

# Study of antibiotics and symbiotic effects on sperm quality using the CASA system



A. El B. Bouchicha, A. Kalem, N. Mimoune\*, S. Djouadi, D. Khelef and R. Kaidi

## Abstract

The objective of the current work was to study *in vitro* sperm quality after antibiotics and symbiotic administration and to evaluate treatment administered before insemination with the aim of reducing artificial insemination failures in goats. This experimental study was carried out at the Animal Reproduction Biotechnology Laboratory (Blida, Algeria). Semen analysis was performed using the Computer-assisted sperm analysis system. In the first experimental approach, we used the antibiotics most commonly administered in the veterinary field for the treatment of subclinical endometritis. A total of eight antibiotics were studied. Each antibiotic tested was co-incubated with frozen goat semen brought from the Centre for Artificial Insemination and Genetic Improvement. For the second experimental approach, we incubated semen with a symbiotic (Symbiovéba). Finally, we selected two antibiotics among those used, namely colistin and cotrimoxazole, and these

were co-incubated with the symbiotic and the semen, to examine possible combinations of antibiotics with symbiotics in the treatment and prevention of uterine infections (broad spectrum synergistic activity). Antibiotics have been shown to have a detrimental effect on the sperm cell, by decreasing sperm motility. The average value calculated on all antibiotics was 18% (as opposed to initial motility of 78% in the control group), with an alteration of the linear speed that would have a negative impact on fertilization. On the other hand, symbiotics had a beneficial effect on spermatozoa motility and vitality. The combination of the symbiotic and colistin proved to be very promising. In conclusion, the use of symbiotics in the treatment of subclinical endometritis at the time of goat insemination is beneficial, and requires greater attention in future research.

**Key words:** *sperm; antibiotic; symbiotic; CASA; semen; subclinical endometritis*

Ahmed El Bachir BOUCHICHA, Institute of Sciences and technology, Tissemsilt University, Laboratory of Biotechnologies related to animal reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb University, Blida, Algeria; Ammar KALEM, Laboratory of Biotechnologies related to animal reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb University, Blida, Algeria; Nora MIMOUNE\*, (Corresponding author, e-mail: nora.mimoune@gmail.com), Laboratory of Biotechnologies related to animal reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb University, Blida, Algeria, National High School of Veterinary Medicine, Animal Health and Productions Laboratory, El-Alia, Algiers, Algeria; Sohaib DJOUADI, Faculty of Nature & Life Sciences, Farm Animal Reproduction Laboratory, University of Tiaret, Algeria; Djamel KHELEF, National High School of Veterinary Medicine, Animal Health and Productions Laboratory, El-Alia, Algiers, Algeria; Rachid KAIDI, Laboratory of Biotechnologies related to animal reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb University, Blida, Algeria

## Introduction

Uterine infections including subclinical endometritis (SCE) have a major economic impact due to their impacts on reproductive performances (Ahmadi et al., 2006; Đuričić et al., 2013; Oruc et al., 2015; Szenci et al., 2018; Asadpour et al., 2020). Fortunately, treatments are available to improve fertility. The preferred choice of the therapy and administration route differ among veterinarians. Therapy is primarily based on local application of antibiotic preparations, and the selection of antibiotics should be based on the determination of antibiotic resistance. Furthermore, antibiotic preparations have different absorption properties and induce multiple interactions within the uterus (Đuričić et al., 2015; Samardžija et al., 2017).

In order to reduce the performance gaps attributable to artificial insemination (AI) failures, for animals with SCE, some authors recommend an antibiotic-based treatment shortly before the act of insemination (Đuričić et al., 2014). In particular, antibiotics have attracted the attention of scientists for their toxic potential on sperm quality. Indeed, *in-vitro* studies have revealed the presence of a significant alteration in sperm mobility parameters (Aral, 2008). Furthermore, spermatozoa are sensitive to oxidative stress caused by polynuclear cells and antibiotics due to their structure rich in polyunsaturated fatty acids (Žura Žaja et al., 2016a,b, 2019a,b).

Our objective was to test alternatives to antibiotics for the treatment of genital infections at the time of natural mating or AI. Particularly, we tested *in vitro* antibiotics and symbiotic effects on sperm motility and vitality of buck semen.

## Material and methods

The study was carried out at the Animal Reproduction Biotechnology

Laboratory of the Veterinary Institute (Saad Dahleb University, Blida 1, Algeria) during the period from June to October 2019.

### Semen

Frozen semen straws from alpine bucks were collected from the National Centre of Artificial Insemination and Genetic Improvement (CNIAAG), Baba Ali, Algiers, Algeria. The volume of each straw was 0.25 mL.

### Antibiotics

In this study, eight antibiotics were tested. They were selected based on the frequency of their use for uterine infection therapy, in particular in the case of SCE: tetracyclines, erythromycin, sulfamethoxazole, gentamycin, colistin, ciprofloxacin, amoxicillin + clavulanic acid, rifampicin.

**Table 1.** Antibiotics tested with an antibiogram

Antibiotics	Abbreviation
Tetracycline	TE
Ciprofloxacin	Cip
Erythromycin	E
Rifamycin	RD
Gentamycin	GN
Co-trimoxazole	CoT [SMX]
Colistin	CT
Amoxicillin + clavulanic acid	AMC

### Symbiotic

The product used in this study is a purely biological additive for veterinary use; SYMBIOVEBA® (MARCOPOLO Environmental Group) consists of medicinal plants (*Taraxacum officinalis*, *Zingiber officinalis*), probiotics (*Lactobacillus* and *Saccharomyces cervicie*), enzymes, and plant extracts.

## CASA system

This system comprises a microscope linked to a digital camera, the whole is associated with a computer equipped with a software for computer analysis of sperm parameters (Sperm class Analyzer; SCA Version 5.4, microptic SL, Spain). This system performs an automatic analysis of videos of moving spermatozoa to generate objective values of the following mobility parameters: curvilinear velocity (VCL), the average path velocity (VAP), linear speed or VSL (Straight-Line Velocity), ALH (Amplitude of Lateral Head displacement), beat cross frequency (BCF).

## Experimental design

Manipulations were carried out under aseptic conditions to avoid any microbial contamination that could alter the results.

## Preparation of solutions

### First part

#### Control preparation protocol

In an Eppendorf flask with 1 mL 0.9% NaCl (saline solution), we added 0.5 ml frozen semen. Alternatively, to save

straws, we added 0.25 mL semen to 0.5 mL 0.9% NaCl. The flask was placed in a water bath at 37°C to avoid thermal shocks for the sperm. The control made it possible to estimate the impact of antibiotics on spermatid capacity.

#### Antibiotic + semen solution preparation protocol

In an Eppendorf flask with 1 mL saline solution, we added 0.5 ml semen, using two straws each with 0.25 mL, and an antibiotic disc (one antibiotic disc for 1 ml saline solution to retain the minimum inhibitory concentration (MIC)).

#### Preparation of samples for analysis

For sperm motility: a few drops of diluted semen (controls) using 10  $\mu$ L, 100  $\mu$ L micropipette were applied to a slide and a coverslip added for analysis on the CASA computer.

For sperm vitality: 10  $\mu$ L diluted semen + 10  $\mu$ L eosins and 20  $\mu$ L nigrosine were prepared on a slide, and left for 3 to 5 minutes before spreading out and leaving to dry. Finally, slides were analysed on a CASA computer.

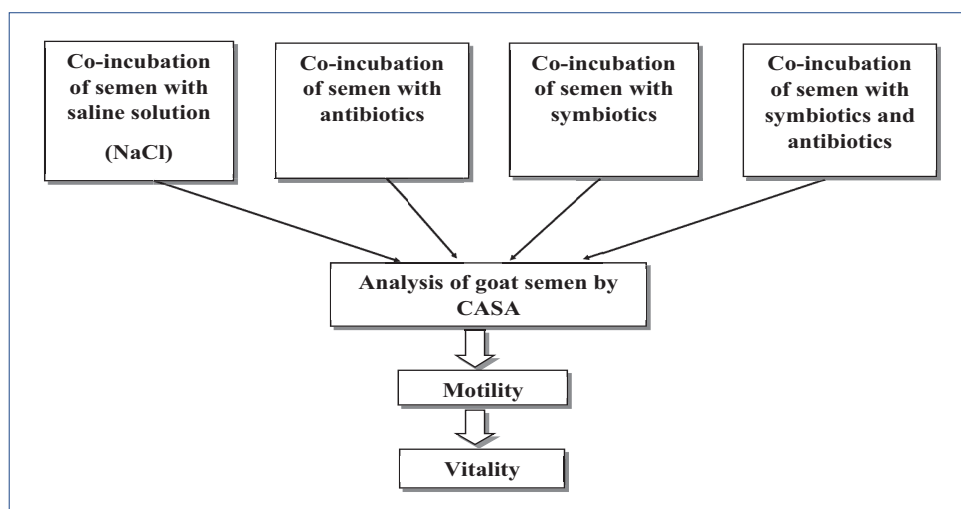


Figure 1. Flowchart of the second part of the study

## Second part

In this part of the study, we examined the symbiotic effect and the results of co-incubation of antibiotics with symbiotic on semen quality (Figure 1).

### Protocol for preparing symbiotic and semen solution

In a labelled Eppendorf flask (sperm + symbiotic), 1 mL NaCl (saline solution) was added with 1 mL symbiotic solution. The solution was mixed using a vortex and placed in a water bath at 37°C.

### Protocol for preparing the sperm solution co-incubated with the symbiotic and the antibiotics

In a labelled Eppendorf flask (sperm + symbiotic + ATB) containing 1 mL NaCl, we added 1 mL symbiotic solution, two seed straws, and an antibiotic disc. This was mixed using a vortex and placed in a water bath at 37°C.

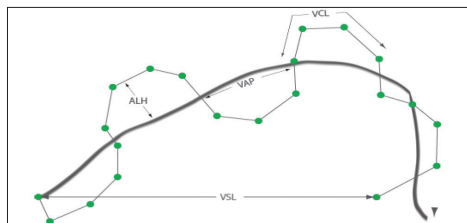
### Microscopic analysis

Microscopic analysis was obtained directly from CASA system which presents objective values of the mobility parameters: curvilinear speed (VCL), average trajectory speed (VTBI), linear speed (VSL), beat cross frequency (BCF) (Figure 2).

### Motility

#### Control analysis

The control analysis was performed at  $T_0$ . Overall spermatozoa movement



**Figure 2.** Diagram of the sperm mobility parameters

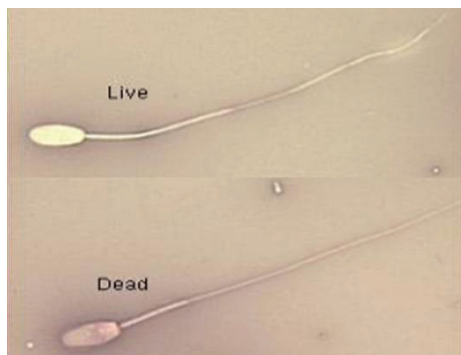
was estimated directly after analysis by observing a drop of sperm under the optical microscope at 10x magnification, Ph1 chamber with green filter, with and a heating plate at 37°C to avoid thermal shock or distortion of the results.

### Analysis of the antibiotic + semen solution

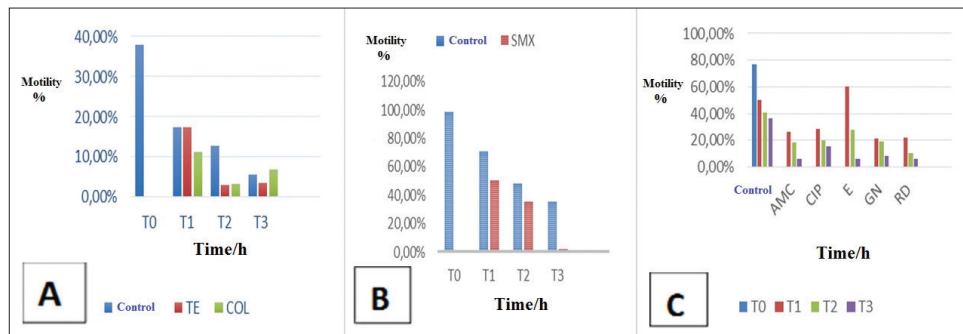
For this analysis, the steps were the same as above. However, the results obtained are quite different. The analysis was then reproduced at  $T_1 = 30$  min,  $T_2 = 1$  h,  $T_3 = 2$  h, and  $T_4 = 3$  h for each of the solutions.

### Sperm Vitality

Sperm vitality was ascertained after calculating the number of dead and living (active) sperm in different fields of the optical microscope at 60x magnification, chamber A without filter (Figure 3).



**Figure 3.** Representation of a colourless live spermatozoa and a pink dead spermatozoa



**Figure 4.** Evolution of motility during the period of co-incubation of spermatozoa with antibiotics (TE, CT, SMX, AMC, CIP, GN, RD) + the control group

## Results

### First part (antibiotics)

#### Motility

The results of motility testing are shown in Figure 4.

#### Figure A

A progressive decrease was seen in the percentage of motility at  $T_1$  and  $T_2$  compared to the control group; this shows that the antibiotics (COL and TE) has a detrimental effect on sperm capacities. A slight increase was observed in  $T_3$ .

#### Figure B

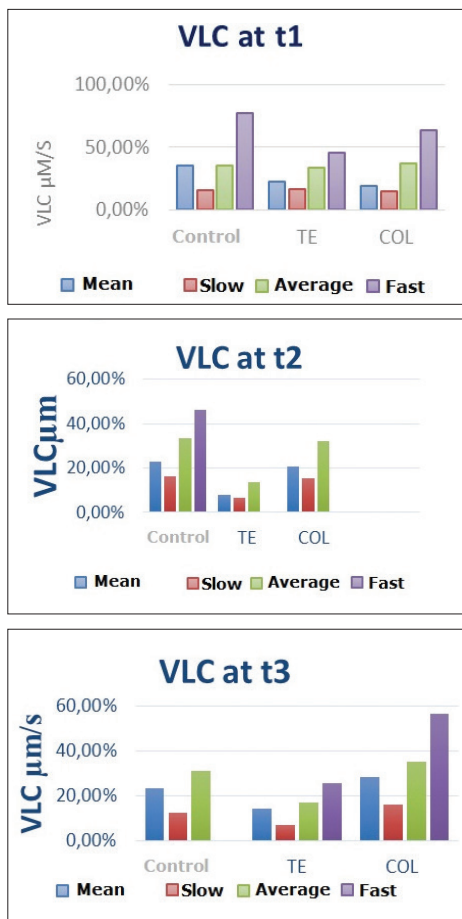
A decrease in the percentage of motility was recorded at  $T_1$ ,  $T_2$ , and  $T_3$  compared to  $T_0$  (control). This is also attributed to the toxic effect of the antibiotic (SMX) on the sperm cell.

#### Figure C

The percentage of motility showed a progressive decrease during co-incubation of the spermatozoa with (AMC, CIP, GN or RD), except for erythromycin where the value is higher.

#### Curve speed (VCL)

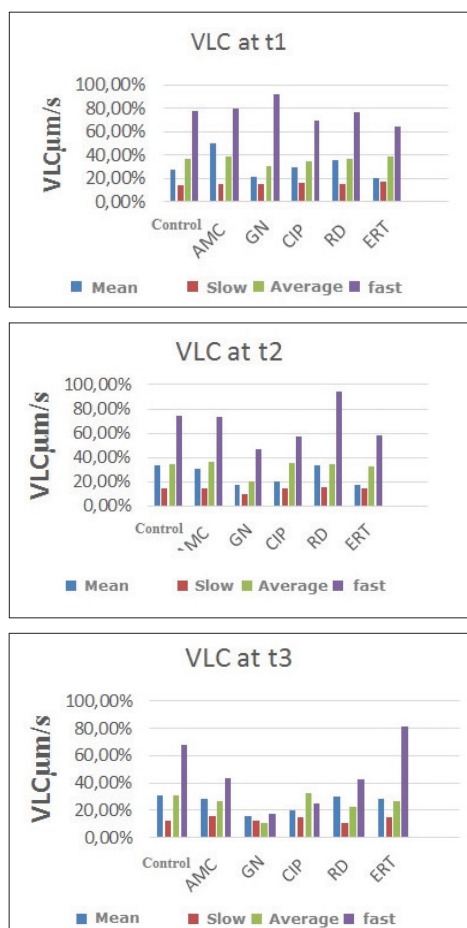
The results are shown in Figures 5, 6, and 7:



**Figure 5.** Evolution of VCL during the period of co-incubation of spermatozoa with antibiotics (TE, COL) + the control group



**Figure 6.** Evolution of VLC during the period of co-incubation of spermatozoa with antibiotics (SMX) + control group



**Figure 7.** Evolution of the VLC during the period of co-incubation of spermatozoa with antibiotics (AMC, GN, CIP, RD, ERT) + the control group

We noticed a decrease in VLC at  $T_1$  and  $T_2$  during co-incubation of sperm with COLs and TEs, and an increase in VLC at  $T_3$  compared to the control VLC (Figure 3). We recorded a decrease in VLC at  $T_1$  and  $T_2$  with SMX compared to the control VLC (Figure 4).

Regarding the results illustrated in Figure 7:

At  $T_1$ : A decrease in VLC was observed during co-incubation with CIP and ERT and a slight decrease with RD compared to the control. On the other hand, an increase was recorded during the period of co-incubation with GN and AMC.

At  $T_2$ : A decrease in VLC was observed with AMC, GN, CIP, ERT, CIP and a significant increase in VLC with RD.

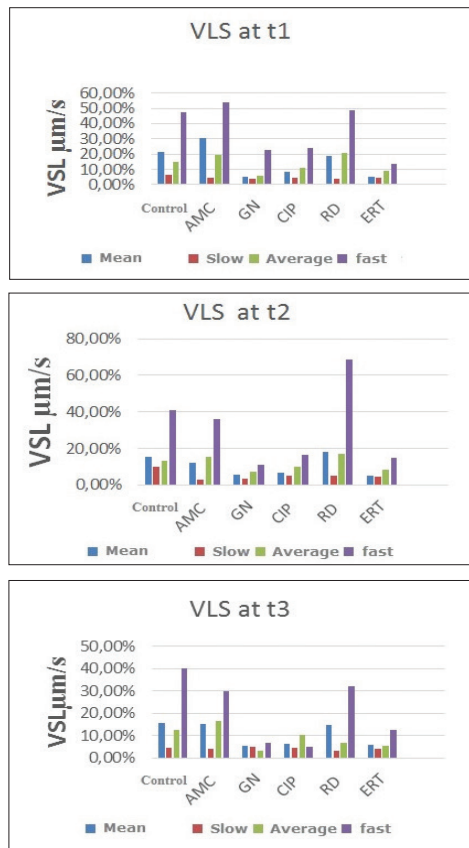
At  $T_3$ : A decrease in sperm VLC was also observed during co-incubation with AMC, GN, CIP, RD, and a marked increase with ERT.

### Linear Speed (VSL)

The values of VSL of semen of the control group and of each antibiotic are represented at the three test times in Figures 8, 9 and 10.

Regarding the results illustrated in Figure 8:

At  $T_1$  and  $T_3$ : We noted a decrease in VSL compared to the control group except for AMC, where the VSL is increased.



**Figure 8.** Evolution of the VSL of spermatozoa co-incubated with antibiotics (AMC, GN, CIP, RD, ERT) as a function of time + control group

At  $T_2$ : There is a significant increase in the VSL of the spermatozoa co-incubated with the ED.

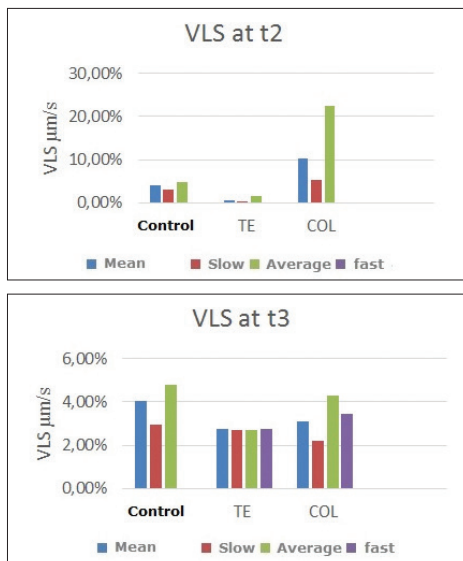
Regarding the results illustrated in Figure 9:

At  $T_1$ : A significant decrease in VSL was recorded compared to the control group.

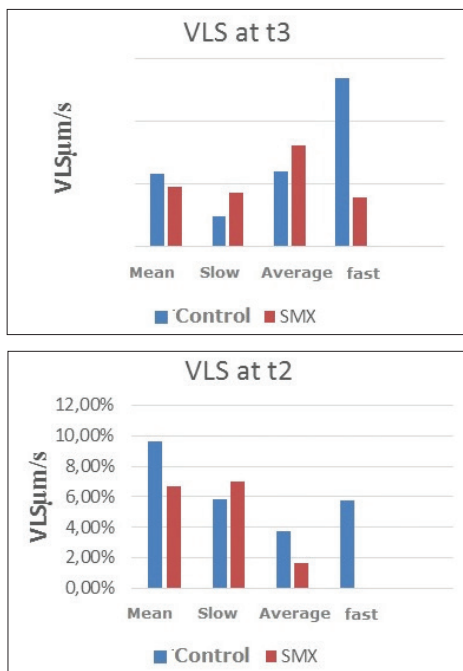
At  $T_2$ : At this stage the VSL is stationary.

At  $T_3$ : We noticed a resumption of the VSL again, especially of semen co-incubated with COL and TE.

The results illustrated in Figure 8 show a decrease in VSL at  $T_2$  and  $T_3$  with SMX compared to the control group.



**Figure 9.** Evolution of the VSL of the spermatozoa co-incubated with the antibiotics (TE, COL) as a function of time + control group



**Figure 10.** Evolution of the VSL of spermatozoa co-incubated with antibiotic (SMX) as a function of time + control group

**Part two**

The results of this part are illustrated by the following tables and figures:

From Table 2 and Figure 11, it can be seen that vitality and motility of spermatozoa co-incubated with the symbiotic was better compared to the control.

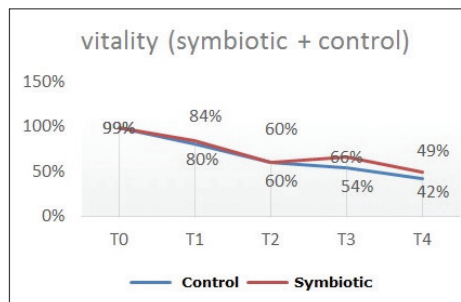
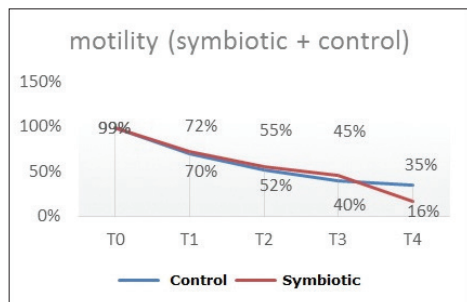
Motility and vitality were also improved when the sperm were co-

incubated together with the symbiotic and colistin (Table 3 and Figure 12).

The evolution of motility and vitality over time was better than when the semen was co-incubated solely with the symbiotic. However, the combination of the symbiotic with cotrimoxazole showed no benefits (Table 4, Figure 13).

**Table 2.** Motility and vitality of the symbiotic and the control

Sample	Control group		Symbiotic	
	Vitality	Motility	Vitality	Motility
Time				
T <sub>0</sub>	69%	74%	69%	74%
T <sub>1</sub>	60%	50%	64%	62%
T <sub>2</sub>	50%	42%	58%	48%
T <sub>3</sub>	44%	30%	50%	38%
T <sub>4</sub>	32%	25%	38%	30%

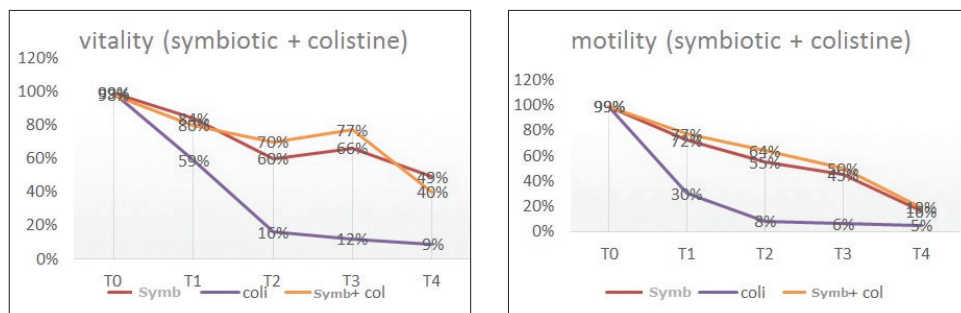


**Figure 11.** Evolution of the motility and vitality of semen co-incubated with the symbiotic, and the control

**Table 3.** Evolution of the motility and vitality with the symbiotic and colistin.

Sample	Symbiotic		Colistin		Symbiotic + Colistin	
	vitality	motility	vitality	motility	vitality	motility
T <sub>0</sub>	99%	99%	99%	99%	98%	99%
T <sub>1</sub>	84%	72%	59%	30%	80%	77%
T <sub>2</sub>	60%	55%	16%	8%	70%	64%
T <sub>3</sub>	66%	45%	12%	6%	77%	50%
T <sub>4</sub>	49%	16%	9%	5%	40%	19%

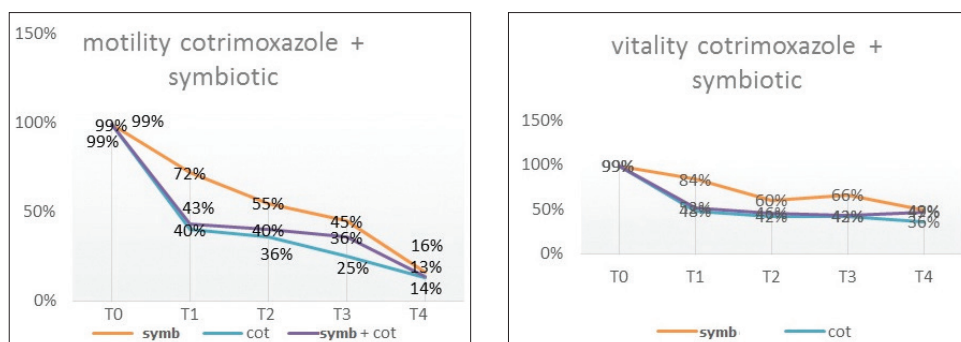




**Figure 12.** Evolution of the motility and vitality of semen co-incubated with the symbiotic and colistin

**Table 4.** Evolution of the motility and vitality of semen co-incubated with the symbiotic and cotrimoxazole (Cot).

Sample	Symbiotic	Cot	Symbiotic + Cot
Time	Motility	Motility	Motility
T0	99%	99%	99%
T1	72%	40%	43%
T2	55%	36%	40%
T3	45%	25%	36%
T4	16%	13%	14%



**Figure 13.** Evolution of the motility and vitality of semen co-incubated with the symbiotic and cotrimoxazole

## Discussion

Subclinical endometritis is treated with antibiotics using two strategies: treat and inseminate at the same time; or treat and let the female rest with insemination taking place after 21 days (Kasimanickam et al., 2005; Leblanc, 2009). Previous

studies *in vivo* and *in vitro* showed that antibiotics negatively affect the sperm cell, and so AI is known to fail if insemination is performed immediately following treatment. Overall, the results presented here demonstrate that the treatment of spermatozoa with antibiotics induced an altering effect on sperm mobility.

The deleterious effect of tetracycline on sperm mobility is well documented in the literature. This antibiotic is responsible for inhibiting the capacitation of sperm and the acrosome reaction. Indeed, its ability to chelate calcium can be very relevant, as calcium is not only involved in the initiation of sperm movement in mature mammals, but it is also crucial for hyperactivation (Yanagamachi, 1994). In addition, this drug is a fluorophore: it binds avidly to human sperm. This connection may actually present a physical obstruction to motility (Ericsson and Baker, 1967). Other studies have also shown that the therapeutic dose of tetracycline induces a toxic sperm and testicular effect in male rats by induction of oxidative stress (Farombi et al., 2008).

Our *in vitro* exposure of spermatozoa to gentamycin MIC induced a significant alteration in their mobility. Studies by Aral et al. (2008) *in vivo* showed the same results. Co-incubation of sperm with erythromycin, ciprofloxacin and sulfamethoxazole demonstrated an alteration of all sperm motility parameters; the same results were noticed for rifampicin and colistin. However, studies carried out by Hargreaves et al. (1998) have shown a significant improvement in sperm mobility. Studies have been reported in the literature on sperm agglutination. This phenomenon was observed during co-incubation with rifampicin and colistin. The effects of these two antibiotics have been studied by several authors; they demonstrated that the agglutination of spermatozoa can lead to a decrease in mobility and poor penetration of the cervical mucus, influence selection in the genital tract, impede capacitation and finally, and inhibit fertilization of the ovum.

Co-incubation with amoxicillin + clavulanic acid induced alteration, though there are no literature data on this combination. The study by Hargreaves et

al. (1998) on amoxicillin alone showed no significant effect on mobility.

The symbiotics co-incubated with semen showed better results than those of the control, and also compared to those obtained with the antibiotics. In fact, the molecule used in our study is an association between a symbiotic, prebiotic and yeasts, which could explain its beneficial effects.

In a previous study, Barbonetti et al. (2011), found that a combination of three selected strains of *Lactobacilli* (*Lactobacillus brevis*, *L. salivarius*, and *L. plantarum*), whose efficacy in the treatment of bacterial vaginosis in the form of vaginal tablets has been recently reported, was effective in preventing lipid peroxidation of sperm induced *in vitro* by a ferrous ion promoter, thus preserving sperm motility and viability. This finding suggests that the vaginal application of symbiotic *Lactobacilli* could protect human sperm from radical oxygen species in the presence of vaginal disorders, thereby improving the fertilization potential of the female host.

According to Maretti and Cavallini (2017), sperm count was improved after treating oligo-astheno-teratospermia. According to Valcarce et al. (2017), sperm motility was significantly improved after treatment with two strains of symbiotics in males with astheno-spermia. The results reported in this study provide preliminary evidence for the possibility of administering symbiotics to improve motility.

## Conclusion

The present study was aimed at studying the impact of antibiotics and symbiotics on sperm mobility and vitality parameters *in vitro*. Antibiotics cause damage to the sperm cell during AI, and during treatments performed on male breeding animals of all species, including humans. The facts were confirmed by our

results obtained by the CASA system; we have demonstrated an alteration of sperm co-incubated with antibiotics. In addition, symbiotics showed a beneficial effect, on both motility and vitality. Overall, the results obtained under our experimental conditions are promising. A symbiotic and antibiotic combination remains to be confirmed, with the ultimate aim of ceasing the indiscriminate use of antibiotics.

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## Studija učinka antibiotika i simbiotika na kvalitetu sperme putem sustava računalno potpomognute analize sperme

Ahmed El Bachir BOUCHICHA, Ammar KALEM, Laboratory of Biotechnologies related to animal reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb University, Blida, Algeria, Institute of Sciences and Technology, Tissemsilt University; Nora MIMOUNE, Laboratory of Biorechnologies related to animal reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb University, Blida, Algeria, National High School of Veterinary Medicine, Animal Health and Productions Laboratory, El-Alia, Algiers, Algeria; Sohaib DJOUADI, Faculty of Nature & Life Sciences, Farm Animal Reproduction Laboratory, University of Tiaret, Algeria; Djamel KHELEF, National High School of Veterinary Medicine, Animal Health and Productions Laboratory, El-Alia, Algiers, Algeria; Rachid KAIDI, Laboratory of Biorechnologies related to animal reproduction (LBRA), Institute of Veterinary Sciences, Saad Dahleb University, Blida, Algeria

Cilj je ovoga rada bio proučiti *in vitro* kvalitetu sperme nakon dodavanja antibiotika i simbiotika te procijeniti terapiju danu nedugo prije osjemenjivanja da bi se smanjio broj neuspješnosti umjetnih osjemenjivanja (AI) u koza. Naša eksperimentalna studija provedena je u Laboratoriju za asistiranu reprodukciju životinja (Blida, Alžir). Analiza sjemena provedena je sustavom računalno potpomognute analize sperme (CASA). Za prvi eksperimentalni pristup rabili smo antibiotike koji se najčešće daju u veterini za liječenje subkliničkog endometritisa (SCE), a proučeno je ukupno osam antibiotika. Svaki ispitanik antibiotik bio je koinkubiran sa zamrznutim sjemenom koza iz Centra za umjetno osjemenjivanje i genetsko poboljšanje (CNIAAG). Za drugi pristup koinkubirali smo spermu sa simbiotikom (Symbiovéba). Na kraju smo izabrali dva antibiotika među onima rabljenima - kolistin i kotrimoksazol.

Da bismo pokušali pronaći moguću kombinaciju antibiotika sa simbioticima u liječenju i prevenciji infekcije maternice (široki spektar sinergizirane aktivnosti) koinkubirali smo ih sa simbiotikom i sjemenom. Pokazalo se da antibiotici imaju negativan učinak na spermije jer su prouzročili smanjenje njihove pokretljivosti. Prosječna izračunata vrijednost na svim antibioticima bila je 18 % (početna pokretljivost kontrolne skupine bila je 78 %) te promjena linearne brzine koja bi imala negativan učinak na oplodnju, dok su simbiotici su imali blagotvorni učinak na pokretljivost i vitalnost sperme. Kombinacija simbiotika i kolistina bila je vrlo obećavajuća što znači, da je uporaba simbiotika u liječenju subkliničkog endometritisa (SCE) u vrijeme umjetnog osjemenjivanja koza korisna, ali ju je potrebno i dalje pratiti i istraživati.

**Ključne riječi:** *sperma, antibiotik, simbiotik, CASA, sjeme, subklinički endometritis*