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FACULTAD DE VETERINARIA

Tesis Doctoral

Programa de Doctorado

Recursos naturales y gestión sostenible

Análisis del efecto de la estacionalidad en la fragmentación del ADN espermático y caracterización de la flora microbiana seminal del caballo

Analysis of the effect of the seasonality on sperm DNA fragmentation and characterization of the seminal microflora in the stallion

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Córdoba, mayo de 2022

TITULO: *Análisis del efecto de la estacionalidad en la fragmentación del ADN espermático y caracterización de la flora microbiana seminal del caballo*

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TÍTULO DE LA TESIS: Análisis del efecto de la estacionalidad en la fragmentación del ADN espermático y caracterización de la flora microbiana seminal del caballo.

DOCTORANDA: Carlota Quiñones Pérez.

INFORME RAZONADO DEL LOS DIRECTORES DE LA TESIS:

D. José Luis Vega Pla, Director del Laboratorio de Investigación Aplicada; D. Francisco Crespo Castejón, Jefe del Centro Militar de Cría Caballar de Ávila; y Dña. Isabel Ortiz Jaraba, Investigadora Postdoctoral del Departamento de Medicina y Cirugía Animal de la Facultad de Veterinaria de la Universidad de Córdoba.

INFORMAN:

Que el trabajo de tesis presentado por Dña. Carlota Quiñones Pérez, titulado "Análisis del efecto de la estacionalidad en la fragmentación del ADN espermático y caracterización de la flora microbiana seminal del caballo", ha sido realizado bajo nuestra dirección y cumple con la normativa reguladora de los Estudios de doctorado de la Universidad de Córdoba para su presentación como compendio de publicaciones.

Los objetivos principales de la misma consistieron en analizar el efecto de la estacionalidad en la fragmentación del ADN del espermatozoide equino, así como en la caracterización del microbioma seminal del caballo. Parte de los

resultados obtenidos durante el desarrollo de la presente Tesis Doctoral han resultado en cinco publicaciones científicas, siendo ésta elaborada por compendio de publicaciones.

Por todo ello, se autoriza la presentación de la tesis doctoral.

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TÍTULO DE LA TESIS: Análisis del efecto de la estacionalidad en la fragmentación del ADN espermático y caracterización de la flora microbiana seminal del caballo.

DOCTORANDA: Carlota Quiñones Pérez.

INFORME RAZONADO DEL TUTOR:

D. Juan Vicente Delgado Bermejo, Catedrático de Genética de la Universidad de Córdoba, RATIFICA que el trabajo de tesis presentado por Dña. Carlota Quiñones Pérez, titulado "Análisis del efecto de la estacionalidad en la fragmentación del ADN espermático y caracterización de la flora microbiana seminal del caballo", cumple con la normativa reguladora de los Estudios de doctorado de la Universidad de Córdoba para su presentación como compendio de publicaciones.

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En primer lugar, esta tesis no hubiera sido posible sin la ayuda de mi tutor y directores de tesis, Juan Vicente Delgado Bermejo, José Luis Vega Pla, Francisco Crespo Castejón e Isabel Ortiz Jaraba. Este es el resultado final de varios años de esfuerzo. El producto es nuestro. Espero que estén orgullosos de él.

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Y como has llegado hasta aquí, también te voy agradecer a ti, lector, el haberte interesado por este trabajo. No importa que no pases de apartado, ya conoces la parte más importante de la tesis: las personas que la han hecho posible.

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ÍNDICE

SUMMARY

DNA fragmentation is a cause of infertility in stallions with apparent normal spermiograms. Factors behind DNA fragmentation are not always fully understood. Besides, seminal flora may have an effect on sperm quality, as well as predisposition to reproductive diseases. The objective of this thesis is to analyze the effect of seasonality on chromatin integrity, and to study seminal microbiota and factors affecting its variability in stallions. In Chapter 1.1 the effect of season (spring and summer) over some sperm quality parameters and fertility is studied. Progressive motility and DNA fragmentation have better values in spring. Also, fertility diminishes at the end of reproductive season. In Chapter 1.2 the objective was to fine-tune DNA fragmentation progress in refrigerated semen doses to different mathematical models in the four seasons of the year. Second order polynomial model better described DNA fragmentation in all seasons. Then, the evolution of DNA fragmentation was compared among seasons. In summer and winter, DNA fragmentation rate was slower than in spring in autumn. Chapter 2 is divided into Chapter 2.1 (Sections 2.1.1 and 2.1.2) and Chapter 2.2. In Section 2.1.1, the seminal microbiota of healthy stallions was characterized using next-generation sequencing. Inter-subject variability was observed. However, there was still four common phyla in all samples: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. Besides, some bacterial families were common and abundant (>5%) in all stallions: Porphyormonadaceae, Corynebacteriaceae, Peptoniphilaceae and Prevotellaceae. In Section 2.1.2 the seminal microbiota assessment was performed in fertile stallions. Fertility was evaluated according to five sperm quality parameters: concentration, total number of spermatozoa, total motility, progressive motility and DNA fragmentation. No relevant inter-subject differences was observed. No correlation was either observed between families and sperm parameters. Chapter 2.2 is a case-study of a seminal flora shift in a contagious equine metritis carrier. It was observed that Corynebacteriaceae was

the dominant family in semen when the animal hosted *Taylorella equigenitalis*. However, Porphyomonadaceae and Bacteroidetes phylum were more abundant when the stallion was free of the agent. In conclusion, according to the results of this thesis, seasonality affects sperm DNA fragmentation in the stallion in cooled-stored semen. It has been shown that the fragmentation rate changes over seasons. Besides, the seminal microbiota in fertile stallions was mainly composed by Porphyormonadaceae, Corynebacteriaceae, Peptoniphilaceae and Prevotellaceae families. No correlation was observed between families and sperm parameters. However, flora composition was affected by the presence of *Taylorella equigenitalis*.

RESUMEN

La fragmentación del ADN espermático es una causa de infertilidad en sementales con espermiogramas normales. Dicha fragmentación está sujeta a diversos factores, no del todo dilucidados. Por otra parte, la microbiota del semen puede tener efecto sobre la calidad del semen o la predisposición a enfermedades. Por tanto, el objetivo de esta tesis es analizar la influencia del factor estacionalidad en la fragmentación espermática; y también estudiar la composición de la microbiota seminal, y su variabilidad entre individuos. En el Capítulo 1.1 se estudia el efecto de la época del año sobre varios parámetros de calidad seminal y la fertilidad. Se observa que la motilidad progresiva y la fragmentación espermática presentan mejores valores al inicio de la época reproductiva que al final. También se observa una disminución de la fertilidad al final de la temporada. En el Capítulo 1.2 se busca ajustar la evolución de la fragmentación espermática en dosis de semen refrigeradas tomadas durante las cuatro estaciones del año a tres modelos matemáticos diferentes. En todas las estaciones, el modelo que mejor define la evolución de la fragmentación en el tiempo es una función polinómica de segundo grado. Una vez elegido este modelo como el mejor, se compara el comportamiento de la evolución entre las estaciones, resultando que durante el verano e invierno la fragmentación evoluciona más rápidamente, en comparación con la primavera y otoño. El Capítulo 2.1 se subdivide en dos apartados. En el 2.1.1 se caracteriza la composición de la microbiota seminal de caballos fértiles empleando técnicas de secuenciación masiva. El resultado muestra una cierta variabilidad entre individuos, distinguir cuatro fila dominantes: Firmicutes, Bacteroidetes, Actinobacteria y Proteobacteria. De entre todos ellos, algunas familias bacterianas muestran ser comunes en todos los individuos y encontrarse en proporciones altas (>5%). Estas familias son Porphyromonadaceae, Corynebacteriaceae, Peptoniphilaceae y Prevotellaceae. En el 2.1.2 se analizan la composición de la microbiota seminal en caballos y cinco parámetros de calidad seminal: concentración, número total de espermatozoides, motilidad total,

motilidad progresiva y fragmentación espermática. No se observan diferencias significativas entre animales, ni correlación entre la abundancia de las familias bacterianas con los parámetros de calidad seminal. En el Capítulo 2.2 se presenta el caso de un cambio en la composición de la microbiota seminal en un semental al revertir su condición de portador del agente causante de la metritis equina contagiosa. Concretamente, la familia Corynebacteriaceae era dominante en el momento en que el semental era portador de *Taylorella equigenitalis*; mientras que Porphyromonadaceae, y en general el filo Bacteroidetes, era el más abundante cuando el agente desapareció. En conclusión, de acuerdo con los resultados de esta tesis, la estacionalidad tiene efecto en la integridad del ADN del espermatozoide equino en dosis de semen refrigeradas, modificando también la velocidad a la que se degrada. Por otra parte, se ha observado que en la microbiota seminal dominan las familias Porphyromonadaceae, Corynebacteriaceae, Peptoniphilaceae y Prevotellaceae. No se ha observado influencia de la composición de la microbiota con los parámetros de calidad seminal; sin embargo, sí se ha observado en el caso de un portador de *Taylorella equigenitalis*.

INTRODUCCIÓN

INTRODUCCIÓN

El análisis de la calidad espermática es una práctica que no ha dejado de crecer en las últimas décadas. Históricamente, la calidad de las dosis seminales se basaba principalmente en la valoración visual del movimiento espermático, a través de la cual se pretendía "predecir la fertilidad" de las mismas. Sin embargo, aunque la calidad espermática así calculada y la fertilidad están asociadas, desde un punto de vista práctico y estadístico, su relación no es lineal. Por esta razón, se ha seguido investigando en otros parámetros de calidad del eyaculado que puedan ayudar a predecir de manera más fiable la fertilidad potencial de las dosis. En este sentido, el análisis de la estacionalidad y del estudio dinámico de la fragmentación del ADN espermático, junto con la caracterización de la microbiota seminal equina, pueden ayudar a comprender mejor la relación existente entre calidad seminal y fertilidad.

Fragmentación del ADN espermático

El desarrollo embrionario normal es dependiente de la integridad del material genético de los gametos masculino y femenino. En este sentido, los espermatozoides son los vehículos que facilitan el transporte del genoma al ovocito (McKinnon, 2011). Es por ello que el núcleo del espermatozoide ha adoptado una arquitectura única, en la cual el ADN se encuentra altamente compactado con la ayuda de unas pequeñas proteínas, de carga positiva, llamadas protaminas. Sin embargo, a pesar de que existen mecanismos de

protección del ADN espermático, es posible encontrar roturas seriadas en la cadena de nucleótidos, lo que da lugar a la "fragmentación del ADN" (SDF).

La molécula de ADN de los espermatozoides puede contener roturas de cadena doble y simple. Un exceso de dichos daños tiene efectos deletéreos en la reproducción, como la disminución del ratio de concepción, o el aumento del número de abortos espontáneos, tanto en la especie humana como en animales (Cortés-Gutiérrez et al., 2014; Gillan et al., 2005; Love & Kenney, 1998; Robinson et al., 2012; Sánchez-Calabuig et al., 2015). Por ello, en la actualidad se considera que la fragmentación espermática es un indicador fiable de la calidad espermática (Simon et al., 2017) y de fertilidad tanto en el hombre como en machos de diferentes especies, caballo incluido (Bungum et al., 2007; Evenson, 2016; López-Fernández et al., 2007b; Morrell et al., 2008; Oleszczuk et al., 2013).

El caballo es una especie estacional de fotoperiodo positivo lo que hace que, en el hemisferio norte, la etapa reproductiva coincida con la primavera y verano (Aurich, 2011; Chemineau et al., 2008; Hoffmann & Landeck, 1999; Janett et al., 2003; Jasko et al., 1991; Magistrini et al., 1987; Wach-Gygax et al., 2017). Las yeguas comienzan a ciclar al inicio de la primavera, siendo de abril a septiembre cuando de forma más habitual se aplican las diferentes técnicas de reproducción asistida, como la inseminación artificial (IA) con semen fresco o refrigerado (Aurich, 2016). Ello hace que la mayor demanda de recogida, procesado y envío de semen ocurra en esa época (Aurich, 2016; Blottner et al., 2001). A pesar de que se considera que los sementales están menos afectados por el fotoperiodo, y que mantienen su fertilidad a lo largo del año (Deichsel et al., 2016; Walbornn et al.,

2017), varios estudios han evidenciado una cierta influencia estacional en la función testicular y en las características del semen (Clay et al., 1987; Hoffmann & Landeck, 1999; Johnson & Thompson, 1983), fragmentación espermática incluida (Blottner et al., 2001; Chemineau et al., 2008; Morte et al., 2008; Ortiz et al., 2017; Wach-Gygax et al., 2017). Los estudios asocian esta disminución con una menor producción de hormonas gonadotropas (FSH y LH) y de hormonas testiculares durante la época no reproductiva.

Como se ha señalado, la fragmentación del ADN también se ve afectada por la estacionalidad (Blottner et al., 2001; González-Marín et al., 2012, p.; Love et al., 2005; Schmid-Lausigk & Aurich, 2014; Wrench et al., 2010). Lamentablemente, la mayor parte de los estudios que respaldan esta hipótesis se han realizado en semen congelado; existiendo poca información con respecto al efecto de la estacionalidad sobre el ADN en semen refrigerado (Aurich, 2016; Wach-Gygax et al., 2017). Entre la información que se dispone en este último supuesto se encuentra el trabajo de (Wach-Gygax et al., 2017), en el que se compararon las variaciones en la fragmentación espermática entre la estación reproductiva y la no reproductiva en semen a partir de las 24 horas de almacenamiento en refrigeración, pero no durante las primeras horas de conservación. Estas primeras horas de conservación son relevantes desde un punto de vista de calidad del eyaculado, ya que se ha demostrado que algunos sementales aumentan su nivel de fragmentación tras las primeras horas de refrigeración, probablemente debido a daños encriptados en el ADN. Consecuentemente, es de interés estudiar la evolución de la fragmentación del ADN espermático entre las

0 y las 24 horas de refrigeración, y compararla con los resultados de fertilidad durante el inicio y final de la época reproductiva (primavera y verano).

Por otra parte, la fragmentación del ADN puede ser evaluada de manera estática o dinámica. La evaluación estática implica medir la SDF en un solo momento, mientras que la dinámica implica evaluar la SDF varias veces durante un periodo de incubación que imite la temperatura del tracto reproductor de la yegua (aproximadamente, 37ºC). Existen cada vez más evidencias que demuestran que es preferible la evaluación dinámica (Cortés-Gutiérrez et al., 2008; Johnston et al., 2016; López-Fernández et al., 2007a; Ortiz et al., 2015), porque permite descubrir daños del ADN ocultos que no se pueden evidenciar mediante una único análisis (Linfor & Meyers, 2002; López-Fernández et al., 2007a). La evaluación dinámica también permite calcular la velocidad de SDF (rSDF), la cual usualmente se ajusta a un modelo lineal de regresión (Cortés-Gutiérrez et al., 2008, p.; López-Fernández et al., 2007a; Urbano et al., 2013). Esta modelización asume que la velocidad de fragmentación es constante a lo largo del tiempo; asunción que no es siempre correcta según algunos autores (Ferreira et al., 2018; Love et al., 2002; Ortiz et al., 2017). Recientemente se ha propuesto el uso del modelo matemático polinómico de segundo grado para evaluar la rSDF (Ortiz et al., 2017). Con este modelo se asume que el ritmo de fragmentación del ADN no es constante a lo largo del tiempo, sino que es más rápido durante las primeras horas (de la Horra Navarro, 2018).

Los principios de la evaluación dinámica de la SDF a 37ºC pueden ser empleados para el semen refrigerado. El concepto y modelo usado es similar, solo cambia la

temperatura de incubación (5ºC en vez de a 37ºC). En la actualidad, no se disponen de estudios sobre la dinámica de fragmentación en semen refrigerado, ni se ha comprobado si dicha dinámica se ve afectada por la estación del año.

Microbiota seminal

No solo la estacionalidad o la refrigeración tienen efecto en los parámetros de calidad seminal. En los últimos años, se ha descubierto el importante papel que juegan las bacterias en el mantenimiento de la homeostasis del individuo en el que residen, tanto en caballos (Barba et al., 2020; Costa & Weese, 2012; Ericsson et al., 2016; LaFrentz et al., 2020; Manguin et al., 2020), como en otras especies domésticas (Ng et al., 2010; Rando, 2012; Wu et al., 2016; D. Zhang et al., 2016). Asimismo, se ha observado que los desequilibrios en la microbiota pueden provocar alteraciones en el lugar que habitan, e incluso en órganos distantes (Al Jassim & Andrews, 2009; Milinovich et al., 2010; Salem et al., 2019).

En general, existen pocos trabajos sobre el efecto de la microbiota en el tracto reproductor en el hombre (Hou et al., 2013; Liu et al., 2014; Weng et al., 2014) y casi ninguno en animales (Al-Kass et al., 2020; Dardmeh et al., 2017; Inatomi & Otomaru, 2018; Javurek et al., 2016; Rosenfeld et al., 2018; Serrano et al., 2020; Wickware et al., 2020). A pesar de ello, ya se ha podido asociar la presencia de ciertas familias bacterianas con diferencias en la calidad seminal (Altmäe et al., 2019; Hou et al., 2013; Tomaiuolo et al., 2020; Weng et al., 2014; J. Zhang et al., 2020), e incluso con los valores de fertilidad (Hou et al., 2013; Kiessling et al., 2008).

Hay pocos estudios sobre microbiota seminal en el caballo, y los que se encuentran se centran sobre todo en bacterias patógenas del aparato reproductor (Al-Kass et al., 2019; Samper, 2009) o en el efecto que ciertos género bacterianos tienen en las tecnología de la reproducción (Moretti et al., 2009; Ortega-Ferrusola et al., 2009; Varela et al., 2018). En cuanto a estudios sobre microbiota seminal basal, existe una primera descripción (Al-Kass et al., 2020) en la que se observó que los resultados eran variables entre individuos. Es por ello, que se postuló que la microbiota podría estar condicionada por factores externos, como el ambiente o la región (Al-Kass et al., 2020). Así, ha surgido la necesidad de disponer de más información sobre la composición de microbiotas seminales normales, para poder conocer mejor los verdaderos factores que determinan su composición.

Tampoco existen demasiados estudios con respecto al efecto de la microbiota en los parámetros de calidad seminal (Altmäe et al., 2019; Dardmeh et al., 2017; Hou et al., 2013; Inatomi & Otomaru, 2018; Tomaiuolo et al., 2020; Weng et al., 2014; J. Zhang et al., 2020). A pesar de dicha escasez, ya se ha demostrado que la presencia de determinados grupos o perfiles bacterianos se asocian con la calidad seminal. De hecho, estos estudios han abierto la puerta a nuevas herramientas terapéuticas potenciales en casos de infertilidad: algunos autores han publicado la utilidad de la prescripción de prebióticos para mejorar la calidad seminal (Dardmeh et al., 2017; Inatomi & Otomaru, 2018; Maretti & Cavallini, 2017; Valcarce et al., 2017). Podría resultar por tanto interesante estudiar si en el caballo también existe alguna asociación entre grupos bacterianos y los valores de diferentes parámetros de calidad seminal.

Por último, existen estudios de microbiota seminal en humanos que demuestran que ciertas composiciones pueden dificultar la supervivencia de algunos agentes causantes de enfermedades venéreas (Altmäe et al., 2019; Korhonen et al., 2017; Mändar et al., 2017; Witkin & Linhares, 2015).

En el caso del caballo, la metritis equina contagiosa (MEC) es una enfermedad muy relevante para la industria, dado que su presencia en el ganado reduce la fertilidad de las yeguas e impone restricciones al comercio. Su agente etiológico es *Taylorella equigenitalis*. Los sementales son portadores de esta bacteria en la parte distal de la uretra, pudiéndose convertir en portadores de larga duración (Schluter et al., 1991) si no se tratan con protocolos de desinfección adecuados (Crowhurst et al., 1979; Timoney, 1996).

Los síntomas varían entre sementales y yeguas. En yeguas, la enfermedad se manifiesta con cuadros de endometritis, cervicitis y vaginitis de intensidad variable, y a veces aparece como una descarga vaginal mucopurulenta. Normalmente se asocia a infertilidad temporal. La recuperación suele ocurrir sin incidentes, pero la yegua puede permanecer como portadora. En el semental es asintomática, pero su presencia puede estabilizarse y dar lugar a un individuo portador.

En los últimos años, esta enfermedad ha recibido una atención renovada debido a la detección de portadores en ganaderías en principio libres de enfermedad (Timoney, 2011). Esto ha despertado dudas sobre el modo de transmisión de la bacteria (Schulman et al., 2013), e incluso sobre la efectividad de los métodos de diagnóstico (Matsuda & Moore, 2003). También se cuestiona si se conocen

enteramente los mecanismos de supervivencia de la bacteria. Durante nuestros estudios sobre la microbiota seminal con técnicas de secuenciación masiva, hemos observado un cambio en la composición de la microbiota en un semental que experimentó una reversión espontánea del estado de portador al de no portador de *Taylorella equigenitalis*. Como parte de esta tesis, se presentarán los resultados obtenidos de dicha observación, y se discutirá brevemente su posible origen.

OBJETIVOS

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Los objetivos planteados en la presente Tesis Doctoral son:

Objetivo 1. Medir la fragmentación del ADN espermático desde las 0 hasta las 24 horas de conservación en refrigeración y compararla con los resultados de fertilidad mediante inseminación artificial al inicio y final de la época reproductiva (primavera y verano).

Este objetivo ha sido abordado en el Capítulo 1.1, en el que se ha evaluado la fragmentación del ADN espermático durante las primeras 24 horas de refrigeración durante la primavera y el verano y se ha comparado con los resultados de fertilidad de las dosis analizadas.

Francisco Crespo, Carlota Quiñones-Pérez, Isabel Ortiz, María Díaz-Jiménez, César Consuegra, Blasa Pereira, Jesús Dorado, Manuel Hidalgo. Seasonal variations in sperm DNA fragmentation and pregnancy rates obtained after artificial insemination with cooled-stored stallion sperm throughout the breeding season (spring and summer). Theriogenology (2020).

Objetivo 2. Tiene dos partes: (a) establecer un modelo matemático que pueda describir de manera fiable la longevidad de la integridad del ADN espermático en el caballo tras su almacenamiento en refrigeración. Y (b) emplear este modelo para evaluar los efectos de la estacionalidad en la fragmentación del ADN espermático equino.

Este objetivo ha sido tratado en el Capítulo 1.2, donde se estableció el mejor modelo matemático para describir la evolución de la fragmentación espermática del semen en refrigeración, y se comparó entre las estaciones del año.

Isabel Ortiz, Carlota Quiñones-Pérez, Manuel Hidalgo, César Consuegra, María Díaz-Jiménez, Jesús Dorado, José Luis Vega-Pla, Francisco Crespo. Comparison of different mathematical models to assess seasonal variations in the longevity of DNA integrity of cooled-stored stallion sperm. Andrologia (2020). 52: e13545.

Objetivo 3. Caracterizar la composición microbiana seminal en caballos sanos y fértiles del sur de España mediante secuenciación masiva. Y evaluar la

relación entre la presencia de las bacterias más abundantes con cinco parámetros de calidad seminal: concentración, número total de espermatozoides, motilidad total y progresiva y fragmentación de ADN.

Este objetivo ocupa el Capítulo 2.1, que incluye:

El Apartado 2.1.1: en el que se describe la composición de la microbiota seminal en caballos fértiles procedentes del sur de España usando secuenciación de nueva generación.

Carlota Quiñones-Pérez, Manuel Hidalgo, Isabel Ortiz, Francisco Crespo, José Luis Vega-Pla. Characterization of the seminal bacterial microbiome of healthy, fertile stallions using next-generation sequencing. Animal Reproduction (2021). 18 (2): e20200052.

Y el Apartado 2.1.2: en el que se evalúa la relación entre las familias bacterianas más abundantes del semen equino con cinco parámetros de calidad seminal: concentración, número total de espermatozoides, motilidad total y progresiva y fragmentación de ADN.

Carlota Quiñones-Pérez, Amparo Martínez, Isabel Ortiz, Francisco Crespo, José Luis Vega-Pla. The Semen Microbiome and Semen Parameters in Healthy Stallions. Animals (2022). 12 (5):534.

Objetivo 4. Comparar las diferencias en la composición de la microflora seminal de un semental en sus estados de portador de *Taylorella equigenitalis* y de no portador.

Este objetivo aparece en el Capítulo 2.2, en el que se describe el cambio en composición de la microbiota seminal en un semental siendo portador de *Taylorella equigenitalis*, y tras su conversión a no portador.

Carlota Quiñones-Pérez, Amparo Martínez, Francisco Crespo, José Luis Vega-Pla. Comparative Semen Microbiota Composition of a Stallion in a Taylorella equigenitalis Carrier and Non-Carrier State. Animals (2020). 10:868.

CAPÍTULOS

CAPÍTULO 1

CAPÍTULO 1.1

Seasonal variations in sperm DNA fragmentation and pregnancy rates obtained after artificial insemination with cooled-stored stallion sperm throughout the breeding season (spring and summer).

Francisco Crespo, Carlota Quiñones-Pérez, Isabel Ortiz, María Díaz-Jiménez, César Consuegra, Blasa Pereira, Jesús Dorado, Manuel Hidalgo, 2020. Theriogenology.

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Seasonal variations in sperm DNA fragmentation and pregnancy rates obtained after artificial insemination with cooled-stored stallion sperm throughout the breeding season (spring and summer)

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ABSTRACT

The aim of this study was to assess seasonal variations during different periods of the breeding season (spring and summer) on stallion sperm DNA fragmentation and *in vivo* fertility associated with cooled-stored semen samples. Ejaculates were collected from eleven stallions and assessed for sperm motility (assessed by computer-assisted sperm analysis) and plasma membrane integrity (evaluated under fluorescence microscopy). Sperm DNA fragmentation (evaluated by the Sperm Chromatin Dispersion test) was assessed in cooled-stored semen at 5 °C for up to 24 h. Artificial insemination was performed throughout the breeding season. Mares were inseminated with cooled-stored semen (up to 24 h) every other day until ovulation. Pregnancy rates per cycle were determined detecting the embryonic vesicle by ultrasonography fifteen days after ovulation. Values (mean ± SD) for progressive sperm motility were significantly higher ($P < 0.05$) in spring (53.57 ± 9.97%) in comparison to summer (41.37 ± 10.81%). No significant differences in plasma membrane integrity were found between seasons ($P > 0.05$). Sperm DNA fragmentation was significantly lower ($P < 0.01$) in spring in comparison to summer after 0h (4.81 ± 1.87% vs. 8.77 ± 5.78%), 6h (9.00 ± 3.19% vs. 18.73 ± 8.22%) and 24h (14.6 ± 4.13% vs. 30.14 ± 9.85%) of cooled-storage. Pregnancy rates per cycle were also significantly higher ($P < 0.01$) in spring (50%) in comparison to summer (37%). There was a moderate negative relationship between positive pregnancies and sperm with fragmented DNA ($r = -0.619$; $P < 0.001$). Semen samples associated with moderate fertility levels (Pregnancy rate < 50%) showed a higher percentage of sperm with fragmented DNA compared to samples obtaining higher fertility levels. In conclusion, seasonal variations were found during the breeding season, obtaining lower sperm DNA fragmentation and higher pregnancy rates in spring. Additionally, samples with the highest proportion of sperm with fragmented DNA showed the lowest fertility levels throughout the breeding season.

1. Introduction

Horses exhibit reproductive seasonality stimulated by increasing daylight length [1], beginning in spring and into summer in the Northern Hemisphere. Mares come into heat in early spring, therefore most of the reproductive technologies are performed from April to September, particularly those involving artificial insemination (AI) with fresh or cooled-stored semen [2]. Thus a major requirement of semen collection and processing for shipping and AI is needed in comparison to the non-breeding season, in which stallion semen is mostly collected to be cryopreserved [2,3]. Mares normally undergo winter anovulatory season, while stallions are less influenced by photoperiod and fertility persists during the year [4,5].

Several studies have compared the reproductive parameters of

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stallions throughout the year, which have resulted in clear evidence of a seasonal influence on testicular function and sperm characteristics [3,6–8]. However, there is little information about changes in cooled-stored sperm parameters between the beginning and the end of the breeding season (i.e. spring *vs.* summer) or the effect of a long-day light programme on cooled-stored sperm parameters [5]. Considering most managers aim to breed mares as soon as possible in the year [2], it is important to determine whether there is any difference between sperm quality and fertility rates between the beginning and the end of the reproductive season, in order to improve the reproductive efficiency.

Protocols used for semen storage are known to adversely affect the quality of sperm [3]. Sperm resistance to storage-damage is also thought to be influenced by the time of the year when the semen collections are performed [9]. While most studies have been carried out on frozen-thawed semen, comparing semen variables after thawing between breeding vs. non-breeding season [3,10] or within the non-breeding season [11], there is comparatively less information available regarding a possible seasonal effect on cooled stored diluted semen [2].

DNA fragmentation has shown to be a reliable indicator of sperm quality in most animal species, including the stallion [12]. Moreover, sperm DNA integrity has been associated with fertility outcomes in retrospective studies performed in humans [13], so it might be considered as an independent predictor of fertility [14,15]. While sperm DNA assessment has been related with fertility in several studies, including stallions [16], a direct comparison between DNA damage and pregnancy outcomes and its relationship to seasonality has yet to be performed in the horse.

Recently, Wach-Gygax, Burger [9] compared variations of DNA damage in cooled-stored stallion semen samples at 24 and 48 h post-storage between breeding and non-breeding season; this study determined a reduction in the quality of cooled-stored semen in midsummer when compared to other seasons. While sperm damage after cooling was recorded from 24h of storage onwards, no information was provided regarding first storage hours. AI with shipped semen is recommended to be performed as soon as possible and most procedures are performed within the first 24 h of cooled-storage. Consequently, the current study measured the sperm DNA fragmentation from 0 to 24h of cooled-storage and compared the fertility outcomes of AI between the early and late periods of the breeding season (spring and summer).

2. Material and methods

2.1. Animals

Ejaculates were obtained from 11 healthy and fertile stallions of different breeds at the equine breeding centre of the Spanish army located in Avila, Spain (40′66 N, 4′70 W). Stallions ranged in age from 7 to 23 years and were included in the reproductive breeding program of the centre. The stallions were housed in individual paddocks and the feeding consisted of alfalfa, commercial concentrate and water “ad libitum”.

2.2. Semen collection

Semen was collected by allowing the stallions to mount a phantom, using a Missouri-model artificial vagina (Nasco, Fort Atkinson, WI, USA). A mare in oestrus was used to induce sexual behaviour. Before starting the breeding season, extragonadal sperm reserves were depleted (daily collection for five days). For the experimental design, two ejaculates were collected from each animal in each of the following periods: spring (April to June) and summer (July to September), obtaining a total of 44 ejaculates. All the stallions included in this study were regularly collected, two or three times per week, in intervals of at least 24 h between semen collections (Monday, Wednesday and Friday) throughout the breeding season (spring and summer periods) according to the breeding program of the center. Each season was divided into two equal sub-periods to ensure that the four ejaculates collected from each stallion were homogeneously distributed along the period of the study.

2.3. Semen processing

Immediately after collection, the gel-free ejaculates were measured for volume (mL) in a test tube. Sperm concentration (x 10^6 sperm/mL) was assessed with a sperm photometer (Minitube, Tiefenbach, Germany) and sperm motility evaluated by Computer-assisted sperm motility analysis (CASA) as described below. Each ejaculate was diluted (≈1:3 v/v) to a final concentration of 50 x 10^6 sperm/mL in a milk-based extender formulated for equine semen (INRA96, IMV Technologies, L'Aigle, France), stabilized to 22 °C and then cooled to 5 °C in an Equitainer (Hamilton Biovet, Ipswich, MA, USA) for 2 h. Semen samples were assessed after 0, 6, and 24 h of cooled-storage. For sperm analysis, each sample was diluted in the same extender to reach a final concentration of 25 x 10^6 sperm/mL, maintained for 10 min at 37 °C and then evaluated as described below.

2.4. Semen evaluation

2.4.1. Computer-assisted sperm motility analysis (CASA)

Sperm motility was evaluated using the Sperm Class Analyzer (SCA v.5.4, Microptic SL., Spain). In brief, 2 μL of each diluted sample was loaded into a microscopic slide (Leja Products, B.V., Nieuw-Vennep, The Netherlands) placed on a heated stage (37 °C) of a phase contrast microscopy. A minimum of 500 spermatozoa were analysed in five to six microscopic fields using the 10 x objective. Acquisition was performed using high-speed digital camera (A312fc, BaslerTM AG, Ahrensburg, Germany) *with a frame rate of 25 frames/s and 25 images captured.* The trajectory of each spermatozoa was determined by the software and the following parameters were recorded: total sperm motility (TM, %; sperm with a mean average path velocity >10 μm/s), progressive sperm motility (PM, %; motile sperm with >65% of the straightness coefficient), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, μm/s), straightness (STR, %), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz).

2.4.2. Plasma membrane integrity analysis

Sperm membrane integrity was assessed using the double stain propidium iodide with acridine orange (Vital Test Kit, Halotech DNA SL, Madrid, Spain) as previously described [17,18]. Briefly, a 10 μL aliquot of diluted semen was mixed with 1 μL propidium iodide and 1 μL acridine orange and evaluated under epifluorescence microscopy (Optiphot 2 Nikon, Japan) using a 460–490 nm excitation filter. At least 200 sperm were counted and the percentage of viable sperm with green emission (intact membrane) was recorded (PMI %).

2.4.3. Sperm DNA fragmentation analysis

The degree of DNA damage was quantified using the Sperm-Halomax kit (Halotech DNA SL., Madrid, Spain) as previously described [19,20]. Briefly, 20 μL of diluted spermatozoa (10–15 x 10^6 spermatozoa/mL) were added to a vial containing low-melting point agarose and mixed. A small aliquot of the agarose–sperm mixture (10 μL) was then spread onto pre-treated slides

(provided in the kit), covered with a glass coverslip and placed in a refrigerator on a cold metallic plate for 5 min. Following solidification, the coverslip was carefully removed and the 'sperm-microgel' slide preparation placed horizontally in 10 mL of the lysing solution provided in the Halomax kit for 5min. The 'sperm-microgel' preparation was subsequently washed in dH_2O for 5min and then dehydrated in a series of ethanol baths (70% and 100%) for 2 min each. DNA damage was visualized under epifluorescence microscopy (40 x objective) after staining the smears with a commercial kit for green fluorescence (Halotech, DNA, SL, Madrid Spain). At least 200 spermatozoa per sample were counted. Sperm showing a large halo of chromatin dispersion were recorded as sperm with fragmented DNA (SDF, %).

2.5. Artificial insemination (AI)

AI was performed with cooled-stored semen samples collected from the eleven fertile stallions following the routine AI program of the breeding centre. The mares were regularly checked and breeding soundness evaluation was performed before AI. Mares with history of reproductive disorders or subfertility were not included in the study. The ovarian activity of the mares was monitored daily before insemination by transrectal palpation and ultrasonography until at least one follicle reached a diameter of 35 mm. After that, the mares were inseminated in the uterine body using a catheter (Minitübe, Tiefenbach, Germany) every other day until ovulation was detected (Day 0). Each insemination dose contained 500 x 10^6 of progressively motile sperm and was prepared in 20 mL of semen extender, slowly cooled into an Equitainer for 2 h and then loaded into a 20 mL sterile syringe. Semen doses were stored for up to 24 h at 5 °C into a refrigerator when insemination was performed on the same center as semen collection or into a styrofoam shipping box with two ice-blocks (Minitube, Tiefenbach, Germany) when semen was shipped [21]. Most of the mares were checked and inseminated by local practitioners after 12 h of semen processing and storage.

2.6. Experimental design

2.6.1. Sperm characteristics and DNA fragmentation of stallion ejaculates analysed prior to cooling during spring and summer

Seasonal variation in the sperm quality parameters assessed in fresh semen samples obtained prior to cooling was compared between spring and summer.

2.6.2. Effect of cooled-storage on stallion sperm DNA fragmentation during spring or summer

Sperm DNA fragmentation was assessed following cooled-storage after 0, 6 and 24 h. The DNA fragmentation index (SDF, %) and the rate of DNA fragmentation (slope of the regression line) obtained throughout the different incubation times (SDF, %/h) were calculated and compared between semen samples collected in spring or in summer.

2.6.3. Relationship between pregnancy rates and sperm DNA fragmentation of cooled-stored semen during spring or summer

At least four estrous cycles from two different mares were inseminated with semen doses from each stallion during Spring or Summer. Pregnancy rates per cycle were determined detecting the embryonic vesicle by ultrasonography fifteen days after ovulation Fertility was calculated per stallion according to the per cycle pregnancy rate, as defined previously by Vidament et al. (2009). The relationship between sperm DNA fragmentation (SDF) and pregnancy rate was assessed and compared between spring and summer periods. Additionally, sperm DNA fragmentation was compared between stallions based on two different levels of fertility: stallions with a pregnancy rate per cycle of 50% or higher were considered as "good fertility" and stallions showing a pregnancy rate per cycle lower than 50% were considered as "moderate fertility" [22,23].

2.7. Statistical analysis

Statistical analysis was performed using SPSS Statistics 22.0 (SPSS Institute Inc. Headquarters, Amonk, NY, USA) and data was presented as mean ± standard deviation (SD). For each variable, normality of the data distribution was assessed using the Kolmogorov–Smirnov test. When values were not normally distributed, results were transformed to a logarithmic scale. A paired samples *t*-test were performed to compare the sperm parameters assessed between seasons. Correlation between SDF and pregnancy rates per cycle was performed using the Pearson coefficient (r value). A linear regression analysis was conducted for values of SDF at different incubation times and the slopes were compared among treatments by ANCOVA using the GraphPad Prism v.6 for Mac OS (GraphPad Software, CA, USA). Scatter plots presented visual image of data. Significant differences were considered when $P < 0.05$.

3. Results

3.1. Sperm characteristics of stallion ejaculates prior to cooling during spring or summer

Sperm parameters were significantly higher ($P < 0.05$) in spring in comparison to summer for PM (53.57 ± 9.97 vs. 41.37 ± 10.81, %) and VCL (72.61 ± 6.65 vs. 65.16 ± 11.00, μm/s). No significant differences were found in the remaining parameters assessed throughout the breeding season (Table 1).

3.2. Effect of cooled-storage on stallion sperm DNA fragmentation during spring and summer

The percentage of sperm with fragmented DNA was significantly lower in spring than in summer after 0 h (4.81 ± 1.87 vs. 8.77 ± 5.78; $P < 0.001$), 6 h (9.00 ± 3.19 vs. 18.73 ± 8.22; $P < 0.001$) and 24 h (14.6 ± 4.13 vs. 30.14 ± 9.85; $P < 0.001$) of cooled-storage (Fig. 1a). In both seasons, the percentage of fragmented sperm DNA significantly increased ($P < 0.001$) after 6 or 24 h of cooled-storage (Fig. 1a). The slope of SDF was also significantly lower ($P < 0.001$) in semen samples collected and cooled in spring compared to summer (Fig. 1b).

3.2.1. Relationship between pregnancy rate and sperm DNA fragmentation of cooled-stored semen during spring and summer

The number of estrous cycles, mares inseminated per stallion and pregnancy rates obtained is presented in Table 2 and Table 3. The pregnancy rate per cycle was significantly higher ($P < 0.001$) in spring (50%) in comparison to summer (37%). The number of mares and estrous cycles assigned to each stallion was higher in spring compared to summer. A negative correlation was found between SDF after 24 h of cooled-storage and pregnancy rate ($r = -0.619$; $P < 0.001$). Stallions with moderate fertility levels (pregnancy rate < 50%) showed a higher percentage of sperm with fragmented DNA at 0 h and 6 h od cooled-storage when compared to stallions with good fertility levels ($P < 0.05$; Table 4).

4. Discussion

In the present study, it has been observed that the sperm quality

Table 1
Comparison of stallion sperm characteristics before cooling between semen samples collected and processed during spring or summer periods.

Sperm parameters	Breading season		*P*-value
	Spring	Summer	
Gel-free volume (mL)	60.23 ± 20.03	53.86 ± 20.05	>0.05
Concentration (x10^6/mL)	173.91 ± 61.23	214.55 ± 86.80	>0.05
Total Motility (%)	90.97 ± 6.78	87.91 ± 8.30	>0.05
Progressive Motility (%)	53.57 ± 9.97	41.37 ± 10.81	<0.001
Plasma Membrane Integrity (%)	86.55 ± 1,01	83.54 ± 14.91	= 0.05
Curvilinear velocity (VCL, μm/s)	72.61 ± 6.65	65.16 ± 11.00	<0.05
Straight line velocity (VSL, μm/s)	37.88 ± 9.96	34.12 ± 10.43	>0.05
Average path velocity (VAP, μm/s)	55.15 ± 10.88	49.70 ± 12.46	>0.05
Linearity (LIN, %)	51.71 ± 11.10	51.66 ± 9.81	>0.05
Straightness (STR, %)	65.55 ± 17.01	68.52 ± 9.04	>0.05
Lateral head displacement (ALH, μm)	2.24 ± 0.30	2.06 ± 0.40	>0.05
Beat cross frequency (BCF, μm)	9.09 ± 1.59	8.36 ± 2.01	>0.05

Results are expressed as mean ± SD.

of stallion, regarding some parameters, is greater at the beginning of the reproductive season. In particular, the fragmentation of DNA from cooled-stored sperm doses was lower in spring in comparison to summer. Interestingly, this lower DNA fragmentation is correlated with a better fertility, obtaining higher pregnancy rates in spring than in summer. In this study, the mares included in the spring and summer periods were different. Most of the mares included in the study were bred early in the breeding season, during the spring period, since most of the breeders wanted foals to be born early in the year for competition purposes. If those mares were not pregnant after several AI they were not bred again until the next year. Only few breeders, usually not involved in competitions, preferred the foaling to occur in a warmer period with more suitable weather, as it is summer. Therefore, differences in per cycle pregnancy rate are not expected to be due to mares with excellent fertility that became pregnant in spring and mares with low fertility that did not became pregnant and were presented again in summer for AI service. As it has been stated, mares with reproductive disorders were not included in the study.

Among the sperm parameters employed to define the sperm quality, progressive motility is considered an indicator of the potential fertility of a stallion, although its correlation with *in vivo* fertility is low [24]. In the assessment of fresh stallion sperm in this study, a higher progressive motility was observed in spring compared to summer. The information available about the influence of the season on the fresh sperm motility is contradictory. While some studies found that there was a reduction in the sperm motility in spring [9,25], others studies showed an increase in sperm motility during this season [10,26], as it is observed in the present study.

The sperm DNA fragmentation index has shown to be an important parameter regarding its correlation with fertility [27–30]. The relationship between seasonal variations and the sperm DNA fragmentation has also been studied with contradictory results. Some authors have not observed any influence of season on DNA fragmentation [31,32], while others have observed a lower sperm DNA fragmentation in winter [3], or during the breeding season [9]. The present study was performed during the breeding season, focusing on the assessment of sperm DNA fragmentation at 0, 6 and 24 h of cooled-storage during spring and summer. It was found that the percentage of sperm with fragmented DNA was significantly lower in spring in comparison to summer at every time assessed for up to 24 h of cooled-storage. In a previous study of stallion sperm, the highest DNA fragmentation was also obtained in summer, particularly in the months of June and July [9]. An increase in the level of sperm DNA damage can occur due to intrinsic and extrinsic factors, including temperature of testis [33]. Spermatogenesis and normal testicular function are both temperature dependent. It has been observed that scrotal heat stress increases the incidence of DNA damage during spermatocytogenesis. At that stage, this heat-stress in the spermatocyte alter the formation of protamine configuration of disulfide bonds later in the spermiogenesis or during epididymal transit; as consequence, the chromatin becomes more susceptible to denaturation [34]. Therefore, the greater DNA fragmentation observed in this study in summer may be related to the thermic stress during spermatogenesis.

In addition, the DNA fragmentation rate (slope of SDF) was higher in the sperm doses cooled-stored in summer. The higher temperatures during summer may contribute to modify the formation of disulfide bonds between protamines in the chromatin

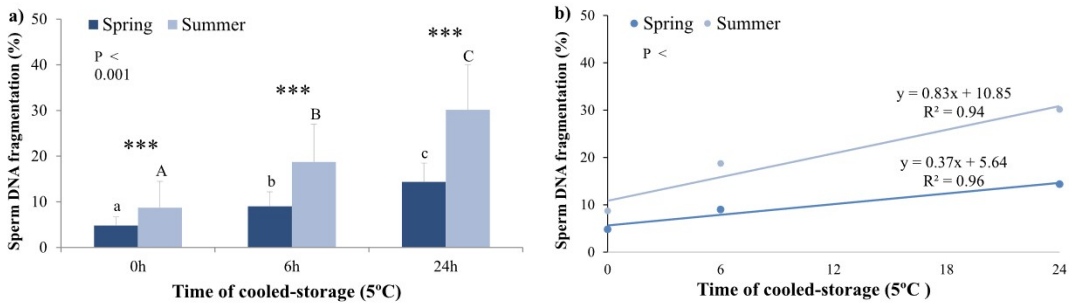

Fig. 1. Effect of cooled-storage at different times on DNA fragmentation of stallion sperm collected in each season. Fig. 1a Differences in sperm DNA fragmentation between spring and summer seasons at each time of cooled-storage ($P < 0.001$; ***). The letter superscripts indicate significant differences among different times of cooled-storage (0, 6 and 24 h) during spring (a-b; $P < 0.001$) or summer periods (A-B; $P < 0.001$). Fig. 1b) Linear regression analysis of the sperm DNA fragmentation rate (slope). Significant differences were found between spring and summer seasons ($P < 0.001$).

Table 2
Average number of mares and estrous cycles assigned per stallion for artificial insemination during spring and summer periods.

Season	Stallions	Average no. mares/stallion	Average no. cycles/stallion	Pregancy rate (%)
Spring	11	23 (5–65)	32 (7–76)	50 ± 9[a]
Summer	11	9 (2–22)	15 (4–34)	37± 9[b]

Results are expressed as mean ± SD; range (minimum and maximum values) is expressed in the brackets. Different superscripts (a-b) indicate significant differences for pregnancy rate per cycle ($P < 0.01$).

Table 3
Description of the number of mares and estrous cycles assigned to each stallion for artificial insemination and pregnancy rates obtained during the breading season.

Stallion	No. Mares			No. Estrous cycles			Pregnancy rate per cycle (%)			Fertility level
	Spring	Summer	Total	Spring	Summer	Total	Spring	Summer	Total	
1	18	9	27	22	13	35	50	38	44	<50
2	25	4	29	49	7	56	37	43	40	<50
3	65	22	87	76	34	110	66	41	54	>50
4	50	19	69	68	24	92	50	54	52	>50
5	5	2	7	7	4	11	43	25	34	<50
6	10	2	12	14	3	17	50	33	42	<50
7	10	8	18	11	11	22	64	45	55	>50
8	24	9	33	42	16	58	45	38	42	<50
9	29	7	36	35	15	50	57	27	42	<50
10	13	5	18	19	12	31	42	25	34	<50
11	10	14	24	16	22	38	44	36	40	<50
Total	**259**	**101**	**360**	**359**	**161**	**520**	**50**	**37**	**43**	

Table 4
Comparison of sperm DNA fragmentation between samples from stallions (n = 11) with different pregnancy rates following AI.

Pregnancy rate (%)	n	Sperm DNA fragmentation of cooled-stored samples (SDF, %)		
		SDF-T0	SDF-T6	SDF-T24
<50	8	7.90 ± 5.02[a]	15.47 ± 8.60[a]	23.37 ± 11.95[a]
>50	3	3.83 ± 1.34[b]	9.58 ± 2.71[b]	19.25 ± 7.12[a]

n = number of stallions; T0, T6, T24: 0, 6 or 24 h of cooled-storage, respectively. Results are expressed as mean ± SD. Different letter superscripts (a-b) indicate significant differences in the same column ($P < 0.05$).

structure, as it was previously observed in stallions exposed to heat stress [34]. During semen cooling, spermatozoa also suffer an alteration on its plasma membrane, which may lead to an increase in proteases activity and sperm apoptosis [35]. These damaged sperm generate large amounts of reactive oxygen species (ROS) [36], which causes an oxidative stress to sperm, inducing different DNA damages: DNA strand breaks, modified bases, DNA cross-links, frame shifts, production of base-free sites and chromosomal rearrangements [13,37]. Therefore, sperm collected in summer would be more vulnerable to these injuries in the chromatin structure, showing a higher DNA fragmentation rate. Further studies are needed to confirm this hypothesis.

Interestingly, during the summer period the values of the sperm DNA fragmentation were higher and the pregnancy rates of mares inseminated with cooled-stored semen were lower. Based on the results of the present study, the higher pregnancy rates in spring can be due to the low DNA fragmentation obtained in this period, since a negative correlation has been observed between them. Actually, the highest DNA fragmentation index, at 0h and 6 h of cooled-storage was presented in semen samples from stallions with moderate fertility after AI (pregnancy rates < 50%). These finding are supported by previous studies where the negative impact on fertility of a highly DNA fragmented sample of sperm was revealed [34,38,39]. The sperm DNA must be decondensed before fertilization, a process dependent on the reduction of disulfide bonds [40]. Sperm with damaged chromatin by heat-stress, present a reduction in the number of disulfide bonds, therefore, affecting the fertilization process [34]. In addition, most of the studies indicate that sperm with fragmented DNA has adverse effects from fertilization onwards, having a negative impact on quality of embryo development, transmission of genetic material to the offspring, early pregnancy loss and pregnancy outcome [12,39].

In conclusion, there were differences on the sperm DNA integrity and *in vivo* fertility of cooled-stored stallion semen throughout the breeding season, obtaining lower sperm DNA fragmentation and higher pregnancy rates in spring, supporting the recommendation to perform semen cooling and AI during early breeding season. Additionally, stallions with the highest proportion of sperm with fragmented DNA showed the lowest fertility levels throughout the breeding season.

CRediT authorship contribution statement

F. Crespo: Formal analysis, Writing - original draft, Project administration. **C. Quiñones-Pérez:** Formal analysis. **I. Ortiz:** Formal analysis, Data curation. **M. Diaz-Jimenez:** Writing - original draft. **C. Consuegra:** Formal analysis, Writing - original draft. **B. Pereira:** Formal analysis, Writing - original draft. **J. Dorado:** Formal analysis, Data curation. **M. Hidalgo:** Formal analysis, Data curation, Writing - original draft, Project administration.

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CAPÍTULO 1.2

Comparison of different mathematical models to assess seasonal variations in the longevity of DNA integrity of cooled-stored stallion sperm.

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Comparison of different mathematical models to assess seasonal variations in the longevity of DNA integrity of cooled-stored stallion sperm

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Abstract

Dynamic assessment of sperm DNA fragmentation (SDF) has shown to give fuller understanding of stallion semen quality; however, there have been limited attempts to use this parameter to investigate seasonal changes in productive functions. The aims of this study were to: (a) establish a reliable mathematical model to describe the longevity of cooled-stored sperm DNA integrity; (b) to examine the effect of seasonal variations on SDF. Ejaculates were cooled to 5°C, and SDF was analysed after 0, 6 and 24 hr of storage. The coefficient of determination (R^2) was calculated after fine-tuning linear (LIN), exponential (EXP) and second order polynomial (POL) models. R^2 was significantly higher ($p < .001$) for POL than for LIN and EXP. The rate of DNA degradation was calculated using the slopes of POL equations. After assessing the rate of change of the POL functions, significant differences between the acceleration of DNA fragmentation were found ($p < .01$) among seasons, being higher for winter and summer than spring and autumn. In conclusion, DNA analysis of stallion sperm fits better to a second order polynomial mathematical model, being spring the best season to collect and process cooled stallion semen in order to maintain the DNA integrity of the stallion sperm.

KEYWORDS

DNA analysis, regression equation, season, semen, stallion

1 | INTRODUCTION

There is a general consensus about the deleterious effect of sperm DNA fragmentation (SDF) in human reproduction outcomes, especially as it relates to miscarriage (Robinson et al., 2012). In fact, the 5th edition of the WHO laboratory manual for the examination and processing of human semen now includes a specific section for the evaluation of DNA integrity (World Health Organization, 2010). These findings have been gradually supported and verified by the discoveries made in other mammals (Cortes-Gutierrez et al., 2014; Gillan, Evans & Maxwell, 2005; Love & Kenney, 1998; Sanchez-Calabuig et al., 2015). It has been shown that several reproductive techniques could affect the integrity of the sperm DNA in different animal species, including artificial breeding technologies in the stallion, such as sperm cooling or cryopreservation, prolonged exposure to semen extenders (Love et al., 2005) and handling conditions of the ejaculate (Gonzalez-Marin, Gosalvez & Roy, 2012).

Despite the equine has been well established as a long-day seasonal breeder (Chemineau et al., 2008; Hoffmann & Landeck, 1999; Janett, Thun, Niederer, Burger, & Hassig, 2003; Jasko, Lein, & Foote,

1991; Magistrini, Chanteloube, & Palmer, 1987; Pickett, Faulkner, Seidel, Berndtson, & Voss, 1976; Wach-Gygax et al., 2017), there are remarkably only a few studies that have exanimated this phenomenon on semen quality, with these studies typically confirming better semen quality in the spring (Blottner, Warnke, Tuchscherer, Heinen, & Torner, 2001; Chemineau et al., 2008; Wach-Gygax et al., 2017). While there is still debate in the literature about the specific impact of season on each parameter, there is, nevertheless, overall agreement that season (photoperiod) does influence sperm quality (Chemineau et al., 2008; Hoffmann & Landeck, 1999), including that of SDF (Blottner et al., 2001; Chemineau et al., 2008; Morte et al., 2008; Ortiz et al., 2017; Wach-Gygax et al., 2017).

With regards to the effect of seasonality on cooled-stored semen, only limited information is available (Deichsel, Schrammel, Aurich, & Aurich, 2016; Wach-Gygax et al., 2017), this is despite the fact that stallion sperm cooling is regarded as standard practice for the storage and transport of semen in this species (Aurich, 2008; Brinsko & Varner, 1992; Loomis, 2001; Varner, 2016). It is also well known that stallion semen cooling affects routine sperm quality parameters and pregnancy rates (Brinkerhoff et al., 2010; Foster et al., 2011; Heckenbichler, Deichsel, Peters, & Aurich, 2011; Henderson, Capewell, & Johnson, 1998; Kiser et al., 2013; Love et al., 2015; Urbano et al., 2013), mostly attributable to the relatively short longevity of cooled-stored semen of approximately 24–48 hr. Other studies have shown that semen cooling increases SDF values after 48 hr of storage (Blottner et al., 2001; Fraser, Strzezek, & Kordan, 2011; Gil et al., 2014; Linfor & Meyers, 2002; Love & Kenney, 1998; Love et al., 2015; Ortiz et al., 2017).

SDF can be evaluated as a static or dynamic parameter, static assessment involves measuring SDF at one time point whereas dynamic assessment involves evaluating the SDF at multiple time points over a period of incubation at a temperature that mimics the female reproductive tract (usually 37°C). There is an increasing body of evidence (Cortes-Gutierrez et al., 2008; Johnston et al., 2016; Lopez-Fernandez et al., 2007; Ortiz et al., 2015) that shows that dynamic assessment has an advantage over static assessment as it can expose hidden or cryptic sperm DNA damage that is not initially revealed using a single time point (Linfor & Meyers, 2002; Lopez-Fernandez et al., 2007). This dynamic assessment also allows the calculation of a rate of SDF (rSDF) which can then be fine-tuned using a linear regression model (Cortes-Gutierrez et al., 2008; Lopez-Fernandez et al., 2007; Urbano et al., 2013). These linear models suppose that the velocity of rSDF is constant, an assumption that some authors claim to be incorrect (Ferreira et al., 2018; Love, Thompson, Lowry, & Varner, 2002; Ortiz et al., 2017). More recently, a new approach to the analysis of rSDF that involves the use of and second order polynomial mathematical models has been established (Ortiz et al., 2017). These two models imply that the velocity of DNA fragmentation is not constant over time (de la Horra Navarro, 2018).

The principles of dynamic SDF assessment at 37°C should be translated into observation of stored preserved diluted semen at refrigerated temperatures. The concepts and models used are similar, except for the temperature of incubation (5°C instead of 37°C). Therefore, the aims of this study were to: (a) establish a mathematical model that could be reliable used to describe the longevity of stallion sperm DNA integrity following cooled storage; (b) use this model to examine the effect of seasonal variations on stallion sperm DNA fragmentation.

2 | MATERIAL AND METHODS

2.1 | Animals and semen collection

Semen was collected from twenty clinically healthy and fertile stallions aged 6–21 years of different breeds (11 Pure Spanish horse, 2 Spanish-Arab, 2 Anglo-Arab, 2 Spanish-Breton, 1 Arabian, 1 Selle-Français, 1 Spanish sport-horse), using an artificial vagina and a phantom for mounting in the presence of a mare in oestrus. Animals were housed at the Equine Breeding Centre of the Spanish Army located in Ávila, Spain (40.66N, 4.70W). Two ejaculates were collected from each stallion in every season for one year to complete the semen samples used in the experimental design (n = 160). Seasons were defined as follows: winter: January to March; spring: April to June; summer: July to September; autumn: October to December. A total number of 160 ejaculates were included in the experimental design. All the experiments were performed in accordance with the Spanish law for animal welfare and experimentation (Decision 2012/707/UE and RD 53/2013).

2.2 | Semen processing and cooling

All the semen samples were evaluated as previously defined by Hidalgo et al. (2017) and had at least a gel-free volume of 20 ml, a sperm concentration over 110 × 10^6 sperm/ml, a progressive sperm motility over 50% and a plasma membrane integrity over 50%. A milk-based extender formulated for equine semen was used for semen evaluation, processing and cooling (INRA96, IMV Technologies, L'Aigle, France).

Immediately after collection, the semen samples were extended to reach a final concentration of 25 × 10^6 sperm/ml, maintained at room temperature (≈22°C) for stabilisation during 10–15 min and then cooled to 5°C in a semen shipping container (Equitainer, Hamilton-Thorn-Research). After 2 hr of cooling, considered as time 0 in the experimental design, samples were stored in a standard refrigerator at 5°C for up to 24 hr for further analysis.

2.3 | Sperm DNA fragmentation analysis

Sperm DNA fragmentation was assessed using the Sperm Halomax Kit (Halotech DNA SL), based on the dispersion of the sperm chromatin as previously described (Lopez-Fernandez et al., 2007). Briefly, an aliquot of the cooled-stored semen was incubated at

37°C for 10 min. After that, 25 µl of diluted sperm was added to a vial containing low melting point agarose. A 10 µl aliquot of the agarose–sperm mixture was spread upon a pre-treated slides provided in the kit, covered with a glass coverslip and placed in a refrigerator at 5°C on a cold metallic plate for 5 min. Following solidification and formation of the microgel, the coverslip was carefully removed and the slide preparation was placed horizontally in 10 ml of the lysing solution provided in the kit for 5 min. The preparation was subsequently washed in distilled water for 5 min and dehydrated in a series of ethanol baths (70% and 90%) of 2 min each. Spermatozoa in the microgel were then stained with the Fluored staining kit (Halotech DNA SL), and DNA damage was visualised under epifluorescence microscopy. A minimum of 200 spermatozoa were counted. The percentage of sperm with fragmented DNA (displaying a large halo of chromatin dispersion) was recorded for further analysis (sperm DNA fragmentation index, SDFi, %).

2.4 | Experimental design

2.4.1 | Experiment 1. Comparison of three mathematical models to assess the sperm DNA fragmentation dynamics in the stallion

Sperm DNA fragmentation analysis was performed for each sample after 0, 6 and 24 hr of cooled storage. SDF values were then analysed using three mathematical regression models for dynamic assessment: linear, exponential and second order polynomial, as previously described (Ortiz et al., 2017). In order to compare which model was the better fit for SDF, the coefficient of determination (R^2) was calculated. Accuracy of the three different regression models was performed by comparing the coefficients of determination obtained in each season.

2.4.2 | Experiment 2. Assessment of seasonal variations in stallion sperm DNA fragmentation dynamics

Dynamics of sperm DNA fragmentation was assessed and compared for seasons using the most accurate regression model, according to the results obtained in *Experiment 1*.

2.5 | Statistical analysis

Normality of the data distribution and homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene test. Values not normally distributed were transformed to a logarithmic scale. Regression coefficients were calculated using Microsoft Excel for Windows 10 and then compared with SPSS 20.0. In Experiment 1, SDFi (%) values (*y* coefficient) at 0, 6 and 24 hr (*x* coefficient) were adjusted to linear, exponential and second order polynomial models. R^2 was calculated for each replicate and compared separately for each season (spring, summer, autumn and winter), among models (linear, exponential and polynomial) with an ANOVA, using animals, ejaculates and seasons as fixed effects.

In Experiment 2, second order polynomial functions were chosen. Since second order polynomial functions are parabolic lines, the derivative function $\frac{d\,SDF}{dt}\left(\frac{\%}{h}\right)$, which assesses the rate of change of the POL functions, was calculated for each season (spring, summer, autumn and winter) (de la Horra Navarro, 2018). Afterwards, a graphic was represented using the rate of change of SDF (%/*h*, DNA fragmentation velocity) of the polynomial function $\frac{d\,SDF}{dt}\left(\frac{\%}{h}\right)$ as *y* coefficient and time (0, 6 and 24 hr) as *x* coefficient. The slopes of these straight lines (DNA fragmentation acceleration $\%/h^2$) were compared between seasons by ANOVA followed Duncan *post hoc* tests.

Significant differences were considered when $p < .05$ in both experiments. Duncan *post hoc* test was carried out to assess differences between seasons.

3 | RESULTS

3.1 | Experiment 1. Comparison of three mathematical models to assess the sperm DNA fragmentation dynamics in the stallion

The dynamics of SDF assessed with each mathematical model (polynomial, linear and exponential regression models) in each season (winter, spring, summer and autumn) is represented in Figure 1. The polynomial regression model showed significantly ($p < .001$) higher values for the coefficient of determination (R^2) in comparison with linear and exponential models, respectively in winter (1.00000 vs. 0.95077 vs. 0.78986), spring (1.00000 vs. 0.98006 vs. 0.84985), summer (1.00000 vs. 0.94846 vs. 0.79825) and autumn (1.00000 vs. 0.97131 vs. 0.87219). Consequently, the polynomial regression model was selected as the most accurate model to analyse in Experiment 2.

3.2 | Experiment 2. Assessment of seasonal variations in stallion sperm DNA fragmentation dynamics

Figure 2 shows the graphical representation of the derivative function of the polynomial regression models for each season. It represents the velocity of fragmentation over time for winter, summer, spring and autumn. These velocities tended to be higher for summer and winter followed by autumn and spring. The slopes of these lines

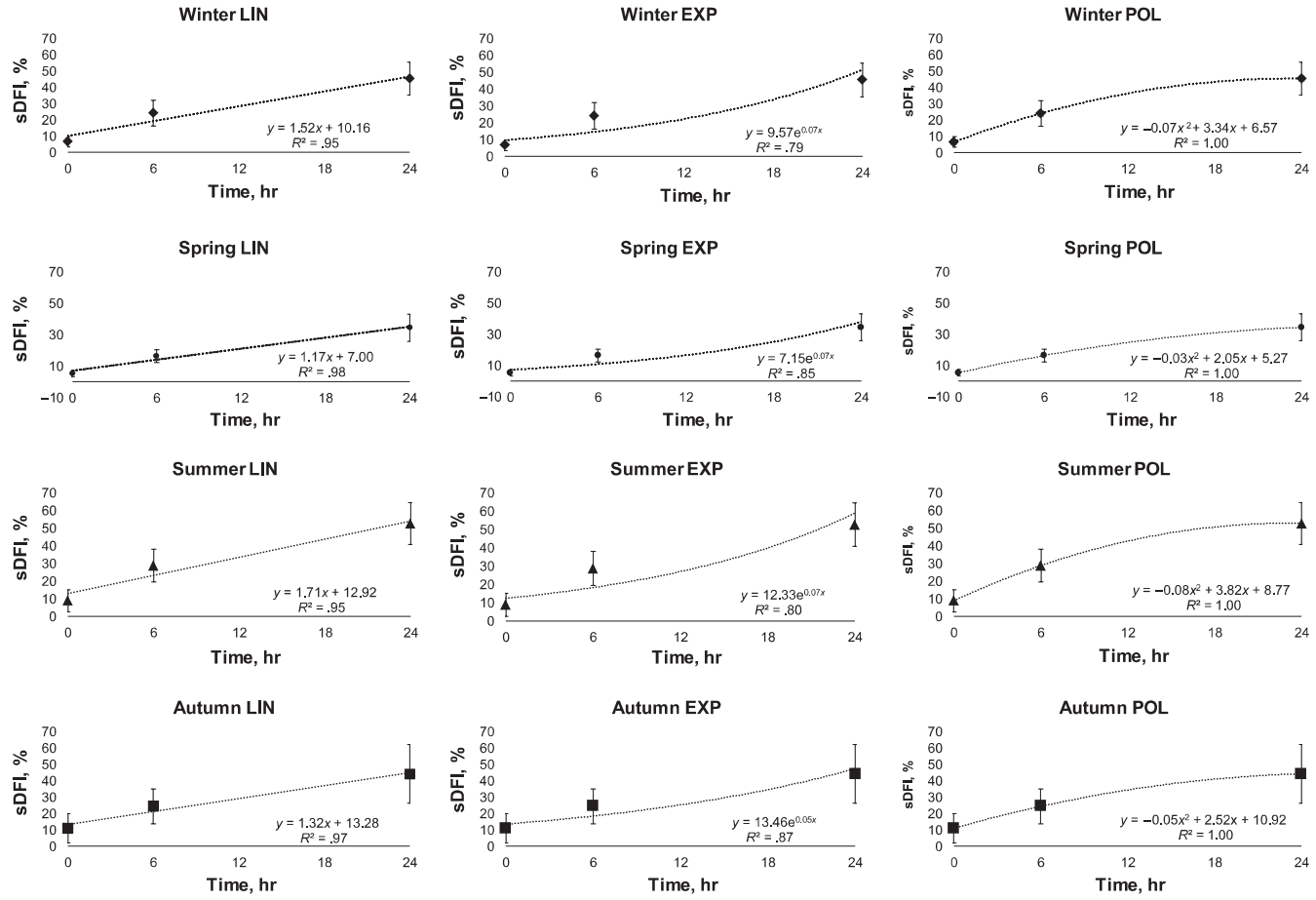

FIGURE 1 Coefficient of determination (R^2) for linear, exponential and second order polynomial models within seasons. EXP, exponential; LIN, linear; POL, second order polynomial; SDFi, sperm DNA fragmentation index

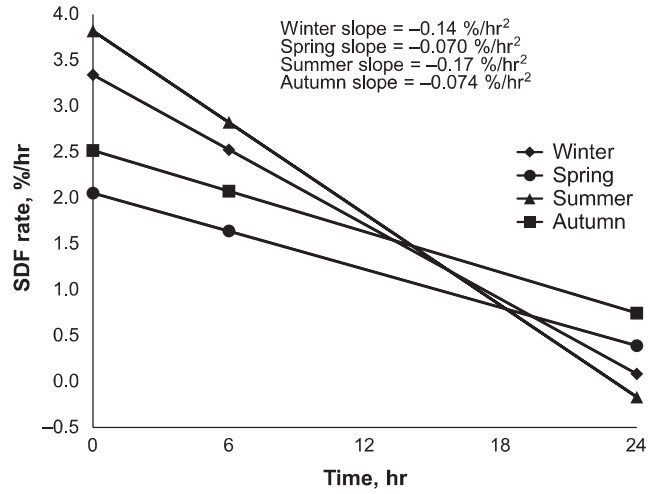

FIGURE 2 Sperm DNA fragmentation acceleration (%/hr^2) in relation to cooling storage hours at 5°C for each season. SDF, sperm DNA fragmentation

represent the rate of change in the velocity of fragmentation (acceleration of fragmentation, aSDF, fragmentation rate/time, %/h^2).

Significant differences between DNA fragmentation accelerations ($p < .01$) were found among winter −0.13565%/h^2 versus summer −0.16620%/h^2 versus spring −0.06933%/h^2 versus autumn −0.07373%/h^2, with summer showing highest acceleration of SDF, followed by winter. Spring and autumn essentially displayed the same degree of deceleration.

4 | DISCUSSION

According to the results of Experiment 1, cooled stallion sperm DNA fragmentation dynamic conformed better to a second order polynomial mathematical model regardless of the season studied. This has also been intuitively described by Ferreira et al. (2018), where no linear regression was observed when making and inter-assay study between different DNA fragmentation methods. In a study conducted by Lopez-Fernandez et al. (2007), it was described that the highest increase in SDF occurred within the first hours after heating frozen and cooled samples up to 37°C. Concurring as well with our results, previous studies made in other species such as boars (Boe-Hansen, Ersboll, Greve, & Christensen, 2005; Fraser & Strzezek, 2004), donkeys (Ortiz et al., 2017) and even koalas (Johnston, Zee, Lopez-Fernandez, & Gosalvez, 2012) indicated that sperm DNA denaturation progression was also quadratic, though boar and koala spermatozoa appear to be more resistant to degradation than

stallion and donkey spermatozoa. Therefore, due to the sperm DNA fragmentation during cooling is not a constant in stallions, as it slows down over time regardless of the season, the DNA analysis fits better to a second order polynomial mathematical model.

However, these results do appear to disagree with the findings showed by Love et al. (2002), where the polynomial correlation of the SDF was only found in subfertile stallions, while the fertile population fitted a linear regression. Additionally, Linfor and Meyers (2002) confirmed that DNA degradation accelerated from 48 hr of cooled-stored and that it followed a linear progression. One of the reasons for the disagreement could be attributed to the method used to analyses sperm DNA. In the studies of Love et al. (2002) and Linfor and Meyers (2002), the sperm chromatin structure assay and comet assay were employed respectively, while the SCD methodology was used by Ferreira et al. (2018) and in this study. Another plausible reason for this discrepancy could be associated with the seminal plasma concentration of samples. Studies have shown that a high seminal plasma concentration leads to a deterioration of sperm motility parameters (Jasko, Moran, Farlin, & Squires, 1991; Varner, Blanchard, Love, Garcia, & Kenney, 1988), which may also explain the damage of other parameters, such as DNA integrity. In the study of Love et al. (2002), no centrifugation process was performed, while in Linfor and Meyers (2002), the final sperm concentration was twice that the one used in our study; both cases could lead to an increases in the seminal plasma concentration. The effect of the breed on the sperm characteristics, including SDF, has been discussed by several authors in other studies such as Greiser, Sieme, Martinsson, and Distl (2020), Labitzke, Sieme, Martinsson, and Distl (2014), Janett, Thun, Niederer, et al. (2003), Greiser et al., 2020, Labitzke et al., 2014, Janett, Thun, Bettschen, Burger, and Hassig, (2003). Although this is an important variable to consider, the irregular distribution of individuals per breed in this study (now detailed in the material and methods section) does not allowed to accurately analyse the importance of this variable. Further studies with a very high number of animals (to decrease the individual effects) a similar number of individuals per breed are needed in order to analyse the effect of breed on the SDF dynamics seasonality. In the present study, longevity of sperm DNA integrity was assessed for up to 24 hr due to this is the period in which shipping doses of cooled-stored stallion semen are commonly used for artificial insemination.

A possible explanation for the quadratic evolution is that DNA fragmentation induced by cooling is produced by the osmolality changes that happen at low temperatures, as well as plasma membrane integrity instability (Lopez-Fernandez et al., 2007). Both of these parameters need some time to produce their effects. So much so that in our study, initial SDFi values after cooling were quite similar to the values obtained in fresh semen. This was also noted by Lopez-Fernandez et al. (2007) in the stallion and it has also been demonstrated in other species (Duru, Morshedi, Schuffner, & Oehninger, 2001; Evenson, Thompson, & Jost, 1994; van der Schans, Haring, Dijk-Knijnenburg, Bruijnzeel, & Daas, 2000); in fact, this was one of the main reasons why DNA fragmentation dynamic studies were developed: to reveal hidden DNA damage that could only be revealed over time. This finding simply means that, as expected, the longer semen samples are kept under refrigeration conditions, the more severe their DNA damage is (Cortes-Gutierrez et al., 2008; Johnston et al., 2016; Lopez-Fernandez et al., 2007).

Previous studies have used a correct-enough methodology to analyse the SDF dynamics, as it is LIN model, this study focuses on developing a new methodology significantly more accurate. This new methodology highlights that the DNA fragmentation process is not linear, as it was assumed before. Experiment 2 simplifies the analysis of a complicated function with a parabolic shape by using its rates of change (derivative functions), which are linear and thus, straight lines. The slopes of these derivative functions represent the acceleration of each function. The analysis of the seasonal effect on stallion SDF reported in Experiment 2 revealed that spring and autumn fragmentation rates were essentially the same, as their slopes were nearly parallel. However, both the SDF values depicted in Figure 1 and the SDF rates showed in Figure 2 are higher in autumn than in spring. Therefore, although the progression (SDF acceleration) is similar in both seasons, the SDF values for spring are always lower than for autumn. On the other hand, summer and winter displayed steeper slopes, meaning that their fragmentation rate was higher. A closer look to Figure 2 reveals that this difference in the rates for each season was not constant over the 24 hr periods of observation. This could be explained because of an accumulation of fragmented DNA at the beginning of their storage, leaving lesser DNA to damage by the end of the observation period. This may also be the reason why all seasons fit quadratic functions. Nevertheless, it is important to consider that the values obtained for the rates of SDF (Figure 2) have been obtained from a model, that is they are expected rather than observed values. Further studies with more frequent assessments for longer periods of time are needed to fit the model as much as possible to obtain more accurate predictions.

Analysing the seasonal effect on sperm DNA fragmentation and taking into account that horses are long-day breeding animals, it is logical to find lower values of DNA fragmentation rate during spring. Accessory glands and endocrine activity change based on the length of the day (Hoffmann & Landeck, 1999; Janett, Thun, Bettschen, et al., 2003; Jasko, Lein, et al., 1991), boosting high-quality sperm production during spring. Some authors considered autumn the best season for cooling (Janett, Thun, Niederer, et al., 2003; Magistrini et al., 1987). This disagrees with our results, as SDF rate is lower in spring. Other studies claim that SDF values are low during winter (Blottner et al., 2001; Wach-Gygax et al., 2017), while in our study, it appears that SDF rate in winter is quite high compared with spring or autumn. This difference may be due to the experimental design or climate variations. The climate in Ávila is classified as Mediterranean Oceanic, and it is determined by its high altitude (1,100 m). Daily thermal amplitude is large (≈12°C), summers are short, hot and sunny, and winters are long, cold and occasionally cloudy. In our study, December, with lower values regarding SDF, is considered part of autumn, which drops the average of sperm with intact DNA during winter. In our study, we observed higher rates of SDF during summer similar

to that described previously (Wach-Gygax et al., 2017). It is usually explained that heat disrupts the insulating action of scrotum, which consequently has a harmful effect over early stages of spermatogenesis (Love & Kenney, 1999). It is also known that sulphide bonds between protamines and DNA get weaker in this season (Wach-Gygax et al., 2017).

In conclusion, the DNA analysis of stallion sperm fits better to a second order polynomial mathematical model, being spring the best season to collect and process cooled semen in order to maintain the DNA integrity of the stallion sperm.

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CAPÍTULO 1.2

Comparison of different mathematical models to assess seasonal variations in the longevity of DNA integrity of cooled-stored stallion sperm.

Isabel Ortiz, Carlota Quiñones-Pérez, Manuel Hidalgo, César Consuegra, María Díaz-Jiménez, Jesús Dorado, José Luis Vega-Pla, Francisco Crespo, 2020. Andrologia.

Animal Reproduction

ORIGINAL ARTICLE

Characterization of the seminal bacterial microbiome of healthy, fertile stallions using next-generation sequencing

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Abstract

High-throughput sequencing studies have shown the important role microbial communities play in the male reproductive tract, indicating differences in the semen microbial composition between fertile and infertile males. Most of these studies were made on human beings but little is known regarding domestic animals. Seminal bacteria studies made in stallions mostly focus on pathogenic bacteria and on their impact on reproductive technology. However, little is known about stallion commensal seminal microflora. That ultimately hinders our capacity to associate specific bacteria to conditions or seminal quality. Therefore, the aim of this study was to characterize the seminal microbial composition of 12 healthy, fertile stallion using next-generation sequencing. Hypervariable region V3 was chosen for bacterial identification. A total of nine phyla was detected. The most abundant ones were Bacteroidetes (46.50%), Firmicutes (29.92%) and Actinobacteria (13.58%). At family level, we found 69 bacterial families, but only nine are common in all samples. Porphyromonadaceae (33.18%), Peptoniphilaceae (14.09%), Corynebacteriaceae (11.32%) and Prevotellaceae (9.05%) were the most representative ones, while the Firmicutes phylum displayed the highest number of families (23, a third of the total). Samples showed high inter-subject variability. Findings previously described in other species notably differ from our findings. Families found in human such as Lactobacillaceae, Staphylococcaceae and Streptococcaceae only represented a 0.00%, 0.17% and 0.22% abundance in our samples, respectively. In conclusion, Porphyromonadaceae, Prevotellaceae, Peptoniphilaceae and Corynebacteriaceae families are highly represented in the seminal microbiome of healthy, fertile stallions. A high variation among individuals is also observed.

Keywords: horse, microbiome, semen, next-generation sequencing.

Introduction

Next-generation sequencing has been used in horses to characterize the bacterial flora of the digestive tract (Costa and Weese, 2012; Ericsson et al., 2016; Su et al., 2020; Daly et al., 2001). These studies brought to light the important role microbial communities play in maintaining the homeostasis of this complex environment not only in the horses (Costa and Weese, 2018) but also in other domestic animal species (Wu et al., 2016; Zhang et al., 2016; Rando, 2012; Ng et al. 2010). More recent studies have shown the interaction between bacterial flora and the host may contribute to the occurrence of laminitis (Milinovich et al., 2010; Al Jassim and Andrews, 2009), colic (Al Jassim and Andrews, 2009; Salem et al., 2019) and stomach ulcers (Al Jassim and Andrews, 2009). It has even described that they are able to induce

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alterations of behavioural and mood status in human beings (Costa and Weese, 2018; Goulet, 2015). These discoveries have led to the use of probiotics as a prophylactic and sometimes therapeutic tool for some digestive conditions in the horse (Coverdale, 2016; Swyers et al. 2008). These findings have opened the door to study the microbiome in new niches, such as lower respiratory tract (Manguin et al., 2020), conjunctive (LaFrentz et al., 2020) or female reproductive tract (Barba et al., 2020; Hou et al., 2013). However, little is known regarding the commensal flora of the male reproductive tract. That ultimately would hinder our capacity to associate specific bacteria to conditions.

There are very few studies of the male reproductive tract microbiome in humans (Hou et al., 2013; Liu et al., 2014; Weng et al., 2014) and practically none in animals (Rosenfeld et al., 2018; Javurek et al., 2016; Wickware et al., 2020; Serrano et al., 2020; Al-Kass et al., 2020). In spite of that, a few studies have already associated the presence of some bacteria families to fertility (Hou et al., 2013; Kiessling et al., 2008). In the studies performed in horses, researchers have mostly focused on the detection and reduction of pathogenic bacteria in the reproductive tract to prevent their spread (Samper, 2009; Al-Kass et al., 2019); whereas some others focused their research on associating bacteria genera to its effect on reproductive technologies (Moretti et al., 2009; Ortega-Ferrusola et al., 2009; Varela et al., 2018). To the best of our knowledge, there is only one article (Al-Kass et al., 2020) describing the seminal microbiome in horses. Results vary among those articles, maybe because microflora may depend on external factors, such as environment or region (Al-Kass et al., 2020). In order to have broader picture of the commensal flora of the stallion reproductive tract, more metagenomic analysis are needed. Therefore, the aim of this study is characterizing the seminal microbial composition of healthy, fertile stallion in the south of Spain using next-generation sequencing.

Methods

Ethical statement

Animals were raised and handled in accordance with the Spanish law for animal welfare (Law 32/07). Animals were not submitted to extra semen extractions for our experiment sample collection nor was their daily workflow interrupted. Samples were not collected for the purpose of the study.

Animals and semen collection

Samples

Samples were obtained from 12 (seven Andalusian and five Arabian) healthy and fertile stallions located in the Equine Breeding Centre of the Spanish Army of Écija (Seville, Spain). Stallions ranged in age from 7 to 24 years and were included in the reproductive breeding program of the Centre. All the stallions were housed in individual boxes with a straw bedding. Faeces were removed from the housing daily. The feeding consisted of oats, commercial concentrate and water *ad libitum*. Animals lived under the same dietary and exercise conditions.

Semen was collected using an in-line gel-filter Missouri artificial vagina, with a mare in estrus as a teaser. An inner disposable plastic liner was used with each animal so to avoid cross-contamination. Semen was regularly collected, two or three times per week, in intervals of at least 24 h between semen collections (Monday, Wednesday and Friday) throughout the breeding season (from March to July). A total of 12 ejaculates were collected (one per stallion) by the end of the month of March. No clinical diseases were reported. All the stallions had physiological values of sperm quality parameters. Mean ± standard deviation values of the following parameters were: volume = 31.4 ± 21.2 ml; sperm concentration = 276.0 ± 95.1 spermatozoa/ml; total motility = 80.4 ± 7.9%; and progressive motility = 37.0 ± 10.1%.

DNA extraction control sample

A pattern was created in order to evaluate the quality of the DNA extraction and its amplification. It was composed of five field strain species. *Rhodococcus equi* and *Taylorella equigenitalis* came from the Microbiology Department of Military Veterinary Centre, (Ministry of Defence, Spain). The other three strain species came from Colección Española de Cultivos Tipo: *Staphylococcus aureus* (ATCC 43300), *Klebsiella pneumoniae* (ATCC 10031) and *Pseudomonas aeruginosa* (CECT 108).

The pattern sample contained $2x10^7$ bacteria, equally distributed among the five species ($4x10^6$ cells each). Counting was performed with a Neubauer chamber. It was submitted to DNA extraction at the same time as the rest of samples.

Next generation sequencing

DNA extraction

Samples were cryopreserved immediately after their extraction. Then, DNA extraction was performed using a ZymoBIOMICS® DNA Miniprep kit (Zymo Research, CA) commercial kit. Samples were previously submitted to a combination of mechanic and enzymatic-digestion cell disruption as described by Bag (Bag et al., 2016). Then, DNA extraction was performed following the manufacturer's instructions.

Library preparation and sequencing:

Amplicons were obtained using an Ion 16S Metagenomics® kit (Thermo Fisher, Waltham, MA). This kit characterizes five different sets of 16S hypervariable regions, V2, V3, V4, V67 and V8. The library was constructed with an Ion Plus Fragment Library kit and amplicons were labelled with an XpressTM Barcode Adapters 1-16 kit. Samples were then pooled using Ion PGM® (Thermo Fisher, Waltham, MA), HiQ Sequencing kit®, Ion 316 v2 BC® chip and sequenced using the Ion 16S™ Metagenomics Workflow in Ion Reporter™ Software. OTUs were obtained from the Ion Reporter server system (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

The Ion Reporter server system was used for data analysis (Thermo Fisher Scientific, 2021). The α-diversity analysis with Chao 1 non-parametric model to confirm that all potential bacteria have been detected (Mira Obrador, 2014). OTUs from hypervariable region V3 was chosen for bacterial identification, as it obtained the highest number of copies. Moreover, it has been suggested to detect a wider range of bacterial species (Fullston et al., 2015). Then, mean values and standard error of the mean were calculated for each phylum. Inter-subject variability was calculated with Bray-Curtis dissimilarity index.

Results

Control

The microbiome is described at a taxonomic family level. The observed proportions in the pattern were 23.1% for Alcaligenaceae, 31.4% for Enterobacteriaceae, 15.5% for Pseudomonadaceae, 27.0% for Nocardiaceae, and 2.7% for Staphylococcaceae. In most of the cases, sequenced families have appeared in the expected proportion (20%). Nevertheless, Staphylococcaceae lowers its proportion (near 2.7%) in Enterobacteriaceae's favour (31.4%).

Microbial abundance and composition

Table 1 shows the number of valid sequences obtained and the number of which that were assigned to different OTUs. The α-diversity analysis show samples were sequenced to the plateau (Figure 1), which means that the analysis have virtually located all bacteria present in the samples (Espinosa, 2019). The β-diversity analysis shows a high inter-subject variability (Figure 2).

Table 1. Number of valid sequence (VS) and mapped sequences (MS) per sample according to V3 results. Numbers correspond to the stallions. Results are expressed as number of copies.

Stallion	VS	MS
1	113651	81924
2	101979	73542
3	142745	111842
4	57464	37400
5	22609	15790
6	60555	45921
7	80441	58957
8	58750	42277
9	53504	39195
10	101524	75815
11	78380	62325
12	76634	47306

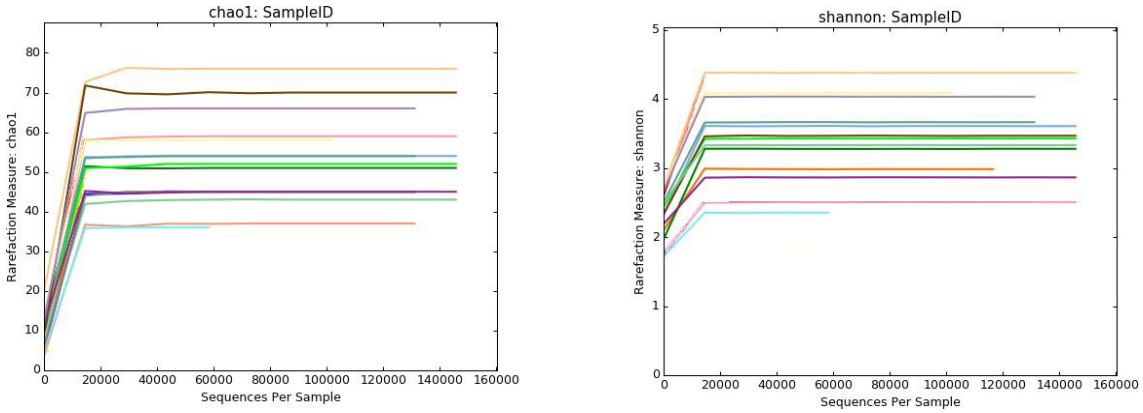

Figure 1. Rarefaction curves using Chao 1 model. All samples reach to the plateau, which is an indicator that all potential families have been detected.

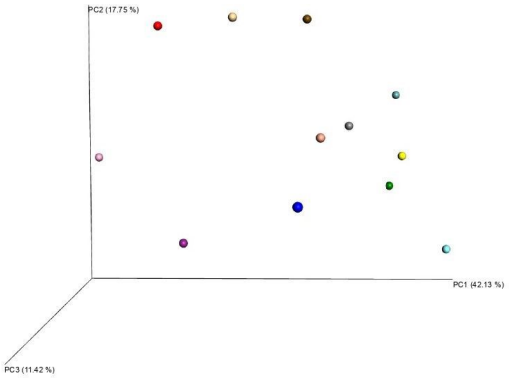

Figure 2. β-diversity using Bray-Curtis dissimilarity index. Samples are uniformly distributed along de spectrum. It was not possible to classify animals into subgroups, which is an indirect indicator of inter-subject variability.

A total of nine phyla were found. The most abundant ones were Bacteroidetes (46.50%), Firmicutes (29.92%) and Actinobacteria (13.58%). The following most common phyla were

Fusobacteria (4.50%), Proteobacteria (4.32%) and Spirochaetes (4.10%), but only Proteobacteria was detected in all horses. Fusobacteria only appeared in five of them, and Spirochaetes in nine. The last three phyla were Synergestes (0.99%), Tenericutes (0.40%) and Chloroflexi (0.10%), which only were present in seven, two and one animals, respectively.

A total of 69 families (Figure 3) were found and nine phyla were found (Figure 4). Only 22 families out of 69 exceed a 1% presence. In addition, only nine appear to be common in all samples. The most common families were Porphyromonadaceae (32.61 ± 18.16%), Corynebacteriaceae (11.05 ± 6.10%), Peptoniphilaceae (13.69± 4.28%) and Prevotellaceae (10.05 ± 10.83%). The following most common families were Clostridiaceae (3.59 ± 4.22%), XI Family, which includes several non-identifiable genera of Clostridia (3.31 ± 5.60%) and Peptostreptococcaceae (3.05 ± 5.33%).

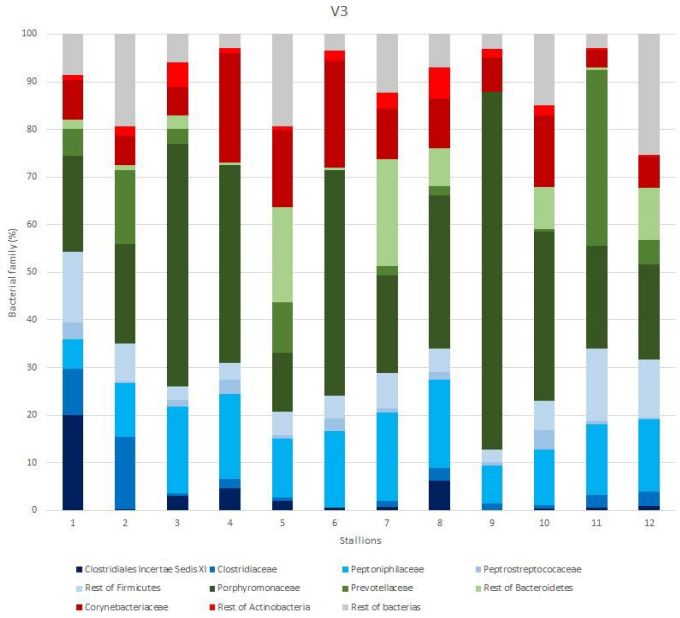

Figure 3. Detailed bacteria family composition of the samples. The results are expressed as percentages (%). Only common families with a relative abundance above 1% are included separately. Sections in blue represents Firmicutes phylum; green represents Bacteroidetes phylum; red represents Actinobacteria phylum; grey represents the rest of phyla.

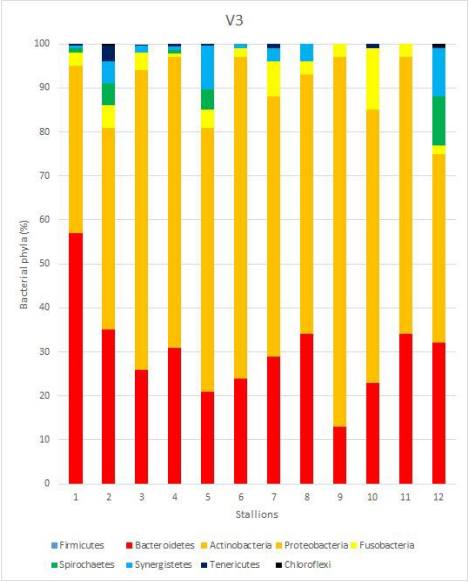

Figure 4. Phyla detected in samples. The results are expressed as percentages (%).

Discussion

In this study, next generation sequencing has been used for characterizing the seminal microbiome of stallions. This technique is more efficient for analysing microbial flora than culture-based methods, especially for hard-to-cultivate species (Zhang et al., 2016). That is why it has been used for characterizing gut microbiome (Su et al., 2020), lower respiratory tract (Manguin et al., 2020), conjunctive (LaFrentz et al., 2020). To the best of our knowledge, there is only one paper analysing the seminal microbiome in stallions with NGS technology (Al-Kass et al., 2020). However, authors have stated that there may be variations due to external factors. (Wickware et al., 2020; Al-Kass et al., 2020; Tomaiuolo et al., 2020). Therefore, it is of the utmost interest to keep exploring seminal metagenomics.

According to our study, there are four main families that represent the seminal microbiome in healthy and fertile stallions: Porphyromonadaceae (Bacteroidetes phylum), Peptoniphilaceae (Firmicutes phylum), Corynebacteriaceae (Actinobacteria phylum) and Prevotellaceae (Bacteroidetes phylum). Our results greatly concur with the ones found in the north of Europe (Al-Kass et al., 2020). However, they plainly differ from studies made in human, where gram-positive bacteria prevail (Hou et al., 2013; Weng et al., 2014). With respect to other species, they also differ from mice (Rosenfeld et al., 2018; Javurek et al., 2016) and ram (Serrano et al., 2020).

Starting with the Bacteroidetes phylum, there is no a general consensus about its function in semen. It has been identified in healthy, fertile men (Hou et al., 2013; Liu et al., 2014), while some authors associated the combination of this family and Prevotellaceae with a higher rate of reproductive inflammatory conditions (Mändar et al., 2015). In any case, the vast majority of human studies leave the presence of Porphyromonadaceae in the background, as its percentage is usually less than that of other families.

The other member from this same phylum is Prevotellaceae, traditionally defined as a natural component of vaginal, oral, cutaneous and digestive microflora. This family has been correlated with low semen quality by some authors (Weng et al., 2014), whereas others have found it to share a niche with healthy flora (Liu et al., 2014; Weng et al., 2014; Mändar et al., 2015). Our findings concur with the latter ones, as the animals in our study do not show clinical signs of disease and have a good semen quality. It is noteworthy to say that Bacteroidetes families scarcely appear in classical references. This is because this phylum is mostly composed of anaerobic gram-negative organisms. These bacteria are laborious to culture and, therefore, have been systematically omitted in culture-based microflora studies. Having pointed that, this family has neither been detected in the horse semen in other NGS studies (Al-Kass et al., 2020).

Regarding Corynebacteriaceae, it has been consistently defined as a natural component of seminal flora in humans (Liu et al., 2014; Weng et al., 2014; Mändar et al., 2015; Ivanov et al., 2009; Jarvi et al., 1996). This concurs with previous studies in the stallion (Al-Kass et al., 2020; Varela et al., 2018;, Althouse et al., 2010; Maasen and Christensen, 1995; Pickett et al., 1999; Varner et al., 1998). Other authors (Kiessling et al., 2008; Mändar, 2013) state that they are commensal bacteria that can become pathogenic when the flora unbalances or when there is a high activity of caspases (Ortega-Ferrusola et al., 2009).

The following four more common families belong to the Firmicutes phylum, Clostridia class: Peptoniphilaceae, Peptostreptococcaceae, Clostridiaceae and XI Family. Studies performed in humans show that these families are clearly rarer than in our case, excepting Peptoniphilaceae (Hou et al., 2013; Sanocka-Maciejewska et al., 2005). The same applies in the case of horses (Al-Kass et al., 2020). Interestingly, another class of this very same phylum, Bacilli, seems to be the most represented one in human semen, with families such as Lactobacillaceae, Staphylococcaceae and Streptococcaceae (Rando, 2012; Ng et al., 2010;, Hou et al., 2013; Weng et al., 2014; Varner et al., 1998; Fullston et al., 2015; Bromfield et al., 2014; Pasing et al., 2013; Rando and Simmons, 2015; Rodgers et al., 2013; Rota et al., 2011; Sharma et al., 2010). In our findings, these families were only represented at 0%, 0.17% and 0.22%, respectively. The only family related to these bacteria with a higher presence in our study was Aerococcaceae (1.23%), which has been associated with infertility cases in humans (Hou et al., 2013).

Conclusion

In conclusion, the equine seminal microbiome is principally represented by Porphyromonadaceae, Peptoniphilaceae, Corynebacteriaceae and Prevotellaceae. A high inter-subject variability is also observed. Our results concur with the ones found in other studies (Al-Kass et al., 2020). However, they differ from studies made in other species (Hou et al., 2013; Weng et al., 2014; Rosenfeld et al., 2018; Javurek et al., 2016; Serrano et al., 2020). Further studies are needed to fully characterise the natural flora composition of stallion reproductive tract.

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Author contributions

CQP: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing; MH: Project administration, Supervision, Validation, Writing – review & editing; IO: Supervision, Writing – review & editing; FC: Supervision, Writing – review & editing; JLVP: Conceptualization, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Apartado 2.1.2

The Semen Microbiome and Semen Parameters in Healthy Stallions. Animals.

Carlota Quiñones-Pérez, Amparo Martínez, Isabel Ortiz, Francisco Crespo, José Luis Vega-Pla, 2022. Animals.

animals

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Article

The Semen Microbiome and Semen Parameters in Healthy Stallions

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Simple Summary: Stallion infertility is a major cause of concern in the horse industry. Despite zootechnics advances, sub- or infertile animals appear in stud farms without a toxic, genetic, or nutritional reason. Recent research in human andrology has opened the door for a new, plausible factor that affects sperm quality: seminal microflora. In recent years, there has been an increasing amount of evidence regarding the relationship between different seminal flora compositions and male fertility. However, little has been studied in veterinary science, including horses. Therefore, the objective of this study was to examine associations with the presence of bacteria families in horse semen with five sperm quality parameters: concentration, total number of spermatozoa, total and progressive sperm motility, and DNA fragmentation. Our study detected a correlation between the presence of the Peptoniphilaceae family and higher total motility and the presence of Clostridiales Incertae Sedis XI and lower progressive motility. These changes in seminal flora may contribute to the idiopathically poorer sperm quality in certain animals. Although further mechanisms behind bacteria–spermatozoa interactions are unknown, these associations are already leading to a new therapeutic approach to infertility: the use of prebiotics, which has already yielded promising results in human andrology.

Abstract: Despite the advances in reproductive technology, there is still a considerable number of low sperm quality cases in stallions. Recent studies in humans have detected several seminal microflora–spermatozoa associations behind some idiopathic infertility cases. However, no studies are available on horses, and there is limited information on the microflora present in stallion ejaculates. Accordingly, the objective of this study was to examine associations to the presence of bacteria families with five sperm quality parameters: concentration, total number of spermatozoa, total and progressive motility, and DNA fragmentation. Samples were cryopreserved after their extraction. High-speed homogenization using grinding media was performed for cell disruption. Family identification was performed via 16S rRNA sequencing. Bacterial families were only considered if the relative abundance was higher than 1%. Only two families appeared to have a correlation with two sperm quality parameters. Peptoniphilaceae correlated positively with total sperm motility, whereas Clostridiales Incertae Sedis XI correlated negatively with progressive motility. No significant differences were found for the rest of the parameters. In conclusion, the seminal microbiome may affect spermatozoa activity. Our findings are based on statistical associations; thus, further studies are needed to understand the internal interactions between seminal flora and cells.

Keywords: microbiome; stallion; sperm quality; motility

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1. Introduction

The success of the equine industry greatly depends on good reproductive outcomes. These outcomes depend on a variety of factors, such as sperm quality. There are objective parameters that assess sperm quality, such as concentration, total motility, or progressive motility [1–3]. Factors affecting these parameters have been subject to large-scale analysis in horse reproductive science [4].

In recent years, the microbiome has proven to have a great impact on the systems they dwell on [5–9]. Unfortunately, the male reproductive tract has not received sufficient attention [10,11]. In the human species, however, some authors have already pointed out the influence of bacteria on semen quality [12–16]. In fact, these studies have opened the door to a potential therapeutic tool in infertility cases, and some authors have already published some positive effects of prebiotics in improving sperm quality [17–20].

Unfortunately, in veterinary science, research focuses on animal experimentation, such as mice [17] or broilers [18]. Regarding stallions, papers usually focus on pathogenic bacteria [21] or on their effect on reproductive technologies [22,23]. Besides, these are usually culture-based studies, which may underestimate the presence of some difficult-to-culture bacteria [24].

To the best of our knowledge, there are no studies evaluating sperm quality and the seminal microbiome in this species. Therefore, the objective of this study was to assess the relationship between the presence of more abundant bacteria and five sperm quality parameters: concentration, total number of spermatozoa, total and progressive sperm motility, and DNA fragmentation.

2. Materials and Methods

2.1. Materials

2.1.1. Sample Collection

All the experiments were performed in accordance with the Spanish law for animal welfare and experimentation (Decision 2012/707/UE and RD 53/2013). Animals belonged to the Equine Breeding Centre of the Spanish Army of Écija. Animals lived in accordance with the Spanish law for animal welfare (Law 32/07). Semen was opportunistically collected during daily work to avoid extra collections.

Samples were collected from 12 clinically stallions (7 Andalusians, 4 Arabs, and 1 Anglo-Arab) in Écija (Seville, Spain) during the breeding season (March–June). Stallions were collected a maximum of 3 times per week, with at least 48 h between collections. Age ranged from 6 to 23 years old, mean 13.3 $\pm$ 5.2 standard deviation (Table 1). Semen collection was performed using a phantom for stallion support, with a mare in estrus to stimulate sexual behavior. Semen was collected using a Missouri-type artificial vagina (Minitüb®, Tiefenbach, Germany) with an in-line filter. In order to prevent contamination, personnel wore gloves during the whole process of collection, preparation, and evaluation of ejaculates. An inner disposable sterile plastic liner was used for each animal. It was internally spread with a sterile, silicon-free commercial lubricant (Vet Gel, Kerbl®, Buchbach, Germany). At the beginning of the breeding season, the penis and prepuce of the stallion were gently washed with warm water to remove smegma excess. No routinary penis preparation prior to collection was performed unless there was smegma accumulation. Animals were housed in individual boxes with straw bedding, fed under the same dietary conditions, and had the same exercise regime. Diet included alfalfa hay, commercial concentrate, and oats.

Each sample was divided into two aliquots to evaluate: (i) Sperm quality: raw semen was extended with INRA96® (IMV, L'Aigle, France) until reaching 25 $\times$ 10^6 sperm/mL to assess sperm parameters (Table 1); and (ii) Microbiome: raw semen was frozen immediately after collection following the method described in [25], prior to analysis using next-generation sequencing as detailed below.

Table 1. Sperm quality analysis: Numbers represent animals. PRE: Andalusian. Aa: Anglo-Arabian. Ar: Arabian. C: sperm concentration (millions of cells/mL). NSPZ: total number of sperm (millions). TM: total sperm motility (%). PM: progressive sperm motility (%). Frag: sperm DNA fragmentation (%).

	Breed	C	NSPZ	TM	PM	Frag
1	Ar	163	6520	80.0	38.0	6.0
2	Aa	79	6715	80.0	40.0	6.7
3	Ar	372	3348	91.0	42.0	4.3
4	PRE	232	9280	70.0	25.0	11.7
5	PRE	227	6810	75.0	25.0	8.3
6	PRE	374	9350	80.0	50.0	3.3
7	PRE	220	12,100	90.0	25.0	5.0
8	PRE	377	3770	77.0	43.0	8.0
9	PRE	392	7840	75.0	25.0	4.0
10	Ar	307	6140	68.0	36.0	7.7
11	PRE	230	8050	85.0	57.0	5.0
12	Ar	339	2712	94.0	38.0	3.0

2.1.2. Control Sample

In order to evaluate the extraction and amplification quality, the pattern sample ZymoBIOMICS Microbial Community Standard® (Zymo Research, Irvine, CA, USA) was included during DNA extraction.

2.2. Methods

2.2.1. Sperm Parameters Evaluation

Sperm concentration was measured using a spectrophotometer (Spermacue®, Minitüb, Tiefenbach, Germany). The total number of spermatozoa was calculated by multiplying the concentration and volume. Then, semen was diluted until reaching an approximate concentration of 25×10^6 sperm/mL in milk-based extender (INRA 96®, IMV Technologies, L'Aigle, France) and placed in a 37 °C water bath. The extender contains fractions of milk micellar proteins, penicillin, gentamicin, and amphotericin B. Extended semen was only used for sperm parameters evaluation.

Sperm motility was evaluated using computer-assisted sperm analysis (Sperm Class Analyzer®, SCA, Microptic SL, Barcelona, Spain) using a 37 °C heated plate and a phase-contrast microscope (Optiphot-2, Nikon®, Tokyo, Japan). Chamber slides were pre-heated at 37 °C and up with the extended samples. Total (TM, %) and progressive sperm motility (PM, %) were evaluated as described by [25]. The minimum number of cells per sample analyzed was 500.

Sperm DNA fragmentation was assessed with the Sperm Halomax kit® (Halotech DNA® SL, Madrid, Spain), as described in [26].

2.2.2. DNA Extraction

DNA extraction was performed using a ZymoBIOMICS® DNA Miniprep (Zymo Research®, CA, USA) commercial kit. Samples had been previously submitted to a combination of mechanic and enzymatic-digestion cell disruption, as described by Bag [27]. Briefly, 100 µL of the raw semen sample was broken down for 1 h with 10 mg/mL lysozyme, 4000 U/mL lysophosphatin, and 25,000 U/mL mutanolysin. Then, samples were mechanically disrupted by high-speed homogenization (5000 rpm for 5 min) in grinding media (0.1 and 0.5 mm-diameter ceramics beads). Then, DNA was extracted following the manufacturer's instructions.

2.2.3. Next-Generation Sequencing Analysis

Next-generation analysis was performed using Ion semiconductor sequencing following the protocol described by Quiñones [28].

Data analysis was performed in the Ion Reporter server system (https://ionreporter.thermofisher.com/ir/secure/home.html) (accessed on 15 October 2021). Hypervariable region V3 was chosen for bacterial identification, as it has been suggested to detect a wider range of bacterial species [29].

2.2.4. Statistical Analysis

Bray-Curtis dissimilarity was calculated among animals (Table 2) using the following formula:

$$BC_{i,j} = 1 - \frac{2 \times B_{i,j}}{A_i + B_j}$$

$BC_{i,j}$ = Bray-Curtis dissimilarity.
$B_{i,j}$ = sum of the lesser count of common families in groups A and B.
A_i = total number of bacterial families in group A.
B_j = total number of bacterial families in group B.

Values range from 0 to 1. Values closer to 1.00 mean more dissimilarity between groups. Statistical analysis was performed using Microsoft Excel® 2013.

Table 2. Bray-Curtis dissimilarity. Values closer to 1.00 mean more dissimilarity between samples. Numbers represent animals. PRE: Andalusian. Aa: Anglo-Arabian. Ar: Arabian. Values range from 0 to 1. Values closer to 1.00 mean more dissimilarity between groups.

	Breed	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Ar	-	-	-	-	-	-	-	-	-	-	-	-
2	Aa	0.21	-	-	-	-	-	-	-	-	-	-	-
3	Ar	0.02	0.21	-	-	-	-	-	-	-	-	-	-
4	PRE	0.20	0.15	0.22	-	-	-	-	-	-	-	-	-
5	PRE	0.32	0.18	0.34	0.15	-	-	-	-	-	-	-	-
6	PRE	0.23	0.22	0.24	0.18	0.21	-	-	-	-	-	-	-
7	PRE	0.46	0.41	0.48	0.31	0.27	0.26	-	-	-	-	-	-
8	PRE	0.22	0.26	0.24	0.26	0.44	0.13	0.35	-	-	-	-	-
9	PRE	0.45	0.40	0.47	0.27	0.22	0.35	0.25	0.46	-	-	-	-
10	Ar	0.45	0.39	0.47	0.36	0.21	0.24	0.10	0.33	0.20	-	-	-
11	PRE	0.42	0.42	0.44	0.36	0.31	0.22	0.04	0.31	0.32	0.22	-	-
12	Ar	0.39	0.32	0.37	0.28	0.19	0.17	0.13	0.25	0.27	0.16	0.12	-

3. Results

Quality control was performed by submitting the ZymoBIOMICS Microbial Community Standard® to the same extraction and analysis process as the rest of the samples. The resulting composition showed minor variations compared to that provided by the manufacturer (Figure 1).

Then, samples were submitted to a sperm quality analysis. Concentration, number of spermatozoa, total and progressive sperm motility parameters, and sperm DNA fragmentation were included. Results are represented in Table 1.

Four common phyla were detected in samples and a total of 74 families. Phyla results are represented in Figure 2.

Then, Bray-Curtis dissimilarity was calculated between animals. Results are represented in Table 2.

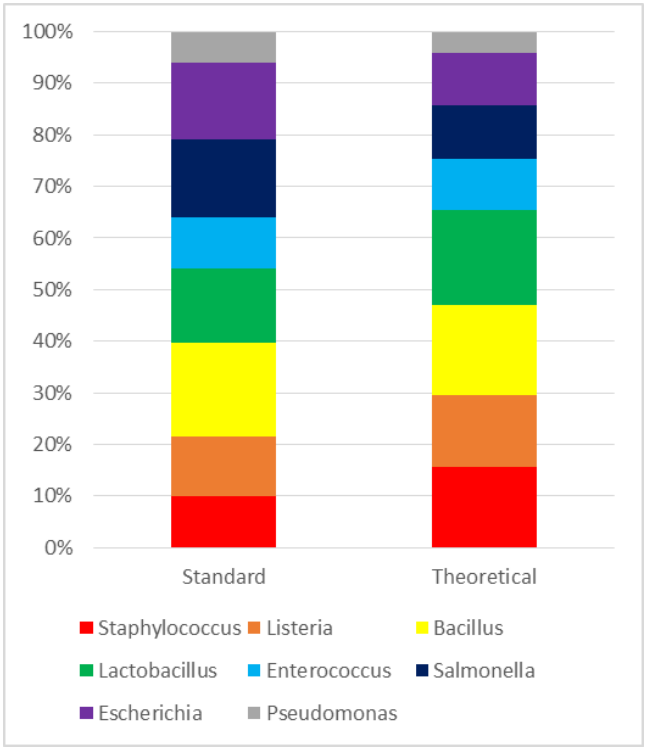

Figure 1. Bacterial composition of standard sample compared to its theoretical composition. Results are expressed as a percentage (%).

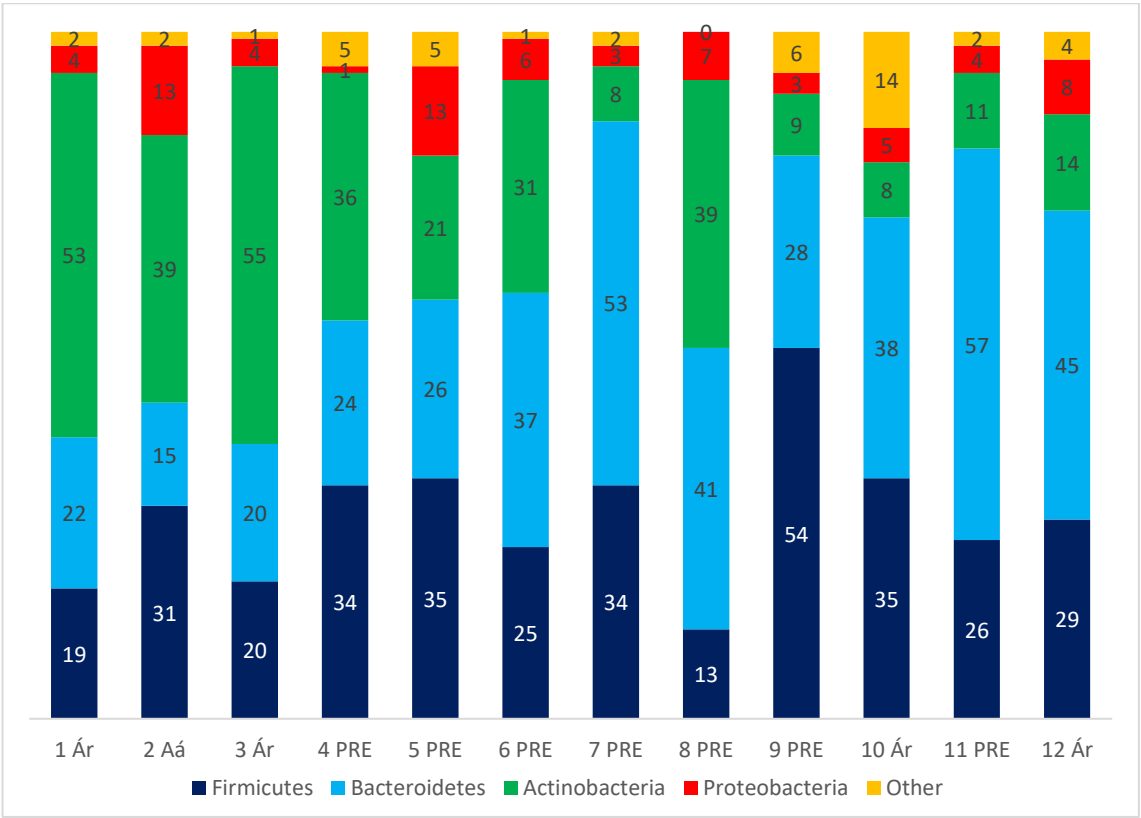

Figure 2. Graphical representation of bacteria the four more abundant phyla in samples. Numbers in X axis represent animals. PRE: Andalusian. Aa: Anglo-Arabian. Ar: Arabian. Numbers inside the bars represent the percentage of abundance of each phyla.

4. Discussion

Our results show that there might be a correlation between some sperm quality parameters and the seminal flora composition of healthy, fertile stallions, in particular, Firmicutes phylum. Although there are some individual differences, the more abundant phyla are common in all animals. Starting with Firmicutes phylum, the literature contains divergent findings regarding the effect of bacterial families on sperm quality. Some authors have highlighted the positive correlation between specific Firmicutes families and good sperm quality. In this regard, *Lactobacillus* gender has been proven to have a protective effect on spermatozoa [13,30–32]. The mechanisms of protection are not fully understood, but they may be related to the antioxidant products exerted by lactobacilli in the extracellular environment [32]. Additionally, the positive effects of lactobacilli supplementation on sperm quality parameters have also been described in humans [19,20], mice [17], and broilers [18]. Stallion semen is not abundant in the *Lactobacillus* genus [10,11], but there are related bacterial families.

However, other authors have found some Firmicutes to have a detrimental effect on sperm parameters. In the literature, we found *Anaerococcus*, a Clostridiales genus, to have a detrimental effect on sperm quality [15,33]. The underlying mechanism needs to be further studied. Another detrimental family is Mycoplasmataceae (specifically, its *Ureaplama* genus) [24,34–36], whose pathogenic activity lies in acrosome damage [35]. This family was not detected in our samples.

A dominant family in fertile stallions is Porphyromonadaceae [11]. According to our results, this family is highly abundant, as it represents almost the whole Bacteroidetes phylum. This family seems to be a natural component in fertile males [15,34]; however, it is not as abundant as it is in horses. Regarding this family, it is necessary to highlight that it is difficult to find in culture-based references, as it is a laborious process to culture bacteria. The other highly dominant family in stallion semen is Corynebacteriaceae [10,11], which has regularly been found in fertile individuals [10,23,37–40]. However, some authors consider it has an opportunistic character [22,33,41]. It has been associated with a higher activity of caspases [22], which is usually linked to apoptosis [42]. Its predominance has also been linked to low motility [43].

Most infertility-related bacteria families belong to the Proteobacteria phylum, particularly to the Gammaproteobacteria class [13,24,35,44]. Enterobacteriaceae is included in this group, which has been found to alter spermatozoa motility [22,35] and the proportion of dead spermatozoa [22]; and Pseudomonadaceae [13,24,35,39,40], which may contain opportunistic pathogenic species [13,45]. In stallions, the presence of the Enterobacteriaceae family typically has a fecal origin [46] and worsens various seminal parameters [22,39,40]. Regarding Pseudomonadaceae, this family has been related to lower values of motility and integrity parameters [23,46,47], while other authors agree to consider it an opportunistic pathogen [40]. However, this family has been regularly found in the semen of fertile stallions [46,48]. The negative impact of these two families has also been found in boars [45]. In our case, Enterobacteriaceae only appeared in one horse (0.19%) and Pseudomonadaceae in just two (0.06% and 0.04%).

Bacteria in stallion semen have long been associated with a detrimental impact on fertility [23] as well as with a lower storage capacity [22]. Our study wanted to show that there may be certain bacterial families that harmoniously dwell in semen.

Regarding the strengths and limitations of our paper, next-generation sequencing is a better tool to characterize the seminal flora, as it overcomes laborious-to-culture bacteria [10,11,28,49]. However, the data process may be more complicated [50]. Finally, we mostly compared our findings with those of experiments carried out on humans, as animal references are extremely scarce. Further studies including a larger number of animals, including subfertile stallions with low sperm quality, are needed in order to find the possible relationship between the seminal microbiome and sperm quality.

5. Conclusions

In conclusion, four common bacterial phyla are present in all the stallions evaluated: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Although proportions vary among individuals, sperm quality values are similar. Further studies are needed to better understand the interactions between seminal flora and sperm quality.

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Institutional Review Board Statement: The Bioethics and Biosafety Committee of the University of Córdoba considers this study is exempt from ethics approval. It considers that it has had no impact on animal welfare because the procedure performed on animals was part of routine animal husbandry and did not involve harm to the animal. The animals used in the study were legally in accordance with the Spanish law for animal welfare (Law 32/07, of 7 November, for the care of animalsin their exploitation, transport, experimentation and slaughter).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

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CAPÍTULO 2.2

Comparative Semen Microbiota Composition of a Stallion in a *Taylorella equigenitalis* Carrier and Non-Carrier State.

Carlota Quiñones-Pérez, Amparo Martínez, Francisco Crespo, José Luis Vega-Pla, 2020. Animals.

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Comparative Semen Microbiota Composition of a Stallion in a *Taylorella equigenitalis* Carrier and Non-Carrier State

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Simple Summary: Contagious equine metritis carriers have become a new cause of concern in horse stud farms. Their detection can result in significant financial loss and force owners to have their animals undergo antibiotic treatment. Current research has not been able to satisfactorily explain the appearance of carriers in agent-free farms. Studies made on microbial flora have given new insights into the diagnosis and treatment of different issues in animal systems. Next-generation sequencing (NGS) is a powerful tool that can draw an accurate picture of microbial flora. Therefore, the aim of this study was to compare the seminal bacterial composition of one stallion before and after being diagnosed with *Taylorella equigenitalis* using NGS. Our results show that the microbial seminal flora visibly changed between the samples analyzed. Corynebacteriaceae, an opportunistic bacterial family, was more common in the infected sample. However, Porphyromonadaceae, a natural component in several tissues, was more abundant in the negative sample. Despite the constraints of a single-case study, these findings can open the door to new therapeutic tools, as flora transplants. Similarly, seminal flora analysis may foresee microbial shifts, letting practitioners take preventive actions before a potential outbreak. Furthermore, these actions would have the extra benefit of reducing the administration of antibiotics to treat an infection.

Abstract: Contagious equine metritis is receiving renewed attention due to the continuous detection of carriers in apparent agent-free farms. Interactions of *Taylorella* with the seminal microflora may be the plausible cause behind these spontaneous changes of the carrier state. Accordingly, the aim of this study was to compare the differences in the seminal microbiome composition of one stallion in the contagious equine metritis carrier state and non-carrier state. Samples were cryopreserved after their extraction. Cell disruption was performed by high-speed homogenization in grinding media. Bacterial families were identified via V3 amplification of the 16S rRNA gene and Ion Torrent sequencing. Only bacterial families with relative abundance above 5% were taken into consideration. The positive sample contained a strong dominance of Corynebacteriaceae (37.75%) and Peptoniphilaceae (28.56%). In the negative sample, the Porphyromonadaceae (20.51%), Bacteroidaceae (19.25%) and Peptoniphilaceae (18.57%) families prevailed. In conclusion, the microbiome seminal composition varies when an individual carries *Taylorella* from when it is free of it. The wider differences were found in the Corynebacteriaceae, Porphyromonadaceae and Bacteroidaceae families. Due to the limitations of a single-case analysis, further studies are needed for a better understanding of the stallion seminal microflora interactions.

Keywords: *Taylorella equigenitalis*; carrier; microbiome; stallion

1. Introduction

Contagious equine metritis (CEM) is a concerning condition in the horse industry, as its presence in livestock reduces fertility soundness in mares and can involve commercial restrictions. Its etiologic agent is *Taylorella equigenitalis*, a bacteria belonging to the Actinobacteria phylum. Stallions host the agent in the distal part of the urethra, becoming a long-timer carrier [1] if they are not submitted to a disinfection standard protocol [2,3].

Symptoms vary in stallions and mares. In mares, the disease manifests with endometritis, cervicitis and vaginitis of variable severity, and it sometimes appears a mucopurulent vaginal discharge. It usually leads to temporary infertility. The recovery is uneventful, but the animal becomes a carrier. There are no symptoms in stallions, but they also become carriers. Official diagnostic methods includes culture and detection of the agent by PCR from swabs taken from predilection sites [4].

Recently, attention has been brought to CEM, as new carriers have been detected in non-symptomatic farms [5]. This has raised questions about the transmission mechanisms of the bacteria [6] or even the effectiveness of diagnostic methods [7]. It has been stated that official methods may not detect low microbial concentration carriers [8,9] or that the nature of this agent makes it difficult to isolate it in culture-based methods [4]. However, next-generation sequencing studies performed in humans have shown that certain seminal microbiome compositions could lower the chance of venereal agents of surviving [10–13].

Our study presents the case of the seminal microbiome shift in a horse that underwent a spontaneous reversion from a *Taylorella equigenitalis* carrier state to a non-carrier one. Therefore, the aim of this study was to compare the differences in the seminal microbiome composition of one stallion in its *Taylorella equigenitalis* carrier and non-carrier state.

2. Materials and Methods

2.1. Sample Collection

The sample collection was performed in the Equine Breeding Centre of the Spanish Army located in Écija under the Chief of Unit authorisation. Animals are handled in accordance with Spanish law for animal welfare (Law 32/07). Animals were not submitted to extra semen extractions for our experiment sample collection nor was their daily workflow interrupted. Samples were not collected for the purpose of the study.

Samples were collected in two different batches. The first batch was composed of semen samples from six stallions. Age ranged from 8 to 18 years old. Samples were collected using an in-line gel-filter Missouri artificial vagina, with mare in oestrus as a teaser. An inner disposable plastic liner was used with each animal to avoid cross-contamination. Animals lived under the same dietary and exercise conditions, with no clinical diseases reported. Official analysis for contagious equine metritis tests [4] were negative in all cases, so animals did not receive any type of treatment. Sperm quality tests were also performed prior to seminal extraction (total volume, gel-free volume, sperm concentration, total motility and progressive motility).

Samples were cryopreserved immediately after their extraction. All samples were then analysed using next-generation sequencing following the process described below. After a beta diversity study, we found out that one of the samples was manifestly different.

This finding led to the collection of a second batch six months afterwards. It was composed of semen samples from 14 different stallions, including the animals from the first batch. Age ranged from 6 to 18 years old in this case. The collection process, environmental conditions and analysing methods post-collection were the same as the ones for the first batch.

2.2. Control Sample

For each of the batches, a pattern sample (ZymoBIOMICS Microbial Community Standard®, Zymo Research, Irvine, CA, USA) was included in order to evaluate the quality of the DNA extraction and its amplification.

2.3. DNA Extraction

DNA extraction was performed using a ZymoBIOMICS® DNA Miniprep kit (Zymo Research, Irvine, CA, USA) commercial kit. Samples were previously submitted to a combination of mechanic and enzymatic-digestion cell disruption as described by Bag [14]. Then, DNA extraction was performed following the manufacturer's instructions.

2.4. Next-Generation Sequencing Analysis

Amplicons were obtained using an Ion 16S Metagenomics® kit (Thermo Fisher, Waltham, MA, USA). This kit characterizes five different sets of 16S hypervariable regions, V2, V3, V4, V67 and V8. The library was constructed with an Ion Plus Fragment Library kit and amplicons were labelled with an Xpress™ Barcode Adapters 1–16 kit. Samples were then pooled using Ion PGM® (Thermo Fisher, Waltham, MA, USA), HiQ Sequencing kit® (Thermo Fisher, Waltham, MA, USA), Ion 316 v2 BC® chip (Thermo Fisher, Waltham, MA, USA) and sequenced using the Ion 16S™ Metagenomics Workflow in Ion Reporter™ Software (Thermo Fisher, Waltham, MA, USA).

Data analysis was performed in the Ion Reporter server system (https://ionreporter.thermofisher.com/ir/secure/home.html). β-diversity was calculated using Bray-Curtis dissimilarity analysis. Hypervariable region V3 was chosen for taxonomic bacterial identification, as it has been suggested to detect a wider range of bacterial species [15].

2.5. Statistical Analysis

Similarity of samples was compared calculating the Bray-Curtis dissimilarity index according to the following formula:

$$BC_{a,b} = 1 - \frac{2C_{a,b}}{S_a + S_b}$$

where a and b are two samples; S_a is the total number of specimens counted on site a; S_b is the total number of specimens counted on site b; and $C_{a,b}$ is the sum of the lesser counts of each bacterial family found in both sites. Value of 0 means the two samples have the same composition, and 1 means the two samples do not share any species.

Bray-Curtis indexes were then compared using Pearson's χ^2 test. Significant values were consider when $p < 0.1$.

3. Results

The resulting composition of the quality control had a minor variation (±5.6% maximum) comparing to the composition provided by the manufacturer.

3.1. Microbial Community Structure Differences Between Batches

Beta diversity analysis was used to compare microbial community structure between seminal samples of different stallions at a sampling point [16]. In the first sample batch, we observed one outlier within the group (Figure 1).

We calculated the Bray-Curtis (B-C) dissimilarity index to determine the degree of similarity among samples. Significant differences were found between the *Taylorella equigenitalis* carrier indexes and the rest of indexes ($p < 0.1$). Results are represented in Table 1.

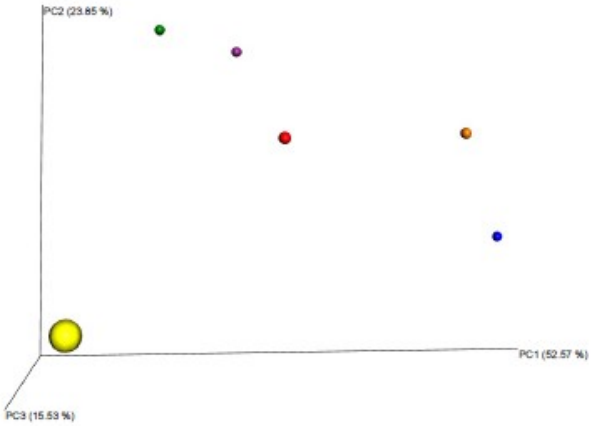

Figure 1. Family β-diversity using Bray-Curtis dissimilarity analysis of the first six-sample batch. There is a group of five samples in the upper-right area of the graph, while one sample is isolated on the down-left corner. The highlighted dot corresponds to *T equigenitalis* positive sample. Significant differences were found between the *Taylorella equigenitalis* carrier composition and the rest of samples ($p < 0.1$).

Table 1. Bray-Curtis dissimilarity indexes for the first batch.

Stallions	1	2	3	4	5	TE+
1	-	-	-	-	-	-
2	0.40	-	-	-	-	-
3	0.57	0.37	-	-	-	-
4	0.51	0.55	0.66	-	-	-
5	0.53	0.51	0.63	0.37	-	-
TE+ *	0.64	0.66	0.64	0.78	0.58	-

Values near 0 means the two samples have the same composition. Values near 1 means the two samples do not share any species. Numbers represent animals. TE+: *Taylorella equigenitalis* carrier. * group of samples with $p < 0.1$.

This sample was reanalysed six months later following the same method within a second batch of 14 samples. This time, the former outlier sample belonged to the main group (Figure 2). Then, we also calculated the B-C dissimilarity index for this batch (Table 2). This time, no significant differences were found between samples ($p > 0.1$).

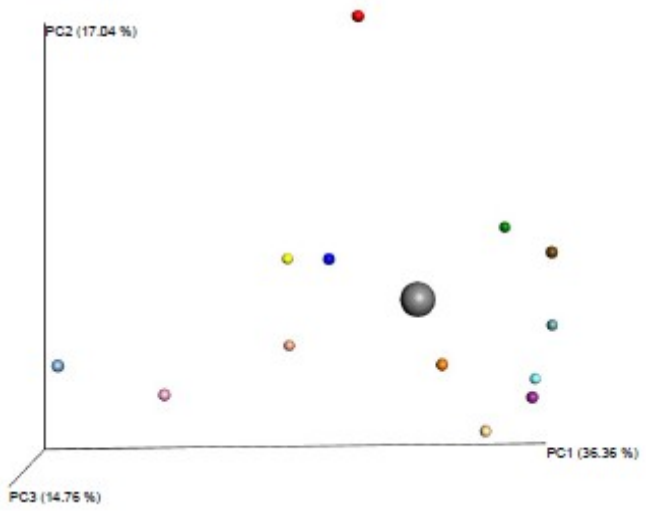

Figure 2. Family β-diversity using Bray-Curtis dissimilarity analysis of the second 14-sample batch. The highlighted dot corresponds to *T equigenitalis* negative sample. No significant differences were found between the *Taylorella equigenitalis* negative sample composition and the rest of samples ($p > 0.1$).

Table 2. Bray-Curtis dissimilarity indexes for the second batch.

Stallions	1	2	3	4	5	6	7	8	9	10	11	12	13	TE−
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	0.39	-	-	-	-	-	-	-	-	-	-	-	-	-
3	0.57	0.37	-	-	-	-	-	-	-	-	-	-	-	-
4	0.51	0.54	0.65	-	-	-	-	-	-	-	-	-	-	-
5	0.54	0.51	0.61	0.38	-	-	-	-	-	-	-	-	-	-
6	0.49	0.54	0.62	0.44	0.25	-	-	-	-	-	-	-	-	-
7	0.53	0.42	0.42	0.67	0.54	0.51	-	-	-	-	-	-	-	-
8	0.54	0.48	0.59	0.38	0.22	0.12	0.53	-	-	-	-	-	-	-
9	0.45	0.47	0.51	0.33	0.15	0.29	0.44	0.34	-	-	-	-	-	-
10	0.60	0.60	0.70	0.14	0.30	0.39	0.68	0.32	0.47	-	-	-	-	-
11	0.52	0.53	0.58	0.49	0.36	0.30	0.52	0.28	0.34	0.42	-	-	-	-
12	0.53	0.39	0.48	0.60	0.47	0.55	0.54	0.55	0.49	0.60	0.54	-	-	-
13	0.45	0.34	0.50	0.61	0.47	0.50	0.34	0.53	0.37	0.61	0.51	0.47	-	-
TE−	0.46	0.46	0.53	0.65	0.37	0.45	0.48	0.45	0.30	0.56	0.34	0.47	0.38	-

Values near 0 means the two samples have the same composition. Values near 1 means the two samples do not share any species. Numbers represent animals. TE−: stallion cleared of *Taylorella equigenitalis*.

3.2. Taxonomic Composition of Seminal Samples

A general taxonomic composition of batches 1 and 2 is represented in Figure 3. The most abundant phyla were Firmicutes and Bacteroidetes in all cases, except for the *Taylorella equigenitalis* carrier (TE+). The two most dominant phyla for TE+ were Firmicutes and Actinobacteria.

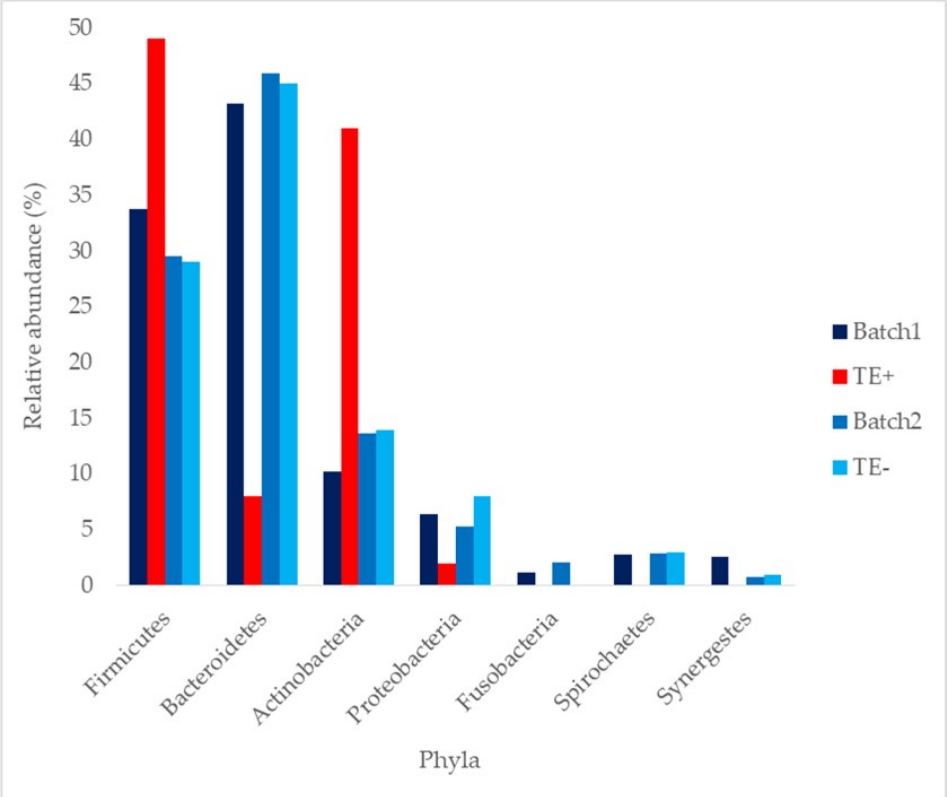

Figure 3. Mean phylum taxonomic composition of batch 1, TE+, batch 2 and TE−. It is observed how TE+ phylum composition highly varies compared with batch 1, batch 2 and TE−. TE+: *Taylorella equigenitalis* carrier. TE−: stallion cleared of *Taylorella equigenitalis*. Results are expressed as percentage (%).

Mean values of batch 1 were 36.33% for Firmicutes phylum, 37.33% for Bactoroidetes; 15.33% for Actinobacteria; 5.50% for Proteobacteria; 1.50% for Fusobacteria; 2.00% for Spirochaetes; and 2.23% for

Synergestes. Mean values of batch 2 were 29.50% for Firmicutes phylum, 45.86% for Bactoroidetes; 13.71% for Actinobacteria; 5.27% for Proteobacteria; 1.86% for Fusobacteria; 2.91% for Spirochaetes; and 0.89% for Synergestes.

3.3. Comparative Taxonomic Composition of TE+ and TE−

Figure 4 shows relative abundance at the family level in samples TE+ and TE− (stallion cleared of *Taylorella equigenitalis*). Only common families with a relative abundance above 5% are represented.

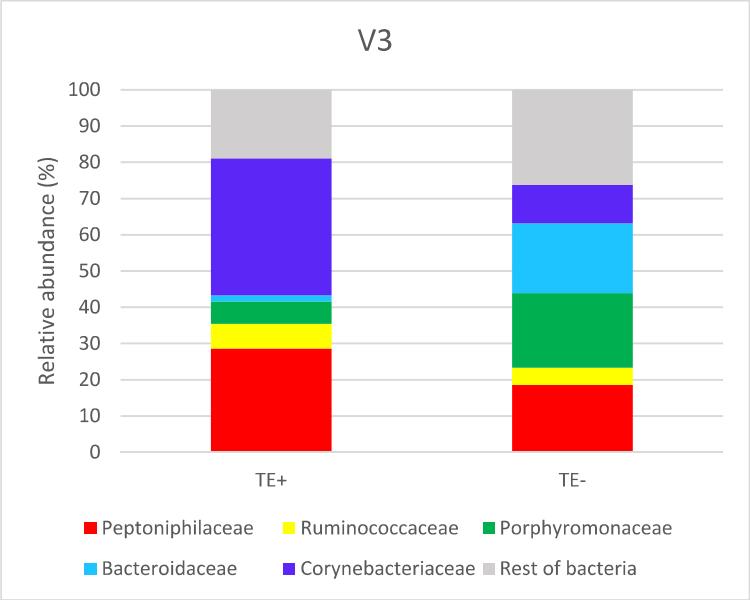

Figure 4. Comparative taxonomic composition of TE+ and TE−. The results are expressed as percentages (%). TE+ corresponds to *T equigenitalis* positive sample and TE− to the stallion cleared of *T equigenitalis*. Only common families with a relative abundance above 5% are included separately.

Taxonomic composition classification revealed that TE+ contained a 0.02% of the Alcaligenaceae family (represented by *Taylorella equigenitalis* species), while TE− showed no remainders of the *Taylorella equigenitalis* species nor its complete family.

As far as general bacterial composition is concerned, TE+ presents a microbial diversity of 32 families, with a strong dominance of Corynebacteriaceae (up to 37.75%) and Peptoniphilaceae (28.56%). The percentage of the following most common bacteria, Ruminococcaceae, only reaches 6.82%. Regarding TE−, it contains 31 different bacteria families, with a dominance of Porphyromonadaceae (20.51%), Bacteroidaceae (19.25%) and Peptoniphilaceae (18.57%). Corynebacteriaceae is the fourth most abundant family with a 10.64% presence. The family composition of TE+ also contrasts with the composition of the rest of samples. Thus, mean values for the families are represented in Table 3.

Table 3. Mean values for Porphyromonadaceae, Bacteroidaceae, Prevotellaceae and Corynebacteriaceae families of the TE+ sample and the first batch of samples without TE+.

Bacterial families	TE+	Rest of Samples
Porphyromonadaceae	6.24	30.07 ± 16.18
Bacteroidaceae	1.68	0.80 ± 0.60
Prevotellaceae	0.08	10.72 ± 6.43
Corynebacteriaceae	37.75	7.87 ± 2.38

Results are expressed as percentage (%) and mean ± standard error of the mean. TE+ corresponds to *Taylorella equigenitalis* positive sample.

4. Discussion

Our results show there is a different microbiome composition when an animal carries *Taylorella equigenitalis* than when it is free of the agent. It has already been proven that some microflora changes in the equine digestive tract can favor the growth of some pathogens [17–19]. In the genital tract, it has also been hypothesized that infections or strange agents may produce changes in the environment around them and, therefore, favor some bacteria families to grow while hindering others [20].

In our case, Actinobacteria phylum highly differs between TE+ and TE− (41% vs. 14%, respectively). The main family of this phylum, Corynebacteriaceae, has been regularly described as a normal component of seminal flora in humans [20–25] as well as in stallions [26–32]. Meanwhile, other authors maintain that it holds an opportunistic nature, and even some have related its presence with a higher caspases activity [33].

Concerning Bacteroidetes phylum, the three most common families have largely varied from the carrier situation to the non-carrier one. Mändar have already pointed out that the presence of Prevotellaceae family alone or combined with Porphyromonadaceae are associated with a higher rate of reproductive inflammatory conditions [23]. Our results may contradict this finding, as they are the predominant group in the non-carrier state, especially Porphyromonadaceae. It is relevant to say that it is not easy to compare the effect of the Bacteroidetes phylum with previous studies, as it is a difficult to culture group and most studies utilized cultured-based methods.

In Mändar [23], it was possible to divide a population in three groups according to their seminal microbiome composition, that time relating the results with their seminal quality. In our case, the division would create two groups: the Corynebacteriaceae family predominant group, representing the contagious equine metritis carriers; and the Bacteroidetes phylum predominant group for the non-carrier stallions. It would be of the utmost importance to dig deeper into the mechanisms underlying these differences, as understanding the seminal microbiome composition can open the door to a future diagnostic or even prophylactic tools.

Finally, it is necessary to indicate some strengths and limitations of this study. Next-generation sequencing overcomes limitations of culture-based diagnostic methods. It can detect fastidious-to-cultivate genera and it is less affected by contamination [34]. In spite of that, NGS also has limitations. For example, differential amplification of primers [35], DNA extraction or interpretation of data [12]. Another limitation of our study would be sample size.

5. Conclusions

The microbiome seminal composition varies when an individual carries *Taylorella equigenitalis* from when it is free of the agent. The wider differences were found in the Corynebacteriaceae family, increased in the carrier case; and the Porphyromonadaceae and Bacteroidaceae families, increased in the non-carrier case. Besides, we have observed it is possible to detect CEM carriers using NGS. However, due to the limitations of a one-subject case report, further studies are needed in order to completely comprehend the interactions that occur in the stallion seminal microflora.

Author Contributions: Conceptualization, C.Q.-P.; formal analysis, C.Q.-P.; writing—original draft preparation, C.Q.-P.; writing—review and editing, A.M., F.C. and J.L.V.-P.; supervision, A.M., F.C. and J.L.V.-P.; visualization, C.Q.-P. All authors have read and agreed to the published version of the manuscript.

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CONCLUSIONES

CONCLUSIONES

Según los objetivos propuestos y los resultados obtenidos, se puede concluir lo siguiente:

Conclusión 1. Existen diferencias entre la integridad del ADN espermático y la fertilidad *in vivo* del semen refrigerado durante la temporada reproductiva, obteniendo una menor fragmentación y mayores tasas de gestación en primavera. Ello respalda la recomendación de emplear el semen refrigerado y realizar la inseminación artificial durante el inicio de la temporada reproductiva. Además, los sementales con mayor porcentaje de fragmentación espermática mostraron unos niveles de fertilidad más bajos a lo largo de la temporada reproductiva.

Conclusión 2. El análisis del ADN espermático equino se ajusta mejor a un modelo matemático polinómico de segundo grado, siendo la primavera la mejor estación para recoger y elaborar dosis de semen refrigeradas en cuanto al mantenimiento de la integridad del ADN espermático equino se refiere.

Conclusión 3. La microbiota seminal equina está formada principalmente por bacterias de las familias Porphyromonadaceae, Peptoniphilaceae, Corynebacteriaceae y Prevotellaceae, existiendo una alta variabilidad interindividual. Estos resultados concuerdan con los hallados en otros estudios, pero no con otras especies animales. También se concluye que la variabilidad entre individuos no se relaciona con cambios en los valores de calidad espermática.

Conclusión 4. La composición de la microbiota seminal del caballo varía entre el estado de portador y no portador de *Taylorella equigenitalis*. Las mayores diferencias se encontraron en la familia Corynebacteriaceae, aumentada en el caso del estado portador; y las familias Porphyromonadaceae y Bacteroidaceae, aumentadas en el estado no portador, Además, se ha observado que es posible detectar el estado portador del agente causante de la metritis equina contagiosa usando secuenciación de nueva generación.

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ÍNDICES DE CALIDAD

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Primera publicación

- Título: *Seasonal variations in sperm DNA fragmentation and pregnancy rates obtained after artificial insemination with cooled-stored stallion sperm throughout the breeding season (spring and summer).*
- Autores (p.o. de firma): *F. Crespo, C. Quiñones-Pérez, I. Ortiz, M. Diaz-Jimenez, C. Consuegra, B. Pereira J. Dorado, M. Hidalgo*
- Revista (año, vol., pag.): *Theriogenology (2020), Vol. 148, 89-94*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2020)*
- Área temática en la Base de Datos de referencia: *Veterinary Sciences*
- Índice de impacto de la revista en el año de publicación del Artículo: 2,740
- Lugar que ocupa/N.º de revistas del Área temática: *21/146 (Q1)*

Segunda publicación

- Título: *Comparison of different mathematical models to assess seasonal variations in the longevity of DNA integrity of cooled-stored stallion sperm.*
- Autores (p.o. de firma): *I. Ortiz, C. Quiñones-Pérez, M. Hidalgo, C. Consuegra, M. Diaz-Jimenez, , J. Dorado, J. L. Vega-Pla, F. Crespo*
- Revista (año, vol., pag.): *Andrologia (2020), Vol. 52, e13545.*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2020)*
- Área temática en la Base de Datos de referencia: *Andrology*
- Índice de impacto de la revista en el año de publicación del Artículo: 0,97
- Lugar que ocupa/N.º de revistas del Área temática: *6/8 (Q3)*

Tercera publicación

- Título: *Characterization of the seminal bacterial microbiome of healthy, fertile stallions using next-generation sequencing.*
- Autores (p.o. de firma): *C. Quiñones-Pérez, M. Hidalgo, I. Ortiz, F. Crespo, J. L. Vega-Pla*
- Revista (año, vol., pag.): *Animal Reproduction (2021), Vol. 18, e20200052*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2020)*

- Área temática en la Base de Datos de referencia: *Agriculture, Dairy and Animal Science*
- Índice de impacto de la revista en el año de publicación del Artículo: 1,807
- Lugar que ocupa/N.º de revistas del Área temática: *30/63 (Q2)*

Cuarta publicación

- Título: *The Semen Microbiome and Semen Parameters in Healthy Stallions*
- Autores (p.o. de firma): *C. Quiñones-Pérez, A. Martínez, I. Ortiz, F. Crespo, J. L. Vega-Pla*
- Revista (año, vol., pag.): *Animals (2022), Vol. 12, 534*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2020)*
- Área temática en la Base de Datos de referencia: *Veterinary Science*
- Índice de impacto de la revista en el año de publicación del Artículo: 2,752
- Lugar que ocupa/N.º de revistas del Área temática: 19*/146 (Q1)*

Quinta publicación

- Título: *Comparative Semen Microbiota Composition of a Stallion in a Taylorella equigenitalis Carrier and Non-Carrier State*
- Autores (p.o. de firma): *C. Quiñones-Pérez, A. Martínez, F. Crespo, J. L. Vega-Pla*
- Revista (año, vol., pag.): *Animals (2020), Vol. 10, 868*
- Base de Datos Internacional o Nacional en las que está indexada: *Journal of Citation Reports, JCR (2020)*
- Área temática en la Base de Datos de referencia: *Veterinary Science*
- Índice de impacto de la revista en el año de publicación del Artículo: 2,752
- Lugar que ocupa/N.º de revistas del Área temática: *19/146 (Q1)*

PRODUCCIÓN CIENTÍFICA

PRODUCCIÓN CIENTÍFICA

Otras aportaciones científicas derivadas de la Tesis Doctoral:

Contribuciones a Congresos Nacionales

- "Mapa del microbioma seminal del caballo"

  C. Quiñones Pérez. VI Congreso Científico de Investigadores en Formación de la Universidad de Córdoba, Córdoba (España). 18-19 enero 2018. Tipo: comunicación oral

- "Análisis descriptivo del microbioma seminal del caballo"

  C. Quiñones Pérez, F. Crespo, A. de Santiago López de Uralde, J.L. Vega Pla. III Congreso de Sanidad Militar, Santander (España). Febrero 2018. Tipo: comunicación oral.

Contribuciones a congresos Internacionales con *meeting-abstracts* publicados en Revistas Indexadas en el JCR

- "Fine-tuning sperm DNA fragmentation dynamics over seasons".

  C. Quiñones-Pérez, F. Crespo, J.L. Vega-Pla, J. Gosálvez, J. Dorado, M. Hidalgo, I. Ortiz. 21st Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). Berna (Suiza), 24-26 agosto 2017. Tipo: Comunicación oral.

  Revista: Reproduction in Domestic Animals, Vol 52 (s3): 29 - 34.

- "First description of the seminal microbiome in healthy stallions and donkeys".

  C. Quiñones-Pérez, F. Crespo, I. Ortiz, J.L. Vega-Pla. 22nd Annual Conference of European Society of Domestic Animals Reproduction (ESDAR). Córdoba (España), 27-29 septiembre 2018. Tipo: póster.

Revista: Reproduction in Domestic Animals, Vol 54 (s3): 53 - 56.

- "Differential seminal microbiome composition in contagious equine metritis carrier and non-carrier stallions".

C. Quiñones-Pérez, F. Crespo, A. de Santiago López de Uralde, I. Ortiz, J.L. Vega-Pla. 24th Annual Conference of European Society of Domestic Animals Reproduction 2021 (ESDAR). Virtual Congress, 11-16 octubre 2021. Tipo: póster.

Revista: Reproduction in Domestic Animals, Vol 57 (s1): 43-129.