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Molecular detection and probiotic treatment for bacteria that cause diarrhea

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Abstract

Background: Misdiagnosis of the pathogen leads to serious consequences. Diarrhea is a worldwide health problem that kills 1.3 million people each year. Diarrhea can be induced by a variety of causes. However, infectious agents like bacteria are among the leading causes. Several resistant bacterial strains are emerging that tend to spread globally; hence, resistance of bacteria to antibiotics is a global health threat. The study aimed to ascertain the *Saccharomyces boulardii* filter's impact on lowering the virulence of the bacterial species that cause diarrhea and the extent of genetic affinity between those organisms.

Methods: 350 stool samples were investigated for all age groups who suffer from diarrhea, using biochemical and molecular methods.

Results: Biochemical diagnosis were 2.28% for *Salmonella* and 0.57% for *Enterobacter aerogenes*, while molecular diagnosis didn't record any percentage for the mentioned bacteria, therefore molecular test was a better way to properly diagnose bacterial species, the causes of diarrhea are bacteria, viruses, parasites, medications, and toxins. The molecular identification of isolates by PCR technique and sequencing of 16S rDNA gene indicated that 12 isolates belonged to the genera *Enterobacter cloacae*, *Cronobacter sakazakii*, and *Aeromonas hydrophila*. The study revealed that there is a difference in the rates of resistance of isolates to some antibiotics before and after exposing them to probiotics. Where the percentage of antibiotic resistance was recorded before exposure to probiotics were piperacilline (91.6%), cefotaxime/clavulanic acid (41.6%), and tetracycline (25%), while the percentage of antibiotic resistance after exposure to probiotics were (58.3%) piperacillin, (0%) cefotaxime/clavulanic acid, and (0%) tetracycline. Where the resistance of bacteria to antibiotics decreases after exposing them to probiotics.

Conclusion: The molecular diagnostic method is more accurate method to diagnose the bacterial species with complete accuracy. The *Saccharomyces boulardii* filter had an effective role in reducing or suppressing bacterial resistance against piperacillin, cefotaxime/clavulanic acid, and tetracycline, while it did not affect the bacterial resistance towards amoxicillin clavulanic acid.



Introduction

Diarrhea is a worldwide health problem that kills 1.3 million people each year, primarily newborns and young children [1]. Diarrhea is a clinical symptom of disrupted intestinal ion transport channel proteins, channels, physical and chemical barriers, resulting in water and electrolyte transport abnormalities in the digestive tract, furthermore, diarrhea can be a symptom of a variety of disorders [1]. Diarrhea is caused by Bile acid disturbance, bacterial infections, carbohydrate malabsorption. Although the death rate linked to diarrhea has decreased dramatically over the years, it is still one of the most prevalent reasons for pediatric emergency department visits, particularly in some low-income Asian and African nations [1]. Diarrhea can be induced by a variety of causes. However, infectious agents like bacteria are among the leading causes, particularly Gram-negative enteric bacterial pathogens [2].

Culture-based microbiological techniques are insufficient to identify and characterize the complex gut microbiome because some gut bacteria are unable to grow in culture [3]. Technology based on sequencing, in particular 16S rRNA-based metagenomic sequencing, has been developed. Some of these problems have been overcome, and microbiome research has evolved by overcoming some of these obstacles [3].

In addition, molecular methods are used to discover bacterial diversity and novel bacterial species [4]. Bacteria are currently being identified using a range of molecular approaches, including polymerase chain reaction (PCR) and the 16S rRNA sequencing technology [5]. Advanced 16S rRNA-based sequencing technology has aided in demonstrating the gut microbiome's vital role in human health [3].

Antibiotics are frequently used in the treatment of diarrhea caused by bacteria; the resistance is emerging as a result of the widespread and indiscriminate use of antibiotics. Several resistant strains are emerging that tend to spread globally [2]. Hence, resistance of bacteria to antibiotic is a global health threat [2].

The constant search for new antibiotics is imperative due to the emergence of antibiotic-resistant bacteria. Appropriate management is an important determinant of treatment success and additionally aids in prevent antibiotic resistance [6]. As well as using of probiotics which are living organisms promote health by preventing or treating infection [7].

One of the most important probiotics is *Saccharomyces boulardii*, also known as baker's yeast, which is a subtype of *Saccharomyces cerevisiae*. While *Saccharomyces boulardii* is generally a non-pathogenic fungus [7], research shows that it can aid in the treatment of diarrhea [7, 8], specifically acute watery

diarrhea [7]. Also, this fungus has been used in probiotics to prevent antibiotic-associated diarrhea [9].

Methods

Collection of samples

A total of 350 stool samples from patients with diarrhea of both sexes and for all age groups were collected at Al- Shatrah General Hospital, the Public Health Laboratory, and Bint Al Huda Hospital in Thi-Qar province /Iraq for the period between October 2022 and January 2023. Stool samples were collected straight into a sterile tube containing peptone water and quickly transported to Al- Shatrah General Hospital's bacteriology laboratory by using a cool box(-20 °C).

Isolation of bacteria

Fecal samples were incubated on peptone water for 24 hours at 37 degrees Celsius, then cultured directly on MacConkey agar and sub-cultured on tetrathionate broth with iodine solution (selective for *salmonella*) for 24 hours at 37 degrees Celsius, and transferred from tetrathionate broth into xylose lysine deoxycholate XLD medium and incubated at 37 degrees Celsius overnight (18–24 hours). After that, the plates made with MacConkey and XLD were examined. The diagnosis was subsequently established utilizing biochemical confirmation. Also, biochemical tests were conducted to look for bacteria other than *Salmonella* [10].

Identification of the isolates by API 20 E System

Analytical profile index for Enterobacteriaceae test is used clinically for the fast identification of Enterobacteriaceae, according to (BioMerieux, France). This test consists of a strip with 20 tiny tubes with an upper and lower opening (cupule and tube) that contain dried material and constitute a biochemical test. Color changes occur in the tubes during incubation or after the reagents are added [10].

Genomic DNA extraction

The genomic DNA of 12 bacterial isolates was extracted from cultures cultured in brain-heart infusion broth for 18-24 hours according to the manufacturer's instructions, where the Geneaid Genomic DNA Purification Kit (UK) was used. Gel electrophoresis was used to detect the isolated genomic DNA, which was stored at -20°C for future use [11].

Amplification of 16 rRNA gene by PCR for bacterial identification

Polymerase chain reaction was used to amplify the gene encoding 16S rRNA using universal primers 27F (5-AGAGTTTGATCCTGGC TCAG-3) and 1492R (5-GGTTACCTTGTTA CGACTT-3). Twenty-five microliters (25µl) PCR reaction mixture contained

13.5µl of master mix (Bioneer / South Korea), 3.5µl of DNA template, 1 µl each of forward and reverse primers, and 6µl of nuclease-free water. Polymerase chain reaction amplification settings were as follows: an initial denaturation phase of 96°C for 5 minutes, followed by 35 cycles of 96°C for 30 seconds, annealing at 56°C for 30 seconds, elongation at 72°C for 1 minutes, and a final extension step at 72°C for 10 minutes [12], [13]. Gel electrophoresis with a DNA ladder (Bioneer / Korea) was used to measure the PCR results.

Sequencing PCR products of 16S rRNA

The amplified 16S rRNA gene products were forwarded to the Macrogen company in South Korea for purification and sequencing. The raw 16S rRNA gene sequences of 12 bacterial isolates were edited with Bioedit and matched with NCBI nucleotide sequence databases using BLAST tools "http://www.ncbi.nlm.nih.gov" to estimate sequence homology and identify bacterial isolates. The program of Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA-7) was used to create the phylogenetic tree [14].

Susceptibility Test

The disc diffusion method was used to test resistance of bacteria to the included antibiotics in this study. Some antibiotic disks have been used to test resistance of bacteria that cause diarrhea [15].

Antibiotic disks were used in the present study: amoxicillin-clavulanic acid (AMC), cefotaxime/clavulanic acid (CEC), tetracycline (TE), piperacilline (PI), amikacin (AK).

Exposure of diarrhea-causing bacteria to a *Saccharomyces boulardii* filter

Saccharomyces boulardii Yeast was activated in Sabouraud Dextrose Agar and incubated at 37°C for 24–48 hours, then it was transferred to peptone water and incubated at 37°C for 5 days, after that the yeast suspension was centrifuged and filtered, and a filter was utilized by combining 4 ml of the filtrate with 16 ml of Muller-Hinton agar and pouring it into the dish [16]. Then, bacteria were cultured from a fresh culture at 24 hours of age, streaking was applied by using a cotton swab on the dish, and it was incubated for 24 hours. Then, a swab of yeast filter exposed bacteria was obtained after the bacterial suspension was adjusted to 0.5 McFarland standards and distributed on a Muller Hinton agar dish. Finally, susceptibility test of the exposed bacteria to the included antibiotics was done on it. The above method is a modification of [17].

Results

Identification of bacteria with different methods

A) Identification by using API 20 E system

After routine morphological and biochemical was performed, all the isolates have been tested by the Analytical profile index for Enterobacteriaceae test (API 20E) for confirmation of the identification. The results showed that of 15 isolates of bacteria were *Enterobacter cloacae* 10(2.85%), *Cronobacter sakazakii* 1(0.28%), *Aeromonas hydrophila* 1(0.28%), *Enterobacter aerogenes* 2(0.57%), *Salmonella* 1(2.28%), Figure(1 and 2) and Table (2) .



Figure 1: Results of examination by the API 20E System for *Salmonella* spp.

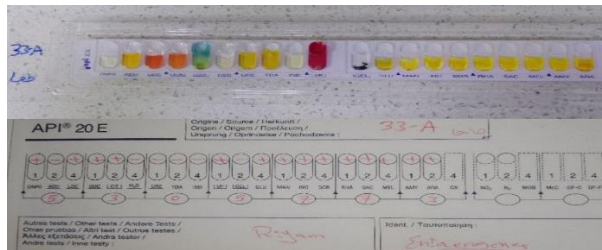


Figure 2: Result of examination by the API 20E System for *Enterobacter aerogenes*.

B) Amplification of 16S rRNA gene

A total of 15 bacterial strains DNA subjected to PCR for 16S rRNA gene amplification figure (3) and sequences table (1). The result was only 12 isolates that represented the bacteria that are of interest to us, as the percentage of *Enterobacter cloacae* 9(2.57%), *Cronobacter sakazakii* 2(0.57%) and 1(0.28%) *Aeromonas hydrophila* tables (2).

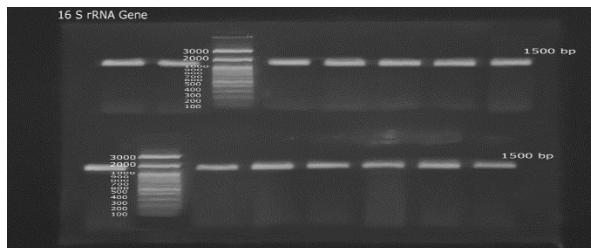


Figure 3: Agarose gel electrophoresis for PCR products of 16S rRNA gene product size (1500 bp) (1.5% agarose, TBE , 70 Volt , 85 mA).

Samples No.	Accession Number In this Study (Sequence size)	16 SrRNA Gene fragment size	Organisms
105	LC765980	(1373 bp)	<i>Enterobacter cloacae</i>
8	LC765981	(1427 bp)	<i>Enterobacter cloacae</i>
18	LC765982	(1427 bp)	<i>Enterobacter cloacae</i>
51	LC765983	(1091 bp)	<i>Enterobacter cloacae</i>
36	LC765984	(1380 bp)	<i>Enterobacter cloacae</i>
39	LC765985	(1423 bp)	<i>Enterobacter cloacae</i>
99	LC765986	(1356 bp)	<i>Enterobacter cloacae</i>
126	LC765987	(1375 bp)	<i>Enterobacter cloacae</i>
76	LC765988	(1379 bp)	<i>Enterobacter cloacae</i>
103	LC765989	(1297 bp)	<i>Cronobacter sakazakii</i>
122	LC765990	(1296 bp)	<i>Cronobacter sakazakii</i>
112	LC765991	(1473 bp)	<i>Aeromonas hydrophila</i>

Table 1: Accession Numbers Submitted at Gene Bank (NCBI) in this study.

A comparison between biochemical and molecular diagnosis

Bacteria were diagnosed using biochemical and molecular diagnostics, and the results of biochemical diagnosis were as follows: *Enterobacter cloacae* 10 (2.85%), *Cronobacter sakazakii* 1 (0.28%), *Aeromonas hydrophila* 1 (0.28%), *Enterobacter aerogenes* 2 (0.57%), and *Salmonella* 1 (2.28%). As for the results of the molecular diagnosis were *Enterobacter cloacae* 9 (2.57%), *Cronobacter sakazakii* 2 (0.57%), and *Aeromonas hydrophila* 1 (0.28%), while this diagnosis didn't record any percentage of *Salmonella* or *Enterobacter aerogenes* table (2).

Species	Biochemical diagnosis		Molecular Diagnostics	
	Number	Percentage%	Number	Percentage%
<i>Enterobacter cloacae</i>	10	2.85	9	2.57
<i>Cronobacter sakazakii</i>	1	0.28	2	0.57
<i>Aeromonas hydrophila</i>	1	0.28	1	0.28
<i>Enterobacter aerogenes</i>	2	0.57	0	0.0
<i>Salmonella</i>	1	2.28	0	0.0

Table 2: Comparison of biochemical and molecular diagnostic outcomes in the identification of bacteria that cause diarrhea

Comparative Phylogenetic Genome Analysis

The phylogenetic tree includes three genera from the same family, enterobacteriaceae; thus, the differences between them are not vast. These genera are *Enterobacter*, *Cronobacter*, and *Aeromonas*.

Enterobacter appeared in four groups, all of which were diagnosed as *Enterobacter cloacae*, as the species (LC765985 *Enterobacter cloacae*, LC765983 *Enterobacter cloacae*, LC765986 *Enterobacter cloacae*) belonging to the group that is located below the reference isolate(NR 102794-2 *Enterobacter cloacae* ATCC) is the closest to it from the rest of the other groups, and they are more genetically close to each other than the closeness that exists between the four species (LC765981 *Enterobacter cloacae*, LC765982 *Enterobacter cloacae*, LC765988 *Enterobacter cloacae*, LC765984 *Enterobacter cloacae*) at the top of phylogenetic tree.

As for the types (LC765990 *Cronobacter sakazakii* and LC765989 *Cronobacter sakazakii*), they are similar to each other. The genus *Aeromonas hydrophila* is very similar to the reference isolate (NR043638-1 *Aeromonas hydrophila* CCM). It appears in the phylogenetic tree that *Cronobacter sakazakii* is more closely related to *Enterobacter cloacae* than *Aeromonas hydrophila*. Figure (4).

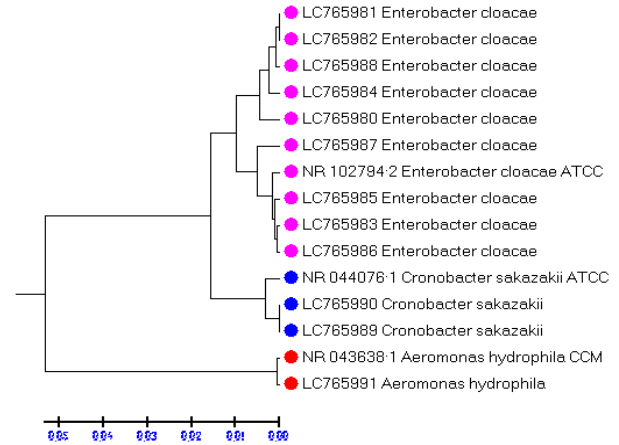


Figure 4: A phylogenetic tree (*Enterobacter cloacae*, *Cronobacter sakazakii*, *Aeromonas hydrophila*)

Effect of *Saccharomyces boulardii* filter on resistance of bacteria to antibiotics

Bacteria were tested for antibiotic susceptibility before exposure to the probiotic filter and after exposure to the probiotic filter. Before exposure to probiotics (*Saccharomyces boulardii* yeast filtre), the resistance of diarrhea-causing bacteria to amoxicillin-clavulanic acid was 100%, piperacilline 91.6%, cefotaxime-clavulanic acid 41.6%, and tetracycline 25%. The percentage of bacterial resistance to antibiotics except for amoxicillin-clavulanic acid differed after exposing them to *Saccharomyces boulardii* yeast filter where the resistance was 58.3% piperacilline , 0% cefotaxime-clavulanic acid , and 0% tetracycline figure (5, and 6) and table (3) .

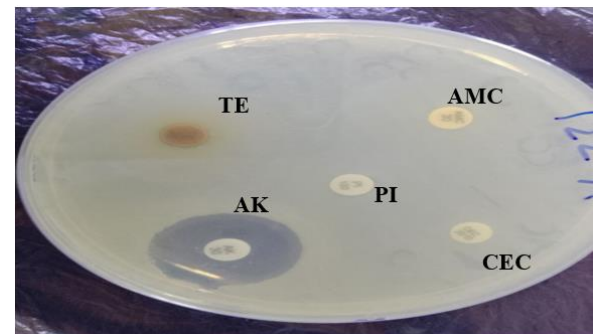


Figure 5: Susceptibility test before exposing of bacteria to probiotic

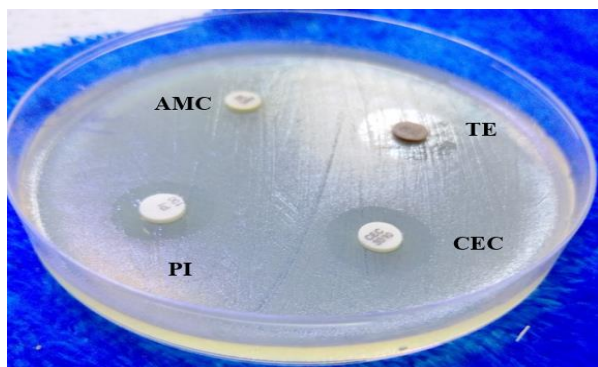


Figure 6: Susceptibility test after exposing bacteria to probiotic.

Antibiotic	susceptibility test before exposure to probiotics			susceptibility test after exposure to probiotics		
	R%	S%	I%	R%	S%	I%
AMC	100	0	0	100	0	0
PI	91.6	0	8.3	58.3	16.6	25
CEC	41.6	25	33.3	0	50	50
TE	25	41.6	33.3	0	66.6	33.3

Table 3: Percentages of isolates resistance to antibiotics before and after exposing them to probiotic.

Discussion

Diarrhea is a widespread health concern that kills and causes harm to thousands of people worldwide. Infectious and non-infectious factors can both cause diarrhea; among the infectious agents, enteric bacterial pathogens play critical roles in the onset or severity of diarrhea [2]. *Salmonella* and *Enterobacter aerogenes* were detected by biochemical diagnosis, but those bacteria weren't noted by molecular diagnosis. The diagnosis of biochemical tests is shaded because they assess gene activity, which is subject to alteration or malfunction based on the conditions that the bacteria are subjected to, making it difficult to identify the proper bacterial type [5]. However, 16S rDNA gene is commonly used to identify bacteria and is regarded as the best tool for studying bacterial phylogeny and taxonomy due to its presence in all bacteria, the function of the 16S rDNA gene has not changed over time, and the length of the 16S rDNA gene is appropriate, in addition to accurate and reliable analysis that resulted in the detection of new bacterial strains when compared to conventional techniques[13]. The group of species (*LC765985 Enterobacter cloacae*, *LC765983 Enterobacter cloacae*, and *LC765986 Enterobacter cloacae*) located at the bottom of the reference isolate (*NR 102794-2 Enterobacter cloacae ATCC*) is farther from the rest of the groups than the group of species at the top of Figure 4 (*LC765981 Enterobacter cloacae*, *LC765982 Enterobacter cloacae*, *LC765988 Enterobacter cloacae*, and *LC765984 Enterobacter cloacae*). The reason may be due to the different places of residence of the patients from whom the bacteria were isolated. It appears in the phylogenetic tree that *Cronobacter sakazakii* is more

closely related to *Enterobacter cloacae* than *Aeromonas hydrophila*. The reason is due to the fact that *Cronobacter* was a species belonging to *Enterobacter* from 1980 to 2007, when it was named by Japanese bacteriologist " Riichi Sakazakii". In 2007, based on recently revised taxonomy, a new genus called *Cronobacter* was created, which contains seven species, including *Cronobacter sakazakii* [18]. The present study showed no changes about resistance of bacteria to amoxicillin-clavulanic acid (AMC) before and after exposing it to probiotic. The bacteria's resistance to amoxicillin-clavulanic acid may be brought on by the indiscriminate and frequent use of this antibiotic, or it may be brought on by bacterial evolution and mutations in genes that increase its resistance. The bacteria's resistance to amoxicillin-clavulanic acid agrees with [19].

As for piperacilline (PI), cefotaxime-clavulanic acid(CEC), and tetracycline (TE) antibiotics, the resistance percentage lowered after probiotic treatment, the reason returns, and as studies have suggested, probiotic bacteria and yeast can act as reservoirs for antibiotic resistance genes [20, 21]. Inappropriate antibiotic use promotes antimicrobial resistance, leading to increased treatment costs and a prolonged hospital stay. Therefore, alternatives were resorted to, which are probiotics [22]. The establishment and viability of probiotics in the intestinal lumen of host organisms is the foundation for human probiotic administration due to the presence of bile, gastrointestinal juices, and digestive enzymes in the stomach, probiotic yeast has a better chance of surviving there. Because of its extraordinary antibacterial capabilities, it can be given to patients as a substitute for antibiotics. Consuming probiotics can help lessen the pathogenicity of harmful bacteria found in the human stomach [20].

Author Contributions

Riyam Mohsen Dakheel Al-Hilali: Preparing materials, Funding acquisition, Data curation, explaining the findings, and article writing.

Hanaa Daaj Khalaf Al-Mozan: the research article proposal, experiment design, Statistical analysis and review & editing.

Competing Interests

The authors declared that there were no conflict of interest.

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