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Research Article

Comparison between the Effect of Secondary Metabolic Products of Nutrient Medium and Modified Medium of Yeast *Saccharomyces boulardii* Against Different Bacterial Species

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Abstract
Background: *Saccharomyces boulardii* yeast are characterized by their resistance to the acidic environment in the stomach and a high ability to survive in these conditions. *S. boulardii* produces metabolites that make it able to resist acidic environments, act as an anti-inflammatory, and stimulate the immune system, as well as its effectiveness against intestinal bacterial pathogens. Therefore, this study aimed to determine the effect of secondary metabolic products of the nutrient media and the modified media of the yeast *S. boulardii* against different bacterial species.
Methods: Yeast *S. boulardii* was obtained from the markets of Anbar city. The strains of the bacterial species isolated from burn patients were provided by the microbiology laboratory at the burn center at Ramadi Hospital, Anbar, Iraq. Bacterial strains *Enterococcus faecalis, Staphylococcus epidermis, Escherichia coli, Serratia marcescens*, and *Pseudomonas aeruginosa* were grown in nutrient agar media for 24 hours and surface spread in petri plate. The antibacterial activities of *S. boulardii* are tested by the agar diffusion method.
Results: In this study, the results indicate that *S. boulardii* yeast when grown in the modified media as the incubation period increases, the active substances that have positive antimicrobial activity against the five bacterial species increase. The results showed that the *E. coli, P. aeruginosa*, and *S. marcescens* bacteria are the most affected by *S. boulardii* yeast filtrate due to the modified media, especially at the incubation period of 168 hours, where the inhibition zone for these bacteria was 19 and 18 mm.
Conclusion: The study concluded that *S. boulardii* yeast proved effective in inhibiting five types of bacteria. In addition, the microbial activity of *S. boulardii* yeast against these bacteria was clear and high when grown in the modified media compared to its growth in the nutrient (natural) media.

Keywords: Secondary Metabolic, Nutrient Medium, Modified Medium, Yeast, *Saccharomyces boulardii*, Bacterial Species.

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1. Introduction

Yeasts are microorganisms and belong to the fungi of the genus Saccharomyces, the most important type of which is *Saccharomyces boulardii*, which is one of the strains of probiotics that have been extensively studied and are used as a preventive and therapeutic agent in most countries of the world (SOUZA et al., 2021; Hadjimbei et al., 2020). Yeasts and their metabolic products are considered one of the world's most important methods of food processing and preservation. They are also used in the fermentation and bread processing process. In addition, yeast metabolic products are important in many commercial fields, including industrial enzymes, medicines, foods, and beverages (Tao et al., 2023; Moslehi-Jenabian et al., 2010). Societies whose populations seek a good, healthy life include probiotics such as yeast *S. boulardii* in their daily nutritional routine. This is because this type of yeast is safe for use in the food and pharmaceutical industry and has proven its ability to preserve the digestive system and protect it from inflammatory processes (Egea et al., 2023; Fu et al., 2023). Also, yeast *S. boulardii* can regulate the antioxidant activity and the antimicrobial activity of intestinal pathogens (Egea et al., 2023; McFarland, 2017; Bagherpour et al., 2018).

Probiotics are defined as living microorganisms that provide health benefits to the host (humans) when consumed in specific and sufficient quantities (Goktas et al., 2021). The strains belonging to the probiotics, such as *S. boulardii*, are characterized by their resistance to the acidic environment in the stomach and a high ability to survive in these conditions. In addition, they cannot be inhibited by bacterial antibiotics and have the ability to harmonize with natural microflora species (Goktas et al., 2021; Tomičić, 2016). In addition, *S. boulardii* yeast has been widely used to treat many diseases as an oral probiotic, including diarrhea resulting from bacterial contamination, colitis, cholera, and other diseases caused by intestinal bacteria (Goktas et al., 2021; Moslehi-Jenabian et al., 2010; Kaźmierczak-Siedlecka et al., 2020). Due to the importance of *S. boulardii* yeast, many studies have focused on the properties of this type of yeast, indicating that *S. boulardii* produces metabolites that make it able to resist acidic environments, act as an anti-inflammatory and stimulate the immune system, as well as its effectiveness against intestinal bacterial pathogens (Goktas et al., 2021; Durmusoglu et al., 2023). Moreover, recent studies have proven that *S. boulardii* produces biologically active secondary metabolites and possesses antimicrobial properties where *S. boulardii* affects the biofilms of species of bacteria pathogenic and resistant to many antibiotics such as *Escherichia coli*, *Serratia marcescens*, and *Pseudomonas aeruginosa* (Isayenko et al., 2019; Fu et al., 2023).

Secondary metabolites produced by *S. boulardii* as antibacterial substances are of great interest for developing new antibacterial preparations or drugs due to their inhibitory activity against multi-resistant pathogenic bacteria. Therefore, the current study aims to determine the effect of the secondary metabolic products of *S. boulardii* yeast in the nutrient medium and the modified medium against some types of bacteria and compare the results to the antibacterial effect of the metabolic of this type of yeast in a different media. In addition, this can be crucial because studies focusing on these topics are rare.

2. Materials and Methods

2.1 Sample Collection

Samples of fresh fruits (grapes, figs, and lychees) were collected from the markets of Anbar city in Iraq to obtain yeast *S. boulardii*. These samples were kept in sterile polythene bags and brought to the laboratory to conduct the yeast isolation process. These samples were preserved before and after analysis. The 34 bacteria samples isolate (*Enterococcus faecalis*, *Staphylococcus epidermis*, *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*) were obtained from the Ramadi hospital burn center in Anbar city, Iraq as shown in Table 1.

Table 1: Shows the bacteria strains used in this study.

Bacteria strains	**Number**	**Source**
Enterococcus faecalis	2	Ramadi hospital burn center
Staphylococcus epidermis	7	
Escherichia coli	10	
Serratia marcescens	10	
Pseudomonas aeruginosa	5	
Total	34	

2.2 Isolation of *S. boulardii* yeast

A gram was isolated from a specific sample of the collected and prepared fruit, then this gram was mixed with 9 ml of sterile peptone water, and a serial dilution of this mixture was made up to 10-5. After that, 1 ml of the diluted yeast isolation mixture is cultured following the pouring technique using Sabouraud dextrose agar medium with a pH of 5.5 (Deak and Beuchat, 1987). Yeast-grown petri plates were incubated at 37 °C for 3-7 days. Finally, morphological characteristics were observed by microscopic examination of yeast *S. boulardii* colonies.

2.3 Isolation and cultivation of bacterial strains

The strains of the bacterial species shown in Table 1 were provided by the microbiology laboratory from the

burn center at Ramadi Hospital. These species have been isolated from various clinical samples. Nutrient agar culture medium was prepared to cultivate isolated bacterial species strains after autoclaving at a temperature of 121°C and a pressure of 15 psi for 15 minutes, then the culture medium was poured into Petri dishes and sterilized. The 0.5 ml of samples were distributed over the surface of the medium in Petri dishes in which the stability of the medium was confirmed. The plates are then incubated at 37°C for 24 hours.

2.4 Identification of yeast *S. boulardii*

To identify *S. boulardii* yeast, biochemical tests were applied, which included utilization of a nitrogen source, utilization of a carbon source, or fermentation of a carbon source, in addition to tests for acid and ester production, urea hydrolysis to isolate the *S. boulardii* yeast, a gelatin liquefaction test, and an H_2S test, as mentioned by (Barnett et al.,1990; O Khidhr & M AL Zubaidy, 2014; Hossain et al., 2020).

2.5 Preparing the modifying nutrient medium for yeast *S. boulardii*

The nutrient medium was prepared according to the manufacturer's instructions by dissolving 30 g in 1000 ml of sterile distilled water, then thawing the 30 g in the medium using heat and continuous shaking for 10 min. After that, the nutrient medium was sterilized for 30 minutes at a pressure of 15 bar at 121 °C. This medium was left in a water bath at 37 °C. After checking and inspecting the temperature of the nutrient medium, the yeast was added by adding the *S. bolardii* capsule, adding 5 ml of 100% alcohol, and incubating this solution for 72 hours at 37 °C. Then, 30 g of sugar is added to the nutrient medium containing yeast *S. bolardii* for every 250 ml. The nutrient medium solution containing yeast was incubated for 168 hours with continuous monitoring and shaking. The yeast filtrate is isolated using filter paper No.1. To obtain the yeast *S. bolardii* extract and its secondary metabolites from the modified nutrient medium solution, this yeast filtrate is isolated by placing it in an ultrasonic device and then centrifuging it for 10 minutes at 5000 rpm. Finally, an extract of yeast *S. bolardii* is obtained with its secondary metabolites, from which 0.22 ml is taken to determine the antibacterial activity against bacterial species isolated from burn injuries.

2.6 Antibacterial activity of yeast *S. bulardii* in a nutrient medium and modified medium against bacterial species

Bacterial strains *E. faecalis, E. coli, S. epidermidis, S. marcescens P. aeruginosa* were grown in Nutrient agar medium for 24 hours and surface spread in petri plate. Three wells (5 mm in diameter) were drilled with a sterile borer in each plate in a Petri plate pre-inoculated with bacteria, and 0.22 ml of *S. boulardii* yeast suspension after centrifugation was placed in three wells. The antibacterial activities of *S. boulardii* are tested by the agar diffusion method which was identified by Attaie et al. (Attaie et al., 1987). The inoculated plates were placed for propagation and incubated for 24 hours at 37°C. After that, the inhibition zones in the plates were examined. The diameters of the inhibition zones were then measured according to the method of Izgu et al. (Izgu et al., 1997). Accordingly, the effect of *S. boulardii* yeast in natural and modified media was determined through bacterial isolates whose zone of inhibition was greater than 6 mm and thus *S. boulardii* yeast had activity against these types of bacteria.

3. Result and Discussion

3.1 Identification of *yeast S. boulardii* by biochemical characteristics

S. boulardii yeast was identified through the application of biochemical tests, and the results shown in Table 2 showed that this yeast species takes advantage of carbon and nitrogen production sources and does not utilize or consume galactose to produce carbon. In addition, the results of yeast isolation were negative regarding the use of lactose and galactose, starch, and nitrates and also negative for urea hydrolysis and gelatin liquefaction test. While the results for this isolate were positive for the rest of the tests, which confirms that this yeast isolate is S. boulardii. These results are consistent with the results of the study conducted by O Khidhr & M AL Zubaidy in Iraq and the study conducted by Hossain et al. in Bangladesh (O Khidhr & M AL Zubaidy, 2014; Hossain et al., 2020).

Table 2: Biochemical tests for identification of *S. boulardii* yeast.

Tests		**Result**
Carbon utilization	Fructose	+
	Glucose	+
	Sucrose	+
	Lactose	-
	Galactose	-
	Starch	-
	Raffinose	+
Nitrogen utilization	Peptone	+
	Nitrate	-
	Aspargine	+
	Ammonium sulfate	+
Ester production		+
Acid production		+
Cyclohexamide Resistance		+
Urea hydrolysis		-
Gelatin Liquefaction Test		-

3.2 Phenotypic and biochemical examinations of bacterial isolates

Bacterial isolates *E. faecalis, E. coli, S. epidermidis, S. marcescens*, and *P. aeruginosa* were identified through phenotypic and biochemical examinations, the results are shown in Table 3. Determining the phenotype of the five types of bacteria consists of first Gram staining to observe each type's specific staining and shape, followed by cell shape and appearance, growth on MacConkey medium and blood culture medium, and growth at 4°C and 42 °C. From Table 3, the results showed that the bacterial isolates *E. faecalis, E. coli*, and *S. epidermidis* was able to produce Gram stain, while the *S. marcescens* and *P. aeruginosa* did not produce Gram stain. All bacterial isolates showed growth on blood agar and all showed growth on MacConkey medium except *S. epidermidis*. All bacterial isolates showed clear growth at temperatures of 42°C, while they did not grow at temperatures of 4°C, except for the *S. marcescens*. These phenotypes were consistent with results reported by several studies (Dolka et al., 2020; Janezic et al., 2013; Monteiro et al., 2016; Pradhan & Tamang, 2019). In addition, some biochemical tests were conducted to identify the types of bacteria. The types of bacteria were subjected to the following biochemical tests: testing for the production of the enzymes (catalase, oxidase, urase) and tests of indole, methyl red, voges proskauer, and citrate utilization (Table 3). All bacterial species produce the enzymes oxidase and catalase, but do not produce the enzyme urase. The results of the examination of E. coli indicated that they did not produce methyl red dye, indole, and citrate utilization. While the S. marcescens, S. epidermidis, and P. aeruginosa isolates produced the citrate enzyme, but E. faecalis isolate did not produce this enzyme. The results regarding biochemical tests in this study are consistent with many studies (Alves et al., 2012; Dolka et al., 2020; Janezic et al., 2013; Monteiro et al., 2016; Pradhan & Tamang, 2019).

Table 3: Phenotypic and biochemical examinations of bacterial isolates.

Test type	***E. faecalis***	***E. coli***	***S. epidermidis***	***S. marcescens***	***P. aeruginosa***
Gram stain	+	+	+	-	-
Cell shape and appearance	Cocci	Pink colonies, smooth	Cocci, spherical cells	Rod-shaped	Rod-shaped
Growing on MacConkey medium	+	+	-	+	+
Growth on blood culture medium	+	+	+	+	+
Growth at 4 °C	-	-	-	+	-
Growth at 42 °C	+	+	+	+	+
Oxidase enzyme	-	-	-	-	+
Catalase enzyme	-	+	+	+	+
Urase enzyme	-	-	+	-	
Indol	-	+	-	-	-
Methyl red	-	+	-	-	-
Voges proskauer	+	+	+	+	-
Citrate utilization	-	+	-	+	+

3.3 Comparison of antibacterial activity of secondary metabolic products of *S. bulardii* in natural media and modified media against bacterial species

The effect of the S. boulardii yeast filter in natural media and modified media against five types of bacteria was determined by conducting an antimicrobial activity test of *S. boulardii* yeast against bacteria pathogens for humans. This is done by culturing *S. boulardii* in a natural and modified media, observing its microbial activity against bacteria when it grows in these two media, and comparing the results. The results showed that the *S. boulardii* yeast filter had antibacterial activity when grown in both media. Still, the inhibition zone of the growth of the bacterial species also depended on the incubation time. The results showed the effect of the S. boulardii yeast filter in the natural and modified media through the inhibition zones for the growth of *E. faecalis, E. coli, S. epidermidis, S. marcescens*, and *P. aeruginosa* as shown in Tables 4 and 5.

Table 4: Antibacterial activity of secondary metabolic products of *S. bulardii* in the natural media against five bacterial species.

Bacterial isolates	***E. faecalis***	***E. coli***	***S. epidermidis***	***S. marcescens***	***P. aeruginosa***
Incubation hours	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**
24	6	6	6	6	6
48	7	8	7	8	7
72	8	7	7	7	8
96	9	8	9	8	8
120	9	8	9	9	9
144	11	10	11	10	10
168	12	12	12	12	12
Control	5.00	5.00	5.00	5.00	5.00

Table 5: Antibacterial activity of secondary metabolic products of *S. bulardii* in the modified media against five bacterial species.

Bacterial isolates	***E. faecalis***	***E. coli***	***S. epidermidis***	***S. marcescens***	***P. aeruginosa***
Incubation hours	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**	**Inhibition Zone (mm)**
24	12	12	12	11	12
48	13	14	13	13	14
72	14	12	13	14	14
96	14	12	13	14	14
120	15	15	15	15	16
144	16	16	16	17	16
168	16	19	16	18	19
Control	5.00	5.00	5.00	5.00	5.00

The results showed that the *S. boulardii* yeast filter had a clear effect on the growth of *E. faecalis*, as the inhibition zone increased with increasing incubation period. On the other hand, it was observed that the effect of the *S. boulardii* yeast filter in the modified media on the growth of *E. faecalis* was clear and big compared to the natural media as shown in Figure 1. Where the inhibition zone was 16 mm after 168 hours of incubation. This clear effect on the growth of *E. faecalis* is explained by the fact that *S. boulardii* yeast cells contain α-d-glucan and β-d-glucan sugars that interact directly with the cell walls of this type of bacteria. Consequently, this interaction causes *E. faecalis* cells to adhere to the outer walls, causing damage and preventing their growth. These results are consistent with the results of a study conducted by Miller et al. when they indicated the possibility of producing quantities of various sugars that have a clear effect on the bacterial species that cause many diseases such as *E. faecalis* (Miller et al., 2014).

Also, Rajkowska et al. reported that the probiotic yeast S. boulardii exhibits antagonistic activity against pathogenic bacteria such as *E. coli*, *E. faecalis*, and *P. aeruginosa* (Rajkowska et al., 2012).

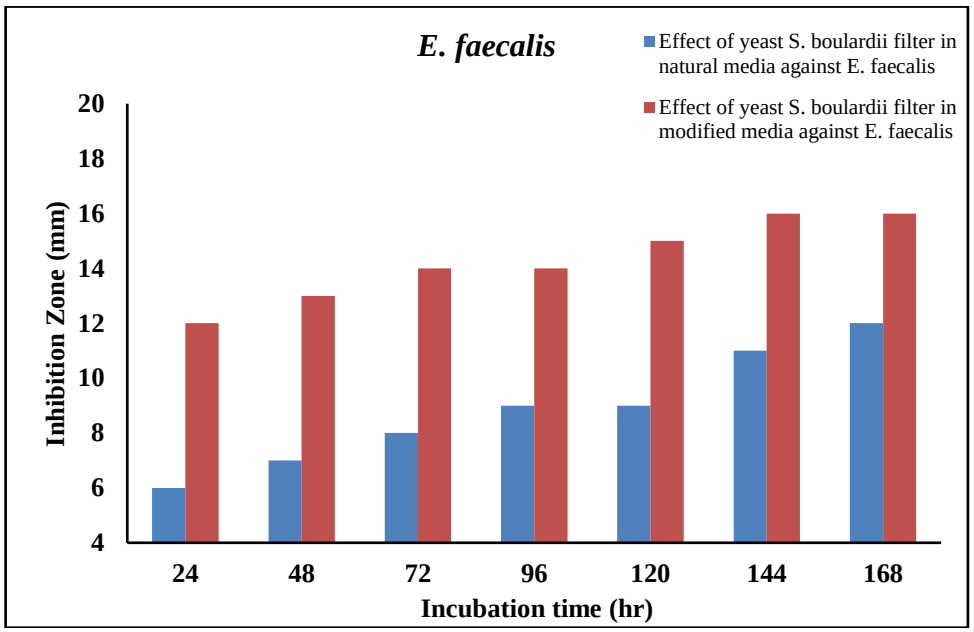

Figure 1: Shows the effect of secondary metabolic products of *S. boulardii* yeast cultivating in natural and modified media against *E. faecalis*.

The results also showed that the effect of secondary metabolic products of *S. boulardii* yeast had a clear effect on the growth of intestinal bacteria *E. coli*, and the effect in natural and modified incubated media was high. The inhibition zone for the effect of yeast filtrate after 168 hours of incubation reached 19 mm. The results proved that the effect of *S. boulardii* yeast in the modified media is greater than in the natural media (Figure 2). This explains that the effect of the yeast filtrate on the growth of *E. coli* is at its highest levels, as the media contains sugars and alcoholic substances. This is because the presence of *S. boulardii* yeast in such a medium makes it able to produce additional substances with a high effect against pathogenic bacteria. In addition, the reason for this may be the production of substances such as mannose by this type of yeast, which has a great ability to adhere to the surfaces of bacterial cells, including *E. coli* bacteria. Thus, its growth is greatly inhibited. These results are consistent with the findings of Jalal and Aziz, they confirmed in their study that the membrane of *S. boulardii* yeast is rich in mannose and that pathogenic bacteria have a high ability to bind to the mannose present in that yeast. This may prevent bacteria from sticking to the surfaces that feed them, which weakens their ability to grow and reproduce (Jalal & Aziz, 2023).

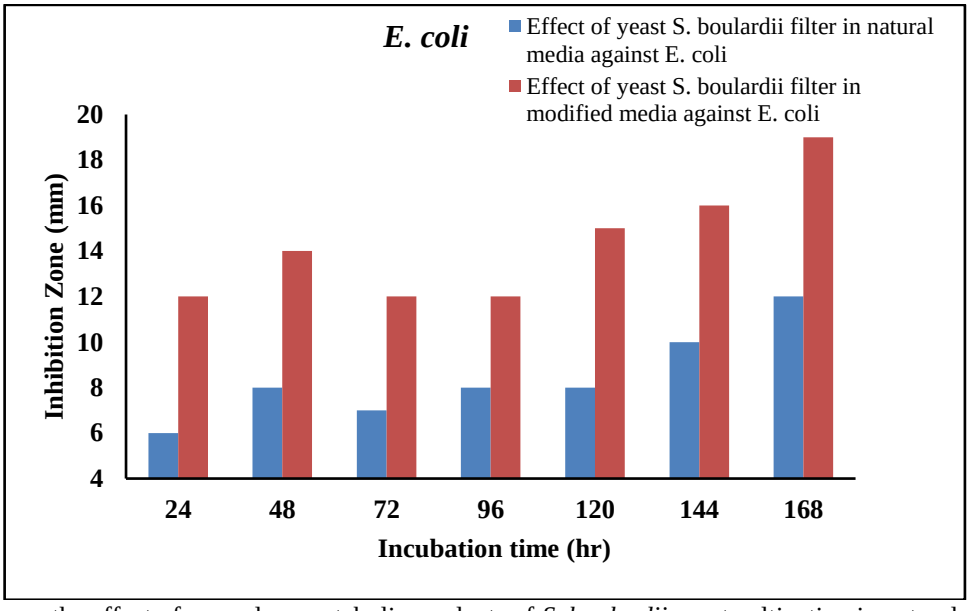

Figure 2: Shows the effect of secondary metabolic products of *S. boulardii* yeast cultivating in natural and modified media against *E.coli*.

Also concerning the effect of *S. boulardii* yeast filtrate in natural media on the growth of *S. epidermidis*, it was a clear effect and the highest effect of the filtrate was 12 mm for the zone of inhibition when using the isolated filtrate after 168 hours, but the effect was higher in the modified media where the zone of inhibition reached 16 mm at the same incubation time, with a clear significant difference as shown in Figure 3. This indicates that yeast uses sugars and alcohols as reinforcing materials to produce substances with a microbial effect against isolated bacterial species, including *S. epidermidis*. These results are consistent with the findings of Ali et al. reported that *S. boulardii* yeast possesses antibacterial activity due to the secretion of capric acid, which contributes to inhibiting the formation of bacterial cell membranes. Thus, preventing the adhesion of bacterial cells to the food medium by destroying their biofilms, which gave results with a higher effect on the bacteria under study (Ali et al., 2012).

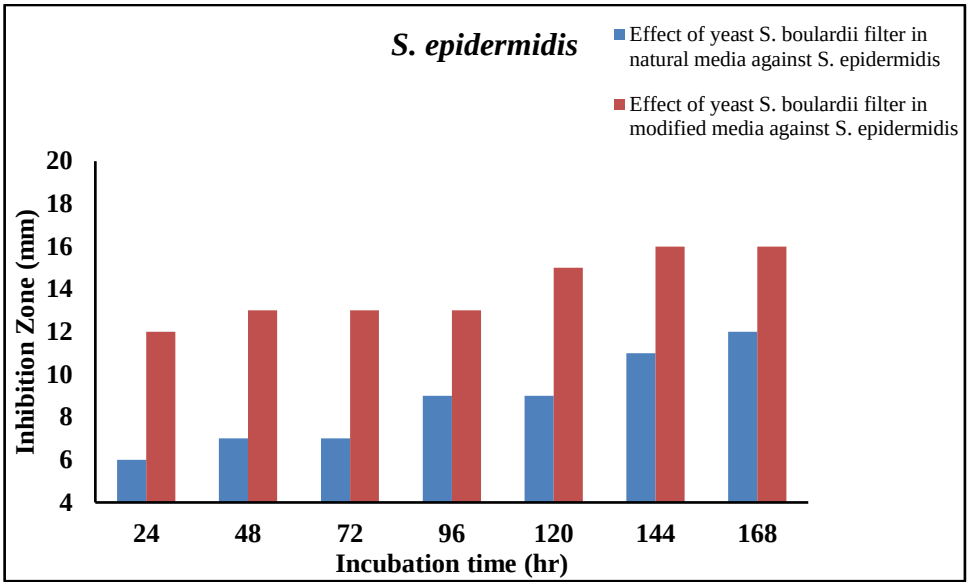

Figure 3: Shows the effect of secondary metabolic products of *S. boulardii* yeast cultivating in natural and modified media against *S. epidermidis*.

Also, the yeast filtrate in both media gave a high killing effect on the bacterial cells of *S. marcescens*, and the results were similar to the effect of the yeast filtrate on the *E. coli* bacteria cells. The reason for this may be as pointed out by Canani et al. in their study, where they stated that the yeast *S. boulardii* produces small peptides, including serine protease, which are capable of inhibiting the toxin activities of bacterial cells and leading to the degradation of toxin receptors present on the surface of the bacterial cell, thus inhibiting their activity and growth (Canani et al., 2011).

The results showed that the *P. aeruginosa* bacteria is one of the species most affected by *S. boulardii* yeast filtrate due to the modified media, especially at the incubation period of 168 hours, where the zone of inhibition reached 19 mm. This confirms a difference in the effect of *S. boulardii* yeast in natural media and modified media on *P. aeruginosa*, as shown in Figure 5, including all bacterial species under this study. This result is consistent with a study conducted by Venkateswarulu et al. It was concluded that *S. boulardii* has a pesticide activity against *P. aeruginosa* bacteria. This is because this yeast secretes substances such as peptides that work to separate bacterial toxins or reduce the sugar levels of bacterial cells and thus prevent their growth (Venkateswarulu et al., 2019).

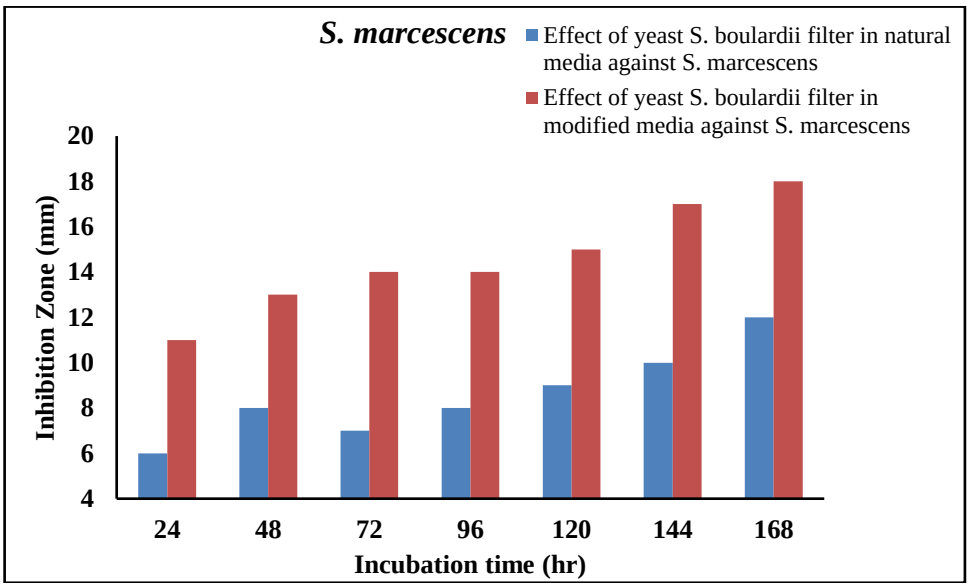

Figure 4: Shows the effect of secondary metabolic products of *S. boulardii* yeast cultivating in natural and modified media against *S. marcescens.*

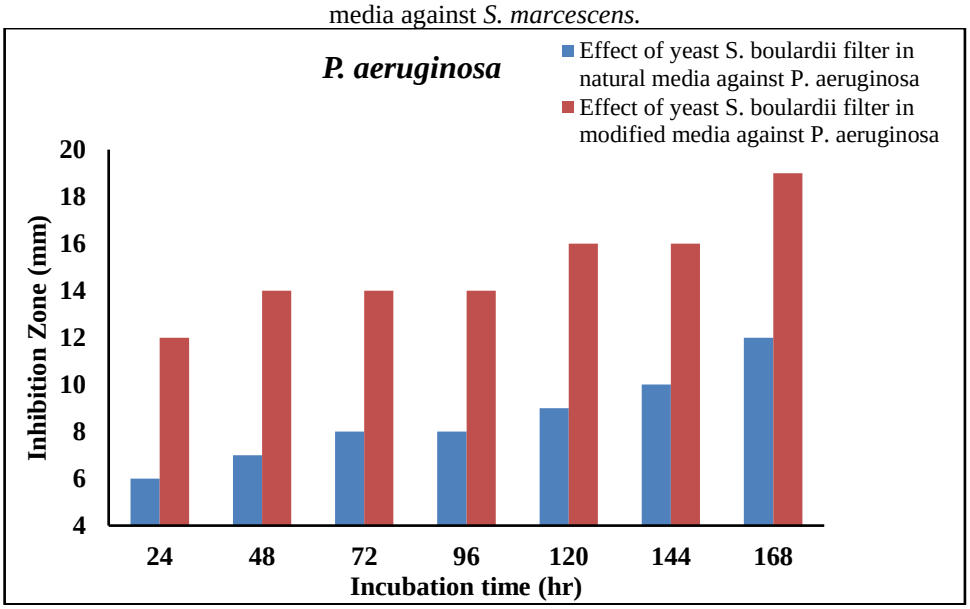

Figure 5: Shows the effect of secondary metabolic products of *S. boulardii* yeast cultivating in natural and modified media against *P. aeruginosa*.

Moreover, the results indicate that of *S. boulardii* yeast in the modified media as the incubation period increases, the active substances that have positive antimicrobial activity against the five bacterial species increase. However the *S. boulardii* yeast had a high antimicrobial activity against *E. coli*, *P. aeruginosa*, and *S. marcescens*, respectively as shown in Figure 6. This may be because yeast consumes nutrients extensively and its cells produce high levels of inhibitory substances that have a high effect against bacterial species such as sugar, enzymes, inhibitory proteins, polysaccharides, and a group of metabolites that have biological activity (gamma aminobutyric acid, 2-hydroxyisocaproic acid, P-aminobenzoic acid, polylactic acid, shikimic acid, tyrosol) (Egea et al., 2023; Fu et al., 2022; Hyrslova et al., 2024).

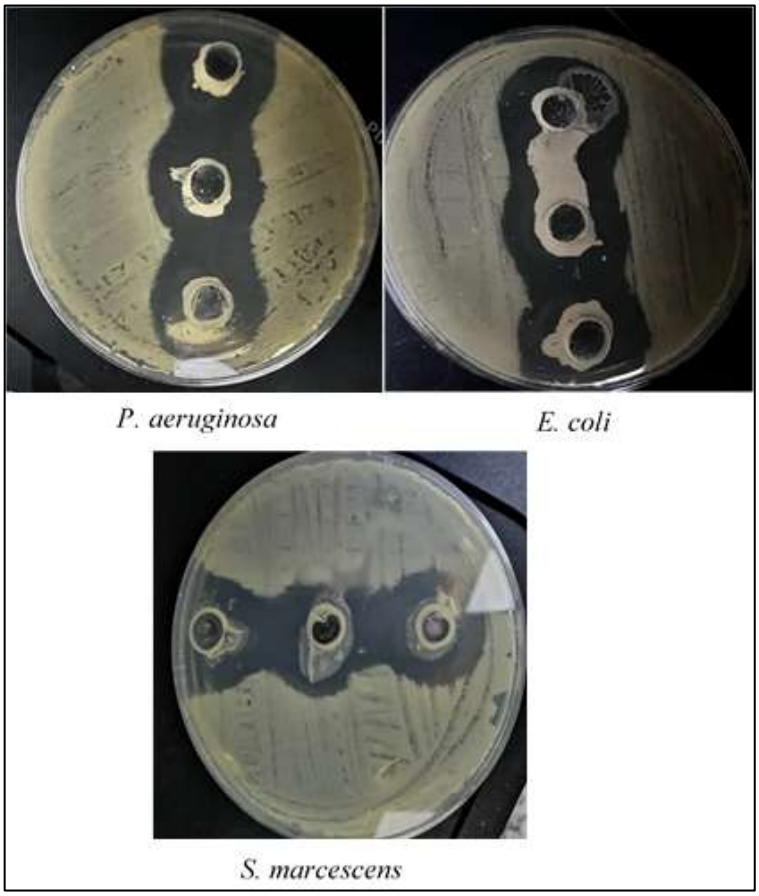

Figure 6: Shows the effect of modified media for *S. boulardii* yeast on isolated bacteria *E. coli*, *P. aeruginosa*, and *S. marcescens*.

4. Conclusion

The current study concluded that *S. boulardii* medical yeast belongs to probiotics and plays an important role as an antimicrobial activity such as bacteria. Also, this yeast proved effective in inhibiting five types of bacteria: *E. faecalis, S. epidermis, E. coli, S. marcescens*, and *P. aeruginosa*. In addition, the microbial activity of *S. boulardii* yeast against the five types of bacteria was clear and high when grown in the modified media compared to its growth in the nutrient (natural) media. One of the most important reasons that increased the activity of *S. boulardii* yeast against bacteria in the modified media is that this media and increasing the incubation period to 168 hours made the yeast cells grow at a high concentration. Thus, yeast produces a group of secondary metabolites that inhibit the growth of bacterial species, such as glucose, enzymes, inhibitory proteins, sugars, and acid metabolites. In addition, the production of these substances that inhibit the growth of bacterial cells by this yeast greatly increases their ability and effectiveness, where the zone of inhibition was 18 and 19 mm for three types of disease-causing bacteria are *E. coli*, *P. aeruginosa*, and *S. marcescens*. This indicates that the activity of *S. boulardii* yeast against these types of bacteria is high and effective. The antibiotic activity of *S. boulardii* against bacteria or when used as an antibiotic to treat diseases caused by several genera of bacteria is likely to be affected by several factors. The most important of which is the nutritional media that allows it to produce many effective metabolites in inhibiting the activity of disease-causing bacteria, and the mechanisms that increase the ability of this yeast to be a powerful therapeutic substance for several diseases. Therefore, the current study recommends the need to explore the relationship between the effect of the biological components of *S. boulardii* yeast and the methods that can be obtained from it in high concentrations to ensure the production of therapeutic drugs for new bacterial strains. Furthermore, these results open horizons for further future studies to apply a set of techniques to obtain components of *S. boulardii* yeast to increase the production of useful antibiotics to treat many bacterial diseases that many humans suffer from.

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