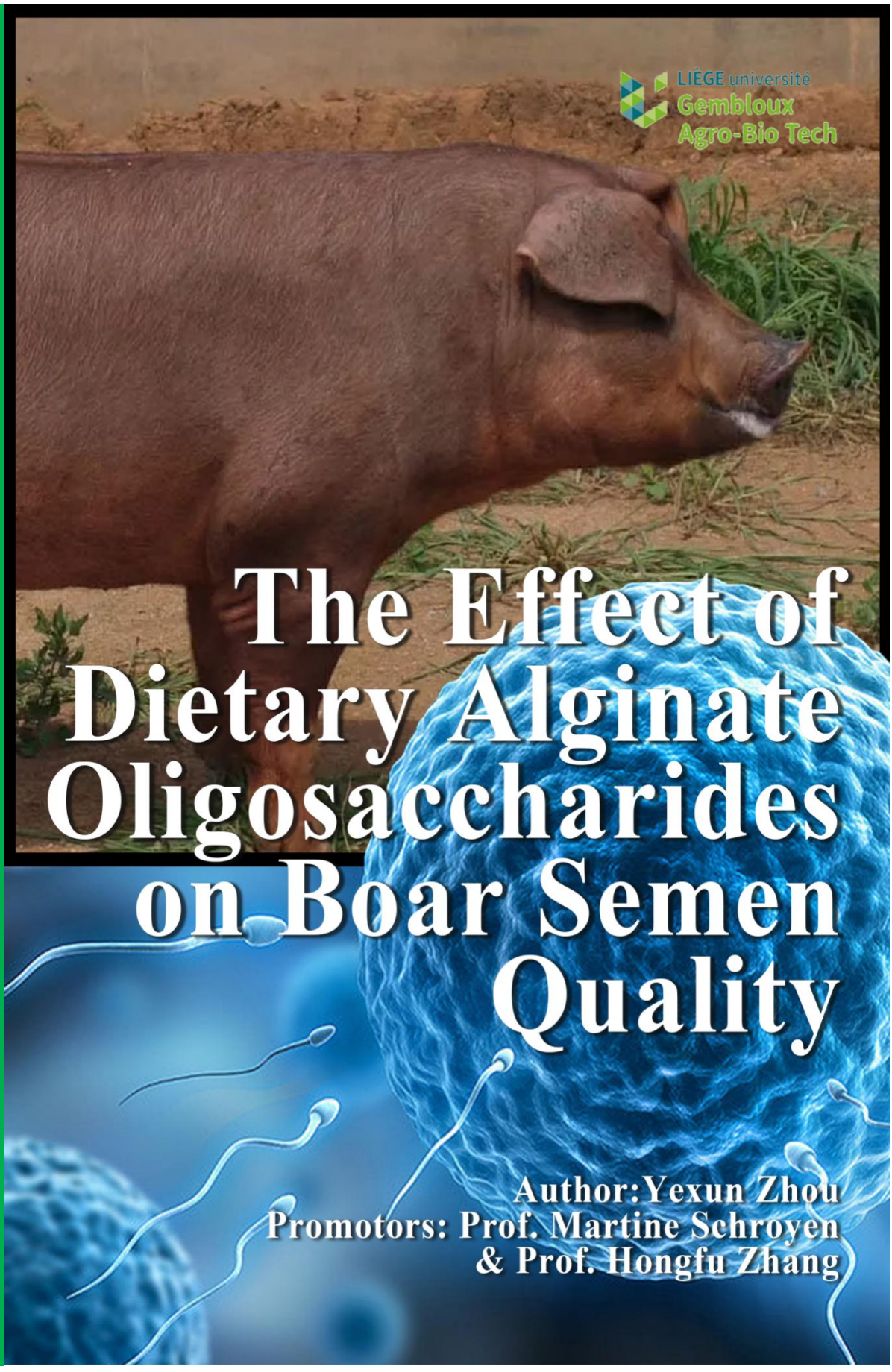

The Effect of Dietary Alginate Oligosaccharides on Boar Semen Quality

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Dissertation originale présentée (ou essai présenté) en vue de l'obtention du grade de doctorat en sciences agronomiques et ingénierie biologique

Promoteurs: Prof. Martine Schroyen & Prof. Hongfu Zhang

Année civile: 2024

Abstract

China is not only the largest pork producer in the world, but also the largest pork consumer. In China, the annual production of pork exceeds 50 million tons, accounting for more than 40% of the worldwide production. Nevertheless, compared with European countries such as The Netherlands and Denmark, the swine raising technology of China is still falling behind. There are many reasons for this delay in production, of which boar semen quality is a particularly critical one. The reproductive capacity of boars not only directly determines the economic benefits, but also affects the reproductive performance of sows in large-scale pig farms. The raw semen of boars should meet the standards of artificial insemination, so that a normal conception rate of sows can be guaranteed. Therefore, it is very important to improve boar semen quality to further ameliorate reproductive performance in swine.

Maintaining the sexual desire of a boar, and increasing its sperm volume, sperm concentration and sperm motility are important objectives for animal husbandry workers in large scale boar studs. There are many factors that affect boar semen quality, such as breeding management, disease status, nutritional level, environment, and age. On the one hand, in the south of China, the summer climate is represented by high temperatures and high humidity levels, especially between June and September, and the boar semen quality and utilization rate decrease significantly in that period. On the other hand, as the age of a boar increases, the semen quality will gradually decrease as well, which results in an increased elimination rate of boars in large-scale boar studs. With the ban on antibiotics, more and more research has been shifted towards natural plant extracts. Alginate oligosaccharide, as a natural plant extract, has multiple biological functions such as immune regulation, and it has antioxidant, anti-inflammatory and even anti-cancer properties. However, the regulation mechanism of AOS on the semen quality of boars is not clear.

In a first experiment in this PhD, it was demonstrated that AOS supplementation improved sperm motility significantly ($P<0.05$). Advantageous blood and sperm metabolites changed. Meanwhile, the gut microbiota diversity changed at both phylum and genus level, and the relative abundance of beneficial microbes improved, while the relative abundance of harmful bacteria reduced. Therefore, AOS improved semen quality by affecting gut microbiota and metabolites in blood and sperm.

In a second experiment in this PhD, everyday temperature and humidity were recorded during the experimental period. The Temperature-Humidity index (THI) was also calculated to proof the boars were in the heat stress condition. AOS significantly improved sperm motility ($P<0.001$) and sperm concentration ($P<0.05$). AOS supplementation also improved the sperm metabolites, changed the composition of gut microbiota, changed the relative abundance of beneficial bacteria, reduced the relative abundance of harmful bacteria, and improved the amount of short chain fatty acids. Proteomic results showed that AOS improved proteins related to spermatogenesis, while decreasing heat shock protein 70 ($P<0.05$) and heat shock protein 90 ($P<0.01$). Therefore, AOS can be used as a feed additive to improve semen quality of boars to enhance reproductive performance under heat stress.

A third experiment focused on AOS in an extended service lifespan context. The results showed that in older boars AOS supplementation significantly improved sperm motility ($P<0.05$) and sperm validity rate ($P<0.001$), and significantly reduced the abnormal sperm rate ($P<0.01$) as well. At the same time, AOS significantly improved the testosterone levels in the blood ($P<0.01$) induced the presence of beneficial metabolites such as adrenic acid ($P<0.05$) and succinic acid ($P<0.05$), and significantly reduced harmful substances such as dibutyl phthalate ($P<0.05$). AOS supplementation changed the composition of gut microbiota, altered the relative abundance of beneficial bacteria, and reduced the relative abundance of harmful bacteria, as well as rise the level of short chain fatty acids, similar as was observed in the other two experiment on younger boars. Therefore, AOS boosted semen quality of aging boars by improving the intestinal microbiota and sperm metabolome. AOS can thus be used as a feed additive to solve the problem of high elimination rate in large scale boar studs.

In summary, this PhD thesis focused on the boar sperm metabolome, sperm proteins and gut microbiota in improving sperm parameters such as sperm motility, sperm concentration, and abnormal sperm rate. The PhD explores the effects of AOS on boar semen quality in different conditions (under heat stress and in aging boars), and provides a theoretical basis for AOS using in swine industry as feed additives.

Key words: Alginate oligosaccharide, semen quality, gut microbiota, sperm metabolites, sperm protein, boars

Résumé

La Chine est non seulement le plus grand producteur de porc au monde, mais aussi le plus grand consommateur de porc. En Chine, la production annuelle de viande de porc dépasse les 50 million de tonnes, soit plus de 40% de la production mondiale. Néanmoins, par rapport à des pays européens comme les Pays-Bas et le Danemark, la technologie chinoise d'élevage porcin est toujours à la traîne. De nombreuses raisons expliquent ce retard dans la production, parmi lesquelles la qualité de la semence de verrat est particulièrement critique. La capacité de reproduction des verrats détermine non seulement directement les avantages économiques, mais affecte également les performances de reproduction des truies dans les élevages porcins à grande échelle. La semence brute des verrats doit répondre aux normes de l'insémination artificielle, afin de garantir un taux de conception normal des truies. Par conséquent, il est très important d'améliorer la qualité du sperme des verrats pour améliorer encore davantage les performances de reproduction chez le porc.

Maintenir le désir sexuel d'un verrat et augmenter son volume de sperme, sa concentration et sa motilité sont des objectifs importants pour les travailleurs de l'élevage dans les élevages de verrats à grande échelle. De nombreux facteurs affectent la qualité du sperme des verrats, tels que la gestion de l'élevage, l'état sanitaire, le niveau nutritionnel, l'environnement et l'âge. D'une part, dans le sud de la Chine, le climat estival est caractérisé par des températures et des niveaux d'humidité élevés, en particulier entre juin et septembre, et la qualité et le taux d'utilisation du sperme de verrat diminuent considérablement pendant cette période. D'autre part, à mesure que l'âge d'un verrat augmente, la qualité du sperme diminue également progressivement, ce qui entraîne une augmentation du taux d'élimination des verrats dans les élevages de verrats à grande échelle. Avec l'interdiction des antibiotiques, de plus en plus de recherches se sont orientées vers les extraits naturels de plantes. L'oligosaccharide d'alginate, en tant qu'extrait naturel de plante, a de multiples fonctions biologiques telles que la régulation immunitaire, et possède des propriétés antioxydantes, anti-inflammatoires et même anticancéreuses. Cependant, le mécanisme de régulation de l'AOS sur la qualité du sperme des verrats n'est pas clair.

Dans une première expérience de cette thèse, il a été démontré que la supplémentation en AOS améliorait significativement la motilité des spermatozoïdes ($P<0,05$). Les métabolites bénéfiques du sang et du sperme ont augmenté. Pendant ce temps, la diversité du microbiote intestinal a changé au niveau du phylum et du genre, et l'abondance relative des microbes bénéfiques a augmenté, tandis que l'abondance relative des bactéries nocives a diminué. Par conséquent, l'AOS a amélioré la qualité du sperme en affectant le microbiote intestinal et les métabolites du sang et du sperme.

Dans une deuxième expérience de cette thèse, la température et l'humidité quotidiennes ont été enregistrées pendant la période expérimentale. L'indice

température-humidité (THI) a également été calculé pour prouver que les verrats étaient dans des conditions de stress thermique. L'AOS a amélioré de manière significative la motilité des spermatozoïdes ($P<0,001$) et la concentration des spermatozoïdes ($P<0,05$). La supplémentation en AOS a également amélioré les métabolites du sperme, modifié la composition du microbiote intestinal, augmenté l'abondance relative des bactéries bénéfiques, réduit l'abondance relative des bactéries nocives et augmenté la quantité d'acides gras à chaîne courte. Les résultats protéomiques ont montré que l'AOS augmentait les protéines liées à la spermatogenèse, tout en diminuant la protéine de choc thermique 70 ($P<0,05$) et la protéine de choc thermique 90 ($P<0,01$). Par conséquent, l'AOS peut être utilisé comme additif alimentaire pour améliorer la qualité du sperme des verrats afin d'améliorer les performances de reproduction sous stress thermique.

Une troisième expérimentation s'est concentrée sur l'AOS dans un contexte de durée de vie prolongée. Les résultats ont montré que chez les verrats plus âgés, la supplémentation en AOS améliorait significativement la motilité des spermatozoïdes ($P<0,05$) et le taux de validité des spermatozoïdes ($P<0,001$), et réduisait également de manière significative le taux de spermatozoïdes anormaux ($P<0,01$). Dans le même temps, l'AOS a augmenté de manière significative les taux de testostérone dans le sang ($P<0,01$), a induit la présence de métabolites bénéfiques tels que l'acide adrénique ($P<0,05$) et l'acide succinique ($P<0,05$), et a considérablement réduit les substances nocives telles que phtalate de dibutyle ($P<0,05$). La supplémentation en AOS a modifié la composition du microbiote intestinal, augmenté l'abondance relative des bactéries bénéfiques et réduit l'abondance relative des bactéries nocives, tout en augmentant le niveau d'acides gras à chaîne courte, comme cela a été observé dans les deux autres expériences sur des verrats plus jeunes. Par conséquent, l'AOS a amélioré la qualité du sperme des verrats vieillissants en améliorant le microbiote intestinal et le métabolome du sperme. L'AOS peut ainsi être utilisé comme additif alimentaire pour résoudre le problème du taux d'élimination élevé dans les élevages de verrats à grande échelle.

En résumé, cette thèse de doctorat s'est concentrée sur le métabolome du sperme de verrat, les protéines du sperme et le microbiote intestinal dans l'amélioration des paramètres du sperme tels que la motilité des spermatozoïdes, la concentration des spermatozoïdes et le taux anormal de spermatozoïdes. La thèse explore les effets de l'AOS sur la qualité du sperme de verrat dans différentes conditions (sous stress thermique et chez les verrats vieillissants) et fournit une base théorique pour l'utilisation de l'AOS dans l'industrie porcine comme additif alimentaire.

Mots clés : Oligosaccharide d'alginate, qualité du sperme, microbiote intestinal, métabolites du sperme, protéine du sperme, verrats

Acknowledgments

I would like to express my heartfelt gratitude to everyone who have provided me with guidance, encouragement, and support throughout my doctoral journey. I am truly grateful for all the help that I have received.

First and foremost, I want to sincerely thank my doctoral supervisors, Prof. Martine SCHROYEN and Prof. Hongfu ZHANG. Your expertise, rigorous academic approach, dedicated guidance and patience have greatly enriched my knowledge and skills. Your unwavering support have kept me on track in my research endeavors. I am deeply grateful for your patience, guidance, and selfless dedication.

Furthermore, I would like to thank for my jury members of my dissertation defense committee (Prof. Jérôme Bindelle, Prof. Jean-Luc Hornick, Prof. Véronique Delcenserie, and Dr. Alice Van den Broeke). Your valuable feedback and suggestions have contributed to the improvement and depth of my research work. I am thankful for your meticulous review and guidance, which allowed me to critically examine my research findings from different perspectives.

I would also want to express my gratitude to thank the lab mates in CAAS, especially Mr. Zeou Wei, you really helped me a lot not only in my lab experiments but also in drawing figures, we were also postgraduate classmates, I really wish you can finish your PhD journey very smoothly. Mr. Xiaohong Wang, you are really a good football teammate, when I felt depressed, you always companied me, and then we could go to the playground to play football together. During the three years of research in Beijing, we have learned from each other and encouraged each other. We have spent an unforgettable time together, including Dr. Guoqi Dang, Dr. Xiaobin Wen, Dr. Chengzeng Luo, Dr. Hui Han. All of you leave a good memory in my mind.

Besides, special thanks to Cécile Russo, Chi Ren, Wanjie Yu, Hang Shu during my stay at the lab of Precision Livestock and Nutrition Unit of Gembloux Agro-Bio tech.

Additionally, sincere gratitude is given to the China Scholar Council (CSC) for the financial support.

Finally, I would like to offer a special thank you to my family. I appreciate my parents Prof. Yuanhang Zhou and Prof. Hongshuai Zhao, for your unwavering support and encouragement. Your love and understanding have been the driving force behind my continuous efforts and aspirations. Thank you for your ongoing support and care. Thanks to my brother Prof. Yang Gao, you give me so many suggestions in my PhD work and guide me a lot as well. What is the most important is that I would like to thank my wife Dr. Huan Leng, during my PhD journey, you give me all your love, understanding and patience, I am not sure if I can complete my PhD journey without your support, so loving you forever.

Yexun Zhou
July, 2024 in Gembloux, Belgium

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List of abbreviations

AOS: Alginate oligosaccharides
CASA: Computer-assisted sperm analysis
T-AOC: Total antioxidant capacity
SOD: Superoxide dismutase
GSH-Px: Glutathione peroxidase
CAT: Catalase
MDA: Malondialdehyde
GSH: Glutathione
IHF: Immunofluorescence staining
SPAG11: Sperm associated antigen 11
SPACA1: Sperm acrosome membrane-associated protein 1
HSP 70: Heat shock protein 70
HSP 90-α: Heat shock protein 90-α
HSP 90-β: Heat shock protein 90-β
PKA: Protein kinase A
ROS: Radical oxygen species
TNF-α: Tumor necrosis factor- α
NO: Nitric oxide
AI: Artificial insemination
PUFAs: Polyunsaturated fatty acids
DHA: Docosahexaenoic acid
IVF: In vitro fertilization
p-AKT: phosphorylated protein kinase B
ZAG: Zn-alpha2 glycoprotein
CatSper 8: Cation channel sperm-associated protein 8
ODF2: Outer dense fiber of sperm tails 2
p-ERK1: phosphorylated extracellular signal-regulated kinase 1
PI3K: Phosphoinositide-3-Kinase
SCFAs: Short chain fatty acids
BAX: Bcl-2-associated X protein

List of Publications

FIRST AUTHOR

Zhou Y，Han H, Xiong B et al. Alginate oligosaccharides increase boar semen quality by affecting gut microbiota and metabolites in blood and sperm. ***Frontiers in microbiology***, 2022.

Zhou Y, Wei Z, Tan J, et al. Alginate Oligosaccharide extends the service lifespan by improving sperm metabolome and gut microbiota in an aging Duroc boars model. ***Frontiers in Cellular and Infection Microbiology***, 2023.

Zhou Y, Chen L, Han H et al. Taxifolin increased semen quality of Duroc boars by improving gut microbes and blood metabolites. ***Frontiers in microbiology***, 2022.

Zhou Y, Wei Z, Tan J, et al. Alginate oligosaccharide supplementation improves boar semen quality under heat stress. ***Journal of Stress Biology***, 2024.

Zhou Y, Wei Z, et al. The role of Alginate oligosaccharide on boar semen quality : A research review. ***International Journal of Biological Macromolecules***, 2024.

Chapter I

General introduction

Chapter 1 General introduction

1.1 Decline of boar semen quality - a big problem in pig production

Excellent breeding boars play important roles in improving their genetic performance. Due to the occurrence of African swine fever, the production and transportation of semen has been becoming a higher biosecurity strategy than the introduction of boars. It is not only the current focus but also the hotspot in the future for improving boar genetic breeds and developing the production performance from all over the world. In China, due to the large population, a large amount of pork is needed, so high-quality boar semen can not only improve the pregnancy rate of sows, but also increase the litter sizes, so as to obtain more pork yield in the production. Therefore, high quality of boar semen is also very important. However, in large-scaled boar studs, there are two main factors that effect boar semen quality. One is heat stress, another one is the aging of boars. In this part, we will talk about these two problems and give potential routes on how to solve them.

1.1.1 Heat stress

Temperature is a prominent environmental factor that necessitates attention with regard to animal production, since it is widely affecting the growth and reproductive performance of mammals (Serviento et al., 2020; Kumar et al., 2021). When the environmental temperature exceeds the limitation that animals tolerate, heat stress reaction often occurs (Belhadj Slimen et al., 2016). The main manifestations are an increased heat production and sweating. Excessive heat stress can lead to irreversible loss of function (Luo et al., 2021). Boar sperm is greatly influenced by heat stress, particularly in terms of sperm motility, average path distance, straight-line velocity, straightness, and linearity (Sui et al., 2022). Moreover, it has an impact on DNA integrity and mitochondrial membrane potential (Calle-Guisado et al., 2017). Once a boar is exposed to a high temperature and high humidity for a long time, his breathing speeds up, the body temperature rises, the sperm motility decreases, and sperm deformity rate increases up till the level of an increased sperm death (Peña et al.,

2021). Due to these increasing problems, the boar will eventually be eliminated, which causes significant economic losses. The best solution is to provide air conditioner in the pig farm, however, consider the economic costs, a solution to deal with this problem in the swine industry is to adjust the feed formula in time, strengthen the feeding management, add anti-heat stress compounds in the feed during summer days, such as electrolytes (McCubbin, 2021), multivitamins (Liu et al., 2016) and prebiotics (Deng et al., 2022). This can be done by adding these compounds to the basal diet or to the drinking water.

1.1.2 Aging

In recent years, research on pig breeding has expanded beyond nutritional demands. Increasing attention is being given to reproductive performance and service lifespan (Poulsen et al.,2020; Plaengkaeo et al., 2021). With the increasing age of boars, the semen quality gradually declines, which results in a reduced service lifespan (Luther and Waberski, 2019). Sperm motility decreases and abnormal sperm rate increases, meanwhile the function of sperm proximity and seminal plasma also decline. Therefore, extending service lifespan in a reasonable manner has important practical significance for the swine industry (Spinaci et al., 2016). Increasing service lifespan can help to reduce breeding costs, increase conception rates and litter sizes, and improve the overall stability of the swine population (D'Allaire et al., 1992; Hoffman and Valencak, 2020). The service lifespan of breeding boars refers to the time starting from the first mating till the boars' elimination (Koketsu and Sasaki, 2009). A study by D'Allaire and Leman has shown that the average service lifespan of boars in the late 20th century was 20 months (D'Allaire and Leman, 1990). In the early 21st century, it was prolonged to 2 years (Knox et al., 2008). In China, the lifespan of breeding boars in large scale boar studs is currently 30 months. There are many factors affecting the service lifespan, such as breed, nutrition and environment (Cassady et al., 2002; Sancho et al., 2004; Akerfelt et al.,2010). Different breeds of boars have different service lifespan, and in general, Duroc has a higher service lifespan than Large White and Landrace due to different genetic parameters

(Plaengkaeo et al., 2021). Nutritional deficiencies can directly affect the reproductive performance of breeding boars, and thus indirectly affect the service lifespan. In the case of high sperm collection intensity, the addition of protein-rich feeds, such as egg white and fish meal (Kong et al., 2021), is a good solution (Louis et al., 1994b). The effects of light intensity on boars can easily be overlooked. Light intensity exposure can affect the process of mammalian spermatogenesis by affecting the synthesis and secretion of melatonin (Turek et al., 1975) Long time light intensity can also improve overall sperm function and reproductive performance of boar ejaculates (Yeste et al., 2016). When not taking the aforementioned factors into account, and having to deal with nutritional deficiencies or a bad light management can negatively impact the semen quality, and decrease sexual desire (Berger et al., 1980), which eventually leads to the elimination of the boars. Therefore, to solve this problem, we can try to interfere through nutritional regulation, by, for example, adding feed additives such as natural plant extracts (Zhou et al., 2022) and fatty acids (Castellano et al., 2010), and determine if this can extend the service lifespan of the boars with an appropriate sperm quality.

1.2 The source and structure of alginate oligosaccharide

Alginate is a natural polysaccharide polymer that exists in the wall of brown algae cells. It is composed of monomers containing carboxyl groups. Alginate has been widely used in pharmaceutical and food industry due to its unique physicochemical properties and beneficial health effects. Its molecular formula is $(C_6H_7O_6Na)n$ (Lu et al., 2022). Alginate connects α-L-Guluronic (G) acid and β-D-Mannuronic acid (M) monomers through an 1,4-glycoside bond forming a long chain polymer. Therefore, there are three different kinds of combinations such as Polymannuronate (PM), Polyguluronate (PG) and Heteropolymer (polyMG). The structure of alginate is shown in Figure 1-1 (Liao et al., 2009). With the continuous deepening research on alginate, it is gradually considered to be a macromolecular compound with anti-coagulation and anti-viral functions. However, due to its large molecular weight and viscosity, it is difficult to cross the cell membrane and multiple

biological barriers, thereby limiting its utilization and development (Pawar and Edgar, 2012). Alginate oligosaccharide (AOS) is a decomposition product of alginate and has received increasing attention due to its low molecular weight, high water solubility, safety, and non-toxicity. (Falkeborg et al., 2014). AOS has a variety of biological activities, including immune regulation (Xu et al., 2014), anti-inflammatory (Zhang et al., 2022a) and antioxidant (Lu et al., 2022). Therefore, AOS is extremely widely used in pharmaceutical research, as well as in the functional food industry and the agricultural field.

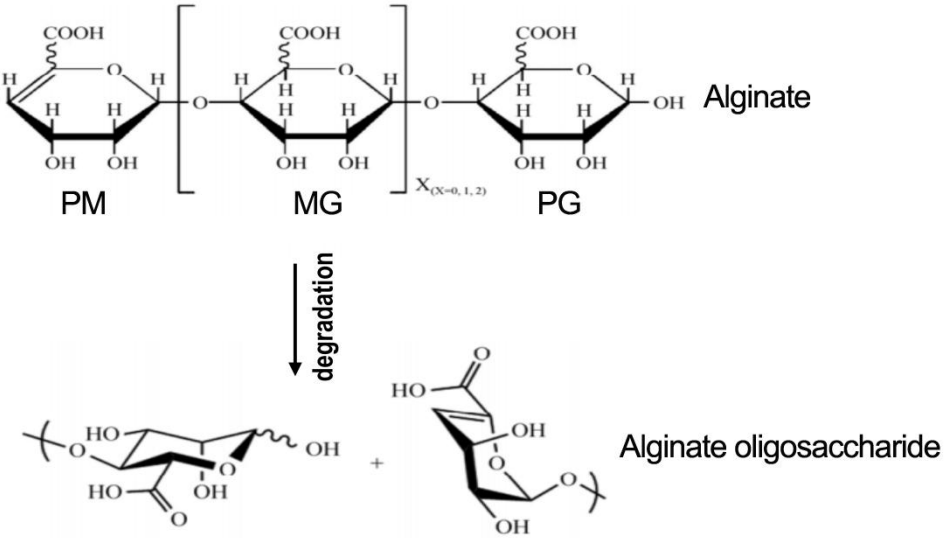

Figure 1-1 The molecular structure of alginate and alginate oligosaccharides, the upper part represents alginate, when using different degradation methods to decompose, the lower part shows the alginate oligosaccharide obtained after degradation.

1.3 The prepared methods of alginate oligosaccharide

There are many extraction methods for AOS, including the chemical degradation method, the physical degradation method and the bio-degradation method. In what follows we will give an overview of the degradation methods, their effectiveness, usability and potential shortcomings.

1.3.1 The chemical degradation method

The chemical degradation method has been widely used in the preparation of AOS, mainly including acid hydrolysis, alkaline hydrolysis and oxidation degradation.

Among them, the acid hydrolysis method is a traditional method for preparing AOS (Bi et al., 2023). The acid hydrolysis method usually uses hydrochloric acid, oxalic acid, formic acid, and sulfuric acid. During the acid hydrolysis reaction, the reaction conditions have a great impact on the aggregation of AOS. At 100°C, using 1 mol/L oxalic acid to prepare AOS, around 30% of alginate is hydrated. This is because alginate is resistant to acid and is not easy to be completely hydrolyzed by an acid (Marais and Joseleau, 2001). With the development of the technology, using 1.5 mol/L methane acid for 8 h (at 100°C), alginate can be completely hydrolyzed (Chandıa et al., 2001). Alkali hydrolysis is another way to prepare AOS. However, under alkaline conditions, the use of β-eliminating reactions can break the glycosidic bond of alginate, which will cause a change in structure, so this method is generally not used. The advantages of acid hydrolysis and alkali solution are their low cost and convenient operation. The disadvantage is that it is easy to produce toxic degradation products and cause environmental pollution. The oxidation degradation method (H_2O_2) is a green method for preparing AOS. There are no double bonds in the prepared AOS. The by-product is H_2O, which avoids pollution to the environment (Mao et al., 2012).

1.3.2 The physical degradation method

There are many physical methods for preparing AOS. Among those commonly used are the ultrasonic method, the ultraviolet radiation method and the microwave radiation method. In terms of energy efficiency, microwave radiation is considered one of the most effective physical degradation methods at present (Mohdy et al.,2017). At the same time, it is not affected by temperature, environment, nor additives. The advantage of the physical degradation method is that the response is rapid and that there is no environmental pollution. However, the cost is high, the degradation efficiency is low, and the response mechanism is not clear, so further studies are warranted.

1.3.3 The bio-degradation method

The bio-degradation method uses microorganisms or specific alginate cracking enzymes to decompose the substrate. In nature, alginate cracking enzymes are widely

distributed, and can be extracted from marine microorganisms, marine animals, fungi and viruses (Helga et al.,2015). Alginate cracking enzymes have a high substrate specificity and are an ideal choice for degrading unique low polysaccharide structures. However, there are currently few research reports on alginate cracking enzymes that have high-level substrate-specificity. Furthermore, rather than isolating the enzymes, intact microorganisms can be used directly to degrade alginate, offering a more straightforward approach compared to enzymatic degradation as there is no need to separate and purify enzymes. The production conditions of microorganisms can be controlled and their production cycle is short. A study by Moen et al. has found that alginate can be degraded in the kelp by mixed microbes (Moen et al., 1997). *Bacteroides uniformis* (Liu et al., 2019a) and *B. Ovatus* (Li et al., 2017a) obtained from human intestine, are microorganisms that can also produce AOS through enzyme secretion.

1.4 The biological activities of AOS

AOS, as a natural plant extract, has multiple biological functions such as immune regulation (Bland et al., 2004), antioxidant (Falkeborg et al., 2014) and anti-inflammatory (Feng et al., 2021). Due to its low molecular weight, high water solubility, safety and non-toxic characteristics (Wang et al., 2021b), it has received high attention from researchers in recent years. The main functions of AOS are described in Figure 1-2.

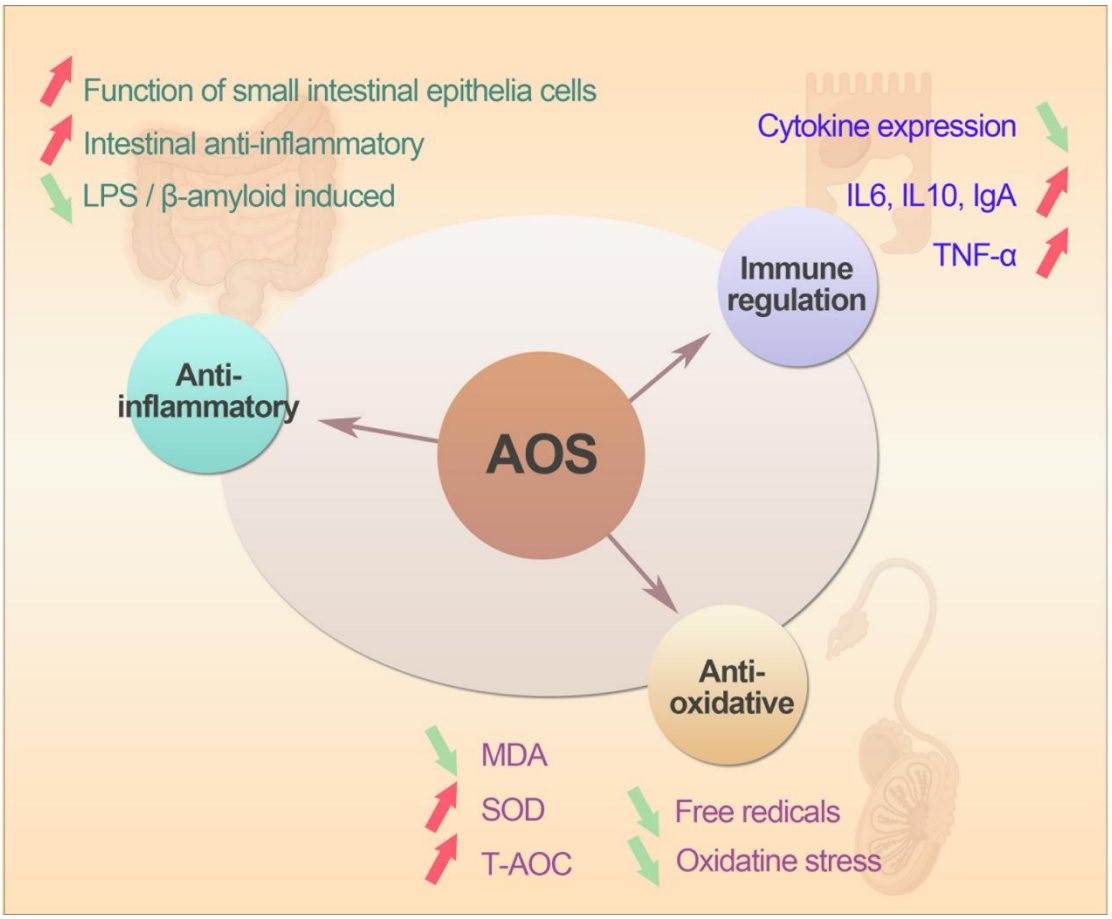

Figure 1-2 The three main biological activities of AOS: immune regulation, antioxidant and anti-inflammatory properties. For the immune regulation, AOS influences cytokine expression. AOS improves the function of small intestinal epithelial cells and helps against intestinal inflammatory challenges such as those induced by LPS. AOS increases the antioxidant indicators SOD and T-AOC, and decreases MDA and ROS. Red arrow shows up-regulated, green arrow shows down-regulated.

1.4.1 Immune regulation properties of AOS

Natural polysaccharides and oligosaccharides have the potential to be immune regulators. One of the key functions of AOS in immune regulation is the ability to induce cytokine expression (Xu et al., 2015). It is found that AOS can increase the concentration of IL6, IL10, and IgA in the serum of weaning piglets, as well as the level of IgA in the small intestine (Wan et al., 2018b), which indicates that AOS has the potential to elevate the levels of immunoglobulins and cytokines, thereby enhancing the immune function of weaning piglets. In a study on macrophages, alginate was seen to induce the secretion of TNF-α, and AOS obtained by alginate cracking augmented the induction activity of TNF-α compared to intact alginate (Kurachi et al., 2005). Using different methods to crack alginate, the obtained AOS

has different chemical groups, and AOS induced cytokine secretion is closely related to its structure. At present, more and more studies are concerned about the relationship between the unsaturated terminal structure of AOS and its activity. AOS obtained by microorganisms to crack alginate contains more unsaturated double bonds on the terminal structure than AOS obtained by acid degradation methods. As unsaturated groups play a key role in the secretion of TNF-α in macrophages, the degradation method used is important (Iwamoto et al., 2005). In addition, a study by Xu et al. has shown that AOS can increase the expression of induced nitric oxide (iNOS) synthase in macrophages, promoting the generation of nitric oxide (NO), and stimulating the generation of reactive oxygen species (ROS) and TNF-α (Xu et al., 2014).

1.4.2 Anti-inflammatory properties of AOS

Inflammation is an important defense mechanism for the body to resist pathogen invasion and it will activate inherent immune cells. However, inflammation will also appear in the process of chronic diseases, such as arthritis and diabetes (Saigusa et al., 2015). AOS can improve the intestinal anti-inflammatory ability of weaning piglets by inhibiting the TLR4/NF-κB and NOD1/NF-κB signaling pathways (Wan et al., 2018a). The anti-inflammatory ability is also related to the preparation methods used for AOS production. AOS prepared through oxidation degradation has a stronger inflammatory inhibitory effect compared to AOS prepared by other methods. AOS extracted by this method can inhibit LPS/β-amyloid-induced neuritis, by promoting β-amyloid phagocytosis effects (Zhou et al., 2015b). This is done by blocking NF-κB and MAPKs signaling pathways. Contrarily, AOS prepared by microorganisms has no anti-inflammatory effects (Zhou et al., 2015a). The anti-inflammatory AOS enhances the function of small intestinal epithelial cells by increasing the proportion and number of various cell clusters of small intestine such as goblet cells (Li et al., 2022b), thereby alleviating small intestinal mucositis induced by Busulfan. Furthermore, a study in IPEC-J2 cells has found that AOS can play a significant anti-inflammatory role through the mannose receptor signal pathway (Zhao et al., 2020c). In addition,

AOS is used as an anti-inflammatory drug in clinical trials. Study has shown that AOS has positive effects in various tested mice models used for treating arthritis (Yin et al., 2024). Its anti-inflammatory activity is related to the regulation of the TLR4 signal pathway provoking inflammation (Hajivalili et al., 2016; Aletaha et al., 2017; Mortazavi-Jahromi et al., 2018).

1.4.3 Anti-oxidative properties of AOS

AOS acts as a very strong antioxidant by effectively removing free radicals. Since AOS prepared by microbial engineering (*Microbulbifer*) mainly consists of disaccharides and trisaccharides, it has the ability to remove free radicals (DPPH and Hydroxyl radical), thereby promoting restoration (Zhu et al., 2016). A Study by Fu et al. has shown that AOS prepared by enzyme degradation can have an stronger antioxidant activity than other methods, the reason for that being its structure of more double bonds (Fu et al., 2021). The antioxidant mechanism of AOS may be linked to a way in which hydrogen ions form a hydrogen bond with free radicals, forming a more stable product, leading to the subsequent removal of free radicals (Hernandez-Marin and Martínez, 2012). In animals, AOS has the effect of resisting oxidative stress and preventing oxidative damage. A study of Tusi et al. has shown that in an oxidative stress model, caused by hydrogen peroxide, AOS can block H_2O_2-induced oxidative stress and caspase-dependent apoptotis in both the endoplasmic reticulum and mitochondria (Tusi et al., 2011). AOS can inhibit the expression of GP91 and 4-hydroxylceride, thereby reducing heart oxidative stress, increasing the survival rate of mice damaged by adriamycin (Guo et al., 2016). In a study by Li et al., AOS could significantly reduce the level of hydrogen peroxide and MDA in mice, thereby reducing ROS production (Li et al., 2019). AOS was also found to improve serum levels of SOD, peroxide and total antioxidant capacity (T-AOC) in weaning piglets, and to reduce the content of MDA in the serum and small intestine (Wan et al., 2018a). In addition, adding AOS to a broiler diet increased the concentration of glutathione peroxidase in the serum, and reduced the content of MDA in the liver, thereby improving the antioxidant capacity in broilers (Zhu et al., 2015). To conclude, AOS

can improve the antioxidant capacity in animals by modulating the enzymatic antioxidant system, thereby preventing lipid peroxidation of animal tissues.

1.5 The characteristics of spermatogenesis and membrane structure

Spermatogenesis is a precise regulation process in the testicular seminiferous tubule. This process exhibits periodicity and continuity (Parrish et al., 2017). From the seminiferous tubule, the semen, that contains 5% of sperm, travels to the epididymis (Castillo et al., 2018). The semen is alkaline, its pH is around 7.2-8.0. It is rich in lipids, sugar and protein. The fructose produced by the seminal vesicle is the main energy substance (Drabovich et al., 2014). It is worth noting that it takes about 45 days for spermatogonia to develop into mature sperm (Costa et al., 2013). Interestingly, the semen quality of boars can be improved through nutritional regulation, and this positive switch can occur after feeding potential favorable additives for 6-7 weeks (Zhou et al., 2022). This provides a theoretical basis for the sample collection of boar experiments with regard to such feed additives (Frankenhuis et al., 1982). The structure and functions of the sperm membrane has a vital impact on sperm vitality and fertilization ability (Blesbois et al., 2005; Guthrie and Welch, 2012). This is mainly due to the phospholipid biomolecules in the sperm of boars, containing 60%-70% unsaturated fatty acids (Wathes et al., 2007). Therefore, adding unsaturated fatty acids to the basal diet of boars can regulate sperm membrane structure and function, thereby improving sperm motility (Liu et al., 2017a). It is worth noting that the sperm membrane component is rich in PUFAS, so it is particularly sensitive to oxidative damage. Studies have shown that when the sperm membrane and mitochondria are damaged by oxidation, the sperm motility and fertilization ability of boars significantly decreases (Nesci et al., 2020; Zhu et al., 2020). Therefore, controlling the oxidative damage in sperm cells under various conditions is an important measure to ensure the semen quality of boars.

1.6 Evaluation parameters and detection methods of boar semen quality

In modern swine breeding, the application of artificial insemination (AI) technology has become more and more common. The fertility of boars will not only directly affect the economic benefits of commercial boar studs, but also indirectly affect the reproductive performance of sows. The fertility of boars can be evaluated with indicators such as sexual libido, semen quality, in vitro fertilization capacity and sow pregnancy rate (Louis et al., 1994a; Schulze et al., 2014). Among these indicators, semen quality is the most important parameter to evaluate boar fertility (Berndtson, 2008; Broekhuijse et al., 2012). Generally, indicators such as sperm volume, sperm concentration, sperm motility, and sperm morphology to evaluate semen quality are used (Ruiz-Sánchez et al., 2006). The amount of sperm volume and sperm concentration determines the total sperm per ejaculation, which is mainly reflected by the spermatogenesis in the testis (Flowers, 1997). The overall sperm count plays a role in influencing both the number of mating sows and pregnancy rate during artificial insemination. Sperm motility and sperm morphology are two core indicators that jointly determine the semen quality and affect the pregnancy rate of sows (Holt et al., 1997). In pig production, when semen is stored for a period at room temperature, there is a decrease in sperm motility and an increase in the rate of abnormal sperm. This phenomenon is the main reason for the decline in boar fertility (Knox et al., 2008). Although the number of input sperm can be increased during artificial insemination to solve the problem of a decreased pregnancy rate, the primary objective when improving semen quality is still to improve sperm motility (Jung et al., 2015). This is also a focal point in the research on boar fertility through nutritional regulation technology. Semen quality is closely related to the spermatogenesis, which is affected by several factors such as genetics, environment and nutritional management (Lopez Rodriguez et al., 2017).

Boar semen quality is directly related to it fertilization ability (Han et al., 2019). At present, semen quality parameters include sperm motility, sperm concentration,

sperm vitality and abnormal sperm rate (Sharma et al., 2015). These indicators will directly or indirectly reflect the quality of sperm. In the evaluation method of boar semen, the traditional method, called hematimetry, using a hemocytometer, determines the sperm number of sperm with a forward movement. Another method is the Giemsa dyeing method to determine the abnormal sperm rate. While these methods are accurate, they consume considerable time and labor (Quirino et al., 2023). The computer-assisted sperm analysis (CASA) system can also provide accurate results and is less time and labor-intensive (Browne et al., 2015). Therefore, the CASA system has obvious advantages for artificial insemination in animal husbandry.

1.6.1 Sperm motility

Sperm motility refers to the ability of sperm to move forward. A study by Mahajan et al. found that sperm motility is an important evaluation parameter for semen analysis. Its percentage can directly affect the quality of semen and its fertilization ability (Mahajan et al., 2015). Therefore, using the CASA system conducting a quantitative analysis of sperm motility can lead to a more accurate assessment. In pig production, sperm motility is generally higher than 75%, and meets the standards for AI (Waberski et al., 2019).

1.6.2 Sperm concentration

Sperm concentration refers to the total number of sperm per ml semen. Sperm concentration as an important indicator of semen quality, as it will directly affect the dilution times in the AI process, thereby affects the pregnancy rate of sows (Chanapiwat et al., 2014). At present, sperm concentration evaluation methods are diverse. As such there is the absorption spectrophotometry method, the hematimetry method, as well as the aforementioned computer assisted CASA method (Lesani et al., 2020). Hematimetry is the most accurate method for evaluating sperm concentration. However, in pig production, CASA analysis can quickly detect sperm concentration and saves a lot of time. Generally, the sperm concentration should reach 10^8 per ml for it to meet the requirements for AI (Morrell, 2019).

1.6.3 Sperm vitality

Sperm vitality refers to the percentage of total sperm that moves forward in the semen. Sperm vitality is closely related to the conception rate (Bibi et al., 2022). The use of the naked eye and calculators to evaluate the percentage of sperm with a straight line motion, is difficult and often susceptible to the influence of human factors, which makes the results highly variable. In contrast, the results of CASA analyzes are more accurate. In pig production, the sperm vitality requires to be 85% to perform AI.

1.6.4 Abnormal sperm rate

Abnormal sperm rate refers to the percentage of deformed sperm in the semen. Abnormal sperm rate is one of the important indicators to establish semen quality. An excessive malformation rate will significantly reduce the fertilization capacity of sperm (Fedder et al., 2007). In the process of fertilization, if the abnormal sperm rate is higher than 18%, the ability of fertilization will be significantly reduced. Sperm malformation can occur in the whole sperm cell, and commonly appears in the sperm head, mid-body or sperm tail. Sperm head malformations mainly include a knobbed acrosome (Kawakami et al., 2012) and a pyriform heads (Buendía et al., 2002); Mid-body abnormalities mainly include segmental aplasia (Oko and Hrudka, 1982) and distal reflex (Callaghan et al., 2016); Tail abnormalities mainly include an abaxial tail (du Plessis and Soley, 2012). In the process of AI, the malformation rate detected by the CASA system should be less than 20% for it to meet the standards (Li et al., 2023).

1.7 The role of AOS on semen quality

The way AOS regulates semen quality is mainly divided into three aspects. Firstly, AOS can improve sperm metabolites. These metabolites include antioxidants, unsaturated fatty acids and amino acids. Secondly, AOS can improve the protein content in sperm, which is usually related to spermatogenesis and sperm apoptosis. Thirdly, AOS can improve the composition of intestinal microorganisms. On one hand, AOS increases the relative abundance of beneficial bacteria, on the other hand,

AOS reduces the relative abundance of harmful bacteria, which both have a positive effect on the intestinal-testicular axis. The effects of AOS on the semen quality of animals are summarized in Figure 1-3.

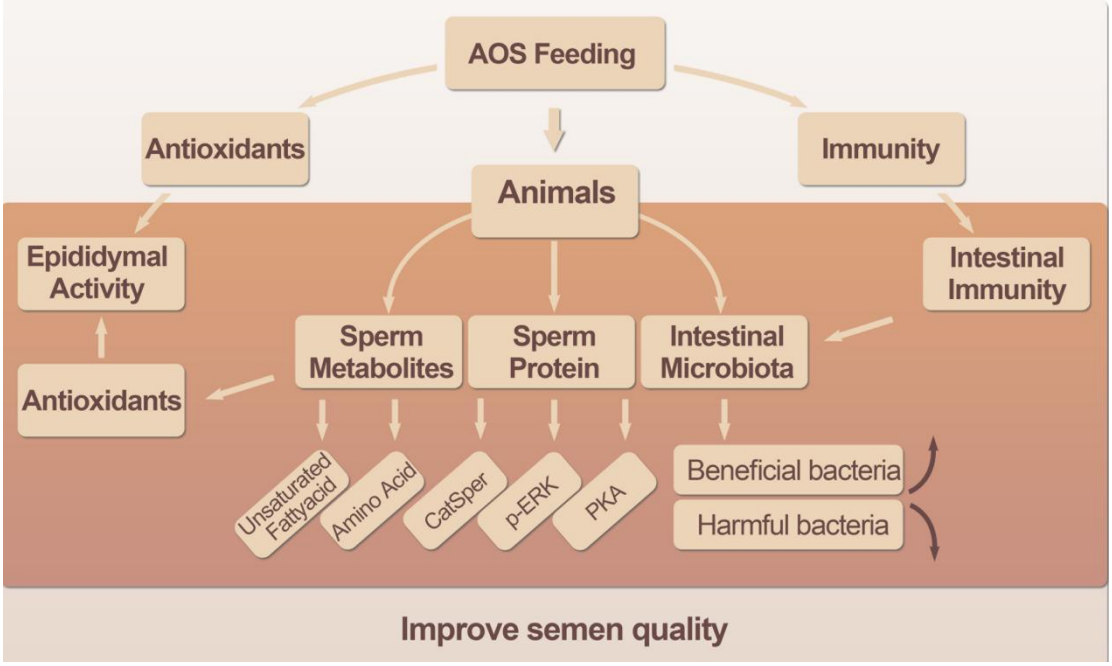

Figure 1-3 The role of AOS in semen quality. AOS is a strong antioxidant which can reduce the ROS in the epididymis. AOS can regulate the immune function of the intestine. Feeding AOS to the animals can improve sperm metabolites such as unsaturated fatty acids, sperm protein such as CatSper, p-ERK, and PKA, and change gut microbiota, as to improve the overall semen quality.

1.7.1 AOS improves sperm metabolites

There are many nutrients in semen that can directly or indirectly regulate semen quality (Salas-Huetos et al., 2019). A Study by Han et al. has shown that adding AOS to the basal diet can significantly improve the sperm metabolome (Han et al., 2022). Among the metabolites are antioxidant substances such as retinol, unsaturated acids such as DHA and EPA, and amino acids such as lysine. A study by Keys and Zimmerman has shown that retinol is a very strong antioxidant able to reduce reactive oxygen species (ROS) (Keys and Zimmerman, 1999). As we all know, sperm will temporarily store and mature in the epididymis (Jones, 1999). At the same time, the mitochondria in the sperm generate a large amount of ROS through the oxidation reactions of the respiratory chain (Chianese and Pierantoni, 2021), which mainly includes oxygen free radicals and hydroxyl radicals. Therefore the retinol can

reduce ROS in the epididymis and improve semen quality (Taşkıran, 2023). Unsaturated fatty acids such as DHA and EPA can also significantly improve semen quality in human. A study has shown that supplementing infertile men with omega-3 fatty acids resulted in a significant improvement in sperm motility and sperm concentration (Hosseini et al., 2019). Another study shows that adding lysine to the basal diet can also increase the semen quality in boars (Chen et al., 2021). These aforementioned sperm metabolites are significantly positively correlated with sperm motility and negatively correlated with abnormal sperm rate. This indicates that AOS can improve semen quality by improving sperm metabolites. Among them, the antioxidant succinic acid significantly increased, while the level of dibutyl phthalate, which is negatively related to spermatogenesis, significantly reduced (Wang et al., 2021a). In addition, AOS can also improve the sperm metabolome to relieve the decline in semen quality caused by heat stress in boars (Johnson et al., 2020), as could be seen by both the increased sperm motility and concentration. Moreover, in a study with mice, it was found that AOS repaired testicular damage caused by busulfan, and improved the sperm metabolites such as retinol, thereby increasing the sperm motility (Zhao et al., 2020d; Zhang et al., 2021b). Therefore, AOS regulates the semen quality through improving the sperm metabolome.

1.7.2 AOS improves sperm proteins

Proteins play a vital role in the spermatogenesis, and the addition of AOS in the feed can increase the level of some specific proteins in the semen, thereby contributing to the improvement of semen quality. The proteins referred to are mainly ZAG, PKA, p-ERK, and CatSper. ZAG is involved in regulating the acrosome reaction of spermatozoa to increase their fertilization ability (Liu et al., 2012). PKA mediates protein phosphorylation, which is important for sperm motility and male fertility because of its necessity in activating sperm capacitation (Baro Graf et al., 2020; Liang et al., 2023). At the same time, in mice, p-ERK can repair testicular damage caused by Biophenol exposure, as well as improve the sperm motility and sperm density, which is beneficial to semen quality (Li et al., 2016). Western blotting

and immunocytochemistry showed that CatSper subunits are present in boar spermatozoa, primarily in the sperm neck, tail and cytoplasmic droplets (Tamburrino et al., 2014). The main mechanism involves that the AOS activates CatSper to promote the entry of calcium ions into sperm, thereby improving sperm motility. Therefore, AOS regulates the semen quality of boars through improving the level of sperm proteins.

1.7.3 AOS changes gut microbes

As the largest immune digestive organ, the intestinal system has emerged as the most popular research hotspot (Mowat and Agace, 2014). The composition of intestinal microorganisms plays an important role in connecting host with diet. Therefore, the intestinal microbes and specific organs or axes between organs, such as the intestinal liver axis (Porras et al., 2017) and intestinal testicular axis (Wang et al., 2023), are more and more closely examined by many experts. Studies have shown that adding AOS can change the relative abundance of intestinal flora, increasing beneficial bacteria (Yan et al., 2022) and reducing harmful bacteria (Han et al., 2021). A previous reasearch of our lab reported that, at the phylum level, *Bacteroidetes* are positively correlated with sperm motility and sperm concentration, however, *Firmicutes* are negatively correlated with sperm motility and sperm concentration (Han et al., 2022). At the genus level, *Butyricicoccus,* as a beneficial bacteria that can produce butyric acid, s beneficial for spermatogenesis (Lin et al., 2022). A Spearman associated analysis also showed that *Butyricicoccus* are positively correlated with sperm motility and sperm concentration (Han et al., 2022). At the same time, AOS can also reduce harmful bacteria such as *Prevotella*. Studies have shown that *Prevotella* appeared to exert a negative effect on sperm quality (Farahani et al., 2021; Zhou et al., 2022). In addition, adding AOS to the mice can increase the content of short-chain fatty acids (SCFAs) in the intestine, and specifically acetic acid and butyric acid are significantly improved (Li et al., 2022b). SCFAs are small molecular compounds which can freely enter the testis through the blood and provide energy for the spermatogenesis (Wu et al., 2024). Feeding AOS to mice was observed to change

the intestinal flora (Zhang et al., 2021a). Using fecal microbiota transplant (FMT) technology, by which bacteria were orally fed to the mice, the sperm motility and relative abundance and colonization of beneficial bacteria such as those belonging to the *Bacteroidetes* phylum and *Bifidobacteriales*, belonging to the *Actinomycetota* phylum, increased in the small intestine (Zhang et al., 2021b). This suggests that AOS can mediate gut microbial changes and improve semen quality in mice. Therefore, AOS can increase the semen quality by changing the microbial composition. On the one hand, this occurs through the increase of beneficial bacteria to promote the production of short-chain fatty acids, thereby promoting the interaction with the intestinal testicular axis. On the other hand, a reduction of intestinal harmful bacteria aims to inhibit the damage of spermatogenesis.

1.8 Concluding remarks and future perspectives

Excellent breeding boars play important roles in improving genetic performance. Due to diseases, such as for instance African swine fever, the production and transportation of semen emerge as a better bio-security measure compared to introducing boars to sows in heat. Improving boar genetic breeds and advancing productive performance is not only the present focal point but also a future hotspot in the swine breeding industry. With the widespread application of artificial insemination technology, the requirements for the quality of boar semen are also increasing.

At present, there are a series of problems that occur in boar studs that need to be solved urgently. One is how to release heat stress of boars in summer, which causes a decrease in semen quality. The other one is how to prolong the service life of older boars in order to reduce the elimination rate.

In this general introduction, we summarized the biological function of AOS, its efficient antioxidant and immune regulatory capacity. AOS can change the composition of intestinal microorganisms to improve semen quality in mice. AOS can also increase protein levels beneficial to spermatogenesis in mice testicles. However, there is limited research available to ascertain whether AOS can increase the semen

quality of boars.

Therefore, the purpose of this PhD thesis was to explore the effect of AOS on boar semen quality. More specifically, we investigated how AOS relieves the decline in semen quality caused by heat stress. We also looked at if and how AOS extends the service lifespan of aging boars in swine production. Addressing these issues could yield substantial economic advantages for the swine industry.

Chapter II

Thesis objectives, hypothesis, and structure

Chapter II. Objectives, hypothesis and outline of the thesis

2.1 Objectives

In this PhD study, our overall objective was to determine if AOS is a good feed additive that can be supplemented to boar diets in order to ensure that boars maintain high quality semen. In this way, the conception rate of sows can be guaranteed during artificial insemination. Specifically, we conducted research in the following three areas:

(1) In what kind of ways does AOS improve the semen quality on middle-aged (24 months old) boars? With this research question we want to investigate if this improvement occurs through gut microbiota and/or sperm metabolites?

(2) Did AOS alleviate the decline of semen quality caused by heat stress after boars were fed AOS for 9 weeks? With this research question we want to identify the effect of AOS in a heat stress model.

(3) How does AOS extend the service lifespan in an aging boars model? With this research question we want to solve the problem of high elimination rates in large scale boar studs.

2.2 Hypothesis

We hypothesize that AOS may have a promoting effect on boar semen quality. The mechanism by which dietary AOS increases boar semen quality might be through changing gut microbiota, sperm metabolites and/or sperm proteins.

2.3 Technical route

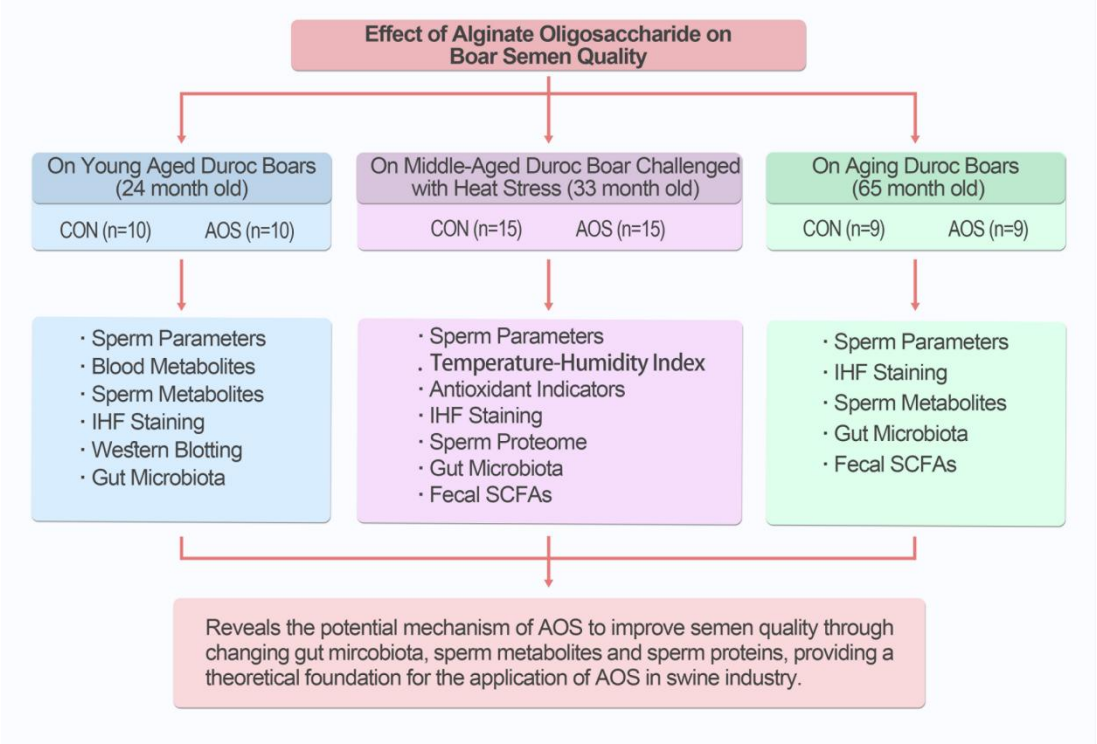

Figure 2-1 The technical route. In this thesis, 3 different kinds of Duroc boar models were used. CASA technology was performed to test sperm parameters. Blood metabolites, sperm metabolites, sperm protein and gut microbiota were all examined in the thesis. IHF staining and WB were used to identify the metabolome data, in order to reveal the potential mechanism of AOS on the semen quality.

2.4 Outline of the thesis

Chapter 1: General introduction

Chapter 2: Thesis objectives, hypothesis and outline of the thesis

Chapter 3 (Article 1): Alginate oligosaccharides increase boar semen quality by affecting gut microbiota and metabolites in blood and sperm. *Han H, Zhou Y, Xiong B, Zhong R, Jiang Y, Sun H, Tan J, Zhang B, Guan C, Schroyen M, Chen L, Zhao Y, Zhang H. 2022. Frontiers in Microbiology 13:982152.*

This chapter shows the effect of AOS fed to 24-month-old boars. Blood, raw semen and feces were combinedly analyzed by multiple omics, and molecular techniques (IHF and WB) were used to validate them, in order to find the potential mechanism by which AOS improves the semen quality.

Chapter 4 (Article 2): Alginate oligosaccharide supplementation improves boar

semen quality under heat stress. *Zhou Y, Wei Z, Tan J, Sun H, Jiang H, Gao Y, Zhang H, Schroyen M. 2024 Stress Biology Sep 3;4(1):37.*

Based on chapter 3, we found that AOS was indeed beneficial to improve semen quality. In the production, heat stress is a serious problem caused boar semen quality decline. It is really important to solve the problem through nutritional regulation. In chapter 4, we therefore used 33-month-old Duroc boars as model challenged by heat stress to reveal the potential mechanism of AOS in alleviating heat stress and improving semen quality. We analyzed this through a combination of multiple omics such as 16S RNA sequencing, sperm metabolome and sperm proteome. The aim was to solve the serious heat stress problem and its negative effect on sperm quality.

Chapter 5 (Article 3): Alginate Oligosaccharide extends the service lifespan by improving sperm metabolome and gut microbiota in an aging Duroc boars model. *Zhou Y, Wei Z, Tan J, Sun H, Jiang H, Gao Y, Zhang H, Schroyen M. 2023. Frontiers in Cellular and Infection Microbiology 13:1308484.*

In the boar production, aging will lead to decline boar semen quality so that the culling rate increased as well. Boars are valuable for the company, it will bring a large amount of economic lost. Therefore it is important to solve the problem through nutritional regulation. In chapter 5, we went a little further in age of the boars and we used 65-month-old Duroc boars as model to reveal that AOS can extend the service lifespan of aging boars. In this chapter we also tested multiple omics, and additional molecular techniques to examine the potential mechanism of AOS on extending service lifespan of aging boars.

Chapter 6: General discussion conclusion and perspectives

2.5 Experimental design

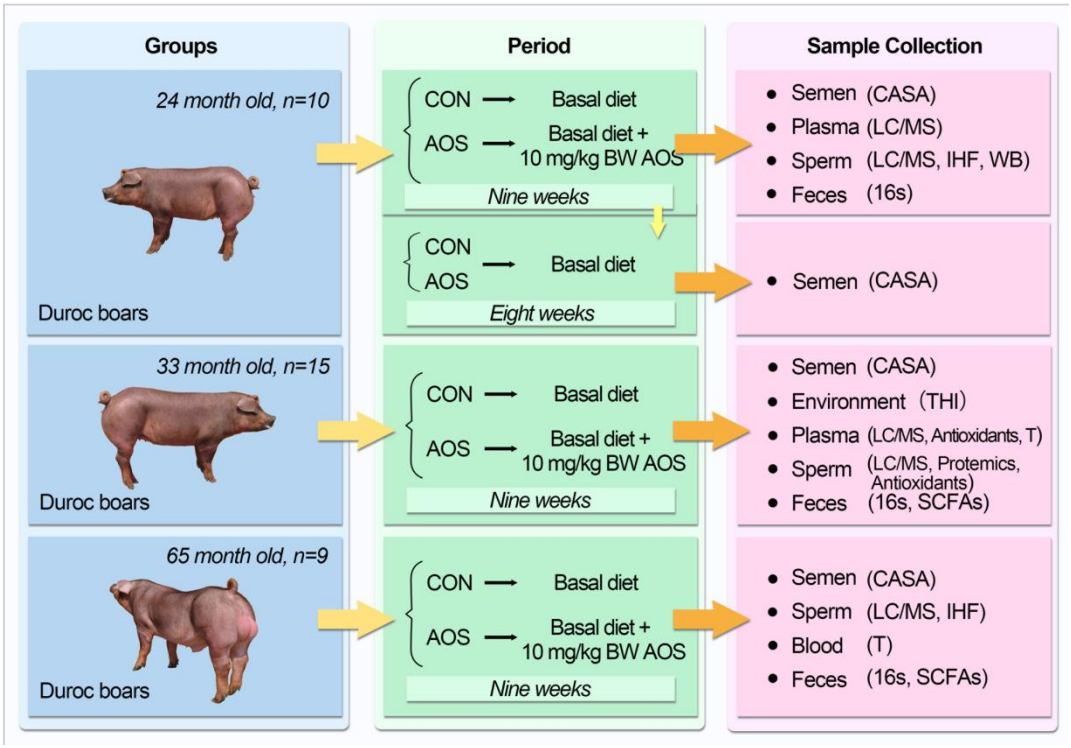

Figure 2-2 The experimental design. There were 3 feeding experiments in the thesis. The first was performed with twenty 24 months old Duroc boars with similar weight that were randomly divided into two groups (AOS group and CON group), 10 boars per group. The AOS group was fed a basal diet with 10 mg/kg AOS and the CON group was fed only the basal diet. The feeding experiment lasted 9 weeks. Then raw semen, blood and feces were collected from all boars. In the next 8 weeks, we also collected raw semen on the same boars and in the same cycle every 5 days to verify the long-term effects of AOS. The second experiment was done with thirty 33 months old Duroc boars, similar in weight and randomly divided into two groups (AOS group and CON group) under heat stress condition, 15 boars per group. The third experiment was performed on eighteen 65 months old Duroc boars, similar in weight, and randomly divided into two groups (AOS group and CON group), with 9 boars per group. These two feeding experiments lasted 9 weeks after which raw semen, blood and feces were collected from all boars.

Chapter III

Alginate oligosaccharides increase boar semen quality by affecting gut microbiota and metabolites in blood and sperm

Chapter III. Alginate oligosaccharides increase boar semen quality by affecting gut microbiota and metabolites in blood and sperm

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Key words: boar, alginate oligosaccharides, sperm motility, sperm concentration, in vitro fertilization, metabolism, gut microbiota

Adapted from the reference: Han H, Zhou Y, Xiong B, Zhong R, Jiang Y, Sun H, Tan J, Zhang B, Guan C, Schroyen M, Chen L, Zhao Y, Zhang H. Alginate oligosaccharides increase boar semen quality by affecting gut microbiota and metabolites in blood and sperm. Front Microbiol. 2022 Aug 22;13:982152. doi: 10.3389/fmicb.2022.982152.

Author Contributions: Yexun Zhou participated in animal husbandry experiments, data collection and analysis, as well as drafting and writing the paper.

3.1 Abstract

Alginate oligosaccharides (AOS), natural polymers from brown seaweeds (such as Laminaria japonica, Undaria pinnatifida, and Sargassum fusiforme), have been reported to possess many beneficial advantages for health. In the current study, after 9 weeks of dietary supplementation, AOS 10 mg/kg group (AOS 10) group increased boar sperm motility from 87.8% to 93.5%, $p < 0.05$. Moreover, AOS10 improved the relative abundances of *Bifidobacterium*, *Coprococcus*, *Butyricicoccus* (1.3–2.3-fold; $p < 0.05$) to increase the beneficial blood and sperm metabolites (1.2–1.6-fold; $p < 0.05$), and important sperm proteins such as gelsolin, Zn-alpha2 glycoprotein, Cation Channel Sperm Associated Protein, outer dense fiber of sperm tails, etc. (1.5–2.2-fold; $p < 0.05$). AOS had a long-term beneficial advantage on boar semen quality by the increase in semen volume (175 vs. 160 ml/ejaculation, $p < 0.05$). AOS may be used as dietary additives for improving semen quality.

3.2 Introduction

Alginate oligosaccharides (AOS), natural biodegradable polymers derived from the degradation of alginate (brown seaweed), are made up of α-L-guluronate (G) and β-D-mannuronate (M) linked by 1, 4-glycoside bonds (Hu et al., 2019). AOS have many biological advantages with the great characteristics: non-immunogenicity, non-toxicity, and biodegradability (Ueno et al., 2012; Moriya et al., 2013; Ruvinov and Cohen, 2016; Pritchard et al., 2017; Han et al., 2019; Hu et al., 2019). AOS can act as anti-inflammation (Moriya et al., 2013), anti-apoptosis (Tusi et al., 2011), anti-proliferation (Tajima et al., 1999), antioxidant activities (Tusi et al., 2011; Ueno et al., 2012; Guo et al., 2016). Recently, dietary AOS improved intestinal cell development and intestinal morphology and barrier function, and stimulating weaned pig growth (Wan et al., 2017, 2018a,b; Zhao et al., 2020a). Thus, AOS have been approved as a safe bio-polymer by the U.S. Food and Drug Administration (reference no.21CFR184.1724) to be applied in pharmaceutical, cosmeceutical, and nutraceutical fields (Park et al., 2016; Hu et al., 2019).

Reproductive biotechnologies, especially artificial insemination (AI), play a vital role in the genetic improvement of pigs and other farm animals (Singh et al., 2021). Moreover, AI not only makes significant contributions to the development of swine production worldwide, it also raises the importance of the reproductive efficiency of boars in pig herds (Tsakmakidis et al., 2010). Semen quality is used as a proxy measure of boar fertility owing to the close correlation between sperm quality with boar fertility, and it creates a desired effect on piglet production in terms of the reproductive performance of sows (Dong et al., 2016). In summary, good semen quality is fundamental for successful AI (Singh et al., 2021).

The production and quality of semen not only depend on intrinsic factors such as breed (Wolf, 2009) and age (Huang et al., 2010), but also on environmental extrinsic factors, for example, temperature, photoperiod, and nutrition (Ciereszko et al., 2000; Yeste et al., 2010; Dong et al., 2016). Boar age and semen quality are factors that are considered in boar culling (Tsakmakidis et al., 2012). Reports show that a maximum semen volume and sperm concentration can be obtained from boars of ≤3.5years of age (Smital, 2009; Tsakmakidis et al., 2012). Hormonal and cellular changes take place in males with aging, which alters sperm quality and fertilization capacity (Araujo and Wittert, 2011). Aging also induces a decrease in testosterone levels, which is involved with intrinsic and extrinsic factors associated with leydig cells (Midzak et al., 2009; Araujo and Wittert, 2011; Tsakmakidis et al., 2012).

Nutrition influences boar libido, sperm output, semen quality, and fertility (Dong et al., 2016; Liu et al., 2017a). It is known that protein levels in the diet affect boar semen quality; both low protein or excessive protein can decrease sperm quality (Louis et al., 1994; Dong et al., 2016). Individual amino acids have potential impacts on semen quality (Ren et al., 2015) as follows: dietary lysine (1.03%) improves boar semen quality compared to 0.86% (Dong et al., 2016); supplementation of threonine benefits ram and boar sperm quality (Wilson et al., 2004); tryptophan significantly improves ram sperm motility (Pichardo et al., 2011; Dong et al., 2016). Furthermore, polyunsaturated fatty acids (PUFAs) have been shown to benefit sperm motility and fertility in human and animal studies (Murphy et al., 2017; Liu et al., 2017a). The

major commercial source of omega-3 fatty acids [specifically docosahexaenoic acid (DHA)] are fish oils, and the most abundant source for linolenic acid is flaxseed (~53%). Chestnut polysaccharides have been discovered to improve the spermatogenesis and semen quality recently (Yu et al., 2020; Sun et al., 2022). Given these significant advantages, this investigation aimed to explore the underlying mechanisms by which AOS enhances boar semen quality.

3.3 Materials and methods

3.3.1 Materials and reagents

AOS (>98%) was from Qingdao BZ Oligo Biotech Co. Eosin, insulin, EGF, cysteine, pyruvate, kanamycin, paraformaldehyde, and Triton X-100 were from Sigma-Aldrich. BSA, goat serum, and TCM-199 medium were from Life Technologies Ltd. Polyvinylidene fluoride (PVDF) membrane was from Merck. E.Z.N.A.® Stool DNA Kit was from Omega Bio-tek Inc. The antibodies were purchased from different companies listed in Table 3-3.

3.3.2 Boars and experimental design

All animal procedures were approved by the Animal Care and Use Committee of the Institute of Animal Sciences of Chinese Academy of Agricultural Sciences (IAS2021-67). Twenty boars (~24months of age; male) were used in this investigation at the artificial insemination center of Yangxiang Joint Stock Company (Guangxi, China; Guo et al., 2020). To select the boars, we first looked at the previous semen quality parameters of around 1200 boars, then the sperm motility was screened and had to be around 85%, while the sperm concentration had to be around 5x10^^{8}/ml, and the abnormal sperm rate required to be less than 20%. There were about 200 boars that met the above criteria, and among them 20 boars were randomly chosen and divided into two groups. The results are shown in Table 3-1. Boar feeding conditions have been previously reported (Wu et al., 2019a). There were two treatments: (1) Control group (CON), 10 boars fed with a basal diet (Table 3-2), and (2) AOS 10mg/kg group (AOS 10), 10 boars fed with a basal diet plus 10mg/kg body weight of AOS. Semen samples were collected after 9-week feeding (Figure 3-1A) by

gloved-hand techniques. After collection, four semen parameters were assessed: semen volume, sperm concentration, sperm motility, and abnormal sperm rate, according to the reported methods (Wu et al., 2019b; Guo et al., 2020). Blood samples were harvested by venipuncture from the hindlimb vein of boars during ejaculations. Each blood sample was then centrifuged at 3000×g for 10min at 4°C to obtain a plasma sample and subsequently stored at −80°C until analysis. Each boar's rectum was massaged to stimulate defecation, and then, fresh feces were collected and stored at −80°C for subsequent microbiota analysis (Guo et al., 2020). The long-term effects of AOS on boar semen quality were determined. After AOS supplementation, all the boars were fed with basal diet (without AOS supplementation) for another 8weeks. The semen was collected every 5 days and the semen quality was analyzed.

3.3.3 Evaluation of spermatozoa motility using a computer-assisted sperm analysis system

Spermatozoa motility and concentration were determined by the computer-assisted sperm assay (CASA) method according to World Health Organization guidelines (WHO, 2010; Zhao et al., 2016; Zhang et al., 2018, 2019). Boar spermatozoa were incubated at 37.5°C for 30min then samples were placed in a pre-warmed counting chamber (MICROPTIC S.L., Barcelona, Spain). The Microptic Sperm class analyzer (CASA system) was used in this investigation. It was equipped with a 20-fold objective, a camera adaptor (Eclipse E200, Nikon, Japan), and a camera (acA780-75gc, Basler, Germany); it was operated by an SCA sperm class analyzer (MICROPTIC S.L.).

3.3.4 Morphological observations of spermatozoa

Boar sperm was stained with Eosin Y (1%; Zhao et al., 2016; Zhang et al., 2018, 2019). Spermatozoa abnormalities were then viewed using a bright-field microscopy (AH3-RFCA, Olympus, Tokyo, Japan) and were classified into head or tail morphological abnormalities: two heads, two tails, blunt hooks, and short tails. The examinations were repeated three times, and 500 spermatozoa per animal were scored.

3.3.5 In vitro fertilization

The procedure for the preparation of porcine oocytes has been reported previously (Redel et al., 2019; Zhou et al., 2020). Porcine ovaries were obtained from a slaughterhouse. Follicular fluid from 3 to 6 mm antral follicles was aspirated with an 18-gauge syringe. Cumulus oocyte complexes (COCs) with uniform cytoplasm and several layers of cumulus cells were selected and rinsed three times in washing medium [TCM-199 medium supplemented with 10% porcine follicular fluid (pFF), 5μg/ml insulin, 10ng/ml EGF, 0.6mM cysteine, 0.2mM pyruvate, and 25μg/ml kanamycin]. Approximately 70 COCs per well were cultured under mineral oil in 4-well plates containing TCM-199 medium supplemented with 10% porcine follicular fluid (pFF), 5μg/ml insulin, 10ng/ml EGF, 0.6mM cysteine, 0.2mM pyruvate, 25μg/ml kanamycin, and 5IU/ ml of each of eCG and hCG. The oocytes were matured for 44h at 38.5°C, 5% CO2 in a humidified incubator.

The in vitro fertilization (IVF) medium Tyrode's albumin lactate pyruvate (TALP) 29 was previously equilibrated for~3h at 38.5°C, with 5% CO2 in air and a humidified incubator until it reached a final pH of 7.4. In vitro mature (IVM) oocytes were mechanically denuded with an automatic pipette, washed in TALP medium, and transferred in groups of 50 oocytes to a 4-well plate (Nunc, Roskilde, Denmark) containing 500μl TALP medium per well. Sperm suspensions were added to the IVF wells at a final concentration of 25×10^3 cells/ml. After a 6-h coculture, the putative zygotes were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS), stained with 1% (w/v) Hoechst 33342 in PBS, and examined under an epifluorescence microscope. The parameters analyzed were the percentage of oocytes penetrated by one or more spermatozoa (Pen, %; Zapata-Carmona et al., 2020).

3.3.6 Boar fecal microbiota analysis

Total genomic DNA of boar feces was isolated using an E.Z.N.A.® Stool DNA Kit (Omega Bio-tek Inc., United States) following the manufacturer's instructions. DNA quantity and quality were analyzed using NanoDrop 2000 (Thermo Scientific, United States) and 1% agarose gel (Zhang et al., 2020).

The V3–V4 region of the 16S rRNA gene was amplified using the primers 338F and 806R with Barcode. The PCR reactions (total 30 μl) included 15 μl Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 mM primers, and 10 ng DNA. The thermal cycle was carried out with an initial denaturation at 98°C, followed by 30 cycles of 98°C for 10 s, 50°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, United States). The sequencing libraries were constructed with NEB Next® UltraTM DNA Library Prep Kit for Illumina (NEB, United States) following the manufacturer's instructions, and index codes were added. Then, the library was sequenced on the Illumina MiSeq 2,500 platform (Illumina, United States) and 300 bp paired-end reads were generated at the Novo gene. Meanwhile, all chimeras were removed (Zhang et al., 2020). The "Core Set" of the Greengenes database3 (DeSantis et al., 2006) was used for classification.

The raw reads obtained were filtered using the Trimmomatic program and then merged using FLASH (version 1.2.7). After that, quality control of the merged reads was performed using the QIIME (version 1.9.1) platform. The quality-filtered sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using UPARSE (version 7.1). Multiple sequence alignment was conducted using the MUSCLE software. The alpha diversity indices were calculated with QIIME (Version 1.7.0; Caporaso et al., 2010). Partial least squares discrimination analysis (PLS-DA) was performed using R software (v2.15.3).

3.3.7 Plasma and sperm metabolites determination

Plasma and sperm metabolites were determined by LC–MS/ MS (Zhang et al., 2020). Boar plasma and sperm were collected and maintained at −80°C. The protein was removed from the samples before LC–MS/MS analysis with ACQUITY UPLC and AB Sciex Triple TOF 5600 (LC/MS) as reported previously (Zhang et al., 2020).

The conditions for HPLC were: ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm), solvent A [aqueous solution with 0.1% (v/v) formic acid], and solvent B [acetonitrile with 0.1% (v/v) formic acid] with a gradient program: 0–2 min,

5%–20% B; 2–4 min, 20%–25% B; 4–9 min, 25%–60% B; 9–17 min, 60%–100% B; 17–19 min, 100% B; 19–19.1 min, 100%–5% B; and 19.1–20.1 min, 5% B. The flow rate was set at 0.4 ml/min and 5 μl was injected. ESI was used in the mass spectrometry program. Progenesis QI v.2.3 (Nonlinear Dynamics, Newcastle, United Kingdom) was used to normalize the peaks. Human Metabolome Database (HMDB), LIPID MAPS (v. 2.3), and METLIN software were used to qualify the data. Furthermore, the data were analyzed with SIMCA software (v. 14.0, Umetrics, Umeå, Sweden) and the KEGG database1 was used for pathway enrichment analysis.

3.3.8 Detection of protein levels and location in spermatozoa using immunofluorescence staining

The methods for IHF of boar sperm have been reported in our previous articles. Boar spermatozoa were fixed in 4% paraformaldehyde for 1 h, then the cells were spread onto poly-L-lysine coated microscope slides and air-dried. After three washings with PBS (5 min each), spermatozoa were incubated with 2% (vol/vol) Triton X-100 in PBS for 1 h at RT. Then, after three washes with PBS, the cells were blocked with 1% (wt/vol) BSA and 1% goat serum in PBS for 30 min at RT, followed by incubation with primary antibodies (1:100; Table 3-2) diluted in blocking solution overnight at 4°C. The following morning, after three washes with PBS Tween 20 (0.5%) the slides were incubated with Alexa Fluor 546 goat anti-rabbit IgG (1,200) for 30 min in darkness at RT. The negative control samples were incubated with a secondary antibody and without a primary antibody. Slides were washed with PBS Tween-20 three times and then incubated with DAPI (4.6-diamidino-2-phenylindole hydrochloride, 100 ng/ml) as a nuclear stain for 5 min. After a brief wash with ddH2O, the slides were covered with an antifading mounting medium (Vector, Burlingame, United States). Fluorescence images were obtained with a Leica Laser Scanning Confocal Microscope (LEICA TCS SP5 II, Germany; Zhao et al., 2016; Zhang et al., 2020).

3.3.9 Determination of protein levels by western blotting

The procedure for Western blotting analysis of boar sperm proteins is reported in our previous publications (Zhao et al., 2016; Zhang et al., 2020). Briefly, sperm cells were lysed in RIPA buffer containing the protease inhibitor cocktail from Sangon Biotech, Ltd. (Shanghai, China). Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology, Shanghai, China). Actin was used as a loading control. The primary antibodies (Abs) are listed in Table 3-2. Secondary donkey anti-goat Ab (Cat no.: A0181) was purchased from Beyotime Institute of Biotechnology (Shanghai, P.R. China), and goat anti-rabbit (Cat no.: A24531) Abs were bought from Novex® by Life Technologies (United States). Fifty micrograms of total protein per sample were loaded onto 10% SDS polyacrylamide electrophoresis gels. The gels were transferred to a polyvinylidene fluoride (PVDF) membrane at 300mA for 2.5h at 4°C. Then, the membranes were blocked with 5% BSA for 1h at RT, followed by three washes with 0.1% Tween-20 in TBS (TBST). The membranes were incubated with primary Abs diluted at 1:500 in TBST with 1% BSA overnight at 4°C. After three washes with TBST, the blots were incubated with the HRP-labeled secondary goat anti-rabbit or donkey anti-goat Abs, respectively, for 1h at RT. After three washes, the blots were imaged.

3.3.10 Statistical analysis

Data are expressed as the mean ± SEM. $P<0.05$ was considered significantly difference. Using the student's t-test (SPSS 21 software) to perform the statistical analyzes. Plots were performed by using GraphPad Prism 8.0.2.

3.4 Results

3.4.1 Impact of AOS on boar semen quality and in vitro fertility potential

After 9 weeks of feeding (Figure 3-1A), the AOS10 group showed significantly increased sperm motility from 87.8% to 93.5% (Figure 3-1B; $p<0.05$). The semen volume per boar per day (204 ml/ejaculation vs. 176 ml/ejaculation; $p=0.612$) and the concentration ($5.6 \times 10^{\wedge 8}$/ml vs. $5.0 \times 10^{\wedge 8}$/ml; $p=0.137$) of sperm also showed an

increase in AOS10 over CON while the difference was not significant (Figure 3-1C,D). Moreover, the in vitro fertilization rate was also higher in AOS10 (58.6%) than in CON (52.1%; Figure 3-1E), however, this was not significant.

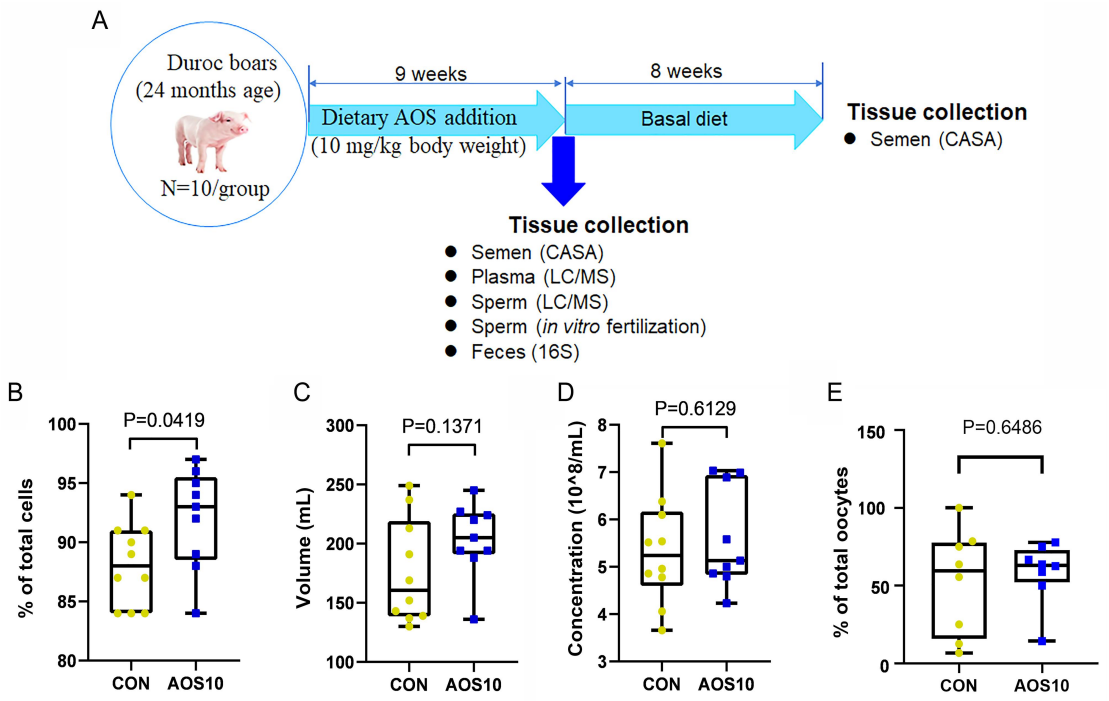

Figure 3-1 The impacts of AOS on boar sperm quality. (A) Study scheme. (B) Spermatozoa motility determined by CASA. Y-axis = % of total cells, X-axis = treatment group (mg/kg body weight). n = 10. (C) Semen volume. Y-axis = volume (ml), X-axis = treatment concentration (mg/kg body weight). n = 10. (D) Sperm concentration. Y-axis = sperm concentration (10^8/ml), X-axis = treatment group (mg/kg body weight). n = 10. (E) In vitro fertilization rate. Y-axis = % of total oocytes, X-axis = treatment group (mg/kg body weight). n = 10.

3.4.2 Effects of AOS on boar sperm quality and sperm metabolism

To understand the mechanisms underlying the AOS improvement of semen quality, we explored the protein levels of important genes for sperm quality. AOS10 increased the protein levels of gelsolin (1.61-fold), p-AKT (phosphorylated protein kinase B; 1.48-fold), protein kinase A (PKA; 1.95-fold), and Zn-alpha2 glycoprotein (ZAG; 2.05-fold) according to IHF detection (Figure2-2A,B; *p <0.05). The data for these protein levels were confirmed by western blotting analysis. Moreover, the levels of the other four sperm proteins such as cation channel sperm-associated protein (CatSper; 2.12-fold), outer dense fiber of sperm tails 2 (ODF2; 1.42-fold), p-ERK1

(phosphorylated extracellular signal-regulated kinase;1.51-fold 1), and Phosphoinositide-3-Kinase (PI3K; 1.47-fold) were also elevated in AOS10 over CON (Figure 2-2C,D; *p <0.05).

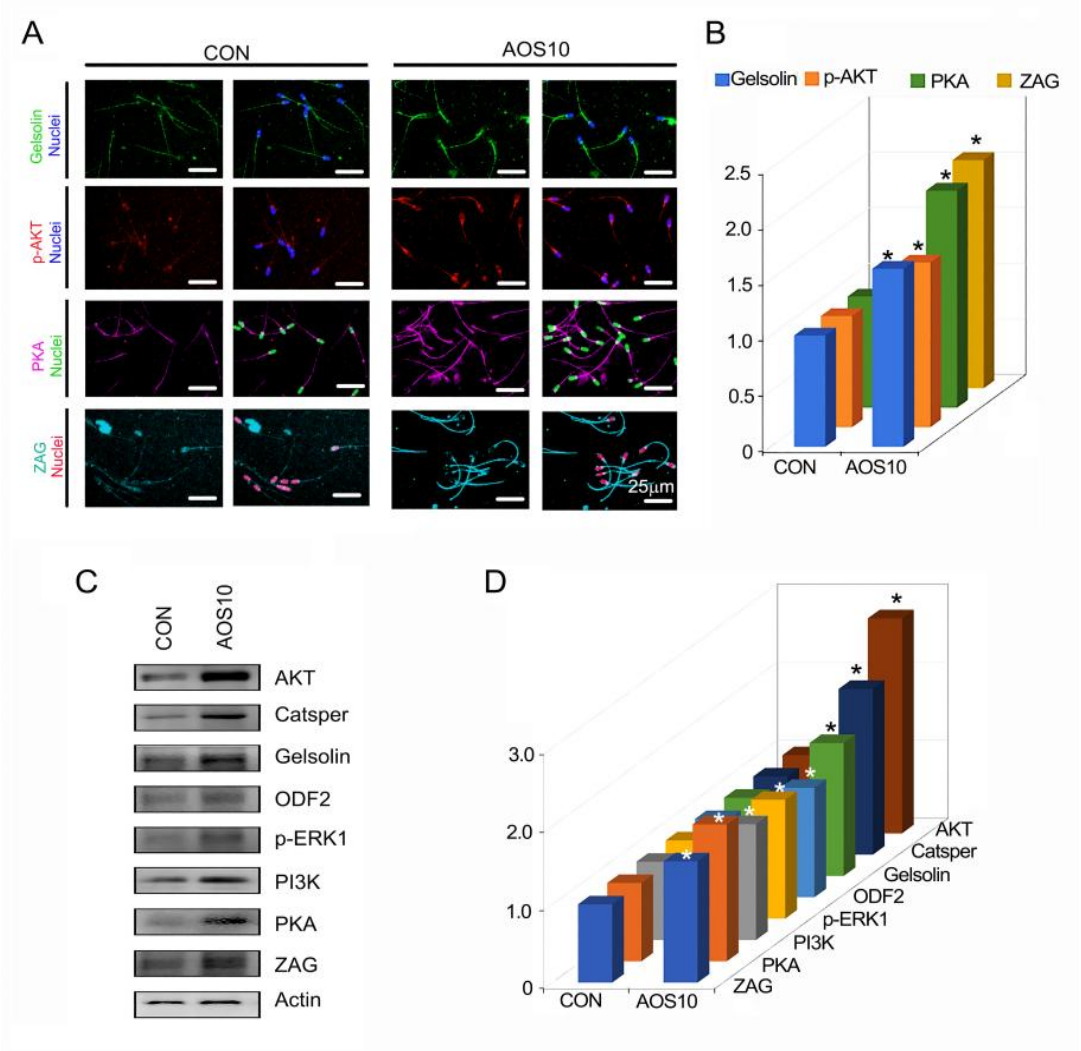

Figure 3-2 The influence of AOS on the protein expression in boar sperm. (A) Sperm protein levels of important genes for semen quality as detected by immunofluorescence staining. N > 6. (B) Quantitative data for immunofluorescence staining. Y-axis = fold change to CON, X-axis = treatment group (mg/kg body weight). *p < 0.05. (C) Sperm protein levels of important genes for semen quality as detected by Western blotting. N > 3. (D) Quantitative data for western blotting analysis. Y-axis = fold change to CON, X-axis = treatment group (mg/kg body weight). *p < 0.05.

There were 1,031 metabolites detected in the boar sperm samples. The OPLS-DA analysis showed that the AOS10 and CON groups were well separated. Twenty-eight metabolites were significantly different in AOS10 compared to CON (Figure3-3A). Nine of the significantly improved metabolites including β-leucine, D-glutamic acid, γ-glutamylthreonine, L-lysine, L-norleucine, L-proline, methionine

sulfoxide, O-acetylserine, and tyrosylglutamate are shown in Figure 3-3B (1.2–1.6folds; $p<0.05$). Some of the significantly decreased metabolites (such as 2-hydroxylfelbamate, 14,15-diHETrE, heptadecanoic acid, and methyl hexadecenoic acid) are shown in Figure 3-3C (5%–10%; $p<0.05$). These 28 metabolites were well correlated with sperm motility, sperm concentration, and sperm volume (Figure 3-3D), with the changed metabolites in AOS10 being positively correlated with these sperm parameters, while the reduced metabolites in AOS10 were negatively correlated. At the same time, the metabolites were well correlated with each other.

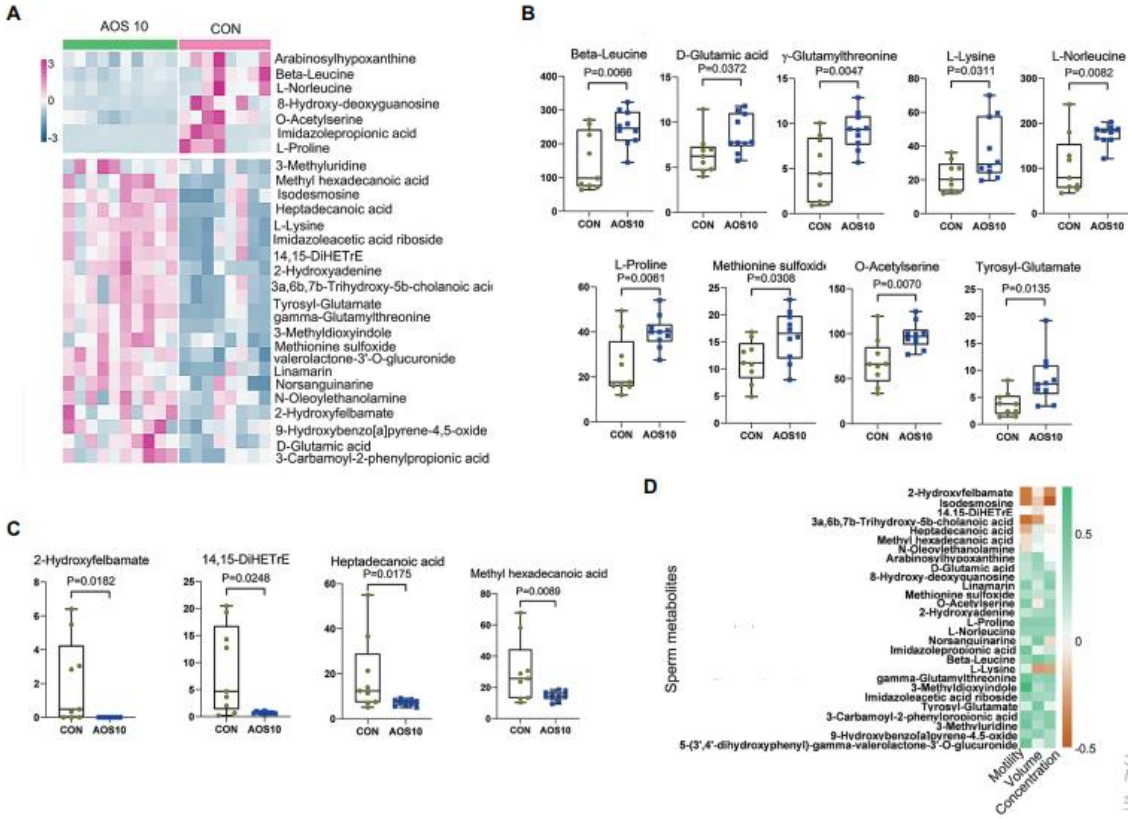

Figure 3-3 The impacts of AOS on boar sperm metabolome. (A) Heatmap of changed sperm metabolites. The sperm metabolites were determined by LC– MS/MS. (B) List of AOS increased sperm metabolites. Y-axis = relative amount, X-axis = treatment group (mg/kg body weight). (C) List of AOS decreased sperm metabolites. Y-axis = relative amount, X-axis = treatment group (mg/kg body weight). (D) Correlation of sperm metabolites and sperm concentration, volume, and motility.

3.4.3 Impact of AOS on boar blood metabolism

There were 1,087 metabolites detected in boar plasma samples. The OPLS-DA analysis showed that the AOS10 and CON groups were well separated. Eighteen metabolites were significantly different in AOS10 compared with CON (Figure 3-4A).

Functional enrichment of these metabolites showed that they were involved in amino acid metabolism and retinol metabolism (Figure3-4B). Some of the significantly changed metabolites are shown in Figure 3-4C. 11-cis-retinol, betaine, 5-hydroxy-indoleacetaldehyde, N1-methyl-2-pyridone-5- carboxamide, and quinolinic acid were increased from 1.5–2.4-fold (p<0.05), while iminoaspartic acid was reduced (45%, p<0.05). At the same time, these metabolites were well correlated with each other. Moreover, the significantly changed blood metabolites and the significantly changed sperm metabolites were well correlated (Figure3-4D).

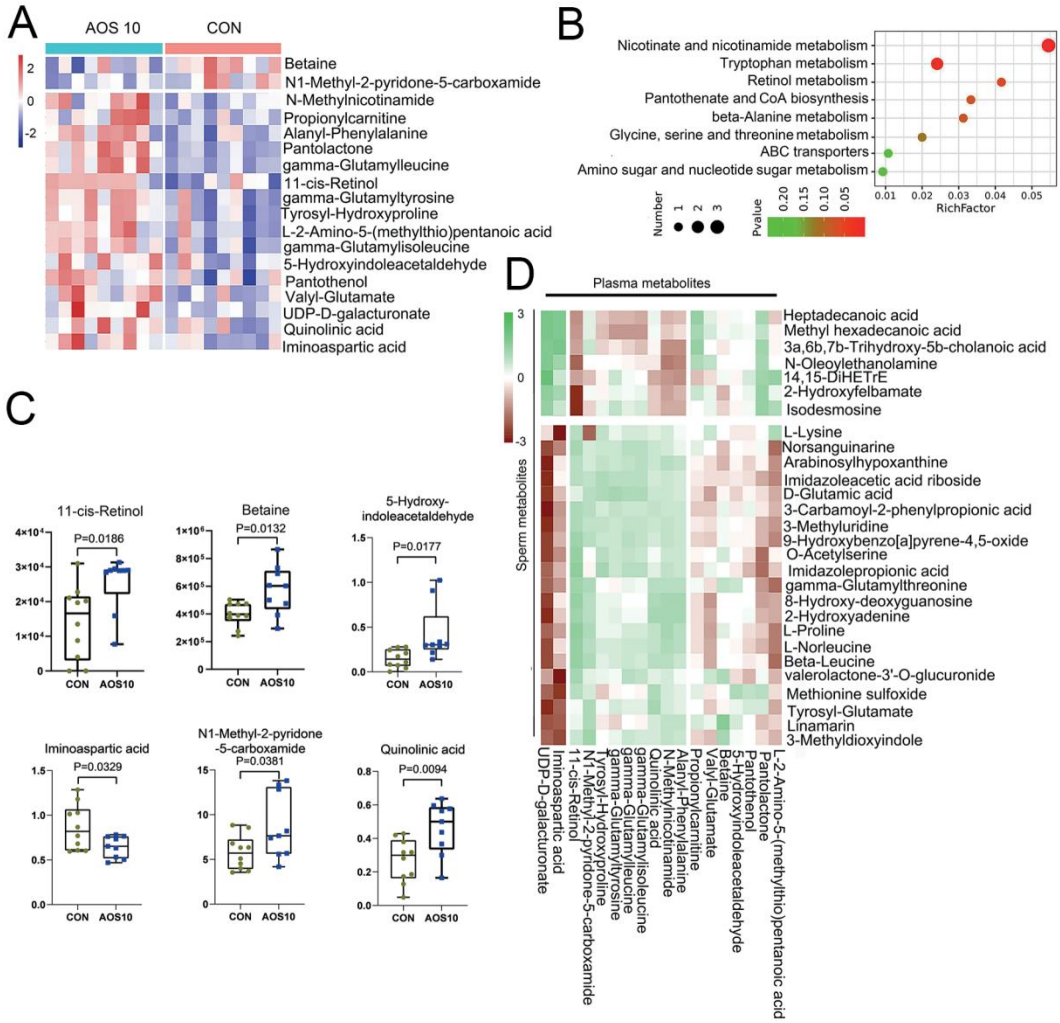

Figure 3-4 The effects of AOS on boar blood metabolome. (A) Heat map of changed blood metabolites. The blood metabolites were determined by LC–MS/ MS. (B) KEGG enriched pathways of changed blood metabolites. (C) List of AOS changed blood metabolites. Y-axis = relative abundance, X-axis = treatment group (mg/kg body weight). (D) Correlation of blood metabolites and sperm metabolites.

3.4.4 Effects of AOS on boar gut microbiota

AOS10 affected the gut microbiota composition (Figure 3-5A). At the phylum level, compared to CON, AOS10 changed the relative abundances of *Bacteroidetes* (1.21fold; $p>0.05$), decreased the levels of *Firmicutes* (Figure 3-5B; $p>0.05$), and increased the ratio of *Bacteroidetes*/*Firmicutes* in boar fecal samples. Moreover, at the class level, the microbiota and sperm motility were well correlated and *Bacteroidetes* was positively correlated with sperm motility; meanwhile, *Firmicutes* was negatively correlated with sperm motility (Figure 3-5C). At the genus level, AOS10 improved the relative abundances of several beneficial bacteria: *Bifidobacterium* (1.51-fold), *Coprococcus* (2.33-fold), and *Butyricicoccus* (1.33-fold). (Figure3-5D; $p>0.05$). Moreover, *Coprococcus* was positively correlated with sperm motility (Figure3-5E), while *Intestinimonas* and *Lactobacillus* were positively correlated with both sperm motility and concentration (Figure 3-5E).

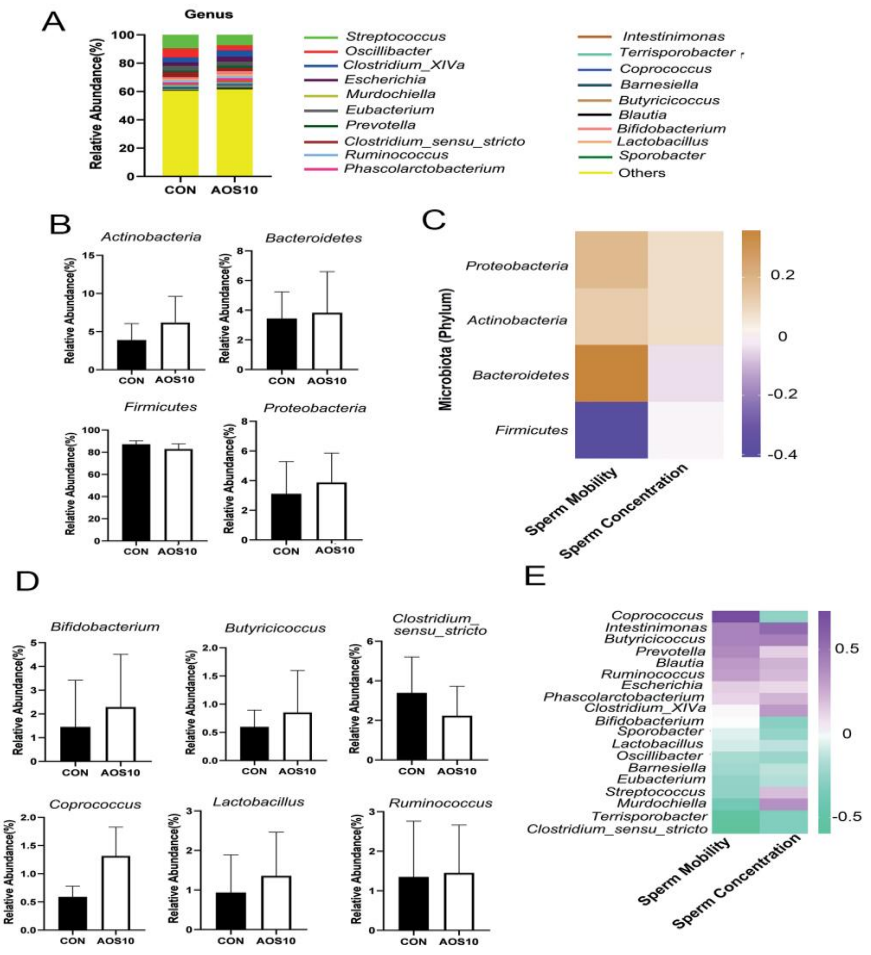

Figure 3-5 The influence of AOS on boar gut microbiota. (A) Differences of bacterial abundance at the genus level. Y-axis = relative abundance, X-axis = treatment group (mg/kg body weight). (B) Differences of bacterial abundance at the class level. (C) Correlation of bacterial abundance at the class level with sperm motility and concentration. (D) Representative differences of bacterial abundance at the genus level. Y-axis = relative abundance, X-axis = treatment group (mg/kg body weight). (E) Correlation of bacterial abundance at the genus level with sperm motility and concentration.

Furthermore, sperm metabolites and fecal microbiota were well correlated (Figure 3-6A). Sperm metabolites increased in the AOS10 group were positively correlated with the beneficial microbiota *Bifidobacterium*, *Coprococcus*, and *Butyricicoccus* (Figure 3-6A). Similarly, blood metabolites and fecal microbiota were well correlated (Figure3-6B), and the blood metabolites in AOS10 were positively correlated with the beneficial microbiota *Bifidobacterium*, *Coprococcus*, and *Lactobacillus* (Figure 3-6B).

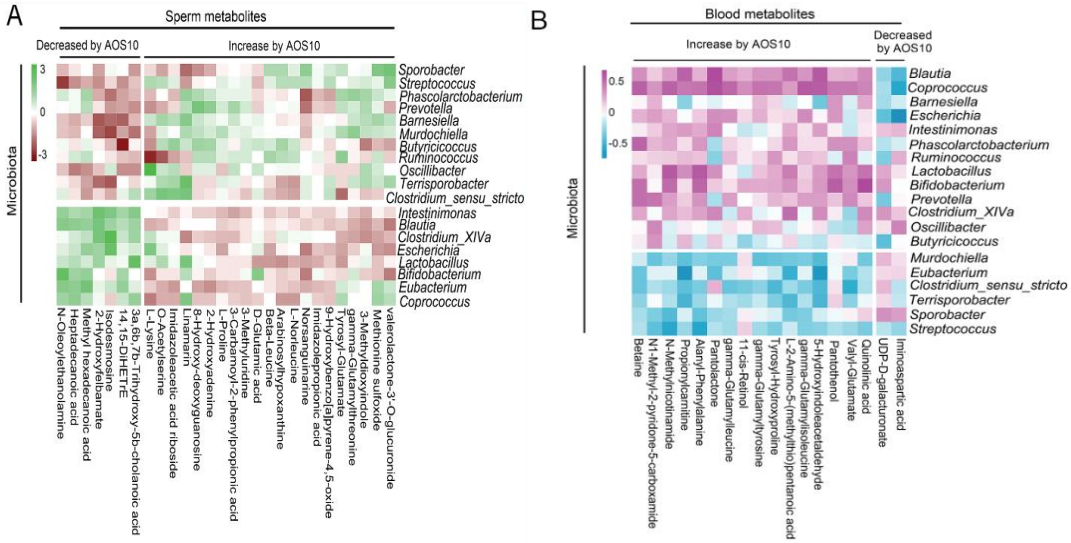

Figure 3-6 (A) Correlation of sperm metabolites with fecal microbiota. (B) Correlation of blood metabolites with fecal microbiota.

3.4.5 Impact of AOS10 on semen quality for a long time

AOS had a long-term beneficial improvement on boar semen quality by the increase in the semen volume (175 vs. 160ml/ ejaculation, $p<0.05$) and sperm motility, while the decrease in the percentage of abnormal sperm after another 2 months on basal diet (without AOS addition; Figure 3-7A–C). The sperm concentration was in an increased trend in AOS10 group compared to CON (Figure3-7D; p =0.185).

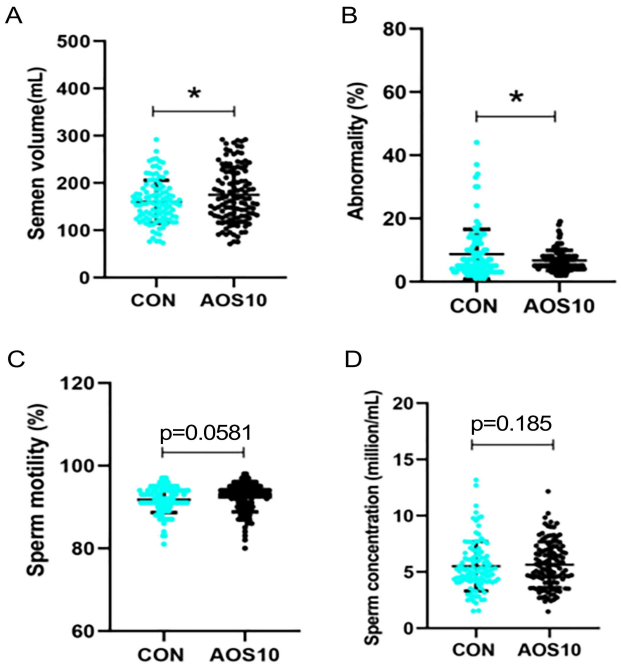

Figure 3-7 Long-term effects of AOS on boar semen quality. After AOS supplementation, boars were fed with basal diet for 8 weeks. Semen quality was determined every 5 days for 2 months (no AOS supplementation). (A) Semen volume. Y-axis = volume (ml), X-axis = treatment group (mg/kg body weight). (B) Abnormal sperm. Y-axis = % of total cells, X-axis = treatment group (mg/kg body weight). (C) Sperm motility. Y-axis = % of total cells, X-axis = treatment group (mg/kg body weight). (D) Sperm concentration. Y-axis = sperm concentration ($10^{\wedge 8}$/ ml), X-axis = treatment group (mg/kg body weight). n = 10, *$p < 0.05$.

3.5 Discussion

AOS have been used in many different perspectives as anti-inflammation (Moriya et al., 2013), anti-apoptosis (Tusi et al., 2011), anti-proliferation (Tajima et al., 1999), and even anti-cancer chemicals (Wan et al., 2017) because of the highly desired natural properties (non-immuno-genicity, biodegradability, and non-toxicity). AOS decrease the production of nitric oxide, prostaglandin E2, and pro-inflammatory cytokines, and block the expression of toll-like receptor 4 and nuclear factor (NF)-κB to prevent neuroinflammation or neurodegenerative diseases (such as Alzheimer's disease; Zhou et al., 2015). Wan and colleagues reported that AOS improved antioxidant levels and increased piglet growth rates (Wan et al., 2017, 2018a,b). AOS benefited intestinal epithelial cell growth and differentiation to improve livestock growth rates (Zhao et al., 2020a). Moreover, AOS improved busulfan disrupted spermatogenesis in mice (Zhao et al., 2020b). In the current investigation, we found

that AOS 10 mg/kg could benefit boar sperm motility. The difference between AOS10 and CON was small, which may be because the boars used in this investigation were at an optimal age for sperm production; therefore, the room for improvement in these parameters was limited. However, all-together, the data suggested that AOS has the potential to improve boar semen quality and fertility. Moreover, the levels of important proteins in boar sperm on AOS group was higher than CON group such as gelsolin, ODF2, PKA, AKT, etc. All these proteins play important roles in sperm function or fertility (Lee, 2012; Liu et al., 2012; Finkelstein et al., 2013; Zhao et al., 2016; Sun et al., 2017). The data here indicated that AOS10 improved boar sperm quality. The data indicated that AOS could be used as a dietary additive for boars to improve their semen quality.

Gut microbiota play many physiological roles not limited to metabolic-related disorders such as obesity and diabetes (Bouter et al., 2017; Liu et al., 2017b), but also including nervous system and reproductive system-related diseases or conditions (Dai et al., 2015). Previously, we found that AOS benefited gut microbiota by the increasing in the “beneficial” bacteria while the decreasing in “harmful” bacteria in murine small intestines to rescue cell development (Zhang et al., 2020). Gut microbiota metabolize nutrients in the intestine and regulate intestinal metabolites to influence the blood metabolome (Tajima et al., 1999; Tusi et al., 2011). In turn, while traveling through body organs, blood metabolites can influence their development or induce disorders (Dai et al., 2015). It is known that metabolic regulation is essential for spermatogenesis (Cheng et al., 2010; Rato et al., 2010, 2012), and cholesterol and lipid homeostasis play a vital role in male fecundity (Cross, 1998; Ergün et al., 2007; Maqdasy et al., 2013; Lu et al., 2016; Kim et al., 2017). Sertoli cells act as nurse cells, providing the nutrients and energy for the process of spermatogenesis. Many other components such as hormones and endogenous or exogenous factors have a synergistic role in the homeostasis of metabolism in the testis and the progression of spermatogenesis (Rato et al., 2012). It has been shown that abnormal lipid metabolism in the reproductive system or blood contributes to human male infertility (Lu et al., 2016; Kim et al., 2017). AOS could improve busulfan-damaged homeostasis of lipid

metabolism in murine blood (Zhang et al., 2020). In the current investigation, the relative abundances of beneficial bacteria were higher in the AOS group compared to the CON group, such as *Lactobacillus*. At the same time, AOS benefited boar sperm and blood metabolites by increasing levels of proline, lysine, retinol, betaine, etc. All the data indicated that AOS can boost boar semen quality through improving the gut microbiota, blood, and testicular metabolites.

The very interesting finding in the current study was that AOS benefited gut microbiota can continuingly improve boar semen quality after AOS treatment followed by 8 weeks of basal diet (without AOS supplementation). Because AOS cannot be directly absorbed into blood to reach the organs to affect their functions (Liu et al., 2019), the beneficial effects of AOS on sperm metabolites and semen quality may be due to the benefited gut microbiota. Otherwise, the effects cannot last for so long time. The data in the current investigation and our previous reports confirm that AOS benefit gut microbiota in mice and boars, and the benefited gut microbiota to increase semen quality.

3.6 Conclusions

In summary, the beneficial impact of AOS on boar semen quality possibly through the positive changes in the gut microbiota and plasma/sperm metabolism was revealed as well as the sperm proteins. These improvements will increase the conception rate and litter sizes of sows, so as to increase the economical supply of porcine meat for global consumption.

Table 3-1. Sperm parameters before animal experiment

Items	CON	AOS10	P value
Sperm motility (%)	85.19± 1.67	86.58±1.32	0.81
Sperm concentration (10^8/ml)	4.3±0.4	4.8±0.65	0.46
Abnormal sperm rate (%)	10.53±1.49	10.03±1.41	0.81

Note: Before the animal experiment start, 20 boars were chosen with similar conditions in order to eliminate the differences between the two groups from the start of the experiment. A P value higher than 0.05 indicated no differences between the two groups.

Table 3-2 Composition and nutrient analysis of basal diet

Ingredient	Content, %
Corn	35.15
Barley	24.83
wheat	15.82
Rice bran meal	9.40
Soybean meal	7.90
Soybean oil	2.00
L-lysine	0.40
Methionine	0.14
Threonine	0.24
Ground limestone	1.44
Monocalcium phosphate	1.21
Sodium chloride	0.48
Premix*	1.00
total	100
Nutrient, %	
Calculated NE, kcal/kg	2240
Crude protein, %	14.50
Crude fat, %	3.22
Crude ash, %	6.18
Crude fiber, %	4.15

*: Premix provided the following minerals per kilogram: 17 mg Cu, 160 mg Fe, 140 mg Zn, 50 mg Mn, 0.50 mg I, 0.50 mg Se, and 0.22 mg Cr.

Table 3-3. Primary antibody information

Gene symbol	Name	Cat. #	Predicted size	Source (Animal)	Company
AKT1	Protein kinase B	bs-0115R	56kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Actin	actin	Ab3280	42kDa	Rabbit (polyclonal)	Abcam
PKA	cAMP dependent protein kinase alpha catalytic subunit	bs-0520R	40kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
P-ERK	phospho-Erk1 (Thr202 + Tyr204)	bs-1645R	43kDa	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
ZAG	Zinc Alpha 2 Glycoprotein	bs-19382R	32kDa	Rabbit	Beijing Biosynthesis Biotechnology CO.
CATSPER	CATSPER	bs-23326R	90kDa	Rabbit	Beijing Biosynthesis Biotechnology CO.
Gelsolin	Gelsolin	bs-1160R	80kDa	Rabbit	Beijing Biosynthesis Biotechnology CO.
ODF2	Cenexin1	bs-10309R	91kDa	Rabbit	Beijing Biosynthesis Biotechnology CO.
p-PI3K	phosphorylated Phosphoinositide 3-kinase	bs-5571R	80kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
p-AKT	phosphorylated AKT	bs-2720R	56kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.

Chapter IV

Alginate oligosaccharide supplementation improves boar semen quality under heat stress

Chapter IV. Alginate oligosaccharide supplementation improves boar semen quality under heat stress

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Key words: Heat stress, Alginate oligosaccharides (AOS), semen quality, gut microbiota, sperm metabolome, sperm proteome, boars

Adapted from the reference: Zhou Y, Wei Z, Tan J, Sun H, Jiang H, Gao Y, Zhang H, Schroyen M. Alginate oligosaccharide supplementation improves boar semen quality under heat stress. *Stress Biology.*

Author Contributions: Yexun Zhou participated in animal husbandry experiments, data collection and analysis, as well as drafting and writing the paper.

4.1 Abstract

Heat stress is a serious problem that affects animal husbandry by reducing growth and reproductive performance of animals. Adding plant extracts to the diet is an effective way to help overcome this problem. Alginate oligosaccharide (AOS) is a natural non-toxic antioxidant with multiple biological activities. This study analyzed the potential mechanism of AOS in alleviating heat stress and improving semen quality in boars through a combination of multiple omics tools. The results indicated that AOS could significantly improve sperm motility (P<0.001) and sperm concentration (P<0.05). At the same time, AOS improved the antioxidant capacity of blood and semen, and increased blood testosterone (P<0.05) level. AOS could improve the metabolites in sperm, change the composition of gut microbiota, increase the relative abundance of beneficial bacteria such as *Pseudomonas* (P<0.01), *Escherichia-Shigella* (P<0.05), *Bifidobacterium* (P<0.01), reduce the relative abundance of harmful bacteria such as *Prevotella_9* (P<0.05), *Prevotellaceae_UCG-001* (P<0.01), and improve the content of short chain fatty acids. Proteomic results showed that AOS increased proteins related to spermatogenesis, while decreasing heat shock protein 70 (P<0.05) and heat shock protein 90 (P<0.01). These results were verified using immunofluorescence staining technology. There was a good correlation among sperm quality, sperm metabolome, sperm proteome, and gut microbiota. In conclusion, AOS can be used as a feed additive to increase the semen quality of boars to enhance reproductive performance under heat stress.

4.2 Introduction

Temperature is a prominent environmental factor that necessitates attention with regard to animal production, since it is widely affecting the growth and reproductive performance of mammals (Serviento et al., 2020; Kumar et al., 2021). When the environmental temperature exceeds the limitation that animals tolerate, the heat stress reaction often occurs (Belhadj Slimen et al., 2016). The main manifestations include increased heat production and sweating. Excessive heat stress can lead to irreversible

loss of function (Luo et al., 2021). Swine are animals that are sensitive to temperature. Due to a thick subcutaneous fat layer, underdeveloped sweat glands and poor thermal regulation, the heat generated as a byproduct of a pig's metabolic processes is difficult to evaporate through the skin (Stombaugh et al., 1973). Research has shown that reproductive traits in pigs are less influenced by genetic factors (Dervishi et al., 2021). On the contrary, they are more influenced by environmental factors (Madsen et al., 2018). Studies have shown that when the average humidity of the pig barn reaches 30% and the average temperature reaches 28℃, heat stress will occur, which will significantly affect the intestinal health and growth performance of pigs (Wegner et al., 2016; Guo et al., 2018; Serviento et al., 2020), especially for boars and sows with large body weight. The body weight of adult boars is around 300kg (Zhou et al., 2022), and when the ambient temperature exceeds 32℃ and lasts a long time, boars with that weight will undergo heat stress, which can reduce the semen quality (Gruhot et al., 2020). The reason for this is two-fold. On the one hand, in the condition of heat stress, the testicular temperature will also rise, which affects the testicular ability to regulate temperature (Thundathil et al., 2012). On the other hand, sperm stored in the epididymis of a heat-stressed boar produces more radical oxygen species through respiration, resulting in a decline in semen quality (Jannatifar et al., 2019). The ideal breeding environment of a large-scale boar stud has a humidity that cannot exceed 50%, and a temperature that cannot exceed 24℃ in China (Gruhot et al., 2020; Sui et al., 2022). Therefore, adopting nutritional regulation to alleviate the decrease in semen quality caused by heat stress has positive significance for the swine industry.

Alginate is a type of polysaccharide polymer that has been widely used in the pharmaceutical and food industries due to its unique physicochemical properties and beneficial health effects (Mrudulakumari Vasudevan et al., 2021). However, due to the low water solubility and high viscosity of alginate, its development and application are limited. Alginate oligosaccharides (AOS) are degradation products of alginate, characterized by a low molecular weight, high solubility in water and non-toxic nature (Lu et al., 2022), and it has received widespread attention in recent years. AOS has multiple biological activities including immune regulation,

anti-inflammatory properties, anti-oxidative effects. One of the key functions of AOS in immune regulation is to induce cytokine activity. Research had also shown that AOS could increase the concentrations of immunoglobulins and IgA in the serum of weaned piglets, thereby enhancing their immune function (Wan et al., 2018b). AOS is a strong antioxidant to scavenge free radicals. Research had found that AOS increased the levels of superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) in the serum and intestine of weaned piglets, while reducing the levels of malondialdehyde (MDA) in the serum and intestine (Wan et al., 2018a). AOS could also inhibit the TLR4/NF-κB signaling pathway to enhance the intestinal anti-inflammatory ability of weaned piglets (Wan et al., 2021b). In our previous research, we found that AOS could improve the sperm motility of mice that were treated with Busulfan, and repaired the damaged testicular tissue (Zhang et al., 2021b). However, there are few reports on AOS in alleviating heat stress and improving boar semen quality. The aim of this investigation was to explore the potential mechanism of AOS in improving semen quality of Duroc boars under heat stress, and to provide a theoretical basis for solving the problem of poor semen quality caused by high temperature in summer.

4.3 Materials and methods

4.3.1 Boars and experimental design

The study was approved by the Animal Care and Use Committee of the Institute of Animal Sciences of Chinese Academy of Agricultural Sciences (access number of IAS2022-13). Thirty Duroc boars with similar age (33 months old), health status and weight (around 300 kg) were selected randomly at the Ya Ji Mountion boar stud which belongs to the Yangxiang Joint Stock Company. The boars were divided into 2 groups randomly, a control group (CON) and AOS group (AOS). The control group (CON) was fed a basal diet (Feed formula is shown in Table 4-1), and boars in the AOS group (AOS) were fed a basal diet with 10 mg/kg body weight AOS (Han et al., 2022). The boars were housed in individual pens and the whole feeding period lasted

9 weeks. During the experiment, the everyday temperature and humidity were recorded at the same time each day (8:00 am and 14:00 pm).

Semen samples were obtained using the hand grip method. Sperm parameters including sperm concentration, sperm motility and abnormal sperm rate were assessed by Computer-assisted sperm analysis (CASA) software according to previous reports (Guo et al., 2020). In order to remove the differences between two groups before the experiment, we examined the semen quality parameters for the 2 semen collection cycles. The results are shown in Table 4-3. The sperm parameters before the experiment (summer and winter) are shown in Table 4-4. A sample of semen was stored at -80°C for further analyzes. During ejaculating, when the boars are immobilized, blood samples were taken from the hind legs. Blood plasma and blood cells were separated by centrifugation at 3000 x g for 10 minutes, after which plasma was transferred to -80°C for further testosterone analyzes. Fecal samples were taken from the rectum, placed in liquid nitrogen, and finally stored in a -80°C freezer for 16S analysis and short chain fatty acids determination.

4.3.2 Temperature-Humidity Index calculated during the experiment

During the experiment, temperature and humidity were recorded every day. The THI was calculated according to the formula THI = 0.8 Ta + RH/100 × (Ta−14.4) + 46.4 (Yin et al., 2022), with Ta (°C) is the ambient temperature; RH (%) indicates the relative humidity. When the THI<74, animals are not in heat stress. When the THI is between 74 to 78, animals are in a state of mild heat stress. When the THI is between 78 to 82, animals are in a state of middle level heat stress. When the THI >82, animals are in serious heat stress. The THI for this experiment is shown in Figure 4-1, which indicates that the boars were in a moderate to serious level of heat stress.

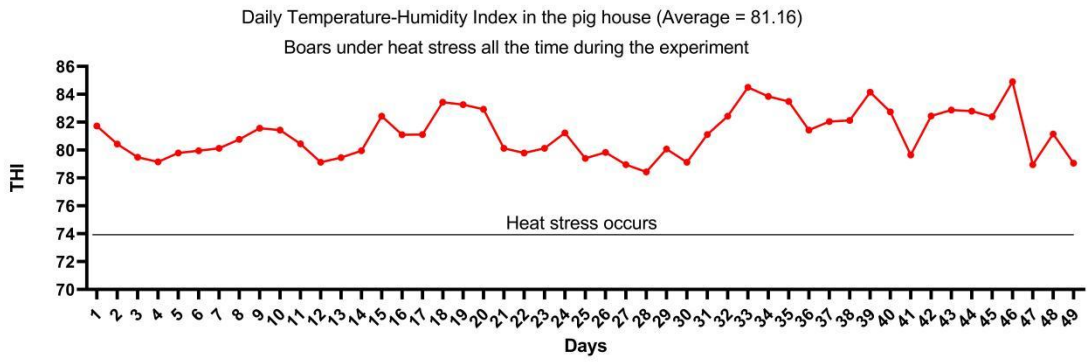

Figure 4-1 THI variation. The y-axis represents the THI. The x-axis represents the number of days of the experiment. The black line indicated a THI of 74. All THI values were higher than this reference for the duration of the experiment.

4.3.3 Sperm parameters analyzed by computer-assisted sperm analysis system

Sperm parameters, including sperm concentration, sperm motility, and abnormal sperm rate, were recorded by a computer assisted sperm analysis (CASA) system (Shanghai Kasu Biotechnology Co., Ltd., Shanghai, China). The evaluated criteria of sperm motility was as follows: grade A fast forward movement > 22 μm s^{-1}; grade B forward movement < 22 μm s^{-1}; grade C curve movement < 5 μm s^{-1}; grade D no movement. The sperm concentration should be more than 10^8/ml, the abnormal sperm rate should be less than 30% (Cao et al., 2011).

4.3.4 Detection of plasma and sperm antioxidant indicators and blood testosterone content

Antioxidant parameters (T-AOC, SOD, GSH-Px, CAT, MDA, GSH, Hydroxyl free radical, Oxygen free radical) in serum and sperm as well as blood testosterone were tested using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China and Beijing Boxbio Science&Technology Co.,Ltd, respectively) following the manufacturer's instructions.

4.3.5 Sperm metabolome assay by LC-MS/MS

Boar sperm (n=6 per group) was taken out from -80°C freezer. The protein

fraction was removed and then the samples were analyzed by LC/MS. An ACQUITY UPLC BEH C18 column (1.7 μm , 2.1 × 100 mm) was employed in both positive and negative modes. Solvent A and solvent B, aqueous solutions containing 0.1% formic acid and 0.1% acetonitrile respectively, were used. The following procedure was performed: 5–20 % B over 0–2 minutes; 20–60 % B over 2–4 minutes; 60–100 % B over 4–11 minutes, keep the composition at 100 % B for 2 minutes, 13–13.5 minutes from 100 % to 5 % B, and hold it at 5% B for 13.5–14.5 minutes. The flow rate was set at 0.4 mL/minute and the column temperature was 45℃. The sperm was stored at 4℃ and each time 5 μL was used to inject. ESI was used in the mass spectrometry program.

4.3.6 Quantitative analysis of boar sperm proteome using TMT labeling

Frozen samples (n=4 per group) were transferred into low protein binding tubes and lysed with 300 μL lysis buffer supplemented with 1mM PMSF. Then samples were further lysed with sonication. The parameters were set as 1s /1s intervals and 80 W for 2 min. After sonication, the samples were centrifuged at 12000 rpm for 10 min at 4°C to remove insoluble particles, and this was repeated once to further exclude precipitation. Protein concentration was determined by BCA assay. The protein samples were aliquoted to store at -80°C.

According to the measured protein concentration, the same quality protein was taken from each sample, and different groups of samples were diluted to the same concentration and volume. Twenty-five mM DTT of corresponding volume was added into the above protein solution to make the DTT final concentration about 5mM, after which this was incubated at 55°C for 30 min. Then, the corresponding volume of iodoacetamide was added to reach a final concentration of 10mM, after which the solution was placed in the dark for 15 min at room temperature. To precipitate the

protein, 6 times of the volume of pre-cooled acetone was added and the sample was placed at - 20 °C for more than four hours or overnight. Subsequently, the sample was centrifuged at 8000g for 10 min at 4°C to collect the precipitate. With respect to the amount of protein, the corresponding volume of enzymolysis diluent (protein: enzyme = 50:1 (m/m), 100 ug of protein and 2 ug of enzyme) was added to redissolve the protein precipitate. The solutions were incubated for digestion at 37°C for 12 h. Finally, samples were lyophilized or evaporated after enzymolysis.

For TMT pro labelling, the lyophilized samples were resuspended in 100 μL 100 mM TEAB (pH8.5) and 40 μL of each sample were transferred into a new tube for labeling. Anhydrous acetonitrile was added to the TMT reagent vial at room temperature. The centrifuged reagents were dissolved for 5 min and mixed for centrifugation and this step was once repeated. Then 10 μL of the TMT pro label reagent was added to each sample for mixing. The composition was incubated at 17°C for 1 hour. Five μL of 5% hydroxylamine was added to each sample and incubated for 15 minutes to quench the reaction. The labeling peptide solutions were lyophilized and kept at -80°C conditions.

The solutions were supplemented with 1% formic acid (FA) to adjust the pH value to 1-3. We utilized a C18-Reverse-Phase SPE Column to desalt the digested peptides. First of all, 1 mL methanol was used to wash the column twice, followed by 0.1% TFA/H2O for 2-3 times. All the samples were loaded on the column. Next, 0.1%TFA/H2O was used to wash the column 3 times. Finally, the peptides were eluted with 90% ACN/H2O (containing 0.1%TFA) 3 times and lyophilized.

Separations were performed on an Agilent Zorbax Extend-C18 column on a 1100 HPLC system. Mobile phases A and B were used for gradient which was set as follows: 98% A over 0-8 minutes; 98%~95% A over 8-8.01 minutes;95%~75% A over

8.01-48 minutes;75~60% A over 48-60 minutes; 60~10% A over 60-60.01 minutes; 10% A over 60.01-70 minutes; 10~98% A over 70-70.01 minutes and98% A over 70.01-75 minutes. Tryptic peptides were separated at a fluent flow rate of 300 μL/min and monitored at 210 nm. Samples were collected for 8-60 minutes, and eluent was collected in centrifugal tubes every minute for 15 minutes. Samples were recycled in this order until the end of gradient. The separated peptides were lyophilized for mass spectrometry.

4.3.7 Analysis of protein levels in boar sperm using immunofluorescence staining (IHF)

The IHF methods for boar sperm has been reported in our previous article (Zhou et al., 2022). The IHF analyzes was done on the sperm samples of all 30 Duroc boar. Primarily, the boar sperm was fixed in 4% paraformaldehyde for 1 hour, then air-dried and spread on slides covered with poly-L-lysine. The slides were washed 3 times with PBS (each time for 5 minutes), and kept in a container with 2% Triton X-100 in PBS to incubate for 1 hour at room temperature. Next, they were washed again 3 times (each time for 5 minutes) with PBS, after which the sperm was blocked with PBS containing 1% BSA and 1% goat serum for 30 minutes at 17℃. The slides were then incubated with a diluted primary antibody (1:100; Table 4-2) overnight at 4°C. The next morning, they were washed three times with PBS which contained 1% BSA, each time for 5 minutes after which the secondary antibody (1:100) was added. Slides were subsequently incubated at 37°C in the dark for 1 hour. After three times washing with PBS solution, Hoechst 33342 was added in order to stain the nucleus of the sperm for 5 minutes at room temperature. Finally, the slides was again washed three times with PBS for 5 minutes each time, after which an accelerator was added and pictures were taken under a fluorescence microscope (LEICA TCS SP5 II, Germany).

The protein positive rate was defined as red sperm/total sperm×100% in the randomly selected view. Each slide was screened 5 times and the resulting average was used to determine the positive rate.

4.3.8 Boar feces microbiota sequencing and short chain fatty acids determination

The protocol for the analysis of fecal microbiota was reported in our previous study (Zhou et al., 2022) (n=15 per group). An E.Z.N.A. ® Stool DNA Kit (Omega Bio-tek Inc., USA) was used to separate total genomic DNA from feces of boars, followed the manufacturer's instructions. NanoDrop 2000 (Thermo Scientific, USA) was used to detect DNA quantity and 1% agarose gel was made to test DNA quality. The primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3–V4 region of the microbial 16S rRNA genes. The PCR was done as described in our previous study (Wan et al., 2021a). The PCR amplification products were extracted and purified from a 2% agarose gel using the AxyPrep DNA Gel Extraction Kit (AXYGEN, New York, NY, United States). The raw reads obtained were filtered using the Trimmomatic program and then merged using FLASH (version 1.2.7). After that, quality control of the merged reads was performed using the QIIME (version 1.9.1) platform. The quality-filtered sequences were clustered into operational taxonomic units (OTUs) with a 97% similarity threshold using UPARSE (version 7.1). Multiple sequence alignment was conducted using the MUSCLE software. The alpha diversity indices were calculated with QIIME (Version 1.7.0; Caporaso et al., 2010). Partial least squares discrimination analysis (PLS-DA) was performed using R software (v2.15.3). We used the Bray-Curtis distance method to calculate β diversity, and the PCoA method to analyze β-diversity.

GC-MS was used to test the concentration of SCFAs in the feces. One g of feces

was weighed into a 1.5ml centrifuge tube, 1ml of double-distilled water was added and the sample was subsequently centrifuged at 10000 rpm for 10 minutes in a low-temperature centrifuge. The supernatant and metaphosphoric acid were mixed in a ratio of 9:1, and centrifuged at 1000 rpm for 10 minutes at 4°C for more than two hours. A 0.45μm filter membrane was used for filtration and the Agilent 6890 gas chromatography was used to analyze the SCFAs (Agilent Technologies, Inc., Palo Alto, CA, United States).

4.3.9 Statistical analysis

All data were expressed as the mean ± SEM. $P<0.05$ was considered as significantly different. Student's t-test was performed for the statistical analyzes. Spearman's correlation analysis was completed by RStudio (version 4.0.3) platform. The plots were made using GraphPad Prism 8.0.2.

4.4 Results

4.4.1 AOS improved boar semen quality under heat stress condition.

As shown in Fig. 4-2A (Study scheme), thirty adult Duroc boars were fed a control diet (CON group) or a diet supplemented with 10 mg/kg body weight of AOS (AOS group) for nine weeks. Daily temperature and humidity from day 1 to day 49 were recorded. The average temperature outside was 32.9°C while inside the pig house was 28.2°C. The average relative humidity was 87.8% (Fig. 4-2B and Fig.4-2 C). Feeding AOS was observed to significantly improve boar sperm motility (Fig. 4-2D; $P<0.001$) and sperm concentration (Fig. 4-2E; $P<0.05$). Moreover, AOS also tended to reduce the abnormal sperm rate (Fig. 4-2F; $P=0.1867$). However, there was no difference in abnormal sperm rate between AOS and CON group.

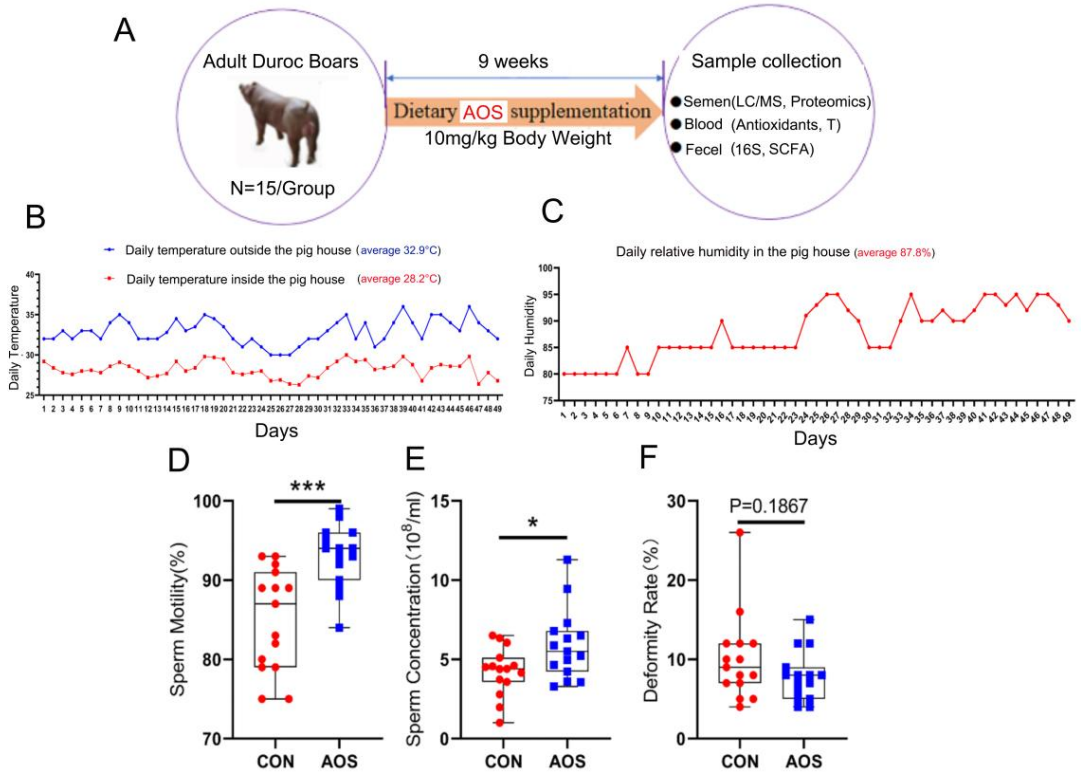

Figure 4-2 Effects of AOS on the semen quality under heat stress. (A) Study design. (B) Temperature variation. The y-axis represents the temperature. The x-axis represents the number of days during the experiment. The blue line represents outdoor temperature, the red line represents indoor temperature. (C) Humidity variation. The y-axis represents the humidity. The x-axis represents the number of days during the experiment. (D) Sperm motility. The y-axis represents the percentage of total cells. The x-axis represents the treatment (n=15/group). ***P<0.001. (E) Sperm concentration. The y-axis represents concentration. The x-axis represents the treatment (n=15/group). *P<0.05 (F) Abnormal sperm rate. The y-axis represents the percentage of abnormal cells. The x-axis represents the treatment. Data were expressed as the mean ± SEM.

4.4.2 AOS improved antioxidant indicators and testosterone content in boar plasma.

Heat stress is usually accompanied by oxidation stress, so for this reason we tested the antioxidant indicators present in boar blood. From Fig. 4-3, it can be seen that there were 6 out of 8 different antioxidant indicators that were significantly different between AOS and CON group, namely hydroxyl free radical (Fig.4-3C; P<0.05), oxygen free radical (Fig. 4-3D; P<0.001), total superoxide dismutase (T-SOD) (Fig. 4-3E; P<0.05), glutathione peroxidase (GSH-Px) (Fig. 4-3F; P<0.001), catalase (CAT) (Fig. 4-3G; P<0.05), glutathione (GSH) (Fig. 4-3H; P<0.05). The T-AOC and MDA levels showed no differences (Fig. 4-3A and B;

P>0.05). AOS can also significantly improve the testosterone content in the plasma (Fig. 4-3I; *P*<0.05). The data indicated that AOS could improve the antioxidant capacity of boars to alleviate heat stress and increase important hormone levels related to semen quality in plasma.

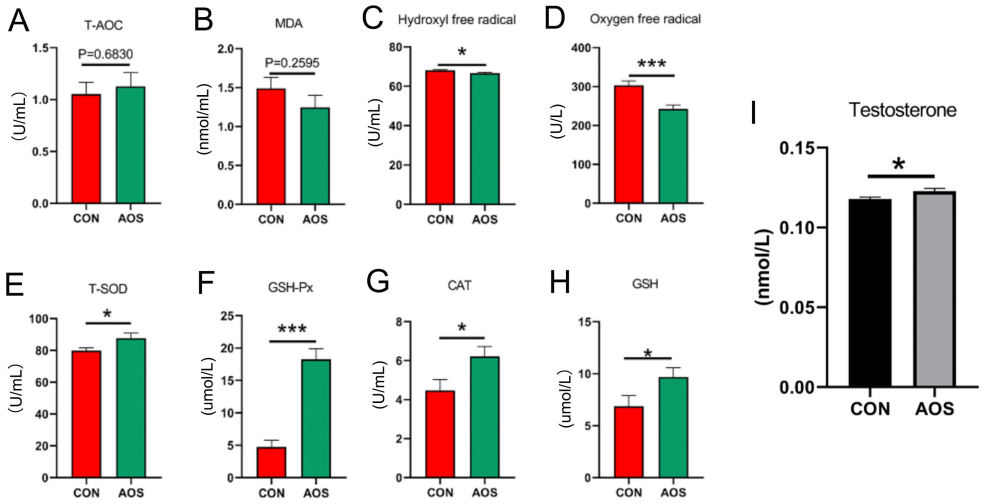

Figure 4-3 AOS improved antioxidant indicators and testosterone content in boar plasma. (A) Blood total antioxidant capacity level. (B) Blood malondialdehyde level. (C) Blood Hydroxyl free radical level. (D) Blood Oxygen free radical level. (E) Blood superoxide dismutase level. (F) Blood glutathione peroxidase level. (G) Blood Catalase level. (H) Blood glutathione Level. (I) Blood testosterone level. Data were expressed as the mean ± SEM. The y-axis represents the amount. The x-axis represents the treatments (n=15/group). ***P<0.001, *P<0.05.

4.4.3 AOS improved the metabolites and antioxidant indicators of sperm.

AOS benefited the sperm metabolites which were determined by LC/MS analysis. We tested a total of 3361 different metabolites, of which 33 were significantly up-regulated and 35 were significantly down-regulated in the AOS group compared to the CON group (Fig.4-4A). Among them, AOS could significantly improve phospholipids in sperm such as LysoPC (17:0/0:0) (Fig.4-4B; *P*<0.001) and LysoPC (0:0/16:0) (Fig.4-4B; *P*<0.001). AOS could also improve amino acids such as Hypotaurine (Fig.4-4B; *P*<0.001) and N-Acetylhistidine (Fig.4-4B; *P*<0.01). Meanwhile, AOS elevated sperm antioxidants such as Triazophos (Fig.4-4B; *P*<0.05)

and Quercetin (Fig.4-4B; P<0.05). The potential metabolic pathways of the changed metabolites were determined by KEGG pathway analysis (Fig.4-4C). AOS was also observed to improve the antioxidant capacity in sperm. The T-AOC level was significantly higher in the AOS group compared to the CON group (Fig.4-4D; P<0.05). Meanwhile, AOS significantly reduced Hydroxyl free radical level (Fig.4-4D; P<0.05). AOS also benefited other antioxidant indicators, but not significantly (Fig.4-4D).

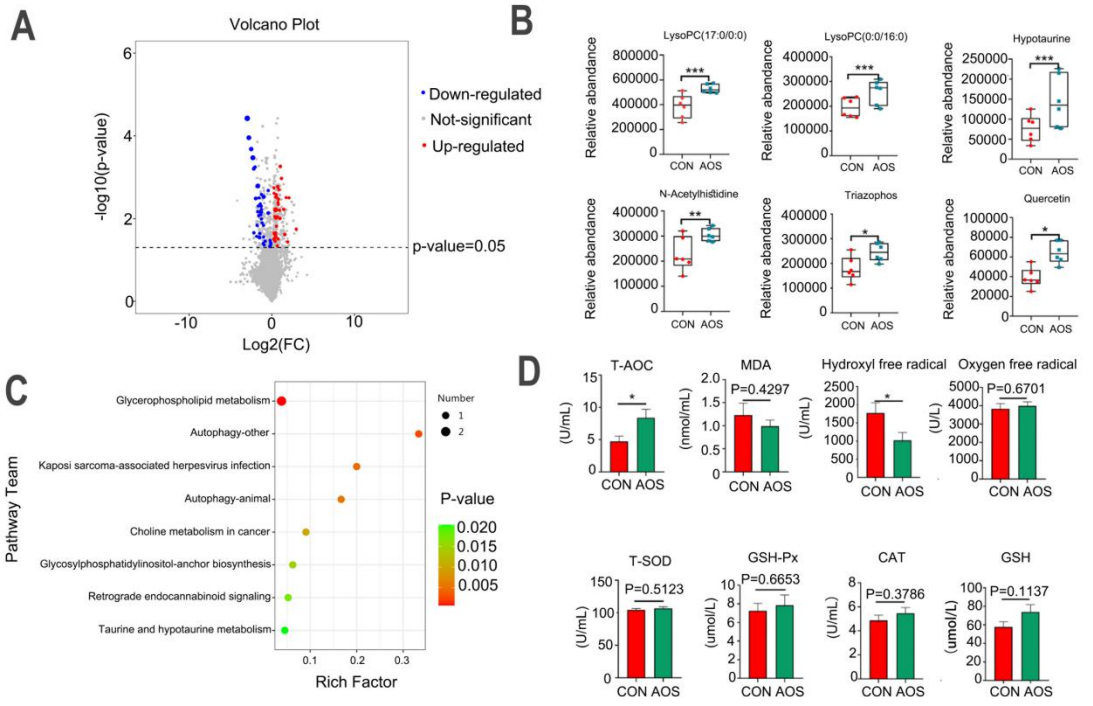

Figure 4-4 AOS improved the metabolites and antioxidant indicators of sperm. (A) Volcano plot of sperm metabolites. (B) Significant difference metabolites in the sperm including LysoPC (17:0/0:0), LysoPC (0:0/16:0), Hypotaurine, N-Acetylhistidine, Triazophos and Quercetin. (C) The top 8 functional prediction metabolic pathways of sperm metabolites. (D) Sperm antioxidant indicators including T-AOC, MDA, Hydroxyl free radical, Oxygen free radical, T-SOD, GSH-Px, CAT and GSH. Data were expressed as the mean ± SEM. The y-axis represents the relative amount. The x-axis represents the treatments. ***P<0.001. **P < 0.01.*P<0.05.

4.4.4 AOS improved sperm proteome to increase semen quality under heat stress condition.

In order to explore how AOS alleviates heat stress and improves semen quality, we conducted a proteomics analysis. We tested a total of 3281 different proteins, of which 12 were significantly up-regulated and 215 were significantly down-regulated

in the AOS group compared to the CON group (Fig.4-5A). Among them we found proteins involved in spermatogenesis that were significantly changed such as Glutamine synthetase (Fig.4-5B; $P<0.05$), Tetraspanin 8 (Fig.4-5B; $P<0.05$), Sperm associated antigen11 (SPAG11) (Fig.4-5B; $P<0.05$), Sulfhydryl oxidase (Fig.4-5B; $P<0.01$), and Sperm acrosome membrane-associated protein 1 (SPACA1) (Fig.4-5B; $P<0.05$). Meanwhile, other proteins related to heat stress were significantly down-regulated such as Heat shock protein 70 (HSP 70) (Fig.4-5B; $P<0.05$), Heat shock protein 90-α (HSP 90-α) (Fig.4-5B; $P<0.01$), and Heat shock protein 90-β (HSP 90-β) (Fig.4-5B; $P<0.01$).The functional prediction of the changed proteins was determined by KEGG pathway analysis (Fig.4-5C). Five categories of KEGG terms could be distinguished to differ between the AOS and CON group such as cellular process, environment, human disease, metabolism and organismal system.

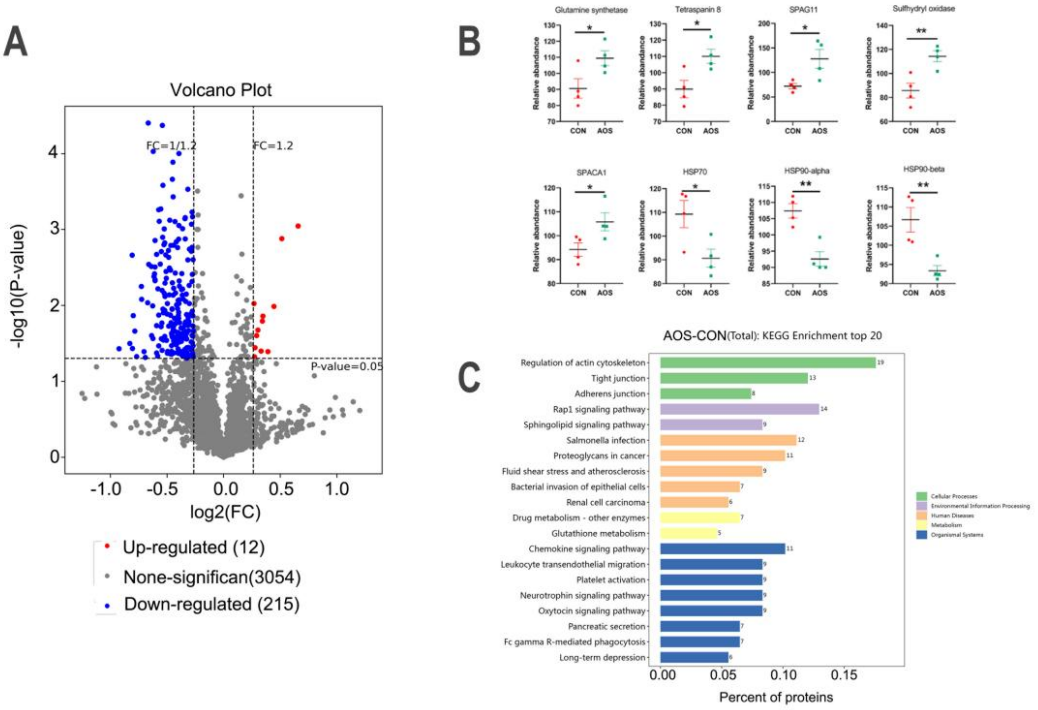

Figure 4-5 AOS improved sperm proteome to increase semen quality under heat stress condition. (A) Volcano plot of sperm proteomics. (B) Significant difference proteins in the sperm including Glutamine synthetase, Tetraspanin8, Sperm associated antigen11 (SPAG11), Sulfhydryl oxidase, Sperm acrosome membrane-associated protein 1 (SPACA1), Heat shock protein 70 (HSP 70), Heat shock protein 90-α (HSP 90-α) and Heat shock protein 90-β (HSP 90-β). (C) The top 20 functional prediction metabolic KEGG pathways of sperm proteins. Data were expressed as the mean ± SEM. The y-axis represents the relative amount. The x-axis represents the treatments (n=4/group). $^{**}P < 0.01$.$^{*}P<0.05$.

4.4.5 AOS improved the level of proteins related to spermatogenesis under heat stress condition.

To understand how AOS improved boar semen quality, the protein levels of CatSper 8, PKA, Bcl, HSP 70 and HSP 90, all proteins important for sperm quality under heat stress, were quantified (Fig.4-6A). Using IHF staining, it could be observed that AOS improved the protein levels of CatSper 8, PKA and Bcl significantly compared to the CON group (Fig.4-6B-D; $P<0.01$). On the other hand, AOS reduced the HSP 70 and HSP 90 level significantly compared to CON group (Fig. 4-6E-F; $P<0.001$).

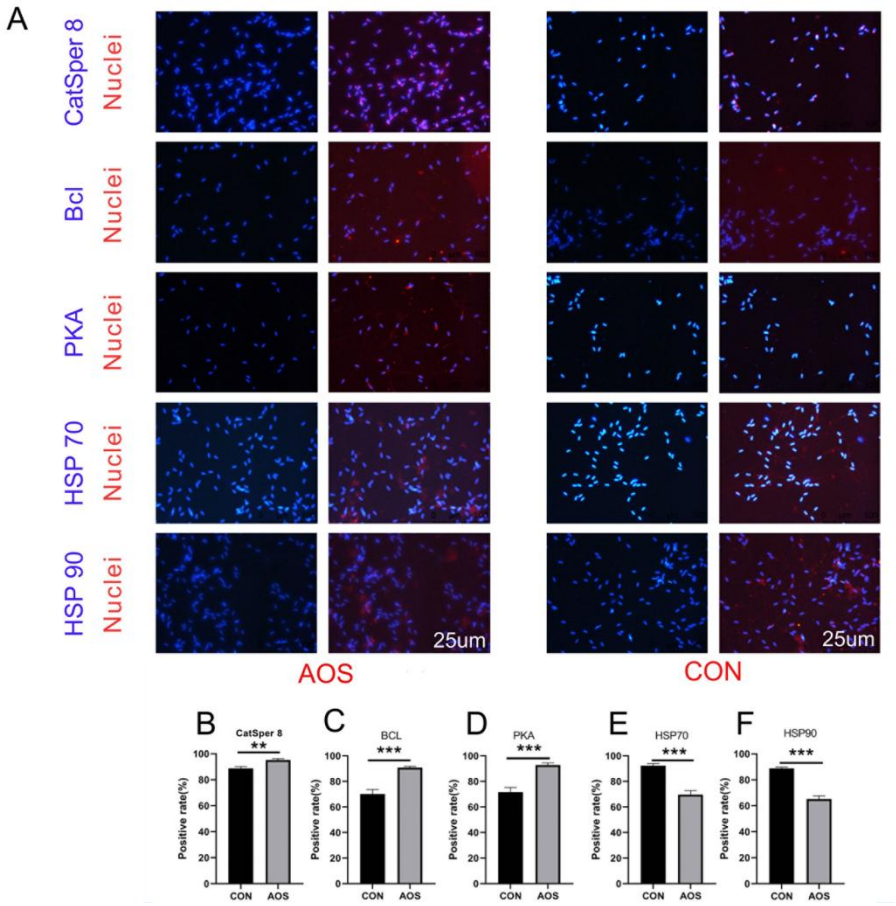

Figure 4-6 AOS improved the protein level related to spermatogenesis under heat stress condition. (A) Immunofluorescence staining (IHF) of Catsper 8, Bcl, PKA, HSP70 and HSP 90. (B) Positive rate of Catsper 8. (C) Positive rate of Bcl. (D) Positive rate of PKA. (E) Positive rate of HSP70. (F) Positive rate of HSP90. Data were expressed as the mean ± SEM. The y-axis represents the amount of Positive rate. The x-axis represents the treatments (n=6/group). ***P<0.001.**P<0.01.

4.4.6 AOS changed microbial composition and improved SCFAs level in boar feces.

To investigate the effect of AOS on boar intestinal microbes, we conducted 16s sequencing and short-chain fatty acids determination in feces. The α-diversity measured using the observed taxa metric (Fig.4-7A; P<0.05) or using the Shannon index (Fig.4-7B; P<0.05) was significantly different. The microbiota composition was also observed to be different between AOS and CON group by PCA analysis (Fig.4-7C). AOS changed the abundance of beneficial microbiota at genus level such as *Enterobacter* (Fig.4-7D; P=0.076; FDR=0.676), *Pseudomonas* (Fig.4-7E; P<0.01; FDR=0.236), *Escherichia-Shigella* (Fig.4-7F; P<0.05; FDR=0.514), and *Bifidobacterium* (Fig.4-7G; P<0.01; FDR=0.407). At the same time, AOS reduced the

abundance of harmful microbiota such as *Streptococcus* (Fig.4-7H; $P<0.05$; FDR=0.514), *Prevotella_9* (Fig.4-7I; $P<0.05$; FDR=0.537), *Prevotella_1* (Fig.4-7J; $P=0.089$; FDR=0.793), *Ruminococcaceae_UCG-002* (Fig.4-7K; $P<0.001$; FDR=0.0347), *Klebsiella* (Fig.4-7L; $P=0.3732$; FDR=0.893), and *Prevotellaceae_UCG-001* (Fig.4-7M; $P<0.01$; FDR=0.312). To analyze the metabolites of gut microbes, we measured the SCFAs in the feces. Acetic acid (Fig.4-7N; $P<0.05$), propionic acid (Fig.4-7O; $P<0.05$), butyric acid (Fig.4-7P; $P<0.05$) were significantly improved in the feces after feeding AOS, others, such as isobutyric acid (Fig.4-7Q; $P=0.4249$), pentanoic acid (Fig.4-7R; $P=0.4691$), isopentanoic acid (Fig. 6S; $P=0.0625$), were also changed but not significantly. The correlation between gut microbiota and SCFA is shown in (Fig.4-7T). Butyric acid had a strong positive correlated with the beneficial bacterium *Pseudomonas* ($P<0.05$) and a strong negative correlated with the harmful bacterium *Prevotella_1* ($P<0.05$).

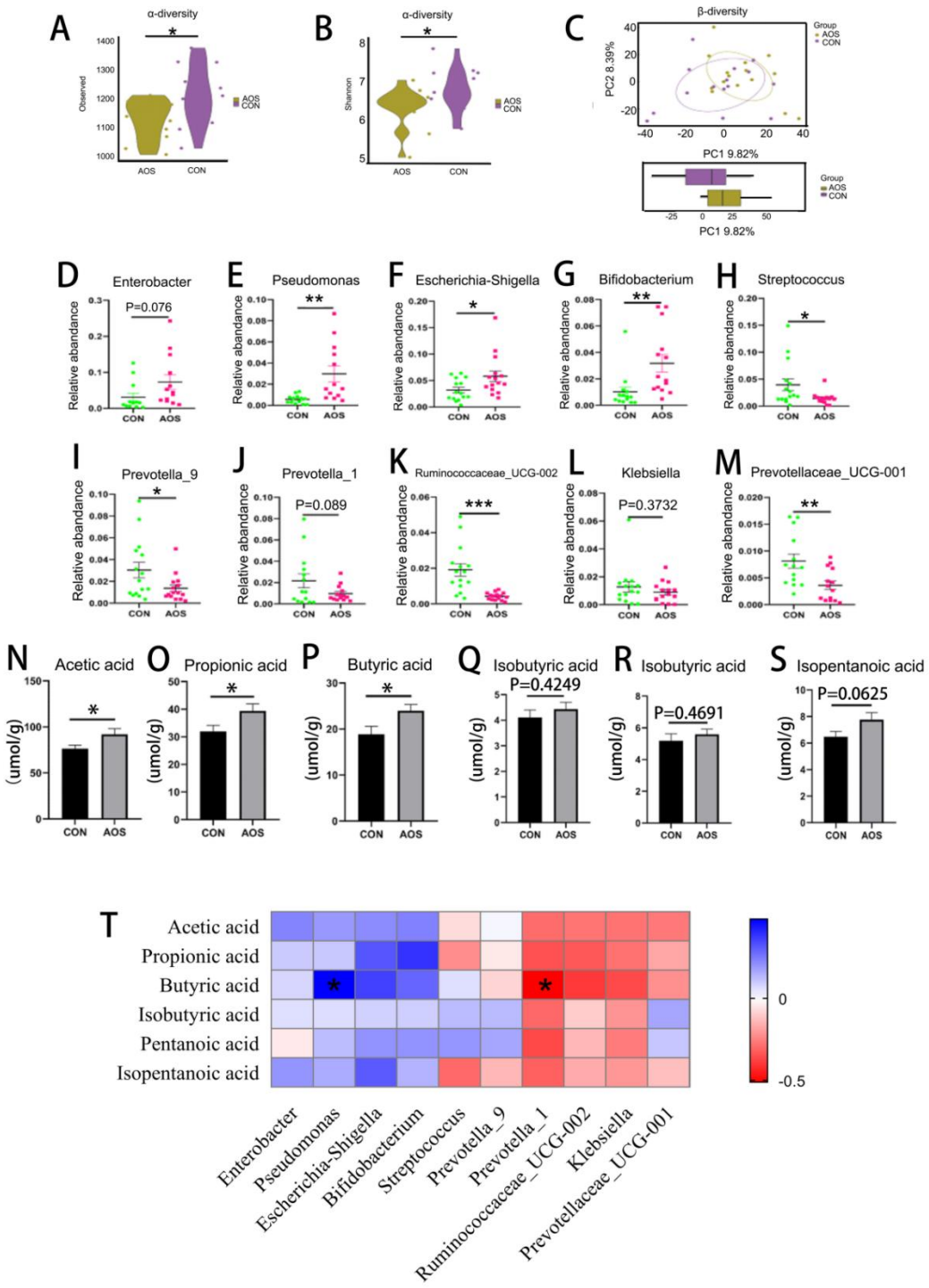

Figure 4-7 Effects of AOS on the fecal microbial composition and SCFAs. (A) α-diversity with Observed. (B) α-diversity with Shannon. (C) β-diversity with PCoA. The relative amount of individual microbiota in feces at Genus level (D-M). Data were expressed as the mean ± SEM. The y-axis represents the relative amount. The x-axis represents the treatments (n=15/group). ***P<0.001. **P<0.01.*P<0.05. (N) Acetic acid level. (O) Propionic acid level. (P) Butyric acid level. (Q) Isobutyric acid level. (R) Pentanoic acid level. (S) Isopentanoic acid level. (T) The correlation between gut microbes and SCFAs. Data were expressed as the mean ± SEM. The y-axis represents the relative amount. The x-axis represents the treatments (n=15/group). *P<0.05.

4.4.7 Spearman correlation among fecal microbes, sperm protein, sperm metabolites and sperm parameters.

The spearman correlation analysis (Fig.4-8) indicated that the fecal microbiota, sperm metabolites, sperm protein and semen parameters were well correlated. Firstly, there was a significant negative correlation between sperm motility and the relative abundance of harmful bacterium *Prevotellaceae_UCG-001*. Next, the abnormal sperm rate was significantly negatively correlated with the N-acetylhistidine and sulfhydryl oxidase. At the same time, the abnormal sperm rate was positively correlated with the level of heat shock proteins. In addition, there was also a good correlation between fecal microorganisms, sperm metabolites and the sperm proteome.

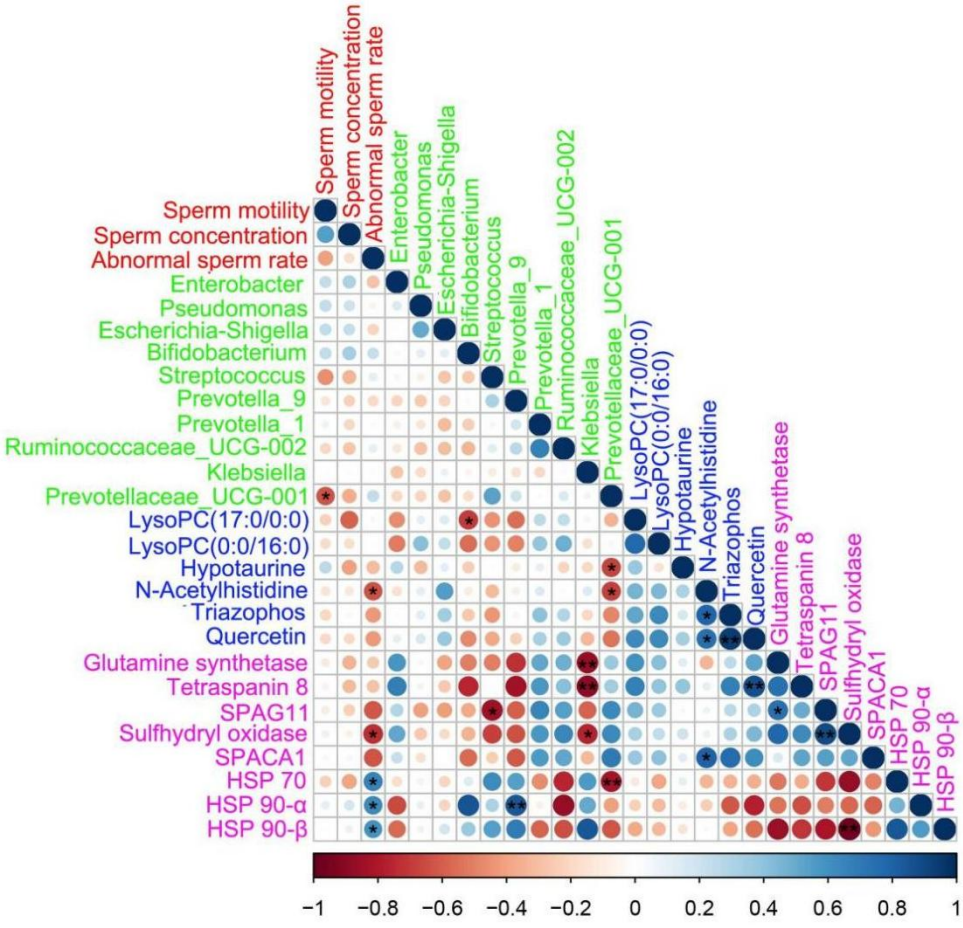

Figure 4-8 Spearman correlations. Correlations among fecal microbes, sperm metabolites, sperm protein and semen quality parameters. Red color represents sperm parameters, green color represents gut microbes, blue color represents sperm metabolites, pink color represents sperm protein. Blue cycles represent positive correlation, red cycles represent negative correlation. The size of the circle represents the strength of the correlation. (larger circle means stronger correlation, the range of value is from -1 to 1). $^{**}P<0.01$.$^{*}P<0.05$.

4.5 Discussion

Alginate Oligosaccharide (AOS) is an oligosaccharide containing 2~25 sugar units, which is obtained from the cleavage of algin at the glycosidic bond or the biosynthesis of its monosaccharide components (Lu et al., 2022). Due to its low molecular weight, high water solubility, safety and non-toxic characteristics (Wang et al., 2021b), AOS has received high attention from researchers in recent years. AOS has a variety of unique biological activities, such as immune regulation (Bland et al., 2004), antioxidative properties (Falkeborg et al., 2014), and anti-cancer activities (Liu et al., 2022). AOS holds significant promise and is currently a focal point of research in several fields including drug development, nutritional health and sustainable agriculture (Pritchard et al., 2017). Reactive oxygen species (ROS), such as oxygen or hydroxyl free radicals, are substances produced by the metabolism under normal physiological and pathological conditions (Su et al., 2019). Excessive accumulation of ROS may however cause harmful oxidative stress, and induce structural (Valko et al., 2007) damages in cells, including harm to proteins (Hawkins and Davies, 2019) and DNA (Marnett, 2000). Excessive oxidative damage can lead to loss of cell function and ultimately result in cell apoptosis (Nordberg and Arnér, 2001). In this study, we found that the content of reactive oxygen species (oxygen free radicals and hydroxyl radicals) in boar blood reduced significantly by feeding AOS, while, at the same time, the content of hydroxyl radicals in boar sperm also reduced significantly. This indicated that AOS could alleviate the degree of oxidative stress caused by heat stress to potentially improve semen quality by reducing the reactive oxygen species in blood and sperm.

Heat stress refers to the sum of non-specific physiological reactions that the body undergoes in a hot environment at high environmental temperatures (Cramer et al., 2022). Heat stress can disrupt antioxidant systems such as SOD (Liu et al., 2021a), CAT (Ouyang et al., 2022), and GSH-px (Chang et al., 2022). Research had shown that in boars exposed to 40°C for 5 hours per day for 8 days, the MDA level was significantly increased in the epididymis, indicated that heat stress caused significant

oxidative stress damage to testicular tissue (Li et al., 2017b). In this study, it was found that the antioxidant indicators SOD, GSH-px, and CAT content in boar serum were significantly improved, while the T-AOC content in boar sperm was also significantly improved, indicating that AOS exerted a strong antioxidant ability. In other studies, it has been found that AOS could alleviate the damage caused by oxidative stress (Saghir et al., 2023), while improving sperm motility of breeding boars (Han et al., 2022). This is consistent with our experimental results. Therefore, we inferred that AOS could alleviate the adverse effects of heat stress on semen quality by exerting strong antioxidant properties. Testosterone in blood can improve sexual desire (van Anders, 2012), which is an important male hormone to promote spermatogenesis (Smith and Walker, 2014). High temperature and high humidity environments can easily lead to a decrease in sexual desire and sperm quality in boars (Chen et al., 2018). However, in this experiment, the testosterone content in the blood was significantly increased by adding AOS. Therefore, AOS could improve the testosterone level under heat stress conditions, thereby positively influencing semen quality.

Several proteins are significant raw material for spermatogenesis (Chalmel and Rolland, 2015). Studies have shown that glutamine synthetase can catalyze the condensation of glutamate and ammonia to form glutamine, which is an amino acid proven to affect mammalian sperm motility (Francou et al., 2012). Tetraspanin 8 is a protein critical to male fertility and found in sertoli cells in the testis (Pradhan et al., 2020). SPAG 11 affects sperm vitality of mammals. Lack of SPAG 11 will lead to abnormal spermatogenesis, which greatly increases the abnormal sperm rate (Liu et al., 2019b; Sangeeta and Yenugu, 2022). SPACA1 is a protein that affects the integrity of the sperm acrosome and plays an important role in the fertilization process (Minami et al., 2020). In this study, we conducted a proteome analysis on boar sperm and found that supplementing diet with AOS significantly improved the protein levels of the aforementioned proteins under heat stress conditions. At the same time, the levels of heat shock protein 70 and heat shock protein 90 significantly decreased. Research has shown that when animals are exposed to high temperatures for a long

time, they synthesize heat shock proteins in order to protect themselves (Thirumalaikumar et al., 2021; Goto et al., 2022). We validated the proteomic results by using IHF and found consistent results. In addition, the content of other proteins related to spermatogenesis increased significantly, such as CatSper 8 (Rahban et al., 2021), PKA (Baro Graf et al., 2020) and Bcl (Cayli et al., 2004). The data indicated that AOS could improve proteins related to spermatogenesis and reduce the content of heat shock proteins, thus alleviate the adverse effects of heat stress on boar semen quality.

In this study, in order to investigate the changes of sperm metabolites under heat stress, we determined the metabolome of boar sperm. We could find that after supplementing with AOS, there were notable improvements in specific sperm metabolites, mainly phospholipids, amino acids and antioxidants, indicating a significant positive change. Previous studies had reported that LysoPC (17:0/0:0) and LysoPC (0:0/16:0) exerted a potential protective role in regulating metabolic disorders (Zhang et al., 2020b). Hypotaurine is an amino acid that can be used to improve the post-thawed Merino ram sperm parameters (Bucak et al., 2013). Triazophos is a strong antioxidant that is used in previous research and led to substantial up-regulation of a male spermatogenesis-associated protein 5-like gene (NlSPATA5) to increase semen quality (Ge et al., 2016). Quercetin is widely reported in many fields, and it can increase testosterone and sperm concentration in mice (Garcia et al., 2023). Quercetin can also ameliorate sperm oxidative stress and inflammation, while preserving sperm morphology and function in rats (Yelumalai et al., 2019). Therefore, AOS can increase the antioxidant content and change the amino acid level in semen to benefit spermatogenesis.

The intestine is the largest digestive organ (Gasbarrini et al., 2008) of the animal body. Gut microbiota is the intermediary between host and diet, thus it plays a vital role in regulating animal health (Bibbò et al., 2016). Research showed that plant extracts can improve the fecundity of male animals by changing their gut microbiota (Zhou et al., 2022). In our previous studies we found that AOS can improve the semen quality of boars and mice by improving gut microbiota and sperm metabolites (Han et

al., 2022; Yan et al., 2022). In this study, we conducted 16s sequencing analysis on boar feces, and examined enriched microbiota species at the genus level. It was found that the relative abundance of four beneficial bacteria related to spermatogenesis increased, while the relative abundance of six harmful bacteria down-regulated. Among them, the beneficial bacterium *Enterobacter* can improve the storage time of boar semen at room temperature (Prieto-Martínez et al., 2014). *Pseudomonas* was beneficial for sperm capacitation and protein phosphorylation of boar spermatozoa (Sepúlveda et al., 2014, 2016). *Escherichia-Shigella* was positively correlated with the synthesis of sex hormones (Ramírez-Acosta et al., 2022). *Bifidobacterium* can be used for patients with asthenozoospermia for 6 weeks, after which semen quality is significantly improved (Valcarce et al., 2017). Meanwhile, *Prevotella* is considered a harmful bacterium in terms of affecting male fertility. Through the study of semen microbial composition and male fertility, it has been noted that *Prevotella* has a negative impact on sperm motility and concentration (Farahani et al., 2021; Lundy et al., 2021; Gachet et al., 2022). *Streptococcus* and *Klebsiella* were the most susceptible bacteria during fertilization, reducing sperm motility and ultimately leading to sperm apoptosis (Zuleta-González et al., 2019). In order to further explore the relationship between gut microbiota and semen quality, we measured short-chain fatty acids in boar feces. Short-chain fatty acids mainly participate in the energy supply of intestinal epithelial cells (Hu et al., 2018) and affected the permeability of intestinal mucosa (Usuda et al., 2021). At present, more and more studies indicated the existence of an intestinal-testicular axis (Hao et al., 2022). Gut microbiota can use carbohydrates to produce short chain fatty acids (Chen et al., 2019; He et al., 2020b). A correlation analysis study pointed out that SCFAs could improve semen quality (Lin et al., 2022). A study by Olaniyi et al. has also shown that SCFAs significantly decreased testicular proprotein convertase subtilisin/kexin type 9 (PCSK9) with a significant reduction in cholesterol and lipid peroxidation as a result. This reduction resulted in enhanced testicular tissue, characterized histologically by the restoration of tissue architecture, seminiferous tubules and spermatogonia, along with improved testicular function, including spermatogenesis and steroidogenesis (Olaniyi et al., 2021). In this study, the

addition of AOS significantly increased the content of acetic acid, propionic acid, and butyric acid, and there was a significant positive correlation between beneficial bacteria *Pseudomonas* and butyric acid content, while harmful bacteria *Prevotella* had a significant negative correlation with butyric acid production. The above data indicated that the potential mechanism of AOS to improve boar semen quality may be to increase the relative abundance of beneficial bacteria in the gut, that on their turn could produce short-chain fatty acids beneficial for spermatogenesis.

4.6 Conclusion

Current research showed that AOS improved boar semen quality during heat stress by improving gut microbiota, sperm metabolome and sperm proteome. Therefore, AOS can be used as a feed additive to solve the problem of decreased semen quality in boars caused by high temperature and humidity in summer.

Table 4-1 Composition and nutrient analysis of basal diet

Ingredient	Content, %
Corn	35.15
Barley	24.83
wheat	15.82
Rice bran meal	9.40
Soybean meal	7.90
Soybean oil	2.00
L-lysine	0.40
Methionine	0.14
Threonine	0.24
Ground limestone	1.44
Monocalcium phosphate	1.21
Sodium chloride	0.48
Premix*	1.00
total	100
Nutrient, %	
Calculated NE, kcal/kg	2240
Crude protein, %	14.50
Crude fat, %	3.22
Crude ash, %	6.18
Crude fiber, %	4.15

*: Premix provided the following minerals per kilogram: 17 mg Cu, 160 mg Fe, 140 mg Zn, 50 mg Mn, 0.50 mg I, 0.50 mg Se, and 0.22 mg Cr.

Table 4-2. Primary antibody information

Name	Cat. #	Source (Animal)	Company
Bcl	bs-0520R	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
PKA	bs-1645R	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Anti-HSP 70	bs-0126R	boar	Beijing Biosynthesis Biotechnology CO.
Anti-HSP 90	Bs-10100R	boar	Beijing Biosynthesis Biotechnology CO.
CATSPER	bs-23326R	Rabbit	Beijing Biosynthesis Biotechnology CO.

Table 4-3. Sperm parameters before animal experiment

Items	CON	AOS	P value
Sperm motility (%)	83.23± 1.79	84.77±1.66	0.88
Sperm concentration (10^^{8}/ml)	4.1±0.6	4.3±0.55	0.69
Abnormal sperm rate (%)	12.59±1.77	10.14±1.68	0.52

Note: Before the animal experiment start, 30 boars were chosenwith similar conditions in order to eliminate the difference between the groups. We chose to look at data of 2 sperm cycles. A P value higher than 0.05 indicated no differences between the two groups.

Table 4-4. Compare with the semen quality parameters between summer and winter

Items	Summer	Winter	P value
Sperm motility (%)	84.22± 1.58	89.27±1.32	<0.001
Sperm concentration (10^^{8}/ml)	4.1±0.6	4.6±0.55	0.462
Abnormal sperm rate (%)	11.30±1.84	7.17±1.79	<0.001

Note: We measured semen quality parameters from July to September (summer) 2021 compared with January to March 2022 (Winter) of 30 boars. The data come from the Yangxiang company, and these 30 boars are the experimental animals in chapter 4.

Chapter V

Alginate Oligosaccharide (AOS) extends the service lifespan by improving sperm metabolome and gut microbiota in an aging Duroc boars model

Chapter V. Alginate Oligosaccharide (AOS) extends the service lifespan by improving sperm metabolome and gut microbiota in an aging Duroc boars model

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Key words: Alginate oligosaccharide, service lifespan, gut microbiota, sperm metabolome, aging Duroc boars

Adapted from the reference: Zhou Y, Wei Z, Tan J, Sun H, Jiang H, Gao Y, Zhang H, Schroyen M. Alginate oligosaccharide extends the service lifespan by improving the sperm metabolome and gut microbiota in an aging Duroc boars model. Front Cell Infect Microbiol. 2023 Dec 5;13:1308484. doi: 10.3389/fcimb.2023.1308484.

Author Contributions: Yexun Zhou participated in animal husbandry experiments, data collection and analysis, as well as drafting and writing the paper.

5.1 Abstract

Alginate oligosaccharide (AOS), as a natural non-toxic plant extract, has been paid more attention in recent years due to its strong antioxidant, anti-inflammatory and even anti-cancer. However, the mechanism by which AOS affects animal reproductive performance is still unclear. The purpose of the study is to use multi-omics technology to analyze the effects of AOS in extending the service lifespan of aging boars. The results showed that AOS can significantly improve the sperm motility (P<0.05) and sperm validity rate (P<0.001) of aging boars, significantly reduce the abnormal sperm rate (P<0.01) by the elevation of the protein levels such as CatSper 8 and protein kinase (PKA) for semen quality. At the same time, AOS significantly improved the testosterone content in the blood of boars (P<0.01). AOS significantly improved fatty acids such as adrenic acid (P<0.05), antioxidants such as succinic acid (P<0.05) in sperm metabolites, significantly reduced harmful substances dibutyl phthalate (P<0.05) which was negative to spermatogenesis. AOS can improve the composition of intestinal microbes, mainly increasing beneficial bacteria *Enterobacter* (P=0.1262), reducing harmful bacteria such as *Streptococcus* (P<0.05), *Prevotellaceae_UCG-001* (P<0.05) and *Prevotellaceae_NK3B31_group* (P<0.05). Meanwhile, short-chain fatty acids in feces such as acetic acid (P<0.05) and butyric acid (P<0.05) were significantly changed. Spearman correlation analysis showed that there was a closely correlation among microorganisms, sperm metabolites and sperm parameters. Therefore, the data indicated that AOS improved the semen quality of older boars by improving the intestinal microbiota and sperm metabolome. AOS can be used as a feed additive to solve the problem of high culling rate in large scale boar studs.

5.2 Introduction

In recent years, the research on breeding pigs is no longer limited to the nutritional demands, more importantly, the problems of reproductive performance and service lifespan had already been focused on more and more widely (Poulsen et al., 2020; Plaengkaeo et al., 2021). Excellent breeding boars can directly affect the

benefits of pig farms. Therefore, the reasonable extending service lifespan has important practical significance for the production of swine industry (Spinaci et al., 2016), such as saving breeding costs, increasing conception rate and litter sizes, improving the stability of the whole swine population (D'Allaire et al., 1992; Hoffman and Valencak, 2020). The service lifespan of breeding boars refers to from the first mating time to culling (Koketsu and Sasaki, 2009). Study had shown that the average service lifespan of boars in the late 20th century was 20 months (D'Allaire and Leman, 1990). In the early 21st century, it was mainly concentrated in 2 years (Knox et al., 2008). In China, the lifespan of breeding boars in large scale boar studs is currently 30 months. However, there are many factors affecting the service lifespan, such as varieties, nutrition and environment (Cassady et al., 2002; Sancho et al., 2004; Akerfelt et al., 2010). These factors made the semen quality worse, decreased sexual desire (Berger et al., 1980), and eventually eliminated. Therefore, we hope to improve the breeding performance of boars through nutritional regulation, determine the appropriate feed formula so as to extend its service lifespan.

Alginate oligosaccharide (AOS) is a natural and non-toxic plant extract that comes from alginate (Li et al., 2022a). Due to its multiple biological functions, such as anti-inflammatory (Feng et al., 2020), and antioxidant (Zhang et al., 2022b), it is currently widely used in the medical area. AOS can activate the specific immune system and inhibit the proliferation of tumor cells by activating macrophages (Saigusa et al., 2015). AOS can not only remove active oxygen, but also significantly reduce the content of lipid peroxidation. At the same time increased the activity of hydrogen peroxide and superoxide dismutase (SOD), thus remove excessive free radicals (Zhao et al., 2020a). Studies have shown that AOS can alleviate the intestinal inflammation of DSS-induced mice, which is conducive to improving the intestinal health of animals (Zhang et al., 2022a; Lu et al., 2023). Our previous study found that AOS can repair the testicular damage of mice induced by Busulfan, thereby improving the semen quality (Zhang et al., 2021a). However, few research reported that AOS can extend the lifespan of aging animals. The purpose of the study is to explore the potential mechanism of AOS to extend the lifespan of aging boars, then provide

theoretical basis for solving the problem of high elimination rates in large-scale boar studs.

5.3 Materials and methods

5.3.1 Boars and experimental design

The animal experiments were followed by the Animal Care and Use Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS2022-24). Eighteen Duroc boars with similar age (65 months old), healthy and body weight (around 300 kg) were selected randomly in the investigation at Ya Ji Mountain boar stud. The Duroc boars were divided into 2 groups randomly, each group included 9 boars namely control group (CON) and AOS group (AOS). Control group (CON) was fed basal diet (Feed formula was shown in Table 5-1), boars in the AOS group (AOS) were fed basal diet with 10 mg/kg body weight (Han et al., 2022) AOS (provided by Qingdao Zhibo Biotechnology Co., Ltd). Each boar were housed in an individual pen and the whole feeding period was lasted for 9 weeks (Figure 5-1A).

In the experiment, we used gloved-hand technology to get the semen samples. After that, sperm parameters including sperm concentration, sperm motility, abnormal sperm rate, sperm volume and sperm validity rate were assessed by CASAII software according to the reported methods (Guo et al., 2020). Blood samples were taken from hind leg veins when they were ejaculating, placed in an anti-coagulated tube. Then, blood samples were centrifuged at 3000 × g for 10 min, transfer the supernatant to a 1.5 ml centrifuge tube, stored the plasma to -80 °C refrigerator until further research. Fecal samples were taken from the rectum by hand, massage the rectum of boars to promote peristalsis and wait for a while to get fresh feces, placed them in liquid nitrogen immediately, finally stored in a -80°C freezer for 16S sequencing analyze and short chain fatty acids test.

5.3.2 Using computer-assisted sperm analysis system (CASAII) to detect sperm parameters

The sperm parameters, including the sperm concentration, sperm motility, abnormal sperm rate, sperm volume and sperm validity rate were analyzed by a

computer assisted sperm analysis (CASAII) system (Shanghai Kasu Biotechnology Co., Ltd., Shanghai, China). The evaluated criteria of sperm motility was as follows: grade A fast forward movement > 22 μm s^{-1}; grade B forward movement < 22 μm s^{-1}; grade C curve movement < 5 μm s^{-1}; grade D none movement (Yeste et al., 2018). The sperm concentration should be more than 10^8/ml, the abnormal sperm rate should be less than 30%. The semen volume should not be less than 50 ml each time. The sperm validity rate should be more than 80% (Cao et al., 2011).

5.3.3 Detection of blood testosterone content

Blood testosterone content was measured by ELISA kits (Beijing Boxbio Science&Technology Co.,Ltd) following the instructions of the manufacturer. Then used the microplate reader (central laboratory) to detect the absorbance value of each sample. Finally, the plasma testosterone content was calculated by using the formula which was given from the instructions.

5.3.4 Sperm metabolome assay by LC-MS/MS

Boar sperm (n=6 per group) was taken out from -80°C fridge. Firstly, the protein was removed from the samples and then analyzed by LC/MS. Next, An ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm) were employed in both positive and negative modes. Solvent A is an aqueous solution containing 0.1% formic acid. Solvent B is an aqueous solution containing 0.1% acetonitrile. The following program was as below: 5–20 % B over 0–2 min; 20–60 % B over 2–4 min; 60–100 % B over 4–11 min, the composition was held at 100 % B for 2 min, then 13–13.5 min, 100 % to 5 % B, and 13.5–14.5 min holding at 5% B. The flow rate was set at 0.4 mL/min and column temperature was 45℃. The sperm was all kept in 4℃ and the volume of the injection was 5 μL. ESI was used in the mass spectrometry program.

5.3.5 Using immunofluorescence staining (IHF) to detect the protein levels in boar sperm

The methods for IHF of boar sperm have been reported in our previous articles (n=9 per group) (Zhou et al., 2022). Primarily, fix the boar sperm in 4% paraformaldehyde for 1 hour, then air-dried the sperm which were spread on the slides

covered with poly-L-lysine. After 3 times (each time for 5 minutes) washing by PBS, the sperm were incubated with 2% Triton X-100 in PBS for 1 hour at room temperature. Next, washing 3 times (each time for 5 minutes) again with PBS, the sperm were blocked with PBS which contained 1% BSA and 1% goat serum for 30 minutes at 17℃, it was then incubated with diluted primary antibody (1:100; Table 5-2) overnight at 4°C. The next morning, washing three times with PBS which contained 1% BSA (the secondary antibody dilution), each time for 5 minutes. Added secondary antibody (1:100) to the diluent and incubated at 37°C in the dark for 1 hour. Washing three times with PBS, 5 minutes each time, added Hoechst 33342 to stain the nuclear, waiting for 5 minutes at room temperature. Washing three times with PBS again for 5 minutes each time, added accelerator, taken pictures under a fluorescence microscope (LEICA TCS SP5 II, Germany).The protein positive rate = red sperm/total sperm×100% in the view which was selected randomly. Each slide choose 5 screen them made a calculation to gain the positive rate.

5.3.6 Boar feces 16s RNA sequencing and short chain fatty acids test

The protocol for the analysis of fecal microbiota was reported in our previous study (Zhou et al., 2022) (n=9 per group).

Using an E.Z.N.A. ® Stool DNA Kit (Omega Bio-tek Inc., USA) to separate total fecal genomic DNA, followed with the instructions of manufacturer. Using NanoDrop 2000 (Thermo Scientific, USA) and 1% agarose gel to detect the DNA quantity and quality respectively. Using primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') to amplify the V3–V4 region of the microbial 16S rRNA genes. The conditions of PCR system and amplification are followed with our previous study (Wan et al., 2021a). The PCR amplification products can be extracted by 2% agarose gel and using AxyPrep DNA Gel Extraction Kit (AXYGEN, New York, NY, United States) which were followed with the instructions to purify them. After that, the sequences that were assigned to the same operational taxonomic units ((OTUs) > 97% similarity).

Concentrations of SCFAs in fecal were measured by using GC-MS. Briefly, fecal samples were weighed into 1.5 ml centrifuge tubes and mixed with 1 ml of ddH2O, homogenized, and centrifuged (10,000 rpm, 10 min, 4°C). A mixture of the supernatant fluid and 25% metaphosphoric acid solution (0.9 and 0.1 ml, respectively) were vortexed for 1 min and centrifuged (1,000 rpm, 10 min, 4°C) after standing in a 1.5 ml centrifuge tube at 4°C for over 2 h. The supernatant portion was then filtered through a 0.45μm polysulfone filter and analyzed using Agilent 6890 gas chromatography (Agilent Technologies, Inc., Palo Alto, CA, United States).

5.3.7 Statistical analysis.

Data are expressed as the mean ± SEM. Differences with a $P<0.05$ were considered significant. The student's t-test (SPSS 21 software) was used to perform the statistical analyzes. Spearman's correlation analysis was completed by the RStudio (version 4.0.3) platform. Plots were performed using GraphPad Prism 8.0.2.

5.4 Results

5.4.1 AOS improved semen parameters and blood testosterone content of aging boars.

As shown in Fig.5-1A (Study scheme), the aging Duroc boars were fed AOS with 10 mg/kg body weight for 63 days. Dietary supplementation of AOS significantly increased the sperm motility (Fig.5-1B; $P<0.05$). Meanwhile, the abnormal sperm rate was significantly lower compared to the CON group (Fig.5-1D; $P<0.01$). In contrast, the sperm validity rate of the AOS group was significantly higher than that of CON group (Fig.5-1F; $P<0.001$). Apart that, the sperm concentration (Fig.5-1C; $P=0.2242$) and sperm volume (Fig.5-1E; $P=0.1527$) had no differences between two groups. Adding AOS in the basal diet was associated to a significantly improve the testosterone content in the blood of the aging boar (Fig.5-1G; $P<0.01$).

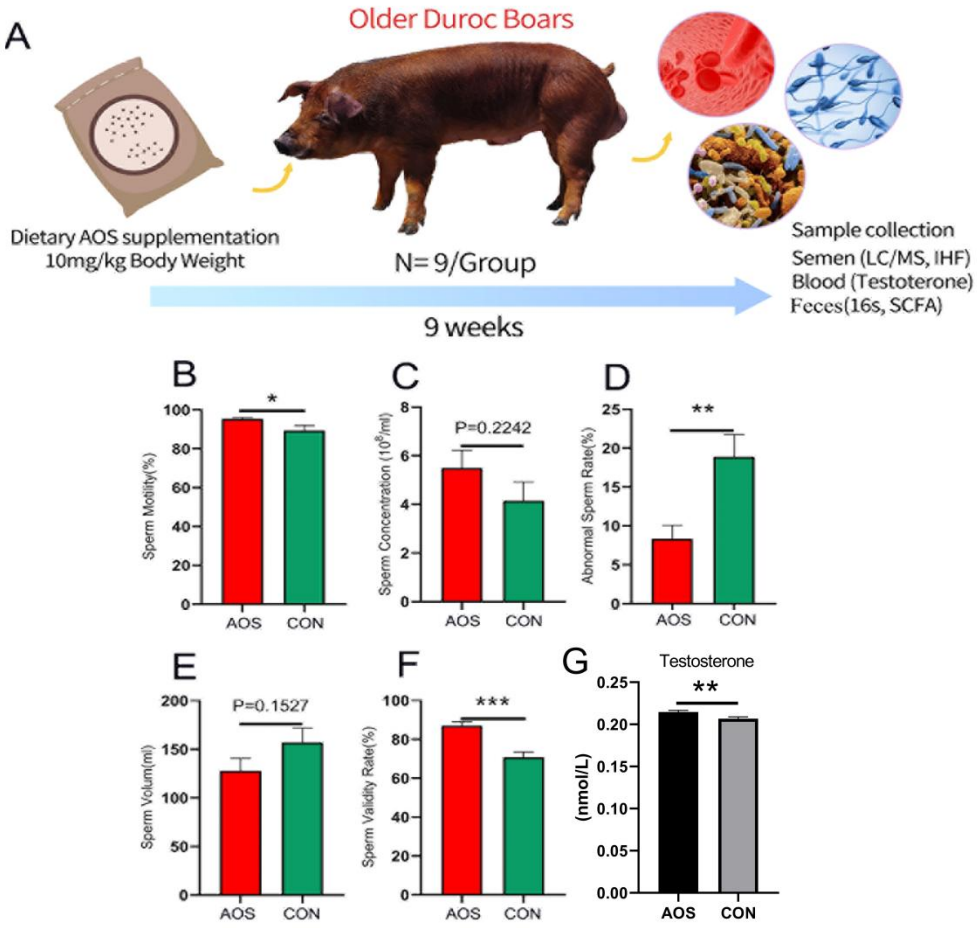


Figure 5-1 AOS improved semen parameters and blood testosterone content of aging boars. (A) Study design. (B) Sperm motility. The y-axis represents the percentage of total cells. The x-axis represents the treatment (n=9/group). *P<0.05. (C) Sperm concentration. The y-axis represents the concentration. The x-axis represents the treatment (n=9/group). P=0.2242 (D) Abnormal sperm rate. The y-axis represents the percentage of abnormal cells. The x-axis represents the treatment (n=9/group).**P<0.01. (E) Sperm volume. The y-axis represents the Volumetric weight. The x-axis represents the treatment (n=9/group). P=0.1527. (F) Sperm validity rate. The y-axis represents the percentage of validity cells. The x-axis represents the treatment (n=9/group).***P<0.001. (G) Blood testosterone content. The y-axis represents the testosterone level. The x-axis represents the treatment (n=9/group). **P<0.01. Data were expressed as the mean ± SEM.

5.4.2 AOS improved the protein that related to spermatogenesis of aging boars.

In order to understand how AOS prolong the lifespan of aging Duroc boars, the protein levels (CatSper 8, PKA, Bcl, Bcl-2-associated X protein) for sperm quality and spermatogenesis were quantified (Fig.5-2A). AOS improved the protein level which reflected the positive rate of CatSper 8 (Fig.5-2B; P<0.001) and PKA

(Fig.5-2D; $P<0.01$) significantly compared to the CON group by IHF staining. At the same time, AOS improved Bcl (Fig.5-2C; $P=0.2830$) and BAX (Fig.5-2E; $P=0.4329$) protein levels, but the differences were not significant.

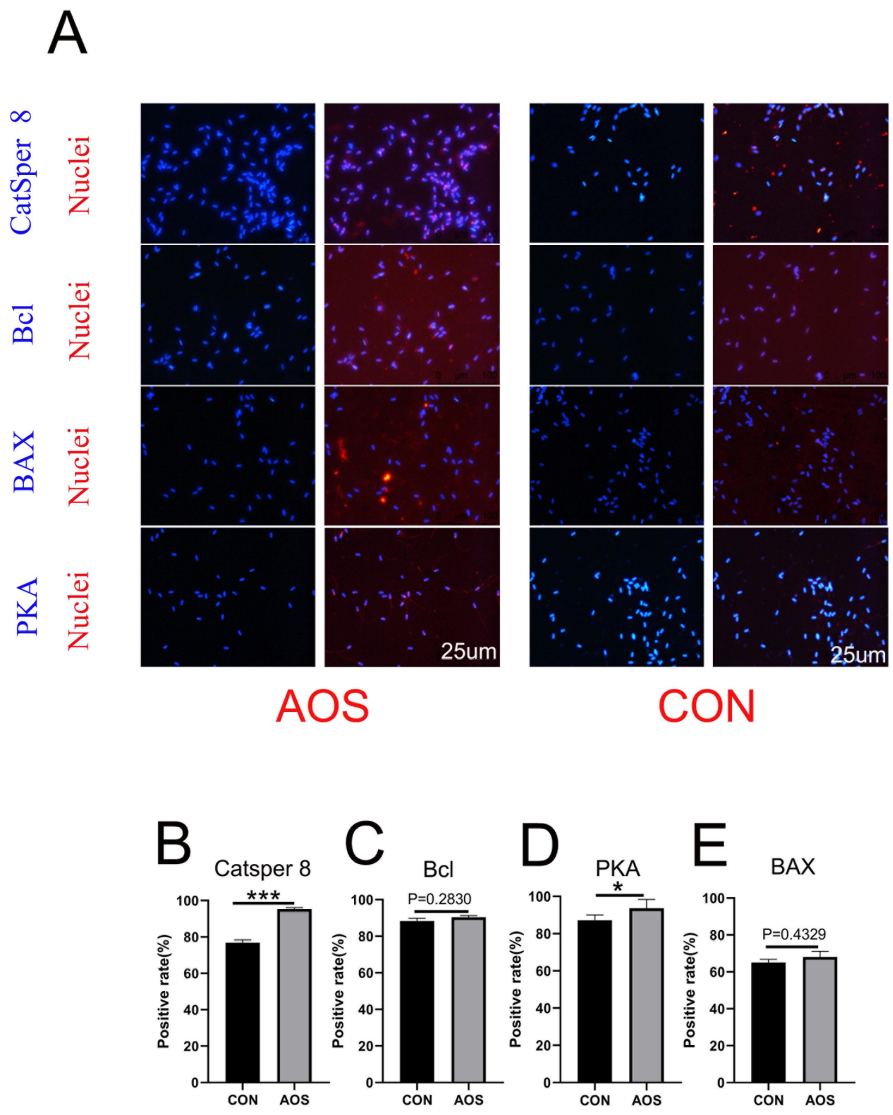


Figure 5-2 AOS improved the protein that related to spermatogenesis of aging boars.(A) Immunofluorescence staining (IHF) of Catsper 8, Bcl, BAX, PKA. (B) Positive rate of Catsper 8. (C) Positive rate of Bcl. (D) Positive rate of BAX. (E) Positive rate of PKA. Data were expressed as the mean ± SEM. The y-axis represents the amount of Positive rate. The x-axis represents the treatments. (n=9/group) ***$P<0.001$.**$P<0.01$.

5.4.3 AOS improved the sperm metabolites of aging boars.

AOS benefited the sperm metabolites which were determined by LC/MS analysis. Firstly, AOS can significantly up-regulated some kind of fatty acids and derivatives in sperm such as butyrylcarnitine (Fig.5-3A; $P<0.05$), propionylcarnitine (Fig.5-3B;

P<0.001), adrenic acid (Fig.5-3C; *P*<0.05) and 4-Trimethylammoniobutanoic acid (Fig.5-3F; *P*<0.001). Secondly, AOS elevated a batch of sperm antioxidants such as succinic acid (Fig.5-3D; *P*<0.05). Thirdly, AOS could significantly reduce harmful metabolites that were related to reproductive function in sperm of aging boars such as dibutyl phthalate (Fig.5-3E; *P*<0.05). Meanwhile, the potential metabolic pathways of the changed metabolites were determined by KEGG pathway analysis. The top 10 pathways (Fig.5-3G-H) showed that the changed metabolites were involved in Lysine degradation (*P*<0.01), GABAergic synapse (*P*<0.05), Oxidative phosphorylation (*P*<0.05), Citrate cycle (*P*<0.05), cAMP signaling pathway (*P*<0.05), Glucagon signaling pathway (*P*<0.05), Alanine, aspartate and glutamate metabolism (*P*<0.05), Central carbon metabolism in cancer (*P*<0.05), Pyruvate metabolism (*P*<0.05) and Sulfur metabolism (*P*<0.05).

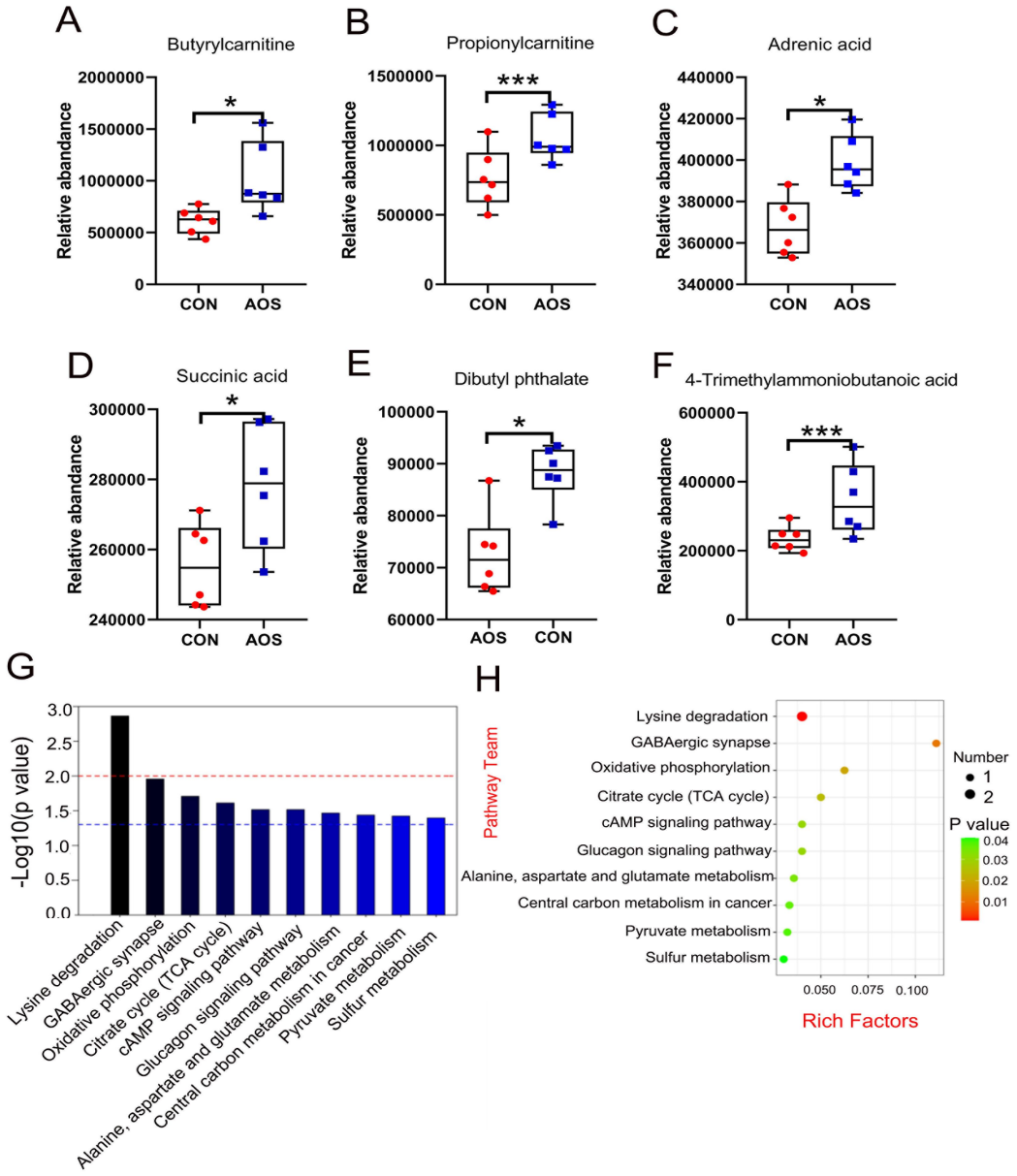


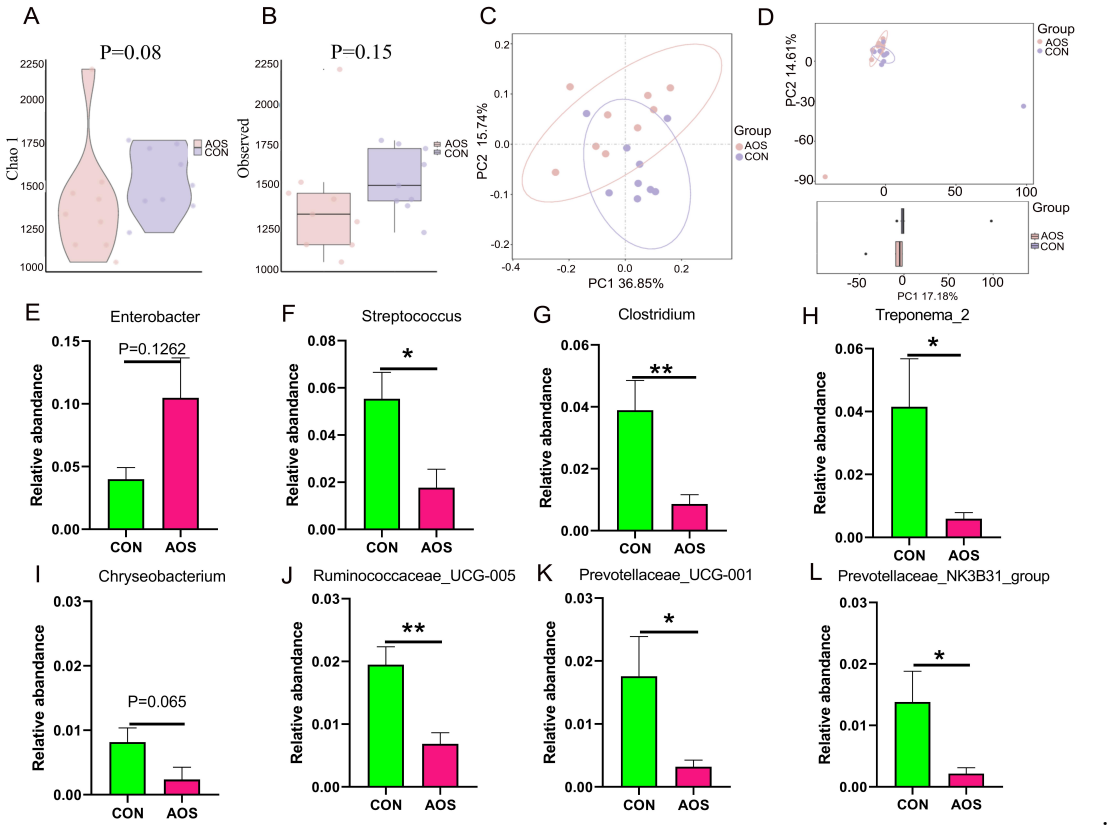
Figure 5-3 AOS improved the sperm metabolites of aging boars. (A) Sperm Butyrylcarnitine level. (B) Sperm Propionylcarnitine level. (C) Sperm Adrenic acid level. (D) Sperm Succinic acid level. (E) Sperm Dibutyl phthalate level. (F) Sperm 4-Trimethylammoniobutanoic acid level. (G) KEGG metabolic pathway histogram (top10), red dotted line means $P< 0.01$, blue dotted line means $P< 0.05$. (H) KEGG metabolic pathway bubble chart (top10). Data were expressed as the mean ± SEM. The y-axis represents the relative amount. The x-axis represents the treatments (n=6/group). ***$P< 0.001$.*$P < 0.05$.

5.4.4 AOS changed microbial composition in the feces of aging boars

To investigate the effect of AOS on intestinal microbes of aging boars, we conducted 16s sequencing on feces. The microbes (β-diversity) were different between AOS and CON group by PCA analyze (Fig.5-4C-D). The α-diversity on the Chao1 (Fig.5-4A; P=0.08) and Observed (Fig.5-4B; P=0.15) level were not changed

much, however, there was a tendency of altering. AOS changed the abundance of beneficial microbiota at genus level such as *Enterobacter* (Fig.5-4E; P=0.1262; FDR=0.672). At the same time, AOS reduced the abundance of harmful microbiota such as *Streptococcus* (Fig.5-4F; P<0.05; FDR=0.492), *Clostridium* (Fig.5-4G; P<0.01; FDR=0.372), *Treponema_2* (Fig.5-4H; P<0.05; FDR=0.537), *Chryseobacterium* (Fig.5-4I; P=0.065; FDR=0.654), *Ruminococcaceae_UCG-005* (Fig.5-4J;P<0.01; FDR=0.102), *Prevotellaceae_UCG-001* (Fig. 5-4K; P<0.05; FDR=0.537) and *Prevotellaceae_NK3B31_group* (Fig.5-4L; P<0.05; FDR=0.531).

Figure 5-4 AOS changed microbial composition in the feces of aging boars. (A) α-diversity with Chao 1 level. (B) α-diversity with Observed level



(C) β-diversity with PCoA level. (D) β-diversity with PCA level. The relative amount of individual microbiota in feces at Genus level (E-L). Data were expressed as the mean ± SEM. The y-axis represents the relative amount. The x-axis represents the treatments (n=9/group). **P<0.01.*P<0.05.

5.4.5 AOS improved the content of short-chain fatty acids in aging boar feces.

To analyze the metabolites of gut microbes, we measured the SCFAs in the feces

of aging boars. Some SCFAs were significantly improved such as acetic acid (Fig.5-5A; $P<0.05$) and butyric acid (Fig.5-5C; $P<0.05$). The propionic acid has a tendency to increase (Fig.5-5B; $P=0.075$). In this experiment, some short-chain fatty acids had also risen, but the differences were not significant, such as isobutyric acid (Fig.5-5D; $P=0.7133$), pentanoic acid (Fig.5-5E; $P=0.4014$) and Isopentanoic acid (Fig.5-5F; $P=0.2006$). There was a good correlation between microorganisms and short-chain fatty acids(Fig.5-5G). *Enterobacter* was significantly positively correlated with acetic acid and propionic acid, respectively. *Ruminococcaceae_UCG-005* was significantly negatively correlated with propionic acid. Meanwhile, *Clostridium* was significantly negatively correlated with butyric acid.

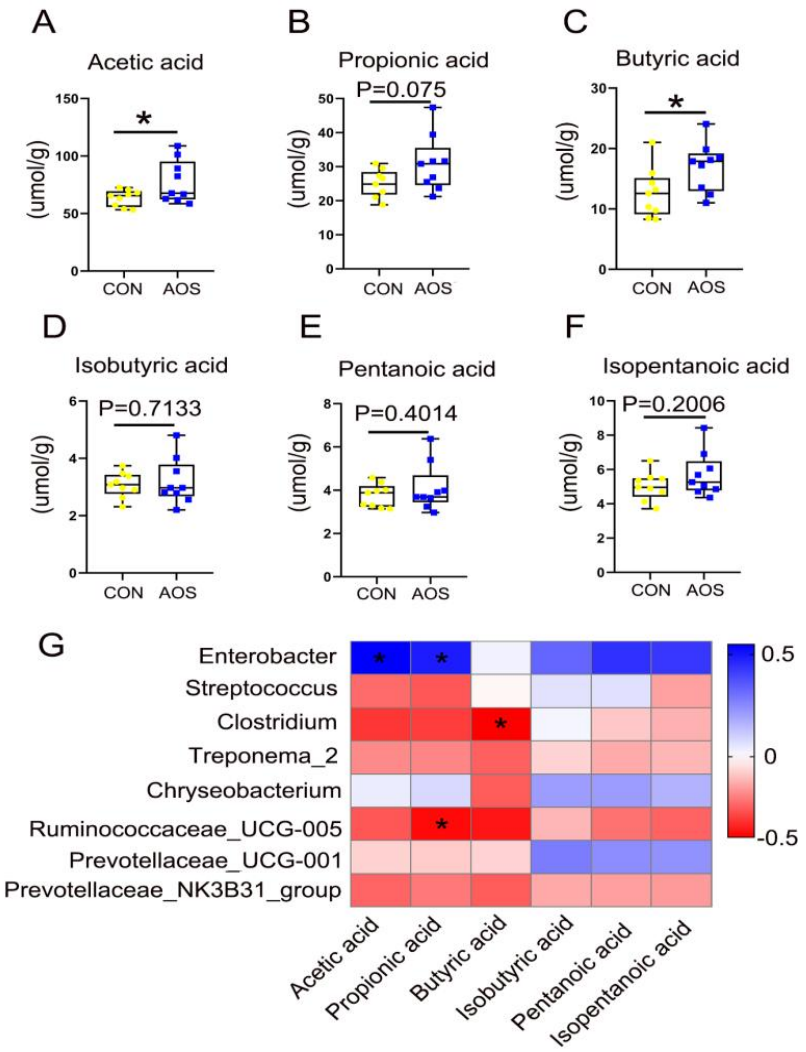


Figure 5-5 AOS improved the content of short-chain fatty acids in aging boar feces. (A) Acetic acid level in feces. (B) Propionic acid level in feces. (C) Butyric acid level in feces. (D) Isobutyric acid level in feces. (E) Pentanoic acid level in feces. (F) Isopentanoic acid level in feces. (G) The correlation ship between gut microbes and SCFAs. Data were expressed as the mean ± SEM. The y-axis represents the relative amount. The x-axis represents the treatments (n=9/group). *P<0.05.

5.4.6 Spearman correlation among fecal microbes, sperm metabolites and sperm parameters.

The spearman correlation analysis (Fig.5-6) indicated that the fecal microbiota, sperm metabolites and semen parameters were well correlated. First of all, the sperm metabolites and gut microbes were well correlated with each other. Secondly, there was a good correlation between sperm metabolites and gut microbes. In terms of semen quality, the decreased metabolite dibutyl phthalate in the AOS group was significantly negatively with sperm concentration. The *Ruminococcaceae_UCG-005* was positively correlated with sperm validity rate and negatively correlated with

sperm volume. Meanwhile, *Prevotellaceae_UCG-001* was positively correlated with sperm validity rate and negatively correlated with abnormal sperm rate.

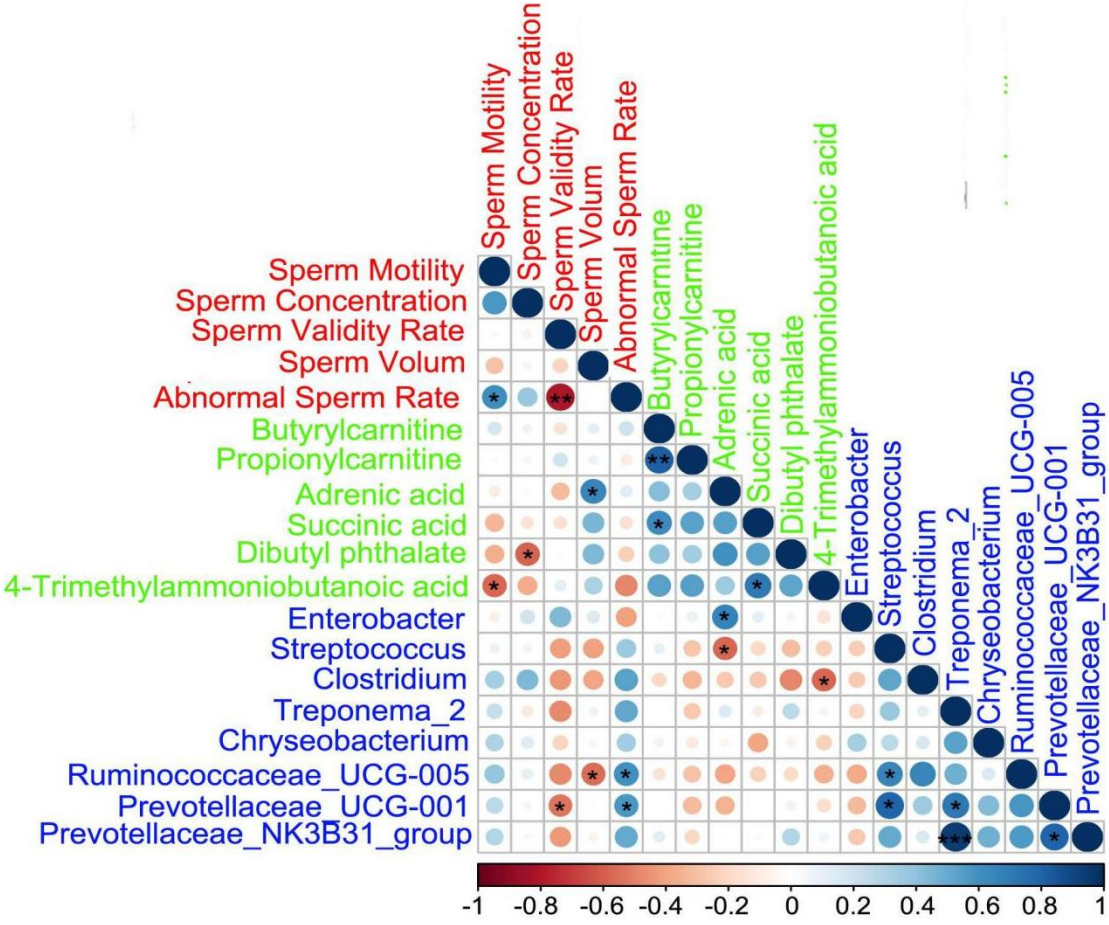


Figure 5-6 Correlations. Correlations among fecal microbes, sperm metabolites and sperm parameters. Color red represents sperm parameters, color green represents sperm metabolites, color blue represents gut microbes. Blue cycles represent positive correlation, red cycles represent negative correlation. The size of the circle represents the strength of the correlation. (larger circle = stronger correlation). ***P*<0.01.**P*<0.05.

5.5 Discussion

As a natural plant extract, AOS has multiple biological functions, which is the most prominent characteristic of antioxidant (Yang et al., 2022) and anti-aging (Feng et al., 2021). According to the literature, AOS can improve the sperm motility of young boars (Han et al., 2022). On the research of others, with mice as an animal experiment model, AOS was seen to repair testicular damage caused by busulfan (Zhao et al., 2020d; Zhang et al., 2021b; Yan et al., 2022), thereby improving sperm quality. In this study, by feeding AOS for aging boars, sperm parameters had been

significantly improved, and the testosterone content in the blood had also been significantly increased. As we all know, testosterone is an important hormone that can regulate spermatogenesis (Smith and Walker, 2014; Ge et al., 2021; Walker, 2021) and promote sexual desire (van Anders, 2012; Uloko et al., 2022), it is a positive effect on improving the lifespan of the aging boars. At the same time, AOS is associated to an improvement of the protein content in boar sperm, such as Catsper 8 and PKA. Studies had shown that CatSper 8 can maintain the chromatin integrity, morphology of sperm (Carlson et al., 2009; Khordad et al., 2022). However, PKA can not only participate in the fertilization process of mammals, especially sperm capacitation (Baro Graf et al., 2020), but also highly associated with sperm motility and apoptosis (Huang et al., 2018; Yan et al., 2020). It can be seen that the potential mechanism of AOS extended the lifespan of the aging boar is to improve the key protein content in the sperm and the level of blood testosterone.

Sperm metabolites play a vital role in the procedure of spermatogenesis (Zhao et al., 2022). Studies had shown that AOS was conducive to improving the blood metabolome of boars, which in turn affects systemic metabolism (Han et al., 2022; Hao et al., 2022). At the same time, AOS was also conducive to improving metabolites in boar sperm. In the experiment of youth boars, AOS could improve some unsaturated fatty acids, amino acids and antioxidants to improve semen quality (Han et al., 2022). In this study, AOS improved the sperm metabolome of aging boars, several kinds of fatty acids and derivatives were significantly increased, such as butyrylcarnitine, propionylcarnitine and adrenic acid. A large number of studies indicated that the content of butyrylcarnitine in the study of Bulls was significantly positively correlated with the reproductive performance (Longobardi et al., 2020). However, in the male reproductive tract, propionylcarnitine was secreted by epididymis, which will protect sperm in semen (Golan et al., 1983). Through the study of metabolome on mice testicular, the content of adrenic acid was significantly positively correlated with testicular function (Lai et al., 2017). AOS could significantly increase the antioxidant content in boar semen such as succinic acid. Studies had shown that succinic acid was a strong antioxidant that improves sperm

motility by reducing the content of ROS in epididymis (Frenkel et al., 1975; Nikolopoulou et al., 1985). In contrast, AOS could also reduce sperm metabolite dibutyl phthalate, which was a substance that was not conducive to spermatogenesis (Czubacka et al., 2021). Study had also shown that dibutyl phthalate induced oxidative stress and impaired spermatogenesis in adult rats (Aly et al., 2016). Therefore, the potential mechanism of AOS extending the lifespan of aging boars was to improve the metabolites in the sperm, thereby improving the semen quality.

As the largest digestive organ of animal body, the intestine has been becoming a research hotspot in recent years (Takiishi et al., 2017; Zhou et al., 2020). As an important medium between the meal and the host, the intestinal microorganism not only regulates the health of the host, but also generates a specific connection with the organs, such as the intestine-liver axis (De Gregorio et al., 2020) and intestine-testicular axis (Zhao et al., 2020b). Therefore, it plays a vital role in human and animals. In the study of young boars as a model, it was found that AOS could improve the composition of intestinal flora. On the one hand, it improved the relative abundance of beneficial bacteria such as *Butyricicoccus* and *Bifidobacterium*, on the other hand, it reduced the relative abundance of harmful bacteria such as *Streptococcus* and *Oscillibacter* (Han et al., 2022). In the research of mice as an animal model, the semen quality had been improved by using fecal microbial transplantation (FMT) technology (Yan et al., 2022; Sheng et al., 2023). In this study, due to the experimental animals were older boars, only one beneficial bacteria had been increased that was *Enterobacter.* Study had shown that *Enterobacter* could improve the storage time of boar semen at room temperature (Prieto-Martínez et al., 2014). At the same time, the *Prevotella* in the research field of reproduction was recognized as a kind of harmful bacteria, which is negatively related to sperm quality (Farahani et al., 2021). In the study, we found that both *Prevotellaceae_UCG-001* and *Prevotellaceae_NK3B31_group* were significantly reduced. Studies had shown that both bacteria above could promote intestinal inflammation and harmful to intestinal health (Huang et al., 2021; Wu et al., 2023). Therefore, AOS extended the service lifespan of the older boars by reducing the relative abundance of harmful bacteria.

Meanwhile, short chain fatty acids have a strong effect in the intestine. It can provide energy for small intestinal epithelial cells, which will affect the permeability of the intestinal mucosa (Hu et al., 2018; He et al., 2020a; Liu et al., 2021b). Intestinal bacteria can use glucose to produce butyric acid, and the energy provided by butyric acid can be used for spermatogenesis (Du et al., 2013; Yan et al., 2022). In this study, by determining the short chain fatty acids in boar feces, we found that the content of acetic acid and butyric acid was significantly increased. The relative abundance of the changed microbes and short chain fatty acids were well correlated. Therefore, the potential mechanism of AOS to improve the semen quality of aging boars is to improve the composition of microbes in the intestine, thereby increasing the SCFAs content to promote the spermatogenesis.

5.6 Conclusion

Adding 10 mg/kg body weight AOS to the diet can extend the service lifespan of aging breeding boars by improving the intestinal microorganisms and sperm metabolites in a Duroc model. Therefore, AOS can be used as a feed additive to reduce the productive problem with high culling rate of boar stud.

Table 5-1 Composition and nutrient analysis of basal diet

Ingredient	Content, %
Corn	35.15
Barley	24.83
wheat	15.82
Rice bran meal	9.40
Soybean meal	7.90
Soybean oil	2.00
L-lysine	0.40
Methionine	0.14
Threonine	0.24
Ground limestone	1.44
Monocalcium phosphate	1.21
Sodium chloride	0.48
Premix*	1.00
total	100
Nutrient, %	
Calculated NE, kcal/kg	2240
Crude protein, %	14.50
Crude fat, %	3.22
Crude ash, %	6.18
Crude fiber, %	4.15

*: Premix provided the following minerals per kilogram: 17 mg Cu, 160 mg Fe, 140 mg Zn, 50 mg Mn, 0.50 mg I, 0.50 mg Se, and 0.22 mg Cr.

Table 5-2. Primary antibody information

Name	Cat. #	Source (Animal)	Company
Bcl	bs-0520R	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
PKA	bs-1645R	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
BAX	Bs-0127R	Rabbit	Beijing Biosynthesis Biotechnology CO.
CATSPER 8	bs-23326R	Rabbit	Beijing Biosynthesis Biotechnology CO.

Chapter VI

General discussion, conclusion and perspectives

Chapter 6 General discussion, conclusion and perspectives

6.1 General discussion

As we mentioned in the introduction, AOS, as a natural plant extract, has multiple biological functions such as immune regulation (Xu et al., 2014), anti-inflammatory (Zhang et al., 2022a), antioxidant (Lu et al., 2022). Due to its low molecular weight, high water solubility, as well as safe and non-toxic characteristics (Falkeborg et al., 2014; Wang et al., 2021b), AOS has received a lot of attention from researchers in recent years. The current research has found that AOS can significantly improve the sperm motility in chapters 3, 4 and 5. However, The improvement in chapter 4 is the biggest compared to chapters 3 and chapter 5, because its original value is the lowest due to the occurrence of heat stress. Compared to chapter 3 and 5, the improvement of sperm concentration is the biggest as well in chapter 4. Additionally, compared to chapter 3 and chapter 4, the abnormal sperm rate dropped the biggest in chapter 5, this may be because AOS had the best effect on aging boars. It is conducive to improving boar semen quality mainly by changes in 4 parameters: blood metabolites, sperm metabolites, sperm protein and gut microbiota. Therefore, in this PhD thesis we will discuss the effect of AOS on boar semen quality in four parts.

6.1.1. The effects of AOS on blood metabolites to increase semen quality

Blood metabolites can be transported to target organs as nutrients. In this PhD work, it was discovered that adding AOS to the basal diet improved the semen quality of Duroc boars through improving the blood metabolome. Various substances such as antioxidants, testosterone and antioxidant indicators were improved in the blood. Therefore, we will discuss the effects of AOS on these three types of blood metabolites. However, we do need to mention that this significant improvement of metabolites is not significant anymore after correction for multiple testing. It's crucial to approach conclusions cautiously, ensuring we maintain a good balance between controlling for false positives and not missing potentially important

findings. Additional research is definitely needed to delve deeper into this subject.

6.1.1.1. AOS improves antioxidants levels in the blood

In chapter 3, AOS improved boar blood metabolites by increasing levels of antioxidant compounds such as retinol and betaine. A study by Maya-Soriano et al. (2013) has shown that retinol might stabilize the sperm acrosomal membrane in situations of oxidative stress and under high temperatures in Friesian bulls (Maya-Soriano et al., 2013). The serum level of retinol was found to reduce sperm DNA fragmentation due to its strong antioxidant activity, in both normospermic and infertile men (Ghyasvand et al., 2015). Betaine improved the integrity of the plasma membranes of sperm tails, suggesting that betaine has a positive effect on sperm motility (Mori et al., 2022). Meanwhile, betaine not only reduced testicular damage due to oxidative stress, but also had a therapeutic effect on sperm deformity under chronic restraint stress in mice (Meng et al., 2022). In chapter 3, we found that retinol and betaine were positively correlated with sperm motility and sperm concentration, and negatively correlated with abnormal sperm rate. This is consistent with the discovery of Maya-Soriano et al and Meng et al that we mentioned above. Therefore, AOS improves boar semen quality through increasing beneficial antioxidant compounds in the blood.

6.1.1.2. AOS improves testosterone levels in the blood

Testosterone in blood can improve sexual desire (van Anders, 2012), and thus, testosterone is an important male hormone to promote spermatogenesis (Smith and Walker, 2014). A study has shown that serum testosterone levels in 3-month-old boys can predict their semen quality as young adults (Scheutz Henriksen et al., 2022). Therefore, testosterone seems to be a biomarker to evaluate semen quality. In chapter 4, we found that adding AOS to the basal diet could up-regulated the blood testosterone level under heat stress. As we know, environments with high temperature and high humidity levels can easily lead to a decrease in sexual desire and decrease in sperm quality in boars (Chen et al., 2018). Nutritional regulation can increase the testosterone level, then increase sperm motility and decrease abnormal sperm rate

(Akomolafe et al., 2022; Huang et al., 2023). This is consistent with the discovery in this PhD.

Aging is also a factor that effects fertility (Jimbo et al., 2022). Older men exhibit notable disturbances in the reproductive axis, with steroidogenesis being impacted much more than spermatogenesis. The endocrine changes, together with morphological and functional alternations of the aging testis, result in decreased testosterone production (Almeida et al., 2017). In chapter 5 of this PhD, we used the aging Duroc boars model, and it was observed that the testosterone content in the blood significantly increased by adding AOS to the diet. A study has shown that nutritional regulation could increase the testosterone content in aging roosters, and that sperm motility and sperm concentration all could be increased significantly due to diet (Qi et al., 2019). That is similar to the results described in this PhD thesis. Therefore, AOS improved the testosterone level under heat stress and aging conditions, thereby positively influencing semen quality of Duroc boars.

6.1.1.3. AOS improves antioxidant indicator levels in the blood

In chapter 4 of this PhD work, we found that the content of reactive oxygen species (oxygen free radicals and hydroxyl free radicals) in boar blood decreased significantly by feeding AOS, indicating that AOS exerted a strong antioxidant ability. A study by Su et al. (2019) reported that reactive oxygen species (ROS), such as oxygen or hydroxyl free radicals, are substances produced by the metabolism under normal physiological and pathological conditions (Su et al., 2019). Excessive accumulation of ROS may cause harmful oxidative stress, and induce structural damages in cells (Valko et al., 2007), including harm to proteins (Hawkins and Davies, 2019) and DNA (Marnett, 2000). Excessive oxidative damage can lead to loss-of-cell-function and ultimately result in cell apoptosis (Nordberg and Arnér, 2001). It is believed that in the epididymis, where sperm is stored, ROS is produced by respiratory action of sperm (O'Flaherty, 2019). Infertile men have higher levels of semen reactive oxygen species (ROS) than fertile men. High levels of semen ROS can cause sperm dysfunction, sperm DNA damage and reduced male reproductive

potential (Jannatifar et al., 2019). Therefore, our study indicated that AOS, by lowering the reactive oxygen species in blood, could alleviate the degree of oxidative stress to potentially improve semen quality.

6.1.2. The effects of AOS on sperm metabolites to increase semen quality

In our research, we found that adding AOS could improve the semen quality of Duroc boars through sperm metabolites, such as antioxidants compounds, fatty acids and amino acids. Therefore, we will discuss the effects of AOS on these three types of sperm metabolites.

6.1.2.1. AOS improves antioxidant compounds in the semen

In chapter 4 of this PhD, AOS improved boar sperm metabolites by increasing levels of antioxidant compounds such as triazophos and quercetin. A study by Ge et al. (2016) has shown that insects treated with triazophos can show substantial up-regulation of a male spermatogenesis-associated protein 5-like gene (NlSPATA5). Reduced expression of NlSPATA5 led to decreased male accessory gland protein content and reproductive system development in brown planthopper (BPH) males (Ge et al., 2016). Quercetin is widely reported in many fields, and it has been seen to increase testosterone and sperm concentration in mice (Garcia et al., 2023). Quercetin can also ameliorate sperm oxidative stress and inflammation, while preserving sperm morphology and function in rats (Yelumalai et al., 2019). So, by feeding these two compounds directly to animals, the fertility and semen quality improved. In this PhD thesis, triazophos and quercetin were negatively correlated with abnormal sperm rate. Although there are few articles reported the mechanism how triazophos and quercetin prevented the sperm, here we can suppose that AOS can promote the synthesis of triazophos and quercetin, which can reduce the ROS in the epididymis due to the effect of antioxidant. In chapter 5, we found that the succinic acid improved in the semen after feeding AOS. As is known, succinic acid is a strong antioxidant that improves sperm motility by reducing the content of ROS in the epididymis (Frenkel et al., 1975; Nikolopoulou et al., 1985). In this PhD thesis, succinic acid was negative correlated with abnormal sperm. Therefore, AOS increased sperm antioxidants to

reduce the abnormal sperm rate, and thus improve semen quality.

6.1.2.2. AOS improves fatty acids and derivatives in the sperm

In this PhD work we used LC/MS to examine sperm metabolites. We found that a portion of fatty acids and derivatives were significantly improved in boar semen due to AOS supplementation. In chapter 3, after adding 10 mg/kg AOS, omega-3 fatty acids DHA and EPA were changed. DHA and EPA can significantly improve semen quality in human (Lass and Belluzzi, 2019) A study by Hosseini et al. (2019) has shown that supplementing infertile men with omega-3 fatty acids resulted in a significant improvement in sperm motility and sperm concentration (Hosseini et al., 2019). Another study showed that feeding AOS to diabetic mice increased the DHA and EPA content in the testicles by increasing the beneficial intestinal bacteria *Lactobacillus* (Yan et al., 2022). Therefore, the underlying mechanisms may be that AOS increased gut-beneficial microbes to elevate sperm DHA and EPA to promote spermatogenesis and thus to ameliorate sperm concentration and motility. In chapter 5, propionylcarnitine and adrenic acid were improved as well by feeding AOS. In the male reproductive tract, propionylcarnitine is secreted by the epididymis, and is present in semen with the role to protect sperm (Golan et al., 1983). Through the study of the metabolome in mice testes, the content of adrenic acid was found to be significantly positively correlated with testicular function (Lai et al., 2017). According to the spearman correlationship analysis performed in chapter 3 and chapter 5, DHA and EPA were positively correlated with sperm motility, and adrenic acid was negatively correlated with abnormal sperm rate. Therefore, AOS improved beneficial sperm fatty acids to increase sperm motility and reduced the abnormal sperm rate.

6.1.2.3. AOS improves amino acids in the sperm

Amino acids are essential substances for spermatogenesis (Dai et al., 2015), as more and more evidence shows that the lack of amino acid is closely related to male infertility (Dong et al., 2016; Saleem et al., 2021). In chapter 3 of this PhD thesis, we found, using LC/MS, that lysine was increased. A study by Chen et al. (2021)

reported that adding lysine to diluted boar semen could increase the sperm motility and acrosome integrity (Chen et al., 2021). Another study reported that lysine could be used as a cryoprotectant to protect post-thaw semen quality in bulls (Tariq et al., 2020). In chapter 4 of this PhD thesis, we found that the level of hypotaurine in sperm improved after feeding AOS. A study by Bucak et al. (2013) has shown that hypotaurine, a derivative of the amino acid cysteine, can be used to improve post-thawed ram sperm parameters (Bucak et al., 2013). In chapter 3 to 5 of this PhD thesis, we have shown that the aforementioned amino acids all were positively correlated with sperm motility. The mechanism is that AOS can modulate the protein interaction through medium (Labre et al., 2018), the amino acids can synthesis protein which can be used for spermatogenesis. Therefore, we can state that AOS improved beneficial sperm amino acids to increase sperm motility.

6.1.3. The effects of AOS on sperm proteins related to spermatogenesis

In the process of spermatogenesis, proteins play an indispensable role because they present vital building blocks for sperm production (Chalmel and Rolland, 2015). PKA activity is essential for vigorous sperm motility and for the resumption of meiosis in oocytes, two events required for successful fertilization (Burton and McKnight, 2007). CatSper has been shown to have a positive correlation with mature sperm function (Wang et al., 2016). Therefore, these proteins are closely related to the reproductive performance of male animals. In the current investigation, we found that 10 mg/kg AOS could benefit boar sperm motility and concentration. Moreover, AOS increased the levels of these aforementioned important proteins, CatSper 8 and PKA, in boar sperm. Studies have shown that CatSper 8 can maintain chromatin integrity, and the morphology of sperm (Carlson et al., 2009; Khordad et al., 2022). Meanwhile, PKA does not only participate in the fertilization process of mammals, especially in the sperm capacitation process (Baro Graf et al., 2020), but it is also highly associated with sperm motility and apoptosis (Huang et al., 2018; Yan et al., 2020). Therefore, the potential mechanism of AOS increased the semen quality of boars by improving key proteins in sperm.

6.1.4. The effects of AOS on boar gut microbiota

The intestine is an important digestive organ in mammals (Gasbarrini et al., 2008). Gut microbiota present the intermediary between host and diet, thus it plays a vital role in regulating animal health. Gut microbiota has many physiological roles, not limited to metabolic disorders such as obesity and diabetes (Bouter et al., 2017; Liu et al., 2017b). Gut microbiota also has a significant involvement in diseases relating the nervous system and reproductive system (Dai et al., 2015). Previously, we found that AOS benefits gut microbiota by increasing beneficial bacteria while it decreases harmful bacteria in murine small intestine to rescue cell development (Zhang et al., 2020a). In chapter 3 of this PhD, we found that adding 10 mg/kg AOS could change the gut microbiota composition at the genus and phylum level, as found by 16s RNA sequencing. Not only the population of beneficial microbiota changed but the amount of harmful bacteria also reduced. However, we do need to mention that this significant increase of beneficial bacteria or down-regulated of harmful bacteria is not significant anymore after correction of multiple testing. It is crucial to approach conclusions cautiously, ensuring we maintain a good balance between controlling for false positives and not missing potentially important findings. Additional research is definitely needed to delve deeper into this subject. In this part, we will discuss the effect of AOS on boar semen quality with regard to both beneficial and harmful bacteria.

6.1.4.1. AOS increases the relative abundance of beneficial bacteria

As an important medium between food and the host, the intestinal microorganisms not only regulate the health of the host, but also generate a specific connection with the organs and the between-organ axes, such as the intestine-liver axis (De Gregorio et al., 2020) and intestine-testicular axis (Zhao et al., 2020b). In chapter 3 to 5, we conducted a 16s sequencing analysis on individual boar feces, and examined enriched microbiota species at the genus level. After feeding AOS for 9 weeks, we found that the relative abundance of beneficial bacteria related to semen quality was higher in the AOS group compared to the CON group. These beneficial

bacteria were the following: *Enterobacter*, *Pseudomonas*, *Escherichia-Shigella*, *Bifidobacterium* and *Butyricicoccus*. A study by Prieto-Martínez et al. (2014) reported that *Enterobacter* can extend the storage time of boar semen at room temperature (Prieto-Martínez et al., 2014). *Pseudomonas* was described being beneficial for sperm capacitation and protein phosphorylation of boar spermatozoa (Sepúlveda et al., 2014, 2016). *Escherichia-Shigella* was positively correlated with the synthesis of sex hormones (Ramírez-Acosta et al., 2022). *Bifidobacterium* was used for patients with asthenozoospermia for 6 weeks, after which semen quality was significantly improved (Valcarce et al., 2017). Limited evidence has been reported regarding the effect *Butyricicoccus* could have on semen quality directly. However, *Butyricicoccus* is a beneficial bacterium that uses carbohydrates to produce short chain fatty acids (SCFAs) (Chen et al., 2019; He et al., 2020b). A correlation analysis study pointed out that SCFAs could improve semen quality (Lin et al., 2022). A study by Olaniyi et al. (2011) has also shown that SCFAs significantly decreased testicular proprotein convertase subtilisin/kexin type 9 (PCSK9) with a significant reduction in cholesterol and lipid peroxidation. This reduction resulted in restoring testicular tissue as seen histologically, with an improved architecture of seminiferous tubules, along with improved testicular function, including spermatogenesis and steroidogenesis (Olaniyi et al., 2021). Meanwhile, in our study, the addition of AOS significantly improved the content of acetic acid, propionic acid and butyric acid, and there was a significant positive correlation between beneficial bacteria and SCFAs. The data above indicated that the potential mechanism of AOS to improve boar semen quality may be to increase the relative abundance of beneficial bacteria in the gut that, on their turn, produce short-chain fatty acids beneficial for spermatogenesis.

6.1.4.2. AOS decreases the relative abundance of harmful bacteria

As we know, gut microbiota is a double-edged sword for humans and animals. On the one hand, some microbes are beneficial to our health as they, for example, promote digestion and absorption of nutrients. On the other hand, some are harmful due to their physiological roles. The elevation of harmful bacteria in the gut can lead

to intestinal disorders resulting in diarrhea and intestinal inflammation, which will affect animal health. In our investigation, we found that the relative abundance of harmful bacteria reduced in AOS group compared to the CON group. These harmful bacteria were the following: *Prevotella*, *Streptococcus*, *Prevotellaceae_UCG-001*, *Prevotellaceae_NK3B31_group*. A study by Zhou et al. (2022) has reported that *Prevotella* was considered a harmful bacterium in terms of affecting male fertility (Zhou et al., 2022). Through the study of semen microbial composition and male fertility, it has been noted that *Prevotella* indeed has a negative impact on sperm motility and concentration (Farahani et al., 2021; Lundy et al., 2021; Gachet et al., 2022). Streptococcus was found to be the most susceptible bacteria during fertilization, reducing sperm motility and ultimately leading to sperm apoptosis (Zuleta-González et al., 2019). In chapter 5, we found that both *Prevotellaceae_UCG-001* and *Prevotellaceae_NK3B31_group* were significantly reduced. Studies have shown that both aforementioned bacteria could promote intestinal inflammation and be harmful for intestinal health (Huang et al., 2021; Wu et al., 2023). Meanwhile, we found that the harmful bacteria showed to have a negative correlation with sperm motility and sperm concentration. On the contrary, there was a positive correlation between harmful bacteria and abnormal sperm rate. Therefore, feeding AOS to the boars could increase the semen quality through decreasing the relative abundance of harmful bacteria.

6.2. Conclusion

In this PhD thesis, we investigated the effects of AOS on boar semen quality in different models of Duroc boars. A supplementation with AOS can improve the semen quality through changing gut microbiota and metabolites in blood and sperm, as well as increasing sperm proteins that related to spermatogenesis. Therefore, we summarize that AOS has a positive effect on intestinal microorganisms, blood metabolites, sperm metabolites and sperm proteins. AOS therefore can improve the semen quality of Duroc boars by these parameters so as to alleviate reproductive problem as occurring in heat stress moments and in an older age when the culling rate

increases.

6.3. Perspectives

AOS is a natural additive with development value. Clarification of the relationship between structure and biological activity of AOS can help to prepare targeted AOS with specific functions, so as to apply in the fields of food, medicine and feed additives. However, several bottlenecks still exist in the preparation and application of AOS. In this PhD, the positive effect of AOS on boar semen quality has been supported. In addition, AOS, as an active substance with anti-inflammatory and immune regulation, can not only remove inflammatory factors in the intestine, but also enhance the immune function of the gut. This is of great value in the research of AOS on the intestinal-testicular-axis. More research is needed to elucidate its specific molecular mechanism in this field. Firstly, the beneficial microbiota that we obtained through 16s RNA sequencing can be separated and purified, then they can be fed orally as probiotics in mice and see if this implication helps against heat stress or aging. The semen parameters can be tested to verify if the beneficial bacteria can improve the semen quality. Progress in this area will be more conducive to the application value of AOS in the prevention and treatment in animal production.

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