in Pakistan (Rahman and Mohsin, 2019), US and EU (Stacy et al., 2019), China (Liu et al., 2009), other countries (Ilenia et al., 2019; José et al., 2019). In this study, vancomycin and ampicillin shown high percentage than gentamycin among *Lactobacillus* locally isolates (Figure 1). Example of antibiotic resistant bacteria such as vancomycin resistant entercocci (VRE) now was an emerging problem in hospital and vancomycin was often described as one of the last resorts against the infection caused by multidrug-resistant pathogens (Tang et al., 2007).

Table 3: Summary of number of *Lactobacillus* isolates against antibiotics

No	Source	Total isolates	Number of *Lactobacillus* Resistant to Antibiotic								
			AM10			V30			G10		
			R	I	S	R	I	S	R	I	S
1	Goat milk	7	2	0	5	1	1	5	0	0	7
2	Cattle milk	11	6	0	5	4	1	6	0	0	11
3	Homemade Yogurt (cattle milk)	8	2	0	6	4	1	3	0	0	8
4	Commercial yogurt	3	0	0	3	2	1	0	0	0	3
5	Human milk	8	6	0	2	5	1	2	5	0	3
	Total number Percentage (%)	37	16 (43.2%)	0	21 (56.8%)	16 (43.2%)	5 (13.5%)	16 (43.2%)	5 (13.5%)	0	32 (86.5%)

Table 4: Summary percentage among *Lactobacillus* isolates from various milk resistant to antibiotics

No.	Source	Total isolates	No of *Lactobacillus* isolates		
			$AM10_R$	$V30_R$	$G10_R$
1.	Human milk	8	6 (75%)	5 (62.5%)	5 (62.5%)
2.	Cattle milk	19	8 (42.1%)	8 (42.1%)	0 (0%)
3.	Goat milk	7	2 (28.6%)	1 (14.3%)	0 (0%)

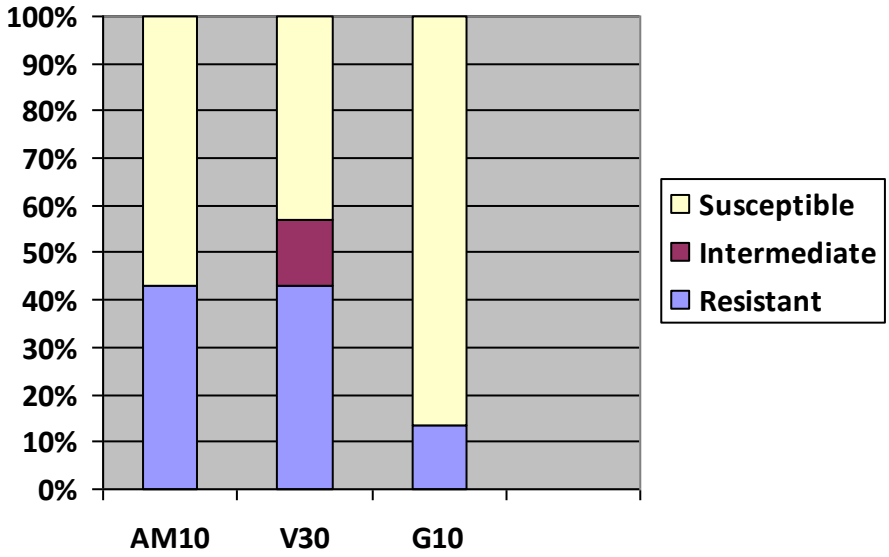

Figure 1: Antibiotic susceptibility analysis was performed on milk-isolated *Lactobacillus* toward antibiotics.

Lactobacillus currently used as probiotics, which confer as live microorganisms that promising give health benefits on the host when administrated in adequate amount. It was used due to the problem associated with the spread of clinical antibacterial resistance among pathogens and also the rises of allergic or inflammatory disease. Antibiotics resistance or susceptible is one of the characteristics that a required for *Lactobacillus* to be considered as probiotics strain in various applications used. It is important to determine and monitor the antibiotic profile either susceptible or resistance in *Lactobacillus spp.* Lately, it shown high resistance level towards different antibiotics observed in *Lactobacillus spp.* is getting importance as it can act as a reservoir of antibiotic resistance genes and can be transferred to other bacteria residing in the human gut horizontally by conjugation, natural transfer and transduction (Chetan et al., 2019). Table 5 shows ampicillin, vancomycin and gentamycin used in a different group and mode of actions the ability of *Lactobacillus* isolates resist to antibiotics class. Despite of *Lactobacillus* safety status, many Lactobacilli have been reported as being antibiotic resistant whereas vancomycin-resistant phenotype is the best-characterized intrinsic resistance mechanisms.

Previously study by Umama et al., 2019 and Chetan et al., 2019 have stated that *Lactobacillus* isolates from human breast milk are resistance towards to many of antibiotics such as amoxillin, chloramphenicol, cephalosporins, oxacillin, aztreonam, meropenem and polymyxin group. *Lactobacillus spp*. were also screened and presented of antibiotic resistance gene such as blaCTX-M and this may happen because exposure of a serious threat in treatment regimen (Umma et al., 2019). Various types of antibiotics are being used worldwide in veterinary sector indiscriminately for promotion of growth and treatment of the livestock. Significant portions of antibiotics are released through milk of dairy animals unaltered and exert serious harmful effects to human health. According to Rahman and Mohsin, 2019, they reported that this emergence and dissemination of antibiotic resistance is accelerated up by indiscriminate and incessant usage of

antibiotics due to uncontrolled infections in hospital settings, community, FPAs and aquaculture. Monitoring usage of antibiotics is lacking information of systemic surveillance data and impact of antimicrobial-resistance-control strategies. It is also important to highlight and getting attention of policymakers for improving infection control measures and implementing laws on restricted use of antibiotics in animals.

Table 5: List of antibiotics used and mode of actions

No.	Name of Antibiotic	Concentration (ug)	Antibiotic groups	Site of actions	Mode of actions
1.	Ampicillin	10	B-lactams	Cell wall synthesis	-Interaction with penicillin binding proteins (PBs). -Disruption of peptidoglycan layer and cell lysis.
2.	Vancomycin	30	Glycopeptides	Cell wall synthesis	-Interaction with D-alanyl-D-alanine termini of peptidoglycan chain. -Prevent the binding of D-anayl subunit wth the PBP.
3.	Gentamycin	10	Aminoglycosides	Protein synthesis	-30S ribosomal subunit. -Misreadings and premature termination of mRNA translation.

From the survey has been done by Taohida et al., 2019 reports that consumers were highly aware about the resistance in pathogenic organisms causing disease in human against antibiotics which it will failure resulting in treatment in hospitals. But however, consumers did not aware about allergic reaction and painful rash with many antibiotics. They showed positive attitude with their awareness regarding effect of antibiotic used in animal feed and human health. In future, consumers become more aware of the importance of good nutrition and health concerned about the antibiotics effect. Probiotic products will be designed more safely, should be inherent and non-transferrable resistance gene (Chetan et al., 2019) and well-documented to provide the consumers with the full health benefits (John et al., 2019). It is recommended that the government and the producer should aware trend of antibiotic resistance and develop new strategies for a prudent use of antibiotics in food producing animals to ensure food safety globally.

Conclusion

In conclusion, most of *Lactobacillus* isolates were resistant to two antibiotics from animal's milk and most of *Lactobacillus* isolated from human's milk are resistance to three antibiotics. This antibiotic profile shown *Lactobacillus* isolates in human and animal's milk are increasing and need a monitoring system to evaluate the evolution of antibiotics especially in food safety aspect.

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