Iraqi Journal of Market Research and Consumer Protection
(2023) 15(1): 45-53
DOI: http://dx.doi.org/10.28936/jmracpc15.1.2023.(4)
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المجلة العراقية لبحوث السوق وحماية المستهلك

STUDY OF THE SYNERGISTIC EFFECT OF PROTEINS PRODUCED FROM *Saccharomyces cerevisiae* WITH LACTOFERRIN AGAINST MULTI RESISTANT DIARRHEAL BACTERIA

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Received 28/ 6/ 2022, Accepted 17/ 8/ 2022, Published 30/ 6/ 2023

ABSTRACT

The research included the isolation of 12 bacterial isolates from stool samples of children with acute watery and bloody diarrhea under the age of five years, as it was possible to obtain 6 isolates of *Escherichia coli*, 3 isolates of *Salmonella typhimurium*, and 3 isolates of *Shigella flexneri*. Vitek2 system, and the inhibitory activity of lactoferrin and killer toxin produced from *S. cerevisiae* against pathogenic bacteria was studied by the well diffusion method, as the results recorded that the killer toxin had the highest inhibitory activity towards pathological bacteria with varying results, and the lowest inhibitory concentration was determined The MIC of the lactoferrin and the killer toxin against the bacterial isolates under study using the turbidity method in the tubes. for antibiotics, as the results showed a The relationship was synergistic due to the activity of lactoferrin with killer toxin in inhibiting the action of pathogenic bacteria, whose inhibitory activity increases with increasing concentration used.

Keywords: Diarrhea, Inhibition activity, Antimicrobial activity, Lactoferrin, Killer toxins, Bakery yeast, Synergistic activity.

دراسة التأثير التآزري للبروتينات المنتجة من خميرة *Saccharomyces cerevisiae* مع اللاكتوفيرين ضد بكتيريا الإسهال المتعددة المقاومة

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الخلاصة

تضمن البحث عزل 12 عزلة بكتيرية من عينات براز اطفال مصابين بالاسهال المائي الحاد والدموي دون سن الخامسة، اذ امكن الحصول على 6 عزلات لبكتريا *Escherichia coli* و3 عزلات لبكتريا *Salmonella typhimurium* و 3 عزلات لبكتريا *Shigella flexneri*، اخضعت جميع هذه العزلات للفحوصات الزرعية والمجهرية والكيمائية الحيوية وشخصت باستعمال نظام Vitek 2، وجرى دراسة الفعالية التثبيطية للاكتوفيرين والسموم القاتلة المنتجة من خميرة *S. cerevisiae* تجاه البكتريا المرضية بطريقة الانتشار في الحفر، اذ سجلت النتائج ان السموم القاتلة كانت اعلى فعالية تثبيطية تجاه البكتريا المرضية وبنتائج متفاوتة، وقد حدد التركيز المثبط الادنى MIC لبروتين اللاكتوفيرين والسموم القاتلة تجاه العزلات البكتيرية قيد الدراسة و باستعمال طريقة العكورة في الانابيب، واثبتت النتائج ان قيمة MIC كانت متباينة حسب تراكيز الرواشح و فعاليتها، كما درست العلاقة التآزرية بين بروتين اللاكتوفيرين والسموم القاتلة تجاه البكتريا المرضية التي تم اختيارها لهذا الاختبار حسب درجة مقاومتها للمضادات الحيوية، اذ اظهرت النتائج ان العلاقة كانت تآزرية بسبب فعالية بروتين اللاكتوفيرين مع السموم القاتلة في تثبيط فعل البكتريا المرضية والذي تزداد فعاليته التثبيطية بزيادة التركيز المستعمل.

الكلمات المفتاحية: الاسهال، الفعالية التثبيطية، الفعالية المضادة للميكروبات، اللاكتوفيرين، السموم القاتلة، خميرة الخبز، الفعالية التآزرية.

INTRODUCTION

Diarrhea is one of the main causes of death among children in various countries of the world, especially those under five years of age **(Aziz, 2015)**. The number of children who are exposed to diarrhea under the age of five years is estimated at a thousand million, and approximately 3.3 million children die annually, the majority of this percentage of children who do not exceed the age of two years, the main cause of deaths resulting from diarrhea is due to dehydration to the body losing the necessary fluids, and diarrhea is one of the important causes of malnutrition, so diarrhea and malnutrition are among the main causes of deaths, and there are multiple causes of diarrhea including bacterial causative agents such as *Escherichia coli*, *Salmonella typhimurium*, *Shigella flexneri* and Campylobacter, including parasitic causative agents, the most important of which are *Entamoeba histolytica* and *Giardia lamblia*, in addition to viral causative agents such as *Rota virus*, *Corona virus* and *Adeno virus*, also, *Candida albicans* is one of the yeasts that cause diarrhea **(Aziz *et al.*, 2016; Aziz *et al.*, 2014; Al-Samarraie, *et al.*, 2008)**. Numerous studies have shown that the lactoferrin molecule consists of two main lobes, and that each lobe is responsible for binding to an iron atom Fe^{+3}, as each mole of lactoferrin can bind to two moles of iron, so the activity of the antibacterial lactoferrin depends on the state of the free protein Iron Apo-LF, as it works to chelate iron from the medium and thus deprive microorganisms of it, which leads to the elimination of them or the cessation of their growth **(Aziz *et al.*, 2017)**, the process of attachment of lactoferrin to iron requires the presence of a negative ion of $-HCO_3$, which is considered essential to bind minerals as its presence greatly facilitates iron saturation, when each positive ion is bound to lactoferrin an atom $-HCO_3$ is added inside the groove of iron binding, and this is the opposite of the action of citrate that chelate iron **(Al-Rikabi *et al.*, 2015)**, therefore, lactoferrin effect the growth of microorganisms that not to need iron through its ability to destroy the outer membrane of G^- bacteria by causing changes in the permeability of the membrane, as it can bind positively charged ions with a valence Binaries such as Mg^{+2} and Ca^{+2}, where the negative charge constant is modified by *Lipopolysaccharides* (LPS) **(Al-Rikabi *et al.*, 2016)**.

Yeast is one of the beneficial microorganisms, and humans used it for the purpose of fermentation. Nowadays, *Saccharomyces cerevisiae* yeast is widely used industrially in bread fermentation and the production of alcohol and cholesterol **(Alsoufi *et al.*, 2017a)**. Yeast is one types of the probiotics that produces inhibitory substances capable of killing the microorganisms present with it in the same medium, which are protein substances with lethal activity that destroy the plasma membrane of the bacterial cell that called killer toxins **(Alsoufi & Aziz, 2022; Alsoufi *et al.*, 2017b; Aziz *et al.*, 2014b)**, this inhibition activity depends on temperature and pH. Yeast also produces phosphatase. Which removes phosphorylated endotoxins and inhibits their cytotoxic effects such as LPS in *E. coli* **(Aziz *et al.*, 2014a)**.

MATERIALS AND METHODS

Bacterial isolates

In this study, 18 bacterial isolates were used from fecal samples of children with acute watery and bloody diarrhea under the age of five years. The samples were obtained from the laboratories of Al-Kadhimiya teaching hospital and the central children's hospital in Baghdad, Iraq.

Diagnosis of bacterial isolates

Bacterial isolates were purified by repeated cultivation and initially diagnosed based on the morphological characteristics of the colonies, their color, height and texture, and were examined microscopically for the purpose of describing the cell shapes after treating them with

a gram stain. Biochemical tests were also conducted based on what was mentioned in **Hamel *et al.* (2020a)** and the final diagnosis using the VITEK 2 System.

Sensitivity test for bacterial isolates

A sensitivity test of the bacterial isolates against 10 different antibiotics was conducted, namely: Rifampicin (RIF 5 µg), Amoxicilln (AMC 30 µg), Ampicillin (AMP 10 µg), Ciprofloxacin (CIP 5 µg), Trimethoprim (TR 5 µg), Cefotaxim (CTX 30 µg), Ceftriaxon (CTR 30 µg), Neumycin (N 30 µg), Tetracycline (TE 30 µg), Chloramphenicol (C 30 µg). The test was conducted using Mueller-Hinton agar, and the inhibition diameter was measured according to **Hamel *et al.* (2020b)**.

Standard turbidity solution-McFarland

McFarland's solution was prepared according to **Khadam *et al.* (2019)**.

Preparing the bacterial inoculum

Bacterial isolates were cultured in tubes containing the nutrient broth medium, then incubated at of 37°C for 18-24 h, after that a plot was made on the surface of the solid nutrient medium with nutrient agar medium by the loop, and the dishes were incubated at 37°C for 24 h. After the end of the incubation period, a few colonies were transferred. purified bacteria at the age of 24 h by means of the loop into tubes containing sterile nutrient broth medium with an amount of 5 mL, then the turbidity of the bacterial suspension was compared with the turbidity of the standard McFarland tube No. 0.5, which is equivalent to a bacterial growth equal to 1.5×10^{8} CFU/mL **(Jabbar *et al.*, 2020)**.

Lactoferrin

It was used lactoferrin from bovine milk, Sigma-Aldrich, Product No. L9507.

Killer toxin

It was obtained it from previous study **Alsoufi & Aziz (2022).**

Estimation of protein

Total protein mg/mL estimation through method of Bradford, (1976) using BSA stock solution at 595 nm.

Inhibitory activity of lactoferrin and killer toxins

The method described by **Alsoufi & Aziz (2017b)** was used to determine the inhibitory activity of lactoferrin and killer toxins against pathogenic bacteria isolates using the well diffusion method.

Minimum inhibitory concentration (MIC) and minimal lethality (MBC) of lactoferrin and killer toxins

The method described by **Saleh *et al.* (2020)** was used to determine the minimum inhibitory concentration (MIC) and minimal lethality (MBC) of lactoferrin and killer toxins against pathogenic bacteria isolates using the Tube method.

Synergistic effect of lactoferrin and killer toxins

The method described by **Hamel *et al.* (2020b)** was used to determine the synergistic effect of lactoferrin and killer toxins towards isolates of pathogenic bacteria using the checker board method, through which it is possible to determine the type of relationship between two antibodies and to determine the lowest concentration of the first antibody with the lowest concentration of the second antibody, which gives an active effect

RESULTS AND DISCUSSION

Isolation and diagnosis

The results showed that obtained of 12 intestinal bacterial isolates from fecal samples of children with acute watery and bloody diarrhea under the age of five years. The culture characteristics of the colonies and microscopic characteristics of the bacterial cells were confirmed and then diagnosed using the Vitek2 diagnostic kit. They were as follows: 6 isolates of *E. coli* and 3 isolates of *S. typhimurium* and 3 isolates of *Sh. flexneri*.

Sensitivity test

The sensitivity of the bacterial isolates was tested towards 10 antibiotics. The results were determined by describing bacteria that are resistant to R or sensitive to S by measuring the diameter of the inhibition zone and comparing that with what was mentioned in **CLSI (2009)**, the results in (Table 1) showed that there is a variation in the resistance of the isolates *E. coli* towards the used antibiotics, as it was noted that all isolates of this bacteria were 100% resistant to AMP and AMC, while they were 100% sensitive to C, CIP and RIF, while their sensitivity to other used antibiotics differed; also, *S. typhimurium* was 100% resistant to AMP and AMC, while it was 100% sensitive to CTR, C, CIP and RIF, and its sensitivity to other used antibiotics differed; while *Sh. flexneri* was 100% resistant to AMP, AMC, TE, and TR, while it was 100% sensitive to C, CIP, and RIF, and its sensitivity to other used antibiotics differed.

Table (1): Resistance and sensitivity of pathogenic bacterial isolates to antibiotics.

Bacterial isolates	Antibiotics									
	CIP	AMP	AMC	RIF	CTX	CTR	TR	C	S	TE
E. coli	S	R	R	S	S	R	S	S	R	S
E. coli	S	R	R	S	S	R	S	S	R	R
E. coli	S	R	R	S	R	S	R	S	S	R
E. coli	S	R	R	S	R	R	S	S	R	S
E. coli	S	R	R	S	R	R	R	S	S	R
E. coli	S	R	R	S	R	S	S	S		S
Sh. flexneri	S	R	R	S	S	R	R	S	S	R
Sh. flexneri	S	R	R	S	R	S	R	S	R	R
Sh. flexneri	S	R	R	S	R	R	R	S	S	R
S. typhimurium	S	R	R	S	R	S	S	S	R	S
S. typhimurium	S	R	R	S	R	S	R	S	R	R
S. typhimurium	S	R	R	S	S	S	R	S	S	R

***R: Resistant**
****S: Sensitive**

These results were consistent with the findings of **Mirzaagha *et al.* (2011); Ren *et al.* (2006)** when they conducted a sensitivity test for a number of antibiotics against intestinal bacteria, as it was noted that antibiotic resistance is high, and perhaps the reason is due to the wide random use of them and the lack of health awareness, as well as the use of some of them as first treatment for children's diarrhea in some hospitals, which led to the development of resistance of bacterial strains to antibiotics, as the bacteria can acquire resistance to the group of broad-spectrum β-lactams by several mechanisms, the most important of which is the plasmid that encodes resistance to the ESBL and AMPC enzymes, and it was also found that there is another type of enzymes called extended spectrum β-Lactamase (ESβLS) work on analyzing the β-lactam ring **(Skurnik *et al.*, 2005)**, and the characteristic of producing β-Lactamase is not an absolute characteristic for all bacterial isolates, as some bacterial isolates may be non-producing β-Lactamase despite their resistance to β-lactam, so bacteria resort to Other mechanisms of resistance to β-lactam antibiotics other than their production of β-Lactamase, such as alteration of outer membrane proteins as a step to change the target site, or change in the permeability barrier of the cytoplasmic membrane **(Sabate *et al.*, 2002)**.

Inhibitory effect of lactoferrin and killer toxin

The results have shown that lactoferrin and killer toxin have an antibiotic effect against Intestinal bacterial isolates (Table 2), the inhibitory effect of them on *E. coli*, *S. typhimurium* and *Sh. flexneri*; as *E. coli* was the most inhibited followed by *Sh. flexneri* then *S. typhimurium*.

Table (2): Inhibition zones of lactoferrin and killer toxin towards the intestinal bacterial isolates.

Bacterial isolates	Inhibition zone (mm)	
	Lactoferrin	Killer toxin
E. coli	24	23
E. coli	22	23
E. coli	25	22
E. coli	24	25
E. coli	23	24
E. coli	22	23
Sh. flexneri	22	21
Sh. flexneri	20	22
Sh. flexneri	20	22
S. typhimurium	18	20
S. typhimurium	19	18
S. typhimurium	18	19

The results of minimum inhibitory concentration (MIC) of lactoferrin and killer toxin (Table 3) showed that the MIC towards *E. coli* isolates appeared at 1.51 mg/mL to Lactoferrin and 5.87 mg/mL to killer toxin, while the MIC results for *S. typhimurium* and *Sh. flexneri* were at 3.025 mg/mL to lactoferrin and 11.75 to killer toxin.

Table (3): Minimum inhibitory concentration (MIC) of lactoferrin and killer toxin towards the intestinal bacterial isolates.

Bacterial isolates	MIC (mg/mL)	
	Lactoferrin	Killer toxin
E. coli	1.51	5.87
E. coli	1.51	5.87
E. coli	1.51	5.87
E. coli	1.51	5.87
E. coli	1.51	5.87
E. coli	1.51	5.87
Sh. flexneri	3.025	11.75
Sh. flexneri	3.025	11.75
Sh. flexneri	3.025	11.75
S. typhimurium	3.025	11.75
S. typhimurium	3.025	11.75
S. typhimurium	3.025	11.75

From the foregoing, it is clear that the results of the MIC are due to the effect of each of the lactoferrin and killer toxin against intestinal bacteria.

Recently, many doubts have been raised about the safety of antibiotics from a health point of view, and their use has become controversial due they have side effects, so attention has been focused on natural sources that do not have toxic effects, which have antimicrobial effect **(Alsoufi & Aziz, 2022; Alsoufi & Aziz, 2021; Alsoufi & Aziz 2019; Al-Soufi, 2015)**. In this regard, **Saleh *et al.* (2020)** indicated that the reason for the high resistance of pathogenic bacteria to these concentrations of filtrate is due to factors affecting MIC, including incubation conditions, culture medium, and inoculum size. The reason for the ability of *S. cerevisiae* yeast to inhibit the growth of pathogenic bacteria is due to its secretion of multiple killer substances, and these killer substances are protein substances that have a highly specific action that depends on a group of environmental factors such as pH, temperature and ventilation, as it was found that the The optimum hydrogen for the production of these substances from yeast ranges

between 4. 2 to 7.4 at 25 to 30 C, and the effectiveness of lethal substances decreases with increasing temperature and pH values, and the mechanism of production of toxins and the immunity of killer cells to them is linked to the presence of genetic elements outside the chromosomal (**Alsoufi & Aziz, 2022**), characterized by the yeast *S. cerevisiae* possesses many mechanisms that explain its ability to influence pathogens, it does not have an effect on invading pathogens directly or by inhibiting some pathogens' virulence facto (**Alsoufi *et al.*, 2017b; Aziz *et al.*, 2014b**). Some of them have no effect on invading pathogens directly or by inhibiting some pathogen virulence factors such as adhesion and production of toxins as well as stimulating the immune system and preventing bacterial toxins from reaching the receptors in the body of the organism and others **(Braij *et al.*, 2022; Aziz, 2015)**.

Synergistic effect of lactoferrin and killer toxin

The synergistic relationship between the lactoferrin at 1.12, 05.6, 025.3, 51.1 and 75.0 mg/mL, and killer toxin at 94, 47, 5.23, 75.11 and 87.5 mg/mL was studied against the bacterial isolates (Table 4).

Table (4): The synergistic effect and minimum inhibitory concentration (MIC) of lactoferrin and killer toxin with partial inhibitory concentration (FIC) for both of them using the board Checker method and the type of relationship for pathogenic bacterial isolates.

Bacterial isolates	MIC for lactoferrin (mg/mL)	Concentration in combined for lactoferrin	MIC for killer toxin (mg/mL)	MIC in combined for killer toxin	FIC	FIC Index
E. coli	51.1	377.0	87.5	467.1	498.0	Synergistic
E. coli	51.1	377.0	87.5	467.1	498.0	Synergistic
Sh. flexneri	05.6	188.0	75.11	935.2	28.0	Synergistic
Sh. flexneri	05.6	188.0	75.11	935.2	28.0	Synergistic
S. typhimurium	05.6	377.0	75.11	87.5	561.0	Synergistic
S. typhimurium	05.6	188.0	25.19	203.1	093.0	Synergistic

The results showed that the relationship was synergistic against pathogenic bacteria, as the lactoferrin is one of the active substances against G^+ bacteria, which facilitates the disruption of the paths and defenses of host cells as well as targeting the organic functions of those cells, and is characterized by its cytotoxic effectiveness due to its possession of a dual charge low molecular weight enables it to penetrate host membranes **(Aziz *et al.*, 2017; Al-Rikabi *et al.*, 2016; Al-Rikabi *et al.*, 2015).** Also, killer toxin that produced from *S. cerevisiae* has a wide inhibitory effect against pathogenic bacteria, due to its acidic nature similar to bacteriocins secreted by bacteria with an action specialized in destroying the plasma membranes of sensitive cells, causing the loss of their cellular contents as well as inhibition The transfer of amino acids, or the inhibitory effect may be due to the secretion of deadly toxins, or due to the production of proteolytic enzymes with a high molecular weight estimated at 54 kDa, which may be the reason for the inhibition of the activity of the catalase enzyme by bacteria (**Alsoufi *et al.*, 2017a; Aziz *et al.*, 2014a**), from the aforementioned, it is clear that the

inhibition growth of *E. coli*, *S. typhimurium* and *Sh. flexneri* attributed to the synergistic effect between lactoferrin and killer toxin

CONCLUSION

The synergistic relationship between lactoferrin and the deadly toxin showed a high inhibitory effect against isolates of intestinal bacteria that cause diarrhea in children, so this relationship can be invested in the pharmaceutical industry as an alternative to antibiotics in this field.

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