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Synergistic Effect of Antibiotics Action Against *Raoultella ornithinolytica* and *Enterococcus faecalis*

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Abstract
The purpose of this study is to provide understanding on the previously undiscovered synergy effects against *R.ornithinolytica* and *E.faecalis.* Hospital infections and the excessive use of antibiotics have led to the emergence of multidrug-resistant bacteria. The total number of antibiotics that are still effective against infections with resistance is gradually declining. Combinations of antibiotics are frequently studied as an alternate method to treat them; thus, it is favoured over monotherapy treatment. In the present study, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were investigated beforehand in order to determine the synergism of antibiotics combination through checkerboard assay method on *E.faecalis* isolate and *R.ornithinolytica* isolate. Both strain that were used in this study were isolated from sewage treatment plant in Johor. The susceptibility profile of both isolates showed that they were resistant rifampicin and ampicilin. On the other hand, tetracyclines acts bactericidal towards *E.faecalis* isolate while chloramphenicol acts bactericidal towards *R.ornithinolytica* isolate. In particular, the fractional inhibitory concentrations (FICI) of antibiotic combinations were determined. All the combinations showed antagonism effects with FICI value more than 4. To conclude, the antagonism between antibiotics may help in developing treatment strategies specifically aimed at delaying the emergence of resistance. However, discovering an effective combination of antibiotics against multidrug resistant must be the prime concern.
Keywords: *Enterococcus faecalis; Raoultella ornithinolytica*; Minimum inhibitory concentration; Minimum bactericidal concentration; Checkerboard assay

Introduction

Antimicrobial resistance (AMR) and multidrug resistance (MDR) are a worldwide public health problem that is spreading due to the dissemination of antibiotic resistance genes (ARG) in diverse settings. The scientific community has recognized the significance of wastewater treatment plants (WWTPs) as hotspots for the spread of AMR and MDR. Antimicrobial resistance (AMR) is becoming more common in formerly susceptible pathogenic microorganisms. The rise of multidrug resistance (MDR) in both pathogenic and non-pathogenic bacterial populations is another linked issue with major public health implications. Several global organisations, including the World Health Organization, the United States Center for Disease Control, the National Academy of Sciences' Institute of Medicine, the Federal Interagency Task Force on Antimicrobial Resistance, the Infectious Diseases Society of America, and numerous other worldwide public health authorities, have identified this as a critical issue of significant concern (Mukherjee et al., 2021).

Raoultella ornithinolytica belongs to the *Enterobacteriaceae* family and is an encapsulated Gram- negative, oxidase-negative, catalase-positive, aerobic, non-motile rod. *R.ornithinolytica* has been detected in water, dirt, insects, fish, ticks, and termites, among other places. This bacterium converts histidine to histamine, resulting in histamine poisoning and cutaneous flushing, also known as scombroid syndrome. Depending on the amount of histamine ingested, this condition might include vomiting, diarrhea, headache, in addition to skin flushing (Hajjar et al., 2020). *Enterococcus faecalis* is a Gram-positive commensal bacterium that may be found in the gut microbiota of a variety of animals. It has evolved into a multi-drug resistant, hospital-acquired pathogen since the introduction of antibiotic treatment (Van Tyne et al., 2013).

Combined antibiotic therapy is preferred over single antibiotic therapy because it has a larger antibacterial range, a lower chance of acquiring antibiotic resistant bacteria, and, most significantly, it can produce synergistic effects. Synergy is a well-defined concept in the world of microbiology in which the use of a paired or triple combination of inhibitory drugs suppresses the development of specific microorganisms with more activity and positive interaction than each agent alone (Tamma et al., 2012). Therefore, this study aims to provide understanding on the previously undiscovered synergy effect of antibiotics action against *E.faecalis* and *R.ornithinolytica* isolated from wastewater treatment plants (WWTPs) sources that has not been investigated before. In addition, it is anticipated that antibiotic combination therapy will shed some light on the most effective antibiotics to use in order to treat bacteria infections and reduce the duration of treatment.

Materials and methods

Generally, two strains from different species which are *Raoultella ornithinolytica* isolate and *Enterococcus faecalis* isolate were cultured from glycerol stock, and antibiotic susceptibility tests were performed by measuring MICs. From MBC, different concentrations of MIC salts were plated on Mueller-Hinton agar (MHA) and classified as bacteriostatic or bactericidal based on their activity. Following that, the synergism of antibiotics agents was determined by double antibiotics combination.

Two isolates of different species, which are *E. faecalis* isolate and *R.ornithinolytica* isolate from influent sewage sample and effluent sewage sample in Mutiara Rini and Taman Selesa, respectively, cultivated from glycerol stock. A sterile loop was used to scrape the frozen bacteria off the surface of the glycerol stock of the isolates that were kept at -80°C. It was then streaked on MHA and incubated for an overnight period at 37°C (Yung, 2021).

To determine the antibiotic susceptibility profile of the strains *R. ornithinolytica* isolate and *E. faecalis* isolate, the Clinical and Laboratory Standards Institute (CLSI) recommended the broth microdilution method on 96-well plates. Ciprofloxacin, chloramphenicol, tetracycline, gentamicin, sulfafurazole, ampicillin and rifampicin were the antibiotics used in the study. Due to their broad spectrum of activity, the antibiotics selected target different aspects of bacterial growth, and resistance is expected to be demonstrated by *E. faecalis* and *R. ornithinolytica* based on previous studies (Yung, 2021).

Following the determination of MIC, antibiotic activity was classified as bactericidal or bacteriostatic by determining MBC, which is the lowest concentration of an antibacterial agent required to kill a bacterium or reduce the viability of the initial bacterial inoculum by 99.9% over an extended period (El-Azizi, 2016). El-Azizi discussed the procedure in detail (2016). In general, 10 µl sections of MIC, 2X MIC, and 4X MIC were collected from 96-well plates cultured with resazurin and distributed onto MHA, where they were incubated overnight. MBC was measured at a concentration where no colony growth was observed on the plate, indicating that the antibiotic was bactericidal; MBC was measured at a concentration greater than 4X MIC, indicating that colony growth was observed on the highest MIC plated, indicating that the antibiotic was bacteriostatic.

By using FICI, a checkerboard experiment including antibiotic MICs was used to investigate the synergistic impact of a double antibiotic combination. The test was carried out on 96-well plates using a two-plate approach, as described by Xu et al. (2018), with minor modifications. In a brief, tetracycline dilutions from 8X MIC to 1/8 MIC were carried out horizontally from right to left (column 8 to 2) using a two-fold serial dilution procedure, with 100 l of mixture discarded at column 2. Ciprofloxacin dilutions ranging from 4X MIC to 1/16 MIC were generated in microcentrifuge tubes and afterwards applied to the designated wells on 96-well plates to obtain varied proportions with tetracycline using a two-fold serial dilution procedure. The MIC of tetracycline was determined in row A, while the MIC of ciprofloxacin was determined in column 1. With growth control and sterility control, columns 11 and12 served as quality control segments. Columns 9 and 10 have been left empty. The bacteria suspension was made in the same way as before and then added to each well to obtain the final inoculum concentration of 5 x 105 CFU/mL. The tray's incubation temperature will be set at 352°C for 16 to 20 hours. To examine colour formation, 5 µl resazurin was added to each well, and the results were evaluated in the same way as before.

Results and discussion

The result obtained as shown in the Table 1 shows that *E.faecalis* isolate is sensitive to all the antibiotics which is gentamicin, tetracycline, sulfafurazole, chloramphenicol and ciprofloxacin except rifampicin and ampicillin. This finding opposes the previous finding from (Gagetti et al., 2019), which stated all enterococci exhibit decreased susceptibility to ampicillin.

The antibiotic susceptibility profile for *R.ornithinolytica* isolate as shown in the Table 1 is similar to *E.faecalis* isolate. *R.ornithinolytic*a isolate is susceptible to all antibiotics except ampicillin and rifampicin. This finding agrees with the previous finding from (Hajjar et al., 2020) which stated *Raoultella spp.* Exhibit intrinsic resistance to ampicillin due to the chromosomally encoded beta-lactamases.

Table 1: Susceptibility profile of *E.faecalis* isolate and *R.ornithinolytica* isolate

Antibiotics	**Minimum inhibitory concentration (ug/ml)**			
	***E.faecalis* isolate**	**Susceptibility**	***R.ornithinolytica* isolate**	**Susceptibility**
Ciprofloxacin	0.007813	S	0.01563	S
Gentamicin	0.03125	S	0.0625	S
Tetracycline	0.0625	S	0.0625	S
Chloramphenicol	0.5	S	0.5	S
Sulfafurazole	1	S	256	S
Rifampicin	4	R	8	R
Ampicilin	32	R	64	R

S: susceptible; I :intermediate; R: resistant.

For *E.faecalis* isolate, all the antibiotics are bacteriostatic antibiotics with positive growth observed at 4X MIC while tetracycline is a bactericidal antibiotic with no growth observed at 4x MIC (Table 2). Based on finding from (Loree & Lappin, 2019), bacteriostatic antibiotics require a functioning host immune system to fully clear overgrowth due to merely inhibiting further growth of bacteria, Due to this effect, there is a lower incidence of toxic shock and more tolerable side effect profile.

Table 2: Minimum Bactericidal Concentrations (MBC) of Antibiotics for *E.faecalis* isolate.

ANTIBIOTICS	**DILUTION**			**MBC (µg/mL)**	**ACTIVITY**
	MIC	2X MIC	4X MIC		
Gentamicin	+	+	+	>0.0625	Bacteriostatic
Tetracyclines	+	+	-	0.25	Bactericidal
Sulfafurazole	+	+	+	>4	Bacteriostatic
Chloramphenicol	+	+	+	>2	Bacteriostatic
Ciprofloxacin	+	+	+	>1	Bacteriostatic
Rifampicin	+	+	+	>32	Bacteriostatic
Ampicilin	+	+	+	>128	Bacteriostatic

* + indicates growth, - indicates no growth.

R.ornithinolytica isolate shares the similar trend where all the antibiotics are bacteriostatic with positive growth at 4x MIC (Table 3). Chloramphenicol is bactericidal antibiotics due to no growth observed at 4X MIC. Chloramphenicol is a bacteriostatic agent against gram-negative bacilli. However, it can act bactericidal when used in high concentration or when used against highly susceptible organism.

Table 3: Minimum Bactericidal Concentrations (MBC) of Antibiotics for *R.ornithinolytica* isolate.

ANTIBIOTICS	DILUTION			MBC (µg/mL)	ACTIVITY
	MIC	2X MIC	4X MIC		
Gentamicin	+	+	+	>0.25	Bacteriostatic
Tetracyclines	+	+	+	>0.25	Bacteriostatic
Sulfafurazole	+	+	+	>1024	Bacteriostatic
Chloramphenicol	+	+	-	2	Bactericidal
Ciprofloxacin	+	+	+	>0.0625	Bacteriostatic
Rifampicin	+	+	+	>32	Bacteriostatic
Ampicilin	+	+	+	>256	Bacteriostatic

* + indicates growth, - indicates no growth

From the result of the checkerboard assay in Table 4, all the combinations of the antibiotics for both isolates show antagonism effects with the FICI value which is greater than 4. The highest FICI value recorded is 258 while the lowest FICI value recorded is 6 which indicates all the combinations has antagonistic effects. According to Ocampo et al., (2014), antagonism between antibiotics may help in developing treatment strategies specifically aimed at delaying the emergence of resistance, even though antagonistic drug combinations are currently discouraged in clinical settings. How to best implement combination therapy in clinical settings will largely depend on the benefits of synergism and the various nontrivial effects of antagonism.

Table 4: Double antibiotic combinations with FICI of each combination

Isolates	Combination			FICI Value	Interpretation
	No	**Antibiotic A**	**Antibiotic B**		
***E.faecalis* isolate**	1	Ampicilin	Gentamicin	66	Antagonism
	2	Ampicilin	Chloramphenicol	66	Antagonism
	3	Ampicilin	Tetracycline	66	Antagonism
***R.ornithinolytica* isolate**	1	Tetracycline	Ciprofloxacin	6	Antagonism
	2	Ampicilin	Chloramphenicol	130	Antagonism
	3	Ampicilin	Tetracycline	258	Antagonism

Conclusion

In conclusion, this study reveals that antagonism between antibiotics may help in developing treatment strategies specifically aimed at delaying the emergence of resistance, even though antagonistic drug combinations are currently discouraged in clinical settings. As the antibiotic resistance problem is growing which consequently creates health concern towards the public, this study indeed plays a crucial role nowadays.

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