Detecting some factors affecting *Salmonella typhimurium* adhesion and inhibition by *Saccharomyces boulardii*

التحري عن بعض العوامل المؤثرة على التصاق وتثبيط بكتريا *Salmonella typhimurium* بواسطة خميرة *Saccharomyces boulardii*

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Abstract

Antibiotic sensitivity of *Salmonella* isolates was investigated, the isolates were sensitive to some antibiotics such as chloramphenicol, imipenem and most of them showed resistance to amoxicillin and naldixic acid. The hydrophobicity of *Salmonella* was investigated by applying BATH test (Bacterial Adherence To Hydrocarbons) using xylene. The range was between (21-90) %, the results showed that five isolates were hydrophobic including sal10 isolate. The surface layer proteins (SLP)of sal10 showed the ability to act as agglutinins by applying haemagglutination test with and without 0.5% mannose, also the bacteria seemed to possess pili -type1 because of its' ability to agglutinate with human blood cells and yeast cells. The agglutination of the bacteria was inhibited in the presence of mannose.The isolate sal10 was adhesive to buccal epithelial cells in an average 52 cell/epithelial cell, while this average decreased to reach 24 cell/ epithelial cell in the presence of *Saccharomyces boulardii* which was able to inhibit 54% of the bacterial adherance.

المستخلص

تم التحري عن حساسية عزلات *Salmonella* لعدد من المضادات الحياتية وقد كانت العزلات حساسة لعدد من المضادات منها chloramphenicol و imipenem وأغلبها مقاومة لعدد من المضادات منها amoxicillin وnaldixic acid . كما تم التحري عن صفة السطح الكارة للماء بطريقة الألتصاق على سطوح المواد الهيدروكاربونية بأستعمال الزايلين وقد تراوحت النسبة المئوية للألتصاق بين (21-90)% وكانت خمسة عزلات كارهه للماء ومنه ا العزلة sal 10 العائدة لنوع *S. typhimurium*. أختبرت قدرة البروتينات السطحية المستخلصة للبكتريا على العمل كعوامل تلازن بأجراء أختبار التلازن الدموي بوجود أو عدم 0.5 % وجود سكر المانوز وكانت هذه البروتينات قادرة على العمل كعوامل تلازن وثبط عملها بوجود المانوز . من جهه أخرى كانت العزلة sal10 تمتلك شعيرات من النمط الأول وقد تم التحري عن ذلك بأستعمال طريقة التلازن الدموي بوجود أو عدم وجود المانوز وطريقة التلازن مع الخمائر بوجود أو عدم وجود المانوز، كانت البكتريا قادرة على التلازن مع خلايا الدم و *S. boulardii* وغير قادرة على ذلك بوجود سكر المانوز . كما كانت العزلة sal 10 قادرة ع لى الألتصاق على سطوح الخلايا الظهارية بمعدل 52 cell/epithelial cell في حين كانت ملتصقة بمعدل 24cell/epithelial cell بوجود *Saccharomyces boulardii* حيث كانت الخميرة قادرة على تثبيط الألتصاق بنسبة 54 %.

Introduction

Salmonella spp. are pathogenic in both humans and animals, they can cause diseases ranging from gastroenteritis to typhoid fever depending on the serotype and the host [1]. Many investigations had been done upon *Salmonella* serotype *Typhimurium* in terms of pathogenesis in comparison with other serotypes [2]. Many investigations had been done upon factors which affect *Salmonella* adhesion: Hydrophobicity of cell surface of bacteria affects directly on its' adhesion, when bacteria is hydrophobic

it is more adhesive [3]. *Salmonella* possess type-1 fimbriae which mediates attachment of bacteria to mannose receptors on mammalian cells [4]. Surface Layer Proteins (SLP) which is a paracrystalline surface also has a role in both adhesion and virulence of many bacteria [5]. As a result of the alarming increase in bacteria resistance including *Salmonella* (which may be due to the random use of antibiotics or the use of antibiotics in the therapy of animals and as growth promoters) [6].Using living ingested microorganisms instead of antibiotics may play a role in prevention and treatment of intestinal infections including salmonellosis [7]. *Saccharomyces boulardii* is the only yeast with proven probiotic efficiency and clinical effects [8]. Many experimental effects of *S. boulardii* had been determined such as: inhibition of enteric pathogens, modulation of immune system, anti-inflammatory effects [9]. This study aimed to examine the adhesion abilities of *Salmonella* by detecting its' cell surface characteristics which helps in understanding its' inhibition by *S. boulardii* a probiotics strain, this represent an important application in biotechnology in the present.

Materials and methods

Isolation and characterization of *Salmonella*

Twenty five isolates suspected to be *Salmonella* were obtained from the Teaching Laboratories of Medical City /Baghdad. These isolates were characterized as *Salmonella* by using biochemical tests [10]. APi 20 E system was used for additional biochemical tests. Serological classification was carried on in Central Public Health Laboratories / Baghdad.

***Saccharomyces boulardii*:** The probiotic strain was obtained from Ultra-levure-inter flora (Biocodex, Inc. /France).

Antibiotic sensitivity test (qualitative disk method): 13 antibiotic disks (Amoxicillin, Ciprofloxacin, Amoxicillin –Clavulonic acid, Imipenem, Gentamicin, Nirtofurtoin, Ceftriaxon, Tetracyclin, Trimethoprim– Sulfamethaxazole, Naldixic acid, Chloramphenicol, Ampicillin, Cephalothin) were used to detect the sensitivity of *Salmonella* isolates [11]. The test was carried on by culturing bacteria at 37C for 18 hr and suspended, homogenized in normal saline and compared with 0.5 standard MacFarland solution and the suspension was cultured on Muller– Hinton agar. The results were compared with National Committee for Clinical Laboratory Standard [11].

Detection of surface hydrophobicity of *Salmonella*

Cell surface hydrophobicity of the bacteria was determined by using Bacterial Adhesion To Hydrocarbons (BATH) test [12]. The bacteria were grown in tryptic soy broth at 37Ċ for 18 hr. The Optical Density (OD) was adjusted to 1 at 600 nm. Xylene was added and mixed with the suspension using the vortex; the aqueous phase was taken by using Pasteur pipette. Hydrophobicity was calculated as following:

$$\text{Hydrophobicity} = [\ O.D_{initial} - O.D_{aqueous\ phase} / O.D_{\ initial}\ ] \times 100$$

Extraction and purification of Surface Layer Proteins (SLP) of *S. typhimurium*

The extraction was done by using the modified Sarkosyl method [13] which includes obtaining overnight culture of bacteria harvested and resuspended in PBS and then treated with bovine deoxyribonuclease , sonicated and centrifuged , supernatant was taken and recentrifuged at 10000 r/min for 1 hr. The pellet was treated with 2%

sakosyl dissolved in 50 mM – Tris HCl and treated further with 10 mM- Tris HCl containing 2% Sodium Dodcyl Sulfate (SDS). The suspension was collected and assessed for protein content.

Detection of surface layer proteins as agglutinins by haemagglutination

The test was carried on by mixing 25µL of extracted SLPs with 25 µL of 10% human erythrocytes which were suspended with or without 0.5% mannose solution on a glass slide [4].

Detection of Type-1 pili in *S. typhimurium*

This test was performed by using two methods:

1. **Agglutination with human RBCs (Haemagglutination) :**
   The test was done as described previously [4] by mixing 25µL of bacterial broth with 25µL of 10% human erythrocytes on a glass slide; the agglutination was examined visually and microscopically.
2. **Agglutination with *S .boulardii* :**
   Yeast was cultured separately in a medium (2% peptone, 1% yeast extract, 2% glucose) with shaking for 18 hr. at 37C̊, centrifuged and resuspended in PBS and were treated with gluteraldehyde and stained with safranine, the agglutination was proceeded as described previously by mixing bacteria with yeasts [14].

Inhibition of the adhesion of *S. typhimurium* by *S. boulardii* on human buccal epithelial cells

1. **Preparation of *S. typhimurium* and *S. boulardii* suspensions**
   Microbial suspensions were prepared by centrifugation overnight of microbial growth in BHI broth, the microbial cells number was adjusted to 1×10^8cell/ml by using viable count method[15].
2. **Preparation of buccal epithelial cells**
   This step was carried on by taking a sterile cotton swab , scraping it on the walls of the buccal cavity suspending and washing by PBS the suspension three times by using centrifugation in 2000 r/min for 5 min [16]. The number of the epithelial cells was adjusted to 1×10^5 cell/ml by using haemocytometer.
3. **Testing the ability of. *S. typhimurium* and *S. boulardii* to adhere** This step was carried on by taking 0.5 ml of each microbial suspension prepared previously and mix it separately with 0.5 ml of epithelial cells suspension and incubated with shaking (70 r/min) at 37C for 60 min, the resulting suspension was washed and resuspeded by centrifugation (2000 r/min) by PBS [16]. One drop was transferred to a clean slide, fixed with methanol then stained by Geimsa stain. Microbial cells that adhered on 50 epithelial cells were counted and their average was calculated.
4. **Testing the ability of *S. boulardii* to inhibit the adhesion of *S. typhimurium*** This step was carried on as described previously [15] by mixing equal volumes of both microbes with epithelial cells (all prepared previously). Inhibition of the adhesion of *S. typhimurium* by *S. boulardii* was calculated as the following:
   Inhibition of adhesion rate % = [(average of *Salmonella* adhesion − its' adhesion in the presence of *Saccharomyces*) / average of *Salmonella* adhesion] ×100.

Results and discussion

Isolation and characterization of *Salmonella*

10 isolates diagnosed as *Salmonella* depending on their biochemical tests which were mentioned previously [10] and the results were confirmed by applying APi 20E system. The ten isolates of *Salmonella* were tested to identify the serotypes to which they belonged to as its' elucidated in Table (1).

Table (1): Serotypes of *Salmonella*

Isolates	Serotype	Source of Isolation
sal 1	*S.ependroff*	stool
sal 2	*S.muenchen*	stool
sal 3	*S.enteritidis*	stool
sal 4	*S.typhi*	blood
sal 5	*S.enteritidis*	stool
sal 6	*S.enteritidis*	stool
sal 7	*S.typhi*	blood
sal 8	*S.enteritidis*	stool
sal 9	*S.anatum*	stool
sal 10	*S.typhimurium*	stool

Antibiotic sensitivity test of *Salmonella*

As it is shown in Figure (1), the highest sensitivity of *salmonella* isolates was observed towards: imipenem (100%) and chloramphinicol (100%), trimethoprim-sulfamethaxazol (90%) followed by tetracycline (80%) and (80%) for ciprofloxacin. A study showed the sensitivity towards chloramphinicol and trimethoprim-sulfamethaxazol was (90%), and it also showed that imipenim was the only effective agent to treat multidrug resistant *Salmonella* [17]. The results showed that all isolate (100%) were resistant to amoxicillin-clavulonic acid, 10% sensitive to naldixic acid, 70% sensitive to cephalothin, 60% sensitive for ampicillin and gentamicin. It was also observed that 30% of the isolates were sensitive to amoxicillin and ceftriaxon and 10% were sensitive to nitrofurantoin. The isolate sal 10 which belongs to the serotype *S. typhimurium* was resistant to penicillins (ampicillin and amoxicillin) and β-lactamase inhibitors combinations (amoxicillin – clavulonic acid) and also resistant to cephalosporins (cephalothin, ceftriaxon) This is due to the production of extended – spectrum β- lactamases, such β- lactamases were described in *Salmonella* [18]. It was observed an emergence of resistance to ciprofloxacin with isolates sal9 and sal 2. A study reported that resistant strains to ciprofloxacin were shown to have mutations in the production of specific amino acids in specific positions in genome , when imipenem is considered according to results to be effective to Salmonellosis treatment, imipenem resistant *Salmonella* isolates have already been reported since 1997 [17].

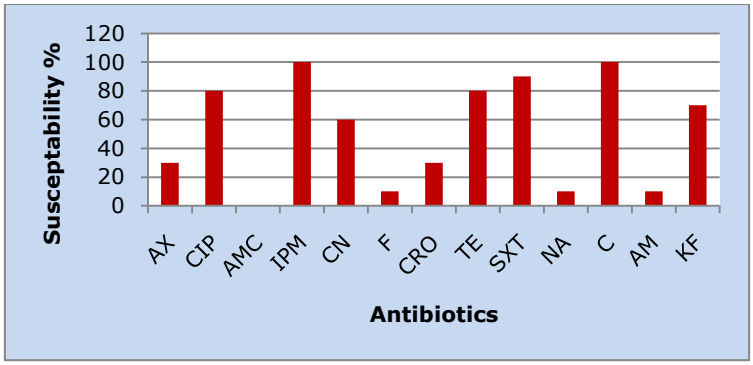


Figure (1): Susceptibility Percentages of *Salmonella* Isolates for Antibiotics
AX :Amoxicillin, CIP:ciprfloxacin, AMC:amoxicillin- clavulonic acid, IPM:imipenem, CN:gentamicin, F: nitrofuratoin, CRO : ceftriaxone, TE: tetracycline , SXT: trimethoprim-sulfamethaxazole , NA:naldixic acid, C:chloramphinicol ,AM:ampicillin,KF:cephalothin

Detection of surface hydrophobicity of *Salmonella*

As it is represented in Table (2), the hydrophobicity percentages ranged between 21 – 90%, the isolates with their hydrophobicity more than 40% are considered hydrophobic [3]. The most hydrophobic isolates were sal 7and sal 10. Xylene was used instead of aliphatic hydrocarbons such as hexadecane, n-octane because it is negatively charged when it is in contact with aqueous solutions including PBS and can detect the less hydrophobic cells [3] Figure (2). In a previous study reported that physiochemical properties (including hydrophobicity) play a major role in initial interaction with host tissue, on the other hand it was found the presence of a relationship between hydrophobicity of *Salmonella* and its' virulence [12]. Therefore there had been attempts to lower the hydrophobicity of *Salmonella* as a method to decrease its' adhesion on surfaces either by using sub inhibitory concentrations of antibiotics, or by adding hydrophilic groups or minerals [19].

Table (2): Hydrophobicity % of *Salmonella* isolates measured at wave length 600 nm

Isolates	Initial Optical Density (O.D $_{initial}$)	Aqueous phase Optical Density (O.D $_{aqueous}$ phase)	Hydrophobicity %
sal 1	1	0.74	26
sal 2	1	0.61	39
sal 3	1	0.66	34
sal 4	1	0.49	51
sal 5	1	0.41	59
sal 6	1	0.76	24
sal 7	1	0.1	90
sal 8	1	0.79	21
sal 9	1	0.5	50
sal 10	1	0.2	80

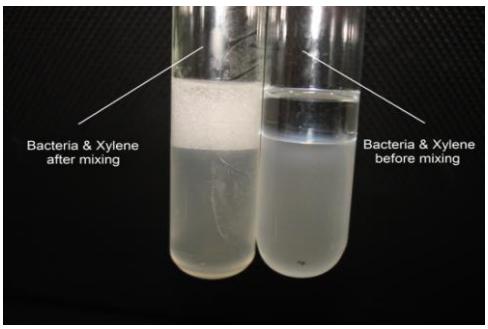


Figure (2): Hydrophobicity Detection Test for *Salmonella*

Detection of surface layer proteins as agglutinins by haemagglutination Many investigations had been done upon *Salmonella* serotype *Typhimurium* in terms of pathogenesis in comparison with other serotypes [2] , so the isolate sal 10 was taken to study its' surface characteristics.

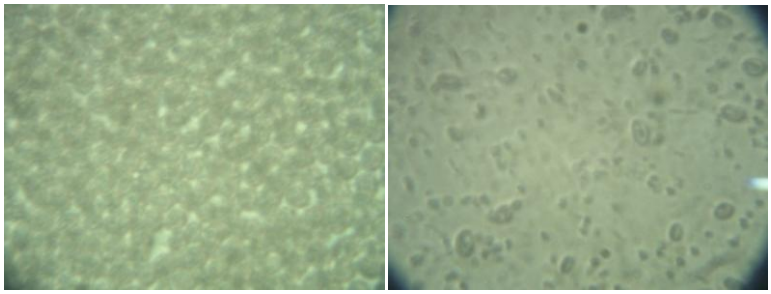
Protein content of the surface layer extract was 0.213µg/ml. The ability of extracted Surface Layer Proteins (SLP) of sal10 to act as agglutinins was observed.

In *Salmonella*, these proteins play an important role in adhesion by acting as adhesins and by possessing mannose sensitive and mannose resistant molecules; SLPs are also required in invasion of the bacteria [20,21]. SLPs are present in all *Salmonella* species (motile and non motile) and have an antigenic properties [13]. This explains the ability of *S. typhimurium* mutants lacking motility and non fimbriated to remain virulent, these mutants have low levels of adhesion to mucosal surfaces but in contrast they remain virulent, this suggests that there are another factors contribute in adhesio (have receptors on mammalian cells) and responsible of virulence [20].

Detection of Type-1 pili in *S. typhimurium*

Type -1 pili test showed the ability of *Salmonella* cells to agglutinate with either human erythrocytes and *S. boulardii* cells and its' inhibition in the presence of mannose. This suggests that *Salmonella* possesses type 1 pili (mannose sensitive) and this explains the inhibition of its' agglutination when mannose was

added Figure (3). This fact agrees with a previous study [14].This type of pili mediates the attachment of bacteria to mannose which enter in the structure of mammalians cell wall [4]. Also as units known as mannans in the yeasts cell wall [22].



A: without mannose **B: with mannose**

Figure (3): Haemagglutination Test of *Salmonella* Isolate (sal 10)

Inhibition of the adhesion of *S. typhimurium* by *S. boulardii* on human buccal epithelial cells

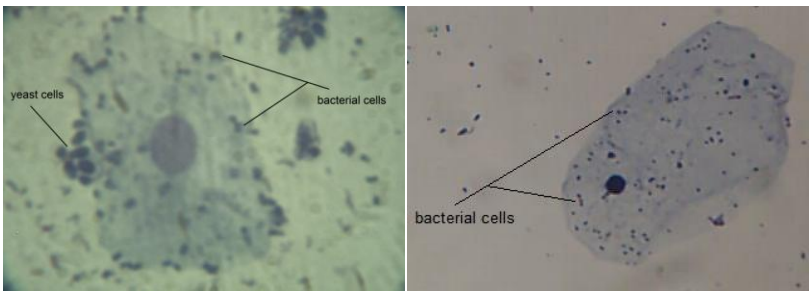
Adherence of pathogenic bacteria to host cells is an important step in virulence, the adhesion of pathogenic bacteria to the gut epithelium is an obligatory step in the infection process [15]. The adhesion average of *S.typhimurium* was 52 cell / epithelial cell whereas the adhesion average of *S.boulardii* was 20 cell / epithelial cell. The adhesion average of both microbes is considered as a good adhesion [15].

In the presence of *S. boulardii*, it had been observed a decrease in the adhesion average of the bacteria to reach 24 cell/ epithelial cell, in another word 54% of the bacterial adhesion had been inhibited by *S. boulardii* (Fig. 4).

S. boulardii has been used in the treatment of different types of diarrheal diseases such as antibiotic – associated diarrhea, traveler diarrhea, acute gastroenteritis and

chronic diarrhea in HIV patients, the mechanism of action of *S. boulardii* in experimental infections had been extensively studied in both animals and invitro assays , these studies indicated that *S. boulardii* may have a protective role and specific activities against various enteric pathogens [23]. The protective role of *S. boulardii* was investigated in previous study against *S. typhimurium* and *Shigella flexneri* in mice, mortality and histipathology revealed a protective effect in yeast treated mice, it was hypothesized that yeast and the bacteria compete for the same adhesion sites [23].

***S.boulardii* also has many other protective effects such as:** delaying the host cell apoptosis of bacteria by decreasing 50% of the number of intracellular bacteria and the changes in the cytoskeleton of the epithelial cells [24]. It was found that *Saccharomyces* was able to colonize and survive in the gastrointestinal tract of germ free mice and also protect the animals against oral infection with *S.typhimurium* [22].The ability of *S.boulardii* to agglutinate with cells of *S.typhimurium* is considered as a protective mechanism against the infection by this pathogen, detection of lectin sites for mannose sensitive adhesion on the outer membrane of the yeast and the irreversible binding pathogens and provides a substitute for drugs used in the treatment of enteric pathogens [25]. As it is mentioned previously D- mannose had been found to be an effective inhibitor of the bacterial adherence. *S.boulardii* cells contains mannose in their cell wall, this mannose may act as an alternative for the attachment of pathogens [25].



A:adhesion of *S.typhimurium* in the presence of *S.boulardii* **B: adhesion of *S.typhimurium***

Figure (4): Inhibition of The Adhesion of *S.typhimurium* by *S.boulardii*

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