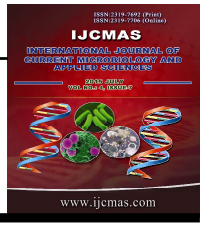
*Int.J.Curr.Microbiol.App.Sci* (2015) 4(7): 35-42

*International Journal of Current Microbiology and Applied Sciences*
***ISSN: 2319-7706*** **Volume 4 Number 7 (2015) pp. 35-42**
**http://www.ijcmas.com**




**Original Research Article**

# CFS and Crude Bacteriocin of *Lactococcus* against Growth and Biofilm Formation for Some Pathogenic Bacteria

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**Keywords**

Biofilm formation, Cell Free Supernatant, pathogenic bacteria

**A B S T R A C T**

Lactic Acid Bacteria (LAB) remains an important group of bacteria, *Lactococcus* is one of the member of these groups, because they are known to exhibit antagonistic activity against pathogenic organisms. This study evaluates the protective effect of *Lactococcus* Cell Free Supernatant (CFS) and Crud Bacteriocin (CB) against growth and Biofilm formation of some pathogenic bacteria. Antimicrobial effect of CFS and CB were tested against growth and biofilm formation of test bacteria by agar well diffusion assay and adhesion reduction test with Trypton Soy Broth. The results implicate that CFS and CB strongly inhibits test bacteria, they were able to inhibit all bacterial isolates with high inhibition zones and with average of 8.5, 20.5 mm respectively, also CFS and CB could reduce the adhesion of bacterial isolates and prevent biofilm formation, the conclusion is that these products (CFS and CB) are very necessary to use them as natural alternative agent because of (1) High bactericidal activity, (2) Do not leave any risk on human health and (3) Do not generate resistant strains like antibiotic.

## Introduction

Lactic acid bacteria (LAB) are a group of gram – positive bacteria including many genera.which produce lactic acid as a major end product, LAB are industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Darsanaki *et al.*, 2012).

*Lactococcus* is one of this group, spherical-shaped, gram – positive bacterium used extensively for industrial production of fermented milk and their product, in human *Lactococcus* has been associated with endocarditis, known as preferred probiotic (probiotic: term, simply means – for life-originating from the Greek words –pro- and –bios-, the most widely definition as: alive microbial feed supplement which beneficially affects the host animal by improving its intestinal balance (Bourouni *et*

*al.*,) with high potential of healing acute disease are generally associated with pathogenic bacteria (Khalid *et al.,* 2011). Bacteriocins are ribosomally synthesized antimicrobial compounds that are produced by many different bacterial species including many members of the lactic acid bacteria, A study of 40 wide –type strains of *Lactococcus* showed that 35 produced nicin (nicinis the only bacteriocin with GRAS (Generally Regarded as Safe) (Rattanachaikunsopon and Phumkhachorn, 2010).

Biofilms are intimate cellular aggregations of one or more organisms on the surface of submerged solid objects that covered by extracellular slimy materials (Renslow *et al.*, 2011), this style of living offers many advantages to the microorganisms that include establishment of adhesion, minimizing desiccation, accumulation of nutrients and protection against harmful materials or drugs (Cuellar-Cruz *et al.*, 2012). Now it is a well- known fact that the biofilm cells show high resistance to antifungal treatments and the host defence mechanisms, and exhibit an excellent ability to adhere to biomaterials (Melean *et al.*, 2010; Cao *et al.*, 2010). Some surface structures of bacterial cells, such as flagella, curelifibers, type I fimbriae and Ag 43 are involved in biofilm formation *E. coli* (Nakao *et al.*, 2012).

There are many researchs for use the LAB generally as antibacterial activity but in the same time did not refer any one of these to use the product of *Lactococcus* (CFS and crude bacteriocine) as natural alternative drugs, so that we preface this working.

## Material and Methods

### Bacterial strains

The microbial strains that including pathogen *E. coli, Klebsiella, Pseudomonas, Staphylococcus* were obtained from high studies laboratory in collage of science, Al – Mustansiriyah university, then sure from their identification by vitic system.

### *Lactococcus* source:

This isolate was from dairy product (local source), it was cultured and grew on De Man Rogosa Sharpe (MRS), identical according to Wood and Holzapfel, (1995) (Wood and Holzapfel, 1995).

### Preparation of cell-free filtrate of *Lactococcus*

We inoculated 10 ml of MRS broth with *Lactococcus* and incubated at 30 $C^{o}$ for 24 h. after incubation, a cell free solution was obtained by centrifuging the bacterial culture at 6000 xg for 15 min. Followed by filtration of the supernatant through 0.2 mm pore size filter thus obtaining cell free filtrate (Allaf *et al.,* 2009).

### Bacteriocin *Lactococcus* production test

This isolate was tested for its ability tobacteriocin production according to Razzak *et al.* (2011).

### Preparation of crude –bacteriocin extract

To obtain to crude –bacteriocineextract, we add NaOH to CFS of *Lactococcus*to get 6.7 molar and put it in water –bath at $80^{0}$C for 10 min (Khalid *et al.,* 2011).

### Biofilm production assay

To assess the biofilm formation potential of the isolates, an overnight culture of each was grown in tryptic soy broth supplemented with 1% sucrose for 18–20 h. at 37$^{o}$C. Tubes were made in duplicate and incubated for 24 h at $37^{0}$C, at 24 h.

The plank tonic suspension and nutrient solutions were aspirated and each tube was washed three times with 300 ml of sterile physiological saline the tubes were vigorously shaken in order to remove all non adherent bacteria, the remaining attached bacteria were fixed with 250 mi of 96% ethanol per tube and after 15 mint tubes were emptied and left to dry each tube then stained for 5 min with 0.2 ml of crystal violet or safranine (according to isolates) excess stain was rinsed off by placing the tubes under running tap water.

Stains were suitable for determining the amount of biofilm, after drying the stained tubes, biofilms were visible as purple or blue spots formed on the sides of each tube (Tahmourespour and Kermanshahi, 2011).

**The effect of CFS and crude acteriocineon growth bacteria:**

CFS and crude bacteriocine were examined for their antibacterial activity by the agar well diffusion as described by Bilkova *et al.* (2011).

**The effect of CFS and crude bacteriocins of *Lactococcus* on biofilm formation**

To assess these effect, an overnight culture of each isolate was grown as remembered in above (6) but here, each of CFS and CB were (prepared an overnight) also added to tubes with tryptic soy broth supplemented with 1% sucrose in the end these tubes divided into 3 groups for each isolate as the following:

1. Inoculated with isolate + CFS
2. Inoculated with isolate + CB
3. Inoculated with isolate just (control).

## Results and Discussion

According to the results obtained, all the isolates tested were sensitive to CFS and crude bacteriocin (CB), the frequency of resistance to the various antimicrobials for all bacteria is presented in table 1 and figures 1, 2, 3 and 4.

Table 1 showed that CFS produced the highest inhibitory effect 13 mm against *Staphylococcus,* whereas the lowest inhibitory rate (Melean *et al.,* 2010) mm belonged to *Klebsiella, E. coli* and *pseudomonas* also could not resistant the CFS in 9, 8 mm inhibition zone respectively. In general, CFS with an average inhibition power of 8.5 mm produced a good capacity for inhibiting of pathogenic bacteria.

In the other side (the same table) CB showed stronger antibacterial properties against Gram positive bacteria *Staphylococcus* (30)mm in compared with Gram negative bacteria *E. coli, Klebsiella, pseudomonas* 12, 17 and 22 mm respectively, and also CB with an average inhibition power of 20.25 mm produced a very good capacity for inhibiting of pathogenic bacteria.

In fact, LAB generally and *Lactococcus* especially produce various compounds such as organic acids, diacetyl, hydrogen peroxide and bacteriocin or bacteriocidal proteins (Sarkono and Sofyan, 2010) and besides organic acid, these could possibly include several heat labil substances, resistant to proteinase K cleavage (Bilkova *et al.,* 2011). Many Researchers relating to relationship of probiotic LAB to pathogenic bacteria found that the high activity is usually attributed to various compound (Mohsen *et al.,* 2013; Baqer *et al.,* 2014; Dhanasekaran *et al.,* 2010; Jatkauskas and Vrotniakiene, 2010; Mirnejad *et al.,* 2013; Panda *et al.,* 2013). In addition to that,

Bacteriocins, extracellularly released antibacterial peptides or proteins, display a limited inhibitory spectrum towards closely related G+ bacteria (Bilkova *et al.*, 2011), these studies agreed with our results in the activity of CFS and CB.

According to the biofilm formation *E. coli* was with highly strong adherent (strong biofilm forming bacterium) and *Klebsiella* was also strongly adherent based on the test tubes results, then *Pseudomonas*, *Staphylococcus*, the effect of products (CFS and CB) on the adherence of pathogenic bacteria in test tubes was assessed by mixed each of CFS or CB alone with each isolate, the results showed the adherence reduction of *Staphylococcus* and *Pseudomonas* to test tubes wells in the presence of CFS and more in the CB product.

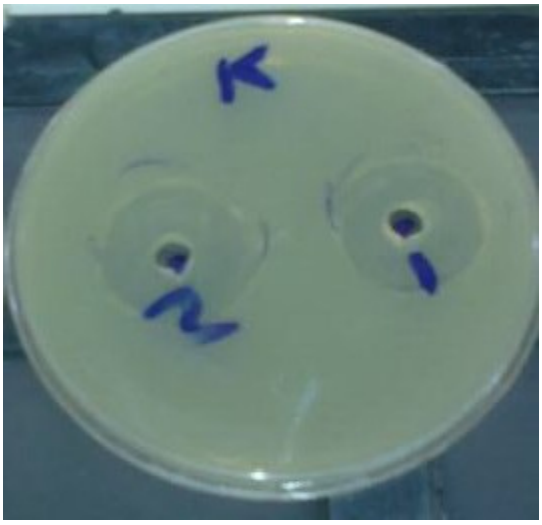


**Figure.1** Antimicrobial activity of *Lactococcus* CFS and CB against *Klebsiella*
1: CFS (Cell Free Supernatant).
2: CB (Crude Bacteriocin)


**Figure.2** Antimicrobial activity of *Lactococcus* CFS and CB against *Staphylococcus*
1: CFS (Cell Free Supernatant).
2: CB (Crude Bacteriocin)

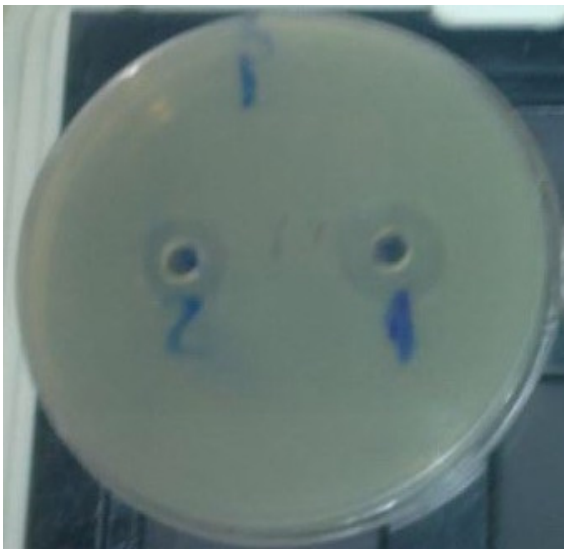


**Figure.3** Antimicrobial activity of *Lactococcus* CFS and CB against *Pseudomonas*
1: CB (Crude Bacteriocin)
2: CFS (Cell Free Supernatant).


**Figure.4** Antimicrobial activity of *Lactococcus* CFS and CB against *E. coli*
1: CFS (Cell Free Supernatant).
2: CB (Crude Bacteriocin)

This adherence reduction for strong biofilm forming bacteria (*Staphylococcus, Pseudomonas*) was higher than (*E.coli, Klebsiella*) and there were not very appear differences between them in the case of CFS effect on all isolates, but adherence reduction for strong biofilm forming was less than CB effect.

**Table.1** Antibacterial activity of CFS and CB of *Lactococcus* isolate against some pathogenic bacteria (Inhibition zones with millimeter)

| Isolate product | CFS | CB |
|---|---|---|
| *Staphylococcus* | 13 | 30 |
| *Klebsiella* | 7 | 17 |
| *E. coli* | 9 | 12 |
| Pseudomonas | 8 | 22 |

Average of inhibition: CFS: 8.5, CB: 20.25

In the other side, The CB effect on adhesion reduction was different in each isolate from other in general CB had more effect on adherence of tested bacteria than CFS (Figures 5 and 6).

**Figure.5** Antimicrobial activity of *Lactococcus* CB against *E. coli* biofilm formation
1. Adhesion cell bacteria (without any addition)
2. Reduction of adhesion after CB addition.

*Lactococcus* was preferred as a probiotic bacterium because of its ability to resistant different conditions like acid resistance and bile salt tolerance and its known probiotic potential (3). According to the results, it is cleared that the presence of *Lactococcus* can cause reduction in the adherence of all isolate that it is probably related to interaction between bacteria, so it is thought that adhesion reduction is likely due to bacterial interactions and colonization of adhesion sites with any probiotic strain before the presence of pathogenic bacteria, also the probiotic strain is able to modify the proportion and extent of the species within the biofilm (Jatkauskas and Vilma, 2013).

They also suggest that the reduction of these pathogenic isolates can be explained either by competition for adhesion sites or growth factors (Giang *et al.*, 2011). In the same time, production of antimicrobial elements, volatile fatty acids, decreasing the pH of the environment and increased or decreased enzyme activity (Ahmed, 2013), all of these factors enter in mechanisms for pathogen inhibition.

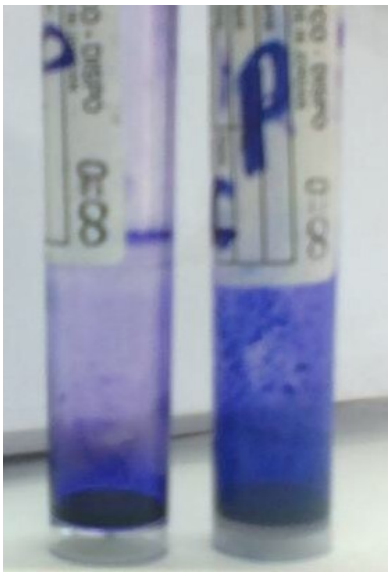

**Figure.1** Antimicrobial activity of *Lactococcus* CFS and CB against *Staphylococcus*

1. Adhesion of cell bacteria (without any addition).
2. Reduction of adherence (after CB addition)

In beside of that Bacteriocins, extracellularly released antibacterial peptides or proteins, display a limited inhibitory spectrum towards closely related G+ bacteria (Bilkova *et al.,* 2011), in our experiments G+ target bacteria (*Staphylococcus*) was more sensitive towards the antibacterial activity of products (CFS, CB) in compared to G- (the rest test bacteria), Authors supposed that this may be due to composition of the cell wall among G+ and G- bacteria, G- bacteria have an outer phospholipid membrane carrying the structural lipopolysaccharide components, this makes the cell wall impermeable to antimicrobial substances, therefore, the cell walls are more complex in lay out, acting as diffusion barrier and making them less susceptible to the antimicrobial agents than that of G+ bacteria (Puttaalingamma and Begum, 2010; Ravaei *et al.,* 2013), the G- bacteria on the other hand are more susceptible, having only an outer peptidoglycan layer which is not effective permeability barrier (Cerezuela *et al.,* 2011; Kreuzer *et al.,* 2012).

In the G+ or G-, Generally, the suggested mechanisms for pathogen inhibition by probiotic include contending for nutrients, production of antimicrobial elements like bacteriocin, the antagonistic actions of these elements are believed to be inhibitors target the cell membrane and depolarize it, and also inhibit synthesis of the cell wall to G+ or G- bacteria (Lefoka, 2009).

The recommendations for this study include:

1. The results of our study showed, that CFS and CB had good antimicrobial effect and could be used widely in production of native probiotic strains, contributing to enhance health in society.
2. Adhesion reduction can be an effective way on decreasing pathogenic potential of bacteria and all of the evidence has shown that probiotic bacteria such as *Lactococcus* can affect the human ecology.

So we recommend that make a natural drug from these probiotic act as alternative drug to antibiotic because of all reasons we remember it in abstracts in our research.

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