

## EFFECT OF THE ADDITION OF PROBIOTICS AS ANTIBIOTIC ALTERNATIVE ON THE QUALITY OF COOLED STORED SEMEN IN RABBITS

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### ABSTRACT

Antibiotics are an essential component of semen extender to avoid a possible depreciation of semen quality. However, antibiotics have been questioned because of an ever-increasing bacterial resistance. In this term, probiotics (*Lactococcus lactis*) can be used as an alternative to antibiotics due to their antimicrobial properties. In this study, the effects of probiotics (*Lactococcus lactis*) as an antibiotic alternative compared to control and antibiotic group (gentamicin-GEN) on semen quality in rabbits was studied. The Tris fructose extender was prepared and the supplemented with antibiotic (AB, 250µg gentamycin) probiotic (pro, 10<sup>9</sup> colony-forming units of *Lactococcus lactis*) per ml of semen or without supplementation as a control and the semen was cooled at 5°C for three days. The results showed that probiotics had the same antibiotic-like effect on the quality of cryopreserved semen, except for noticeable differences in sperm motility and gross and vital abnormalities. In conclusion,

**probiotics ( $10^9$  colony-forming units of *Lactococcus lactis*) improved semen quality over time thus it could be used as safe alternative to conventional antibiotics in rabbit semen extenders.**

**Keywords:** sperm, probiotic, antibiotic alternative, rabbit.

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## INTRODUCTION

Artificial insemination (AI) is a highly effective assisted reproductive technology utilized in animal breeding across the world. Despite the use of strong sanitary precautions throughout the collection and manipulation operations, semen from healthy reproductive males of different animal species contains bacteria originating from natural colonization in the male tract and the surroundings (Waberski et al., 2019). As a result, international rules recommended the inclusion of antibiotics in semen extenders to avoid bacterial development. On the other hand, the excessive antibiotic usage in several fields raised global concerns due to the emergence of microbial resistance which is emerging as one of the serious issues globally (Da Costa et al., 2013 ).

Semen infected with bacteria such as Enterobacteriaceae can negatively impact the outputs of AI (Althouse and Lu., 2005). Even if the majority of them are harmless bacteria, high levels can have a deleterious impact on semen physiochemical properties (Úbeda et al., 2013; Biesta-Peters et al., 2019; Marco-Jiménez et al., 2020). Bacterial resistance to commonly administered antibiotics, as well as the global dissemination of resistance genes, has become a severe public health issue in recent years. Bacteria might counteract antimicrobial effects in a variety of ways,

including enzyme modification, change of target binding sites, active efflux pumps, and reduced membrane permeability (Agyare et al., 2019). This resistance may arise from spontaneous mutations as well as through the horizontal transfer of mobile genetic elements from other bacteria, phages, and/or the transmission of resistance genes via the environment (Sharma et al., 2016). There, some alternatives to conventional antibiotics in semen extenders, such as colloid centrifugation (Nicholson et al., 2000; Martínez-Pastor et al., 2021) or removal of seminal plasma (Ramires Neto et al., 2015), have been examined in many species. However, these procedures need an increase in the processing time of semen with various effects on semen quality. Other techniques for inhibiting bacterial growth include the use of active compounds such as EDTA (Finnegan and Percival, 2015), chitosan (Rabea et al., 2003; Sahariah and Másson, 2017), nanoparticles (Rudramurthy et al., 2016), peptides (Bahar & Ren, 2013; Mahlapuu et al., 2016) and aminopeptidase inhibitors (Dickneite et al., 1985), and probiotic (Bactria) (García-Galán et al., 2020). Previous studies have demonstrated that lactic acid bacteria (LAB) of the genus, *Lactobacillus* spp., may restrict the development of pathogenic bacteria. This can be due to the ability of (LAB) to compete for nutrient resources, stimulate the host immune system, and produce hydrogen peroxide, acetic and lactic acids, reducing pH (Eschenbach et al., 1989).

Accordingly, the main objective of this study was to determine the effects of adding (*L. lactis*) probiotics as an antibiotic alternative on the quality of cooled preserved rabbit sperm.

## MATERIALS AND METHODS

This study was carried out at the nanobiotechnology and microbiology laboratory (Nanoencapsulation and Animal Physiology Unit) and the Laboratory of Rabbit Physiology Research, Faculty of

Agriculture, Animal and Fish Production Department, Alexandria University, Egypt.

### **Semen extender and treatments**

#### **Fabrication of probiotics**

The probiotic strain *Lactococcus lactis* ATCC 11454, Microbiological Resource Center, Faculty of Agriculture, Ain Shams University) were selected as probiotic strains. According to the MIRCEN-provided information, the bacterial strain was isolated from Anchu mash. For mass production of (*L. lactis*) bacteria strain, De Man, Rogosa, and Sharpe (MRS, Merck KGaA, Darmstadt, Germany), agar and broth media were autoclaved at 120° C and 1–1.5 atm for 15 min. strain of (*L.lactis*) bacteria was grown in De MRS agar plates at 37°C for 48 h. Then, the resultant colonies of (*L. lactis*) were inoculated in MRS broth under anaerobic conditions at 37°C for 48 h. Wet biomass was harvested by centrifugation at 5000 rpm for 15 min at 25°C. The bacterial biomass was washed three times with saline and re-suspended in MRS broth plus 15% glycerol (v/v) and kept at -80°C (Hashem et al., 2021).

### **Animal husbandry and experimental design**

#### **Animal management**

Five sexually mature and fertile V-Line male rabbits were in this study. Rabbits aged 30-36 weeks and 3.00-3.5 kg body weight. Rabbits were housed in a naturally ventilated lighted rabbit try under similar management and hygienic conditions and were individually kept in galvanized wire cages (60 cm×55 cm×40 cm) equipped with feeders and automatic drinkers. Rabbits were fed a basal pelleted diet that covers daily maintenance requirements according to (NRC, 1977).

### **Semen collection**

Before starting the semen collection for the experimental period, the bucks were trained for collection of semen by an artificial vagina. Semen was collected in the morning (8 am) by an artificial vagina (40-41°C) using a teaser doe. After semen collection, any gel plug was removed.

Semen was collected twice weekly in replicate. Strict attention was paid to the hygiene of collection equipment and semen samples were collected into sterile tubes. Semen samples were collected (2 months) twice a week for six weeks (from January to February). After collection, determine the physical properties of semen samples, ejaculate volume, semen color, and sperm concentration, progressive motility (%), dead sperm and abnormal sperm percentage in the following procedures. (Theau-Clément et al., 2005).

### **Extender preparation**

**TRIS base Extender:** TRIS-citric acid fructose (TCF) was prepared and used as The Tris extender contained 38.0125 g of Tris-hydroxymethyl-aminomethane, 21.6550 g of monohydrated citric acid, and 5.9993 g of fructose dissolved in 1000 ml of distilled water (Roca et al., 2000; Oztemel et al., 2005; Abo-Elsoud et al., 2019).

The pooled semen sample was splitted into four aliquots and diluted with (1:10) (semen: extender) of the following extenders:

Control: Tris without supplement.

Tris AB: Tris + Antibiotic (Gentamycin 250µg / ml DS)

Tris Pro: Tris + Probiotic (*Lactococcus Lactis*)  $10^9$  Colony-Forming  
Units / mL in DS

#### **Cooling 5 ° C-**

All diluted was transferred into the cold beaker, and allowed to reach the stable degree 5°C in about 1-1.5 hours to control the time of cooling diluted semen in the cold cup, ice cubes were added to the beaker when the temperature of the water in the beaker reached 20° C, so should be below 5° C in a controlled manner, this can be done by the aid of sensitive thermometer to determine the degree of the temperature.

#### **Evaluation of semen quality**

Semen collection and diluted samples were cooled at 5° C for three days. Cooled samples were examined for total motility, percentage of live sperm, and abnormality, every 12, 24, 36, 48, 60, 72 h (**Viudes-De-castro et al., 2021**).

**Motility:** The percentage of sperm motility (% of progressive motility) was evaluated in several microscopic fields for each semen sample by visual examination at 40 × magnifications using a light microscope with a heated stage with classifications of subjective assessments ranging from 0% to 100%. (**Theau-Clément et al., 2005; Abo-Elsoud et al., 2019**).

**Dead sperm percentage:** it was determined by placing 1-2 drops of the fresh semen sample and 1-2 drops of eosin-nigrosine (pre-warmed) on a clean Slide, then an edge of the second slide applied to mix the semen sample with stain and also used to drag the mixture along the surface of clean slide after she smears was dried examination had been done under a light microscope at (400X). Eosin is used to stain the dead sperms, whereas nigrosine is used to stain the background, so the sperm cells were classified

according to the staining pattern into complete or partial, purple-stained sperm cells, which were considered non-viable; and unstained sperm cells, which were considered viable.

The stain was prepared as the following, The active ingredient of the stain. Eosin –y- 1.67gm and 2. 10 gm of nigrosine. Dissolving in 100ml double distilled water followed by mixing, boiling, and filtration. The eosin-nigrosine stain was pre-warmed at 37° C (water bath), and the Microscope of the study had a heated stage.

**Sperm abnormalities percentage:** The slide used for counting at least 200 sperm to determine dead sperm percentage is also used to estimate abnormal sperm percentage. The study of sperm abnormalities focused on identifying head, and tail abnormalities under a light Microscope (400X) (**Theau-Clément et al., 2005**)

### **Statistical analysis**

The Statistical Package for the Social Sciences (SPSS, Version 26) was used for analysis. The Generalized Linear Model (GLM) method uses the following model:  $y_{ij} = \mu + T_i + e_{ij}$ , in which  $y_{ij}$  = the observed value of the dependent variable;  $\mu$  = the overall mean;  $T_i$  = the fixed effect of the treatment, and  $e_{ij}$  = the residual error. Comparisons between treatment means were performed using Duncan's multiple-range test. Significance was set at  $p < 0.05$ .

## RESULTS

### Evaluation of semen quality parameters

#### progressive motility

The effects of antibiotic (gentamycin) and probiotic (*Lactococcus lactis*) addition to extender on spermatozoa progressive motility of rabbit at different cooling storage times are illustrated (**Table 1**). The results showed that the addition of probiotic (Pro) significantly improved progressive motility compared with the control (Con) and antibiotic (AB) groups ( $P < 0.05$ ).

**Table 1:** Effect of antibiotic and probiotic additive to extender after cooling at 5°C for 12, 24, 36, 48, 60 and 72 h on progressive motility sperms.

Treatment	H12	H24	H36	H48	H60	H72
Con	59.2 <sup>b</sup>	55.7 <sup>c</sup>	52.3 <sup>c</sup>	49.8 <sup>c</sup>	42.8 <sup>c</sup>	38.2 <sup>b</sup>
AB	66.5 <sup>b</sup>	65.2 <sup>b</sup>	59.8 <sup>b</sup>	60.5 <sup>b</sup>	56.3 <sup>b</sup>	52.7 <sup>ab</sup>
Pro	79.2 <sup>a</sup>	77.0 <sup>a</sup>	74.2 <sup>a</sup>	74.0 <sup>a</sup>	67.5 <sup>a</sup>	62.8 <sup>a</sup>
SEM	0.019	0.017	0.018	0.018	0.021	0.025
p-value	0.001	0.001	0.001	0.001	0.001	0.001

Con = Control (basic extender); AB= Antibiotic (extender enriched with 250µg gentamycin/ml in diluted semen); Pro = probiotic (extender enriched with 10<sup>9</sup>colony-forming units of *Lactococcus lactis* /mL in diluted semen); SEM = standard error of mean. <sup>a,b,c</sup> Means within columns with different letter superscripts are significantly different based on statistical analysis ( $p \leq 0.005$ ).

#### Viability

The effects of antibiotic and probiotic addition to extender on spermatozoa viability of rabbit at different cooling storage times are



illustrated (Table 2). The percentage of viable sperm was significantly improved in AB and Pro groups compared to the Con group ( $P < 0.05$ ). This improvement was observed during the experimental period with the highest values in the (Pro) group.

**Table 2:** Effect of antibiotic and probiotic additive to extender after cooling at 5°C for 12, 24, 36, 48, 60 and 72 h on viability sperms.

Treatment	H12	H24	H36	H48	H60	H72
Con	63.8 <sup>c</sup>	59.0 <sup>c</sup>	58.3 <sup>b</sup>	59.5 <sup>b</sup>	57.7 <sup>c</sup>	55.8 <sup>b</sup>
AB	80.0 <sup>ab</sup>	74.3 <sup>b</sup>	75.8 <sup>a</sup>	72.8 <sup>a</sup>	70.2 <sup>b</sup>	68.7 <sup>a</sup>
Pro	85.7 <sup>a</sup>	85.2 <sup>a</sup>	84.7 <sup>a</sup>	81.3 <sup>a</sup>	80.3 <sup>a</sup>	79.2 <sup>a</sup>
SEM	1.979	2.282	2.382	2.243	2.027	2.238
p-value	0.001	0.001	0.001	0.002	0.001	0.001

Con = Control (basic extender); AB= Antibiotic (extender enriched with 250µg gentamycin/ml in diluted semen); Pro = probiotic (extender enriched with 10<sup>9</sup>colony-forming units of Lactococcus lactic /mL in diluted semen); SEM = standard error of mean. <sup>a,b,c</sup> Means within columns with different letter superscripts are significantly different based on statistical analysis ( $p \leq 0.005$ ).

### Abnormality

The effects of antibiotic and probiotic addition to extender on spermatozoa abnormality of rabbits at different cooling storage times are illustrated (Table 3). the percentage of abnormal sperm was significantly decreased in the AB and pro groups compared to the Con group. This reduction was observed from H24 and along the cooling storage period.

**Table 3:** Effect of antibiotic and probiotic additive to extender after cooling at 5°C for 12, 24, 36, 48, 60 and 72 h on total abnormality (%).

Treatment	H12	H24	H36	H48	H60	H72
Con	32.7	36.0 <sup>a</sup>	38.3 <sup>a</sup>	43.8 <sup>a</sup>	48.5 <sup>a</sup>	51.5 <sup>a</sup>
AB	27.0	26.8 <sup>b</sup>	28.2 <sup>b</sup>	32.3 <sup>b</sup>	36.3 <sup>b</sup>	39.7 <sup>b</sup>
Pro	21.8	22.0 <sup>b</sup>	23.7 <sup>b</sup>	26.2 <sup>b</sup>	30.3 <sup>b</sup>	32.8 <sup>b</sup>
SEM	1.870	1.432	1.466	1.669	1.756	1.690
p-value	0.240	0.001	0.001	0.001	0.001	0.001

Con = Control (basic extender); AB= Antibiotic (extender enriched with 250µg gentamycin/ml in diluted semen); Pro = probiotic (extender enriched with 10<sup>9</sup>colony-forming units of *Lactococcus lactic* /mL in diluted semen); SEM = standard error of mean. <sup>a,b,c</sup> Means within columns with different letter superscripts are significantly different based on statistical analysis (p≤0.005).

## DISCUSSIONS

During collection and/or sampling, microbial contamination of ejaculates is often observed, even if all the requirements for aseptic and antiseptic handling of semen are met (Bielanski., 2007). Duracka et al., (2019) reported that the bacterial microflora in rabbit semen was primarily represented by *Enterobacteriaceae*, *Pseudomonas*, *Clostridium* and *Streptococcus*. Bacteria contamination, such as *Enterobacteriaceae* family might adversely affect the quality of semen used for AI (Althouse and Lu, 2005). Studies in rabbits showed that the semen samples that suffer from significant contamination showed reduced semen quality and subsequently fertilization unsuccess (Dalimata and Graham., 1997; Gliozzi et al., 2003).

As semen collection is not a completely sterile process, antibiotics ABare commonly added to extenders used for artificial insemination or in-vitro fertilization in order to control possible microbial contamination during semen processing (Morrell and Wallgren., 2014). Since AB themselves may be toxic to spermatozoa (Zeh et al., 2012; Azawi & Ismaeel, 2012), and because of an alarmingly increasing bacterial resistance (Karakalpakis et al., 2018) there is an urgent need to find alternatives to conventional AB to be used in animal reproduction biotechnologies (Morrell and Wallgren, 2014). Recent studies have emphasized using antibiotic alternatives on cooled stored semen in rabbits.

The probiotic used in this study was selected upon a strong body of evidence on their beneficial antimicrobial and antimicrobial properties, bacteria belonging to genera *Lactobacillus* and *Bifidobacterium* have been shown to decrease the levels of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,20-azino-bis (ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radicals. Kim et al., (2022) reported that the scavenging capacity of *Lactobacillus* is due to the action of bacterial exopolysaccharides, antioxidant enzymes, bioactive peptides, and manganese ions. These bacterial elements can directly scavenge free radicals, chelation of metal ions, and reduce ascorbate autoxidation (Benkerroum et al., 2000; Khemariya et al., 2017).

The antimicrobial activity of probiotics lactobacilli used in this study may be due to the production of secondary metabolites, such as bacteriocin, lactic acid, and hydrogen peroxide (intracellular ROS-mediated cell damages) (Ruizet al., 2009). For example, *Lactococcus* lactic produce bacteriocin nisin, which has substantial antimicrobial activity against bacterial pathogens and fungi, such as *Escherichia coli*,

*Staphylococcus aureus*, *Pseudomonas* spp., *Candida albicans*, and *Aspergillus niger* (Thanjavuret al., 2022).

Our results show that probiotics (*Lactococcus lactis* ssp *lactis*) could improve the sperm motility, abnormality, and overall viability of extended rabbit semen stored cooling for 71 hr. A similar observation was reported by Duracka et al., (2019) and Abood and Aliawy (2023).

Results indicated comparatively good total motility rates for the first 12 hours of storage period than 72 hours. On the other hand, with the time of storage, the motility rates reduced substantially at a very high rate on the other time. As sperm motility is an important indicator of semen health, sperm must travel and reach the point of fertilization. Therefore, preserving the motility of spermatozoa is a major challenge in spermatology works.

## CONCLUSION

In conclusion, we demonstrated in this study that the addition of probiotic ( $10^9$ colony-forming units of *Lactococcus lactis*) to semen extender improves semen quality over time and could be used as an alternative to conventional antibiotics in rabbit semen extenders.

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## تأثير إضافة البروبيوتيك كبديل للمضادات الحيوية على جودة السائل المنوي المحفوظ بالتبريد في الارانب

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### الملخص العربي

المضادات الحيوية عنصر أساسي أثناء تخفيف السائل المنوي وذلك لتجنب انخفاض جودة السائل المنوي، ومع ذلك، فقد أدى الاستخدام المستمر للمضادات الحيوية إلى زيادة المقاومة البكتيرية، ولذلك يمكن أن يتم استخدام البروبيوتيك (*Lactococcus lactis*) كبديل للمضادات الحيوية بسبب خصائصه المضادة للميكروبات. في هذه الدراسة، تم دراسة تأثير البروبيوتيك كبديل للمضادات الحيوية مقارنة بمجموعة الكنترول والمضاد الحيوي (الجنتاميسين) على جودة السائل المنوي في الارانب. تم تحضير مخفف التريس فركتوز وتم توزيع المعاملات بإضافة ٢٥٠ ميكروجرام من الجنتاميسين و ١٠<sup>٩</sup> مستعمرة من البروبيوتيك لكل مل سائل منوي مخفف أو بدون إضافات (الكنترول) وتم تبريد السائل المنوي لمدة ٣ أيام على درجة حرارة ٥٥ م. أظهرت النتائج أن البروبيوتيك كان له نفس التأثير المماثل للمضاد الحيوي على جودة السائل المنوي المحفوظ بالتبريد، باستثناء اختلافات ملحوظة في حركة الحيوانات المنوية والتشوهات الكلية والحيوية. وبناء على ما سبق فإن إضافة البروبيوتيك ١٠<sup>٩</sup> مستعمرة من *Lactococcus lactis* يحسن من

جودة السائل المنوى بمرور الوقت، وبالتالي يمكن استخدامه كبديل امن للمضادات الحيوية في مخففات السائل المنوى للأرانب.

**الكلمات الدالة:** الحيوان المنوى، البروبيوتيك، بديل المضاد الحيوى، الارانب