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Synergetic effects of *Bacillus subtilis* and antibiotics against *Alcaligenes faecalis*

Nawfal H. Aldujaili[1], Ahmed Shandookh Hameed[2], Shaima R. Banoon[3]*

ABSTRACT

Aim: The use of beneficial bacteria in conjunction with antibiotics may decrease the effects of dysbiosis initiated by the antibiotics, and maximize the assistance of probiotics in the gut in terms of exclusion and immune stimulation. Samples of *Alcaligenes faecalis* from burns were nominated for detection and identification according to morphological and biochemical tests, the VITEK-2 Compact System, and molecular sequencing by 16S rRNA. **Materials and Methods:** Antibiotic susceptibility was ascertained by determining the inhibition length of *A. faecalis* against three antibiotics from varied classes (based on the VITEK 2), which consequently showed resistance to ceftriaxone (10 mm), high resistance to trimethoprim–sulfamethoxazole (SXT) (8 mm), and sensitivity to imipenem (20 mm). The sensitivity of *A. faecalis* to *Enterococcus faecium*, *Streptococcus thermophilus*, and *Bacillus subtilis* was determined using Mueller-Hinton agar (MHA) with wells of diffused agar. **Results:** *B. subtilis* performed best against *A. faecalis* isolates compared to *E. faecium* and *S. thermophilus*, where the area of inhibition was 28 mm. This study dealt with the enhancement of the inhibition zone of antibiotics (imipenem, SXT, and ceftriaxone) by *B. subtilis* against *A. faecalis.* **Conclusion:** The potentiation of antimicrobial activity was observed by imipenem in combination with *B. subtilis* (32 mm) and *B. subtilis* with ceftriaxone antibiotics (20 mm). The inhibition action was not affected by SXT (8 mm).

KEY WORDS: 16S rRNA, *Alcaligenes faecalis,* Antibiotics, *Bacillus subtilis,* Synergetic effects

INTRODUCTION

Emerging infectious diseases (EIDs) are caused by pathogens that (I) have increased in incidence, geography, or host range; (II) have changed pathogenesis; (III) have newly evolved; or (IV) have been newly discovered or recognized.[1] EIDs are influenced by complex factors including ecology, human behavior, globalization, microbial adaptation, and public health infrastructure. Antibiotic resistance is an example of a kind of microbial adaptation and change.[2,3]

Alcaligenes faecalis is a Gram-negative rod that is positive for oxidase, aerobic nonfermentative, and nonencapsulated and is a member of the family Alcaligenaceae. It exists in soil and water, as well as in the human intestine and hospital environments. Most infections caused by *A. faecalis* are opportunistic. UTIs, keratitis, endophthalmitis, skin and soft-tissue infections, bacteremia, meningitis, wound infections, and peritonitis in patients with peritoneal dialysis have been associated with *A. faecalis*, which causes infection infrequently. Most reported infections initiated by the organism have been nosocomial, occurred as a result of the contamination of hospital apparatus or liquids, and happened in immunocompromised humans.

Outbreaks of nosocomial pseudobacteremia associated with *A. faecalis* have occurred in neonatology and pediatric units.[4] Management can be challenging in such cases due to significant resistance to common antibiotics.[5-7] The use of beneficial bacteria in conjunction with antibiotics may decrease the effects of dysbiosis initiated by the antibiotics, and maximize the assistance of probiotics in the gut in terms of exclusion and immune stimulation.

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MATERIALS AND METHODS

Isolations and Identification of *A. faecalis*

A. faecalis samples from burns were isolated and examined for further analysis. All isolates were

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subjected to Gram staining, colony morphology and biochemical tests, and assessment by VITEK-2 Compact System.[8]

Molecular Identifications of *A. faecalis*

Genomic DNA was extracted and purified from isolates according to a previous study.[9] The PCR reaction mixture was 1 µL (10 µM) of primers 27F and 1492R and 5 µL genomic DNA; the volume was adjusted to 25 µL of nuclease-free water. PCR cycles were: 93°C, 3 min with 34 cycles; 31 s at 93°C; 61 s at 55°C; 100s at 72°C; and 3 min at 72°C. Separation of PCR fragments was conducted with 1.3% agarose, and 16S rDNA was then sequenced.[9]

The 16S rRNA gene was subjected to nucleotide sequencing with an automated DNA sequencing machine at the Bio-Service Unit of the National Science and Technology Development Agency. The 16S rRNA gene nucleotide sequences were aligned with the nucleotide sequences of strains retrieved from GenBank using the software CLUSTAL X (version 1.82). Mega6 was used for the overall editing and analysis. Nucleotide sequences were compared for similarity to the nucleotide sequences in NCBl. Nucleotide sequences were accomplished using the BLASTN tool against the nucleotide sequence database.[10-12]

Antimicrobial Sensitivity Test

Antibiotic sensitivity of *A. faecalis* was evaluated through the disk diffusion method with antibiotic disks (Bauer *et al*., 1966) (Kiehlbauch *et al*., 2000). Bacterial suspensions were inoculated onto Mueller-Hinton agar (MHA). Imipenem (10 µg), ceftriaxone (30 µg), and trimethoprim–sulfamethoxazole (SXT) (1.25/23.75 µg) were placed on the MHA and incubated at 37°C for 18 h.

Probiotic Strains

Probiotic strains (*E. faecium*, *S. thermophilus*, and *Bacillus subtilis*) obtained from a commercial pharmacy were suspended in 5 mL of suitable media and conditions and kept at 37 °C for 24 h.

Susceptibility of Tested Strains

Wells of MHA were occupied with a single concentration and the plates were incubated for 18 h. Similar experiments were repeated with using a disk soaked with compounds for 24 h. The experiment was monitored the following day, and outcomes were equated with inhibition action of the disk antibiotic.

Susceptibility Test of Combination Antibiotic and *B. subtilis*

The synergistic action of combined *B. subtilis* and antibiotic was assessed through DDM test (Kiehlbauch *et al*., 2000). *B. subtilis* inoculum was arranged by inoculating with BHI at 37°C for 18 h. The MHA Petri dishes were planted with antibiotic disks saturated with *B. subtilis* with the antibiotic disk as the control. MHA Petri dishes were incubated at 4°C for 1 h. The MHA Petri dishes were incubated at 37°C for 18 h and the inhibition diameter was measured.

Determination of MIC

MICs were measured through microdilution assay with information from CLSI. Dilutions of *A. faecalis* (0.5−0.0321 µg/mL) were primed by combining ordinary antibiotic solution in the MHB. *A. faecalis* inoculum 100 µL (5×10^5 CFU/mL) was mixed with 100 µL of *B. subtilis* in the wells. The final solution of each well was 200 µL. The microtiter was maintained at 37°C for18 h. (CLSI, 2018).

RESULTS AND DISCUSSION

Identification Using VITEK2

A. faecalis was primarily identified according to its morphological properties. On blood agar, colonies are white, smooth, convex, and glistening, and tend to spread at the outer border of the colonies. It is oxidase-positive, citrate-positive, and catalase-positive. The isolates of *A. faecalis* were recognized by the VITEK 2 system with a probability of 91%.

16S rDNA Sequencing

Isolates were identified through 16sRNA sequencing. PCR showed one band with 1275 bp. Isolates were recognized with 100% certainty to be *A. faecalis*. The partial sequence was detected and registered in GenBank as an *A. faecalis* strain (KUFA3) under accession number (MN055689.1), as shown in Figure 1. This study diagnosed and isolated this pathogen for the first time from burns in Iraq.

Antibiotic Susceptibility Testing

Inhibition areas near antibiotic disks were identified with CLSI (2018). *A. faecalis* was screened against three antibiotics from different classes according to the results of the VITEK 2 system [Table 1].

A. faecalis showed [Figure 2] resistance to ceftriaxone (10 mm) and SXT (8 mm), sensitivity to imipenem (20 mm), and resistance to ceftriaxone (10 mm). Ceftriaxone is the most commonly recommended third-generation cephalosporin in health procedures.[13]

Probiotic Susceptibility Testing

The sensitivity of *A. faecalis* to *E. faecium*, *S. thermophilus*, and *B. subtilis* was assessed using MHI. *B. subtilis* inhibited (28 mm) *A. faecalis* more than the other strains [Table 2 and Figure 3].

```
>MN055689.1 Alcaligenes faecalis strain KUFA3 16S ribosomal RNA gene, partial sequence
TGCAGTCGAACGGCAGCGCGAGAGAGCTTGCTCTCTTGCGCGAGCGAGTGGCGGACGGGTGAGTAATATA
TCGGAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCCCTACGGGGG
AAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAGTTGGTGGGGTAAA
GGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCG
TGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTACCTCCTAATACGAGGT
ACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTG
CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCA
GGGCTCAACCTTGGAACTGCATTTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGT
GTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAATACTGAC
GCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCA
ACTAGCTGTTGGGGCCGTTAGGCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGG
TCGCAAGATTATAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGAT
GCAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAAAGCCGAAGAGATTTGGCCGTGCTCGCAAGA
GAACCGGAAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCCTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTGTCATTAGTTGCTACGCAAGACCACTCTAAGAGACTGCCGGTGACAACCGGAGGAA
GGTGGGGAAGAAGTCAGGTCTCATGGCCCTTAGGGGGAGGGCTTC
```

Figure 1: *Alcaligenes faecalis* strain (KUFA3) under accession number (MN055689.1) in GenBank

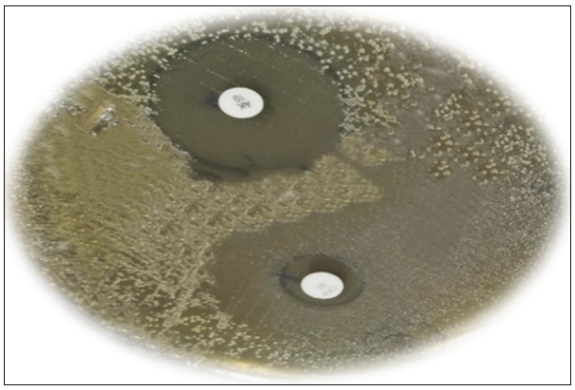

Figure 2: Susceptibility of *Alcaligenes faecalis* to imipenem antibiotic

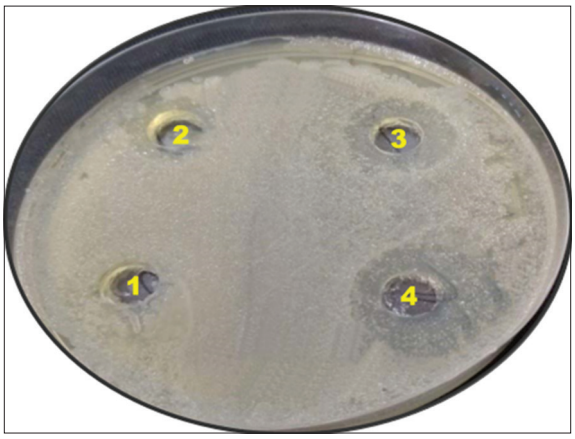

Figure 3: Sensitivity of *Alcaligenes faecalis* for control (1), *E. faecium* (2), *S. thermophilus* (3), and *Bacillus subtilis* (4)

Table 1: Antibiotic susceptibility of *Alcaligenes faecalis* against three types of antibiotic disk

	Antibiotics inhibition zones (mm)		
	IPM	**SXT**	**CRO**
Alcaligenes faecalis	20	8	10

IPM: Imipenem, SXT: Trimethoprim–sulfamethoxazole, CRO: Ceftriaxone

Sensitivity Test by Antibiotic and *B. subtilis*

The synergistic action of antibiotics and combinations of antibiotics and *B. subtilis* were estimated by DDM test, referring to the NCCLS [Table 3 and Figure 4].

The inhibition action of imipenem (IPM) was greater in combination with *B. subtilis* (20 mm), while the inhibition diameter of *B. subtilis* with SXT (8 mm) had an antagonist action. Ceftriaxone did not affect *A. faecalis* [Table 3].

Detection of MIC for *B. subtilis* Against Multidrug-resistant (MDR) *A. faecalis*

MIC was 1/2 to 1/32 µg/mL. *B. subtilis* showed the best performance, inhibiting *A. faecalis* at 0.125 µg/mL.

DISCUSSION

The problem of antimicrobial resistance (AMR) has been verified as being more complex and daunting than originally envisaged, compounded by general concerns such as urbanization, poverty, and global economic activity mechanisms. The solutions developed to address AMR must therefore be broadly focused. Over 80% of antibiotics are applied to livestock to promote growth or prevent infection, leading to the development of MDR, which can be diffused to humans through dangerous meat treatment procedures. These antibiotics may arrive to humans through water, leading to changes in the gut microbiome.[14] In general, 50% of antibiotic recommendations are unsuitable (in terms of indication, spectrum, dose, or duration).

The present study showed that the inhibition actions of *B. subtilis* were greater than those of antibiotics against MDR *A. faecalis.* Chauhan showed that the inhibition actions of probiotics were greater than those of antibiotics.[15] *B. subtilis* can secrete antimicrobials and assist in pathogen removal, and also has immunomodulation abilities.[16-18]

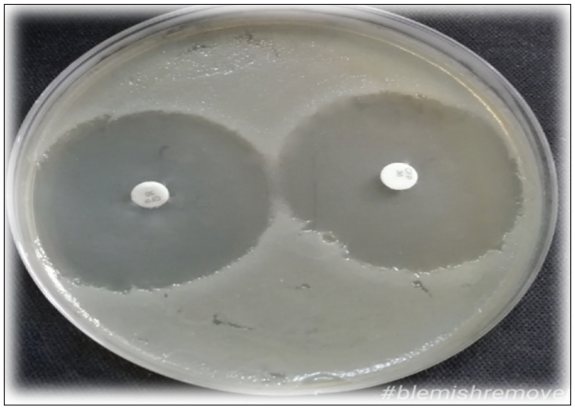

Figure 4: Sensitivity of *Bacillus subtilis* with imipenem against *Alcaligenes faecalis*

Table 2: *Enterococcus faecium*, *Streptococcus thermophilus*, and *Bacillus subtilis* activity against *multidrug-resistant* *Alcaligenes faecalis*

	Inhibition diameter (mm)		
	Enterococcus faecium	***Streptococcus thermophilus***	***Bacillus subtilis***
Alcaligenes faecalis	12	21	28

Table 3: Sensitivity of antibiotics, *Bacillus subtilis*, and combinations against *A. faecalis*

Drug	Inhibition zone (mm)
B. subtilis	28
Imipenem	20
Ceftriaxone	10
Trimethoprim–sulfamethoxazole	8
B. subtilis+IPM	32
B. subtilis+CRO	20
B. subtilis+ SXT	8

The effectiveness of a mixture of *B. subtilis* and antibiotics was shown to be better than that of antibiotics only.[19]

Synergism was detected in doxycycline and probiotics.[20,21] It was noted that the inhibition action with beneficial bacteria and aztreonam was greater than that of ciprofloxacin, meropenem, or amikacin alone. Synergistic action was also found between aztreonam and probiotics.[19,21] The type of antibiotic and beneficial bacteria is an essential factor in generating synergistic or antagonistic actions. When an antibiotic is used independently, the bacterial tolerance is superior to that of the combination. Probiotics increase diffusion and enhance sensitivity.[20,22]

The inhibition action of bacteria by means of a combination of gentamicin plus *L. salivarius* was improved in comparison with that of the combination of gentamicin plus *L. reuteri*.[15]

However, many outstanding issues regarding antimicrobial agents remain to be further investigated: (i) Strains within a species may be resistant, while others may be sensitive to a specific antimicrobial agent; (ii) a strain that is resistant to antimicrobial agents may have some cells that are sensitive; (iii) a strain can be resistant to one antimicrobial agent and sensitive to an analogous type of antimicrobial agent; (iv) bacterial cells of a strain creating single antimicrobials can be sensitive to other antimicrobials; and (v) bacterial spores of a strain that is sensitive to an antimicrobial agent may develop sensitivity propagation.[23]

The partial DNA sequencing of 16SrRNA is the most accurate, subtle, and precise technique for use in the identification of bacteria.[24,25]

B. subtilis is an aerobic, spore-forming bacterium that is able to survive transit through the acidic environment of the stomach and colonize the intestine even in the presence of antibiotics.[26] *Bacillus* spp. have an extensive active metabolome that includes antimicrobial metabolites such as bacteriolytic enzymes, bacteriocin, and antibiotics.[27,28]

AUTHORS' CONTRIBUTIONS

The authors contributed equally. All authors have read and agreed to the published version of the manuscript.

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