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PROBIOTIC AND BOVINE MASTITIS ASSOCIATED PATHOGENS

By

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ABSTRACT

A total of 112 quarter milk samples (QMS) were collected from cows and subjected to California Mastitis Test (CMT) for detection of subclinical mastitis where 85.71% of examined quarter's milk samples reacted positively to CMT. Somatic cell count (SCC) was measured and showed that, the mean standard error of SCC was (548.13 x103±24.96) cells/ ml of examined quarter milk samples. *S*taphylococcus *aureus* was isolated from 40.62% of positive CMT bovine QMS. Antagonistic activity of probiotic lactobacilli (*Lactobacillus acidophilus* and *Lactobacillus plantarum*) against *S. aureus* isolated from bovine mastitis. Cell Free Supernatant (CFS) of probiotic lactobacilli in well diffusion method had inhibitory effect. The effect of *L. acidophilus* on 20 isolated *S. aureus* strains was 2strains (10%) gave (++++), 2 strains (10%) gave (+++), 6 strains (30%) gave (++) and 10 strains (50%) gave (+) and the effect of *L. plantarum* was, 2 strain (10%) gave (+++), 8 strains (40%) gave (++) and 10strains (50%) gave (+). The effect of lactobacilli in co-aggregation method showed that, the high co-aggregation percentage of *L. acidophilus* and *L. plantarum*on 20 strains of *S. aureus* was 94.63% and 94.61% respectively the effect of *L. acidophilus* on 20 isolated *S. aureus* strains by modified double layer was 4 strains (20%) gave (+++),7 strains (35%) gave (++) and 9 strains (45%) gave (+) and the effect of *L. plantarum*was, 2 strain (10%) gave (+++), 5 strains (25%) gave (++) and 13strains (65%) gave(+).

INTRODUCTION

Mastitis is the most prevalent affection in dairy herds world-wide and it is responsible for several loses as decreased milk production and if the case was not treated it would affect the next lactation season **(Seegers *et al.,* 2003)**. Mastitis may also affect the composition of produced milk. However, the severity of the induced changes depends mainly on the causing agent and the inflammatory response **(Duffield, 2000**; **Fetrow *et al.,*2000 and Pyörälä, 2003)**. Regarding its effect on public health, mastitis has a vital importance as it is associated with many zoonotic diseases that are transmitted through **(APHA, 1993).**

The California Mastitis Test (CMT) has an important role in the dairy herd monitoring programs as a simple, inexpensive and rapid screening test to detect cows with intramammary infections (IMI) that are caused by major pathogens **(Sargeant *et al.,* 2001)**.When CMT is being regularly performed as a control measure, significant lower risks of subclinical mastitis were being observed **(Busato *et al.,* 2000)**.

Somatic Cell Counts (SCC) are used as an international standard used to measure milk quality. During mastitis the major increase is in SCC**.** Responses to major pathogens varies from cow to other however, it is not possible to differentiate between the types of pathogens by SCC alone **(Harmon, 1994 and Khalil, 2007)**. **Schukken *et al.,* (2003)** stated that SCC can be used for monitoring udder health and milk quality. Meanwhile **Haas *et al.,* (2004)** estimated the association between pathogen-specific cases of clinical mastitis (CM) and somatic cell count (SCC) patterns based on deviations from the typical curve for SCC during lactation and compared with associations between pathogen-specific CM and lactation average SCC.

In dairy animals, *S. aureus* still remains one of the most significant organisms associated with clinical and subclinical bovine mastitis worldwide **(Saei *et al.,* 2009; Luini *et al.,* 2015; Carfora *et al.,* 2016**; **Singh *et al.*, 2016).**

In spite of increasing frequency of isolation of coagulase-negative staphylococci (CNS) from the bovine mammary glands, coagulase-positive *S. aureus* is still recognized worldwide as a major pathogen causing subclinical intramammary infection in dairy cows **(Turutoglu *et al.*,2002)**. **Burvenich *et al.,* (2003)** investigated the major causes of mastitis. They stated that intramammary infections of dairy cows with Gram-positive bacteria such as *S. aureus* cause sever production losses that were induced by an increase in somatic cell count **(El-Sayed *et al.,* 2006; El - Zubeir and El - Owni, 2006; Amir and Tanveer, 2007).**

Probiotics administration is considered an alternative method for the prevention and treatment of infection. So preventive treatment with probiotic would decrease antibiotic usage.

Probiotics does not cause negative influence on normal gut microflora **(Serikbayeva *et al.,* 2005 and Reid *et al.,* 2006)** and provides 'healthy bacteria' including *Lactobacillus acidophilus* strains. Probiotic lactobacilli have great potential to produce antimicrobial compounds that inhibit and control pathogenic bacteria **(ShirleyandJean, 2010)**.

Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have stated that, the use of probiotic foods provide health benefits and strains are safe **(FAO/WHO, 2001)**.

The objective of this study is to screene the antagonistic properties of some probiotic strains against the most prevalent bacteria in bovine subclinical mastitis. The investigation was conducted through four phases:

Phase I: Applying California Mastitis Test (CMT) on collected milk samples from healthy and subclinical mastitic cows.

Phase II: Laboratory measurement of somatic cells by means of Milk Scan Apparatus (Milk Soma Counter).

Phase III: Bacteriological examination of milk samples and biochemical identification of *S. aureus*.

Phase IV: Evaluation of the antagonistic effect of some probiotic strains (*L. acidophilus and L. plantarumss. plantarum*) against mastitic associated pathogen; *S. aureus* using well diffusion method, co-aggregation method and modified double layer method.

MATERIAL AND METHODS

Samples for California Mastitis Test (CMT):

A total number of 112 quarter milk samples were collected from 30 apparently healthy Holstein Friesian lactating cows with apparently healthy udder after washing the udder, teats and hands of the veterinarian with running water and soap. Then the area was dried with clean towel. The udder, teats, tester hands were then disinfected by alcohol 70% to insure that there is no external contamination **(Kerro-Dego and Tareke, 2003)**. The first strips of milk were discarded, and then fifty ml. samples of milk were collected from each quarter.

All collected samples were subjected to California Mastitis Test (CMT) **(Schalm and Noorlander,1957)** and positive quarter milk samples for CMT were examined for somatic cell count (SCC) by means of milk scan apparatus **(Zecconi *et al.,* 2002)**, as well as for bacteriological examination to determine the main causative organisms of subclinical mastitis **(Koneman *et al.,*1992)**. Quarter milk samples were incubated for 18-24 hr at 37ºC then centrifuged at 3000 rpm for 20 minutes.The cream and supernatant were discarded and a loop full was taken from milk sediment then cultivated on the surfaces of Baird-Parker medium and Sheep blood agar **(Quinn *et al.,* 1994 and 2002).** The plates were incubated at 37°C for 24 - 48 hours. Purified isolates of *S. aureus* Gram's positive were subjected to further identification morphologically and thermostable nuclease and coagulase detection according to **Lancette and Tatini, (1993)**. Further biochemical identification was carried out according

to **Collee *et al.,* (1996) and Koneman *et al.,* (1992)** then Polymerase chain reaction (PCR) was carried out.

Strains used of probiotics:

Two types of probiotic were used. These strains were obtained from microbiological resources center (Cairo MIRCEN), Faculty of Agriculture Ain Shams University. The two strains are; *Lactobacillus acidophilus* (DSM 20079) and *Lactobacillus plantarum* ss. *plantarum* (DSM 20174). The two strains were examined and confirmed.

Identification and purification of *Lactobacillus*:

Lactobacillus spp. were identified on the basis of Gram staining, catalase growth on de Mann Rogosa and Sharpe (MRS) broth

Preparation of cell free supernatant:

Cell Free Supernatant (CFS) was prepared by, each probiotic *Lactobacillus* was cultivated in MRS broth for 24 h at 37°C. CFS was obtained by centrifuging then filtration through a 0.2 µm pore size filter **(Nowroozi *et al.*2004)**.Inhibitory activity of CFS of probiotic *lactobacilli* was determined by well diffusion method according to **Anas *et al.,(*2008)**.Co-aggregation method was done according to **Svetoslav *et al.*, (2009).** Modified double layer method according to **Tagg and McGiven, (1971).**

RESULTS

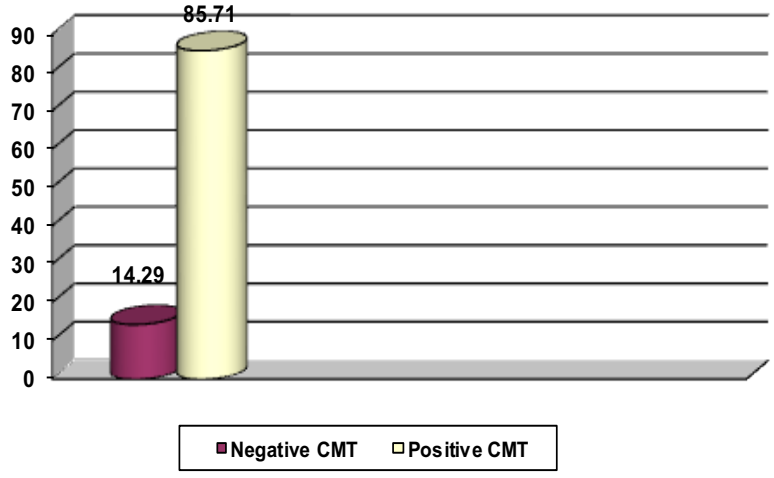


Fig. (1): The frequency distribution of Mastitis in QMSusing CMT.

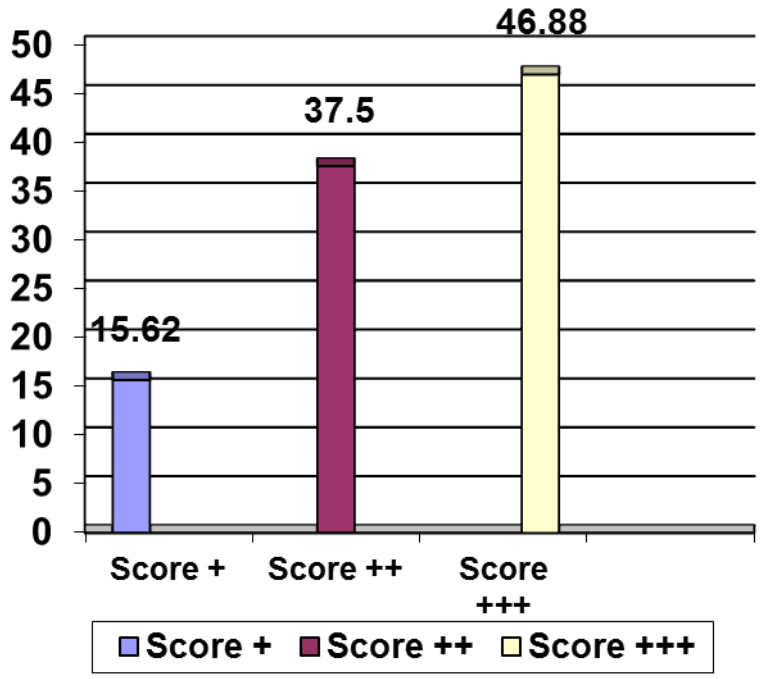


Fig. (2): Illustration of subclinical mastitis scores by using CMT.

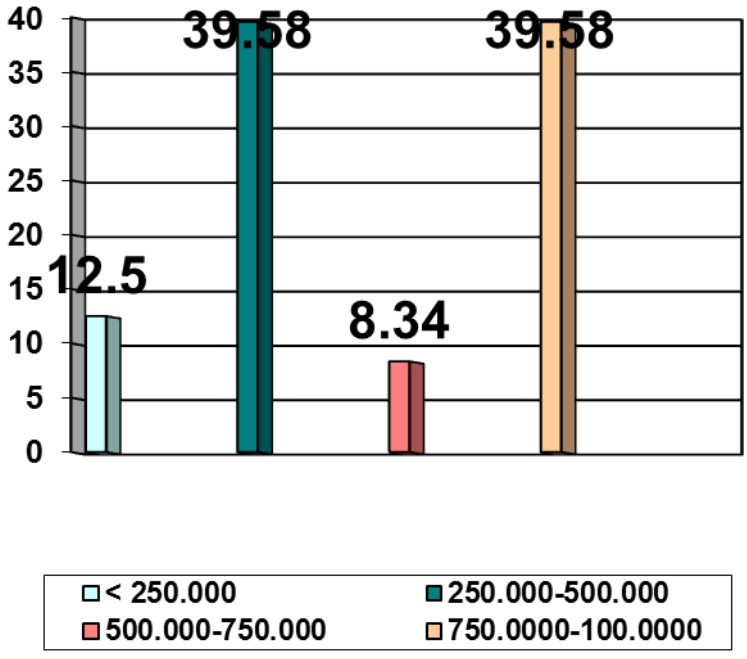


Fig. (3): Frequency distribution of SCC in examined QMS (No. = 96).

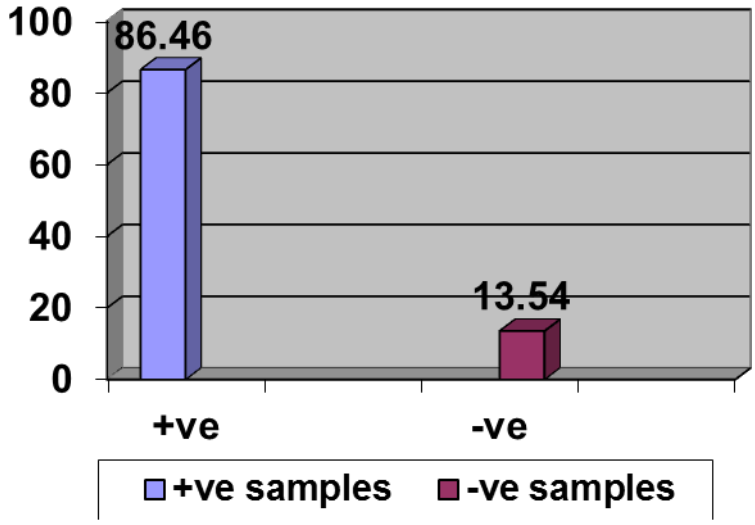


Fig. (4): Prevalence of Gram-positive cocci in the examined QMS.

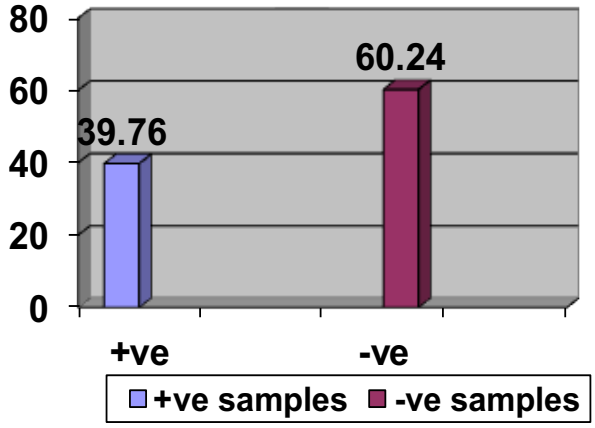


Fig. (5): Results of isolates according to hemolytic activity.

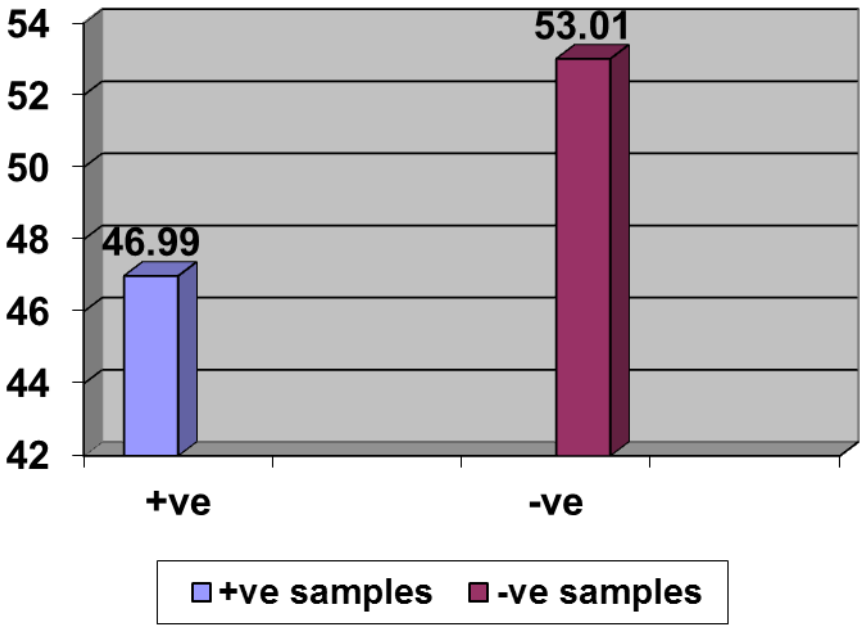
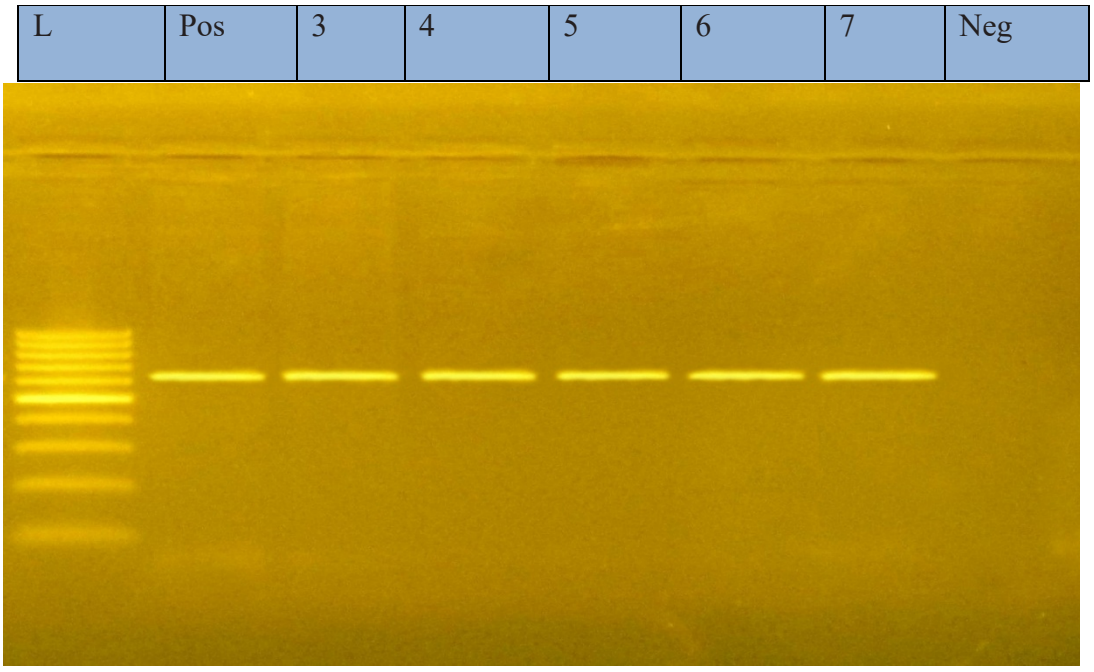


Fig. (6): Prevalence of *S .aureus* isolates of Gram-positive cocci.



Agarose gel electrophoresis showing amplification of 638 bp. fragment of clfA gene of *Staphylococcus aureus*. Lane (1): DNA ladder (1000 bp.) Lane (2): control positive Lane (3-7): samples Lane 8: negative control

(Table 1): The inhibitory effect of probiotic *Lactobacillus acidophilus* and *Lactobacillus plantarum* ss. *Plantarum* against BM *S. aureus*

BM *S. aureus*	Inhibition zone diameter (mm) by well diffusion method		Co-aggregation %		Inhibition zone diameter (mm) by modified double layer	
	L. acidopillus	*L. plantarum*	*L. acidopillus*	*L. plantarum*	*L. acidopillus*	*L. plantarum*
1	++	+	++	++	++	++
2	+	+	**92.93**	**71.77**	++	+
3	+	++	**75.39**	**85.42**	+	++
4	+++	++	**94.63**	**92.94**	+++	++
5	+	++	**90.96**	**94.26**	+++	+
6	++++	+++	**78.10**	**69.49**	+++	+++
7	++	++	**91.88**	**64.91**	+	+
8	+	+	**93.94**	**82.86**	++	+
9	++	+	**90.50**	**91.59**	+	+
10	+	+	**71.20**	**93.44**	++	+++
11	+	++	**67.72**	**73.27**	+	+
12	++	+	**81.53**	**83.84**	+	+
13	++	+++	**70.91**	**92.52**	++	+
14	+	+	**83.20**	**76.17**	++	+
15	+	+	**95.25**	**84.71**	+	+
16	++++	++	**90.13**	**91.72**	+	++
17	+	+	**89.20**	**94.29**	++	+
18	+++	++	**74.52**	**77.63**	+++	++
19	++	+	**87.47**	**94.61**	+	+
20	+	++	**80.31**	**74.44**	+	+

Diameter of inhibition zone (mm):21-22= ++++; 19-20= +++; 17-18= ++; 15-16= +

DISCUSSION

Subclinical mastitis is considered to be most vital importance to the public health due to its association with manyzoonotic diseases in which the milk mayact as a vehiclefor transmission of infectious agents **(Guillemette *et al.,* 1996)**. More attention has been focused for the diagnosis of subclinical mastitis.The application of screening tests lead to earlier detection of subclinically infected quarter and as an aid in the selection of dairy animals either for production or therapy **(Zaki *et al,* 2008)**.

CMT has a useful role in dairy herd monitoring programs as a screening test and widely used diagnostic tool to detect cows with Intra-mammary infection (IMI) caused by major pathogens **(Brito *et al.,* 1997; Sargeant *et al.,* 2001and Kivaria *et al.,* 2006)**.

Our study revealed that, the prevalence of subclinical mastitis in 112 quarter milk samples collected from lactating cows of CMT was 96 (85.71%) of apparently normal quarter milk samples reacted positively to CMT while 16 (14.29 %) of examined quarter milk samples reacted negatively.

The level of subclinical mastitis is due to a number of factors which include the level of hygiene during and after milking,aswell asnutritionalstatus oftheanimals**(AbdEl-Fatah, 2001)**. Nearly similar findings were obtained by **Mulei, (2000) and Dego and Tareke (2003)** while higher results were obtained by **Kivaria *et al.* (2004, 2006).** Lower findings were recorded by **Karimuribo *et al.*,(2005) and Sori *et al.*,(2005) and Tyagi *et al.*,(2013)**. This lower finding reflects the good hygienic measures of these farms.

Consulting the results of the severity of subclinical mastitis in 96 quarter milk samples, it is evident that, score (+), score (++) and score (+++) constituted 15.62%, 37.50% and 46.88 % of examined samples respectively.

Nearly similar results were obtained by **Abebe *et al.*,(2010); Bedane *et al.*,(2012) and Fosgate *et al.*, (2013).** Lower percentages were recorded by **Lakew *et al.*,(2009) and Sarker *et al.*, (2013);** while higher results were recorded by **Vianni and Nadder-Filho (1990) and Kivaria *et al.*, (2006).**

The obtained results showed that, the minimum, the maximum and the mean ± S.E. of SCC were ($178x10^3$), ($984x10^3$) and ($548.13\ x10^3 \pm 24.96$) cells / ml of the examined quarter milk samples respectively.

The highest frequency distribution of SCC was (39.58%) samples lied in the range between 25×10^4 – $<50 \times 10^4$cells/ml and 75×10^4 – $<10 \times 10^5$cells/ml; the lowest one was (8.34%) samples which lied in the range between 50×10^4 – $<75\times 10^4$cells/ml; 12.50% of samples lied in less than$< 25 \times 10^4$cells/ml. These findings agree with those reported by **Khalil, (2007) and Abd-El Fatah, (2004)**.

From the obtained results, it is clear that SCC is considered to be an important parameter for assessing mammary health status in lactating animals.Thus SCC in bovine mammary gland secretions was analyzed to measure the degree of inflammation. In this concern, the bovine subclinical mastitis is characterized by an increased SCC, and is generally used as diagnostic

criterion to identify subclinical infected cows. As well as SCC proved to be a useful tool for monitoring the existence of problem infection, but it must be complemented with bacteriologic culture of milk **(Paape *et al.,* 2000 and Park *et al.,* 2007)**.

The results given illustrated that Gram-positive cocci was isolated from 86.46% of examined positive CMT quarter milk samples. While the given results cleared that *S. aureus* was identified as 46.99 % of these isolates. The prevalence of *S. aureus* was 40.62% of positive CMT bovine quarter milk samples. The present results agree with a certain extent with those reported by **Jian-ping *et al,* (2009) and Shekhan, *et al*. (2011)**.

Lower prevalence of *S. aureus* was reported by **Mørk *et al.,* (2010); Giannatale *et al.,* (2011) and Persson *et al.,* (2011)**. In several other studies, higher prevalence for *S. aureus* was found by **Rall *et al.,* (2008); Ateba *et al.,* (2010); Cheng *et al.,* (2010) and Olde Riekerink *et al.,* (2010).**

The present study proved that 39.76% % of suspected *S. aureus* strains isolated from bovine milk had β-hemolytic activity on blood agar and 60.24% non-hemolytic.

Higher results of β hemolytic activity was recorded by **Tyagi *et al.* (2013)** whom reported that on blood agar, 48 (70.58 %) isolates showed β- hemolysis. Such result disagrees with those of the present study and disagreed with **Arshad *et al*., (2006**) whom found that all *S. aureus* isolates were ß-hemolytic.

Detection of clumping factor A coagulase gene by PCR was carried out in all different *S. aureus* isolates of the study, all of them showed expression of clfAgene. From the obtained results of clfAgene PCR amplification of (638 bp product) fragment of clfAgene of *S. aureus*, Lane(1):DNAladder(1000 bp.) Lane (2):control positive Lane (3-**7):**samples,Lane 8: negative control. The present study showed that, the antibacterial effect of cell free supernatant (CFS) of probiotic lactobacilli by well diffusion method on 20 strains of bovine mastitis *S. aureus* (BMS). It was clear from the obtained results that, the effect of *L. acidophilus* on 20 isolated *S .aureus* strains was, 2 strain (10%) gave (++++), 2 strains (10%) gave (+++), 6 strains (30%) gave (++) and 10 strains (50%) gave (+). It was also cleared from the obtained results that, the effect of *L. plantarumss. plantarum* on 20 isolated *S. aureus* strains was, 2 strain (10%) gave (+++), 8 strains (40%) gave (++) and 10 strains (50%) gave (+).

The obtained results agreed with those of **Soleimani *et al.* (2010)** who admitted all used probiotic lactobacilli have an antagonistic activity against *S. aureus*. On the other hand the obtained result agrees with **Bilge and Sumru (2005)** who announced that antimicrobial

sub-stances produced by *Lactobacillus* have a great potential for inhibiting the growth of pathogenic microorganisms.

The obtained results showed that, the antibacterial effect of probiotic lactobacilli by co-aggregation method on 20 strains of bovine mastitis *S .aureus* (BMS). It was cleared from the obtained results that, the high co-aggregation percentage of *L. acidophilus* on 20 isolated *S. aureus* strains was 94.63%, while that obtained by *L. plantarumss. plantarum*on 20 isolated *S. aureus* strains was 94.61%.

Our study showed that, the antibacterial effect of probiotic lactobacilli by modified double layer method on 20 strains of bovine mastitis *S. aureus* (BMS) on 20 strains of bovine mastitis *S. aureus* (BMS). It was cleared from the obtained results that, the effect of *L. acidophilus* on 20 isolated *S .aureus* strains was, 4 strains (20%) gave (+++), 7 strains (35%) gave (++) and 9 strains (45%) gave (+). It was cleared from the obtained results that, the effect of *L. plantarumss. plantarum*on 20 isolated Staphylococcus aureus strains was, 2 strain (10%) gave (+++), 5 strains (25%) gave (++) and 13 strains (65%) gave (+). The given results agree with those of **Soleimani *et al.* (2010) and Mami *et al.* (2008).**

The data obtained are able to confirm the potential capacity of probiotics to inhibit selected pathogens associated mastitis (*S. aureus*) and suggest that, the probiotic strains might be a promising candidate for the development of new strategies of mastitis control in bovines.

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البروبيوتكس و الميكروبات المرضيه المصاحبه بالتهاب الضرع في الفصيله البقريه

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إن مرض التهاب الضرع من اكثر الامراض شيوعا فى قطعان الابقار الحلابه و على الرغم من أن مرض التهاب الضرع الغير ظاهرى لا يؤدى إلى تغيرات مرئية فى اللبن او الضرع الا انه يعتبر من اكثر المشاكل الاقتصادية واسعة الانتشار و المسببه لخسائر جسيمه فى انتاجية ونظرا لاهميته البالغه فقد حظى تشخيص التهاب الضرع الغير ظاهرى باهتمام العديد من الدراسات.ومن الميكروبات المسببه لمرض التهاب الضرع الغير ظاهري الميكروب العنقودى الذهبى تم إجراء اختبار الكاليفورنيا للكشف عن التهاب الضرع الغير ظاهرى.وقياس عدد الخلايا الجسيمية و دراسة علاقتها بحدوث التهاب الضرع .

التعرف على المسببات البكتيريه لمرض التهاب الضرع خاصة البكتريا الاكثر شيوعا و التى تشمل الميكروب العنقودى الذهبى. تم عزل وتصنيف الميكروب العنقودى الذهبى

قد تم تصنيف معزولات الميكروب العنقودي الذهبي باستخدام باستخدام تكنولوجيا PCR (تفاعل البلمرة المتسلسل) وتعتبر هذه التكنولوجيا هي الأحدث علي الإطلاق حيث أتجه أليها العالم الآن لكونها وسيلة سريعة ودقيقة لتصنيف الميكروبات علاوة علي انها طريقة لإيجاد العلاقات الجينية بين الميكروبات. وقد اثبتت النتائج عند دراسة تأثير البروبيوتكس (لاكتوباسيلس أسيدوفيلس و لاكتوباسيلس بلانتارم) على نمو الميكروب العنقودى الذهبي

تم قياس مقدار تثبيط نمو الميكروب العنقودى الذهبي بواسطة بروبيوتكس لاكتوباسيلس أسيدوفيلس عن طريق well diffusion method و اجريت على 20 ميكروبات عنقوديه ذهبيه و اظهرت النتائج 10% بنسبة تثبيت (++++) و 10% بنسبة تثبيت (+++) و 30% بنسبة تثبيت (++) و 50% بنسبة تثبيت (+). تم قياس مقدار تثبيط نمو الميكروب العنقودى الذهبي بواسطة بروبيوتكس لاكتوباسيلس بلانتارم عن طريق well diffusion method و اجريت على 20 ميكروبات عنقوديه ذهبيه و اظهرت النتائج 10% بنسبة تثبيت (+++) و 40% بنسبة تثبيت (++) و 50% بنسبة تثبيت (+). علما بان (++++) تعني تثبيت بمقدار21 او 22 و (+++) تعني تثبيت بمقدار 19 او 20 مم و (++) تعني تثبيت بمقدار 17 او 18 مم و (+) تعني تثبيت بمقدار 15 او 16 مم.

تم قياس مقدار تثبيط نمو الميكروب العنقودى الذهبي بواسطة بروبيوتكس لاكتوباسيلس أسيدوفيلس عن طريق co-aggregation method و اجريت على 20 ميكروبات عنقوديه ذهبيه و اظهرت النتائج ان اعلى نسبة مئويه لل co-aggregation هي 94.63%. تم قياس مقدار تثبيط نمو الميكروب العنقودى الذهبي بواسطة بروبيوتكس لاكتوباسيلس بلانتارم عن طريق co-aggregation method و اجريت على 20 ميكروبات عنقوديه ذهبيه و اظهرت النتائج ان اعلى نسبة مئويه لل co-aggregation هي 94.61%.

تم قياس مقدار تثبيط نمو الميكروب العنقودى الذهبي بواسطة بروبيوتكس لاكتوباسيلس أسيدوفيلس عن طريق modified double layer method و اجريت على 20 ميكروبات عنقوديه ذهبيه اظهرت النتائج 20% بنسبة تثبيت (+++) و 35% بنسبة تثبيت (++) و 45% بنسبة تثبيت (+). تم قياس مقدار تثبيط نمو الميكروب العنقودى الذهبي بواسطة

بروبيوتكس لاكتوباسيلس بلانتارم عن طريق modified double layer method و اجريت على 20 ميكروبات عنقوديه ذهبيه و اظهرت النتائج 10% بنسبة تثبيت (+++) و 25% بنسبة تثبيت (++) و 65% بنسبة تثبيت (+). علما بان (++++) تعني تثبيت بمقدار 21 او 22 و (+++) تعني تثبيت بمقدار 19 او 20 مم و (++) تعني نثبيت بمقدار 17 او 18 مم و (+) تعني تثبيت بمقدار 15 او 16 مم.

أخيراً أثبتت الدراسة أن بكتيريا لاكتوباسيلس أسيدوفيلس و لاكتوباسيلس بلانتارم هى البديل الآمن لإستعمال المضادات الحيوية فى علاج ووقاية الحيوانات من الاصابة بمرض التهاب الضرع الغير ظاهرى