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THE EFFECT OF INTRAVAGINAL PROBIOTIC USE IN IMPROVING THE REPRODUCTIVE EFFICIENCY OF AWASSI EWES

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Abstract

This study examined the topical use of locally isolated probiotic bacteria using direct injections and with vaginal sponges in order to compare their effects with a commercial probiotic on enhancing the reproductive efficiency of ewes. Twenty Awassi ewes aged 2.5 - 4 years with an average weight of 47 ± 1.62 kg were used. All the ewes underwent estrus synchronization using vaginal sponges, and were randomly divided into four groups, each with five replicates. The first was the control group, the second (T1) had the vagina directly injected with locally isolated *Enterococcus faecium* before sponge insertion, the third (T2) had the same bacteria injected with the vaginal sponges, and the fourth (T3) had the commercial probiotic injected with the vaginal sponges. The results showed a significant decrease in estrogen levels in all probiotic treatments compared to the control group, while progesterone levels were not affected by the topical probiotic use in the vagina. There was also a significant reduction in total aerobic bacterial count when the vagina was directly injected with locally isolated bacteria on the 7th and 14th days after sponge insertion. Additionally, injecting locally isolated bacteria with vaginal sponges improved the physical appearance of the vaginal sponges after removal, as well as the reproductive efficiency parameters, which were

highest in the group treated with locally isolated bacteria injected with vaginal sponges. It can be concluded that injecting locally isolated lactic acid bacteria into the vaginas of ewes, either directly or with vaginal sponges, reduces the adverse effects associated with sponge insertion, which typically causes heavy mucous secretions after removal, and thus can improve the reproductive efficiency parameters in ewes.

Keywords: Intravaginal probiotic, Enterococcus faecium, Estrus synchronization, Ewes.

تأثير استخدام المعزز الحيوي داخل المهبل في تحسين الكفاءة التناسلية للنعاج العواسية

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الخلاصة

أجريت هذه الدراسة لتقييم الاستخدام الموضعي لبكتريا المعزز الحيوي المعزولة محلياً بطريقتي الحقن المباشر، وداخل الاسفنجات المهبلية، ومقارنة تأثيرها مع المعزز الحيوي التجاري في تحسين الكفاءة التناسلية للنعاج. استخدمت عشرون نعجة من النعاج العواسي بعمر (2.5–4 سنوات) وبمتوسط وزن (47 ± 1.62) كغم، أجريت لجميعها عملية تزامن الشبق بطريقة الاسفنجات المهبلية. وزعت النعاج عشوائياً على أربع مجاميع لكل مجموعة خمسة مكررات. كانت المجموعة الأولى مجموعة سيطرة (Control)، والمجموعة الثانية حقن المهبل مباشرة ببكتريا *Enterococcus faecium* المعزولة محليا قبل دفع الاسفنجات (T1)، أما المجموعة الثالثة فقد حقنت بنفس البكتريا مع الاسفنجات المهبلية (T2)، في حين حقنت النعاج في المجموعة الرابعة بالمعزز الحيوي التجاري مع الاسفنجات المهبلية (T3). أظهرت نتائج الدراسة انخفاضاً معنوياً في مستوى هرمون الاستروجين في جميع معاملات المعزز الحيوي عند المقارنة مع مجموعة السيطرة، بينما لم يتأثر مستوى هرمون البروجسترون بالاستخدام الموضعي للمعزز الحيوي في المهبل. وأشارت النتائج إلى أن أعداد البكتريا الهوائية الكلية قد انخفض معنوياً عند حقن المهبل مباشرة بالبكتريا المعزولة محلياً في اليومين السابع والرابع عشر بعد دفع الاسفنجات. كما بينت النتائج ان حقن البكتريا المعزولة محلياً مع الاسفنجات المهبلية قد حسن من صفات الشكل الظاهري للاسفنجات المهبلية بعد إزالتها، وكذلك الحال بالنسبة لمعايير الكفاءة التناسلية التي كانت أعلاها في معاملة حقن البكتريا المعزولة

محلياً مع الاسفنجات المهبلية. نستنتج من الدراسة الحالية أن حقن بكتريا حامض اللاكتيك المعزولة محلياً من مهبل النعاج الوالدة سواء كان بصورة مباشرة أو حقنها مع الاسفنجات المهبلية يقلل من الآثار السلبية المرافقة لدفع الاسفنجات التي تشهد افرازات مخاطية كثيرة بعد إزالتها، وبالتالي يمكن أن يحسن من معايير الكفاءة التناسلية لدى النعاج.

كلمات مفتاحية: المعزز الحيوي، Enterococcus faecium، تزامن الشبق، العواسي.

Introduction

Estrus synchronization is an important reproductive strategy to raise sheep reproduction (9) levels by increasing the number of lambing, potentially up to two per year (39). The most common method in estrus synchronization in sheep is the use of vaginal sponges soaked with progesterone (35). The body treats vaginal sponges as foreign objects, causing vaginal infections through mucus accumulation, pus discharge, and unpleasant odors due to the increase in bacteria, especially opportunistic ones. Additionally, vaginal fungal infections cause vaginal inflammation (26), leading to increased numbers of opportunistic Enterobacteriaceae family bacteria such as *E. coli* and *Klebsiella*. *Staphylococcus aureus* has been identified as the leading cause of purulent vaginitis in ewes, indicated by purulent vaginal discharge during sponge removal, and also produces cellular and tissue changes in the vaginal wall (13).

Vaginal infections are often treated with topical antibiotics such as enrofloxacin to reduce the harmful effects of vaginal inflammation. However, the use of antibiotics in such cases is not recommended due to bacterial resistance and the production of more dangerous new generations (14). Increasing concerns over the disadvantages of widespread antibiotic use in sheep farming has prompted researchers to propose the topical use of various probiotic strains to prevent reproductive tract infections in ruminants (22). Several studies have indicated the positive effects of treating vaginal infections associated with the use of vaginal sponges through topical probiotics. These are administered directly into the reproductive tract rather than orally and rely on their high adhesion capacity to the lining of the reproductive canal. This feature is one of the most essential criteria for topical probiotics in the reproductive tract (16).

The topical use of lactic acid bacteria provides health benefits to the reproductive system against bacterial infections by lowering pH values (32) through the secretion of short-chain fatty acids that compete with pathogenic bacteria for oxygen and nutrients competing for binding sites on the tissue lining, secreting extracellular enzymes, and producing bacteriocins that inhibit the growth of pathogenic bacteria (40). All these mechanisms reduce the number of pathogenic bacteria that cause vaginal infections, which in turn improves vaginal health and increases the reproductive efficiency of ewes. This research used locally isolated bacteria as a topical probiotic within vaginal sponges and compared it with injections of similar bacteria before sponge insertion and the injection of commercial probiotic bacteria within the sponges to study their effects on the reproductive efficiency of ewes.

Materials and Methods

Before conducting the field experiment, *Enterococcus faecium* bacteria were isolated from the vaginas of postpartum ewes in the laboratories of the Department of Food Sciences, College of Agriculture, University of Anbar. These bacteria were characterized and identified phenotypically, biochemically, and genetically, and their potential use as a probiotic was studied. The study was conducted at the Douar Research Station/General Authority for Agricultural Research, affiliated with the Ministry of Agriculture. The study involved 20 local ewes aged between 2.5 - 4 years and weighing approximately 47 kg on average. The sponges were inserted on 10 April 2023 for 14 days (Reformulate) according to the established protocol, after which they were removed and the ewes injected with the PMSG hormone (500 IU). On the 15th day following sponge insertion, rams were introduced to the experimental groups at a ratio of one ram per group, and they remained with the ewes for five days. Pregnancy was checked using an ultrasound device (sonar) on the 50th day after the hormone treatment was withdrawn. The ewes were fed according to the station's standard protocol of two regular meals, morning and evening, and free water access. The feeding was uniform for all treatments. Ewes with twin births were isolated in special pens away from the others, and the gestation period lasted 155 days, during which all lambing occurred. The ewes were divided into four treatment groups, each consisting of five replicates (ewes), as follows:

- Group 1: No probiotic treatment (Control).
- Group 2: Direct vaginal injections of locally isolated *Enterococcus faecium* before sponge insertion (T1).
- Group 3: Vaginal sponges injected with locally isolated *Enterococcus faecium* (T2).
- Group 4: Vaginal sponges injected with commercial probiotic bacteria (T4).

Blood samples were drawn from the jugular vein using a 10 ml medical syringe on days 0, 7, and 14 of the experiment. The blood was placed in tubes without anticoagulants, and the serum separated from the cellular component to measure estrogen levels according to method (38) and progesterone levels according to method (8) using an ELISA device. The total aerobic bacterial count in the vagina was determined using the plate-pour technique after taking swabs from the vaginal lining, performing serial dilutions, and culturing them on nutrient agar. The cultures were incubated, and the number of colonies for each dilution was counted the following day. Additionally, reproductive parameters were measured to assess the reproductive efficiency of the ewes according to (3), which included estrus, fertility, pregnancy, twinning, conception, birth, and fecundity rates. The odor and physical appearance of the vaginal sponges were also observed after removal from the vagina.

The statistical analysis for estrogen and progesterone hormone levels and the total aerobic bacterial counts was conducted using a one-way analysis of variance (ANOVA). The general linear model (GLM) was employed using the SAS program version 9.0. Significant differences between means were tested using Duncan's multiple range test at a significance level of ($P \leq 0.05$) (6).

Results and Discussion

Table 1 shows an apparent fluctuation in estrogen levels among the different treatments compared to the control group. The control group significantly outperformed all other treatments on each day in terms of estrogen levels.

Table 1: Mean value ± SE of estrogen levels (pg/ml) in ewes' blood.

Day	Control	T1	T2	T3
0	269 ± 5.29^{a}	159 ± 3.84^{c}	191 ± 5.29^{b}	179 ± 7.68^{b}
7	283 ± 3.17^{a}	183 ± 8.08^{c}	205 ± 6.38^{b}	197 ± 5.60^{bc}
14	303 ± 4.48^{a}	197 ± 5.92^{c}	224 ± 9.26^{b}	241 ± 6.42^{b}

Data in the same row with different superscripts differ significantly at probability value (P≤0.05).

This might be due to the differences in measurement times and the interruption of the hormone's pulse in the body. Estrogen is synthesized and secreted by the theca interna cells of the ovarian follicles, which generally produce androgens from cholesterol. These androgens are then converted to estrogen by the theca interna cells (18). The maintenance and continuation of pregnancy up to birth primarily depend on the hormonal balance between progesterone and estrogen during gestation. Any change in this hormonal balance during pregnancy can lead to embryo absorption or miscarriage (4).

Additionally, the higher estrogen levels could be attributed to removing the inhibitory effect of progesterone on the hypothalamus and pituitary gland, which stimulates the production of gonadotropins and follicular growth, thereby increasing estrogen levels (17). The results of this study, where the administration of the probiotic through vaginal sponges or direct injection of bacteria into the vagina led to a decrease in estrogen concentration, do not align with those of (28), who indicated that administering probiotics to cows has beneficial effects on their reproductive performance. This discrepancy might be because dietary supplements, including probiotics, improve energy balance during pregnancy and lactation. However, this study's results differ, potentially because estrogen is a steroid hormone synthesized from cholesterol and is not stored in the body (15). Most studies on the effects of probiotics indicate that probiotics work to reduce cholesterol and triglyceride levels in the body by stimulating the bile salt hydrolase enzyme, which hydrolyzes the amide bond and detaches bile salts, leading to a reduction in cholesterol levels (31), and consequently, in the concentration of steroid hormones, including estrogen.

Table 2 indicates that the progesterone levels show no significant differences between the treatments on all the days when blood was drawn from the ewes.

Table 2: Mean value ± SE progesterone levels (ng/ml) in ewes' blood.

Day	Control	T1	T2	T3
0	0.326 ± 0.078*	0.550 ± 0.050	0.530 ± 0.060	0.490 ± 0.111
7	0.480 ± 0.144	0.603 ± 0.054	0.816 ± 0.078	0.680 ± 0.017
14	0.676 ± 0.123	0.680 ± 0.066	0.926 ± 0.044	0.803 ± 0.074

The maintenance and continuation of pregnancy until birth primarily depend on the appropriate hormonal balance, and any changes in it can lead to embryo absorption or miscarriage (4). There are fluctuations in progesterone levels, which are known to rise

and fall according to the physiological state, rising to their highest levels when pregnancy is constant in females. The non-significant results for progesterone levels align with those obtained by (2), who found no significant differences in progesterone levels when administering Lactobacilli bacteria to cows. Most previous studies indicate that administering probiotics to ruminants before and after breeding periods increases reproductive efficiency, due possibly to the stimulation of the immune system or improvements in health during the process (28).

Table 3 shows a significant decrease in the total aerobic bacterial count in Treatment 1 (T1), where the vagina was injected with locally isolated *Enterococcus faecium* before inserting the sponges, compared to the other treatments, on the 7th and 14th days after inserting the vaginal sponges.

Table 3: Mean value ± SE of total aerobic bacterial count (log CFU/ml) in the ewes' vaginas.

Day	Control	T1	T2	T3
0	5.31 ± 0.586	4.99 ± 0.715	5.87 ± 0.320	6.50 ± 0.345
7	7.20 ± 0.619^{a}	5.01 ± 0.202^{b}	7.16 ± 0.236^{a}	6.79 ± 0.398^{a}
14	8.03 ± 0.531^{a}	6.11 ± 0.290^{b}	8.00 ± 0.153^{a}	6.33 ± 0.329^{a}

Data in the same row with different superscripts differ significantly at probability value (P≤0.05).

This can be attributed to the presence of vaginal infections caused by these sponges upon insertion, as confirmed by (24), who noted that inserting vaginal sponges in ewes leads to vaginal infections within days of insertion. It is well known that antibiotic therapy often disrupts the microbial balance in the vaginal environment (1), where lactobacilli bacteria usually predominate. This imbalance may lead to increased growth of pathogenic bacteria after treatment at the expense of lactobacilli bacteria (37). The results of this study indicate a significant decrease in the vaginal microbial community in ewes injected with locally isolated bacteria compared to the other treatment groups. This can be explained by the locally isolated bacteria reducing excessive growth of pathogenic bacterial genera and other microorganisms on the 7th and 14th days following sponge insertion. (21) indicated that vaginal injection of Lactobacillus reuteri and Lactobacillus rhamnosus bacteria contributed to a greater reduction in Candida in the vagina compared to common antifungal agents by inhibiting the gene expression of PHR1 and ALS12 genes involved in Candida biofilm formation. Another study comparing metronidazole treatment with Lactobacillus bulgaricus vaginal injections found that latter gradually reduced excessive growth of various bacteria in the vagina, such as Gardnerella vaginalis, Prevotella, Atopobium, Sneathia, and Megasphaera, while promoting the relative abundance of lactobacilli bacteria (20). These results are linked to the restoration of microbial balance facilitated by lactobacilli bacteria in the vagina through several mechanisms, including promoting its pre-existing growth in the vaginal lining, secreting hydrogen peroxide, lactic acid, bacteriocins, all of which prevent the overgrowth of the aforementioned disease-causing microorganisms, and encourage the re-establishment of lactobacilli dominance over other microbial species (19).

Table 4 clearly shows the distinct differences among the treatments in the visual appearance and odor of the vaginal sponges after removal from the vagina. The control treatment differed from the others in terms of color, with brown predominating. As for odor, the sponges had a foul smell. The best appearance and odor were observed in treatments 1, 2, and 3, characterized by white and creamy color and a natural odor (Figure 1).

Table 4: Effect of treatments on vaginal sponge characteristics after removal.

Group	Sponge Number	Color			Odor	
		White	Creamy	Brown	Fetor	Normal
Control	5	1	1	3	5	0
T1	5	3	2	0	1	4
T2	5	4	1	0	0	5
T3	5	3	2	0	1	4

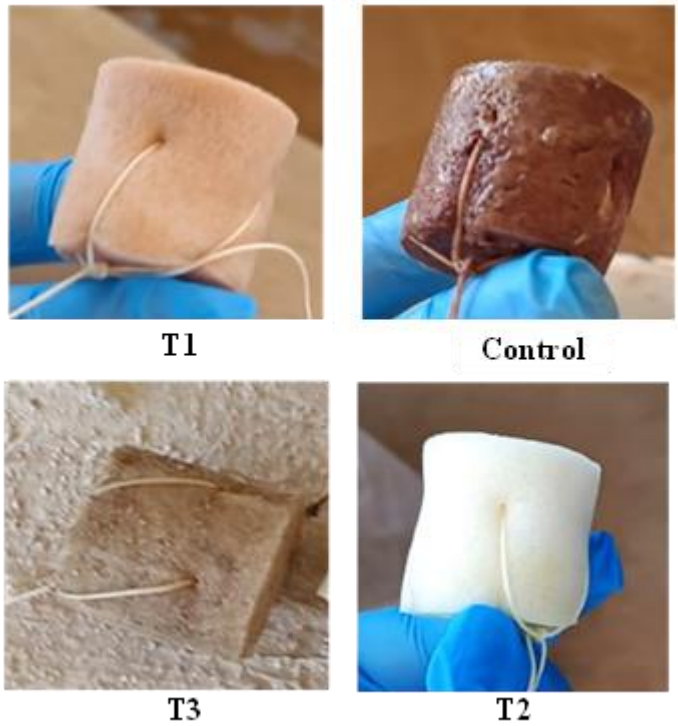

Figure 1: Vaginal sponges after the synchronization period.

The reason behind this could be the probiotic added to the sponges, which acts as an antioxidant and antibiotic, reducing vaginal inflammations caused by the sponges and thus maintaining an infection-free uterine environment by preventing bacterial accumulation and mucous formation around the sponges. Mucous would create a suitable environment for bacterial adherence around the sponges. The use of vaginal sponges can alter the composition of typical vaginal microorganisms, causing an abnormal foul discharge upon sponge removal. According to (29), the sponges retain vaginal secretions, resulting in abnormal secretions upon their removal thus changing the composition of natural vaginal bacterial microorganisms and allowing opportunistic pathogenic bacteria to grow, requiring antibiotic treatment (32).

As noted from Table 5, the second treatment, which included administration of the isolated bacteria via vaginal sponges, produced the best results for the reproductive parameters examined (pregnancy, birth, twinning, fertility, culling, and fertilization rates) by recording the highest numbers. This was followed by the third treatment, which included administration of the commercial probiotic through vaginal sponges. This indicates a relationship between the administration of probiotic bacteria via vaginal sponges and improvements in the reproductive efficiency of ewes in general by creating a suitable environment in the uterus and reducing bacterial numbers, especially pathogenic bacteria. There was a marked decrease in microbial count when probiotic bacteria were administered via vaginal sponges.

Table 5: Effect of experimental treatments on reproductive efficiency criteria.

Group	Number of Ewes	Pregnancy Rate		Birth Rate		Twinning Rate		Fertility Rate		Conception Rate		Fecundity Rate	
		n	%	n	%	n	%	n	%	n	%	n	%
Control	5	3	60	3	60	0	0	3	60	2	40	3	60
T1	5	4	80	4	80	0	0	4	80	1	20	4	80
T2	5	5	100	5	100	7	140	5	100	5	100	5	100
T3	5	4	80	4	80	6	120	4	80	1	20	5	100

Vaginal sponges can cause irritation and local inflammation of the vaginal tissue, along with an increase in mucous secretions (23). Although vaginal environments under normal circumstances inhibit the proliferation of disease-causing bacteria and fungi, controlling environmental factors such as temperature, humidity, pH, and nutrient availability cannot be achieved when using vaginal sponges (29). The use of vaginal sponges increases the levels of opportunistic bacteria such as *E. coli*, *Klebsiella*, *Staphylococcus spp*, and *Streptococcus spp*, resulting in the accumulation of foul-smelling purulent secretions (14). Additionally, the sponges cause cellular and tissue changes in the vaginal lining (13). With the removal of the sponge, abnormal vaginal secretions are eliminated, and the numbers of pathogenic microorganisms in the vaginal microbiome decrease (36). Although the vaginal microbiome returns to its normal state seven days after sponge removal (30), most opportunistic bacteria leave adverse effects on the reproductive efficiency of ewes. The vaginal sponges negatively impact sperm viability after insemination, thus adversely affecting pregnancy rates (25). Therefore, preventive measures, such as cleaning the materials used in vaginal sponge applications, are critical for mitigating the adverse effects of vaginal sponge use (11).

Reproductive efficiency criteria are crucial in sheep breeding due to the increased need to produce large numbers of offspring per female. Pregnancy losses are among the major problems faced in sheep breeding due to various factors, such as the use of vaginal sponges and the accompanying infections (7). Many previous studies have linked improved reproductive efficiency in ewes to the administration of probiotics. (27) indicated that administering a probiotic comprising several strains of bacteria through food contributed to increased pregnancy rates in cows. They attributed this to the ability of the probiotic bacterial strains to withstand stomach acidity and bile secretions and migrate to organs outside the digestive system, including the vagina, to

reduce reproductive tract infections, and consequently improve reproductive efficiency.

Similar results were obtained by (7), who found that the best birth and twinning rates, as well as the lowest abortion rates, were in sheep fed a diet containing different levels of probiotics. Several strategies are adopted to prevent vaginal infections associated with the use of vaginal sponges, the most common being the addition of antibiotics to counteract the adverse effects of vaginal sponges (10). Some studies report that the most effective antibiotic for reducing vaginal infections is enrofloxacin (12). Nevertheless, treating vaginal infections with antibiotics has many adverse effects, including the presence of antibiotic residues in milk (34), the spread of antibiotic-resistant bacterial strains, and disruption of the microbial balance in the vagina (32). Therefore, probiotics are generally proposed as an alternative to antibiotics for treating vaginal infections associated with the use of vaginal sponges.

Bacterial strains used as probiotics, especially lactic acid bacteria, contribute to increasing reproductive efficiency by protecting the vagina through various mechanisms. These include competing with pathogenic bacteria for binding sites on the vaginal lining, thus preventing their attachment, producing antimicrobial substances such as hydrogen peroxide, maintaining a low pH through acid production, and stimulating anti-inflammatory cytokine responses in the lining (33). All types of lactic acid-producing bacteria can produce small protein molecules known as bacteriocins, which are antimicrobial agents that inhibit the growth of bacteria, fungi, and possibly even nucleated cells (5). Moreover, intensive lactic acid production lowers the pH to less than 4.5 in ewe vaginas (37). Perhaps the main reason for reducing the numbers of pathogenic microorganisms and vaginal secretions associated with the use of vaginal sponges is the decrease in pH (13), thus improving the vaginal and reproductive health of ewes, which in turn enhances their reproductive efficiency standards. This is confirmed by the results of this study, which indicated that the best reproductive standards were observed in ewes injected with vaginal sponges contaminated with *Enterococcus faecium* bacteria isolated primarily from their vaginas.

Conclusions

The topical use of the locally isolated probiotic in the vagina led to a significant reduction in the level of estrogen, due probably to a decrease in cholesterol and triglycerides in the body, but did not affect progesterone levels. Additionally, it resulted in a decrease in the total number of aerobic bacteria, which tend to increase with the use of vaginal sponges and cause an increase in mucous secretions after the sponges are removed. This had a positive effect on the appearance and odor of the sponges, consequently leading to a noticeable improvement in the reproductive efficiency parameters of the ewes.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Author 1: methodology, writing and original draft preparation; Author 2: supervisor; Author3: co-advisor.

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The authors declare no conflict of interest.

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