BACTERIOSPERMIA AND PORCINE REPRODUCTION: EFFECTS ON SPERM QUALITY AND FERTILITY POTENTIAL OF BOAR SPERMATOZOA

Lilian Sepúlveda Muela

Universitat de Girona

Doctoral Thesis

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Universitat de Girona

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CERTIFIQUEN:

Que la Tesi Doctoral titulada "**Bacteriospermia and porcine reproduction: effects on sperm quality and fertility potential of boar spermatozoa**", presentada per na Liliana Sepúlveda Muela per optar al grau de Doctor per la Universitat de Girona, s'ha dut a terme sota la seva direcció i, considerant aquesta acabada, autoritzen la seva presentació perquè sigui jutjada per la Comissió corresponent.

I, perquè així consti i tingui els efectes oportuns, signen aquest document.

Dr. Sergi Bonet Marull

Dra. Eva Bussalleu Muntada

Girona, juliol de 2016

Als meus **pares** i a la meva **germana**

A tu, **Òscar**

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Caminante, son tus huellas

el camino, y nada más;

caminante, no hay camino:

se hace camino al andar.

Al andar se hace camino,

y al volver la vista atrás

se ve la senda que nunca

se ha de volver a pisar.

Caminante, no hay camino,

sino estelas en la mar.

Antonio Machado

List of Papers

This Thesis Dissertation is presented as a compendium of three papers:

PAPER I

Sepúlveda, L., Bussalleu, E., Yeste, M., Torner, E., and Bonet, S. 2013. **How do different concentrations of *Clostridium perfringens* affect the quality of extended boar spermatozoa?** *Animal Reproduction Science* 140: 83– 91. (doi: 10.1016/j.anireprosci.2013.04.013).

Animal Reproduction Science has an impact factor of **1.897** and it is situated in the **first quartile** in the Agriculture, Dairy & Animal Science category (2012 Journal Citation Reports® Science Edition).

PAPER II

Sepúlveda, L., Bussalleu, E., Yeste, M., and Bonet, S. 2014. **Effects of different concentrations of *Pseudomonas aeruginosa* on boar sperm quality.** *Animal Reproduction Science* 150(3-4):96-106. (doi: 10.1016/j.anireprosci.2014.09.001).

Animal Reproduction Science has an impact factor of **1.581** and it is situated in the **first quartile** in the Agriculture, Dairy & Animal Science category (2013 Journal Citation Reports® Science Edition).

PAPER III

Sepúlveda, L., Bussalleu, E., Yeste, M., and Bonet, S. 2016. **Effect of *Pseudomonas aeruginosa* on sperm capacitation and protein phosphorylation of boar spermatozoa**. *Theriogenology* 85(8):1421-31 (doi: 10.1016/j.theriogenology.2015.12.025)

Theriogenology has an impact factor of **1.798** and it is situated in the **first quartile** in the Veterinary Sciences category (2014 Journal Citation Reports® Science Edition).

Abbreviations & Symbols

µL	Microlitre (10^{-3} mL)
µM	Micromolar
µm	Micrometre (10^{-3} mm)
Abs	Antibodies
ACRBP	Acrosin-binding protein
AI	Artificial insemination
AIJ	Ampullary-isthmic junction
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
AR	Acrosome reaction
BCF	Beat cross frequency
BME or β-met	2-Mercaptoethanol, β-mercaptoethanol or 2-Hydroxy-1-ethanethiol
BSA	Bovine serum albumine
BTS	*Beltsville* thawing solution
ºC	Degree Celsius
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphat
CASA	Computer assisted sperm analysis
CFU	Colony forming units
CM	Capacitating medium
CO_2	Carbon dioxide
CP	Central pair of microtubule doublets of the axoneme
DPBS	*Dulbecco's* Phosphate Buffered Saline without calcium and magnesium
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (examples)
et al.	*et alii* (and others)
etc.	*Et cetera* (and other things)
EU	European Union
EV	Electronic volume
FAOSTAT	Statistics Division of the Food and Agriculture Organization of the United Nations
Fig.	Figure
FL1, FL2, FL3	Flow cytometer photodetectors 1, 2 and 3
FSC	Forward-scatter
g	Gram
x *g*	Centrifuge force (g-force units)
h	Hour(s)
HCO^{3-}	Bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
i.e.	*id est* (that is)
IVC	*In vitro* capacitation
IVF	*In vitro* fertilisation
KCl	Potassium chloride
kDa	Kilodalton
Kg	Kilogram
L	Litre
LC	Fibrous sheath
LIN	Linearity index percentage
LN	Liquid nitrogen
LPS	Lipopolysaccharides
M540	Merocyanine 540
MCTs	Minimum contamination techniques
mg	Milligram
M-II	Metaphase II
min	Minutes
mL	Millilitre
mm	Millimetre
mM	Millimolar (mmol/litre)
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
MS	Mitochondrial sheath
MW	Molecular weight
NaCl	Sodium chloride
NaF	Sodium fluoride
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Disodium hydrogen phosphate

$Na_3C_6H_5O_7 \cdot 2H_2O$	Sodium citrate tribasic dihydrate
Na_3VO_4	Sodium orthovanadate
ODF	Outer dense fibres
OEC	Oviductal epithelial cells
OMDA	Outer microtubule doublets of the axoneme
p32 or sp32	Acrosin-binding protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
pH	Power of hydrogen
PI	Propidium iodide
PKA	Adenyl cyclase cAMP-dependent protein kinase A
PM	Plasma membrane
PMOT	Percentage of spermatozoa with a progressive motility
PMSF	Phenylmethylsulfonyl fluoride
PPs	Protein phosphatases
PS	Phosphatidylserine
pSer	Protein phosphorylation in serine residues (phosphoserine)
pThr	Protein phosphorylation in threonine residues (phosphothreonine)
PTK	Protein tyrosine kinases
pTyr	Protein phosphorylation in tyrosine residues (phosphotyrosine)
PVA	Polyvinyl alcochol
PVDF	Polyvinylidene fluoride
QS	*Quorum sensing*
UTJ	Utero-tubal junction
s *or* sec	Seconds
SACY	Soluble adenylyl cyclase
SAF	Sperm agglutinating factor
Ser	Serine amino acid
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SIF	Sperm immobilising factor
Spz	Spermatozoa/ spermatozoon
SSC	Side scatter
STR	Straightness index percentage
SYBR®-14	Commercial name of nucleic acid synthetic dyes
TBS	Tris buffer saline
TBST	Tris buffer saline + Tween 20
Thr	Threonine amino acid
ToxA	Exotoxin A
TR	Transverse ribs
TRIS	Tris(hydroxymethyl)aminomethane
T3SS	Type III secretion system
Tyr	Tyrosine amino acid
TMOT	Percentage of total sperm motility
v	Volume
VAP	Average velocity
VCL	Curvilinear velocity
VSL	Straight-linear velocity
w	Weight
WOB	Oscillation index percentage
YP1	Yo-Pro-1
ZP	Zona pellucida

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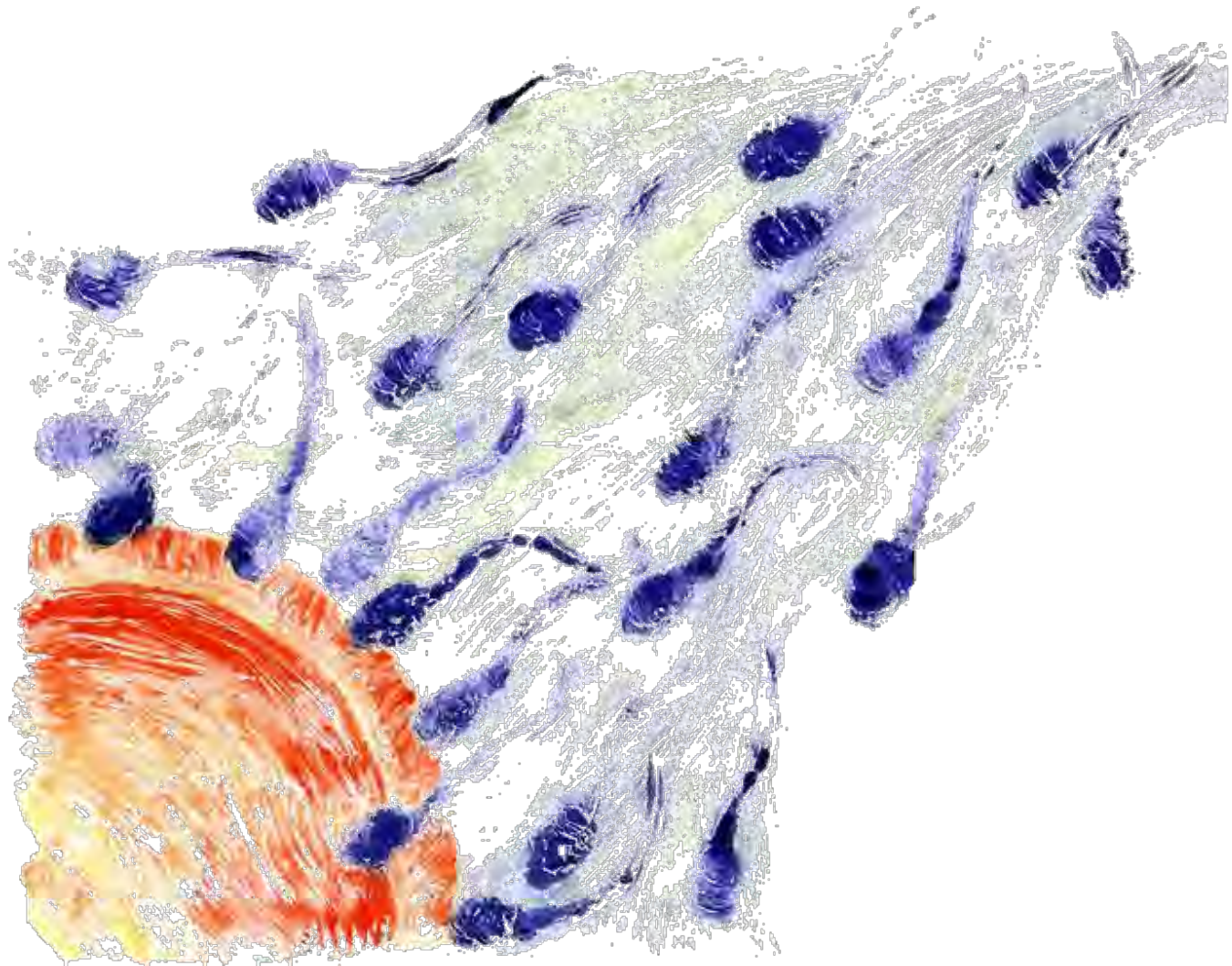


Abstract

The present Thesis Dissertation is focused upon one of the main concerns to pig breeders: the presence of contaminant bacteria in boar semen. The implementation of artificial insemination (AI) in the swine industry has exponentially grown over the last decades, as it exhibits several advantages such as low boar-to-sow ratio and, since direct contact between pigs is avoided, a better sanitary control of boar semen can be achieved. However, the same advantages that initially prompted AI development can also imply negative aspects. Despite the application of strict hygienic measures during collection and manipulation processes, boar semen normally harbours a broad range of microorganisms. The effects of such contamination have been reported to highly impair boar sperm quality, like sperm motility, morphology, and acrosome and membrane integrity, and to have repercussions on the recipient sow and the reproductive performance. The majority of these reports are mainly focused on the impact of enterobacteria as bacterial contaminants, since bacteria belonging to the family *Enterobacteriaceae* are the most frequently isolated bacteria from boar semen; nevertheless, not only enterobacteria can be found in boar semen, but a great variety of other bacteria can also be commonly detected, including anaerobes, although their effects on boar sperm have not been largely studied. Therefore, since in AI one ejaculate is used to inseminate several sows, bacterial contamination in a given ejaculate can cause severe economic losses in the swine industry. Against this background, the present work has been devised to reach three aims: 1) to evaluate the effects that *Clostridium perfringens*, an anaerobic bacterium highly prevalent in the swine industry, causes on boar semen during storage at 15ºC (common storage temperature of boar semen) and incubation at 37ºC (to mimic the approximate body temperature); 2) to assess the consequences that contamination with *Pseudomonas aeruginosa*, an aerobic bacterium that is commonly isolated from boar semen, causes upon boar sperm stored at 15-17ºC; and 3) to determine the effects of *P. aeruginosa* on the ability of boar sperm to achieve their fertilising competence.

For aims 1 and 2, semen samples were inoculated with different concentrations (10^2-10^8 CFU/mL) of the corresponding bacteria, and then incubated at 15ºC-17ºC **(PAPERS I and II)** and 37ºC **(PAPER I)**. Regarding the effects of *C. perfringens* on boar sperm quality during 11 days at 15ºC and 96h at 37ºC (aim 1), results obtained showed a decrease ($P < 0.05$) in the percentage

of total sperm motility, progressive motility and viability of boar spermatozoa especially at the highest infective concentrations (10^7 and 10^8 CFU/mL). This bacterial damaging effect, however, was not observed in the case of sperm morphology. Similar results were also obtained for the second aim (**PAPER II**); in comparison to values obtained for the negative control, *P. aeruginosa* also caused significant ($P < 0.05$) reductions in sperm viability, sperm motility, and sperm's acrosome membrane integrity at the highest bacterial concentrations ($2x10^7$ and $2x10^8$ CFU/mL) throughout the 11 days of storage at 15-17ºC. These deleterious effects provoked by *P. aeruginosa* were caused without an acidic environment, as seminal pH in all treatments remained similar ($P > 0.05$) to the negative control.

Since the effects of bacterial contamination on boar sperm capacitation were yet to be reported, the third paper (aim 3) sought to evaluate the effects of different concentrations of *P. aeruginosa* ($3x10^4$–$3x10^8$ CFU/mL) on different indicators of the capacitation status after *in vitro* capacitation (IVC). With this purpose, different loads of *P. aeruginosa* were inoculated to boar sperm in a capacitating medium and co-incubated for 3h at 38.5ºC 5% CO_2. Results obtained by flow cytometry and computer assisted sperm analysis (CASA) demonstrated that *P. aeruginosa* causes a significant ($P < 0.05$) and concentration-dependent decrease in the percentage of viable sperm with low membrane lipid disorder, as well as in all motility parameters assessed during the IVC procedure. Furthermore, SDS-PAGE and immunoblot analyses revealed that the presence of this bacterium also led to a reduction in the levels of tyrosine, serine and threonine protein phosphorylation, again in a concentration-dependent manner. The reduction observed in the tyrosine phosphorylation levels of p32, a well-known indicator of the capacitation status in boar sperm, was the most remarkable variation observed. Unlike the aforementioned results obtained in **PAPER II**, all changes caused by *P. aeruginosa* upon boar sperm capacitation were not only observed at the highest infective concentrations (i.e. 10^7-10^8 CFU/mL), but rather at far lower bacterial loads ($3x10^4$-10^6 CFU/mL).

Altogether, this Thesis demonstrates that the presence of *P. aeruginosa* (>10^4-10^6 CFU/mL) in boar semen negatively affects their longevity and potential fertilising ability, since different sperm quality parameters, as well as the ability of boar sperm to become fully capacitated, are jeopardised. In addition, these results also emphasise the importance of applying hygienic measures during the processes of collection and manipulation of boar semen, as much as the evaluation of the bacterial load in doses intended to AI to determine the shelf-life storage of such doses and avoid the usage of those with low sperm quality.

Resum

La present Tesis doctoral se centra en un dels principals problemes que la indústria porcina ha d'afrontar: la presència de bacteris contaminants en el semen de porcí. Durant les últimes dècades l'ús de la inseminació artificial (IA) en el sector porcí ha crescut exponencialment, atès que presenta diversos avantatges respecte a la munta natural. D'entre aquests avantatges cal destacar que la IA utilitza una baixa proporció de mascles envers les femelles, i que amb aquesta tecnologia s'evita el contacte directe entre animals; així doncs, es pot controlar millor l'estat sanitari del semen porcí. Tanmateix, els mateixos avantatges que han permès el desenvolupament de la IA també poden presentar aspectes negatius. Malgrat l'aplicació de mesures higièniques durant els processos de recol·lecció i manipulació del semen de porc, aquest acostuma a contenir un ampli ventall de microorganismes. Diversos estudis han demostrat que aquest tipus de contaminació provoca greus danys sobre la qualitat espermàtica, afectant, per exemple, a la motilitat, la morfologia, i a la integritat de membrana i de l'acrosoma de l'espermatozoide; també s'ha vist pot tenir repercussions negatives sobre les femelles receptores i el rendiment reproductiu. La gran majoria d'aquests estudis s'han centrat en analitzar l'impacte que tenen els enterobacteris com a contaminants bacterians, degut a que els bacteris pertanyents a la família *Enterobacteriaceae* són els que es troben més freqüentment en les ejaculacions de porcí; nogensmenys, en el semen de porcí no només s'hi troben enterobacteris, sinó que s'acostuma a detectar-hi una gran varietat de bacteris, incloent fins i tot bacteris anaerobis, els efectes dels quals no han estat gaire estudiats. Per tant, com que en la IA una mateixa ejaculació és utilitzada per inseminar diverses truges, la contaminació bacteriana d'una ejaculació en qüestió pot provocar gran pèrdues econòmiques a la indústria porcina. Seguint aquest context, el present treball ha estat dissenyat per tal d'assolir tres objectius: 1) avaluar els efectes que *Clostridium perfringens*, un bacteri anaerobi amb una elevada prevalença dins de la indústria porcina, provoca sobre el semen de porc a 15ºC (temperatura típica d'emmagatzematge del semen porcí) i a 37ºC (per mimetitzar la temperatura corporal aproximada); 2) analitzar les conseqüències que la contaminació per *Pseudomonas aeruginosa*, un bacteri aerobi comunament aïllat en el semen de porc, causa sobre els espermatozoides de porcí emmagatzemats a 15-17ºC; i 3) determinar els efectes que té *P. aeruginosa* sobre la capacitat de l'espermatozoide de porcí d'adquirir la seva capacitat fecundant.

Per tal d'assolir els objectius 1 i 2, diferents mostres seminals van ser inoculades amb diferents concentracions (10^2-10^8 UFC/mL) del bacteri corresponent, i posteriorment incubades a 15-17ºC (ARTICLES I i II) i a 37ºC (ARTICLE I). En referència als efectes que *C. perfringens* causa sobre la qualitat espermàtica durant 11 dies a 15ºC i 96h a 37ºC (objectiu 1), els resultats obtinguts mostren una reducció ($P < 0.05$) del percentatge de motilitat espermàtica total, motilitat progressiva i de viabilitat espermàtica, especialment evident a altes concentracions bacterianes (10^7-10^8 UFC/mL). A diferència d'aquests paràmetres, la morfologia espermàtica no es va veure afectada per la presència del bacteri. Pel que fa al segon objectiu, es van obtenir uns resultats molt similars als obtinguts amb *C. perfringens*. Els resultats assolits amb *P. aeruginosa* mostren que aquest bacteri, en comparació als valors del control negatiu, també causa una reducció significativa ($P < 0.05$) de la viabilitat i la motilitat espermàtiques, així com de la integritat de l'acrosoma dels espermatozoides durant els 11 dies d'emmagatzematge a 15-17ºC. Aquests efectes van ser notables, de nou, a altes concentracions bacterianes ($2x10^7$ i $2x10^8$ UFC/mL). A més, també s'ha demostrat que aquest efecte nociu de *P. aeruginosa* no es produeix a causa d'una acidificació del medi, atès que el pH seminal de tots els tractaments avaluats es va mantenir amb valors similars ($P > 0.05$) al del control negatiu durant tot el període experimental.

Com que els efectes que té la contaminació bacteriana sobre la capacitació espermàtica de porcí no han estat avaluats amb anterioritat, el tercer objectiu (ARTICLE III) d'aquesta Tesi és determinar els efectes que tenen diferents concentracions de *P. aeruginosa* ($3x10^4$–$3x10^8$ CFU/mL) sobre diferents indicadors de l'estat de capacitació de l'espermatozoide de porc després d'un procés de capacitació *in vitro* (CIV). Per tal d'assolir aquest objectiu, es van inocular diferents concentracions infectives de *P. aeruginosa* a mostres espermàtiques diluïdes amb un medi de capacitació, i posteriorment van ser incubades durant 3h a 38.5ºC i 5%CO_2. Els resultats obtinguts per citometria de flux i per l'anàlisi computeritzada de l'esperma (CASA) demostren que aquest bacteri causa una disminució significativa ($P < 0.05$), i dependent de la concentració, del percentatge d'espermatozoides viables amb un baix desordre lipídic de membrana, així com dels diferents paràmetres de motilitat avaluats durant el procés de CIV. Tanmateix, les anàlisis per SDS-PAGE i western blot revelaren que *P. aeruginosa* també provoca reduccions dels nivells de fosforilació proteica en residus de tirosina, serina i treonina en funció de la concentració bacteriana. Quant als canvis en els nivells de fosforilació, la variació més important causada per aquest bacteri és la reducció de la fosforilació en tirosines de la proteïna p32, un conegut indicador de l'estat de capacitació espermàtica en porcí. A diferència dels resultats prèviament

obtinguts per aquest bacteri (ARTICLE II), els efectes de *P. aeruginosa* sobre la capacitació són evidents a partir de concentracions bacterianes de $3x10^4$-10^6 UFC/mL.

En conjunt, aquesta Tesi demostra que la presència de *P. aeruginosa* (>10^4-10^6 UFC/mL) en el semen de porcí afecta negativament tant la longevitat com la fertilitat potencial de l'espermatozoide porcí, ja que afecta indistintament a diferents paràmetres de qualitat espermàtica i a la capacitat dels espermatozoides per assolir el seu complet estat de capacitació. Addicionalment, aquests resultats també emfatitzen la importància de l'aplicació de mesures d'higiene durant els processos de recol·lecció i manipulació del semen de porc, així com la necessària avaluació de la càrrega bacteriana en aquelles dosis destinades a ser utilitzades per IA, per tal de limitar el seu temps d'emmagatzematge i evitar l'ús de dosis amb baixa qualitat espermàtica.

Resumen

La presente Tesis doctoral se centra en uno de los principales problemas con los que industria porcina tiene que lidiar: la presencia de contaminantes bacterianos en el semen de porcino. Durante las últimas décadas el uso de la inseminación artificial (IA) en la industria porcina ha crecido de manera exponencial, debido a que ésta presenta diversas ventajas frente a la monta natural. De entre estas ventajas cabe destacar que en la IA se utiliza una baja proporción de machos frente a hembras y que, debido a que esta tecnología evita el contacto directo entre animales, se puede llevar a cabo un mejor control sanitario del semen de cerdo. Sin embargo, estas mismas ventajas que han permitido el desarrollo de la IA también presentan aspectos negativos. A pesar de la aplicación de mesuras higiénicas durante los procesos de recolección y manipulación del semen porcino, éste acostumbra a estar contaminado por un amplio abanico de microorganismos. Diversos estudios han demostrado que este tipo de contaminación provoca graves daños sobre la calidad espermática, afectando, por ejemplo, a la motilidad, a la morfología, y a la integridad de membrana y del acrosoma del espermatozoide; también se ha visto que puede tener repercusiones negativas sobre las hembras receptoras y el rendimiento reproductivo. La gran mayoría de estos estudios se han centrado en analizar el impacto que tienen las enterobacterias como contaminantes bacterianos, dado que las bacterias pertenecientes a la familia *Enterobacteriaceae* son las que se encuentran más comúnmente en los eyaculados porcinos; sin embargo, en el semen de cerdo no solamente se detectan enterobacterias, sino también otro tipo de bacterias, incluso bacterias anaerobias, los efectos de las cuales no han sido muy estudiados. Por consiguiente, puesto que en la IA un mismo eyaculado es usado para inseminar diversas cerdas, la presencia de contaminación bacteriana en un eyaculado en cuestión puede provocar grandes pérdidas económicas en la industria porcina. Siguiendo este contexto, el presente trabajo ha sido diseñado para lograr tres objetivos: 1) analizar los efectos que *Clostridium perfringens*, una bacteria anaerobia altamente presente dentro de la industria porcina, provoca sobre el semen de cerdo a 15ºC (temperatura típica de almacenaje del semen porcino) y a 37ºC (para mimetizar la temperatura corporal aproximada); 2) examinar las consecuencias de la contaminación seminal con *Pseudomonas aeruginosa*, una bacteria aerobia frecuentemente presente en el semen de cerdo, sobre el esperma porcino almacenado a 15-17ºC; y 3) determinar los efectos que tiene *P. aeruginosa* sobre la capacidad del espermatozoide de cerdo de adquirir su capacidad fecundante.

A fin de alcanzar los objetivos 1 y 2, diferentes muestras seminales fueron inoculadas con distintas concentraciones (10^2-10^8 UFC/mL) de la bacteria correspondiente e incubadas a 15-17ºC (**ARTÍCULOS I y II**) y a 37ºC (**ARTÍCULO I**). En referencia a los efectos que *C. perfringens* causa sobre la calidad espermática durante 11 días a 15ºC y 96h a 37ºC (objetivo 1), los resultados obtenidos muestran una reducción (P <0.05) del porcentaje de motilidad espermática total, de motilidad progresiva, y de viabilidad espermática, especialmente evidente a altas concentraciones bacterianas (10^7-10^8 UFC/mL). Contrariamente a estos resultados, la morfología espermática no se vio afectada por la presencia de las bacterias. En lo relativo al segundo objetivo, los resultados obtenidos fueron muy similares a aquellos conseguidos con *C. perfringens.* Los resultados alcanzados con *P. aeruginosa* muestran que esta bacteria, en comparación con los valores del control negativo, también causa una significativa (P < 0.05) reducción en los parámetros de viabilidad y motilidad espermáticas, así como de la integridad del acrosoma de los espermatozoides durante los 11 días de almacenaje a 15-17ºC. De nuevo, estos efectos fueron evidentes a altas concentraciones bacterianas ($2x10^7$ y $2x10^8$ UFC/mL). Además, los resultados del **ARTÍCULO II** también demostraron que los efectos de *P. aeruginosa* no eran producidos a causa de una acidificación del medio, dado que el pH seminal de todos los tratamientos analizados se mantuvo similar (P > 0.05) al del control negativo durante todo el período experimental.

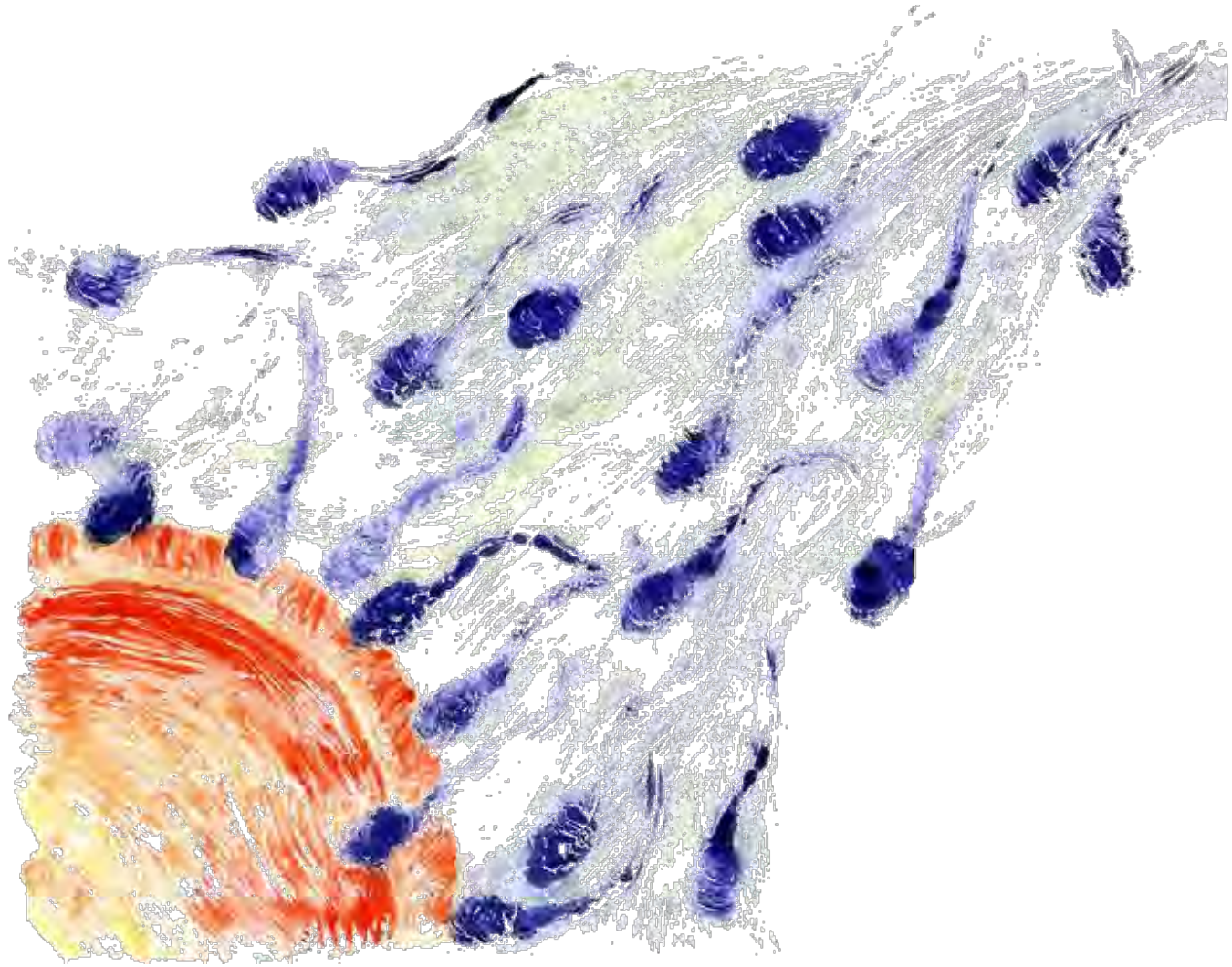
Puesto que los efectos que tiene la contaminación bacteriana sobre la capacitación espermática en porcino no han sido evaluados con anterioridad, el tercer objetivo (**ARTÍCULO III**) de esta Tesis pretende determinar los efectos que tienen diferentes concentraciones de *P. aeruginosa* ($3x10^4$–$3x10^8$ CFU/mL) sobre distintos indicadores del estado de capacitación del esperma de cerdo después de un proceso de capacitación *in vitro* (CIV). Por esta razón, diferentes concentraciones infectivas de *P. aeruginosa* fueron inoculadas a distintas muestras espermáticas diluidas en un medio de capacitación para posteriormente ser incubadas durante 3h a 38.5ºC y 5% CO_2. Los resultados obtenidos mediante citometría de flujo y el análisis computarizado del semen (CASA) demuestran que esta bacteria provoca una disminución significativa (P < 0.05) y dependiente de la concentración del porcentaje de espermatozoides viables con un bajo desorden lipídico de membrana, así como de los diferentes parámetros de motilidad evaluados durante el proceso de CIV. Asimismo, los análisis por SDS-PAGE y western blot revelaron que *P. aeruginosa* también provoca reducciones de los niveles de fosforilación proteica en residuos de tirosina, serina y treonina en función de la carga bacteriana. En lo concerniente a los cambios de los niveles de fosforilación proteica, la variación más significativa

causada por esta bacteria es la reducción de la fosforilación de tirosinas de la proteína p32, un conocido indicador del estado de capacitación espermática en porcino. A diferencia de los resultados previamente obtenidos para esta bacteria (**ARTÍCULO II**), los efectos de *P. aeruginosa* sobre la capacitación son notorios a partir de concentraciones bacterianas de $3x10^4$-10^6 UFC/mL.

En conjunto, la presente Tesis demuestra que la presencia de *P. aeruginosa* (>10^4-10^6 UFC/mL) en semen de cerdo afecta negativamente tanto la longevidad como la fertilidad potencial del esperma de cerdo, debido a la afectación de diferentes parámetros de calidad espermática y de la capacidad de los espermatozoides de lograr su completo estado de capacitación. Adicionalmente, estos resultados también resaltan la importancia que tienen tanto la aplicación de medidas de higiene durante los procesos de recolección y manipulación del semen de cerdo, como la necesaria evaluación de la carga bacteriana en aquellas dosis destinadas a ser utilizadas para IA, con tal de limitar su tiempo de almacenado y evitar el uso de dosis con una baja calidad espermática.

SECTION I

BIBLIOGRAPHIC REVISION



Thesis Outline

The present Thesis project is focused on the impact that bacterial contamination, a major concern to swine industry, causes on boar sperm quality and reproductive performance. It is written as an article compendium and divided into three main sections, including the set of papers and the concluding remarks. It also provides a summary and its translation into Catalan and Spanish.

The **first section** is an extensive bibliographic revision of the main points concerning this Thesis: 1) swine industry, 2) porcine reproduction, and 3) bacteriospermia. In this section, the main objectives of the present Thesis are also described.

The **second section** describes the methodology, as well as the results obtained in the present investigation. This section comprises three different papers, each one organised as follows: a specific introduction related to the aims of the study; the material and methods used; the results obtained; and a detailed discussion together with the conclusions of the paper.

Paper I studies how different concentrations of the anaerobe bacterium *Clostridium perfringens* affect boar sperm quality during storage at 15ºC and incubation at 37ºC.

Paper II describes the effects that different loads of *Pseudomonas aeruginosa* provoke on sperm motility, sperm viability, acrosome integrity and pH of extended boar semen, as well as its growth dynamics during storage at 15ºC.

Paper III is focused on determining the impact that contamination with *Pseudomonas aeruginosa* has on the fertilising ability of boar sperm. Particularly, it describes the effects that this bacterium has on the motility and viability of boar sperm incubated under capacitating conditions, along with its repercussion on membrane integrity and protein phosphorylation of these sperm during and after *in vitro* capacitation.

The **third section** of this Thesis dissertation deals with the most important findings obtained in these three aforementioned papers and discusses them as a whole; it also summarises the main conclusions derived from this Thesis project.

Finally, at the end of this Thesis, the reference list is found.

Bibliographic Revision

1. GENERALITIES ON SWINE INDUSTRY

1.1. The domestic pig

The domestic pig (*Sus scrofa scrofa* or *Sus scrofa domesticus*; Linnaeus, 1758) is one of the animals belonging to the genus *Sus* within the *Suidae* family. Its domestication dates back to ~9000 years before present, originating from the Eurasian wild boar (*Sus scrofa*) (Giuffra *et al.*, 2000). Particularly, these mammals follow an omnivorous diet and, depending on the breed, adults can range from 50 to 350 Kg of weight and 0.9 to 1.5 m height. The actual life span of domestic pig can fluctuate from 6 to 15 years, even though their mean breeding life is only of 3-4 years (Casas, 2010).

Regardless the existence of hundreds of different pig breeds throughout the world, such as Large-White, Landrace or Duroc, all sperm samples used in the present Thesis came from Piétrain boars (Figure 1).

Figure 1. Piétrain boar

Piétrain breed owes its name to a small village located in the province of Barbant (Belgium), and, although its exact origin remains controversial, it started becoming popular during the 1950's, especially due to the quality of its meat. Piétrain boars are very muscular, so they generate high percentages of lean meat with a low fat index; the mean yield is around 72-73%; the percentage of ham and loin is about 56-58%, and the thickness of dorsal fat has, approximately, 20 mm. On the contrary, although being prolific, this breed has a low reproductive performance (around 10

piglets are born alive, and only 7-8 are weaned, approximately), and poor milk production due to the under-developed mammary glands of the sows. Moreover, their heart/body ratio is very low; such characteristic frequently causes heart failure, which leads to a higher mortality rate when compared to other pig breeds. For all these reasons, Piétrain boars are used in cross-breeding programmes using sows from other breeds, such as Hampshire, Duroc and hybrids to improve carcass quality (Buxadé, 1984).

1.2. Pork industry

Nowadays, pork meat is the most consumed meat in many countries. In fact, according to the *Statistics Division of the Food and Agriculture Organization of the United Nations* (FAOSTAT), in 2013 pork meat accounted for the 36.42% of all meat produced all over the world, being the top producers Asia (57%) and Europe (24%); in the latter case, Germany and Spain were the first and second largest pig producers, respectively (**Figure 2**). Actually, Spain was placed in forth position of worldwide pig meat production, preceded only by China, USA and Germany (**Figure 3**).

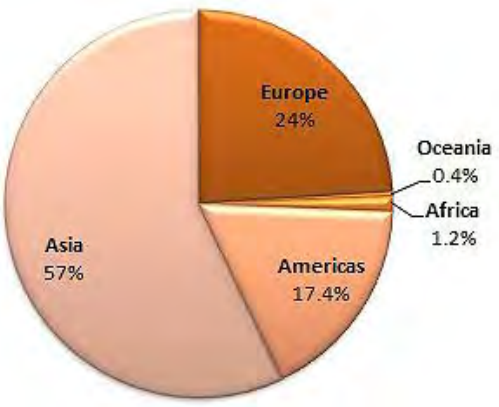


Figure 2. Pig meat production in 2013. (Modified from FAOSTAT. August 2015. Available via: <http://faostat3.fao.org/browse/Q/QL/E>).

Since pork industry is the highest meat sector, efficient pork production with high quality standards is of very importance, especially in those countries where swine industry is a primary income. Consequently, research in new pig breeding biotechnologies to improve quality traits, as well as the study of those factors that currently cause economic loses, such as bacterial

contamination, are needed with the main goal of improving pork quality for its selling and consumption.

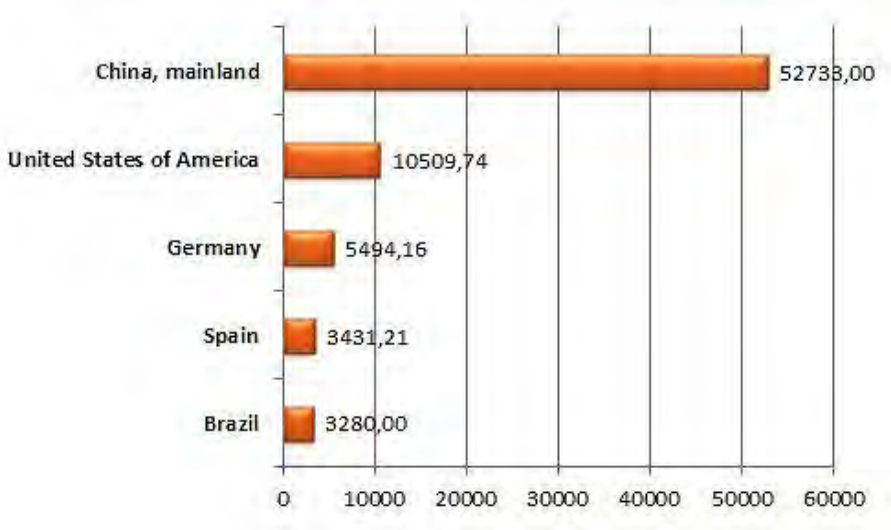


Figure 3. Largest worldwide pig producers in 2013. Numbers mean 1000 tonnes of carcass weight. (Modified from FAOSTAT. August 2015. Available via: <http://faostat3.fao.org/browse/Q/QL/E>).

2. PORCINE REPRODUCTION

2.1. Boar spermatozoa

The boar spermatozoon is an elongated and highly specialised haploid cell of approximately 45 µm long divided into three different morphological regions: the head, the connecting piece (neck) and the tail. Each region is related to different functions and are all surrounded by a continuous lipid bilayer called plasma membrane (Bonet *et al.*, 2000) (**Figure 4A**).

2.1.1. The head

The **head**, oval and bilaterally flattened, measures around 7 µm long, 3.7 µm wide and 0.4 µm thick. The two faces of the head are not identical: while one side is completely flat, the other presents a half moon-shaped apical protuberance of 0.4 µm wide and 1.2 µm long. Due to its tiny dimensions, the sperm head contains only two organelles: the nucleus and the acrosome.

Likewise, the disposition of the acrosome divides the sperm head into two major regions: the acrosomal region (anterior part of the head), and the post-acrosomal region (posterior part of the head) (Bonet *et al.*, 2000; Briz and Fàbrega, 2013).

The *nucleus*, which comprises most of the sperm head, contains highly condensed and electron-dens chromatin fibres. Despite being 6.6 µm long, its thickness varies from 220 nm in the acrosomal region to 320 nm in the post-acrosomal region, due to the absence of the acrosome in the latter region (Briz and Fàbrega, 2013).

The *acrosome* is a specialised cellular vesicle originated from the Golgi complex, and is located in the acrosomal region and over the nucleus, covering around the 80% of its length. The acrosomal region is divided into three distinguishable acrosomal segments: **1)** the apical segment (apical ridge), located in the anterior part of the acrosome, is the most expanded zone of the vesicle; **2)** the principal segment, which encompass the majority of the acrosomal region and, **3)** the equatorial segment, located in the posterior part of the acrosome (Fàbrega, 2012; Briz and Fàbrega, 2013) (**Figure 4B**).

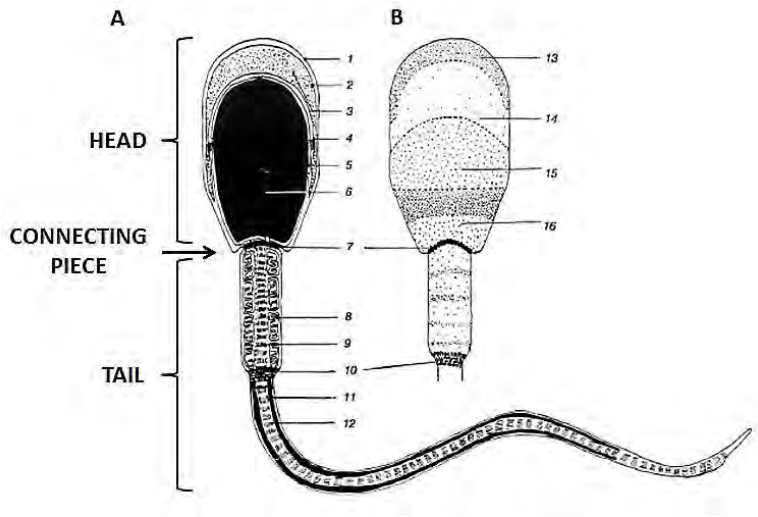


Figure 4. Schematic diagram of boar spermatozoon (modified from Gadella *et al.*, 2008**). (A)** Sectional view of the sperm cell. Solid lines represent membrane bilayers. **1:** plasma membrane; **2:** outer acrosomal membrane; **3:** acrosomal matrix; **4:** inner acrosomal membrane; **5:** nuclear envelope; **6:** nucleus; **7:** connecting piece (neck); **8:** midpiece; **9:** axoneme and mitochondria; **10:** *annulus* (Jensen's ring); **11:** fibrous sheath and **12:** axoneme and outer dense fibres. **(B)** Surface view of sperm head subdomains. **13:** apical ridge; **14:** principal segment; **15:** equatorial segment and **16:** post-acrosomal region.

Although the acrosomal membrane is continuous, the region overlying the anterior part of the nucleus is called inner acrosomal membrane, whereas the region in contact with the inner

surface of the plasma membrane is the outer acrosomal membrane (Figure 4A). The acrosomal matrix, enclosed inside both acrosomal membranes, contains a great variety of chemically different molecules, such as mucopolysaccharides, proteins, lipids and, especially, the hydrolytic enzymes, like acrosin, necessary for the sperm to penetrate through the egg membranes and fuse the oocyte (Knobil and Neill, 2005; Fàbrega, 2012; Briz and Fàbrega, 2013). The acrosomal content is released by exocytosis in the so-called *acrosome reaction* (AR) when the sperm gets in contact with the egg (Gadella and Evans, 2011).

2.1.2. The connecting piece

The **connecting piece** (or neck) of the boar spermatozoa is the linking segment between the base of the sperm nucleus and the first mitochondria of the tail. The most important feature of this structure is that here is where the axoneme origins to form the sperm tail. The axoneme consists of two central microtubules surrounded by nine microtubules doublets (9+2); it extends across the full length of the sperm tail (Bonet *et al.*, 2000; Knobil and Neill, 2005; Briz and Fàbrega, 2013).

2.1.3. The tail

The **tail** (flagellum) is the region responsible for propelling the spermatozoon following a helical forward movement, and also for helping the spermatozoon to penetrate the egg coat. It is divided into three distinguishable regions: the midpiece, the principal piece, and the short end piece (Boj *et al.*, 2015) (Figure 5A).

The *midpiece* is characterised by presenting an axonemal structure surrounded by the mitochondrial sheath, which is formed by several elongated mitochondria, helically arranged around the axoneme, responsible for providing the energy needed for promoting the flagellar movement (Bonet *et al.*, 2000; Briz and Fàbrega, 2013) (Figure 5B). This region extends from the connecting piece to the *annulus* (Jensen's Ring), a ring-like structure that separates the midpiece from the main piece and that avoids the displacement of mitochondria (Guan *et al.*, 2009).

The *principal piece* is the longest portion of the tail, covering the area between the *annulus* and the terminal piece. It follows the same structure as the midpiece, with the exception

of the mitochondrial sheath. In this case, the mitochondrial sheath is replaced by the fibrous sheath (Eddy *et al.*, 2003; Turner, 2003) (Figure 5C).

The *end piece* is the last and shortest portion of the sperm tail. It has no accessory cytoskeletal structures; it is only composed of a simple axonemal structure (Briz and Fàbrega, 2013) (Figure 5D).

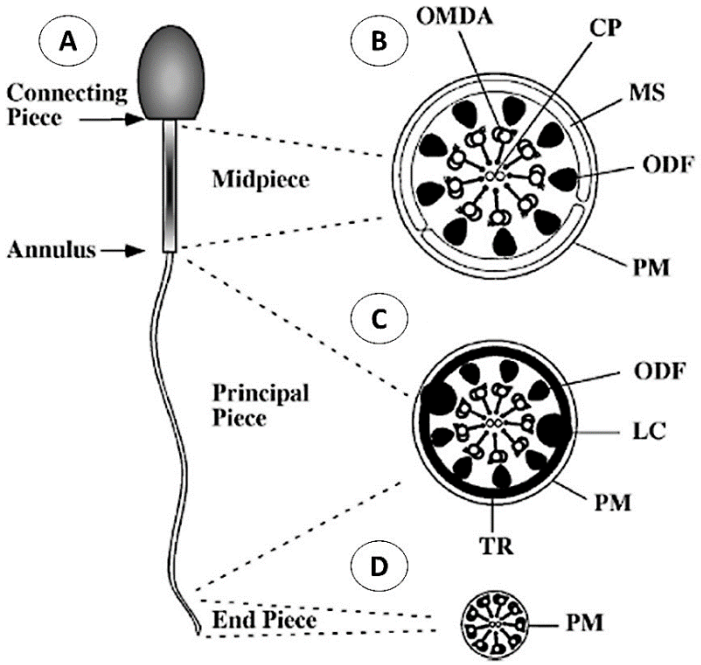


Figure 5. Schematic representation of a mammalian sperm and the ultrastructure of the flagellum (modified from Turner, 2003). **(A)** Regions of the sperm flagellum. **(B)** Schematic cross-section through a representative segment of the midpiece. **(C)** Schematic cross-section through a representative segment of the principal piece. **(D)** Schematic cross-section through a representative segment of the end piece. The 9 outer microtubule doublets of the axoneme (OMDA); central pair of microtubule doublets (CP); mitochondrial sheath (MS); plasma membrane (PM); fibrous sheath (LC); transverse ribs (TR).

2.2. Boar ejaculate

During the process of ejaculation, sperm are released from the epididymis and mixed with secretions from the accessory sex glands, i.e. seminal plasma, thus constituting the boar ejaculate. Seminal plasma contains a broad range of proteins, amino acids, carbohydrates and lipids whose functions are to nourish and maintain sperm survival and metabolism. Boar ejaculates usually vary between 150-300 mL of volume, depending on the individual

characteristics and environmental conditions, have a pH around 7.4 and an osmolality of 290-300 mOsm/Kg, approximately (Yeste, 2008; Casas, 2010; Sancho and Vilagran, 2013).

The ejaculate is commonly divided into three main fractions (Yeste, 2008; Casas, 2010; Fàbrega, 2012; Sancho and Vilagran, 2013):

- **Pre-spermatic fraction**: This fraction is constituted by secretions produced by the prostrate, seminal vesicles and bulbourethral (or Cowper) glands; it lubricates and cleans the urethra for the further sperm passage. Its volume is around 10-15 mL, and normally does not contain spermatozoa.

- **Spermatic rich fraction:** The volume of this fraction is about 70-100 mL, and is composed, mainly, by secretions from the prostate and the seminal vesicles. It also contains a high sperm concentration, ranging between 0.5-1x10^9 spz/mL (80-90% of total sperm), and shows a milky appearance. This fraction is the one used to prepare seminal doses destined to Artificial Insemination (AI).

- **Post-spermatic fraction:** This fraction has a pale white appearance and a low sperm concentration (below 10^6 spz/mL), and consists of, approximately, 150-200 mL of secretions from the prostate, the seminal vesicles and the bulbourethral glands. Accordingly, this fraction contains a large amount of seminal plasma, which acts as a sperm stimulator/activator; however, as sperm cells must achieve a dormant state to better preserve sperm quality traits during storage, it is not recommended to collect this fraction when preparing seminal doses intended to AI. Moreover, the secretion from the bulbourethral glands has a gelatinous consistency, since it is involved in the formation of the "mucous plug" that seals the female genital tract, thus preventing sperm backflow in natural mating (Badia *et al.*, 2006).

2.3. Sperm transport through the female genital tract and storage

In boars, unlike other species, during natural mating and conventional artificial insemination, sperm is deposited directly into the cervix of the sow, so, since the vaginal region is bypassed, the transport through the female reproductive tract is guaranteed (Suarez and Pacey, 2005; Rath *et al.*, 2016). Three crucial events take place inside the female genital tract, specifically in the

oviduct: sperm reservoir, sperm capacitation and fertilisation. Once inside the female reproductive system, either by natural mating or AI, sperm begin their journey to reach the site of fertilisation by passing through different environments and obstacles in order to select the best gametes. Therefore, during this transit, large amounts of sperm are lost due to semen backflow and/or to sperm phagocytosis from polymorphonuclear neutrophils (PMNs) present in the uterus (Katila, 2012; García-Vázquez *et al.*, 2015; Hernández-Caravaca *et al.*, 2015; Rath *et al.*, 2016). Those sperm that overcome these barriers bind to oviductal epithelial cells (OEC) in the utero-tubal junction (UTJ) and caudal isthmus of the oviduct, thereby forming the sperm reservoir. This reservoir provides a safe environment for the spermatozoa, as they are protected from the female immune system, and maintains them viable and uncapacitated for at least 36-40h, until ovulation occurs (Yeste, 2013a; Tienthai, 2015). Once ovulation takes place, spermatozoa are gradually released towards the venue of fertilisation, i.e. the ampullary-isthmic junction (AIJ), in small amounts to prevent polyspermy, and become capacitated (Tienthai *et al.*, 2004; Tienthai, 2015). The environment in the AIJ is lower in cholesterol and higher in albumin and bicarbonate (Harrison *et al.*, 1996), which makes it suitable for sperm capacitation. *In vivo* fertilisation studies have demonstrated that the requirements for sperm capacitation are due to the temporal pattern between mating or AI and fertilisation (Vadnais *et al.*, 2007).

2.4. Sperm capacitation

Ejaculated sperm are unable to fertilise the egg; hence, before reaching the oocyte, sperm must first reside within the female reproductive tract for a period of time (from hours to days) and undergo a sequence of events to acquire its fertilising ability (Yeste, 2013b). This process, known as “Sperm Capacitation” was first described in 1951 in rats and rabbits by Austin and Chang, respectively, in independent investigations (Austin, 1951; Chang, 1951). Sperm capacitation can be defined as the events taking place in both the head and the tail that enable the spermatozoa to gain flagellar hyperactivation, interact with the oocyte zona pellucida (ZP) and undergo acrosome reaction (AR), prerequisites for a proper ZP penetration and fusion with the oolemma (Visconti and Kopf, 1998; Naz and Rajesh, 2004; Gadella *et al.*, 2008; Gadella, 2010; Gadella and Boerke, 2016). In boar, the completion of this process, i.e. when the spermatozoa is able to bind to the ZP and trigger the AR, takes about 2-6h (Imai *et al.*, 1979; Vadnais *et al.*, 2007; Botto *et al.*, 2010; Fàbrega, 2012). Principal events occurring while sperm is under the capacitation process include sperm membrane destabilisation, increase of calcium influx,

hyperactive movement and activation of second messenger pathways (Fàbrega, 2012). Lipid-binding components present in the oviductal fluid cause a remodelling of the plasma membrane architecture due to cholesterol removal, which consequently provokes lipid scrambling and an increase in membrane fluidity. These events, in turn: **1)** trigger the activation of ion channels; **2)** increase the influx of calcium and bicarbonate, with the subsequent increase of cyclic adenosine monophosphate (cAMP) levels; and **3)** activate signalling pathways, such as the sperm-specific adenyl cyclase cAMP-dependent protein kinase A (PKA). All these events will lead to changes in flagellar motility to provide the motile thrust needed to reach and penetrate the egg, to an increase in tyrosine phosphorylation of sperm proteins, and will render spermatozoa ready to undergo AR and thus penetrate the oocyte (Flesch and Gadella, 2000; Tardif *et al.*, 2001, 2004; Harayama, 2013; Yeste, 2013b) **(Figure 6)**.

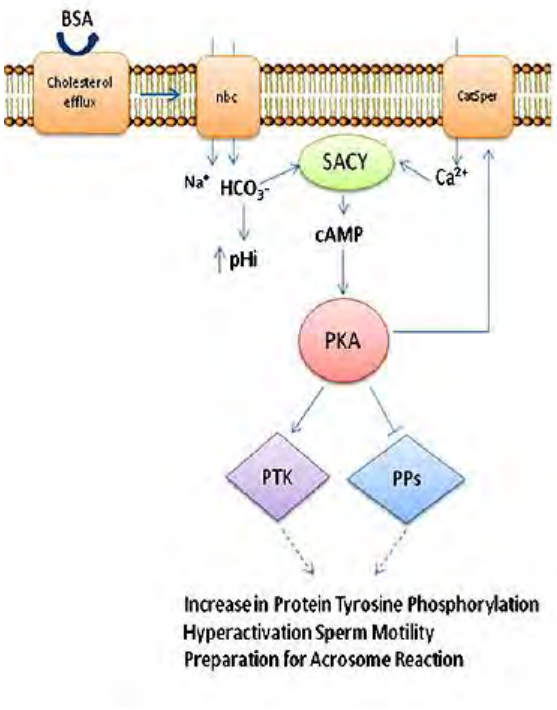


Figure 6. Molecular basis of events associated with capacitation (from Signorelli *et al.*, 2012). Overall pathway is modulated by cholesterol removal, which causes an influx of Ca^{2+} and HCO^{3-}. These events, in turn, trigger the activation of intracellular signalling pathways, like the cAMP/PKA dependent pathway, leading to an increase in Tyr phosphorylation, sperm motility hyperactivation and acrosomal exocytosis, hallmarks required for the sperm to acquire fertilising capacity (BSA: bovine serum albumin; SACY: soluble adenylyl cyclase; PKA: adenylyl cyclase cAMP-dependent protein kinase A; PTK: protein tyrosine kinases; PPs: protein phosphatases; pHi: internal pH).

Mature spermatozoa are highly specialised cells incapable of neither transcriptional nor translational activity. Hence, post-translational modifications through protein phosphorylation/dephosphorylation, mediated by kinases and phosphatases, are of highly

importance in regulating different cellular processes, including sperm motility, ZP recognition, AR and capacitation (Flesch *et al.*, 1999; Asquith *et al.*, 2004; Naz and Rajesh, 2004; Jha *et al.*, 2006). During capacitation, proteins can be phosphorylated at serine, threonine and/or tyrosine residues; nevertheless, reports dealing with serine/threonine phosphorylation are scarce (Yeste, 2013b). Related to this, it has been observed a direct relationship between protein phosphorylation in tyrosine residues and sperm capacitation in different mammalian species, such as mouse (Visconti *et al.*, 1995b), human (Carrera *et al.*, 1996; Luconi *et al.*, 1996), goat (Chatterjee *et al.*, 2010), bull (Vadnais *et al.*, 2007) and boar (Flesch *et al.*, 1999; Bravo *et al.*, 2005). In fact, it has been reported in mammals that inhibition of protein tyrosine phosphorylation prevents sperm capacitation, AR and *in vitro* fertilisation (IVF), which may be causes of infertility (Signorelli *et al.*, 2012; Naresh and Atreja, 2015). Several proteins have been described to be tyrosine phosphorylated during sperm capacitation (Kalab *et al.*, 1998; Flesch *et al.*, 1999; Fàbrega *et al.*, 2011); notwithstanding, the tyrosine phosphorylated protein that appears after sperm capacitation and that has been considered a an indicator of true capacitation in boar is a protein band of 32 kDa (Tardif *et al.*, 2001; Bravo *et al.*, 2005; Dubé *et al.*, 2005; Ramió-Lluch *et al.*, 2012b). This protein, known as p32, sp32 or ACRBP, is an acrosin-binding protein located in the acrosome of boar sperm that has been shown to participate in acrosin maturation promoting proacrosin conversion into acrosin (Baba *et al.*, 1994; Dubé *et al.*, 2005; Sun *et al.*, 2013; Dong *et al.*, 2015), an important protein for sperm-oocyte interaction and fertilisation (Urch and Patel, 1991).

As previously stated, *in vivo* sperm capacitation takes place inside the female genital tract; however, it can also be performed *in vitro* by using chemically defined media, mimicking the electrolytic composition of the oviductal fluid (Visconti *et al.*, 1998). Nevertheless, prior incubation under capacitating conditions, sperm suspensions must be washed to remove the sperm decapacitation factors adhered to the sperm surface during epididymal maturation and ejaculation. This would suggest that the process of sperm capacitation is intrinsically regulated by the sperm itself rather than by the female reproductive tract, although this might have a modulatory influence (Visconti and Kopf, 1998; Yeste, 2013b). Despite the existence of many different capacitating media (CM) supporting mammalian sperm capacitation, they typically contain energy sources (pyruvate, lactate, glucose) to maintain viability; bovine serum albumin (BSA); calcium; and sodium bicarbonate; all components present in the female reproductive tract and that have been proven to be essential for *in vitro* capacitation (IVC) (Visconti *et al.*, 2002; Tsai *et al.*, 2010; Gadella and Boerke, 2016). It has been pointed out that BSA serves as a

sink for the removal of cholesterol from the plasma membrane, which accounts for the membrane fluidity changes concomitant with capacitation (Visconti *et al.*, 2002; Naz and Rajesh, 2004). Furthermore, the role of calcium and bicarbonate in sperm capacitation is of highly importance; it has been observed that both are involved in the increase in cAMP observed during capacitation, which triggers all the events leading to an increase in protein tyrosine phosphorylation, hyperactive movement and the acrosome reaction (Visconti *et al.*, 1995a, 1995b; Yeste *et al.*, 2015a) (Figure 6). However, the ionic requirements for sperm capacitation are species-specific. Dubé *et al.* (2003) described that the presence of extracellular calcium in the CM is an absolute prerequisite for protein tyrosine phosphorylation and the increase in intracellular calcium levels concomitant with boar sperm capacitation. Nevertheless, in other species, concretely in human, extracellular calcium negatively modulates sperm protein tyrosine phosphorylation (Carrera *et al.*, 1996; Luconi *et al.*, 1996; Baker *et al.*, 2004). On the other hand, bicarbonate has been considered a key effector in promoting IVC; it has been observed *in vivo* that there are different levels of extracellular bicarbonate along the male and female reproductive tracts, being higher in the site of ovulation. This might suggest that bicarbonate plays a role in supressing/promoting sperm capacitation (Visconti and Kopf, 1998; Yeste, 2013b). In fact, it has also been involved in the remodelling of the plasma membrane architecture, in the increase in sperm motility, and in the migration of seminolipids from the apical head to the equatorial domain occurring during capacitation (Visconti *et al.*, 1998; Naz and Rajesh, 2004; Yeste, 2013b). Seminolipids stabilise the plasma membrane and protect uncapacitated spermatozoa from undergoing AR when bicarbonate levels are low; however, when bicarbonate levels are higher, they are translocated to the equatorial region, so their protective role diminishes (Witte and Schäfer-Somi, 2007; Yeste, 2013b; Gadella and Boerke, 2016).

2.5. Fertilisation

Once spermatozoa released from the sperm reservoir are capacitated, they are able to fertilise the oocyte. Mammalian fertilisation, the process by which the female and male gametes fuse together to produce a diploid zygote that will constitute a new individual (Signorelli *et al.*, 2012), includes various sequential steps (Figure 7):

1. Sperm penetration through the cumulus layer

2. Specific binding to the oocyte's ZP. This binding induces a calcium influx that triggers the fusion of the plasma membrane with the outer acrosomal membrane, thus releasing the hydrolytic and proteolytic content of the acrosome (i.e. AR) that will digest the ZP, and rendering the inner acrosomal membrane exposed
3. Sperm penetration through the ZP, by means of hyperactive sperm motility and the activity of the acrosome content, and its subsequent entrance into the perivitelline space
4. Binding and fusion of the sperm and oocyte membranes

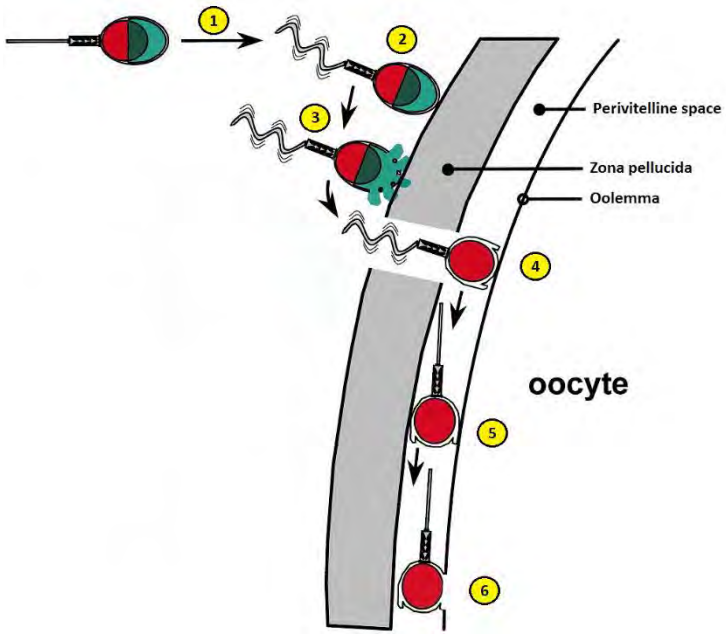


Figure 7. Events leading to mammalian fertilisation (modified from Flesch and Gadella, 2000). Freshly ejaculated sperm cells are **(1)** capacitated and **(2)** hyperactivated; both events are required to bind and penetrate the ZP. **(3)** Binding of sperm cells to the ZP triggers the acrosome reaction and acrosomal enzymes are secreted. **(4)** Hydrolytic and proteolytic enzymes secreted from the acrosome lyse the ZP and, subsequently, sperm cells penetrate the ZP, enter the perivitelline space and bind to the oolemma, firstly by the apical tip, and then, laterally **(5)**. Finally, spermatozoon fuses with the oocyte **(6)** and gets all incorporated into it.

It is worth mentioning that the plasma membrane of the equatorial region does not fuse with the outer acrosomal membrane, and seems to have an important role in the gamete fusion (Flesch and Gadella, 2000) (**Figure 7**). Unlike sperm capacitation, which is a reversible process, AR is irreversible and an absolute requirement for fertilisation. Premature acrosome exocytosis or sperm cells with altered acrosomes will not be able to penetrate through the ZP and bind to the oolemma, thus preventing fertilisation to occur (Flesch and Gadella, 2000; Vadnais *et al.*, 2007).

Ovulated mature oocytes are known to be arrested at metaphase-II (M-II); they only proceed into next meiosis steps when fertilised, in a process known as "oocyte activation". This process is triggered upon fertilisation by the release of intracellular Ca^{2+} stored in the endoplasmic reticulum into the ooplasm (Amdani *et al.*, 2015; Yeste *et al.*, 2015b). Oocyte changes occurring during this period include: cortical granule exocytosis (i.e. cortical reaction), which hardens the ZP to avoid polyspermy; pronuclear formation; maternal mRNA recruitment; cytoskeletal arrangements; resumption of meiosis; and the initiation of embryonic gene expression (Flesch and Gadella, 2000; Gadella and Evans, 2011; Yeste, 2013c; Amdani *et al.*, 2015; Yeste *et al.*, 2015b). Therefore, Ca^{2+} release is crucial for both successful oocyte activation, and for a proper early embryonic development.

3. BACTERIOSPERMIA

3.1. Artificial Insemination: state-of-art

The utilisation of AI in the swine industry dates back to the beginning of the XXth century, when Ivanow tried his first attempts in Russia (Ivanow, 1907, 1922). Over the subsequent years, its practise spread to other countries such as USA, Japan and UK (Mckenzie, 1931; Ito *et al.*, 1948; Polge, 1956). However, its true expansion did not occur since the 1980's, when insemination protocols were standardised for its commercial application. Nowadays, it is practised in over 90% of pig farms in European countries, USA and Canada (Johnson *et al.*, 2000; Rodríguez-Gil and Estrada, 2013). This great development in the implementation of AI in the pork industry was prompted by the multiple advantages that AI exhibits when compared to natural mating. In short, these advantages include: low boar-to-sow ratio, which lessens cost of housing large number of boars; genetic dissemination of superior boar genes both nationally and internationally, thus increasing the possibilities of crossbreeding; a superior sanitary control due to the implementation of hygienic measures during the collection and processing of boar semen, hence reducing disease transmission; and the fact that results obtained by using AI are equivalent or even better than those associated to natural service (Gadea, 2003; Maes *et al.*, 2008; Althouse and Rossow, 2011; Rodríguez-Gil and Estrada, 2013).

Spermatozoa are contained in the seminal plasma, which protects and supplies them with the necessary nutrients for the high metabolic demands of sperm transport through the female genital tract. In the ejaculate, this elevated metabolic activity can only be sustained for a short period of time. Therefore, in order to maintain and prolong *in vitro* sperm quality and fertilising ability for days, ejaculates intended to AI are diluted in semen extenders. Those buffers provide energy to sperm cells for metabolic maintenance (e.g. glucose, fructose); protection against cold shock (e.g. BSA); control of pH to neutralise metabolic wastes (e.g. TRIS, HEPES, sodium citrate…); control of osmotic pressure (e.g. NaCl, KCl); a chelating agent for blocking the action of calcium as a mediator of capacitation and AR (e.g. EDTA); and antibiotics to avoid the growth of microorganisms (Johnson *et al.*, 2000; Gadea, 2003; Casas, 2010) (**Table 1**).

Table 1. Composition of the most commonly used semen extenders in AI (modified from Gadea, 2003)

Composition (g/L)	IVT	Kiev	BTS	Zorlesco	MRA	ZORPVA	Reading	Modena	Androhep
Glucose	3.0	60	37	11.5	+	11.5	11.5	25[a]	26
Sodium citrate	24.3	3.7	6.0	11.7	+	11.65	11.65	6.90	8.0
EDTA		3.7	1.25	2.3	+	2.35	2.35	2.25	2.4
Sodium bicarbonate	2.4	1.2	1.25	1.25	+	1.75	1.75	1.00	1.2
Potassium chloride	0.4		0.75		-		0.75		
Acetylcysteine	0.05								
Hepes									9.0
BSA				5	+			3.00	2.5
Tris				6.5	-	5.5	5.5	5.65	
Citrate				4.1	-	4.1	4.1	2.00	
Cysteine				0.1	+	0.7	0.7	0.05	
Trehalose							1		
PVA						1	1		
Potassium acetate					+				
MOPS					+				
mOsm	290	380	330	240	290	275	300	282	309
pH		7.2	7.2		6.9			6.9	6.8

[a]: glucose monohydrate

BTS (Pursel and Johnson, 1975); Zorlesco (Gottardi *et al.*, 1980); IVT (Du Mesnil du Buisson and Dauzier, 1959); Kiev (Plisko, 1965); Modena (Moretti, 1981); Androhep (Weitze, 1990); MR-A (Martín Rillo, 1984); ZORPVA (Cheng, 1985); Reading (Revell and Glossop, 1989)

Semen extenders are classified into short-term extenders, which preserve fertility for a maximum of 3 days, and long-term extenders, which allow sperm preservation for up to 15 days (Bussalleu and Torner, 2013; Casas and Flores, 2013; Pinart and Puigmulé, 2013; Rodríguez-Gil and Estrada, 2013). The former are commonly used in local commerce, when seminal doses destined to AI are used within a short time lapse right after collection, while the latter allow national or international trading, when the site of semen production is far away from the site of insemination. Furthermore, long-term extenders also permit conducting sanitary control tests

of seminal doses before its use, and more exhaustive quality analyses (Gadea, 2003; Bussalleu and Torner, 2013). Today, probably the most used semen extender throughout the world is the *Beltsville Thawing Solution* (BTS). This solution was initially conceived by Pursel and Johnson (1975) as a medium for thawing frozen sperm, but then was adapted to refrigerated semen (Johnson, 1998). The special characteristic of this medium is that it contains small amounts of potassium, which is believed to maintain intracellular concentrations of this ion at physiological levels, thus avoiding the intracellular potassium exhaustion related to sperm motility impairment (Alvarez and Storey, 1982). According to Vyt *et al.* (2004), the choice of semen extender is crucial to better preserve sperm quality, as it depends upon the storage time.

It is well-known that boar spermatozoa are very sensitive to cold shock, which occur when sperm are rapidly cooled from body temperature to temperatures below 15ºC. This phenomenon causes cellular damage, including irreversible depression of sperm motility and metabolic activity, membrane impairment, and acrosome disruption; therefore, cold shock compromises cell viability and fertility of the processed and stored semen (White, 1993; Zeng and Terada, 2001). Specifically, this susceptibility to chilling injury seems to be related to the lipid composition of the membrane bilayer. Boar sperm plasma membrane contains a high number of unsaturated phospholipids, which confer the cell membrane a high fluidity, and a low amount of sterols, such as cholesterol, whose role is to give stability to the cell membrane, thus counteracting/complementing the phospholipids action (Casas and Flores, 2013). In this system, temperature dropping causes a restriction of lateral movement of membrane phospholipids, which results in a transition from a fluid to a gel phase. Considering that membrane phospholipids show different transition temperatures, some unsaturated phospholipids become jellified earlier than others; as a consequence, separation of lipid phases may occur. This event is associated to an irreversible clustering of membrane proteins that leads to an altered membrane function (Johnson *et al.*, 2000; Gadea, 2003; Schulze *et al.*, 2013). The presence of sterols and saturated phospholipids in the plasma membrane composition are believed to prevent the formation of gel at lower temperatures, as they are rigidifying components of the membrane (Blesbois *et al.*, 2005). Hence, the fact that boar sperm have a high ratio of unsaturated:saturated phospholipids and a low cholesterol content, makes boar spermatozoa more sensible to cold-shock than other species (Bamba and Miyagawa, 1992; Casas and Flores, 2013). Therefore, in order to avoid cooling injury and to prolong its life-span, boar seminal doses intended to AI are stored in temperatures ranging 15-17ºC to reduce its metabolism.

Semen collection in farm animals is mainly performed by using an artificial vagina or the well-known "gloved-hand" technique; thus, this process is far from being executed under sterile conditions (Althouse and Lu, 2005; Bussalleu and Torner, 2013). In fact, there are multiple contamination sources along with the collection and manipulation processes. These sources can be classified as either from animal or non-animal origin (**Table 2**):

Table 2. Sources of bacterial contamination in boar semen (modified from Althouse and Lu, 2005)

Animal origin	Non-animal origin
Faeces/urogenital infections	Water sources
Preputial fluids	Plant matter (i.e., feed, bedding)
Skin/hair	Air/ventilation systems
Respiratory secretions	Sinks/drains
Personnel	Laboratory material

In order to reduce/avoid semen contamination, boar studs need to implement general hygienic measures, generally known as minimum contamination techniques (MCTs) (**Table 3**).

Table 3. Standard minimum contamination technique protocol for boar studs (from Kuster and Althouse, 2016)

Boar preparation/semen collection
• Preputial hair should be kept trimmed around the preputial opening • Double gloving should be used, with the outer glove discarded after preparation of the boar, allowing for a clean gloved hand for grasping the penis • Disposable vinyl gloves or a hand disinfectant should be used during semen collection to minimise contamination of semen and reduce risk of cross-contamination between boars • Clean preputial opening and surrounding area (if needed) with a single-use disposable wipe • Manually evacuate preputial fluids before exteriorising penis for semen collection • Hold penis perpendicular to boar to minimise preputial fluid contamination of the semen and the semen collection vessel • Divert initial jets of an ejaculate (i.e., pre-spermatic fraction) and gel fractions from the semen collection vessel • Dispose of rubber band and filter/gauze before passing collected semen through to the laboratory for further processing
Semen processing/laboratory and barn sanitation
• Encourage single-use disposable products when economically feasible to minimise cross-contamination • When using reusable laboratory materials (i.e., glassware, plasticware, plastic tubing, containers, and so forth) which cannot be heat/gas sterilised or boiled, clean these reusables initially using a laboratory-grade detergent (residue free) with water, followed by a distilled water rinse, and finally through a 70% alcohol (nondenatured) rinse. Allow sufficient time and proper ventilation for complete evaporation of residual alcohol on the reusable. Rinse reusables with a semen extender before their first use of the day • Disinfect countertops and contaminated laboratory equipment at the end of processing day with a moist residue-free detergent and rinse • Floor should be mopped at the end of the day with a disinfectant (e.g., phenolic, formalin product) • Break down bulk products into smaller, daily-use quantities immediately after opening • Ultraviolet lighting can be installed to aid in sanitising reusables and laboratory surfaces; however, safety precautions need to be integrated to prevent exposure to personnel • Boar housing should be put on a maintenance schedule that will minimise buildup of organic material and surface moisture • The semen collection area and collection dummy should be thoroughly cleaned and disinfected at the end of each collection day

These sanitary recommendations are mainly focused on preventing contamination during the process of collection and processing of boar semen. For example, boar abdomen, preputial opening and surrounding areas should be cleaned prior semen collection; and disinfection of reusable laboratory material and equipment, as well as semen collection area and laboratory surfaces, should be performed at the end of each collection/processing day (OIE, 2011; Kuster and Althouse, 2016). Furthermore, stud personnel should also practice good hygiene and general sanitation guidelines, such as wearing clean protective garments (hair nets, boots and overalls) provided on site by the stud, and use protective gloves throughout all areas of the stud to avoid contamination of material, semen and/or extender (Althouse, 2008).

Despite the existence of all these hygienic recommendations to minimise microbiological risk, ejaculates are commonly contaminated with a broad range of microorganisms, including viruses, fungi and bacteria. In fact, several studies have tested both raw and extended semen for bacterial contamination in the lasts years. Two studies performed in Italy and Cuba reported that 63% and 75%, respectively, of raw ejaculates assessed were positive for bacterial growth (Maroto Martín *et al.*, 2010; Bresciani *et al.*, 2014). Additionally, retrospective and prospective studies in extended porcine semen from samples submitted to the University of Pennsylvania Reference Andrology Laboratory (USA), detected bacteriospermia in 31.2% of samples in 2002-2003 (Althouse and Lu, 2005), in 28.6% of samples in 2004-2005, in 17% of semen doses in 2005 and in 26% of samples in 2006 (Althouse *et al.*, 2008). Similar results were also obtained in field investigations in Germany and Austria, over a 4-year period (2010-2013) study (Schulze *et al.*, 2015). In this latter particular case, bacterial contamination was found in 88 out of 344 (26%) of extended seminal doses analysed, and in 18 out of 24 (66.7%) of the boar studs included in the study; however, in only 4.5% (8 of 88) of contaminated seminal doses, the bacterial species detected from the extended semen were also isolated in the corresponding raw ejaculate. This would suggest that most of bacterial contamination is due to semen processing, as represented in **Figure 8**.

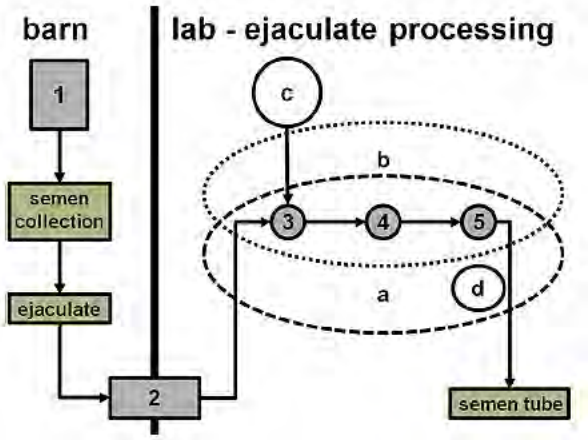


Figure 8. Hygienic critical control points during boar semen production (from Schulze *et al.*, 2015). **(1)** heating cabinets; **(2)** ejaculate transfer; **(3)** extenders; **(4)** inner face of dilution tank lids; **(5)** dyes for differentiation of boar lines; **(a)** manual operation elements (e.g. keyboards, telephone…); **(b)** laboratory surfaces; **(c)** ultrapure water treatment plants; **(d)** sinks or drains.

To control bacteriospermia below a threshold, one of the most used strategies is to include antibiotics in the semen extenders formulation. However, an excessive confidence is given to this method of bacterial control. Antibiotics are normally toxic to sperm (Morrell and Wallgren, 2011a), so, following both national and international guidelines such as the European Council directive 90/429/EEC (see reference list) and the World Organisation for Animal Health (Office International des Epizooties) (OIE, 2011), there is an existing propensity of using a cocktail of broad spectrum antimicrobials in semen extenders at lower concentrations than those required if used individually, in order to reduce cytotoxicity (Morrell and Wallgren, 2014). Nevertheless, since pigs are currently bred by AI, large amounts of semen extenders containing antibiotics are used in the pork industry, fact that can cause the emerging of antibiotic resistance. Actually, according to several studies, a great number of bacteria frequently isolated from ejaculates of domestic livestock, including the boar, are currently resistant to the most common antibiotics added in semen extenders (Althouse and Lu, 2005; Yániz *et al.*, 2010; Bolarín Guillén, 2011; Morrell and Wallgren, 2014). Antibiotic resistance is normally associated to genetic determinants, and can be intrinsic, i.e. innate of the bacterium, or acquired. Acquired antibiotic resistance might be due to acquisition of exogenous resistant genes via gene transfer between microorganisms by plasmids, transposons, integrons and bacteriophages, and/or to genetic mutation and selection (Giedraitienė *et al.*, 2011). Major mechanisms of antibiotic drug resistance include, among others, 1) reduced permeability to the antibiotic, 2) gene encoding enzymes that destroy the antibacterial agent, 3) efflux pumps to extrude the agent from the bacterial cell before exerting its effect, and 4) mutations or genes for special metabolic pathways

leading to drug target alterations (Tenover, 2006; Zhang, 2007). Notwithstanding, bacteria can also be transiently resistant to antibiotics in absence of a genetic change (phenotypic resistance). This antibiotic resistance is mostly related to the growth in biofilms, to the physiological condition of a particular bacterium, such as the case of resting cells that are less susceptible to antibiotics, and even to the metabolic state of the bacterial cell (Zhang, 2007; see Corona and Martinez, 2013 for review). Therefore, if preservative antibiotics are ineffective in controlling contaminant bacteria, the extenders composition turns out to be a potential medium where bacteria can flourish, even at low temperatures (Althouse *et al.*, 2000).

Like sperm, bacteria are unable to regulate their own temperature, so they are also susceptible to chilling injury. In this case, the degree of viability loss not only depends on the bacterial membrane lipid composition, but also on the growth temperature (McElhaney, 1984). Farrell and Rose (1968) demonstrated that a strain of *Pseudomonas aeruginosa* grown at 30ºC showed a rapid loss of viability when chilled rapidly to 0º or -2ºC; on the contrary, this diminution of viability due to rapid cooling was not observed when this bacterium was grown at 10ºC. Therefore, bacteria are much less sensitive to temperature changes than sperm because, unlike them, they do have the necessary intracellular organelles to modify plasma membrane fluidity and to produce cold inducible proteins in order to adapt to temperature changes (Barria *et al.*, 2013). Consequently, the lower temperatures used to reduce the metabolic activity and to induce dormancy in sperm cells in order to prolong their longevity, can also favour the growth of bacteria, as they can adapt to temperature changes (Althouse, 2008). Thus, taking into account that bacteria not only can survive but thrive in semen extenders, as reported in Althouse and Lu (2005), the detrimental effects that bacterial contamination may have on both sperm quality and sow are a main concern to pig breeders, as it can be exacerbated during storage. In addition, potential risk factors, aside from the initial amount of bacterial contaminants present in the raw ejaculate, may involve a delayed cooling-rate of freshly processed semen samples (Althouse *et al.*, 1998), higher temperature than recommended during sperm transport and storage (Aurich and Spergser, 2007), prolonged storage, and the extender's composition (Johnson *et al.*, 2000). For example, both bacterial generation times and antimicrobial kill-times can be modified by extender pH and storage temperature (Althouse and Lu, 2005; Althouse *et al.*, 2008). For all these reasons, bacterial control remains mandatory in liquid preserved semen doses, and finding alternatives to antibiotics usage in semen extenders to control bacterial contamination would be beneficial to improve both sperm quality and survival, and to avoid the emergence of antibiotic resistance.

3.2. Impact of bacterial contamination

3.2.1. Effects of bacteriospermia on sperm quality and fertilisation potential

Over the years, the effects that bacteriospermia, defined as the presence of bacteria in semen fluid samples, cause over sperm quality have been seriously underestimated by personnel working in boar studs. Generally, it is widely accepted that a boar with a good general health status subsequently produces semen of good quality; however, as semen collection and processing are not sterile procedures, semen is commonly contaminated with a broad range of microorganisms, which may lead to sub-optimal reproductive performance. Furthermore, not only those bacterial agents that cause urogenital tract infections contribute to the decrease in sperm quality, but also those non-pathogenic bacteria responsible for colonisation and contamination of the male urogenital tract (Cottell *et al.*, 2000).

Effects of contaminant bacteria on sperm quality are manifold and have been described by many authors and in several different species, such as ram (Yániz *et al.*, 2010), bull (Akhter *et al.*, 2008; Smole *et al.*, 2010), boar (Althouse and Lu, 2005; Bussalleu *et al.*, 2011; Ubeda *et al.*, 2013; Prieto-Martínez *et al.*, 2014), human (Diemer *et al.*, 1996; Huwe *et al.*, 1998; Schulz *et al.*, 2010; Fraczek *et al.*, 2012) and stallion (Varner *et al.*, 1998; Aurich and Spergser, 2007; Morrell *et al.*, 2014). Detrimental effects provoked by different bacterial contaminants include: sperm motility and membrane integrity impairment (Althouse, 2008; Bussalleu *et al.*, 2011; Ubeda *et al.*, 2013; Fraczek and Kurpisz, 2015), ultrastructural morphological alterations (head, mid-piece and tail) and/or degenerative acrosome exocytosis (Köhn *et al.*, 1998; Diemer *et al.*, 2000; Zan Bar *et al.*, 2008; Prieto-Martínez *et al.*, 2014), mitochondrial activity diminution (Fraczek *et al.*, 2012), increase in DNA fragmentation and phosphatidylserine (PS) externalisation (Villegas *et al.*, 2005; Kaur and Prabha, 2013; Fraczek *et al.*, 2015), and sperm agglutination (Monga and Roberts, 1994; Ubeda *et al.*, 2013), among others (see Fraczek and Kurpisz, 2015 for review). It has also been documented that acidification of sperm handling medium due to bacteriospermia leads to an alteration of sperm physiology that can compromise sperm survival (Althouse *et al.*, 2000; Purdy *et al.*, 2010; So *et al.*, 2011; Prieto-Martínez *et al.*, 2014). All those reported effects have been shown to be bacterial concentration-dependent. However, this issue is still controversial because some authors have reported that the presence of bacterial strains in semen does not usually causes sperm quality impairment (Cottell *et al.*, 2000; Pasing *et al.*, 2013); in fact, spermicidal effects may depend on the boar, the bacterial type, specie and strain

concerned, as well as its concentration (Maes *et al.*, 2016). **Figure 9** shows the relative size of contaminant bacteria in proportion to boar sperm.

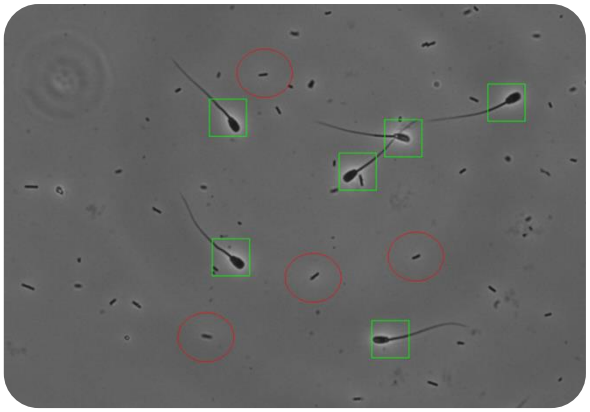


Figure 9. Boar sperm (green squares) and several contaminant bacteria (some marked by a red circle)

Boar ejaculates normally contain between 10^3-10^5 Colony Forming Units (CFU)/mL (Althouse and Lu, 2005; Morrell and Wallgren, 2011a), although bacterial contamination can reach concentrations of up to 10^9 CFU/mL (Althouse *et al.*, 2000). A sperm:bacteria ratio of 1:1 has been determined by several authors to be the threshold level for inducing detrimental effects on both sperm quality and fertility (Althouse, 2008; Yániz *et al.*, 2010; Prieto-Martínez *et al.*, 2014); nevertheless, other studies have reported that a sperm:bacteria ratio as low as 100:1 is sufficient to cause detrimental effects on sperm (reviewed in Kuster and Althouse, 2016), meaning that the amount of bacteria necessary to cause undesirable effects is species related. Accordingly, Althouse *et al.* (2008) estimated that the amount of bacteria necessary to produce detrimental effects to a standard seminal dose containing $3x10^9$ sperm is $3x10^7 – 3x10^9$ bacteria. The majority of studies regarding the effects of bacteriospermia on sperm quality are performed by using fresh extended semen; however, as reported in equine and bovine by Corona and Cherchi (2009) and Bielanski *et al*. (2003), respectively, bacteriospermia can also affect frozen semen, as most microorganisms can survive storage at low temperatures, including in liquid nitrogen (LN; -196 ºC); semen extenders may act as stabilisers at freezing temperatures. Indeed, it has also been shown that cross-contamination of pathogens via LN can also occur (Bielanski, 2012).

The harmful effects of microbial contamination on spermatozoa can be caused by direct contact of bacteria with sperm, and/or by cell-associated and extracellular factors secreted by bacteria, like lipopolysaccharides (LPS) (Zhang *et al.*, 1998; Villegas *et al.*, 2005; Okazaki *et al.*,

2010), *quorum sensing* molecules (Rennemeier *et al.*, 2009), α- and β-haemolysins (Schulz *et al.*, 2010; Fraczek and Kurpisz, 2015), and soluble spermatotoxic factors such as the sperm immobilising factor (SIF) (Prabha *et al.*, 2010, 2011; Kumar *et al.*, 2011) or the sperm agglutinating factor (SAF) from *E. coli* and *Staphylococcus aureus* (Kaur *et al.*, 2010; Prabha and Kaur, 2012; Kaur and Prabha, 2013). Sperm surface is rich in glycoproteins susceptible to bacterial-sperm receptor-ligand interactions mediated by attachment organelles such as pili and fimbriae (Monga and Roberts, 1994). For example, Diemer *et al.* (2000) reported that *E.coli* caused massive morphological alterations, leading to sperm immobilisation and impaired acrosomal function, by direct attachment to spermatozoa; on the contrary, Schulz *et al.* (2010) and Prabha *et al.* (2010) showed that altered function of human sperm (motility, viability, mitochondrial function…) was not only attributable to direct contact but also to soluble factors secreted by *E.coli*. In other non-bacterial microorganisms, this tendency was also observed, as Tian *et al.* (2007) demonstrated that fungus *Candida albicans* also caused sperm motility and viability impairment, as well as ultrastructural damage in human sperm by both direct contact and/or virulence factors secreted.

The fertilising competence is usually measured by evaluating sperm quality (motility, morphology, functional integrity…), and the percentage of high quality sperm per dose (Johnson *et al.*, 2000). Spermatozoa are complex cells that require to fulfil several criteria to achieve a proper fertilisation, like the attainment of the hyperactivated state, membrane and acrosome intactness, the ability to capacitate, a normal complement of DNA, and the ability to bind to the zona pellucida (Gillan *et al.*, 2005). Therefore, if some of these criteria become altered or cannot be accomplished due to damaging effects caused by bacterial contamination, sperm function is consequently compromised and sperm fertilisation ability jeopardised (Villegas *et al.*, 2005). Taking this into account, microbial contamination is an important parameter that should be analysed in the quality control of semen.

3.2.2. Effects of bacteriospermia on the sow

Aside from the damaging effects that bacteriospermia has on sperm quality and fertilisation, it may also cause adverse effects when entering into the female reproductive tract. During natural mating, the female genital tract is normally exposed to bacterial contamination from the male, so it has developed its own natural defence mechanisms to cope with bacterial onslaught. Therefore, it is reasonable to think that a certain degree of bacterial contamination in extended

seminal doses could also be tolerated. Nevertheless, when using AI, semen might be deposited in a non-physiological part of the female reproductive tract, especially when using intrauterine (IU) and deep intrauterine insemination (DIU), thus bypassing the first immune defence barrier of the recipient female (Morrell and Wallgren, 2011a, 2014). Deleterious effects that bacteria may cause on inseminated females include: an increase in returns to oestrus and post-insemination vulvar discharges (Althouse *et al.*, 2000; Moreira *et al.*, 2013), pregnancy failure, endometritis, embryonic loss due to direct invasion of the embryo by the pathogen, abortion, clinical disease and infections in sow herds (Maes *et al.*, 2008; Knox, 2016), and reduced litter size (Maroto Martín *et al.*, 2010). Hence, although the risk of disease transmission while using AI is minimal, the impact of bacterial contamination can be enormous, causing major economic loses to the swine industry especially if large numbers of sows are inseminated using the same contaminated ejaculate sample (Maes *et al.*, 2008).

A study involving five farms that artificially inseminated sows with contaminated semen reported an increase in returns to oestrus of 17-100%, depending on the farm (Althouse *et al.*, 2000). This increased return rate seemed to be related to the storage time of seminal doses prior to insemination, as the only farm reporting a 100% return rate (total pregnancy failure) used contaminated semen samples stored for >48h (Althouse *et al.*, 2000). In a similar fashion, a case report of inseminating 21000 sows using extended semen contaminated with *Achromobacter xylosoxidans*, a *Pseudomonas*-like bacterium, coming from the water distillation system of a boar stud, reported white purulent vulvar discharges within 3 weeks post-breeding with a prevalence of 8-15%, and reduced conception rates by 6-12%. In those sows suffering from vulvar discharges, this bacterium also caused endometritis and was isolated from the uterus of affected sows (Payne *et al.*, 2008). In addition, a study by Maroto Martín *et al.* (2010) described a negative correlation between the presence of spermagglutinating *E. coli*, alone or in combination with not more than two other bacteria, and litter size in a concentration-dependent manner. In fact, although the World Organisation for Animal Health (the former Office International des Epizooties, OIE) (OIE, 2001) established a value of 5×10^3 CFU/ml as a limit for the bacterial presence in bovine semen samples, this study from Maroto Martín *et al.* (2010) revealed that inseminating sows using boar extended semen with more than 3.5×10^3 CFU/mL, resulted in a reduction in litter size of 2.53-3.35 pigs/litter. However, it is worth stressing that the value set by the OIE only serves as a guide, since the animal species concerned in its recommendation is other than porcine. On the other hand, Sone (1990) reported that AI using boar semen contaminated with three different bacteria (*E. coli, Staphylococcus* spp. and

Pseudomonas spp.) during oestrus did not caused alterations related to conception rate, number of embryos or litter size, and the endometrial morphology seemed to be normal; furthermore, no bacteria were detected from the uterus. However, those sows inseminated with contaminated semen during the luteal phase presented an oedematous uterus or pyometra, and large numbers of bacteria were isolated from those uteri (Sone, 1990). Therefore, this would imply that sows uterus is more resistant to bacterial infection during oestrus.

3.3. Bacterial agents

The majority of microorganisms commonly detected in boar semen are aerobic gram-negative bacteria especially belonging to the *Enterobacteriaceae* family, although species from the *Pseudomonaceae* family also appear in a regular basis (Schulze *et al.*, 2016). In fact, according to different studies, at least 25 different bacterial genera have been identified as common bacterial flora in boar ejaculates, being *Escherichia coli, Proteus* spp., *Serratia* spp., *Enterobacter* spp., *Klebsiella* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Pseudomonas* spp. the most frequently isolated (Table 4). Generally speaking, given that anaerobes are sensitive to semen preservation conditions, their presence in semen samples has not been widely studied (Eggert-Kruse *et al.*, 1995); notwithstanding, the presence of anaerobes in semen of stallion (Corona and Cherchi, 2009), human (Eggert-Kruse *et al.*, 1995; Damirayakhian *et al.*, 2006) and boar (De Grau *et al.*, 2006; Maroto Martín *et al.*, 2010) is not exceptional. In fact, it has been pointed out that the aerotolerant anaerobe *Clostridium perfringens* is highly prevalent throughout the swine industry (Baker *et al.*, 2010); in accordance, it is likely to isolate this bacterium from boar semen as a consequence of seminal contamination during collection and/or manipulation of ejaculates.

The most extensively studied bacterial contaminant in human and livestock semen is *E. coli*, probably because it is a major causative agent of urogenital and male accessory sex glands infections, such as acute prostatitis, in human (Auroux *et al.*, 1991; Diemer *et al.*, 1996, 2000), equine (Varner *et al.*, 1998) and porcine (Althouse *et al.*, 2000). Male genitourinary tract infections can affect different areas of the male reproductive tract, e.g. testis, epididymis…, thus affecting sperm at different levels: during its development, maturation and/or transport via the seminal pathway. Therefore, urogenital infections have been considered as a substantial cause

of male infertility; actually, this problem accounts for approximately 5-15% of cases of male infertility in human (Diemer *et al.*, 2003; Schulz *et al.*, 2010).

Table 4. Microorganisms commonly isolated from boar semen samples (modified from Althouse and Lu, 2005)

Tamuli *et al.* (1984)	Danowski (1989)	Dagnall (1986)	Sone *et al.* (1989)	Althouse and Lu (2005)	Maroto Martín *et al.* (2010)
Escherichia coli	*Staphylococcus* spp.	*Citrobacter* spp.	*Pseudomonas* spp.	*Enterococcus* spp.	*E. coli*
Pseudomonas spp.	*Pseudomonas* spp.	*Pseudomonas* spp.	*Micrococcus* spp.	*Stenotrophomonas maltophilia*	*Proteus* spp.
Bacillus spp.	*E. coli*	*Corynebacterium* spp.	*Staphylococcus* spp.	*Alcaligenes xylosoxidans*	*Staphylococcus* spp.
Staphylococcus spp.	*Citrobacter* spp.	*Streptococcus* spp.	*Klebsiella* spp.	*Serratia marcescens*	*Streptococcus* spp.
Klebsiella spp.	*Providencia* spp.	*E. coli*	*E. coli*	*Acinetobacter lwoffii*	*Klebsiella* spp.
Proteus spp.	*Neisseria* spp.	*Actinomyces*-like spp.	*Citrobacter* spp.	*E. coli*	*Serratia* spp.
Enterobacter spp.	*Proteus* spp.	*Bacteroides* spp.	*Proteus* spp.	*Pseudomonas* spp.	*Enterobacter* spp.
Pasteurella spp.		*Lactobacillus* spp.	*Actinomyces* spp.	*Comamonas testosteroni*	*Pseudomonas* spp.
Citrobacter spp.		*Acinetobacter* spp.	*Serratia* spp.	*Klebsiella* spp.	Anaerobes
		Bacillus spp.	*Enterobacter* spp.	*Providencia rettgeri*	
		Actinobacillus spp.	*Bacillus* spp.	*Burkholderia cepacia*	
		Staphylococcus spp.	*Streptococcus* spp.	*Enterobacter cloacae*	
		Flavobacterium spp.		*Corynebacterium* spp.	
		Klebsiella spp.		*Pasteurella multocida*	
		Micrococcus spp.		*Proteus mirabilis*	
		Proteus spp.		*Streptococcus suis*	

Most of bacterial contaminants usually found in boar ejaculates are not considered as primary pathogens in swine; they are considered as a part of normal microflora and responsible for colonisation and contamination of the male urogenital tract, rather than causing infection. However, these non-pathogenic bacteria can also negatively influence sperm quality and lead to a low reproductive performance, especially at high concentrations (Maes *et al.*, 2008; Fraczek and Kurpisz, 2015), or even become opportunistic pathogens in immune-compromised and susceptible individuals, thus producing genital infections and disease (Sone, 1990; Althouse *et al.*, 2000; Maroto Martín *et al.*, 2010). Moreover, due to the proximity of the intestinal tract, infections can also be caused by bacteria belonging to the normal intestinal flora (Bergsten *et al.*, 2005).

3.3.1 *Clostridium perfringens*

As stated in section 3.3, the presence of anaerobes in semen from mammals is not exceptional. Yet, no study has investigated whether anaerobic bacteria can survive and/or proliferate in boar sperm and how their presence could affect sperm quality. This question is discussed in **PAPER I**.

Clostridium perfringens is a gram-positive, catalase and oxidase-negative, rod-shaped, non-motile, spore-forming and anaerobic bacterium belonging to the family *Clostridiaceae* (genus *Clostridium*) with a cell size of 0.3-2 µm x 1.5-20 µm; however, contrary to other clostridia, it tolerates oxygen tensions that are inhibitory to others (Holt *et al.*, 1994; Heikinheimo, 2008; Songer, 2010). It is a fermentative organism that grows rapidly in media containing carbohydrates, thereby generating hydrogen and carbon dioxide (CO_2) that help maintaining the anaerobic environment. Its optimal growth temperature is 43-45ºC, although growth is supported in a temperature range of 12-50 ºC (Labbe, 1989; Rood and Cole, 1991; Li and McClane, 2006). This bacterium is widely distributed throughout the environment, especially in soil and water, and is also commonly found in the gastrointestinal tract of both human and animals; nevertheless, under appropriate conditions, this bacterium can become pathogenic, thus causing histotoxic and enteric diseases in both human and animals, such as diarrhoea or necrotic enteritis (Rood and Cole, 1991; Madigan *et al.*, 2004; Morris and Fernández-Miyakawa, 2009). Moreover, it is a highly prevalent microorganism in the swine industry, where causes enteric disease, predominantly in neonatal piglets, that may consequently lead to preweaning mortality and economic losses. In fact, it has been observed that clostridial spores can persist in the faecal matter and environment of pigs; therefore, semen contamination during collection and processing, and the spread of this bacterium among animals is a major issue difficult to restrain (Baker *et al.*, 2010).

C. perfringens isolates are classified into five toxinotypes (A-E) based on the production of four so-called major toxins: alpha (α), beta (β), epsilon (ε) and iota (ι) **(Table 5)**. Notwithstanding, *C. perfringens* virulence does not only rely on these 4 toxins, but also in a wide array of more than 15 different toxins not currently used in the classification of this microorganism. Among these other toxins, some pathologically important ones, such as the enterotoxin (CPE) and beta2 (CPB2), can be found (Songer, 1996; Songer and Uzal, 2005; Uzal and McClane, 2011). Toxins are secreted into the environment during the exponential growth phase, with the exception of the CPE; this toxin is produced and accumulated in the cytoplasm

during sporulation and until bacterial lysis, when both the spore and CPE are released (Petit *et al.*, 1999; Varga *et al.*, 2004; Paredes-Sabja and Sarker, 2009; Songer, 2010; Briggs *et al.*, 2011). Each toxinotype is associated with a specific disease in both human and animals (Rood and Cole, 1991; Petit *et al.*, 1999; Songer, 2010) (Table 5). *C. perfringens* types A and C are the principal enteric pathogenic clostridia in swine (Songer and Uzal, 2005).

Table 5. Toxinotypes and diseases associated with *Clostridium perfringens* (Rood and Cole, 1991; Petit *et al.*, 1999; Smedley *et al.*, 2004; Heikinheimo, 2008; Bokori-Brown *et al.*, 2011)

Toxinotype	Major toxin	Associated disease	
		Humans	Animals
A	α	Gas gangrene Food-poisoning[a] Antibiotic-associated diarrhoea[a] Necrotic enteritis (infants) Sporadic diarrhoea[a] Sudden infant death syndrome[a]	Enterotoxaemia (various animals) Necrotic enteritis (fowl, poultry) Enteritis (various animals)[a,b]
B	α, β, ε		Dysentery in new-born lambs Enterotoxaemia (sheep, calves, foals) Haemorrhagic enteritis in neonatal calves and foals
C	α, β	Necrotic enteritis	Enterotoxaemia (calves, piglets, lambs) Struck (fatal enterotoxaemia in sheep) Necrotic enteritis (piglets, lambs, calves, foals) Enteritis (pig)[b]
D	α, ε		Enterotoxaemia (sheep, calves, goats) Pulpy kidney disease (fatal enterotoxaemia in lambs)
E	α, ι		Enterotoxaemia (calves, rabbit, sheep)

[a] Enterotoxin (CPE)-producing strains
[b] β2 (CPB2)-producing strains in pig

Although *C. perfringens* virulence has not been fully elucidated, most of these clostridial toxins seem to act on the cell membrane, either by causing membrane disruption, such as the activity of the α-toxin, a phospholipase C sphingomyelinase, or by pore formation, like CPE, β- , ε- and beta2 toxins, which leads to alterations in membrane permeability. On the contrary, ι-toxin acts intracellularly ADP-ribosylating actin and, thus, disrupting cellular cytoskeleton. Principal biological activities of these toxins include cytolytic, cytotoxic, dermonecrotic, enterotoxic and lethal properties (Perelle *et al.*, 1993; Titball, 1993; Katahira *et al.*, 1997; Nagahama *et al.*, 1997; Naylor *et al.*, 1998; Petit *et al.*, 1999; Morris and Fernández-Miyakawa, 2009; Sakurai *et al.*, 2009; Briggs *et al.*, 2011; Uzal and McClane, 2011; Popoff, 2014) (Table 6). *C. perfringens* also produces hydrolytic enzymes, like proteases and neuraminidases, which, besides degrading extracellular substrates to provide nutrients for bacterial growing, they seem to act synergistically with membrane-damaging toxins during cell disruption (Petit *et al.*, 1999).

Therefore, rather than being a function of a single toxin, *C. perfringens* virulence is multifactorial.

Table 6. Biological activity of *C. perfringens* major toxins (Songer, 1996; Petit *et al.*, 1999; Morris and Fernández-Miyakawa, 2009)

Toxin	Biological activity
α	Phospholipase/sphingomyelinase C, haemolytic, cytolitic, dermonecrotic, lethal
β	Cytolitic, dermonecrotic, haemorrhagic necrosis intestinal mucosa, lethal
ε	Protease-activated prototoxin, oedema, central nervous system tocixity, increases intestinal permeability, dermonecrotic, lethal
ι	Disruption of actin cytoskeleton and cell barrier integrity, dermonecrotic, enterotoxic, lethal
β2 (CPB2)	Cytolitic, haemorrhagic necrosis intestinal mucosa, lethal
CPE	Cytotoxic, erythematous, water and ions leakage by enterocytes (diarrhoea), enterotoxic, lethal

For the present Thesis Dissertation (**PAPER I**), 5 different commercial *C. perfringens* strains, identified as CECT 4110, CECT 4647, CECT 822, CECT 376 and CECT 486, from the Spanish collection of microbiological type cultures (www.cect.org) were used. These strains were chosen because they expressed, as confirmed by PCR, most of the major toxins of *C. perfringens* toxinotypes: α, β, ε, β2 and CPE.

3.3.2. Pseudomonas aeruginosa

Pseudomonas aeruginosa is one of the most commonly isolated bacteria from mammalian ejaculates, such as human (Rehewy *et al.*, 1979; Berktas *et al.*, 2008), boar (Althouse and Lu, 2005; Maroto Martín *et al.*, 2010), bull (Eaglesome *et al.*, 1995; Akhter *et al.*, 2008) and stallion (Pickett *et al.*, 1999; Aurich and Spergser, 2007), and is a well-known agent of causing urinary transmitted infection (Mittal *et al.*, 2009). Although the effects that this bacterium produces on sperm quality and fertility have been reported in bull (Smole *et al.*, 2010), stallion (Tiago *et al.*, 2012) and human (Huwe *et al.*, 1998; Berktas *et al.*, 2008), to our knowledge, no study about the effects of *P. aeruginosa* on boar sperm quality and fertility had been performed so far. For this reason, the present Thesis has addressed this issue in **PAPERS II** and **III**.

P. aeruginosa is an aerobic, gram-negative, catalase and oxidase-positive, rod-shaped, nonsporulated and polarly flagellated bacterium that belongs to the *Pseudomonadaceae* family (genus *Pseudomonas*); it measures 0.5-1 µm in width and 1.5-4 µm in length (Bergan, 1981; Madigan *et al.*, 2004; Ruiz Martínez, 2007). This bacterium is a highly ubiquitous microorganism due to its metabolic versatility, which confers a remarkable ability to adapt and thrive in a great

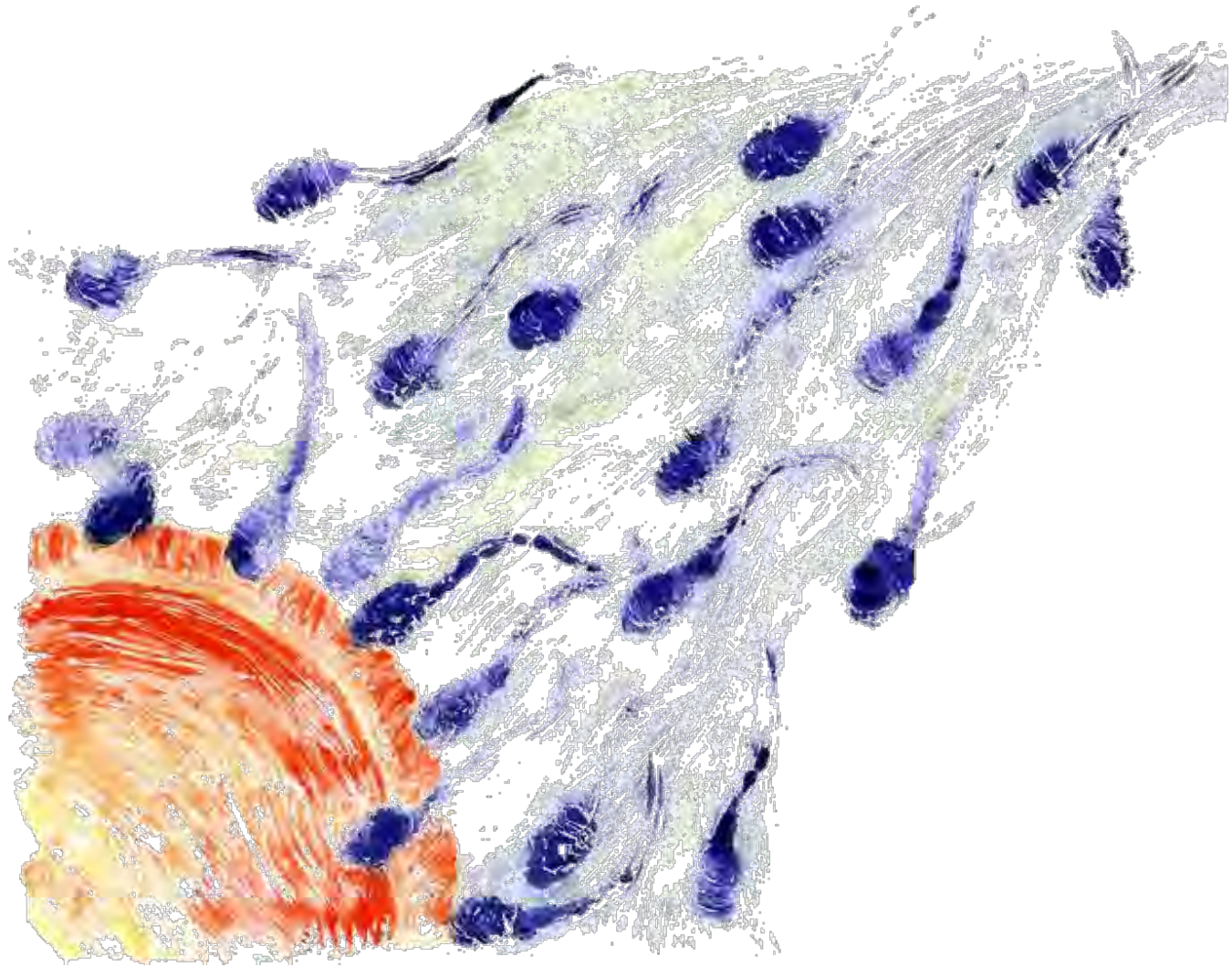
variety of environments, such as lakes, soils, plant matter and clinical settings. Particularly, it has minimal nutrient requirements and can subsist using different carbon sources for energy; some strains are even able to utilise nitrogen as a terminal electron acceptor to respire under anaerobic conditions. Moreover, *P. aeruginosa* is also resistant to the most common antibiotics, including, but not limited to, amoxicillin, ampicillin, gentamicin or tyolisin, and is capable of growing in a wide variety of temperatures (4-42ºC), although its optimal range is 30-37ºC (Ruiz Martínez, 2007; Kung *et al.*, 2010; LaBauve and Wargo, 2012; Bresciani *et al.*, 2014; Tümmler *et al.*, 2014). In addition to have the ability to survive in different environments, it is an opportunistic pathogen of a broad range of different organisms, including plants, animals and human, and can cause disease, such as respiratory disease, urinary tract infections and venereal disease, especially when their resistance to infection is low (Bergan, 1981; Madigan *et al.*, 2004; Battle *et al.*, 2009; Bleves *et al.*, 2010; LaBauve and Wargo, 2012; Tiago *et al.*, 2012; de Oliveira *et al.*, 2013).

This microorganism is capable of developing several different virulence factors that are classified depending on its mode of action: **1)** bacterial cell surface virulence factors, **2)** secreted virulence factors, and **3)** those related to the type III secretion system. Flagella, pilis, LPS and alginate are included among bacterial cell associated factors **(1)**, and act mainly as adhesins, anchoring *P. aeruginosa* to the colonised cell (Kipnis *et al.*, 2006; Ruiz Martínez, 2007). Secreted factors **(2)** consist of pyocyanin (oxidative reactive blue pigment metabolite characteristic of *P. aeruginosa* that increases reactive oxygen species), pyoverdine (siderophore that regulates the secretion of other virulence factors, such as exotoxin A), alkaline protease, protease IV, elastase, phospholipase C and exotoxin A (ToxA), among others (Kipnis *et al.*, 2006; Bleves *et al.*, 2010; Filloux, 2011). These factors are secreted either by a one-step secretory system, which secretes proteins directly from the cytoplasm to the cell surface (type I and VI secretion systems), or via a two-step secretory system, where secreted proteins do a stopover in the periplasm prior its externalisation (type II and V secretion system) (Bleves *et al.*, 2010; Filloux, 2011). To mention a few, these secreted factors can induce cell death and/or tissue damage by lysis of structural proteins (Kipnis *et al.*, 2006; Ruiz Martínez, 2007). The most important toxin in this category is ToxA, which has been reported to have a major role in *Pseudomonas* spp. toxicity by depressing host response to infection and inhibiting host cell protein synthesis (Davinic *et al.*, 2009; Bleves *et al.*, 2010). Although *P. aeruginosa* pathogenicity is multifactorial, its major determining virulence factor is the type III secretion system (T3SS) **(3)**. This secretion system is shared with other gram-negative bacteria, and acts as a mechanism of direct injection of toxins

into the host cell cytoplasm through a syringe-like apparatus. The T3SS encompasses four effector cytotoxins (ExoS, ExoT, ExoY and ExoU), which hijack different cellular pathways, thus causing several effects such as disruption of normal cytoskeletal organisation in host cell and cell death. Among these toxins, the one showing higher cytotoxic power is ExoU (Kipnis *et al.*, 2006; Hauser, 2009; Bleves *et al.*, 2010; Filloux, 2011; Perdu *et al.*, 2015). The presence of the genes encoding these effectors may vary from one strain to another, but it has been observed that *exoU* and *exoS* genes are mutually exclusive, i.e. strains carrying *exoU* does not carry *exoS*, and vice versa (Wolfgang *et al.*, 2003; Kipnis *et al.*, 2006; Filloux, 2011). ExoU-producing strains cause a rapid necrotic death, whereas ExoS-producing strains are somehow internalised in the cell host, leading to a delayed cell death similar to apoptosis (Bleves *et al.*, 2010). In addition to these virulence factors, *P. aeruginosa* has also the ability to form biofilms and to use the mechanism of *quorum sensing* (QS). The phenomenon of QS is a global regulatory system depending on cell population density that enables bacteria to coordinately regulate the expression of responsive genes by means of secretion and detection of signalling molecules (autoinducers). The detection of a threshold concentration of autoinducers in a bacterial population initiates the simultaneous expression of QS-controlling genes in the majority of the bacterial population. Physiological processes regulated by QS are diverse, including biofilm formation, motility and the expression of virulence factors (Miller and Bassler, 2001; Willcox *et al.*, 2008; Rennemeier *et al.*, 2009; Kumar *et al.*, 2016). Therefore, these two mechanisms (biofilm formation and QS) also have a role in *P. aeruginosa* pathogenicity by allowing the colonisation of different surfaces, and the regulation of genes coding for virulence factors, respectively (Kipnis *et al.*, 2006; Mittal *et al.*, 2009).

In order to fulfil the objectives addressed in **PAPER II** and **PAPER III**, the commercial strain identified as CECT 4145 from the Spanish collection of microbiological type cultures was used. This strain was selected as it is positive for the secreted factors pyocianin, pyorubin and pyoverdin, and it is routinely used for sterility testing.

Objectives



Objectives

Based on all stated in the previous section, this Thesis dissertation has three main objectives according to the challenges that currently concern the utilisation of boar seminal doses with bacteriospermia. In response to the following objectives, three papers, named at the end of each aim, have been published:

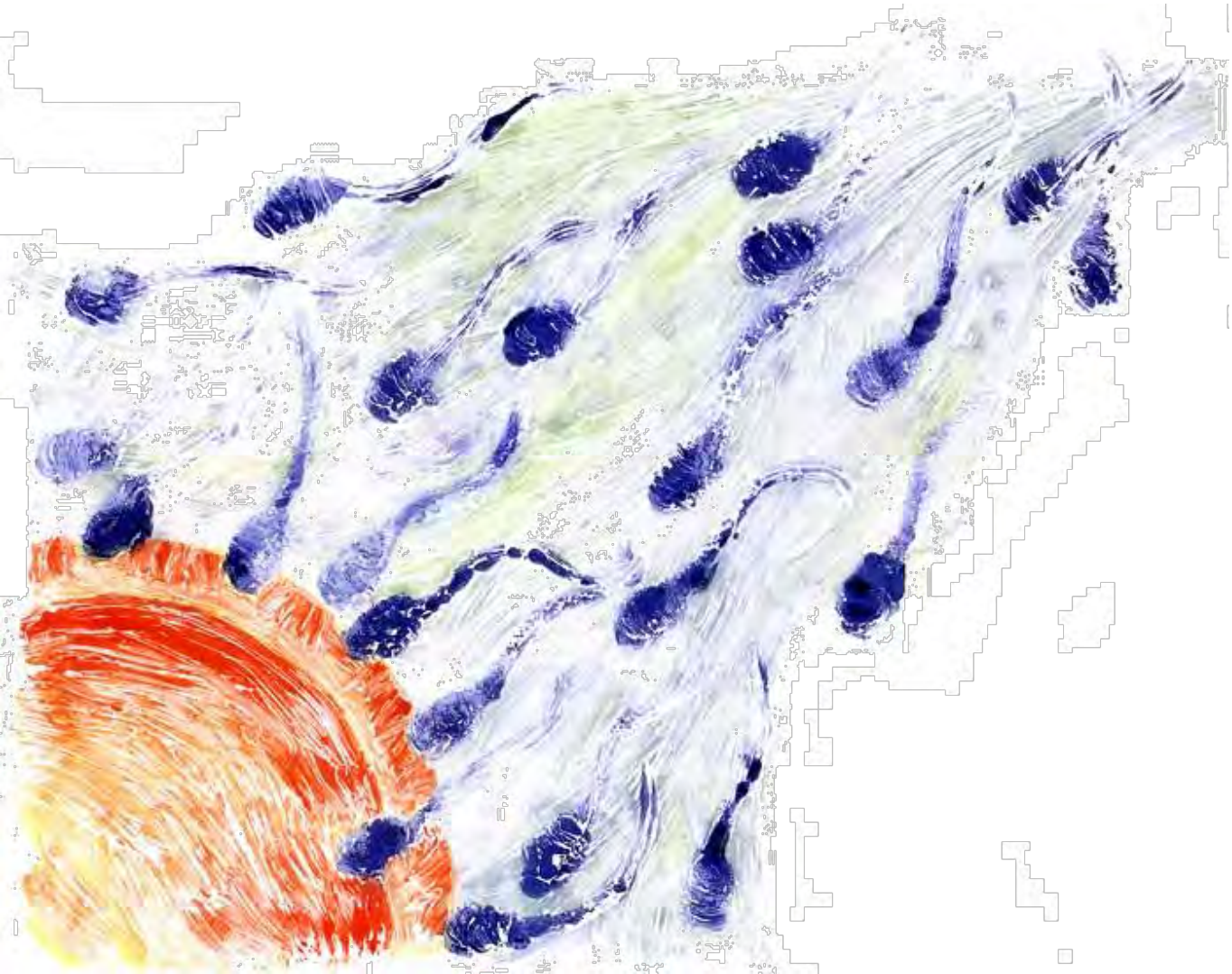
1. To evaluate the effects that *Clostridium perfringens* causes on sperm motility, sperm morphology, and sperm viability of extended boar spermatozoa at approximately body animal temperature (37ºC) and at typical storage temperature in AI centers (15ºC). **PAPER I**

2. To assess if contamination with *Pseudomonas aeruginosa* alters sperm motility, sperm viability, acrosome integrity and pH of extended boar semen during storage at 15ºC. **PAPER II**

3. To determine the effects that contamination with *Pseudomonas aeruginosa* produces on sperm motility and viability of boar sperm incubated under capacitating conditions, as well as to elucidate its role on membrane integrity and protein phosphorylation of boar sperm during and after *in vitro* capacitation. **PAPER III**

SECTION II

ARTICLE COMPENDIUM



Paper I

How do different concentrations of *Clostridium perfringens* affect the quality of extended boar spermatozoa?

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How do different concentrations of *Clostridium perfringens* affect the quality of extended boar spermatozoa?

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ABSTRACT

Bacteriospermia in boar fresh and extended semen is a frequent finding that produces alterations on sperm quality and, consequently, causes economic losses in artificial insemination (AI) centres. The present study sought to evaluate the effect of different infective concentrations of *Clostridium perfringens* on boar sperm quality, assessed as sperm motility (CASA), morphology and viability, through 11 days of storage at 15 °C (experiment 1), and after 96 h of incubation at 37 °C (experiment 2). With this purpose, different seminal doses were artificially inoculated with different infective concentrations of *C. perfringens*, ranging from 10^2 to 10^8 cfu mL^{-1}. The negative controls were non-inoculated doses. Sperm quality was checked after 0, 1, 2, 3, 4, 7, 8, 9, 10 and 11 days of storage at 15 °C in experiment 1, and after 0, 24, 48, 72 and 96 h at 37 °C in the second experiment. Moreover, the presence/absence of bacteria was detected by PCR analyses during both experiments at different time points.

In both experiments, sperm morphology of inoculated samples did not differ from the negative control. Conversely, detrimental effects on sperm viability and motility were observed after 24 h of incubation/storage at the highest infective concentrations in both experiments. The deleterious effects observed because of the presence of *C. perfringens* in semen emphasise the relevance of detecting bacteria in extended doses destined to AI. So, this study suggests that the evaluation of bacterial contamination in semen is a procedure that should be routinely applied while assessing sperm quality in AI centres to avoid the use of doses with low sperm quality and the possible spread of bacterial contaminants.

1. Introduction

Nowadays, artificial insemination (AI) is widely used in swine industry. Unlike natural mating, AI increases the genetic diversity in sow herds and decreases the costs to feed and house large number of boars, all with a minimal risk of disease transmission (Althouse and Rossow, 2011; Clements, 2011). The presence of bacteria in semen is a frequent finding in fresh and extended boar ejaculates (Althouse and Lu, 2005). Bacteria adhere to the sperm surface producing ultrastructural changes at the level of the midpiece, plasma membrane and acrosome (Diemer et al., 1996) that can reduce sperm motility and viability and provoke a premature acrosome reaction (Diemer et al., 1996; Kohn et al., 1998; Zan Bar et al., 2008). Therefore, sperm function is compromised by bacterial contamination (Villegas et al., 2005). Moreover, the contamination of extended semen with high bacterial loads can produce reduced conception rates, shorter lifespan of semen doses, early embryonic or foetal death due to the direct invasion of the embryo by the pathogen, endometritis, and systemic

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infection and/or disease in the recipient female resulting from the transmission of the pathogen by semen (De Grau et al., 2006; Maes et al., 2008). So, although the risk of disease transmission using AI may be minimal, the economic looses that semen contaminated with pathogens causes to swine industry can be enormous (Maes et al., 2008).

Related to this, it is worth mentioning that contamination of semen can be due to systemic and/or urogenital tract infections in the boar (Althouse and Lu, 2005) or also due to the hands of the technician performing the collection. The equipment used during semen collection and processing also plays an important role in contamination (De Grau et al., 2006).

The majority of contaminants found in extended seminal doses destined to artificial insemination (AI) are not considered primary pathogens in swine. Most of them are gram negative bacteria, especially from the Enterobacteriaceae family (Althouse and Lu, 2005). Traditionally, given that anaerobes are sensitive to semen preservation conditions, their presence in semen samples has not been widely studied (Eggert - Kruse et al., 1995). However, the presence of anaerobes in semen of horse, human and boar is not exceptional (Corona and Cherchi, 2009; Damirayakhian et al., 2006; Maroto Martín et al., 2010). In this study we have worked with the aerotolerant anaerobe gram positive bacterium *Clostridium perfringens*, which can survive and proliferate in oxygen tensions that are inhibitory for other clostridia (Joclik and Willett, 1991). *C. perfringens* has a wide distribution in the environment, as well as in the intestinal flora of both animals and humans. This bacterium can act as an opportunistic pathogen causing different diseases, such as diarrhoea or necrotic enteritis in young piglets (Morris and Fernández-Miyakawa, 2009), and is highly prevalent throughout the swine industry (Baker et al., 2010), so semen contamination during its collection or processing can occur.

To minimize the effects of bacterial load on extended semen, antimicrobials with bactericidal or bacteriostatic activity are usually included in the semen extender formulation (Althouse et al., 2000; Yániz et al., 2010). However, too much reliance is placed on this method of bacterial control, because some studies have demonstrated that over the 90% of bacteria isolated from extended semen are resistant to the most used antibiotics in extended semen (Bolarín Guillén, 2011).

Against this background, the aim of this study was to determine how different bacterial loads of *C. perfringens* can affect the sperm quality of extended boar semen when incubated at 37 °C and 15 °C. With this aim, two experiments were performed simultaneously, one at 15 °C for 11 days and the other at 37 °C for 96 h.

2. Material and methods

2.1. Semen collection

Thirteen seminal doses coming from thirteen healthy and sexually mature Piétrain boars, aged from 9 to 12 months and submitted to a collection frequency of twice a week, were used in the study. All the samples were manually collected using the gloved-hand technique. After removing the gelatinous fraction of the semen using a gauze filter under high hygienic measures, the sperm rich-fraction was immediately diluted 1:6 (v:v) in a long-term extender (Duragen®, Magapor, Zaragoza, Spain) to a final concentration of 3×10^7 spermatozoa mL^{-1}, divided into 90 mL semen doses and cooled at 15–17 °C. Finally, two seminal doses were transported within 24 h post-collection to the laboratory inside an insulated container held at 15–17 °C.

Upon arrival, all the seminal doses were assessed for semen quality parameters (performed as described below) and, according to the boar semen quality criteria (total motile spermatozoa > 80%; morphologically normal spermatozoa > 85%; spermatozoa with intact acrosome and mitochondrial sheath > 80%), all thirteen seminal doses were qualified for the present study.

2.2. Bacterial inoculation of extended semen

Different pathogenic *C. perfringens* strains purchased from Spanish collection of microbiological type-cultures (CECT, Valencia, Spain) and identified as CECT 4110, CECT 4647, CECT 822, CECT 376 and CECT 486, were used in this study. Strains were cultured in liquid Liver Broth medium (Conda/Pronadisa, Madrid, Spain) for 36–48 h in a universal oven (MEMMERT UNB 200, Schwabach, Germany) at 37 °C and under anaerobic conditions. After assessing the cell concentration with a spectrophotometer (SmartSpec™ Plus, Bio-Rad, California, USA) at a wavelength of 600 nm (optical density, OD_{600}), the culture was diluted with Ringer solution (Scharlau, Barcelona, Spain) up to 10^3 cfu mL^{-1}. Then, 9 mL of semen were taken and inoculated with 1 mL of each bacterial dilution. Thus, the final infective concentrations, as reported by Bussalleu et al., 2011, were: tube A (inoculated with a bacterial concentration of 10^8 cfu mL^{-1}), tube B (with 10^7 cfu mL^{-1}), tube C (with 10^6 cfu mL^{-1}), tube D (with 10^5 cfu mL^{-1}), tube E (with 10^4 cfu mL^{-1}), tube F (with 10^3 cfu mL^{-1}), tube G (with 10^2 cfu mL^{-1}) and tube H (with 1 mL of Ringer solution; this was the negative control tube). The tubes were stored at 15 °C for 11 days in a Z-15 storage chamber (Magapor®, Zaragoza, Spain) in experiment 1, and at 37 °C for 96 h in a universal oven (previously mentioned) in experiment 2. All this procedure was repeated 13 times ($n = 13$) inoculating only one strain in each replicate.

2.3. Sperm quality analyses

After inoculation, an aliquot of each tube (A, B, C, D, E, F, G and H) containing semen inoculated with *C. perfringens* was taken on days 0, 1, 2, 3, 4, 7, 8, 9, 10 and 11 in the experiment at 15 °C, and at 0 h and every 24 h over a 96 h time period in the experiment carried out at 37 °C. Sperm motility and sperm morphology were assessed using a computer-assisted sperm analysis system (SCA® 2002 Production, Microptic, Barcelona, version 2002). Sperm motility, evaluated after 20 min of incubation at 37 °C, was established as counts of motile/immotile spermatozoa and results expressed as the percentage of motile spermatozoa. For each infective concentration, three counts/replicates, of minimum 1000 spermatozoa, were performed.

In the assessment of sperm morphology (also using the computer program SCA® 2002 Production), spermatozoa were previously fixed with pre-warmed 2% formaldehyde in PBS for 5 min at room temperature, and then 5 μL of each semen sample were placed on a slide and mounted with a cover slip. Afterwards, spermatozoa were classified as mature spermatozoa, spermatozoa with a proximal or distal droplet, spermatozoa with head and/or tail anomalies and agglutinated spermatozoa (spermatozoa were considered agglutinated when there were at least three or more spermatozoa attached to one another). For each infective concentration, three counts/replicates of 100 spermatozoa were performed.

Sperm viability was assessed by fluorescence microscopy through the simultaneous evaluation of the state of the nucleus, the integrity of the mitochondrial sheath and the state of the acrosome. The technique used was a multiple staining with the following fluorochormes: propidium iodide (Sigma, USA), bisbenzamide Hoechst 33258 (Sigma), MitoTracker®Green FM (Molecular Probes®, Leiden, The Netherlands) and SBTI – Alexa Fluor® 488 Conjugate (Molecular Probes®) (Bussalleu et al., 2005; Pinart et al., 2006). Fluorescence microscopy images were visualized on a Zeiss Axio Imager Z1 Microscope (Carl Zeiss, Göttingen, Germany) using a Rhodamina filter (excitation spectra: 546/12 nm; emission spectra: 575–640 nm) and an Alexa Fluor 4800 filter (excitation spectra: 485/20 nm; emission spectra of 515–565 nm). Viable spermatozoa showed an intact plasma membrane, nucleus, acrosome and mitochondrial sheath; spermatozoa showing anomalies in at least one of these sperm compartments were considered as non-viable. For each infective concentration, three counts/replicates of 100 spermatozoa were performed.

The presence of *C. perfringens* was tested by using a conventional and a multiplex PCR, both developed in our laboratory (Bussalleu et al., 2013), on different days after inoculation.

2.4. Statistical analysis

Data were managed using SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and Origin Pro 8.0 software (OriginLab Corp., Northampton, Massachusetts, USA). Sperm quality and function parameters (viability and acrosome and mitochondrial sheath integrity, motility and morphology) were considered as dependent variables, while each ejaculate coming from each boar was treated as a biological replicate. The variables were first tested for normality (Kolmogorov-Smirnov) and for homoscedasticity (Levene), and prior to inoculating the semen samples one-way analysis of variance (ANOVA) was used to examine whether the ejaculates were significantly different. No differences ($P > 0.05$) were observed among ejaculates in terms of sperm quality.

Individual effect of boars was removed by calculating per ejaculate, experiment and time point, a ratio between the value of each sperm parameter in a given infective concentration and the value of the same sperm parameter in negative control (e.g. % viable spermatozoa at a concentration of 10^2 cfu mL^{-1} (boar 1 and day 1) per % viable spermatozoa in negative control (boar 1 and day 1)). Although the results are presented in the tables as raw, the statistical analyses were performed using ratio data. Following the first and second experiments, data on ratios were recalculated using the arcsine square root (x) transformation in each case. Then, data were tested using a repeated measures ANOVA (where the incubation/refrigeration time was the intrasubject factor, the bacteria concentration was the intersubject factor and each sperm parameter was the dependent variable). A post hoc Sidak test was used for multiple comparisons.

In all statistical analyses the significant level was set at 5%. Results are expressed as means ± standard error of the mean (SEM).

3. Results

In the present study, we conducted two assays, thereby testing how different *C. perfringens* loads affected boar sperm quality at two different temperatures, 15 °C and 37 °C, during 11 days and 4 days respectively.

3.1. Sperm motility

Regarding the percentage of total motile spermatozoa (TMOT), Fig. 1 shows the results for the first experiment conducted at 15 °C for 11 days (in this and the other figures three infective concentrations (10^3, 10^4 and 10^5 cfu mL^{-1}) are not depicted, since they did not show significant differences when compared to the effects observed in the 10^2 cfu mL^{-1} treatment). Twenty-four hours post inoculation, a significant decrease in the percentage of total motile spermatozoa was observed in the tubes inoculated with 10^8 cfu mL^{-1} and 10^7 cfu mL^{-1} compared to the others (i.e. negative control, 10^2, 10^3, 10^4, 10^5 and 10^6 cfu mL^{-1}). After 48 h of storage at 15 °C, the highest inoculated tube (10^8 cfu mL^{-1}) showed a significant ($P < 0.05$) and drastic decrease in the percentage of total motile spermatozoa. The tubes containing 10^6 cfu mL^{-1} and 10^7 cfu mL^{-1} also showed a significant decrease ($P < 0.05$) compared to the negative control, although it was lower than the inoculated tube with 10^8 cfu mL^{-1}. Nevertheless, this tendency was also observed in the 4th day of storage and remained until the end of the experiment. Finally, at the end of the experiment, the tubes with the highest bacterial loads (10^8 and 10^7 cfu mL^{-1}) had hardly any motile spermatozoa. The tube containing an infective concentration of 10^6 cfu mL^{-1} presented a significant reduction ($P < 0.05$) in the percentage of total motile spermatozoa compared to the negative control and to the tubes inoculated with lower loads ($P < 0.05$), but showed higher total sperm motility than the tubes containing 10^7 cfu mL^{-1} and 10^8 cfu mL^{-1}.

Fig. 2 shows the evolution of the percentage of total motile spermatozoa in the inoculated tubes in the second experiment, performed during four days at 37 °C. After 24 h of incubation at 37 °C, the tube containing 10^8 cfu mL^{-1} had a drastic and significant decrease, so that less than 5% of motile spermatozoa remained. The tube containing 10^7 cfu mL^{-1} also showed a highly significant decline in the percentage of total motile spermatozoa, but it was significantly lower compared to that observed in the

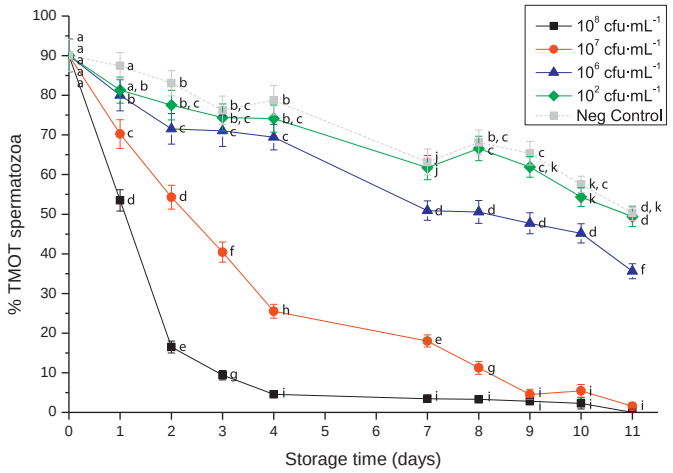

Fig. 1. Percentage of total motile spermatozoa over the storage period (11 days) at 15 °C (experiment 1) after inoculating different infective concentrations (10^2–10^8 cfu mL^{-1}) of *C. perfringens*. Different superscripts (a–k) mean significant differences ($P < 0.05$) between treatments and incubation times (treatment × time point).

highest inoculated tube (10^8 cfu mL^{-1}). Forty-eight hours after inoculation the tubes containing 10^7 and 10^8 cfu mL^{-1} showed a significant decrease ($P < 0.05$) in the percentage of total motile spermatozoa (there were no motile spermatozoa) in comparison with the others tubes. After 72 there were no motile spermatozoa in any tube, including the negative control.

When the percentage of progressive motile spermatozoa (PMOT) was analysed in the experiment carried out at 15 °C for 11 days (Fig. 3), there was a marked reduction in the tube with the highest *C. perfringens* load during the first two days of storage ($P < 0.05$). After 3 days of storage, the tube with 10^7 cfu mL^{-1} also showed a significant ($P < 0.05$) diminution in the percentage of progressive motile spermatozoa compared to the non-inoculated tube and the rest of treatments. However, its decrease was significantly lower ($P < 0.05$) compared to the highest inoculated tube (10^8 cfu mL^{-1}). This trend was observed until the ninth day post inoculation. At this time point and up to the end of the experiment, no progressive motile spermatozoa were found in these two treatments (10^8 cfu mL^{-1} and 10^7 cfu mL^{-1}).

Finally, Fig. 4 shows the evolution of the percentage of progressive motile spermatozoa in experiment 2 (37 °C), and, as we can see, these results are similar as those obtained in Fig. 2. Again, a reduction in the percentage of progressive motile spermatozoa was observed throughout the incubation period, the decrease being the highest in the tubes containing 10^7 and 10^8 cfu mL^{-1} after 24 and 48 h of inoculation.

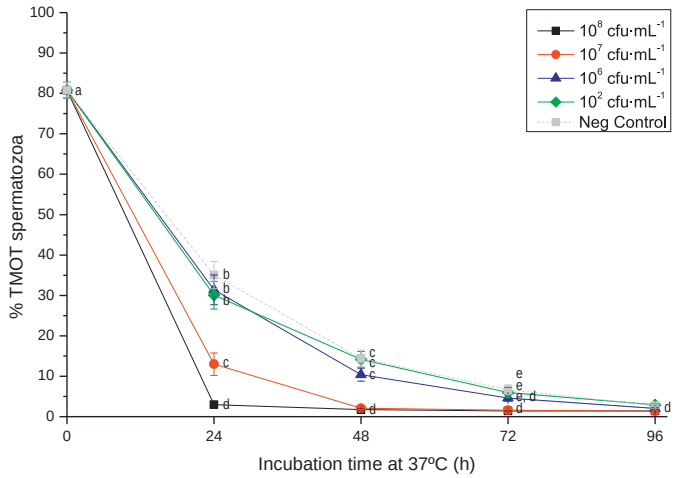

Fig. 2. Percentage of total motile spermatozoa over the incubation period (96 h) at 37 °C (experiment 2) after inoculating different infective concentrations (10^2–10^8 cfu mL^{-1}) of *C. perfringens*. Different superscripts (a, b, c, d) mean significant differences ($P < 0.05$) between treatments and incubation times (treatment × time point).

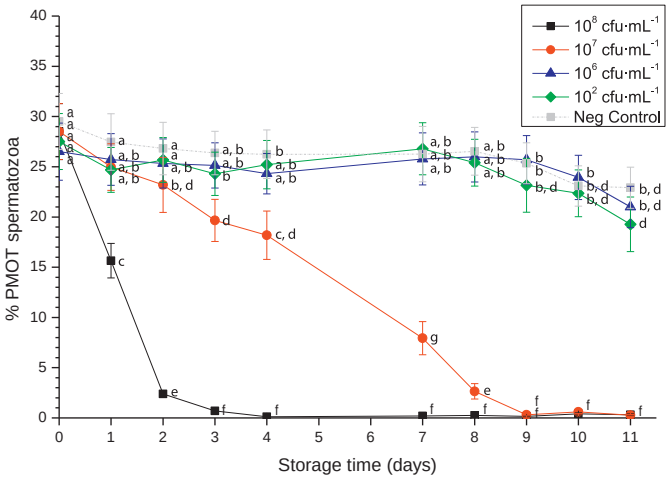

Fig. 3. Percentage of progressive motile spermatozoa over the storage period (11 days) at 15 °C (experiment 1) after inoculating different infective concentrations (10^2–10^8 cfu mL^{-1}) of *C. perfringens*. Different superscripts (a–g) mean significant differences ($P < 0.05$) between treatments and incubation times (treatment × time point).

3.2. Sperm viability and acrosome and mitochondrial sheath integrity

Fig. 5 shows the percentage of viable spermatozoa observed in experiment 1 (15 °C). Twenty-four hours after inoculation, the percentage of viable spermatozoa in the highest inoculated tube (10^8 cfu mL^{-1}) was significantly lower ($P < 0.05$) when compared to the other treatments and the negative control. After 3 days of storage, a decrease in the viability was also observed in the tube containing 10^7 cfu mL^{-1}; however, it was significantly lower compared to the inoculated tube with 10^8 cfu mL^{-1}. This tendency remained until the day 8 of storage, when these two infective concentrations showed no significant differences between them but their viability continued significantly lower compared to the negative control and the rest of treatments. At the end of the experiment, when there was approximately only 10% of viable spermatozoa left.

In the second experiment, carried out at 37 °C (Fig. 6), the negative control (non-infected tube) and the tubes inoculated with a bacterial concentration ranging from 10^2 to 10^5 cfu mL^{-1} showed a decrease in the percentage of viable spermatozoa 24 h after the start of the experiment. However, sperm viability in these inoculated concentrations was similar to the negative control. Contrarily, sperm viability in the other inoculated tubes (10^6, 10^7 and 10^8 cfu mL^{-1}) decreased significantly ($P < 0.05$) with regard to negative control and the other treatments, the highest inoculum (10^8 cfu mL^{-1}) being the most deleterious. After 48 h of inoculation there were significant differences when comparing the tubes containing 10^8 cfu mL^{-1} and 10^7 cfu mL^{-1} with the other ones (including the negative control). Seventy-two hours post inoculation, the sperm

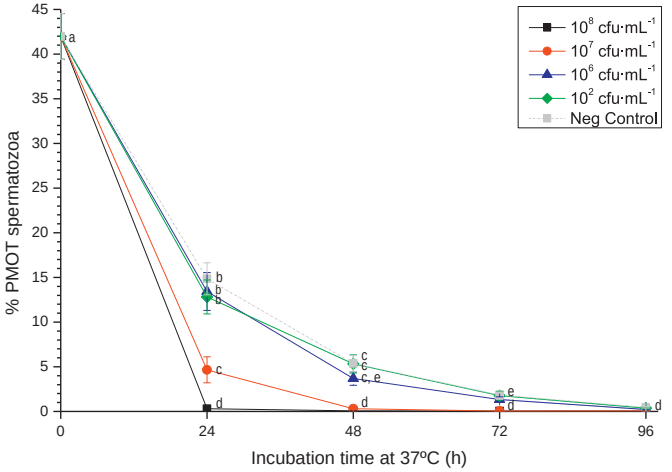

Fig. 4. Percentage of progressive motile spermatozoa over the incubation period (96 h) at 37 °C (experiment 2) after inoculating different infective concentrations (10^2–10^8 cfu mL^{-1}) of *C. perfringens*. Different superscripts (a–e) mean significant differences ($P < 0.05$) between treatments and incubation times (treatment × time point).

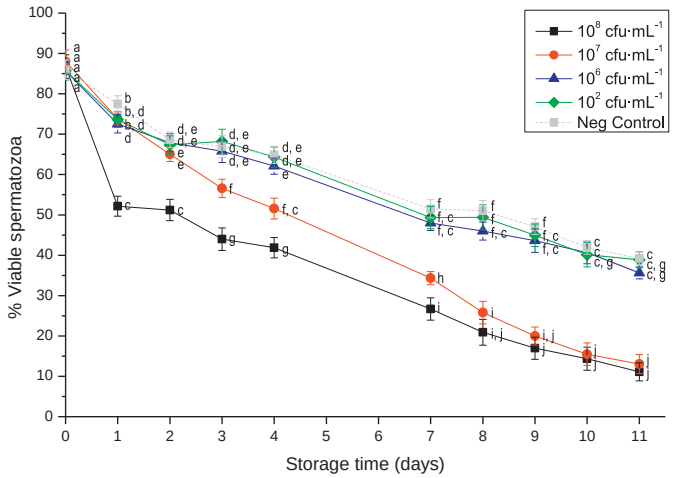

Fig. 5. Percentage of viable spermatozoa over the storage period (11 days) at 15 °C (experiment 1) after inoculating different infective concentrations (10^2–10^8 cfu mL^{-1}) of *C. perfringens*. Different superscripts (a–j) mean significant differences ($P < 0.05$) between treatments and incubation times (treatment × time point).

viability of the tube inoculated with 10^8 cfu mL^{-1} was significantly ($P < 0.05$) lower than the other tubes. Finally, after 96 h there were practically no viable spermatozoa in any tube.

3.3. Sperm morphology

As far as sperm morphology is concerned, there were no significant changes ($P > 0.05$ in all cases) in any treatment along both experiments (e.g.: day 4 at 15 °C: control tube 94.9% ± 1.9 of normal spermatozoa, 10^8 tube 94.8% ± 2.2 of normal spermatozoa; day 4 at 37 °C: control tube 93.1% ± 1.9 of normal spermatozoa, 10^8 tube 94.3% ± 1.9 of normal spermatozoa). However, a high sperm agglutination was observed in both experiments, especially in the highest inoculated tubes incubated at 37 °C just from 24 h post inoculation and until the end of the experimental period (data not shown).

In all cases, the presence of bacteria was confirmed by PCR analysis at each relevant time point and in both experiments (Bussalleu et al., 2013).

4. Discussion

The collection of boar semen for AI purposes is not a sterile procedure, since there are multiple point sources of contamination during the process: boar ejaculate passes through the testis (spermatozoa) and accessory glands (seminal plasma), and comes into contact with different environments, like the epididymis, urethra and prepuce (Bonet et al., 2013). Furthermore, recipients used for semen collection and processing can also constitute a source of contamination (Pérez Llano et al., 2001).

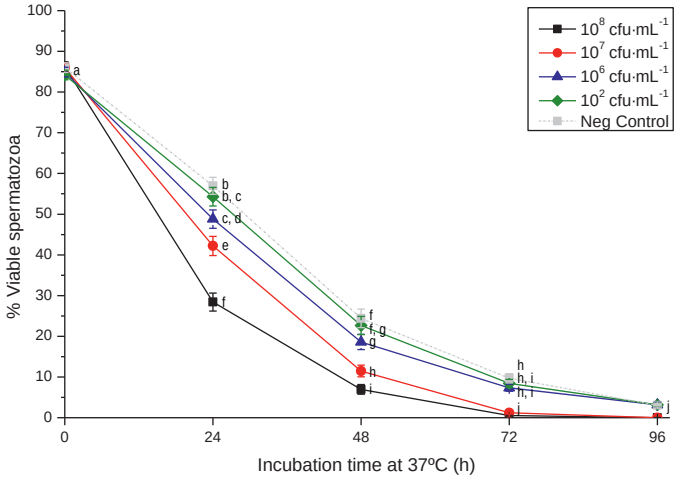

Fig. 6. Percentage of viable spermatozoa over the incubation period (96 h) at 37 °C (experiment 2) after inoculating different infective concentrations (10^2–10^8 cfu mL^{-1}) of *C. perfringens*. Different superscripts (a–j) mean significant differences ($P < 0.05$) between treatments and incubation times (treatment × time point).

Boar ejaculates usually contain 10^4–10^5 cfu mL^{-1} (Sone, 1990). As previously mentioned in the introduction, the most frequent boar sperm contaminants are aerobic bacteria belonging to the Enterobacteriaceae family (Althouse and Lu, 2005). Even most of them are non-pathogenic bacteria, they can negatively influence sperm quality and longevity if present in high concentrations. However, boar semen can also become infected with other bacterial pathogens that may be transmitted by semen (Maes et al., 2008). The most common way of spreading diseases in a boar stud is through direct or close boar-to-boar contact (Althouse and Rossow, 2011). Nevertheless, transmission of bacterial pathogens into a sow herd can also occur by AI (Althouse and Rossow, 2011; Maes et al., 2008), thus provoking economic loses to swine industry.

Despite anaerobic bacteria have been isolated from boar ejaculate (De Grau et al., 2006; Maroto Martín et al., 2010), no study has investigated whether anaerobic bacteria can survive in boar sperm and how their presence affects sperm quality. In this study we aimed to determine the effect of an anaerobic bacterium, *C. perfringens*, on boar sperm quality in case of accidental contamination, as *C. perfringens* has a wide distribution in the environment and swine industry and can act as an opportunistic pathogen (Baker et al., 2010; Morris and Fernández-Miyakawa, 2009). With this purpose, two experiments were performed. In the first experiment, our aim was to follow how different infective concentrations of *C. perfringens* affected boar sperm quality when samples were preserved by refrigeration at 15 °C in a long-term extender. This experiment allowed us to mimic the conditions of AI centres, which use seminal doses that are conserved at 15 °C. The second experiment sought to determine the effect of the same bacterium on boar sperm quality after incubation at 37 °C, to mimic what happens within male and female reproductive tracts. However, this approach needs more research in the near future, since the animal's immune system must be taken into account to better address how these *C. perfringens* loads affect sperm quality at corporal temperature. The range of bacterial load inoculation (from 10^8 cfu mL^{-1} to 10^2 cfu mL^{-1}) in both experiments was chosen because boar ejaculates have been reported to range in concentrations up to 10^9 cfu mL^{-1} (Althouse et al., 2000). Nevertheless, as previously reported by Bussalleu et al., 2011 with *E.coli*, the effects of a bacterial load of 10^8 cfu mL^{-1} on boar sperm quality have proven to be extremely deleterious.

Results obtained in the present work show that there was no affectation on boar sperm morphology in the two experiments carried out, probably because the provoked changes by *C. perfringens* cannot be seen using optical microscopy. Related to this, previous studies have demonstrated that *E. coli* damages sperm ultrastructure (Diemer et al., 2000b, 1996), but these damages can only be observed when using electron microscopy rather than conventional optical microscopy (Diemer et al., 2000a). Thus, this might explain why sperm morphology parameters remained unaffected in our analysis. However, further studies should address whether *C. perfringens* also induces ultrastructural changes.

Regarding sperm motility and viability, *C. perfringens* has demonstrated to be highly harmful in both experiments, especially in the highest inoculated tubes, suggesting that the effects of *C. perfringens* on boar sperm quality are time- and concentration-dependent (Althouse et al., 2000; Bussalleu et al., 2011; Huwe et al., 1998). These effects resulted to be more significant at 37 °C than at 15 °C in both parameters. In both cases the reduction of sperm quality may be attributable to the attachment of bacteria to the sperm plasmalemma inducing alterations, as has been reported in humans (Diemer et al., 2000b). Another suitable explanation may be related to the release of certain toxins that might affect the sperm plasma membrane integrity, as has been demonstrated in the case of *E. coli*, whose haemolysin seems to be involved in the molecular mechanism that produces the membrane disruption of human sperm (Diemer et al., 2003; Schulz et al., 2010). It is well-known that *C. perfringens* produces different toxins. Toxins α, β, ε and CPE interact with the cell membrane provoking membrane disruption or pore formation (Briggs et al., 2011; Naylor et al., 1998; Petit et al., 1999; Robertson et al., 2011; Uzal and McClane, 2011). The ι-toxin, however, acts intracellularly catalysing the ADP-ribosylation of actin monomers, which leads to cell rounding and death (Petit et al., 1999; Sakurai et al., 2009). Nevertheless, the aforesaid effects have been mostly studied in epithelial cells. So further studies are needed to determine whether the effects provoked by *C. perfringens* are caused by sperm-bacteria binding or toxin secretion, because, as far as we know, no reports have previously investigated the mechanism of action of *C. perfringens* on boar sperm.

In both experiments, great sperm agglutination was also observed, probably associated to bacterial adherence to spermatozoa, as Kohn et al. (1998) and Sone (1990) have pointed out in previous studies. Again, further studies involving electron microscopy analyses are required to confirm these suppositions.

In the present work the most adverse effects were seen at 37 °C. It must be taken into account that at this temperature sperm metabolism is greatly active, so the absence of motile spermatozoa at the end of the second experiment (at 37 °C) may be explained by a presumable depletion of energy sources (Barbonetti et al., 2010). On the other hand, *C. perfringens* has its optimal growth temperature at 37 °C and the extenders used contain plenty of nutrients, which are not only indispensable for sperm survival but also adequate for allowing bacterial growth (De Grau et al., 2006). This also suggests that the concentration/combination of antibiotics used in commercial long-term extenders is not able to prevent bacterial growth completely when the infective concentration is too high and/or when the environment temperature is higher than 15–17 °C. Furthermore, bacterial metabolism is lower and less efficient at 15 °C and bacteria must also adapt to the novel temperature and environment (Althouse, 2008). Thus, this fact would explain why the effects of *C. perfringens* on boar sperm quality were not seen immediately, similarly to previous reports involving other bacteria (Althouse et al., 2000; Bussalleu et al., 2011). Moreover, another element that would explain diminished sperm quality is acidification of the medium due to bacterial metabolism, which is harmful for sperm survival (Pérez Llano et al., 2001).

Commercial extenders used for semen storage maintain sperm function and survival in vitro, and contain antimicrobials to minimise the undesirable effects of bacterial contamination. However, it has been reported that there is a high number of bacteria resistant to the antimicrobials present in boar extenders (Bolarín Guillén, 2011). Thus, as seen with the results obtained in these two experiments, *C. perfringens* could survive in the doses despite the presence of antimicrobials, even if the adverse effects are only seen at high inocula (10^8 cfu mL^{-1} to 10^6 cfu mL^{-1}). Contrarily, in previous studies with the aerobic bacteria *E. coli*, the deleterious effects on boar sperm quality were already seen at an infective concentration of 10^3 cfu mL^{-1} (Bussalleu et al., 2011); and an infective concentration higher than 3.5×10^3 cfu mL^{-1} in boar semen has been reported to produce a significant reduction in litter size (Maroto Martín et al., 2010). Consequently, differences could also be explained by the fact that *C. perfringens* would need a greater spermatozoa:bacteria ratio to produce more adverse effects than *E. coli*.

In conclusion, this work reveals that the aerotolerant anaerobe bacterium *C. perfringens* reduces boar sperm quality when present in high concentrations in extended seminal doses destined to AI. Therefore, doses destined to AI programmes should be tested routinely for the presence of bacterial contaminants like *C. perfringens*, prior to their use, to avoid the use of semen doses with diminished quality and the spread of bacterial contaminants. However, further studies must be carried out to evaluate the effects of *C. perfringens* on sperm ultrastructure and the role of the toxins released by this bacterium in affecting boar sperm quality.

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Paper II

Effects of different concentrations of *Pseudomonas aeruginosa* on boar sperm quality

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Effects of different concentrations of *Pseudomonas aeruginosa* on boar sperm quality

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ABSTRACT

Bacteriospermia in boar ejaculates is a frequent finding that compromises the sperm quality and, consequently, causes economic losses in swine industry. The present study sought to evaluate the effect of different concentrations of *Pseudomonas aeruginosa* on boar sperm quality over a storing period of 11 days at 15–17 °C. Ten commercial seminal doses coming from post-pubertal and healthy boars were artificially inoculated with different infective concentrations of *P. aeruginosa*, ranging from 2×10^8 to 2×10^4 cfu/mL. Negative controls were non-inoculated doses. Sperm quality, assessed as sperm motility (CASA), sperm viability, acrosome integrity and pH, as well as the bacterial growth, were checked after 0, 1, 2, 4, 7, 9 and 11 days of storage at 15–17 °C. Results obtained showed significant decreases in the percentages of total and progressive sperm motility, sperm viability and acrosome integrity in the greatest infective concentrations (2×10^7 and 2×10^8 cfu/mL), when compared to the negative control. In contrast, there was no effect on seminal pH throughout the experiment. Results indicate the presence of *P. aeruginosa* in boar semen, apart from being a potential source for the spread of infectious diseases and harmful impact on sows, negatively affects the longevity and fertilizing ability of boar sperm when present in high concentrations. Thus, *P. aeruginosa* causes deleterious effects on boar sperm quality during liquid storage at 15–17 °C, thus strict hygienic measures must be implemented in boar studs to minimize bacterial concentration of semen doses.

1. Introduction

Over the last decades, artificial insemination (AI) techniques have been extensively used in countries with intensive pig production due to their advantages over natural mating, such as genetic dissemination, control of sexually transmitted diseases and lesser costs of housing large numbers of boars (Rodríguez-Gil and Estrada, 2013). The worldwide application of AI was rapidly adopted due to the ability to store semen for more than 10 days at 15–17 °C, which enables the trade both nationally and internationally (Gerrits et al., 2005; Guérin and Pozzi, 2005; Rodríguez-Gil and Estrada, 2013). However, it is well known that the process of semen collection in farm animals is a procedure that is not sterile due to multiple sources of contamination during the collection and manipulation processes (Bussalleu and Torner, 2013). These sources of contamination are classified into those from animal and non-animal origin, such as feces, preputial cavity fluids, water or ventilation system (Althouse and Lu, 2005; Althouse, 2008; Aurich and

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Spergser, 2007; Thibier and Guerin, 2000; Varner et al., 1998; Yániz et al., 2010). This fact is the primary concern to pig breeders because of the consequences that can result from these factors when using AI.

Several studies have shown that bacterial contamination might affect semen quality and longevity during preservation and storage and may lead to infection of the female reproductive tract after insemination (Maes et al., 2008; Sepúlveda et al., 2013; Sone et al., 1989). Adverse effects of different bacteria upon sperm quality include decreases in sperm motility and viability (Bussalleu et al., 2011; Huwe et al., 1998; Ubeda et al., 2013; Sepúlveda et al., 2013), morphological alterations and/or degenerative acrosome exocytosis (Diemer et al., 1996; Köhn et al., 1998) among others. This diminution in sperm quality can underlie reductions in reproductive performance. In addition, contaminated semen is also a vehicle for pathogen transmission and may also cause infertility by leading to endometritis, embryonic or fetal death and other clinical diseases and infections (Maes et al., 2008; Maroto Martín et al., 2010). The impact of contaminated semen can, therefore, be great and may lead to major economic losses for the swine industry.

To minimize the effects of bacterial load on extended semen, antimicrobials are usually included in the semen extender formulation (Althouse et al., 2000; Yániz et al., 2010). However, complete elimination cannot be assured, because many bacteria are resistant to the antimicrobials that are commonly used in semen extenders (Bolarín Guillén, 2011; Maes et al., 2008). Hence, regular monitoring for bacterial contamination of extended semen samples may be an important component of a control program (Althouse, 2008; Maes et al., 2008).

In boar ejaculates, typical bacterial concentrations range from 10^3 to 10^5 cfu/mL (Althouse and Lu, 2005; Morrell and Wallgren, 2011), even though there are previous reports showing that bacterial contamination can vary in concentrations of up to 10^9 cfu/mL (Althouse et al., 2000). Nevertheless, in some studies, bacterial loads greater than 10^3 cfu/mL are detrimental to sperm quality and reduces litter size (Chung et al., 2013; Maroto Martín et al., 2010). The majority of the contaminants usually present in boar ejaculates are not considered as primary pathogens in the swine industry. However, these bacteria can negatively influence sperm quality especially if present at high concentrations or if a predominant bacterium is present, thereby inhibiting the growth of others (Althouse et al., 2000; Berktas et al., 2008; Smole et al., 2010). In different studies, at least 25 different genera have been detected as common boar semen contaminants, which are predominantly Gram-negative bacteria belonging to the *Enterobacteriaceae* family (Althouse and Lu, 2005; Dagnall, 1986; Danowski, 1989; Sone et al., 1989; Tamuli et al., 1984). According to Bresciani et al. (2014) and Maroto Martín et al. (2010), the most frequently isolated bacteria from boar semen are *Escherichia coli*, *Proteus* spp., *Serratia* spp., *Enterobacter* spp., *Klebsiella* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Pseudomonas* spp. To our knowledge, the effects of *Pseudomonas aeruginosa* on boar sperm quality are still unknown. *P. aeruginosa*, a Gram-negative rod-shaped bacterium belonging to the *Pseudomonadaceae* family, is widely distributed around the natural environment, especially abundant in water and soils. It is an ubiquitous and opportunistic pathogen for animals and humans that can cause disease when resistance to infection is low (Bergan, 1981; De Oliveira et al., 2013).

In previous studies, effects of different concentrations of *E. coli* (Bussalleu et al., 2011) and *Clostridium perfringens* (Sepúlveda et al., 2013) on boar sperm quality were examined, because it is important to identify and evaluate the agents that are present in greater concentrations in boar semen doses and pose the main risks for the swine industry, not only those of intrinsic pathogenicity. The knowledge of the effects of bacteria on boar sperm quality is, therefore, a useful tool to avoid the use of contaminated doses that may cause economic and health problems. For this reason, the aim of the present study was to evaluate how different concentrations of *P. aeruginosa*, a bacterium commonly found in semen, affect the quality of boar spermatozoa when stored at 15–17 °C for 11 days, to mimic the conditions in AI centers and to determine the concentration at which the effects become detrimental.

2. Material and methods

2.1. Semen samples

Ten sperm samples from 10 healthy and sexually mature Pietráin boars, aged from 9 to 12 months, were used to conduct this study. All boars were submitted to a collection frequency of twice a week using the gloved-hand technique. The semen was filtered through gauze under hygienic conditions to remove the gelatinous fraction. The sperm-rich fraction was immediately diluted 1:6 (v:v) in a long-term extender (Duragen®, Magapor, Zaragoza, Spain), divided into 90 mL semen doses and stored at 15–17 °C. One seminal dose was transported refrigerated to the laboratory within 24 h post-collection. No fertility problems were previously associated with processing of semen at the AI station.

Upon arrival, sperm samples were assessed for semen quality variables as subsequently described (sperm motility, morphology, viability and concentration). Sperm concentration was assessed using the Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and averaged $44.3 \times 10^6 \pm 2.64$ spermatozoa/mL. Sperm morphology was evaluated using a computer-assisted sperm analysis (CASA) system equipped with the Sperm Class Analyser® software (SCA® 2002 Production Module, Microptic, Barcelona, version 2002). According to the boar semen quality criteria (total motile spermatozoa >80%; morphologically normal spermatozoa >85%; viable spermatozoa >75%) (Sancho and Vilagran, 2013; Yeste et al., 2013), all seminal doses were qualified for the study.

2.2. Bacterial inoculation

2.2.1. Origin and preparation of bacteria

The strain of *P. aeruginosa* identified as CECT 4145 and obtained from Spanish collection of microbiological type cultures (CECT, Valencia, Spain) was used in the study. The presence of antibacterial agents in extended

semen is regulated by the European Union (Council Directive 90/429/EEC). The bacteria used in this study have been tested for antibiotic susceptibility by disk diffusion assay according to CLSI standards (CLSI, 2012) and were shown to be susceptible to streptomycin and spectinomycin, two of the antibiotics approved by the Council Directive 90/429/EEC.

The strain was cultured in Luria-Bertani (LB) medium (Tryptone 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L, pH 7.2) at 37 °C for 18–24 h in a shaking water bath (MEMMERT waterbath WNB 7-45 coupled with the shaking device 14/22, Schwabach, Germany). All *Pseudomonas* isolates were collected by centrifugation at 4800 × *g* for 10 min and washed with *Beltsville thawing* solution (BTS) (glucose 37 g/L, sodium citrate 5.88 g/L, sodium bicarbonate 1.26 g/L, EDTA 1.25 g/L, potassium chloride 0.75 g/L, pH 7.2) at 4800 × *g* for 10 min. Subsequently, bacteria were gently resuspended with 1 mL of BTS and the cell concentration was assessed with a spectrophotometer (SmartSpec™ Plus, Bio-Rad, California, USA) at a wavelength of 600 nm (optical density, OD600). All bacterial isolates averaged $32 \times 10^8 \pm 8.12$ cfu/mL. Soon after, the bacterial culture was adjusted to a concentration of 2×10^9 cfu/mL, and serially diluted with BTS up to 2×10^5 cfu/mL. Then, 2 mL of each infective dilution were added to five different falcon tubes.

2.2.2. Preparation and inoculation of semen

After analyzing the sperm concentration, semen was added to each of the above-mentioned falcon tubes to achieve a final sperm concentration of 2×10^7 spz/mL in a total volume of 20 mL. Each falcon tube was subsequently brought to the final volume of 20 mL with BTS. Thus, the final infective concentrations of *P. aeruginosa* used in the study were: 2×10^4, 2×10^5, 2×10^6, 2×10^7 and 2×10^8 cfu/mL. The negative control tube was a 20-mL solution containing semen at a final sperm concentration of 2×10^7 spz/mL and BTS. All tubes were stored at 15–17 °C for 11 days in a Z-15 storage chamber (Magapor®, Zaragoza, Spain).

2.3. Growth dynamics

To elucidate the growth dynamics of *P. aeruginosa* over the experimental period, growth patterns were determined for the different treatments tested in the study. At time 0 and at days 1, 2, 4, 7, 9 and 11, 100 μL of each infective concentration/control tube (diluted in Ringer's solution [Scharlau, Barcelona, Spain] if necessary) were plated per duplicate on *Brain Heart Infusion* (BHI) agar plates (Conda/Pronadisa, Madrid, Spain). Colonies were directly counted after incubating the plates at 37 °C for up to 48 h.

2.4. Sperm quality analyses

Before the addition of bacteria (time 0) and after 1, 2, 4, 7, 9 and 11 days post-inoculation, different sperm quality variables were evaluated.

2.4.1. pH

The pH of each sample was measured using a Crison pH-meter 507 (Crison Instruments, Barcelona, Spain). To avoid contamination between treatments, the electrode was thoroughly washed before and after each measurement with distilled water. Three measures per treatment were performed in each time point.

2.4.2. Sperm motility

Total and progressive sperm motilities were assessed using a computer-assisted sperm analysis (CASA) system equipped with the Sperm Class Analyser® software (SCA® 2002 Motility Module, Microptic, Barcelona). After 20 min of incubation at 37 °C, 20 μL of semen were placed on a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) and observed at 100× magnification under phase-contrast microscopy (Olympus BX41; Olympus Europa GmbH, Hamburg, Germany; negative phase contrast). Results were expressed as percentage of total or progressive motile spermatozoa on the total number of evaluated sperm cells. Three counts of at least 1000 spermatozoa were evaluated.

2.4.3. Flow cytometry analyses

Flow cytometric analyses were conducted to evaluate two sperm functional variables: sperm viability (membrane integrity) and acrosome integrity, using a Cell Lab Quanta SC Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with an argon ion laser (488 nm) and standard optics. Green fluorescence from SYBR-14 and PNA-FITC was read with the FL1 photodetector (525 ± 30 nm), while red fluorescence from PI was detected with the FL3 photodetector (670 ± 30 nm). The forward scatter (FS) system was replaced by electronic volume (EV), based on the Coulter principle for volume assessment, which is simply the volume of electrolyte displaced by a cell suspended in an electrolyte solution. This system detects changes in electrical resistance and enables accurate and sensitive cell size measurements. Electronic volume and side scatter (SS) dot plots gave physical properties of size and granularity which allowed the gating of sperm-specific events and excluded debris and aggregates from the analysis. Ten thousand events were collected per sample and replicate, with a flow rate of 200 cells/s. Each analysis was repeated three times in independent tubes per sample and variable. Data were analyzed using Cell Lab Quanta SC MPL Analysis Software v 1.0 (Beckman Coulter).

2.4.3.1. Sperm viability (SYBR-14/PI). Sperm viability was assessed by evaluating the membrane integrity using the fluorochromes SYBR-14 and Propidium iodide (PI) (LiveDead® Sperm Viability Kit L-7011; Molecular Probes Inc., Eugene, OR, USA). For this purpose, after 1 h of incubation at 38 °C, samples were diluted with BTS to a final concentration of 1×10^6 spz/mL. Aliquots of 500 μL from diluted samples were incubated with 0.5 μL of SYBR-14 working solution (final concentration of 100 nM) at 38 °C for 10 min in darkness, and then with 2.5 μL of PI (final concentration of 12 μM) for 5 min, under the same conditions, before cytometric analyses. After the evaluation, three sperm populations were identified: (1) viable

green-stained spermatozoa (SYBR-14$^+$/PI$^-$), (2) non-viable red-stained spermatozoa (SYBR-14$^-$/PI$^+$) and (3) non-viable green- and red-stained spermatozoa (SYBR-14$^+$/PI$^+$). In the SYBR-14$^-$/PI$^-$ quadrant, non-sperm particles were found (debris). Sperm viability was calculated as the mean of three measures performed per sample (mean ± S.E.M.).

2.4.3.2. Acrosome integrity (FITC-PNA/PI). Acrosome integrity was evaluated by staining spermatozoa with the lectin *Arachis hypogaea* peanut agglutinin labeled with fluorescein isothiocyanate (FITC-PNA, L7381; Sigma-Aldrich, St. Louis, MO, USA) and PI. After 1 h of incubation at 38 °C, aliquots of 500 μL from samples diluted with BTS to a final concentration of 1×10^6 spz/mL, were incubated with 1.5 μL of FITC-PNA working solution (3 μg/mL) at 38 °C for 5 min in the dark. Then, the aliquots were stained with 2.5 μL of PI (final concentration of 12 μM) for 5 min under the same conditions prior to the cytometric analysis. After the assessment, three different sperm populations were observed: (1) viable spermatozoa with intact acrosome membrane (FITC-PNA$^-$/PI$^-$), (2) non-viable spermatozoa with damaged plasma membranes (FITC-PNA$^+$/PI$^-$; FITC-PNA$^-$/PI$^+$) and (3) non-viable spermatozoa with exocytosis of the acrosome membrane (FITC-PNA$^+$/PI$^+$). In the case of PNA-FITC/PI staining, data obtained from flow cytometry experiments were corrected according to the procedure described by Petrunkina et al. (2010). Acrosome integrity was calculated as the mean of three measures performed per sample (mean ± S.E.M.).

2.5. Statistical analyses

Statistical analyses were conducted using IBM® SPSS®19.0 (IBM Corp.; Chicago, IL) and Origin Pro 8.0 for Windows statistical packages (OriginLab Corp., Northampton, MA).

All data obtained from evaluation was first tested for normality (Shapiro–Wilk test) and then for variance homogeneity (Levene test), and motility data were recalculated using the arcsine square root (x) transformation to match the parametric assumptions. Next, a linear mixed model for repeated measures was used. In this model, each evaluated parameter (% viable spermatozoa, % total motile spermatozoa, % progressive motile spermatozoa, % acrosome intact spermatozoa, seminal pH, bacterial growth) was the dependent variable, time of storage at 15–17 °C was the intra-subject factor, the bacterial load (control, 2×10^4, 2×10^5, 2×10^6, 2×10^7, 2×10^8) was the fixed-effect factor and the ejaculate was the random-effect factor. Pair-wise comparisons were made through a t-test with Bonferroni correction. Data are shown as percentages and mean ± standard error of the mean (S.E.M.). Each ejaculate was considered as an independent observation, and the minimal level of significance was set at $P < 0.05$ in all statistical analyses.

3. Results

In all the figures, the infective concentrations 2×10^4 and 2×10^6 cfu/mL are not depicted because there were no significant differences at these concentrations when compared to the effects observed in the 2×10^5 cfu/mL treatment.

3.1. Growth dynamics

Fig. 1 shows the growth dynamics of *P. aeruginosa* during 11 days of storage at 15–17 °C. Twenty-four hours after inoculation and until the end of the experiment, there were differences with all treatments ($P < 0.05$) compared to the negative control, with the exception of the tubes with 2×10^4 and 2×10^5 cfu/mL at Day 2. Hence, the tubes artificially inoculated with *P. aeruginosa* had greater bacterial growth than the negative control throughout the study. However, at Days 1, 7, 9 and 11, there were not only differences ($P < 0.05$) with all treatments compared to the control, but also among these groups (e.g. control tube and the tubes inoculated with 2×10^5 and 2×10^7 cfu/mL, had bacterial counts of $1.96 \times 10^6 \pm 0.99$, $1.02 \times 10^8 \pm 0.26$ and $7.38 \times 10^8 \pm 0.97$ cfu/mL, respectively, at Day 7). Bacterial growth also increased as incubation time progressed as exhibited from Day 1 for the tubes inoculated with 2×10^7 and 2×10^8 cfu/mL, and from Day 2 for the rest of treatments. This tendency was maintained until the end of the experimental period. Interestingly, the fact that at Day 2 the tubes inoculated with 2×10^4, 2×10^5, 2×10^6 and 2×10^7 cfu/mL of *P. aeruginosa* did not show significant differences when compared to the values obtained at Day 1 probably implies that these treatments were under an adaptation or lag phase. Therefore, according to the results obtained in the present study, the growth dynamics of *P. aeruginosa* are time- and concentration-dependent.

3.2. pH

Table 1 shows, as mean ± S.E.M., the effect of *P. aeruginosa* on seminal pH. No differences ($P > 0.05$) were found for both, storage time or bacterial load factors.

3.3. Sperm motility

Figs. 2 and 3 show the results obtained after assessing the effects of different bacterial loads on total and progressive sperm motility, respectively. *P. aeruginosa* affected sperm motility in a both time- and concentration-dependent manner. There was also an interaction ($P < 0.05$) between these two factors (i.e. bacterial load × storage time).

3.3.1. Total sperm motility

There were no differences ($P > 0.05$; Fig. 2) between treatments and the negative control after 24 h of storage at 15–17 °C (e.g. 97.67 ± 3.67 and 97.83 ± 3.88% of motile spermatozoa in control tube and 2×10^7 cfu/mL tube, respectively). At Day 2, only the tube containing 2×10^8 cfu/mL of *P. aeruginosa* (79.83 ± 2.93%) was different ($P < 0.05$) compared to the negative control (94.88 ± 3.57%) at the same time point. From Days 4 to 9, the percentages of total motile spermatozoa in the tubes inoculated with 2×10^7 and 2×10^8 cfu/mL were less ($P < 0.05$) than those found in the negative control and with the other treatments, the reduction caused by the bacterial load of 2×10^8 cfu/mL being the most detrimental. Nevertheless,

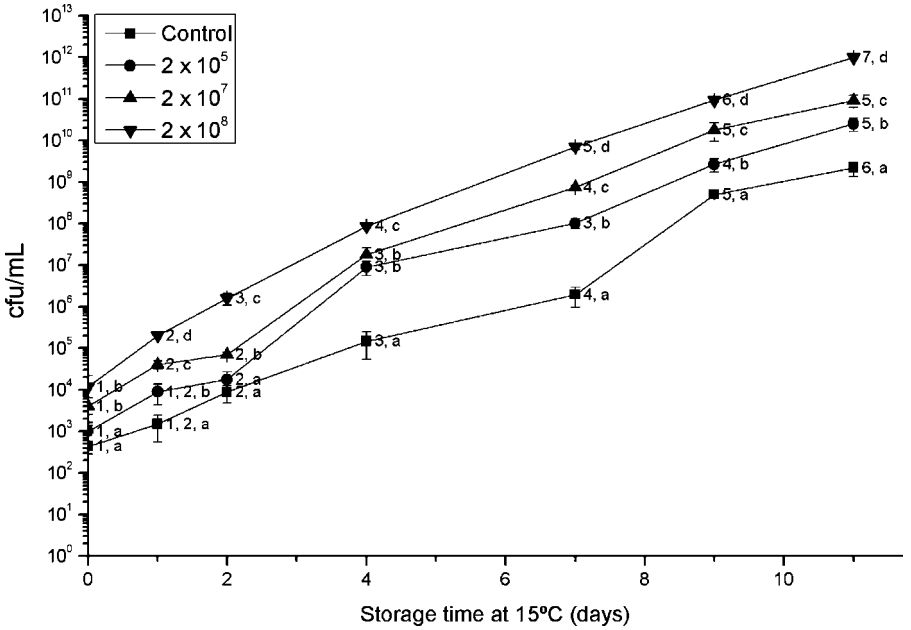

Fig. 1. Growth dynamics of different concentrations (cfu/mL) of *P. aeruginosa* during 11 days of storage at 15–17 °C (mean ± S.E.M.). Different letters (a–d) mean significant differences ($P < 0.05$) between treatments at the same time point, whereas different numbers (1–7) indicate differences ($P < 0.05$) within a given bacterial load throughout the storage period.

no differences ($P > 0.05$) were observed between treatments and the negative control at the end of the experiment (Day 11). Furthermore, treatments 2×10^4, 2×10^5 and 2×10^6 cfu/mL did not differ either between treatments or as compared to the negative control during the entire experiment.

Similar to the results obtained from the effect of different bacterial loads, the effects of the storage time on total sperm motility were not observed until Day 2. After 48 h of inoculation, the greatest inoculated tube (2×10^8 cfu/mL) (79.83 ± 2.93%) was different ($P < 0.05$) compared to the values obtained for the same treatment at Day 0 (99.90 ± 3.45%). This tendency was maintained until the end of the experimental period. For the rest of treatments, however, these differences were observed from Day 4 to 11.

3.3.2. Progressive sperm motility

With regard to the results obtained after evaluating progressive sperm motility, Fig. 3 shows differences ($P < 0.05$) between the negative control (70.19 ± 3.19% of progressive motility) and the tube containing 2×10^8 cfu/mL of *P. aeruginosa* (49.89 ± 2.06%) at Day 2. This tendency was maintained until Day 9. From Days 4 to 7, not only was the tube with the greatest inoculation different ($P < 0.05$) when compared to the negative control, but treatment with 2×10^7 cfu/mL was also different. At Day 9, however, the tube with 2×10^7 cfu/mL did not differ from the other treatments, except for the tube containing 2×10^8 cfu/mL. In addition, at the end of the experimental period (Day 11), and as observed in the case of total motile spermatozoa, no significant differences were observed among treatments.

As far as the storage time effects are concerned, a decrease ($P < 0.05$) was observed in the tube containing 2×10^8 cfu/mL (49.89 ± 2.06%) when values on Day 2 were compared to the values acquired at the beginning of the experiment (72.63 ± 3.21%). After 4 days of storage, the rest of treatments, including the negative control (56.52 ± 2.72%), also resulted in a significant reduction in percentage of progressive motile spermatozoa when compared to the results obtained at Day 0 (72.63 ± 3.21% in control tube). For all treatments, this tendency was maintained until the end of the storing time.

Table 1
Effect of different infective concentrations of *P. aeruginosa* on pH of boar semen over 11 days of storage at 15–17 °C (mean ± S.E.M.).

Storage time (days)	Control	2×10^4 cfu/mL	2×10^5 cfu/mL	2×10^6 cfu/mL	2×10^7 cfu/mL	2×10^8 cfu/mL
0	7.7 ± 0.2	7.6 ± 0.3	7.6 ± 0.4	7.6 ± 0.4	7.6 ± 0.3	7.5 ± 0.4
1	7.5 ± 0.3	7.4 ± 0.4	7.4 ± 0.3	7.4 ± 0.3	7.4 ± 0.2	7.3 ± 0.4
2	7.8 ± 0.3	7.7 ± 0.4	7.7 ± 0.3	7.7 ± 0.3	7.6 ± 0.3	7.6 ± 0.4
4	7.9 ± 0.4	7.8 ± 0.4	7.8 ± 0.4	7.8 ± 0.4	7.7 ± 0.4	7.7 ± 0.4
7	7.7 ± 0.2	7.7 ± 0.3	7.7 ± 0.4	7.7 ± 0.2	7.6 ± 0.4	7.7 ± 0.4
9	7.8 ± 0.3	7.8 ± 0.3	7.7 ± 0.4	7.7 ± 0.3	7.7 ± 0.3	7.8 ± 0.4
11	7.8 ± 0.4	7.8 ± 0.4	7.7 ± 0.4	7.8 ± 0.4	7.7 ± 0.3	7.8 ± 0.3

No differences ($P > 0.05$) were found between treatments.

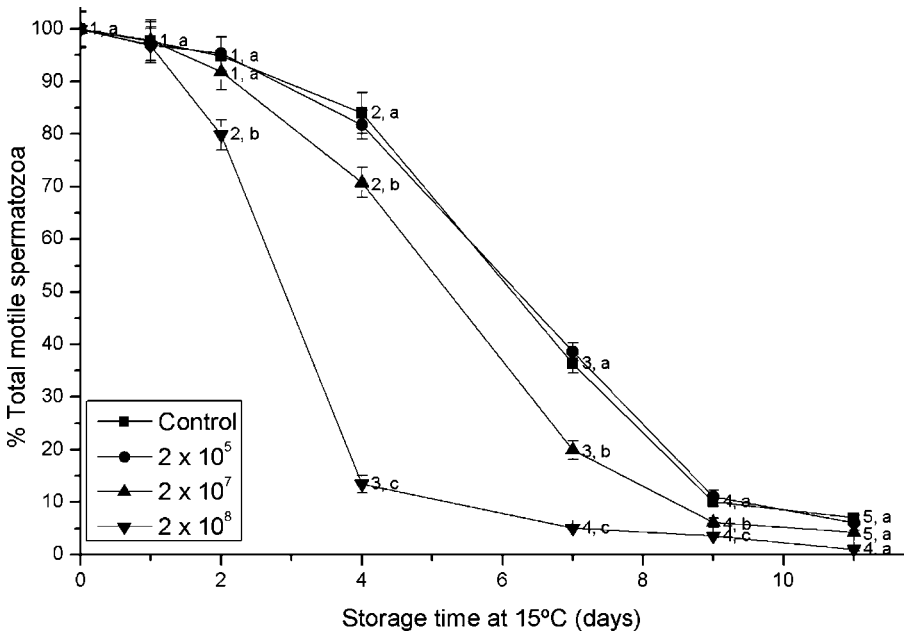

Fig. 2. Effect of different infective concentrations of *P. aeruginosa* on total sperm motility over 11 days of storage at 15–17 °C (mean ± S.E.M.). Different letters (a–c) indicate differences ($P < 0.05$) between treatments at the same time point, whereas different numbers (1–5) indicate differences ($P < 0.05$) within a given bacterial load throughout the storage period.

3.4. Sperm viability

Sperm viability was evaluated by assessing the membrane integrity through SYBR-14/PI co-staining. Similar to the results for sperm motility, there was an effect ($P < 0.05$, Fig. 4) of both time and bacterial concentration. Again, there was an interaction ($P < 0.05$) between bacterial load and storage time from the statistical model.

After 24 and 48 h of storage at 15–17 °C, the percentage of viable spermatozoa in the tubes inoculated with the greatest number of bacteria (2×10^7 and 2×10^8 cfu/mL) was less ($P < 0.05$) than that of the negative control (e.g. 61.28 ± 2.16% in the tube with 2×10^7 cfu/mL; 51.82 ± 1.80% in 2×10^8 cfu/mL and 69.74 ± 2.24% in the negative control, after 48 h). This tendency was maintained until the end of the experimental period for the

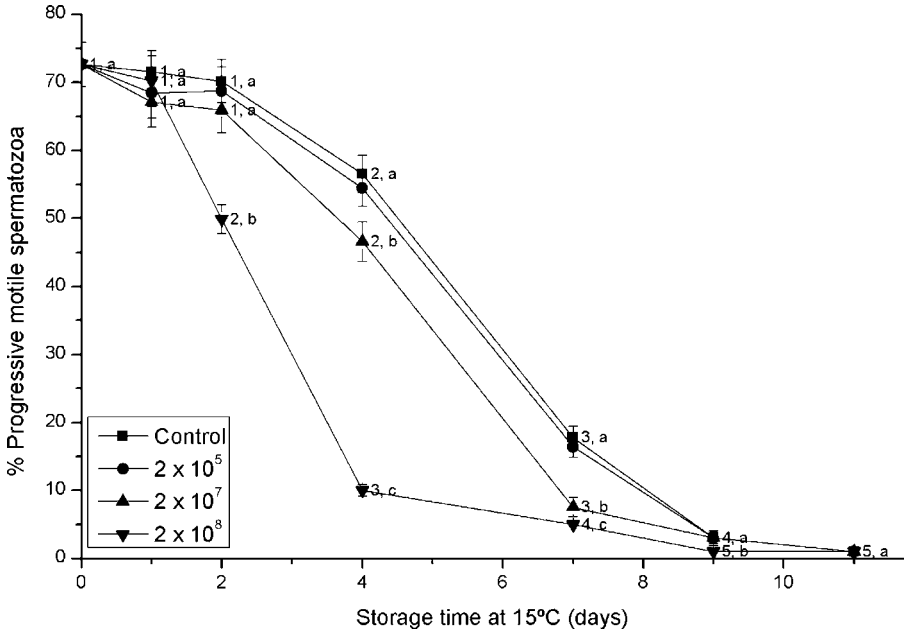

Fig. 3. Effect of different infective concentrations of *P. aeruginosa* on progressive sperm motility over 11 days of storage at 15–17 °C (mean ± S.E.M.). Different letters (a–c) indicate differences ($P < 0.05$) between treatments at the same time point, whereas different numbers (1–5) indicate differences ($P < 0.05$) within a given bacterial load throughout the storage period.

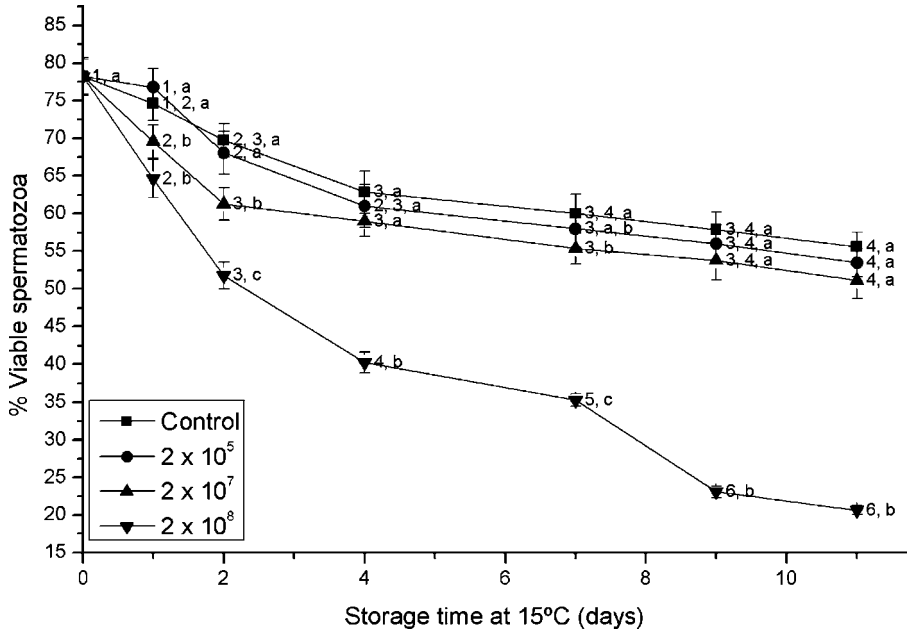

Fig. 4. Effect of different infective concentrations of *P. aeruginosa* on sperm viability over 11 days of storage at 15–17 °C (mean ± S.E.M.). Different letters (a–c) indicate differences ($P < 0.05$) between treatments at the same time point, whereas different numbers (1–6) indicate differences ($P < 0.05$) with a given bacterial load throughout the storage period.

tube containing 2×10^8 cfu/mL of *P. aeruginosa*. In fact, the reduction seen in this tube (2×10^8 cfu/mL) was significant from the very beginning. The other treatments (i.e. 2×10^4, 2×10^5, 2×10^6 cfu/mL), however, were not different when compared to the negative control during the first 2 days of storage, and the rest of the experimental period. From Days 2 to 7, the tube containing 2×10^7 cfu/mL also had lesser ($P < 0.05$) percentages of viable spermatozoa than the negative control. Nonetheless, this decrease in the percentage of viable spermatozoa with the 2×10^7 cfu/mL treatment was of a lesser extent than that with the 2×10^8 cfu/mL treatment, as the 2×10^8 cfu/mL treatment had a lesser percentage of viable spermatozoa than 2×10^7 cfu/mL during all the storage periods. For example, at Day 4, the tube inoculated with 2×10^7 cfu/mL had a percentage of viable spermatozoa of $59 \pm 2.04\%$, whereas the tube with 2×10^8 cfu/mL had $40.25 \pm 1.39\%$.

Interestingly, from Day 1, for the tubes inoculated with 2×10^7 and 2×10^8 cfu/mL, as well as from Day 2, for the rest of treatments including the negative control to the end of the experiment, all treatments had a lesser ($P < 0.05$) percentage of viable spermatozoa than those observed in the negative control at Day 0. This tendency was magnified as the storage time increased, especially with the treatments where bacterial inoculations were greater (2×10^8 cfu/mL).

3.5. Acrosome integrity

Fig. 5 shows the results of evaluating sperm acrosome integrity through PNA-FITC/PI co-staining. Both storage time and *P. aeruginosa* concentration damaged ($P < 0.05$) the acrosome integrity in a dependent manner.

Twenty-four hours after inoculation, differences ($P < 0.05$) were found in the tubes with greater bacterial inoculations (2×10^8 cfu/mL) ($68.81 \pm 2.80\%$ of acrosome and membrane intact spermatozoa) when compared to the other treatments and the negative control ($76.02 \pm 2.98\%$). This tendency was maintained until the end of the experiment, but the reduction of acrosome and membrane intact spermatozoa (PNA-FITC$^-$/PI$^-$) with this treatment was especially noticeable from Days 0 to 7. The inoculated tube with 2×10^7 cfu/mL had a percentage of membrane-intact spermatozoa that was less ($P < 0.05$) than the negative control from Days 2 to 11, but this decrease was not as pronounced as that where the bacterial inoculation was 2×10^8 cfu/mL. For example, at Day 4, the tube inoculated with 2×10^7 cfu/mL had a percentage of membrane-intact spermatozoa of $60.73 \pm 2.67\%$, whereas for the tube with 2×10^8 cfu/mL and the negative control this percentage was of 38.36 ± 1.90 and $72.30 \pm 2.74\%$, respectively. The rest of treatments (2×10^4, 2×10^5 and 2×10^6 cfu/mL) did not have differences ($P > 0.05$) when compared to the negative control at any of the time points evaluated, except for Day 4. After 24 h of storage at 15–17 °C and to the end of the experimental period, however, the percentage of membrane-intact spermatozoa in the tubes with the greatest bacterial inoculations (2×10^7 and 2×10^8 cfu/mL) was reduced ($P < 0.05$) when compared to the values observed at Day 0. For the rest of treatments, including the negative control, these differences ($P < 0.05$) were detected after 48 h of storage and continued to exist to the end of the experiment. However, this reduction was particularly prominent for the tube inoculated with a *P. aeruginosa* concentration of 2×10^8 cfu/mL.

4. Discussion

The utilization of artificial insemination (AI) techniques has grown exponentially over recent decades until

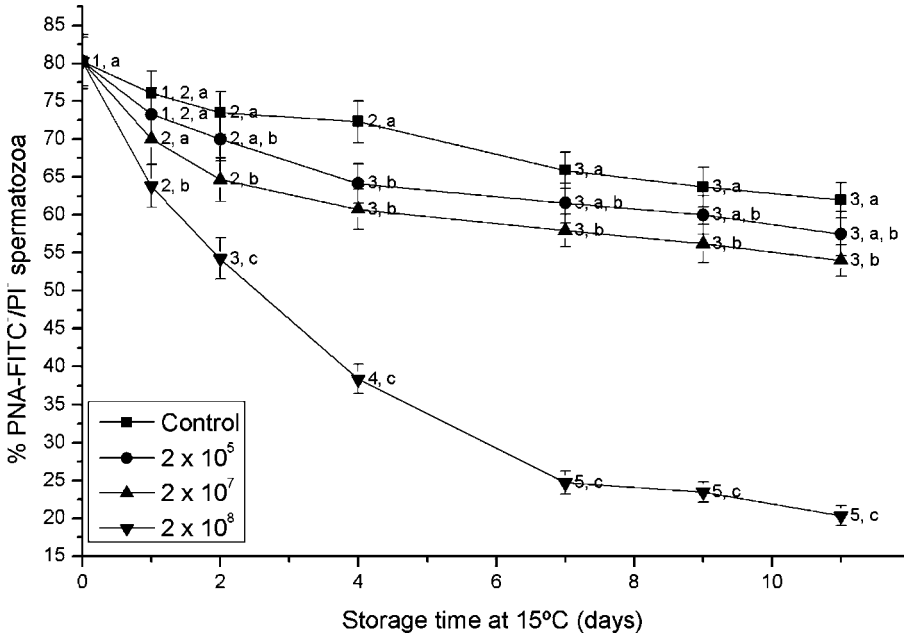

Fig. 5. Effect of different infective concentrations of *P. aeruginosa* on sperm acrosome integrity over 11 days of storage at 15–17 °C (mean ± S.E.M.). Different letters (a–c) indicate differences ($P < 0.05$) between treatments at the same time point, whereas different numbers (1–5) indicate differences ($P < 0.05$) for a given bacterial load throughout the storage period.

becoming the most widely used reproductive technology in the swine industry. In European countries, USA and Canada, over 90% of the sows are bred using this procedure (Rodríguez-Gil and Estrada, 2013). One of the main advantages, among others, of using AI over natural mating for breeding farm animal species, is to minimize, if not eliminate, uterine contamination and, consequently, avoid the spread of diseases (Vaillancourt et al., 1993). It is normally accepted that when the general health status of the boar is good, the subsequent semen is also of good quality. However, it is well documented that the ejaculate of boars harbors a broad range of organisms that can have spermicidal effects on spermatozoa, thus altering the quality and longevity of the semen and leading to suboptimal reproductive performance in sow herds (Maes et al., 2008). In fact, several studies have reported the detrimental effects that bacterial contamination exert on spermatozoa of rams (Yániz et al., 2010), bulls (Smole et al., 2010), stallions (Aurich and Spergser, 2007; Ortega-Ferrusola et al., 2009), humans (Huwe et al., 1998; Schulz et al., 2010) and boars (Bussalleu et al., 2011; Prieto-Martínez et al., 2014).

The present study was conducted with extended semen artificially inoculated with different concentrations of *P. aeruginosa*. The aim of the present research was to determine how different concentrations of *P. aeruginosa* affected boar sperm quality when samples were preserved in a long-term extender. For achieving this goal, all treatments were stored at 15–17 °C for 11 days after bacterial inoculation so as to mimic preservative conditions of AI centers. The range of bacterial load inoculation (from 2×10^4 to 2×10^8 cfu/mL) in the present experiment was chosen because although it has been reported that boar ejaculates usually contain 10^3–10^5 cfu/mL (Althouse and Lu, 2005; Morrell and Wallgren, 2011), some studies have shown that ejaculates can range in concentrations of up to 10^9 cfu/mL (Althouse et al., 2000). Nevertheless, as previously reported for *E. coli* (Bussalleu et al., 2011), *C. perfringens* (Sepúlveda et al., 2013) and *Enterobacter cloacae* (Prieto-Martínez et al., 2014), a bacterial load of 10^8 cfu/mL has been proved to result in extensive damage to boar sperm and thus reduce the quality of these germ cells.

It is also important to take into account that the *P. aeruginosa* strain used in the present study was not isolated from boar semen but purchased from a commercial company. Consistent with the study of Smole et al. (2010), since differences in bacterial strains studied may have led to discrepancies in previous results, this might also occur if the present study was reproduced with another *P. aeruginosa* strain. However, this would not likely change the primary conclusions of the present study.

In the present research, effects of different bacterial loads on sperm motility, sperm viability, acrosome integrity and pH were evaluated, which altogether can be used as predictors of fertility. Sperm motility is an important indicator of boar sperm quality for AI centers and is widely used as an indicator of active metabolism and undamaged membranes (Pinart and Puigmulé, 2013; Sutkeviciene et al., 2009). In the present study, significant reductions in both total and progressive sperm motility were seen after 48 h of incubation at 15–17 °C in the tubes inoculated with the greatest number of bacteria. These results are consistent with those obtained by Huwe et al. (1998) where the inhibitory effect of *E. coli* on sperm motility was detected when the sperm/bacteria ratio was around 1:1 (i.e. approximately 2×10^7 cfu/mL). Besides, this reduction increased as incubation time progressed similar to results of the present research. Therefore, *P. aeruginosa* affects boar sperm motility, as do other bacteria such as

E. coli or *E. cloacae* (Berktas et al., 2008; Bussalleu et al., 2011; Prieto-Martínez et al., 2014).

Sperm viability and acrosome integrity are key factors in the evaluation of sperm quality for predicting the fertilizing ability of the spermatozoa (Gillan et al., 2005; Yeste et al., 2010). Sperm viability can be measured by evaluating the integrity of the sperm plasma membrane, which intactness is a prerequisite for correct sperm metabolism and function (Pinart and Puigmulé, 2013). An intact acrosome is also required for sperm penetration through the zona pellucida of the oocyte and the subsequent fusion with the plasma membrane (Gadea, 2005). Therefore, when a given spermatozoon suffers a premature acrosome exocytosis, it is not capable of subsequently fertilizing the oocyte. In the present study, for both variables (sperm viability and acrosome integrity), there was a diminution mainly when inoculations with bacteria were greatest (2×10^7 and 2×10^8 cfu/mL). Unlike sperm motility, the reduction was already evident at 24 h post-inoculation. Thus, data from the present study indicated the presence of *P. aeruginosa* affects both viability and acrosome integrity. The present results are consistent with those from previous studies performed with different bacteria, such as *E. coli* or *Serratia marcescens*, among others (Chung et al., 2013; Fraczek et al., 2012; Sepúlveda et al., 2013; Ubeda et al., 2013; Yániz et al., 2010), and highlight the impact that bacterial contamination can have on the fertilizing ability of seminal doses intended for AI.

It is well documented that pH is capable of influencing boar sperm physiology (Purdy et al., 2010). Commercial extenders pH usually range from 6.8 to 7.3 (Althouse et al., 2000) although the pH of fresh boar semen is normally 7.4 ± 0.2. These differences are explained by the fact that once seminal pH is reduced, a decrease in both the metabolism and motility of the sperm is observed due to a reduction in the internal pH (Gadea, 2003). This is the mechanism used to prolong the lifespan of sperm during liquid storage. Acidification of sperm handling medium due to bacterial contamination, which is harmful for sperm survival, has been previously described for other bacterial species like *E. cloacae* or *E. coli* (Prieto-Martínez et al., 2014; So et al., 2011). However, the results obtained in the present research are in agreement with those obtained by Althouse et al. (2000), as the spermicidal effect was detected even without an acidic environment.

To explain the detrimental effects provoked by *P. aeruginosa* on boar sperm quality, it is important to take into account that the pathogenesis of this bacterium not only depends on the direct contact with spermatozoa, but also on a large number of cell-associated and extracellular factors, which allows it to adapt to different environments. These virulence factors have an important and pathological role for the colonization and survival of *P. aeruginosa* as well as for tissue invasion (Ben Haj Khalifa et al., 2011; Kipnis et al., 2006). In a study performed by Kaur et al. (1988), it was shown that the *P. aeruginosa* secreted factor elastase, which is known to cause tissue damage, produced spermicidal effects in human, bull and rat spermatozoa. Moreover, it has also been reported that Gram-negative bacteria release lipopolysaccharides (LPS) during both bacterial growth and death (Zhang et al., 1998). Lipopolysaccharides are components of the outer bacterial wall and act as endotoxins binding directly to the sperm, thereby suppressing functional activities, such as motility (Okazaki et al., 2010). In addition to these virulent factors, it is commonly accepted that *P. aeruginosa* use Quorum sensing (QS) to regulate physiological activities, including pathogenicity (Kipnis et al., 2006; Rennemeier et al., 2009). According to this, Rennemeier et al. (2009) reported in humans that soluble quorum sensing molecules of *P. aeruginosa* may have detrimental effects on spermatozoa and can provoke acrosome exocytosis. These reports are consistent with the reduction observed in sperm motility, sperm viability and acrosome integrity in the present study. However, these detrimental effects may also be attributable to the attachment of bacteria to the sperm plasmalemma, thus inducing alterations, as has been reported in humans (Diemer et al., 2003; Köhn et al., 1998). For this reason, further studies are needed to ascertain explanations for *P. aeruginosa* virulence, because there have been no previous reports of which we are aware regarding the mechanism of action of *P. aeruginosa* on boar sperm.

Spermicidal effects of *P. aeruginosa* in the present research were not detected immediately after bacterial inoculation, but after 24 or 48 h. These findings are in agreement with those obtained by Althouse (2008), who proposed that after the introduction of bacteria to a novel environment, a period of adaptation occurs. Results of the present study indicate that the tubes inoculated with 2×10^4, 2×10^5, 2×10^6 and 2×10^7 cfu/mL of *P. aeruginosa* undergo a lag phase, i.e. adaptation phase, in the first 48 h. Once adaptation has been accomplished, bacteria start growing exponentially. Previous studies also support these findings (Bussalleu et al., 2011; Prieto-Martínez et al., 2014; Sepúlveda et al., 2013).

To minimize the damaging effects caused by bacteria, there are different strategies to maintain bacterial contamination below a threshold. Enhancing hygienic measures during semen collection and processing, and including antibiotics in the extender formulation are the most common practices (Althouse, 2008). However, too much reliance is placed on the use of antibiotics as a method for bacterial control. Some studies have demonstrated that the majority of bacteria isolated from extended semen are resistant to the most common antibiotics included in boar extenders (Althouse and Lu, 2005; Bolarín Guillén, 2011) and that these resistance genes can be exchanged between species (Duijkeren et al., 2005). When the preservative antibiotic is ineffective in controlling the strains of contaminant bacteria, the extender becomes a culture broth in which bacteria can flourish (Althouse et al., 2000). Against this background, there is a current need to find new strategies for reducing the use of antibiotics. Okazaki et al. (2010) reported that the addition in semen extenders of the polycationic antibiotic Polymyxin B (PMB) neutralizes the endotoxic activity of LPS by binding to them, thereby improving sperm quality. Moreover, a study performed by Morrell and Wallgren (2011) showed that the utilization of the single-layer centrifugation (SLC) technique with the boar-specific colloid formulation, Androcoll^{TM}-P, reduces considerably the bacterial load without damaging the spermatozoa. Some studies have found promising

results to improve some aspects of the sperm quality and to reduce the use of antibiotics for avoiding the emergence of antibiotic-resistant strains of bacteria. However, further studies are needed in this regard.

In conclusion, the present study reveals that the presence of the Gram-negative bacterium *P. aeruginosa* in boar semen stored at 15–17 °C reduces sperm quality when bacterial numbers are present in greater concentrations. Strict hygienic measures must, therefore, be implemented in boar studs and AI centers to minimize the bacterial contamination of semen doses, which can affect both their viability and longevity, thus causing economic loses in swine industry. Furthermore, studies are needed to fully understand the mode of action by which *P. aeruginosa* exert virulence in boar spermatozoa.

Conflict of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgements

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Paper III

Effect of *Pseudomonas aeruginosa* on sperm capacitation and protein phosphorylation of boar spermatozoa

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Effect of *Pseudomonas aeruginosa* on sperm capacitation and protein phosphorylation of boar spermatozoa

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ABSTRACT

Several studies have reported the detrimental effects that bacteriospermia causes on boar sperm quality, but little is known about its effects on IVC. Considering that, the present study sought to evaluate the effects of different concentrations of *Pseudomonas aeruginosa* on different indicators of capacitation status (sperm viability, membrane lipid disorder, sperm motility kinematics, and protein phosphorylation of boar spermatozoa) after IVC. Flow cytometry and computer assisted sperm analysis (CASA) revealed that the presence of *P aeruginosa* in boar sperm samples, mostly at concentrations greater than 10^6 CFU/mL, is associated with a significant ($P < 0.05$) decrease in the percentages of both sperm membrane integrity and sperm with low membrane lipid disorder, and also with a reduction in sperm motility kinetic parameters when compared with results obtained from the control sample, which presented the typical motility pattern of capacitated-like boar spermatozoa. Moreover, Western blot results also showed significant ($P < 0.05$) changes in the levels of tyrosine, serine, and threonine protein phosphorylation because of bacterial contamination, the decrease in phosphotyrosine levels of p32, a well-known marker of IVC achievement in boar sperm, being the most relevant. Indeed, after 3 hours of IVC, phosphotyrosine levels of p32 in the control sample were 3.13 ± 0.81, whereas in the tubes with 10^6 and 10^8 CFU/mL were 1.05 ± 0.20 and 0.36 ± 0.07, respectively. Therefore, the present study provides novel data regarding the effects of bacterial contamination on boar sperm, suggesting that the presence of *P aeruginosa* affects the fertilizing ability of boar sperm by altering its ability to accomplish IVC.

1. Introduction

The process of collecting semen in boars is far from being a sterile procedure because of multiple sources of bacterial contamination existing along with the collection procedure [1]. Consequently, freshly ejaculated boar semen is commonly contaminated with a broad range of microorganisms, which can compromise sperm quality and function, and cause major problems in the recipient female after artificial insemination [2,3]. In addition, several studies have shown that bacteria impair sperm quality by causing morphologic alterations, degenerative acrosome exocytosis [4–6], sperm agglutination, and decreases in sperm motility and membrane integrity [5,7–9]. These alterations lead to suboptimal reproductive performance [3].

Bacterial contamination in fresh semen usually ranges from 10^3 to 10^5 colony-forming units (CFU) per milliliter [10,11], although these loads can fluctuate and reach concentrations of up to 10^9 CFU/mL [12]. Despite the presence of antibiotics in the semen extender formulation, both nutrient-rich semen extenders and storage temperature (15 °C–17 °C) favor the survival of microorganisms in boar semen. Many bacteria have become resistant to the most

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common antibiotics used in semen extenders (see [10,13,14] for review), so that they can thrive during the storage period, thus increasing the initial bacterial load and magnifying the negative effects of bacterial contamination.

Sperm must undergo biochemical and physiological changes, known as "sperm capacitation," to acquire the ability to bind and penetrate the egg [15–18]. Sperm capacitation involves phospholipid changes in the plasma membrane and cholesterol efflux, activation of ion channels, and increases in intracellular calcium and cAMP levels, as well as changes in flagellar activity and protein phosphorylation [15,19–22]. Because mature spermatozoa are highly specialized cells incapable of neither transcriptional nor translational activity, post-translational modifications through protein phosphorylation-dephosphorylation are of high importance in regulating different cellular processes, such as cellular growth and sperm capacitation [17,23,24]. Proteins can be phosphorylated at serine, threonine, and tyrosine residues and, specifically, phosphorylation on tyrosines has been clearly reported as a steady indicator of sperm capacitation [15]. Indeed, inhibition of tyrosine phosphorylation has been reported to prevent sperm capacitation, acrosome reaction, and IVF [25]. Although this process takes place inside the female's genital tract, sperm can also become capacitated *in vitro* by using defined media that attempt to mimic the composition of the oviductal fluid [26–28].

Several reports have widely studied the effects that these bacterial contaminants might exert on boar sperm quality [2,8,11]; however, none of them have considered the potential effect that bacteria may have on sperm capacitation. In this regard, it is worth noting that although Maroto Martín et al. [3] found that a bacterial concentration of 3.5×10^3 CFU/mL significantly reduced litter size, previous studies conducted in our laboratory showed that the detrimental effects of *Escherichia coli*, *Enterobacter cloacae*, *Clostridium perfringens*, and *Pseudomonas aeruginosa* on boar sperm quality were only apparent at the highest bacterial concentrations (10^7 and 10^8 CFU/mL) [5,7,9,29]. Although knowing the spermicidal effects of bacteria is essential to establish a quality threshold for seminal samples intended to artificial insemination, it is also important to understand the potential effects that these loads may cause on sperm fertilizing ability. Moreover, it has been reported that contamination during IVF is related to lower IVF outcomes in human [30–32]. Therefore, the aim of the present study is to address whether the presence of *P aeruginosa*, a bacterium commonly found in boar ejaculates, in concentrations similar to the aforesaid works (from 10^4–10^8 CFU/mL) affects the ability of boar sperm to capacitate *in vitro*, especially at those bacterial loads in which the damages on sperm membrane integrity are not apparent ($\geq 10^7$ CFU/mL).

2. Materials and methods

2.1. Materials

All chemicals used in the present study were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.2. Boar semen collection

All animal procedures were performed following the guidelines established by the Animal Welfare Directive of the Regional Government of Catalonia (Generalitat de Catalunya, Spain), which conform to European Union Regulation 2010/63/UE. All seminal doses were purchased from a commercial local farm (Grup Batallé, Girona, Spain), and thus no animal was manipulated by us.

Sperm samples came from 21 healthy and sexually mature Pietráin boars, all of them being of proven fertility. All boars, aged 9 to 12 months, were submitted to a collection rhythm of twice a week using the gloved-hand technique. Sperm rich fraction was diluted with a commercial long-term sperm extender (Duragen, Magapor, Zaragoza, Spain) to a final concentration of 3×10^7 sperm/mL. This diluted fraction was then split into seminal doses of 90 to 100 mL containing a total of 3×10^9 sperm each and stored at 15 °C to 17 °C. One dose per boar was sent to our laboratory within 24 hours after collection. Upon arrival, seminal doses were pooled (3 doses per pool per day; $n = 7$) and sperm quality of each pool (sperm motility and viability) was evaluated, as described in the following sections, to check whether the doses were qualified for the study (total motile spermatozoa >80%; progressive sperm motility >60%, viable spermatozoa >75%) [33].

2.3. Origin and preparation of bacteria

The strain CECT-4145 of *P aeruginosa* (CECT, Valencia, Spain) was cultured in Luria–Bertani medium (10 g/L tryptone [Laboratorios Conda S.A., Madrid, Spain], 5 g/L yeast extract (Liofilchem S.R.L., Roseto d.A., Italy), 10 g/L NaCl [Labbox Labware S.L., Barcelona, Spain], pH 7.2) at 37 °C to 38 °C for 18 to 24 hours in a shaking water bath (Memmert water bath WNB 7-45 coupled with the shaking device 14/22; Schwabach, Germany). Fifty milliliters of bacterial culture were centrifuged at $3860 \times g$ for 15 minutes at 15 °C to 17 °C and washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL, Invitrogen, Barcelona, Spain) without calcium and magnesium at $3860 \times g$ for 15 minutes at 15 °C to 17 °C. The resulting pellet was gently resuspended in 2 mL of DPBS, and cell concentration was assessed with a spectrophotometer (UV-1600PC; VWR International, PA, USA) at a wavelength of 600 nm (optical density, OD600). Subsequently, bacterial concentration was first adjusted to 3×10^9 CFU/mL and then serially diluted up to 3×10^5 CFU/mL in a capacitating medium (CM) (20 mM HEPES, 112 mM NaCl, 3.1 mM KCl, 5 mM glucose [Panreac Química S.L.U., Barcelona, Spain], 21.7 mM sodium L-lactate, 1 mM sodium pyruvate, 0.3 mM Na_2HPO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 4.5 mM $CaCl_2$, 5 mg/mL BSA [Roche Diagnostics, Basel, Switzerland], pH 7.4; [27]).

2.4. Inoculation of semen and IVC procedure

Seminal pools were assessed for concentration using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). Then, volumes of semen corresponding to a final sperm concentration of 3×10^7 spermatozoa/mL in a total volume of 20 mL were added into four different falcon

tubes, which were then centrifuged at 600 × *g* for 5 minutes at 15 °C to 17 °C. Resultant pellets were further washed with DPBS at 600 × *g* for 5 minutes at 15 °C to 17 °C, to obtain clean sperm samples, and subsequently resuspended in 18 mL of CM. Immediately after, 2 mL of each of the aforementioned infective concentrations were added into the corresponding falcon tube to obtain the final infective concentrations of *P aeruginosa* used in the study (3×10^4, 3×10^6, 3×10^7, and 3×10^8 CFU/mL). Hence, each treatment consisted of 20 mL of CM at a sperm concentration of 3×10^7 spermatozoa/mL and inoculated with a specific infective concentration.

All infective concentrations, plus two controls, were incubated at 38.5 °C in a humidified 5% CO_2 atmosphere for 3 hours. These controls were a 20-mL solution containing CM without bacteria but with sperm at a final concentration of 3×10^7 spermatozoa/mL (referred subsequently as control tube), and a tube with 20 mL of CM inoculated with 3×10^8 CFU/mL of *P aeruginosa* but without sperm (referred subsequently as control containing no sperm).

Aliquots of each treatment, except for the control containing no sperm, were taken at 0, 2, and 3 hours of incubation for assessing the IVC status through the evaluation of sperm motility, plasma membrane integrity (sperm viability), and membrane lipid disorder.

2.5. Sperm motility

Sperm motility was assessed using the motility module of Integrated Sperm Analysis System (v1.2; Proiser R + D, Valencia, Spain). Briefly, 20 μL of sperm cells were placed on a Makler counting chamber (Sefi-Medical Instruments) and observed at a magnification of ×100 with a negative phase-contrast microscopy (Olympus BX41; Olympus Europa GmbH, Hamburg, Germany); aliquots at time 0 were preheated at 37 °C for 20 minutes before sperm motility analysis. Fifty consecutive digitalized photographic frames were acquired per field in a time lapse of 1 second and three replicates of at least 1000 spermatozoa were evaluated per sample. The following kinematic sperm motility parameters were recorded: progressive motility (PMOT, %), curvilinear velocity (VCL, micrometers per second), straight-linear velocity (VSL, micrometers per second), average path velocity (VAP, micrometers per second), linearity index (LIN, %), straightness index (STR, %), oscillation index (WOB, %), amplitude of lateral head displacement (ALH, micrometer), and beating frequency (BCF, Hertz), as described in Yeste et al. [34]. Results are expressed as the percentages of the mean ± standard error of the mean.

2.6. Flow cytometry analyses

Flow cytometry analyses were conducted to evaluate plasma membrane integrity and membrane lipid disorder in all samples and time points (0, 2, and 3 hours). In both assays, samples were firstly diluted and adjusted to 1×10^6 spermatozoa/mL with *Beltsville Thawing Solution* (37 g/L glucose, 5.88 g/L sodium citrate, 1.26 g/L sodium bicarbonate, 1.25 g/L EDTA, 0.75 g/L potassium chloride, 0.05 g/L kanamycin, pH 7.2) in a final volume of 0.5 mL and then stained with the corresponding combination of fluorochromes, following the protocols described subsequently.

Sperm viability was evaluated by measuring the plasma membrane integrity through SYBR-14/propidium iodide (PI) assay (Live-Dead Sperm Viability Kit L-7011; Molecular Probes Inc., Eugene, OR, USA). Samples diluted to 1×10^6 sperm/mL were incubated with 0.5 μL of SYBR-14, at a final concentration of 100 nM, for 10 minutes at 38 °C in darkness, and then counterstained with 2.5 μL of PI (final concentration of 12 μM) for 5 minutes at the same temperature. Membrane-intact spermatozoa were positive for SYBR-14 and negative for PI (SYBR-14^+/PI^-).

Membrane lipid changes were determined by costaining sperm samples with Merocyanine 540 (M-540) and YO–PRO-1 (YP1, Y-3603; Molecular Probes). Briefly, diluted samples were incubated for 10 minutes in darkness at 38 °C with 1.3 μL of M-540 and 0.5 μL of YP1 at final concentrations of 2.6 μM and 25 nM, respectively [35]. After analysis, four different sperm populations were observed: (1) nonviable spermatozoa with low membrane lipid disorder (M-540^-/$YP1^+$), (2) nonviable spermatozoa with high membrane lipid disorder (M-540^+/$YP1^+$), (3) viable spermatozoa with low membrane lipid disorder (M-540^-/$YP1^-$), and (4) viable spermatozoa with high membrane lipid disorder (M-540^+/$YP1^-$). Obtained data were corrected according to the procedure described by Petrunkina et al. [36].

Information about flow cytometry analyses performed in the present study is provided according to the recommendations of the International Society for Advancement of Cytometry [37]. In all cases, samples were evaluated using Cell Lab Quanta SC Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) through excitement with an argon ion laser (488 nm) set at a power of 22 mW. For each particle, physical properties of size and granularity were measured and plotted as electronic volume (equivalent to forward scatter) and side scatter, which allowed the gating of sperm-specific events and excluded subcellular debris (particle diameter, <7 μm) and cell aggregates (particle diameter, >12 μm). Green fluorescence from SYBR-14 and YP1 was detected through the fluorescence filter photodetector (dichroic mirror [or beam splitter], DRLP: 550 nm; band-pass filter, BP: 525 nm; and detection width: 505–545 nm), whereas red fluorescence from PI and M-540 was detected with the FL3 photodetector (DRLP: 600 nm; long-pass filter, LP: 670 nm; and detection width: 655-685 nm). Signals were logarithmically amplified and photomultiplier settings were adjusted to each particular staining method. A minimum of 10,000 events per replicate were evaluated at a flow rate of 200 cell/s and analyzed using the Cell Lab Quanta SC multi-platform loader Analysis Software v1.0 (Beckman Coulter). Each assessment was repeated three times per sample and parameter, before calculating the corresponding mean ± standard error of the mean.

2.7. Protein extraction and quantification

After IVC, all treatments (including both controls) and a noncapacitated fresh sperm sample were pelleted at 600 × *g* for 10 minutes at 4 °C, and washed twice with DPBS at the same centrifugation conditions. Supernatants were

carefully discarded and the resultant pellets were stored at −20 °C until protein extraction.

Total protein extraction was carried out using a 1% (v:v) Triton X-100 lysis buffer with 150 mM NaCl, 50 mM Tris (pH 8.0), 1 mM of phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and a protease inhibitor cocktail at 1:100 (v:v). Frozen pellets were resuspended with 300 μL of ice-cold lysis buffer and sonicated thoroughly (50% amplitude, 20 long lasting pulses) (SONOPLUS ultrasonic homogenizer HD 2070; Bandelin, Berlin, Germany) to obtain homogenized suspensions. Immediately after sonication, samples were placed on ice for 30 minutes and then centrifuged at 10,000 × *g* for 15 minutes at 4 °C. Supernatants were meticulously collected, quantified by detergent compatible Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and stored at −20 °C until used.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analysis

For SDS–PAGE separation, 10 μg of each protein sample were diluted 1:2 in 2X Laemmli reducing buffer with 5% (v:v) β-mercaptoethanol (Bio-Rad Laboratories, Inc.), boiled for 3 to 5 minutes at 90 °C, and loaded onto 1.0 mm SDS–PAGE gels. Stacking gels contained 5% acrylamide (v:v) (Bio-Rad Laboratories, Inc.), whereas separating gels contained a 8% to 16% gradient of acrylamide (v:v). After running the gels at constant voltage (10 mA for stacking gels and 20 mA for separating gels), proteins were blotted onto polyvinylidene fluoride membranes (Immobilon-P; Millipore, Darmstadt, Germany) for 2 hours at 120 mA. Membranes were subsequently washed with Tris-Buffered Saline (TBS)-Tween 20 (TBST; 10 mM Tris, 150 mM NaCl, 0.05% [v:v] Tween 20 [Panreac], pH 7.3) for 10 minutes and then blocked with blocking solution, consisting of 5% (wt/vol) BSA in TBST, overnight at 4 °C with agitation. Blocked membranes were then briefly washed with TBST and incubated with the corresponding primary antibody (anti-phosphotyrosine [anti-pTyr], anti-phosphoserine [anti-pSer], or anti-phosphothreonine [anti-pThr]) under agitation for 1 hour at room temperature.

To detect tyrosine-phosphorylated protein residues, membranes were incubated with an anti-pTyr mouse antibody (4G10 Platinum anti-pTyr, 05–1050; Millipore) diluted 1:1500 in blocking solution (v:v); for serine-phosphorylated residues, membranes were incubated with an anti-pSer mouse antibody (anti-pSer clone 4A4, 05–1000; Millipore) diluted 1:2000 in blocking solution (v:v); and for threonine-phosphorylated protein residues, membranes were incubated with an anti-pThr mouse antibody (anti-pThr antibody clone 20H6.1, 05–1923; Millipore) diluted 1:1000 in blocking solution (v:v). After washing membranes four times for 5 minutes each in TBST, they were incubated with a horseradish peroxidase (HRP)-conjugated polyclonal anti-mouse antibody (P0260; Dako Denmark A/S, Glostrup, Denmark) diluted 1:5000 in blocking solution (v:v) at room temperature with agitation for 1 hour.

Reactive bands were visualized with a chemiluminiscent substrate (Immobilon Western Chemiluniniscent HRP Substrate; Millipore) and scanned using G:BOX Chemi XL 1.4 (SynGene, Frederick, MT, USA) and GeneSys image acquisition software v1.2.8.0 (SynGene). Protein bands from scanned images were semiquantified through Quantity One v4.6.9 software package (Bio-Rad Laboratories, Inc.). Protein quantification was expressed as the total signal intensity inside the boundary of a band measured in pixel intensity units (density, square millimeter) minus the background signal, considered as 0 (white) [38]. α-Tubulin was used as an internal standard to normalize blotted protein content for each band after stripping–reprobing membranes. Shortly, membranes were stripped under agitation for 40 minutes at 38 °C with a buffer containing 0.2 M glycine and 0.05% (v:v) Tween 20 (pH 2.2). After washing and blocking, stripped membranes were subsequently incubated with an anti–α-tubulin mouse antibody (anti–α-tubulin antibody clone DM1A, MABT205; Millipore) diluted 1:1000 in blocking solution (v:v) for 1 hour at room temperature and under agitation, and then with a HRP-conjugated polyclonal anti-mouse antibody (P0260; Dako Denmark A/S) diluted 1:5000 in blocking solution (v:v) at the same conditions. Reactive bands were visualized as previously described. Phosphotyrosine, pSer, and pThr levels were normalized by determining the following ratio:

$$Protein\ content = \frac{band\ of\ interest(intensity)}{\alpha - tubulin\ band(intensity)}.$$

2.9. Statistical analyses

Statistical analyses were conducted using a statistical software (IBM SPSS Ver 21.0; SPSS Inc., Chicago, IL, USA). Data (x) were tested for normality (Shapiro–Wilks test) and homogeneity of variances (Levene test) to check whether they accomplished the parametric assumptions. All data, except percentages of $M540^{-}$/YO–PRO-1^{-}, SYBR-14^{+}/PI^{-}, percentages of progressive motile sperm, VCL, VSL, VAP, and WOB, were transformed with arcsin $\sqrt{x}$ or $\sqrt{x}$ transformation, and when data were not transformed, nonparametric tests were used. A general mixed model (incubation time: intrasubject factor; infective concentration: intersubject factor) was run followed by the Sidak *post hoc* test. Friedman and Wilcoxon tests were used as nonparametric alternatives. Level of significance was set at $P \leq 0.05$ in all cases.

3. Results

Figure 1A and Table 1 show the results obtained after assessing the effects of *P aeruginosa* on different sperm motility parameters along the incubation period and the achievement of IVC. Motility parameters included in the analysis were PMOT, VSL, VCL, VAP, LIN, STR, WOB, BCF, and ALH.

Regarding the control tube, as expected, results showed a progressive decrease in PMOT and an increase in most of sperm kinetic parameters (VSL, VCL, VAP, LIN, STR, BCF, and ALH) along the incubation under capacitating conditions, which is the typical motility pattern of capacitated-like boar spermatozoa [39]. These increases-decreases,

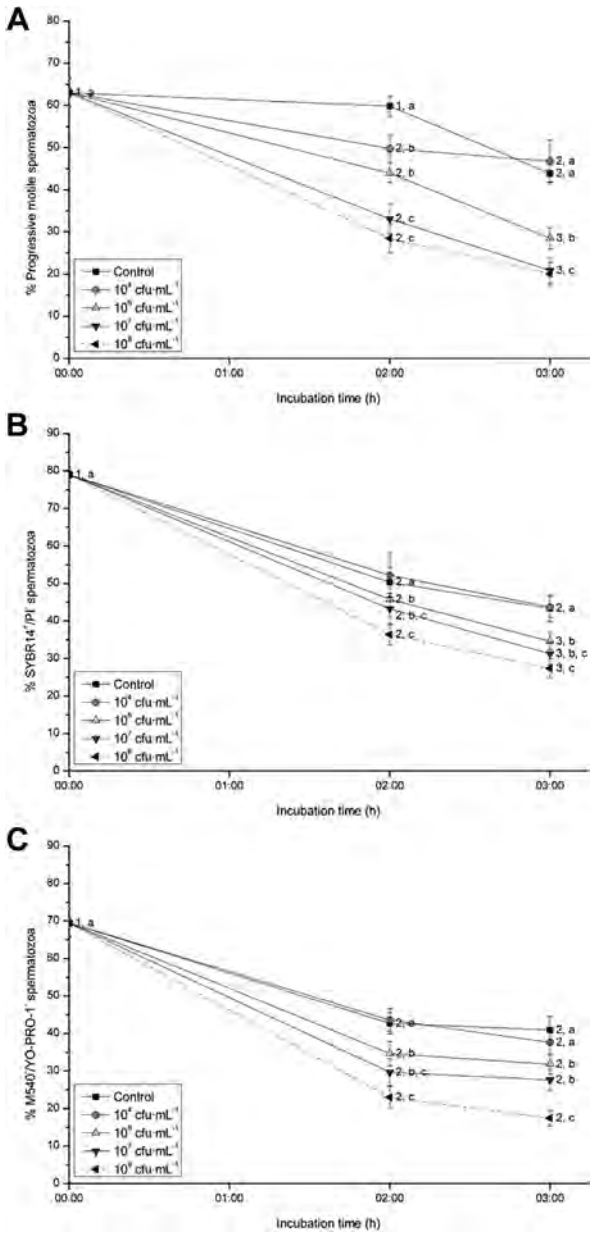

Fig. 1. Effect of different concentrations of *Pseudomonas aeruginosa* on (A) progressive sperm motility, (B) sperm viability, and (C) on the percentage of sperm with low membrane lipid disorder (M-540^{-}/YO–PRO-1^{-}) during IVC (mean ± standard error of the mean). Different superscripts (a–c) indicate significant differences ($P < 0.05$) between treatments at the same time point, whereas different numbers (1–3) mean significant differences ($P < 0.05$) within a given bacterial load throughout the experimental period. The control tube contains capacitated sperm sample without bacteria. Representative images for seven separate experiments ($n = 7$) are shown. CFU, colony-forming unit.

however, were not observed in all treatments evaluated: treatments with 10^7 to 10^8 CFU/mL for VSL and LIN, the tube with 10^4 CFU/mL for WOB, and all inoculated treatments in the case of VCL and VAP exhibited no significant ($P > 0.05$) differences during the IVC procedure when compared with data obtained at the beginning of the experiment (0 hours). As far as differences among treatments at each time point are concerned, Figure 1A and Table 1 show that after 2 hours of IVC, percentages of PMOT and values of VSL, VCL, and VAP in tubes ranging from 10^4 to 10^8 CFU/mL were significantly ($P < 0.05$) different from those observed in the control tube. Nevertheless, these changes became magnified as the bacterial concentration increased, the tube containing 10^8 CFU/mL being, in all cases, the one that showed the lowest results. This tendency persisted after 3 hours in the case of PMOT (except for the tube with 10^4 CFU/mL). However, for VSL, VCL, and VAP, only the highest inoculated tube (10^8 CFU/mL) was different from the control tube at this time point. In a similar fashion to that observed for VSL, VCL, and VAP, effects of *P aeruginosa* on the percentages of STR and WOB, after 2 hours of experiment, can be seen in lower bacterial concentrations (10^6–10^8 CFU/mL) than results obtained at 3 hours, when only tube containing 10^8 CFU/mL was significantly different from the control tube. For the percentage of LIN, however, only the tube with 10^8 CFU/mL was different from the negative control throughout the IVC procedure. Unlike other sperm motility parameters, BCF and ALH only showed lower values compared with the control tube on treatment with 10^8 CFU/mL after 2 hours, and on treatments with 10^7 to 10^8 CFU/mL after 3 hours, respectively. Hence, on the whole, bacterial contamination affected boar sperm motility parameters after IVC in a concentration-dependent manner, especially at high bacterial concentrations.

Plasma membrane integrity during IVC was evaluated through SYBR-14/PI costaining. Figure 1B shows the effects that different loads of *P aeruginosa* exerted on viable spermatozoa during IVC. Detrimental effects of *P aeruginosa* were evident 2 hours after the IVC procedure began. At this time point, tubes contaminated with 10^6, 10^7, and 10^8 CFU/mL showed a significantly ($P < 0.05$) lower percentage of viable sperm than the tube with 10^4 CFU/mL and the control tube, the reduction of sperm viability in the 10^8 CFU/mL treatment being also of a lesser extent ($P < 0.05$) than that of the tube with 10^6 CFU/mL. This tendency was maintained after 3 hours of IVC; however, damaging effects were significantly ($P < 0.05$) greater when compared with data obtained at 2 hours in the tubes with 10^6 to 10^8 CFU/mL. Therefore, again, effects were both time and concentration dependent.

Regarding membrane lipid disorder, this parameter was assessed by M-540 and YP1 staining during IVC. Impact of *P aeruginosa* on the percentage of viable sperm with low membrane lipid disorder is shown in Figure 1C. As with sperm viability, its effects were both time and concentration dependent. After 2 hours of IVC, the percentage of viable spermatozoa with low membrane lipid disorder was significantly ($P < 0.05$) lower in the tubes with 10^6, 10^7, and 10^8 CFU/mL when compared with the treatment with 10^4 CFU/mL and the control tube, the treatment with the highest bacterial load being again significantly ($P < 0.05$) different from that of the tube with 10^6 CFU/mL. This trend was more striking at the end of the IVC procedure, when the tube containing 10^8 CFU/mL exhibited a percentage of viable spermatozoa with low membrane lipid disorder not only significantly ($P < 0.05$) lower than the tubes with 10^4,

Table 1
Motility parameters of contaminated and noncontaminated boar sperm samples measured with CASA during IVC (at 0, 2, and 3 h of incubation).

Motility parameters	Treatment	0 h	2 h	3 h
VSL (μm/s)	Control	$54.82 \pm 7.82^{1,a}$	$126.87 \pm 20.73^{2,a}$	$88.93 \pm 16.87^{3,a}$
	10^4 CFU/mL	$54.82 \pm 7.82^{1,a}$	$85.20 \pm 15.23^{2,b}$	$81.18 \pm 16.52^{1,2,a}$
	10^6 CFU/mL	$54.82 \pm 7.82^{1,a}$	$85.31 \pm 15.56^{2,b}$	$69.27 \pm 12.07^{1,2,a,b}$
	10^7 CFU/mL	$54.82 \pm 7.82^{1,a}$	$73.44 \pm 16.45^{1,b,c}$	$64.50 \pm 12.95^{1,a,b}$
	10^8 CFU/mL	$54.82 \pm 7.82^{1,a}$	$58.03 \pm 11.74^{1,c}$	$48.46 \pm 10.39^{1,b}$
VCL (μm/s)	Control	$109.69 \pm 14.17^{1,a}$	$181.26 \pm 25.25^{2,a}$	$149.54 \pm 19.77^{1,2,a}$
	10^4 CFU/mL	$109.69 \pm 14.17^{1,a}$	$133.76 \pm 15.66^{1,b}$	$138.39 \pm 16.92^{1,a,b}$
	10^6 CFU/mL	$109.69 \pm 14.17^{1,a}$	$143.48 \pm 22.18^{1,a,b}$	$127.73 \pm 13.34^{1,a,b}$
	10^7 CFU/mL	$109.69 \pm 14.17^{1,a}$	$132.35 \pm 16.32^{1,b}$	$118.38 \pm 11.78^{1,a,b}$
	10^8 CFU/mL	$109.69 \pm 14.17^{1,a}$	$116.03 \pm 14.09^{1,b}$	$106.73 \pm 13.68^{1,b}$
VAP (μm/s)	Control	$89.14 \pm 13.77^{1,a}$	$150.72 \pm 24.81^{2,a}$	$110.11 \pm 19.84^{1,2,a}$
	10^4 CFU/mL	$89.14 \pm 13.77^{1,a}$	$101.22 \pm 17.01^{1,b,c}$	$100.02 \pm 18.86^{1,a}$
	10^6 CFU/mL	$89.14 \pm 13.77^{1,a}$	$105.48 \pm 18.58^{1,b,c}$	$87.55 \pm 13.77^{1,a,b}$
	10^7 CFU/mL	$89.14 \pm 13.77^{1,a}$	$92.15 \pm 17.65^{1,b,c}$	$80.63 \pm 13.45^{1,a,b}$
	10^8 CFU/mL	$89.14 \pm 13.77^{1,a}$	$75.85 \pm 13.25^{1,c}$	$65.16 \pm 11.87^{1,b}$
LIN (%)	Control	$49.80 \pm 1.42^{1,a}$	$68.29 \pm 2.00^{2,a}$	$57.30 \pm 2.96^{3,a}$
	10^4 CFU/mL	$49.80 \pm 1.42^{1,a}$	$62.35 \pm 3.68^{2,a}$	$57.20 \pm 4.56^{2,a}$
	10^6 CFU/mL	$49.80 \pm 1.42^{1,a}$	$57.47 \pm 3.26^{2,a,b}$	$52.33 \pm 4.08^{1,2,a,b}$
	10^7 CFU/mL	$49.80 \pm 1.42^{1,a}$	$52.75 \pm 5.63^{1,a,b}$	$51.66 \pm 4.74^{1,a,b}$
	10^8 CFU/mL	$49.80 \pm 1.42^{1,a}$	$48.05 \pm 3.99^{1,b}$	$43.56 \pm 3.39^{1,b}$
STR (%)	Control	$63.94 \pm 2.67^{1,a}$	$84.31 \pm 0.72^{2,a}$	$80.03 \pm 1.11^{2,a}$
	10^4 CFU/mL	$63.94 \pm 2.67^{1,a}$	$83.62 \pm 1.15^{2,a}$	$80.51 \pm 2.06^{2,a}$
	10^6 CFU/mL	$63.94 \pm 2.67^{1,a}$	$80.27 \pm 1.25^{2,a,b}$	$77.51 \pm 2.35^{2,a,b}$
	10^7 CFU/mL	$63.94 \pm 2.67^{1,a}$	$77.02 \pm 3.60^{2,b,c}$	$77.43 \pm 2.73^{2,a,b}$
	10^8 CFU/mL	$63.94 \pm 2.67^{1,a}$	$74.47 \pm 2.26^{2,c}$	$72.52 \pm 2.51^{2,b}$
WOB (%)	Control	$78.72 \pm 3.40^{1,2,a}$	$80.96 \pm 2.40^{1,a}$	$71.38 \pm 2.99^{2,a}$
	10^4 CFU/mL	$78.72 \pm 3.40^{1,a}$	$74.30 \pm 3.48^{1,a,b}$	$70.68 \pm 4.28^{1,a}$
	10^6 CFU/mL	$78.72 \pm 3.40^{1,a}$	$71.29 \pm 3.30^{1,2,b,c}$	$66.65 \pm 3.62^{2,a,b}$
	10^7 CFU/mL	$78.72 \pm 3.40^{1,a}$	$67.18 \pm 4.72^{2,b,c}$	$65.65 \pm 3.94^{2,a,b}$
	10^8 CFU/mL	$78.72 \pm 3.40^{1,a}$	$63.60 \pm 3.59^{2,c}$	$59.51 \pm 2.72^{2,b}$
BCF (Hz)	Control	$14.11 \pm 0.52^{1,a}$	$18.42 \pm 0.19^{2,a}$	$18.62 \pm 0.33^{2,a}$
	10^4 CFU/mL	$14.11 \pm 0.52^{1,a}$	$18.82 \pm 0.40^{2,a}$	$18.56 \pm 0.58^{2,a}$
	10^6 CFU/mL	$14.11 \pm 0.52^{1,a}$	$18.83 \pm 0.32^{2,a}$	$17.91 \pm 0.39^{2,a}$
	10^7 CFU/mL	$14.11 \pm 0.52^{1,a}$	$17.70 \pm 0.74^{2,a,b}$	$17.40 \pm 0.65^{2,a,b}$
	10^8 CFU/mL	$14.11 \pm 0.52^{1,a}$	$16.62 \pm 0.63^{2,b}$	$15.79 \pm 0.85^{2,a,b}$
ALH (μm)	Control	$1.99 \pm 0.15^{1,a}$	$2.83 \pm 0.24^{2,a}$	$2.70 \pm 0.21^{2,a}$
	10^4 CFU/mL	$1.99 \pm 0.15^{1,a}$	$2.41 \pm 0.14^{2,a}$	$2.51 \pm 0.13^{2,a,b}$
	10^6 CFU/mL	$1.99 \pm 0.15^{1,a}$	$2.52 \pm 0.16^{2,a}$	$2.35 \pm 0.13^{2,a,b}$
	10^7 CFU/mL	$1.99 \pm 0.15^{1,a}$	$2.38 \pm 0.12^{2,a}$	$2.18 \pm 0.08^{1,2,b}$
	10^8 CFU/mL	$1.99 \pm 0.15^{1,a}$	$2.33 \pm 0.17^{2,a}$	$2.18 \pm 0.17^{1,2,b}$

Different letters (a–c) indicate differences ($P < 0.05$) between treatments at the same time point, whereas different numbers (1–3) indicate differences ($P < 0.05$) within a given bacterial load throughout the experimental period. The control tube contains capacitated sperm sample without bacteria.
Abbreviations: ALH, amplitude of lateral head displacement; BCF, beating frequency; CASA, computer assisted sperm analysis; CFU, colony-forming unit; LIN, linearity index; STR, straightness index; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-linear velocity; WOB, oscillation index.

10^6 CFU/mL and the control tube, but also lower than the tube with 10^7 CFU/mL. Notwithstanding, unlike the results obtained for sperm viability, no differences ($P > 0.05$) regarding membrane lipid disorder were observed when comparing the percentages acquired after 3 hours of IVC with the ones recorded after 2 hours in all treatments, including the control tube.

As far as protein tyrosine phosphorylation is concerned, Figure 2A (a) shows a pTyr pattern of nine main reactive bands. Their expression was normalized and semi-quantified with α-tubulin (Table 2). The reactive bands identified in the blots had, approximately, the following molecular weights: 74, 66, 49, 40, 37, 34, 32, 23, and 12 kDa. In most of these bands, the negative effects of *P aeruginosa* on pTyr levels were already seen at a concentration of 10^6 CFU/mL. Hence, in bands corresponding to 66, 49, 40, 37, 34, 32, and 12 kDa, the tubes with 10^6 to 10^8 CFU/mL expressed a significantly ($P < 0.05$) lesser pTyr content than the control tube. On the contrary, bands with 74 and 23 kDa began to show a lower extent of pTyr in treatments with 10^7 and 10^4 CFU/mL, respectively. In all cases, pTyr levels decreased their expression as bacterial load gradually increased, the tube containing 10^8 CFU/mL being again that exhibited the lowest values.

As seen in Figure 2A (a), the band corresponding to 32 kDa was absent in noncapacitated samples (lane 1); however, it appeared after the IVC procedure (lanes 2–6). Therefore, the appearance of the tyrosine-phosphorylated protein p32 is an indicator of IVC achievement. The expression pattern obtained in the lane corresponding to the control containing no sperm (lane 7) was different from those obtained in sperm samples; however, to avoid confusing statements, only those bands identified in either noncapacitated samples (lane 1) or control tube (lane 2) were analyzed in the present study.

With respect to the pSer pattern obtained through Western blot analysis, Figure 2B (a) shows the expression of 11 main reactive bands, normalized and semiquantified

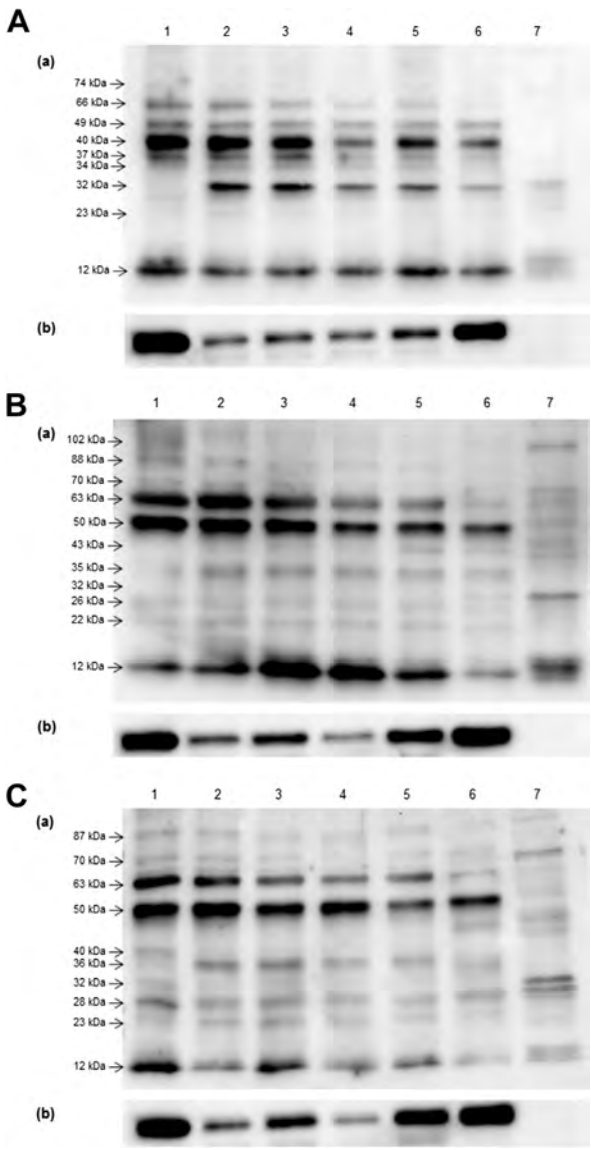

Fig. 2. Protein phosphorylation pattern on (A [a]) tyrosine, (B [a]) serine, and (C [a]) threonine residues in contaminated and noncontaminated boar sperm samples. Arrows indicate the main reactive bands discussed in the present work. Lanes 1 to 7 correspond to fresh noncapacitated sperm sample, control tube (capacitated sperm sample without bacteria), 10^4 CFU/mL treatment, 10^6 CFU/mL treatment, 10^7 CFU/mL treatment, 10^8 CFU/mL treatment, and the control containing no sperm, respectively. α-Tubulin was used as a standard to normalized total blotted protein content (A(b), B(b), and C(b)). Representative images for seven separate experiments (n = 7) are shown. CFU, colony-forming unit.

with α-tubulin (Table 2). Molecular weights of the main reactive pSer bands identified were of about 102, 88, 70, 63, 50, 43, 35, 32, 26, 22 and 12 kDa. The effects of *P aeruginosa* on pSer expression levels were of a lesser extent than those observed for pTyr levels. As shown in Table 2, in most bands (88, 70, 32, 26, and 22 kDa) the only treatment significantly ($P < 0.05$) different from the control tube was the tube containing 10^8 CFU/mL. In contrast, the effects of bacterial contamination started to be evident at a concentration of 10^6 CFU/mL in bands of 63, 50, and 12 kDa and at a concentration of 10^7 CFU/mL in the 35-kDa band. As observed for changes in pTyr levels, the reduction observed in the pSer expression of these bands became greater as the bacterial load increased, with the exception of the bands of 102 and 43 kDa, in which pSer levels were not significantly different among treatments. The expression pattern obtained in the lane corresponding to the control containing no sperm (lane 7) was different from those obtained in sperm samples. As with the pTyr pattern, only those bands related to sperm, i.e., identified in either noncapacitated samples (lane 1) or control tube (lane 2), were analyzed in the present study.

Finally, Figure 2C (a) exhibits that 10 main reactive bands were identified from the pThr pattern obtained through the Western blot analysis. Their expression was also normalized and semiquantified with α-tubulin (Table 2). Main reactive blotted protein bands identified had, approximately, the following molecular weights: 87, 70, 63, 50, 40, 36, 32, 28, 23, and 12 kDa. In a similar fashion to that observed in the expression patterns of pTyr and pSer, pThr expression of these bands also lessened as the bacterial load increased, with the exception of the bands corresponding to 87 and 40 kDa, in which pThr levels were not significantly ($P > 0.05$) different among treatments. This reduction observed in the pThr levels, however, was not as pronounced as that described for pTyr and pSer. In this case, the effects of *P aeruginosa* after IVC were mainly seen at the two highest infective concentrations (10^7–10^8 CFU/mL). In the bands corresponding to 63, 32, 28, and 23 kDa, both treatments showed pThr levels significantly ($P < 0.05$) lower when compared with the control tube, and in the rest of bands (70, 50, 36, and 12 kDa) only the tube with 10^8 CFU/mL was different from the control tube.

The expression pattern obtained in the lane corresponding to the control containing no sperm (Fig. 2C [a]) was different from those obtained in sperm samples. Again, as with the pTyr and pSer patterns, only those bands related to sperm, i.e., identified in either noncapacitated samples (lane 1) or control tube (lane 2), were analyzed in the present study. Therefore, the band identified in lane 6 between bands of 40 and 50 kDa was not included in the present analysis.

4. Discussion

As previously stated, sperm capacitation involves modifications regarding membrane fluidity and sperm motility, among others [39–41]. In the present study, as far as membrane fluidity and sperm motility is concerned, a reduction in both low membrane lipid disorder and sperm motility kinematics concomitant with bacterial contamination, especially at high concentrations, has been observed. Despite the bibliography concerning the effects of bacteria on sperm capacitation is quite limited, these results match with previous studies, in which contamination with different bacteria caused an increase in phospholipid scrambling of the plasma membrane [42] and a reduction in sperm motility kinematics in different mammal species, such as ram [43], stallion [44], and boar [8]. Although these previous reports were performed

Table 2
Protein phosphorylation levels (Tyr, Ser, and Thr) in contaminated and noncontaminated boar sperm samples after α-tubulin normalization (mean ± standard error of the mean).

Phosphorylated residue	Band (kDa)	Control tube	10^4 CFU/mL	10^6 CFU/mL	10^7 CFU/mL	10^8 CFU/mL
pTyr	74	0.44 ± 0.16^{a}	$0.15 \pm 0.06^{a,b}$	$0.22 \pm 0.09^{a,b}$	0.14 ± 0.04^{b}	0.09 ± 0.02^{b}
	66	1.10 ± 0.33^{a}	0.50 ± 0.12^{a}	0.29 ± 0.07^{b}	0.29 ± 0.06^{b}	0.13 ± 0.03^{c}
	49	1.80 ± 0.62^{a}	1.02 ± 0.26^{a}	0.49 ± 0.10^{b}	0.43 ± 0.08^{b}	0.32 ± 0.04^{b}
	40	3.35 ± 1.03^{a}	2.02 ± 0.48^{a}	1.13 ± 0.24^{b}	0.88 ± 0.11^{b}	0.53 ± 0.10^{c}
	37	1.76 ± 0.51^{a}	1.03 ± 0.20^{a}	0.59 ± 0.10^{b}	$0.48 \pm 0.10^{b,c}$	0.30 ± 0.06^{c}
	34	0.50 ± 0.07^{a}	0.50 ± 0.11^{a}	0.31 ± 0.04^{b}	0.18 ± 0.03^{c}	0.14 ± 0.02^{c}
	32	3.13 ± 0.81^{a}	1.77 ± 0.33^{a}	1.05 ± 0.20^{b}	0.77 ± 0.15^{b}	0.36 ± 0.07^{c}
	23	0.58 ± 0.15^{a}	0.30 ± 0.05^{b}	$0.20 \pm 0.05^{b,c}$	0.15 ± 0.03^{c}	0.13 ± 0.04^{c}
	12	1.29 ± 0.29^{a}	$0.90 \pm 0.40^{a,b}$	0.67 ± 0.13^{b}	$0.40 \pm 0.14^{b,c}$	0.25 ± 0.07^{c}
pSer	102	0.06 ± 0.10^{a}	0.06 ± 0.04^{a}	0.06 ± 0.09^{a}	0.04 ± 0.05^{a}	0.05 ± 0.03^{a}
	88	0.37 ± 0.23^{a}	0.21 ± 0.06^{a}	$0.15 \pm 0.09^{a,b}$	$0.10 \pm 0.04^{a,b}$	0.08 ± 0.03^{b}
	70	0.57 ± 0.26^{a}	0.37 ± 0.04^{a}	$0.42 \pm 0.16^{a,b}$	$0.21 \pm 0.07^{a,b}$	0.20 ± 0.05^{b}
	63	2.48 ± 0.49^{a}	$1.53 \pm 0.32^{a,b}$	$0.94 \pm 0.12^{b,c}$	0.68 ± 0.16^{c}	0.26 ± 0.04^{d}
	50	2.70 ± 0.51^{a}	$2.23 \pm 0.59^{a,b}$	$1.17 \pm 0.15^{b,c}$	$0.88 \pm 0.26^{c,d}$	0.63 ± 0.08^{d}
	43	0.52 ± 0.30^{a}	0.35 ± 0.06^{a}	0.32 ± 0.16^{a}	0.22 ± 0.12^{a}	0.15 ± 0.06^{a}
	35	1.16 ± 0.19^{a}	1.11 ± 0.39^{a}	$0.68 \pm 0.19^{a,b}$	$0.41 \pm 0.14^{b,c}$	0.23 ± 0.06^{c}
	32	0.34 ± 0.09^{a}	0.39 ± 0.08^{a}	0.33 ± 0.06^{a}	$0.23 \pm 0.07^{a,b}$	0.17 ± 0.02^{b}
	26	0.69 ± 0.28^{a}	0.56 ± 0.14^{a}	0.42 ± 0.14^{a}	$0.26 \pm 0.07^{a,b}$	0.15 ± 0.05^{b}
	22	0.58 ± 0.23^{a}	0.60 ± 0.30^{a}	0.45 ± 0.19^{a}	$0.24 \pm 0.09^{a,b}$	0.14 ± 0.06^{b}
	12	2.93 ± 0.37^{a}	3.02 ± 1.30^{a}	1.55 ± 0.27^{b}	$1.22 \pm 0.37^{b,c}$	0.47 ± 0.20^{c}
pThr	87	0.32 ± 0.20^{a}	0.16 ± 0.09^{a}	0.16 ± 0.04^{a}	0.11 ± 0.06^{a}	0.09 ± 0.03^{a}
	70	0.47 ± 0.17^{a}	$0.29 \pm 0.12^{a,b}$	$0.26 \pm 0.06^{a,b}$	$0.20 \pm 0.04^{a,b}$	0.16 ± 0.04^{b}
	63	1.62 ± 0.51^{a}	$1.02 \pm 0.26^{a,b}$	$0.94 \pm 0.43^{a,b}$	0.61 ± 0.13^{b}	0.22 ± 0.04^{c}
	50	1.58 ± 0.47^{a}	1.37 ± 0.32^{a}	$1.29 \pm 0.58^{a,b}$	$0.78 \pm 0.25^{a,b}$	0.58 ± 0.11^{b}
	40	0.48 ± 0.23^{a}	0.55 ± 0.2^{4a}	0.36 ± 0.08^{a}	0.33 ± 0.13^{a}	0.19 ± 0.03^{a}
	36	0.89 ± 0.34^{a}	0.59 ± 0.19^{a}	0.52 ± 0.14^{a}	$0.35 \pm 0.10^{a,b}$	0.22 ± 0.07^{b}
	32	0.41 ± 0.03^{a}	0.39 ± 0.03^{a}	$0.34 \pm 0.08^{a,b}$	0.21 ± 0.02^{b}	0.20 ± 0.03^{b}
	28	1.16 ± 0.78^{a}	0.40 ± 0.08^{a}	$0.34 \pm 0.07^{a,b}$	0.24 ± 0.05^{b}	0.16 ± 0.05^{b}
	23	1.41 ± 1.04^{a}	$0.38 \pm 0.12^{a,b}$	$0.38 \pm 0.13^{a,b}$	0.20 ± 0.05^{b}	0.16 ± 0.06^{b}
	12	3.03 ± 1.65^{a}	1.61 ± 0.58^{a}	1.54 ± 0.14^{a}	$0.94 \pm 0.13^{a,b}$	0.47 ± 0.05^{b}

Different superscript letters (a–c) mean significant differences ($P < 0.05$) between infective concentrations within rows. The control tube contains capacitated sperm sample without bacteria.
Abbreviations: CFU, colony-forming unit; pThr, phosphothreonine; pSer, phosphoserine; pTyr, phosphotyrosine.

under storage conditions (stallion at 5 °C; ram and boar at 15°C–17 °C), they could serve us as a starting point because, under capacitation conditions (38.5 °C), bacteria are more metabolically active than at lower temperatures [45].

Sperm capacitation can be attained *in vitro* by using defined media. Although composition of media may differ between works, there are three components (BSA, Ca^{2+}, and bicarbonate) that are involved in promoting IVC in mammals [17,26]. In this regard, it is important to stress out that in the present study IVC was achieved by using a CM without bicarbonate, a well-known and potent capacitation inductor in most mammalian species, including boar [46]. However, in agreement with previous studies [27,28,39,47,48], our results have confirmed through evaluation of sperm viability, motility, membrane lipid disorder, and specially tyrosine phosphorylation of p32 that IVC in boar sperm can be achieved without bicarbonate. Therefore, the present results suggest that, unlike other species such as human or mice [49,50], bicarbonate is not a mandatory component for CM to accomplish IVC in boar sperm.

In different mammal species, sperm capacitation is associated with an increase in protein phosphorylation, which has been considered as a marker of late capacitation-like events [15]. Indeed, in humans, protein phosphorylation has been shown to correlate with the capacity of sperm to bind to the oocyte zona pellucida, and alterations in Tyr phosphorylation have been related to subfertile subjects [51], and, in boar, it has also been associated with the regulation of sperm motility [52] and with the tolerance of sperm to frozen storage [53]. Thus, it is suggested to have a significant role in fertilization. The present work showed a protein pTyr pattern mostly in accordance with other studies conducted in boar sperm after IVC [24,54,55]. As indicated, the expression levels of the tyrosine-phosphorylated proteins detected in this work significantly decreased as the bacterial load increased, the tube with the highest bacterial load being that presented the lowest levels. According to the earlier studies, most of the bands identified (12, 34–74 kDa) are tyrosine-phosphorylated proteins present in both capacitated and uncapacitated sperm extracts. Some of them are constitutive bands, even present in immature spermatozoa (34–49 kDa) [54–56]. Hence, to determine the biological effects of the reduction in the pTyr levels because of bacterial contamination observed in the present study, further studies are needed to characterize these proteins and understand the role of their tyrosine phosphorylation in fertilization.

On the other hand, two of the bands identified in Western blots corresponded to important proteins implicated in sperm capacitation-function. Dubé et al. [57] first identified a tyrosine-phosphorylated protein of 23 kDa that appeared concomitantly with capacitation. Later on, this protein was described as a chain 1–phospholipid

hydroperoxide glutathione peroxidase (PHGPx; *Sus scrofa*) of 21 kDa [56], which may correspond to the protein band of 23 kDa described here. Phospholipid hydroperoxide glutathione peroxidase is a 20 to 22 kDa monomer protein enzyme that protects biomembranes from oxidative stress and has been found in high levels in mammalian testis [58,59]. During sperm maturation, this enzyme switches to an enzymatically inactive, disulfide cross-linked, and insoluble structural protein of the sperm mitochondrial capsule [60]. Although the effects of tyrosine phosphorylation of PHGPx are still not known, previous data suggest that it may represent an important event in the signaling cascade associated with capacitation, affecting the regulation of sperm hyperactivation, the mitochondrial function, or both. Therefore, modifications of the tyrosine levels caused by *P aeruginosa* may imply a modulation of the PHGPx action.

The other protein band identified in the Western blots, and the most important for the present study, is the band corresponding to 32 kDa. Several previous studies in boar sperm have reported the appearance of a protein band of 32 kDa concomitant with capacitation in a calcium-dependent manner [21,47,61–63]. This protein, known as either p32 or sp32, is a proacrosin binding protein located in the acrosome of boar sperm that has been shown to participate in acrosin maturation promoting proacrosin conversion into acrosin [62,64–66], an important protein for sperm–oocyte interaction and fertilization [67]. Therefore, the reduction in the expression levels of p32 when increasing the bacterial load observed here may imply that bacterial contamination with *P aeruginosa* decreases the ability of boar sperm to attain IVC.

During capacitation, proteins are not exclusively phosphorylated on tyrosine residues, but also on serine and threonine residues [15]. However, reports dealing with Ser/Thr protein phosphorylation are scarce. As the present work, some reports on human [68,69], hamster [70], and boar [47] have described a set of proteins that are phosphorylated at serine and/or threonine residues and whose phosphorylation levels mostly increase in a time-dependent manner during sperm capacitation. Nevertheless, Alnagar et al. [71] observed a reduction in serine phosphorylation levels on five proteins during IVC in boar. Thus, the dynamics of serine and threonine phosphorylation in boar are still unclear.

Several reports have elucidated that capacitation not only increased Ser/Thr protein phosphorylation, but also induced a subcellular shift in localization of phosphorylated sperm proteins that can be associated with the regulation of processes required for a successful fertilization [47,68,72,73]. Moreover, it has been observed that fertilization antigen-1, which is involved in zona pellucida binding and acrosome reaction, is Ser/Thr and Tyr phosphorylated during sperm capacitation in humans [68], and boar sperm motility seems to be modulated by pSer levels of glycogen synthase kinase-3α [74]. Thus, it is suggested that Ser/Thr phosphorylation is involved in sperm motility and acrosome reaction. Indeed, some reports have reported that several proteins phosphorylated at serine residues may have implications in sperm function [75,76]. For this reason, the study of all phosphorylation changes (Tyr, Ser, and Thr) is necessary to fully understand all changes that boar sperm undergoes during capacitation and to comprehend the potential effects that bacterial contamination may cause.

In the present work, we also observed a decrease in pSer/pThr expression levels when increasing bacterial concentrations. Most of the proteins appeared to be phosphorylated in both serine and threonine residues, and some also in tyrosine residues, suggesting that these proteins are phosphorylated by dual-specificity kinases [70]. However, these are just speculations because we cannot guarantee the exact molecular weight of the protein bands detected, so the identity of these proteins remains unknown. Consequently, identification of these proteins may shed further light on the possible function of such dephosphorylation pattern observed because of bacterial contamination during IVC. It must be taken into consideration that the present study is a preliminary insight into the effects of bacteriospermia on the fertilizing competence of boar spermatozoa; therefore, these results must be evaluated from a conservative point of view, as further studies are needed to tackle this issue more deeply.

4.1. Conclusions

Taking together all the capacitation-like changes observed in the study (the reduction in the protein phosphorylation levels, especially tyrosine phosphorylation of p32 when increasing the bacterial load, with the reduction in sperm motility kinematics, sperm viability, and membrane lipid disorder), our results indicate that bacterial contamination with *P aeruginosa* greater than 10^4 to 10^6 CFU/mL decreases the ability of boar sperm to become fully capacitated, thus affecting the sperm fertilizing ability. Because most of the studies performed in boar are conducted at 15 °C to 17 °C, the real impact of bacteria on sperm fertilizing ability is unknown. Thus, although the female factor is excluded from the present study, these results open a door to explain, for example, why allegedly safe concentrations of bacteria in boar sperm cause the reduced litter size observed in the study of Maroto Martín et al. [3]. Nevertheless, further studies to characterize the phosphorylated proteins detected here are warranted to comprehend the biological effects that these changes may have on sperm capacitation and fertilizing ability.

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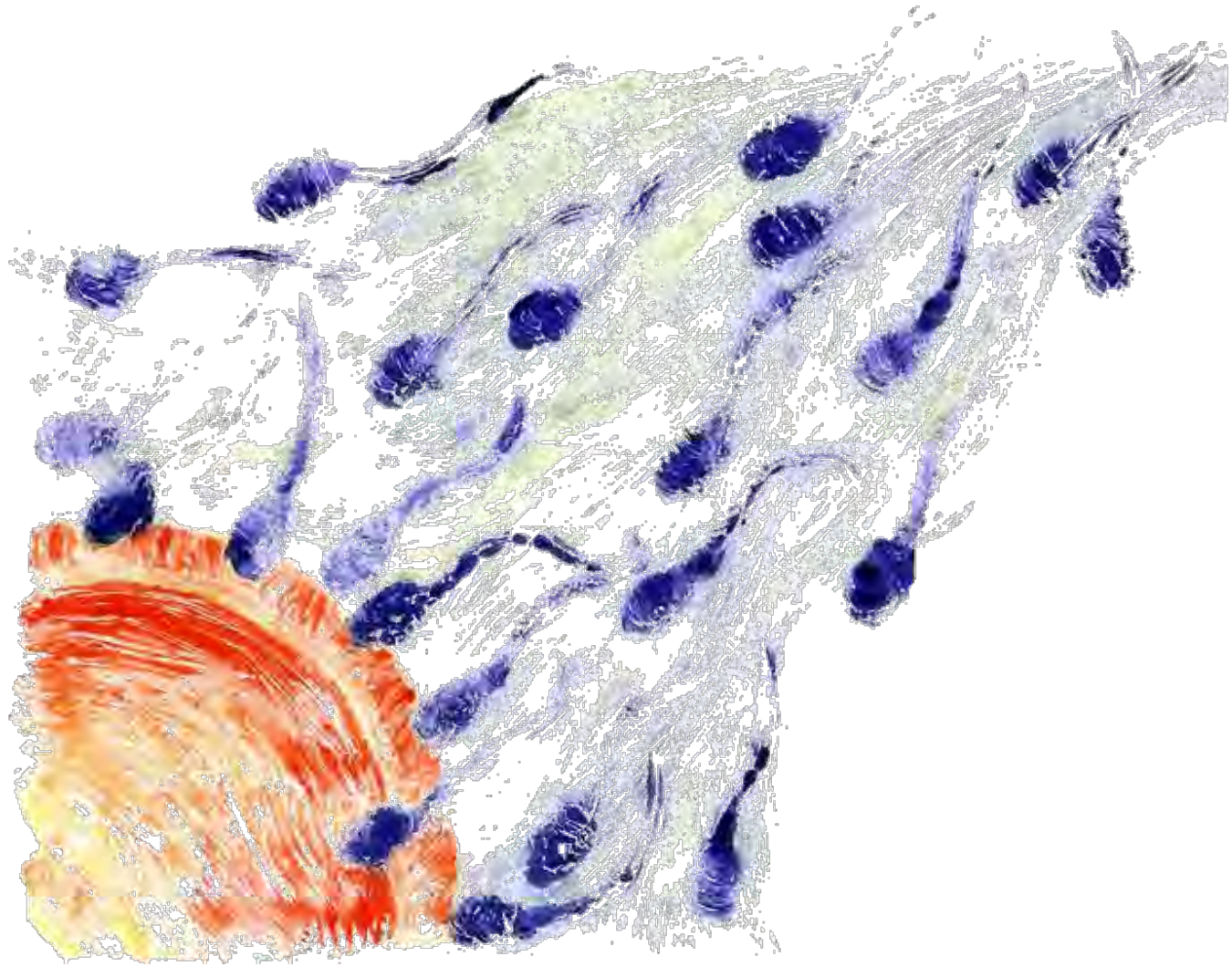
SECTION III

GENERAL DISCUSSION & CONCLUSIONS



General Discussion

The use of AI has grown exponentially since the 1980's until becoming the most practised assisted reproductive technology throughout the world, on account of the development of efficient semen extenders, and the standardisation of AI techniques to be used on-farm (Rodríguez-Gil and Estrada, 2013; Knox, 2016; Roca *et al.*, 2016). Nowadays, in European countries, USA, Latin America and Canada more than 90% of sows are bred using AI techniques. In East Asian countries, although the number of sows bred using this technology is quite lower, there is a mounting tendency in the use of AI; in Philippines, this method is employed in 50% of the cases, whilst in Taiwan and Thailand 70% of the sows are currently inseminated by AI (Maes *et al.*, 2008, 2016; Rodríguez-Gil and Estrada, 2013). One of the main goals of utilising this technique is to reduce, if not eliminate, uterine contamination and venereal disease transmission on breeding farms, as direct contact between pigs, the most common way of bacterial transmission, is avoided (Vaillancourt *et al.*, 1993; Althouse and Rossow, 2011). Nevertheless, when using AI, several sows can be inseminated with the same ejaculate, since one ejaculate is processed and diluted to generate various insemination doses. Problems rise when this single ejaculate is contaminated with different microorganisms, which, subsequently, can be transmitted to the sow herd and cause infection, negative effects on sperm quality, and sub-optimal reproductive success, to name a few. Therefore, the adoption of AI is a double-edged sword because, although the risk of transmission using AI is minimal, repercussions for the swine industry can be manifold, especially if large numbers of sow herds are involved (Knox, 2016; Maes *et al.*, 2016).

Non-ejaculated sperm in healthy boars is sterile, as testes, epididymes and accessory sex glands are regarded as free of microorganisms; notwithstanding, during ejaculation sperm come into contact with different environments that may contain different bacteria, such as the urethra and prepuce, which make sperm no longer germ-free (Bonet *et al.*, 2013). Indeed, while the primary source of bacterial contamination is the boar itself, multiple point sources of contamination have been identified during the process of collection and manipulation of boar semen; consequently, ejaculated boar sperm normally harbours a broad range of microorganisms (Althouse and Lu, 2005). As previously stated in Section I (bibliographic revision) of the present Thesis, most of the bacterial contaminants present in boar semen are not

considered as primary pathogens in swine, but rather as a part of the normal microflora; however, they can become pathogenic in susceptible swine, or if present at high concentrations (Sone, 1990; Althouse *et al.*, 2000). Despite being non-pathogenic, the presence of such bacteria has been related to detrimental effects, as contaminant bacteria can highly affect the quality and longevity of spermatozoa and, consequently, the reproductive performance of the sow herd (Althouse and Lu, 2005; Maes *et al.*, 2008; Kuster and Althouse, 2016).

In the context outlined here, the present Thesis has been addressed to study the effects of two different bacteria frequently found in boar ejaculates on the quality and the fertilising ability of boar sperm during liquid storage. As a result, a total of three papers have been published and this Thesis has been submitted as a compilation of articles. Accordingly, **PAPER I** aimed to evaluate how different infective concentrations of the anaerobic bacterium *Clostridium perfringens* affect the quality of boar sperm during 11 days of storage at 15ºC, and during four days (96 h) of incubation at, approximately, body animal temperature (37ºC). In order to compare and contrast the impact of anaerobic and aerobic bacteria on sperm quality, **PAPER II** sought to assess the effects of different loads of *Pseudomonas aeruginosa*, an aerobic bacterium, on boar sperm quality during storage at 15ºC. It is important to mention that *P. aeruginosa* is more frequently isolated from porcine semen than *C. perfringens* due to its oxygen requirements. Finally, as the effects of *P. aeruginosa* on sperm quality *per se* had already been discussed in **PAPER II**, **PAPER III** was conducted to move one step forward ahead. Concretely, this study was performed to analyse the effects that *P. aeruginosa* causes on the ability of boar sperm to achieve capacitation, a prerequisite for a successful fertilisation.

As previously mentioned in Section I, several studies have evaluated the effects that bacterial contamination cause upon sperm quality in different animal species. Since obligate anaerobes can neither proliferate nor survive in oxygen tensions due to its metabolic necessities, most of these reports are focused on dealing with aerobic bacteria; notwithstanding, the presence of aerotolerant anaerobic bacteria in semen is not unusual (Eggert-Kruse *et al.*, 1995; Damirayakhian *et al.*, 2006; De Grau *et al.*, 2006; Corona and Cherchi, 2009; Maroto Martín *et al.*, 2010). To the best of our knowledge, no study had already investigated whether anaerobic bacteria could survive in extended boar semen. As *Clostridium perfringens*, an aerotolerant anaerobic bacterium, is highly prevalent throughout the swine industry (Baker *et al.*, 2010), it is highly plausible to isolate it from boar semen by reason of accidental contamination during sperm collection and/or processing. Hence, trial experiments performed in **PAPER I** aimed at

assessing the effects of different concentration of *C. perfringens* on extended boar semen during **1)** storage at 15ºC, to mimic storage conditions at which semen doses are exposed to in AI centres; and **2)** incubation at 37ºC, to emulate the impact that this bacterium may have on both the male and female reproductive tracts. On the other hand, **PAPER II** was conducted in a similar way to **PAPER I** but, in this case, an aerobic bacterium was used. Particularly, the bacterium used in this paper was *Pseudomonas aeruginosa*, which, after *E. coli*, is one of the most frequently isolated bacterium from boar semen (see Section I). In fact, while the vast majority of gram negative bacteria detected in boar semen belong to the *Enterobacteriaceae* family, the next bacterial family in terms of abundance is *Pseudomonodaceae* (Schulze *et al.*, 2015). Accordingly, as no previous study had investigated this issue, **PAPER II**, following the same fashion as **PAPER I**, sought to analyse the impact of different bacterial concentrations of *P. aeruginosa* on boar sperm during storage at 15-17ºC, which is the common range of temperature for boar semen storage.

It is important to mention that the strains of *C. perfringens* and *P. aeruginosa* used in the present Thesis were not isolated from semen samples, but purchased from a commercial Spanish company (CECT; Spanish collection of microbiological type cultures). In a preliminary study involving all *C. perfringens* strains used in **PAPER I**, the effects of each strain on boar sperm quality were assessed individually as well as collectively. Results obtained showed that no significant differences regarding sperm quality were observed neither between them (single strains) nor when compared to the sample inoculated simultaneously with a mixture of the five strains. Therefore, although differences may exist between strains, including the wild-type and the cultured one, main conclusions of this work would have not been different if other bacterial strains were used.

In current AI practices, the prediction of sperm fertilising ability has a great economic importance because it leads to the selection of those boars with good reproductive performance. Classical laboratory methods of semen evaluation usually measure volume, sperm concentration, sperm viability, sperm motility and sperm morphology (Gadea, 2005; Sancho and Vilagran, 2013). Nevertheless, most of these traits alone are poor in predicting fertility outcomes, as only those samples with markedly poor quality can be identified. Therefore, combination of assays can provide better assessment of the quality of the ejaculate in question (Gadea *et al.*, 2004; Petrunkina *et al.*, 2007; Sancho and Vilagran, 2013; Knox, 2016). To improve sperm quality analyses, new standardised and objective procedures of *in vitro* seminal analysis

and new assays for the evaluation of sperm functionality have been developed in the last years. The introduction of computer-assisted semen analysis (CASA) systems and the evaluation of the plasma membrane integrity or capacitation status using objective tools like flow cytometry (FC), which have been reported to better predict the fertilising competence of sperm cells, are some of these new assays (Gadea *et al.*, 2004; Gadea, 2005; Broekhuijse *et al.*, 2012a, 2012b; Sancho and Vilagran, 2013).

Sperm motility is one of the most frequently parameters assessed in the conventional spermiogramme, as it is a simple, quick and inexpensive evaluation. Although it is commonly believed to be a fundamental semen quality characteristic, whether sperm motility in pigs is related to fertility results is a controversial subject. Results in the literature concerning correlations between sperm motility and pig fertility outcomes are contradictory (Berger *et al.*, 1996; Holt *et al.*, 1997; Tardif *et al.*, 1999; Hirai *et al.*, 2001; Pérez-Llano *et al.*, 2001; Gadea, 2005; Sutkeviciene *et al.*, 2009; Yeste *et al.*, 2010; Broekhuijse *et al.*, 2012a, 2012b). This inconsistency might be due to experimental differences; in some studies sperm motility is assessed by eye, which is subject to great intra- and inter-observer variability, while others use a more standardised approach like CASA systems. Nevertheless, although the relation between this sperm quality parameter and boar fertility is not clear, it is broadly accepted that non-motile or poorly motile spermatozoa are unable of reaching and fertilising the oocyte (Tardif *et al.*, 1999; Turner, 2003; Hernández-Caravaca *et al.*, 2015).

Regarding the effects caused by *C. perfringens* **(PAPER I)**, results showed that detrimental effects for both TMOT and PMOT were seen just after 24h of incubation/storage at both temperatures (15ºC and 37ºC) and at the highest infective concentrations (10^6-10^8 CFU/mL). On the contrary, damaging effects for *P. aeruginosa* **(PAPER II)** were observed after 48h of storage at 15ºC. In both papers, these effects were both time- and concentration- dependent. These results are consistent with previous studies, where bacterial contamination also caused a substantial reduction of sperm gross motility in different animals, such as stallion (Aurich and Spergser, 2007), human (Huwe *et al.*, 1998; Diemer *et al.*, 2003; Fraczek and Kurpisz, 2015), bull (Smole *et al.*, 2010), ram (Yániz *et al.*, 2010) and boar (Althouse *et al.*, 2000; Bussalleu *et al.*, 2011; Ubeda *et al.*, 2013; Prieto-Martínez *et al.*, 2014). Therefore, fertilising capacity of seminal doses contaminated with bacteria may be jeopardised, as the effects on sperm motility show; if sperm motility is below recommended ranges (<60% of progressive motility) (Sancho and Vilagran, 2013), the probability of reaching and penetrating the oocyte diminishes.

Another parameter tested in both studies was sperm viability. Sperm viability is commonly measured by evaluating the integrity of the plasma membrane, since its intactness is pivotal for sperm interactions with other cells and their environment, as well as for sperm metabolism and function (sperm capacitation, AR,…) (Hossain *et al.*, 2011; Pinart and Puigmulé, 2013). Hence, sperm plasma membrane integrity is a key determinant factor in the assessment of sperm quality and a prerequisite for successful fertilisation, because of its relationship with *in vivo* and *in vitro* fertility (Sutkeviciene *et al.*, 2009; Yeste *et al.*, 2010). Even though sperm viability was analysed by different techniques in **PAPER I** and **PAPER II** (fluorescence microscopy and flow cytometry, respectively), a clearly and significant reduction in sperm viability, mainly at the greatest bacterial inoculums (10^7 and 10^8 CFU/mL), after 24h of storage/incubation was observed in both papers. Nonetheless, effects caused by *C. perfringens* at 15ºC were greater than those provoked by *P. aeruginosa*; from the 4th day of storage onwards the only treatment showing significant differences when compared to the control in **PAPER II** (*P. aeruginosa*) was the highest inoculated tube (2x10^8 CFU/mL), while in **PAPER I** (*C. perfringens*) the tube with 10^7 CFU/mL was also significantly different after 3 days of storage and until the end of the experiment. Again, these results were time- and concentration-dependent, and are in agreement with previous reports in human (Fraczek *et al.*, 2012), ram (Yániz *et al.*, 2010) and boar (Bussalleu *et al.*, 2011; Chung *et al.*, 2013; Ubeda *et al.*, 2013; Prieto-Martínez *et al.*, 2014), where different bacteria, such as *E.coli* or *Enterobacter cloacae*, were used.

In **PAPER I** results showed that most adverse effects on boar sperm quality were observed when sperm was incubated with *C. perfringens* at 37ºC. It must be taken into consideration that sperm metabolism at 37ºC is greatly active, so energy sources are depleted earlier than at 15ºC (Barbonetti *et al.*, 2010). This may explain the lower results obtained for both sperm motility and viability, even in the control group. Moreover, since extenders contain abundant nutrients and 37ºC of temperature perfectly allows the growth of *C. perfringens*, the initial bacterial load and the toxin production may rapidly augment while being incubated. Therefore, damaging effects caused by this bacterium are magnified during incubation. Similarly, results obtained in **PAPER II** demonstrated that *P. aeruginosa* was also able to grow at 15-17ºC, thus also increasing the bacterial concentration along the experimental period. This would also suggest that *C. perfringens* may also grow at 15ºC. However, as Althouse *et al.* (2008) observed for *Achromobacter xylosoxidans* and *Serratia marcescens*, bacterial generation times are temperature-dependent, being longer at 15ºC than at 37ºC, because 15ºC is not their optimal

growth temperature. Bearing this in mind, it is highly probable that the adverse effects caused at 37ºC by *C. perfringens* were related to a higher bacterial growth than at 15ºC.

Considering that only boar sperm with intact acrosome can initiate the binding and penetration to the ZP and the subsequent fusion with the oocyte, the acrosome status of living cells is also a highly important parameter to predict sperm fertility (Fazeli *et al.*, 1997; Gadea, 2005; Hossain *et al.*, 2011). For this reason, a premature or degenerative acrosome exocytosis makes the spermatozoon no longer able to fertilise the oocyte (Spinaci *et al.*, 2010). Furthermore, the percentage of acrosome-intact spermatozoa in a given ejaculate has been consistently recorded as a reliable indicator for *in vitro* and *in vivo* fertilisation success or failure in humans (Abu Hassan Abu *et al.*, 2012). In the present Thesis, this parameter was directly evaluated in **PAPER II** by using FC, an objective and reliable tool. Results showed a diminution in the percentage of acrosome-intact sperm, again, at higher inoculated samples ($2x10^7$ and $2x10^8$ CFU/mL) and, unlike results obtained for sperm motility in the same paper, after only 24h of storage. Once again, effects were time- and concentration-dependent, as acrosome impairment was aggravated by higher bacterial loads as well as during storage time. According to these results, several authors have also pointed out that premature acrosome exocytosis is one of the main consequences of bacterial contamination in mammalian spermatozoa in both *in vitro* and *in vivo* studies (Köhn *et al.*, 1998; Zan Bar *et al.*, 2008; Rennemeier *et al.*, 2009; Fraczek *et al.*, 2012; Chung *et al.*, 2013; Prieto-Martínez *et al.*, 2014).

Sperm morphology has been associated with both *in vitro* and *in vivo* fertilisation, as it is related to sperm dysfunction (Abu Hassan Abu *et al.*, 2012). In agreement with Bussalleu *et al.* (2011) working with *E. coli* and Prieto-Martínez *et al.* (2014) with *E. cloacae*, and unlike other sperm quality parameters assessed in the present Thesis, **PAPER I** demonstrated that sperm morphology remained unaltered after co-incubation with *C. perfringens* at 15 and 37ºC. These results are in discrepancy with other reports in human, in which sperm morphology was clearly affected by *E. coli* contamination by means of ultrastructural sperm alterations at the level of the midpiece, plasma membrane and acrosome (Diemer *et al.*, 1996, 2000). This inconsistency might be explained by the fact that ultrastructural changes can only be observed by electron microscopy rather than using conventional optical microscopy (Diemer *et al.*, 2000), which is the method used in **PAPER I** and in the studies of Bussalleu *et al.* (2011) and Prieto-Martínez *et al.* (2014). However, a great agglutination was actually observed while increasing concentrations in **PAPER I** and **PAPER II**, as others authors have also pointed out (Sone, 1990; Köhn *et al.*, 1998;

Prieto-Martínez *et al.*, 2014). Due to results obtained regarding sperm morphology in **PAPER I**, this parameter was not assessed in **PAPER II**.

PAPER I and **PAPER II** demonstrated that detrimental effects on boar sperm quality occur after 24 or even 48h of incubation, thus suggesting that bacteria need to undergo through an adaptation or acclimation phase before causing sperm impairment. In fact, this tendency has also been observed in other studies (Bussalleu *et al.*, 2011; Prieto-Martínez *et al.*, 2014), and, as stated in Althouse (2008), is explained by the fact that after the introduction into a novel environment, bacteria need some time to recover from the physical damage or shock caused by transfer and to put its own metabolic machinery in motion; once acclimation is fulfilled, bacteria start growing again. Moreover, as some authors have also described (Althouse, 2008; Yániz *et al.*, 2010; Prieto-Martínez *et al.*, 2014), inhibitory effects on boar sperm quality were seen from a sperm/bacteria ratio of 1:1 in both **PAPER I** and **PAPER II**. Nonetheless, while results in **PAPER I** showed that damaging effects on boar sperm at 37ºC were mostly seen at concentration higher than 10^7 CFU/mL, previous reports in boar with *E. coli* demonstrated that loads greater than 10^3 CFU/mL in sperm incubated at 37ºC (Bussalleu *et al.*, 2011), and AI with semen containing at least $3.5x10^3$ CFU/mL (Maroto Martín *et al.*, 2010) were enough to cause detrimental effects on boar sperm quality and a reduction in litter size, respectively. With this being said, at this specific temperature *C. perfringens* seem to need a greater sperm:bacteria ratio than *E. coli* to be deleterious. Therefore, damaging effects on sperm quality seem to depend upon the bacterial species and the temperature.

The exact mechanisms by which *C. perfringens* and *P. aeruginosa* cause all the damaging effects observed in **PAPER I** and **PAPER II** remain largely unexplained; notwithstanding, it can be hypothesised that it may be related to direct contact with the sperm cell. Different studies using *E. coli* postulated that the attachment of bacteria to spermatozoa, via mannose-binding type 1 adhesion molecules, induces ultrastructural alterations and damage in the plasma membrane and other structures of human sperm cells, as well as sperm agglutination, thus resulting in sperm quality impairment (Auroux *et al.*, 1991; Wolff *et al.*, 1993; Diemer *et al.*, 2000, 2003; Schulz *et al.*, 2010). However, this mechanism may not be the only one, since bacterial cells also produce cell-associated and extracellular factors, such as toxins, that they release into the environment and contribute to their pathogenesis and survival. Actually, some reports have elucidated the impact of virulent molecules from *E. coli* and *P. aeruginosa* on mammalian sperm. According to Diemer *et al.* (2000) and Schulz *et al.* (2010), among the factors released by *E. coli*,

one that seems to be involved in the molecular mechanism responsible for diminished plasma membrane integrity and loss of sperm motility in human is haemolysin. Indeed, Schulz *et al.* (2010) verified that soluble factors secreted by *E. coli* cause a reduction in mitochondrial membrane potential, motility and viability of human sperm. Moreover, the studies portrayed by Prabha *et al.* (2010) and Kaur and Prabha (2013) are in agreement with the study of Diemer *et al.*, (2000). All these studies observed that two factors secreted by non-agglutinating and agglutinating *E. coli* strains, known as sperm immobilization factor (SIF) and sperm agglutinating factor (SAF), respectively, have detrimental effects on human sperm. Such effects are related to sperm immobilisation and apoptotic-like changes that appear to be related to sperm ultrastructural alterations, as revealed by scanning electron microscopy (Prabha *et al.*, 2009, 2010). As far as *P. aeruginosa* is concerned, a study performed by Rennemeier *et al.* (2009) demonstrated that *quorum sensing* (QS) molecules released by this bacterium provoke multiple damage on human spermatozoa; sperm motility loss, premature acrosome reaction and even sperm cell death are some examples. Moreover, other bacterial products from *P. aeruginosa* that could contribute to the deleterious effects observed in the present Thesis are the secreted factor elastase and lipopolysaccharides (LPS). A study performed by Kaur *et al.* (1988) demonstrated that the spermicidal effects (motility impairment and sperm agglutination) caused on human, bull and rat spermatozoa by different microorganisms isolated from cervix of infertile women, including *P. aeruginosa*, were mainly attributable to the secreted factor elastase. LPS are the major outer surface membrane components present in almost all gram-negative bacteria and are released into the environment as a result of cell division, death, or antibiotic treatment. They act as endotoxins binding directly to sperm and inducing damaging effects on sperm quality, like motility impairment and apoptosis (Zhang *et al.*, 1998; Rosenfeld and Shai, 2006; Okazaki *et al.*, 2010). It has been shown that both pig and mouse sperm express the Toll-like receptors (TLR) 4, which are stimulated by LPS (Okazaki *et al.*, 2010). In immune cells, this stimulation promotes the activation of the NF-κB pathway, which triggers the expression of several genes involved in immune responses or the apoptotic pathway (Takeda and Akira, 2005; Kawai and Akira, 2007). Hence, a study reported by Fujita et al. (2011) showed that LPS can exert apoptotic effects in sperm by activating the TLR4 signaling pathways, thus suggesting that sperm may respond to bacterial infection in semen by direct TLR-regulated mechanisms. Therefore, if preservative antibiotics included in semen extenders are efficient in killing gram-negative bacteria, LPS concentrations likely increase during storage due to bacteriolysis, thus amplifying the negative effects caused on sperm.

In regard to *C. perfringens*, to the best of our knowledge, literature concerning about the effects of extracellular and cell-associated factors of *C. perfringens* on mammalian sperm is quite limited, if not absent. Notwithstanding, several studies have indicated that treatment of fowl sperm with neuraminidases from *C. perfringens* prior to intravaginal insemination resulted in a reduced fertility, without affecting sperm functional integrity (sperm motility and viability), presumably due to alterations of the sperm glycocalyx. On the contrary, when hens were inseminated intramagnally, this effect was not observed (Froman and Thurston, 1984; Froman and Engel, 1989). Later on, Steele and Wishart (1996) demonstrated in chicken that neuraminidase treatment removes sialic acid residues from the sperm glycocalyx, causing a severe diminution in the sperm migration through the vagina; hence, sperm were not able to reach and populate the sperm storage tubules when inseminated intravaginally, fact that explained the reduced fertility. Those authors hypothesised that this inhibition of migration is caused by an increase of antigenicity in the vagina due to separation of sialic acid from sperm surface, which may confer protection against immunological attack.

Another mechanism whereby sperm quality can be impaired after bacterial contamination is acidification of the sperm handling medium. In freshly ejaculated boar semen pH usually ranges from 7.2 to 7.6, as in other body fluids; pH below these values gradually decreases both sperm metabolism and motility due to a reduction in intracellular pH (Johnson *et al.*, 2000; Gadea, 2003). Thus, in order to prolong life-span of seminal doses, sperm are normally extended in diluents with a corresponding pH varying between 6.8-7.3 (Althouse *et al.*, 2000; Gadea, 2003; Bussalleu and Torner, 2013). Different studies using different bacteria, like *E. cloacae, S. marcescens* or *E. coli*, have reported that bacterial metabolism can cause a pH reduction to acidic levels of sperm handling medium, which is harmful for sperm survival (Althouse *et al.*, 2000; Acosta, 2010; So *et al.*, 2011; Prieto-Martínez *et al.*, 2014); however, this mechanism has been discarded to account for the sperm quality impairment observed in the presence of *P. aeruginosa* in **PAPER II** as, in agreement with Althouse *et al.* (2000), negative effects caused by this specific bacterium are not related to an acidic environment.

Overall results shown in **PAPER I** and **PAPER II** emphasise the impact that both aerobic and anaerobic bacteria have on boar sperm quality and, therefore, fertilising ability of boar sperm stored at 15-17ºC and incubated at 37ºC, especially when bacteria are present at high concentrations. In addition, these effects seem to rely on both storage time and bacterial concentration. Consequently, routine bacterial analyses of semen doses are of highly

importance in order to foresee the magnitude that a specific bacterial load may have, and to establish a limiting storage time, since the higher the initial bacterial load is, the lower the storage time should be (Althouse *et al.*, 2000; Acosta, 2010; Goldberg *et al.*, 2013; Bryła and Trzcińska, 2015). Further studies are also required to elucidate the exact mechanisms by which both bacteria (*C. perfringens* and *P. aeruginosa*) cause these undesirable effects upon boar sperm quality.

Most of the existing papers dealing with the effects of bacteriospermia on mammalian sperm are mainly focused on evaluating classical sperm quality parameters, in order to detect alterations in sperm that evidence a reduction of fertility. However, several issues still remain unaddressed. For example, what does it happen with the functional capacity of the spermatozoa? Do bacteria have any effect on that? To gain the fertilising aptitude, i.e. the ability to bind and further penetrate the oocyte, mature mammalian sperm must first undergo through the process of capacitation, which ultimately modulates flagellar activity and renders the sperm apical-head plasma membrane fusogenic (Rodriguez-Martinez, 2007). This process, therefore, is a pre-requisite for proper fertilisation, and is also a good fertility predictor (Gadea, 2005). Related to this, previous reports observed that the presence of bacteria during IVF processes in human was associated to lower IVF outcomes (e.g. oocyte degeneration, suboptimal fertilisation rates and impaired embryonic development) (Huyser *et al.*, 1991; Shalika *et al.*, 1996; Steyaert *et al.*, 2000). To the best of our knowledge, no study has previously discussed the impact that bacteriospermia has on boar sperm capacitation; hence, as the effects of *P. aeruginosa* on boar sperm quality *per se* have already been studied in **PAPER II**, the purpose of **PAPER III** was to describe the repercussion that this bacterium has upon boar sperm capacitation, parameter assessed as changes in membrane fluidity, motility kinematics and protein phosphorylation.

Results presented in **PAPER III** provide an integrated study of ejaculated and *in vitro* capacitated boar spermatozoa contaminated with bacteria by using complementary techniques such as FC, immunoblotting and CASA analyses. It is well known that during sperm capacitation, the fluidity of sperm's plasma membrane rises due to a decrease in the packaging of membrane phospholipids, which results in a reduced stability of the plasma membrane (Gadella *et al.*, 2008; Fàbrega *et al.*, 2012). The present results showed that contamination with *P. aeruginosa* causes a significant and concentration-dependent drop in the percentage of viable sperm with low membrane lipid disorder, especially notorious at high bacterial loads. This alteration in the

capacitation process is supported by results obtained regarding sperm motility kinematics; in this case, bacterial contamination provoked a reduction in all motility parameters assessed when compared to those values showed by the bacteria-free capacitated sperm sample. Therefore, this paper demonstrates for the first time that the ability of boar sperm to achieve their fertilising ability (specifically, to become fully capacitated) is jeopardised by bacterial contamination. In fact, previous studies performed under storage conditions partially agree with our results, since also reported alterations associated to bacterial contamination in the plasma membrane architecture of human sperm (phospholipid scrambling) (Fraczek *et al.*, 2012) and in sperm motility kinematics in ram (Yániz *et al.*, 2010), stallion (Aurich and Spergser, 2007) and boar (Ubeda *et al.*, 2013); however, these studies were performed under storage conditions, rather than under *in vitro* capacitating conditions. In addition, obtained results regarding sperm viability are in agreement with those obtained in **PAPER II**, as the percentage of viable spermatozoa in contaminated samples is significantly reduced during the IVC procedure in a time- and concentration-manner.

One of the most important changes occurring during the capacitation process, and considered as a marker of late capacitation events, is protein phosphorylation (Yeste, 2013b). As stated in Section I, protein phosphorylation seems to be involved in different processes that are of highly importance for proper mammalian fertilisation, such as sperm motility (Si and Okuno, 1999; Holt and Harrison, 2002; Turner, 2003) or the binding capacity of spermatozoa to oocyte's ZP (Liu *et al.*, 2006); therefore, it is well accepted that protein phosphorylation has a physiological role on fertilisation. For all these reasons, the present Thesis has evaluated the effect of bacterial contamination on tyrosine, serine and threonine protein phosphorylation, concluding that *P. aeruginosa* causes a decrease in Tyr/Ser/Thr phosphorylation levels in most of the proteins identified in **PAPER III**.

First of all, it must be pointed out that the attainment of a feasible IVC in the present Thesis was achieved by using a capacitating medium (CM) without bicarbonate. Bicarbonate is a well-known and potent sperm capacitation inductor in most mammalian species, including porcine (Yeste, 2013b). Nevertheless, the present study has demonstrated by evaluating sperm viability, motility, membrane lipid disorder and, specially, tyrosine phosphorylation of p32, that IVC can be achieved without bicarbonate in boar sperm; some previous studies in boar also obtained these results (Ramió *et al.*, 2008; Ramió-Lluch *et al.*, 2012a, 2012b, 2014; Yeste *et al.*, 2015a). Thus, although bicarbonate is a potent capacitation factor in other species such as

human or mice (Visconti *et al.*, 1995a; Battistone *et al.*, 2013), it is not an absolute requirement to accomplish IVC in boar sperm. In our experimental conditions, BSA acted as the sole inductor of sperm capacitation.

In **PAPER III**, the study of the phosphotyrosine expression pattern of sperm proteins after IVC of sperm extracts contaminated with *P. aeruginosa* revealed the presence of nine main reactive bands (74, 66, 49, 40, 37, 34, 32, 23 and 12 kDa); these bands may correspond to those obtained in other studies in boar without contaminant bacteria (Kalab *et al.*, 1998; Fàbrega *et al.*, 2011; Kwon *et al.*, 2014). Fàbrega *et al.* (2011) studied the phosphotyrosine pattern of boar sperm proteins during maturation, ejaculation and IVC, and described a set of phosphorylated proteins only present in immature sperm (93, 66 and 45 kDa), in mature sperm from cauda and ejaculated sperm (76, 23, 12 kDa), in both mature and immature sperm, i.e. constitutive bands, (49, 40, 37, 30, 26 and 25 kDa), and those only present in capacitated sperm extracts (28 and 20 kDa). In the case of proteins from mature sperm and constitutive bands, their phosphotyrosine expression increased after IVC (Fàbrega *et al.*, 2011). Similarly, Kwon *et al.* (2014) detected a significant increase in four tyrosine phosphorylated proteins (18, 26, 34 and 36 kDa) as a result of boar sperm capacitation. The study of Kalab *et al.* (1998) also described a set of proteins constitutively tyrosine phosphorylated (44, 40, 38 and 34 kDa); however, contrarily to the studies of Fàbrega *et al.* (2011) and Kwon *et al.* (2014), these proteins were phosphorylated at the same level in fresh and capacitated sperm. Therefore, protein bands detected in **PAPER III** probably correspond to those previously observed in the aforesaid works. The present study resulted in lowered phosphotyrosine expression levels of sperm proteins as bacterial contamination increased. These results, however, are in disagreement with the results obtained in human with *Chlamydia trachomatis* (Hosseinzadeh *et al.*, 2000). In this case, co-incubation of sperm with *C. trachomatis* yielded to an increase in sperm protein tyrosine phosphorylation levels after IVC. These divergences may be due to the fact that *C. trachomatis* is an obligate intracellular pathogen. Although these results are opposed, both microorganisms somehow affect sperm capacitation, as both modify the tyrosine phosphorylation patterns. Moreover, another study detected two sperm membrane proteins from capacitated boar sperm cells of 35 and 46 kDa, and with a high ZP-binding affinity after being tyrosine phosphorylated (Flesch *et al.*, 2001). These proteins may or may not correspond to the proteins identified in **PAPER III** with 34 and 49 kDa approximately, but suggest that, as the present study determined that contamination with *P. aeruginosa* causes a decrease in the levels of tyrosine phosphorylation after IVC, bacterial contamination compromises the ability of boar sperm to achieve the

fertilising competence. Therefore, the characterisation of these sperm proteins will help us to elucidate the biological impact of the reduction in the phosphotyrosine levels due to bacterial contamination, and to understand its role in fertilisation.

Western Blot analyses showed that two of the proteins identified in the present study only had phosphotyrosine residues after the IVC procedure; these proteins are of, approximately, 23 and 32 kDa. Taking into consideration previous literature on proteins being tyrosine phosphorylated during sperm capacitation, the band of 23 kDa may correspond to the phosphoprotein of 23 kDa first identified in Dubé *et al.* (2003) that appeared concomitantly with sperm capacitation, and later on described as the phospholipid hydroperoxide glutathione peroxidase (PHGPx) in *Sus scrofa* (21 kDa) (Dubé *et al.*, 2004) and hamster (19 kDa) (Nagdas *et al.*, 2005). PHGPx is a selenoprotein of 20-22 kDa responsible for the protection of membranes against oxidative damage, since reduces lipid hydroperoxide generated in cell membranes (Imai and Nakagawa, 2003; Imai *et al.*, 2003). This protein is especially found in high levels in mammalian testis, as it is expressed by spermatids; during sperm differentiation (namely, maturation), this protein switches to an enzymatically inactive structural protein of the midpiece mitochondrial sheath (Ursini *et al.*, 1999; Nagdas *et al.*, 2005; Puglisi *et al.*, 2005; Jung *et al.*, 2015). Indeed, it has been described that insufficient content of PGHPx in sperm of human and mice is related to male infertility, due to motility impairment and structural abnormalities (Imai *et al.*, 2001; Nagdas *et al.*, 2005). Downstream effects of tyrosine phosphorylation on PGHPx during sperm capacitation are not yet known; however, it is suggested that it can reactivate the catalytic activity of PHGPx, regulate the stability of the mitochondrial sheath or participate in the signalling cascade associated with capacitation. Therefore, it seems to be implicated in the regulation of the hyperactivation of sperm motility and/or mitochondrial function (Nagdas *et al.*, 2005).

The band identified with an approximated molecular weight of 32 kDa could correspond to the calcium-dependent phosphoprotein of 32 kDa described to be tyrosine phosphorylated during boar sperm capacitation and considered as a steady indicator of IVC achievement in boar (Tardif *et al.*, 2001, 2003; Bailey *et al.*, 2005; Dubé *et al.*, 2005; Ramió-Lluch *et al.*, 2012b). This protein is known as p32, sp32 or also as Acrosin-binding protein (ACRBP). ACRBP is specifically located in the acrosome of germ cells, as it has been described in spermatozoa from human (Ono *et al.*, 2001), mouse (Arcelay *et al.*, 2008; Tardif *et al.*, 2012), guinea pig (Baba *et al.*, 1994), stallion (Kim *et al.*, 2015) and pig (Polakoski and Parrish, 1977; Dubé *et al.*, 2005; Vilagran *et al.*,

2013, 2016), and it is modulated via tyrosine phosphorylation. ACRBP seems to have a role in acrosin maturation, an important protein for sperm-oocyte interaction and fertilisation (Urch and Patel, 1991). ACRBP has been shown to bind to the proacrosin zymogen (55-53 kDa), thereby delaying its maturation; once ACRBP is tyrosine phosphorylated during capacitation, it activates the autocatalytic conversion of proacrosin into mature acrosin (35 kDa) (Baba *et al.*, 1994; Kanemori *et al.*, 2013; Sun *et al.*, 2013). Furthermore, this protein seems to be implicated in the packaging and assembly of the acrosomal matrix proteins during spermatogenesis and, since it has been described that acrosomal contents are dispersed more slowly without acrosin activation, it also appears to be an important regulator of proteolytic processing events during acrosomal matrix disassembling (i.e. AR) occurring during fertilisation (Baba *et al.*, 1994; Foster, 2013; Kanemori *et al.*, 2013). Therefore, modifications/reductions of the phosphotyrosine levels observed for the proteins of 23 kDa and 32 kDa, concomitant with the presence of *P. aeruginosa*, may imply that bacterial contamination have a significant negative impact upon the action of PHGPx and ACRBP, thus compromising the fertilising ability of boar sperm.

Despite the existence of several reports dealing with Tyr phosphorylation, only a few studies have been published on the phosphorylation of Ser/Thr residues, reflecting the difficulties in performing such studies. One of the main reasons for this inequality, in contrast to reports dealing with Tyr phosphorylation, may be that good-quality antibodies recognising Ser/Thr phosphoproteins have only become available in the last years (Alnagar *et al.*, 2010). Moreover, data available show that the dynamics of Ser/Thr phosphorylation are also quite unclear. The study performed by Naz (1999), working with human sperm, was one of the first works describing Ser/Thr phosphorylation. The author observed that at least six groups of proteins (18, 35, 43-55, 94, 110 and 190 kDa) were Ser/Thr phosphorylated, and some even also Tyr phosphorylated, in the course of sperm capacitation and after exposure to ZP. This tendency was also observed in other studies working with human (Kong *et al.*, 2009), hamster (Jha and Shivaji, 2002) and boar (Ramió-Lluch *et al.*, 2012b; Yeste *et al.*, 2014) spermatozoa; in contrast, Alnagar *et al.* (2010) observed opposite results: a reduction in Ser phosphorylation on five proteins (105, 96, 90, 64 and 55) during boar sperm capacitation.

Although data regarding Ser/Thr phosphorylation are scant, some studies have described that there are sperm proteins highly important for sperm function that are Ser/Thr phosphorylated. For instance, Naz (1999) described in human that the fertilisation antigen-1 (FA-1) involved in ZP binding and AR, is Ser/Thr and Tyr phosphorylated during sperm capacitation,

and Aparicio *et al.* (2007) demonstrated that porcine sperm motility is regulated by pSer levels of glycogen synthase kinase-3α (GSK3). Furthermore, sperm capacitation not only causes an increase in protein phosphorylation, but also a subcellular movement of phosphoproteins in the sperm head and tail that may be involved in the regulation of necessary processes for a proper fertilisation (Naz, 1999; Adachi *et al.*, 2008; Fàbrega, 2012; Ramió-Lluch *et al.*, 2012b). In fact, Adachi *et al.* (2008) suggested that post-acrosomal Ser/Thr phosphorylated proteins are involved in suppression of the AR in boar spermatozoa *in vitro*, and the study performed by Ramió-Lluch *et al.* (2012b) described the appearance of serine phosphorylation in the acrosome area during IVC, which greatly increased during *in vitro* acrosome exocytosis.

Some studies with human (Naz *et al.*, 1991) and boar (Fàbrega *et al.*, 2011) sperm also demonstrated that sperm capacitation, in both human and boar, and the exposure to ZP proteins in human, produce an increase in tyrosine phosphorylation in the acrosomal region of the sperm head. Other studies in human, monkey, hamster, rat and mouse also described an intense tyrosine phosphorylation during capacitation in the sperm tail, thus suggesting a role in the acquisition of hyperactivated motility (reviewed in Naz and Rajesh, 2004). All these data imply the involvement of protein phosphorylation in processes crucial for the development of the fertilising capacity, such as sperm motility and AR. Therefore, as results in **PAPER III** show that bacteria have a negative impact on Tyr/Ser/Thr phosphorylation levels after IVC, we can suggest that several steps involved in the acquisition of the fertilising capacity might be compromised by the presence of *P. aeruginosa*. Additional studies analysing the localisation of sperm phosphoproteins following IVC and in the presence of other contaminant bacteria are warranted to better address the impact of bacteriospermia on fertilising capacity.

All three protein phosphorylation patterns described in **PAPER III** are quite similar, since most of the protein bands detected seem to be phosphorylated at more than one amino-acid residue (Tyr, Ser and Thr); accordingly, these results would suggest that most of the proteins detected were phosphorylated by dual-specificity kinases. Nevertheless, this supposition must be carefully analysed from a conservative angle considering that the exact molecular weights, i.e. the identity, of the proteins detected in the present Thesis remain to be clarified. Therefore, further studies focusing on the identification of these proteins (via, for example, 2D electrophoresis and MALDI-TOF analyses) will help to comprehend and elucidate the repercussion of such dephosphorilation on sperm function.

In view of the results obtained in **PAPER III** regarding sperm motility kinematics, membrane fluidity, sperm viability and protein phosphorylation (especially pTyr of p32), we can conclude that, in seminal doses, *P. aeruginosa* not only affects sperm quality, but also the fertilising ability of spermatozoa. Hence, these results suggest that the utilisation of seminal doses with concentrations of bacteria that *a priori* do not have detrimental effects on boar sperm quality (<10^7 CFU/mL; **PAPER II**) may not be appropriate, as bacteria affect the capacity of boar sperm to acquire *in vitro* sperm capacitation. Furthermore, these results with *P. aeruginosa* might shed light into explaining why concentrations of *E. coli* as low as 3.5 x 10^3 CFU/mL caused a reduction in litter size in the study of Maroto Martín *et al.* (2010), since negative effects upon sperm quality are observed from a bacterial concentration of 10^4-10^6 CFU/mL. Bearing this in mind, additional studies involving the characterisation and location of phosphoproteins are warranted to fully understand the biological effects of bacterial contamination on boar sperm capacitation and fertilising competence.

Considering all the potential effects that bacteria may have upon boar sperm quality and fertilising ability, as shown in the present Thesis Dissertation, there are different strategies to maintain the bacterial concentration below a threshold level, so fertility is not highly affected (Althouse *et al.*, 2000). The first, and most feasible option, is to enhance the hygienic measures and practice general sanitation protocols during semen collection and processing, such as the implementation of automated semen collection (Aneas *et al.*, 2008). The second option is to place a mandatory shelf-life time to limit the storage of extended semen doses and, subsequently, restrict bacterial multiplication, which, eventually, can overcome the extender's buffering capacity. The third and the most used option is to include preservative antibiotics with a broad-spectrum activity in the semen extender formulation (Althouse *et al.*, 2000; Yániz *et al.*, 2010). However, this practice of bacterial control is not totally efficient and may be even unsustainable because some studies have demonstrated that over the 90% of bacteria isolated from extended semen are resistant to the most used antibiotics in extended semen (Althouse and Lu, 2005; Bolarín Guillén, 2011). Furthermore, it has also been shown that resistant genes can be interchanged between different bacterial species, which aggravate the situation (Duijkeren *et al.*, 2005). Considering that, nowadays there is a current need to find supportive and alternative new strategies for improving semen quality during storage and for reducing the use of antibiotics and, consequently, the emergence of antibiotic-resistant strains. Morrell and Wallgren (2011a) found promising results to reduce the antibiotic usage; they reported that it is

plausible to obtain bacteria-free sperm samples, or sperm samples with a lesser extent of bacterial load, and without having detrimental effects on sperm quality by using the Single Layer Centrifugation (SLC) technique with the boar-specific colloid formulation, Androcoll TM-P, just right after semen collection. The application of this technique not only reduces the bacterial load, but also improves the sperm quality of the samples by increasing the proportions of viable and morphologically normal spermatozoa exhibiting linear motility. Therefore, the incorporation of SLC in boar semen processing, despite being impractical from an industrial point of view, is a viable and feasible option that would be beneficial to improve the shelf-life time of doses destined to AI, to increase the biosecurity of those doses, and to reduce the use of antibiotics in semen extenders (Morrell and Wallgren, 2011b). Anyway, other strategies are currently under development. The male genital tract and other cellular sources such as epithelial cells, phagocytic cells, etc, produce endogenic substances which are essential components of the early innate immune response of vertebrates, invertebrates, bacteria, fungi and plants, due to their antimicrobial activities; these substances are known as antimicrobial peptides (AMPs) (Reddy *et al.*, 2004; Rosenfeld and Shai, 2006; Chatterjee *et al.*, 2016). During infection, microorganisms are exposed to AMPs; however, levels of resistance to AMPs in natural microbial populations are normally low, as most cationic AMPs do no induce resistance *in vivo* (Hancock, 1997). Accordingly, several efforts have been made, and are being made, during the last years to design and use exogenic AMPs as alternatives to conventional antibiotics. Since bacterial cell membranes are rich in negatively charged lipids and LPS, the latter just in gram-negative bacteria, cationic AMPs initiate electrostatic interactions that lead to disruption of the membrane barrier function and, thus, to bacterial cell killing (Reddy *et al.*, 2004; Speck *et al.*, 2014). Although promising results have been described, AMPs usually exert dose- and time-dependent toxic effects on mammalian sperm (Schulze *et al.*, 2016), which made them also interesting as contraceptive agents (Reddy *et al.*, 2004; Schulze *et al.*, 2016). Therefore, in general, further studies are needed to improve some aspects of the sperm quality and to reduce the use of antibiotics for avoiding the emergence of antibiotic-resistant strains of bacteria in boar extended semen.

In conclusion, the present Thesis dissertation provides new insights into the effects of two microorganisms (*C. perfringens* and *P. aeruginosa*), highly prevalent throughout the swine industry, on boar sperm physiology by means of multiparametric studies, a combination of conventional sperm quality parameters and molecular assessments **(PAPER I and II)**. Findings

about motility kinematics, membrane lipid disorder, and phosphotyrosine, phosphoserine and phosphothreonine expression in sperm co-incubated with *P. aeruginosa* during the capacitation process (**PAPER III**) disclose novel information about the impact of contaminant bacteria on the acquisition of the sperm's fertilising ability/competence. The precise manner how such bacteria-related alterations in sperm quality and capacitation achievement are produced is not yet fully established. Therefore, further studies are needed to better address the effects of bacteria on boar sperm functionality and male fertility. Results provided herein highlight the importance of applying strict hygienic measures during the processes of collection and manipulation of boar semen, as well as routine microbiological quality analyses of doses intended to AI in order to avoid using seminal doses with higher bacterial loads and the subsequent economic loses. In addition, the present research also emphasises the urgency of finding alternatives other than the addition of antibiotics to semen extender's formulation, and to control bacterial growth in extended boar semen.

General Conclusions

The present Thesis Dissertation concluded that:

1. The presence of the anaerobe *Clostridium perfringens* in boar seminal doses stored at 15ºC for 11 days and incubated at 37ºC for 96h causes a significant reduction in both the percentage of total and progressive sperm motility at high bacterial loads (10^6-10^8 CFU/mL). These effects are already evident after 24h of incubation/storage.

2. Contamination of semen doses with *C. perfringens* at concentrations higher than 10^7 CFU/mL also results in a significant decline in the percentage of viable sperm after 24h of incubation/storage at both temperatures.

3. Bacterial contamination with the aerobe *Pseudomonas aeruginosa* produces a notable reduction in the percentage of total and progressive motile sperm during storage at 15-17ºC during 11 days. This effect is evident after 48h of storage and only at the highest bacterial concentrations (> 10^7 CFU/mL).

4. Percentages of viable and acrosome-intact sperm are also affected by the presence of *P. aeruginosa* during storage. In both cases, the negative impact is apparent just after 24h of storage and only at the highest bacterial loads (10^7-10^8 CFU/mL), but more especially at 10^8 CFU/mL.

5. *P. aeruginosa* is able to grow during 11 days of storage at 15-17ºC while being diluted in extended semen.

6. Detrimental effects caused by *P. aeruginosa* on boar sperm quality are not related to an acidic environment, since seminal pH in all treatments remained steady and similar during the whole 11 days experimental period.

7. The *in vitro* capacitation procedure demonstrated that the presence of *P. aeruginosa* provokes a significant diminution in the percentage of sperm membrane integrity, viable

sperm with low membrane lipid disorder and motility kinematics during and after IVC, mostly at concentrations higher than 10^4-10^6 CFU/mL.

8. Different phosphorylation patterns for tyrosine, serine and threonine residues in samples containing *P. aeruginosa* were characterised after IVC. The majority of the identified proteins have a lesser extent of Tyr, Ser and Thr phosphorylation at bacterial loads higher than 10^6 CFU/mL, although one band shows differences at concentrations as low as 10^4 CFU/mL.

9. The most remarkable protein identified, whose tyrosine phosphorylation is affected by bacterial contamination, is ACRBP (p32), a widely known indicator of the boar capacitation status. The phosphorylation state of this specific protein is affected from a bacterial concentration of 10^6 CFU/mL onwards.

10. The alterations in membrane lipid disorder, motility kinematics and tyrosine phosphorylation of p32, clearly imply that bacterial contamination with *P. aeruginosa* affects the ability of boar sperm to achieve their capacitated state.

11. Since most adverse effects on sperm quality during storage were notable from bacterial loads of 10^7 CFU/mL onwards when using either *C. perfringens* or *P. aeruginosa*, results derived from the IVC experiment reveals that lower concentrations can also have detrimental effects on the reproductive performance.

12. All the detrimental effects caused by either *C. perfringens* or *P. aeruginosa* are time- and concentration-dependent, meaning that greatest damaging effects are observed at the highest bacterial concentration (10^8 CFU/mL) and at the end of the experimental period.

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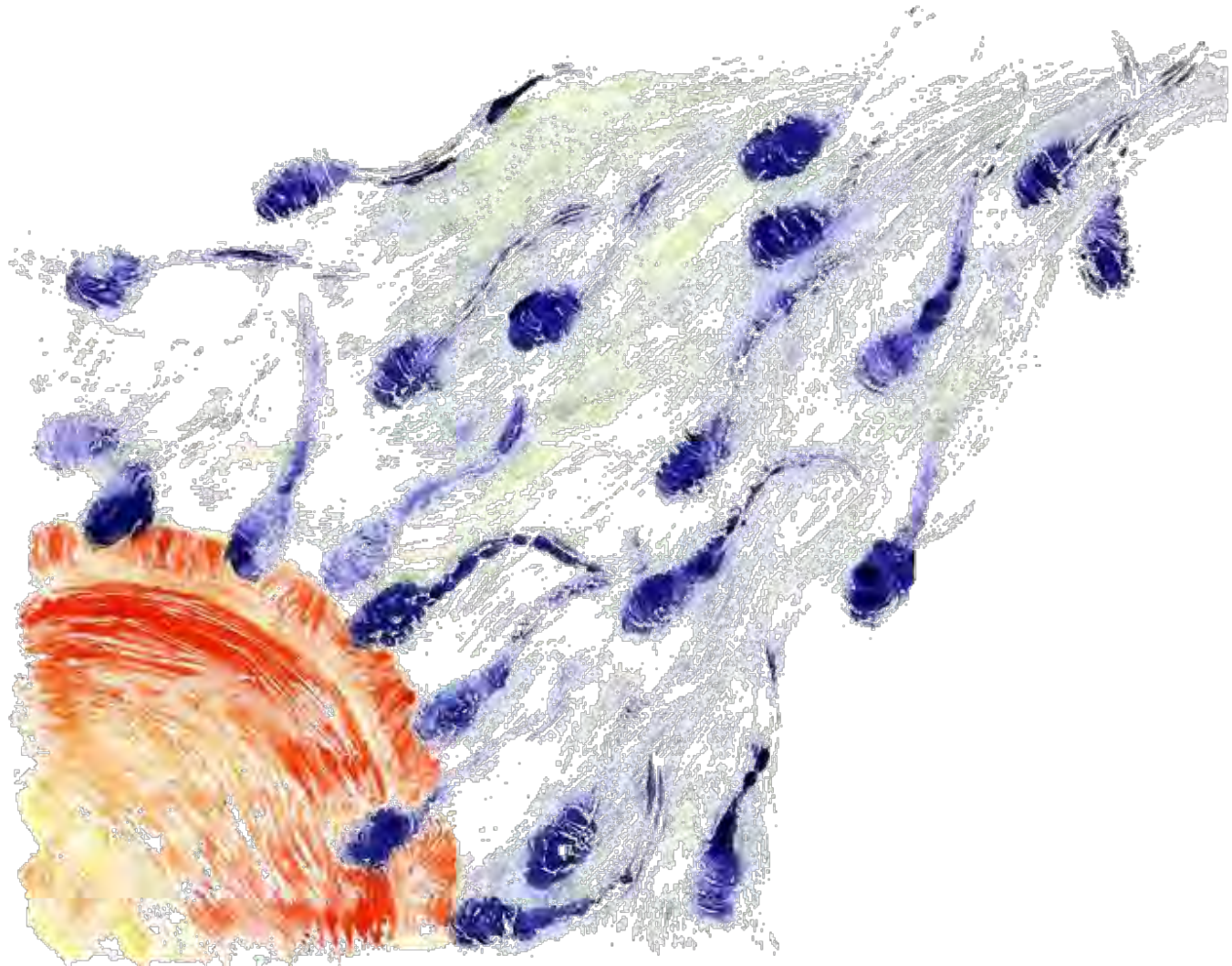

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