

## IMPACT OF INTRAVAGINAL PROBIOTICS INOCULATION ON REPRODUCTIVE PERFORMANCE OF HOLSTEIN DAIRY CATTLE DURING TRANSITION PERIOD

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### ABSTRACT

In this study we tested the hypothesis that intravaginal infusions of lactic acid bacteria (LAB) around calving would improve uterine involution and reproductive performance of dairy cows. The probiotic culture used in this study was a mixture of 2 LAB strains isolated previously from the vaginal tract of healthy pregnant heifers. Forty pregnant 2<sup>nd</sup> lactation dairy cows 2 wk before the expected day of calving were assigned to one of 2 groups, first group was the control group (C) receiving 1 mL of media only, and the 2<sup>nd</sup> group was the treated group (T) receiving 1 mL of media enriched with probiotic bacteria at 10<sup>10</sup> to 10<sup>12</sup> cfu / treatment. Intravaginal infusions were performed once during wk -2, -1, +1, +2, +3, and +4 relative to parturition with probiotic bacteria. All cows were observed for reproductive performance and reproductive diseases until next conception using ultrasonography. The incidence of uterine infections in the multiparous cows were measured. Days open, conception rate at 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> insemination and repeat breeders (%) were calculated. Incidence of purulent and foul-smelling discharges on +1, +2, +3 and +4 wk relative to parturition and numbers of cows in the clean-up program were measured, cervix diameters (C.D) and uterine horn diameters (U.H.D) were measured at days (22-29) and days (38-45) postpartum. Ovarian structures and endometritis were determined. Results revealed that uterine and cervical involution of postpartum dairy cows were earlier in cows treated with LAB. Cows showed purulent and fowl smelling discharges are fewer in number in treated group than control, also data showed that percentage of cows in the cleanup program was higher in the control group (40%) than in the treated group (20%). The averaged insemination number per conception was lesser in the treated group (2.55) than control (4). The conception rate at 1<sup>st</sup> insemination and 3<sup>rd</sup> insemination was higher in the treated group (35%, 20%) than in control one (25%, 10%), and the % of repeated breeder cows was lower in the treated group (30%) than control (40%), finally days open in the treated group (116.4) was much lower than in the control (154.72). Therefore it can be conclude that, intravaginal treatment of transition dairy cows with a mixture of lactic acid bacteria (LAB) could improve uterine involution, lower the incidence rates of uterine infections, and improve the reproductive performance.

**Key words:** Reproductive performance, uterine involution, Probiotics, Dairy cattle

### INTRODUCTION

Transition dairy cows are susceptible to uterine infections due to the compromised immunity around calving and substantial bacterial contamination in the uterus immediately after calving. Uterine infections affect 1 in 2 dairy cows after parturition, infertility related to uterine infections has become the main reason for a cow to be culled from the herd (Rajala-Schultz and Gröhn, 1999). So far, there have been no effective approaches to treat uterine infections.

The vaginal tract of dairy cows harbors various bacteria including aerobic, facultative anaerobic, and obligatory anaerobic ones (Wang *et al.*, 2013). Plate culture analysis has shown that 15 Enterococcus and Staphylococcus are the predominant bacteria of the vaginal tract of healthy heifers, followed by Enterobacteriaceae and Lactobacilli (Otero *et al.*, 2000). During gestation, the cervix is closed with a mucus plug, isolating the bacteria harboring vagina and the sterile uterine body. After parturition, the cervix is open to allow the drainage of intrauterine fluid, which also provides a passage for bacteria to ascend into the uterine body via the vaginal tract.

To our knowledge, there is no known effective treatments or prophylactic medications against uterine infections. Although various intrauterine antibiotics such as oxytetracycline® and cephalirin benzathine® are currently used to treat cows, their

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efficiency is not proven and concerns about drug residue in milk and carcass, or bacterial acquisition of drug-resistance have limited their widespread use (Galvão, 2011). Presently, there are no intrauterine antibiotics approved for utilization in dairy cows in the U.S (Galvão, 2011). Only systemic ceftiofur® is approved for treatment of cows with metritis. Although systemic administration of ceftiofur® could lower the incidence of metritis, it does not improve the reproductive performance (Risco and Hernandez, 2003).

Infusion of povidone iodine® also has been stopped due to its ineffectiveness, impeding of phagocytic activity of leukocytes in the uterus and triggering of endometrial necrosis (Azawi, 2008).

Intramuscular PGF2 $\alpha$  has been reported as a promising therapy in treating endometritis cows. It has a direct effect on flushing out bacteria from the uterus by stimulating myometrial contractions and enhancing immune responses (Lewis, 2003). However, a meta-analysis to previous studies demonstrated only a marginal benefit of PGF2 $\alpha$  on reproductive performance (Burton and Lean, 1995).

Recently, there is an increasing interest of using lactic acid bacteria (LAB) or probiotics to treat vaginal inflammatory conditions (Reid and Bruce, 2003; Ametaj *et al.*, 2014). Nader-Macías *et al.* (2008) found that LAB strains isolated from vaginal tract have a strong capability of producing H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub>-generating lactobacilli from the vaginal tract of cattle have the potential to be utilized as probiotics, among which *Lactobacillus gasseri* CRL1421 is reported to have the greatest capacity to inhibit *Staphylococcus aureus* by generating H<sub>2</sub>O<sub>2</sub> and lactic acid (Otero and Nader-Macías, 2006). A few strains of LAB (mainly *Lactobacillus fermentum*) isolated from cow's vaginal mucus have been reported to be able to inhibit the growth of *Actinomyces pyrogens* in vitro, a recognized pathogen isolated from mastitic cows, which hold great potential to be used as probiotic product to prevent metritis (Otero *et al.*, 2006). *Pediococcus acidilactici* isolated from healthy pregnant dairy cows has exhibited inhibitory effect on *Lactobacillus innocua* and *Enterococcus faecalis* by the production

of pediocin (Wang *et al.*, 2013). A previous study reported that 6 times treatment around calving with a cocktail of 3 LAB, isolated from the vaginal tract of healthy cows, lowered the incidence of purulent vaginal discharges and improved conception rates of multiparous cows (Ametaj *et al.*, 2014), they hypothesized that lowering the number of treatments around calving from 6 times to 2 or 3 treatments might give the same beneficial effects to the cows. Therefore the objectives of this study were to test whether intravaginal treatment of transition dairy cows with a mixture of lactic acid bacteria (LAB) can lower the incidence rates of uterine infections, improve uterine involution and improve the reproductive performance.

## MATERIALS AND METHODS

Vaginal samples were obtained from pregnant Holstein heifers from Sanad farm (on Cairo-Ismailia desert road). The animals that had no history of metritis infection were selected. The vulvar area was washed with povidone-iodine and water. A disposable speculum was inserted into the vagina to swab the posterior area. Samples were collected and transported to the microbiology laboratory of Ain Shams University.

### Microbial strains and growth condition of the used probiotics

Three isolates were isolated from vagina of pregnant Holstein heifers revealed on 2 bacterial strains characterized and identified to be propagated and used as a probiotic supplementation. Lactobacilli isolates were grown on MRS broth (Oxoid) while Streptococci isolates were grown on M17 broth (Difco), after that the broth media incubated for 24 h at 37 °C. The strains were activated two or three times in order to obtain high biomasses in the stationary phase.

### Genetic identification of isolated and tested strains

The isolates were identified according to 16S rRNA in Sigma Company (Germany) by the automated sequencer (Table 1).

**Table 1:** The genetic identification of the tested probiotics stains.

Probiotic strain	Isolation source	Isolation media
<i>Lactobacillus farraginis</i> strain NRIC 0676 16S ribosomal RNA gene, partial sequence, NR 041467.1	Pregnant heifers vaginal mucous	MRS
<i>Lactobacillus rhamnosus</i> strain NBRC 3425 16S ribosomal RNA gene, partial sequence, NR 113332.1	Pregnant heifers vaginal mucous	M17

### Experimental animals and design

The probiotic culture used in this study was a mixture of 2 LAB strains isolated previously from the vaginal tract of healthy pregnant heifers. Forty pregnant 2<sup>nd</sup> lactation dairy cows 2 wk before the expected day of calving were assigned to one of 2 groups, 1<sup>st</sup> group was the control group (20 cows) receiving 1 mL of media only, and the 2nd group was the treated group (20 cows) receiving 1 mL of media enriched with probiotic bacteria at 10<sup>10</sup> to 10<sup>12</sup> cfu / treatment. The LAB infused in the vagina of the treated cows in our experiment are commensal bacteria identified and isolated from healthy vaginal tracts of pregnant heifers as previously described by Wang *et al.* (2013) and Ametaj *et al.* (2014). Intravaginal probiotics infusions were performed once per week during wk -2, -1, +1, +2, +3, and +4 relative to parturition. All cows were observed for reproductive performance and reproductive diseases until next conception. The incidence of uterine infections in the multiparous cows were measured. Days open, conception rate at 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> insemination and repeat breeders % were calculated. Incidence of purulent and foul-smelling discharges on +1, +2, +3 and +4 wk were measured, cervix diameters and uterine horn diameters were measured in days (22-29) and (38-45) post-partum. The numbers of cows in the clean-up program were measured. Ovarian structures and endometritis were determined.

Research was conducted at Sanad farm. Animals used in this experiment were kept in the same conditions of housing. All cows were in the 2<sup>nd</sup> parity, data about age, health condition and reproduction have been taken from farm protocol.

### Ultrasound examination

Done by Unit of Radiology and Ultrasonography, Animal Reproduction Research Institute. Ultrasound scanning for reproduction have been performed

starting from 22 to 45 days postpartum through two periods. First period was 22–29 days, 2<sup>nd</sup> was 38–45 days postpartum. In this study it is been used diagnostic ultrasound electronic linear scanner. Examination technique is similar to standard trans-rectal manual examination. After removing feces from the rectum, we placed probe intra-rectally to obtain image of the uterus using portable, battery operated and B-mode ultrasound scanner. To obtain data of uterine horn diameter and cervix diameter, we have used, freeze“ mode and after that „caliper“ option at the edges of viewed tissues to get precise data about searched uterine dimensions. Obtained data about tissue dimensions were calculated. A Sonosite ultrasound (Sonosite, M- turbo, USA) fitted with (5-10)-MHz, L52x linear-array probe was used to obtain images of the cervix, uterine body and horns, follicles, and corpora lutea on ovaries of all cows according to (Melendez *et al.*, 2004).

### Statistical analysis

The various data were subjected to ANOVA procedure for a complete randomized design and performed according to Snedecor and Cochran (1982). Analysis of variance, by Duncan's test using, and least significant difference were applied to the data to test for differences between treatments using a computer program 'COSTAT', Significance was declared at P = 0.05. A trend was considered to exist if 0.05 < P < 0.10.

## RESULTS

### Incidence of purulent and/or foul smelling discharges

Results showed that, cows showed purulent and foul smelling discharges are fewer in no. in treated group than control, although no. of cows showed difficult labor and/or retained placenta was higher in the treated group (Table 2).

**Table 2:** Incidence of purulent or foul smelling discharges

Group	Total cows no.	No. of cows showed dystocia and/or retained placenta	No of cows with purulent and /or foul smelling discharges	Incidence of purulent and/or foul smelling discharges
Control	20	2	12	60%
Treatment	20	4	4	20%

### Ultrasound scanning for reproductive tract (22-29 day postpartum)

The results showed that, the cervical diameter, right horn and left horn diameter decreased in the treated group than control, statistically there is no significant differences between treatment and

control (P<0.05), only the average of cervix diameter tend to be significantly (P<0.09) lesser in the treated group than control. Cows in treated group showed lower percent of endometritis and pyometra than control group (table 3 and figure 3).

**Table 3:** Ultrasound scanning for reproductive tract (22-29 day postpartum)

Group	Cervix diameter (average) Cm Mean±SD	Right horn diameter (Average) Cm Mean±SD	Lift horn diameter (Average) Cm Mean±SD	Cows showed endometritis (E.)	% of endometritis	Cows showed pyometra	% of Cows showed pyometra
Control	3.67±0.45	3.2±1.8	3.08±1.91	6/19	31.6	1	5.26
Treatment	3.41±0.43	2.53±0.48	2.54±0.39	1/16	6.25	0	0
Sign.	0.09	0.16	0.27	-	-	-	-

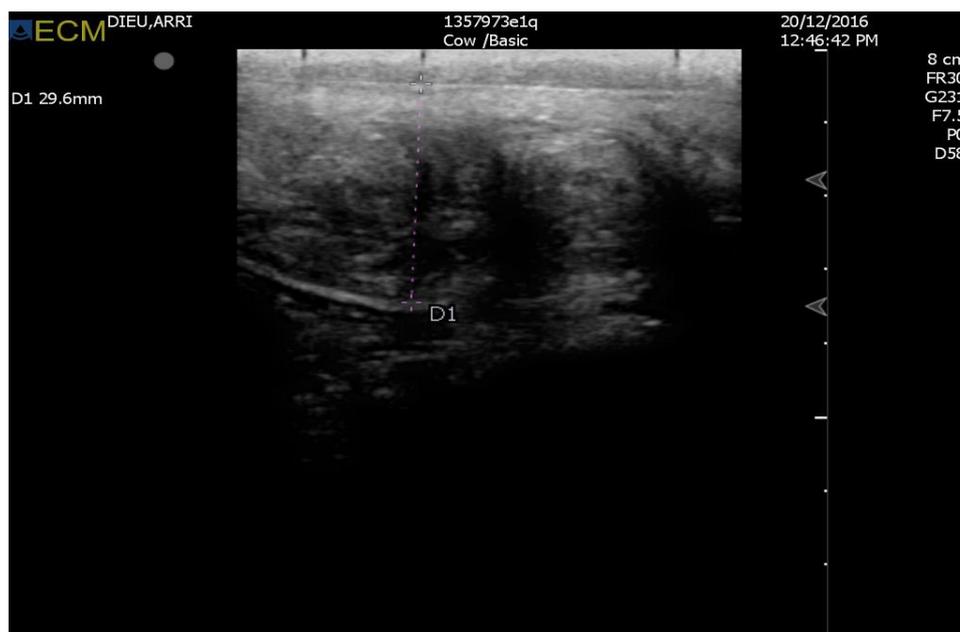
**Ultrasound scanning for reproductive tract (38-45 day postpartum)**

Results showed that the cervical diameter, right horn and lift horn diameter were decreased in the treated group than control, statistically, there is no

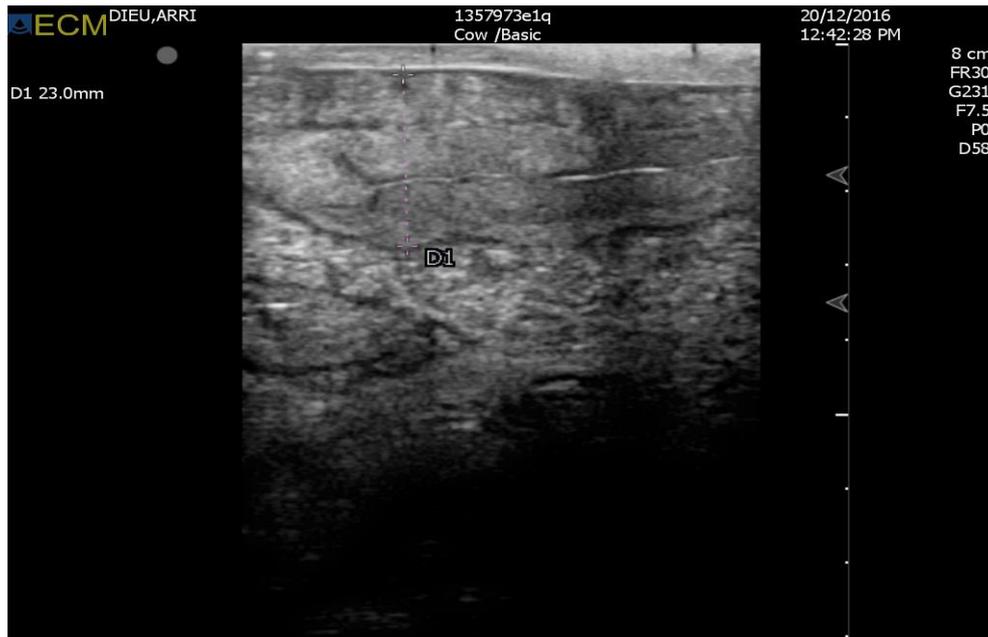
significant differences among treatments (P<0.05), also treated group showed lower percent of endometritis, pyometra and ovarian cysts than control (Table 4, figure 1 and 2).

**Table 4:** Ultrasound scanning for reproductive tract (38-45 day postpartum)

Group	Cervix diameter (average) Cm (Mean±SD)	Right horn diameter (Average) Cm (Mean±SD)	Lift horn diameter (Average) Cm (Mean±SD)	Cows showed endometritis (E)	% of Endometritis	Cows showed pyometra	% of pyometra	Ovarian cyst	% of Ovarian cyst
Control	3.6±0.55	2.91±1.168	2.77±1.91	5/17	29.4%	1/17	0.06%	2/17	1.2%
Treatment	3.31±0.49	2.54±0.48	2.33±0.39	2/16	12.5%	0	0%	0	0%
Sign.	0.16	0.22	0.27	-	-	-	-	-	-



**Figure 1:** Ultrasound image showing cervix diameter (38-45 days postpartum)



**Figure 2:** Ultrasound image showing endometritis (38-45 days postpartum)



**Figure 3:** Ultrasound image showing uterine horn diameter of normal uterus (22-29 days postpartum)

### Reproductive parameters

Results showed that percentage of cows in the cleanup program was higher in the control group (40%) than in the treated group (20%), the averaged insemination no. per conception was decreased in the treated group (2.55) than control, conception rate at 1<sup>st</sup> insemination and 3<sup>rd</sup> insemination was higher

in the treated group (35%, 20%) than control (25%, 10%), the no. of repeat breeder cows was lower in the treated group (30%) than control (40%), finally, days open in the treated group (116.4) was much lower than in the control group (154.72) with no significant ( $P < 0.05$ ) between the two groups as shown in table 5.

**Table 5:** Effect of intravaginal probiotics inoculation on reproductive performance

Group	No of cows in the cleanup program before 1 <sup>st</sup> insemin.	No of insemin. /conception	Conception rate at 1 <sup>st</sup> insemin.	Conception rate at 2 <sup>nd</sup> insemin.	Conception rate at 3 <sup>rd</sup> insemin.	% of repeat breeders (more than 3 insemination /conception)	Days open Mean±SD
Control	8/20 (40%)	4	25%	25%	10%	8/20 (40%)	154.72±138 (15 cows)
Treatment	4/20 (20%)	2.55	35%	15%	20%	6/20 (30%)	116.4±42.13 (20 cows)
Sign.	-	-	-	-	-	-	0.26

## DISCUSSION

The results of this study revealed that intravaginal administration of LAB confers a health benefit to the reproductive tract against bacterial infections of dairy cows, results revealed that cows showed purulent and fowl smelling discharges were fewer in number in treated group than control, also the percentage of cows in the cleanup program was higher in the control group (40%) than in the treated group (20%), although number of cows showed difficult labor or retained placenta was higher in the treated group which refers to the prophylactic and treatment effect of the used vaginal probiotics. These results agreed with a previous study reported that 6 doses around calving with a cocktail of 3 LAB isolated from the vaginal tract of healthy cows lowered the incidence of purulent vaginal discharge of multiparous cows (Ametaj *et al.*, 2014).

According to (Williams *et al.*, 1995) time interval from parturition to the end of involution lasts 23 – 42 days. In the same study it was found that the greatest number of cows (51, 4%) completed involution during period from 29 to 35 days postpartum. The most intensive involution changes are developing up to 25 days postpartum, when uterine horn diameter is 20 – 40 mm and both horns are approximately same size (Leslie 1983), In research of Leslie (Leslie 1983) uterine involution in nursing cows has ended already after 15–25 days postpartum. In this study greatest number of cows has ended uterine involution in time period from 22–29 days. Results in table 3 and 4 also declared that treated group had better reproductive involution than control although the non-significance shown between treatments. Cervical diameter, right horn and left horn diameter decreased in the treated group than control which confirm earlier uterine involution in the treated group, also treated group showed lower percent of endometritis, ovarian cysts and

pyometra which confirm the prophylactic and treatment effect of the used vaginal probiotics.

Bacterial infections of the uterus are commonly present in the postpartum dairy cows, which are associated with histological lesions and inflammation of the uterine tissue (Sheldon *et al.*, 2006). The lower incidence rates of metritis and total uterine infections in the treated group obtained in this study confirm previous finding that intravaginal LAB lowers purulent vaginal discharges in the treated cows (Ametaj *et al.*, 2014). *Lactobacillus* spp., such as *L. rhamnosus* GG, *L. rhamnosus* GR-1, *L. fermentum* RC-14, and *L. acidophilus* are well-known for their ability to maintain and restore a normal vaginal microflora and therefore have been used to prevent and treat urogenital infections in women (Reid *et al.*, 2001; Gardiner *et al.*, 2002; Reid *et al.*, 2003). *Lactobacillus* spp. has been administered directly in the vagina attenuating or treating symptoms of vaginal infections (Reid *et al.*, 1995; Hilton *et al.*, 1995). This treating effect can be explained that infusion of LAB in the vaginal tract of cows increased concentrations of IgA in the vaginal mucus. Secretory IgA (sIgA) is recognized as the most important mucosal immunoglobulin of mucosal tissues, there are reports demonstrating that commensal bacteria can stimulate the production of IgA with the involvement of local epithelial cells and dendritic cells (DCs) (Thomas and Versalovic, 2010). In addition, Boullier *et al.* (2009) found that sIgA was able to dampen the inflammation at mucosal tissues. The LAB-treated cows had greater concentrations of sIgA in the vaginal tract on wk 0 (immediately after calving), +1 and +2. This was probably due to the invasion of pathogenic bacteria into the reproductive tract during this period, as Kaila *et al.* (1992) found that *Lactobacillus* could promote the development of sIgA specific-antibody producing cells and therefore enhance the secretion of local sIgA in the presence of pathogenic bacteria.

The averaged insemination no. per conception was lesser in the treated group than control, also treated group had better conception rate at 1<sup>st</sup> insemination and 3<sup>rd</sup> insemination than control group, these results were in agreement with (Ametaj *et al.*, 2014) Who reported that 6 doses around calving with a cocktail of 3 LAB isolated from the vaginal tract of healthy cows improved conception rates of multiparous cows. The no. of repeat breeder cows decreased in the treated group than control, finally days open in the treated group was much lower than control and agreed with (Deng *et al.*, 2015) who found that two doses vaginal infusion of LAB decreased the number of days from calving to conception than control by 40 d (110 vs. 150 d).

## CONCLUSION

It can be concluded that, intravaginal treatment of transition dairy cows with a mixture of lactic acid bacteria (LAB) can lower the incidence rates of uterine infections, improve uterine involution and improve the productivity of reproduction.

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## تأثير التلقيح المهبلي لبكتريا البروبيوتك على الاداء التناسلي لأبقار الهولشتين الحلابه

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في هذه الدراسة تم اختبار فرضية ما اذا كان استخدام بكتريا حمض اللاكتيك مهبليا من الممكن ان يكون لها تأثير ايجابي على الاداء التناسلي وتحسين التفاعل الرحمي (عودة الرحم لحجمه الطبيعي بعد الولادة) في الأبقار الحلابه. وكان البروبيوتك المستخدم في هذه الدراسة خليط من عدد ٢ سلالة تم عزلها من قناة المهبل لعجلات عشار في حالة صحية جيدة. تم اختيار عدد اربعين بقرة موسم ثاني اسبوعين قبل موعد الولادة المنتظر ، وتم تقسيمهم عشوائيا الى مجموعتين ، المجموعة الاولى كانت المجموعة الضابطة (٢٠ بقرة) ، تم تلقيحها مهبليا ب 1ml media فقط ، والمجموعة الثانية (٢٠ بقرة) تم حقنها ب ١ مللي media محملة ب عدد ١٠<sup>١٠</sup> الى ١٠<sup>١٢</sup> cfu لكل تلقيحها ، تم عمل الحقن المهبلي في الاسابيع الاخير والقبل الاخير من الولادة والاسابيع الاول والثاني والثالث والرابع بعد الولادة ، تم متابعة جميع الأبقار تناسليا حتى حدوث الاخصاب التالي، تم حساب كل من معدل الاصابات الرحمية ، الفتره المفتوحه حتى حدوث الاخصاب بعد الولادة ، نسبة الاخصاب عند التلقيح الاولى والثانية والثالثة ونسبة الأبقار ذات الشياخ المتكرر (اكثر من ثلاث تلقيحات). كما تم حساب نسبة الأبقار التي اظهرت افرازات رحمية صديديه في الاسابيع الرابع الاولى من الولادة، ونسبة الأبقار التي دخلت برنامج علاجي لتنظيف الرحم، وكذلك قياس قطر عنق الرحم وقطر قرني الرحم في الفتره ٢٢-٢٩ والفتره ٣٨-٤٥ من الولادة. كما تم تحديد المجسمات التي على المبيض ونسبة التهابات الرحمية. واطهرت البيانات ان معدل التفاعل الرحمي بعد الولادة افضل في المجموعه المعالجه من حيث قطر عنق الرحم وقطر قرني الرحم. كما كانت نسبة الأبقار التي اظهرت افرازات مهبليه صديديه اقل في المجموعه المعالجه بالبروبيوتك عن المجموعه الضابطة ، كما ان نسبة الأبقار التي دخلت برامج علاجية لتنظيف الرحم بعد الولادة كانت اقل في المجموعه المعالجه من مثيلتها في المجموعه الضابطة، كما كان عدد التلقيحات اللازمه للاخصاب في المجموعه المعالجه اقل من المجموعه الضابطة ، كذلك كانت نسبة الاخصاب عند التلقيح الاولى والثالثه اعلى في المجموعه المعالجه، كما كانت نسبة الأبقار ذات الشياخ المتكرر اقل في المجموعه المعالجه ، كما كانت الفتره المفتوحه بعد الولادة بدون اخصاب اطول في المجموعه الضابطة عنها في المجموعه المعالجه. لذلك من الممكن استخلاص ان العلاج بالتلقيح المهبلي في الفتره الانتقاليه من الولادة بخليط من بكتريا حمض اللاكتيك من الممكن ان تكون تأثير ايجابي على خفض نسبة الاصابات الرحمية وتحسين معدل التفاعل الرحمي ورفع معدل الخصوبه.

الكلمات الداله: الاداء التناسلي ، التفاعل الرحمي ، البروبيوتك ، الأبقار الحلابه