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RESEARCH ARTICLE

Prevalence of Heavy Metals and Antibiotic Resistance of Solid Waste in a Landfill

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ABSTRACT - Antibiotics, heavy metals, and related antibiotic resistance bacteria (ARB) in municipal solid waste (MSW) landfills have aroused more attention due to their potential risk to the ecosystems and public healthcare. However, their contents and relationships have yet to be systematically understood in a landfill setting in Malaysia. Therefore, the prevalence of heavy metals and antibiotic resistance were observed in this study. This study selected solid waste samples of fresh or aged MSW from two representative garbage hills from Jabor-Jerangau landfills, Kuantan, Pahang. The samples were tested for ICP-MS for the concentration of heavy metals and diffusion discs for antibiotic resistance. The total content of measured heavy metals between new and aged garbage hills ranged from 0.2 mg/kg (Ni) to 108.19 mg/kg (Fe) and 0.01 mg/kg (Cu) to 31.92 mg/kg (Fe), respectively. No significant difference ($p>0.05$) between the new and aged MSW indicated time, and any treatments available for MSW did not affect its concentration. Fe was observed as the most concentrated heavy metal in these landfills. All isolates (100%) in fresh MSW were resistant to erythromycin, penicillins G, tetracyclines and sulfamethoxazole. However, 50% of the isolates in aged MSW were resistant to the tested antibiotics, whereas all isolates in aged MSW have susceptibility to sulfamethoxazole $p<0.05$. The data gained from this study can be used to plan appropriate treatment for both contaminants. This baseline information is critical for guiding future research, waste management policies, and public health interventions to address the growing concern of antibiotic resistance.

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1. INTRODUCTION

The aggressive economic development in Malaysia has caused increased waste production. In 2020, 4.0 million tonnes of scheduled waste were reported, where 57.1% (2.3 million tonnes) were contributed from the electrical and electronics, power plant, metals refinery and chemical industries, and 31.4 thousand tonnes (0.78%) came from clinical waste. Clinical waste increased by 7.5% in 2020 compared to the previous year [1]. Electronic products, electroplating waste, painting waste, used batteries, and other sources, when discarded with municipal solid waste, increase the accumulation of heavy metals in dumpsites. Unfortunately, deposited heavy metals cannot be degraded easily, so they are typically found more significantly in solid wastes on the grounds. Consequently, heavy metals become potential polluters of soils, surface water and groundwater [2]. It affects human health, but its increment is also linked to the proliferation of antibiotic resistance, specifically antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) [3].

The heavy metals accumulating in the landfill can trigger resilience and cause a rise of bacteria with antibiotic resistance and expanded virulence [4]. Heavy metals can be co-selecting agents and promote antibiotic resistance [3]. It can ease the creation and spread of integron-like protein structures. Antibiotic resistance has been identified as an emerging pollutant across the globe. This is due to a few factors, such as the improper use and disposal of antibiotics for humans, livestock or aquaculture and the persistence in water and soil [5]. For example, 30% to 50% of incorrect antibiotic prescriptions were given in the United States [6]. This improper waste ends up in landfills, water bodies, and drains, contaminating the soil and posing a variety of toxicities to humans and uncontrolled exposure to microorganisms, which end up in landfills [7]. The landfill may become an artificial reservoir for antibiotic resistance by accelerating its development and expansion [8]. World Health Organization (WHO) declared that antibiotic resistance is a human health crisis that will threaten the latest pharmaceutical technology and may lead to the primary cause of death by 2050 [3]. However, this is not limited to humans; antibiotic resistance can also be widespread in aquatic and soil ecosystems, often due to human waste and agricultural runoff [9].

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The correlation between heavy metals in landfills and antibiotic resistance is increasingly evident through several mechanisms. Gram-negative bacteria, particularly those in the phylum Proteobacteria and the family Enterobacteriaceae, possess a significant capacity to host plasmids with co-selection potential. This means that exposure to a single stressor ARGs or heavy metal resistance genes can induce tolerance to both, facilitating the development of multidrug resistance [3]. For example, *Pseudomonas aeruginosa*, a human pathogen linked to pneumonia, has shown co-resistance to mercury, cadmium, and cobalt alongside antibiotics like penicillin and amphenicol [10]. A review by Nguyen et al. [3] emphasized that heavy metals such as zinc and cadmium frequently co-occur with resistance to multiple antibiotics in various environmental studies. Supporting this, *Escherichia coli* strains resistant to zinc have demonstrated strong resistance to antibiotics such as ampicillin, nalidixic acid, and Tetracycline [11]. Given this, analyzing the heavy metal content in landfill solid waste is critical, as it can provide insights into environmental factors that contribute to the development and spread of antibiotic resistance in these settings.

Although some research on antibiotic resistance in Malaysia has focused on rivers [12], animals [13-15], soils or sediment [16-18], leachate [19, 20], landfill [21, 22], and water effluents from clinical waste and aquaculture [23], limited information is available regarding the correlation between antibiotic resistance and heavy metals in solid wastes. As a result, more data is needed to comprehensively understand antibiotic resistance at landfills in Malaysia. Therefore, this research aims to study the prevalence of antibiotic resistant bacteria and heavy metals in solid waste.

2. METHODS AND MATERIAL

2.1 Sampling Sites

This study collected two solid waste samples from the Jabor-Jerangau Landfill Site. Jabor-Jerangau Landfill Site covers an area of about 60.4 hectares, and it can hold 500 tons of waste daily. The samples were collected at S1 (fresh solid waste) and S2 (aged solid wastes) (Figure 1). The samples were randomly picked on the hill of the selected landfill and kept in the capped bucket. They were stored at room temperature in the laboratory.

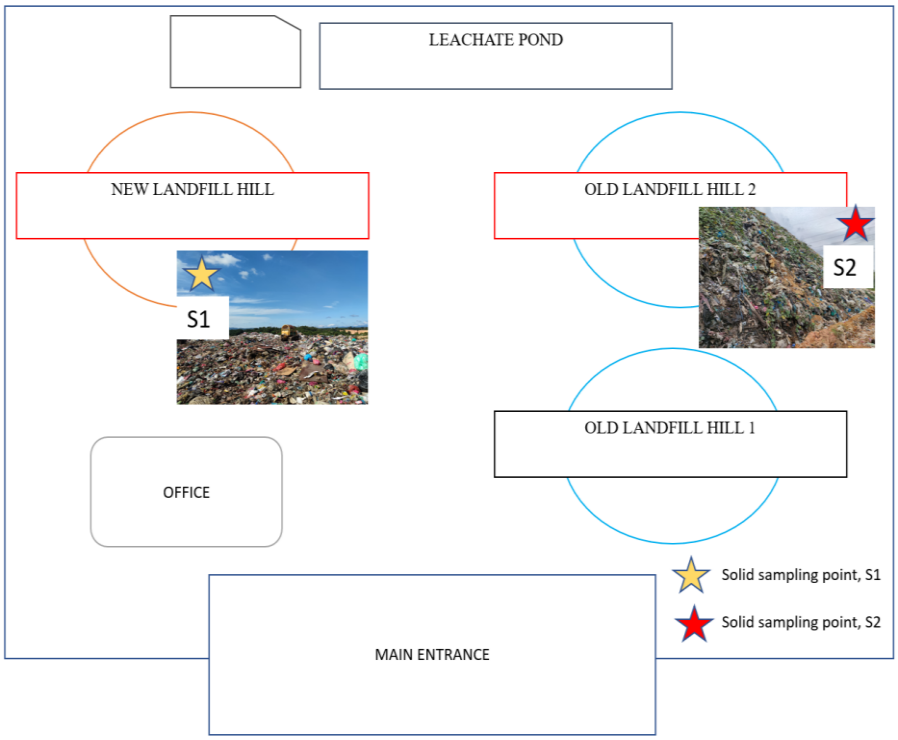

Figure 1. Sampling points

2.2 Sample Preparation for Heavy Metals Analysis

10 g of each S1 and S2 were mixed and homogenized before being blended in the conventional blender separately. Then, approximately 0.1 g of each blended sample was transferred into the digestion vessel. 2 ml of nitric acid and 6 mL of hydrochloric acid were poured into the vessel, and all samples were microwave digested for 24 mins in 4 different steps. The treatment was conducted as presented in Table 1. The microwave-assisted acid digestion was conducted using the microwave digestion system (PreeKem WX-6000 M). Then, all samples were cooled until 50 ℃. 5 mL of all samples were withdrawn separately and diluted with deionized water until the volume reached 50 mL [24, 25]. Fine particles were still present in the digested sample after the digestion; hence, all samples were filtered using vacuum filtration with 0.45μm microporous membrane filter. Then, all samples were sent to the Toxicological Laboratory, UMPSA, for ICP-MS analysis of cadmium (Cd), lead (Pb), nickel (Ni), copper (Cu), chromium (Cr), arsenic (As), manganese (Mn), zinc (Zn) and iron (Fe). The sample treatments were done in duplicate for all samples.

Table 1. Microwave-assisted acid digestion treatment for solid waste

Solvent	Volume, 0.1g/mL	Step	Temperature, ℃	Pressure, atm	Time, min
HNO_3	2	1	150	15	3
HCl	6	2	180	25	3
		3	200	30	3
		4	200	35	15

2.3 Isolation of Antibiotic Resistance Bacteria

10 g of each sample from solid waste was blended in the conventional blender to obtain a homogenous mixture with a sterile 85% saline solution. Then, 1g of each sample was enriched in 9 mL Lysogeny broth (LB broth) for 24 hours at 37 ℃. Next, serial dilution was performed on the enriched samples from 10^{-1} to 10^{-6}. Dilution of 10^{-2} to 10^{-5} was used to enumerate the bacteria in the sample, where 0.1mL of the diluted sample was spread on the MacConkey agar. The plates were labelled accordingly and incubated at 37 ℃ for at least 8 to 12 hours. The colony morphology was characterized for the suspected bacteria, and each colony forming was known as one colony forming unit (CFU). Next, the suspected colony was further purified and streaked on the MacConkey agar. The plates were labelled accordingly and incubated at 37 ℃ for 24 hours. The pure colony on the plate was selected, enriched in tryptic soy broth for 48 hours for further testing, and streaked on Eosin methylene blue (EMB) agar, a selective and differential media. The plates were labelled and incubated at 37 ℃ for 24 hours. Observation on the colony was observed after. All steps were done in the laminar flow, and all samples were duplicated.

2.4 Identification of The Bacteria

Two biochemical analyses were conducted to identify the sample's isolated bacteria. First, the IMViC test was performed. The pure colony from the MacConkey media were used for this test. Tryptone broth media, Methyl Red Voges-Proskauer medium and Simmons citrate agar was prepared for the test and sterilized for 15 mins at 121 ℃. Then, the pure culture was inoculated into all four tubes containing each prepared medium. All tubes were incubated at 37 ℃ for 24 hours (Indole and Citrate Utilization Test) and 48 hours (Methyl Red Voges-Proskauer). After 24 hours, the mentioned tubes were taken out, 0.3 mL of Kovac's reagent was added to the tryptone broth media, and observation was done. After 48 hours, the Methyl Red Voges-Proskauer medium was taken out, and four drops of methyl red indicator were dropped into one of the tubes for the methyl red test. For the Voges-Proskauer test, two drops of alpha naphthol (6g/100 ml of 95% ethyl alcohol) were followed by three drops of 40% KOH. The tube was shaken vigorously every 2-3 mins, and observation was made 1 hour later. All tests were done in duplicate.

Then, the RapID^{TM} ONE system test kit consisting of RapIDTM ONE Panels and RapIDTM ONE Reagent was used to identify the bacteria species. The colony of all samples were dissolved in sterile 85% saline solution until it matched 2 MacFarland turbidity. Then, 2 mL of the inoculum mixtures were suspended into the panel until a uniform level was achieved between all panels in a tilted manner opposite the cavities. Then, slowly, the panel was tilted toward the cavities, and the inoculum mixture was into the cavities. All inoculated samples were incubated for 12 hours at 37 ℃. Observation was done for all cavities, and two drops of RapID Spot Indole Reagent were added to cavity 18 (ADON/IND). Results were recorded not exceeding 2 mins.

2.5 Antibiotic Susceptibility Testing (Disc Diffusion Method)

0.5 MacFarland standard was prepared and checked for turbidity ranging from 0.08 to 0.13 at 625 nm. The tryptic broth cultures were diluted with 85% saline solution until they matched the 0.5 MacFarland standard. Then, the adjusted inoculums were smeared on the MH agar using a sterile cotton swab. 30 μg tetracycline, 15 μg erythromycin, 10 μg penicillin g and 100 μg sulfamethoxazole antibiotic discs were used and aligned on the inoculated plates. The plates were sealed and incubated for 18 hours at 37 ℃. The inhibition zones were observed and measured. All test was done in duplicate. The results of antibiotic susceptibility testing were compared using interpretive categories and zone diameter breakpoints for *Enterobacteriaceae* (Table 2) from the Clinical and Laboratory Standard Institute [26].

Table 2. Interpretive categories and zone diameter breakpoint for *Enterobacteriaceae*

Antibiotic	Interpretive categories and Zone diameter breakpoint (mm)		
	Susceptible (S)	Intermediate (I)	Resistant (R)
Erythromycin, 15µg	≥ 13	-	≤ 12
Penicillin G, 10µg	≥ 17	14-16	≤ 13
Tetracycline, 30 µg	≥ 15	12-14	≤ 11
Sulfamethoxazole, 100 µg	≥ 17	13-16	≤ 12

2.6 Statistical Analysis

Data were analyzed and descriptive statistical data were generated using Microsoft Excel 2019 (Microsoft Corp., Redmond, WA, USA). Significant changes in the heavy metal concentrations between fresh and aged solid waste samples were compared using a t-test (R studio). Data were analyzed, and descriptive statistical data were generated using Microsoft Excel 2019 (Microsoft Corp., Redmond, WA, USA). Significant changes in the inhibition zones between bacteria isolates were compared using a t-test. The Spearman correlation test was performed to test the correlation between the heavy metal concentrations and the diameter of the diffusion disc.

3. RESULTS AND DISCUSSION

3.1 Heavy Metal Analysis

In general, the amount of heavy metals in municipal solid waste at the Jabor-Jerangau landfills site did not exceed the legal limits except for Fe, manganese, and arsenic (Table 3). Although the concentration of heavy metals between old and new garbage hills was insignificant ($p>0.05$), there is 66% confidence that the concentration of heavy metals in the older garbage hill was lower than that of the new garbage hill. This is similar to the related study by Pascual et al. [27], where the heavy metal content of compost made from urban MSW is lower than the statutory limits. For Cd, Pb, Ni, Cu, Cr, As, Mn, Zn and Fe, the typical metals concentrations in MSW compost collected from multiple US composting facilities are 2.6, 2.9, 34.8, 154, 215, 248 & 503 mg/kg respectively. The study on the MSW Łubna Landfill revealed that heavy metal concentrations were within safe limits where the groundwater contained cadmium (0.0005 mg/L), lead (0.008 mg/L), zinc (0.113 mg/L), copper (0.017 mg/L), and chromium (0.044 mg/L). Soil and plant samples also showed low metal levels, reflecting effective containment through vertical barriers and drainage systems [28].

Table 3. Heavy metal concentration between fresh solid waste and aged solid waste

Heavy metals	Mean concentration, mg/kg				Environmental Quality Act 1974 (leachate, mg/L)
	Fresh solid wastes	%	Aged solid wastes	%	
Chromium (Cr)	0.18	0.17	0.05	0.2	-
Lead (Pb)	-0.02	0.00	-0.01	0.0	0.10
Zinc (Zn)	0.45	0.41	0.41	1.2	2.00
Copper (Cu)	0.04	0.04	0.01	0.0	0.20
Manganese (Mn)	0.43	0.39	0.29	0.9	0.20
Iron (Fe)	108.19	98.78	31.92	97.2	5.00
Cadmium (Cd)	-0.03	0.00	-0.03	0.0	0.01
Nickle (Ni)	0.02	0.02	0.00	0.0	0.20
Arsenic (As)	0.21	0.19	0.16	0.5	0.05

Among the heavy metals, Fe showed the highest concentration in fresh solid waste (99%) and aged solid waste (97%). The other heavy metals showed less than 0.5% in new samples and 1.2% in aged solid wastes. Similar observations were recorded in eight landfills in Malaysia (Sungai Sabai landfill; 0.631 mg/kg, Sungai Wangi landfills; 0.397 mg/kg, Sungai Kertas dumpsite; 0.443 mg/kg, Taman Beringin landfill; 0.194mg/kg, Air Hitam Sanitary landfill; 0.770 mg/kg, Dengkil Inert Waste landfill; 0.251 mg/kg, Jeram Sanitary landfill; 0.154 mg/kg and Krubong landfill; 0.323 mg/kg) where the Fe were the highest concentration amongst other heavy metals in the landfills [16]. When comparing the unsanitary open landfills and sanitary landfills, the unsanitary landfills scored the highest Fe among the landfills and the lowest Fe content coming from landfills at sanitary level with treatment facilities [16]. Fe is not classified as a dangerous or poisonous metal to humans, and its concentration may be related to environmental conditions and landfill area backdrop, which significantly impact the accumulation of certain heavy metals and their content [29]. Fe is abundant in construction and demolition waste, metal scraps, and electronic waste, all commonly disposed of in landfills. Oxidation and reduction reactions under landfill conditions often enhance the mobility and concentration of Fe [30].

The concentrations of Mn and As exceeded the specified limit by more than 100%. Mn and As are also commonly found in materials like batteries, paints, and steel products, frequently disposed of in landfills. Organic waste and biodegradation can mobilize Mn, releasing it into the leachate or solid waste matrix. Usually, the highest content of heavy metals in the MSW is Cu and Zn, sourced from accumulating an assortment of plastic bags, papers, toilet tissue, packaging materials, cigarette packs, and human/animal hairs [31], but we did not observe this study. A higher concentration of these metals in waste might put selection pressure on antibiotic resistance. It may linked to the abundance of ARGs and antibiotic concentrations [25]. Even if the heavy metal concentrations remain within legal limits, their potential toxicity to surrounding bacteria remains uncertain, as there is no established threshold for metal toxicity in environmental microorganisms. Heavy metals and bacteria interact in nature, and they have a variety of pathways by which they might interact with microorganisms [32].

3.2 Bacteria Enumeration

For the fresh solid samples, the mean number of colonies for each duplicate in 10^{-2}, 10^{-3} and 10^{-4} are 118 colonies, 102 colonies and 86 colonies (Table 4), respectively. The morphology of the colonies was observed. There was no similarity in their morphology, indicating multiple species of gram-negative bacteria in the samples. In 10^{-2}, the colonies formed are uniformly round, translucent, white, shiny, and non-lactose fermenters. Meanwhile, 10^{-3} dilution plates showed similar bright pink colonies (lactose fermenter) but in different shapes and margins, shiny and moist. As for 10^{-4}, there is a mixture of similar colonies from the 10^{-2} dilution culture plate and newly observed mucoid colonies. The mucoid colonies have a pinkish-white centre and are very slimy and moist. Based on the morphology in these plates, the suspected gram-negative bacteria are either *Klebsiella* or *Enterobacter* due to its mucoid shape showing its capsules using lactose on the media.

Table 4. Colonies with different dilution factors in fresh and aged solid waste

Dilution factor	Fresh solid samples			Aged solid samples		
	Number of colonies Mean ± SD	Morphology		Number of colonies Mean ± SD	Morphology	
10^{-2}	118 ± 14.14	M:	smooth	166 ± 29.70	M:	smooth
		C:	translucent pink		C:	translucent pink
		E:	raised		E:	raised, undulate
		T:	slimy, moist		T:	slimy, moist
		S:	uniformly round		S:	round, punctiform
10^{-3}	102 ± 13.44	M:	lobate, round	228 ± 21.92	C:	translucent pink
		C:	bright pink		E:	raised, undulate
		E:	flat, raised		M:	smooth
		T:	shiny, moist		T:	slimy, moist
		S:	round, irregular		S:	round, punctiform
10^{-4}	86 ± 16.26	M:	smooth	78 ± 7.78	C:	translucent pink
		C:	translucent pink, pinkish-white centre		E:	raised, undulate
		E:	raised		M:	smooth
		T:	slimy, moist, mucoid		T:	slimy, moist
		S:	uniformly round		S:	round, punctiform

SD: standard deviation, M: margin, C: color, E: elevation, T: texture, S: shape

As for aged solid waste samples (Table 4), the mean number of colonies for each duplicate in 10^{-2}, 10^{-3} and 10^{-4} are 166 colonies, 228 colonies and 78 colonies, respectively. All colonies formed are raised or undulated on the surface media with smooth, translucent pink colonies except for 10^{-2}; the colour of the colony is brighter than on another plate. These plates have two types of size colonies; some are round, and some are punctiform. No bright pink colonies in this sample indicated the absence of lactose fermenter bacteria. The enumeration of the gram-negative bacteria using selective and differential media such as MacConkey (MAC) helped in culturing only gram-negative bacteria and limiting the growth of any gram-positive bacteria in the solid waste samples. The enumeration of bacteria indicated the possible bacteria that existed in the sample. Lactose fermentation creates organic acids, primarily lactic acid, which lowers the agar's pH. In acidic settings, MAC incorporates a pH indicator that becomes pink. Lactose-fermenting gram-negative (lactose fermenters) will produce pink colonies, whereas non-lactose fermenters will produce off-white opaque colonies [33].

3.2 Bacteria Identification Using Differential Media

The isolates from the streak media were streaked again on the differential media, EMB media, for further identification. EMB medium acted as a colour indicator between non-lactose fermenter and lactose fermenter bacteria. As indicated in the table, the colonies in fresh solid waste at 10^{-2} seemed dark purple but lacked the green metallic sheen that covered the colonies. This showed that the colonies on the specified plate were non-lactose fermenters. The morphology of the fresh solid waste isolates for dilutions 10^{-3} and 10^{-4} was quite equivalent, with a darker colony and a green metallic sheen around the bacterium development. Such colour and shape are caused by bacterial activity in the medium digesting lactose, reducing the pH of the media and increasing dye intake by the colonies. Yet, the fact that the green metallic sheen does not adequately cover the colonies shows that the bacteria is either a slow lactose fermenter or that the colony being inoculated on the media is not pure. Meanwhile, a little green metallic sheen can be seen in aged solid waste at 10^{-2}, when the colonies seemed flat and black. Because of the minor amount of green sheen on the colonies, they may be slow lactose fermenters. Two plates from aged solid waste in 10^{-3} and 10^{-4} produced purple and pinkish colonies, which indicates that the bacteria are non-lactose fermenters.

3.2 Bacteria Identification Using Biochemical Analysis

IMViC and Remel RapID™ ONE System consist of various biochemical tests that test the bacterial activity and will be categorized based on their corresponding behaviour. Table 5 shows the identification of isolated colonies from fresh wastes using the IMViC test and the Remel RapID™ ONE System. The IMViC test identified two dominant Gram-negative bacteria, *Proteus mirabilis* and *Enterobacter cloacae*. *P. mirabilis* was positive for methyl-red and citrate but could not be confirmed by the Remel RapID™ ONE System. Conversely, *E. cloacae* were consistently identified and confirmed across dilutions, showing positive results from both methods. However, discrepancies highlight the importance of additional confirmation steps for certain bacterial species.

Table 5. Identification of isolated colony from fresh solid wastes sample using IMViC Test and Remel RapID™ ONE System

Sample	Dilution factor	IMViC Test				Potential Gram-negative bacteria	Remel RapID™ ONE System (confirmed)
		Indole	Methyl-Red	Voges Proskauer	Citrate		
a	10^{-2}	-	+	-	+	*Proteus mirabilis*	Unidentified
b		-	+	-	+	*Proteus mirabilis*	Unidentified
a	10^{-3}	-	-	+	+	Enterobacter	*Enterobacter cloacae*
b		-	-	+	+	Enterobacter	*Enterobacter cloacae*
a	10^{-4}	-	-	+	+	Enterobacter	*Enterobacter cloacae*
b		-	-	+	+	Enterobacter	*Enterobacter cloacae*

a: First duplicate b: Second duplicate

Table 6 illustrates bacterial identification results from aged solid waste samples using the IMViC test and the Remel RapID™ ONE System. The IMViC test detected multiple potential Gram-negative bacteria, including *C. freundii*, *P. mirabilis*, and *Salmonella*, across various dilutions (10^{-2} to 10^{-4}). *C. freundii* was confirmed by the Remel RapID™ ONE System only in the 10^{-2} dilution, while all other isolates remained unidentified despite the IMViC results. Though confirmation was not achieved, *P. mirabilis* and *Salmonella* were consistently indicated as potential bacteria in higher dilutions (10^{-3} and 10^{-4}). Most samples' methyl-red and citrate-positive results suggest active acid production and citrate utilization as a carbon source, characteristic of many gram-negative bacteria in nutrient-limited environments.

Table 6. Identification of isolated colony from aged solid wastes sample using IMViC Test and Remel RapID™ ONE System

Sample	Dilution factor	IMViC Test				Potential Gram-negative bacteria	Remel RapIDTM ONE System (confirmed)
		Indole	Methyl-Red	Voges Proskauer	Citrate		
a	10^{-2}	-	+	-	+	*Citrobacter*	*Citrobacter freundii*
b		-	+	-	+	*Proteus mirabilis*	
a	10^{-3}	-	+	-	+	*Salmonella*, *Citrobacter*	Unidentified
b		-	+	-	+	*Proteus mirabilis*	
a	10^{-4}	-	+	-	+	*Salmonella*, *Citrobacter*	Unidentified
b		-	+	-	+	*Proteus mirabilis*	

a: First duplicate b: Second duplicate

3.2 Antibiotic Susceptibility Testing

Table 7 summarizes the antibiotic susceptibility of bacterial isolates from fresh and aged solid waste based on the disc diffusion method. All isolates from fresh solid waste were resistant to all antibiotics; meanwhile, 50% were resistant to aged solid waste. In the fresh solid waste samples, bacteria such as *P. mirabilis* and *E. cloacae* exhibited high resistance to most tested antibiotics. These isolates showed no inhibition zones for penicillin G and sulfamethoxazole, indicating complete resistance, while their inhibition zones for erythromycin and tetracycline were small (6–8 mm), also reflecting resistance. In contrast, isolates from aged solid waste, including *C. freundii* and unidentified bacteria, demonstrated varying resistance profiles. They were completely resistant to erythromycin and penicillin G but showed moderate susceptibility to tetracycline (inhibition zones of 35–37 mm) and high susceptibility to sulfamethoxazole (31–34 mm). One isolate from aged waste also displayed intermediate susceptibility to penicillin G, suggesting reduced antibiotic

resistance compared to fresh solid waste. These differences indicate that bacteria in fresh waste experience higher selection pressure due to higher antibiotic concentrations, while aged waste reflects attenuation of resistance over time.

Table 7. The inhibitory zone of all isolates with their susceptibility strength: susceptible, intermediate or resistant

	Dilution	Sample	Bacteria	Diameter of Inhibition zone, mm			
				Erythromycin 15 µg	Penicillin G 10 µg	Tetracycline 30 µg	Sulfamethoxazole 100 µg
Fresh solid waste	10^{-2}	a	*Proteus mirabilis*	8	0	8	0
		b		7	0	8	0
	10^{-3}	a	*Enterobacter cloacae*	7	0	6	0
		b		6	0	7	0
	10^{-4}	a		0	0	0	8
		b		0	0	0	7
Aged solid waste	10^{-2}	a	*Citrobacter freundii*	0	0	0	34
		b		0	0	0	31
	10^{-3}	a	Unidentified	0	0	36	32
		b		0	0	37	33
	10^{-4}	a		0	15	35	34
		b		0	14	35	31

a= First duplicate b= Second duplicate

Susceptible | Intermediate | Resistant

It has been observed that aged solid waste has become more susceptible to antibiotics than fresh solid waste (Table 7). This may be due to the landfill's environment undergoing physical and chemical changes over time. For example, the accumulation of heavy metals can be toxic to the bacteria, or the lack of oxygen in the landfill layers limits bacterial activity. These environmental stressors selectively eliminate resistant bacteria and reduce bacterial populations [34]. This observation aligns with Xu et al. [32], who also found that the abundance of antibiotic resistance genes (ARGs) was significantly higher in young waste than in old waste in a Chinese landfill, suggesting that ARG contamination decreases as solid waste ages. Meanwhile, all isolates in the fresh solid waste samples were resistant to all antibiotics tested, possibly due to the high level of antibiotics in the fresh solid waste from medical and household waste. These antibiotics create selective pressure and higher mobile genetics elements that favour the survival of resistant bacteria [35]. Fresh solid waste may contain higher organic matter and nutrients that will support the growth of different bacterial populations, including ARB [36].

Several studies confirmed that *P. mirabilis* is commonly found in various environmental and hospital settings with increasing antibiotic resistance [37, 38]. Proteus infections have gained considerable attention in the recent decade due to the establishment of resistance in some species to some antibiotics, particularly many β-lactams. *P. Mirabilis* does not manufacture any chromosomally encoded β-lactamase, resulting in resistance to all β-lactams for the wild-type phenotype [39]. We also observed that the resistance to the penicillin G was more than 80%. *E. cloacae* is also found to be resistant to landfill and abattoir wastewater. A study in a large-scale landfill in China found that resistance genes for sulfamethoxazole and tetracycline (tetO, sulI) were widespread in waste layers [40]. Meanwhile, Onuoha 2016 [41] observed that their wastewater from abattoirs contained *E. cloacae* strains highly resistant to penicillin G, tetracycline, erythromycin, and sulfamethoxazole. This suggests that *E. cloacae* in the environment could also develop resistance to these antibiotics. In our study, we found 100% resistance to all tested antibiotics.

Citrobacter freundii showed susceptibility toward sulfamethoxazole, which indicated the antibiotic is still functioning, restricting *Citrobacter freundii* growth. *Citrobacter freundii* can harbour a wide variety of β-lactamases and is resistant uniformly to all first-generation cephalosporins, ampicillin and tetracycline [42]. The resistance observed from the susceptibility testing supports this study. *Citrobacter freundii* and some other *Citrobacter spp*. harbor chromosomal AmpC-type β-lactamases that can inactivate third-generation cephalosporins, which in this case sulfamethoxazole, but it stayed susceptible to the antibiotic, which means the isolated *Citrobacter freundii* still did not acquire that ability [43]. Some studies confirmed that *Citrobacter freundii* resisted penicillin, erythromycin, and tetracycline, among other antibiotics from lakes in Udaipur, India [44]. Other than that, the isolated *C. freundii* from broiler chicken farms were resistant to erythromycin (77%), sulfamethoxazole (50%), and tetracycline (59%) [45] and also found resistant in fish, with penicillin G (67%), erythromycin (65%), tetracycline (63%), and sulfamethoxazole (13%) [46] as well as in ducks in Bangladesh with resistance genes encoding tetracycline (tetA, tetB), sulfonamides (sul1, sul2), and beta-lactams (blaTEM-1)[47].

The heatmap (Figure 8) reveals a strong correlation between heavy metal concentrations and antibiotic resistance, with several metals showing statistically significant ($p < 0.05$). It indicates that Cr, Cu, Ni, and Fe exhibit the most significant negative correlations with penicillin G, tetracycline, and sulfamethoxazole, indicating that higher

concentrations of these metals drive increased bacterial resistance to these antibiotics. This suggests that co-selection mechanisms may be at play, where resistance genes for metals and antibiotics are carried together on mobile genetic elements such as plasmids. In contrast, Cr and Fe show significant positive correlations with erythromycin, meaning higher concentrations of these metals may increase bacterial susceptibility to erythromycin rather than resistance. These findings suggest that heavy metal pollution contributes to multidrug resistance, particularly for penicillin G, tetracycline, and sulfamethoxazole, through genetic adaptation and efflux pump activation. The strong correlation between metal contamination and antibiotic resistance highlights the urgent need for environmental monitoring and regulation of heavy metal discharge to mitigate the spread of antibiotic-resistant bacteria. Future research should focus on identifying the specific genetic mechanisms responsible for this resistance and developing targeted strategies to reduce the impact of metal pollution on public health.

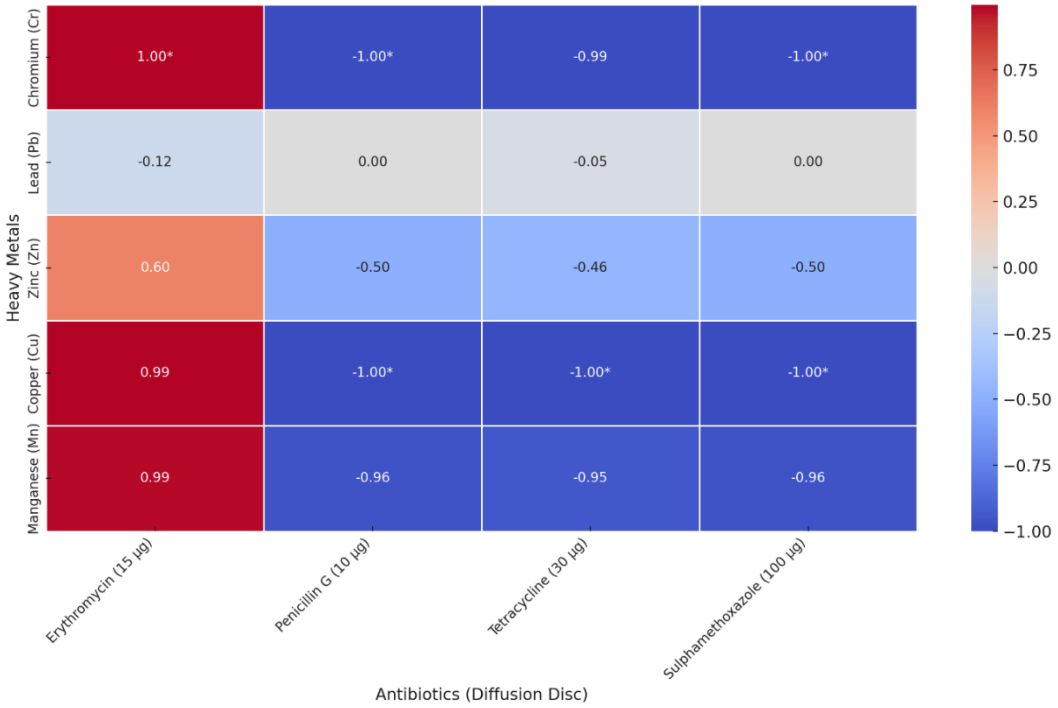

Figure 8. Correlation between heavy metal concentrations and antibiotic diffusion disc
*Indicates $p<0.05$

The limitation of this study lies in the inability of the Remel RapID™ ONE System to confirm the majority of isolates, underscoring its restricted capability in identifying the diverse bacterial populations present in aged solid waste. These results emphasize the complexity of microbial diversity in such environments and highlight the importance of employing multiple methods for accurate bacterial identification. For a more comprehensive analysis of antibiotic resistance, techniques such as qPCR or sequencing should be utilized to detect resistance genes and characterize bacterial profiles more effectively.

4. CONCLUSION

The presence of heavy metals and antibiotic resistance in Jabor-Jerangau landfills is justified in this study. Between two separate garbage hills (fresh and aged), the concentrations of eight heavy metals (chromium, lead, zinc, copper, manganese, iron, cadmium, nickel, and arsenic) were measured. The difference in heavy metal concentrations between fresh and aged solid waste samples is negligible, and the concentrations do not exceed the legal limit. When different heavy metal concentrations were compared, iron was the most prevalent among the other heavy metals in old and new solid waste samples. Hazardous heavy metals, including lead, cadmium, and chromium, are undetectable and account for less than 0.5% of overall heavy metal concentrations. Resistance to erythromycin, penicillin G, tetracycline, and sulfamethoxazole has been identified in 12 bacterium isolates from fresh and old solid waste. Antibiotic resistance was found in fresh and old samples, with the latter showing more susceptibility. *Enterobacter cloacae* isolates were resistant to Erythromycin, penicillin G, and Tetracycline but responsive to sulfamethoxazole. Although the resistances are unique, they can be passed on to other bacteria, both *P. mirabilis* and *E. cloacae* resistant to all tested antibiotics. Future studies can focus on the amount of antibiotics in landfills and the correlation and contribution of antibiotics, ARBs, ARGs, and heavy metals in landfills. This study serves as a baseline to understand antibiotic resistance in Malaysia, particularly within the context of bacterial populations found in solid waste environments. This baseline information is critical for guiding future research, waste management policies, and public health interventions to address the growing concern of antibiotic resistance.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

N. N. A. Manaf (Investigation; Methodology).

N. Mahmud, H. F. Ahmad, M. F. F. Asras, N. F. H. Nordin and N. S. Khalid (Writing – review & editing).

N. A. Sabri (Conceptualization; Writing – review & editing; Supervision).

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