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The role of Lactic acid bacteria in combination with conventional antibiotics against bacterial species isolated from genital tract of cattle

Likaa H. Khamees[1], Ammar R. Algburi[2], Zahid I. Mohammed[3]
[1,3] University of Diyala, College of Veterinary Medicine
[2] University of Diyala, College of pure sciences
Corresponding author: zahidma1977@yahoo.com

Abstract

Numerous microorganisms are present in the cow's reproductive system from an early age. Other pathogenic microorganisms can occasionally enter and become inhabit cows' genital tracts, which can cause reproductive disorders that impair fertility. Vaginal samples were collected from cows in different fields of animal in Baquba city and from Veterinary clinic in Kanaan district during the period of October 2021to January 2022. Eighty one (81) local adult post calving were examined. The collected swabs or (loopfull) were submitted to culture by Streaking on to blood agar and MacConkey agar then incubated at thirty seven Celsius for 24-48 hr.From 81cows, all isolates were examined, the highest bacterial strain was isolated *Staphylococcus* spp. 75/268 (28%), followed by *Pseudomonas* spp. 61 (22.7%), *E.coli* 49 (18.3%), *Sphingomonas* spp. 46 (17.1%), *Kocurea* spp. 23 (8.6%), and *Granulicatella* spp. 14 (5.2%). *Lactobacillus planrerum* had positive effect and caused bacterial growth inhibition of for *Pseudomonas* spp., *Kocurea* spp. and *granulicatella* spp. While, *Lactobacillus acidophilus* had positive effect and caused inhibition in bacteria growth of *Staphylococcus* spp., *pseudomonas* spp., *kocurea* spp., and *Granulicatella* spp. When CFS of LAB was combined with different types of conventional antibiotic discs had growth inhibition effects against many types of bacterial isolates.

Key words: genital tract, cow, antibiotic, sensitivity, bacteria.

Introduction

High reproductive performance is a factor that affects the productivity of dairy and beef cows and is essential to keep farms economically viable. Inadequate nutrition, ineffective reproductive management, and a poor energy balance during the postpartum period are some of the multifactorial causes of poor reproductive efficiency. These factors might negatively affect the immune system and open the door for pathogen invasion. Clinical and subclinical endometritis in cows is the key factor contributing to its adverse impact on fertility (SEM). A higher rate of infertility and the prevalence of SEM harm cows (1). Numerous studies provide credence to the idea that the presence of bacteria in the reproductive tracts of dairy and beef cattle results not only from external colonization but also from a hematogenous transmission mechanism. Galvao and associates (2). Additionally, they demonstrated in their investigations that the hematogenous route is an effective method of uterine pathogen infection. There is a drive to create adaptable antimicrobial medicines with minimal resistance-inducing potential in addition to employing traditional antibiotics to treat these illnesses (3). The genital tract are particularly exposed to various infections, mostly, with bacteria that are resistant to many types of antibiotics. Therefore, it is necessary to find the alternative solutions or treatments to control these infections. This study aimed to assessment antimicrobial potential of Cell Free Supernatants (CFS) of LAB and

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evaluation of antimicrobial combinations of LAB CFS with the commonly prescribed antibiotics against the most isolated bacteria from genital tract in cows.

Materials and Method:

Area of the study :This study was conducted in Baquba city/ Diyala province .

Samples collection and processing: vaginal samples were collected from cows in different fields of animal in Baquba city and from Veterinary clinic in Kanaan district. Vaginal and uterine swabs were collected after restraining the animal and securing its tail, the perennial region was washed with disinfectant solution and water .sterilized vaginal speculum and disinfectant free hand, and swabs with transport media were used in collecting. Immediately, all collecting samples shifted to microbiology laboratory to detection (4).Selective and deferential media were used to inoculation and cultivation of samples as, MacConkey agar, Mannitol salt agar and chocolate agar, all prepared according to Manufacturer's instructions to enhance a chance of growth different types of prevalent and fastidious pathogens. The inoculated media were incubated at 37C^{o} for 24 - 48 hrs (5). under aerobic and anaerobic conditions and the growth was observed after every 12 hours till 48 hours post inoculation, after that the isolates are purified in selective Culture media.

Preparation of Culture Media:

All culture media (MacConkey agar, blood agar, Nutrient broth, EMB agar, , BHI broth, Nutrient agar) were prepared according to company manufacturing instructions and sterilization were completed done by autoclave at (121^{o}C, 15 lb / inch^{2} for 15 min). Muller – Hinton agar used for the sensitivity test**,** According to manufactures instructions , dissolving 38 grams of powder in liter, of distill water and sterilized by the autoclave at 121 ^{o}C for fifteen minutes, then cool to 45 ^{o}C and held in water bath until used (5).

Isolation and Identification of bacteria:

Colonies identified as discrete on MacConkey ,EMB, and blood agars were examined macroscopically carefully for detect the color, shape and the hemolysis on blood agar ; and consistency of these colonies.

By used the appropriate biochemical tests , culture media and Gram staining according to standard procedures. For conclusive bacteriology, the VITEK2 system was utilized to validate bacterial identification (6).

Identification of Isolates:

Macroscopically and colonial identification : The colony were examined by naked eye for characterization after the isolates cultivated on MacConkey and Blood agar and incubated for 24-48 hours at 37 ^{o}C. By observing the morphology of the colonies on the plate (macroscopically), for colonial appearance in accordance with (6).

Gram Stain:

Gram staining of colonies according to (7).

Using Vitek System to Identification of bacteria (Confirmation Test) as described by (8).

To examined isolates the standard as the protocol requiring ; a 0.50 McFarland inoculums. one loop of culture from the stock agar was cultured on agar 24hours ; the bacterial suspension prepared in normal saline and about (1-2 $\times 10^{8}$ CFU/ml) of suspension was prepared. A pure single colony was taken from the bacteria and diluted in 3 milliliters of saline solution in a sterile tube placed on a special stand. The solution was measured using the VITEC 2 DENSICHEK so that turbid is equal to the McFarland constant 0.5 which is equivalent to x 1.5 cells/ml 10^{8}. The stuck was put in VITEK 2 Cassette for gram-negative bacteria . VITEK 2 Cassette was placed in the

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device and the bacteria were diagnosed depend on 64 biochemical tests.

Preservation of Isolate:

short term and long term preservation : according to the procedure of (6) and (9).

Potential of cell free supernatant (CFS) of some spp. Of Lactic Acid bacteria (LAB) against isolated bacteria:

The potential of CFS derived from selected local LAB (*Lactobacillus acidophilus ,and Lactobacillus planterum)* were subjected to various treatment, were tested for inhibition of growth of isolated bacteria in this study, the effects of the CFS on various growth parameters.

Antimicrobial Activity:

Antimicrobial activity test done by used the disk diffusion susceptibility method .The disks diffusion susceptibility method is practical and simple and Was well standardized. The test is done by following steps according to kirby Bauer method as showed by (10). The isolates were screened for resistance to the following antimicrobial agents as a table (1)

Table (1): antibiotics disc used and their concentration in study

Antibiotic name	Abbreviation	Concentration	Classification and family	Nature of work	origin
Ampicillin	AMP10	10 µg	Beta-Lactum aminopenicillin family	Inhibitor for cell wall synthesis by inhibition of transpeptidase enzyme which needed by bacteria to make cell wall	Liofilchem (Italy)
Gentamicin	CN-10	10 mcg	Aminoglycoside family	Disrupt the ability of bacteria to protein synthesis	Bioanalyse (Turkey)
Metronidazol	MET-30	30 mcg	Class of Nitroimidazole	Inhibit nucleic acid synthesis by forming nitrose radicals which disrupt DNA synthesis	Bioanalyse (Turkey)
Oxytetracyclin	TE-10	10 mcg	Tetracycline family	Interfere with protein synthesis	Bioanalyse (Turkey)
Cefotaxime	CTX-30	30 mcg	3rd generation of cephalosporin (beta-lactamus)	Interfere with cell wall synthesis	Bioanalyse (Turkey)
Cefixeme	CFM-5	5 µg	3rd generation of cephalosporin(beta lactamus)	Disrupt bacterial cell wall resulting bacterial death	Bioanalyse (Turkey)
Cloxacillin	CX^5	5 mcg	Beta lactamus penicillin familly	Interfere with cell wall synthesis	Titan biotech (India)
clindamycin	DA2	2 mcg	Lincosamide class	Block bacteria from protein synthesis by inhibition ribosomal translocation	Condalab (Spanish)

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The zones of growth inhibition that around each of the antibiotic disks are calculated at the nearest millimeter and then the zone diameters of each drugs are interpreted using the parameter published by the Clinical and Laboratory Standards Institute (CLSI) are "qualitative, in that a category of susceptibility (susceptible, intermediate, or resistant) ". Last step; comparing zone sizes with standard criteria of(11) and (12). isolates of Lactobacillus were obtained from one of the educational laboratories in Baquba. Where it was cultivated and used in the study.

Statistical analysis: Data were calculated by SPSS for windows TM version 24.0. Statistical analysis of data was performed using chi square (13).

Results:

Level of bacterial contamination:

The results revealed that out of 81 samples were collected, 32 (39.5%) were without clinical signs or in subclinical cases of bacterial contamination from genital tract; while 49 (60.5%) were contaminated and had clinical signs, from samples 268 bacteria were isolated, as double isolates 20/268 (7.5%) identified in 10 cows, as three isolates 117 (43.6%) in 39 cows, four isolates 116 (43.3%) in 29 cows and five isolates 15 (5.5%) in 3 cows.

Isolates of bacterial strains in collected samples:

From 81cows, all isolates were identified, the highest bacterial strain was *Staphylococcus* spp. 75/268 (28%), followed by *Pseudomonas* spp. 61 (22.7%), *E.coli* 49 (18.3%), *Sphingomonas* spp. 46 (17.1%), *Kocurea* spp. 23 (8.6%), and *Granulicatella* spp. 14 (5.2%) (Table 2.)

Table: 2. Numbers and percentages of Bacterial isolates in the study

Bacterial isolates	No. of isolates	Percentage
Staphylococcus spp.	75	(28%)
Pseudomonas spp.	61	(22.7%)
E.coli	49	(18.3%)
Sphingomonas spp.	46	(17.1%)
Kocurea spp.	23	(8.6%)
Granulicatella spp.	14	(5.2%)
Total	268	100%

Characterizations of Isolates :

a. separation and distinguishing of bacteria: Separation and distinguishing of isolates were depended on forms of colonies upon the agar which used in the study (color and shape) in addition to their odor, biochemical tests and used VITEK 2 system to identify of bacteria from genitalia of cows.

b. VITEK 2 system for bacterial strains identification

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This sys. Was used to emphasize a definitive diagnosis of isolates that which; *Staphylococcus* spp., *Pseudomonas* spp., *E.coli, Sphingomonas* spp., *Kocurea* spp.,*and Granulicatella* spp.

Table (3) measuring of zone of inhibition (mm) CFS only with isolated bacteria in mm.

Type of bacteria	*L.acidophillus*	*L.planterum*
E coli 1	-	-
E coli 2	-	-
E coli 3	-	-
E coli 4	-	-
Staph.	-	7
pseudo	16	12
Sphingo1	-	-
Sphingo2	-	-
Koc.	26	22
Gran	18	14

combination antibiotic discs with lactic acid bacteria (LAB) for detection ability of inhibition of bacterial growth:

The measuring of inhibition zones of these combinations were measured and listed in (Tables 4 and 5).

Lactobacillus planterum alone with bacterial isolates, was showed positive effect and caused bacterial growth inhibition against *Pseudomonas* spp., *Kocurea* spp., and *granulicatella* spp. but there was no effect and not inhibition bacteria growth against *E.coli* (1,2,3, and 4), *Staphylococcus* spp., and *Sphingomonas* 1 and 2 (table 4).

Table 4: measuring of diameter of inhibition zone in millimeter (mm) for antibiotics with *L.planterum*

Type of bacteria	AMP10 +L.p	CN-10 +L.p	MET-30 +L.p	TE-10+L.p	CTX-30+L.p	CFM-5+L.p	CX5 +L.p	DA2+L.p
E. 1	16	10	-	-	38	28	-	-
E. 2	17.5	11.5	-	-	37	28.5	-	-
E. 3	9	24	-	17	31	20	-	-
E. 4	16.5	9	-	-	38	28	-	-
Staph.	23	20	-	25	-	-	-	15
pseudo	14	31	-	25	27	-	-	-
Sphingo1	42	24	-	28	47.5	26	-	33.5
Sphingo2	13	33	-	30	12	-	-	32
Koc.	31	48	19	33.5	37	33	-	11
Gran	38	29	-	36	36	24	8.5	39

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While *Lactobacillus acidophilus* had positive effect and caused inhibition in bacteria growth against *Staphylococcus* spp., *pseudomonas spp*., *kocurea* spp., and *Granulicatella spp.* but had negative effect without inhibition of bacterial growth with *E.coli* (1,2,3, and 4), and *Sphingomonas* (1 and 2).

Table: 5 measuring of diameter of inhibition zone in millimeter (mm) for antibiotics with *L.acidophillus*

Type of bacteria	AMP10+ L.a	CN-10+ L.a	MET-30+ L.a	TE-10+ L.a	CTX-30+ L.a	CFM-5+ L.a	CX5 +L.a	DA2 +L.a
E. 1	18	16	-	-	33	23.5	-	-
E. 2	19	15	-	-	33.5	24	-	-
E. 3	8	22	-	16	31	21	-	-
E. 4	18.5	17	-	-	32	23	-	-
Staph.	25	25	-	22	-	-	-	17
pseudo	11	31	-	28	14	-	-	-
Sphingo1	45	19	-	30	44	28.5	-	37
Sphingo2	11	32	-	32	11	-	-	30
Koc.	30	30	24	36	43	33	34	20
Gran	38	26	-	39	36	25	-	42

Our data showed that *Pseudomonas* were all resistant to the tested of antibiotics used (MET, CFM, CX, and DA.) even after mixing with CFS of *L.plantarum* or ***L.acidophillus.*** However, when CFS was combined with antibiotics, the highest zone of inhibition in some antimicrobial combinations included AMP, TE and CTX.

Also,, when antibiotics were mixed with CFS of *Lactobacillus* spp., an increase in zone of inhibition of AMP and TE were noticed but the zones were variable regarding to CN and CTX. While *Pseudomonas* were resistant to DA, CFM. MET, and CX.

E. coli (1, 2,3 and 4) were sensitive to combination **CFS of *L.plantarum* or *L.acidophillus* with antibiotic discs** CN, TE, and CFM. Furthermore, we identified that *E. coli* (1,2,3,and 4) was resistant to CFS with MET, CX, and DA (Table 5).

The data showed that the best antibiotics action after combination with **CFS of *L.plantarum* on *Sphingomonas1* were** CN and CTX. While the best antibiotics action after combination with **CFS of *L.acidophillus* on *Sphingomonas1* were** AMP, CFM, DA and CN. Furthermore, we identified that ***Sphingomonas1*** was resistant to CFS with MET, CX, and DA.

On another hand, ***Sphingomonas 2 was sensitive to*** combination **CFS of *L.plantarum or L.acidophillus with*** AMP, CN, TE and CTX antibiotics due to zone of inhibition, but there are resistant to CFS with MET, CFM, and CX. The results referred that ***Staphylococcus*** was sensitive to combination **CFS of *L.plantarum* or *L.acidophillus* with antibiotic discs of** TE, also slightly effects of AMP, CN, and DA on this bacteria. Furthermore, we identified that ***Staphylococcus*** was resistant to CFS with MET, CFM, CX, and CTX.

In regards to TE, CFM, MET, AMP, DA and CN that combination with **CFS of *L.plantarum* or *L.acidophillus,* Kocurea bacteria was** sensitive while it was resistant to CX. ***Granulicatella*** was resistant to CFS with MET, while sensitive to DA, AMP, TE, and CFM caused bacterial growth inhibition when

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combination of antibiotic with **CFS of *L.plantarum* or *L.acidophillus*** (Table 5).

Discussion:

The genital microbes are thought to originate from the environment or different organs such as the rumen, skin, rectum or feces, the vagina is considered to be the main source of endometrial microfauna, especially during the times when the cervical lumen is less restricted at estrus, breeding or parturition (2).Interestingly, these microbes frequently populate the genital tract of cows, leading to reproductive diseases that perturb fertility. A comparison of the results from this study reveals that the proportions of the predominant microbial populations differ significantly between individuals. (14). In this study twelve cows were pregnant (3-8 months of gestation) with previous case history of reproductive disorders with range of pregnancy period 3-8 months of gestation, while 15 cows were suffering from reproductive disorders that caused infertility. The range of cows ages were 1-10 years old. High colonizing rate of genital tract of heifers and multiparous cows was found to be due to different bacterial species (15).The antibacterial properties of LABs had created interest in their use as cleaning agents, biocides, and antimicrobial agents, LAB are being released into the environment and this raises concerns about their effects on microbes in the receiving ecosystems.In the present study, the methods of sample collection, transportation, and culture media used permitted the isolation of a large diversity of aerobic and anaerobic agents, in addition to the limitations of culture-dependent techniques. Lactobacilli produces lactic acid, which regulates vaginal pH and inhibits the proliferation of pathogens (16). In turn, symbiotic bacteria utilize the secretions of the genital tract such as mucus sugar and proteins as a source of essential nutrients (17). Lactic acid also induces acidification of milieu within the vagina, which interferes with intracellular functions, leading to microbial elimination (18).It should be noted that Mechanisms of pathogens inhibition by LAB-probiotics include (i) production of inhibitory compounds, (ii) prevention of the pathogens adhesion, (iii) competition for nutrients, (iv) modulation of the host immune system, (v) improvement of nutrient digestibility, feed conversion, and (vi) reduction of toxin bioavailability.Interestingly, (19) and (20) reported and argued on the use of lactic acid bacteria (LAB)-probiotics in promoting the growth and reproduction performances and the survival rate and health status of animals and potentially usable as antibiotics replacers because of their multifaceted functions

Conclusions: The majority of bacterial contaminants isolated from the vaginal samples of cows are *Staphylococcus* spp, *Pseudomonas* spp., *E.coli*. *Sphingomonas* spp., *Kocurea* spp and *Granulicatella* spp. which showed a considerable resistance to the many antibiotics investigated in this study. These contaminates, the bacterial isolates, have a variety of virulence factors, the most importantly is biofilm formation, which enhance bacterial colonization in the reproductive tract and increase their antibiotic resistance . Also Lactobacillus spp. possess several natural antimicrobial molecules making them attractive candidates for preventing the pathogenic biofilm formation.

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