



Original Research Article

Effect of *Lactobacillus salivarius* metabolites against *Staphylococcus aureus* producing Phenol-Soluble Modulins (PSMs)

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A B S T R A C T

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In this research, a total of (50) Methicillin resistant *Staphylococcus aureus* isolates were tested for the presence of the *psm-mec* genes by PCR . On the other hand, the antibacterial activity of *Lactobacillus salivarius* metabolites included concentrated and unconcentrated supernatant (acidic and neutrized) and bacteriocin were tested against *S.aureus* producing Phenol-Soluble Modulins (PSMs). Also inhibitory effect of metabolites were tested on colony spreading of PSM producer isolates for (24 and 48)hour of incubation. Results demonstrated presence of *psm-mec* gene in 90% of tested MRSA isolates, the PSM producer isolates showed resistance to the neutralized concentrated cell free supernatant (CFS) and bacteriocin, while showed sensitive to the acidic concentrated and unconcentrated CFS with inhibition diameter ranged(13-19) mm. The acidic concentrated and unconcentrated CFS had good inhibition activity against colony spreading, especially acidic concentrated CFS with higher diameter of colonies reached 7mm after 48h of incubation compared with (15-16)mm for control.

Introduction

Lactobacillus salivarius is Gram-positive bacteria, anaerobic rods , non spore forming , widespread in human mouth, especially in saliva, as well as in intestinal tract of humans and animals (Caralampopoulos and Rastall ,2009). *Lactobacillus* species are the main part of the normal flora of fish (Ghanbari *et al.*, 2009). *L.salivarius* one of the species that isolated from the intestines of fish (Ogunshe and Olabode, 2009 ; Talpur *et al.*, 2012).These bacteria are portentous probiotics(O'Shea *et al.*,2011).

Probiotics are live micro-organisms which

metabolites have several antimicrobial effects, that inhibit Gram-positive and Gram-negative bacteria (Neville and O'Toole, 2010).The antimicrobial compounds that responsible for inhibition of various pathogenic microorganisms including bacteriocins, organic acids, diacetyl, hydrogen peroxide (Bamidele *et al.*,2013).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important human pathogen capable of causing diseases both health care and community-associated infections

(Watkins *et al.*,2012). The ability of *S. aureus* to cause infections depends on the production of virulence factors , Among the most these factors especially in the community-associated Methicillin resistant *S. aureus* (CA-MRSA) lineages, are the so-called phenol-soluble modulins (PSMs) (Tsompanidou *et al.*,2013). These PSMs are amphipathic, short, α -helical peptides that have multiple roles in staphylococcal pathogenesis, causing lysis of RBCs and WBCs and have biosurfactant properties, contributing to biofilm development and the dissemination of biofilm-associated infections, all PSM-types have ability to lysis neutrophils from different species equally efficient and induced membrane damaging effects(Löffler *et al.*,2010; Peschel and Otto, 2013). This study focus on the detection of the *psm-mec* genes in locally MRSA isolates and detection of the inhibitory effect of *L.salivarius* metabolites against growth and colony spreading of *S.aureus* producing Phenol-Soluble Modulins (PSMs).

Materials and Methods

Bacterial isolates

Isolate of *Lactobacillus salivarius* was isolated from intestine of Iraqi fish , then identified through out cultural , microscopical and biochemical test according to (Kandler and Weiss,1986;Hammes and Vogel,1995;Carr *et al.*,2002). Methicillin resistant – *Staphylococcus aureus* (MRSA) isolates were collected from cutaneous samples from patients who were admitted to Baghdad hospitals. The isolates were inoculated a CHROM agar MRSA plate. The results were read after 24 and 48 h of incubation at 35°C for indicating MRSA.

DNA Preparation and PCR

Reactions of PCR with specific primers

were performed to identify *psm-mec* genes of each MRSA isolates according to (Chatterjee *et al.*,2011). DNA template was prepared as described by (Olsvik and Strockbin, 1993). (25 μ l) of PCR amplification mixture contained deionized sterile water,(12.5) μ l Green Go *Taq* Master Mix pH (8) (Promega,USA) . All PCR products were analyzed by electrophoresis through 1% agarose gels.

Preparation of *Lactobacillus salivarius* Supernatant

Lactobacillus salivarius isolate was grown in MRS broth with pH 6.2 at 37°C for 24 h. After incubation , cell free supernatant(CFS) was obtained by centrifugation at 10,000 rpm for 10 min. Supernatant was taken and sterilized by Millipore filter (0.02 μ m) and was concentrated (Lievin *et al.*,2000). The CFS was neutralized by 1N NaOH and used for another assay.

Purification of bacteriocin produced by *Lactobacillus salivarius*

The supernatant which obtained in above step was adjusted to pH 6.2 by adding 1 N NaOH to remove the influence of organic acids (Svetoch *et al.*,2011).The crude bacteriocin was precipitated with 80% ammonium sulphate saturation. The precipitate was dialysed against 20 mM potassium phosphate buffer (pH 7.0) for 12 h at 4°C (Devi *et al.*, 2013) .

Antibacterial activity of *Lactobacillus salivarius* metabolites

Lactobacillus salivarius metabolites including acidic concentrated and unconcentrated cell free supernatant(CFS) ,neutralized concentratd CFS and bacteriocin were screened for their inhibitory activities against *S. aureus* producing PSM, using agar well diffusion-

method. Plates were prepared by spreading approximately 10^5 cfu/ml culture broth of indicator bacterial isolate on nutrient agar surface. The agar plates were left for about 15 min before aseptically dispensing the 50 μ l of each metabolite samples into the agar wells already bored in the agar plates. The plates were then incubated at 37°C for 18 - 24 h. Zones of inhibition were measured and recorded in millimeter diameter.

Effect of *Lactobacillus salivarius* metabolites on colony spreading of *S. aureus*

The colony spreading assay was performed as method described by Kaito and Sekimizu (2007) with some modification. To detect colony spreading of the *S. aureus* isolates producing PSM, the isolates were grown in nutrient broth for 24 h. After 24 h of growth, equal amounts of cells were spotted onto the brain heart infusion (BHI) agar, incubated for (24 and 48)h at 37°C. Colony spreading was detected by measuring the diameter of the resulting transparent zone.

To detect the effect of *Lactobacillus salivarius* metabolites on colony spreading of *S. aureus*, 1ml of each metabolites included acidic unconcentrated and concentrated CFS, neutralized CFS were spread separately on BHI plates, after drying equal amounts of *S. aureus* cells were spotted onto the plates. After incubation at the same condition above, the diameter of the resulting transparent zone of each treatment were measured and compared with control (plates without metabolites).

Results and Discussion

In this research 50 Methicillin resistant *S. aureus* isolates were tested for the presence of the *psm-mec* genes by PCR. Our results

demonstrated presence of *psm-mec* gene in 90% of tested MRSA isolates. The PSM toxin -mec is coupled in its link to an antibiotic resistance element. This allows acquisition by *S. aureus* strains of antibiotic resistance with virulence determinants (Queck *et al.*, 2009). Result of our study shows the *psm-mec* genes is found in wide range among Methicillin – resistant *S. aureus*. The genes that carry resistance to methicillin are located on mobile genetic elements called staphylococcal cassette chromosome *mec*, from 21 – 67 kbp in size (Chatterjee *et al.*, 2011).

The pH of fermentation CFS for *L. salivarius* in current study reached to 4.5, while the pH of control remain without change, a low pH of CFS indicates the production of organic acids by *L. salivarius* which led to this lower. CFS was neutralized with NaOH to remove the role of acids and then concentrated, precipitated by 80% ammonium sulphate to concentrate proteins, followed by dialysis as a step of partially purification of bacteriocin produced by *L. salivarius*. Many bacteriocins of lactic acid bacteria are not produced in high quantities by the producer strain, wherefore it is very necessary to concentrate the supernatant that contains the antimicrobial agents at the initial steps (Vera Pingitore *et al.*, 2007).

The antibacterial activity of acidic concentrated and unconcentrated CFS, neutralized concentrated CFS and bacteriocin from *L. salivarius* isolated from fish intestine were evaluated against *S. aureus* producing PSM. The PSM producer isolates showed resistance to the neutralized concentrated CFS and bacteriocin, while showed sensitive to the acidic concentrated and unconcentrated CFS with inhibition diameter ranged (13-19) mm (Table 1). Here, the results showed that the organic

acids produced by *L.salivarius* isolate the effective metabolites and it is responsible for inhibition of PSM producer isolates. These results agree with Bamidele *et al* (2013), they reported that *L. salivarius* CFS had effect against MRSA and they showed that organic acids were responsible for the inhibitory effect but the neutralized CFS did not have any inhibition activity. *L. salivarius* produce organic acids like lactic acid which can inhibit pathogenic bacteria (Neville and O'Toole , 2010). Fahad and Hala (2011) observed that the acidic CFS of

Lactobacillus inhibited lipase and biofilm production by MRSA. OShea *et al.* (2011) showed that the effect of neutralized supernatant from *L. salivarius* was found to be bordered. Nawaz *et al.* (2009) showed that bacteriocins are the compounds with a lower antimicrobial activity against MRSA , they found bacteriocin of only one strains from fifty strains had inhibition activity. Bacteriocin activity was depressed after the step of ammonium sulfate protein precipitation of the CFS(Svetoch *et al.*,2011) .

Table.1 Antibacterial activity of *L. salivarius* metabolites against *S.aureus* produced PSM

Treatments	Inhibition Zone Diameter(mm)	
	<i>S.aureus</i> isolates	
	No.1	No.2
Acidic unconcentrated CFS	13	13
Acidic concentrated CFS	19	18
Neutralized CFS	-	-
Bacteriocin	-	-

(-): No inhibition zone

Table.2 Inhibitory effect of *L. salivarius* metabolites on colony spreading of *S.aureus* produced PSM

Treatments	Diameter of colony spreading zone(mm)			
	Incubation Time(hour)			
	24		48	
	<i>S.aureus</i> (1)	<i>S.aureus</i> (2)	<i>S.aureus</i> (1)	<i>S.aureus</i> (2)
Control (without metabolites)	12	11	16	15
Acidic unconcentrated CFS	10	9	12	10
Acidic concentrated CFS	6	7	7	7
Neutralized CFS	12	11	13	12

On the other hand, the inhibitory effect of *L.salivarius* metabolites on colony spreading of *S.aureus* isolates producing PSM were detected on BHI agar and the diameter of colony spreading zone was measured. Results showed that the higher diameter of the colonies appeared on control plates was 16mm after 48h of incubation, the acidic concentrated and unconcentrated CFS had good inhibition activity against colony spreading, especially acidic concentrated CFS after 24h and 48h of incubation with higher diameter reached 7mm, while no inhibition activity of neutralized concentrated CFS was observed at 24h of incubation, but it is had inhibitory effect on colony spreading after 48h of incubation comparative of control (Table 2). For another study the colony spreading of *S.aureus* on solid media was 6 mm in diameter (Kaito and Sekimizu, 2007).

Colony-spreading activity of *S. aureus* need the *psma* operon and the *agr* locus up-regulates *psma* to catalyze colony spreading (Omae *et al.*, 2012). CA-MRSA strains have higher colony-spreading capability than HA-MRSA strains. This variation in colony spreading capability is featured to the *psm-mec* gene, this gene in *S. aureus* suppresses its colony spreading activity (Omae *et al.*, 2014). The capability of PSMs to confirm spreading is necessary for *S.aureus* to transfer from a biofilm and to settle the moist surface (Tsompanidou *et al.*, 2013).

The conclusion of our results that the *psm-mec* gene presence in 90% of tested MRSA isolates, these isolates had the ability to spread on surface. The acidic concentrated and unconcentrated CFS from *L. salivarius* isolated from fish intestine had higher inhibition activity against growth and colony spreading of

PSM producer isolates and the organic acid being responsible for this inhibition activity.

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