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ANTIMICROBIALS IN A RABBIT SEMEN EXTENDER: EFFECTS ON REPRODUCTION

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Abstract: The use of fluoroquinolone antibiotics was legally restricted by the European Commission in March 2019. Since the extender for rabbit semen Galap® contained this antibiotic, it became necessary to modify it. The purpose of this study was to search for another molecule, based on its antimicrobial activity and also on the conservation, motility and fertility of semen diluted with the new extender. Several bacterial strains were isolated from 10 poor-quality ejaculates, including *Enterococcus* spp., *Staphylococcus aureus* and *Proteus* spp. They were then tested for antimicrobial susceptibility. Out of 15 antibiotics evaluated, gentamicin was the one targeting such bacteria. *In vivo* tests were then carried out to assess the effects of this antibiotic change on sperm parameters. Up to 26 pools of good quality semen (total motility >70%) were diluted in original Galap® or in this extender with gentamicin. Ejaculates were analysed on the day (D) of collection and up to 6 d of storage at +17°C. After 24 h storage, the motility in the new extender was reduced by 7.7% compared to the original; this decrease did not worsen by storing. After 6 d of storage, no difference between the two media was detected. A total of 360 females were then inseminated with pools of 4 to 5 good quality semen diluted 1:10 in both extenders, following the routine protocol of rabbit semen processing centres. There was no difference in female fertility or prolificacy between both formulations. In conclusion, these preliminary results suggest that the addition of gentamicin to this extender is useful and also has no adverse effect on fertility or prolificacy.

Key Words: rabbit, semen extender, bacterial load, motility, fertility.

INTRODUCTION

The breeding of rabbits for meat has long been of agronomic interest, due in particular to the rabbit's reproductive behaviour and its economic profitability (Lebas *et al.*, 1997). Since the 1980s, rabbits have been bred in groups and the use of artificial insemination (AI) has become widespread. The success of AI in rabbits depends on the reproductive performance of the female, the male and the skill of the operators. The main factors related to the quality of the semen are fertility in females (proportion of pregnant females after AI), and prolificacy (number of offspring per litter), as pointed out by IRRG (2005). Indeed, a correlation between sperm motility, concentration and female reproductive performance has been demonstrated in several studies (Brun *et al.*, 2002; Theau-Clément *et al.*, 2016).

The bacterial load in semen is another determinant of the quality of AI doses. Mercier and Rideaud (1990) and Duracka *et al.* (2019), in their preliminary studies, reported that the commensal bacterial community in rabbit semen can contain *Enterobacter* spp., *Pseudomonas* spp. and *Enterococcus* (Group D *Streptoccocus*) species. However, poor

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animal health and inadequate collection and handling of semen can lead to abnormal microbial load, as could be widely demonstrated in studies conducted for swine production (Althouse *et al.*, 2000, 2008, Althouse and Lu, 2005). High bacterial contamination of the semen has an adverse effect on the quality of semen (Gadea, 2003; Morrell and Wallgren, 2014). Bacteria produce toxic metabolites leading to DNA fragmentation and a decrease in sperm viability (Koppers *et al.*, 2008, Fraczek and Kurpisz, 2015). They can also lead to sperm agglutination, resulting in a decline in sperm motility (Althouse *et al.*, 2008; Bussalleu *et al.*, 2011). We know that AI with contaminated and poor-quality doses has an impact on female health and fertility in the sow. It can cause systemic inflammation and disease to the female (Maes *et al.*, 2008) and might reduce litter size (Maroto Martín *et al.*, 2010).

Therefore, the choice of semen extender is critical. To limit possible microbial contamination during semen processing, antibiotics are added to the extender (Morrell and Wallgren, 2014). Due to the regulation established in the European Union in 2019, through the Pharmacovigilance Risk Assessment Committee of the European Medicines Agency's (EMA) (Francisco, 2018), we considered the need to change the antibiotic composition of Galap®, while maintaining the quality of the diluent in sperm functions.

In this study, we carried out (a) preliminary trials to identify potential candidate antibiotics for substitution of enrofloxacin in Galap® extender; (b) an evaluation of their efficacy on rabbit sperm bacterial communities; and (c) the design of a new antibiotic formulation of this extender for the preservation of sperm motility and fertility.

MATERIAL AND METHODS

Experimental design

Several trials were performed in this study to identify alternative antibiotic(s) to the original Galap® antibiotic formula containing penicillin, streptomycin and enrofloxacin (Figure 1). First, we measured the sensitivity of 18 bacterial

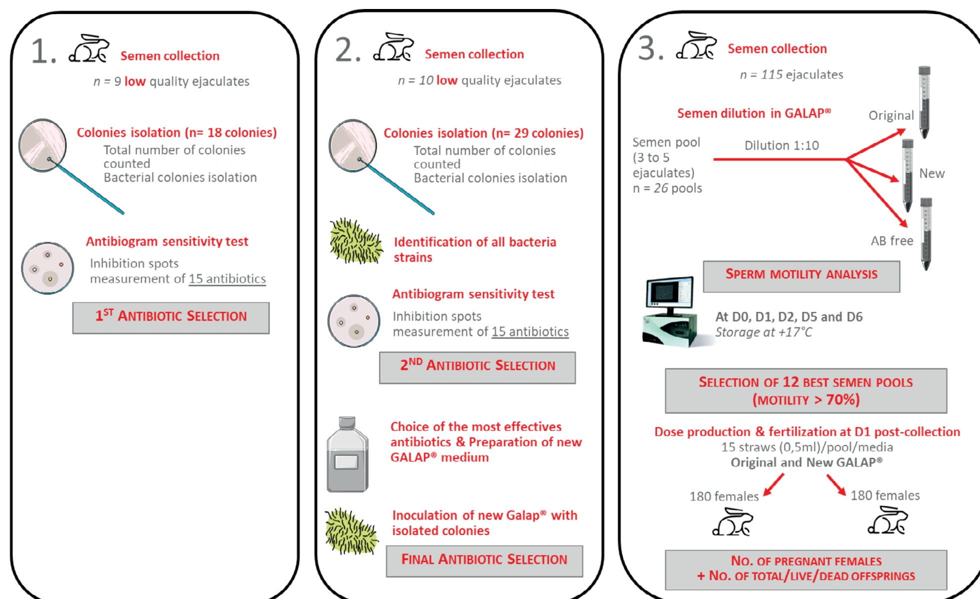


Figure 1: Experimental design. Scheme of (1) the microbial isolation of 9 low quality ejaculates and first antibiogram sensitivity test. Scheme of (2) the second microbial isolation and identification of 10 low quality ejaculates, and second antibiogram sensitivity test. Scheme of (3) the dilution and pool of several ejaculates in Original, New or Antibiotic (AB) Free Galap® extender to analyse the total sperm motility from day (D) 0 to 6 post-collection.

colonies, isolated from 9 poor-quality ejaculates, to several antimicrobials. After identifying the most effective antimicrobials, we repeated the experiment on 10 other poor-quality ejaculates and isolated a total of 29 bacterial colonies. The colonies were identified to determine which ones had acquired resistance to the antimicrobials. Antibiotics with the largest activity spectra were diluted individually or in combination with other antibiotics in the extender and were incubated with the 29 isolated bacterial colonies. The bacterial proliferation was observed after 24, 48 and 72 h of incubation, by optical density measurement, to choose the candidate antibiotic combination to replace the original antibiotic formula. Lastly, we diluted and choose a total of 12 pools of 3 to 5 ejaculates. This allowed us to verify (i) the absence of bacterial growth in the semen pools and (ii) the absence of toxic effect on sperm motility and on fertility.

Animals

We used a total of 475 rabbits (115 males and 360 females *Oryctolagus cuniculus*) of the Hycole line, aged 6 to 24 mo, at 5 to 7 kg body weight. The rabbits were healthy and underwent the following medical prophylaxis: myxomatosis and viral haemorrhagic disease vaccination and deworming. Bucks and females were kept in individual cages at room temperature (+20 to +23°C), under a natural photoperiod (January to October 2020). All rabbits were fed with a diet containing 16.2% crude protein, 15.5% crude fibre, 3.4% fat, 1.35% calcium, 8.2% ash, 0.63% phosphorus and 0.26% sodium as feed, in addition to fresh water *ad libitum*. No change of feeding was done during the experiment. The handling of the rabbits and the experimental protocol were carried out in strict compliance with the ethical guidelines for animals formulated by the British Society of Animal Science (Jarvis *et al*., 2005) and with the sanitary approval of semen processing centres given by the French commission *Charte Fénalap* (National Federation of Rabbit Producers Groups).

Bacterial load analysis

The analyses of the bacterial communities in ejaculates and the antibiograms were carried out using samples of low quality semen. In a first test, 9 low quality ejaculates, with a score of 5 out of 9 maximum in mass motility, with presence of debris and dust, based on Brun *et al*. (2002) were collected, using a lubricated and pre-heated (+45°C) artificial vagina (Collap, IMV Technologies, L'Aigle, France). The ejaculates were placed in laminoculture using the URITEST N ready-to-use kit (Liofilmchem, Roseto degli Abruzzi, Italy). They were inoculated on a dip slide, before insertion of the slide into the different culture media in the kit (MacConkey agar, Cetrimide Agar and CLED Agar). The total microbial population was revealed with CLED Agar (*Escherichia coli, Proteus* spp., *Klebsiella* spp., *Candida* spp., *Pseudomonas* spp., *Enterococcus* spp. and *Staphylococcus* spp.), the Gram-negative bacteria (*Escherichia coli, Proteus* spp., *Klebsiella* spp., *Pseudomonas* spp.) count on the MacConkey Agar and the count of *Pseudomonas* spp. on the Cetrimide Agar. Colonies were counted after 24 h of inoculation at +36°C. A preliminary antibiotic sensitivity test was performed on isolated colonies to identify potential antibiotic substitutes for enrofloxacin in Galap®. To isolate colonies, part of their surfaces was taken with a pointed needle and cultured in saline solution. The Kirby-Bauer method (disc-diffusion antibiotic sensitivity test) in Petri plate coated with Mueller-Hinton Agar (composed of hydrolysate of casein, beef extract, starch and agar, allowing the growth of each bacterial colony identified), detailed in the protocol of the American Society for Microbiology in 2009, was then followed (Hudzicki, 2009). The Mueller-Hinton certificate of analysis available online (Code 100611ZA, Batch 111255, VWR International, Leuven, Belgium) shows that this composition allows the growth of control strains (*Escherichia coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC® 27853, *Enterococcus faecalis* ATCC® 29212, and *Staphylococcus aureus* ATCC® 29213 were used as quality control strains). Sterility tests were performed after incubation at + 30-35°C for 48 hours and + 20-25°C for 48 hours (no growth observed). We also checked the sterility at 7 days after incubation in the same conditions (no growth observed). The concentration of bacterial suspensions from the colonies was adjusted with the McFarland method (Hudzicki 2009) to 0.5 McFarland standard, i.e. 1.10^8 colony forming units (CFU)/mL of bacteria. After spreading bacteria with a swab, antibiotic discs were placed in the culture medium (Liofilmchem or Mast Group Ltd Mast House, Derby Rd, Bootle L20 1EZ, UK) at different concentrations (enrofloxacin 5 µg, amikacin 30 µg, doxycycline 30 µg, florfenicol 30 µg, gentamicin 10 and 30 µg, kanamycin 30 µg, lincomycin 15 µg, marbofloxacin 5 µg, neomycin 30 µg, penicillin G 1IU, polymyxin B 300 µg, spectinomycin 100 µg, tobramycin 30 µg and tylosin 30 µg). The inhibition spots of the antibiotic discs were measured and analysed after incubation of the plates at +35°C for 24 h

(aerobiosis), according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute Inc. (CLSI).

In a second test, 10 new poor-quality ejaculates were collected to isolate the bacteria again following the same protocol. Antibiograms were repeated to validate the previous ones and to start identifying the antibiotic candidates of the study. All isolates (29 in total) were biochemically identified to detect those that had acquired resistance to the antibiotics, using the Urin system Plus kit (Liofilmchem, Roseto degli Abruzzi, Italy). This colorimetric kit allows identification of the bacterial strains (*Escherichia coli, Proteus* spp., *Providencia* spp*., Pseudomonas* spp*.,* KES Group (*Klebsiella* spp*., Enterobacter* spp*., Serratia* spp.)*, Enterococcus* spp, *Staphylococcus aureus, Candida* spp.) present in colonies, after incubation of the ejaculate in different suspension following the commercial protocol.

In a third test, antibiotics with the broadest activity spectra were diluted individually or in combination with other antibiotics in Galap® and their concentrations of use were selected. Since the optical density (OD) is correlated with the quantity of bacteria in this extender, the bacterial proliferation in the different media was analysed by spectrophotometry. Galap® media were inoculated with 29 identified bacterial colonies. On the day of inoculation, bacterial concentration of the samples was standardised to 10^8 CFU/mL by performing absorbance (optical density, OD) readings at 595 nm and adjusting the OD to 0.08. The samples were then incubated at +15°C and the OD was measured once again after 24, 48 and 72 h of incubation. The number of samples with OD less than, equal to or greater than those obtained in semen samples diluted in antibiotic-free extender was reported.

In a fourth test, a field trial was carried out to validate the new antibiotic composition of the extender. A total of 10 randomised pools of 3 to 5 ejaculates from rabbit males routinely used in production in a AI centre in France were diluted (1:10) in three different compositions of extender —antibiotic-free, original extender (with enrofloxacin) and extender with different antibiotic composition— based on the bacteriological analysis results (e.g., gentamicin 0.3 g/L, or gentamicin 0.3 g/L+amikacin 0.2 g/L). Bacterial analyses of these samples were performed by a veterinary laboratory (Labovet Conseil, Beaupréau, France). After a serial dilution of the samples up to 1/10000, allowing us to estimate the bacterial concentration, they were inoculated on a nutritive medium (Agar-agar compound, commercial composition not communicated, routinely used for the culture of microbiota present in rabbit semen) in Petri dish at +30°C for 48 h. At the end of the incubation, traces or development of bacteria colonies were analysed.

Semen collection for motility analysis and dose packaging

The semen of 105 males, collected regularly for dose production (twice a week), was harvested in the facilities of the Hycole semen processing centre in France, in the morning. Immediately after collection, the gel was removed by using a plastic pipette, since it contains high concentrations of citric acid (Holtz and Foote, 1978). The quality of the ejaculate was first assessed based on appearance. The semen must have a milky or aqueous white appearance (based on the protocol of Daniel and Renard, 2010). Any ejaculate with yellow or brownish traces of urine or blood was removed. Ejaculates were pooled in groups of 4 to 5 to eliminate individual variations between males (for a final volume of at least 3 mL). The pools were diluted (1:10) in 15 mL sterile conical tubes (final volume of 10 mL) in different pre-warmed (+ 37°C) Galap® media (IMV Technologies, L'Aigle, France): 1 mL of semen in an antibiotic-free medium (*AB free*), 1 mL of semen in a medium with the original antibiotic composition (*Original*) and 1 mL of semen in a medium with the new antibiotic composition (*New*).

After one hour of equilibration time at +23°C (Daniel and Renard, 2010), 1.5 mL from each semen pool was stored in 2 mL sterile tubes and kept in the fridge at +17°C to monitor semen quality during storage time (at day 0 or collection day immediately after the equilibration time, and then at day 1, 2, 5 and 6 of storage after collection). The quality of up to 26 semen pools was analysed after dilution of 200 µL of each pool in the appropriate media (1:4 in antibiotic-free, original or new extender). Diluted pooled semen was incubated for 10 min at +37°C. Then, sperm motility and concentration were analysed in a Leja slide chamber (Leja, IMV Technologies group, GN Nieuw-Vennep, Netherlands), using a computer-assisted semen analyser (IVOS II, Hamilton Thorne, Beverly, USA). Percentages of total motile and progressive spermatozoa, velocity on average path (VAP), curvilinear velocity (VCL), velocity on a straight line (VSL) and linearity (LIN=VSL/VCL) were also calculated. Two chambers per samples were analysed (frame rate=60, frame acquired=45, with a minimum of 8 frames per chamber).

The other 8.5 mL of the semen pools were used to carry out the insemination doses in 0.5 mL straws (IMV Technologies, L'Aigle, France) with a total of around 24 million sperm cells per dose (484.5±122.2 million spermatozoa/mL in raw ejaculates) (Theau-Clément *et al.*, 2016). Doses were prepared only from semen pools with a total motility above 70% (Daniel and Renard, 2010) and diluted in the medium with the original antibiotic composition (original) or new antibiotic composition (gentamicin 0.3 g/L; *New*). A total of 12 pools diluted in the 2 different media were stored for 24 h (day 1) at + 17°C and then used for the insemination of 15 females each (360 females in total).

Artificial insemination

A total of 360 multiparous rabbits (parity between 2 to 11), distributed in two French breeding farms (180 per farm), were inseminated in batches with one dose: 180 total females (90 per farm) were inseminated with the semen diluted in the original extender; 180 total females (90 per farm) were inseminated with the new formula. Inseminations were carried out in January 2020 in one farm and in November 2020 in another. Services were performed using a curved plastic sheath (IMV Technologies, L'Aigle, France). Immediately after AI, each female received an intramuscular injection of 5 µg of lecirelin (0.2 mL of Reproreline® 25 µg/mL, Vetoquinol, France) to induce ovulation (Daniel and Renard, 2010). At parturition, various parameters were measured including the number of kindling females, as well as the total number of offspring, alive and dead, to evaluate the fertility rate, fecundity and prolificacy.

Statistical analysis

Data were presented as mean±standard error and with box plot. Data presented on box plot have the five-number summary (minimum, first quartile Q1, median, third quartile Q3 and maximum), outliers and mean represented with a rhombus. A Chi-square test and a multi-comparison via an exact Fisher test were used to interpret the presence/absence of bacterial colonies. Bacterial proliferation using absorbance measurement was analysed by a Friedman's test followed by a multiple comparison with Wilcoxon test for paired samples. A mixed model following a GLMM procedure was used to analyse the difference between the different antibiotic compositions of Galap® media and the semen quality, fertility, fecundity and prolificacy. For motility analysis, the extender and/or day of storage after semen collection were included as the fixed effects, with semen pool as random effect. For fertility analysis, the extender and parity order (1st group from parity 1 to 3; 2nd group from 4 to 11) were included as the fixed effects, and semen pool as the random effect. Statistical analyses were performed on R software, version 1.1.463 (R Core Team, 2014). A significant difference in the results was considered when $P<0.05$.

RESULTS

Sensitivity of bacterial colonies from poor-quality semen to various antibiotics

The inoculation of 9 poor-quality ejaculates allowed us to identify up to 18 bacterial colonies. With some of these colonies we performed 9 antimicrobial susceptibility testing. These tests showed the interest of the study, as only 22.2% of the bacterial colonies tested were sensitive to enrofloxacin, present in the original extender (data not shown, but available). Of the 9 antibiotics tested, only 2 antibiotics were effective, with 83.3% of the bacterial colonies sensitive (15/18): amikacin 30 µg and gentamicin 10 µg (data not shown). Isolated colonies were 3.7 times more sensitive to amikacin 30 µg or gentamicin 10 µg than to enrofloxacin 5 µg.

To confirm these results, we repeated the experiment based on 10 other poor-quality ejaculates. After isolation of 29 bacterial colonies, we performed 15 antimicrobial susceptibility tests. In this instance, the results showed that 44.8% of the bacterial colonies tested were sensitive to enrofloxacin, which was twice the sensitivity observed in the first test. Some bacterial colonies showed statistically increased resistance to other antibiotics, such as penicillin G (1 IU) with only 17.2% sensitivity and 82.8% resistance (P=0.011) or lincomycin 15 µg and tylosin 30 µg, with both 24.1% sensitivity and 75.9% resistance (P=0.044) (Table 1). Of the 15 antibiotics, only 2 antibiotics were effective with more than 70% of the bacterial colonies sensitive. These were the same antibiotics previously selected, namely amikacin 30 µg (72.4% of sensitivity) and gentamicin 10 µg (82.8%) and 30 µg (89.7%) (Table 1). Although isolated colonies were 1.6 times more sensitive to amikacin 30 µg than to enrofloxacin 5 µg, they were significantly more

Table 1: Antibiograms made from poor-quality ejaculate samples. Number (no.) and percentage (%) of bacterial colonies isolated after ejaculate laminoculture, that are susceptible, moderately susceptible (intermediate) and resistant to different antibiotics (n=10 ejaculates and 29 colonies tested), according to the generic cutoff values of the CLSI or EUCAST Standard.

	Resistant		Intermediate		Sensitive		
ANTIBIOTICS (µg)	no.	%	no.	%	no.	%	*P*-value
enrofloxacin 5	12	41.4	4	13.8	13	44.8	
amikacin 30	7	24.1	1	3.4	21	72.4	ns
doxycyclin 30	19	65.5	0	0.0	10	34.5	ns
florfenicol 30	9	31.0	3	10.3	17	58.6	ns
gentamycin 10	5	17.2	0	0.0	24	82.8	ns
gentamycin 30	1	3.4	2	6.9	26	89.7	0.005
kanamicin 30	10	34.5	0	0.0	19	65.5	ns
lincomicin 15	22	75.9	0	0.0	7	24.1	0.044
marbofloxacin 5	13	44.8	0	0.0	16	55.2	ns
neomicin 30	11	37.9	1	3.4	17	58.6	ns
penicilin G 1 IU	24	82.8	0	0.0	5	17.2	0.001
polimyxin B 300	16	55.2	3	10.3	10	34.5	ns
spectinomycin 100	13	44.8	2	6.9	14	48.3	ns
tobramycin 30	8	27.6	1	3.4	20	69.0	ns
tylosin 30	22	75.9	0	0.0	7	24.1	0.044

Statistical analysis were done based on enrofloxacin group as control (ns=not significant).

sensitive to gentamicin 30 µg than to enrofloxacin, with 2.0 times more sensitive colonies and 12.1 times fewer resistant colonies (Table 1).

The bacterial strains contained in the 10 ejaculates and the 29 isolated colonies were then identified. All ejaculates contained at least *Enterococcus* spp., *Staphylococcus aureus* or both; 50% had *Proteus* spp. and bacteria from KES group (*Klebsiella* spp., *Enterobacter* spp., *Serratia* spp.), and 40% had *Pseudomonas* spp. (Table 2). Our kits also allowed us to detect *Candida* spp. in 50% of the ejaculates (Table 2). In the 29 colonies isolated, 58.6% of them had *Staphylococcus aureus*, 31.0% *Candida* spp., 41.4% *Proteus* spp., 37.9% *Enterococcus* spp. and 17.2% were other strains (*Pseudomonas* spp., *Klebsiella* spp., and so on) (Table 2). None of the ejaculates contained *Escherichia coli.*

We then tested the sensitivity of each of the 29 identified colonies to antibiotics, especially amikacin and gentamicin. Among 7 resistant colonies to amikacin 30 µg, all had at least one *Enterococcus* spp. and some had also *Staphylococcus aureus*, *Proteus* spp., *Pseudomonas* spp. and *Candida* spp. (Table 3). In the presence of 10 µg gentamicin, more than 5 colonies were resistant (Table 3). All of them had *Enterococcus* spp. as well, and 3 of them had at least one

Table 2: Number and percentage of microbial strains found in poor-quality ejaculates or colonies isolated.

	Pseudomonas spp.	*Enteroccoccus* spp.	*Candida* spp.	*Staphylococcus aureus*	*Proteus* spp.	KES	*Enteroccoccus* spp. and/or *Staphylococcus aureus*	Total
No. ejaculates (n=10)	4	7	5	7	5	5	10	10
% ejaculates	40.0	70.0	50.0	70.0	50.0	50.0	100.0	10
No. colonies (n=29)	9	9	11	19	14	5	24	29
% colonies	27.6	37.9	31.0	58.6	41.4	17.2	82.8	29

KES: *Klebsiella-Enterobacter-Serratia*, n=10 ejaculates, n=29 colonies.

Table 3: Microbial identification of strains resistant to amikacin and gentamicin. The different strains were identified from colonies isolated in ejaculates (n=10 ejaculates; 29 isolated colonies analysed).

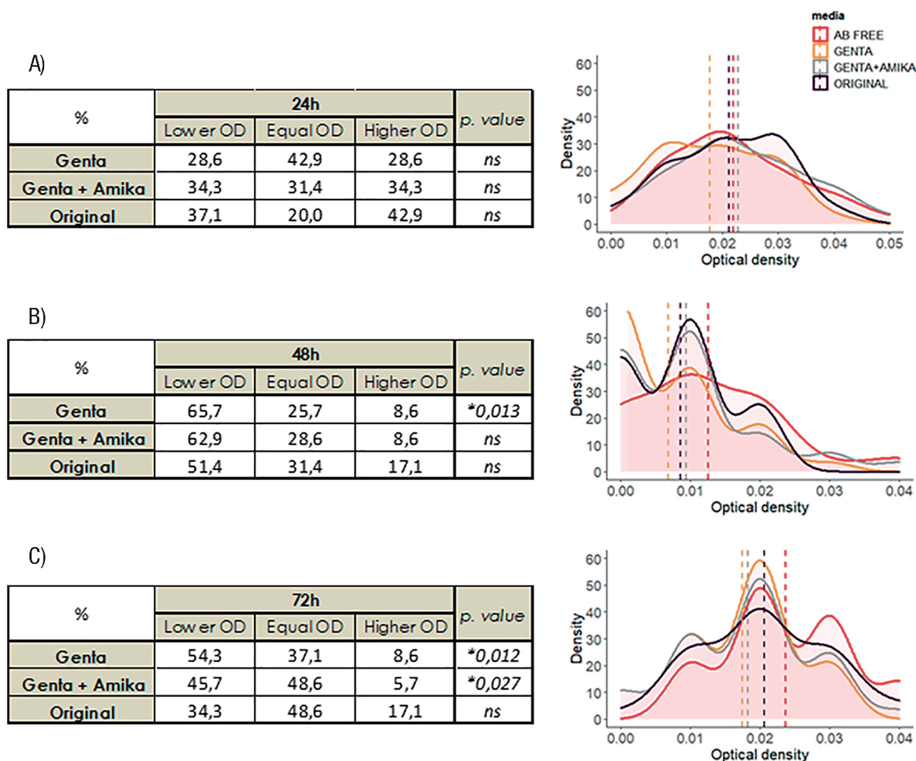
Ejaculate nb Colony nb	*Pseudomonas* spp.	*Enterococcus* spp.	*Candida* spp.	*Staphylococcus* aureus	*Proteus* spp.	Others spp.
Resistant colonies to amikacin 30 µg						
134.2		✓				
134.3		✓				
258.2		✓				
377.1		✓				
547.2		✓	✓		✓	
548.3		✓		✓		
618.2	✓	✓		✓	✓	
Total	1	7	1	2	2	0
Resistant colonies to gentamycin 10 µg						
134.2		✓				
258.2		✓				
377.1		✓				
548.3		✓		✓		
618.1	✓	✓	✓	✓	✓	KES
Total	1	5	1	2	1	1
Resistant colonies to gentamycin 30 µg						
134.2		✓				
Total	0	1	0	0	0	0

KES: *Klebsiella-Enterobacter-Serratia*.

Staphylococcus aureus. By increasing the concentration of gentamicin to 30 µg, one Enterococcus colony out of the 11 identified (i.e. 9.1% resistance) remained resistant. This colony was also resistant to amikacin 30 µg (Table 3).

Therefore, we chose to improve our analysis by incubating these same 29 colonies in (i) antibiotic-free Galap® extender (*AB free*); (ii) the original extender containing enrofloxacin (*Original*); (iii) antibiotic-free extender supplemented with 0.3 g/L gentamicin (*Genta*) or (iv) antibiotic-free extender supplemented with 0.3 g/L gentamicin and 0.2 g/L amikacin (*Amika*). Since it is impossible to determine an equivalent solution concentration (in g/L) from the antibiotic discs (in µg), these concentrations were chosen based on the average concentration commonly used in pig semen dilution media (0.33 g/L in Bresciani *et al.*, 2014, 0.25 g/L in Waberski *et al.,* 2019). Absorbance was measured to determine the bacterial concentration in the different paired samples.

After 24 h of incubation of the colonies in the different media, no significant differences were observed (mean OD: *AB free*: 0.022±0.011; *Original*: 0.021±0.010; *Genta*: 0.018±0.011; *Genta + Amika*: 0.023±0.012) (Figure 2.A). After 48 h of incubation, more than half of the samples diluted in the original extender (51.4%) had a lower OD than in the medium without antibiotics, indicating a lower but not significant bacterial growth (P=0.185) (Figure 2.B). Two-thirds of the samples diluted in medium supplemented with amikacin and /or gentamicin (62.9% and 65.7% respectively) had a lower OD than the medium without antibiotics (mean OD: *AB free*: 0.012±0.011; *Original*: 0.008±0.007; *Genta*: 0.007±0.008; *Genta + Amika*: 0.009±0.009). Gentamicin significantly limited bacterial proliferation compared to the medium without antibiotic (P=0.013) (Figure 2.B). On the other hand, the addition of amikacin did not significantly decrease bacterial proliferation compared to the medium without antibiotic and the medium with gentamicin only (P=0.174 and 0.24 respectively). No difference was observed between the extender supplemented with amikacin and/or gentamicin and the original extender.



A)

%	24h			p. value
	Lower OD	Equal OD	Higher OD	
Genta	28,6	42,9	28,6	*ns*
Genta + Amika	34,3	31,4	34,3	*ns*
Original	37,1	20,0	42,9	*ns*

B)

%	48h			p. value
	Lower OD	Equal OD	Higher OD	
Genta	65,7	25,7	8,6	**0,013*
Genta + Amika	62,9	28,6	8,6	*ns*
Original	51,4	31,4	17,1	*ns*

C)

%	72h			p. value
	Lower OD	Equal OD	Higher OD	
Genta	54,3	37,1	8,6	**0,012*
Genta + Amika	45,7	48,6	5,7	**0,027*
Original	34,3	48,6	17,1	*ns*

Figure 2: Efficiency of antibiotic formulations after dilution in Galap® extender, on the proliferation of bacterial colonies isolated from poor-quality ejaculates. Bacterial colonies isolated from the ejaculate laminoculture were inoculated into antibiotic-free Galap® (AB *free*), with gentamicin (*Genta*: 0.3g/L), gentamicin and amikacin (*Genta+Amika*: 0.3 g/L+0.2 g/L respectively), and Original Galap® (*Original*). Percentage of inoculations with an OD lower, equal or higher to inoculations made in an antibiotic-free medium and density plots representing the distribution of absorbances of the diluted samples in the different media (dashed line=mean); after (A) 24 h, (B) 48 h and (C) 72 h of culture (n=29 bacterial colonies). Statistical analyses were performed on the average absorbances (ns=not significant, **P*-value<0.05, *P*-value indicates the difference compared to the antibiotic-free medium.).

After 72 hours of incubation, samples diluted with amikacin and/or gentamicin had again a significantly lower mean optical density than in the antibiotic-free medium (mean OD: *AB free*: 0.024±0.009; *Genta*: 0.017±0.008; *Genta + Amika*: 0.018±0.009; *Original:* 0.020±0.009, *P*=0.027 and 0.012 respectively) (Figure 2.C). Gentamicin 0.3 g/L was as effective on its own as in combination with amikacin (*P*=1) and was as effective as the original medium (*P*=0.503). We did not contemplate the existence of antagonisms when combining those two antibiotics. Therefore, the new Galap® medium was supplemented only with gentamicin 0.3 g/L.

Identification of the semen bacterial load

To validate the new extender containing gentamicin 0.3 g/L, bacterial development in pooled semen from males routinely used in production was analysed. Traces of bacterial growth, corresponding to a very low bacterial development in the lowest dilution, not countable in colony, were observed in 3 out of 10 pools of semen diluted in the antibiotic-free medium. However, no traces of bacterial development were found in semen diluted either in the original extender or in the new, with less than 10^3 CFU/mL, thus respecting the standard established in the Rabbit AI centre charter. There was no significant difference in semen bacterial load among the three media (*P*=0.23).

Analysis of semen motility parameters over the post-semen collection time

The remainder of the study focused on the effects of gentamicin 0.3 g/L in Galap® medium (*New*) on sperm parameters. At collection day (D0), ejaculates showed a total motility percentage of 77.7±10.2% (*AB free*: 78.3±8.6%; *Original*: 78.9±8.8%; *New*: 78.2±8.4%) (Figure 3.A) and a progressive sperm percentage of 38.2±7.3% (*AB free:* 38.6±8.5%; *Original*: 39.8±6.5%; *New*: 38.9±6.6%) (Figure 3.B). Sperm motility was analysed at different times up to 6 d after collection.

After 24 h of storage at +17°C (D1), the percentage of total motile sperm stored in the new formula was significantly lower (7.7%) than that of sperm stored in the original (*Original:* 74.2±10.7%; *New*: 66.5±9.7%; *P*=0.0001) (Figure 3.A), whereas no significant difference was observed in the proportion of progressive sperm between both media (Figure 3.B). At day 2 of storage (D2), the difference (7.8%) was still significant between the two antibiotic formulas (*Original*: 64.9±10.9%; *New:* 57.1±14.4%; *P*=0.0028) (Figure 3.A). The new formula was nevertheless not significantly different from the antibiotic-free medium (*AB free*: 59.1±12.3%; *P*=0.5032). After 5 d of storage (D5), the gap of 7.6% in the percentage of motile spermatozoa between the two media was maintained (*Original:* 49.9±16.1%; *New*: 42.3±14.8%) (Figure 3.A). However, the difference was no longer significant at day 6 (D6) because of the high variability observed between pools of semen diluted in the original medium (*Original:* 46.5±20.5%; *New*: 40.8±14.8%; *P*=0.5307) (Figure 3.A).

A significant decline of 5.3% in progressive spermatozoa also occurred at D2 in the new medium compared to the original (*Original:* 27.8±10.7%; *New*: 22.5±12.7%; *P*=0.0137) (Figure 3.B). However, a higher difference of 9.5% was observed over the 5 d of storage (*Original:* 27.2±12.1%; *New*: 17.7±9.9%; *P*=0.0116). At D6, the difference was no longer significant (*Original*: 23.4±11.9%; *New*: 16.2±7.2%; *P*=0.1238) (Figure 3.B).

Regarding the velocity parameters assessment, at D0, semen diluted in the antibiotic-free medium and in the new antibiotic formula showed significantly faster velocity of the motile and progressive sperm (VAP, VCL, VSL and LIN) than in the original extender (Figure 4). This difference was no longer observed after 24 h of storage (D1) between the original and the new formula (Figure 4). From day 2 to day 5 of storage, the motile and progressive velocity average pathway (VAP), curvilinear (VCL) and straight-line velocities (VSL) were significantly lower in the new extender than in the original and antibiotic-free extender (Figure 4). The linearity was similar in both original and new formula. At 6 days of storage, the significant differences previously demonstrated were no longer observed, except in the progressive VAP and VSL motility between the original and the new extender formula (Figure 4).

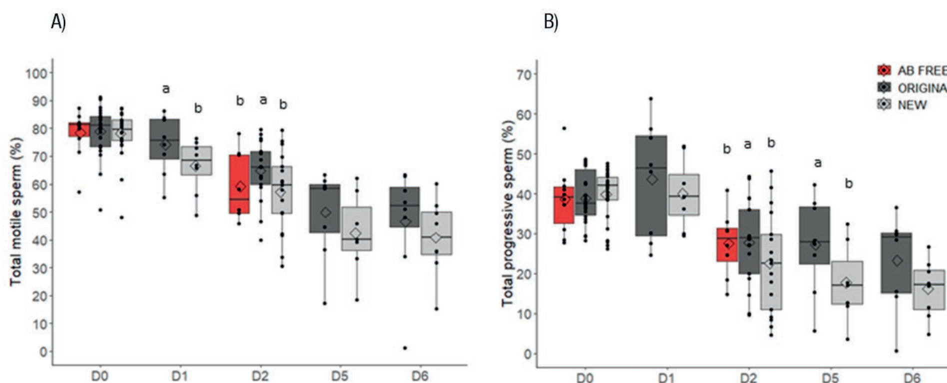


Figure 3: Sperm motility analysis in Galap® extender. (A) Percentage of total motile spermatozoa and (B) percentage of total progressive spermatozoa, after semen dilution in different Galap® extender: antibiotic-free (AB free), original (Original) and new with gentamicin (New). Semen was analysed at different days after collection (D0, D1, D2, D5 and D6). n=8 to 26 semen pools were analysed. Different lower case letters (a and b) indicates significant difference (*P*-value<0.05).

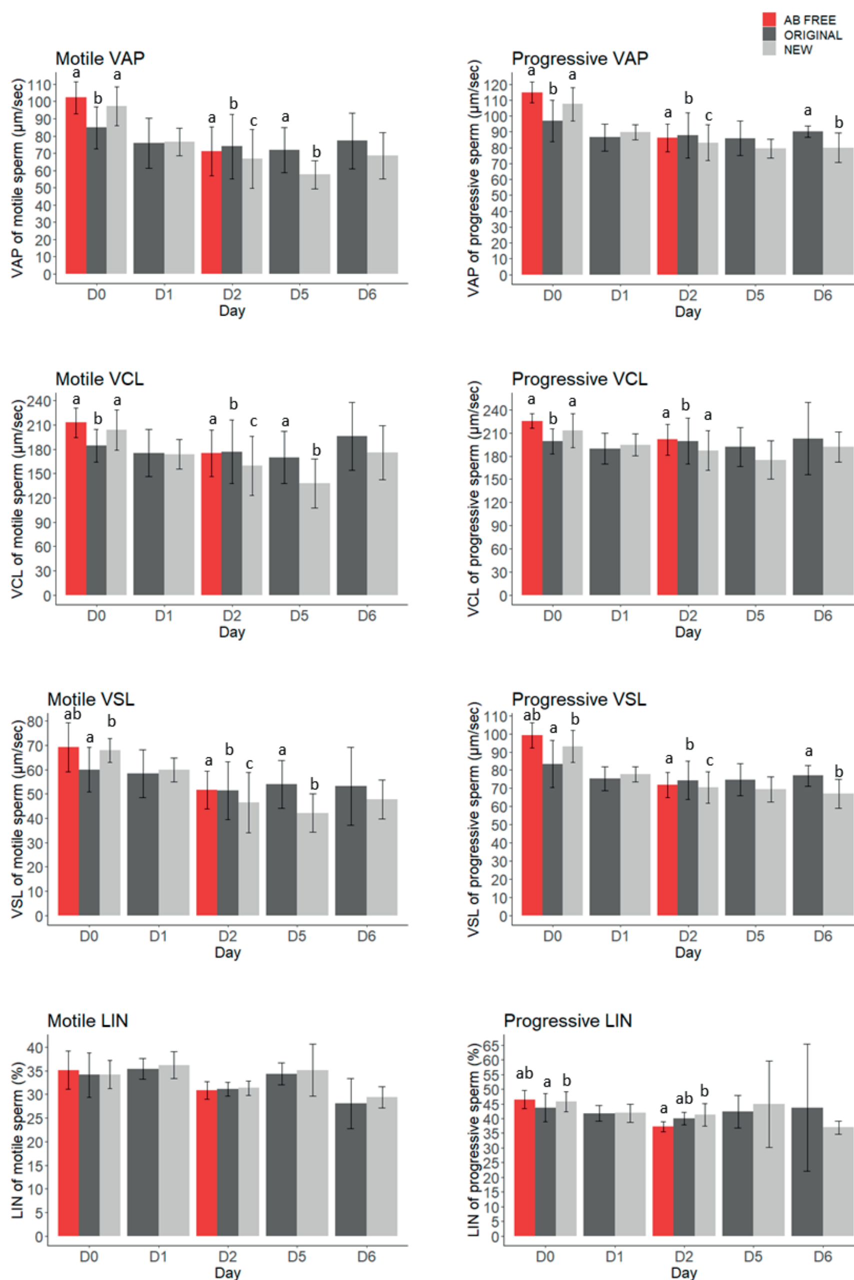


Figure 4: Sperm velocity analysis in Galap® extender. Graphs showing means±standard error of different velocity parameters of motile and progressive spermatozoa (VAP: Velocity average pathway, VCL: Curvilinear velocity, VSL: Straight-line velocity, LIN: Linearity (ratio VSL/VCL)). Semen was evaluated after semen dilution in different Galap® media: antibiotic-free (AB *free*), original (*Original*) and new with gentamicin (*New*), at different day after collection (D0, D1, D2, D5 and D6). n=8 to 26 semen pools were analysed. Different lower case letters (a, b and c) indicates significant difference (P<0.05).

Effect of the new Galap® antibiotic composition on fertility and prolificacy

Several parameters reflecting the reproductive performance of female rabbits inseminated with semen diluted in the different antibiotic compositions of the extender were analysed. The number of females pregnant after AI was recovered, providing a fertility rate assessment. Taking into account the total number of pregnant females at parity divided by the number of inseminated females, without considering semen pools, the fertility rate after AI with semen diluted in the original formula and the new Galap® formula was similar, with 75.6 and 75.3% pregnant females, respectively (*P*=0.867) Considering the 12 semen pools that each allowed the insemination of 15 females (presented in the Figure 5.A.), the analysis of fertility showed no significant differences between media either (*Original*: 84.9±11.6%; *New*: 85.9±9.5%; *P*=0.7868) (Figure 5.A).

Prolificacy was then assessed by the ratio of the number of kits delivered to the number of kindling females. All females combined, without considering semen pools, a total of 11.3 total kits per litter were obtained with the original extender and 11.7 total kits per litter with the new formula, i.e. 0.4 more kits per litter, without significant differences (considering semen pools, average kits per litter: *Original*: 11.3±1.5; *New*: 11.6±1.13; *P*=0.315) (Figure 5.B). Equally, the corrected prolificacy (number of live kits delivered per pregnant female) was not significantly different between the two groups (considering semen pools, average number of alive kits per litter: *Original*: 10.5±1.4; *New*: 10.8±1.3*;* *P*=0.198).

The last indicator evaluated was the female fecundity, which is related to fertility and prolificacy. It was assessed by the ratio of the number of kits delivered to the number of inseminated females. As with previous reproductive parameters, it was also not significantly affected by change in the antibiotic composition of the extender (*P*=0.479). The *New* extender yielded 8.8 kits per AI on average, which means 0.3 extra kits per AI than the original extender. Considering all semen pools, the average fecundity reached 8.5±2.4 kits per AI in the *Original*, and 8.8±2.0 kits per AI in the *New* (Figure 5.C). The corrected fecundity, taking into account the number of liveborn kits, showed similar results, with 8.0 live kits per AI in the *Original* group on average, and 8.3 live kits per AI in the *New* (considering semen pools, average number of live kits per AI: *Original:* 7.95±2.22; *New*: 8.26±2.14; *P*=0.303; data not shown).

DISCUSSION

Contamination of the semen by bacteria of animal origin (urine, faeces, hair), environmental (air, feed, litter) or linked to poor harvesting conditions (Althouse and Lu, 2005; Althouse, 2008; Althouse *et al.*, 2008), requires the use of dilution media containing antibiotics that limit bacterial proliferation. However, the regular use of the same antibiotic or antibiotic cocktail in this type of medium could favour the development of antibiotic resistance. Of greater importance

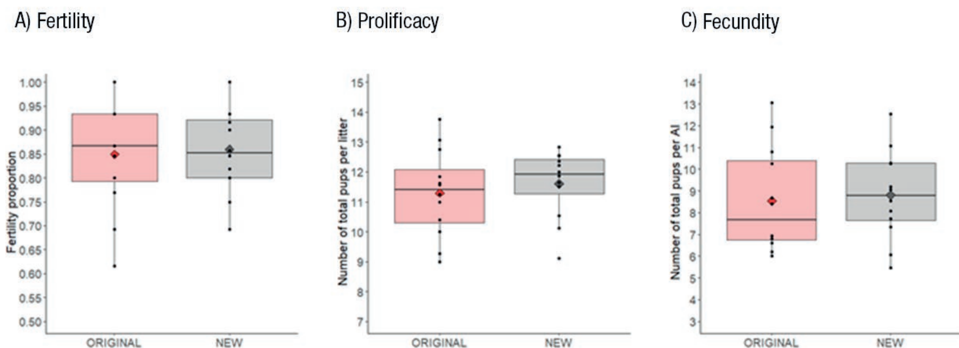


Figure 5: Female reproductive parameters after AI with semen diluted in Original Galap® extender and New extender. (A) Female fertility; (B) female prolificacy; (C) and female fecundity. Females were inseminated with semen diluted in the original or new Galap® media. Twelve semen pools were used to inseminate females.

than making the medium ineffective, is that antibiotic resistance remains today one of the most serious fears for global human health. The use of certain critical antibiotics (latest generation), such as the enrofloxacin present in the original Galap® formula, is advised to be limited to human use for public health protection. We have therefore sought to replace the current antibiotic cocktail present in the original formula with other antibiotics.

Several bacterial strains were identified in this study in rabbit semen, most commonly *Enterococcus* spp. (Group D *Streptoccoccus*) and *Staphylococcus aureus*, but also *Enterobacter* and *Pseudomonas* spp., which is in accordance with previous studies (Mercier and Rideaud, 1990; Duracka *et al*., 2019). This study showed that two antibiotics could replace the original antibiotic mixture with a wider bacterial spectrum than enrofloxacin and penicillin combined. Although amikacin 30 µg showed interesting efficiency against the different bacterial strains identified with antibiograms in this study, gentamicin 30 µg had the broadest bacterial spectrum, by targeting nearly 83% of the bacterial strains identified. Gentamicin inhibited the growth of 90.9% of the *Enterococcus* spp. and 100% of the *Staphylococci aureus* and *Protei* spp. identified in this study. As this antibiotic has the advantage of good stability in aqueous solution for more than 12 months (Berendsen *et al*., 2011), we sought to validate an extender with the addition of 0.3 g/L of gentamicin alone (*New* Galap®). Gentamicin addition in the extender has shown to prevent the bacterial development on its own, at the same level as the original extender formula. However, our results showed a large variability in the sensitivity of isolated colonies to some antibiotics, especially to enrofloxacin, with a susceptibility that doubled between the first (n=18 colonies) and the second test (n=29 colonies). This variability is due to the fact that few colonies were used in the first preliminary test. No variability in colony susceptibility to amikacin and gentamicin was observed between the two tests. However, it shows the interest in performing other antibiograms on a larger number of bacterial strains before validating gentamicin as the candidate antibiotic for substitution of enrofloxacin in the *Original* extender formula.

To determine the impact of the change in antibiotic composition of the extender for semen preservation, the quality of semen diluted in different types of extender (*antibiotic-free*, *Original* or *New* antibiotic formula) was evaluated at different post-collection times. On the day of collection, the total and progressive motility of the pooled ejaculates was identical between the different formulas. However, sperm velocities were significantly higher in the *antibiotic-free* and in the *New* formula with gentamicin than the *Original*. After 24 h of storage, we showed that sperm motility was lower in the *New* than in the *Original*, without affecting the proportion of progressive spermatozoa and motility. The progressive sperm population was negatively impacted after 2 d of storage in the *New* extender. These decreases in semen motility were also observed up to 5 d of storage, with the same difference.

These results corroborate the study carried out on stallion semen by Aurich and Spergser (2007). Indeed, the extender with gentamicin, although preventing bacterial development, negatively affects sperm motility but is not worsened by storage time. Gloria *et al*. (2014) suggested that the toxicity of gentamicin is related to the cooling phase of the semen, as no reduction in sperm parameters (velocities and progressive motility) was found after dilution and incubation of the semen at room temperature (Gloria *et al*., 2014). However, no further explanation has been given in the literature and this hypothesis remains to be proven. Motility have been shown to be a determining factor in female fertility and prolificacy (Brun *et al*., 2002; Lavara *et al*., 2005; Theau-Clément *et al*., 2016). AI results showed no negative impact of this loss of motility on the reproductive performance of females. The *New* antibiotic composition medium does not impact the female fecundity, and would even allow giving 0.3 extra kits per AI. However, a larger study would be necessary to show the significance of this result. Since the concentration and total number of motile sperm cells are critical to the success of AI, Theau-Clément *et al*. (2016) demonstrated that fertility, prolificacy and fecundity do not increase significantly above 20 million total sperm or 17 million motile sperm per dose. Based on the 66% motile sperm cells observed in the extender with gentamicin, at 24 h post-collection, packaging doses with around 25 million sperm cells allowed the minimum 17 million motile sperm cells required to be obtained and to reach the observed optimised fertility rate.

CONCLUSION

In this study, we have shown the effectiveness of gentamicin on rabbit semen extender, in relation to the antimicrobial spectrum and aqueous solubility. However, dilution of semen in Galap® extender with gentamicin causes a slight drop in sperm motility within 24 h post-collection. In addition to performing ejaculate pools and heterospermic dosing

to limit the individual effect of males, diluting semen to a final concentration of 50 million spermatozoa/mL can provide an appropriate balance between motility and potential *in vitro* toxicity of the antibiotic to maintain the best female fertility performance. Indeed, under these conditions, the new extender with gentamicin showed the same effectiveness as the original extender. The substitution of the antibiotic cocktail of the original formula with gentamicin showed, in this preliminary study, no negative effect on female reproductive performances, and therefore does not lead any economic damage for rabbit breeders. However, other studies comparing seasonal and farm effect could be necessary to gain more evidence of the use of gentamicin.

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