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INHIBITION OF PSEUDOMONAS AERUGINOSA BY BACTERIOCIN PRODUCED BY LACTOBACILLUS FERMENTUM

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ABSTRACT

Ninty-four clinical samples were collected as swabs from cows suffered from different types of wounds in Baghdad governorate. Samples were cultured on different media and biochemically tested for identify the type of bacteria that colonize in the wounds and to isolate and identify *P. aeruginosa*. The results revealed that out of 94 samples collected from wounds, 87 bacteria isolate (90.55%) were grow bacterial growth and consider positive samples, while negative growth appear to be 7.4%. The profile of bacteria in the cultured samples revealed bacteria as fellow: *P. aeruginosa* 36 (36.78%) was the most common isolate followed by S. aureus 22(25.28%), E. coli 21(24.1%), K. pneumoniae 5 (5.7%), P. mirabilis 3(3.4%). Thirty six P. aeruginosa isolates were tested for antibiotic sensitivity. All isolate (100.0%) are resistant to Ampicilin-Sulbactam, Ticracillin-pyostacine Ticracillin-,clavulnic acid. and Ticracillin, these isolates show high resistance to Cotrimoxazol 94.4%, Cefepim 77.7% , Ceftazidime 66.6%, Imipenem 63.8%, Meropenem 61.1% while show low resistance to Gentamicin 44.4%, Tobramycin 38.8%, Piperacillin-pyostacine 27.7%, Piperacillin-Tazobactam 30.5%, Piperacillin- Tazobactam-pyostacine 33.3%, Colistin 33.3%, Ciprofloxacin 22.2% and Amikacin 22.2%, and among these antibiotics, Amikacin and Ciprofloxacin was the most effective antibiotic against P. aeruginosa isolates.

Keywords: Pseudomonas Aeruginosa, Bacteriocin, Lactobacillus Fermentum**.**

INTRODUCTION

Pseudomonas aeruginosa, is an opportunistic pathogen gram negative bacilli ,that have a distinctive ability to cause a remarkable morbidity and mortality according to severe diseases which infected the respiratory tract, blood stream, gastrointestinal tract (Olejnickova *et al.*, 2014).

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Probiotics are living microorganisms that have a benefit health effect when given in adequate amounts to the host (Corr *et al*., 2009). The benefits of probiotics are according to their multifactorial ability to produce postbiotics like bacteriocins, exopolysaccharides short-chain fatty acids, etc. or interaction with the microflora of the intestine (Cizeikiene *et al*., 2013; Bamidele *et al*., 2013). Bacteriocins are peptides synthesized in the ribosome and have effect against closely related bacterial strains and are found in three type 1, II, or III (Cleveland *et al*., 2001; Kemperman *et al*., 2003). The bacteriocins syntheses by

different species of Lactobacillus are type II bacteriocins, that are small, heat stable proteins and consider cell membrane-harmful ability (Corr *et al*., 2007).

MATERIAL AND METHOD

Sample collection.

Samples were collected from 94 wounded cow in Baghdad province for the period from December 2020 to May 2021. wound specimens were collected by cotton swabs from the deep lesions.

Conventional Bacteriological Diagnosis

Macroscopically the obtained swabs were cultured and colonies recovered were diagnosed microscopically (Gram stain), and different biochemical tests were done according to standard methods (Madigan and Martinko, 2005; Chapin, 2007)

Sample culturing

All specimens first inoculated on MacConkey agar and blood agar. All these agar were incubated aerobically at 37°C for 24hrs. Different types of bacteria were identified based on their ability to stain, color, shape, size, odor, producing some pigments, transparency and mucoid properties of the colonies growing on Blood agar and MacConkey agar plates, and some biochemical tests were achieved by conventional identification methods, as well as the commercial kits API 20E and API Staph for gram negative bacteria and for gram positive bacteria respectively (BioMerieux, France).

Antibiotic Susceptibility

Thirty six *P.aeruginosa* isolates are tested for antimicrobial sensitivity, by means of ATB–PSE5 kit (Biomerieux) according to standard protocols.

Antibacterial activity of crude bacteriocin determination:

The activity of crude bacteriocin against *P.aeruginosa* was performed using the agar gel diffusion method (Shanks *et al*., 2012). *Lactobacillus fermentum* was cultured in deMan, Rogosa, and sharp broth for 24 h at 37 °C and centrifuged (9000g for 10 min / 4 °C). After that, adjusted the pH of the supernatant to 6.8 with 1 mol/L NaOH and this store as crude bacteriocin.

Identifying the nature of crude bacteriocin

The nature of crude bacteriocin was assessed by following: 200 µL of crude bacteriocin incubating with 20 µL of proteolytic enzymes (proteinase K; pH 7.0 and trypsin; pH 7.0 at a final concentration of 1 mg/mL). incubated for 5 hours at 37 °C, after that antimicrobial activity was measured by using agar well diffusion test where untreated CFS consider as control. (Sharma *et al*., 2018).

RESULTS

Bacterial Isolation

Out of 94 samples obtained wounds, 87 samples (90.55%) were growing in an aerobic culture (positive samples), while 7.4% have no growth. The profile of bacteria in the cultured samples revealed bacteria as fellow: *P. aeruginosa* 36 (36.78%) was the most common isolate followed by *S. aureus* 22(25.28%), E. coli 21(24.1%), *K.pneumoniae* 5(5.7%), P. mirabilis 3(3.4%). The domination of the *p.aureoginosa* in wound abscess seen in many other study cattle (Saleh *et al*., 2016; Neamah, 2017; Hammond *et al*., 2010). The absence of the growth in some samples may be because of uninfected wound with aerobic bacteria or the sample taken from an early wound.

Biochemical tests

Biochemically, *Staphylococcus* is catalase positive, and this simple test can differentiate *Staphylococcus* from *Streptococcus*. Oxidase test helps in differentiating *Staphylococcus*, which gives positive result, from *Micrococcus*, which

gives negative reaction. Differentiation among species of *Staphylococcus* was achieved by the coagulase test, in which only *S. aureus* gives positive reaction, also this bacterium is Arginine dehydrogenase positive, Urease positive and utilize most carbohydrate, and the results are shown in table(1) and, figure (1).

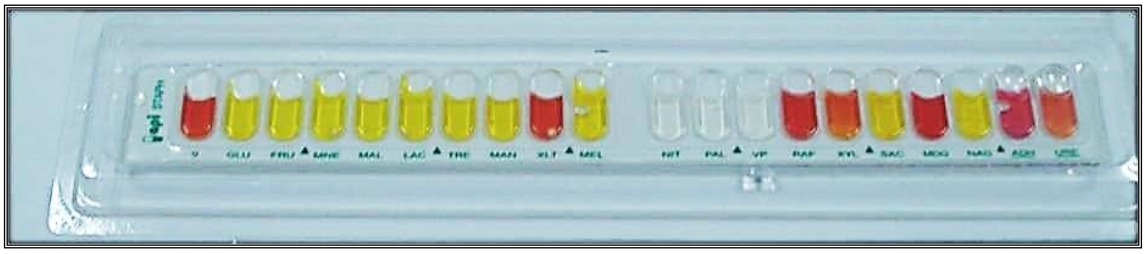

Figure 1: Api Staph strip used for *S. aureus* identification.

Table1: Biochemical tests of API Staph system used for identifying gram positive isolates.

			RESULT	
TESTs	**SUBSTRATE**	**REACTIONS \ ENZYMES**	**NEGATIV E**	**P OSITIVE**
0	**No substrate**	**Negative control**	**red**	**–**
GLU·	**D-Glucose**	**(Positive control)**		
FRU	**D-Fructose**			
MNE	**D-Mannose**		**red**	**yellow**
MAL	**Maltose**			
LAC	**Lactose**	**Acidification due to carbohydrate Utilization**		
TRE	**Q-Trehalose**			
MAN	**Q-Mannitol**			
XLT	**Xylitol**			
MEL	**D-Melibiose**			
NIT	**Potassium nitrate**	**Reduction of nitrate to nitrite**	**NIT' + NIT 2/10 min**	
			colorless-light pink	**Red-pink**
PAL	**β-naphthyl-acid phosphate**	**Alkaline phosphatase**	**ZYM A + ZYM B/1 0 min**	
			yellow	**Violet-pink**
VP	**Sodium pyruvate**	**Acelyl - methykarbinol production**	**VP 1 +VP2/10min**	
			colorless	**violet-pink**
RAF	**Raffinose**	**Acidification due to carbohydrate utilization**	**Red**	**Yellow**
XYL	**Xylose**			
SAC	**Sucrose**			
MDG	**α-methyl-D-qlucoside**			
NAG	**N-acetyl-glucosamine**			
ADH	**Arginine**	**Arginine dihydrolase**	**yellow**	**orange-red**
URE	**Urea**	**Urease**	**yellow**	**red-violet**

-Negative, +positive, V variable

Gram Negative Bacteria

Microscopical and Morphological Diagnosis

- *Klebsiella pneumoniae* is Gram-ve, straight rods, non-motile, appeared as single or double chains and colonies on MacConkey agar were large mucoidal and pink (lactose fermenting) with irregular edges and exhibited no hemolysis on blood agar.

- *Escherichia coli* is Gram-ve bacilli, motile , appeared on MacConkey agar as small pink colonies (lactose fermenting) with regular edges. The bacteria on blood agar have the ability to β-hemolysis after 24 hour of incubation.

- *Proteus mirabilis* appeared as Gram-ve bacilli, motile and colonies on MacConkey agar were small in size and pale (lactose non-fermenting). They were recognized by swarming movement and rotten fish odor. This bacterium exhibited no hemolysis on blood agar.

- *Pseudomonas aeruginosa* is Gram negative rods, motile and on MacConkey agar colonies shows as small circular convex with pale color (non-fermenting to lactose), the colonies appear as either smooth or rough with regular edges. They also have an odor like a rotten-potato odor sometime, and almost all isolates produced a β-hemolysis on blood agar. *Pseudomonas* agar consider a selective medium for *Pseudomonas* genus this bacterium was able to grow on and also some isolates were able to produce pigments as pyocianin and the fluorescent pigment pyovridin (Figure 2). This bacterium also was capable of growth at 4°C and 42°C.

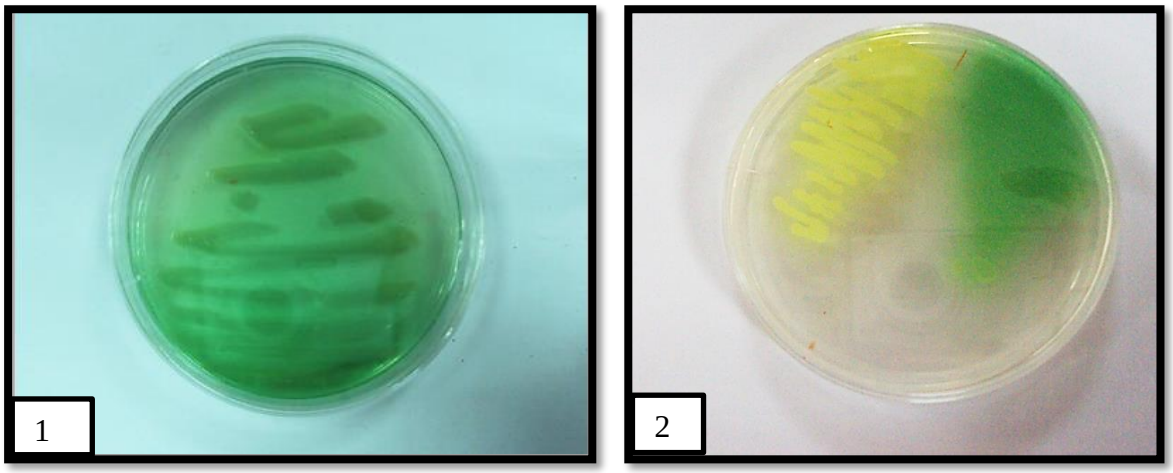

Figure 2: 1. *Pseudomonas aeruginosa* isolate producing pyocyanin pigment on *Pseudomonas* isolation agar. **2**. Two *P. aeruginosa* isolates growing on *Pseudomonas* isolation agar; one of them produced blue-green pigment (pyocyanin) and the other produced yellow-green fluorescent pigment (pyoverdin).

Table 2: Cultural and Biochemical tests used for identifying Bacterial isolates from burn and wound infections.

Test / Bacteria	Gram stain	Catalase	Oxidase	Hemolysine	Growth at 42°C	Growth at [illegible]	Growth on Psedomonas agar	Motility	Lactose fermentation	API20E system	Coagulase	APISTAPH system
P.aeruginosa	-	+	+	V	+	+	+	+	-	+	/	/
K.pneumoniae	-	+	-	-	/	/	/	-	+	+	/	/
E.coli	-	+	-	+	/	/	/	+	+	+	/	/
P.mirabilis	-	+	-	-	/	/	/	+	-	+	/	/
S.aureus	+	+	+	+	/	/	/	-	/	/	+	+

+ positive,-Negative ,V variable, / not done, ND: not detected by API20E system termed others.

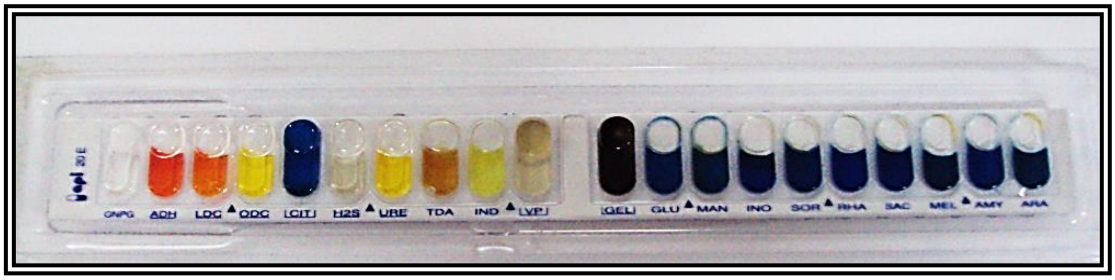

Figure 3: Api 20E strip used for identifying *P.aeruginosa. K.pneumoniae E.coli and P.mirabilis*

Table 3: Biochemical tests of API 20E system used for identifying gram negative isolates.

Test / Bacteria	*P.aeruginosa*	*K.pneumoniae*	*E.coli*	*P.mirabilis*
ONPG	-	+	+	-
ADH	+	-	-	-
LCD	-	+	+	-
ODC	-	-	+	+
CIT	+	+	-	V
H2S	-	-	-	+
URE	-	+	-	+
TDA	-	-	-	+
IND	-	-	+	-
VIP	-	+	-	-
GEL	V	-	-	V
Sugars fermentation/Oxidation				
GLU	- / V	+	+	+
MAN	-	+	+	-
INO	-	+	-	-
SOR	-	+	-	-
RHA	-	+	+	-
SAC	-	+	+	+
MEL	-	+	+	-
AMY	-	+	-	-
ARA	-	+	+	-

Negative, +positive, V variable

Antibiotic Resistance of *P. aeruginosa*

Pseudomonas aeruginosa inherently have the ability to resistant to most type of pencillins, cephalosporines, sulfonamides and nalidixic acid. *P.aeruginosa* is naturally susceptible to aminoglycosides, antipseudomonal penicillins, cephalosporins, Quinolones and carbapenems. In the treatment of disease caused by *P.aeruginosa* aquired antibiotic resistance is a common phenomenon (Giliene *et al.*, 2007).

Thirty six *P. aeruginosa* isolates were tested for antibiotic sensitivity. All isolate (100.0%) are resistant to Ampicilin-Sulbactam, Ticracillin-pyostacine Ticracillin-.clavulnic acid. and Ticracillin, these isolates show high resistance to Cotrimoxazol 94.4%, Cefepim 77.7% , Ceftazidime 66.6%, Imipenem 63.8%, Meropenem 61.1% while show low resistance to Gentamicin 44.4%, Tobramycin 38.8%, Piperacillin-pyostacine 27.7%, Piperacillin- Tazobactam 30.5%, Piperacillin- Tazobactam-pyostacine 33.3%, Colistin 33.3%, Ciprofloxacin 22.2% and Amikacin 22.2%, and among these antibiotics, Amikacin and Ciprofloxacin was the most effective antibiotic against *P. aeruginosa* isolates (Table 4).

Table 4-7: Antibiotic sensitivity test for *Pseudomonas aeruginosa* isolates.

Antibiotics	*P. aeruginosa* isolates (Number = 36)					
	Resistant		Intermediate		Sensitive	
	No.	%	No.	No.	%	No.
Ampicilin-Sulbactam	36	100.0	-	-	-	-
Ticracillin-Pyostacine	36	100.0	-	-	-	-
Ticracillin- Clavulnic acid	36	100.0	-	-	-	-
Ticracillin	36	100.0	-	-	-	-
Piperacillin	13	36.1	-	-	23	63.8
Piperacillin-Pyostacine	10	27.7	-	-	26	72.2
Piperacillin-Tazobactam	11	30.5	-	-	25	69.4
Piperacillin-TazobactamPyostacine	12	33.3	-	-	24	66.6
Cefepim	28	77.7	-	-	8	22.2
Imipenem,	23	63.8	-	-	13	36.1
Meropenem,	22	61.1	7	19.4	7	19.4
Ceftazidime	24	66.6	-	-	12	33.3
Amikacin	8	22.2	-	-	28	77.7
Gentamicin	16	44.4	-	-	20	55.5
Tobramycin	14	38.8	-	-	22	61.1
Ciprofloxacin	8	22.2	-	-	28	77.7
Colistin	12	33.3	-	-	24	66.6
Cotrimoxazol.	34	94.4	-	-	2	5.5

It was observed that the crude bacteriocin of *Lactobacillus fermentum* inhibited the *P. aeruginosa* growth, an inhibition zone 14 in maxim and an average inhibition zone of 12.5 among all 36 isolate Figure (4).

Probiotics proved to produced postbiotics that was exopolysaccharides, short- chain

fatty acids, etc. and have an inhibitory ability to many pathogens such *Listeria monocytogenes*, *Clostridium perfringens*, *Salmonella enterica*, and *Escherichia coli* and thereby reduce the risk correlated with probiotics even in host with immunocompromised system(Patel and Denning., 2013; Kareem *et al*., 2014; Kareem *et al*., 2016).

Figure (4):-The inhibition zone of crude bacteriocin on some isolate of *P.aureoginosa*

Certain bacteria with pathogenic potential can be inhibited from growing by lactic acid bacteria. (de Vrese and Marteau, 2007) It is currently known that a number of lactobacilli strains may prevent other bacteria from growing in vitro. (Shanahan, 2006), It has been demonstrated that Lactobacillus acidophilus and Lactobacillus rhamnosul prevent the growth of Helicobacter pylori, a significant cause of intestinal ulcers. (deVrese *et al*., 2003).

In vitro studies have indicated that many strains of LAB have been shown to inhibit growth and metabolic activity as well as the adhesion to intestinal cells of enteropathogenic bacteria (Salmonella, Shigella, E.coli, Vibrio cholera) Mach *et al*.,1999 and Reid *et al*., 2003 refer that the LAB have a potential activity against different pathogenic bacteria like *S. typhimurum; E.coli* .

Postbiotics (i.e. bacteriocin) are known to have both bactericidal and bacteriostatic effects because bacteriocin leads to degradation of cellular DNA, pore formation in cell membrane, disruption through specific cleavage of 16S rRNA, and inhibition of peptidoglycan synthesis (Blanc and Todorov 2011; Li *et al.*, 2014). Sharma *et al*., 2018 showed effect of bacteriocin on the ability of P.aureoginosa biofilm formation.

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تثبيط بكتريا الزائفة الزنجارية بواسطة البكتروسين المنتج من بكتريا LACTOBACILLUS FERMENTUM

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تم جمع أربعة وتسعون عينة سريرية على شكل مسحات من الأبقار المصابة بأنواع مختلفة من الجروح في محافظة بغداد. تم زرع العينات على أوساط مختلفة واختبارها كيميائياً للتعرف على نوع البكتيريا الملوثة للجروح ولعزل وتشخيص *P. aeruginosa*. أظهرت النتائج أنه من أصل ٩٤ عينة تم جمعها من الجروح، تم عزل ٨٧ بكتيريا (٩٠,٥٥%) نمواً بكتيرياً واعتبرت عينات إيجابية، في حين ظهر نمو سلبي بنسبة ٧,٤. كشف تشخيص البكتيريا في العينات المزروعة عن نسب مختلفة لعدة أنواع من البكتريا كالتالي : ٣٦ عزلة لبكتريا *P. aeruginosa* بنسبة (36.78%) وكانت العزلة الأكثر شيوعًا تليها ٢٢ عزلة لبكتريا *S. aureus* بنسبة (25.28%) ،ثم ٢١ (٢٤,١٪) عزلة لبكتريا E. coli ،ثم ٥ عزلات لبكتريا *K. pneumoniae* بنسبة (٥,٧٪)،وأقلها كانت ٣ عزلات (٣,٤٪) *P. mirabilis*.كما تم اختبار ستة وثلاثين عزلة من *P. aeruginosa* لحساسيتها للمضادات الحيوية وأظهرت جميع العزلات (١٠٠,٠%) مقاومة لـ Ampicilin-Sulbactam، Ticracillin-pyostacine، Ticracillin-، clavulnic acid و التيكراسيلين. أظهرت هذه العزلات مقاومة عالية للكوتريموكسازول بنسبة ٩٤,٤%، سيفيبيم ٧٧,٧%، سيفتازيديم ٦٦,٦%، إيميبينيم ٦٣,٨%، ميروبينيم ٦١,١% بينما أظهرت مقاومة منخفضة للجنتاميسين ٤٤,٤%، توبراميسين ٣٨,٨%، بيبيراسيلين-بيوستاسين ٢٧,٧%، بيبيراسيلين- تازوباكت ٣٠,٥%، بيبيراسيلين- تازوباكتام- بيوستاسين ٣٣,٣%، كوليستين ٣٣,٣%، سيبروفلوكساسين ٢٢,٢% وأميكاسين ٢٢,٢%، ومن بين هذه المضادات الحيوية كان أميكاسين وسيبروفلوكساسين أكثر المضادات الحيوية فعالية ضد عزلات P. aeruginosa.