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Evaluation of bacteriospermia after the cryopreservation process of boar semen samples

(Avaluació de la bacteriospèrmia després del procés de criopreservació de semen de porcí)

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RESUM

El semen criopreservat és molt útil per a la inseminació artificial (IA), ja que permet emmagatzemar el semen durant un període il·limitat de temps des de mesos a anys, fins que sigui utilitzat. No obstant això, en la indústria porcina només l'1% de les IAs es realitzen amb semen criopreservat a causa de la baixa taxa de fertilitat que presenten les mostres descongelades. Un dels factors que afecten directament la qualitat i l'emmagatzematge dels espermatozoides és, entre d'altres, la contaminació bacteriana, denominada com bacteriospèrmia. Els bacteris poden tenir un efecte nociu en els espermatozoides causant, entre altres: aglutinació d'espermatozoides, acrosomes malmesos, reacció prematura de l'acrosoma i baixa motilitat i viabilitat espermàtica. A més, si els bacteris són patògens, hi ha el risc de transmetre una malaltia a la truja. La bacteriospèrmia és un tema que s'ha estudiat en mamífers amb semen fresc i amb semen criopreservat. No obstant això, en porcí a dia d'avui no s'han trobat articles que abordessin la contaminació bacteriana en mostres congelades (o congelades-descongelades) d'espermatozoides. En aquest estudi s'ha realitzat una avaluació de la bacteriospèrmia després de la descongelació espermàtica. Les mostres de semen pertanyien a quatre porcs diferents i havien estat criopreservades en nitrogen líquid (LN_2) durant tres anys dins de palles de plàstic segellades. Es van avaluar quatre palletes de cada mascle per la detecció de contaminació bacteriana. Es van aïllar un total de setze colònies, de les quals tretze (81.25%) van poder ser identificades, mitjançant la reacció en cadena de la polimerasa (PCR) i el kit *BBL ™ Crystal ™ Enteric / Nonfermenter*. Els resultats van mostrar que la contaminació es va produir en totes les mostres, sent més freqüents els bacteris Gram-negatius (68.75%). Els contaminants aïllats van ser *Staphylococcus* spp. (18,75%), *Streptococcus* spp. (12,5%), *Burkholderia cepacia* (25%), *Yersinia pseudotuberculosis* (6,25%), *Yersinia enterocolitica* (6,25%), *Pasturella aerogenes* (6,25%) i fongs (6,25%). Això, semblaria demostrar que els bacteris presents a les mostres de semen són capaços de suportar el procés de criopreservació. No obstant, no es pot determinar quin és l'origen d'aquests bacteris i només es pot hipotetitzar mitjançant revisió literària.

RESUMEN

El semen criopreservado es muy útil para la inseminación artificial (IA), ya que permite almacenar el semen durante un período ilimitado de tiempo desde meses a años, hasta que sea utilizado. Sin embargo, en la industria porcina sólo el 1% de las IAs se realizan con semen criopreservado debido a la baja tasa de fertilidad que presentan las muestras una vez descongeladas. Uno de los factores que afectan directamente la calidad y el almacenamiento de los espermatozoides es, entre otros, la contaminación bacteriana, denominada como bacteriospermia. Las bacterias pueden tener un efecto nocivo en la célula espermática causando, entre otros: aglutinación de espermatozoides, acrosomas dañados, reacción prematura del acrosoma y bajas tasas de motilidad y viabilidad espermática. Además, si las bacterias son patógenas, existe el riesgo de transmitir una enfermedad a la cerda. La bacteriospermia es un tema que se ha estudiado entre los mamíferos con semen fresco y con semen criopreservado. Sin embargo, en porcino hasta esta fecha, no se encontraron artículos que abordaran la contaminación bacteriana en muestras congeladas (o congeladas-descongeladas) de esperma. En este estudio se ha realizado una evaluación de la bacteriospermia después de la descongelación espermática. Las muestras de semen pertenecían a cuatro cerdos diferentes y habían sido criopreservadas en nitrógeno líquido (LN_2) durante tres años dentro de pajas de plástico selladas. Se evaluaron cuatro pajitas de cada verraco para la detección de contaminación bacteriana. Se aislaron un total de dieciséis colonias, de las cuales trece de las dieciséis (81.25%) pudieren ser identificadas usando la reacción en cadena de la polimerasa (PCR) y el kit *BBL ™ Crystal ™ Enteric / Nonfermenter*. Los resultados mostraron que la contaminación se produjo en todas las muestras, siendo más frecuentes las bacterias Gram-negativas (68.75%). Los contaminantes aislados fueron *Staphylococcus* spp. (18,75%), *Streptococcus* spp. (12,5%), *Burkholderia cepacia* (25%), *Yersinia pseudotuberculosis* (6,25%), *Yersinia enterocolitica* (6,25%), *Pasturella aerogenes* (6,25%) y hongos (6,25%). Esto, parecería indicar que las bacterias presentes en las muestras de semen son capaces de soportar el proceso de criopreservación. No obstante, no se puede determinar cuál es el origen de estas bacterias, y solo puede ser hipotetizado mediante revisión bibliográfica.

ABSTRACT

Cryopreserved semen is very useful for artificitial inemination (AI) since it enables the semen to be stored for unlimited period of time ranging from months to years until it needs to be used. However, in the swine industry only 1% of the AIs are conducted with cryopreserved semen due to the low fertility rate of thawed semen samples. One of the factors that affect directly the quality and storage of sperm is, among others, bacterial contamination, referred as bacteriospermia. Bacteria can have detrimental effects upon the sperm cell causing, among others: sperm agglutination, damaged acrosomes, premature acrosome reaction and low motility and viability rates. Moreover, if the bacteria are pathogenic there is a risk of passing a disease to the sow. Bacteriospermia, is an issue that has been studied among mammals with fresh and cryopreserved semen. Nonetheless, with boar up to this date no articles have been found addressing bacterial contamination in cryopreserved (or frozen-thawed) semen samples. In this study an evaluation of bacteriospermia after sperm thawing. The semen samples belonged to four different boars and they had been cryopreserved in liquid nitrogen (LN_2) for three years inside sealed plastic straws. Four straws, each from a boar were assessed for bacterial contamination. A total of sixteen colonies were isolated, which a total of thirteen out of sixteen (81.25%) could be identified by using the Polymerase chain reaction (PCR) and the BBL™ Crystal™ Enteric/Nonfermenter kit. The results showed that contamination occurred in all samples, being Gram-negative bacteria (68.75%) more prevalent. The contaminants isolated were *Staphylococcus* spp. (18.75%), *Streptococcus* spp. (12.5%), *Burkholderia cepacia* (25%), *Yersinia pseudotuberculosis* (6.25%), *Yersinia enterocolitica* (6.25%), *Pasturella aerogenes* (6.25%) and yeast (6.25%). Thus seems to show that bacteria present in semen samples are able to withstand the cryopreservation process. However, it cannot be determined which source of contamination might be, and it can only be hypothesised by literary review.

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GLOSSARY

µL	Microliter
ACTE	Androhep Cryoguard Thaw Extender
AI	Artificial Insemination
AMPs	Antimicrobial Peptides
ART	Assisted Reproduction Technologies
BC	Before Christ
bp	Base pair
BTS	Belstville Thawing Solution
CPA	Cryoprotectant Agent
DNA	Deoxyribonucleic acid
EU	European Union
FAO	Food And Agriculture Organization of the United Nations
FT	Frozen – Thawed
ICAI	Intracervical Insemination
LB	Luria Bertrani
LEY	Lactose – Egg – Yolk
LEYGO	LEY – Glicerol – Orvus Es Paste
LN_2	Liquid Nitrogen
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry
MCTs	Minimum Contamination Techniques
mL	Millilitre
MM	Multiplex Mix
ºC	Degree Celsius
PCR	Polymerase Chain Reaction
SLC	Single Layer Centrifugation
TCM	Traditional Culture Methods
UV	Ultraviolet

1. INTRODUCTION

1.1. THE PIG INDUSTRY

1.1.1. An overview

Since their domestication around 4900 BC in China – and later on in 1500 BC in Europe (EU) – pigs (*Sus scrofa domesticus*) have played an important role in the production of food for human consumption **[1]**. Many countries throughout the world continue to depend on pig meat as an important food source **[2]**.

The world's pig industry (including both fresh and processed pig meat) has been rising since the 1970s, and global production surpassed 110 million tons in 2014 **[3]**. According to the Food and Agriculture Organization (FAO) of the United Nations, pig meat (pork) is the most consumed meat in the world and accounts for more than 36% of the world meat intake according to the data from 2012 (Figure 1) which shows that pork is an important part of the human diet throughout the world **[2, 4, 5]**.

Currently, China is the biggest pig meat producer around the world followed by the EU – being the later the biggest exporter of pig meat and pig meat products –, the United States and Brazil **[4, 6, 7]**.

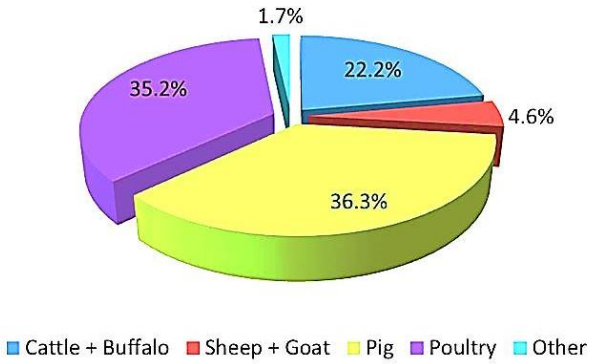

Figure 1: Sources of the world's meat supply in 2012. Pig meat (pork) it's the most consumed meat, followed by poultry (35%) and beef (22%) (**Extracted from Fao.org, 2016) [4].**

Furthermore, based on FAO records in the past 10 years, pig production has increased from 73 to 94 million metric tons[1] (MT) according to Gerrits et al., (2005) **[2]**. In addition, it is expected that the demand for pork will increase to 125 million metric tons by 2020.

The increase in the productivity in the swine industry is a response to the constant growth in human population, consequently has increased dramatically the need for pork in the market, demanding an upsurge in pig production **[8]**. Thus has basically occurred by the development and the adoption of new technologies in practically all areas, such as genetics, nutrition, management, sanity and reproduction **[9]**. Undoubtedly, in the reproduction area, artificial insemination (AI) has represented an enormous progress since the beginning of the 70-ies as this technique caused a great impact on the increment of the swine production industry **[2, 9]**. AI is a fundamental tool for facilitating pig production by increasing reproductive efficiency and achieving global improvements in fertility, genetics, labor and herd health **[8]**. Overall, the implantation of this method substantially facilitates the reproductive management of herds with high number of sows **[9]**.

[1] Equal to 1000 kilogram (Kg) also called tonne (t)

1.2. ARTIFICIAL INSEMINATION

AI belongs to a group of technologies commonly known as "assisted reproduction technologies" (ART), whereby offspring are generated by facilitating the meeting of gametes (spermatozoa and oocytes) **[10]** and consists of the manual placement of diluted semen, containing viable spermatozoa, into the female reproductive tract by a method other than natural mating **[8, 10]**. Its main priority is to pass on more quickly the desirable characteristics of the male and to achieve more progeny than if the animal is mated with females in a natural fashion **[10]**.

Currently AI is the most commonly used ART in livestock, revolutionising the animal breeding industry during the 20th century. It is by far, the most common method of breeding domestic livestock, such as pigs (more than 90% in Europe and North America), dairy cattle and turkeys (approximately 80% in Europe and North America and almost 100% in intensive production, respectively) **[10]**. Among different techniques of AI, the intracervical insemination (ICAI) is the most used in technified farms; in ICAI, semen is deposited in the cervix and spermatozoa are transported until the ampulla of the uterine tube, the place where fecundation occurs **[9]**.

Initially, AI appeared in order to provide genetic improvement of the animals and to solve sanitary problems such as controlling the spread of disease **[9, 10]**. Later on, the implementation of semen extenders, which contain antibiotics, has also helped to prevent the transmission of bacterial diseases during AI **[10]**. Today commercial production efficiency and profit are tied to the use of AI **[8]**, since this technique represents a significant improvement in both productive and economical aspects, 1) by preventing the spread of infectious or contagious diseases, that can be passed on when animals are in close contact or share the same environment; 2) allowing the utilization of semen from males of high genetic merit for superior females, increasing thus the rate of genetic development and production gain by accelerating the diffusion of desirable traits, with animals being in different geographic locations, or at different times; 3) and finally, it is a powerful tool when linked to other reproductive biotechnologies such as sperm sexing and sperm cryopreservation, the latter enabling breeding between animals even after the male´s death **[10]**.

However, AI also has some disadvantages; for instance some males can shed virus in semen without clinical signs of disease (these males are known as "shedders"). Moreover, some bacterial pathogens are resistant to the antibiotics in semen extenders or can avoid their effects by forming bio-films thus being susceptible to be transmitted via semen **[10]**.

Pre-requisites for AI consist of supplies of semen, reliable methods for oestrus detection in the female and means of inserting the semen into the female reproductive tract. Semen collection, in most domestic animals such as bull, ram and stallion is collected through an artificial vagina whereas in boars is usually collected by manual stimulation known as the glove handed method. Semen can either be used after collection ("fresh"), after storage at a reduce temperature (stored also known as "chilled" or "cooled" semen) or after freezing and thawing ("frozen-thawed" (FT)) semen is obtained by cryopreservation) **[11]**.

The pig industry uses mostly liquid semen either "fresh" or "chilled" (that has been diluted with extender[2] formulas and stored for one to several days at 16 – 18º) over cryopreserved semen as the latter shows a lower fertility rate compared to liquid semen (60% vs. 85% respectively). Nonetheless, cryopreserved semen it Is very useful for AI since it enables the semen to be stored for unlimited period of time ranging from months to years until it's needed for AI **[11, 12]**.

[2] The term extender, or diluent, refers to an aqueous solution used to increase the volume of the ejaculate until that of the required dose, in which antibiotics are added since components present in the diluents can promote the growth of bacteria **[15]**

1.3. CRYOPRESERVATION

FT semen – or cryopreserved semen – is achieved through cryopreservation (ice equilibrium – freezing) **[13]**, a multi-step protocol which maintains cellular life for an extended period of time at sub-zero temperatures **[14]**. The first protocols for boar sperm cryopreservation were developed in the 1970s with the Hülsenberg (5 mL packaging straws) (Westerndorf et al. (1975) **[16]**) and Beltsville (freezing pill) (Pursel and Johonson, (1975) **[17]**) methods. They have gone under some modifications, in the last two decades, in order to optimize the quality of the FT sperm **[12]**. The more significant changes are in 1) the packing method, substituting the 5 mL straws and the freezing pills, for 0.5 mL straws and FlatPacks® which allow a homogenous sperm freezing and thawing; 2) the freezing and thawing speed, with existence of a control – rate freezing curve and specific thawing speeds for the packaging method used; 3) and the addition of bio-freezers that allow an uniform freezing of a large number of straws **[12]**.

The protocol used nowadays, described in Bonet et al. (2006) **[12]**, includes the following steps:

1. ***Collection and manipulation of the ejaculate***
   Beginning at collection, the rich fraction of the ejaculate must be extended usually with Belstville Thawing Solution (BTS) at the same temperature as the ejaculate (37ºC) and then cooled to 17°C in order to preserve the spermatozoa integrity **[12]**. This dilution and temperature lowering helps to preserve the spermatozoa since it reduces their metabolic activity that can only be maintained over a limited period **[12, 14, 15]**. The extended ejaculate is distributed in aliquot tubes of 50 mL and centrifuged (at 800 x g for 10 min) at 17 °C to remove seminal plasma and concentrate the sperm cells **[12, 14]**.

2. ***Cooling and freezing diluents***
   The sperm pellet is diluted to the desired concentration in cooling diluting media (LEY) being one of its components, among others, egg yolk and also an antibiotic; the pellet is cooled from the 17ºC to 5 °C and held over for 3h to allow for membrane equilibration prior to freezing; the egg yolk protects the spermatozoa since they are extremely sensitive to temperatures below 12 – 15ºC. The freezing phase of cryopreservation begins with the addition of freezing diluting media (LEYGO) in which incorporates a CPA (i.e. glycerol), no antibiotics and a detergent (Equex STM) to achieve the desired freezing volume and concentration **[12, 14]**.

3. ***Packaging and freezing***
   Finally, the sperm is then packaged in straws[3] at 5ºC with an automated or semi-automatic filling and sealing machine, and placed into a computer controlled-rate freezing system (bio-freezer) or manually over liquid nitrogen (LN_2) vapours to freeze the contents of the straws. Following freezing, the samples are stored at -196°C in tanks filled with LN_2 will be kept there until needed for AI **[12, 14]**.

4. ***Sperm thawing***
   When the straws are needed, they are moved from the LN_2 tank and immersed in a water bath at 37ºC for 20s in agitation with the help of tweezers, previously cooling the pointed tip in LN_2 in order to avoid thermic shock; The content of the straws is then transferred inside a tube containing the thawing extender (1:3; semen volume: thawing extender volume) previously warmed to 37ºC in the water bath **[12]**. In this project two different thawing extenders were used: 1) Beltsville Thawing Solution (BTS) commonly used in the boar IA since 1975 in freezing and thawing **[18]**, a 2) Androhep Cryoguard Thaw Extender (ACTE) an extender designed especially for thawing boar semen samples, and commercialized by Minitub® **[18]**.

[3] Boar sperm package can either be in a “mini” FlatPack® (5 mL) or normal (0.7 mL), and 0.5 mL labelled straws, being the latter the one used more frequently; this system allows an individual identification and the ability to ship domestically and internationally **[12]**.

However, spermatozoa of various species differ in their ability to withstand cryopreservation **[11]**. For instance, boar cryopreserved semen, in contrast with chilled semen, faces problems related to sperm survival, damage and fertility, resulting in lower farrowing rates and litter size obtained after insemination with FT **[20]**, making the use of FT sperm doses unattractive for commercial pig breeders **[11, 12]**; The low fertility rate of FT boar spermatozoa could be associated to the damage that the spermatozoa undergoes during the freezing-thawing process, which lowers de fertility capacity, since boar spermatozoa are especially susceptible to cold shock **[19]**, limiting the progress towards more intensive use of AI with cryopreserved boar semen **[20]**; according to Yeste (2016) **[5]** only 1% of all artificial inseminations (AI) conducted worldwide are made using frozen – thawed boar sperm.

Nevertheless, frozen semen is: 1) the vehicle for international transport of genetic material **[20]**, in other words, it enhances the transportation of genetic material across countries reducing geographical barriers **[11, 12]**, 2) allows to have a higher sanitary control **[13]**, and 3) permits having a stock of semen available for periods of low production **[13]** and 4) overall it is fundamental for the preservation of genetic resources and improve the genetic progress **[12, 13]**. The world's pig population is being consistently upgraded through the utilisation of frozen semen, while slaughter pig production is improved locally through the use of liquid-stored semen. However, the usefulness of frozen-stored semen from boars of high genetic merit for repopulation after natural disasters, such as serious disease outbreaks, cannot be underestimated **[20]**.

1.4. BACTERIOSPERMIA

There are many factors that can affect directly the quality and storage of sperm being one of the most important, among them, the bacterial contamination. Currently the frequent finding of bacteria in semen is referred as bacteriospermia **[21]**, a recurrent problem faced among mammals such as boar, bull, stallion and even human since most ejaculates collected form healthy animals are contaminated with bacteria to some extend **[22 – 26]**. It is a major concern for most semen production laboratories, as it has adverse effects on semen quality and hence subsequent fertility rates **[27]**, resulting in reductions in reproductive performance, which may lead to major economic losses for the swine industry **[28]**. Due to the ubiquitous presence of bacteria, there are high chances of contamination semen during its collection (which is far from being a sterile procedure **[29]**), processing and preservation **[27]**.

Generally, semen from a healthy boar does not contain bacteria; however the preputial diverticulum and the skin/hair of the boar do as well as the barn and collection environment which can contaminate the collector's hands or collection container **[23]**. Bacterial contamination in different breeding mammals can be divided in two main sources; non animal sources and animal sources (see Table 1) **[21, 30]**. Moreover, extenders with ingredients of animal origin (i.e.: egg yolk or milk) can also be a source of bacteria, resulting in the contamination of semen **[31]**.

Table 1: Sources of bacterial contamination modified from Althouse et al. (2005) [32]

Animal origin	Non – animal
Fecal	*Tap water*
Preputial cavity fluids	*Purified water (e.g., water lines or holding tanks)*
Skin/Hair	*Plant matter (i.e., feed, beeding)*
Respiratory secretions	*Air/ventilation system*
Human/Personnel (e.g., skin, hair, respiratory secretions)	*Skin/drains*
	Laboratory
	Equipment

1.4.1. Most frequently isolated bacteria

In boar semen the majority of contaminants are Gram-negative bacteria, with a large percentage belonging to the family Enterobacteriaceae. Many bacterial species have been isolated from semen as it can be seen in Table 2. The majority of these bacteria are not considered primary pathogens in swine [32].

Table 2. Common bacteria isolated from boar raw ejaculate modified from Althouse et al. (2005) [32]

Tamuli et al. [33]	Danowski [34]	Dagnall [35]	Sone et al. [36]	Bresciani et al. [30]
E. coli	*Staphylococcus* spp.	*Citrobacter* spp.	*Pseudomonas* spp.	*Escherichia coli*
Pseudomonas spp.	*Pseudomonas* spp.	*Pseudomonas* spp.	*Micrococcus* spp.	*Staphylococcus epidermidis*
Bacillus spp.	*E. coli*	*Corynebacterium* spp.	*Staphylococcus* spp.	*Serratia marcescens*
Staphylococcus spp.	*Citrobacter* spp.	*Streptococcus* spp.	*Klebsiella* spp.	*Proteus* spp.
Klebsiella spp.	*Providencia* spp.	*E. coli*	*E. coli*	*Pseudomonas* spp.
Proteus spp.	*Neisseria* spp.	*Actinomyces-like* spp.	*Citrobacter* spp.	*Streptococcus* spp.
Enterobacter spp.	*Proteus* spp.	*Bacteroides* spp.	*Proteus* spp.	*Staphylococcus aureus*
Pasteurella spp.		*Lactobacillus* spp.	*Actinomyces* spp.	
Citrobacter spp.		*Acinetobacter* spp.	*Serratia* spp.	
		Bacillus spp.	*Enterobacter* spp.	
		Actinobacillus spp.	*Bacillus* spp.	
		Staphylococcus spp.	*Streptococcus* spp.	
		Flavobacterium spp.		
		Klebsiella spp.		
		Micrococcus spp.		
		Proteus spp.		

However, this bacterial contamination is not only observed in raw undiluted ejaculates but also in extended semen samples. According to Bresciani et al. (2016) [30] 62.5% of raw and a 79% of extended ejaculates had bacterial contamination, mainly due to the resistance that bacteria have develop to the antimicrobials used in the semen extenders [32].

Among the different genera and species the ones that are found more frequently in both extended and raw semen are: *Escherichia coli, Pseudomonas* spp., *Staphylococcus* spp., *Proteus* spp., *Serratia* spp., *Enterobacter* spp., *Klebsiella* spp., *Streptococcus* spp., and occasionally also *Clostridium* spp. [30, 32 – 37].

1.4.2. The effect of bacterial contamination

Generally, the effect of bacteria on boar semen depends on the type of bacteria and their concentration [21]. Most microorganisms detected in boar ejaculates are considered (to some extent) non-pathogenic [22, 37 – 39], and they seem to have little effect on fecundity under natural mating conditions [37, 40], as the female reproductive tract has developed its own defence mechanisms to cope with bacteria from the semen [37, 41, 42]. But when such semen is used in AI, there is a possibility that the bacteria will grow and multiply in the extended semen [32].

Bacterial contamination can lead to infertility problems especially if bacteria are present in high concentrations [21] due to the spermicidal effect that bacteria exert upon the sperm cell [32]. High levels of bacterial contamination are associated with a high incidence of sperm-to-sperm

agglutination, damaged acrosomes, premature acrosome reaction, poor sperm motility and viability **[22, 29]** thus having a reduced shelf life (i.e., decreased sperm longevity) of the extended semen product **[22, 29, 32, 38]**. If bacterial contamination is left uncontrolled, the final result is a decreased reproductive performance of swine females **[22, 29, 38]**. The existence of a possible competition between bacteria and spermatozoa for the nutrients present in the seminal plasma might also contribute to a negative effect on sperm quality **[43]**.

Furthermore, the insemination with contaminated semen may be associated with vulvar discharge and returns to estrus, reduced litter size, or embryonic or fetal death, endometritis, systemic infection and/or disease in recipient females **[22]** – since some bacteria can be pathogenic and cause various clinical diseases conditions causing damage to the sow – **[23]**.

1.4.3. How to control bacteriospermia

1.4.3.1. Minimum contamination techniques (MCTs)

Due to the various sources of contamination (see Table 1), Althouse et al. (2008) **[43]** elaborated a general sanitation protocol known as minimum contamination techniques (MCTs) in order to reduce (or avoid) bacterial contamination. Currently applied in the swine industry, these MCTs seek to prevent the presence of bacteria during its collection, processing and preservation by establishing sanitary guidelines for stud personnel, animal housing, handling (semen collection and processing) and laboratory.

For instance, personnel should use clean protective garments (i.e.: boots/shoes, hair nets, caps…) as well as protective gloves if they are involved or in contact with the semen collection and processing and any materials used in them. Proper cleaning and drying of the ventral abdomen of the boar plus trimming the hair surrounding the preputial orifice – getting rid of the organic matter accumulated in the area – can reduce the bacterial load originating from the stud donor.

Furthermore, animal housing should be put on a regular sanitary maintenance schedule, followed by the removal of organic matter and cleaned with a broad spectrum disinfectant; in the laboratory countertops, floor and contaminated equipment have to be disinfected at the end of the day **[43]**.

1.4.3.2. Antimicrobials in semen extenders

Semen extenders are primarily designed to nurture and prolong sperm viability; however these same attributes are what make them a potential medium in where contaminant bacteria can flourish **[32]** since the nutrients in the extender, such as glucose, and the storage temperature (15 – 16Cº) of chilled semen allow the development of Gram-negative bacteria, like *E. coli* and *Pseudomonas* spp. **[15, 44]**. Therefore, even under hygienic and sanitary conditions the presence of bacteria results unavoidable, a fact that makes antimicrobials a necessary component of semen extenders.

According to Alhouse et al. (2005) **[32]** the most common antibiotics used nowadays in porcine semen extenders are gentamicin sulphate (an aminoglycoside), ampicillin (β – lactam) and Lincomycin hydrochloride (lincosamides). The concentration of antimicrobials and which ones are added are both stipulated by national and international guidelines **[45]**; more specifically in the European Union (EU) they are stipulated by the Council Directive 90/429/EEC **[42]**. The use of antibiotics has become an extended and well established practice for the collection, processing and preservation of sperm, but complete elimination of bacteria cannot be guaranteed, since bacteria can develop antibiotic resistance which in turn, can be transferred to other bacteria in other host species **[45]**.

Moreover, it has been reported that the use of antibiotics, like gentamicin, might produce adverse effects on sperm's motility and function **[22, 45]**; despite that, they are still used because their beneficial effects on controlling bacterial contamination are greater than their detrimental effects on boar sperm quality.

Due to the development of antibiotic-resistant strains in semen samples when antibiotics are added to semen extenders, other methods have been studied in order to improve sperm quality and survival.

1.4.3.3. Other methods

- **Single Layer Centrifugation (SLC)**

An alternative that has been considered to reduce the use of antibiotics in semen extenders is by physical separation of the bacteria present in the semen samples right after collection through colloid centrifugation **[42]**.

In breading animals the use of a Single Layer Centrifugation (SLC) has been tested through a species-specific colloid formulation: Androcoll^{TM} – "X" [4] **[42]**. This product not only increases the quality of sperm samples – selecting only the spermatozoa with high motility and normal morphology – but it has been demonstrated that this technique is also able to separate sperm cells from bacterial contaminants in boar **[42]** and stallion **[46]** reducing the bacteria in samples.

- **Synthetic Antimicrobial Peptides (AMPs)**

Antimicrobial peptides (AMPs) are of notable interest, due to their broad spectrum of antimicrobial activity, being able to rapidly kill their target cell and the low tendency for the development of bacterial resistance **[47]**. For these reasons they have been studied as potential substitutes to antibiotics in extended semen in boar. Speck et al. (2014) **[40]** and Schulze et al. (2014) **[48]** reported that two cyclic antimicrobial peptides combined with small amounts of gentamycin were effective against bacterial growth offering a possible solution to decrease the use of antibiotics.

1.4.4. Cryopreservation and bacteriospermia

Studies of the current state of knowledge on bacterial contamination before and after cryopreservation are sparse. They have been primarily carried out in stallion **[49]**, bull **[24]** and human **[50]** but up to our knowledge not with boar, which only studies examining the contaminants of both extended and raw semen have been published **[30, 32, 38]**.

According to Garcia et al. (1981) in human **[50]** bacterial contamination was largely unaffected by cryopreservation; practically all the bacteria isolated from human fresh semen samples were also recovered from frozen semen in liquid nitrogen, as well as with stallion FT semen based on Corona et al. (2009) **[49]** study. However, Prado et al. (2014) **[24]** reported that in bull there was a reduction of the bacterial contamination in FT semen compared to fresh samples.

Furthermore, the bacteria isolated differ among animals. For example, in bull and human FT samples where bacteria were isolated, *E. coli* (12%) was the most frequently found **[24, 50]**. Meanwhile in stallion the microbial agents identified were mostly *Enterobacter-coccus* spp. (15%), *Pseudomonas* spp. (6.25%), *Stenotrophomonas maltophila* (6.25%) and anaerobic bacteria like *Propionibacterium granulosum* (7.5%) and *Clostridium* spp. (3.75%) **[50]**.

[4] Androcoll^{TM} – "X" is a Single Layer Centrifugation (SLC) medium that allows for whole ejaculates to be processed in one easy step; the letter "X" stands for the first initial of the species (i.e. "P" = porcine) **[42]**.

2. OBJECTIVE

The main goal of this work is to evaluate the presence of bacteria after thawing cryopreserved boar sperm samples.

The specific objectives are:

- To evaluate the presence or absence of bacterial growth in anaerobic and aerobic conditions of frozen – thawed (FT) sperm samples.

- To identify which bacteria are present in boar frozen – thawed (FT) sperm samples through a Polymerase Chain Reaction (PCR) technique or BLL Crystal identification kit.

3. MATERIALS AND METHODS

3.1. Previous work - Bacterial glycerol stocks

In this project, bacterial evaluation and identification was carried out by using 16 bacterial glycerol stocks that were already elaborated and stored at -80ºC previously to this project. Each glycerol stock contained a different colony isolated from thawed semen straws (0.5 mL) – from 4 different boars – that had been cryopreserved for 3 years.

The thawing process – the same for each straw – consisted of transferring a straw with the help of tweezers from the N_2L, in which was kept, to a water bath at 37ºC and left there for 20s. Afterwards, the straw was dried and the sealed end cut, followed by the placement of the straw in a tube (previously warmed to 37ºC in the water bath) which contained the thawing extender (1:3; v:v) being BTS the extender used for the bacterial colonies that were further incubated under aerobic conditions, and ACTE the one for the bacterial colonies incubated under anaerobic conditions (sealed with parafilm). The content inside the straw was released inside the tube by cutting the other straw's end.

To obtain the colonies of the 4 males, for each male, 100 µL of thawed semen were plated into a Luria Bertrani (LB) (Conda Pronadisa, Madrid, SP) agar plate to be incubated under aerobic conditions, and 100 µL onto a LB agar plate to be incubated under anaerobic conditions; counts were done at 24 and 72 hours to check for bacterial growth. The colonies that grew in each agar plate were re-isolated and once a pure growth obtained were kept with glycerol and LB, under aseptic conditions and, kept at -80ºC allowing long-term storage of the bacterial sample until needed. The colony, which male and conditions in which the colony grew (anaerobic or aerobic) were conveniently labeled on the eppendorf.

3.2. Bacterial incubation and growth

Bacterial identification was mainly performed using the polymerase chain reaction (PCR) technique (see section 3.3.). However, in order to facilitate the process, bacteria were discerned between two major categories of bacteria, Gram-positive and Gram-negative, by using Gram staining. Thus narrowed down which primers had to be used in each sample; for instance samples that were Gram-positive only primers for Gram-positive bacteria would be used (see section "*Oligonucleotide primer selection*" p. 3).

Previous to the identification, under sterile conditions bacteria were inoculated from each of the 16 bacterial stocks (-80ºC) in an LB agar plate and in an eppendorf with LB broth (2mL) to be used, each of them, for different purposes (see Figure 2.). They were then incubated over 48h at 37ºC under aerobic and anaerobic conditions, based on which conditions was the colony first isolated.

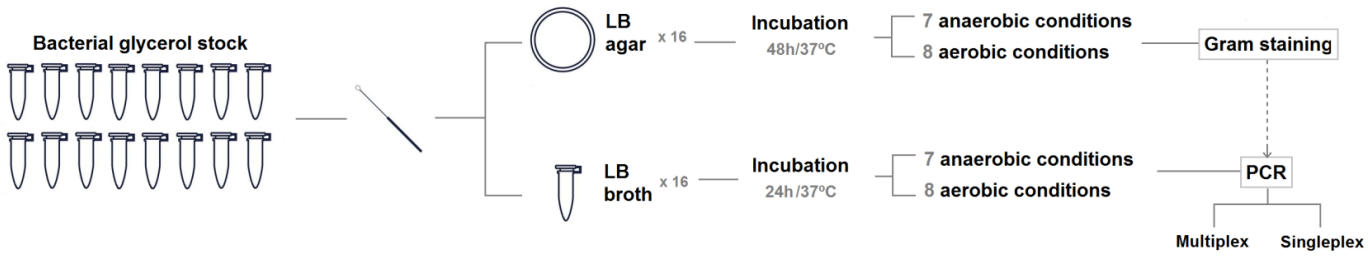

Figure 2. Diagram of bacterial glycerol stock processing, incubation and identification of the samples. Bacteria were transferred from each of the 16 bacterial stocks (-80ºC) with an inoculation loop in an LB agar plate and in an eppendorf with LB broth (2mL), to be incubated and afterwards identified.

3.3. Bacterial identification

3.3.1. Gram staining

The gram staining process consisted of smearing with and inoculation loop a clean glass slide with a loopful of bacterial culture grown for 48h from an LB agar plate. The slide was then heat fixed in the Bunsen's flame; the process was repeated for each colony.

The heat fixed bacterial smear was then flooded in a staining tray with a series of different stains: 1) crystal violet, 2) Lugol's iodine, 3) alcohol-acetone and 4) safarine; all stains were left on for 1 minute each, and with washes of water in between stains to remove the stain excess. Each glass slide, once dried, was then placed on the microscope, and observed under 1000X (Leica Microsystems, Leica DM750, SP) objective with the addition of immersion oil.

3.3.2. Polymerase chain reaction (PCR) technique

3.3.2.1. Genomic DNA extraction

For the identification of the colonies, two type of PCRs were performed, a singleplex PCR (see section "*Oligonucleotide primer selection*" p. 10) in which each reaction contains one primer pair to amplify one target, and a multiplex PCR (see section "*Singleplex PCR assay*" p. 11) in which multiple primer pairs are used to amplify multiple targets in one reaction allowing to save time, reagents and samples.

Before initiating the PCR, bacterial DNA must be isolated from the LB broth samples. Herein, genomic DNA was extracted using the QIAcube® (QIAgen, Ontario, CA) (see Figure 3) which performs a fully automated purification of nucleic acids up to 12 samples in around 50 min. The bacteria that were grown for 48h in LB broth (2mL) aerobically and anaerobically (see Figure 2) were centrifuged and the supernatant was removed. The resulting eppendorf contained the cell pellet, which was placed into the QIAcube® shaker (see Figure 3. Image 2) c.). Further setup for the QIAcube®, previous to the DNA extraction, included the placement of the novel rotator adapters in the centrifuge, the reagents in the reagents bottle rack, proteinase K in the microcentrifugate tube slots and the filling of the tip racks.

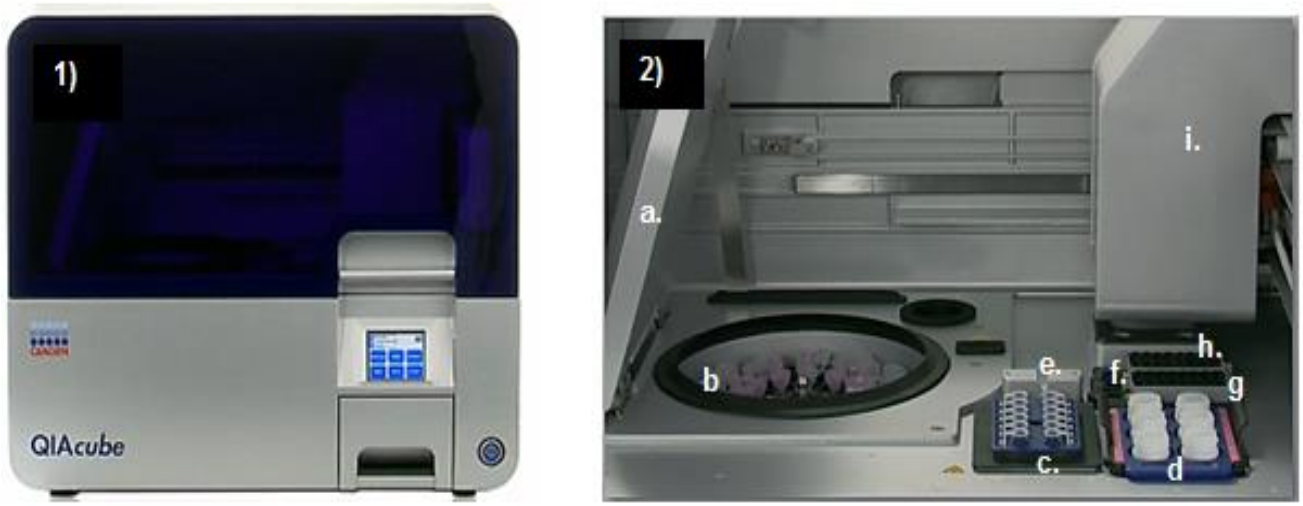

Figure 3. 1) QIAcube® automated sample prep. 2) Internal view of the QIAcube® robotic workstation. The features that it has are shown through the different series of numbers: a. Centrifuge lid; b. Centrifuge; c. Shaker; d. Reagent bottle rack; e. Tip sensor; f. Microcentrifuge tube slots; g. Tip racks; h. Disposal slots for tips and columns; i. Robotic arm (Extracted from QIAgen, 2009 **[51]**).

The protocol selected from the QIACube® for bacterial DNA extraction was: genomic DNA > Blood and Tissue > Bacterial pellet; before the process started a fully automated check to ensure the correct loading of the worktable was done by the QIACube® [51].

Inside the QIAcube® the samples were lysed in the orbital shaker, and then transferred to a spin column in a rotor adaptor. Nucleic acids bonded to the purification resin of the QIAgen spin column and were washed to remove contaminants [51]. Finally the spin column was transferred to a centrifuge tube for elution of purified nucleic acids, the DNA was measured in NanoDrop spectrophotometer (Thermo Scientific, Inc, Madrid, SP) and it was kept at 4ºC until needed.

3.3.2.2. Oligonucleotide primer selection

In this work, the primers that were chosen for the PCR identification were the ones that amplified for the genres and species of bacteria that are reported to be isolated more frequently in both raw and extended boar semen (see Introduction, section 1.4.1. Table 2), ending up with a selection 11 bacteria. The primer's sequence, the target, the PCR product's size (bp) and references, are listed in Table 3. All the primers were obtained from previous studies [52 – 56] and purchased at Invitrogen (Invitrogen, ThermoFisher Scientific, Madrid, SP).

Table 3. List of oligonucleotide primers used for bacterial identification

Bacteria	Gene	Primer set sequences (5´→3´)	Product size (bp)	Source
E. coli	*uidA*	F: CTG GTA TCA GCG CGA AGT CT R: AGC GGG TAG ATA TCA CAC TC	556	[52]
K. pneumoniae	*ntrA*	F: CAT CTC GAT CTG CTG GCC AA R: GCG CGG ATC CAG CGA TTG GA	90	[52]
Proteus mirabilis	*tuf*	F: TCT ACT TCA CAC GTA G R: TTC TAA CAG CTC TTC A	240	[52]
Salmonella typhimurium	*viaB*	F: GTT GCA GTA GGA CAT CAG R: GTC CGT AGT TCT TCG TAA G	157	[52]
E. cloacae	*atpd*	F: CGA GAG CCT GUT GCT G R: GAT TGG CTG ACC CAA T	180	[52]
***Citrobacter* spp.**	*16S rRNA*	F: GCT CAA CCT GGG AAC TGC ATC CGA R: AGT TCC GGC CTA ACC GCT GGC AA	529	[52]
Entereobacteria	*16S rRNA*	F: GGCGGCAGGCCTAAC R: CAGGCAGTTTCCCAGACATTACT	100 – 103	[53]
***Pseudomonas* spp.**	*16S rRNA*	F: GAC GGG TGA GTA ATG CCT A R: CAC TGG TGT TCC TTC CTA TA	618	[54]
E. coli	*16S rRNA*	F: CCCCCTGGACGAAGACTGAC R: ACCGCTGGCAACAAAGGATA	401	[55]
***Staphylococcus* spp.**	*16S rRNA*	F: CCT ATA AGA CTG GGA TAA CTT CGG G R: CTT TGA GTT TCA ACC TTG CGG TCG	791	[56]
***Streptococcus* spp.**	*16S rRNA*	F: GCG TGC CTA ATA CAT GCA A R: TAC AAC GCA GGT CCA TCT	207	[57]

3.3.2.3. Conventional multiplex PCR assay

On the one hand, a conventional multiplex PCR assay was carried out for those Gram-negative colonies – based on Gram staining – in groups of three organisms in two sets, since the product size of the 6 genes chosen would not allow their detection in a single multiplex reaction, as stated by Anbazhagan et al., (2010) [52]. The first set consisted of primers to identify *E. coli, P. mirabilis* and *S. typhimurium.* and the second set had the primers for *K. pneumoniae, Citrobacter* spp. and

E. cloacae. The PCR amplification reaction mixture was carried out in 50µL reaction volumes in 0.5 mL thin-walled PCR tubes which contained for both sets: 5 µL DNA, 5 µL of each primer and 25µL PCR MM and 10µL of nuclease free H_2O from the QIAgen Multiplex PCR Kit® (QIAgen®, Hilden, DE). Moreover, a negative control with the addition of water instead of DNA was used and, a positive control whenever possible was performed.

PCR amplification was done with a PCR thermal cycler (GeneAmp® PCR System 9700 thermocycler, ThermoFisher Scientific, Madrid, SP) with the following PCR cycling temperatures: initial denaturalization at 95ºC for 10 min followed by 35 cycles of denaturalization at 95ºC for 45s; primer annealing at 58ºC for 45s, primer extension at 72ºC for 90s and the final extension at 72ºC for 10 min **[52]**.

3.3.2.4. Singleplex PCR assay

On the other hand, for those colonies that were Gram-positive, a singleplex PCR assay was performed for the detection of *Staphylococcus* spp. and *Streptococcus* spp. Furthermore, since the multiplex PCR proposed for Gram-negative bacteria failed to identify any colony, (see Results p. 16 – 17) a singleplex PCR was opted for the identification of Entereobacteria genus, *E. coli,* and *Pseudomonas* spp.

The PCR amplification reaction mixture for *Staphylococcus* spp. and *Streptococcus* spp. was prepared in 50µL reaction volumes in 0.5mL thin-walled PCR tubes by mixing 5µL DNA, 5µL of each primer (Forward and Reverse) and 25µL PCR M (Master Mix) and up to 50 µL of nuclease free H_2O from the Promega PCR Master Mix® kit (Promega, Wisconsin, US). And for Entereobacteria, *E. coli* and *Pseudomonas* spp. the reaction mixture contained 5µL DNA, 5µL of each primer (Forward and Reverse) and the master mix was prepared with 1µL Taq, 1µL dNTPs, 4µL $MgCl_2$, 5µL 10xNH_4 and 24µL of nuclease free H_2O from the BIOTAQ™ DNA Polymerase (Bioline, Madrid, SP) in a reaction volumes of 50µL. For each singleplex PCR, a negative control was prepared by the addition of 5µL DNA of water (nuclease free H_2O) instead of DNA; positive controls for Entereobacteria, *E. coli* and *Pseudomonas* spp. were also prepared. The PCR amplification was performed with a PCR thermal cycler (GeneAmp® PCR System 9700 thermocycler, ThermoFisher Scientific, Madrid, SP) with the appropriate PCR cycling temperatures, differing for each bacterium (see Table 4).

Table 4: PCR thermal cycling temperatures for bacteria for the conventional PCR assay.

Bacteria	Initial denaturaliza-tion	Denaturalization	Primer annealing	Primer extension	Final extension	Source
Entereobacteria	95ºC for 10min.	95ºC for 15s	60ºC for 60s	72ºC for 30s	72ºC for 7min	**[53]***
		25 cycles				
***Pseudomonas* spp.**	95ºC for 2min.	94ºC for 20s	54ºC for 20s	72ºC for 40s	72ºC for 60s	**[54]**
		25 cycles				
Escherchia coli	98ºC for 8min.	95ºC for 30s	58ºC for 30s	72ºC for 30s	72ºC for 7min	**[55]**
		20 cycles				
***Streptococcus* spp.**	94ºC for 4min.	94ºC for 45 s	64ºC for 45 s	72ºC for 45 s	72ºC for 5min	**[57]**
		20 cycles				
***Staphylococcus* spp.**	94ºC for 3min.	95ºC for 90s	58ºC for 60s	72ºC for 60s	72ºC for 10min	**[56]**
		35 cycles				

***Note:** The thermal cycling temperatures for Entereobacteria have been slightly modified from Sen et al. (2001) **[53]**.

3.3.2.5. Detection of the DNA products

For the detection of the DNA products, an E-Gel® iBase™ electrophoresis System device with an E-Gel™ system with SYBR Safe™ 2% agarose was used (Invitrogen,ThermoFisher Scientific, Madrid, SP). This allowed to obtain results in a short lapse of time and eliminated the need to prepare agarose gels, buffers and to stain gels.

In each well, 20 µL of prepared sample with 3 µL of loading buffer were loaded. The first two wells contained respectively an E-Gel® 1 Kb Plus DNA Ladder and an E-Gel® High Range DNA marker (Invitrogen,ThermoFisher Scientific, Madrid, SP). The addition of a second MwM was to make sure that the results could be read and interpreted without difficulties, since the E-Gel® 1 Kb Plus DNA Ladder had shown sometimes poor or non-band staining in previous gels, making it difficult the interpretation of the results.

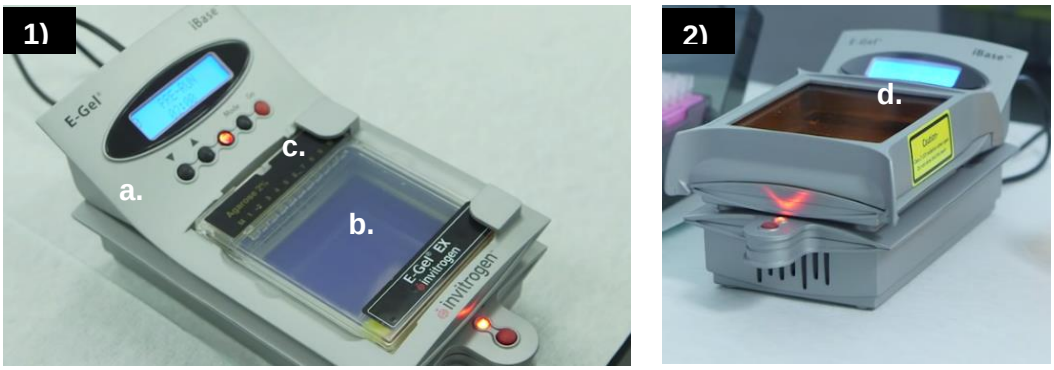

Figure 4. 1) E-Gel® iBase™ electrophoresis system device without the E-Gel® Safe Imager™. 2) Complete E-Gel® electrophoresis system. Its features are shown through the different series of numbers: a. E-Gel® Pre-Cast Gel & iBase™; b. E-Gel™ with SYBR Safe™ 2% agarose; c. 1 – 12 E-Gel® wells ; d. E-Gel® Safe Imager™ (Extracted from TermoFisher, 2017 **[58]**).

3.3.3. BBL™ Crystal™ Enteric/Nonfermenter Kit

As not all Gram-negative bacteria could be identified by PCR (see Results pp. 15 – 20) an identification with the BBL™ Crystal™ Enteric/Nonfermenter (E/NF) Identification (ID) System Kit (BD, Microbiology Systems, Madrid, SP) was carried out. This system allows the identification of aerobic Gram-negative bacteria that belong to the family *Enterobacteriaceae* as well as some of the more frequently isolated glucose fermenting and nonfermenting Gram-negative bacilli **[59]**.

The procedure consisted of the inoculation of a pure colony into a tube with BBL Crystal fluid under aseptic conditions. Those colonies had been previously inoculated from each glycerol stock and plated in an LB agar plate and incubated for 24h in anaerobic or anaerobic conditions depending on the colony. The BBL tube was then vortexed for approximately 10 – 15 s and the contents of the tube were poured into the kit's base. The inoculum was rolled gently along the track base's filling the wells and covered with a lid to be incubated for >24h at 37ºC (see Figure 5). After the incubation each colony panel was read based on a colour reaction chart. The resulting profile was entered on a PC where the BBL Crystal ID System Electronic Codebook was installed to obtain the identification of each colony. Furthermore, for a complete identification, it was necessary to perform Indol (BD, Microbiology Systems, Madrid, SP) and oxidase tests (BD, Microbiology Systems, Madrid, SP).

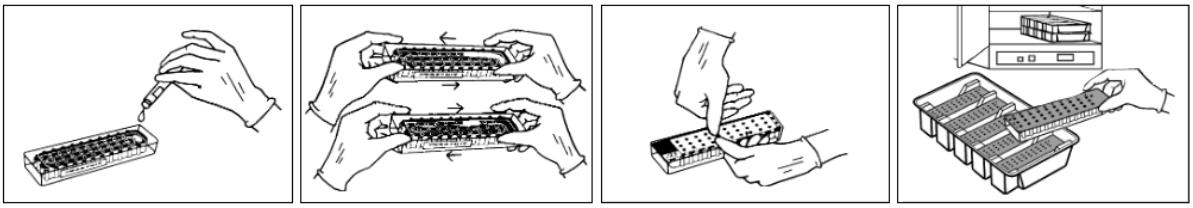

Figure 5. Procedure for the detection of Gram-negative bacteria species using the BBL™ Crystal™ Enteric/Nonfermenter Identification System. (Extracted from BD, 2017 **[59]**)

3.4. Ethics and sustainability

In this experiment, it must be said that ethics and sustainability guidelines were followed at all times; for the sample's obtainment, manipulation, and for the proper elimination of the material used as well as the residues generated.

The obtainment of the boar's semen samples was done by the gloved hand technique. This is a well-established practice for the semen collection which does not have negative effect on the boar and it is a safe and practical procedure for the animal to obtain semen. The boars used in this study were treated accordingly to the"*Recomendación relativa a los cerdos"* **[60]** established by "*El Comité Permanente del Convenio Europeo de Protección de los Animales en Explotaciones Ganaderas*" in which is stated that the pigs are properly housed, with access to sun light, are regularly fed and can see/interact with other pigs within the facility.

All the residues generated during the manipulation of the samples and the material used, were eliminated by disposing them in their specific container in order to avoid any harm to the laboratory facilities, environment or health.

The solely purpose of this work, was to provide information about the contaminants that can be recovered from cryopreserved semen samples, which has not been investigated yet (up to our knowledge). The presence of bacteria in frozen samples is a risk in the swine industry and can cause economic losses, as bacteria can exert negative effects on sperm quality and also can transmit diseases. Bacteriological controls should be performed, before, during and after cryopreservation before using it for artificial insemination (AI).

4. RESULTS

4.1. Types of colonies isolated

After thawing the semen samples of all four males and plating them into LB agar plates, several colonies were observed after an incubation period of 72h at 37ºC. Contamination appeared on the plated samples with the visible growing of different colonies on the agar. These colonies were isolated and labelled for their identification (see Table 5); a 56.25% of the colonies grew under aerobic conditions and a 43.75% under anaerobic conditions, isolating a total of sixteen different types of colonies.

Table 5. Colony types of bacteria in LB agar plate cultured under aerobic and anaerobic conditions for 72h for the straws of each male.

MALE	AEROBIC COND. (BTS EXTENDER)		ANAEROBIC COND. (ACTE EXTENDER)	
	Nº colony types 72h	Colony types	Nº colony types 72h	Colony types
Male #1	3	C#1, C#2 and C#3	3	C#7, C#8 and C#9
Male #2	3	C#4, C#5 and C#6	2	C#10 and C#11
Male #3	1	C#12	1	C#15
Male #4	2	C#13 and C#14	1	C#16

Note: The different colonies isolated were labelled with the code "C#X" "C" standing for colony and "X" for the number of the new found colony

4.2. Bacterial identification

4.2.1. Gram staining results

Bacterial isolates were first identified with Gram staining, thus providing preliminary results on whether the bacteria present in the colonies were Gram-positive or Gram-negative, differentiating them from yeast cells; the results obtained showed that 31.25% of the colonies were formed by Gram-positive bacteria (C#5, C#6, C#9, C#12 and C#16), 68.75% were Gram-negative bacteria (C#1, C#2, C#3, C#7, C#8, C#10, C#11, C#12, C#13, C#14 and C#15) and 6.25% were yeast; the latter was found in one colony (C#4), which was excluded for the PCR identification since it was not a bacterium. Moreover, the colony C#12 was positive for both Gram-negative and Gram-positive bacteria; to rule out the possibility that it was not an error with the interpretation of the staining in the microscope, it was loaded in both Gram-positive and Gram-negative PCRs for its identification.

4.2.2. PCR gel results

Note: If not mentioned otherwise, in Figures 6 – 11, the assignments for each MwMs' band are as follows: **E-Gel® 1 Kb Plus DNA Ladder:** Band 1, 2.000bp; Band 2, 1.650bp; Band 3, 1.000bp; Band 4, 850bp; Band 5, 650bp; Band 6, 500bp; Band 7, 400bp; Band 8, 300bp; Band 9, 200bp; Band 10, 100bp. And **E-Gel® High Range DNA marker:** Band 1, 10.000bp; Band 2, 4.000; Band 3, 2.000bp; Band 4, 800bp; Band 5, 400bp. They are numerated in the gel with number from 1 to 10. Other bands than the ones appearing on lane 1 and 2 are labelled with letters (i.e.: a, b, c...).

Species and genus identification of the Gram-negative/positive colonies was carried out with a Multiplex and Singleplex PCR. The results obtained for each PCR are presented below. However, with the PCR technique not all the colonies were able to be identified, as a 66.67% of them remained unknown after the PCR.

Multiplex PCR results for Gram-negative bacteria

- **Enterobacteriaceae**

In order to identify those colonies that based on Gram staining results were Gram-negative, a Multiplex PCR for Entereobateriaceae was carried out in groups of 3 organisms in two sets of primers to identify *E. coli, P. mirabilis, S. typhimurium*, *K. pneumoniae, Citrobacter* spp. and *E. cloacae*.

For the first set (*E coli, P. mirabilis, S. typhimurium.*) only the positives controls amplified, with bands of ~500bp (band a.) in lane 10, corresponding to *E. coli* (556bp), and ~200bp (band b.) in lane 11 for *P. miriabilis* (240bp) (See Figure 6 Gel 1).

For the second set (*K. pneumoniae, Citrobacter* spp. and *E. cloacae*) only 3 colonies (C#14, C#12 and C#3) displayed bands on the gel. The colonies C#14 and C#12 (Figure 6.Gel 2), showed a band of ~800bp (bands a.) and a band of ~400bp (bands b.). Whereas the colony C#3 (Figure 7 Gel 1) showed 3 bands at ~1700bp (band a.), ~800bp (band b.) and ~500bp (band c.) respectively. However, these bands did not belong to any of the bacteria that were expected to see since they differed in product size. Only the C#3 with the band of ~500bp (band c.) could belong to *Citrobacter* spp. (525bp).

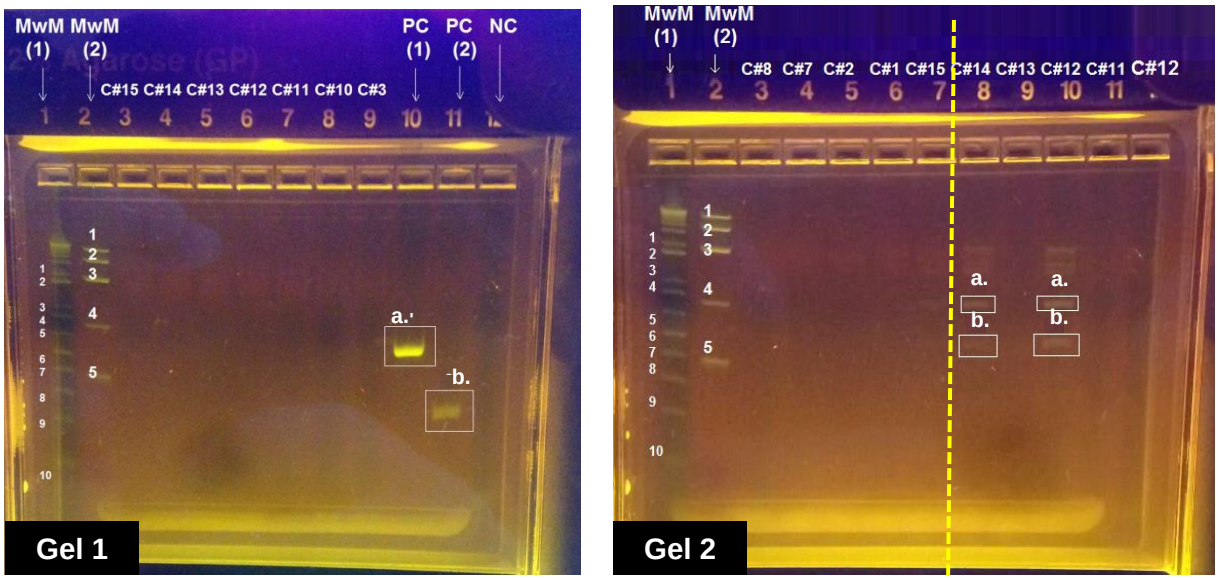

Figure 6. PCR results for *E. coli, P. mirabilis*, *S. typhimurium (first set) K. pneumoniae, Citrobacter* spp. and *E. cloacae (second set)* by multiplex PCR in 2% agarose gel. In Gel 1) begins the multiplex PCR for the first set. Lane 1, E-Gel® 1 Kb Plus DNA Ladder (1 – 10); Lane 2, E-Gel® High Range DNA marker (1 – 5); Lane 3, C#15; Lane 4, C#14; Lane 5, C#13; Lane 6, C#12; Lane 7, C#11; Lane 8, C#10; Lane 9, C#3; Lane 10, Positive control 1 (PC1) *E. coli* with a band of ~500bp (band a.); Lane 11, Positive control 2 (PC2) *P. mirabilis* with a band of ~200bp (band b.); Lane 12, negative control (NC). **In Gel 2) ends the multiplex PCR for the first set and starts the PCR for the second set.** Lane 1, E-Gel® 1 Kb Plus DNA Ladder; Lane 2, E-Gel® High Range DNA marker; Lane 3, C#8; Lane 4, C#7; Lane 5, C#2; Lane 6, C#1. In Lane 7 for the second set C#15; Lane 8 C#14 with a band of ~800bp (a.) and a band of with a band of ~400bp (b.); Lane 9, C#13; Lane 10, C#12 800bp (a.) and a band of with a band of ~400bp (b.); Lane 11, C#11; Lane 12, C#10.

Therefore, most of the samples loaded in the gel did not show any bands, which lead to believe that no amplification occurred. However, the few samples that did show bands, they had a different size (bp) to the ones that were expected to be obtained. Thereby, in an attempt to identify some of the Gram-negative colonies isolated, it was decided to carry out three more singleplex PCRs for the detection of: *Pseudomonas* spp., *E. coli* (16srRNA) and Entereobacteria, due to the lack of results obtained with the Enterobacteriaceae multiplex PCR (see Results, pp. 19-20).

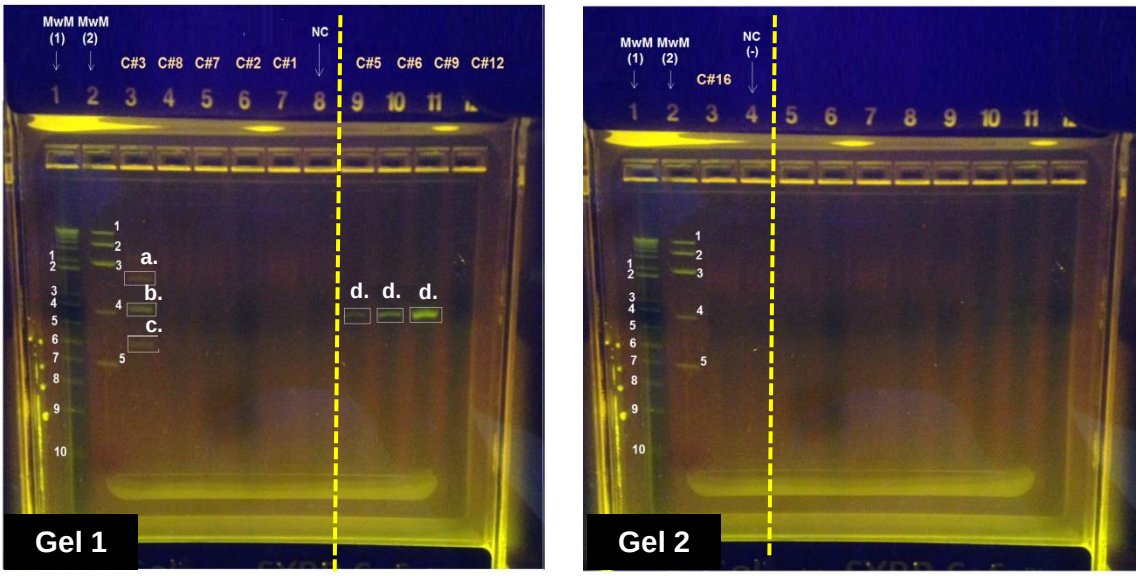

Figure 7. PCR results for *K. pneumoniae, Citrobacter* spp. and *E. cloacae* by multiplex PCR in 2% agarose gel and singleplex PCR bands for *Staphylococcus spp*. In Gel 1) ends the multiplex PCR for the first set and starts the PCR for the second set. Lane 1, E-Gel® 1 Kb Plus DNA Ladder (1 – 10); Lane 2, E-Gel® High Range DNA marker (1 – 5); Lane 3, C#3 with bands of ~1700bp, ~800bp and ~500bp (a., b. and c. respectively); Lane 4, C#8; Lane 5, C#7; Lane 6, C#2; Lane 7, C#1; Lane 8 Negative control (NC). And from Lane 9 in gel 1 begins de singleplex PCR for *Staphylococcus spp*. Lane 9, C#5 with a band of ~800bp (d.); Lane 10 C#6 with a band of ~800bp (d.); Lane 11, C#9 with a band of ~800bp (d.); Lane 12, C#12. **In Gel 2) ends the singleplex PCR for *Staphylococcus spp.*** Lane 1, E-Gel® 1 Kb Plus DNA Ladder; Lane 2, E-Gel® High Range DNA marker; Lane 3, C#16; Lane 4, Negative control (NC).

Singleplex PCR results for Gram-positive bacteria

- ***Staphylococcus* spp. and *Streptococcus* spp.**

The identification for those colonies that were Gram-positive a Singleplex PCR was carried out to detect *Staphylococcus* spp. and *Streptococcus* spp. In the case of *Staphylococcus* spp. the 16SrRNA gene was amplified in the colonies C#5, C#6 and C#9 showing a band in the region ~800bp corresponding to the molecular weight expected for *Staphylococcus* spp. which is 791bp (See Figure 7 Gel 1. band d.). The colonies C#12 and C#16 were the only ones that did not amplify for *Staphylococcus* spp. Therefore the amplification mixture of *Streptococcus* spp. for these two colonies was loaded in a new gel, where a band at ~200bp. was observed (See Figure 8 band a.), corresponding to the molecular weight expected for *Streptococcus* spp. of 207bp.

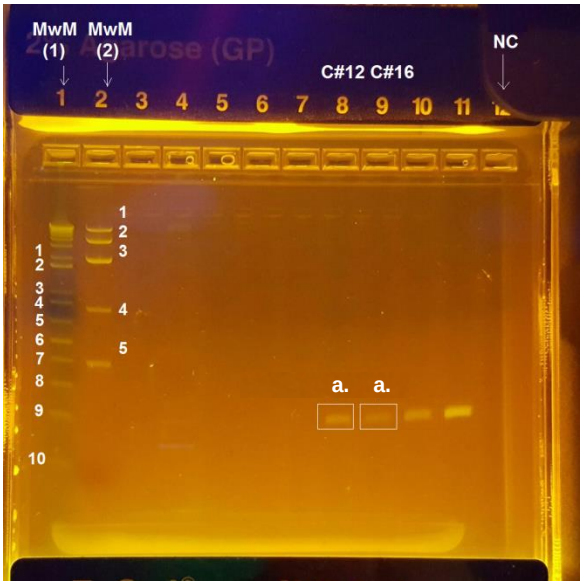

Figure 8. PCR results for *Streptococcus* spp. by singleplex PCR in 2% agarose gel. Lane 1, E-Gel® 1 Kb Plus DNA Ladder (1 – 10); Lane 2, E-Gel® High Range DNA marker (1 – 5); only the lanes for 8, C#12; 9, C#16 and Lane 12 with a band of ~200bp (a.), which contained the negative control (CN) belong to this study, the rest lanes are loaded with samples from another project.

Singlepex PCR results for Gram-negative bacteria

- ***Pseudomonas* spp., *E. coli (16srRNA)* and Entereobacteria**

The Singleplex PCRs for *Pseudomonas* spp., *E.coli* (16srRNA) and Entereobacteria showed similar results. In all of them, none of the samples loaded amplified for the target genes of each bacterium. Only the positive controls in the case of *Pseudomonas* spp. (See Figure 9. Gel 1 and 2) and *E.coli* (16srRNA) (See Figure 10. Gel 1 and 2) were seen. *Pseudomonas* spp. had a band of ~600bp in lane 11 (band a.) corresponding to the positive control (618 bp) and Entereobacteria had a band for its positive control in lane 11 in Figure 10 Gel 1 and lane 9 in Gel 2 (~400bp (band a.) for *E. coli* (16srRNA) (401bp)).

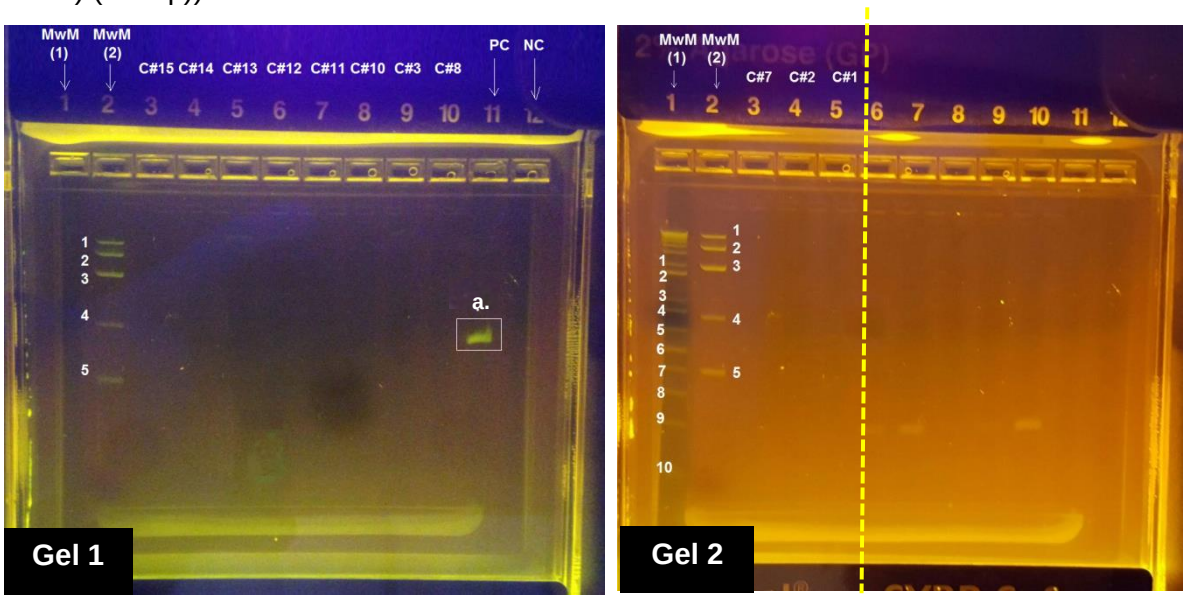

Figure 9. PCR results for *Pseudomonas* spp. by singleplex PCR in 2% agarose gel. In Gel 1) ends the multiplex PCR for the first set and starts the PCR for the second set. Lane 1, E-Gel® 1 Kb Plus DNA Ladder (no bands visible); Lane 2, E-Gel® High Range DNA marker (1 – 5); Lane 3, C#15; Lane 4, C#14; Lane 5, C#13; Lane 6, C#12; Lane 7, C#11; Lane 8, C#10; Lane 9, C#3; Lane 10, C#8; Lane 11, Positive control (PC) *Pseudomonas spp.* with a band of ~600bp (a.) and Lane 12, negative control (NC). **In Gel 2) continues the singleplex PCR for *Staphylococcus spp.*** Lane 1, E-Gel® 1 Kb Plus DNA Ladder; Lane 2, E-Gel® High Range DNA marker; Lane 3, C#7; Lane 4, C#2 and Lane 5

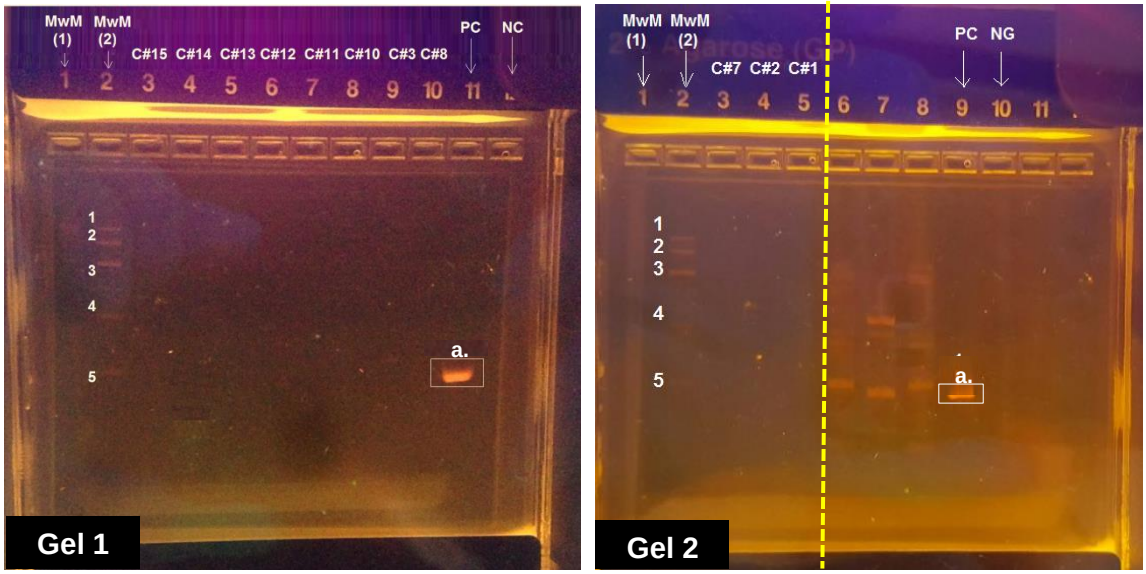

Figure 10. PCR results for *E. coli (16srRNA)* by singleplex PCR in 2% agarose gel. In Gel 1) begins the single PCR *E. coli (16srRNA).* Lane 1, E-Gel® 1 Kb Plus DNA Ladder (no bands visible); Lane 2, E-Gel® High Range DNA marker (1 – 5); Lane 3, C#15; Lane 4, C#14; Lane 5, C#13; Lane 6, C#12. Lane 7, C#11; Lane 8, C#10; Lane 9, C#3; Lane 10, C#8; Lane 11, positive control (PC) *E. coli* (16srRNA) with a band of ~400bp (a.) and Lane 12, negative control (NC). **In Gel 2) ends the singleplex PCR for *E. coli (16srRNA).*** Lane 1, E-Gel® 1 Kb Plus DNA Ladder (no bands visible); Lane 2, E-Gel® High Range DNA marker (1 – 5); Lane 3, C#7; Lane 4, C#2; Lane 5, C#1 and Lane 9, positive control (PC) with a band of ~400bp (a.) and Lane 10, negative control (NC). The rest of lanes from 6 - 8 and 11 - 12 belonged to another project different to this one.

However, for the Singleplex PCR for Entereobacteria not only none of the samples amplified for the target gene (16srRNA) but neither did any of both positive controls (See Figure 11). This concluded to determine that this PCR did not work and the rest of the Gram-negative samples were not loaded (C#8, C#7, C#2 and C#1) to determine whether they belonged or not to Entereobacteria.

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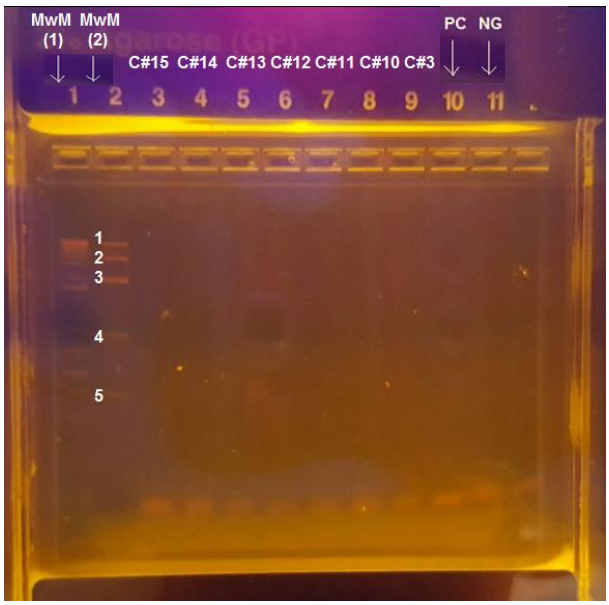

Figure 11. PCR results for Entereobacteria by singleplex PCR in 2% agarose gel. Lane 1, E-Gel® 1 Kb Plus DNA Ladder (1 – 10); Lane 2, E-Gel® High Range DNA marker (1 – 5); Lane 3, C#15; Lane 4, C#14; Lane 5, C#13; Lane 6, C#12. Lane 7, C#11; Lane 8, C#10; Lane 9, C#3; Lane 10, positive control for Entereobacteria; Lane 11, negative control negative control (NC).

Gram-negative bacteria were not able to be identified with any PCR neither Multiplex nor Singleplex. Only Gram-positive bacteria colonies were successfully identified for *Staphylococcus* spp. (C#5, C#6 and C#12) and *Streptococcus* spp. (C#9 and C#16).

4.2.3. BBL™ Crystal™ Enteric/Nonfermenter kit results

Despite carrying out several PCRs for the identification of Gram-negative bacteria, no results were obtained, which lead to use a BBL™ Crystal™ Enteric/Nonfermenter identification kit (see Methodology, section *"BBL™ Crystal™ Enteric/Nonfermenter Kit"*) to identify those Gram-negative colonies. As a result, 7 out of 11 colonies were identified as *Burkholderia cepacia* (C#3, C#12 – 14), *Pasteurella aerogenes* (C#8), *Yersinia pseudotuberculosis* (C#1) and *Yersinia enterocolitica* (C#11), while 4 out of 11 colonies remained unidentified (C#2, C#7, C#10 and C#15).

4.3. Global results for bacterial identification

Taking into account all the results obtained for bacterial identification of the colonies, with Gram-staining, PCRs, and the BBL™ Crystal™ Enteric/Nonfermenter kit, it was found that a total of 11 out of 16 colonies were able to be identified finding, 13 different microorganisms. These results are compiled in Table 6.

Table 6. Frequency of microorganisms isolated in frozen-thawed boar semen in this study

Microorganisms	Nº of isolates (n)	Percentage of isolates (%)	Method of detection
***Staphylococcus* spp.**	3	18.75	PCR
***Streptococcus* spp.**	2	12.5	PCR
Burkholderia cepacia	4	25	Kit
Yersinia pseudotuberculosis	1	6.25	Kit
Yersinia enterocolitica	1	6.25	Kit
Pasturella aeurogenes	1	6.25	Kit
Yeast	1	6.25	Gram staining
Total	**13**	**81.25**	**-**

From the sixteen colonies isolated in the thawed straws and kept in the bacterial glycerol stocks, one of them resulted to be a yeast 6.25% (1 out of 16) and the rest of the colonies were from bacteria (93.75%). In the case of bacteria, a 68.75% (11 out of 16) were identified. However, the total number of microorganisms identified was higher (13) than the number of colonies isolated (11); this is because one of the colonies turned out to be a co-culture of two bacterial species / genus, and not a pure isolate. This colony was C#12; in Gram-staining it was reported that this specific colony was positive for both Gram-negative and Gram-positive bacteria, which was already an indication that maybe more than one bacterium formed the colony. With the PCR and with the BBL™ Crystal™ kit identification, it was determined that the colony was formed by *Burkholderia cepacia* and *Streptococcus* spp. Nonetheless, in regards of the rest of the colonies, four Gram-negative colonies could not be identified (6.25%), and the remaining colonies were positive for pure culture of a single bacteria species / genera since only one bacterium was identified.

Moreover, with the bacterial identification it was found out that some of the bacteria are in fact anaerobic facultative (*Staphylococcus* spp., *Streptococcus* spp., *Pasteurella aerogenes, Yersinia enterocolitica and Yersinia pseudotuberculosis*); only *Burkholderia cepacia* is a strict aerobe. Hence, some of the colonies that grew under aerobic conditions were not strict aerobes and are capable of growth under both aerobic and anaerobic conditions (see Table 7). Thus, the percentage of 56.25%, given for aerobes is relative (see, Results, section "*Types of colonies isolated*" p.16).

Table 7. Anaerobic facultative bacteria identified with the BBL™ Crystal™ Enteric/Nonfermenter kit

ANAEROBIC FACULTATIVE BACTERIA			
AEROBIC COND. (BTS EXTENDER)		**ANAEROBIC COND. (ACTE EXTENDER)**	
C#1	*Yersinia pseudotuberculosis*	**C#8**	*Pasteurella aerogenes*
C#5	*Staphylococcus* spp.	**C#9**	*Staphylococcus* spp.
C#6	*Staphylococcus* spp.	**C#11**	*Yersinia enterocolitica*
C#12	*Streptococcus* spp.	**C#16**	*Streptococcus* spp.

5. DISCUSSION

Many factors affect directly on the quality and storage of sperm, being one of the most important bacterial contamination, referred as bacteriospermia **[21]**, as it is a documented risk to reproductive performance when using extended semen for artificial insemination **[23]**. Bacteriospermia is a major concern for most semen production laboratories due to its adverse effects on semen quality and consequently, on fertility **[27]**. Different bacteria have been recovered in fresh boar semen, attributing its contamination to animal and non-animal sources **[23]**. The aims of this project were to evaluate the presence of bacterial contaminants in both aerobic and anaerobic conditions of cryopreserved sperm straws from 4 different boars, after thawing, and identify which bacteria were present through Polymerase Chain Reaction (PCR) technique and the Enteric/Nonfermenter ID Kit. Furthermore, up to our knowledge, no studies have been done regarding the relationship between bacteriospermia and boar frozen-thawed semen to report whether, bacteria are found after thawing and to identify which.

Herein, it was found that contamination occurred in all samples with the grown of 16 colonies, which 56.25% of them grew under aerobic conditions and 43.75% in anaerobic conditions. Aerobic contamination is frequently found in the extended ejaculates **[37]**, in contrast to anaerobes which, in boar semen are less frequently found, around ~1% according to Maroto et al (2016) **[61]** and Gączarzewicz et al. (2016) **[37]**. However, in our study some of the colonies that grew under aerobic conditions were not strict aerobes and were capable of growing under both aerobic and anaerobic conditions, which makes the percentage of 56.25% given to the aerobes relative. Gram staining results showed, that almost all samples were contaminated with bacteria (93.75%), being Gram-negative bacteria more prevailing (68.75%) than Gram-positive bacteria (31.25%). This results are similar to those obtained from other authors (Kuster et al. (2016) **[23]**), and Gączarzewicz et al. (2016) **[37]**); all of them reported Gram-negative bacteria as the most commonly recovered type of bacteria from boar extended doses. Only one sample out of the sixteen (6.25%) was contaminated with yeasts, which can also be found in extended boar semen due to the lack of antifungals and the presence of glucose in the diluent that can be used to their growth **[62]**.

For bacterial identification, a PCR technique was performed by using primers to detect the most frequent bacteria isolated in boar semen, in contrast to most studies in which bacterial isolates are not identified by molecular techniques, but by standard microbiological procedures. However, with our molecular approach, some issues were encountered. For instance, Gram-negative bacteria could not be identified with the Multiplex PCR for Entereobacteriacea, since no bands were seen in the gels for the first set of primers (*E. coli*, *K. pneumoniae*, *Proteus mirabilis*) nor the second set (*Salmonela typhumurium*, *E. cloacae*, and *Citrobacter* spp), in most of the eleven Gram-negative colonies, except for the positive controls for *E. coli* and *Proteus mirabilis,* thus indicating that the PCR for the first set worked but none of the colonies loaded were formed by these bacteria. Only bands for three colonies appeared in the second set of the PCR for Enterobacteriaceae (C#3, C#12 and C#14), but had different sizes from the bands that were expected, not matching with any of the primer products. Furthermore, C#3 had a band which had a similar molecular weight to *Citrobacter* spp. but was discarded due to the results that were obtained later on, when the BBL™ Crystal™ Enteric/Nonfermenter kit was used to identify these 11 colonies. Thus, was unexpected since these bacteria from the Enterobacteriaceae family, are frequently recovered from raw and extended boar samples (especially the genera *Eschericha* spp., *Enterobacter* spp., *Klebisella* spp. and *Proteus* spp.) **[23]**. An explanation for the mismatching bands obtained in the second set of primers, could be, that the size difference was a result of primer dimerization or primer's inespecificity. The latter, could be an option, but it is not applicable in this case, since there were no positive controls for these bacteria to contrast the three bands obtained. If we had more time for this study, the bands would have been sent to sequentiate, and we would have found out what those bands belonged to. In our case it was opted to carry out three more Singleplex PCRs to detect Entereobacteria, *Pseudomonas* spp. and *E. coli* (16srRNA) for the eleven Gram-negative colonies, but no results were obtained, although in these cases the positive control worked.

Some Gram-negative colonies were finally identified with the BBL™ Crystal™ Enteric/Nonfermenter kit, obtaining that 25% were *Burkholderia cepacia,* 6.25% *Yersinia pseudotuberculosis,* 6.25% *Pasturella aeurogenes,* 6.25% *Yersinia enterocolitica* and 6.25% was not able to be identified, which could be attributed to limitations of the procedure **[59]**. Nevertheless, Gram-positive bacteria could be identified successfully through Singleplex PCR; 18.75% of the colonies in the samples were *Staphylococcus* spp. and 12.5% were *Streptococcus* spp.

Staphylococcus spp. (18.75%), *Streptococcus* spp. (12.5%) and *Burkholderia cepacia* (25%) were the most frequently bacteria isolated in our frozen-thawed semen samples. These results are consistent with those of other authors (Kuster et al. (2016) **[23]** Gączarzewicz et al. (2016) **[37]**) who concluded that among the different bacteria the identification in boar semen of *Staphylococcus* spp., *Streptococcus* spp. and *Burkholderia cepacia* occur frequently along with *Klebisella* spp*., Proteus mirabilis, Pseudomonas* spp*. and E. coli* but the lasts ones were not identified in this study. The least frequently bacteria found in this study were *Yersinia pseudotuberculosis* (6.25%)*, Yersinia enterocolitica* (6.25%) and *Pasturella aerogenes* (6.25%)*. Yersinia* spp., in contrast to *Pasteurella aerogenes,* has been not reported to be commonly recovered from boar semen samples **[23]**.

The source of contamination in our samples is hard to assess since there was no data of the fresh samples used in this study; bacterial contamination could, for instance, came from the boar or during its processing and manipulation in the laboratory. According to Schulze et al. (2016) **[63]** in only 4.5% of contaminated AI doses, the respective bacteria were also isolated from the raw ejaculate, suggesting that the contaminations occurred within the laboratory surroundings as a result of poor hygienic practices in the use of utensils in semen collection preparations. Therefore, here the source of bacterial contamination can only be hypothesized by literary review.

On the one hand, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are pathogens, which are responsible for foodborne zoonosis in humans in Europe **[64]**; these two bacteria have been reported in both pigs and wild boars. Based on Arrausi-Subiza et al. (2016) **[64]**, *Yersinia pseudotuberculosis* (25%) and *Yersinia enterocolitica* (33.3%) are highly prevalent among wild boars in Spain (specifically in the Basque Country) with *Yersinia enterocolitica* being the most frequently found. The main sources of contamination of these two bacteria are the tonsils and intestinal contents of clinically healthy pigs, as most of them asymptomatic carriers of *Yersinia* spp. **[65]**. Therefore, since these bacteria are prevalent in pigs, the source of contamination of our samples with *Yersinia* spp. could be the animal itself. Unfortunately, no articles were found regarding a possible source of contamination for *Pasturella aerogenes.*

Burkholderia cepacia is commonly found in soil or water and can live for long periods in humid environment **[66]**. Furthermore, it has been also recovered from extended semen samples due to its resistance to a wide range of antibiotics (i.e.: Amoxicillin, Ampicillin, Spectinomycin + Lincomycin, Gentamicin, Neomicin, Penicilin and Sulfazotrim) **[66]**. The source of contamination of *Burkholderia cepacia* in the extended semen was investigated by Nazaré et al. (2017) **[66]** and Darwin et al. (2003) **[67]** by culturing those areas of the laboratory and equipment that were in contact with the semen extender. They discovered that this bacteriumwas present in the water systems used in the barn to make the semen extender, (from powder semen extender) and the equipment used for semen collection. Furthermore, Nazare et al. (2017) **[66]** found out that the presence of *Burkholderia cepacia* in extended semen was caused by one of the personnel members that had a respiratory infection and unintentionally contaminated the extender. In this study, it is hypothesized that *Burkholderia cepacia* was already present in the semen extender, previous to cryopreservation, due to its resistance to antibiotics. Nonetheless, it is not possible to determine the exact source of contamination in the semen extender.

On the other hand, *Staphylococcus* spp. and *Streptococcus* spp. are found on the skin and mucosa of healthy animals as well as in the gut and respiratory tract **[45]**. Thus, the source of contamination could be either the boar or the personnel during semen collection or processing, even after thawing, making it harder to assess the origin without knowing if this bacteria was present in the extended or raw semen of our samples.

Based on the results obtained, there is a high chance that the majority of the bacteria identified were already present in the extended semen and survived the cryopreservation process, since most of the contaminants that were recovered from frozen semen after thawing, are frequently isolated in boar extended semen. Previous studies carried out in other mammals like human **[50]**, stallion **[49]** and bull **[24]** – but not boar – mentioned how bacteria were largely unaffected by cryopreservation. In these studies the authors assert that pathogenic and non-pathogenic bacteria can survive in LN_2 (-196ºC) for a long time and they possess a relatively high cryotolerance **[49]**. This is, due to the fact that ingredients (i.e.: milk, serum, sucrose and other sugars) used in semen extenders and also in embryo culture media act as stabilizers for microorganisms at freezing temperatures. Moreover, the most common CPs applied in cryopreservation (i.e.: glycerol, DMSO, ethylene glycol, propylene glycol…) also not only efficiently protect the sperm cells but also the bacteria and viruses from cryoinjuries **[68]**. Garcia et al. (1981) **[50]**, who carried out a bacteriological evaluation of semen samples from fertile men before and after freezing (to -196ºC) using 10% glycerol as a protective medium, reported the presence of aerobic bacteria in all the ejaculates examined, and practically all bacteria isolated from the fresh ejaculates were also recovered in frozen semen samples. In bull Najee et al. (2012) **[69]** evaluated the contamination of frozen semen from imported bulls by using culture methods and biochemical tests for bacterial identification and also evaluate antibiotic sensitivity of the isolates. In their results a 55% of the samples were contaminated, and they found out that the majority were Gram-negative bacteria, isolating *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa.* Moreover, the antibiotic sensitivity test determined that most of bacteria isolated from frozen semen were resistant to most antibiotics frequently used in semen extenders like Penicillin, Streptomycin, Gentamycin, Trombycin and Amoxicillin in a percentage ~100% and for both bacteria. This resistance is suggested to the excessive use of antibiotics in semen extenders. However, Prado et al. (2014) **[24]** studied the presence of bacteria before and after cryopreservation of bull semen, and reported that in bull there was a reduction of the bacterial contamination in frozen-thawed semen compared to fresh samples. In stallion, Cora et al., 2007 **[49]** mentioned how important it is to verify the microbial contamination in order to find out the chance of transmission of pathology to the mare in AI, especially since stallions' penis and prepuce are inhabited by commensal or even pathogenic bacteria that can contaminate the stallion's ejaculate and the resistance of equine metritis (CEM) to cryopreservation. Unfortunately similar to boar, bacteriological surveillance of frozen semen is little applied in other species. Therefore, the same authors carried out a study to report the current state of knowledge on bacterial contamination in cryopreserved semen stored in liquid nitrogen. They obtained that bacterial contamination happened in 83.6% of the samples and a 3.8% of isolates were not able to be identified with the conventional methods that they employed. The most frequent isolated bacteria were *Micrococcus* spp. (28.8%), *Pseudomonas* spp. (6.3%), *Pseudomonas aeruginosa* (2.5%), *Stenotrophomonas malophila* (6.3%), *Propinibacterium grunulosum* (7.5%), *Clostridium* spp. (3.8%) and fungi (17.6%) **[49]**.

In order to reduce the risk of bacteria contaminants, bacterial control strategies are needed: antibiotics are currently used in semen extenders to control and eliminate bacteria present in the semen, but their elimination cannot be guaranteed since bacteria have developed antibiotic resistance and these bacteria later on can survive to cryopreservation **[47]**. Nowadays, other methods are studied for bacteria removal to reduce the contaminants without creating bacterial resistance such as gradient centrifugation, and AMPs **[48]**. Althouse et al. (2008) **[43]**, due to the existence of various sources of contamination elaborated a general sanitation protocol known as minimum contamination techniques (MCTs) with the purpose of reduce bacterial contamination by the establishment of guidelines for stud personnel, animal housing, handling and laboratory management.

Generally, most of the studies found – as seen in the ones mentioned above – analyzing semen contaminants rely on traditional culturing methods (TCM), using differential culturing to determine the presence and type of bacteria and bacterial identification is done with standard microbiological procedures (i.e.: growth and colonial characteristics, Gram staining, cellular morphology, catalase and oxidase reactions, coagulase test and haemolysin production) **[30**, **70]**. In contrast, the PCR method the samples go directly from the primary enrichment into PCR process, shortening the time results to hours instead of days **[70]**. It is worth mentioning that primer selection plays an important role; in this study some issues have been encountered with the primers that were used did not amplify some the colonies isolated. Other authors have found another way to identify microorganisms contaminating the sample, the method they use is the MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry), which has shown to be easier, faster, and more reliable than analysis with DNA-based methods **[71]**. This technique consists of obtaining a mass spectrum of the microorganism's proteins and peptides – from whole cells, or cell lysates – generated by the measurement of the time that takes for the ions (produced by UV light ionization of the sample) to reach the detector **[71, 72]**. The mass spectrum obtained is then compared and matched with those present in the reference database. Protein profiles, mostly represented by ribosomal proteins, vary considerably and are characteristic for each bacterial species, making possible to determine not just the genus but the species of the bacterium as well as other microorganisms like fungus **[73]**. This methodology could be applied in the swine industry, when monitoring / analyzing semen samples; it is already been applied in the analysis of microorganisms in cryopreserved semen and culture media used in the *in vitro* production of bovine embryos **[71]**. It also showed successful results for microorganism's identification in bovine semen obtained with a short span of time (15 – 20min for sample) **[71]**.

In conclusion, there is a high chance that bacteria can survive the process of cryopreservation. Thus can be a risk for the sow herd and also sperm quality. Among mammals the bacteria species that are recovered from frozen semen differ, but the majority of them are Gram-negative. In order to prevent bacterial contamination hygiene measures need to be taken. Moreover, bacteriological controls of the semen samples before they are used for AI should be done. In addition, a study in swine regarding which bacteria are present in raw, extended and cryopreserved semen would be very useful. It would allow seeing if the same bacteria that are found in raw / extended semen are the same to the ones that survive the cryopreservation process. This would give an idea of which bacteria are more resistant to the antibiotics used in the extender (by comparing raw – extended semen) and to the cryopreservation technique. Moreover, in the case that more bacteria were recovered from frozen semen, it would suggest that there are critical points during the sample's processing in the laboratory as mentioned by Schulze et al. (2016) **[63]**, and not only during its collection. Furthermore, the detection of contaminants through new molecular techniques can provide a more specific, inexpensive and less time consuming results than the routine laboratory techniques for microorganism identification that are more time consuming.

6. CONCLUSIONS

- Bacterial contaminants were recovered in all of semen frozen-thawed, samples indicating that there is a high chance that they survived the cryopreservation process. Yeast was also detected in one sample.

- Most of the bacteria that were isolated after thawing are similar to the ones found in extended boar semen. However, herein the Gram-negative bacteria from the family Entereobactericeae that were identified differed from those species / genera that are found more frequently.

- Gram-negative bacteria (68.75%) are more prevalent than Gram-positive bacteria (31.25%) in frozen-thawed semen.

- The more frequently isolated bacteria in frozen thawed semen were *Staphylococcus* spp., *Streptococcus* spp. and *Burkholderia cepacia,* followed by *Yersinia pseudotuberculosis, Yersinia entereocolitica* and *Pasturella aeurogenes.*

- PCR technique for bacterial identification in frozen-thawed samples provides a specific detection method, although the primers' choice is crucial for its effectiveness. Moreover, the use of bacterial kits for identification can be quite time consuming, but a good complementing tool for identification.

- In order to reduce the risk of bacteria contaminants, bacterial control strategies need to be emphasized during the collection of the semen, processing and after cryopreserving them through periodical bacteriological controls of one straw for male after freezing.

BIBLIOGRAPHY

1. Leon, C. F., & Moeller J. S. (2009). Overview of the world swine and pork production. In Lal, R. (ed.), *Agricultural Science: Encyclopedia of life support systems* (1st ed., pp. 194–197). Oxford, U.K: Eolss.

2. Gerrits, R., Lunney, J., Johnson, L., Pursel, V., Kraeling, R., Rohrer, G., & Dobrinsky, J. (2005). Perspectives for artificial insemination and genomics to improve global swine populations. *Theriogenology*, *63*(2), 283–299. doi: 10.1016/j.theriogenology.2004.09.013

3. Lynn, J. (2017). *All About The Pork Industry*. *WorldAtlas*. Retrieved 17 June 2017, from http://www.worldatlas.com/articles/all-about-the-pork-industry.html

4. *FAO's Animal Production and Health Division: Meat & Meat Products*. (2017). *Fao.org*. Retrieved 17 June 2017, from http://www.fao.org/ag/againfo/themes/en/meat/backgr_sources.html

5. Yeste, M. (2016). Introduction to the special issue on swine reproduction. *Theriogenology*, *85*(1), 2–3. doi:10.1016/j.theriogenology.2015.10.024

6. *Pigmeat - Agriculture and rural development - European Commission*. (2017). *Agriculture and rural development - European Commission*. Retrieved 17 June 2017, from http://ec.europa.eu/agriculture/pigmeat/index_en.htm

7. *Swine Meat Production by Country in 1000 MT CWE - Country Rankings*. (2017). *Indexmundi.com*. Retrieved 17 June 2017, from http://www.indexmundi.com/agriculture/?commodity=swine-meat&

8. Knox, R. (2016). Artificial insemination in pigs today. *Theriogenology*, *85*(1), 83–93. doi: 10.1016/j.theriogenology.2015.07.009

9. Paulino da Costa, E., Assis da Costa, A., Martins Pereira, E., and Guerino Macedo, G. (2011). Artificial insemination in swine. In Manafi, M (ed.), *Artificial insemination in Farm Animals* (1st ed., pp. 95–97). Rijeka, Croatia: Intech Open Access.

10. Morrell, J. (2011). Artificial insemination: Current and Future Trends. In Manafi, M. (ed), *Artificial insemination in farm animals* (1st ed., pp. 1–2). Rijeka, Croatia: Intech Open Access.

11. Morrell, J. (2011). Artificial insemination: Current and Future Trends. In Manafi, M. (ed), *Artificial insemination in farm animals* (1st ed., pp. 3–6). Rijeka, Croatia: Intech Open Access.

12. Bonet, S., Martínez, E., Rodríguez, J., & Barrera, X. (2006). *Manual de técnicas de reproducción asistida en porcino* (1st ed., pp. 185 - 199). Girona, Spain: Universitat de Girona. Servei de Publicacions

13. Casas, R., I. (2010). *A practical approach on boar sperm cryodamage. Morphofunctional and immunocytochemical study of cryopreserved boar sperm intended for use in artificial insemination.* (Unpublished master's thesis). University of Girona, Spain.

14. Ringwelski, J.M. (2013). *The effect of interval between inseminations and semen handling on in-vivo and in-vitro fertility of froze-thawed boar sperm* (Unpublished master's thesis). University of Urbana, Illinois.

15. Gadea, J. (2003). Review: semen extenders used in the artificial insemination of swine. *Spanish Journal Of Agricultural Research*, *1*(2), 17 – 27. doi: 10.5424/sjar/2003012-17

16. Westendorf, P., Richter, L., & Treu, H. (1975). Zur Tiefgefrierung von Ebersperma. Labor-und Besamungsergebnisse mit dem Hülsenberger Pailetten-Verfahren. [Deep freezing of boar sperma. Laboratory and insemination results using the Hülsenberger paillete method]. *Dtsch Tierarztl Wochensch*r, *82*, 261-267.

17. Pursel, V.G., & Johnson, L.A. (1975). Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *Journal of Animal Science*, *40*, 99-102. doi 10.2527/jas1975.40199x

18. Giner, V., M. (2013). *Estudi comparatiu de l'efete de dos diluents de descongelació sobre la qualitat espermática de dosis seminals de porcí criopreservades.* (Unpublished master's thesis). University of Girona, Spain.

19. Roca, J., Gil, M., Hernandez, M., Parrilla, I., Vazquez, J., & Martinez, E. (2004). Survival and Fertility of Boar Spermatozoa After Freeze-Thawing in Extender Supplemented With Butylated Hydroxytoluene. *Journal Of Andrology*, *25*(3), 397-405. doi:10.1002/j.1939-4640.2004.tb02806.x

20. Johnson, L., Weitze, K., Fiser, P., & Maxwell, W. (2000). Erratum to "Storage of boar semen". *Animal Reproduction Science*, *64*(1-2), 133 – 134. doi: 10.1016/s0378-4320(00)00200-1

21. Sánchez Caycho, K. (2016). Importancia de la detección de bacterias en semen porcino destinado a inseminación artificial (IA). *Sector Pecuario: Aves Y Cerdos*, (4), 19 - 22. Retrieved from http://www.revistasectorpecuario.com/assets/revista_sector_pecuario_04_16.pdf

22. Morrell, J. & Wallgren, M. (2014). Alternatives to Antibiotics in Semen Extenders: A Review. *Pathogens*, *3*(4), 934–946. doi: 10.3390/pathogens3040934

23. Kuster, C.E., & Althouse, G.C. (2016). The impact of bacteriospermia on boar sperm storage and reproductive performance. *Theriogenology, 85*(1), 21–6. doi: 10.1016/j.theriogenology.2015.09.049

24. Prado, E., & Pérez, R. (2005). Flora bacteriana del semen de toro antes y después de la congelación. *Revista Electrónica De Veterinaria REDVET*, *5*(10), 1-7. Retrieved from http://www.veterinaria.org/revistas/redvet/n101005/100508.pdf

25. Malmgren L., Olsson E.E., Engvall A., & Albihn A. (1998). Aerobic bacterial flora of semen and stallion reproductive tract and its relationship to fertility under field conditions. *Acta Veteterinaria Scandinavica*, *39,* 173–182. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9787481

26. Moretti, E., Capitani, S., Figura, N., Pammolli, A., Federico, M., Giannerini, V., & Collodel, G. (2008). The presence of bacteria species in semen and sperm quality. *Journal Of Assisted Reproduction And Genetics*, *26*(1), 47-56. doi: 10.1007/s10815-008-9283-5

27. Sannat, C., Nair, A., Sahu, S., Sahasrabudhe, S., Kumar, A., Gupta, A., & Shende, R. (2015). Effect of species, breed, and age on bacterial load in bovine and bubaline semen. *Veterinary World*, *8*(4), 461-466. doi: 10.14202/vetworld.2015.461-466

28. Sa, S., Choi, S., Kim, H., Cho, K., Hong, J., & Kim, D. et al. (2014). Effect of Different Inoculation Concentration of Escherichia coli on Boar Sperm Quality and Reproductive Performance in Sow. *Reproductive & Developmental Biology*, *38*(4), 159–163. doi: 10.12749/rdb.2014.38.4.159

29. Goldberg, A., Argenti, L., Faccin, J., Linck, L., Santi, M., & Bernardi, M. et al. (2013). Risk factors for bacterial contamination during boar semen collection. *Research In Veterinary Science, 95*(2), 362– 367. doi: 10.1016/j.rvsc.2013.06.022

30. Bresciani, C., Cabassi, C., Morini, G., Taddei, S., Bettini, R., & Bigliardi, E. (2014). Boar Semen Bacterial Contamination in Italy and Antibiotic Efficacy in a Modified Extender. *Italian Journal Of Animal Science*, *13*(1), 83–87. doi: 10.4081/ijas.2014.3082

31. Chenoweth, P. & Lorton, S. (2014). Applied Andrology in Water Buffalo: Bacteriospermia and its Control. In Chenoweth, P. and Lorton, S. (ed), *Animal andrology: Theories and Applications* (1st ed., pp. 391– 392). Wallingford, Connecticut: CABI.

32. Althouse, G.C. and Lu, K.G. (2005). Bacteriospermia in extended porcine semen. *Theriogenology, 63(2)*, 573–584. doi: 10.1016/j.theriogenology.2004.09.031

33. Tamuli, M.K., Sharma, D.K., Rajkonwar, C.K. (1984). Studies on the microbial flora of boar semen. *Indian Veterinary Journal*, *68*, 858 – 61.

34. Danwonski, K.M. (1989). *Qualitative and quantitative investigation of the germ content in boar semen and the antibiotic sensivity of the prevailing germ spectrum*. (Published doctoral thesis). Tierarztliche Hochschule, Hannover.

35. Dagnall, G.J.R. (1986). *An investigation of the bacterial flora of the preputial diverticulum and of the semen of boars.* (Published doctoral thesis). Royal Veterinary Collage, Hertfordshire.

36. Sone, M., Kawarasaki, T., Ogasa, A., Nakahara, T. Effect of bacteria-contaminated boar semen on the reproductive performance. (1989). *Journal of Animal Reproduction*, *35*(3), 159 – 164. doi: 10.1262/jrd1977.35.159

37. Gączarzewicz, D., Udała, J., Piasecka, M., Błaszczyk, B., & Stankiewicz, T. (2016). Bacterial Contamination of Boar Semen and its Relationship to Sperm Quality Preserved in Commercial Extender Containing Gentamicin Sulfate. *Polish Journal Of Veterinary Sciences*, *19*(3). doi: 10.1515/pjvs-2016-0057.

38. Althouse, G.C., Kuster, C.E., Clark, S.G. & Weisiger, R.M. (2000). Field investigations of bacterial contaminants and their effects on extended porcine semen. *Theriogenology, 53*(5), 1167 – 1176. doi: 10.1016/s0093-691x(00)00261-2

39. Maes, D., Nauwynck, H., Rijsselaere, T., Mateusen, B., Vyt, P., de Kruif, A. & Van Soom, A. (2008) Diseases in swine transmitted by artificial insemination: an overview. *Theriogenology*, *70*(8), 1337 – 1345. doi: 10.1016/j.theriogenology.2008.06.018

40. Speck, S., Courtiol, A., Junkes, C., Dathe, M., Müller, K., & Schulze, M. (2014). Cationic Synthetic Peptides: Assessment of Their Antimicrobial Potency in Liquid Preserved Boar Semen. *Plos ONE*, *9*(8), e105949. doi: 10.1371/journal.pone.0105949

41. Dalin, A., Kaeoket, K., & Persson, E. (2004). Immune cell infiltration of normal and impaired sow endometrium. *Animal Reproduction Science*, *82-83*, 401-413. doi: 10.1016/j.anireprosci.2004.04.012

42. Morrell, J.M. & Wallgren, M. (2011) Removal of bacteria from boar ejaculates by Single Layer Centrifugation can reduce the use of antibiotics in semen extenders. *Animal Reproduction Science, 123*(1-2), 64 – 69. doi: 10.1016/j.anireprosci.2010.11.005

43. Althouse, G. C. (2008). Sanitary procedures for the production of extended semen. *Reproduction Domestical Animals*, *43*(2), 374 – 378. doi: 10.1111/j.1439-0531.2008.01187.x

44. Yeste, M. (2017). State-of-the-art of boar sperm preservation in liquid and frozen state. *Animal Reproduction Science, 14*(1)*,* 69–81*.* doi:10.21451/1984-3143-ar895

45. Morrell, J. (2017). *Antimicrobials in Boar Semen Extenders A Risk/Benefit Analysis*. *Antimicrobials in Boar Semen Extenders A Risk/Benefit Analysis*. Retrieved 17 July 2017, from https://www.omicsonline.org/open-access/antimicrobials-in-boar-semen-extenders--a-riskbenefit-analysis-antimicro-1000107.php?aid=68711

46. Morrell, J.M., Klein, C., Lundeheim, N., Erol, E. & Troedsson, M.H. (2014). Removal of bacteria from stallion semen by colloid centrifugation. *Animal Reproduction Science*, *145*(1-2), 47 – 53. doi: 10.1016/j.anireprosci.2014.01.005

47. Wang, S., Zeng, X., Yang, Q., & Qiao, S. (2016). Antimicrobial Peptides as Potential Alternatives to Antibiotics in Food Animal Industry. *International Journal Of Molecular Sciences*, *17*(5), 603. doi: 10.3390/ijms17050603

48. Schulze, M., Junkes, Christof., Mueller, P., Speck, S., Ruediger, K., Dathe, M. & Mueller, K. (2014). Effects of Cationic Antimicrobial Peptides on Liquid-Preserved Boar Spermatozoa. *PLoS ONE, 9*(6), e100490. doi: 10.1371/journal.pone.0100490

49. Corona, A., & Cherchi, R. (2009). Microbial quality of equine frozen semen. *Animal Reproduction Science*, *115*(1-4), 103-109. doi: 10.1016/j.anireprosci.2008.11.016

50. Garcia, A., Sierra, M.F. & Friberg, J. (1981). Survival of bacteria after freezing of human semen in liquid nitrogen. *Fertil Steril, 35*, 549–551. doi:10.1016/s0015-0282(16)45499-3

51. *QIAGEN - Discover the virtual QIAcube world*. (2017). *Qiagen.com*. Retrieved 17 June 2017, from https://www.qiagen.com/media/product-tools/flash/qiacube/360/index.html

52. Anbazhagan, D., Gausillia, K. G., Mansor, M., Ong, S.G.Y., Yasmin, Y. M., & Devi, S. S. (2010). multiplex polymerase chain reaction (PCR) assays for the detection of Enterobacteriaceae in clinical samples. *Journal of Clinical Microbiology, 4*(11), 1186-1191. Retrieved from http://www.academicjournals.org/ajmr

53. Sen, K., & Asher, D. (2001). Multiplex PCR for detection of Enterobacteriaceae in blood. *Transfusion*, *41*(11), 1356-1364. doi:10.1046/j.1537-2995.2001.41111356.x

54. Spilker, T., Coenye, T., Vandamme, P. and LiPuma, J. (2004). PCR-Based Assay for Differentiation of Pseudomonas aeruginosa from Other Pseudomonas Species Recovered from Cystic Fibrosis Patients. *Journal of Clinical Microbiology*, 42(5), pp.2074-2079. doi: 10.1128/JCM.42.5.2074–2079.2004

55. Wang, G., Clark, C., & Rodgers, F. (2002). Detection in Escherichia coli of the Genes Encoding the Major Virulence Factors, the Genes Defining the O157:H7 Serotype, and Components of the Type 2 Shiga Toxin Family by Multiplex PCR. *Journal Of Clinical Microbiology*, *40*(10), 3613-3619. doi:10.1128/jcm.40.10.3613-3619.2002

56. Mason, W., Blevins, J., Beenken, K., Wibowo, N., Ojha, N., & Smeltzer, M. (2001). Multiplex PCR Protocol for the Diagnosis of Staphylococcal Infection. *Journal Of Clinical Microbiology*, *39*(9), 3332-3338. doi: 10.1128/JCM.39.9.3332-3338.2001

57. Meiri-Bendek, I., Lipkin, E., Friedmann, A., Leitner, G., Saran, A., Friedman, S. and Kashi, Y. 2002. A PCR-Based Method for the Detection of Streptococcus agalactiae in Milk. J. *Dairy Science*, 85(7):1717-1723. doi: 10.3168/jds.S0022-0302(02)74245-8

58. *E-Gel Electrophoresis System | Thermo Fisher Scientific*. (2017). *Thermofisher.com*. Retrieved 17 June 2017, from https://www.thermofisher.com/es/es/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/e-gel-electrophoresis-system.html

59. BD. (2010). BBL Crystal™ Identification Systems Enteric/Nonfermenter ID Kit: User manual. Retrieved from http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.370.4250&rep=rep1&type=pdf

60. COMITÉ PERMANENTE DEL CONVENIO EUROPEO DE PROTECCIÓN DE LOS ANIMALES EN EXPLOTACIONES GANADERAS (T-AP). (2005) (pp. 1 - 17). Retrieved from http://www.mapama.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/recomendacioncerdos_tcm7-5571.pdf

61. Maroto Martín, L., Muñoz, E., De Cupere, F., Van Driessche, E., Echemendia-Blanco, D., Rodríguez, J., & Beeckmans, S. (2010). Bacterial contamination of boar semen affects the litter size. *Animal Reproduction Science*, *120*(1-4), 95-104. doi: 10.1016/j.anireprosci.2010.03.008

62. Ciornei şt.g., Roşca P., Drugociu D., (2012) – Bacterial and fungal burden in boar semen. *Research Journal of Biotechnology*, Vol. *7*(4), 23 – 27. Retrieved from https://www.researchgate.net/publication/282675759_Bacterial_and_Fungal_Burden_in_Boar_Semen

63. Schulze, M., Ammon, C., Rüdiger, K., Jung, M., & Grobbel, M. (2015). Analysis of hygienic critical control points in boar semen production. *Theriogenology*, *83*(3), 430-437. doi: 10.1016/j.theriogenology.2014.10.004

64. Arrausi-Subiza, M., Gerrikagoitia, X., Alvarez, V., Ibabe, J., & Barral, M. (2015). Prevalence of Yersinia enterocolitica and Yersinia pseudotuberculosis in wild boars in the Basque Country, northern Spain. *Acta Veterinaria Scandinavica*, *58*(1). doi: 10.1186/s13028-016-0184-9

65. Laukkanen, R., Martinez, P., Siekkinen, K., Ranta, J., Maijala, R., & Korkeala, H. (2008). Transmission of Yersinia pseudotuberculosis in the Pork Production Chain from Farm to Slaughterhouse. *Applied And Environmental Microbiology*, *74*(17), 5444-5450. doi: 10.1128/aem.02664-07

66. *Reproductive failure*. (2017). *pig333.com*. Retrieved 2 July 2017, from https://www.pig333.com/clinical-case-of-the-world/reproductive-failure_5452/

67. Darwin, L., & Reicks, D. (2003). Bacterial contamination and semen quality. In *Leman Swine Conference* (pp. 169 - 170). Minnesota: University of Minnesota. Retrieved from https://conservancy.umn.edu/handle/11299/146365

68. Bielanski, A., & Vajta, G. (2009). Risk of contamination of germplasm during cryopreservation and cryobanking in IVF units. *Human Reproduction*, *24*(10), 2457-2467. doi: 10.1093/humrep/dep117

69. Najee H.B, Al-Shawii A.M, Abd-Al Rahman L.Y. Bacterial contamination of imported bulls frozen semen (2012). *Al-Anbar Journal* of *Veterinary Sciences*, *5*(1), 54–60. Retrieved from http://www.iasj.net/iasj?func=fulltext&aId=61760.

70. Bambusch, L. (2017). *Traditional Culture Methods or PCR: Which is Right for You? - InstantLabs*. *InstantLabs*. Retrieved 11 July 2017, from http://instantlabs.com/traditional-culture-methods-or-pcr-which-is-right-for-you/

71. Zampieri, D., Santos, V., Braga, P., Ferreira, C., Ballottin, D., & Tasic, L. et al. (2013). Microorganisms in cryopreserved semen and culture media used in the in vitro production (IVP) of bovine embryos identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). *Theriogenology*, *80*(4), 337-345. doi: 10.1016/j.theriogenology.2013.04.020

72. De Carolis, E., Vella, A., Vaccaro, L., Torelli, R., Spanu, T., & Fiori, B. et al. (2014). Application of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *The Journal Of Infection In Developing Countries*, *8*(09), 1081-1088. doi :10.3855/jidc.3623