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Identification of bull semen microbiota and possible alternatives for antibiotics in semen extenders

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Abstract

The use of 16S rRNA sequencing has revolutionised research on the microbiome. However, few studies using this method have been done on semen from healthy bulls. Such information is required to optimise addition of antibiotics to semen extenders to inhibit growth of bacteria. With the increasing emergence of antibacterial resistance, it is important to identify alternatives to antibiotics in semen preservation. Possible solutions are to use plant-based substances that exhibit antimicrobial properties or to separate spermatozoa from bacteria in semen samples. The aims of this thesis were to identify the seminal microbiome of healthy bulls, to assess individual differences and their influence on fertility as well as seasonal differences in the occurrence of bacteria and their possible impacts on sperm quality. A second aim was to explore alternatives to antibiotics to reduce bacteria, using plant extracts or by physical removal of bacteria from semen samples. The studies revealed individual differences in bull seminal microbiota that may be correlated with fertility. Seasonal differences were observed in the bacteria in commercial semen samples; however, no significant associations were found between specific bacteria and sperm quality. When evaluating alternatives to antibiotics, we noted that curcumin extract at a concentration of 5% reduced the bacterial count and had a slight positive effect on bull sperm kinematics without negatively affecting sperm viability. Single layer centrifugation (SLC) effectively removed bacteria from bull semen samples while simultaneously selecting good quality spermatozoa. More studies are needed to investigate the influence of certain bacteria on bull sperm quality. The effects of combinations of plant-based substances on bacterial reduction and sperm quality could be investigated, and also further possibilities with SLC. These methods could contribute to reducing the development of antimicrobial resistance.

Keywords: 16S sequencing, male fertility, season, sperm quality, bacteria, plant-based substances, Single Layer Centrifugation

Identifiering av mikrobiota i tjursperma och möjliga alternativ till antibiotika i spädningsvätska

Abstract

Spermaprover innehåller bakterier och därför tillsätts vanligtvis antibiotika till spädningsvätskorna som används vid spermakonservering. Kunskapen om mikrobiomet i tjurens seminalplasma är dock bristfällig. Användningen av 16S rRNA-sekvensering har öppnat nya möjligheter för att utforska bakteriella mikrobiom. Få studier med denna metod har gjorts på sperma från friska tjurar trots att kunskap om mikrobiomet krävs för att optimera tillsatsen av antibiotika. Med ökande antibiotikaresistens är det även viktigt att identifiera alternativ till antibiotika. Möjliga lösningar är att använda växtbaserade ämnen med kända antimikrobiella egenskaper, eller att separera spermier från bakterier i ett spermaprov. Syftet med denna avhandling var att identifiera mikrobiomet i sperma från friska tjurar i relation till individuella skillnader, säsong, samt spermiekvalitet och fruktsamhet. Ytterligare mål var att undersöka alternativ till antibiotika för bakteriell reduktion. Resultaten visade att det var individuella skillnader i mikrobiotan och i antalet bakterier i tjursperma, skillnader som kan vara korrelerade med fertilitet. Dessutom observerades säsongsmässiga skillnader i fördelningen av bakteriefyla och släkten i kommersiella tjurspermaprover. Det var dock inga signifikanta samband mellan specifika bakterier och spermiekvalitetsparametrar. Curcuminextrakt i en koncentration av 5% minskade bakterieantalet och hade en positiv effekt på tjurspermiers kinematik utan att negativt påverka spermiernas livskraft. Single Layer Centrifugering (SLC) avlägsnade effektivt bakterier från tjurspermaprover samtidigt som spermier med bäst kvalitet selekterades fram. Resultaten visar att det skulle vara intressant med fortsatta studier avseende effekterna av bakterier på spermiekvalitet. För att minska behovet av antibiotika skulle det vara intressant att undersöka effekten av olika kombinationer av växtbaserade ämnen på bakterieförekomst och spermiekvalitet samt att ytterligare utforska möjligheten att använda SLC för att separera spermier från bakterier. Dessa metoder har potential att bidra till minskad utveckling av antimikrobiell resistens.

Keywords: 16S-sekvensering, fertilitet, årstid, spermiekvalitet, bakterier, växtbaserade substancer, Single Layer Centrifugering

Dedication

To my parents, for their love and support
To my teachers, for everything I learned
To the animals

"Somewhere, something incredible is waiting to be known."
Carl Sagan

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Cojkic, Aleksandar*, Adnan Niazi, Yongzhi Guo, Triin Hallap, Peeter Padrik, and Jane M. Morrell. Identification of bull semen microbiome by 16S sequencing and possible relationships with fertility. Microorganisms 9, no. 12 (2021): 2431.

II. Cojkic, Aleksandar*, Adnan Niazi and Jane M. Morrell. Seasonal differences in bull semen microbiota and sperm quality parameters. (Submitted Manuscript).

III. Cojkic, Aleksandar, Ingrid Hansson, Anders Johannisson, and Jane M. Morrell*. Effect of Some Plant-Based Substances on Microbial Content and Sperm Quality Parameters of Bull Semen. International Journal of Molecular Sciences 24, no. 4 (2023): 3435.

IV. Cojkic, Aleksandar*, Ingrid Hansson, Anders Johannisson, Eva Axnér and Jane M. Morrell. Single layer centrifugation as a method for bacterial reduction in bull semen for assisted reproduction. (Submitted Manuscript).

The contribution of Aleksandar Cojkic to the papers included in this thesis was as follows:

I. Was involved in conceptualisation, planning and design of the experiment and adapting the methodology and investigation, data curation. He had the main responsibility of writing the original draft, corresponding with the journal and revising the article under supervision.

II. Was involved in conception and design of the work, acquisition, performed most of the laboratory work and a part of statistical work, analysis and interpretation of the data; drafting and revising the manuscript.

III. Was involved in the experimental design and planning, performed most of the laboratory work. He performed the statistical analysis of the results under supervision. Was responsible for writing the manuscript, and preparing the final version of the article.

IV. Was involved in the experimental planning and design, performed most of the laboratory work. Performed the statistical analysis and writing the manuscript.

Other papers that are not included in this thesis were as follows:

I. Cojkic, Aleksandar*, and Jane M. Morrell. Animal Welfare Assessment Protocols for Bulls in Artificial Insemination Centers: Requirements, Principles, and Criteria. Animals 13, no. 5 (2023): 942.

II. Cojkic, Aleksandar*, Moa Skarin, Marina Falk, Ingrid Hansson, Jane M. Morrell. The effect of freezing and storage time on bacteria in bull semen, Cryobiology, 109 (2022), 52-53,

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Abbreviations

AI	Artificial insemination
ALH	Amplitude of lateral head displacement
AO	Acridine orange
BCF	Beat cross frequency
CASA	Computer Assisted Sperm Analyses
CFU	Colony forming unit
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
FC	Flow Cytometry
HE	Hydroethidine
DCFDA	2, 7 -dichlorodihydrofluorescein diacetate
HO	Hoechst 33258
LIN	Linearity
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry
NG	No bacterial growth
PCA	Plate count agar
PM	Progressive motility
SCSA	Sperm chromatin structure assay

SD	Standard deviation
SLC	Single Layer Centrifugation
Spz	Spermatozoa
STR	Straightness
SVA	Statens Veterinärmedicinska Anstalt (National Veterinary Institute)
TM	Total motility
TNE	Tris-sodium chloride and ethylene-diaminetetraacetic acid
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WOB	Wobble

1. Introduction

1.1 Background

Antimicrobial resistance (AMR) started to be of considerable concern worldwide with the publication of the Swann report (Swann Committee 1969) on antibiotic-resistant bacteria. The rapidity with which AMR can spread between bacteria, particularly methicillin-resistant strains (Catry *et al*. 2010), in different animal species increases the need for critical action for its control. The main reason for the development of AMR is the nontherapeutic use of antibiotics. Therefore, there is now a concerted effort globally to combat the development of AMR by restricting antibiotics to therapeutic uses only, by choosing the appropriate antibiotics for their pharmacokinetic properties and sensitivity to the specific, isolated bacteria (Teale & Moulin 2012). The production of dairy products, meat, and eggs requires the maintenance of healthy animal populations. Therefore, effective management of bacterial infections is crucial to ensure the well-being of the human population. However, the emergence of AMR poses a significant threat to animal husbandry due to the decreased effectivity to treat bacterial infections (Tiseo *et al*. 2020). According to recent research, the increased use of antimicrobials in animal production can be attributed to intensive farming practices aimed at satisfying growing global food demand (Tiseo *et al*. 2020). Consequently, the issue of AMR is complex as it involves the interplay of humans, animals, and the environment, which is consistent with the One Health approach (Eriksen *et al*. 2021). Furthermore, addressing AMR requires a comprehensive and integrated approach that involves collaboration between the World Health Organization (WHO), the Food and

Agriculture Organization (FAO), and the World Organization for Animal Health (OIE) (Aslam *et al*. 2021).

One of the less noticed, non-therapeutic uses of antibiotics is in semen processing for artificial insemination (AI), where significant quantities of antibiotics are used. The antibiotics to be added and their concentrations in semen for international trade are specified by government directives, such as the European Union (EUR-Lex 2021/880). Because large quantities of semen are needed for AI, the amounts of antibiotics currently used in semen extenders are correspondingly large. In 1999, 264×10^6 bull semen samples were produced worldwide (Thibier & Guerin 2000). Production of commercial semen doses is thought to have nearly doubled since then. Even if this level of usage had remained constant, it represents an annual usage of 66×10^3 L of antibiotic-containing extender, which could contribute to antimicrobial resistance in bacteria found in animals or the environment, with subsequent transfer to bacteria in human beings.

Quite apart from the non-therapeutic use of antibiotics, their efficacy against microorganisms commonly contaminating semen is being questioned. Microorganisms are now being detected in semen despite the presence of antibiotics. Bacteria could be cultured from 11 out of 30 commercial frozen bull semen doses containing unspecified antibiotics (Zampieri *et al*. 2013). Moreover, gentamicin, spectinomucin, tylosin and lincomycin did not control bacterial growth in bull semen (Gloria *et al*. 2014), although no growth occurred in semen samples containing ceftiofur/tylosin or ofloxacin. In addition, Guimaraes *et al*. (2015), cultured at least 15 species of microorganisms in 20 frozen–thawed stallion semen doses containing amikacin.

Alternatives to antibiotics are clearly needed for use in semen extenders; this theme forms the basis of the present project.

1.2 Bacterial contamination of bull semen

1.2.1 Origin of bacteria in bull semen samples

It is very difficult to collect semen samples that do not contain some bacteria. The primary source of bacterial contamination in semen samples is the mucosa of the reproductive tract, which is colonised by microbes from the environment and from the animals themselves. Therefore, the cleanliness

of the environment in which the animals are maintained, plays an important role in the extent of bacterial contamination. The bacterial content of semen samples was influenced by the frequency of changing the bedding; the more frequently bedding was changed, the less bacterial contamination was present (Pickett *et al*. 1999). In addition to the environment, animal body hygiene during semen collection is required. Hazard Analysis and Critical Control Points (HACCP) systems developed for bulls in Artificial Insemination Centers (AIC) have identified 12 processing steps in frozen bull semen production (Goularte *et al*. 2015). Microbiological hazards play an important role in six of them: in the hygiene of the bull prepuce (normal microflora), semen collection (environmental microorganisms), sample identification and delivery at the double glass window (cross-contamination), two-step cooling (microbial growth), and stabilization (microbial growth), and in straw packing and identification (cross-contamination) (Figure 1).

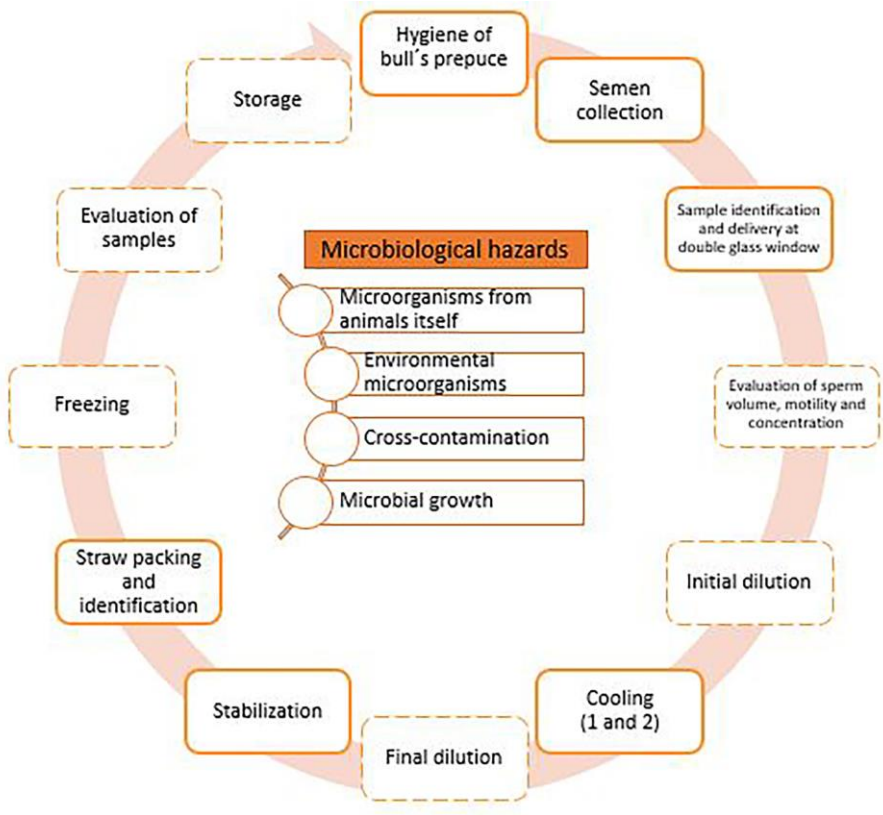

Figure 1. Flow chart of bull semen production and microbiological hazards that could affect semen quality. The steps that are framed by a solid line are those where microbiological hazard plays an important role.

The preventive measures for reducing potential hazards in the majority of cases are staff training about hygiene, and periodical evaluation of antibiotics efficiency (cooling steps) in the cryopreservation process by evaluating AMR of bacteria in semen. Implementation of such a system (HACCP) over a period of six months showed a significant reduction in bacteria and improved post-throwing sperm motility, membrane integrity, and acrosome integrity (Goularte *et al*. 2018). Furthermore, the identification of specific and non-specific microorganisms was a hygiene aspect of storage and use of semen for artificial insemination (Thibier & Guerin 2000) and should be done as a quality control procedure.

1.2.2 Effect of bacterial contamination on sperm quality

Bacteria have direct and indirect influences on sperm quality parameters. The direct bacterial influence, depending on the species of bacteria and their number, is the production of metabolic byproducts and toxins, which reduce the quality of semen doses by affecting spermatozoa. Previous reports emphasis the negative effects of certain bacteria on the number of spermatozoa (Oghbaei *et al*. 2020), sperm motility (Del Porto *et al*. 1975), membrane integrity and acrosome reaction (Cagnoli *et al*. 2020), and sperm DNA fragmentation rates (Gonzalez-Marin *et al*. 2011). Furthermore, microorganisms transmitted via semen were reported to cause infertility (Givens & Marley 2008). However, until recently, evaluation of the whole seminal microbiome of healthy bulls had not been conducted to identify potential pathogenic bacteria in subclinically infected bulls. This knowledge could help in the exclusion of these animals from production and stop bacterial transmission to females causing infection of the urogenital tract, which can indirectly disrupt the neuroendocrine axis that regulates reproduction (Sheldon & Owens 2017). Research on the interaction between bacteriospermia and neuroendocrine regulation of male reproduction has not been conducted.

The indirect influence of bacteria in semen is in competition with spermatozoa for nutrients, since the semen extender used to maintain sperm viability also acts as a nutrient medium for bacterial growth. Production of metabolic byproducts by bacteria also affects sperm quality. Cooling is an additional method, apart from adding antibiotics, to decrease and/or inhibit bacterial growth. For most bacterial species that live in or on mammals, the optimum temperature range is between 30 and 40 °C; these mesophilic bacteria usually stop growing below 15 °C; however, some bacteria have shown potential to multiply below this temperature (Nedwell 1999). Furthermore, bacterial growth was observed even after thawing frozen semen samples containing antibiotics. Cojkic *et al*. (2022), observed that freezing bull semen samples with 15% glycerol at -80 °C, to evaluate bacterial survival during time, showed that total bacterial count was not affected by low temperature but a variable effect was observed on bacterial survival depending on species. To avoid the negative effects of these bacteria on semen quality, the time between thawing and insemination should be kept to a minimum. Another preventive measure is hygiene during the AI process. However, to prevent bacterial contamination of the bull semen doses used

for international trade, the EU Council has stipulated that antibiotics must be added to the semen extenders, as well as the antibiotic activites and doses. These regulations state (EUR-Lex 2021/880):

> *"Where antibiotics or a mixture of antibiotics are added, their bactericidal activity must be at least equivalent to that of the following mixtures in each ml of semen may be added to semen or contained in semen diluents: (a) a mixture of lincomycin-spectinomycin (150/300 µg), penicillin (500 IU) and streptomycin (500 µg); or (b) a mixture of gentamicin (250 µg), tylosin (50 µg), lincomycin-spectinomycin (150/300 µg); or (c) a mixture of amikacin (75 µg) and divekacin (25 µg)"*

However, the non-therapeutic use of antibiotics in small doses increases the risk of the development of antimicrobial resistance (Gloria *et al*. 2014).

Furthermore, some national regulations regarding the addition of antibiotics to semen samples that would be used within the country do not demand that antibiotics need to be added if the semen would be used for AI soon after cooling on the same premises. However, it is common practice to use antibiotics if the semen samples require transport to other premises for AI.

1.2.3 Bacterial resistance to antibiotics in semen extender and risk for potential spreading

Antimicrobial resistance (AMR) is a pressing issue, and there is ongoing debate among researchers regarding the minimum level of antibiotic exposure required to induce AMR. While some researchers consider that even low levels of exposure can cause AMR (Johansson *et al*. 2004), others contend that even therapeutic concentrations have this effect (Dugassa & Shukuri 2017). However, the contribution of the duration and extent of exposure to resistance induction is widely acknowledged. Despite the presence of antibiotics, bacteria could be cultured from bull (Kilburn *et al*. 2013) and boar semen (Bresciani *et al*. 2014). Gloria *et al*. (2014) observed that gentamicin, tylosin, spectinomycin and lincomycin did not control bacterial growth in bull semen, although no growth occurred in semen samples containing ceftiofur/tylosin or ofloxacin. Another study (Goularte *et al*. 2020) on microorganisms in bull semen reported bacterial resistance to

penicillin, and most bacteria showed resistance to tylosin and lincomycin. Frozen stallion semen containing amikacin in extenders showed bacterial growth after thawing (Guimaraes *et al*. 2015).

The appropriate disposal of antibiotic-containing substances is critical in preventing the spread of AMR (Anwar *et al*. 2020), as incorrect disposal may lead to resistance in environmental bacteria that can subsequently be transferred to humans or animals (Martínez & Rojo 2011). Therefore, ensuring that unused semen extenders and doses are not poured down the drain is crucial. The addition of antibiotics to semen extenders is not aligned with current recommendations for the judicious use of antimicrobial substances given the increasing incidence of AMR (Ungemach *et al*. 2006). Although certain bacteria found in semen have been linked to endometritis, there is no definitive evidence of a therapeutic need for antibiotics in semen extenders, particularly if strict attention is paid to hygiene protocols during the collection and processing of semen (Bennett 1986; OIE 2019).

The transmission of resistance genes between bacteria in different host species is a significant concern, as it can occur both with and without the involvement of environmental bacteria (Young *et al*. 2019). For instance, if AMR develops in bacteria in a host animal, these resistant bacteria may spread to humans. The transmission of diseases and AMR from animals to humans can occur through various routes, with the food-borne route being the most important (Hull *et al*. 2021). However, it is also possible for resistance genes to be transferred from livestock to environmental bacteria and then to humans. Additionally, animal-associated methicillin-resistant *Staphylococcus aureus* (MRSA) can cause infections in humans after transmission (Crespo-Piazuelo & Lawlor 2021). Notably, this transfer is not only “one-way”, and there is evidence of continued resistance in farm animals due to contact with human carriers (Crespo-Piazuelo & Lawlor 2021). Therefore, to protect both animal and human populations, it is advisable to limit the development of AMR in animals. One way to achieve that is to identify bacteria in semen samples, their count and antimicrobial susceptibility to different antibiotics.

1.3 Bacterial identification

Historically, different methods of bacterial identification were developed and used in medical research as well as in clinical work, revealing the

advantages and disadvantages of their use. In addition to accurate bacterial identification, these methods need to be fast, easy to use, and economical. Traditional culture-dependent methods are time-consuming, and not all bacteria can be identified using such methods, because different bacteria demand different conditions for their growth, such as agar, temperature, level of CO_2 etc. A method that is reliable and much quicker than conventional technique used worldwide for identification of bacteria and cover some of these specifications for identification is Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Mass spectrometry based on the MALDI-TOF means that the bacterial isolate is adsorbed to some type of carrier material (matrix). The isolate is then irradiated with laser UV light, so that the molecules in the bacteria are broken into positively charged fragments (ionization), which are thrown towards a detector. The time it takes for the fragment to reach the detector (time of flight) is measured. The time is dependent on fragment size and charge (Croxatto *et al*. 2012). A disadvantage of this method is that (i) it is still necessary to culture bacteria, and (ii) bacterial identification depends on the information provided in the database. Most databases contain data on pathogenic bacteria relevant to veterinary and human medicine (Croxatto *et al*. 2012). This is a limiting factor in general bacteriology, where there is a lack of information about environmental bacteria. In contrast, 16S rRNA sequencing to identify bacterial DNA is capable of identifying both live and dead bacteria even at low levels; no culture is needed, and it is able to provide a higher specific identification (Janda & Abbott 2007). However, identifying non-viable bacteria provides limited information on their possible effect on the tissue organism. On the other hand, 16S rRNA sequencing has low phylogenetic power at the species level and a poor discriminatory power for some genera. However, identifying bacteria by these methods, even at the genus level, can help in choosing an antibiotic with a known narrow spectrum (gram-negative vs. gram-positive bacteria), thereby decreasing the risk of developing AMR by using broad-spectrum antibiotics.

1.4 Alternatives to antibiotics in semen extender

1.4.1 Plant based substances

Throughout history, mankind has used plants for medical purposes for treating wounds and burns (Guerriero *et al*. 2018). This knowledge is the basis for the development of modern medicine and pharmacology, and plant extracts have been used as a basis and source for many drugs. Plant secondary metabolites are the main compounds responsible for the pharmacological activity. Based on their properties, the main groups of plant secondary metabolites used in medicine are alkaloids, phenolic compounds, and terpenoids. Some of the properties linked to these compounds are antioxidant, antimicrobial, antiviral, antitumor, analgesic, anti-inflammatory, antimutagenic, and antiparasitic characteristics. Ros-Santaella & Pintus (2021) reviewed 45 plant species that affect sperm function during semen storage. The authors also described the possible toxic effects and antimicrobial properties of plant extracts. Specifically for semen samples, different plants have shown positive effects on sperm morphology, motility, plasma membrane and acrosome integrity, antioxidant activity, reduced lipid peroxidation, mitochondrial activity, DNA integrity, viability, and fertilization ability. Only Sickle-leaved albizia (*Albizia harveyi*) showed positive effects on all bull sperm parameters (Sobeh *et al*. 2017); however, pomegranate (*Punica granatum*) had a positive effect on 7 of 10 bull sperm parameters. Furthermore, *Albizia harveyi* did not show any toxicity, whereas the toxic effect of *Punica granatum* was dose-dependent. However, the antimicrobial properties of these plants have not yet been evaluated.

In addition to evaluating the topical effect, the ideal property for potential antibacterial ingredients in extenders used for sperm cryopreservation is that they should have a broad antibacterial spectrum. Furthermore, sperm additives should not interfere with fertility or evoke antibacterial resistance, should have high stability and activity potential at low storage temperatures, and should be economical and easy to apply (Schulze *et al*. 2016; Schulze *et al*. 2020). Because killed bacteria remain in the sperm suspension, their intracellular contents and/or cell wall substances can have negative effects on sperm cells. If these requirements cannot be fulfilled, it may be better to remove and/or reduce the bacteria in semen samples.

1.4.2 Colloid centrifugation

Nicholson *et al*. (2000), conducted a study in which they were able to improve sperm quality and reduce bacteria in human sperm samples using density centrifugation. In veterinary medicine, an adapted method using only one layer of colloid, named "Single Layer Centrifugation" (SLC), has been used for bacterial reduction in boar (Morrell & Wallgren 2011a), stallion (Al-Kass *et al*. 2019), and dog (Luño *et al*. 2020) semen samples. Apart from the effective reduction of bacteria, SLC improved sperm quality in these species (Johannisson *et al*. 2009; Morrell & Wallgren 2011b; Dorado *et al*. 2013), but also in buck (Jiménez-Rabadán *et al*. 2012) and bull (Goodla *et al*. 2014). Initially, the SLC consisted of processing a small amount of semen samples in 15 mL tubes (Morrell & Wallgren 2011b), but subsequently the method was extended to process larger volumes of semen, such as 50 mL tubes for stallion semen samples (Morrell & Nunes 2018) and 500 mL tubes for boar semen samples (Morrell *et al*. 2011). Furthermore, a scaled-up version of SLC was modified by inclusion of a 5 mL plastic semen straw, inserted through a hole in the cap of a 50 mL tube before loading the semen on the colloid, to facilitate retrieval of the sperm pellet after centrifugation without re-contamination. A study on stallion semen samples using this method (Morrell *et al*. 2014) resulted in removal of 68% to 100% of the bacteria, compared to the original method, which reported a bacterial removal rate of 93% (Varela *et al*. 2018).

Based on the study by Al-Kass *et al*. (2019), it might be unnecessary to add antibiotics to stallion semen samples if they can be processed using SLC. There are three reasons for this: (i) SLC could remove most of the bacteria from semen samples; (ii) the remaining bacteria in these samples did not have a negative effect on sperm quality parameters during the storage period; and (iii) adding antibiotics did not improve sperm quality. In fact, the sperm DNA fragmentation index was actually increased in samples containing antibiotics whereas DNA integrity was retained in the corresponding samples without antibiotics.

Although these studies on removal of bacteria from stallion, boar and dog semen using SLC are interesting, as yet no studies on the possible use of SLC for bacterial removal from bull semen samples have been performed.

2. Aims

The general aim of this thesis was to investigate the seminal microbiota of healthy bulls and to find alternatives to antibiotics in semen extenders.

Study I: To characterise bull semen microflora from raw semen and determine possible links to fertility

Study II: To investigate one of the factors potentially influencing bacteria in bull semen, i.e. season of year

Study III: To investigate antibacterial effect of pomegranate, curcumin and ginger on bacteria in bull semen and on sperm quality

Study IV: To evaluate removal of bacteria by colloid centrifugation and the effect of residual bacteria on bull sperm quality.

3. Materials and methods

A general review of material and methods is described in this section. For more information on specific studies, please see papers I-IV.

3.1 Study design

The studies for this thesis took place at the Swedish University of Agricultural Science (SLU), SciLifeLab, SNP&SEQ Technology Platform, Uppsala University and SciLifeLab in Stockholm, Sweden.

Study I: Metagenomic analysis was used to identify bacteria in bull semen from 18 Holstein bulls housed in Estonian AICs. Semen samples were split; one part was used for bacteriology analyses (DNA extraction and 16S rRNA sequencing), while the remaining part was processed and used for artificial insemination and evaluation of fertility performance (Figure 2.).

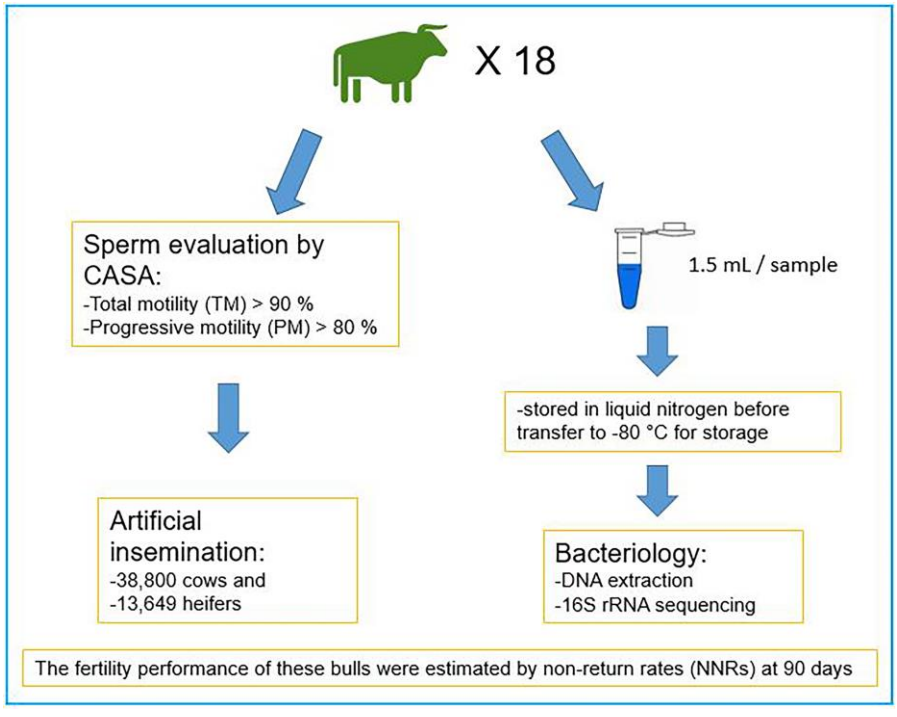

Figure 2. Experimental design for Study I; in total, semen samples from 18 bulls were used for identification of semen microbiota by 16S rRNA sequencing and to evaluate fertility potential.

Study II: Semen was collected from 18 Swedish Red dairy bulls housed at Viking Genetics bull stud (Skara) in Sweden in the period 2010 to 2012. Straws of frozen semen were selected from each bull in three consecutive seasons (winter, spring and summer), one straw per season. Each straw was used for bacterial identification by 16S rRNA sequencing and for evaluation of sperm quality by Computer Assisted Sperm Analyses (CASA) and Flow Cytometry (FC) (Figure 3).

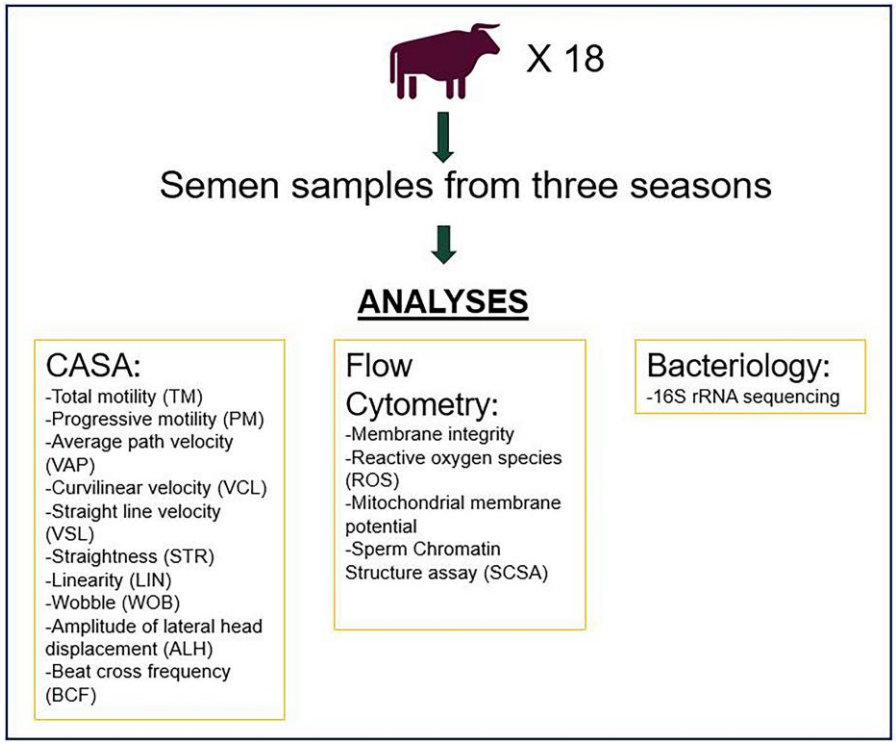

Figure 3. Experimental design for Study II; Semen samples from 18 bulls, collected in three seasons (Winter, Spring and Summer), were used for bacteriological identification by 16S rRNA sequencing, with sperm quality evaluation by CASA and Flow cytometry.

Study III: The antimicrobial effect of pomegranate powder, ginger, and curcumin extract in two concentrations was tested on the microbiota in bull semen after exposure for <2 h and 24 h. In total 30 ejaculates were used (3 ejaculates per bull). The effect of these substances on sperm quality parameters was evaluated by CASA and FC (Figure 4).

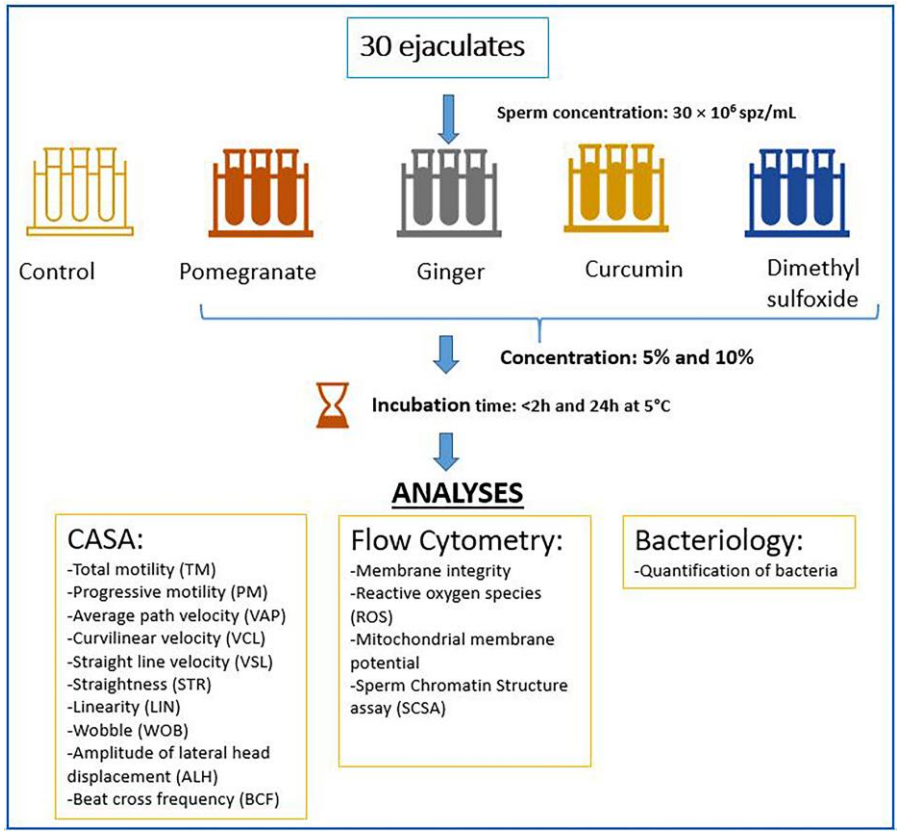

Figure 4. Experimental design for Study III; Incubation time and analyses after adding various plant-based substances to bull semen 24 h after collection. In total, semen samples from 10 bulls were used (three ejaculates per bull).

Study IV: Two densities of colloid for SLC density (high and low) were used to evaluate their influence on bacterial reduction in bull semen. Bacterial culture was performed, followed by bacterial identification using MALDI-TOF MS (Figure 5).

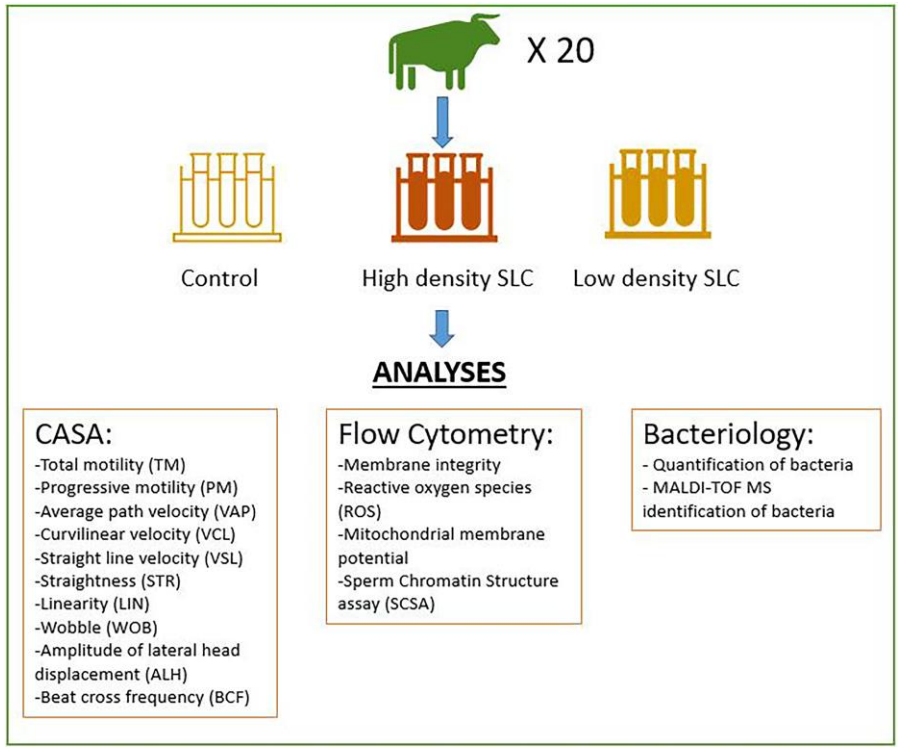

Figure 5. Experimental design for Study IV; Effect of Single Layer Centrifugation of bull semen through a colloid on bacterial count and sperm quality. Two densities of colloid were compared (high and low). In total, semen sample from 20 bulls were used.

3.2 Ethical approval

Study I: Semen collection with an artificial vagina is a routine agricultural practice and, therefore, does not require ethical approval according to Estonian law (Teataja). Therefore, no ethical permission was required for the study.

Study II: Commercial semen samples were used, kindly donated by Viking Genetics (Skara, Sweden).

Study III and IV: No ethical approval is needed in Sweden for bull semen collection using an artificial vagina. The bulls were housed at a commercial stud in Sweden according to standard husbandry conditions for this species.

3.3 Animals and samples

Study I: Semen samples were collected from 18 Holstein bulls at the Animal Breeders' Association of Estonia, Raplamaa, Estonia, where animals were kept according to national and international regulations. The age of the bulls ranged from 3 to 10 years. The animals were prepared for semen collection by allowing them to follow each other in a circular chute for 30–40 min, and to make false mounts by jumping on the bull in front to increase libido, before a sterilized artificial vagina with a sterilized graduated collecting tube was used for the semen collection. Within a minute after collection, collected ejaculates were passed to the laboratory for further processing. All semen collection procedures were performed with sterile equipment and aseptic measures to avoid semen contamination.

Study II: For this study, commercial semen doses were used. Ejaculates were collected from 18 Swedish Red dairy bulls, aged between four and seven years old, housed at Viking Genetics bull stud (Skara) in the period 2010 to 2012. The straws were thawed in a water-bath, at 37 °C for 15 s before further analyses were performed.

Study III and IV: Ejaculates were collected from dairy bulls (Swedish red and Holstein Frisian), aged 1 to 4 years old, housed at Viking Genetics, Skara, Sweden, using an artificial vagina according to the standard husbandry method. The semen was extended 1:1 in Andromed extender free of antibiotics (AndroMed® CSS one-step 200mL, Minitüb GmbH, Tiefenbach, Germany), before transportation to the Swedish University of Agricultural Sciences (SLU), overnight at 6 °C, in an insulated box containing a cold pack. For study III, semen samples from 10 dairy bulls were collected twice a week during October-November 2021. In total, 30 ejaculates were used in this study (three ejaculates per bull). For study IV, 20 dairy bulls were used for semen collection, three times during one week from each bull (n=60 ejaculates in total).

3.4 Preparation of Pomegranate powder, Curcumin and Ginger extract

Study III: The ginger extract and curcumin used in this study were obtained from Sigma-Aldrich GmbH (Stockholm, Sweden), while the pomegranate powder was commercially available from WellAware Granatäpple (Uppsala, Sweden). The test substances were prepared

according to the described protocol (Paper III) and added to diluted semen samples to achieve a final proportion of 10% and 5%. The solvents for pomegranate, curcumin and ginger were peptone water, ethanol (70%) and Dimethyl sulfoxide (DMSO), respectively.

3.5 Colloid centrifugation

Study IV: Colloid centrifugation was carried out according to Goodla *et al*. (2014), using Bovicoll of density 1.104 g/mL (High) or 1.0325 g/mL (Low). Briefly, the sperm concentration in the semen samples was adjusted to 50×10^6/mL for SLC, with 15 mL of sample being layered on top of 15 mL of Bovicoll in a centrifuge tube. After centrifugation at 300 g for 20 min at ambient temperature (approximately 23 °C), the supernatant was removed and the sperm pellet was resuspended in fresh AndroMed without antibiotics.

3.6 Semen quality evaluation

3.6.1 Sperm concentration

Study I, III and IV: Sperm concentration was evaluated using a Nucleocounter-SP 100 (ChemoMetec, Allerød, Denmark) according to the manufacturer´s instructions.

Study II: The sperm concentration was adjusted to 69×10^6 spermatozoa per mL (spz/mL) during preparation of the commercial semen doses at the bull stud.

3.6.2 Computer assisted sperm analysis (CASA)

Study II, III and IV: Sperm motility parameters were evaluated using a SpermVision analyzer (Minitüb GmbH, Tiefenbach, Germany) connected to a Zeiss microscope with a heated stage (38 °C). Semen samples were equilibrated to room temperature before motility analysis. Sperm motility was analyzed in eight fields (at least 850 spermatozoa in total) using the SpermVision software program version 3.8, with settings adjusted for bull spermatozoa. A 5-µL aliquot of the semen sample was placed on a warm slide, covered with an 18 × 18 mm cover slip (VMR, Leuven, Belgium) and the following parameters were analyzed: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), average path velocity

(VAP, μm/s), straight line velocity (VSL, μm/s), linearity (LIN, as VSL/VCL), straightness (STR, as VSL/VAP), wobble (WOB, as VAP/VCL), beat cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, μm). Images were obtained at 200 × magnification using a phase contrast microscope. Particles with an area ranging from 20 to 100 μm^2 were identified as sperm cells and were included in the analysis. Spermatozoa were considered as immotile if the area under curve (AOC) was < 5, BCF < 0.2, and VSL < 0.2; they were considered to be locally motile if the straight-line distance (DSL) covered was < 4.5 μm.

3.6.3 Flow cytometry

Flow cytometry (FC) analysis was performed using a FACSVerse™ flow cytometer (BD Biosciences, Becton Dickinson and Company, San Jose, CA, USA). The lasers used to excite the fluorescent stains were a blue laser emitting at 488 nm and a violet laser emitting at 405 nm. Green fluorescence (FL1) was detected with a band-pass filter (527/32 nm), as was orange fluorescence (FL2, 586/42 nm); red fluorescence (FL3) was collected using a 700/54 nm band-pass filter, while blue fluorescence (FL5) was collected using a 528/45 nm band-pass filter. The data obtained were further analyzed using FCS Express 5 software (De Novo, Glendale, CA, USA). Aliquots of samples were diluted to a sperm concentration of approximately 2 million spermatozoa/mL with 300 μL of buffer B. The SCSA samples were prepared separately (see below).

3.6.4 Sperm membrane integrity (MI)

Study II and III: Assessment of MI was carried out after staining with SYBR14 and propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA). In this assay, PI can only penetrate damaged sperm membranes, whereas SYBR14 can pass into all sperm cells. The SYBR14 dye was diluted 50 times with Buffer B and 1.2 μL was added to each sperm sample. The sperm samples were also stained with 3 μL of 2.4 mM PI followed by incubation for 10 min at 37 ºC. For study III, aliquots were stained and evaluated at <2h and 24h after the semen arrived at SLU.

3.6.5 Reactive Oxygen Species (ROS)

Study II and III: The stains used to assess ROS were (i) Hydroethidine (HE; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) to detect superoxide ($SO^{\cdot}O_2^{-}$), (ii) 2`,7` -dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) for hydrogen peroxide (H_2O_2), while (iii) Hoechst 33258 (HO) was used to permit the simultaneous differentiation of living and dead cells. Aliquots (300 µL) of semen extended to a concentration of approximately 2×10^6 spz/mL with Buffer B were stained with 3 µL of HO (40 mM), 3 µL HE (40 mM) and 3 µLDCFDA, (2 mM). The samples were gently mixed and incubated at 38 °C for 30 min before analysis. Using the dot-plots for HO/HE and HO/DCFDA fluorescence, the following populations were quantified: ROS Live SO^{-} ; ROS Live SO^{+}; ROS Dead SO^{+}; ROS Dead $H_2O_2^{-}$; ROS Dead $H_2O_2^{+}$; ROS Live $H_2O_2^{-}$ and ROS Live $H_2O_2^{+}$. For study III, aliquots were stained and evaluated at two time points.

3.6.6 Mitochondrial membrane potential (MMP)

Study II and III: Assessment of MMP was done by adding 1.2 µL of tetraethylbenzimidazolylcarbocyanine iodide (JC-1 stock 3 mM) to 300 µL sperm aliquots and incubating for at least 30 min at 38 °C before spermatozoa with high and low MMP were quantified.

3.6.7 Sperm Chromatin Structure Assay (SCSA)

Study II, III and IV: SCSA was used to evaluate sperm chromatin integrity using acridine orange (AO) metachromatic dye. The sperm concentration of the samples was adjusted to approximately 2 million spermatozoa/mL by mixing aliquots (50 µL) with buffer containing 0.01 M Tris-HCl, 0.15 M sodium chloride and 1 mM EDTA (pH 7.4; TNE buffer). After snap-freezing in liquid nitrogen, the samples were stored at -80 °C until analysis. On the day of analysis they were thawed on ice and an aliquot (10 µL) was mixed with 90 µL of TNE, and 200 µL of acid-detergent solution. Exactly 30 s later, the sample was stained by adding 600 µL of AO staining solution, and %DFI was analyzed within 3–5 min.

3.7 Bacteriological analyses

3.7.1 Metagenomic analyses

DNA extraction

Study I and II: The DNA extraction was performed in the Clinical Sciences Research Laboratory at SLU using an AllPrep DNA/RNA/miRNA Universal Kit Cat No./ID 80224 (GIAGE, Germantown, Philadelphia, USA) following the manufacturer´s instructions for the simultaneous purification of genomic DNA and total RNA, including miRNA from cells. For Study I, the purity and concentration of the DNA were tested using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham (HQ), MA, USA). The DNA purity was considered adequate when the 260/280 ratio was between 1.7 and 1.9 and the concentration was between 5.34 and 10.97 ng/µL. The DNA samples were stored at −80 °C until further preparation. For Study II the purity and concentration of the DNA was tested using Qubit 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, Eugene, Oregon, USA) with the Quantitation range of 0.1 – 120ng. The DNA samples were stored at -80°C until further preparation. Before sending for 16S rRNA amplification and sequencing, all semen samples were adjusted to 0.4 ng/µL of DNA with elution buffer.

16S rRNA amplification and sequencing

Study I and II: A two-step amplification protocol was used for the preparation of the V3–V4 16S gene region for Illumina sequencing. The details of the primers used and the cycling protocols are presented in Table 1.

The reaction mix for the first PCR in Study I consisted of 4 ng of the DNA template, 0.25 µM Pro 341F and the same volume of Pro 805R primers as well as 1 µg/µL BSA and 1 × Phusion Taq ready-to-use mix. For Study II the reaction mix contained the same substances with the addition of 0.5 µL DMSO in the PCR reaction mix. More details about the second PCR reaction can be found in Table 1 and Papers I and II.

Table 1. Primer combination and thermal cycling conditions used to quantify the 16S rRNA.

	Illumina Primer 16S rRNA	**Sequences (5'-3')**	**Terminal Cycling**
Study I	341F	CCTACGGGAGGCAGCAG	(98 °C 3 min); (98 °C 30 s, 55 °C 30 s, 72 °C 40 s) × 25; 72 °C 10 min; 10 °C hold
	805R	GACTACNVGGGTATCTAATCC	
Study II	341F	ACACTCTTTCCCTACACGACGCTCTTCCG ATCTCCTACGGGNGGCWGCAG	(98 °C 3 min); (98 °C 30 s, 55 °C 30 s, 72 °C 45 s) × 8; 72 °C 5 min; 10 °C hold
	805R	GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTGACTACHVGGGTATCTAATCC	

16S Profiling

Study I and II: The analysis of the 16S rRNA sequencing data was performed using Nextflow pipeline ampliseq v.1.1.2 (https://github.com/nf-core/ampliseq). Briefly, the raw sequencing was quality checked initially, followed by the trimming of the primer sequences from the reads. The raw sequencing data were cleaned from source contamination and then denoised, dereplicated and filtered for chimeric sequences. The denoised paired-end reads were truncated from position 229 (forward) and 215 (reverse) and were merged with at least a 20 bp overlap, resulting in exact amplicon sequence variants (ASVs). These ASVs were taxonomically classified from the phylum to species level, and clustered following removal of the ASVs classified as mitochondria or a chloroplast in origin. Only the ASVs with a minimum read frequency ≥5 in at least one sample were retained for further analysis. For more details about methodology see Paper I and Paper II.

3.7.2 Bacterial Quantification

Study III and IV: The total number of viable aerobic bacteria in the semen samples was analyzed according to NMKL 86, 5 Ed., 2013, with slight modifications. In brief, each sperm sample was diluted 1:1 (v/). In Study III, a 2-fold serial dilution was prepared until 1/4 of the sperm sample was obtained. In Study IV, non-serial dilution was done as follows: 1 mL of diluted sample was pour plated into a petri dish and mixed with melted plate count agar (PCA). Each sample was evaluated in duplicate. The total number of CFU was calculated using a colony counter (Gerber instruments, Im Langhag, Switzerland) and the number of bacteria were calculated according to ISO 7218:2007. Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations.

3.7.3 MALDI-TOF MS bacterial identification

Study IV: For bacterial identification, 1 mL of sperm sample from each treatment group (A, B and C) was mixed with the same amount of peptone diluent. Then, 1 mL of this mix was transferred to cattle blood agar plates which were incubated at 37 ± 1 °C and examined for bacterial growth after 24 hours. Bacterial colonies of different macromorphologies were re-cultured on new blood agar plates and incubated in similar conditions. The

colonies from the pure culture were then identified at the species level by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Billerica, MA, USA). Score values between 2.0 and 3.0 were considered accurate at both genus and species levels, whereas score values between 1.7 and 2.0 were considered reliable only at the genus level.

3.8 Statistical analyses

Study I and II: The data analysis was performed using R v.3.3.1 software. For the microbiome data, Pearson correlations were calculated between ASVs (Study I and II), A linear discriminant analysis effect size (LEfSe) analysis of the microbial abundance between two fertility bull groups (Study I), differential abundance of ASVs was calculated with DESeq2 v 1.38.3 (Love *et al*. 2014) using Wald test (Study II). For the sperm quality parameters (Study II), analysis of variance was used; the statistical model included the fixed effect of season and the random effect of bull.

Study III and IV: The statistical program IBM Statistic SPSS 26 was used. Differences in CASA parameters, FC results between groups and bacterial count between ejaculates were analysed using different parametric (MANOVA in Study III, Two-way ANOVA in Study IV) and non-parametric tests (Kruskal Wallis test in Study IV). Descriptive statistics were used to present bacteriological results in both studies.

4. Results

The results for Studies I – IV are presented in full in the published articles or submitted manuscripts, and summarised as follow:

4.1 Study I – Bull semen microbiota and fertility

In total, 107 bacterial genera were identified in 18 bull semen samples. The 20 most frequently seen bacterial genera, starting with the most frequent, were: *Porphyromonas, Fusobacterium, Ruminococcaceae UCG-010, Fastidiosipila, Ruminococcaceae UCG-005, Cutibacterium, Histophilus, Oceanivirga, Corynebacterium, Campylobacter, W5053, Dyella, Staphylococcus, Lawsonella, Helcococcus, Bacteroides, Capnocytophaga, Curvibacter, Kingella* and *Enhydrobacter*. The bacterial taxa enumerated in the top 20 for each bull displayed substantial heterogeneity. Furthermore, there were marked disparities among bulls with respect to the number of the bacterial genera detected (ranging from 12 to 89) and bacterial abundance (ranging from 27,827 to 247,273).

There were negative correlations between several bacterial genera (Figure 5), particularly among the top 20 genera. In addition, *Curvibacter, Rikenellaceae* RC9-gut-group and *Dyella* spp. (brown spots) were negatively correlated in most cases.

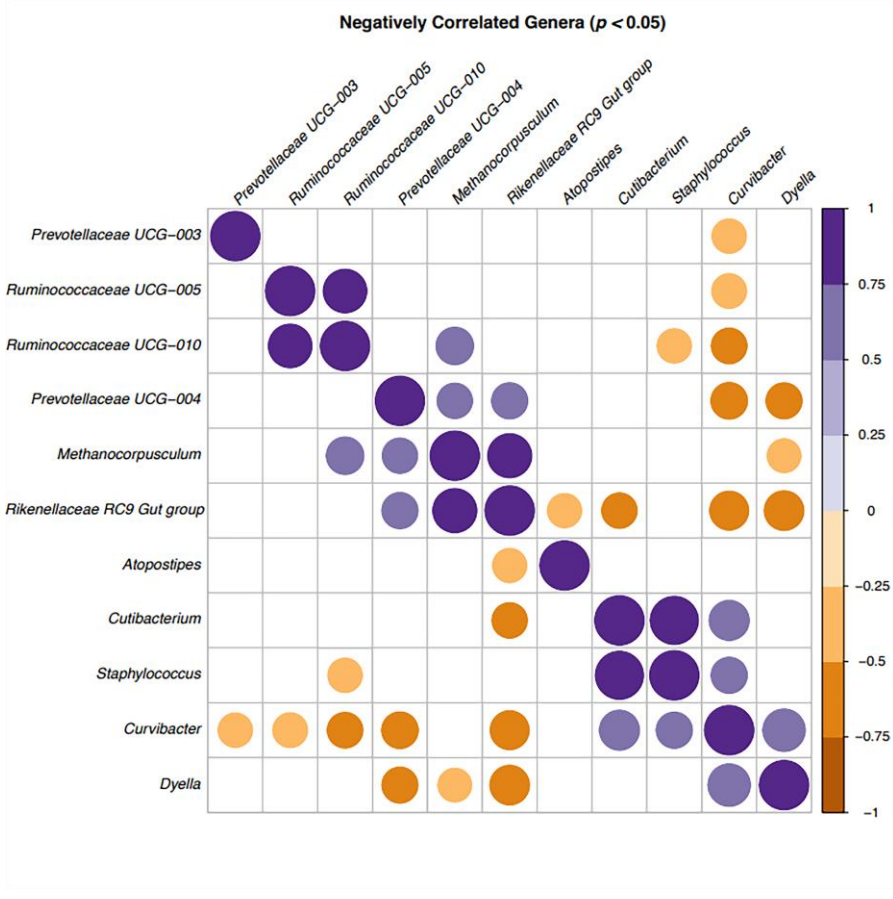

Figure 6. Correlation matrix of the bacteria in bull semen identified by 16S rRNA sequencing showing the significant correlations ($p < 0.05$) between genera: positive (purple) and negative (brown). Blank cells indicate non-significant correlations (Cojkic *et al*. 2021).

The bar plot displayed in Figure 7 show the taxa comprising both genus and family levels, which exhibited noteworthy dissimilarities between the fertility groups. The identification of such taxa was facilitated by a linear discriminant analysis effect size (LEfSe) analysis, utilizing a log-10 transformed linear discriminant analysis (LDA) threshold score of 2.0 and a significant p-value <0.05. The amplicon sequence variant (ASV), characterized by a higher LDA score, was deemed to be more crucial, according to LEfSe, in distinguishing between low and high individuals. Notably, two genera, *W5053* and *Lawsonella*, were more abundant in the low fertility group.

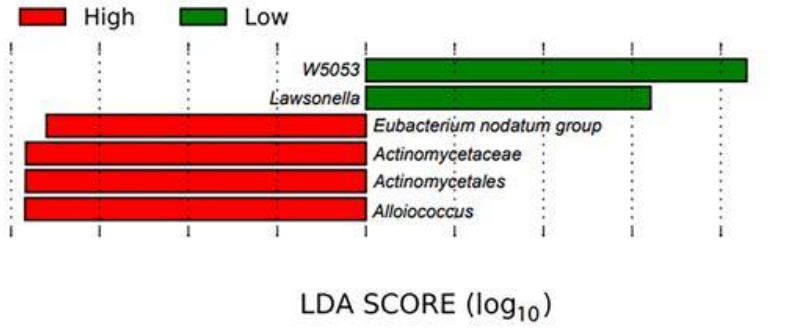

Figure 7. Linear discriminant analysis effect size plot. This plot shows the enriched taxa that were significantly different between the high fertility (red) and low fertility (green) bulls (Cojkic *et al.* 2021).

4.2 Study II – seasonal effect on bull semen microbiota and sperm quality

4.2.1 16S rRNA sequencing results

A total of 1,729 ASVs were identified across all the samples. In total, 28 phyla and 388 genera were identified in the 54 bull semen samples in three seasons. In winter, spring and summer, 20, 21, 20 phyla and 234, 211, 233 genera, respectively were identified. The six phyla that were consistently detected across all seasons are shown in Table 2. Notably, a greater predominance of the phylum Proteobacteria (now named Pseudomonadota) (64.85%) was observed in summer than in winter (58.03%) or spring (49.94%). Conversely, Firmicutes (now named Bacillota) displayed a lower abundance in summer (19.94%) than in winter (32.42%) and spring (32.00%). The phyla Actinobacteriota, Bacteroidota, Campylobacterota, and Fusobacteriota demonstrated a relatively uniform distribution among the seasons. However, the phylum Patescibacteria (now named Candidatus) was present in both winter (0.31%) and summer (0.56%), while Acidobacteriota was only detected in spring (0.39%).

Only nine bacterial genera were present in all seasons (Table 2), and evident differences in the relative abundance of bacterial genera were discernible throughout all seasons. The genus *Acidocella*, identified as the most abundant, displayed a similar distribution in winter (28.28%) and

spring (27.22%) compared to summer (19.32%). *Bacillus* demonstrated a comparable distribution in spring (3.19%) and summer (2.79%), in contrast to winter (10.39%). Conversely, *Histophilus* was present at a lower abundance in winter (2.03%) than in spring (6.97%) or summer (6.10%). Nevertheless, *Burkholderia* was found to be relatively uniform in abundance across winter (12.40%) and spring (12.27%) compared to summer (7.98%). Notably, the presence of *Streptococcus* varied significantly among seasons. The remaining bacterial genera, *Campylobacter*, *Geobacillus*, *Lactobacillus*, and *Pseudomonas*, showed similar levels of abundance (<3%) throughout all seasons.

Table 2. Phylum and Genera present in all seasons.

	Winter	**Spring**	**Summer**
	Phylum (%, n=6)		
Proteobacteria	58.03	49.94	64.85
Firmicutes	32.42	32.00	19.94
Actinobacteriota	2.56	4.09	4.84
Bacteroidota	2.92	6.09	4.83
Campylobacterota	1.22	2.40	1.32
Fusobacteriota	1.71	4.09	2.75
	Genus (%, n=9)		
Acidocella	28.28	19.32	27.22
Bacillus	10.39	2.72	3.19
Burkholderia	12.40	7.98	12.27
Campylobacter	1.22	2.40	1.27
Geobacillus	2.28	2.99	1.27
Histophilus	2.03	6.10	6.97
Lactobacillus	1.97	1.56	1.12
Pseudomonas	1.68	2.42	1.11
Streptococcus	6.60	4.08	3.87

* *n* denotes number of samples

The correlations between bacteria varied across different seasons. There was a negative correlation between bacteria during winter and summer, but not during spring. The phylum Proteobacteria was the sole bacterium found to exhibit a negative correlation with Firmicutes (winter) and Actinobacteriota (summer). However, in spring, Proteobacteria did not exhibit any correlation with other bacterial genera. Furthermore, positive correlations between bacteria showed differences across seasons.

4.2.2 Sperm quality results

The sperm CASA results during the three seasons are presented in Paper II. A trend towards significance was observed for VCL (P=0.08) between winter and summer, as well as for LIN (P=0.09) between spring and summer. The remaining kinematic parameters did not exhibit significant seasonal differences. For the Flow cytometry parameters, the mean proportion of spermatozoa with high respiratory activity showed seasonal variation: with 43.55% in winter, 41.18% in spring, and 49.92% in summer. A trend towards significance (P=0.09) was observed between spring and summer. However, the mean values for spermatozoa with low respiratory activity, living, dying, and dead spermatozoa and %DFI displayed no significant seasonal differences. The mean values of the ROS production during the three seasons are presented in detail in Paper II. Only live H_2O_2 positive sperm cells showed significant differences (P = 0.05) between winter and spring, as well as between spring and summer (P < 0.05) (Figure 8). The remaining ROS subpopulations did not show any significant seasonal variations.

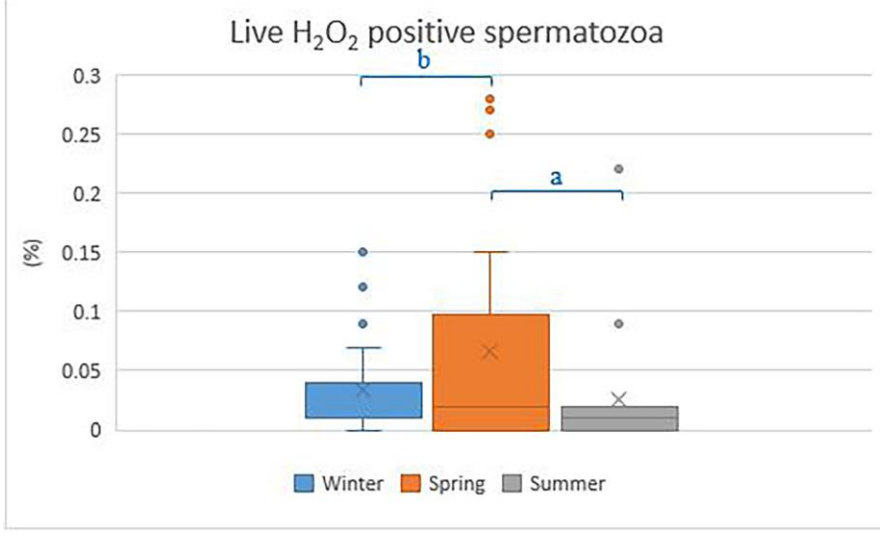

Figure 8. Flow cytometry results of Live Hydrogen peroxide (H_2O_2) producing spermatozoa in different seasons (winter, spring and summer). Letters denote significant differences between groups of sample: a—p < 0.05; b—p = 0.05

4.3 Study III – Plant-based substances and bull semen, effect on bacteria and sperm quality

4.3.1 Sperm quality analyses

Sperm kinematics were evaluated by CASA while sperm viability was analysed by Flow cytometry.

CASA - Computer-assisted sperm analysis

At Time 1 (< 2h), the addition of Pomegranate (P) 5% did not significantly impact on the CASA parameters. However, the administration of Ginger (G) 10%, and Dimethylsulfoxide (D) 10% and 5%, resulted in a significant negative effect on CASA parameters. Concentrations of both Curcumin (C) and Pomegranate (P) 10% had a negative effect on some kinematic parameters, although the proportion of motile sperm remained unchanged. After 24 h of incubation (Time 2), both Pomegranate samples (10% and 5%) and Ginger (G) 10% demonstrated a decrease in CASA parameter values compared to the control. On the other hand, the remaining substances did not negatively influence CASA parameters; in fact, Curcumin (C) 5% was associated with an increase in the values of most CASA parameters after 24 hours of exposure.

Flow cytometry

Substances Ginger (G) 10% and 5%, Dimethylsulfoxide (D) 10% and 5% had a negative influence on all sperm quality parameters except for chromatin integrity at Time 1. However, Pomegranate (P) 10% exhibited a negative influence on mitochondrial membrane potential and chromatin integrity, while Pomegranate (P) 5% had a negative impact only on chromatin integrity. However, Curcumin 10% and 5% did not have any deleterious effects on sperm quality. Similar results were obtained after 24 h (Time 2) of exposure.

Quantification of bacteria

Considerable variation in the number of viable aerobic bacteria (CFU/mL) was noted between individual bulls. However, the majority (80%) of the semen samples exhibited an average bacterial count lower than 100 CFU/mL before exposure to the various plant substances. A reduction in CFU was commonly observed across all animals with increased exposure

time. Furthermore, a decrease in the number of CFU was observed for all tested substances, including the control group, during exposure (Table 3).

At Time 1, the most significant reduction in bacterial count was observed after introducing Dimethylsulfoxide (DMSO) 5% (54%) and Ginger (G) 10% (42%). Conversely, both concentrations of Pomegranate (P) and Curcumin 10% demonstrated the lowest bacterial reduction. A similar trend was observed at Time 2.

Table 3. Number of bacteria (CFU/mL) in the semen before (control) and after exposure of different substances at Time 1 and Time 2 of incubation at 5 °C (Cojkic *et al*. 2023).

Substances	Time 1 Mean (Range) Log CFU/mL	Time 1 Reduction[a] CFU/mL (%)	Time 2 Mean (Range) Log CFU/mL	Time 2 Reduction[a] CFU/mL (%)	Reduction Time 1 vs Time 2 CFU/mL (%)
Control	2.27 (0.3 – 3.2)		2.00 (0.0 – 3.1)		46%
P 5%	2.16 (0.0 – 3.3)	22%	2.01 (0.0 – 3.3)	-3%	28%
P 10%	2.15 (0.0 – 3.1)	25%	1.98 (0.0 – 3.2)	5%	31%
G 5%	2.12 (0.0 – 3.2)	29%	1.91 (0.0 – 3.0)	20%	39%
G 10%	2.03 (0.0 – 3.2)	42%	1.84 (0.0 – 3.0)	31%	35%
C 5%	2.10 (0.0 – 3.2)	32%	1.91 (0.0 – 3.1)	20%	37%
C 10%	2.18 (0.0 – 3.3)	20%	1.98 (0.0 – 3.2)	6%	37%
D 5%	1.93 (0.0 – 3.1)	54%	1.79 (0.0 – 2.9)	38%	27%
D 10%	2.13 (0.0 – 3.1)	28%	1.74 (0.0 – 2.9)	46%	59%

Abbreviation: P –pomegranate powder, G – ginger, C – curcumin, D - dimethyl sulfoxide, CFU - Colony forming units; a Note. The percentage reduction between control and individual tested substances is calculated on the absolute values and not on the log-transformed values and presented in columns Time 1 Reduction and Time 2 Reduction. Bacterial reduction during storage is calculated on the absolute values within the row; presented in column Reduction Time 1 vs Time 2.

4.4 Study IV – Use of Single Layer Centrifugation (SLC) in bacterial removal from bull semen

4.4.1 Bacteriology

In total, 49 bacterial species were identified. Only 8 bacterial species appeared in all ejaculates (*Alkalihalobacillus clausii, Bacillus licheniformis, Bacillus pumilus, Corynebacterium xerosis, Enterobacter cloacae, Micrococcus luteus, Proteus mirabilis* and *Staphylococcus sciuri*) while 11, 10 and 9 species were identified on only one occasion, i.e. first, second or third ejaculate, respectively. There was variability in the total bacterial count across ejaculates. The highest count of different macromorphological bacterial colonies was present in the first ejaculate (n=90) collected after a 96-hour period of abstinence, followed by the second (n=75) and third (n=73) ejaculates, which had shorter periods of abstinence (48 and 24 hours, respectively) (Figure 9). Moreover, the total number of species varied between the groups. However, the number of bacteria categorized under "no identification possible" increased with a decrease in the period of abstinence and with the number of species that could be identified (Figure 9).

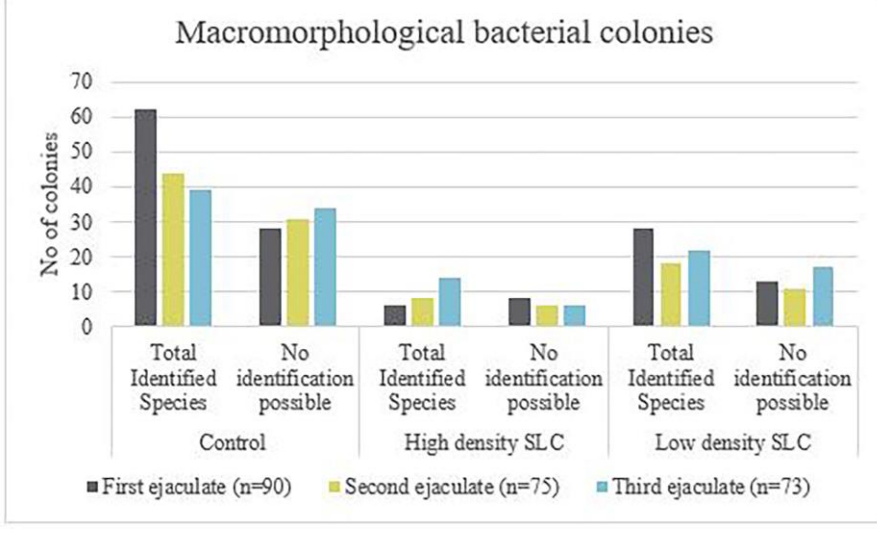

Figure 9. The number of bacterial species identified in bull semen samples by MALDI-TOF MS in different ejaculates and different treatment groups.

Furthermore, variation in the count of different macromorphological bacterial colonies was noted both among bulls and within ejaculates from the same bull (Figure 10).

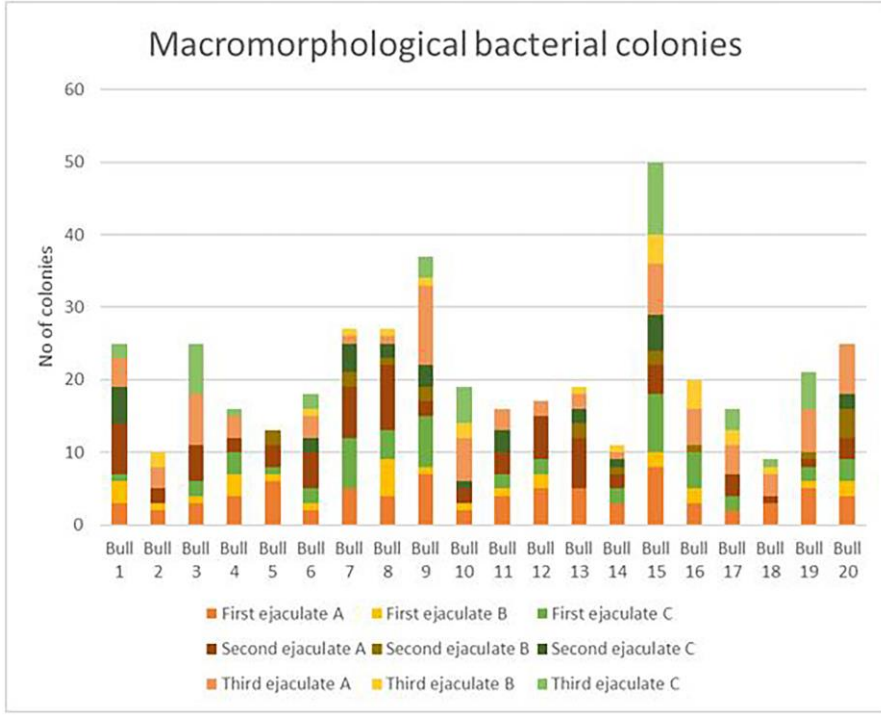

Figure 10. Number of macromorphologically different bacterial colonies between bulls and ejaculates.

A decrease in bacterial count was observed between the Control group (A) and the High (B) and Low (C) density SLC groups, with the High density colloid being effective in removing nearly all *Bacillus* spp. and *Proteus* spp. (Figure 11).

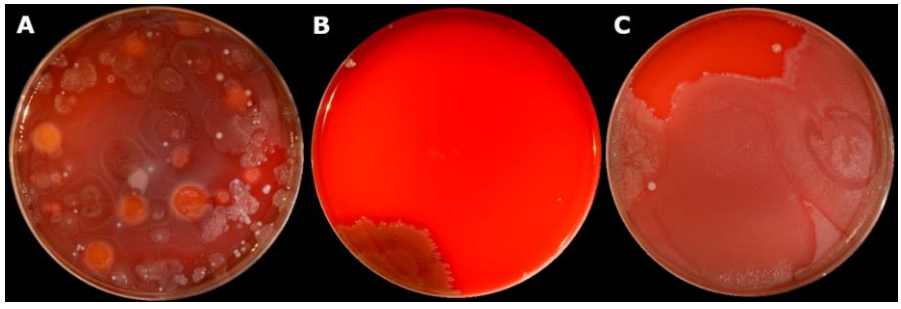

Figure 11. Bacterial growth from bull semen samples on blood agar plates. A represents a Control semen sample, while B and C are sperm samples after High and Low Colloid centrifugation, respectively.

Differences in bacterial count were evident among all groups. The High density SLC group exhibited the lowest bacterial count, with a subset of samples displaying no bacterial growth. However, the number of viable aerobic bacteria (CFU/mL) was low from the beginning; 90% of the bulls had an average bacterial count lower than 200 CFU/mL. Furthermore, the bacterial count (Log CFU/mL) did not differ between first, second and third ejaculates.

4.4.2 Sperm quality parameters

There were significant differences in almost all CASA parameters between groups. A significant difference in straightness (STR) and wobble (WOB) was observed between the Control and High density colloid SLC group. However, there were no differences in linear velocity (LIN) across treatments. Furthermore, the DNA fragmentation index was lower in both colloid groups compared with control, while no significant differences were observed between High and Low SLC samples (Table 4).

Table 4. CASA and Flow cytometry parameters of bull semen samples after colloid centrifugation (Mean ± SD)

	Control	High density SLC	Low density SLC
	CASA (n = 6)		
TM	40.39 ± 14.34[a]	80.04 ± 10.33[ac]	51.86 ± 15.18[c]
PM	31.99 ± 13.64[a]	69.75 ± 10.36[ac]	42.66 ± 16.96[c]
VAP	33.33 ± 3.49[ac]	71.21 ± 9.88[a]	61.38 ± 9.20[c]
VCL	56.35 ± 5.61[ac]	137.99 ± 18.77[a]	116.57 ± 25.32[c]
VSL	20.79 ± 3.65[ac]	60.16 ± 10.70[a]	47.91 ± 7.91[c]
STR	0.62 ± 0.10[b]	0.83 ± 0.04[b]	0.78 ± 0.06
LIN	0.37 ± 0.07	0.43 ± 0.06	0.42 ± 0.09
WOB	0.59 ± 0.05[c]	0.51 ± 0.05[c]	0.53 ± 0.06
ALH	2.92 ± 0.33[bc]	4.07 ± 0.41[c]	4.36 ± 1.24[b]
BCF	17.28 ± 0.95[ac]	29.08 ± 2.54[c]	25.48 ± 2.68[a]
TM	40.39 ± 14.34[a]	80.04 ± 10.33[ac]	51.86 ± 15.18[c]
	Flow cytometry (n = 20)		
DFI (%)	5.61 ± 1.82[bc]	4.04 ± 1.34[c]	4.59 ± 1.87[b]

Abbreviations: CASA—Computer Assisted Sperm Analysis, SLC – Singe-layer centrifugation, TM—Total motility, PM—Progressive motility, VCL—curvilinear velocity, VAP—average path velocity, VSL—straight line velocity, LIN—linearity, STR—straightness, WOB—wobble, BCF—beat cross frequency, ALH—amplitude of lateral head displacement, SD – Standard deviation, n – number of samples; Superscript letters within a row denote significant differences between groups of sample: a—$p < 0.001$; b—$p < 0.01$; c—$p < 0.05$.

5. Discussion

The project focused on (i) the identification of bacteria present in semen samples of healthy bulls and their effects on fertility; (ii) seasonal effects on bull semen microbiota and their potential influence on sperm quality parameters; (iii) methods used to identify bacteria; and (iv) possible alternatives for antibiotics in semen extenders for artificial insemination. For the identification of bacteria in bull semen, 16S rRNA sequencing and MALDI-TOF MS were used, while plant-based substances and a physical method (SLC through a colloid) were used to separate spermatozoa from bacteria in semen.

5.1 Metagenomic analysis of bull semen microbiota

In Study I, 16S rRNA sequencing was used for identification of bacteria in raw bull semen samples and there correlation with fertility results while in Study II, the same method was used for identification of bacteria in commercial bull semen samples collected from the same bulls in three seasons, and a potential connection with semen quality parameters was evaluated.

5.1.1 Bacteria and bull fertility

The aim of Study I was to investigate potential correlations between bacteria in the male reproductive tract and semen fertility, rather than to analyze the effects of these bacteria on sperm quality, which is a different topic. For detail information about bacteria identified in this study as well as their origin, please see Paper I.

Our findings revealed two bacterial genera, *W5053* and *Lawsonella*, which were enriched in semen samples from the low-fertility bull group.

These genera were among the top 20 most abundant bacterial genera. *Lawsonella* spp., are gram-positive, non-spore-forming, partially acid-fast, anaerobic, catalase-positive, and pleomorphic bacteria (Bell *et al*. 2016) that have been isolated from human abscesses, representing a new genus (*Lawsonella clevelandensis gen. nov., sp. nov.*). The genus *W5053* is also a novel bacterium, although more comprehensive information is needed, and with *Lawsonella* was observed to be present at a higher abundance with other identified bacteria in HIV-infected patients without chronic respiratory diseases (Flygel *et al*. 2020) compared to patients with some form of chronical respiratory diseases. Further research is required to determine the origin of these two bacterial genera in semen and their impact on sperm quality in bulls with a reduced fertility potential.

Furthermore, several bacterial genera in this study were negatively correlated with the genus *Curvibacter* in most cases, followed by *Rikenellaceae* RC9-gut-group, *Dyella*, *Staphylococcus*, and *Cutibacterium*. Deines *et al*. (2020), documented that the coexistence of microbial species is influenced by host-bacteria interactions, including the host-environment, and bacteria-bacteria interactions. Their study indicated that the competitive effect of the bacteria depends on their direct contact. Furthermore, the authors discussed that rare microbial community members might be relevant for achieving a "native community composition and carrying capacity". Subsequent research would be of interest to investigate the potential interactions between the bacteria identified in bull semen with reduced fertility and the complex interactions between host bacteria and the environment.

5.1.2 Seasonal effect on bull semen microbiota and sperm quality

The study regarding seasonal effect on bull semen microbiota and sperm quality (Study II) aimed to explore whether seasonal changes affect the microbiome of bull semen and a potential link with sperm quality. Although seasonal differences in bacterial diversity and some sperm quality parameters were observed, specific links between bacteria and sperm quality could not be determined.

Escherichia-Shigella was overrepresented in all semen samples with an approximately equal distribution, suggesting contamination. A detailed discussion of the possible sources of contamination is provided in Paper II. Despite the fact that *Escherichia-Shigella* was present in all samples, the

diversity in community type cannot be disregarded since the bacteria present or absent in different samples were not observed in the negative PCR control.

The five phyla present in each season in the present study (Actinobacteria, Bacteriodetes, Firmicutes, Fusobacteria, and Proteobacteria) were also identified in the prepuce (Wickware *et al*. 2020) and semen samples (Medo *et al*. 2021) of healthy bulls in various countries, as well as in human seminal plasma (Yang *et al*. 2020). These findings led us to conclude that the seminal microbiota is not specific to geographic areas in bulls and, at the same time, raises the question of interspecies similarity in semen microbiota. Moreover, the ratio of the top five phyla presented in this study differed between seasons. This study is the first to perform a metagenomic analysis of the microbiota in commercial bull semen samples, assessing seasonal variations in sperm quality and its association with bacterial composition. Other studies have assessed the seminal microbiome in other species in different seasons; in a study of goat semen microbiota (Moce *et al*. 2022), slight differences between the breeding and non-breeding seasons were observed.

At the genus level, the top 20 genera differed between the studies of bull semen microbiome (Wickware *et al*. 2020; Cojkic *et al*. 2021; Koziol *et al*. 2022), with *Fusobacterium* and *Histophilus* being the only two genera identified as being in the top 20 in all studies. The reasons for the differences at the genus level could be (i) sampling time, (ii) type of ejaculates (raw vs. diluted), and (iii) location.

Differences were observed in the number of bacterial genera with a relative abundance >1% among the seasons. To date, no metagenomic studies have evaluated seasonal variations in the bacterial composition of bull semen, except for one study that assessed the effect of season on bacterial count in bull semen samples (Sannat *et al*. 2016). These authors stated that season influenced the total viable bacterial count in fresh semen samples. Furthermore, differences between bull breeds were also observed. In addition to the nine bacterial genera identified across all seasons in our study, four were exclusive to spring and summer, with samples collected in winter exhibiting different genera. This suggests that temperature influences the type of bacteria colonizing the reproductive tract mucosa.

The results of the present study revealed correlations between different bacterial species during two seasons. In winter and summer, the Proteobacteria phylum exhibited a negative correlation. The negatively correlated bacteria in this study were the same phyla as identified in a study

of bacterial composition in patients with obstructive and non-obstructive azoospermia (Chen *et al*. 2018); however, a negative effect of these bacteria on bull sperm quality in our study was not observed, nor were they identified in low-fertility bulls in our previous study (Cojkic *et al.* 2021).

Seasonal variations in sperm quality parameters in this study were consistent to some extent with previously published findings for bulls maintained under similar conditions (Valeanu *et al*. 2015). The only significant difference observed between the seasons in our study was for live hydrogen peroxide-producing cells, which were more abundant in spring than in winter or summer. Various climatic factors have been assessed previously, both independently (Brito *et al*. 2002) and in combination (Llamas-Luceno *et al*. 2020). However, contradictory outcomes have been reported even in the same geographic region but at different times. This indicates that factors other than climate could also influence sperm quality, for example husbandry factors such as feeding and bedding.

5.2 Effect of plant based substances on bacterial count and sperm quality

The Study III aimed to investigate the potential use of three plant-based substances with reported antimicrobial activity as additives to bull semen extenders to prevent bacterial growth. Our findings demonstrated that exposure to pomegranate, curcumin, and ginger had an adverse impact on sperm motility, particularly after a 24-hour exposure (Time 2).

Pomegranate improved sperm quality when added to semen extenders (El-Sheshtawy *et al*. 2016) or as a food supplement (Türk *et al*. 2008). In addition, it has antibacterial properties, with the total phenolic content strongly correlated with its effectiveness in this regard (Howell & D'Souza 2013). The level of polyphenolic compounds and antimicrobial activity can vary depending on the plant part and extraction method used. In addition, different pomegranate varieties have varying levels of antimicrobial activity (Duman *et al*. 2009). In our study, both P 5% and P 10% had a negative impact on CASA parameters after 24 h of exposure. However, flow cytometry results showed that P 5% preserved sperm quality parameters at Time 2, although they were decreased after exposure to P 10% at Time 2.

Ginger was used as an additive in semen extenders, having positive effects on sperm quality and fertility potential in thawed ram semen (Merati

& Farshad 2020). In addition, ginger had antimicrobial activity against pathogenic and multidrug-resistant bacteria (Mohamedin *et al*. 2018). However, our study found a negative effect of ginger on sperm motility and viability, which may be due to the use of DMSO to dissolve the ginger, since DMSO also negatively affected sperm quality parameters.

Previous studies have shown that curcumin has potent protective and antioxidative properties. It has been tested as a supplement when added to semen prior to cryopreservation, leading to significant dose-dependent positive effects on sperm samples from different animal species (Chanapiwat & Kaeoket 2015), but apparently not on the bull semen samples in our study. Curcumin showed a protective effect on sperm quality parameters during incubation (Tvrda *et al*. 2016), which is in agreement with the results of our study, where curcumin did not have a negative effect on any sperm quality parameter.

Possible reasons for the lack of a positive effect of the tested substances on sperm quality parameters is discussed in detail in Paper III. Regardless of treatment, a decline in bacterial count (CFU/mL) over time was observed in this study, and there was a low initial bacterial count in most of the bull semen samples. However, an antibacterial effect of curcumin in sperm samples with a bacterial reduction of over 30% was observed at Time 1 (< 2h) of incubation using a concentration of C 5%, which, to the best of our knowledge, has not been reported previously.

5.3 Single Layer Centrifugation as a physical method for bacterial reduction in bull semen

Study IV aimed to assess the efficacy of colloid centrifugation (SLC) using high or low density colloids for bacterial removal from bull semen samples. Sperm quality parameters and bacterial load were evaluated in the sperm samples after SLC. Differences were seen in the number of bacterial species in ejaculates collected after different periods of abstinence from the same individuals.

The bacterial species identified and the number of bacteria present varied between individual bulls and within different ejaculates of the same bull. This is consistent with a previous study by González-Marín *et al*. (2011), who found that some bacterial strains were present in multiple ejaculates from the same bull. The bacterial species found in all ejaculates in this study

were the same as those identified by Mitra *et al*. (2016), in a study on commercial frozen bull semen, indicating that these bacteria may be able to survive cryopreservation even in the presence of antibiotics in the semen extender. The bacterial genera isolated from all ejaculates in the present study were *Bacillus* spp., *Corynebacterium* spp., *Micrococcus* spp., *Proteus* spp., and *Staphylococcus* spp.

The bacterial count varied between bulls and ejaculates, although initial loads were generally low. This observation is consistent with findings from a previous investigation of Swedish dairy bulls at the same facility, which attested to the continuous implementation of stringent hygiene protocols during semen collection (Cojkic *et al*. 2023). Although the initial bacterial load was low, there was a significant decrease in the bacterial count after centrifugation for both colloids compared to the controls. These results are consistent with those of earlier studies conducted on stallions (Morrell *et al*. 2014), boars (Morrell *et al*. 2019), and dogs (Luño *et al*. 2020), where SLC was employed to reduce bacterial count. In addition, there was a difference in the bacterial reduction between the high- and low-density colloid SLC groups in our study.

As previously observed, SLC enhanced sperm quality, as evidenced by improvements in motility and %DFI, with a more pronounced effect observed in the High density colloid group. Both the high and low treatment groups exhibited significantly higher values of CASA kinematics, except LIN, compared to control samples. The highest CASA values were observed in the High density SLC group, followed by the Low density group, and finally, the control group. These findings differ from those of a previous experiment conducted on bull semen (Goodla *et al*. 2014), which reported no significant difference between SLC and Control groups in terms of total and progressive motility. However, the sample size in our study (n=6) was smaller than that reported by Goodla *et al*. (2014) (n=60). In our study, the results of SCSA indicated that both high- and low-density colloids reduced the proportion of sperm cells with damaged chromatin compared to the control, with no significant difference between the High and Low density colloid groups. A previous study found that the presence of bacteria in commercial bull semen samples is linked to an increase in DNA fragmentation (Gonzalez-Marin *et al*. 2011). Their study demonstrated that chromatin damage was associated with bacterial growth and varied among bulls.

Our results with colloid centrifugation indicated the possibility to separate spermatozoa of good quality from most of the bacteria in bull semen, thus avoiding the need to add antibiotics to the semen extender. Therefore, potential spermatotoxic effects would be avoided and the possibilities of AMR development could be reduced. Furthermore, separating spermatozoa from opportunistic bacteria decreases the risk for pathology in inseminated females as well as decreasing the risk of spreading AMR. Both of these aspects would have benefits for cattle breading by decreasing the costs of throwing away contaminated semen, or treatment of infected females, and avoiding a prolonged calving-to-conception interval. However, SLC should not be a substitute for attention to hygiene in semen collection and processing, or in animal husbandry in general.

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Popular science summary

Artificial insemination (AI) in cattle breeding is a commonly used technique that has existed for more than 60 years and is still a critical tool in modern livestock production. Briefly, semen from health-tested selected bulls is collected under hygienic conditions and used for insemination of certain cows. The benefits of AI in cattle breeding are numerous. First, it allows farmers to use the genetic material from superior bulls and thus improve the quality of the calves produced. Moreover, AI allows farmers to improve the efficiency of their breeding programs by reducing the time and resource required for natural breeding (a larger number of cows can be inseminated from an individual bull, and semen from different bulls can be used on various cows to obtain the best genetic combinations). Another significant advantage of AI is the ability to reduce the spreading of disease, by allowing farmers to use semen from disease-free animals. In cattle breeding, freezing of semen samples at low temperature (minus 196°C) is a standard procedure for semen preservation and storage, and therefore thawed semen samples are commonly used for AI in most countries except in Ireland and New Zealand. Extremely low storage temperature also helps in the control of bacterial growth since some, but not all, bacteria do not survive freezing under these conditions. However, identifying the semen microbiome of healthy male animals is crucial for excluding the risk of transmitting potentially pathogenic bacteria when fresh semen samples are used, or when they survive freezing, and also for inhibiting bacteria that could decrease sperm quality during storage. Identification of the bacteria in semen can be done using advanced sequencing techniques, which can identify the presence of various bacteria and their relative abundance in semen. By identifying the bacteria in the semen of healthy animals,

researchers can establish what is normal and identify any changes that may be associated with disease or poor reproductive outcomes.

Antibiotics are commonly used in extenders for bull semen to prevent the growth of bacteria during processing. However, there is growing concern about the risk of developing antimicrobial resistance. First, it can lead to the selection of resistant bacteria in the semen and in the reproductive tract of the female. These resistant bacteria can then be transmitted to other animals or humans through contact with contaminated semen or other bodily fluids. Moreover, this use of antibiotics in extenders can lead to the development of antibiotic-resistant genes in the bacteria present in the semen. These genes can then be transferred to other bacteria in the inseminated female or in the environment, potentially leading to the development of new antibiotic-resistant strains.

In addition to the use of natural antimicrobial agents and physical treatments, improving semen quality through proper semen handling and storage practices can also reduce the need for antibiotics in bull semen extenders. For example, proper hygiene practices during semen collection and processing can reduce bacterial contamination, while adequate semen storage and transportation conditions can reduce the risk of bacterial growth. However, there is still a need to deal with the residual bacteria that are present and it is essential to ensure that the use of antibiotics is done in a responsible and judicious manner to prevent the development of antimicrobial resistance and protect both animal and human health.

To address the risk of developing antimicrobial resistance due to the use of antibiotics in extenders, alternative strategies were explored in the present project. One approach is to use natural antimicrobial agents, such as plant extracts. These natural agents have been shown to have antibacterial properties and can be used as an alternative to antibiotics. However, any antibacterial substance that is added to semen must not be harmful to spermatozoa. According to our findings, curcumin can be used to reduce bacterial count in bull semen and at the same time to preserve sperm quality parameters. Another approach is to use physical methods to reduce bacterial growth; such as filtering through special media to remove bacteria from semen before storage and transportation. Such a method was successful in removing almost all *Bacillus* and *Proteus* bacteria and selecting the best quality spermatozoa.

It should be noted that if alternatives are to be used, we need to provide credible evidence that they are effective in inhibiting bacteria so that we can lobby regulatory organisations to change the laws regarding antibiotics in commercial semen samples.

Populärvetenskaplig sammanfattning

Artificiell insemination (AI) är en vanlig teknik i nötkreatursuppfödning som har funnits i mer än 60 år och som fortfarande är ett viktigt verktyg i modern boskapsavel. Kortfattat samlas sperma under hygieniska förhållande från hälsotestade utvalda tjurar och används för insemination av kor. Fördelarna med AI i boskapsavel är många. För det första tillåter det jordbrukare att använda det genetiska materialet de bästa utvalda tjurarna och på så sätt förbättra kvaliteten på de producerade kalvarna. Dessutom gör AI det möjligt för jordbrukare att förbättra effektiviteten i sina avelsprogram genom att minska den tid och de resurser som krävs för naturlig avel (ett större antal kor kan insemineras från en enskild tjur, och sperma från olika tjurar kan användas på olika kor för att få de bästa genetiska kombinationerna). En annan betydande fördel med AI är möjligheten att minska spridningen av sjukdomar, genom att använda sperma från tjurar som är hälsotestade och fria från sjukdomar. Inom boskapsuppfödning är nedfrysning av sperma vid låg temperatur (minus 196°C) en standard procedur för bevarande och lagring av sperma. Frysta-upptinade spermadoser används vanligtvis för AI i de flesta länder, utom i Irland och Nya Zeeland. Den extremt låga lagringstemperaturen hjälper också till att kontrollera tillväxten av bakterier eftersom vissa bakterier, men inte alla, inte överlever frysning under dessa förhållanden. Att identifiera sammansättningen av bakterier i sperman, spermamikrobiomet, hos friska handjur är dock avgörande för att utesluta risken för överföring av potentiellt patogena bakterier, bakterier som kan orsaka sjukdomar när färsk sperma använd, eller när de överlever frysning, och även för att hämma tillväxten av bakterier som kan påverka spermiekvaliteten negativt under lagringsperioden. Identifiering av bakterier i sperma kan göras med hjälp av avancerade sekvenseringstekniker, som kan upptäcka förekomsten av olika

bakterier och deras relativa förekomst i sperma. Genom att identifiera bakterierna i sperma från friska djur kan forskarna fastställa vad som kan betraktas som normalt och identifiera eventuella förändringar som kan vara förknippade med sjukdom eller dåliga reproduktionsresultat.

Antibiotika används ofta i de spädningsvätskor som används vid frysning av tjursperma för att förhindra tillväxt av bakterier. Det finns dock en växande oro för utveckling av antibiotikaresistens. För det första kan det leda till urval av resistenta bakterier i sperman som förs vidare till honans fortplantningsorgan. Dessa resistenta bakterier kan sedan överföras till andra djur eller människor genom kontakt med förorenad sperma eller andra kroppsvätskor. Dessutom kan denna användning av antibiotika i spädningsvätskor leda till utvecklingen av antibiotikaresistenta gener i de bakterier som finns i sperman. Dessa gener kan sedan överföras till andra bakterier i den inseminerade honan eller i miljön, vilket kan leda till utvecklingen av nya antibiotikaresistenta stammar.

Förutom användningen av naturliga antimikrobiella medel och fysiska behandlingar, kan förbättrad spermakvalitet genom korrekt spermahantering och lagringsmetoder också minska behovet av antibiotika i spädningsvätskor som används vid frysning av tjursperma. Till exempel kan korrekta hygienrutiner under spermauppsamling och bearbetning minska bakteriell kontaminering, medan lämpliga spermalagrings- och transportförhållanden kan minska risken för bakterietillväxt. Det är viktigt att säkerställa att användningen av antibiotika görs på ett ansvarsfullt och klokt sätt för att förhindra utvecklingen av antimikrobiell resistens och skydda både djurs och människors hälsa.

För att ta itu med risken att utveckla antimikrobiell resistens på grund av användningen av antibiotika i spädningsvätskor, undersöktes alternativa strategier i detta projekt. Ett tillvägagångssätt är att använda naturliga antimikrobiella medel, såsom växtextrakt. Dessa naturliga medel som har visat sig ha antibakteriella egenskaper skulle kunna användas som alternativ till antibiotika. Antibakteriella substanser som tillsätts sperma får dock inte vara skadliga för spermier. Enligt våra resultat kan curcumin användas för att minska antalet bakterier i tjursperma samtidigt som spermiekvaliteten bevaras. Ett annat tillvägagångssätt är att använda fysiska metoder för att minska bakterietillväxt; såsom att filtrera sperman genom speciella medier för att avlägsna bakterier. Filtrering med så kallad ”Single Layer Centrifugation” (SLC) var framgångsrik för att ta bort nästan alla *Bacillus*-

och *Proteus*-bakterier samtidigt som spermierna med bäst kvalitet selekterades fram.

Det bör noteras att om alternativ till antibiotika ska användas, behöver vi tillhandahålla trovärdig bevisning om alternativens bakteriehämmande effekt så att vi kan påverka reglerande organisationer att ändra lagar som rör antibiotika i kommersiella spermaprover.

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I

microorganisms

MDPI

Article

Identification of Bull Semen Microbiome by 16S Sequencing and Possible Relationships with Fertility

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Abstract: Reports on the use of 16S sequencing for the identification of bacteria in healthy animals are lacking. Bacterial contamination of bull semen can have a negative effect on the sperm quality. The aims of this study were threefold: to identify bacteria in the semen of healthy bulls using 16S sequencing; to investigate the differences in the bacterial community between individual bulls; and to establish if there was a relationship between the bacteria isolated and bull fertility. Semen from 18 bulls of known fertility was used for the DNA extraction and 16S sequencing; 107 bacterial genera were identified. The differences in the amplicon sequence variants (ASVs) and the numbers of genera between bulls were noted. Negative correlations ($p < 0.05$) between several bacterial genera with *Curvibacter*, *Rikenellaceae RC9-gut-group* and *Dyella* spp. were seen. Other negatively correlated bacteria were *Cutibacterium*, *Ruminococcaceae UCG-005*, *Ruminococcaceae UCG-010* and *Staphylococcus*, all within the top 20 genera. Two genera, *W5053* and *Lawsonella*, were enriched in bulls of low fertility; this is the first time that these bacteria have been reported in bull semen samples. The majority of the bacteria were environmental organisms or were species originating from the mucous membranes of animals and humans. The results of this study indicate that differences in the seminal microbiota of healthy bulls occur and might be correlated with fertility.

Keywords: bull semen; bacteria; 16S sequencing; sperm quality; fertility

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1. Introduction

One of the main reasons for introducing artificial insemination into animal breeding was to prevent the spread of infectious diseases between males and females [1]. Apart from the urogenital tract itself as a primary source of pathogens [2], there are critical points in semen processing where sperm samples can be contaminated with different types of microbiota [3–5]. Therefore, antibiotic use in semen for artificial insemination (AI) is considered to be essential. Different types of antibiotics, singly and in combination, are added to semen worldwide with the aim of decreasing or preventing microbial growth. Antibiotics used in bull semen cryopreservation are mainly based on the regulations given by government directives, e.g., the European Union [6]. However, despite the addition of antibiotics, bacteria can still be isolated from bull semen after thawing [7]. In addition, there is considerable concern about the emergence of antibiotic-resistant bacteria, particularly methicillin-resistant strains [8]. The identification of bacteria in semen samples is a primary step to the rational use of antibiotics and could be helpful in developing the next steps in the control of microbial contamination of bull semen [3].

The presence of bacteria in semen does not equate with infection and limited microbial activity does not necessarily affect sperm quality [9]. However, bacteria can have a direct negative affect on sperm quality, depending on the species and number. Negative

correlations were reported between certain bacteria and sperm motility [10], viability, membrane integrity and acrosome reaction [11], sperm DNA fragmentation rates [12,13] and the total number of spermatozoa [14,15]. Givens and Marley [16] reported the presence of microorganisms that cause infertility and/or are transmitted via semen. This knowledge can facilitate the identification and exclusion of subclinically infected bulls from production. Bacteria present in the urogenital tract can influence fertility in cows [17]. Postpartum uterine disease caused by pathogenic bacteria not only reduces fertility but also decreases productivity [18]. Postpartum metritis and clinical endometritis caused subfertility in cows by increasing the time to first insemination, delayed conception and increasing the calving to conception interval [19,20]. Furthermore, postpartum clinical endometritis can cause infertility and consequently results in involuntary culling [21]. Marey et al. [22] reported that infections extending from the uterus to the oviduct induce an immune system disbalance that interferes with fertilization. Apart from an effect on the uterus itself, a uterine infection can lead to disruption in the secretion of gonadotropins [23], disruption in ovarian follicle growth and function [24] and a reduction in the oocyte quality [25] (cited from Sheldon and Owens [18]).

In contrast to these extensive studies in females, the presence and influence of bacteria on sperm quality and fertility in bulls have not been investigated to any great extent. However, the types of bacteria present in semen in various species such as stallions [2,26], boars [27] and rams [28] have been described as well as reports of the influence of bacteria on fertility and sperm quality parameters in humans [29,30]. Interest in the isolation and identification of the main bacteria in bull semen causing infertility began decades ago [31] using culture and identification by means of appearance and biochemical properties; other methods of identification were introduced as the science developed. In general, reports on the use of 16S sequencing for the identification of bacteriospermia in healthy animals are lacking, especially in bulls. The identification of individual opportunistic bacteria is of interest due to the possible transmission between bulls and from bulls to cows, causing economic losses to cattle production. However, there are few studies in which a full microbiological profiling of bull semen has been achieved.

The aims of the present study were to identify the bacteria in bull semen via 16S rRNA sequencing and to investigate the individual differences in bacterial type and number in bulls at a semen collection station. An additional aim was to examine the relationships between the bacterial community and the overall fertility of the bulls.

2. Materials and Methods

2.1. Animals and Semen Collection

Ejaculates were obtained from 18 Holstein bulls at an AI center (Animal Breeders' Association of Estonia, Raplamaa, Estonia) where animals were kept according to national and international regulations. The age of the bulls ranged from 3 to 10 years. The bulls were kept on dry bedding (new sawdust added 4× /24 h) and, if necessary, cleaned by brushing before the semen collection. Semen collection with an artificial vagina is a routine agricultural practice and, therefore, does not require ethical approval according to Estonian law [32]; the bulls at the AI station were not considered to be experimental animals. Therefore, no special ethical permission was required.

The bulls were prepared for semen collection by allowing them to follow each other in a circular chute for 30–40 min; the bulls were able to make false mounts by jumping on the bull in front but the chute was not wide enough for the bulls to turn round. A sterilized artificial vagina lubricated with Bovivet Gel (Jørgen Kruuse A/S, Langeskov, Denmark) and a sterilized graduated collecting tube were used for the semen collection, which took place approximately 10 m from the laboratory separated by a glass wall. Within a minute after the collection, the sterile graduated tube containing the collected ejaculate was separated from the artificial vagina and passed to the laboratory personnel through a small hatch in the glass wall. All semen collection procedures were performed with sterile equipment and aseptic measures to avoid semen contamination. In the laboratory,

an aliquot of 1 mL semen was transferred to an Eppendorf tube above an alcohol burner and stored in liquid nitrogen before transfer to −80 °C for storage until the bacterial DNA extraction was performed. The remainder of the semen samples were processed and used for the evaluation of the sperm motility by CASA. All samples with a total motility >90% and a progressive motility >80% were used for the routine inseminations. The CASA results are presented in Supplementary Table S1. The fertility performance of these bulls was estimated by non-return rates (NRRs) at 90 days post-AI, based on the outcome from the first insemination. In total, 48,469 females were inseminated, comprising 34,800 cows and 13,649 heifers. Miglior et al. [33] defined NRRs as the proportion of cows that are not seen in estrus again after insemination and are, therefore, considered to be potentially pregnant.

2.2. DNA Extraction

The DNA extraction was performed in the Clinical Sciences Research Laboratory at SLU using an AllPrep DNA/RNA/miRNA Universal Kit Cat No./ID 80224 (GIAGE, Germantown, Philadelphia, USA) following the manufacturer´s instructions for the protocol of the simultaneous purification of genomic DNA and total RNA, including miRNA from cells. In total, 10 µL of semen was used for the DNA extraction to reach the maximum amount of 1×10^7 cells, spermatozoa and bacteria according to the protocol. The sperm concentration was evaluated using a Nucleocounter SP100 (ChemoMetec, Allerød, Denmark) according to the manufacturer´s instructions. According to our calculations, 10 µL of semen contained on average 5×10^6 spermatozoa although the bacterial number was not calculated. All samples were centrifuged; the supernatant was removed and only pelleted cells were used. The purity and concentration of the DNA were tested using a NanoDrop 8000 Spectrophotometer (Thermo Scientific, Waltham (HQ), MA, USA). The DNA purity was considered adequate when the 260/280 ratio was between 1.7 and 1.9 and the concentrations were between 5.34 and 10.97 ng/µL. The DNA samples were stored at −80 °C until further preparation.

2.3. 16S rRNA Amplification and Sequencing

A two-step amplification protocol was used for the preparation of the V3–V4 16S gene region for Illumina sequencing. The details of the primers used and the cycling protocols are presented in Table 1. The reaction volume of the first step was 15 µL containing 4 ng of the DNA template, 0.25 µM Pro 341F and the same volume of Pro 805R primers with Nextera adaptor sequences (Illumina Inc., CA, USA) as well as 1 µg/µL BSA and 1 × Phusion Taq ready-to-use mix (New England Biolabs, MA, USA). For the second step, the reaction volume was 30 µL and contained 3 µL of the purified DNA template from the first PCR step, 0.20 µM tagged F and R primers with Nextera adaptor sequences and 1 µg/µL BSA and 1 × Phusion Taq ready-to-use mix. Both PCR steps were performed in duplicate and the reactions were pooled and purified between the steps using SeraMag Magnetic Carboxylate Modified particles in a ratio of 1:1. An Agilent Bioanalyzer was used for the quality check. The amplicons were eluted in 10 mM Tris with a pH of 8.5 and stored at −20 °C. The samples were sent for sequencing to SciLifeLab, SNP&SEQ Technology Platform, Uppsala University. Paired-end sequencing was performed on a MiSeq system (Illumina, San Diego, CA, USA) using kit V2. Approximately 40,000 to 60,000 paired-end reads of 250 bp length were obtained for all samples except the control sample (sterile water), which yielded only 13 reads after sequencing.

Table 1. Primer combination and thermal cycling conditions used to quantify the 16S rRNA.

Primer 16S rRNA	Sequences (5′-3′)	Terminal Cycling	Reference
341F	CCTACGGGAGGCAGCAG	(98 °C 3 min); (98 °C 30 s, 55 °C 30 s, 72 °C 40 s) × 25; 72 °C 10 min; 10 °C hold	[34]
805R	GACTACNVGGGTATCTAATCC	(98 °C 3 min); (98 °C 30 s, 55 °C 30 s, 72 °C 45 s) × 8; 72 °C 5 min; 10 °C hold	

2.4. 16S Profiling

The analysis of the 16S rRNA sequencing data was performed using Nextflow pipeline ampliseq v.1.1.2 (https://github.com/nf-core/ampliseq, accessed on 24 November 2021). Briefly, raw sequencing reads were quality checked initially using FastQC [35] followed by the trimming of the primer sequences from the reads using cutadapt v.2.7 [36]. The raw sequencing data were cleaned from source contamination by running BLAT against the cow reference genome *Bos taurus* 8 available in the UCSC genome browser (https://genome.ucsc.edu/, accessed on 24 November 2021). The sequencing reads were denoised, dereplicated and filtered for chimeric sequences using DADA2 [37]. The denoised paired-end reads were truncated from position 229 (forward) and 215 (reverse) after a manual visualization of the sequencing error profile; all other reads shorter than the cutoff values were dropped. The truncated sequences were merged with at least a 20 bp overlap, resulting in exact amplicon sequence variants (ASVs). These ASVs were taxonomically classified from the phylum to species level clustered with 99% similarity using the SILVA v.132 database [38] by applying a Naive Bayes classifier implemented in QIIME 2 [39] trained on the preprocessed database. Following the taxonomic classification of the ASVs, the ASVs classified as a mitochondria or a chloroplast were removed. Only the ASVs with a minimum read frequency $\geq$5 in at least one sample were retained for a further analysis.

2.5. Statistical Analysis

The data analysis was performed using R v.3.3.1 software. Pearson correlation coefficients were calculated between the bacterial genera using the cor.test function in the R environment, with $p < 0.05$ being considered significant. The plotting was carried out in R using corrplot v.0.9. A linear discriminant analysis effect size (LEfSe) analysis of the microbial abundance between low and high fertility bulls was performed to detect the differences between the two groups and characterize the biomarkers. The groups were divided based on the NRR where the NRRs were <51% and >51% for low and high fertility bulls, respectively.

3. Results

The total amount of DNA in the pool was 1320 ng with a ratio of absorbance at A260/A280 at 1.89 and A260/A230 at 2.1 and a concentration of 24 ng/µL. In total, 107 bacterial genera were identified in 18 bull semen samples. The 20 most frequently seen bacterial genera are presented in Figure 1.

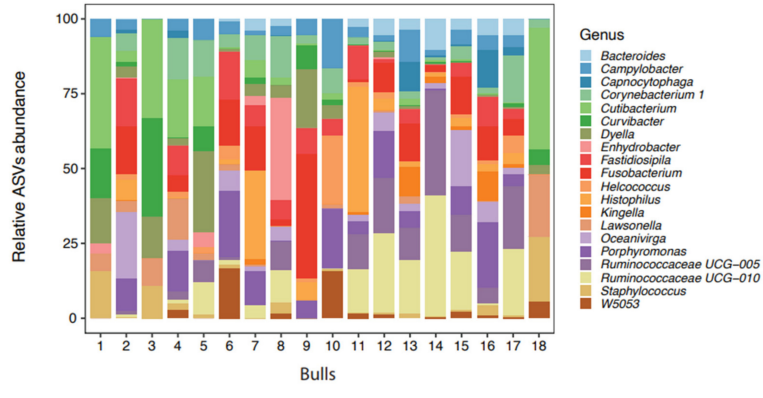

Figure 1. Distribution of the 20 most abundant bacteria genera in the semen from 18 bulls (amplicon sequence variants) identified by 16 rRNA sequencing.

The bacteria listed among the top 20 for each bull varied considerably; there was also a considerable variation between bulls in both the number of bacterial genera present (ranging from 12 to 89) and in the bacterial count (ranging from 27,827 to 247,273) (Table 2). The highest number of genera identified was in the sample from bull 12, which contained 89 bacterial genera of which 19 came from the 20 most frequently seen. In contrast, Bull 3 was the least colonized, with only 12 bacterial genera present.

Table 2. Number of top 20 and total genera, counts, total counts and non-return rate (NRR, %) per bull.

	Top 20 Genera	Counts	Total Genera	Total Counts	NRR (%)
Bull 1	7	50,127	13	60,678	48.9
Bull 2	20	101,453	69	149,773	48.3
Bull 3	5	17,157	12	56,692	51.4
Bull 4	17	53,773	47	95,193	55.1
Bull 5	12	16,089	31	27,827	61.7
Bull 6	16	188,695	47	247,273	45.9
Bull 7	16	61,305	37	92,055	48.9
Bull 8	17	74,382	49	129,282	52.1
Bull 9	10	53,248	24	70,528	50.4
Bull 10	12	95,853	26	114,379	62.1
Bull 11	18	95,136	82	175,372	51.8
Bull 12	19	70,932	89	130,601	55.2
Bull 13	18	115,553	77	183,004	50.5
Bull 14	18	64,435	76	118,757	47.6
Bull 15	18	102,459	83	170,084	54
Bull 16	19	146,754	53	198,532	52.6
Bull 17	18	68,109	86	141,152	52.3
Bull 18	9	116,226	16	137,258	37

Top 20 genera: 20 most frequently seen genera in all samples; counts: read counts of genera per bull present in the top 20; total genera: number of identified genera per bull; total counts: read counts of genera per bull of all genera per bull; NRR: non-return rate.

The 20 most frequently seen bacterial genera, starting with the most frequent, were: *Porphyromonas*, *Fusobacterium*, *Ruminococcaceae UCG-010*, *Fastidiosipila*, *Ruminococcaceae UCG-005*, *Cutibacterium*, *Histophilus*, *Oceanivirga*, *Corynebacterium 1*, *Campylobacter*, *W5053*, *Dyella*, *Staphylococcus*, *Lawsonella*, *Helcococcus*, *Bacteroides*, *Capnocytophaga*, *Curvibacter*, *Kingella* and *Enhydrobacter*, which are presented in Figure 1 as the relative ASV abundance.

There were negative correlations between several bacterial genera (Figure 2). The majority of the bacteria that showed negative correlations were from the top 20 genera (Figure 1) and included *Curvibacter*, *Cutibacterium*, *Dyella*, *Ruminococcaceae UCG-005*, *Ruminococcaceae UCG-010* and *Staphylococcus* spp. In addition, *Curvibacter*, *Rikenellaceae RC9-gut-group* and *Dyella* spp. (brown spots) were negatively correlated in most cases.

In the bar plot (Figure 3), the taxa (including the genus and family) with significant differences between the groups were detected by a LEfSe analysis with a log-10 transformed LDA (linear discriminant analysis) threshold score of 2.0 and a significant p-value <0.05. The ASV with a higher LDA score indicated that the ASV was more important according to the LEfSe in discriminating between the low and high individuals. Two genera, *W5053* and *Lawsonella*, were enriched in the low fertility group.

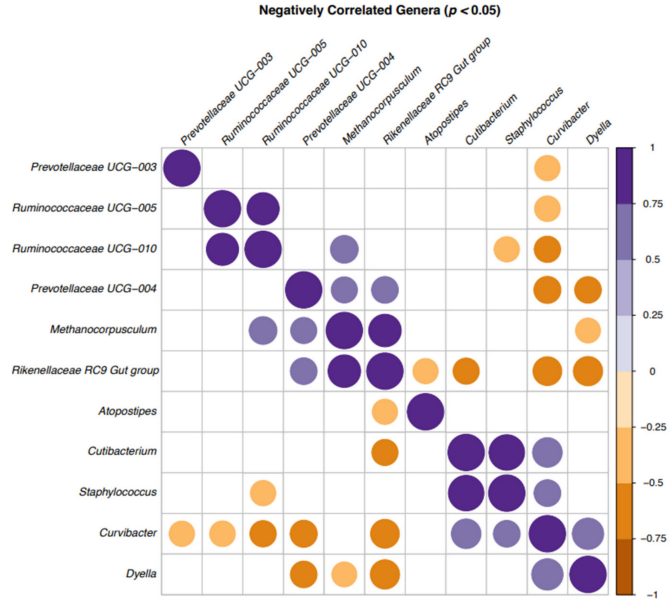

Figure 2. Correlation plot of the bacteria in bull semen identified by 16S sequencing in a correlation matrix showing the significant correlations ($p < 0.05$) between genera: positive (purple) and negative (brown). Blank cells indicate non-significant correlations.

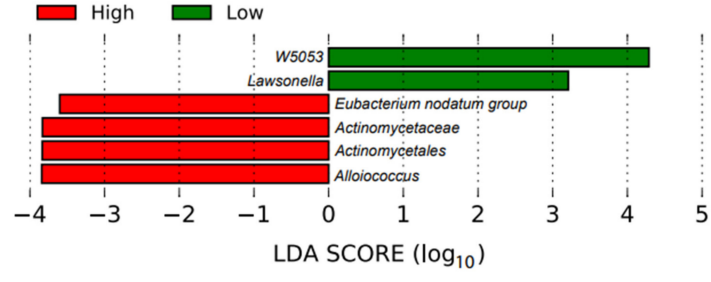

Figure 3. Linear discriminant analysis effect size plot. This plot shows the enriched taxa that were significantly different between the high fertility (red) and low fertility (green) bulls.

4. Discussion

In this study, 16S sequencing of bull semen microbiomes was performed with the aim of identifying the most common bacteria in the semen of healthy bulls. The determination of the most common bacteria genera can enable the development of appropriate control methods.

The isolation and identification of bacteria by commercial microbiological methods are more difficult compared with metagenomics analyses. First, it may not be possible to isolate all the bacteria in a sample. The isolation and identification of bacteria by culture-dependent methods require different culture conditions for different type of bacteria such as the media, temperature, presence or absence of oxygen and time of incubation. The

growth of a few bacteria may be inhibited by competition between bacterial species or bacterial overgrowth. Traditional culture-dependent morphological identification methods are time-consuming and do not enable all bacteria to be identified [40]. Although the development of MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometry can help in the process of bacterial identification after culturing, the possibility of identifying isolates depends on the information in the database for the instrument [2]. In contrast, 16S sequencing does not require a bacterial culture and enables the identification of large numbers of bacteria present in a sample [26].

To our knowledge, this is the first comprehensive metagenomic study of the seminal microbiome of healthy bulls. During the introduction of artificial insemination as an animal husbandry technique, there was a need to inhibit bacterial growth in bull semen [41]. Later, culture-dependent studies were conducted to isolate the bacteria responsible for bull infertility [42] and this technique was also used in more recent studies of the microbiological evaluation of frozen semen samples [43]. During the past few years, the identification of individual bacteria from preputial mucosa [44,45] and bull semen [46] was performed with metagenomic methods. Interestingly, there were no significant correlations between the preputial microbial community of bulls of different ages, breed, diet or co-housing [47]. In other species, several studies on culture-independent methods of identification of the normal microbiota in semen have been reported. Stallion semen microbiota was studied by conventional methods of identification as well as MALDI-TOF [2] and recently by 16S sequencing [26]. Al-Kass et al. [26] reported that large numbers of bacterial genera could be identified in stallion semen using metagenomic analyses by 16S sequencing. Based on those studies, variations in bacteria genera and their number between individual stallions and between countries were identified. However, the researchers agreed that the identified bacteria in both stallions and bulls [26,47] were mostly environmental in origin.

In order to understand the composition of the bull semen bacterial community, it is important to discuss its potential sources. Three of the most common genera identified in our study were *Porphyromonas*, *Fusobacterium* and *Ruminococcaceae UCG-010* respectively. This finding is in agreement with the study of Wickware et al. [47] where *Porphyromonas* and *Fusobacterium* were the most abundant genera in the preputial microbiota of Hereford bulls. These two genera, together with *Bacterioides,* were among the most abundant genera from the upper respiratory and oral mucosal membranes of healthy calves in the first month of life [48,49]. In contrast, the anaerobic bacteria *Porphyromonas*, *Fusobacterium* and *Fastidiosipila* were highly prevalent in most cases of lameness caused by foot lesions [50]. Their presence in semen is probably due to their common occurrence in the environment and subsequent colonization of mucosal membranes.

In the study of Klein-Jöbstl et al. [49] on the microbiota of newborn calves and their mothers, *Ruminococcaceae* was the most abundant type in cow fecal and vaginal samples. In the same study, *Enhydrobacter* was the most dominant in the colostrum on the first day postpartum; this bacterium appeared in our top 20 isolated bacteria, albeit in low numbers. *Ruminococcaceae UCG-005* and *Ruminococcaceae UCG-010,* ranked in the top 10 isolated genera in our study, were also present in the samples of healthy skin in the studies of Bay et al. [50]. Coryneform bacteria *Corynebacterium* and *Cutibacterium* are distributed in the environment in soil and water [51]. These bacteria, as well as *Staphylococci*, are commensals and colonizers of the skin and mucous membranes in animals [52]. *Dyella*, *Helicococcus*, *Capnocytophaga* and *Kingella* are commensal bacteria isolated from the human respiratory tract [53] as well as the oral cavity of humans and animals [54] and are part of the skin flora [55]. Although they are considered to be commensals, all of them were isolated from a patient with severe clinical symptoms; this is the first report of their occurrence in bull semen. Their influence on sperm quality parameters is unknown.

Histophilosis is a common disease in North American cattle in the form of septicemia with a high risk of infection and sudden death in calves. A pathogenic form of *Histophila somni* was isolated from the prepuce of a healthy bull and from the vagina of cows with a clinical manifestation of granular vulvovaginitis and abortion [56]. Bovine genital campy-

lobacteriosis caused by *Campylobacter fetus veneralis* or *Campylobacter fetus fetus* is a venereal disease of cattle characterized by infertility, mucopurulent endometritis, early embryonic death and occasionally abortion in systemically healthy cows. As well as venereal transmission, *Campylobacter fetus fetus* can be transmitted by AI in contaminated semen as well as by contaminated instruments. Infections in young bulls can be transient in contrast to older animals with established chronic infections, which may be due to differences in the preputial and penile epithelial surfaces of the lumen and within the crypts in the older animals and the microaerophilic environment that deeper crypts may provide. *Campylobacter* is one of the most demanding bacteria to culture due to its requirement for microaerophilic or anaerobic conditions as well as the need to be cultured immediately after sampling [57]. Therefore, it may be missed when culturing under conventional microbiological conditions. Cagnoli et al. [11] described a significant negative influence of both *Campylobacter fetus veneralis* and *Campylobacter fetus fetus* bacteria species on bull semen quality parameters.

Metagenomic analyses, especially 16S rDNA sequencing, allows the identification of bacteria with unusual phenotypic profiles, rare bacteria, slow growing bacteria and bacteria that cannot be cultured. Furthermore, 16S sequencing can facilitate the definitions of the etiologies of infectious diseases as well as aiding clinicians to choose the most effective antibiotics and determining the duration of the treatment. However, the interpretation of the results can be challenging even for clinical microbiologists [40].

Farahani et al. [58] conducted a systematic review study and meta-analysis of bacteria identified from fertile and infertile men and their influence on sperm quality and fertility parameters. Major differences in the bacterial presence of fertile and infertile men were identified with different sperm quality parameters as well as the negative and positive effects of the individual bacteria on these parameters. Apparent positive effects of *Lactobacillus* spp. on the sperm morphology in addition to a protective effect against *Pseudomonas* and opportunistic pathogens were highlighted. Boud et al. [9] reported that *Pasteurella* spp. abundance was increased in sperm samples with poor motility. Although the latter study showed that the bacterial content might not have an influence on the fertility of men, specific bacterial genera had an impact on sperm morphology and motility. There are few studies on the influence of the bacterial community in semen on sperm quality and fertility outcomes in veterinary medicine. There are a few studies of bacterial influence on sperm quality parameters but usually fertility data are lacking [59]. Cryopreservation does not necessarily reduce the bacterial count. In the study of Reda et al. [60], the bacterial content of cryopreserved semen was evaluated. Their study showed a negative effect of an increased bacterial content on sperm motility, viability and morphology with differences between ejaculates although no differences between the bulls were detected. When boar semen was stored for five days in the presence of an antibiotic, there was an increase in the number of certain bacteria, which was associated with a decrease in sperm motility [61].

In this study, we were interested in the potential associations between the bacteria in the male reproductive tract and the overall fertility of the semen from these bulls. We were not studying the effects of these bacteria on the sperm quality during subsequent storage, which is a different topic. Our results showed that two genera, *W5053* and *Lawsonella*, were enriched in the semen samples from the low fertility bull group. These genera were also present among the 20 most abundant bacteria. Bell at al. [62] described *Lawsonella* spp. as Gram-positive, partially acid-fast, non-spore-forming, anaerobic, catalase-positive and pleomorphic bacteria. Three strains were isolated from human abscesses, which were determined to represent a novel genus (*Lawsonella clevelandensis gen. nov., sp. nov.).* Genus *W5053* is also a novel bacteria; more comprehensive information is lacking. However, a higher abundance of both genera were seen in HIV-infected patients without chronic respiratory diseases [63]. The presence of these two bacterial genera in the semen samples of the bulls with a lower fertility potential is of interest for future research with regard to the origin and potential microbial influence on the sperm quality.

The diversity, number and interaction between the bacteria found in this study put 16S rDNA sequencing high on the list of the methods of choice for the diagnostics of bacterial

contamination based on its objectivity and reliability. As most of the bacteria present in bull semen samples originate from the environment or from the mucosa of animals and humans, there is a need for a more effective management of the critical control points during semen collection. However, as 16S sequencing provides information about the presence of bacterial DNA in samples and not specifically about bacterial viability, it can only indicate the likely presence of the bacteria rather than the actual cause of a fertility issue. A possible interaction between the bacteria found in bull semen with a low fertility potential and host bacteria complex interactions would be of interest in future research.

Braga et al. [64] reported that the bacteria of different genera have an influence on microbial community modulation. This microbial coexistence occurs via chemical mediators among bacteria and also between microbes and hosts [65]. Such an interaction may cause alterations in the host physiology [64].

Deines et al. [66] also reported that a host-bacteria interaction, i.e., a host-environment and a bacteria–bacteria interaction, influences the coexistence of microbial species. This study showed that the competitive effect of *Curvibacter* depends on direct contact and indicated that rare microbial community members might be relevant for achieving a native community composition and carrying capacity. Although the genus *Curvibacter* was first mentioned by Ding and Yakota [67], who described three species isolated from well water as the source of origin, there is no previous documentation of its presence in bull semen or other sources. That *Curvibacter* was negatively correlated with other bacterial genera in most cases in this study indicated that this bacterium could be the focus for further research on the influence of the semen microbiota on the fertility of healthy bulls.

The second most negatively correlated bacteria in this study was *Rikenellaceae RC9-gut-group*. It belongs to the *Rikenellacea* family that are recently identified bacteria described as being challenging to culture [68]. This bacterial genus was previously identified in the digestive tract and fecal samples of different animals but not in other types of samples including bull semen. An increased abundance of this bacteria was found in an inflamed human digestive tract but there was no direct indication of their association with disease [69]. The study of Bálingt A et al. [70] showed that *Rikenellaceae RC9-gut-group* with other bacteria increased the sensitivity of the gut to inflammation. Based on the fact that these organisms can be challenging to culture, our current knowledge about these bacteria is based on the information gained from large scale sequencing studies.

The results of the present study indicated that differences in the bacterial microbiota of healthy bulls occur and might be associated with the fertility potential of the bull. Most of the identified bacteria were environmental in origin, indicating that a focus on how bulls are housed and how the semen is processed could help to reduce the bacterial abundance in commercial semen doses. The processing of bull semen should always be performed with a high level of hygiene and microbiological control.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9122431/s1: Table S1: CASA results for the sperm samples from 18 bulls.

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III

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Article

Effect of Some Plant-Based Substances on Microbial Content and Sperm Quality Parameters of Bull Semen

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Abstract: The rapid emergence of antibacterial resistance requires alternatives to antibiotics to be found, including for semen preservation. One of the possible alternatives would be to use plant-based substances with known antimicrobial effects. The objective of this study was to test the antimicrobial effect of pomegranate powder, ginger, and curcumin extract in two concentrations on bull semen microbiota after exposure for <2 h and 24 h. An additional aim was to evaluate the effect of these substances on sperm quality parameters. The bacterial count in semen was low from the beginning; however, a reduction was present for all tested substances compared with control. A reduction in bacterial count in control samples was also observed with time. Curcumin at a concentration of 5%, reduced bacterial count by 32% and was the only substance that had a slight positive effect on sperm kinematics. The other substances were associated with a decline in sperm kinematics and viability. Neither concentration of curcumin had a deleterious effect on sperm viability parameters measured by flow cytometry. The results of this study indicate that curcumin extract at a concentration of 5% can reduce the bacterial count and does not have a negative influence on bull sperm quality.

Keywords: bull semen; pomegranate; ginger; curcumin; antibacterial effect

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1. Introduction

During human history, plant extracts have been used in medicine, pharmaceutical industries and nutrition, as well as for cosmetic and other purposes. The main pharmacological activity of the plants comes from their secondary metabolites; terpenoids, such as phenolic compounds, alkaloids, and sulphur-containing compounds [1]. These chemical compounds were found to protect the plant itself against potential pathogens. Apart from this antimicrobial activity, phytochemicals possess numerous characteristics such as antioxidants, antiviral, antiparasitic, antitumor, antimutagenic, anti-inflammatory, and analgesic properties. Consumption of these plants, or their extracts, as a source of antioxidants showed promising results in patients with fertility disorders [2] as well as for the improvement of reproductive performance in animals and humans.

Good sperm quality depends on many factors that can be classified as environmental and/or endogenous in origin. One of these factors, originating from both sources, is bacterial contamination of semen. Bacterial presence in semen samples negatively influences sperm quality on many levels, decreasing sperm motility and viability, inducing premature acrosome reaction, or causing sperm aggregation. However, these effects are associated with the presence of particular microbes in semen samples [3]. The presence of these microbes in semen is unavoidable because semen collection is not a sterile process, even though strict hygiene measures are taken during semen collection. Therefore, European legislation (European Council Directive EU-2019/6) [4] requires that antibiotics must be added to commercial semen doses for artificial insemination to inhibit microbial growth. Despite achieving a reduction in bacterial growth, many antibiotics have a negative effect

on sperm survival. Therefore, a combination of several antimicrobial agents at low concentrations is recommended. However, this non-therapeutic use of antibiotics in smaller doses can still lead to the development of antimicrobial resistance [5].

A possible alternative to adding antibiotics to the semen could be adding plant-based substances with a known antibacterial effect on different bacterial genera. Various plant extracts showed some antimicrobial properties by increasing the activity of several antioxidative enzymes. One example is pomegranate (*Punica granatum* L.) juice that at various concentrations showed a positive effect on sperm quality parameters after chilling and cryopreservation of bull semen samples [6]. Furthermore, antimicrobial activity of arils from different pomegranate varieties was observed against several bacteria [7]. Curcumin (*Curcuma longa*) extract has been shown to have a positive effect on boar spermatozoa [8], as well as on thawed goat sperm parameters [9]; it also exhibited protective properties on bull spermatozoa by the preservation of motility, mitochondrial activity, and antioxidant characteristics during induced oxidative stress [10] and during cryopreservation [11]. The antibacterial effect of curcumin on *Staphylococcus aureus* has been discussed in detail [12]. In the study by Merati and Farshad [13], adding extract of ginger (*Zingiber officinale*) resulted in improved quality and fertility of frozen-thawed ram epididymal spermatozoa.

Schulze et al. [14] defined criteria that a potential sperm additive needs to meet to be considered as an antimicrobial alternative. These include having broad spectrum antimicrobial action, absence of sperm toxicity, no interference with fertility, high stability, high activity at common semen storage temperatures, low potential to evoke bacterial resistance, ease of application, and economic feasibility. With these criteria in mind, the objective of this study was to test the antibacterial effects of three plant-based substances; pomegranate powder, curcumin, and ginger extract, when added to bull semen extenders at different concentrations. A further aim was to evaluate the effect of the tested substances on sperm quality parameters.

2. Results

Pomegranate powder (P) 5% did not affect CASA (Computer-assisted sperm analysis) parameters at Time 1, whereas ginger (G) 10% and DMSO (Dimethylsulfoxide) (D) 10% and 5% had a significant negative effect on CASA parameters (Table 1). Curcumin (C) in both concentrations, as well as P 10%, had a negative effect on some kinematic parameters although the proportion of motile sperm did not change.

Table 1. CASA parameters of bull semen samples after exposure to various plant-based substances at Time 1.

	Control	P 10%	P 5%	G 10%	G 5%	C 10%	C 5%	D 10%	D 5%
TM	74.95 ± 13.34	74.91 ± 12.83	77.50 ± 11.01	64.49 ± 15.42 ^{b}	68.61 ± 11.90	73.43 ± 13.93	72.54 ± 12.15	61.96 ± 14.68 ^{c}	63.16 ± 15.92 ^{b}
PM	72.56 ± 13.62	72.04 ± 13.58	74.90 ± 11.44	61.61 ± 15.48 ^{b}	65.66 ± 12.23	70.17 ± 14.29	70.04 ± 12.28	58.90 ± 14.87 ^{c}	60.83 ± 15.78 ^{b}
VAP	64.44 ± 10.13	60.45 ± 6.65	65.19 ± 6.91	62.63 ± 8.58	58.81 ± 7.72 ^{a}	64.28 ± 10.27	56.48 ± 9.28 ^{b}	56.13 ± 6.57 ^{b}	50.11 ± 5.17 ^{c}
VCL	119.21 ± 24.20	115.48 ± 14.44	121.07 ± 17.79	122.03 ± 20.76	107.58 ± 17.76	124.08 ± 25.62	101.93 ± 21.30 ^{b}	105.05 ± 15.56 ^{a}	89.37 ± 11.29 ^{c}
VSL	50.23 ± 8.35	45.28 ± 6.80 ^{a}	51.28 ± 5.96	43.05 ± 4.79 ^{c}	44.95 ± 6.09 ^{b}	46.35 ± 7.26	43.12 ± 7.95 ^{c}	39.71 ± 4.85 ^{c}	37.24 ± 4.56 ^{c}
STR	0.78 ± 0.05	0.74 ± 0.06 ^{b}	0.78 ± 0.046	0.69 ± 0.05 ^{c}	0.76 ± 0.04	0.72 ± 0.06 ^{c}	0.76 ± 0.04	0.70 ± 0.05 ^{c}	0.74 ± 0.05 ^{b}
LIN	0.42 ± 0.06	0.39 ± 0.06 ^{b}	0.42 ± 0.06	0.35 ± 0.04 ^{c}	0.42 ± 0.04	0.38 ± 0.06 ^{c}	0.42 ± 0.05	0.37 ± 0.04 ^{c}	0.41 ± 0.04
WOB	0.54 ± 0.04	0.52 ± 0.04 ^{a}	0.54 ± 0.05	0.51 ± 0.03 ^{c}	0.54 ± 0.03	0.52 ± 0.04 ^{a}	0.55 ± 0.04	0.53 ± 0.03	0.56 ± 0.03
ALH	4.38 ± 0.95	4.40 ± 0.62	4.35 ± 0.70	5.09 ± 0.94 ^{c}	4.18 ± 0.76	4.84 ± 1.02 ^{a}	4.01 ± 0.71	4.55 ± 0.78	3.68 ± 0.65 ^{c}
BCF	25.24 ± 2.79	7.28 ± 10.03 ^{b}	23.21 ± 20.44 ^{a}	18.96 ± 14.33 ^{c}	29.95 ± 18.6 ^{b}	32.52 ± 25.01 ^{c}	40.04 ± 21.86 ^{b}	35.47 ± 19.09 ^{c}	35.37 ± 16.97 ^{c}

Abbreviations: P—pomegranate powder, G—ginger, C—curcumin, D—Dimethyl sulfoxide, TM—Total motility, PM—Progressive motility, VCL—curvilinear velocity, VAP—average path velocity, VSL—straight line velocity, LIN—linearity, STR—straightness, WOB—wobble, BCF—beat cross frequency, ALH—amplitude of lateral head displacement; Superscript letters denote significant differences within a row compared with control: a—$p < 0.05$; b—$p < 0.01$; c—$p < 0.001$.

After 24 h of incubation, both P samples (10% and 5%) and G 10% showed decreased values for CASA parameters compared with the control. The remaining substances did not negatively influence the CASA parameters; in fact, C 5% increased the values of most CASA parameters after 24 h of exposure (Table 2).

Table 2. CASA parameters of bull semen samples after exposure to various plant-based substances at Time 2.

	Control	P 10%	P 5%	G 10%	G 5%	C 10%	C 5%	D 10%	D 5%
TM	37.75 ± 22.95	7.28 ± 10.04 ^{c}	23.21 ± 20.44 ^{b}	18.96 ± 14.33 ^{c}	29.95 ± 8.60	32.52 ± 25.01	40.06 ± 21.86	35.47 ± 19.09	35.37 ± 16.97
PM	33.13 ± 23.66	5.19 ± 8.78 ^{c}	19.01 ± 20.12 ^{b}	15.64 ± 13.21 ^{c}	26.29 ± 17.63	29.81 ± 24.06	37.09 ± 21.28	32.31 ± 18.75	32.04 ± 16.66
VAP	41.43 ± 11.45	24.48 ± 14.20 ^{c}	32.33 ± 11.77 ^{b}	35.43 ± 10.34	37.41 ± 9.41	41.66 ± 6.58	40.30 ± 7.36	40.50 ± 8.01	36.94 ± 9.85
VCL	74.87 ± 24.16	38.55 ± 22.08 ^{c}	55.79 ± 20.13 ^{c}	60.17 ± 19.36 ^{b}	65.44 ± 18.11	72.11 ± 15.54	69.23 ± 13.88	71.83 ± 17.46	65.61 ± 19.22
VSL	30.20 ± 9.41	18.18 ± 11.51 ^{c}	23.74 ± 9.99 ^{a}	24.06 ± 7.79 ^{b}	27.40 ± 7.40	30.38 ± 5.28	30.51 ± 6.57	27.53 ± 6.47	26.09 ± 7.32
STR	0.71 ± 0.07	0.61 ± 0.29 ^{a}	0.70 ± 0.15	0.65 ± 0.14	0.73 ± 0.07	0.72 ± 0.05	0.75 ± 0.05	0.67 ± 0.07	0.68 ± 0.15
LIN	0.40 ± 0.06	0.40 ± 0.22	0.41 ± 0.12	0.39 ± 0.10	0.42 ± 0.06	0.43 ± 0.06	0.44 ± 0.05	0.38 ± 0.06	0.38 ± 0.10
WOB	0.56 ± 0.05	0.53 ± 0.26	0.56 ± 0.13	0.57 ± 0.13	0.57 ± 0.05	0.58 ± 0.08	0.58 ± 0.04	0.57 ± 0.05	0.54 ± 0.11
ALH	3.16 ± 0.95	2.09 ± 1.40 ^{c}	2.61 ± 1.18	2.82 ± 0.91	2.78 ± 0.83	3.12 ± 0.62	2.85 ± 0.61	3.26 ± 0.64	2.81 ± 0.89
BCF	20.21 ± 2.90	14.14 ± 7.78 ^{c}	17.52 ± 4.25	18.31 ± 4.86	19.65 ± 2.98	20.52 ± 2.92	20.81 ± 2.39	19.06 ± 2.37	18.89 ± 4.49

Abbreviations: P—pomegranate powder, G—ginger, C—curcumin, D—Dimethyl sulfoxide, TM—Total motility, PM—Progressive motility, VCL—curvilinear velocity, VAP—average path velocity, VSL—straight line velocity, LIN—linearity, STR—straightness, WOB—wobble, BCF—beat cross frequency, ALH—amplitude of lateral head displacement; Superscript letters within a row denote significant differences compared with control: a—$p < 0.05$; b—$p < 0.01$; c—$p < 0.001$.

Substances G 10%, G 5%, D 10%, and D 5% had a negative influence on all FC (Flow cytometry) parameters except chromatin integrity at 0 h (Table 3), apart from P 10%, which had a negative influence on mitochondrial membrane potential and chromatin integrity, and P 5%, which had a negative influence only on chromatin integrity. Curcumin 10% and 5% did not have a deleterious effect on sperm quality parameters. Similar results were observed after 24 h of exposure (Table 4).

Table 3. Flow cytometry results of bull semen samples at Time 1 after exposure to various plant-based substances.

	Control	P 10%	P 5%	G 10%	G 5%	C 10%	C 5%	D 10%	D 5%
MI Living	78.77 ± 12.65	75.97 ± 13.17	76.16 ± 13.30	11.03 ± 11.86 ^{c}	26.92 ± 17.29 ^{c}	75.24 ± 13.90	75.97 ± 14.31	8.56 ± 9.27 ^{c}	24.06 ± 14.34 ^{c}
MI Dying	3.75 ± 6.96	1.76 ± 3.71	0.82 ± 0.54	23.76 ± 13.95 ^{c}	15.07 ± 12.48 ^{c}	0.91 ± 0.84	0.59 ± 0.30	18.24 ± 13.65 ^{c}	10.07 ± 7.77 ^{b}
MI Dead	17.48 ± 11.50	22.27 ± 12.53	23.02 ± 13.24	65.21 ± 15.76 ^{c}	58.01 ± 17.78 ^{c}	23.85 ± 13.67	23.44 ± 14.28	73.20 ± 14.76 ^{c}	65.87 ± 15.16 ^{c}
ROS Live SO^{-}	60.83 ± 16.04	59.50 ± 12.32	63.26 ± 13.19	10.15 ± 13.40 ^{c}	19.15 ± 14.43 ^{c}	59.79 ± 15.24	61.10 ± 14.15	7.87 ± 10.03 ^{c}	18.55 ± 15.39 ^{c}
ROS Live SO^{+}	12.27 ± 11.50	10.35 ± 4.53	9.34 ± 3.60	58.62 ± 18.01 ^{c}	50.06 ± 17.04 ^{c}	10.91 ± 5.15	9.93 ± 4.26	59.32 ± 17.46 ^{c}	48.74 ± 20.20 ^{c}
ROS Dead SO^{+}	21.22 ± 12.62	24.24 ± 12.12	22.41 ± 12.10	22.14 ± 12.35	21.35 ± 11.85	23.73 ± 13.50	23.45 ± 12.66	23.40 ± 12.21	23.07 ± 11.94
ROS Dead $H_2O_2^{-}$	28.08 ± 12.82	31.35 ± 12.29	29.32 ± 12.44	48.33 ± 13.30 ^{c}	47.17 ± 12.53 ^{c}	31.52 ± 14.10	30.87 ± 13.13	50.14 ± 14.39 ^{c}	46.60 ± 11.22 ^{c}
ROS Dead $H_2O_2^{+}$	0.11 ± 0.26	0.03 ± 0.02	0.03 ± 0.01	0.06 ± 0.03 ^{c}	0.04 ± 0.02 ^{c}	0.10 ± 0.11	0.05 ± 0.04	0.03 ± 0.01 ^{c}	0.43 ± 2.23 ^{c}
ROS Live $H_2O_2^{-}$	71.8 ± 12.89	68.61 ± 12.30	70.65 ± 12.44	51.61 ± 13.31 ^{c}	52.78 ± 12.53 ^{c}	68.37 ± 14.08	69.07 ± 13.12	49.82 ± 14.39 ^{c}	52.77 ± 11.28 ^{c}
ROS Live $H_2O_2^{+}$	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.01	0.01 ± 0.02	0.00 ± 0.01	0.19 ± 1.01
MMP High RA	67.23 ± 13.07	59.12 ± 19.97 ^{a}	65.99 ± 15.57	12.24 ± 11.07 ^{c}	22.28 ± 14.52 ^{c}	66.88 ± 13.72	67.62 ± 13.91	11.63 ± 13.36 ^{c}	23.33 ± 13.57 ^{c}
MMP Low RA	32.77 ± 13.07	40.88 ± 19.97 ^{a}	34.01 ± 15.57	87.76 ± 11.07 ^{c}	77.72 ± 14.52 ^{c}	33.12 ± 13.72	32.38 ± 13.91	88.37 ± 13.36 ^{c}	76.67 ± 13.57 ^{c}
DFI %	9.27 ± 4.68	22.20 ± 11.23 ^{c}	22.11 ± 11.77 ^{c}	9.34 ± 4.04	9.62 ± 4.22	9.50 ± 4.38	9.41 ± 4.32	9.75 ± 4.43	9.49 ± 4.25

Abbreviations: Control; P—pomegranate powder, G—ginger, C—curcumin, D—Dimethyl sulfoxide; MI—Membrane integrity, ROS—Reactive oxygen species, SO—Superoxide, MMP—Mitochondrial membrane potential, RA—Respiratory activity, DFI—DNA fragmentation index; Superscript letters within a row denote significant differences compared with control: a—$p < 0.05$; b—$p < 0.01$; c—$p < 0.001$.

Table 4. Flow cytometry results of bull semen samples at Time 2 after exposure to various plant-based substances.

	Control	P 10%	P 5%	G 10%	G 5%	C 10%	C 5%	D 10%	D 5%
MI Living	76.76 ± 13.72	68.57 ± 17.20 ^{a}	73.03 ± 14.14	9.28 ± 11.73 ^{c}	27.60 ± 18.03 ^{c}	73.31 ± 14.28	74.05 ± 13.53	8.07 ± 9.26 ^{c}	25.54 ± 17.33 ^{c}
MI Dying	1.98 ± 2.42	3.62 ± 9.15	1.97 ± 3.50	14.68 ± 10.55 ^{c}	12.58 ± 7.93 ^{c}	1.43 ± 1.05	0.97 ± 1.10	8.79 ± 5.70 ^{b}	8.80 ± 14.72 ^{b}
MI Dead	21.26 ± 14.38	27.81 ± 14.45	25.00 ± 13.97	76.04 ± 15.42 ^{c}	59.82 ± 19.70 ^{c}	25.26 ± 14.00	24.98 ± 13.42	83.14 ± 11.15 ^{c}	65.66 ± 20.00 ^{c}
ROS Live SO^{-}	59.89 ± 14.35	52.06 ± 15.25	52.77 ± 16.65	8.61 ± 12.06 ^{c}	21.55 ± 16.54 ^{c}	57.28 ± 17.55	56.83 ± 18.07	7.66 ± 10.65 ^{c}	17.85 ± 15.60 ^{c}
ROS Live SO^{+}	12.00 ± 6.39	15.40 ± 6.47	15.00 ± 7.68	51.99 ± 16.36 ^{c}	44.12 ± 17.35 ^{c}	11.90 ± 5.5	11.74 ± 5.23	54.67 ± 16.35 ^{c}	45.98 ± 16.37 ^{c}
ROS Dead SO^{+}	21.63 ± 12.9	24.30 ± 13.33	25.81 ± 12.46	27.28 ± 11.41	23.39 ± 11.53	25.78 ± 15.37	25.14 ± 15.13	26.63 ± 11.26	24.50 ± 12.08
ROS Dead $H_2O_2^{-}$	31.01 ± 14.51	37.77 ± 4.57	38.03 ± 14.5	62.71 ± 12.97 ^{c}	53.33 ± 12.04 ^{c}	41.64 ± 19.64	36.59 ± 17.94	59.21 ± 14.34 ^{c}	56.12 ± 12.66 ^{c}
ROS Dead $H_2O_2^{+}$	0.09 ± 0.14	0.03 ± 0.02 ^{b}	0.03 ± 0.02 ^{b}	0.05 ± 0.03	0.04 ± 0.01 ^{a}	0.11 ± 0.14	0.06 ± 0.07	0.02 ± 0.01 ^{c}	0.02 ± 0.01 ^{c}
ROS Live $H_2O_2^{-}$	68.90 ± 14.54	62.19 ± 14.58	61.93 ± 14.5	37.23 ± 12.97 ^{c}	46.30 ± 11.97 ^{c}	58.25 ± 19.69 ^{b}	63.34 ± 17.94	40.77 ± 14.34 ^{c}	43.85 ± 12.66 ^{c}
ROS Live $H_2O_2^{+}$	0.00 ± 0.01	0.00 ± 0.01	0.01 ± 0.01	0.00 ± 0	0.00 ± 0	0.00 ± 0	0.01 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
MMP High RA	40.57 ± 26.34	26.59 ± 18.22 ^{b}	34.80 ± 21.45	5.82 ± 5.03 ^{c}	14.82 ± 15.06 ^{c}	36.93 ± 28.19	42.03 ± 25.88	9.80 ± 16.63 ^{c}	21.05 ± 23.28 ^{c}
MMP Low RA	59.43 ± 26.34	73.41 ± 18.22 ^{b}	65.20 ± 21.45	94.18 ± 5.03 ^{c}	85.18 ± 15.06 ^{c}	63.07 ± 28.19	57.97 ± 25.88	90.20 ± 16.63 ^{c}	78.95 ± 23.28 ^{c}
DFI %	20.46 ± 26.79	23.56 ± 12.56	22.53 ± 11.37	36.99 ± 32.54 ^{a}	35.87 ± 33.61	25.21 ± 30.41	20.17 ± 26.51	39.70 ± 29.64 ^{b}	41.10 ± 32.70 ^{b}

Abbreviations: Control; P—pomegranate powder, G—ginger, C—curcumin, D—Dimethyl sulfoxide; MI—Membrane integrity, ROS—Reactive oxygen species, SO—Superoxide, MMP—Mitochondrial membrane potential, RA—Respiratory activity, DFI—DNA fragmentation index; Superscript letters denote significant differences within a row compared with control: a—$p < 0.05$; b—$p < 0.01$; c—$p < 0.001$.

There was considerable variation in the number of viable aerobic bacteria (colony forming units per mL; CFU/mL) between individual bulls, although the majority (80%) of bulls had an average bacterial count lower than 100 CFU/mL before exposure to the different plant substances. Reduction in CFU with exposure time was common for all tested animals (Figures 1 and 2 for Time 1 and Time 2, respectively).

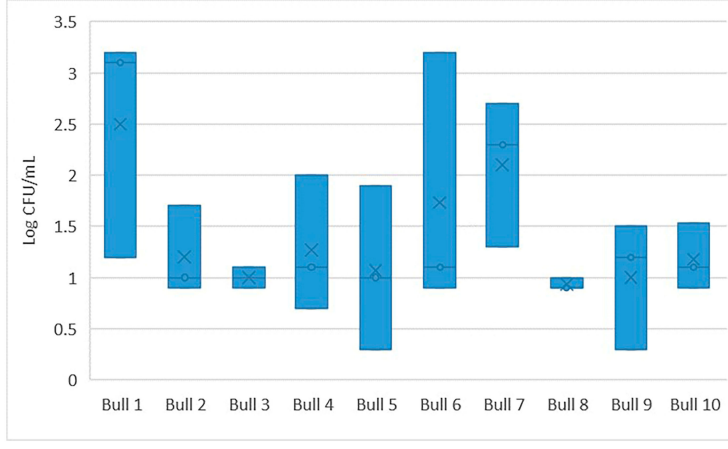

Figure 1. Number of bacteria (Log CFU/mL) in control samples (n = 3) for individual bulls at Time 1.

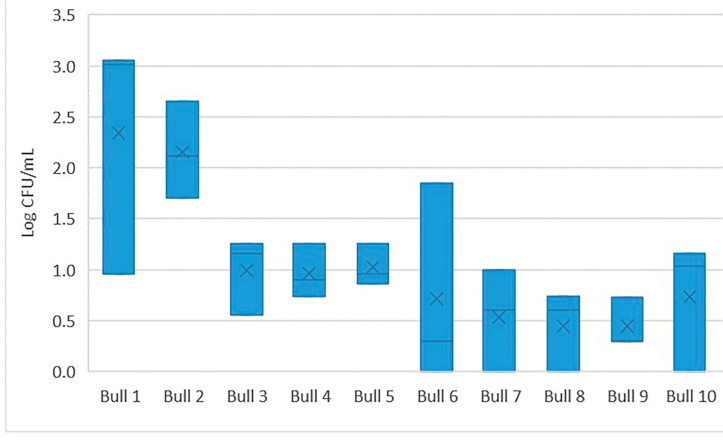

Figure 2. Number of bacteria (Log CFU/mL) in control samples (n = 3) for individual bulls at Time 2.

A reduction in the number of CFU during exposure was also observed for all tested substances as well as controls (Table 5).

The greatest bacterial reduction was observed after adding DMSO 5% (54%) and G 10% (42%) at Time 1. On the other hand, both P concentrations and C 10% showed the least bacterial reduction. A similar trend was seen at Time 2.

Table 5. Number of bacteria (CFU/mL) in the semen before (control) and after exposure of different substances at Time 1 and Time 2 at 5 °C.

Substances	Time 1 Mean (Range) Log CFU/mL	Time 1 Reduction [a] CFU/mL (%)	Time 2 Mean (Range) Log CFU/mL	Time 2 Reduction [a] CFU/mL (%)	Reduction Time 1 vs. Time 2 CFU/mL (%)
Control	2.27 (0.3–3.2)		2.00 (0.0–3.1)		46%
P 5%	2.16 (0.0–3.3)	22%	2.01 (0.0–3.3)	−3%	28%
P 10%	2.15 (0.0–3.1)	25%	1.98 (0.0–3.2)	5%	31%
G 5%	2.12 (0.0–3.2)	29%	1.91 (0.0–3.0)	20%	39%
G 10%	2.03 (0.0–3.2)	42%	1.84 (0.0–3.0)	31%	35%
C 5%	2.10 (0.0–3.2)	32%	1.91 (0.0–3.1)	20%	37%
C 10%	2.18 (0.0–3.3)	20%	1.98 (0.0–3.2)	6%	37%
D 5%	1.93 (0.0–3.1)	54%	1.79 (0.0–2.9)	38%	27%
D 10%	2.13 (0.0–3.1)	28%	1.74 (0.0–2.9)	46%	59%

Abbreviations: P—pomegranate powder, G—ginger, C—curcumin, D—dimethyl sulfoxide, CFU—Colony forming units; [a] Note: The percentage reduction between control and individual tested substances is calculated on the absolute values and not on the log-transformed values and presented in columns Time 1 Reduction and Time 2 Reduction. Bacterial reduction during storage is calculated on the absolute values within the row; presented in column Reduction Time 1 vs. Time 2.

3. Discussion

The present study was conducted to determine whether three plant-based substances with reported antimicrobial activity could be used in semen extenders for bull semen to inhibit bacterial growth.

Our results showed that some of the plant-based additives, pomegranate, curcumin, and ginger, had a detrimental effect on sperm motility, particularly after exposure for 24 h. Oxidative stress, which often arises during semen storage, significantly reduces sperm function and compromises sperm fertilizing ability by inducing oxidative damage to proteins, lipids, and nucleic acids [15]. A positive relationship was reported between the antioxidant capacity of seminal plasma and both sperm concentration and total motility [16]. Moreover, cryopreservation was shown to damage sperm structure and thus sperm function. Sperm storage, on the other hand, leads to the overproduction of ROS (Reactive oxygen species), thus to oxidative stress, compromising sperm integrity and fertilizing ability. Addition of a semen extender during preservation leads to a dilution of the antioxidant capability of the seminal plasma. Several compounds have been added to semen during preparation to increase antioxidative properties. Plant extracts showed promising results in the preservation of semen as a source of antioxidants; this effect is both economic and organic. Another factor that causes a decline in sperm parameters during semen storage is bacterial contamination of the semen prior to cryopreservation [17].

Pomegranate is a polyphenol-rich fruit and, as such, has been used for its antioxidative and antibacterial properties [18]. Besides its positive effect on sperm quality when added to semen extender [6] or as food supplement [19], pomegranate has been tested for its antibacterial properties against several bacteria; there was a strong correlation between total phenolic content and antimicrobial activity [20]. The level of polyphenolic compounds in pomegranates depends on the part of the plant used and which extraction method was performed. Duman et al. [7] reported that antimicrobial activity even differs between different pomegranate varieties. For these reasons, we chose to test a commercially available pomegranate powder with known active substances. In the study by El-Sheshtawy et al. [6], five concentrations (10%, 20%, 30%, 40%, and 50%) of pomegranate extract were added to a semen extender and the effect on bull semen quality parameters was evaluated. Concentrations of 10% and 20% improved frozen-thawed semen quality; the highest concentration had a deleterious effect on sperm quality. However, neither P 5% nor P 10 % had an effect on CASA parameters and there was no indication of an effect on sperm quality from the flow cytometry results. In contrast, in our study, a deleterious effect of both P 5% and P 10% on CASA parameters was noted after 24 h of exposure. Interestingly, flow cytometry results for P 5% at Time 2 (after 24 h exposure to the test substances) indicated that sperm

quality parameters were preserved although they were decreased for P 10% compared with controls.

Ginger, as an additive to semen extenders, has been reported to have a positive effect on spermatozoa, improving quality and fertility potential of ram thawed semen [13]. It had a strong antimicrobial activity against opportunistic pathogenic and multi-resistant bacteria [21,22]. However, our results showed a negative effect of ginger on both motility and viability parameters of the spermatozoa. This could be due to dissolving the ginger extract in DMSO, which also had a negative effect on sperm quality parameters in the present study. It was previously considered that DMSO would have a protective effect during sperm cryopreservation, by penetrating and dehydrating the spermatozoa to minimize intracellular ice formation [23], but it caused damage during processing of buffalo spermatozoa [24]. Increasing the DMSO concentration in the semen extender significantly decreased the proportion of intact acrosomes. The results of our study are in agreement with these findings, since G 10% and 5%, as well as D 10% and 5%, had a negative effect on all FC parameters except on chromatin at 0 h, compared with P 10% and 5%, which only negatively influenced chromatin integrity. In the study by Farshad et al. [25], DMSO concentrations were lower than in the present study, since we added enough DMSO to dissolve the ginger extract. The negative effect of ginger was probably due to the DMSO in which it was dissolved. Similar results were observed when the antibacterial effect of these substances was tested. Both G and DMSO had a strong antibacterial effect compared with other substances.

Curcumin was previously tested as a supplement when added to semen prior to cryopreservation, based on its potent protective and antioxidative properties [8]. The same conclusion was reached in previous studies conducted on sperm samples from different animal species; curcumin had significant dose-dependent protective and antioxidative characteristics [8]. However, there was no such effect of curcumin on bull spermatozoa in our study. This could be due to the use of 70% ethanol to dissolve curcumin powder compared with other studies where DMSO was used. In the study by Tvrdá et al. [26], curcumin in different concentrations was able to prevent the decrease of some sperm quality parameters during incubation, compared with controls. However, curcumin at either concentration in our study was the only substance that did not have a negative effect on any sperm quality parameters; most of the CASA parameters were not adversely affected by C 5% after 24 h of exposure. A bacterial reduction of more than 30% was observed when C 5% at < 2 h of incubation was used. To our knowledge, there are no published results about such an antibacterial effect of curcumin in sperm samples.

The reason for the lack of a positive effect of the tested substances on sperm quality parameters could be due to our samples being tested for the first time at 24 h after semen collection. During transport, the semen samples were extended in the ratio 1.1; which could be insufficient to meet the metabolic needs of the spermatozoa, even at the low temperature. On the other hand, the condition of the packets containing the semen samples was not strictly controlled during transport to the laboratory. There is evidence that different handling conditions of human semen samples negatively affected all sperm quality parameters with time [27]. Irrespective of the extender and the storage conditions used, semen handling and preservation negatively affected sperm quality [17]. The same authors concluded that the concentration of the plant substance to be used is influenced by the extraction method and that the same extract concentration can have different effects on sperm quality due to the sperm preservation method used (refrigeration vs. cryopreservation). However, in the present study, a reduction in bacterial count (CFU/mL) with time was observed, irrespective of treatment, even though the number of bacteria was low from the beginning in most of the bull semen samples. All tested substances were reported to have an antibacterial effect on the particular cultured bacterial strains [28]; however, no information about the antibacterial effect on the same bacteria in semen samples was available. For some other plant-based substances, which were tested for their antibacterial effect in semen samples, an interspecies variation was found. An example is rosmarinic

acid that was reported to have an antibacterial effect in boar semen [29,30] but did not have such an effect in bull semen [31]. Therefore, there may be some components of bull semen that prevent the antibacterial action of rosmarinic acid that is reported to occur when added to boar semen.

Bacteria negatively affect sperm quality, by competing directly with spermatozoa for nutrients supplied by the semen extender, or by the production of toxic metabolic byproducts and endotoxins [32]. Government and regional directives stipulate which antibiotics, and what concentrations, should be added to semen doses for international trade [4]. Usually, the concentrations used are lower than therapeutic ones. The main reason for this low dose and the use of several antibiotics is to avoid the toxic effect of some antibiotics on spermatozoa and to provide a wider range of antimicrobial protection. When a combination of antibiotics is used, an adequate antimicrobial effect can be achieved with no apparent toxic effect on spermatozoa [33]. However, even this sub-therapeutic dose of antibiotics can lead to the development of antimicrobial resistance. It would be interesting to see whether a combination of the plant-based substances mentioned here could achieve an antimicrobial effect without sperm toxicity.

4. Materials and Methods

4.1. Semen Collection, Proceeding and Exposure to the Tested Substances

Semen samples from 10 dairy bulls (9 Swedish Red and 1 Holstein Friesian), aged 1 to 4 years, were collected twice a week using an artificial vagina according to the standard husbandry method, during October–December 2021, giving a total of 30 ejaculates (3 ejaculates per bull). The bulls were housed under standard husbandry conditions at VikingGenetics, (Skara, Sweden). The semen was extended 1:1 in Andromed extender free of antibiotics (AndroMed® CSS one-step 200mL, Minitüb GmbH, Tiefenbach, Germany), before transportation to the Swedish University of Agricultural Sciences (SLU), overnight at 6 °C, in an insulated box containing a cold pack. Further processing of semen samples and analyses were performed in the laboratories at the Swedish University of Agriculture Sciences, as presented in Figure 3 and described in the text.

Sperm concentration was measured using a Nucleocounter-SP 100 (Chemometec, Allerød, Denmark) as follows: 50 µL of semen sample were mixed with 5 mL reagent S100 (Chemometec, Allerød, Denmark) to disrupt sperm membranes. This mixture was then loaded into a cassette containing the fluorescent dye propidium iodide. Thereafter, the cassette was inserted into the reading chamber of the fluorescence meter. The sperm concentration appeared on the display. Based on the sperm concentration of each sample, sufficient Andromed extender free of antibiotics was added to give a final concentration of 65×10^6 spermatozoa per mL (spz/mL).

Aliquots of semen samples were exposed to pomegranate, curcumin, or ginger for up to 2 h (Time 1) and 24 h (Time 2) before evaluating sperm quality and an antibacterial effect of the substance. After removing the first aliquots for evaluation at Time 1, the remaining samples were stored at 5 °C until the next day.

Semen collection with an artificial vagina is a routine agricultural practice and, therefore, does not require ethical approval in Sweden; the bulls at the AI (Artificial Insemination) station were not considered to be experimental animals.

4.2. Preparation of Pomegranate, Curcumin and Ginger

Ginger extract and curcumin were purchased from Sigma-Aldrich GmbH (Stockholm, Sweden), and commercially available pomegranate powder WellAware Granatäpple; Uppsala, Sweden was used. All tested substances were prepared as described and subsequently added to give a final proportion of 10% and 5% in diluted semen samples containing 30×10^6 spz/mL, to test their antioxidative and antimicrobial properties. Pomegranate powder (20 g) was dissolved in 100 mL distilled water and mixed using a magnetic stirrer for 20 min. The choice of 20% pomegranate solution was based on a pilot experiment in which this proportion was demonstrated to give the best antimicrobial effect on pure

bacterial colonies isolated from bull semen [34]. After mixing with a magnetic stirrer, the reconstituted pomegranate solution was centrifuged in 50 mL sterile plastic test tubes for 5 min at 4000× *g* on 22 °C followed by filtration using 0.25 µm pore size filter (Minisart® Syringe Filter, Göttingen, Germany) to remove all potential microorganisms and particles.

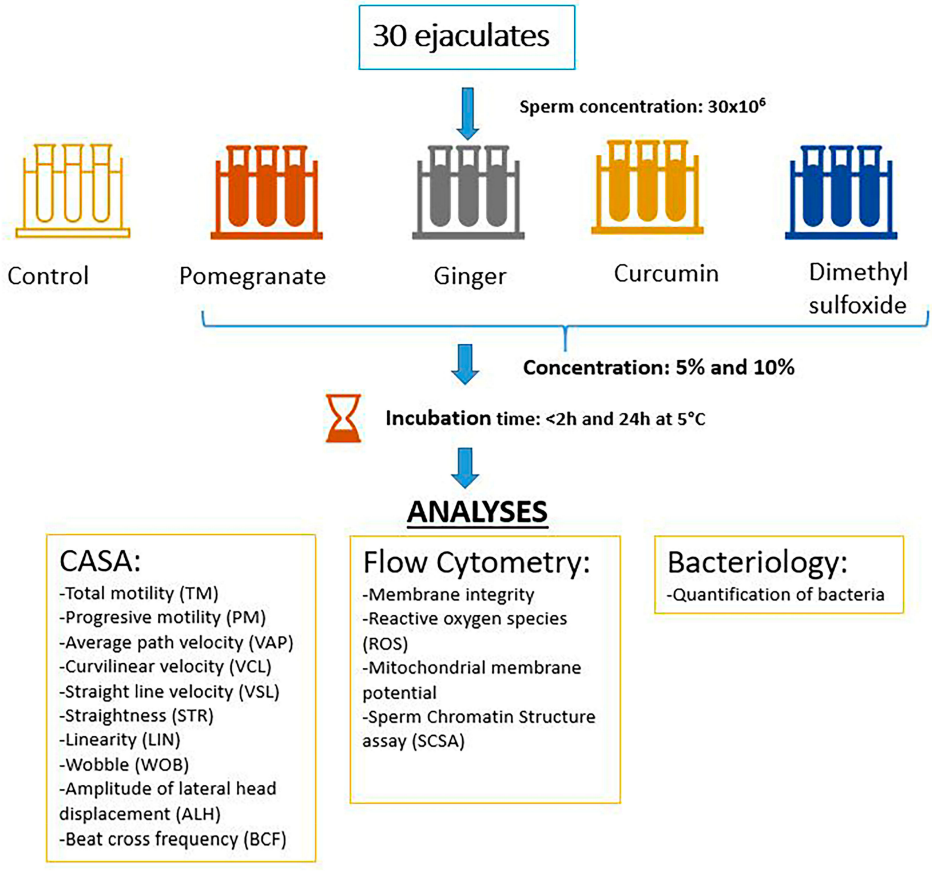

Figure 3. Incubation time and analyses after adding various plant-based substances to bull semen 24 h after collection. In total, semen samples from 10 bulls were used (three ejaculates per bull).

Curcumin (Sigma-Aldrich GmbH, Stockholm, Sweden; 5 mg) was dissolved in 500µL ethanol (70%) in a plastic test tube, and was then diluted to 15 mL with peptone water (Dilucup) to a concentration of 667 µg/mL. Thereafter, 5 mL aliquots were centrifuged for 5 min at 4000× *g* and 22 °C; the supernatant was used.

Ginger extract (250 µL) was dissolved in 6.0 mL dimethyl sulfoxide (DMSO) in a plastic test tube. Thereafter, the mixture was diluted to 13 mL with peptone water and 5 mL aliquots were centrifuged for 5 min at 4000 G and 22 °C. The supernatant was filtered through a 20 Minisart® Syringe Filters (0.25 µm pore size) to eliminate large particles in the ginger dilution.

In this study, curcumin was dissolved in 70% ethanol according to the suggestion of the manufacturer (Sigma-Aldrich GmbH, Stockholm, Sweden). For ginger, DMSO was

suggested as solvent due to its low toxicity, and its ability to dissolve both organic and inorganic compounds [35]. The ginger extract used in this study could not be dissolved in either ethanol or in peptone water, and therefore DMSO was used. The percentages of solvents in the final sperm sample mixtures were 0.33% and 0.17% for ethanol and 4.62% and 2.31% for DMSO, in the 10% and 5% groups, respectively. A previous study [36], evaluated the effect of various solvents on bacterial growth, determining the Minimum Inhibitory Concentration (MIC) of various antimicrobials; the authors stated that ethanol is generally considered safe below 3%. However, the authors did suggest that this cannot be accepted as a general fact for all test organisms. The final concentration of ethanol in semen samples in our study was lower than 0.5%.

4.3. Assessment of Sperm Motility Using CASA

Motility evaluation was performed using a SpermVision analyzer (Minitüb GmbH, Tiefenbach, Germany) connected to a Zeiss microscope with a heated stage (38 °C). Semen samples were equilibrated to room temperature before motility analysis. Sperm motility was analyzed in eight fields (at least 850 spermatozoa in total) using the SpermVision software program version 3.8 with settings adjusted for bull spermatozoa, in a 5-µL aliquot of the semen sample on a warm slide covered with an 18 × 18 mm cover slip (VMR, Leuven, Belgium). The following parameters were used to analyze motility: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), linearity (LIN, as VSL/VCL), straightness (STR, as VSL/VAP), wobble (WOB, as VAP/VCL), beat cross frequency (BCF, Hz), and amplitude of lateral head displacement (ALH, µm). Images were obtained at 200 × magnification using a phase contrast microscope. Particles with an area ranging from 20 to 100 μm^2 were identified as cells and were included in the analysis. Spermatozoa were considered as immotile if the area under curve (AOC) was < 5, BCF < 0.2, and VSL < 0.2; they were considered to be locally motile if spermatozoa covering a straight-line distance (DSL) was < 4.5.

4.4. Flow Cytometry

Flow cytometric (FC) analysis was performed using a FACSVerse™ flow cytometer (BD Biosciences, Becton Dickinson and Company, San Jose, CA, USA). A blue laser emitting at 488 nm and a violet laser emitting at 405 nm were used to excite the fluorescent stains. Green fluorescence (FL1) was detected with a band-pass filter (527/32 nm), as was orange fluorescence (FL2, 586/42 nm); red fluorescence (FL3) was collected using a 700/54 nm band-pass filter, while blue fluorescence (FL5) was collected using a 528/45 nm band-pass filter. The data obtained in the sperm chromatin structure assay were further analyzed using FCS Express 5 software (De Novo, Glendale, CA, USA).

4.4.1. Assessment of Membrane Integrity Using Flow Cytometry

Evaluation of sperm plasma membrane integrity was performed using SYBR14 and propidium iodide (PI) (Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA). Aliquots of each sample were adjusted to a sperm concentration of approximately 2×10^6 sperm cells/mL in 300 µL of buffer B (patent pending; J.M. Morrell and H. Rodriguez-Martinez). Thereafter, 0.5 µL of 1 mM SYBR14 was diluted 50 times with Buffer B and 1.2 µL was added to each sperm sample. The sperm samples were also stained with 3 µL of 2.4 mM PI. The stained aliquots were incubated at 38 °C for 10 min before evaluation was performed and the proportions of membrane intact, membrane damaged, and intermediate populations were enumerated.

4.4.2. Assessment of Reactive Oxygen Species

Hydroethidine (HE; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) and 20, 70 -dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) were used to detect superoxide (O^{2-}) and hydrogen peroxide (H_2O_2),

respectively, while Hoechst 33258 (HO) was added to permit the simultaneous differentiation of living and dead cells. Aliquots (300 µL) of semen extended to a concentration of approximately 2×10^6 spermatozoa/mL (spz/mL) with Buffer B were stained using each of the following: 3 µL of HO (40 mM), 3 µL HE (40 mM), and 3 µL DCFDA, (2 mM). The samples were gently mixed and incubated at 38 °C for 30 min before analysis. Using dot-plots for HO/HE and HO/DCFDA, the following populations were quantified: ROS Live SO^-; ROS Live SO^+; ROS Dead SO^+; ROS Dead $H_2O_2{}^-$; ROS Dead $H_2O_2{}^+$; ROS Live $H_2O_2{}^-$; and ROS Live $H_2O_2{}^+$.

4.4.3. Mitochondrial Membrane Potential

Sperm samples were diluted with Buffer B in order to obtain a concentration of 2.5×10^6 spz/mL. In order to evaluate the mitochondrial potential, 1.2 µL JC-1 (stock 3 mM) was mixed with 300 µL sperm aliquot and incubated for at least 30 min at 38 °C before analyses. JC-1 fluorescence was measured in the FL1 and FL2 channels of the flow cytometer. A total number of 10,000 cells was evaluated and classified as percentages in two distinct groups: sperm cells with high respiratory activity emitting orange florescence, and low respiratory activity emitting green florescence.

4.4.4. Sperm Chromatin Structure Assay

Chromatin integrity was evaluated using the sperm chromatin structure assay (SCSA). The test uses the metachromatic dye acridine orange (AO) to assess the susceptibility of sperm deoxyribonucleic acid (DNA) to acid-induced denaturation. The DNA fragmentation index (%DFI) is calculated and expressed as the proportion of cells with a high ratio of denatured, single stranded DNA; %DFI = (red fluorescence/[green fluorescence + red fluorescence]) × 100. The procedure, media preparation, buffers, and solutions used in the assay have been described in detail previously [37]. Briefly, 50 µL of the semen sample was mixed with the same amount of Tris, sodium chloride, and ethylene-diaminetetraacetic acid buffer (TNE buffer) and immediately transferred to a liquid nitrogen container for snap-freezing. The samples were stored at −80 °C until analysis. They were thawed on ice, an aliquot (10 µL) was mixed with 90 µL of TNE, and 200 µL of acid-detergent solution. Exactly 30 s later, the sample was stained by adding 600 µL of AO staining solution. The stained samples were analyzed within 3–5 min of AO staining.

4.5. Bacterial Quantification

The total number of viable aerobic bacteria in the semen samples was analyzed according to NMKL 86, 5 Ed., 2013, with slight modifications. In brief, 1 mL of each sperm sample was transferred to 1 mL of diluent. The samples were homogenized using a vortex (Saveen & Werner, Malmö, Sweden). The diluent was prepared at SLU using 1 g peptone and 8.5 g NaCl per liter Milli-Q H2O and was autoclaved at 121 °C for 15 min. Plate count agar (PCA) (Oxoid, Basingstoke, UK) was melted in boiled water and thereafter placed in a 48 °C water bath to keep it liquid until required. A 2-fold serial dilution was prepared until 1/4 of the sperm sample was obtained, 1.0 mL from each dilution was pour plated into a petri dish measuring 9 cm in diameter and mixed with 10–15 mL melted PCA. When the agar in the petri dishes had solidified, a further 10–15 mL of PCA was added to each dish as an over layer to avoid swarming and facilitate enumeration, and the petri dishes were moved gently to distribute the bacteria evenly over the plates. After solidification of the agar, the plates were incubated at 30 °C for 72 h. The number of viable bacteria was quantified from the plates with more than 10 but less than 250 colonies. The total number of CFU was calculated from three successive dilutions using a colony counter (Gerber instruments, Im Langhag, Switzerland).

The total bacterial count was assessed in aliquots taken 2 h after adding the plant-based substances (Time 1) and again after 24 h (Time 2).

4.6. Statistical Analysis

The model contained bulls, exposure time, and substances as fixed factors. Interactions were tested between different bulls, substances and time, but were removed if there was no significance. Differences between control and tested substances for CASA and Flow cytometry sperm quality parameters were analysed by MANOVA Dunnett's multiple comparison test (IBM Statistic SPSS 26), with three levels of significance ($p < 0.05$, $p < 0.01$ and $p < 0.001$). Data are presented as mean ± standard deviation (SD). Descriptive statistics were used to present bacteriological results.

5. Conclusions

A reduction in bacterial count was present for all tested substances. The tested substances in different concentrations did not themselves have a negative effect on sperm quality parameters, considering the deleterious effect of DMSO as a solvent. Curcumin at a concentration of 5% was the only substance that had a slight positive effect on sperm motility parameters and a noticeable bacterial reduction (32%), at Time 1. Further studies on combinations of plant-based substances could be conducted to decrease individual toxic effects and increase beneficial effects on sperm quality and an antibacterial effect on bull semen microbiome.

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The bull seminal microbiome was studied to understand its potential effect on sperm fertility; the effect of season on the presence of microorganisms was also determined. Possible alternatives to antibiotics to inhibit these bacteria were investigated, namely the antibacterial effects of the plant-based substances curcumin, ginger and pomegranate, as well as their effects on sperm quality. Finally, colloid centrifugation was assessed for its ability to remove bacteria physically from semen samples without having a deleterious effect on sperm quality.

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