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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* filtrate 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 diagnoses were 0.28% for *Salmonella* and 0.57% for *Enterobacter aerogenes*, while molecular diagnosis did not 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 rRNA gene indicated that 12 isolates belonged to the species *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 piperacillin (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 a more accurate approach for bacterial species identification in this study. The *Saccharomyces boulardii* filtrate 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, particularly 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; 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 (4 °C).

Isolation of bacteria

Fecal samples were incubated in peptone water for 24 hours at 37 °C, then cultured directly on MacConkey agar and sub-cultured on tetrathionate broth with iodine solution (selective for *salmonella*) for 24 hours at 37 °C, and transferred from tetrathionate broth into xylose lysine deoxycholate XLD medium and incubated at 37 °C 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 (bioMérieux, 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 16S rRNA gene by PCR for bacterial identification

Polymerase chain reaction was used to amplify the gene encoding 16S rRNA using universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3). 25 µL PCR reaction mixture contained 13.5 µL of master mix (Bioneer / South Korea), 3.5 µL of DNA template, 1 µL each of

forward and reverse primers, and 6 µL 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 minute, 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 MEGA X (version 10) 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: AMC, CEC, TE, and piperacillin (PI).

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

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 the filtrate was utilized by combining 4 mL of the filtrate with 16 mL of Mueller–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 filtrate exposed to bacteria was obtained after the bacterial suspension was adjusted to 0.5 McFarland standards and distributed on a Mueller 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 out of 350 stool samples, 15 isolates were identified 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 (0.28%) (Figure 1 and 2; Table 2).

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

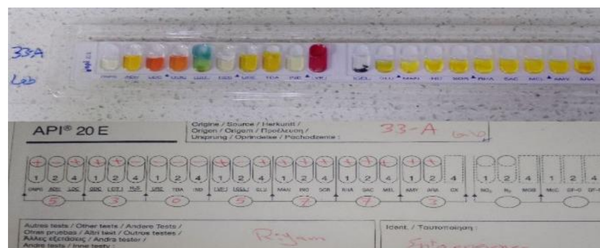

Figure 2: Result of examination by the API 20E System for *Enterobacter aerogenes.*

B) Amplification of 16S rRNA gene

A total of 12 bacterial isolates DNA were 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 *Salmonella* 1(0.28%) *Aeromonas hydrophila* Table (2).

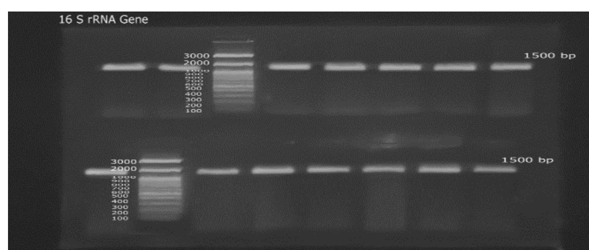

Figure 3: Agarose gel electrophoresis for PCR products of 16S rRNA gene product size (1500 bp) (1.5% agarose, TBE, 70 V, 85 mA).

Samples No.	Accession Number In this Study	16S rRNA Gene	Organisms

	(Sequence size)	fragment size	
105	LC765980	(1373 bp)	*Enterobacter cloacae*
8	LC765981	(1427 bp)	*Enterobacter cloacae*
18	LC765982	(1427 bp)	*Enterobacter cloacae*
31	LC765983	(1091 bp)	*Enterobacter cloacae*
36	LC765984	(1380 bp)	*Enterobacter cloacae*
39	LC765985	(1423 bp)	*Enterobacter cloacae*
99	LC765986	(1356 bp)	*Enterobacter cloacae*
126	LC765987	(1375 bp)	*Enterobacter cloacae*
76	LC765988	(1379 bp)	*Enterobacter cloacae*
103	LC765989	(1297 bp)	*Cronobacter sakazakii*
122	LC765990	(1296 bp)	*Cronobacter sakazakii*
112	LC765991	(1473 bp)	*Aeromonas hydrophila*

Table 1: Accession Numbers Submitted at GeneBank (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 (0.28%). 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%
Enterobacter cloacae	10	2.85	9	2.57
Cronobacter sakazakii	1	0.28	2	0.57
Aeromonas hydrophila	1	0.28	1	0.28
Enterobacter aerogenes	2	0.57	0	0.0
Salmonella	1	0.28	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: *Enterobacter*, *Cronobacter*, and *Aeromonas*. Thus, the differences between them are not vast.

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).

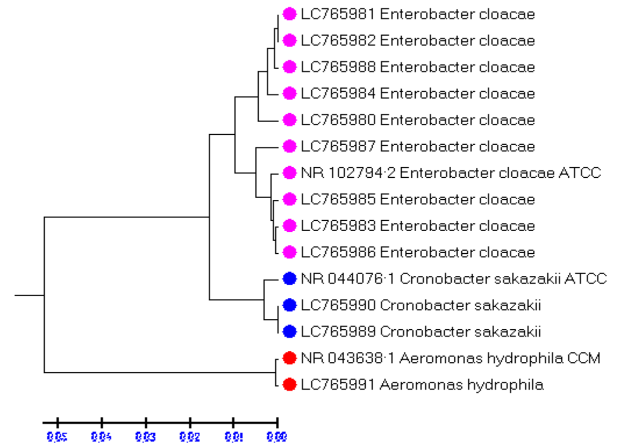

Figure 4: A phylogenetic tree (*Enterobacter cloacae, Cronobacter sakazakii, Aeromonas hydrophila*)

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

Bacteria were tested for antibiotic susceptibility before exposure to the probiotic filtrate and after exposure to the probiotic filtrate. Before exposure to probiotics (*Saccharomyces boulardii* yeast filtrate), the resistance of diarrhea-causing bacteria to amoxicillin-clavulanic acid was 100%, piperacillin 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 filtrate where the resistance was 58.3% piperacillin, 0% cefotaxime-clavulanic acid, and 0% tetracycline figure (5, and 6) and Table (3).

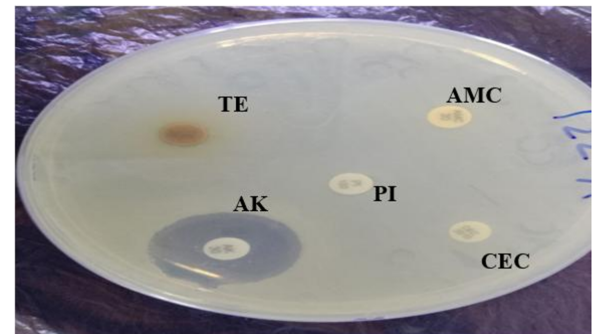

Figure 5: Susceptibility test before exposing bacteria to probiotic

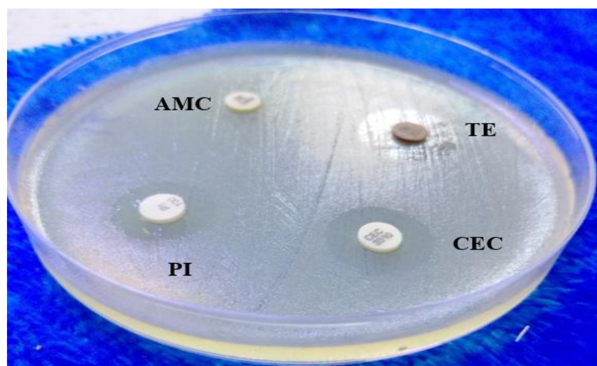

Figure 6: Susceptibility test after exposing bacteria to probiotic.

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	16.7	25
CEC	41.6	25	33.3	0	50	50
TE	25	41.6	33.3	0	66.7	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 rRNA 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 rRNA gene has not changed over time, and the length of the 16S rRNA 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 piperacillin (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 conflicts of interest.

References

1. Li Y, Xia S, Jiang X, Feng C, Gong S, Ma J, Fang Z, Yin J, Yin Y. Gut microbiota and diarrhea: an updated review. Frontiers in cellular and infection microbiology, (2021); 11: 625210.

2. Beyene AM, Gezachew M, Mengesha D, Yousef A, Gelaw B. Prevalence and drug resistance patterns of Gram-negative enteric bacterial pathogens from diarrheic patients in Ethiopia: A systematic review and meta-analysis. Plos one, (2022); 17(3): e0265271.
3. Shahi SK, Zarei K, Guseva NV, Mangalam AK. Microbiota analysis using two-step PCR and next-generation 16S rRNA gene sequencing. JoVE (Journal of Visualized Experiments), (2019); (152):e59980.
4. Li H, Zhong Q, Wirth S, Wang W, Hao Y, Wu S, Zou H, Li W, Wang G. Diversity of autochthonous bacterial communities in the intestinal mucosa of grass carp (C tenopharyngodon idellus)(V alenciennes) determined by culture-dependent and culture-independent techniques. Aquaculture Research, (2015); 46(10): 2344-59.
5. Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S, Nadarajan R, Brodie EL, Lynch SV. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. PloS one, (2015); 10(2): e0117617.
6. Kato H, Hamada Y. Amikacin Therapy in Japanese Pediatric Patients: Narrative Review. International Journal of Environmental Research and Public Health, (2022); 19(4): 1972.
7. Idea SC, Nazar B, Hall S. Role of Probiotics Saccharomyces Boulardii in The Management of Acute Watery Diarrheas in Children. Journal of Medicine, Physiology and Biophysics, (2018); 43: 81-84.
8. Al-zaidi RE, Al-Mozan HD, Alrikabi NJ. Eukaryotic probiotic Saccharomyces boulardii application in clinical trails: a review. International Journal of Pharmaceutical Quality Assurance. 2020;11(1):163-8.
9. Atıcı S, Soysal A, Cerit KK, Yılmaz Ş, Aksu B, Kıyan G, Bakır M. Catheter-related Saccharomyces cerevisiae Fungemia Following Saccharomyces boulardii Probiotic Treatment: In a child in intensive care unit and review of the literature. Medical mycology case reports, (2017); 15: 33-5.
10. AL-Lami WA, Mezal EH. Detection some Antimicrobial Resistance Genes in Salmonella sp. Isolated from Chicken Meat. University of Thi-Qar Journal of Science, (2021); 8(1): 90-100.
11. Hussein KR, Al-Temimy N, Widaa QH, Nasser MH. *Eschershia coli* integrons and cfr, procfr genes identified from samples of autistic children's stools. University of Thi-Qar Journal of Science, (2023); 10(1 (SI)): 1020.
12. Miyoshi T, Iwatsuki T, Naganuma T. Phylogenetic characterization of 16S rRNA gene clones from deep-groundwater microorganisms that pass through 0.2-micrometer-pore-size filters. Applied and environmental microbiology, (2005); 71(2): 1084-8.
13. ALYOUSIF NA. Distribution, occurrence and molecular characterization of Bacillus related species isolated from different soil in Basrah Province, Iraq. Biodiversitas Journal of Biological Diversity, (2022); 23(2): 679-686.
14. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution, (2018); 35(6): 1547.
15. Humphries R, Bobenchik AM, Hindler JA, Schuetz AN. Overview of changes to the clinical and laboratory standards institute performance standards for antimicrobial susceptibility testing, M100. Journal of clinical microbiology, (2021); 59(12): 10-128.
16. Qazangi,M.O.M. Practical experiments in microbiology. 1th Edition, Iraq, University of Baghdad – Science of Agriculture, (2017): 1-138.
17. Al-Mozan, H.D.K. Molecular detection of enterotoxins genes in Staphylococcus aureus isolated from clinical and dairy samples. A thesis submitted to the college of science/ University of Basrah, (2021):1-131.
18. khalaf Al-aawadi K. Phenotypic and Molecular Identification of Cronobacter sakazakii Isolated from Clinical Specimens in Thi-Qar Province. University of Thi-Qar Journal of Science, (2022); 9(1): 62-7.
19. Qin, X., Yang, M., Cai, H., Liu, Y., Gorris, L., Aslam, M.Z., Jia, K., Sun, T., Wang, X. and Dong, Q., 2022. Antibiotic resistance of Salmonella Typhimurium monophasic variant 1, 4,[5], 12: i:-in China: a systematic review and meta-analysis. Antibiotics, 11(4), p.532.
20. Abid R, Waseem H, Ali J, Ghazanfar S, Muhammad Ali G, Elasbali AM, Alharethi SH. Probiotic yeast Saccharomyces: Back to nature to improve human health. Journal of Fungi, (2022); 8(5): 444.
21. Czerucka D, Piche T, Rampal P. yeast as probiotics– Saccharomyces boulardii. Alimentary pharmacology & therapeutics, (2007); 26(6): 767-78.
22. Pais P, Almeida V, Yılmaz M, Teixeira MC. Saccharomyces boulardii: what makes it tick as successful probiotic?. Journal of Fungi, (2020); 6(2): 78.

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