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Article

Total aseptization of boar semen, to increase the biosecurity of reproduction in swine

Ştefan Gregore CIORNEI[1], Dan DRUGOCIU[1], Liliana CIORNEI[1], Mihai MAREŞ[2], Petru ROŞCA*[1]

[1] Iasi University of Life Sciences (IULS), Faculty of Veterinary Medicine, Department Clinics, Reproduction, M. Sadoveanu Alee, no 6, 700489-Iași, Romania
[2] Iasi University of Life Sciences (IULS), Faculty of Veterinary Medicine, Department of Public Health, Microbiology, M. Sadoveanu Alee, no 6, 700489-Iași, Romania
* Correspondence: petru1065@yahoo.com

Abstract: The aim of the study is to establish the complete microbiological profile of boar semen and to choose the most effective antiseptic measures in order to control and optimize AI reproduction in pigs. More than one hundred semen samples were collected and analyzed from several pig farms. The microbiological profile of ejaculates was determined by determining the degree of contamination of fresh semen and after dilution with specific extenders. The bacterial and fungal load of fresh boar semen recorded an average value of 82.41/0.149x10^3CFU/mL, while after diluting the ejaculates the contamination value was 0.354/0.140 x10^3CFU/mL. 23 bacterial and fungal species were isolated, the most common being *Candida parapsilosis/sake* (92%), *Escherichia coli* (81.2%). Modification of the sperm collection protocol (HPBC) reduced contamination in raw sperm (by 49.85% in bacteria and by 9.67% in fungi). The load in bacteria and filamentous fungi can be controllable, but not in levuras fungi. In some Fluconazole-added extenders (12.5mg%), ensures the solution of this problem, and even increase in sperm progressivity (8.39%) for at least a 12-hour shelf life after dilution. The validation of the experiment was done by obtaining the sow fertility rate after AI.

Keywords: Bacteria, Fungus, Boar semen, Progresivity, Fluconazole, Biosecurity of AI

1. Introduction

Studying the factors that may influence the biological value of semen subjected to preservation and artificial insemination (AI) is of great importance within the assisted reproduction biotechnologies in pigs. Of these factors, an important role is attributed to the presence of microbial flora [5,9,12,28]. Changes of semen in terms of metabolism may be caused by various bacteria types and their number per volume unit [5,13]. The presence of bacteria in extended semen is harmful for sperm viability and represents an infection risk for the inseminated sows [4,33]. Microorganisms have direct and indirect deleterious effects on sperm, altering their motility and fecundant capacity. Fernandez et al. (2001), stated that there is a close correlation between sperm agglutination and level of semen contamination, and also that sperm agglutination might affect the fertility of inseminated sows. A diverse bacterial flora could decrease semen quality during preservation [1, 16].

The most frequent bacteria types detected in boar semen are *E. Coli, Pseudomonas spp., Staphylococcus spp.* and *Proteus spp.* [1,2,13]. Some authors identified 56 strains: 40 strains were detected in freshly collected and 16 in the extended semen. *Staphylococcus spp*. have been identified in 75% of the raw semen samples, and 20% of the extended semen samples. *Streptococcus spp*. were identified in 60% and 10%, *E. coli* in 60% and 0%, *Pseudomonas spp.* in 30% and 25% and *Micrococcus spp.* in 50% and 0% [5]. The presence of bacteria in stored semen demonstrated that current antibiotics are not fully efficient against them.

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Several sources of contamination have been described, both of animal and non-animal origin. The first source of contamination is the boar (preputial fluid, skin, hair, feces on the legs). The second source of contamination is the equipment used for semen collection and examination before processing [1,2,10]. Semen collection in farm animals is not a sterile procedure, some bacterial flora contaminating the semen [1,2,4,15,36,44]. Dias et al. (2000), reported different germ loads depending on the hygiene and the method that was used for semen collection. Thus, in case of a strictly hygienic collection, sperm contamination was between 490 and 975 CFU/mL, while in case of a normalcollection, between 14.6×10^3 and 18.8×10^3 CFU/mL [36].

Most commercial extenders for boar semen contain the same groups of antibiotics, more exactly aminoglycosides such as gentamicin and lincosamides [5,17,44], to which some bacteria present in the semen become resistant [2]. Therefore some bacteria survive the preservation process and are present in the insemination doses before AI.

To overcome the problem of semen contamination, some authors [1,12], recommend maintaining good hygiene and permanent application of a periodic disinfection protocol. In addition, it is necessary to use extenders that contain combinations of antibiotics that are effective against bacterial flora present in the semen and to conduct regular microbiological screening of boar semen in the swine industry to avoid the use of poor quality sperm [36]. Various products have been delivered in this regard, with acceptable efficacy. However, extenders with antifungal capacity have not been developed yet.

The literature in poor in information about fungal contamination of boar semen and its persistence during storage [11,31]. The usual conditions of storage before AI (liquid state at +17°C for several days) represent excellent conditions for the development of fungi, and the extenders do not contain antifungal substances. The attention was concentrated on bacteria, so little is known about the fungal contamination of boar semen and its effects on seminal parameters.

The identification of yeast and filamentous fungi in preserved semen confirms the idea that the antibiotics do not influence their presence and development [10]. Both in raw and extended semen, a number of fungi was identified. However, not enough studies regarding the degree of fungal contamination of boar semen have been performed, which justifies once again the appropriateness of this research.

2. Results

Microbiological spermogram

The bacterial and fungal load of fresh boar semen recorded an average value of 82.41 / 0.149 $x10^3$ CFU/mL, while after diluting the ejaculates the contamination value was 0.354 / 0.140 $x10^3$ CFU / mL. The average microbiological profile in raw and diluted semen samples is presented in Table 1.

Table 1

Microbiological profile in fresh and diluted semen in a two swine farm in Romania

The origin of semen samples	Bacterial burden (10^3 CFU/mL)		Fungal burden (10^3 CFU/mL)	
	Raw semen	Diluted semen	Raw semen	Diluted semen
Farm 1	65.49	0.120	0.103	0.089
Farm 2	99.33	0.588	0.194	0.192
average	**82,41**	**0,354**	**0,149**	**0,140**

For raw semen, the number of bacteria and fungi varied between $22.4x10^3$ and $118.20x10^3$ CFU/mL, with a mean of $65.49x10^3$ CFU/mL in farm 1, while in farm 2, the av-

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erage number registered a higher value 99.33x10³ CFU/mL, and the variation limits were between 74.60x10³ and 130.05x10³ CFU/mL. From the all (101) semen samples, only 8 (7.9%) were undetectable contaminated (total viable aerobic count less < 10¹ CFU/mL). After dilution and 12 hours storage at 17°C, the average bacterial burden became 0.12x10³ CFU/mL (limits ranging between 0.08 x10³ and 0.31x10³ CFU/mL) in farm 1, and 0.58x10³ CFU/mL (limits ranging between 0.14 x10³ and 1.20 x10³ CFU/mL) in farm 2, respectively. As it is shown in Table 1, the number of fungi per mL remained quite stable after the semen dilution, indicating a particular condition that must be evaluated. In farm 2, the degree of contamination was higher, apparently due to poor hygiene of sperm collection (boar-operator-hall). Critical points of contamination were identified and described below in another experiment.

Concerning the qualitative aspects of semen contamination, the microbiological tests have highlighted a variety of bacterial and fungal species. Thus, in freshly collected semen, a total number of 14 bacterial and 9 fungal genera have been identified (table 2). The species isolated with higher frequency were *Escherichia coli* (81.2% of samples) followed by *Staphylococcus* species (*aureus zooepidemicus, intermedius, hyichus*) (72.3%), *Pseudomonas aeruginosa* (63.4%), *Enterococcus* (*faecium, faecalis*) and *Streptococcus* (*suis*, other species) (45.5%), *Proteus vulgaris* (35.6%). Other bacterial species like *Tatumella ptyseos, Pantoea spp., Shigella spp., Yersinia (enterocolitica, rukeri, pseudotuberculosis)* and *Serratia (ficaria, marcescens)* were identified with a lower frequency (26.7%). *Bacillus* (*subtilis, cereus, megaterium*), *Arcanobacterium pyogenes* and *Actinomyces suis* were identified in 10.9% of the samples, and *Klebsiella pneumoniae* in 6.9%.

Table 2

Frequency of bacterial and fungal species isolated from boar semen

	Gen	Species	Freqvency of isolations %
Bacteria	Escherichia	coli	81,8
	Staphylococcus	aureus, zooepidemicus, intermedius, hiyicus	72,7
	Pseudomonas	aeruginosa	63,6
	Streptococcus	suis	45,4
	Proteus	vulgaris	36,4
	Bacillus	subtilis, cereus, megaterium	27,3
	Tatumella	ptyseos	27,3
	Pantoea	spp.	27,3
	Arcanobacterium	pyogenes	27,3
	Shiqella	spp.	27,3
	Actinomyces	suis	18,2
	Yersinia	enterocolitica, ruckeri, pseudotuberculosis	18,2
	Serratia	ficaria, marcescens	18,2
	Klebsialla	pneumoniae	9,1
Fungus	*Cladosporium*	*cladosporoides*	36,6
	Penicillium	*spp.*	63,6
	Fusarium	*spp.*	36,6

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	Aspergillus	*spp.*	63,6
	Mucor	*racemosus*	45,5
	Alternaria	*alternata*	18,2
	Geotrichum	*candidum*	72,7
	Acremoniu	*spp.*	18,2
	Candida	*parapsilosis, sake*	92,0

Among fungal species, the yeasts belonging to *Candida* genus were the most prevalent in semen samples (*Candida parapsilosis* was isolated in 92% of samples). Other fungi were encountered in lower proportions: *Geotrichum candidum* (72.3%), *Aspergillus spp*. and *Penicillium spp*. (63.3%), *Mucor racemosus* (45.5%), *Cladosporium cladosporioides* and *Fusarium spp.* (36.6%), *Acremonium spp.* and *Alternaria alternata* (17.8%). Important to note is that after dilution and 12 hours storage at 17°C, yeasts like *Candida parapsilosis* were identified in more 90% of AI doses.

Identify sources of sperm contamination

Before the introduction of a boar in the collecting room, the CFU (bacteria and fungi)/m^3 of atmospheric air had lower values ($30.9x10^3$ m^3/air) compared to those obtained after collecting ($44.2x10^3$ m^3/air), (table 3) .

Table 3

Microbiological load in the air collection room before and after the procedure for collecting sperm from boars

m3 / air	**Before collection**		**After collection**	
		Total		**Total**
Bacteria	$19.7 x10^3$	**$30.9x10^3$**	$26.8 x10^3$	**$44.2 x10^3$**
Fungus	$11.2 x10^3$		$17.3 x10^{-2}$	

The flow of use of boars in the collection room in this farm is 5-7/ day. Contamination of semen is dependent on the load of the external environment and the state of hygiene of the genital tract. Thus, depending on the number of colonies present, we consider that the pollution can be low (+), medium (++) or massive (+++) in the samples worked (table 4). The most common sources of contamination of a semen are the foreskin, the atmospheric air in the collecting room and the laboratory equipment used [39].

Table 4

Bacterial and fungal contamination points and degree of contamination by CFU number

	Bacteria	**Fungus**
The air in the collection room	**++**	**+++**
Working equipment	**+/-**	**+/-**
Prepuce	**+++**	**+++**

+/- low contamination and inconsistency

++ massive contamination

+++ moderate to high contamination

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Due to spraying in the atmosphere of the room, dust and microorganisms settle quickly and those present on the skin and limbs of the boar will adhere to it and will not rise into the atmosphere. The microbiological load of the atmospheric air in the collecting room will be reduced in this way.

After the application of HPBC-protocol it was observed that after obtaining the seminal material and its dilution both the number of bacteria and that of the fungi are much lower (table 5). Thus the bacteriological load of the raw sperm decreased by 49.85% (from 62.39x10^3 to 31.29x10^3 CFU/mL), and in the one after dilution it decreased by 87.2% (from 0.125x10^3 to 0.016x10^3 CFU/mL) . But the fungal load followed a smaller reduction of 9.67% (from 0.115x10^3 to 0.140x10^3 CFU/mL) in raw semen and 9.15% (from 0.153x10^3 to 0.139x10^3 CFU mL) after dilution and these represented by yeast fungi.

Table 5

Microbiological spermogram before and after the use of hygiene protocol and the biosecurity of sperm collection (HPBC)

	Bacterial burden (10^3 CFU/mL)		Fungal burden (10^3 CFU/mL)	
	Raw semen	Diluted semen	Raw semen	Diluted semen
Before (HPBC)	62.39	0.125	0.155	0.153
After (HPBC)	31.29	0.016	0.140	0.139
CFU evulution (%)	**-49,85%**	**-87,2%**	**-9,67%**	**-9,15%**

Antifungal aseptization of sperm by potentiated extenders

The effect of different concentrations of Fluconazole on sperm extenders (A and B) are given by calculating the average of the most important types of sperm motility in Table 6. The mean values of the control samples (M) for extender A were 80.3% in motility (M%) (with limits between 72% and 82%), and the progressivity (P%) was 39.3%, (with limits between 36% and 42%). In the case of extender B, the mean was 75.6% (with limits between 47% and 77%), for total motility, and 30.6% (with limits of 30% and 32%) for progressivity. Concentration of 25mg% Fluconazole (E1) on extender A produced an M% of 72.0% and P% of 39.0%, and at extender B: M of 64.3% and P of 20.3%. While a concentration of 12.5% Fluconazole (E2) on extender A produced an M% of 80.6% and P% of 42.6%, and at extender B: M of 73.0% and P of 29 ,3%.

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Table 6

Assessment of compatibility between semen extenders and different concentrations in fluconazole

	Extender A		Extender B	
	M%	P%	M%	P%
M	80.3	39.3	75.6	30.6
E1	72.0	39.0	64.3	20.3
E2	80.6	42.6	73.0	29.3
(%) M vs E1	*-11,5%*	*-0,7%*	*- 29,1%*	*-50,2%*
(%) M vs E2	*+0,37%*	*+8,39%*	*-3,5%*	*-4,4%*

M% - total sperm motility, P%- total sperm progresivity, E1 – Fluconazole 25mg%, E2- Fluconazole 12,5mg% M- martor

Testing semen extenders added with Fluconazole by determining sperm progressivity in storage dynamics

To observe the effect of the antifungal on the quality of sperm (mobility indices) during the preservation of insemination doses, we followed the total motility and progression in dynamics for 48 hours (table 7).

Table 7

Testing semen extenders added with Fluconazole by determining sperm progressivity in storage dynamics

		T 1		T 2		T 3	
		M%	P%	M%	P%	M%	P%
V1	**M**	82	42	70	27	47	14
	E1	82	47	73	33	46	17
	E2	84	46	65	27	42	12
V2	**M**	78	36	69	36	70	36
	E1	63	31	61	21	51	22
	E2	78	39	65	33	46	20
X	**M**	80	39	69,5	31,5	58,2	25
	E1	72,5	39	67	27	48,5	19,5
	E2	81	42,5	65	30	44	16

M- control lot, E1- experimental lot 1 (fluconazole 25 mg%), E2 - experimental lot 2 (fluconazole 12.5%). M% - total motility. P% - progressivity (fertilizing capacity). T 1-3 - semen preservation time at + 17˚C, at 12, 24 and 48 h, V1- V2 –boars ejaculates, x- average.

At 12 hours after dilution, the motility (M%) of the samples from the control group (M) had values between 82% and 78% with an average of 80%. Of the sperm with total motility, an average of 39% of them had forward movements (Progressivity), the limits being between 42% and 36%. Preservation leads to a steady but sustained decrease in M% and P%.

Within the group (E1-25mg% Fluconazole) an average value of 72.5% was obtained for M%, lower than M (80%), instead, the average percentage of P% registered equal values (39%). The variation limits were between 47% and 31%.

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The mobility parameters studied in dynamics registered progressive decreases as the conservation duration increased in both control and experimental groups. Thus, the determinations performed at 24 hours after dilution (T2), showed average values of motility (M%) in the experimental groups (E1 and E2), significantly lower (67% and 65%, respectively) than those recorded in the control (69.5%). P% was (27% and 30%) vs 31.5%.

The results of the determination performed at 48 hours (T3) show lower average values of progressivity (P%) at E1 and E2 (19.5% and 16%), compared to the mean value at control (25%). The decrease of the average values of M% and P%, registered at both L.E., both at 24 and 48 hours after dilution, correlates with the decrease of the values of these parameters also at L.M., decrease which is achieved in a smaller proportion.

Validation of additive extenders by AI on the farm

For a better appreciation of our research, the effect of the new extender formulas on the reproduction indices was also followed. The aim was to observe the effects of the antifungal in the extender (12.5% Fluconazole) on the fecundity and prolificacy of the sows, the results are presented in table 8. The prolificacy of the lots is slightly different, finding a higher value in the control group. The average number of piglets obtained at calving was 9.125 in L.M., 0.43 less than the average per calving in L.E. (9,062). For the control group the total number of piglets obtained from all sows was 146, with one piglet less than those obtained in the experimental group (145).

Table 8

Fertility and prolificity of AI sows with Fluconazole additive extenders

	No.gilts A.I. (n)	Techinch of I.A.	Sperm/dose $X10^9$	Fecundity		Prolificity	
				Nr.	%	Total Piglets(n)	Average/ farroing
L.M.	20	E.C.	3,5	16	80	146	9,125
L.E.	20	E.C.	3,5	16	80	145	9,062

3. Discussion

All the examined ejaculates were considered to be normal for each parameter of the spermogram. No appearance or pH inconformity was detected. The average volume of the ejaculates after filtration was 344.35 mL. Semen concentration varied between $0.18x10^9$ and $0.51x10^9$ spermatozoa/mL, with a mean of $0.36x10^9$ spermatozoa/mL, motility ranged between 65 and 86%, so the quality of semen was quite high. Other organoleptic parameters used in boar semen assessment were also normal taking in consideration the evaluation criteria described by Knox R.V. (2004) [25].

The average bacterial burden determined for all farms in our study ($82.4x10^3$ CFU/mL in raw and $0.354x10^3$ CFU/mL in diluted semen) is similar to that reported by others [13,41], and depends on the hygiene of semen collection and handling process and also on antibiotic resistance of the microbiota from a particular farm. Values are lower than those obtain by manual collection with double glove ($384x10^3$ CFU/mL) or by automatic collection ($349x10^3$ CFU/mL) [27]. Similar results have been published for other species. For example, Wiersbonski S. (1981) found that the bacterial load of bull semen used for A.I., varies between $1.5x10^6$ and $6.5x10^6$ CFU/mL [45]. Rota et al. (2011) emphasized the presence of bacteria and fungi in stallion semen and other reproductive segments (presence and distribution) [35].

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High bacterial count in AI doses has been associated with a decline of sperm quality [43], reduction of fertility [28] and transmission of pathogens into the females [26]. Therefore, current legal guidelines require the addition of antibiotics to each AI dose (Council Directive, European Union, 90/429/EEC). However, the added antibiotics are often no longer sufficient to stop bacterial growth in AI doses as antimicrobial resistance becomes more widespread [39,40]. Lately, many efforts in the search for promising possibilities to replace antibiotics in liquid-preserved boar semen have been made to reduce the development of multi-resistant bacteria by minimizing the selection pressure of antibiotics. Approaches include, but are not limited to: colloid centrifugation [32], supplementation of antimicrobial peptides [8,20,34,40] addition of algae extracts [21] and low temperature preservation [20,29,45] or development of a new antimicrobial concept in aditiona the extenders [24].

In both diluted and raw semen there were identified different kinds of fungi. The literature concerning the fungal contamination of boar semen is relatively poor and it could be a justification for the opportunity of our study. Concerning the origin of fungal contaminants, we can identify two sources: the indoor air and the boar's foreskin. Generally, mold species like *Mucor*, *Aspergillus*, *Penicillium*, *Cladosporium*, *Acremonium*, *Fusarium*, *Alternaria* etc. are airborne fungi, while yeasts like *Candida parapsilosis* are skin commensals. The airborne fungi may occur in semen in different concentrations dependent on their indoor level and we can reduce them by a rigorous hygiene of the collection and processing rooms. The yeasts' concentration in semen samples is quite constant indicating an internal source of contamination.

Semen contamination was detectable in more than 90% of samples, only about 8% of samples exhibiting values less than 10 CFU/mL. Some authors reported similar or higher percentages in their papers [3,16,28]. Dias C.P. et al. (2000) report different loads of germs depending on hygiene and collection method. Using a collection process with strict hygiene, semen contamination was between 490 and 975 CFU/mL, while using a current collection procedure it ranges between $14.6x10^3$ and $18.8x10^3$ CFU/mL [15].

Most commercial extenders designed for boar semen dilution contain antibiotics belonging to the same group (*i.e.* aminoglycosides like gentamicin and lincomycin) [5,17,44] some bacteria present in semen could be resistant to them [2,3]. In our study, the decrease of bacterial burden was extremely efficient after dilution (more than $2\log_{10}$), indicating a strong antibacterial effect of the extenders. On the contrary, even the ratio used for fresh semen and extender was approximately 1:7, the fungal burden did not decrease after dilution and 12 hours storage. Moreover, the yeasts like *Candida parapsilosis* have multiplied during the storage period due to a favorable chemical composition of the semen extenders (all of them contain glucose and other sugars). This fact is strongly indicative for the lack of antifungal activity of the extenders that contain only antibiotics and no fungistatic or fungicide substances.

Some genera of bacteria and fungi have been identified in the air in the room, but also in the foreskin and in the freshly harvested semen. We assume that the genera identified in the seminal material, being present in the other samples analyzed, come from the external environment. Contamination occurs with the contact of the sperm wave with the air and the collection equipment.

The increase in the number of microorganisms is due to the movements of boars which, through the state of arousal and agitation, given by the sexual reflexes, they entrain the sedimented microorganisms and those present on the skin, hair and limbs. Microorganisms and dust accumulate and float in the atmosphere of the room. Due to the peculiarities of ejaculation in the boar and of the collection equipment (filter container), the sperm wave enriched with such particles when passing through the air, also entails those already sedimented on the filter, thus producing its contamination.

Establishing the most important places where sperm can be microbially contaminated creates real possibilities for limiting the contamination of semen with germs. By

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applying the HPBC boar semen collection protocol, it confirms its success by reducing sperm contamination from both external and internal sources.

The yeasts, being determined constantly and relatively in the same number, we consider that they have an internal origin of sperm contamination, instead, the filamentous fungi that come from external sources of contamination, are missing. Which makes us say that by following the collection protocol, completed by us and supplemented by the observance of hygiene conditions, it is possible to reduce the sperm CFU at critical points of contamination.

After identifying and removing the critical points of sperm contamination during the collection process, we can appreciate that the microorganisms left in the sperm have endogenous origin being represented in their vast majority by yeasts. For more effective aseptization, an antifungal preparation (Fluconazole) was introduced into the formula of the extenders used.

The Computerized Sperm Analysis System provides information about the mobility of each sperm cell, by processing electronic images of sperm, reconstructs the trajectory: each sperm cell, simultaneously and objectively evaluates each component of the sperm so that even minor changes in their mobility can be detected. Depending on the type of chambers used (Leja, Mofa) there may be differences in reporting [6,14,22,23].The CASA method has proven to be more accurate and correct, and therefore more objective [7]. After Ibănescu et al. the CASA system describes that the type of analysis chamber used at CASA may have a different effect on the values obtained.

The differences between control group M and experimental groups E1 and E2 are calculated and presented as percentages in table 5.

In the group with 25 mg% Fluconazole (E1) for extender A the average value of motility (M%) (72.0%) was -11.5% lower than the average of the control group (80.3%), and for progressivity P% by -0.7% (39% vs 39.3%), for extender B the average value of M% was -29.1% lower (64.3% vs 75.6%), and for P% with -50.7% (20.3% vs 30.6%).

In the group with 12.5 mg% Fluconazole (E2) for extender A the average value of M% compared to the control group was 0.37% higher (80.3% vs 80.6%), and for P% 8, 39% higher (39.3% vs 42.6%), for extender B the average value of M% was 3.5% lower (75.6% vs 73%), and for P% lower by 4.4% (30.6% vs. 29.3%).

It appears that the concentration of 25 mg Fluconazole in both extenders (A and B) has a toxic effect on sperm leading to decreased indices of mobility (M% and P%). In contrast, the concentration of 12.5mg% Fluconazole in extenders has a totally different effect, in EB there was a small decrease in both P% and M%, in EA there was a metabolic activation of sperm P% increased more than M%.

Fluconazole at a concentration of 12.5 mg% in extender A exerts a stimulating role on sperm at least for a storage period of 12 hours, after dilution, at a temperature of + 17°C.

Following the artificial insemination of the sows in the respective groups, a number of 4 sows, both from L.M. as well as from L.E., manifested the return to heat syndrome (Table 8). Thus, the fecundity of the experimental group was 80%, a value identical to that of the control group (80%), so we can say that there are no visible changes in the processes of fertilization and nesting.

Thus, we can say that changing the formula of extenders by adding fluconazole at a concentration of 12.5 mg / l, brings a real benefit especially in the case of sperm heavily contaminated with yeast from the foreskin during harvest, by offering better possibilities for aseptic of semen intended for preservation, and the action of the antifungal does not adversely affect the quality of sperm or reproductive evidence.

The technique of collecting boar semen is well known; a decisive role in the contamination of the sperm is represented by the hygiene of the process and the observance of the respective stages. However, the microbiological load in the sperm persists even after dilution of the semen. Regarding the qualitative microbiological spermogram of the

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diluted seminal material and destined for preservation and insemination, an appreciable proportion has yeasts compared to bacteria, an aspect that also corresponds to the quality of the extender, respectively the possibility of total aseptic extender on microorganisms.

For a better aseptization of the seminal material, we propose and recommend the introduction in the extender formula in addition to antibiotics and an antifungal preparation, without significantly altering the longevity and fertilizing capacity of the sperm.

The lack in the domestic and international literature of data on contamination and the possibility of fungal aseptization of boar semen during conservation, led to the motivation to try to modify or supplement the formula of extenders used to preserve boar semen.

4. Materials and Methods

All procedures involving animals were carried out in accordance with guidelines and regulations according to the European Commission Directive for Pig Welfare, and IULS and Farmers.

Pig farms

The studies were carried out in three pig farms in Romania distributed geographically as follows: Farm 1 in the N-E, Farm 2 in the S-E, Farm 3 in the S-V. All research protocols were staged and carried out in succession over several years, but before the outbreak and evolution of African Swine Fever in Europe. Farms are commercial complexes with a large flow of animals and any influence on breeding rates can positively/negatively influence the farm's economy. Breeding management in pig farms plays an important role in producing the number of piglets for fattening and slaughter.

4.1. Biological material

In all farms, the genetic material used for breeding was represented by boars and sows of high genetic value, which cross-produce and deliver line piglets from fatteners. All conditions of specific biosecurity, helminthization, nutrition and well-being were met. The maintenance and exploitation of the animals was optimal, in accordance with European standards. At the time of each experiment, the animals were in clinical health.

4.2. Reproduction organization

Within the farms there is a reproductive laboratory where andrological and gynecological activity is planned. After a well-established daily schedule, sows are identified in the heat, the required number of doses for AI / AI repetition is calculated, a sufficient number of boars are distributed for collecting, ejaculates are collected, doses for AI and storage are produced. The technological process of collection - examination - dilution - preservation - insemination of semen were generally similar.

4.2.1. Boar semen collection. The main method of sperm collection was used - the manual method with collector cup and filter. The protocol took place in specially designed spaces called: the collection room, equipped with a mannequin. The success of the harvest and the quality of the ejaculates varied between farms, depending on the ability and training of the operators. Before collecting the sperm, the operators have the obligation to groom the foreskin and to empty the preputial adivertic of the boars.

4.2.2. Examination of ejaculate quality. All samples were examined according to the standard methodology [25] grouped into two categories: General examinations (organoleptic, macroscopic) and Special examinations (microscopic). Appearance was evaluated in a transparent glass, examining the degree of turbidity, possible presence of the blood or other unusual colors. The smell was evaluated directly over the recipient of semen, searching for the usual smell, the presence of urine or other unexpected odors. Concentration was assessed using the AccuRead Sperm Counter (IMV-Technologies, France) [10,19]. Randomly, and when needed, the quality of the ejaculates was also evaluated by the CASA system (Computer Assisted Sperm Analysis) [23].

4.2.3. Dilution of semen. Compliant ejaculates were diluted with commercial extenders that ensure long-term sperm viability 5-7 days (Extender A) and medium extender, 3-5 days (Extender B). Extenders differ depending on the composition and shelf

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life at + 17°C. The semen extenders were chosen as the best / used on the market at that time [19,25].

4.2.4. Preparation of AI doses and their preservation. After the classical examinations and the determination of the volume and concentration, the dilution ratio was calculated / indicated automatically. For each insemination dose, 3.5 billion ($3.5x10^9$) sperm were assigned in a volume of 80 ml. Those that will be used to repeat AI were stored in incubators at a temperature of + 17°C, up to 5 to 7 days, depending on the type of sperm extender, [36].

2.5. Artificial insemination.

In this complex study, AI of sows were also performed, in order to follow the reproduction parameters such as fecundity and prolificacy. The scoring method was classic, with the fixation of the endocervical catheter, after [19,25].

4.3. Microbiological spermogram

The research was performed on 20 boars used for artificial insemination from Farm 1 and Farm 2. For the microbiological examination the samples were taken from freshly harvested semen (raw) and semen after dilution and its distribution in doses for preservation (over 100 tests). The samples were collected in sterile vials and individualized (for identification) with the serial number on the boar's ear tag. The determinations were made on freshly cornered semen and after dilution and 12 hours storage at 17°C.

4.3.1. Quantitative determinations (CFU).

The semen samples (3 mL) were collected aseptically from each ejaculate before and after dilution, and transferred into a sterile container until the test beginning. The total viable aerobic count, also known as total number of viable bacteria and fungi was assessed using the serial dilution method and incubation in aerobic conditions. A series of ten-fold dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the semen samples was performed using tubes containing phosphate buffer saline (Biokar, France). From each dilution, six volumes of 100 µL were plated on six petri dishes containing solid media. Three of them contained Tryptone Soy Agar (Biokar, France) and were used for the enumeration of bacteria after an incubation of 24 hours at 37°C and the others containing Sabouraud Chloramphenicol Agar (Biokar, France) were used for the enumeration of fungi after an incubation of 3 days at 25°C. Finally, the bacterial and fungal burden in raw and diluted semen was calculated and expressed as CFU/mL, according to methods by APHA [30,42].The total viable aerobic count per mL consists of CFU number of bacteria and CFU number of fungi.

4.3.2. Qualitative determinations (typification of bacterial and fungal genera)

In order to differentiate the bacterial species contaminating both the raw and diluted semen, each sample was plated onto Columbia Agar with 5% (v/v) sheep blood and Mac-Conkey Agar respectively. The plates were incubated in aerobic conditions at 37°C for 24 hours. After this period, one colony of each morphotype was transferred onto Tryptone Soy Agar and re-incubated for 24 hours at 37°C in order to obtain a fresh culture ready for identification. The identification of bacteria isolates was performed using Gram stain and ID32E, ID32GN, ID32STAPH and ID32STREP (bioMérieux, France). Filamentous fungi also called molds were identified on the basis of macroscopic and microscopic features using the primary cultures onto Sabouraud Chloramphenicol Agar. The yeasts were identified by biochemical tests using ID32C strips (bioMérieux, France) [30,42].

4.4. Identifying the sources of sperm contamination and eliminating them

4.4.1. Critical points of sperm contamination

In order to identify the critical points of contamination, an experiment was carried out in Farm 2 where the possible sources of contamination (internal and external) were followed. The samples were taken from the freshly collected semen, from the mucosa of the external genitalia, from the foreskin wash, from the apparatus and from the atmosphere of the collection room. The Koch sedimentation method was used to determine the number of bacteria in the air of the room/m^3, and Omelianski's formula was used for fungi according to methods proposed by APHA [30,37,42].

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4.4.2. Possibilities to optimize the decrease of contamination in critical points by HPBC protocol

The hygiene protocol and the biosecurity of sperm collection (HPBC). As a precursor to the boar semen collection techniques, the mist spray-fog of lightly decontaminating substances was introduced 15 minutes before the start of the collection. The operator equipped with two latex gloves cleans the toilet and sanitizes the foreskin region, obligatorily empties the foreskin diverticulum of secretions and urine. Then remove the first glove and start collecting with the remaining glove. The sanitation and control tests were resumed before and after the application of this strict hygiene protocol of the sperm collection process, although they made a number of 80 determinations.

4.5. Antifungal aseptization of sperm by potentiated extenders

For more effective aseptization, the introduction of an antifungal drug (Fluconazole) into the commonly used formula extenders was investigated. The antifungal introduced was chosen according to the antifungal and water solubility results, and its minimum concentration was given by the minimum inhibitory dose (0.5 mg / L) obtained by ATB-Fungus3 antifungals (Bio Merieux, France).

The research was carried out on freshly collected semen, from ten boars used for artificial insemination from farm 2. Two extenders A (long preservation time) and B (medium) were used, which were added with different concentrations of Flu: Lot E1 25 mg%, Lot E2 12.5mg% and compared with control lots (without Flu). The assessment of sperm compatibility was determined by assessing motility 12 hours after dilution by CASA, (HT-USA), with the methodology of working on Leja chamber [18,22]

4.6. Testing semen extenders added with Fluconazole by determining sperm progressivity in storage dynamics

The experimental protocol aimed at evaluating some sperm parameters in the dynamics of semen preservation, of semen extenders with Fluonazole. Ejaculates of two boars from farm 1 were used, after resuming the methodology of the previous experiment (M, E1 F-25 mg%, E2 F-12.5 mg% in extensors A and B). Doses of semen as per AI (80mL) were stored in a thermostat (17C) for 3 days (average storage of doses on farms). At 12, 48 and 72 hours the motility (M% and P%) was evaluated with the CASA system, Telos software version 14 (IMV France) and the methodology of the Mofa chamber [6,14,23].

4.7. Validation of additive extenders by AI on the farm

The research was carried out on farm 3. The experimental group (LE) was 20 sows, inseminated with 3.5 x10^9 sperm/80 ml extender with 12.5 mg% fluconazole, and the control (LM) 20 sows sown with 3.5 x10^9 sperm/80 ml simple extender. The groups were made up of sows that spontaneously manifested their estrous phase, and were artificially seeded by the classical method. The conditions of maintenance, feeding, hygiene and comfort were identical for the respective lots.The gestational diagnosis was established by ultrasound 21 days after the date of sowing. The device used is a portable Agroscan with a fixed probe of 3.5 and 5 MHz.

5. Conclusions

Boar semen collected in usual conditions contains a series of aerobic germs, bacteria and fungi. The antibiotics used in commercial extenders and a rigorous hygiene during semen collection and processing can significantly reduce the bacterial contamination of A.I. doses. However, the fungal contamination persists in diluted semen stored at 17°C for 3-7 days, the yeasts representing the microorganisms constantly isolated in such samples. They cannot be removed from the semen either by addition of antibiotics or by optimizing the collection process hygiene. Fluconazole-added extenders (12.5mg%), ensures the solution of this problem, and even there is a stimulating role on sperm with an increase in sperm progressivity (+ 8.39%) for at least a 12-hour shelf life after dilution.

Similar to G. Althouse who described and introduced the term “bacteriospermia” term, referring to isolated and identified bacteria in fresh and diluted semen, in aur study ca introduce the term “mycospermia”, referring to fungal burden in semen.

Author Contributions:

Conceptualization, C.S.G; methodology, C.L.; R.P. and M.M.; software, C.S.G.; validation, D.D. and M.M.; investigation, C.S.G., and R.P.; resources, R.P, and C.S.G.; writing—original draft preparation, C.S.G.; writing—review and editing, C.L. and supervision, D.D. M.M. and R.P. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement:
Not applicable.
Data Availability Statement:

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The authors declare no conflict of interest.

Sample Availability:

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