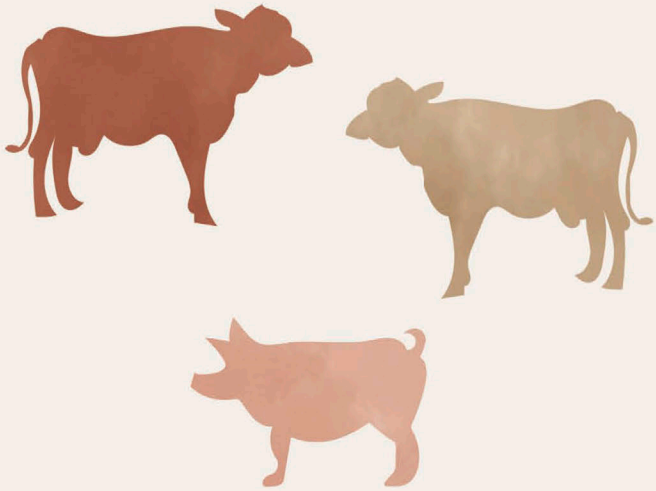
Semen Refinement

MICROFLUIDIC SEPARATION TECHNIQUES
FOR THE REMOVAL OF MICRO-ORGANISMS FROM
SEMEN FOR THE VETERINARY INDUSTRY



Tanja
Hamacher

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DISSERTATION

to obtain
the degree of doctor at the Universiteit Twente,
on the authority of the rector magnificus,
prof. dr. ir. A. Veldkamp,
on account of the decision of the Doctorate Board
to be publicly defended
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by

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in Düsseldorf, Germany

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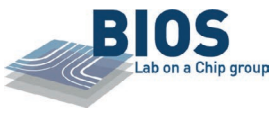

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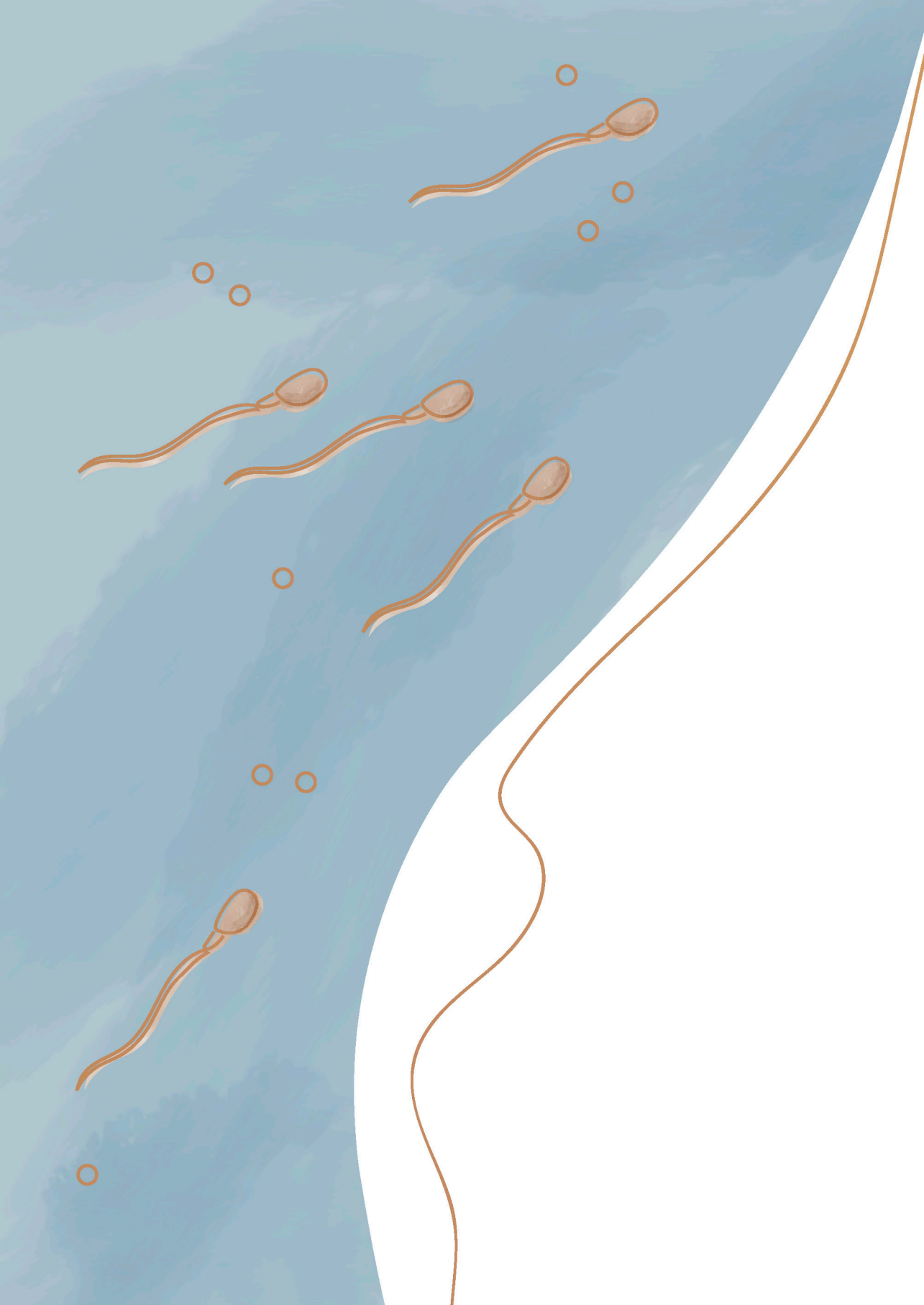

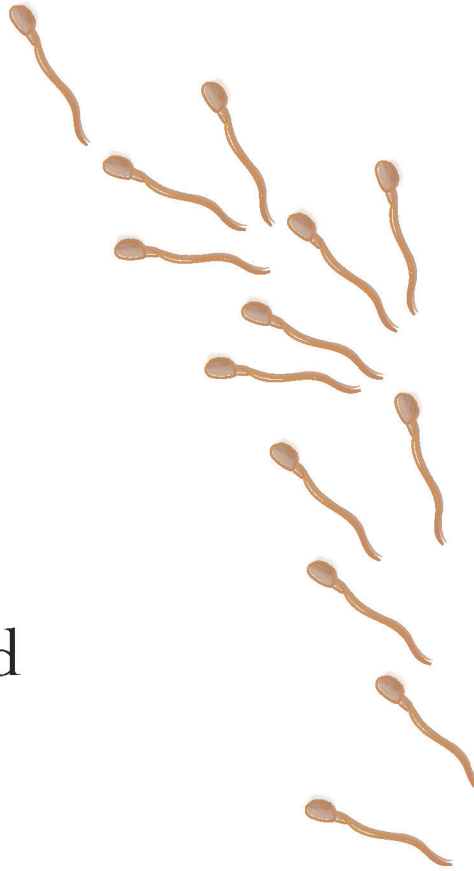

1.

Motivation and outline

Abstract

This chapter introduces the motivation and outline of the research presented in this thesis. In the veterinary industry, artificial insemination is the common fertilization technique. However, semen contaminated with micro-organisms can lead to disease transmission. Additionally, the presence of micro-organisms in semen decreases semen quality. By implementing a micro-organism removal step in routine semen processing, semen quality can be maintained and the biosecurity of artificial insemination will be increased.

1.1. Artificial insemination and biosafety

Artificial insemination (AI) is a procedure in which spermatozoa are introduced into the cervix or uterus at the time of oestrus, to achieve pregnancy through fertilization[1]. It is the most widely used reproduction technology for farm animals. In the pork production in the European Union (EU) and United States of America (USA), 95% and 90% of all fertilizations are carried out with AI, respectively[2]. In the dairy production worldwide, at least 130 million cattle are artificially inseminated every year[3]. AI is also applied to other species, such as horses[4], sheep[5] and turkeys[6]. Originally, AI was introduced to prevent the spread of disease by avoiding physical contact between animals[7].

It can be imagined that AI must fulfill several requirements such as breeding with high genomically evaluated genes, improving animal welfare and being economically beneficial. Therefore, companies and corporations have been founded to organize breeding programs worldwide. The market leaders, not restricted to the Netherlands but also in Europe, are Topigs Norsvin and the Coörperatie Rundveeverbetering B.V. (Cooperative Cattle Improvement, CRV B.V.) for swine and cattle reproduction, respectively. The international operating breeding company Topigs Norsvin produces more than 9 million semen doses annually, which results in more than 100 million slaughter pigs every year[8]. CRV B.V. produces approximately 11 million semen doses, referred to as straws, worldwide[9] of which 3 million doses were sold in 2020 in the Netherlands[10]. As a cooperation, CRV B.V. is owned by more than 25 000 farmers from the Netherlands and Flanders, the northern region of Belgium[9].

The two breeding companies Topigs Norsvin and CRV B.V. founded with Holding Technopolis Twente B.V. the company Semen Refinement B.V.. The aim of Semen Refinement B.V. is to increase the success rate of AI by improving the semen quality in the veterinary industry. The success of AI is mostly determined by the semen concentration, the number of spermatozoa in the seminal fluid and semen quality[1], which is subdivided into the sperm movement and shape, whose professional terminologies are the motility and morphology, respectively. Ideally, the semen consists of spermatozoa with normal morphology and high motility. In fresh porcine and bovine semen, on average more than 85% of the spermatozoa are motile and more than 70% of the spermatozoa are morphological intact[11]. The semen quality is reduced either by the presence of poor-quality spermatozoa[12–14], which are abnormal in morphology or immotile, or by the presence of other substances, such as cell debris and micro-organisms[15–20]. Therefore, two PhD projects were defined within Semen Refinement: 1) Improving the semen quality by removing abnormal and immotile spermatozoa, and 2) the removal micro-organisms from semen. The latter project is presented in this dissertation.

Sources of microbial contamination of semen are the donor animal, the environment and the personnel managing the animal or processing the collected semen[21]. Therefore, hygienic semen collection and processing is a key attention point. Individual hygiene and good sanitation do much to prevent contamination caused by semen handling, but still occur in approximately a quarter of the semen doses[22]. The negative impacts on sperm quality caused by micro-organisms include, among others, decreased sperm motility and viability[23,24], as well as sperm abnormalities[15]. This can result in embryonic or fetal death, reduced litter size and disease in recipient females[15,25]. Apart from the negative biological and animal welfare effects, disease transmission and outbreak lead to severe economic costs[26]. An example of such a disease outbreak is the epidemic of classical swine fever (CSF) in the Netherlands in 1997-1998[27]. During this period 429 herds were infected with CSF and had to be eradicated. Another 1300 herds were slaughtered to prevent further spread of the disease. In total 12 million pigs were killed in connection with the CSF outbreak. It was estimated that an economic loss of $2.3 billion (≈ €2.5 billion) was caused by the CSF epidemic in the Netherlands[28].

Current semen processing and screening techniques remove and/or screen for micro-organisms, such as bacteria and viruses, in semen (figure 1.1). A mixture of antibiotics is routinely applied to semen to kill bacteria preventing them from reducing semen quality (figure 1.1A)[29]. However, the emergence of bacterial strains that are antibiotic-resistant, is caused by the overuse of antibiotics[30]. The use of antibiotics in AI is nowadays legally obligated[29], but there is a desire to find alternatives to maintain the effectiveness of antibiotics in human medicine and veterinary industry. The World Health Organization (WHO) recommends "an overall reduction in use of all classes of medically important antimicrobials in food-producing animals"[31]. Viruses are not affected by antibiotics, and therefore, semen is screened for viruses using polymerase chain reaction (PCR) or enzyme-linked immune sorbent assay (ELISA) (figure 1.1B)[17]. For each type of virus, an individual screening test is performed, because each type requires an individual primer set or antibody. This results not only in high screening costs (up to €150,- per test), but also in labor-intensive and time-consuming work performed by personnel (3-12 working days) (MLWJ Broekhuijse, Topigs Norsvin, CRV BV, personal communication, 2019). Additionally, in the case of porcine semen, best fertilization rates are achieved with fresh semen doses[32], limiting the possibility to perform a screening test prior to insemination. Another drawback of a screening technique is that it is an endpoint measurement; a positive tested semen sample must be discarded and cannot be used for AI.

The aim of this thesis is to develop a microfluidic device which removes potential bacteria and viruses from collected semen while maintaining semen quality. Such a device can replace the current screening techniques and can reduce or eliminate the use

of antibiotics. The physical removal of micro-organisms from semen increases the biosecurity of AI in the veterinary industry.

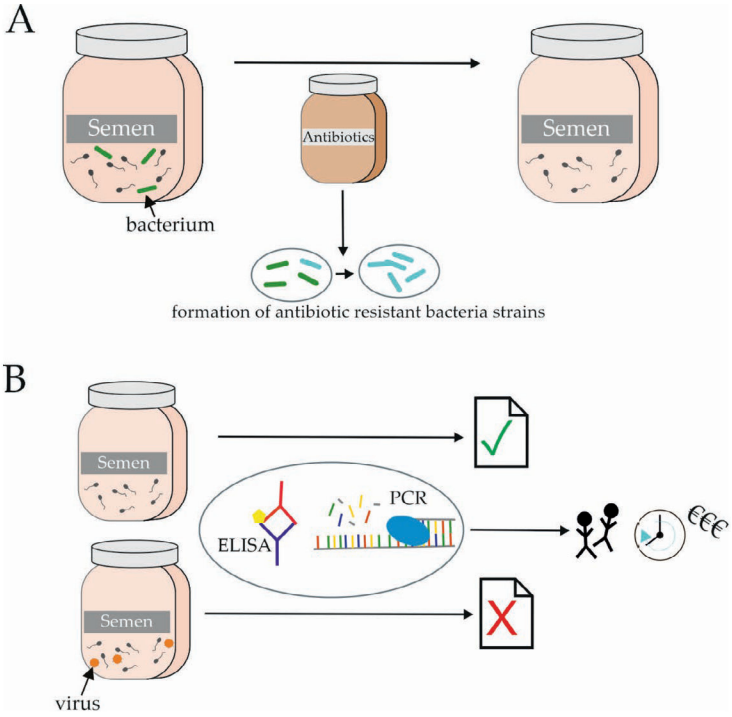

Figure 1.1: A) The spread of bacteria with AI is prevented by adding antibiotics to semen. However, the overuse of antibiotics causes the formation of antibiotic resistant bacteria strains. B) Semen is screened for viruses with ELISA and/or PCR. Semen sample tested positive for viruses are discarded (X), whereas virus-free semen is used for AI (✓). The usage of screening techniques is limited because these tests are labor-intensive, time-consuming and expensive.

1.2. Thesis outline

In **Chapter 2**, background information is provided from the various disciplines united by the aim of this thesis. Current semen processing techniques in AI centers producing boar and bull semen are described. The negative effects of micro-organisms present in semen and current micro-organism prevention procedures are summarized. A literature review of potential separation techniques summarizes current trends, challenges and limitations.

In **Chapter 3**, the effect of microfluidic processing on porcine and bovine spermatozoa is investigated. The estimated experienced shear stresses during ejaculation are compared to shear stress experienced during microfluidic processing. Sperm viabilities after processing with a simple, straight channel microfluidic chip and a pinched flow fractionation chip have been compared to conventional centrifugation and flow cytometry.

In this dissertation research, two potential techniques were explored to remove micro-organisms from semen; pinched flow fractionation and acoustophoresis. **Chapter 4** presents pinched flow fractionation for the removal of viruses from porcine semen. To achieve acceptable separation efficiencies, the chip design and flow parameters were optimized. **Chapter 5** suggests the improvement of semen quality by combining the membrane integrity test based on hypo-osmotic swelling (HOS) with subsequent cell separation accomplished by pinched flow fractionation.

The second applied separation technique is acoustophoresis. **Chapter 6** presents the removal of micro-organisms from semen with a benchtop instrument called AcouWash (AcouSort AB, Sweden). Although the chapter mainly focuses on the removal of bacteria from semen, a short insight into virus removal from semen is also provided. Additionally, the viability of spermatozoa after acoustophoresis processing has been examined. To become an attractive micro-organism removal technique implemented in routine semen processing for the veterinary industry, the sample throughput of the current AcouWash instrument must be increased. In **Chapter 7**, the gap between the current and desired state is analyzed and several suggestions to close the gap are proposed.

In **Chapter 8**, the results and outcomes presented in this thesis are summarized, discussed and several ideas for future research are suggested.

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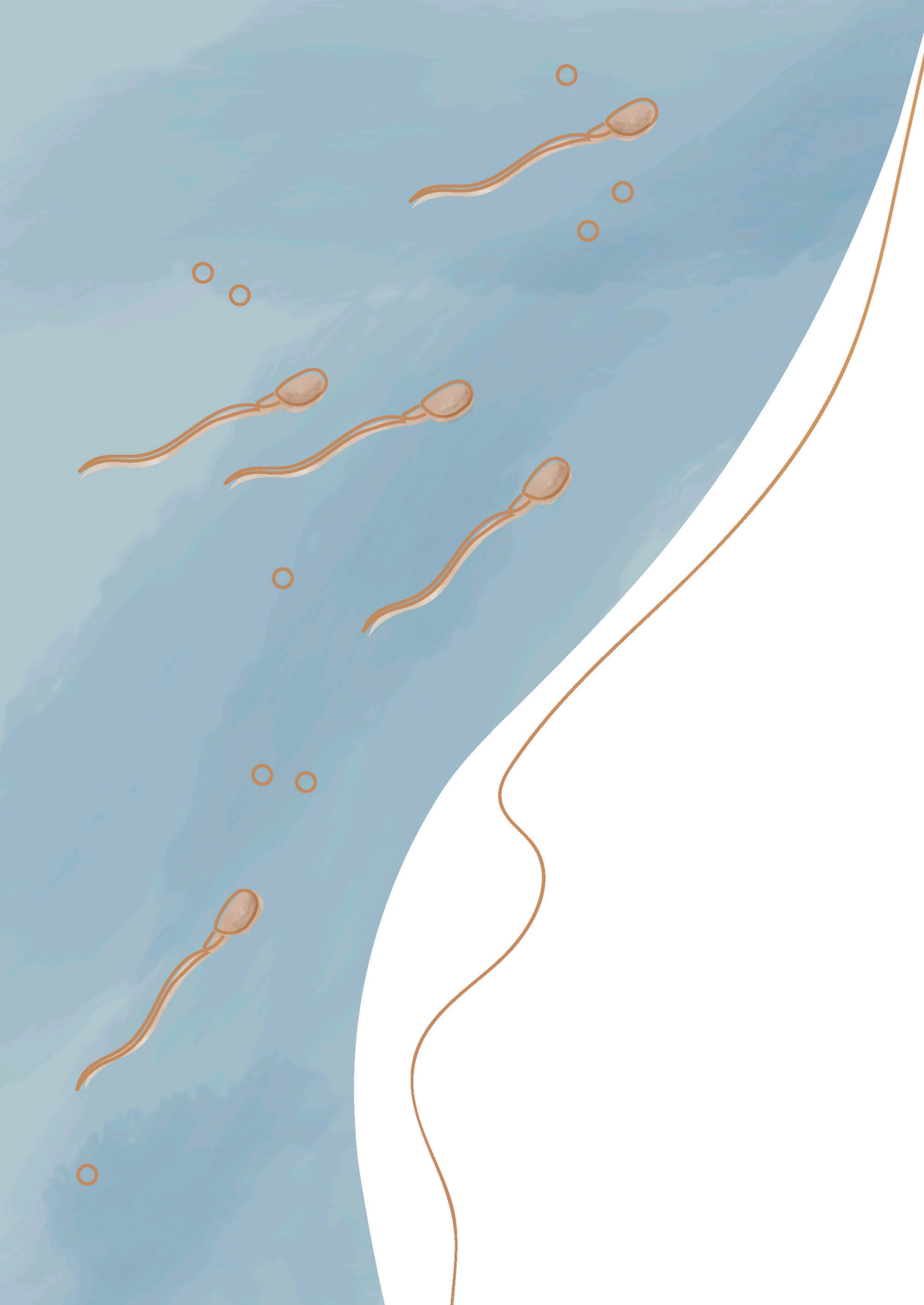

2.

Semen, micro-organisms, and (microfluidic) separation techniques

Abstract

The removal of micro-organisms from semen with microfluidics overlaps various research fields. This chapter provides an overview, so that readers from various disciplines can receive necessary background information. Artificial insemination centers collect and process semen before it is transported to the recipient farm. The presence of micro-organism in semen is unwanted because diseases can be transmitted. Therefore, semen samples are screened for viruses and antibiotics are added to kill bacteria. The physical removal of micro-organism is an alternative to current processing and screening techniques. Potential macroscale separation techniques to remove micro-organism from semen are filtration and density gradient centrifugation. Microfluidics is an alternative to macroscale separation. Microfluidics has gained high interest in studying and manipulating cells, as its dimensions correspond to the cells' size, it is easy to use, and devices can be produced in large-scale. Many microfluidic sorting techniques can be implemented to remove micro-organism from semen. Up to date, a limited number of devices has been proposed to refine semen. Therefore, microfluidic devices used to remove micro-organisms from blood are also considered. Finally, the challenge of high-throughput separation techniques for the aim of this thesis and with respect to current microfluidic separation techniques is discussed.

2.1. Semen and artificial insemination centers

In Europe and North America artificial insemination (AI) is the gold standard technology to produce offspring in the pork and cattle industry[1,2]. Semen of one male can be used to inseminate a multitude of females worldwide. Other benefits of AI are reducing animal transportation and costs[1–3]. In the following section, semen, its production as well as processing procedure at AI centers in the Netherlands are summarized with a special focusing on porcine and bovine semen.

2.1.1. Semen of domestic animals

Semen consists of a heterogeneous suspension of spermatozoa and seminal plasma, which are mixed during ejaculation[2,4]. The seminal plasma provides proteins, amino acids, and carbohydrates to support the viability and motility of spermatozoa. The ejaculation volume and sperm concentration differ between species; boar ejaculates have a volume of 150-300 ml with a concentration of 300 million cells/ml[5], whereas bull ejaculates have an average volume of 10 ml with a sperm concentration of 1 billion cells/ml[6]. The total sperm count of porcine semen is with up to 100 x 10^9 cells about ten times more than the total bovine sperm count. In table 2.1, several ejaculation characteristics of boars and bovine are summarized.

Table 2.1: Typical dimensions and characteristics of porcine and bovine spermatozoa/ejaculates.

Characteristic					
		Dimension	References	Dimension	References
Spermatozoon	Whole spermatozoon	43 - 45 µm long	[5]	73 µm long	[7]
	Head	7.0 µm long, 3.7 µm wide, 0.4 µm thick		8.8 µm long, 5 µm wide, 0.5 µm thick	
	Middle piece and tail	37.4 µm long, diameter 0.2 - 0.7 µm		64 µm long, diameter 0.3 - 0.7 µm	
Ejaculate	Volume	300 ml	[5,8,9]	10 ml	[4,6,10]
	Sperm concentration	250 x 10^6 – 300 x 10^6 /ml		1 x 10^9 /ml	
	Total sperm count	75 x 10^9 – 100 x 10^9		10 x 10^9	
	Ejaculation time	378 s (6 min)	[8]	5 s	MLWJ Broekhuijse, CRV BV, personal communication, 2019

A spermatozoon (ancient Greek: seed and living being) is an extraordinary cell in the sense that it is elongated and can move actively. The sperm morphology of domestic animals is similar, but the dimensions vary slightly. Depending on the species of origin, a spermatozoon is approximately 60 μm long and 8 μm wide. It has three distinct morphological regions, the head, the middle piece and the tail (figure 2.1)[5]. The head contains the nucleus in which the genetic information in the form of DNA is stored and components for sperm-oocyte recognition and fusion. The shape of the head is oval flattened and approximately 7 μm long, 4 μm wide and 0.5 μm thick[5,7]. A small neck connects the head to the middle piece, which contains mitochondria to produce the required energy. The tail is responsible for the cell motility and thus the movement of the cell. The cylindrical tail has a total length of approximately 45 μm and the tail diameter decreases from the neck to the tip from 0.7 μm to 0.2 μm[5,7]. Table 2.1 summarizes dimensions reported for porcine and bovine spermatozoa. A bovine spermatozoon is 74 μm long[7] and thereby longer than a porcine spermatozoon (43-45 μm)[5].

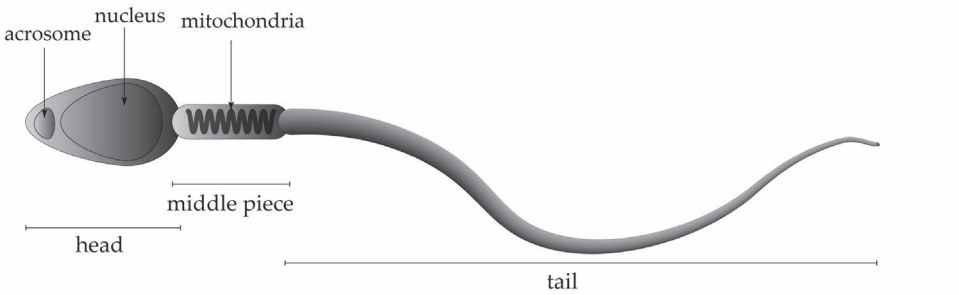

Figure 2.1: Schematic representation of a spermatozoon.

2.1.2. Semen collection at AI centers

The commercial semen production of domestic animals is legally regulated in the Europe Union (EU)[11,12]. Boars and bulls are introduced to AI centers after quarantine and undergo health controls on a regular basis to prevent the spread of diseases. Also the semen collection and processing are put under strict regulations to diminish the contamination with micro-organism and to reassure animal health. Table 2.2 summarizes several semen production parameters such as produced doses, quantity, and sperm quality.

Porcine semen: At the AI center, the semen of boars is collected and transported to the laboratory. First a 1:1 dilution is made with a semen extender, a specific diluent to preserve semen quality, within 15 minutes after collection. Semen motility and concentration are determined with a Computer Assisted Semen Analysis (CASA)

system. Random samples are manually controlled for morphological abnormalities. The final dilution step is performed to acquire the desired concentration of 20 million spermatozoa/ml. On average 41 AI doses are produced from one boar ejaculate. In figure 2.2 a photo of a porcine semen dose is shown. The doses are stored and transported at 17 ± 1°C. Those fresh semen doses are transported to the farms, where they are placed in a transport serving hatch to allow insemination during the next days. (MLWJ Broekhuijse, Topigs Norsvin and CRV BV, personal communication, 2020)

Bovine semen: Also, the bovine semen is collected in the AI center and diluted 1:1 within 15 minutes after collection. At the laboratory, several semen characteristics such as motility and concentration are determined using a CASA system. The semen is diluted to a concentration of 80 million spermatozoa/ml and straws are prepared. A photo of a straw is shown in figure 2.2. On average 600 straws are prepared from one bovine ejaculate. Subsequently, the semen is cooled and equilibrated to 5 °C and then frozen to -196 °C to store the semen cryopreserved in liquid nitrogen. The advantage of storing the semen cryopreserved is the long shelf life. (MLWJ Broekhuijse, Topigs Norsvin and CRV BV, personal communication, 2020)

Table 2.2: Semen production of porcine and bovine semen: doses, quantity and sperm quality. (MLWJ Broekhuijse, Topigs Norsvin and CRV BV, personal communication, 2021)

		Topigs Norsvin (porcine semen)	**CRV (bovine semen)**
Doses per year	worldwide	9 million doses	11 million straws
	the Netherlands	3 million doses	3 million straws
Storage temperature		Fresh (17 °C)	Cryopreserved (-196 °C)
Number of doses/ejaculate		≈ 41	≈ 600
Dose	Volume	1 dose = 80 ml	1 straw = 0.25 ml
	Sperm concentration	1 dose = 1.5 billion cells	1 straw = 15- 25 million cells
Motility	Fresh	87 ± 8% (N=69 433, 2020)	85% (N=4 159, 2020)
		After 3 days: 84 ± 9% (N=51 451, 2020)	Post thaw: at least 40%, 57% (N=3 863, 2020)

2.1.3. Conventional sperm analysis and processing techniques

During semen production and preparation in the laboratory, it is essential to determine several quantitative and qualitative parameters of the collected semen sample to reassure high quality semen and efficient AI.

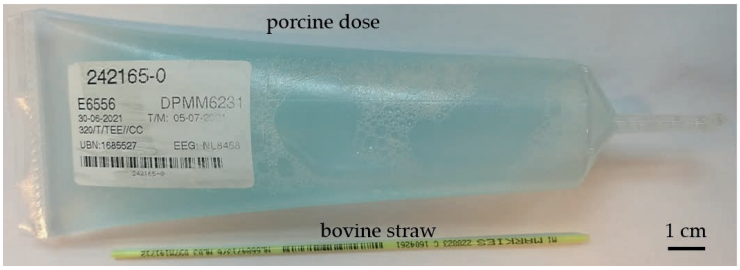

Figure 2.2: Porcine semen dose and bovine semen straw as it is sent to the recipient farms. Each day the semen dose is dyed in a distinct color, so that the farmer can discriminate doses from different days more easily. For example, on Wednesdays the semen is dyed blue. Bovine semen is cryopreserved and is sent to the recipient farms in form of straws. Transport will always take place in liquid nitrogen; thawing of the semen takes place at the farm.

One parameter is the total number of spermatozoa in an ejaculate, which is determined based on the sperm concentration and ejaculate volume. The total number of spermatozoa in an ejaculate appoints the number of doses prepared from that ejaculate.

The semen quality is evaluated by the sperm motility, the percentage of motile cells and sperm morphology. The cut-off motility for fresh porcine and bovine semen samples is 70%. The semen motility is either classified manually using microscopic semen motility assessment or objectively using CASA (figure 2.3A). The CASA system has several benefits to microscopic analysis and has therefore become widely used in AI centers. With the CASA system, the motility analysis has become faster, more accurate and objective in contrast to manual motility assessment. Additionally, more detailed data of sperm motility is generated[5]. The CASA system relies on optical analysis of consecutive images[13]. After a sample of fixed volume is loaded into the calibrated counting chamber, the motion of spermatozoa is measured based on point relocation. Besides concentration and total motility, more motility properties such as curvilinear velocity (VCL) are determined. Today many manufacturers supply CASA systems, all using different software. Typical motility data obtained from porcine and bovine semen in AI centers in the Netherlands are presented in table 2.2. Fresh porcine semen and bovine semen motility is on average 87% and 85%, respectively (MLWJ Broekhuijse, Topigs Norsvin and CRV BV, personal communication, 2021). Just before the semen is used for AI, the motility is decreased; after three days porcine semen has a motility of 84 ± 9% (2020) and bovine semen had an average post thaw motility of 57% (2020) (MLWJ Broekhuijse, Topigs Norsvin and CRV BV, personal communication, 2021).

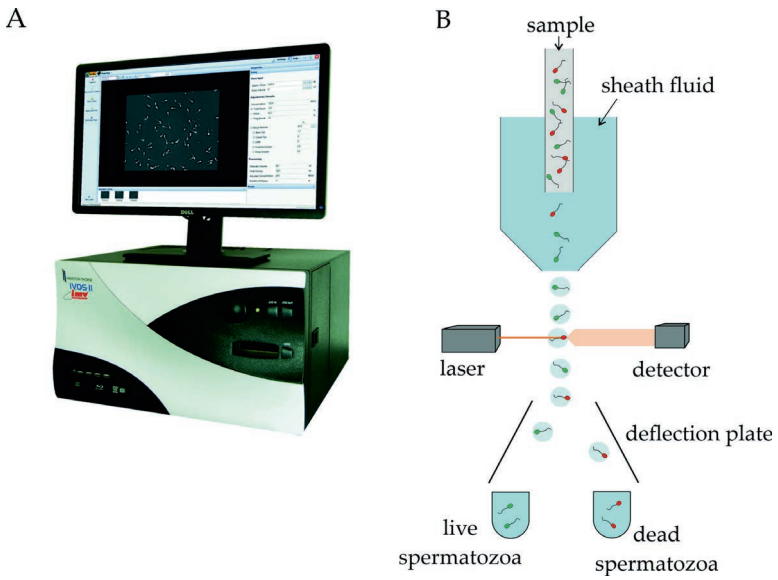

Figure 2.3: A) The IVOS II (IMV Technologies, Osseo, MN, USA). The CASA system used for dose productions by Topigs Norsvin and CRV. (adapted from [191]) B) Principle of FACS: the fluorescent signal of stained spermatozoa is determined for each cell. With a live/dead staining, spermatozoa can be separated based on viability.

Another commercially available method to analyze spermatozoa is flow cytometry. Fluorescence labeling and subsequent analysis with flow cytometry is performed to analyze for example sperm membrane integrity. A common combination is the viability assay using SYBR 14, which stains spermatozoa with an intact membrane, and propidium iodide (PI), which stains damaged membranes[14]. A flow cytometer is an automated system, which measures fluorescence intensity of individual cells and can thus determine the percentage of spermatozoa with an intact membrane. With a fluorescence activated cell sorting (FACS) system, spermatozoa can be separated based on the staining (see figure 2.3B). In contrast to the motility analysis with CASA, not every spermatozoa sample is analyzed for sperm membrane integrity using flow cytometry[5]. Widely applied, but still expensive, is FACS for sex sorted semen, which is described in more in detail later on (Section 2.4.3.1).

2.2. Micro-organism in semen

AI was introduced to prevent the spread of diseases by avoiding transportation of animals and physical contact between animals, because infectious micro-organism are transferred by close contact or sharing the same environment. Nevertheless, micro-organism, such as viruses and bacteria, may be present in semen and can contaminate the receiving animal or reduce the semen quality. Many preventive actions are performed to minimize the occurrence of micro-organisms in semen[15]. The introduction

of new animals in the AI center is strictly controlled. For example, new animals are quarantined for up to 60 days and their health status is evaluated. The AI center must follow strict biosecurity regulations regarding the facilities and personnel of the center. Some examples of biosecurity regulations are the location of the AI center needs to be at a certain distance from other animals of the same species, employees and visitors are not allowed to have contact with pigs or cattle a few days before entering the center, at entering employees must shower, and the water quality is controlled on a regular basis. Another important prevention is monitoring the health status of the animals. In case of clinical symptoms, semen must not be collected. The semen and blood of the animals are tested for micro-organisms on a regular basis. Also, vaccinations are applied to protect the animals for specific viruses.

2.2.1. Viruses in semen

The term virus (Latin: poison, venom, slimy fluid) was first mentioned by the Dutch scientist Martinus Beijerinck at the end of the 19th century[16]. Due to the small size of viruses, 20 to 500 nm, it is impossible to see the particles under a light microscope. Various researchers have described viruses as small microbes that are infectious and require living cells. With the invention of electron microscopy, viruses were visualized and became a distinct class of microbes. Viruses consist of genetic material (in the form of DNA or RNA) encapsulated by a coat of protein subunits. In contrast to bacteria, viruses do not belong to the class of cells, as they do not contain any organelles. Therefore, a virus penetrates a cell and uses the cell's organelles for replicating and translating the virus' genetic information. During that process, the host cell is usually killed. Based on shape, viruses are classified in four types, which are illustrated in figure 2.4A. Most virus types are either polyhedral or spherical. The size of the viruses found

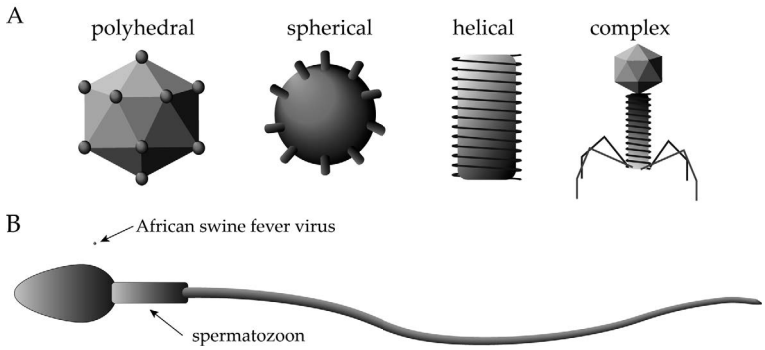

Figure 2.4: A) Viruses are classified in four groups. B) Size relation of spermatozoon and an African swine fever virus.

in porcine and bovine semen ranges from 10 to 400 nm. So, a virus is at least fifteen times smaller than the head of a spermatozoon (see figure 2.4B).

Swine fever viruses have gained media attention worldwide. Well known swine fever virus types are the classical swine fever (CSF) virus, the African swine fever (ASF) virus, as well as the H1N1 virus. During the epidemic of CSF in the Netherlands in 1997-1998, the transmission of CSF was among others proved to be spread via the semen of contaminated boars[17], since for an infected breeding herd other causes such as contact and distance to other infected herds could be excluded. The only contact for this herd was the purchased semen from one of the infected boar centers. Eventually, CSF was detected in two boar centers. Additionally, the first infections started in the sows which were inseminated with semen from infected boars. This resulted in the eradication of 1680 herds with a total of 12 million pigs who received semen from the two boar centers during the infection time and an economic loss of $2.3 billion[17,18]. After the CSF epidemic, research regarding the seminal viruses' transmission, prevention and screening of viruses in semen has been started. A current threat is ASF virus[19]. Many European countries reported first occurrences of the disease in 2016[20]. In Eastern Europe, the disease is mainly spread among wild boars. Between 2016 and 2020 more than 14 000 and 4 000 outbreaks in the domestic swine industry have been reported worldwide and in Europe, respectively[20]. In September 2020, also the first ASF cases among wild boars were reported in the east of Germany[21]. Having high densities of wild boar populations, leads to an increased risk of disease spread to domestic pig populations. Several measures have been proposed to prevent and control the spread of ASF. The already implemented biosecurity and import regulations on farms prevent the contamination of domestic pigs[19,22]. Furthermore, import of pork products from affected areas should be prevented and properly disposed[22]. Also vaccinating domestic pigs would help, but till now no vaccine has been approved. In contrast to CSF and ASF, which have both spread among the pig populations, the H1N1 virus has been transmitted to humans. The latter virus originates from the swine influenza A virus and has been identified in 2009 in Mexico and caused a pandemic among humans worldwide[23,24]. It was estimated that about 200 million people were infected, but the death rate was lower than 1%[25]. In comparison, the bird flu (H5N1) has infected about 850 humans since 2003 with a death rate about 50%[25]. The assumed risk of H1N1 virus is, that if it combines with other virus types, such as H5N1, a new mutated virus version will spread among humans with high death rate[25–27].

Numerous types of viruses have been identified in semen of livestock. Table 2.3 summarizes the viruses detected in porcine and bovine semen, that are listed by the World Organization for Animal Health (OIE)[28]. Although more viruses have been

identified in semen, this list provides an overview of the most relevant viruses. Several review articles describe the effect and impact of specific virus types on AI of porcine[15,29–31] and bovine[32–34] semen. Most viruses present in semen are not found in the spermatozoa itself, but in the seminal plasma or in other cellular components[35–37].

Table 2.3: Overview of viruses found in porcine and bovine semen of diseases listed by the OIE[28].

Virus type	Species		Size (nm)	EU legislation	References
					
African swine fever (ASF) virus	X		200	X	[25,26]
Aujeszky's disease virus	X			X	[29]
Bluetongue virus		X	70		[40,41]
Bovine herpes virus (BHV) or Infectious bovine rhinotracheitis (IBR)		X	170	X	[42,43]
Bovine viral diarrhea virus		X	50	X	[44,45]
Classical swine fever virus (CSF)	X		40-60	X	[17,39]
Foot-and-mouth disease (FMD) virus	X	X	25-30	X	[29,44,46]
Japanese encephalitis virus	X		40-50		[29,47]
Lumpy skin disease virus		X	250		[44,48]
Porcine reproductive and respiratory syndrome (PRRS) virus	X		45-80		[29,49]
Rinder pest virus		X	120-300		[33,50]
Swine vesicular disease virus	X		28		[38,51]
Transmissible gastroenteritis virus	X		80		[29,52]

The preventive measures taken by the AI centers can still not guarantee that every sample is virus free, since animals shed viruses before they show symptoms. Several methods have been applied to detect viruses in semen, namely virus isolation, bioassays, antibody detection and nucleic acid detection. Virus isolation and bioassays can detect viable viruses involving virus filtration and cell culture for the host cells. As a result, the techniques have many limitations such as time-consuming procedure, high costs, sample contamination and low throughput and are not routinely applied in AI centers. However, the detection of antibodies against viruses is fast and simple. Antibodies are formed as a defense mechanism by the body and mark viruses as external particles. Enzyme-linked immunosorbent assay (ELISA) is the mostly applied antibody detection method. A drawback of this method is that viruses are present in semen before the formation of antibodies, thereby resulting in a negative result at early onset of the

disease. As a result, the detection of antibodies is a suitable method to monitor herd immunity instead of disease status. The nucleic acid detection in the form of polymerase chain reaction (PCR) has obtained acceptance as it is known to be a sensitive and specific method. Currently, PCR is commercially implemented to screen semen samples for viruses. PCR combines the amplification and detection of nucleic acid and can be applied to both DNA and RNA viruses. Various PCR methods have been developed to detect viruses in porcine and bovine semen. An example is the real-time PCR test developed by van Rijn *et al.*[53]. Their method can detect five important viruses in porcine semen. Although PCR and ELISA are faster than bioassays, these techniques are still labor-intensive as well as time-consuming (3-12 working days), high in costs (up to 150€/test) and individual parameters must be designed and optimized for each virus type (MLWJ Broekhuijse, Topigs Norsvin, CRV, personal communication, 2019).

The European Union has legally obligated virus isolation tests for trade in the EU or import into the EU. For example, it is covered in Part 5 Chapter 1 of Regulation 2020/686/EEC that "5 % (with a minimum of five straws) of each quantity of semen taken from a donor animal at any time *must be* submitted to a virus isolation test for foot and mouth disease (FMD) with negative results"[12], if the donor has not been vaccinated against FMD. Several treaties with countries outside the EU must be followed when exporting or importing semen. In contrast to cryopreserved bovine semen, fresh porcine semen is used for AI. The virus screening test results for fresh porcine semen are not available prior insemination; therefore, it cannot be assured that porcine semen is virus free.

2.2.2. Bacteria in semen

Bacteria, single-celled organisms, are the oldest form of life and appeared 4 billion years ago[54]. Before the discovery of bacteria, it was known that infections could be transmitted from person to person. In the late 17th century, the Dutch trader Antoni van Leeuwenhoek was able to describe and draw "animalcules", which later became known as bacteria[55]. Bacteria are mostly about 2 μm in length, slightly smaller than eukaryotic cells, and contain all necessary organelles to self-replicate genetic information. Throughout the time, many mutations have evolved bacteria to adapt to almost all environments, resulting in infectious species, which can harm other species. Nowadays it is known that bacteria played a key role in the evolution of all species. For example, billions of bacteria help cattle to digest grass[56] and the importance of microbiome in the gut is getting attention in human medicine[57]. Bacteria have distinct types of shapes, such as roundish or elongated (figure 2.5A). The size of most bacteria ranges between 0.5 to 5 μm and are slightly smaller than spermatozoa (figure 2.5B).

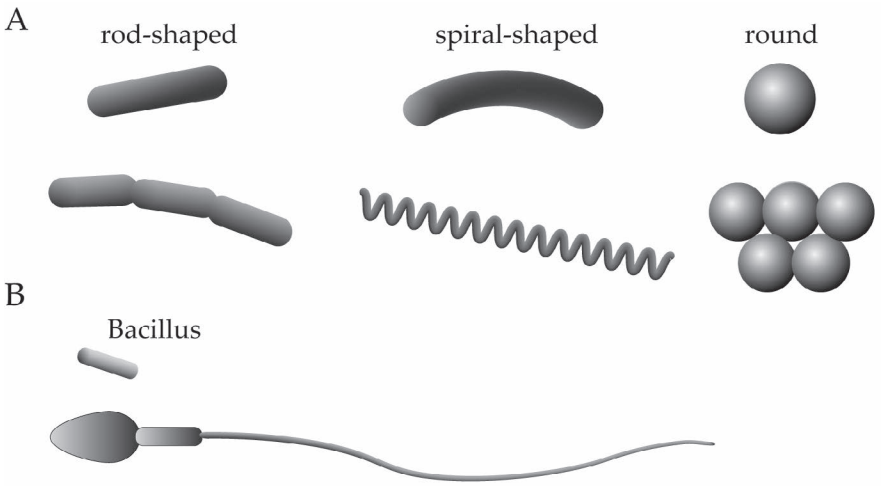

Figure 2.5: A) Bacteria are classified in three shape types. B) Size relation of spermatozoon and the bacteria type bacillus.

Table 2.4: Overview of bacteria identified in porcine and bovine semen. The most important infectious bacteria are listed by the OIE[28]. During sample processing the semen sample may be contaminated with other bacteria types as well. (several species (spp))

Bacteria type	Animal type		Shape	Size (µm)		OIE list	References
				Length	Width		
Bacillus spp	X	X	rod	1-10	0.4-1.8	X	[61,62]
Brucella spp	X	X	rod	0.6-1.5	0.5-0.7	X	[63–65]
Campylobacter fetus		X	spiral	0.5-5	0.2-0.9	X	[66,67]
Chlamydia spp	X	X	round	0.25 - 0.6		X	[68–70]
Clostridium perfringens	X		rod	1.3-19.0	0.6-2.4		[71]
Coxiella burnetti		X	rod	0.2-1.0		X	[72,73]
Escherichia coli (E.coli)	X		rod	2.0	0.5		[58,74]
Leptospirosa	X	X	spiral	6-20	0.1		[66,75,76]
Mycobacterium spp	X	X	rod	1.5-4	0.3-0.5	X	[77–79]
Mycoplasma mycoides		X	-	<1 µm		X	[66]
Pasteurella spp	X	X	rod	1.0-2.0	0.3-1.0		[58,68,80]
Tri trichomonas foetus		X	spiral	5-25		X	[66]

Although many preventive measures are in place, it is impossible to obtain a sterile semen dose. Numerous studies have reported that bacterial contaminations are present in up to 30% of the tested boar ejaculates[58–60]. For example, Schulze *et al.*[60] have shown that 26% of the extended porcine seminal doses contained bacteria, but in only 4.5% of these contaminated doses bacteria were found in the raw ejaculate. This suggests that most bacterial contaminations occur during semen processing. A broad range of bacteria have been isolated from porcine and bovine semen samples. Table 2.4 summarizes the most important bacteria detected in porcine and bovine semen. Several infectious bacteria are also listed by the OIE[28]. Apart from the bacteria causing infections, bacteria associated with microflora were also isolated in semen and have similar negative effects on semen quality.

The effect of several types of bacteria on semen quality has been studied by inoculating bacteria in semen samples and monitoring the sperm motility, which was repeated after a few storage days. Figure 2.6 shows the results for the spermatozoa motility over storage period with the presence of *Escherichia coli* (*E.coli*) or *Clostridium perfringens*[81]. Comparable results were obtained for other bacteria such as *Pseudomonas aeruginosa*[82] and *Enterobacter cloacae*[83] in porcine semen. The motility significantly decreases with the presence of high bacteria concentration (10^8 cfu/ml) after a few days. This trend continues and the motility decreases below 20% after 9 days of storage. Reducing the bacteria concentration in semen samples has a positive effect on sperm motility and therefore increases semen quality.

Antibiotics, which are added to semen extenders, are routinely applied to semen to kill bacteria. The broad range of bacteria contaminating a semen sample, provokes the necessity of a broad range of antimicrobials. The overuse of antibiotics is known to cause antibiotic resistant bacterial strains not only in human healthcare but also in AI of domestic animals. Several studies have reported the presence of antibiotic resistant bacteria in semen[84,85]. Laws from the European Union[12] and guidelines from the World Organization of Animal Health[86] control and limit the use of antibiotics in AI on a national and international level. Nevertheless, the use of antibiotics in semen is obligated, because no alternative to antibiotics has been proven to be as efficient as antibiotics. For porcine and bovine semen the following combination of antibiotics must be added to every milliliter diluted semen: 500 μg streptomycin, 500 IU penicillin, 150 μg lincomycin and 300 μg spectinomycin[12]. An alternative antibiotic mixture is the combination of 75 μg amikacin and 25 μg divekacin[12].

Proposed alternatives to conventional antibiotics are novel antimicrobials such as antimicrobial peptides[87–90]. These peptides interfere with the prokaryotic membrane of the bacterium and induce a change of membrane potential, so that the compound

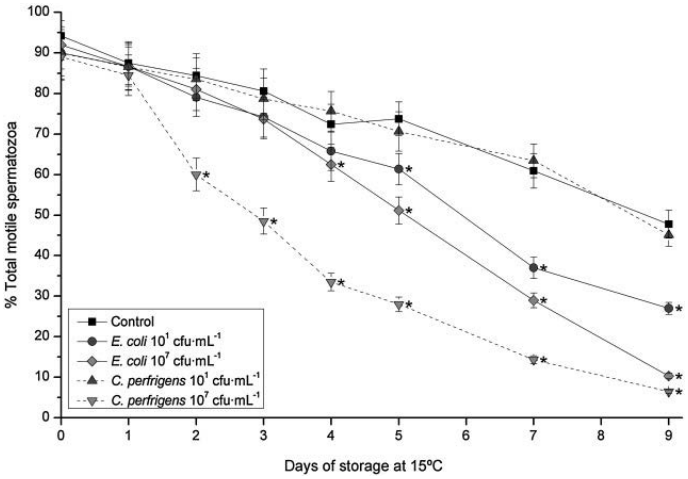

Figure 2.6: Percentage of total motile spermatozoa over storage period after incubation with high and low concentrations of *E. coli* or *Clostridium perfrigens* in porcine semen. Superscript (*) means significant difference ($P<0.05$) between the results obtained for each treatment with the control. (Reprinted from the original work by Pinart *et al.*[81], Copyright 2016, with permission from Elsevier B.V..)

exchange through the membrane is destructed and cell death is provoked. Compared to conventional antibiotics, the development of antibiotic resistant bacteria is reduced with the use of antimicrobial peptides. The current limits are high production costs, lower stability and lower activity *in vivo*, which prevent the commercial use of antimicrobial peptides[89].

2.3. Requirements of the desired separation technique

Before investigating possible separation techniques for the removal of micro-organism from semen, it is important to set the requirements for the desired separation process. The following requirements have been set with the breeding companies Topigs Norsvin and CRV B.V..

In the veterinary industry, the semen is processed in the AI centers. The removal of micro-organism will be implemented in the current processing program if the desired separation efficiencies and processing time are achieved. The desired processing time and separation efficiencies are summarized in table 2.5. The desired processing time is 10 to 30 minutes as several ejaculates must be processed within one day. Most of the calculations presented in this thesis are based on a processing time of 10 minutes. A processing time of 10 minutes results in a sample throughput of 30 ml/min and 1 ml/min for porcine and bovine semen, respectively. The sperm recovery is required to be as high as possible with a minimum of 90%. To completely inhibit the spread of diseases, the virus removal is desired to be 100%. For the bacteria removal, 90% is acceptable, as most

bacteria do not transmit the disease, but decrease semen quality. Another important requirement is that semen quality is retained ensuring high fertilization rates and good health of the offspring. Therefore, sperm motility and field fertility rates should not be less than the ones recently reported.

Table 2.5: Requirements for the removal of micro-organism from porcine and bovine semen. (farrowing rate (FR); total number born (TNB); non return rate 56 days (NRR-56)*[1])

		Boar ejaculate	Bull ejaculate
Desired processing time (min)		10 (to 30)	10 (to 30)
Throughput (ml/min)		30	1
Throughput (cells/min)		9×10^9	1×10^9
Sperm recovery (%)		>90%	
Sperm motility	**Compared to unprocessed sample**	< 5% less	
	Cut-off criteria	≥ 70%	
Virus removal (%)		100%	
Bacteria removal (%)		>90%	
Field fertility rates		FR: 86% [91] TNB: 16 [91]	NRR-56: 60-65% [92]

Besides these requirements, many more requirements can be set for the removal of micro-organisms from semen. Some examples are: the risk for recontamination needs to be minimized, the manual processing steps and processing costs should be as low as possible and it should be user-friendly for trained laboratory assistants.

2.4. Physical removal of micro-organisms based on particle size

The physical removal of micro-organism can provide an alternative to commercially used methods to supply semen free of micro-organisms and to prevent the spread of diseases. As spermatozoa are larger in size than viruses and bacteria found in semen (figure 2.4B and 2.5B), a separation principle based on size seems to provide a promising solution. Several macroscale and microscale techniques have been investigated to separate micro-organisms from (body) fluids based on particle size. In the following sections, these techniques have been reviewed. Micro-organism removal from blood for diagnostic purposes is the most common application found in literature. When available,

*[1] Non return rate 56 days: the percentage of animals not returned (non-return) to insemination at 56 days (8 weeks) after the first insemination

an application for the separation of spermatozoa is provided. For each technique, the benefits and limitations are discussed.

2.4.1. Macroscale separation techniques

2.4.1.1. Filtration

Filtration is a physical method used for the separation of suspended particles with a porous membrane, also called filter. The pore size is the most important characteristic of this method and determines the cut-off value for the particle separation; particles smaller than the pore size pass the filter, whereas particles larger than the filter are deposited. Particle aggregation and particle packing affect the filtration process.

Filtration has been applied for the removal of micro-organism from water for a long time[93,94]. Many waterborne diseases and the resulting outbreaks of cholera and typhoid fever have been constrained with the filtration of water in the end of the 19th century. To date various filter systems have been developed to remove bacteria and viruses from water. The first modern filtration systems of water used sand (slow/rapid sand filtration) to remove micro-organisms. Nowadays many filtration systems use micro- or nanofilters and ultracentrifugation. Both bacteria and viruses are removed with a reduction percentage of more than 99,99%[93].

Filtration with a vertical filter design, in which liquid is pulled through a horizontally placed filter by gravity (figure 2.7A), is a beneficial method to separate larger particles from smaller particles or from a suspension. For the separation of smaller particles from larger particles, such as the separation of micro-organism from semen or blood, filtration has the major drawback of filter cake formation. A filter cake consists of several particle layers deposited on the filter surface[95,96] (figure 2.7A). These particles do not pass the filter because of their size. The space between the random positioned particles allows fluid and smaller particles to pass. The filtration cake itself starts to filtrate solid particles. When the thickness of the filtration cake becomes too thick, the resistance is too high, and the filtration stops. This means that the filter must be cleaned or backwashed. Especially, for the separation of micro-organism from semen and blood this is undesirable as the large amount of cells present in the sample will stimulate the formation of filtration cakes[97].

With column filtration spermatozoa can also be washed. This method uses a matrix, in which debris is entrapped, whereas target cells pass the matrix[5,98]. Column filtration has been shown to separate motile spermatozoa from a semen sample, thereby removing immotile spermatozoa[99], leukocytes[100] and acrosome damaged spermatozoa[101]. So viable and motile spermatozoa pass the column matrix, whereas immotile spermatozoa and other debris are entrapped in the column matrix. This filtration technique uses the self-

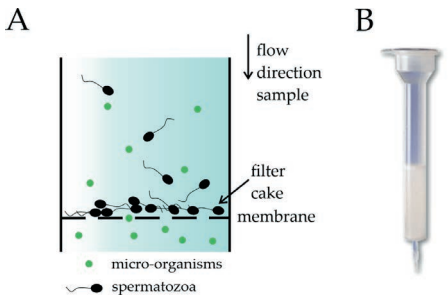

Figure 2.7: A) Principle of filtration: Smaller particles pass the filter, whereas larger particles remain on the filter. If the number of larger particles on the filter surface increases, a filter cake is formed. B) An example of a gel filtration column (Bio-Rad, Hercules, CA, USA). (adapted from [192])

motion of spermatozoa and the filtration characteristics of the column material. Various column material are (commercially) available and consist mostly of gel or glass wool (figure 2.7B). The material and sample combination determines whether separation is based on size, hydrophobicity, and/or charge. Reported results are inconsistent because many different protocols and column materials have been used. On one hand, it has been shown that the sperm morphology and viability is increased, whereas the motility is not altered after column filtration[102,103]. On the other hand, results have also reported an improved motility after column filtration[104,105]. However, the sperm recovery (~60%) was low[104–106].

Column filtration is a user-friendly method and could be easily implemented at AI centers to improve semen quality taking the necessary equipment into account[5]. Filtration columns processing large volumes, which is an important demand of AI centers, have not been developed till now. Each column type and application have an individual processing protocol. For best semen quality enhancement, several column filtration steps would be needed, which increases processing time enormously. Additionally, the low sperm yield is a huge limit for commercial use for AI of veterinary animals.

2.4.1.2. Density gradient centrifugation

Density gradient centrifugation (DGC) has widely been applied to process semen samples thereby improving semen quality. The most important applications of DGC are the separation of motile and morphological normal cells and the removal of micro-organism[107]. DGC is mainly used when smaller volumes are used such as for *in vitro* fertilization (IVF). A colloid, a mixture of particles suspended in another substance, is added to the sample (figure 2.8A). This is followed by a centrifugation step to separate particles with larger and smaller densities with respect to the density of the colloid. Single-layer centrifugation (SLC), in which only one layer of colloid is needed, is a

simplification of DGC, which has additional benefits such as higher volume processing and increased labor-friendliness[107].

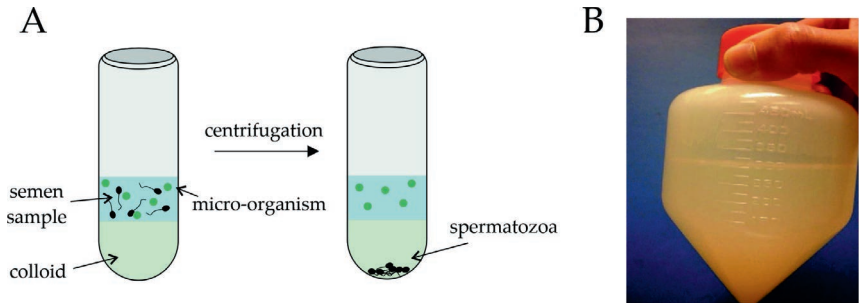

Figure 2.8: A) The principle of DGC to separate spermatozoa from micro-organism: The gradients and sample are inserted into the centrifugation tube. After centrifuging the spermatozoa are found in the bottom layer, whereas the micro-organism and debris are found in the top layer. B) Tubes for large volume processing have been developed. (Reprinted from the original work by Morel *et al.* [108], Copyright 2011, with permission from Creative Commons). A semen sample comprising of 150 ml can be centrifuged in one tube.

For the separation of live and dead spermatozoa, the semen sample with a sperm concentration of 100 x 10^6 cells/ml is layered on the colloid. The tube containing the colloid and sample is centrifuged at 300 x g for 20 minutes. The supernatant including dead spermatozoa and colloid are removed. Then the live spermatozoa are harvested and diluted in a semen extender. Sample volumes of up to 150 ml can be processed in a single tube allowing high-throughput processing (figure 2.8B)[108]. Motility of porcine semen was increased from 68% to 82% after SLC processing with a yield of 80-86%[108]. Overall processing time of one boar ejaculate is approximated to be within half an hour.

The removal of both viruses and bacteria from spermatozoa with SLC has been investigated mainly for porcine, human and stallion semen[107,109,110]. Blomqvist *et al.* have shown to remove 99% of porcine circovirus type 2 from porcine semen using a combination of SLC and "swim-up" procedure[111]. The swim-up procedure uses the self-motion of spermatozoa at body temperature (figure 2.9)[5,98]. After centrifuging a fresh semen sample, a swim-up diluent is added on top of the semen sample. When incubating the semen sample, motile spermatozoa "swim" to the top layer and can be collected.

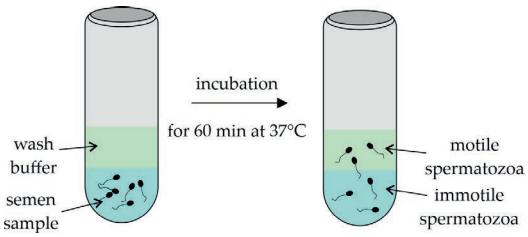

Figure 2.9: Principle of the swim-up procedure: in a tube, the semen sample is covered with wash buffer and incubated. Motile spermatozoa swim to the top of the liquid, whereas immotile spermatozoa stay at the bottom of the tube. Then the motile spermatozoa are aspirated.

The combined method of SLC and "swim-up" is labor-intensive, as it includes three processing steps consisting of two SLC steps and one "swim-up" procedure and a total processing time of approximately 1.5 hours. The sperm motility after processing was 72% ± 23% compared to the control group with a motility of 80% ± 16%[111]. The sperm yield of 45% ± 20% is low and a huge drawback of this method[111]. Equivalent results were obtained using SLC to remove Equine Arteritis Virus of equine semen[112]. The virus titers significantly decreased and the best results were obtained with a double SLC procedure. However, the virus was not completely eliminated. The double SLC method did not have a negative impact on sperm motility; but still lead to a low sperm yield of 43 ± 15%[112]. The processing time of the double SLC procedure is approximately one hour for 15 ml of extended equine semen. SLC has been also applied for porcine semen. The removal of bacteria from boar ejaculates with SLC was tested by Morrell *et al.*[113]. Samples were collected without the addition of antibiotics and processed with SLC. In 6 out of 10 samples, bacteria were completely removed, whereas in the other 4 samples the number of bacteria was reduced to different extents varying from a bacteria reduction of 10% to 50%. The authors mention that flagellated bacteria are difficult to remove from the semen sample by SLC. Similar results were obtained for the removal of bacteria from stallion semen[110,114].

Promising results for the removal of micro-organisms from semen have been reported, but the commercial use of SLC is hindered by some limitations of this technology. A huge drawback of SLC is the sample contamination during manual processing[107]. Sterile processing requires the necessity of laminar air flow benches, which are usually not available in AI centers, and good training of the personnel. Also, several processing steps are needed; for example, the removal of viruses requires at least two SLC processes. This makes SLC a labor-intensive method, as the removal of the supernatant and the cell pellet are performed manually. Additionally, the sperm yield is low, because about half of the spermatozoa are lost during processing, although sperm motility is retained or increased. Another drawback is the need of species related colloids. For every species, an individual colloid must be developed to ensure good separation.

2.4.2. Microscale separation techniques

Microfluidics is a fast-emerging field dealing with the flow of liquids inside micrometer-sized channels, which match the size range of cells. Microfluidic devices have been applied to many biological applications, including biological and chemical analysis, and to both molecular and medical diagnostics[115,116]. The separation of particles based on their size is feasible with microfluidics and for this both active and passive principles exist. Active principles are those based on an external applied force or gradient, such as electrical or acoustic forces, whereas passive principles do not use such external forces.

Often these passive separation principles rely on the motion of fluids and the forces exerted on particles immersed in the fluid, known as hydrodynamic forces.

Before reviewing microfluidic separation techniques, an important characteristic of microfluidics will be explained: the flow profile of microfluidics is typically laminar. At low flow rates and small channel dimensions, the flow follows parallel streamlines (figure 2.10). Additionally, most presented applications use pressure-driven flows which have a parabolic flow profile. Therewith, particles diluted in the fluid do not deviate from their initial streamline. In laminar flow, fluid flow is dominated by viscous forces[117]. An example of laminar flow in daily life is pouring honey. In contrast to the smooth, predictable flow profile of laminar flow, the flow profile of turbulent flow is unstable and chaotic and dominated by inertial forces[117]. A typical example of a turbulent flow profile is water flow in a river.

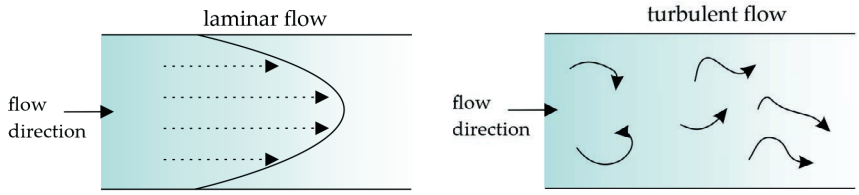

Figure 2.10: Schematic illustration of laminar and turbulent flow profiles.

2.4.2.1. On-chip microfiltration

In 2.4.1.1. filtration was already described as a macroscopic filtration technique. It is a versatile and easy to use separation technique and, therefore, these filters have also been incorporated into microfluidic chips. The principle of on-chip filtration is the same as it is on macroscale: the separation is based on the pore size of the filter. Microfilters have been divided into several types, namely membrane (figure 2.11A), pillar (figure 2.11B), weir (figure 2.11C), and cross-flow filtration (figure 2.11D). These four microfilters are all mechanical based systems. A special type of filter, which can only function at the microscale and in laminar flow field, is the H-filter® (Micronics) (figure 2.11E). The H-filter® system uses the diffusive properties of different sized particles[118]. Smaller particles diffuse faster from the sample containing fluid into the buffer solution and are therefore separated from larger particles.

Many on-chip microfilters have been proposed to separate blood components, such as the separation of erythrocytes and white blood cells from whole blood or for plasmaphoresis[116,119–121]. An application of virus removal from blood was proposed by Wang *et al.*[122]. In their chip an incorporated membrane microfilter with a pore size of 2 µm was able to remove human immunodeficiency virus (HIV) from whole blood with

an efficiency of up to 90%, while recovering 82% of the erythrocytes and 90% of of white blood cells. On-chip microfilters have also been applied to sort and preconcentrate bacteria. A microfiltration system controlled by valves has been shown to separate 81% of *E.coli* at a flow rate of 120 µl/min[123]. At lower flow rates, *E.coli* was preconcentrated up to 536%.

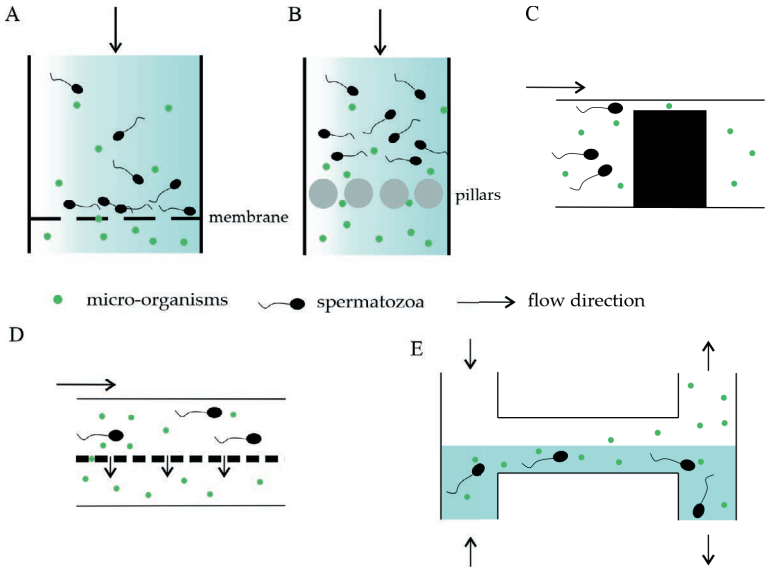

Figure 2.11: Schematic illustrations of microfilter types: A) membrane (side view), B) pillar (side view), C) weir (side view), D) cross flow (side view) E) H-filter (top view).

As on-chip microfiltration is similar to filtration, the technique has the same benefits and limits. The separation of different sized particles leads to the formation of filter cakes, fouling and clogging. Reversing the flow direction periodically has been proposed to reduce the formation of fouling, thereby cleaning the filter[124]. However, the addition of an additional step in the separation process, increases the complexity of the microsystem. Most applications for this type of filter are small-volume point-of-care diagnostics rather than for the separation of particles from large volumes.

H-filter chip devices have been used to separate motile from immotile spermatozoa[125,126]. In contrast to the conventional H-filter® systems based on particle diffusion, the separation of spermatozoa with this filter is based on the motility of the spermatozoa. Immotile spermatozoa stay in the initial streamlines, whereas motile spermatozoa move, thereby deviating from their initial streamlines. Motile sperm purity was nearly 100% with yields around 40%[125]. A similar device was combined with electrical counting to detect motile spermatozoa which do not deviate from their initial streamlines, allowing the differentiation of motile and immotile semen samples[126]. As these systems make use

of the sperm self-motion, sperm exhaustion decreases the semen fertility and the sample throughput is with approximately 1 µl/min low.

2.4.2.2. Pinched-flow fractionation

This principle is based on the sudden broadening of a channel immediately after a pinched segment[127]. A schematic representation of pinched flow fractionation (PFF) is shown in figure 2.12. In the pinched segment, the particles are focused on the sidewall with a sheath flow. The center of smaller particles is closer to the channel wall than the center of larger ones. At the boundary between the pinched and the broadened segments, the larger particles are forced toward the center of the channel, whereas smaller particles are forced toward the channel wall because of the laminar flow, making separation based on size possible.

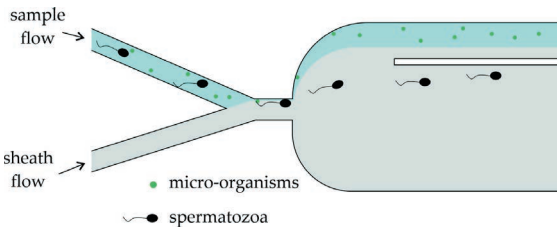

Figure 2.12: Schematic representation of PFF. After the particles are pinched to the channel wall in the small segment of the chip, they follow the streamlines according to their size. This effect is amplified in the broadened segment leading to particle separation.

Liu *et al.* proposed a device to separate epithelial cells from spermatozoa for forensic analysis[128]. Results have shown that 41% of the spermatozoa were separated with a purity of 97%. A 50 µl sample was processed within 30 minutes. Berendsen *et al.* have used PFF to separate spermatozoa from erythrocytes[129]. An erythrocyte removal of 90% and a sperm recovery of 95% was achieved using a sample flow rate of 0.17 µl/s. Depending on the desired separation characteristics, the flow rates can be adjusted.

PFF is known to be simple and inexpensive regarding usage and fabrication, and it does not require an external field. However, the precise flow control of both sample and sheath buffer is particularly important, because it determines separation efficiency. Another drawback is the sample throughput due to the pinching of the sample flow and the subsequent dilution with the sheath buffer in the broadened segment.

2.4.2.3. Inertial microfluidics

Inertial microfluidics is based on transient flow; the transition regime between laminar and turbulent flow. In this transient flow state both inertia and the viscosity of a fluid must be considered[130]. The induced inertial lift force and the particle motion can be used to manipulate particles. The inertial lift force is directed away from the wall and decays with distance from the wall, whereas the shear gradient is directed to the wall and

increases with distance to the wall (figure 2.13). These two forces determine the equilibrium position of particles in a channel with defined dimensions at moderate Reynolds number. The magnitude of the lift force depends on particle size; the inertial lift force pushes larger particles to its equilibrium position, whereas smaller particles are less effected by the inertial lift force[130].

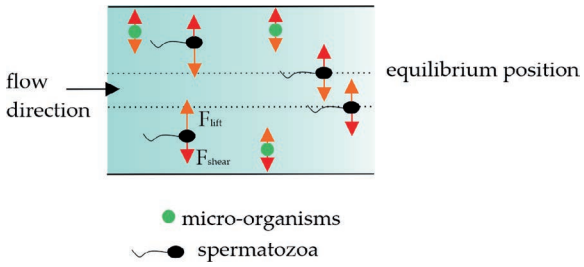

Figure 2.13: Particles experience two forces perpendicular to the flow direction in inertial microfluidics. The interaction of the wall lift force (F_{lift}) and shear gradient (F_{shear}) determine the equilibrium position of a particle. The larger the particle, the larger the effect of the wall lift force.

Bacteria were separated from red blood cells using inertial focusing in a straight channel and subsequently the separation was amplified with expanding channels[131]. The larger red blood cells were more affected by the inertial lift force than the bacteria, which were almost unaffected. More than 80% of the bacteria were removed after processing the sample twice with the proposed chip and the red blood cell yield was 82%[131]. A high-throughput version of this principle with 40 parallel straight channels and a flow rate of 8 ml/min was subsequently proposed and resulted in a blood cell yield of 90%[131]. A similar device with a straight channel and a flow rate of 60 µl/min was able to recover 73% of the bacteria from whole blood and separated 92% of the white blood cells[132]. No data for the separation of red blood cells was shown. Wu *et al.* designed a chip in such a way that a curved flow is formed using an acting and protecting sheath flow[133]. In that curved flow, inertial forces push red blood cell to an equilibrium position and the smaller bacteria remain in the original flow. At a flow rate of 18 µl/min, 98% of the bacteria were removed with a purity of 99.7%. Recently, a device with periodic contractions of a curved channel has recovered at least 80% of *Klebsiella pneumonia* (*K. pneumonia*), a gram-negative bacteria type, and at least 40% of *Streptococcus pneumonia* (*S. pneunomiae*), a gram-positive bacteria type, from whole blood at sample flow rates of 400 µl/min[134].

Dean flow fractionation, one special type of inertial microfluidics, is based on centrifugal forces in a curvilinear microchannel[135]. Mostly, a spiral channel is used to separate various sized particles (figure 2.14). When a fluid flows under certain flow conditions through the spiral channel, the balance of inertial lift forces and Dean drag forces determine a particle position in the cross section of the channel. Inertial lift forces push

particles away from the wall, whereas Dean drag forces the particles to move along lateral vortices. Both forces depend on particle size, but to different extents. The equilibrium positions of particles can be determined based on particle size, flow characteristics and channel dimensions[135].

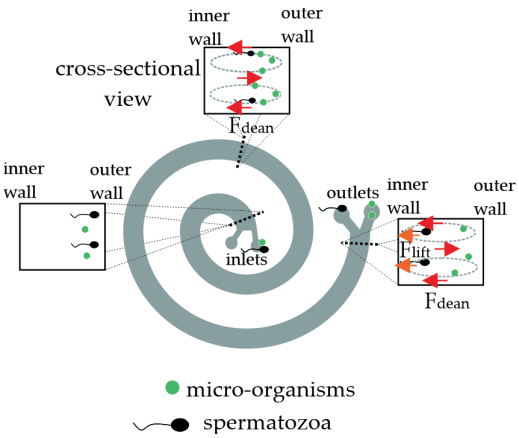

Figure 2.14: Principle of Dean flow fractionation. All particles are forced to move in lateral vortices by the Dean drag force (F_{dean}). The balance of inertial lift force (F_{lift}) and F_{dean} pushes particles to the inner wall before exiting the chip, whereas smaller particles exit the chip at the outer wall.

Hou *et al.* have used such a spiral channel to separate bacteria from blood cells[136]. The spiral channel and flow characteristics induce the blood cells to be at the inner wall of the channel and bacteria to stay at the outer wall of the channel before separation. For various bacterial loads of *E. coli*, a bacterial recovery of at least 65% was achieved. No data on the efficiency of blood cell removal is given. A higher bacteria separation (up to 90%) from whole blood at a sample flow rate of 50 µl/min was achieved with a similar spiral channel by Iyengar *et al.*[137] with an erythrocyte removal ranging between 82-97% depending on the sample concentration. Similarly, Son *et al.*[138] and Jafek *et al.*[139] have used Dean flow fractionation to separate red blood cells and white blood cells from spermatozoa, respectively. Son *et al.* claim to remove 99% of the red blood cells, whereas more than 80% of the spermatozoa were recovered with a sample flow rate of 520 µl/min ($5x10^6$ cells/min)[138]. Jafek *et al.* separated white blood cells from spermatozoa with a flow rate of 1.6 ml/min and reached a sperm recovery of 89% while removing 82% of the white blood cells[139]. It has to be noted that only 28 of the 130 samples contained enough white blood cells to quantify separation. A spiral channel with a trapezoidal cross-section has been used to enrich spermatozoa from heterogeneous cell suspensions[140]. The authors claim that the use of a trapezoidal cross-section results in higher separation efficiencies. Best separation results were achieved at a flow rate of 1.1 ml/ml. The sperm recovery was 96%, whereas epithelial cells (86%), white blood cells (approximately 95%) and erythrocytes (approximately 75%) were removed.

Inertial microfluidics is a relatively new field in microscale manipulation. As these inertial effects become important at moderate Reynolds numbers, a higher flow rate is usually applied to process the sample. To process larger sample volumes, parallelization of several devices is a necessity, which already has been proposed. The inertial effects

depend highly on channel dimensions, particle size and flow characteristics. These parameters must be considered at all development stages, such as design and testing, of a device. For example, to guarantee successful particle separation, the required channel dimension has to be theoretically calculated based on flow characteristics and particle sizes. Small deviations from optimal flow characteristics can have an impact on particle separation.

2.4.2.4. Deterministic lateral displacement

In deterministic lateral displacement (DLD) particles are separated according to the particles' trajectory through a channel containing a specific arrangement of posts[141,142]. The post size, post shape, and succeeding row position with respect to the previous row determine the critical diameter. Particles smaller than the critical diameter follow the main streamline through the center of the channel in a more or less straight flow (figure 2.15). Particles larger than the critical diameter change their streamline when passing a post, because their particles' center is outside the main streamline[141].

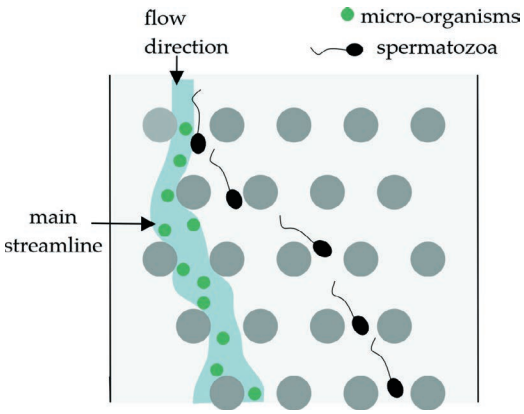

Figure 2.15: Principle of DLD. Particles move around posts arranged in a channel. The smaller particles follow the main stream line, whereas the larger particles change the streamline and are thereby separated from the smaller particles.

The particle shape plays a role in designing DLD devices. In contrast to most bioparticles, bacteria and spermatozoa have a non-spherical shape. An important design suggestion is to include protrusions and grooves in the post shape as suggested by Zeming *et al.*[143]. By incorporating these grooves and protrusions, elongated particles rotate and follow the streamlines with respect to their largest dimension. With I-shaped pillars, Zeming *et al.* were able to separate 100% erythrocytes from whole blood[143]. In a follow up study the authors investigated, whether different shaped bacteria can be separated[144]. With I-shaped pillars, 100% of rod-shaped and rod-liked bacteria were separated from their surrounding fluid[144]. Also spherical bacteria separation was achieved, but the distribution in the output channels was broader than for the non-spherical bacteria. Pariset *et al.* proposed to cascade two DLD devices for the separation of bacteria from blood and cancer cells[145]. They used round pillars, so that the blood cells and cancer cells deviate from the main streamline, whereas bacteria stay in the main streamline. After both DLD processing steps, 99.8% of the cancer cells, 93% of the erythrocytes were

depleted, while recovering 23% of the bacteria. A DLD device with circular posts was used to differentiate subpopulations of *S. pneumonia* based on size and morphology; single cocci were separated from cocci chains and non-encapsulated cells were separated from encapsulated cells[146].

The main design parameters of DLD, which are post shape, post size and array arrangement, are interchangeable[141,147]. On the one hand, this enables a broad variety of applications, but on the other hand, it also impedes finding the optimal device leading to the best separation results. Therefore, detailed understanding of the design parameters and their effect on the critical diameter is needed. Another often opposed issue is particle clogging due to the channel resistance or particle interaction with each other or with the wall[141,147]. Typical DLD sample throughput is in the range of a few microliters per minute[141,148], which is small when comparing it to other microfluidic separation techniques. Parallelized DLD devices have already been reported, which can process sample volumes of up to 100 ml/hour[149,150]. An advantage of using DLD is, that it can be combined with separation techniques using external forces such as dielectrophoresis, acoustophoresis and flow cytrometry[141,148].

2.4.2.5. Acoustofluidic separation

As the names suggests, acoustofluidic separation combines acoustic forces with microfluidic flow characteristics[151]. A straight channel with the sample aligned on the outside wall and a cell-free medium in the center is required for acoustofluidics (figure 2.16). Perpendicular to the channel, an ultrasound device actuates an acoustic wave. The channel diameter is designed in such a way, that a standing acoustic wave is formed between the channel walls. The induced primary acoustic radiation force is proportional to the particle size; larger particles experience a higher force and move faster to the pressure node in the center of the channel compared to smaller particles, which are less

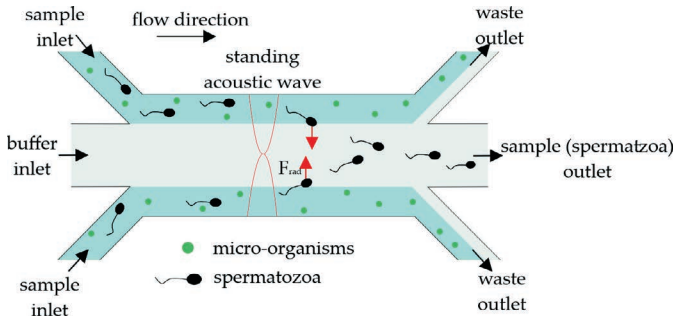

Figure 2.16: Schematic representation of the principle of acoustofluidic separation. The acoustic radiation force induced by the standing acoustic wave scales with particle size. The larger cells, in this application the spermatozoa, move faster towards the pressure node in the middle of the channel than the micro-organism.

affected. The laminar flow profile of microfluidics enables the particle separation, because the larger particles continue to move along the centerline of the channel, whereas smaller particles remain in their fluid stream[151].

Acoustofluidic separation has successfully been applied for the removal of bacteria from blood[152–154]. A tilted-angle acoustic wave by Li *et al.* achieved a bacteria removal of 96% and a red blood cell recovery of 96%[155]. A high-throughput device has been developed by Ohlsson *et al*.[152]. Depending on what is desired, various settings can be used to achieve either high blood cell recovery (99.99%), high bacteria removal (99.7%) or high sample throughput. For high sample throughput, 12.5 minutes are needed to process 1 ml of whole blood with blood cell removal of 99% and bacteria recovery of 90%. The separation of sub-micron particles such as viruses using acoustofluidic separation has been studied as well. Dengue viruses were separated from lymphocytes with a virus purity of 70% and a lymphocyte purity of 98%[156]. The used microfluidic chip deviates slightly from the previously described chips used for acoustofluidic separation. Instead of one channel, a thin silicon wall separated the sample containing channel from a second fluid channel. The ultrasound wave passes both channels and thus both channels contribute to the formation of pressure nodes. In that way a pressure node is not formed in the center of the sample containing channel. This simplifies the sample channel because a H-filter configuration with two sample outlets can be used.

Although acoustofluidic separation has not been applied for sperm handling yet, the equivalent trapping technique – acoustic trapping – has been shown to differentiate spermatozoa from other biological components in the field of forensic analysis and sexual assault[157,158]. In short, spermatozoa, forming the male fraction, are trapped by an acoustic standing wave, while other biological components originating from the female victim, such as free DNA, pass the acoustic field. In this way, the DNA profile of the suspect can be determined. In a first study, it has been shown that the obtained purity of male and female fraction with acoustic trapping is comparable to differential extraction, a commonly used purification technique in forensic analysis[157]. Recently, a prototype for the acoustic differential extraction of spermatozoa from sexual assault victims has been presented[158]. Spermatozoa were successfully trapped and purified from a sample containing a 40-fold excess of female epithelial cells over spermatozoa.

The separation of particles with acoustofluidics has several benefits, such as high-throughput processing and high removal efficiency of bacteria. The effect of acoustic forces on cell viability has been investigated for short and long term acoustic applications[159]. For separation applications, cells are shortly exposed to the radiation force and so far, the effects on cell viability are small and therewith negligible[160,161]. In contrast to other active separation principles, acoustofluidics is therefore known as a

gentle cell manipulation technique. For the previously described applications low frequency ultrasound waves (100 kHz -10 MHz) were used. Some limits of the active separation principle acoustofluidics are a higher complexity of the microfluidic device and fabrication process compared to passive separation principles. Moreover, for sub-micron particles, an additional force becomes dominant, namely the acoustic streaming force[162]. Acoustic streaming induces the sub-micron particles to move in steady vortices close to the channel wall when using frequencies for micron-sized particle separation. Therefore, the manipulation of submicron particles is hampered by the effect of acoustic streaming, which probably caused the low virus purity of 70%. To reduce the effect of acoustic streaming, the frequency of the acoustic wave can be increased. For example, higher frequencies acoustic waves have been used to separate extracellular vesicles from blood[163,164].

2.4.2.6. Dielectrophoretic separation

Another active separation technique is dielectrophoresis (DEP), which uses electrical forces to manipulate particles[165]. When a polarizable particle is placed into a nonuniform electric field, the particle is moved by an electrokinetic force (figure 2.17). This force, also known as DEP force, depends on various properties of the particle such as its size and morphology. Living cells can also be discriminated based on membrane morphology or internal conductivity. Apart from cell separation, DEP has been also applied to trap cells[166,167].

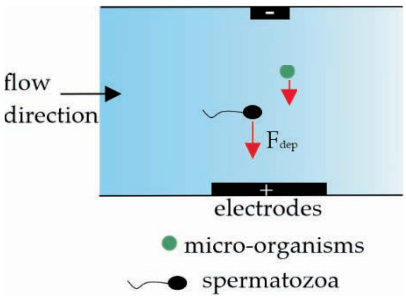

Figure 2.17: Principle of positive DEP separation. The DEP force (F_{dep}) is induced by a nonuniform electric field and differs in direction and magnitude depending on cell size and morphology.

A microfluidic chip with integrated microelectrodes was used to sort and concentrate bacteria from blood[168]. At a flow rate of 1 µl/min and an alternating current (AC) potential of 12 V at a frequency of 500 kHz the red blood cells experienced a higher DEP mobility and were repelled, whereas the smaller bacteria experienced a lower DEP and were collected in the lower subchannel. Approximately 80% of the bacteria and 98% of the red blood cells were in the two subchannels. Additionally, bacteria were distinguished using surface-enhanced Raman scattering. Another bacteria enrichment method has been proposed by Cai *et al.* and is based on a sequential combination of an H-filter for sample desalination and micro-organism trapping with DEP[169]. Bacteria capture efficiency of bacteria spiked human blood was on average 80%. For micro-organism detection, an enrichment method might be appropriate. De Wagenaar *et al.* have proposed the use of

differential impedance analysis followed by DEP sorting to separate 3 μm beads and spermatozoa[170]. No separation characteristics were reported. The sample concentration was 0.02-0.025 μl/min with a sperm concentration of 2 x 10^6 cells/ml.

The use of an active separation principle such as DEP has a few disadvantages. Electrodes are integrated in the microfluidic chip, which increases the complexity and costs of the fabrication. Applying an active force on living cells is usually a risk as the cells may be damaged. So far, the blood sample concentration for DEP was diluted, whereas the bacteria concentration was higher compared to a realistic situation.

2.4.3. Other separation techniques

Besides separation techniques based on particle size, other separation principles have been developed to sort out micro-organisms and/or to isolate spermatozoa. These techniques are summarized with respect to separation principle, application, as well as throughput.

2.4.3.1. Labeling

Labelling is one of the well-known separation techniques of cells for various diagnostic applications such as cancer cell detection[171] and micro-organism isolation[97]. Using chemical or physical interactions, the micro-organisms are attached to other particles which can be removed more easily from the solution and cells. For the attachment of bacteria, several chemical groups, molecules and ligands have been investigated. Some examples are lectins[172], Zn-dipicolylamine[173] and heparin sulfate[174]. For the removal of the labelled micro-organisms, various techniques such as sedimentation, capture on surfaces and filtration have been proposed.

One of the widely used labeling techniques is separation using superparamagnetic beads. Two studies have shown that about 90% of *E.coli* could be separated from whole blood using labelling and magnetic separation[172,173]. In the context of semen preparation techniques, labeling and magnetic sorting has been used to remove apoptotic spermatozoa from samples and thereby increasing semen quality[175–177]. An example is that magnetic sorting with Annexin V increases sperm motility and decreases capacitation status of cryopreserved bovine semen[178]. Some attempts have been made to use magnetic activated cell sorting on chip with an H-shaped based device[179,180].

Flow cytometry is one of the most well-known cell characterization principles. Fluorescently labelled spermatozoa are subjected to a laminar fluid flow and are deflected based on the presence of the label. The use of a DNA-specific stain and the small difference in DNA content due to the X or Y chromosome enables sex-based sorting[181,182]. In the veterinary industry, sex-based sorting of spermatozoa is desired to

make dairy and pork production more efficient. Sex-sorted semen is commercially available, but the costs are too high to be applied daily. Furthermore, flow cytometry decreases porcine and bovine sperm viability to 89% and 80%, respectively[183,184] and many cells are lost during the separation process. Also, the throughput of flow cytometry is limited to 70 000 events/s due to electronic processing[185].

Labelling of micro-organisms has the limitation of specificity. Most known labels attach to a few types of bacteria, but not to all of them. To remove all types of micro-organisms from a sample, a label must fulfill the requirements of attaching to all micro-organisms, high binding affinity and good binding kinetics. Labelling is more suitable for the detection of specific micro-organism in contrast to separating micro-organisms from cells. An example is a microfluidic device developed to detect bovine viral diarrhea virus using antibodies[186]. Also, the throughput of labeling, especially for microfluidic devices, is limited, because mixing, binding kinetics and separation must be considered.

2.4.3.2. Separation principles based on sperm motility and interaction

In contrast to other mammalian cells, spermatozoa are special in the sense that they can actively move. The direction of movement is triggered by various biological substances known as chemoattractants, temperature (thermotactic) gradients, as well as orientation against the fluid flow (rheotaxis)[5,187]. A variety of microfluidic devices based on one or a combination of multiple of the named concepts were proposed to assess spermatozoa behavior and sort based on motility. Several reviews have discussed these microfluidic devices in detail and can be found in references[188–190]. To separate micro-organisms from spermatozoa using spermatozoa motility and interaction for an application in livestock has several limitations including limited throughput and sperm fatigue.

2.4.4. Sample throughput

While setting up the requirements for the desired micro-organism removal technique (Section 2.3, table 2.5), it has been revealed that the desired processing time of one ejaculate is 10 minutes. The resulting necessary sample throughput is 30 ml/min (9×10^9 cells/ml) and 1 ml/min (1×10^9 cells/ml) for porcine and bovine semen, respectively. Macroscale separation techniques have been already applied to process semen for the veterinary industry. Although good separation efficiencies have been achieved, sperm yields were with approximately 50% too low, the processing is labor-intensive and there is a considerable risk of recontamination. Microscale separation techniques may be an alternative, but high-throughput processing is still one of the field's challenges. In table 2.6, the previous reported (microfluidic) separation techniques are summarized with respect to throughput and separation efficiencies. Most methods have a sample throughput of a few microliter per minute. Some techniques, such as acoustophoresis

and inertial microfluidics have been optimized to achieve good separation efficiencies while processing 400 µl/min or even more than 1 ml/min. When considering current achieved throughput rates with microfluidics, it is revealed, that achieving a processing time of 10 minutes for one (boar) ejaculate requires optimization and improvements.

Table 2.6: Throughput and separation efficiencies of various reported (microfluidic) separation techniques.

Method	**Reference**	**Sample flow rate**	**Particle** (#/min)	**Sample and separation efficiency**
Microfiltration	Wang *et al.* [122]	~300 µl/min	1.5 x 10^9 cells/min	Virus recovery (90%) from erythrocytes (82%) and white blood cells (90%)
	Ryzhkov *et al.* [123]	120 µl/min	6 x 10^5 bacteria/min	*E.coli* separation from nanoparticles (81%)
		2 µl/min	1 x 10^4 bacteria/ml	*E.coli* preconcentration (536%)
	Cho *et al.* [125]	0.5 µl/min	*NA*	Motile cells (100% purity, yield 40%) from semen sample
	Segerink *et al.* [126]	1 µl/min	*NA*	Differentiation of motile and immotile semen sample
Pinched flow fractionation (PFF)	Liu *et al.*[128]	1.7 µl/min	2 166 cells/min	Sperm (recovery 41%, purity 97%) from epithelial cells
	Berendsen *et al.*[129]	10 µl/min	51 x 10^6 erythrocytes/min	Erythrocytes (90%), spermatozoa (95%)
Inertial microfluidics	Mach *et al.*[131]	200 µl/min	10 x 10^6 erythrocytes/min	Bacteria (80%), erythrocytes (82%)
		8 ml/min	400 x 10^6/min	Blood cells (90%)
	Faridi *et al.*[132]	60 µl/min	300 x 10^6 erythrocytes/min	Bacteria (73%), white blood cells (92%)
	Wu *et al.* [133]	18 µl/min	4 x 10^6 cells/min	Bacteria (98%) from whole blood; purity 99.87%
	Lu *et al.*[134]	400 µl/min	1 x 10^8 erythrocytes/ml	*K. pneumonia* (87%) and *S. pneumonia* (42%) from erythrocytes
	Hou *et al.*[136]	150 µl/min	~7.5 x 10^5 erythrocytes/min	Bacteria (65%) from blood
	Iyengar *et al.* [137]	50 µl/min	5 x 10^8 erythrocytes/ml	*E.coli* bacteria (90%), erythrocyte (97%)
		50 µl/min	2.5 x 10^9 erytrocytes/ml	82% bacteria removal, erythrocyte removal (82% removal)

	Son *et al.*[138]	520 µl/min	~5 x 10^6 cells/min	Erythrocytes (99%) from spermatozoa (80%)
	Jafek *et al.* [139]	1600 µl/min	Clinically relevant for human applications	Spermatozoa (89%) from white blood cells (83%)
	Vasilescu *et al.* [140]	1100 µl/min	1 x 10^7 - 1.2 x 10^7 cells/min	Spermatozoa (96%), epithelial cells (86%), white blood cells (95%), erythrocytes (75%)
Deterministic lateral displacement (DLD)	Ranjan *et al.*[144]	0.1 µl/min	1 x 10^3 bacteria/ml	100% bacteria deviation from main channel
	Pariset *et al.*[145]	200 µl/min	~2 x 10^7 cells/min	Bacteria recovery (23%) Erythrocytes removal (93%) Cancer cell removal (100%)
	Beech *et al.*[146]	0.02 µl/min	*NA*	Bacteria size and morphology
Acoustofluidics	Li *et al.*[155]	1 µl/min	~10^5 cells/min	Bacteria recovery (96%) from erythrocytes (96%)
	Ohlsson *et al.*[152]	400 µl/min	4 x 10^8 cells/min	Bacteria recovery (90%) from erythrocytes (99%)
	Fong *et al.*[156]	450 µl/min	5 x 10^4 cells/ml	Virus purity (70%) from lymphocytes (98%)
Dielectrophoresis	Cheng *et al.*[168]	1 µl/min	500 cells/min 500 CFU/min	Bacteria (~80%) from erythrocytes (98%)
	De Wagenaar *et al.* [170]	0.02-0.025 µl/min	40-50 cells/min	*NA*

2.5. Conclusion

AI is a common procedure in the veterinary industry to produce offsprings. The presence of micro-organism in semen bears the risk to transmit diseases and to reduce semen quality. Before the semen is used for insemination, antibiotics are currently legally obliged to be added to semen. For the presence of viruses, semen is screened regularly with PCR, which is labor-intensive and time-consuming. An alternative to current micro-organism prevention methods is the physical removal of micro-organisms from semen. Previous applied macroscale techniques, such as filtration and centrifugation, have achieved high separation efficiencies, but low sperm yields. Microfluidics has been widely applied for cell separation/manipulation and micro-organism removal from blood. Microfluidics holds high potentials in achieving high separation efficiencies and sperm yields. One of the challenges of applying microfluidics for the removal of micro-organism from semen is the achievement of high-throughput processing, a common challenge experienced in microfluidics.

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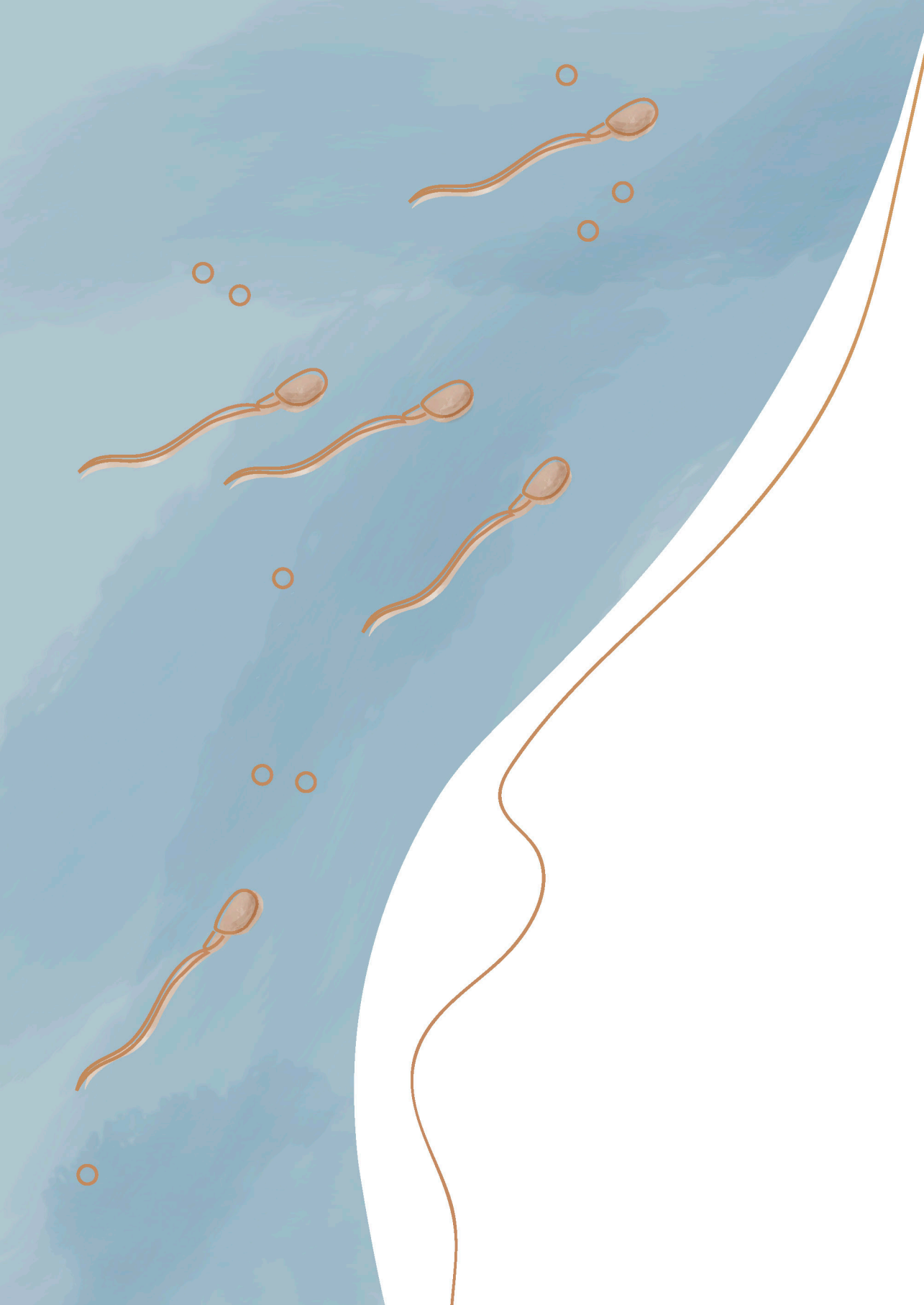

3.

Effect of microfluidic processing on the viability of porcine and bovine spermatozoa

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Abstract

The use of microfluidics in artificial reproductive technologies for manipulation or assessment of spermatozoa is unique in the sense that it is not always an endpoint measurement and the sample may be used afterwards. During microfluidic processing, spermatozoa are exposed to shear stress, which may harm viability and functioning of spermatozoa. The shear stresses during general microfluidic processing steps were calculated and compared to estimated shear stresses during ejaculation. The viability of porcine and bovine spermatozoa after microfluidic processing was studied and compared to the typical handling method (centrifugation) and to a control (the sample in a tube at the same temperature). The porcine spermatozoa showed a small but significant decrease in viability of 6% after microfluidic handling. Bovine spermatozoa proved to be less susceptible to shear stress and were not significantly affected by microfluidic processing. These data indicate that the impact of microfluidic processing on the viability of porcine and bovine spermatozoa is less than the literature values reported for flow cytometry and comparable to the impact of centrifugation.

3.1. Introduction

Artificial reproductive technologies (ART), such as artificial insemination (AI), are commonly used to support the mechanism of fertilization, both for couples with fertility problems and in the veterinary industry[1]. Approximately 9% of all couples in developed countries have infertility problems, of which 56% are looking for medical care[2]. In the veterinary industry worldwide, professional farms breed approximately 90% of pigs and 80% of dairy cattle using AI[1]. For example, for pork production the European Union and the USA use 95% and 90% AI respectively[3]. Part of the success of ART is determined by semen quality. Poor quality spermatozoa, such as morphologically abnormal or immotile spermatozoa, and the presence of external substances, for example, other cells, debris and micro-organisms, reduce the success rate of ART[4].

To control the success rate of ART in the veterinary industry, semen quality assessment is performed by determining semen characteristics such as sperm count, morphology and motility. The success rate of ART in clinics and veterinary industry can be increased by improving the quality of the sample by selecting only 'good' spermatozoa. Established techniques in clinics and/or veterinary industry include among others computer-assisted sperm analysis (CASA)[5,6], flow cytometry[7,8], density gradient centrifugation[9,10] and swim-up[10,11]. However, these techniques are time consuming, expensive and require trained personnel.

Microfluidics is a fast-emerging field, dealing with the flow of liquids inside micrometer-sized channels, which match the size range of cells. Microfluidics can provide advantages over conventional semen processing techniques such as standardization, low costs and ease of visualization. In the field of AI, microfluidic devices have been applied to study, analyze, select and sort spermatozoa[12–14]. A drawback of microfluidics is the presence of shear stress which is known to reduces mammalian cell viability and affects cell physical and biological properties[15–17]. Shear stress is defined as the force exerted per unit area by the flowing fluid in a non-uniform velocity field.

In many biomedical applications of microfluidics, such as in blood diagnostics, endpoint measurements are used. After microfluidic processing, the sample is no longer useful and therefore discarded[18–20]. Hence, the negative effects of microfluidic processing on the cells used for these measurements are not important. However, in the context of ART, viable spermatozoa are needed for successful fertilization. It is essential that the effect on semen quality is minimal while processing, since it is no endpoint measurement. Only when the semen viability and motility after microfluidic processing are preserved, the success rate of ART is retained. Although many microfluidic chips have been proposed

to improve semen quality[12,13,21], so far no systematic study on the viability of spermatozoa after microfluidic processing has been performed.

Recently, high throughput microfluidic processing of spermatozoa has gained increasing interest. An example is the separation of spermatozoa from erythrocytes using a spiral channel by Son *et al.*[22]. In the spiral channel (150 μm channel width and 50 μm channel height), the motile spermatozoa were forced to the outer channel wall with a flow rate ranging from 0.10-0.52 ml/min, where they were exposed to shear stress (estimated 27-139 N/m^2). The separated spermatozoa were, however, not tested for viability or motility. Wu *et al.*[23] separated spermatozoa of different motilities based on the cell's swimming abilities in a retarding flow field. A flow field ($0.3x10^{-3}$ ml/min, estimated shear stress 0.033 N/m^2) was needed to carry the spermatozoa to the separation zone. Also, here it is not known whether the spermatozoa processing harmed sperm viability and motility. De Wagenaar *et al.*[24] have developed a chip, which focuses spermatozoa using dielectrophoresis (DEP) and sorts morphologically abnormal spermatozoa from normal ones based on a difference in the cells impedance curve. The preliminary data suggest a minimal effect of DEP on the integrity of the plasma membrane at frequencies above 10 MHz at 3 or 6 V potential. Pinched flow fractionation (PFF) for the separation of spermatozoa from epithelial cells and erythrocytes has been presented by Liu *et al.*[25] and Berendsen *et al.*[26], respectively. The separation mechanism in PFF is based on the sudden broadening of the channel after the pinched segment[27]. To achieve separation, it is necessary to align the cells to the sidewall of the pinched segment. Due to the shape, alignment and flow in the pinched segment, the cells encounter relatively high shear stress compared to other areas of the chip. Berendsen *et al.*[26] studied the viability after separation with PFF and reported a viability of 88 ± 6% (N=3).

For proof-of-principles, porcine spermatozoa are often used as a model for human spermatozoa[28,29], because human spermatozoa are not widely accessible due to variations in legislation. Moreover, microfluidic processing of spermatozoa has gained attention in the veterinary industry. Li *et al.* and Sano *et al.* have applied a microfluidic sperm sorter based on sperm motility for the selection and production of dairy cattle and porcine embryos, respectively[30,31]. Their results have shown that *in vitro* fertilization after sperm selection is more successful compared to their control groups. However, for *in vivo* fertilization, separation via the self-motion of spermatozoa is not desired, because the spermatozoa may be exhausted after being separated. Also, the throughput is very limited. In non-motility based separations, a high flow rate is desirable to obtain an acceptable throughput, which exposes the cells to a higher shear stress.

In this chapter, we test the impact of microfluidic processing on the viability of spermatozoa. Various parts of a microfluidic set-up can impose shear stress on

spermatozoa. The sample is introduced into the microfluidic chip and collected after microfluidic processing with connection tubing. The amount of shear stress in a microfluidic chip is determined by the dimensions of the channel (cross section) and the flow rate in the chip. In general, the tubing is longer compared to the length of the microfluidic chip and therefore the cells are for a longer time exposed to shear stress in the tubing, even though the shear stress in the tubing is commonly lower than in the microfluidic chip due to the larger diameter. Therefore, we have systematically studied the viability of porcine and bovine spermatozoa after being processed with microfluidic chips and connection tubing. For general purposes, a microfluidic chip with a straight channel was used and as a special case a chip with PFF. Constrictions similar to the pinched segment of a PFF device are used in other microfluidic devices such as a flow cytometers and Coulter counters[29,32,33]. Furthermore, in our investigation the shear stresses in the tubing and chips used were calculated and compared to the shear stress during ejaculation.

3.2. Materials and methods

3.2.1. Chip design and fabrication

Two microfluidic chips were used for the experiments. One chip had a straight channel with 300 μm width and 50 μm height (length (L) 2 cm), and one was a 50 μm high PFF chip, with 100 μm wide inlets, 50 μm wide pinched section and 2500 μm wide broadened section (total length of 8 mm) (figure 3.1). The chips were designed using CleWin software (version 5.0.12.0). Master molds for polydimethylsiloxane (PDMS) fabrication were produced by standard photolithography. A 50 μm layer of SU-8 (Microchem, Berlin, Germany) was spun and developed on a 4" silicon wafer.

Chips were fabricated using PDMS (Sylgard 184, Dow Corning, Midland, MI, USA) in a 10:1 v/v ratio of base versus curing agent. PDMS was poured onto a silicon wafer, degassed and cured at 60 °C overnight. After curing, microfluidic inlets and outlets were punched using a Harris Uni-Core puncher (tip inner diameter (ID) 1.0 mm, Ted Pella Inc., Redding, CA, USA). The chips were bonded to glass microscope slides after activation by oxygen plasma using a plasma cleaner (model CUTE, Femto Science, Hwaseong-Si, South Korea).

3.2.2. Sample preparation

Fresh porcine semen (breed: Tempo (Topigs Norsvin breeding line), AIM the Netherlands, Vught, the Netherlands) and fresh bovine semen (breed: Holstein, CRV, Arnhem, the Netherlands) were obtained at a concentration of 20 x 10^6 cells/ml and 89 x 10^6 cells/ml respectively. Fresh porcine semen was stored at 17 °C and used within three days. Fresh bovine semen was stored on ice for one day. Before the semen was processed,

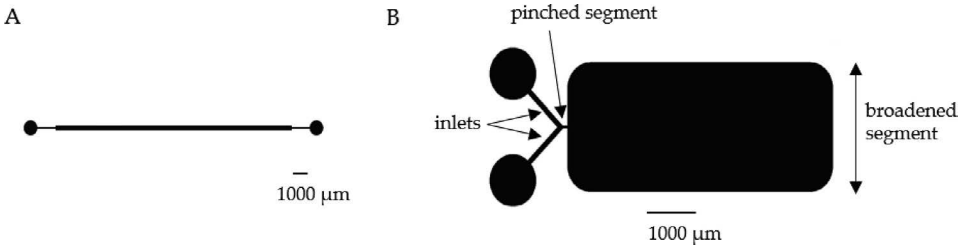

Figure 3.1: Schematic representation of the microfluidic chips used to process spermatozoa. a) straight channel with 300 µm width and 50 µm height (length 2 cm) b) PFF chip, with 100 µm wide inlets, 50 µm wide pinched section and 2500 µm wide broadened section (total length of 8 mm).

porcine semen was diluted with Solusem extender (AIM Worldwide, Vught, the Netherlands) to concentrations of 10 x 10^6 cells/ml (for Group 3) and 4 x 10^6 cells/ml (for Groups 0-2, 4). The bovine semen was diluted with Optixcell® extender (IMV technologies, L'Aigle, France) to concentrations of 44 x 10^6 cells/ml (for Group 3) and 18 x 10^6 cells/ml (for Groups 0-2, 4).

3.2.3. Microfluidic/Chip processing

The in- and outlets of the chip were connected to containers using fused silica capillaries (Polymicro Technologies, ID 100 µm, outer diameter (OD) 360 µm, L 10 cm, Molex, Surrey, UK) and Tygon tubing (ND 100-80, ID 250 µm, OD 760 µm, L 20 cm, Saint-Gobain Performance Plastics, Akron, OH, USA). A pressure pump (MZ flows, Fluigent, Le Kremlin-Bicêtre, France) was connected to the sample and buffer containers. The pressure pump was used to apply the flow through the chip.

Shortly before use, the chips were oxygen plasma treated using a plasma cleaner (model CUTE, Femto Science, Hwaseong-Si, South Korea) and became hydrophilic. The chips were rinsed and incubated with poly(L-lysine)-grafted-poly(ethylene glycol) (PLL-g-PEG, SuSoS, Dübendorf, Switzerland) at a concentration of 100 µg/ml in deionized (DI) water for at least 15 minutes. Subsequently, the sample and the buffer solution were introduced. Flow was induced by applying the desired pressures to the sample and buffer solution. At the outlet, the processed sample was collected.

Four experimental setups were tested (see figure 3.2 and table 3.1): 1) a set with only the tubing connected (applied pressures: 200, 400, 600, 800 and 1000 mbar; porcine semen sample concentration: 4 x 10^6 cells/ml; bovine semen sample concentration: 18 x 10^6 cells/ml), 2) a straight microfluidic channel with in- and outlet tubing (applied pressures: 200, 400, 600, 800 and 1000 mbar; porcine semen sample concentration: 4 x 10^6 cells/ml; bovine semen sample concentration: 18 x 10^6 cells/ml), 3) a pinched flow channel with in- and outlet tubing (sample/sheath pressures in mbar: 200/200, 200/300, 400/400, 400/600,

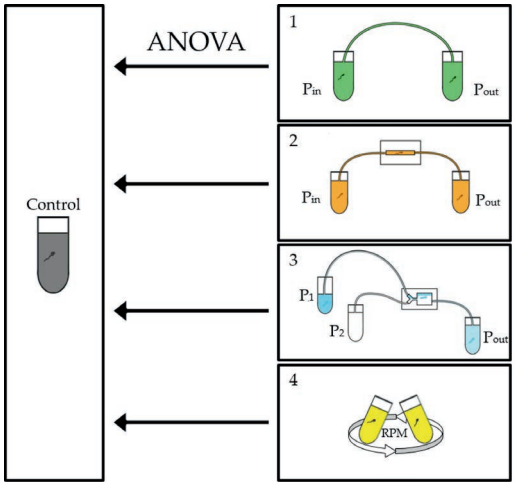

Figure 3.2: Schematic overview of the three different tested conditions for microfluidic processing of 1) tubing, 2) straight channel, 3) PFF channel, and 4) centrifuge for both bovine and porcine spermatozoa. P_{in} (or P_1 (sample) and P_2 (buffer)) were varied, while P_{out} was kept at atmospheric pressure. Each sample group was compared to the control with ANOVA to determine the significance of the change in viability. Features are not to scale. Pressure (P)

600/600, and 600/800; porcine semen sample concentration: $10x10^6$ cells/ml bovine semen sample concentration: 44 x 10^6 cells/ml), and 4) centrifugal forces at different speeds for 15 minutes using the Minispin Plus (Eppendorf, Hamburg, Germany) (relative centrifugal forces (RCF): 700, 1500 and 3000 x g (3230, 4729 and 6688 rounds per minute (rpm)); porcine semen sample concentration: 4 x 10^6 cells/ml; bovine semen sample concentration: 18 x 10^6 cells/ml). All experiments were performed at room temperature to minimize the swimming behavior of the spermatozoa. Each setup was assessed three times with the same conditions (N=3). Exceptions (N=2) are the experiments performed with porcine semen and a straight channel (pressure: 600 mbar) as well as porcine semen and pinched flow channel (sample/sheath pressures in mbar: 200:300, 400:600), because the third measurements counted less than 200 spermatozoa and were therefore excluded. For the centrifugation of porcine spermatozoa, a different control sample was used than for the other experimental setups.

3.2.4. Viability staining

The influence of shear stress from the tubing and the chips on the viability of the spermatozoa was assessed with a SYBR 14/Propidium Iodide (PI) live/dead staining. The spermatozoa were incubated in a 1000x dilution of SYBR 14 (stock 1 mM, ex/em 488/518 nm, Life Technologies, Eugene, OR, USA) for 20 minutes and a 100x dilution of PI (stock 2.4 mM, ex/em 535/617 nm, Life Technologies, Eugene, OR, USA) for 5 minutes at room temperature. For each sample, 20 µl were deposited onto a glass slide. Images were taken

with the EVOS M5000 (ThermoFisher Scientific, Waltham, MA, USA). The number of live and dead cells was manually counted, and the percentage of live cells was determined by dividing the number of live cells by the total number of cells. Per sample, at least 200 spermatozoa were counted to have statistically relevant data[21]. To obtain the effect of the chip on the viability of the cells, the percentage of viable spermatozoa in the processed samples was normalized by dividing it by the percentage of viable spermatozoa of the control sample (the diluted sperm sample which was kept in the container and not processed). The mean normalized viabilities and the corresponding standard deviations were determined.

3.2.5. (Statistical) Analysis

Normal distribution was tested using the Shapiro-Wilk test, which is an appropriate test for small sample sizes. A one-way between-groups analysis of variance (ANOVA) with planned comparisons was conducted to explore the effect of microfluidic processing on the viability of spermatozoa. An ANOVA analysis compares the variances between the different groups. A between-groups ANOVA is applied when different participants are present. Here, the "participants" are the individual experimental conditions (N=2-6). The effects of various microfluidic processing procedures are compared to the control group. Therefore, a planned comparison was used to overcome 'power' issues[34]. A positive one-tail test is more powerful in this context, as it is impossible to achieve higher viability after microfluidic processing.

The samples were divided into five groups according to the experiment (Group 0: control; Group 1: tubing; Group 2: chip with straight channel; Group 3: PFF chip; Group 4: centrifugal forces). The groups obtained after microfluidic processing (Groups 1-3) and after exposure to centrifugal forces (Group 4) were compared with the control group (Group 0) (figure 3.2). The significance level was chosen to be 0.05.

3.3. Results and discussion

3.3.1. Flow and shear stress calculations

The wall shear stresses during ejaculation and the microfluidic processing as performed in this chapter were calculated. Detailed information about the calculations can be found in the *Supporting Information* 3.6.1 Table 3.1 shows the calculated flow rates and wall shear stresses during ejaculation, flow cytometry and microfluidic processing. The peak shear stress represents the highest shear stress of each experiment. The duration is the time the cells were exposed to the peak shear stress.

During ejaculations the Reynolds numbers do not exceed 2000, so the flow is laminar. The spermatozoa were exposed to a wall shear stress of 3.4 N/m^2 and 0.65 N/m^2 during

porcine and bovine ejaculation, respectively. The highest shear stress in the tubing experiments (up to 24 N/m^2) was induced by the capillary and its small surface area. The shear stress induced by the Tygon tubing was small (up to 1.5 N/m^2) compared to the highest shear stress, but the spermatozoa were exposed to this shear stress for a longer time period. Depending on the applied pressure and the resulting flow rate, the wall shear stress during microfluidic processing with a straight channel varied from 0.17 to 11 N/m^2. These shear stresses occurred in the connection tubing rather than in the chip itself. The shear stress in the chip varied between 1.7 and 8.6 N/m^2, which was lower than the shear stress in the capillary. Both wall shear stresses during ejaculation and microfluidic processing, were in the same order of magnitude. The wall shear stress in the pinched section of the PFF device (15-51 N/m^2) was a magnitude higher than the wall shear stress of the tubing and straight channel. This resulted from the smaller channel width of the pinched section compared to the tubing diameter and width of the straight channel. Although the wall shear stress in the PFF device was high, the duration of this wall shear stress was short (less than a millisecond) compared to the other durations of a few seconds.

In another comparison, we have calculated the shear stress during flow cytometry. In the veterinary industry, flow cytometry is used to sort spermatozoa to obtain sex-selected semen[35]. Therefore, the cells must be viable after being sorted by flow cytometry. The normalized sperm viability after flow cytometry reported in literature is 89 ± 3%[36] and 80 ± 3%[37] for porcine and bovine, respectively. We have estimated the shear stress in flow cytometers to be between 2.2-79 N/m^2 (see *Supporting Information* 3.6.1. for estimations on processing velocities and dimensions) which is in the same range as the shear stress of microfluidic processing.

The shear rate and the cross sectional area between the spermatozoon and the channel wall determine the applied force. In this application, spermatozoa flow within the liquid through a microfluidic channel. Usually, spermatozoa are oriented in such a way, that the tip of the head is in front and the tail is dragged behind. The head of a spermatozoon is oval flattened. Therefore, the area opposing the shear stress is either the larger flat surface area or the smaller thin surface area. In case of the flat surface, the experienced applied force is larger than for the thin surface area.

Table 3.1: Calculated shear stress and its duration during porcine and bovine ejaculation as well as during microfluidic processing with tubing and a chip with a straight channel. For both ejaculation and microfluidic processing, the shear stresses were in the same order of magnitude. The highest shear stress occurred in the capillary part of the tubing or in the pinched segment of the PFF chip. The longest duration was in the Tygon tubing, where the shear stress was low.

	Flow rate (ml/min)	Shear stress (N/m^2)	Duration time (s)
Porcine ejaculation	40	3.4	3.6
Bovine ejaculation	1.2×10^2	0.65	9.8
Flow cytometer		2.2-79	≈ few sec

Microfluidics	Pressure (mbar)	Total flow rate (ml/min)	Peak shear stress (N/m^2) (duration time (s))	Longest exposure shear stress (N/m^2) (duration time (s))
Tubing	200	2.8×10^{-2}	4.8 (1.7)	0.30 (21)
	400	5.6×10^{-2}	9.5 (0.84)	0.61 (11)
	600	8.4×10^{-2}	14 (0.56)	0.91 (7.0)
	800	0.11	19 (0.42)	1.2 (5.3)
	1000	0.14	24 (0.34)	1.5 (4.2)
Chip with straight channel	200	4.0×10^{-3}	2.2 (7.3)	0.17 (9.1)
	400	7.8×10^{-3}	4.4 (3.6)	0.28 (4.6)
	600	1.2×10^{-2}	6.6 (2.4)	0.42 (3.0)
	800	1.6×10^{-2}	8.8 (1.8)	0.56 (2.3)
	1000	2.0×10^{-2}	11 (1.5)	0.70 (1.8)
PFF chip	200/200	1.8×10^{-2}	15 (8.3×10^{-4})	0.20 (33)
	200/300	2.3×10^{-2}	18 (6.6×10^{-4})	0.25 (26)
	400/400	3.7×10^{-2}	29 (4.1×10^{-4})	0.39 (16)
	400/600	4.6×10^{-2}	36 (3.3×10^{-4})	0.49 (13)
	600/600	5.5×10^{-2}	44 (2.8×10^{-4})	0.59 (11)
	600/800	6.7×10^{-2}	51 (2.4×10^{-4})	0.69 9.3)

3.3.2. Viability of spermatozoa after microfluidic processing

The effect of microfluidic processing on the viability of spermatozoa was studied by processing spermatozoa with various parts of a microfluidic set-up, namely the connection tubing, a microfluidic chip with a straight channel and a microfluidic chip with PFF. Various pressures were applied while running the spermatozoa through the systems. Figure 3.3 shows representative images of live/dead stained spermatozoa after microfluidic processing. The viability of the control group was 83 ± 5.8% for porcine spermatozoa and 88 ± 3.1% for bovine spermatozoa. In figure 3.4 the normalized

viabilities of bovine and porcine spermatozoa after microfluidic processing with various applied pressures and centrifugation with various centrifugal forces are shown. It was investigated if an increase in applied pressure or centrifugal force decreases the viability. This possible trend was not observed. Under all four experimental conditions, the viabilities were similar to the other test conditions. For that reason, the normalized average viabilities for each experimental condition were determined (figure 3.5).

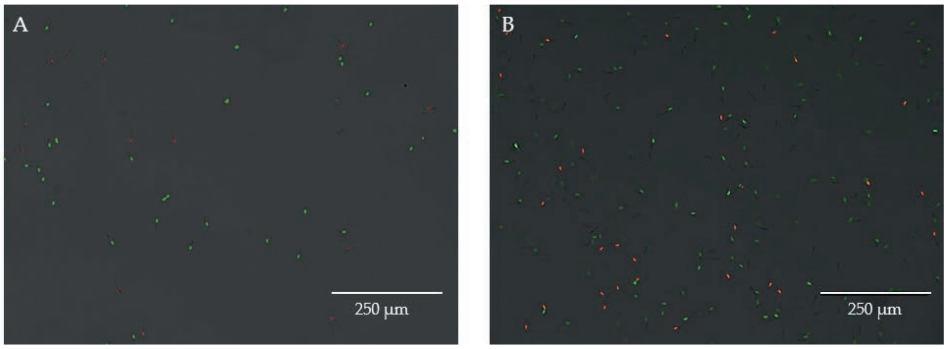

Figure 3.3: Representative images of porcine (a) and bovine (b) spermatozoa treated with live/dead staining after being processed with the microfluidic chip (straight channel, 1000 mbar). Live and dead cells are represented in green and red, respectively.

The normalized averaged viabilities of the porcine and bovine spermatozoa were between 88 - 98% and 97 - 103%, respectively, and were very similar to the control group (100%). This also held for PFF, where the spermatozoa were exposed to the highest shear stress. The lowest viability of 88% viability in porcine semen was still high. When taking the standard deviation into account, at first sight it appeared that the effect of the microfluidic chips is negligible. Especially the bovine semen seemed not to be affected by the microfluidics processing. The results, however, suggest that porcine semen is more susceptible to processing than bovine semen. Similarly, it has been shown that cryopreservation causes more negative effects on porcine than on bovine spermatozoa[38,39]. Differences in physiochemical and biochemical semen characteristics between these animal species may be the reason for this unequal susceptibility[40].

To find additional evidence to the visual observations, statistical analysis was performed. The Shapiro-Wilk test showed that the normalized viabilities showed a normal distribution ($p>0.05$). An ANOVA with planned comparison was applied to test whether there was an impact on viability after microfluidic processing compared to the control group. For porcine spermatozoa, viability in the groups with microfluidic processing was significantly ($F_{1,47} = 5.12$, $p = 0.014$) lower than the control group. The average decrease in viability of 6% seemed, therefore, to be significant. Only the difference for the control with group 5 (centrifugal forces) was not statistically significant ($F_{1,13} = 1.13$, p

= 0.15). There was no significant difference in viability between processing with tubing, a straight channel or PFF (p = 0.41). In each of these sets of experiments, the semen was flushed through the tubing to reach the chip. These results could indicate that the spermatozoa are damaged in the tubing before reaching the chip. Therefore, the effect of microfluidic tubing processing has been investigated by varying tubing parameters such

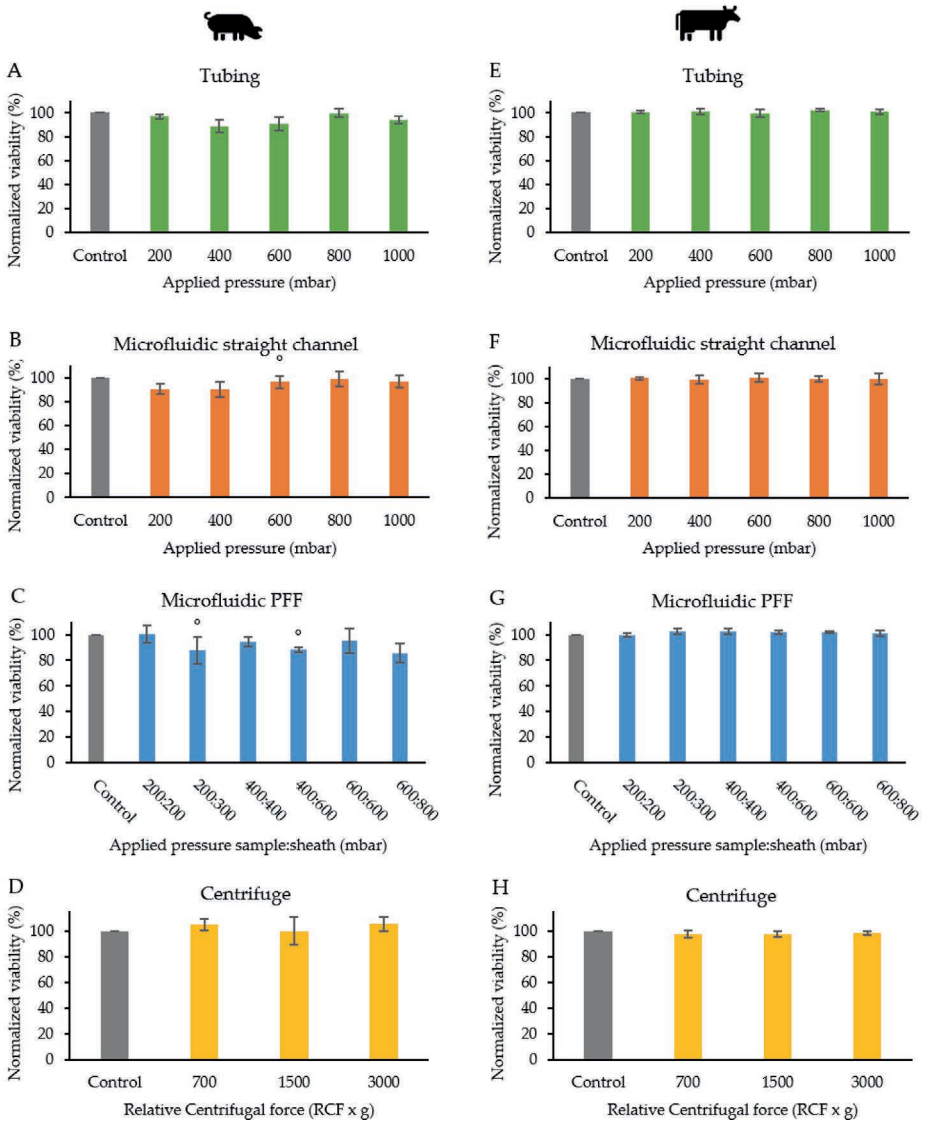

Figure 3.4: The percentage of normalized viability after microfluidic processing of porcine (left) and bovine (right) spermatozoa with connection to tubing (a&e), microfluidic chip with a straight channel (b&f), a pinched flow fractionation (PFF) chip (c&g) and centrifugation (d&h). Error bars=1 SD, N= 3 (° N=2). In all experimental conditions no trend in viability decrease with increasing applied pressure/centrifugal force was observed (p > 0.05).

as tubing type and length (*Supporting Information* 3.6.2). The results show that tubing had no visual nor statistical effect on the sperm viability. The average decrease in viability to 94 ± 7% was lower than the decrease in viability when using a flow cytometer as reported in the literature, where the normalized viability after sorting was 89 ± 3%[36]. Note that this decrease in viability could be affected by the biological variation between species and our relatively small sample size.

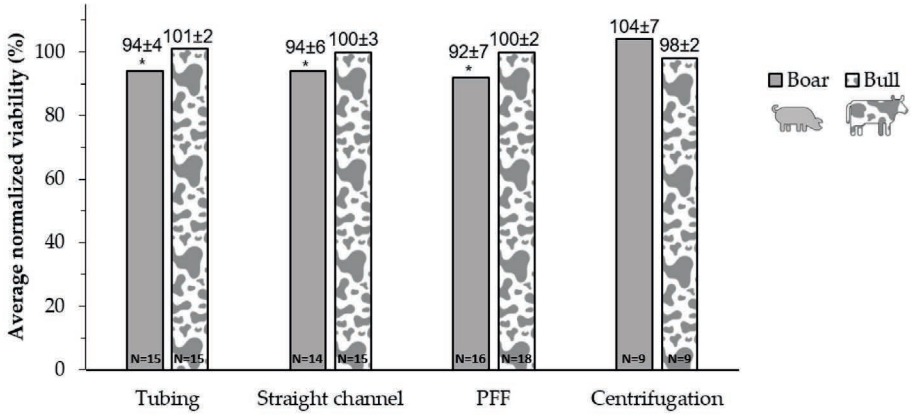

Figure 3.5: Average normalized viability (± 1 SD) of the porcine and bovine spermatozoa after (microfluidic) processing. The viability of spermatozoa after microfluidic processing was almost 100% when taking the standard deviation into account. Therefore, the effect of microfluidic processing on the viability of spermatozoa is negligible. * indicates $p<0.05$.

For bovine spermatozoa, the groups with microfluidic processing did not differ significantly ($F_{1,67}=0.09$, $p=0.38$) from the control group. This indicates that the viability of the bovine spermatozoa was not affected by microfluidic processing. The results represented in this chapter show higher viability than in a study using flow cytometry. The value for the normalized viability (calculated from the absolute viability reported for "bulk sorting", in which all spermatozoa were counted) was 80 ± 3%[37].

In contrast to viability measurements, the motility and morphology of spermatozoa provide important information about sperm condition. Observations have shown that after processing the spermatozoa were intact and showed no morphological difference compared to the control group. To prevent inter-animal differences from occluding the viability results, we have used one semen donor. It is unknown whether these semen donors were representative for the population, but the control semen was collected from a porcine and bovine which are used in routine semen processing for AI. Both donors have high fertility recording (Topigs Norsvin, Vught, the Netherlands; CRV, Arnhem, The Netherlands). For further research, it is recommended to test various semen donors

and to quantify the motility and morphology of spermatozoa after microfluidic processing.

3.4. Conclusions

Over the years, microfluidic analysis and processing of spermatozoa have gained more interest. For the intended applications, it is essential that the spermatozoa are not damaged by the processing and remain viable for insemination. We estimated the shear stress on bovine and porcine spermatozoa during ejaculation and compared it to the calculated shear stress during general microfluidic processing steps. The shear stress is comparable to the natural shear stress during ejaculation. We then studied the viability of spermatozoa after microfluidic processing. The porcine spermatozoa showed a small but significant decrease in viability of 6%. Bovine spermatozoa revealed to be less susceptible; it was concluded that it is not significantly affected by microfluidic processing. This data indicates that microfluidic processing has less influence on the viability of porcine and bovine spermatozoa than literature has reported for flow cytometry.

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3.6. Supporting Information

3.6.1. Flow and shear stress calculations

The flow rate during ejaculation was calculated using $Q = \frac{V}{t}$ (1), where Q is the flow rate (m^3/s), V the volume (m^3) and t the ejaculation time (s). The average flow velocity v (m/s) is calculated by $v = \frac{Q}{\pi a^2}$ (2) with a (m) the radius of the vas deferens. To assess laminar flow conditions, the Reynolds number Re was calculated using the equation $Re = \frac{\rho v 2a}{\eta}$ (3), where ρ is the density of semen (kg/m^3) and η is the semen viscosity (Pa s). During ejaculation the flow is laminar, if the Reynolds number does not exceed 2000. The assumptions used to calculate the flow rate, average flow velocity, Reynolds number and shear stress during boar and bovine ejaculation are shown in table 3.2.

Table 3.2: Assumptions used for shear stress calculation during ejaculation.

Animal species	Semen viscosity (Pa s)	vas deferens radius (mm)	Semen density (kg/m^3)	Semen volume (ml)	Ejaculation time (s)
Boar	0.004 [1]	1.0 [2]	1020 [3]	250 [4, 5]	378 [4]
Bull	0.004 [6]	2.5 [7]	1053 [8]	10 [5, 9]	5 [*1]
Diluted sample	0.004		1000		

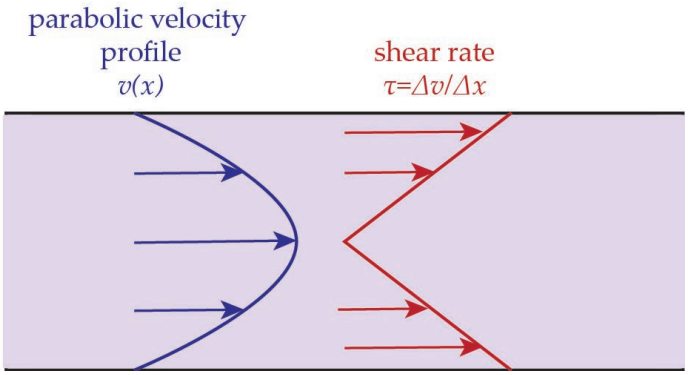

Figure 3.6: In a microfluidic channel with a pressure induced flow the velocity profile is parabolic. The shear rate is the first derivative of the velocity profile.

Pressure-induced flow, which is the type of flow usually used in microfluidics, has a parabolic velocity profile. The first derivative of a parabolic velocity profile is linear and represents the shear rate (figure 3.6). Therefore, the highest shear stress τ (N/m^2) occurs at the channel walls and is known as wall shear stress. The wall shear stress depends on

*1 MLWJ Broekhuijse, CRV BV, personal communication, 2019

the fluid viscosity η (Pa s), channel dimensions (height h (m) and width w (m) /radius a (m)) and average fluid velocity v (m/s):

$$\tau = \frac{6\eta v}{h} \text{ (4) (for a rectangular channel, where h<w and w<<L) and}$$

$$\tau = \frac{4\eta v}{a} \text{ (5) (for a cylinder-like channel)}^{10,11}.$$

The average flow velocity v (m/s) during microfluidic processing was determined by $v = \frac{\Delta P}{AR_h}$(6), where ΔP is the applied pressure difference (Pa), A is the area (m^2) and R_h is the hydraulic resistance (Pa s/m^3). For a circular cross-section the hydraulic resistance was determined using $R_h = \frac{8\eta L}{\pi a^4}$ (7) with η the viscosity (Pa s) (treated as water at room temperature, because of dilution), L the tube length (m) and a the tube radius (m). For a rectangular cross-section the hydraulic resistance was calculated with $R_h = \frac{12\eta L}{h^3 w(1-0.63(\frac{h}{w})}$ (8) with h the height (m) and w the width (m). The hydraulic resistance of various microfluidic devices in series are computed as the sum of the individual hydraulic resistances. Equations 4 and 5 were used to calculate the shear stress in a microfluidic chip with a straight channel and the tubing, respectively[11].

The previously mentioned formulas were used to calculate the flow rate, average flow velocity, Reynolds number and shear stress during ejaculation (table 3.4). The Reynolds number does not exceed 2000, so the flow is laminar. The spermatozoa are exposed to a shear stress of 3.37 and 0.65 N/m^2 during boar and bull ejaculation, respectively. For microfluidic processing, the semen samples are highly diluted and therefore approximate the viscosity and density of water. Similarly, the average flow velocity and shear stress during microfluidic processing with connection tubing, a chip with a straight channel and a PFF chip were calculated (table 3.4 and 3.5). Shear stress during microfluidic processing is in the same order of magnitude compared to ejaculation. Due to the smaller cross-section of microfluidic devices, the average flow velocity is higher for microfluidic processing compared to ejaculation.

The shear stress of flow cytometry was calculated based on the specifications of the Novocyte Benchtop (ACEA Biosciences. Inc., San Diego, CA, USA)[12]. Assuming a sample flow of 5-120 µl/min, a sheath flow of 6.5 ml/min, and flow cell dimensions of 290 µm wide and 170 µm height, the shear stress is approximated to be between 2.20-78.99 N/m^2.

Table 3.4: Calculated flow rate, average flow velocity, Reynolds number and shear stress during boar and bull ejaculation.

	Flow rate Q (ml/s)	**Average flow velocity v (m/s)**	**Reynolds number Re**	**Shear stress τ (N/m^2)**
Boar ejaculation	0.66	0.21	107	3.37
Bull ejaculation	2.00	0.10	134	0.65

Table 3.5: The average flow velocity and shear stress were calculated for microfluidic connection tubing and microfluidic chips with a straight channel and PFF. With increasing pressure, the average flow velocity and the shear stress increases.

Experiment	**Applied pressure (mbar)**	**200**	**400**	**600**	**800**	**1000**
Tubing	Average flow velocity (mm/s)	9.51	19.03	28.54	38.05	47.56
	Peak shear stress (N/m^2)	4.76	9.51	14.3	19.0	23.8
Chip with straight channel	Average flow velocity (mm/s)	14.4	28.7	43.1	57.5	71.8
	Peak shear stress (N/m^2)	2.19	4.39	6.58	8.78	10.8
Chip with PFF	**Applied pressure (mbar)**	**200: 200**	**200: 300**	**400: 400**	**400: 600**	**600: 600**	**600: 800**
	Average flow velocity (mm/s)	121.0	151.3	242.0	302.5	363.0	423.5
	Peak shear stress (N/m^2)	14.52	18.15	29.04	36.30	43.56	50.82

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3.6.2. The effect of microfluidic tubing processing on the sperm viability

The effect of microfluidic connection tubing on the viability of spermatozoa has been further studied. The connection tubing consisted of both a silica capillary (inner diameter (ID)=100 μm, length (L)=10 cm) and Tygon tubing (ID=250 μm, L=25 cm). In order to investigate the influence of each tubing component on the sperm viability, spermatozoa were processed with only a silica capillary and only a length of Tygon tubing. Additionally, the effect of varying the tubing length, tubing inner diameter, and tubing with PLL-PEG coating was tested. Various pressures were applied while running the spermatozoa through the tubing.

Figure 3.7 shows the viability of bovine and porcine spermatozoa after processing with connection tubing with various applied pressures. It was investigated if an increase in applied pressure decreases the viability. This possible trend was not observed. Under all experimental conditions the viabilities are similar to the other test conditions. For that reason, the normalized average viabilities for each experimental condition were determined (figure 3.8). Also, the normalized average viabilities are similar for each experimental condition. No difference was observed when taking the type of tubing, tubing length and coating into account.

To find additional evidence to the visual observations, statistical analysis was performed. A one-way between-groups analysis of variances (ANOVA) with planned comparison was applied to test whether there is an impact on viability after tubing processing compared to the control group. A statistically non-significant difference at a one-tail test with $p<0.05$ level between the control group and the groups with tubing processing was obtained: $F_{7,63}=5.12$, $p=0.35$.

All in all, it has been shown that microfluidic tubing processing has no impact on sperm viability. None of the tested tubing properties such as tubing type, tubing length, inner diameter and coating was significantly different from the control group.

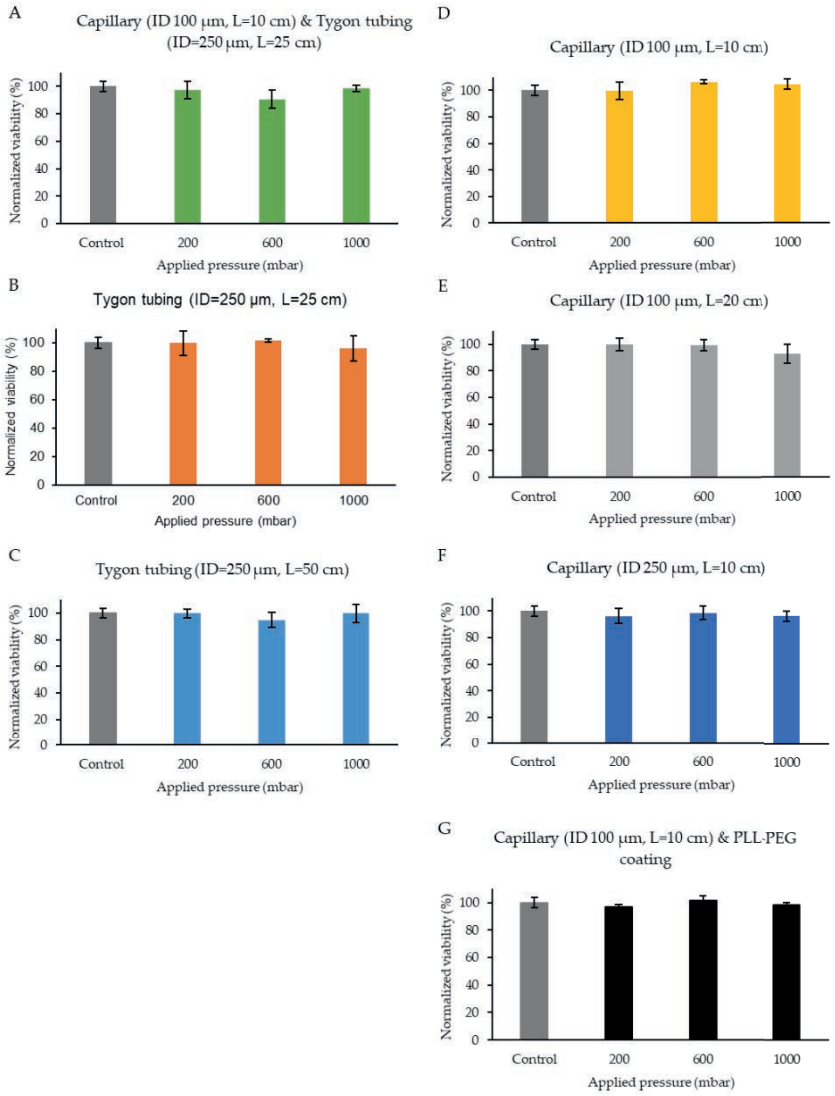


Figure 3.7: The percentage of normalized viability after tubing processing of porcine spermatozoa with various parts of the microfluidic connection tubing: a silica capillary (inner diameter (ID)=100 µm, length (L)=10 cm) and Tygon tubing (ID=250 µm, L=25 cm (a), Tygon tubing (ID=250 µm, L=25 cm) (b), Tygon tubing (ID=250 µm, L=50 cm) (c), silica capillary (ID=100 µm, L=10 cm) (d), silica capillary (ID=100 µm, L=20 cm) (e), silica capillary (ID=250 µm, L=10 cm) (f), silica capillary (ID=100 µm, L=10 cm) and PLL-PEG coating (g). Error bars=1 SD, N=2 or 3. In all experimental conditions no trend in viability decrease with increasing applied pressure force was observed.

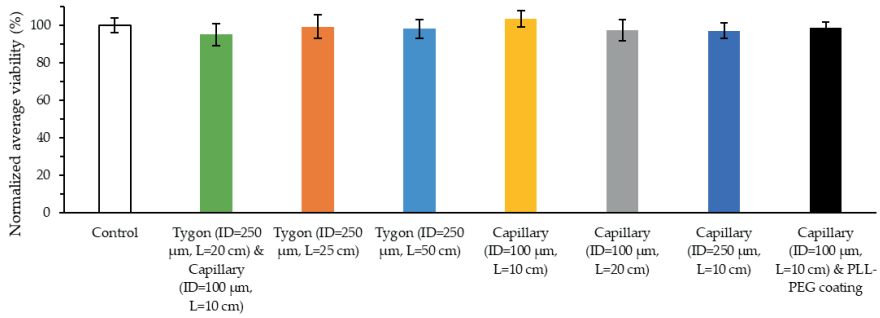
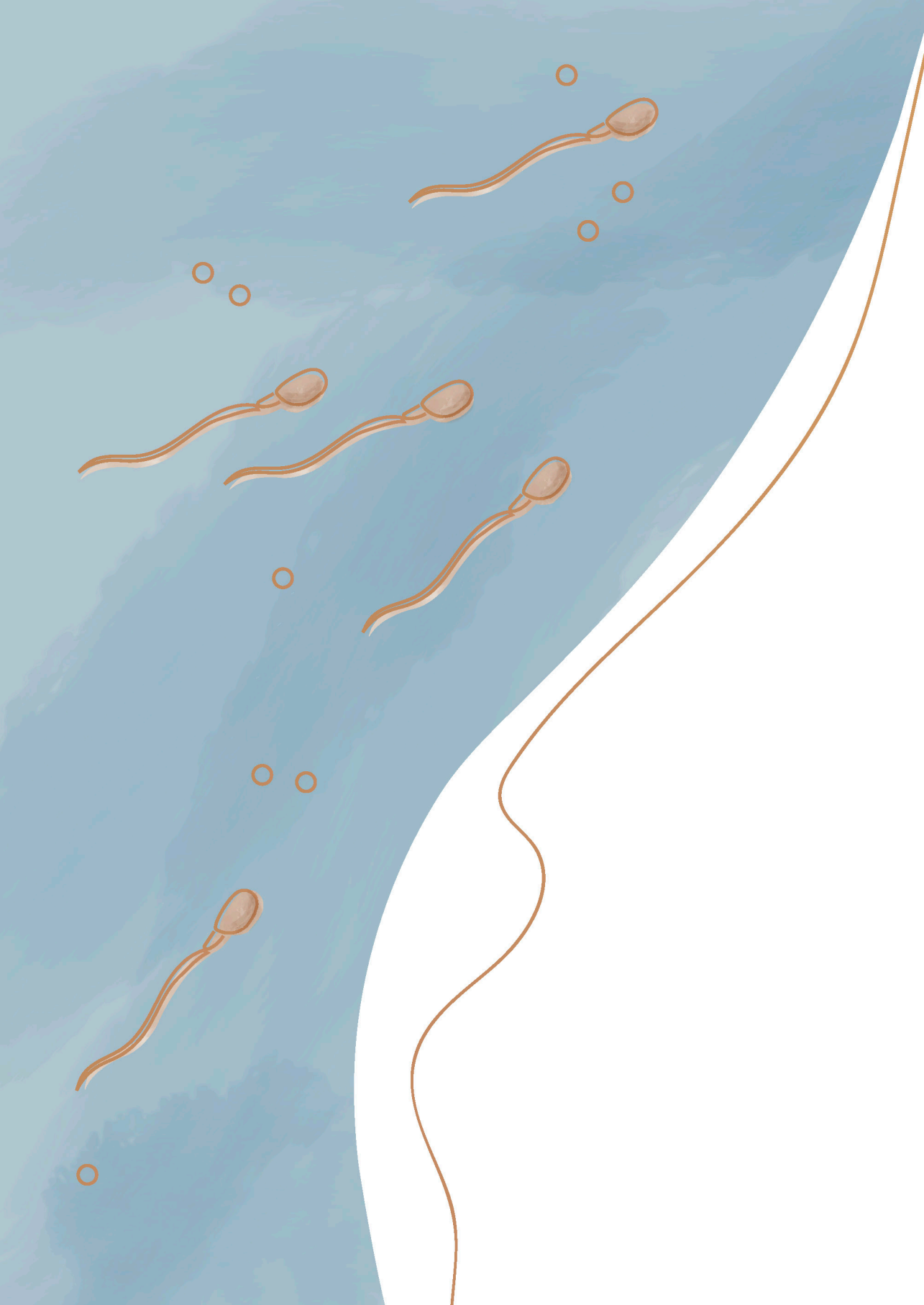
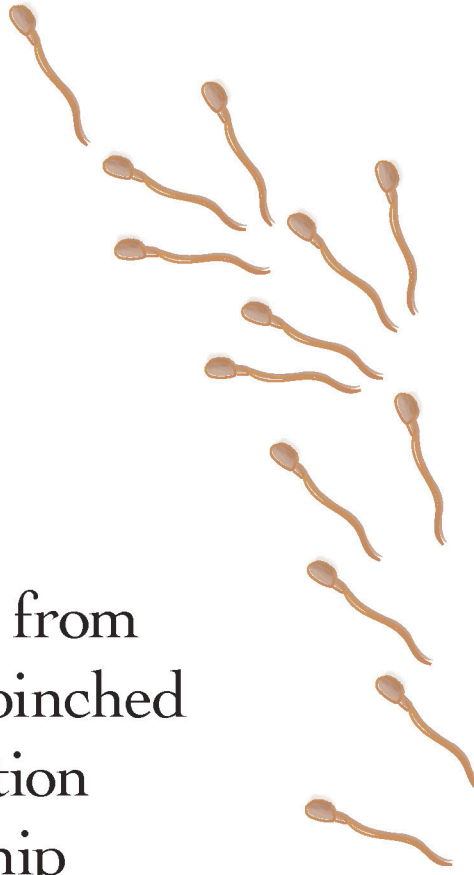


Figure 3.8: Normalized average viability (± 1 SD) of the spermatozoa after (microfluidic) connection tubing processing. The viability of spermatozoa after microfluidic processing is almost 100% when taking the SD into account. Therefore, the effect of microfluidic tubing on the viability of spermatozoa is negligible. No statistical difference between the experimental conditions and control group was found.





4.

Virus removal from semen with a pinched flow fractionation microfluidic chip

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Abstract

Nowadays pigs are bred with artificial insemination to reduce costs and transportation. To prevent the spread of diseases, it is important to test semen samples for viruses. Screening techniques applied are enzyme-linked immunosorbent assays and/or polymerase chain reaction, which are labor-intensive and expensive methods. In contrast to the current used screening techniques, it is possible to remove viruses physically from semen. However, existing methods for virus removal techniques have a low yield of spermatozoa. Therefore, we have developed a microfluidic chip that performs size-based separation of viruses and spermatozoa in boar semen samples, thereby having the potential to reduce the risk of disease spreading in context of artificial insemination in the veterinary industry. As the head of a spermatozoon is at least twenty times larger than a virus particle, the particle size can be used to achieve separation, resulting in a semen sample with lower viral load and of higher quality. To achieve the size separation, our microfluidic device is based on pinched-flow fractionation. A model virus, cowpea chlorotic mottle virus, was used and spiked to porcine semen samples. With the proposed microfluidic chip and the optimized flow parameters, at least 84 ± 4% of the model viruses were removed from the semen. The remaining virus contamination is caused by the model virus adhering to spermatozoa instead of the separation technique. The sperm recovery was 86 ± 6%, which is an enormous improvement in yield compared to existing virus removal techniques.

4.1. Introduction

The introduction of artificial insemination (AI) in the veterinary industry was revolutionary. Besides reducing the cost of animal transportation and the use of semen from superior males for a multitude of females, the spread of diseases was minimized[1,2]. Nowadays, AI is the gold standard reproduction technology; approximately 90% of pigs in Europe and North America are bred using AI[2,3].

Although the spread of diseases is minimized by AI, micro-organisms can still be found in semen and their presence has a major influence on the semen quality[4–6]. The negative impact on sperm quality caused by viruses includes decreases in sperm motility and viability[7] as well as sperm abnormalities[8,9]. Furthermore, one infected boar can transfer a disease to a multitude of sows which can lead to a disease outbreak and severe economic costs[4]. An example is the epidemic of classical swine fever (CSF) in the Netherlands in 1997-1998. During this epidemic, the semen of infected boars was used for AI leading to CSF outbreaks in farms, whose only contact with CSF was via the contaminated semen[10]. This outbreak caused the eradication of 12 million pigs and the economic loss was estimated to be $2.3 billion[11,12]. To prevent another epidemic caused by virus-infected semen, new laws and regulations strictly control the biosafety and animal health of boar centers and pig farms for semen trade and import within the European Union[13,14]. Special agreements with selected countries outside the European Union has been made to control semen import and export[15].

Currently, both enzyme-linked immunosorbent assays (ELISA) and polymerase chain reactions (PCR) are used to detect the presence of viruses in semen[6]. These techniques are costly (up to €150 per test), labor intensive and time consuming (3-12 working days), since these tests are performed by external providers instead of by the AI centers themselves (MLWJ Broekhuijse, Topigs Norsvin, CRV BV, personal communication, 2020). Due to the long screening times, virus screening test results of porcine semen are usually not available prior to insemination, because necessary waiting time reduces semen quality[16,17].

Numerous viral pathogens have been identified in animal ejaculates. Most of the viruses can be found free in the seminal plasma or in somatic cellular components and not in the sperm fraction[18–21]. With the implementation of a virus removal processing step during daily AI laboratory procedure, the viral load and therewith the risk of disease transmission is decreased. Since the chance of disease transmission is related to the viral load, lowering this will facilitate safer insemination with fresh semen, which is currently used for AI of sows.

To reduce the viral load, it could be interesting to separate spermatozoa form the viruses. A combination of single-layer centrifugation (SLC) and "swim-up" procedure has been proposed to remove porcine circovirus type 2 (PCV2) from semen[22]. The total sperm yield was 45 ± 20% and the sperm motility was 72 ± 23% with respect to unprocessed sperm motility of 80 ± 16%[22]. This method claims to reduce more than 99% of PCV2 from the semen samples. Similarly, a double SLC procedure has been proposed to remove equine arteritis virus from equine semen[23]. The mean yield of motile spermatozoa was 52 ± 16% and the virus level was reduced, but the exact virus reduction was not stated[23].

The poor sperm yield of the previously mentioned virus removal techniques, can be improved by using a microfluidic device to separate viruses from spermatozoa. Microfluidics is a fast-emerging field dealing with the flow of liquids inside micrometer-sized channels[24]. The advantage of a separation technique based on microfluidics is that microfluidic processes can be automated and standardized, resulting in less manual performed processing steps.

Many different principles to separate spermatozoa from other particles or cell types using microfluidic devices have been reviewed[25–27]. Microfluidic approaches are both aimed at increasing sperm motility and separation of spermatozoa from different cell types. An example of devices aimed at separation is the spiral channel proposed by Son *et al.*, which focuses particles based on their size and in this way motile spermatozoa are separated from both immotile spermatozoa and erythrocytes[28]. The sperm recovery was 81% with an erythrocyte removal of 99%. Liu *et al.*[29] and Berendsen *et al.*[30] used pinched flow fractionation (PFF) to remove epithelial cells and erythrocytes from spermatozoa, respectively. Liu *et al.* achieved a sperm recovery of 41 ± 3% with a purity of 97 ± 2%[29], whereas Berendsen *et al.* have reached a sperm recovery of 94 ± 8% with an erythrocyte removal of more than 90%[30].

The size separation technique PFF is based on the sudden broadening of a channel immediately after a pinched segment[31]. A sample flow and a sheath flow coincide at the pinched segment, where the particles are focused onto the sidewall in case of a higher sheath than sample flow rate. The width of the sample fluid in the pinched segment should be smaller than the bigger particles present in the sample, which are the spermatozoa in our application. By choosing the flow rates in an appropriate manner, the system will operate in the laminar flow regime, which causes the streamlines to divert into the broadened segment without crossing each other. This allows the particles to effuse according to their initial position in the pinched segment, where the larger particles follow the streamlines more to the center of the channel, whereas smaller particles follow the streamlines closer to the channel wall. This allows for a separation based on each particle's size and deformability. Due to the differences in size between

spermatozoa (head: 7 μm long, 4 μm wide; length: 45 μm [32]) and viruses (10 - 400 nm [6]), the size-based separation principle PFF could, in principle, be used to separate viruses from spermatozoa. PFF is advantageous in contrast to other separation methods, such as labelling with DNA stains, of which contradictory statements have been made regarding the toxicity[33–35], or motility-based methods, which can cause sperm exhaustion[36]. Besides that, PFF does not seem to be harmful to the cells, since we have previously shown that the effect of PFF on the viability of boar and bull spermatozoa, is less than or similar to the current used processing techniques such as centrifugation and flow cytometry (Chapter 3)[37].

To our knowledge, for the first time a microfluidic device is presented that can efficiently separate viruses from porcine spermatozoa to decrease the virus load prior AI. As a virus model cowpea chlorotic mottle viruses (CCMVs) were used, which have a similar size (28 nm) to typical viruses found in semen[38]. The chip design and flow parameters were optimized to achieve high sperm recovery while separating most of the viruses. With a virus cleaning step implemented in the daily processing flow of porcine semen, the biosecurity of AI with porcine semen is improved.

4.2. Materials and methods

4.2.1. Microfluidic chip: design and fabrication

The microfluidic chip has a typical PFF design, of which a schematic illustration is shown in figure 4.1A, consisting of two inlets, a pinched segment, a broadened segment and two outlets. The two parallel inlets, one for the sample (inlet 1) and one for the buffer (inlet 2), conjunct at the pinched segment (width (w_P) 50 μm, length 100 μm). The angle of the boundary between the pinched and broadened segment is 180° and the corners of the broad segment are rounded, which prevents the adherence of air bubbles and spermatozoa, because there is almost no fluid flow in the corners. The broadened segment width (w_b) is 1100 μm (design I) or 2200 μm (design II). After a length of 1500 μm in the broadened segment, the separation channel with a width of 45 μm branches off to outlet 1, whereas the broadened segment ends in outlet 2. The device height was designed to be 50 μm.

The chips were designed using CleWin software (version 5.0.12, WieWeb software, Hengelo, The Netherlands) and master molds were produced using standard photolithography. The polydimethylsiloxane (PDMS) chips were fabricated in a 10:1 v/v ratio of base versus curing agent (Sylgard 184, Dow Corning, Midland, MI, USA). PDMS was poured onto a silicon wafer, degassed, and cured at 60 °C for three hours. After curing, microfluidic inlets and outlets were punched using a Harris Uni-Core puncher (tip inner diameter (ID) 1.0 mm, Ted Pella Inc., Redding, CA, USA). The chips were

bonded to glass microscope slides after activation by oxygen plasma using a plasma cleaner (model CUTE, Femto Science, Hwaseong-Si, South Korea).

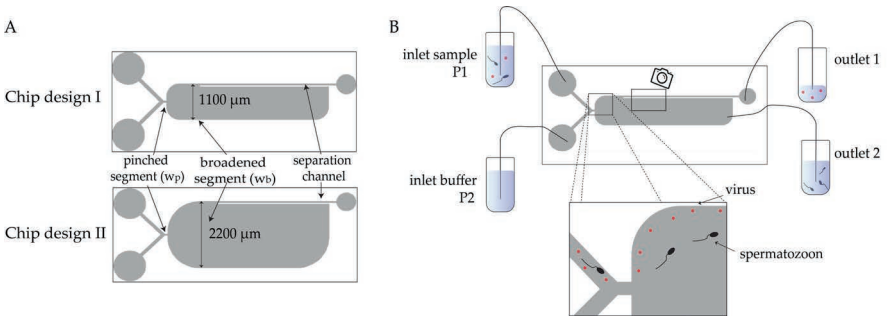


Figure 4.1: A) Schematic representation of the two chip designs. The difference between both designs is the width of the broadened segment. B) Schematic representation of the PFF set-up. The flow of the microfluidic chip is controlled by a pressure pump which is connected individually to the sample and buffer inlets. Particles are pinched and separated to follow different streamlines according to their size. The larger particles (spermatozoa) follow the streamlines further away from the channel wall and exit the chip at outlet 2. The smaller particles (viruses) are closer to the channel wall and exit the chip at outlet 1 into the waste container.

4.2.2. CCMV preparation and characterization

All chemicals described in this section were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands) unless stated otherwise. The CCMV were grown in cowpea plants. After 14 to 18 days, the virus was isolated and purified as described by Verduin[39,40] and adapted by Comellas Aragonès[38]. In short, the harvested leaves were homogenized with cold pH 4.8 buffer containing 0.2 M sodium acetate, 10 mM ascorbic acid and 10 mM disodium ethylenediaminetetraacetic acid (EDTA). The homogenate was squeezed through a cheesecloth and the filtrate was centrifuged (12 000 x g, 10 min, 4 °C) to remove the bigger particles. The supernatant containing CCMV was precipitated with polyethylene glycol (PEG)-6000 (10% w/v) by centrifugation (12 000 x g, 15 min, 4 °C). After reconstitution of the pellet in virus buffer (0.1 M sodium acetate buffer pH 5.0 containing 1 mM sodium azide and 1 mM disodium EDTA), CCMV was centrifuged for 16 h at 145 000 x g at 10 °C in 37.5% cesium chloride with a Sorvall WX80 ultracentrifuge in a vertical rotor TV-1665 (Thermo Fisher Scientific, Waltham, MA, USA). This creates a gradient with a CCMV band at a density of 1.36 g/L. After collection CCMV is dialyzed 3x with 300 ml virus buffer and stored at 4 °C.

CCMV were fluorescently labelled with Atto 647 Hydroxysyccinimide (NHS) ester and Atto 488 NHS ester. Before, CCMV was dialyzed with encapsulation buffer consisting of 5 mM magnesium chloride ($MgCl_2$) in phosphate-buffered saline (PBS) pH 7.4. The buffer

was refreshed twice. The Atto-NHS dye was dissolved in oxygen free, dry DMSO and mixed in a 1:0.03 (w/w) ratio with CCMV in PBS. To remove the excess dye, CCMV was dialyzed with encapsulation buffer and refreshed twice. CCMV was stored in encapsulation buffer at 4 °C. The CCMV concentration and degree of labeling were determined by measuring the absorption of CCMV with a nanodrop system (NanoDrop One, Thermo Fisher Scienfic, MA, USA). For CCMV labelled with Atto 647 NHS (CCMV-647), absorption at 260 nm and 644 nm were measured. CCMV-647 concentration was 3.3 mg/ml, and the degree of labelling was 0.025 dyes/capsid protein. For CCMV labelled with Atto 488 NHS (CCMV-488), absorption at 260 nm and 488 nm were measured. CCMV-488 concentration was 2.4 mg/ml, and the degree of labelling was 0.03 dyes/capsid protein. The size distribution of CCMV was measured with dynamic light scattering (DLS) using a Microtrac Nanotrac Wave W3043. Viscosity and refractive index of water and the refractive index of CCMV (1.54) were used.

4.2.3. Semen sample preparation

Fresh boar semen at a concentration of 20 x 10^6 cells/ml was obtained from a local artificial insemination center (Varkens KI Twenthe, Fleringen, the Netherlands). The semen was stored at 16 °C and used within four days. The samples were diluted with Solusem Bio+ (AIM Extender, AIM Worldwide, Vught, the Netherlands).

4.2.4. Experimental setup

A schematic representation of the microfluidic set-up is shown in figure 4.1B. Both chip inlets were connected to a container using fused silica capillaries (Polymicro Technologies, ID 100 μm, outer diameter (OD) 360 μm, length (L) 9 cm, Molex, Surrey, UK) and Tygon tubing (ND 100-80, ID 250 μm, OD 760 μm, L 20 cm, Saint-Gobain Performance Plastics, Akron, OH, USA). The flow for the inlets was controlled with a pressure pump consisting of two Flow-EZ modules (LineUp Series, Fluigent, Le Kremlin-Bicêtre, France) with a p-cap connector (Fluigent, Le Kremlin-Bicêtre, France). The sheath pressure was 400 mbar (≈64 μl/min), and the sample pressure was varied between 28-32 mbar (≈1.5-2.8 μl/min). The outlet tubing was chosen such that 2-3% of the flow exited at outlet 1. Outlet 1 was connected to a container using fused silica capillaries (L=11 cm) and Tygon tubing (L=25 cm). Outlet 2 was connected to a container with Tygon tubing (L=10-13 cm).

Shortly before use, the chips were hydrophilized using a plasma cleaner (model CUTE, Femto Science, Hwaseong-Si, South Korea). The chips were then rinsed and incubated with poly(L-lysine)-grafted-poly (ethylene glycol) (PLL-g-PEG, SuSoS, Dübendorf, Switzerland) at a concentration of 100 μg/ml in deionized (DI) water for at least 15 minutes to prevent particle and cell adhesion. After coating, the chip was connected to

the pressure pump. Sample and buffer solution, the latter one Solusem Bio+ for all experiments, were introduced via inlets 1 and 2, respectively. Flow was induced by applying the desired pressures to the sample and buffer inlet. At the outlets, the processed sample was collected in Eppendorf tubes.

4.2.5. Experimental procedure

CCMVs in microfluidic chip: A PFF microfluidic chip with a broadened segment of 1100 µm (chip design I) was used. CCMVs were diluted in Solusem Bio+ to 370 ng/ml. After introducing the CCMV sample and Solusem Bio+ as a sheath buffer into the chip, the desired pressures were applied. The applied sheath buffer pressure was constant (400 mbar; 65 µl/min), and the applied sample buffer pressures were 24 (1.5 µl/min), 26 (1.8 µl/min) and 32 mbar (2.8 µl/min). The flow rate ratios for 24, 26, and 32 mbar were 44, 37, 24:1 (total flow/sample flow), respectively. The ratio of outlet 1 (waste) flow/total flow was 2.4%.

Separation of spermatozoa from CCMVs: Separation experiments were performed with chip design I (broadened segment width 1100 µm) and chip design II (broadened segment width 2200 µm). The sample consisted of spermatozoa at a concentration of 10 x 10^6 cells/ml and 74 ng/ml CCMVs in Solusem Bio+. Solusem Bio+ was also used as sheath buffer.

For the comparison of the separation efficiency of chip design I with chip design II, CCMVs labeled with Atto 647 NHS ester were used. The applied sheath pressure was 400 mbar (design I: 65 µl/min, design II: 64 µl/min) for both designs and the sample pressures were 27 (2.5 µl/min) and 30 mbar (2.4 µl/min) for design I and II, respectively. The flow rate ratio for chip design I was 27 (total flow/sample flow) and for chip design II was 28 (total flow/sample flow). Outlet 1 tubing was 10 and 13 cm for chip design I and II, respectively, so that for both designs 2.8% of the total flow were collected in outlet 1.

For the other separation experiments performed with chip design II, CCMVs labeled with Atto 488 NHS ester were used. Outlet 2 tubing was 12, 12.5 or 13 cm long, so that 3.0%, 2.7%, 2.5% of the total volume were collected at outlet 1. The sheath buffer was for all experiments 400 mbar, which is equivalent to a flow rate of 64 µl/min and the sample pressure was 30 mbar (≈2.5 µl/min).

For a constant outlet 1 (waste) removal rate of 2.7% with chip design II, different sample buffer pressures were applied, namely 28 mbar (2.1 µl/min), 30 mbar (2.5 µl/min), and 32 mbar (2.8 µl/min). With a sheath buffer of 400 mbar (64.5 µl/min), the flow rate ratios were 32, 27 and 24 (total flow/sample flow) for a sample pressure of 28, 30 and 32 mbar, respectively.

4.2.6. Separation analysis

The flow profile of CCMVs in the PFF chip was analyzed using fluorescence microscopy. Fluorescence images were taken from the separation channel and broadened segment with the EVOS microscope as shown in figure 4.1. The obtained fluorescent images were analyzed using a Matlab script (Matlab 2017b, Mathworks, Natick, MA). Exemplary images of the figures obtained with the Matlab script can be found in *Supporting Information* 4.6.1. In short, the images were processed with Gaussian noise removal (low-pass Wiener filter) and the image intensity values were saturated. Then the image intensity values of a line orthogonal to the outer channel wall were plotted and the width of the fluid flow containing CCMVs was determined.

The concentrations of CCMVs in the sample and in both outlets were determined with an EnSpire multimode plate reader (Pelkin Elmer, Waltham, MA, USA). Samples were pipetted in black FLUOTRAC™ 600 96-well-plates (Greiner Bio-one, Essen, Germany) and measured in the multimode plate reader in fluorescence mode (λ_{ex}=495 nM, λ_{em}=520 nm, 50 flashes). The fluid collected in outlet 1 (≈20 µl) was diluted and 100 µl was used to measure the fluorescence intensity. The fluid collected in outlet 2 (≈ 500 µl) was directly used and the fluorescence intensity of 250 µl was measured. For both sample volumes, calibration lines were used to determine the CCMV concentration from the fluorescence intensity (*Supporting Information* 4.6.2). The CCMV removal is defined as the percentage of CCMVs present in outlet 1 relative to the CCMVs present in both outlets. The CCMV concentration in weight was transformed to a particle concentration with CCMV weight (4.6×10^6 avogram/CCMV particle, equivalent to 7.6×10^{-21} kg/CCMV particle)[41]). The limit of detection was 0.01 ng/ml (1.3×10^9 CCMVs/ml).

The spermatozoa collected in outlet 1 and 2 were manually counted using a Neubauer counting chamber. A volume of 10 µl from the fluid collected at the outlet 1 was deposited onto a Neubauer chamber. For each experiment, at least 100 spermatozoa were enumerated depending on the cell concentration. It was corrected for the difference in obtained volumes from outlet 1 and 2. The sperm recovery is defined as the percentage of spermatozoa present in outlet 2 relative to the spermatozoa in both outlets.

4.2.7. Statistical analysis

Independent t-tests were performed to compare the separation qualities of the experiments with a constant outlet 1 (waste) removal rate of 2.7%. The separation qualities obtained for a sample pressure of 28 mbar were compared to the separation qualities of 30 and 32 mbar. The significance level of the two-tailed test was chosen to be 0.05.

4.3. Results and discussion

4.3.1. CCMV characterization

To investigate if CCMVs stay intact in Solusem Bio+, the dilution medium for porcine semen, the size distributions of CCMVs in Solusem Bio+, and CCMVs in encapsulation buffer were measured using DLS. CCMVs were diluted in Solusem Bio+ to a concentration of 740 ng/ml. The structure of CCMV is dependent on pH and salt concentration. The pH of Solusem Bio+ is with 7.2 neutral and similar to the pH of the encapsulation buffer (pH 7.4). The size distribution of CCMVs in Solusem Bio+ and CCMVs in encapsulation buffer with the standard deviations were 26.5±9.8 nm and 26.7±10.6 nm, respectively. The respective size distribution graphs obtained with DLS are shown in the *Supporting Information* 4.6.3. The pH and the CCMV size distribution in Solusem Bio+ were like the values obtained in encapsulation buffer, confirming that the CCMVs stayed intact in Solusem Bio+ and were also comparable to CCMVs in virus buffer (27.7 nm).

4.3.2. CCMVs in microfluidic chip

The flow behavior of the fluorescently labelled model virus was followed by a fluorescent microscope in the PFF chips to investigate if separation is technically feasible. The CCMVs were diluted to a concentration of 370 ng/ml in Solusem Bio+. The CCMV concentration was chosen to be higher than realistic virus concentrations to guarantee the detection of the fluorescence signal. The diluted CCMVs were investigated with chip design I (broadened segment width: 1100 μm) and the sample pressures were 24, 26 or 32 mbar, whereas the sheath buffer pressure was constant (400 mbar). With the fluorescent microscope, images were taken from the position where the separation channel branches off the broadened segment (figure 4.2A-C). After image processing, intensity profiles (figure 4.2D-F) orthogonal to the outer channel wall were obtained, which indicate the width of the CCMV containing fluid flow. The intensity profiles show that with increasing sample pressure, the fluid flow containing CCMVs broadens. With an increase in sample pressure, the sample flow rate increases and the flow rate ratio decreases when the sheath buffer pressure is constant. The CCMV flow width for sample pressures of 24 and 26 mbar was smaller than the separation channel width of 45 μm and CCMVs exited the chip at outlet 1. When the sample flow was getting too high, the fluorescent image and intensity plot show that CCMVs exit also at outlet 2 and separation from larger particles was not provided. The flow rate ratio in the pinched segment with width w_P is linearly amplified in the broadened segment with width w_b by w_b/w_P[31]. With this relationship, the fluid width in the broadened segment can be calculated and compared with the experimental results. The theoretical determined width of the CCMV fluid flow is 25 and 30 μm for 24 and 26 mbar, respectively, and therefore below the

width of the separation channel of 45 µm. For a sample pressure of 32 mbar, the CCMV fluid width is 46 µm and efflues also to outlet 2, which is in accordance with the experimental results. The theoretical values can deviate slightly from experimental results, because design parameters are not perfectly translated to the chip. The position of the separation channel wall overlaps with the CCMV containing fluid for a sample pressure of 32 mbar. The width of the position of the separation channel wall was designed to be 45 µm, but the actual width of the PDMS chip can deviate slightly due to the processing steps. Furthermore, during image processing pixels were converted to distance, which can also cause some inaccuracy. Nevertheless, with the intensity profiles, it can be distinguished whether the model virus flow out of the chip via outlet 1 (waste) or outlet 2.

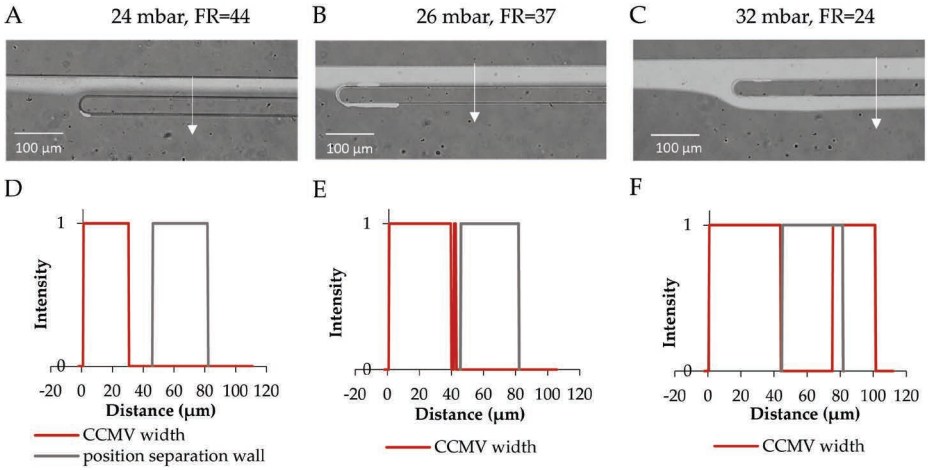
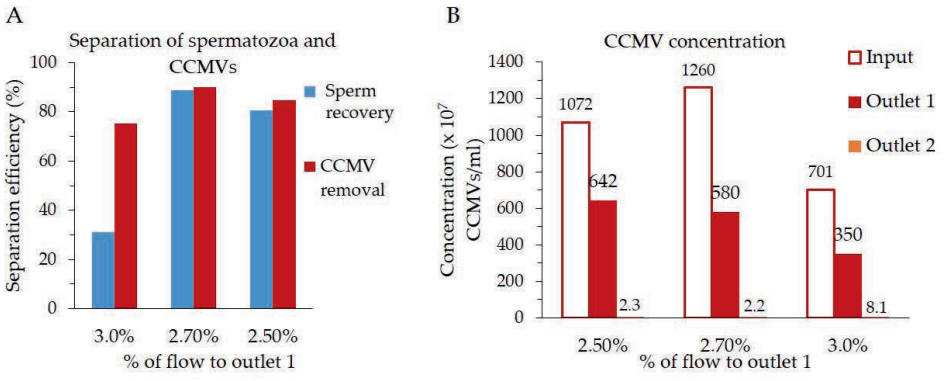


Figure 4.2: Merged brightfield and fluorescent images (A-C) and intensity profiles (D-F) of Atto 647 labelled CCMVs in PFF chip with broadened segment width of 1100 µm (design I): Sample pressure was varied (A&D: 24 mbar; B&E: 26 mbar; C&F: 32 mbar) with constant sheath buffer pressure (400 mbar). With increasing sample pressure, the fluid stream containing CCMVs broadens. (FR: flow rate ratio of total flow/sample flow; red arrow: line of intensity plot)

4.3.3. Separation of spermatozoa from viruses

We optimized the sperm recovery while maintaining the CCMVs removal, by comparing the separation efficiencies of both chip designs and investigating the effect of the fluid removal ratios of both outlets. A wider broadened segment width improves the separation efficiency because the effluent position of the spermatozoa and viruses is farther apart from each other (*Supporting Information* 4.6.4). This also corresponds to the previously mentioned linear amplification relationship of w_b/w_p. Therefore, chip design II, which had a broadened segment width of 2200 µm, was used for the next experiments.

The optimal fluid removal ratio must be found, as this parameter also determines the separation efficiency. With a higher fluid removal ratio, it is expected that more spermatozoa exit the chip at the waste outlet, whereas with a low fluid removal ratio, the virus may also exit the chip at outlet 2. Figure 4.3A presents the obtained separation efficiency with respect to the CCMV removal and sperm recovery. For a fluid removal ratio of 3.0%, most spermatozoa are lost, whereas with a fluid removal ratio of 2.7% almost 90% of the spermatozoa are collected in outlet 2. A higher fluid removal ratio means, that the flow to outlet 1 is higher and with a fluid removal of 3.0% many spermatozoa exit the chip at outlet 1. For all experiments, more than 75% of the CCMVs were removed from the spermatozoa. The best CCMVs removal of 89% was achieved with 2.7% of flow to outlet 1. In figure 4.3B, the CCMV concentrations in the outlets are shown. The concentrations in outlet 1 were more than two order of magnitude higher compared to the concentrations obtained in outlet 2. The CCMV concentration in outlet 1 is lower than the input concentration, which therefore cannot be traced back to the separation efficiency of CCMVs. There are several explanations for this dilution. The largest impact on the dilution is that a third of the volume exiting at outlet 1 is the buffer solution. This can be seen by the CCMV flow width, which is approximately a third of



C

% of flow to outlet 1	CCMV flow width (µm)
2.5%	28 ± 7
2.7%	33 ± 2
3.0%	29 ± 2

Figure 4.3: Separation efficiency for different fluid removal ratios of chip design II. A) Sperm recovery and CCMV removal. B) The CCMV concentration after the separation in both outlets. C) The CCMV flow width from the outer wall in the broadened segment (multiple images were taken during one experiment; ± X, in which X represents 1 SD, N≥3). With all fluid removal ratios, CCMV are collected in outlet 1 (waste outlet). The best sperm recovery was achieved with a fluid removal ratio of 2.7%. (sheath pressure 400 mbar; sample pressure: 30 mbar; N=1)

the separation channel width. Additionally, approximately 20% of the viruses are lost during processing with the microfluidic chip and tubing (*Supporting Information* 4.6.5). With all fluid removal ratios, most CCMVs were separated from spermatozoa, because the CCMV fluid width is smaller than the separation channel width of 45 µm (figure 4.3C). While performing the experiments, spermatozoa were fluorescently visible under the microscope, although they are not known to be autofluorescent. It has been examined, that CCMV and the dye adhere to spermatozoa (*Supporting Information* 4.6.6), which justifies some of the fluorescent signal obtained from samples collected at outlet 2.

The best separation with chip design II was obtained with a fluid removal rate of 2.7%, because this fluid removal rate achieved both the highest sperm recovery and virus removal. The separation efficiency was further investigated by varying the pressure for the sample flow. The individual separation efficiencies with sample pressure of 28 mbar (1.8 µl/min), 30 mbar (2.0 µl/min), and 32 mbar (2.4 µl/min) are shown in the *Supporting Information* 4.6.7. When using a sample pressure below 28 mbar, the flow rate ratio was too high, and the sample flow was blocked by the sheath flow. The results of independent t-tests between 28 mbar and the other applied pressures did not report a statistical difference for the sperm recovery and CCMV removal rates (*Supporting Information* 4.6.7). In figure 4.4 the separation efficiencies are summarized. Sperm recovery of 86 ± 6% and CCMV removal of 84 ± 4% were achieved.

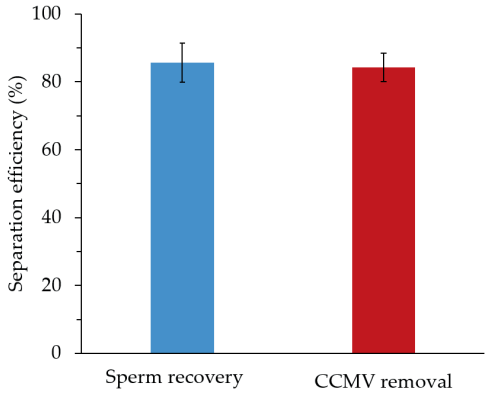


Figure 4.4: Separation efficiency with chip design II and 2.7% fluid removal ratio. (Error bars=1 SD, sheath pressure 400 mbar; sample pressure: 28-32 mbar; N=9)

Up to date only a few microfluidic separation techniques were applied to purify spermatozoa from other types of cells such as blood cells and epithelial cells. Dean flow fractionation, which uses inertial forces in a spiral channel, was applied to separate erythrocytes and white blood cells from spermatozoa[28,42]. Sperm recoveries of 80%[28] and

89%[42] were achieved, while removing 99% of the erythrocytes[28] and 82% of the white blood cells[42]. Dean flow fractionation was further improved with a channel of a trapezoidal cross-section[43]. With this device, the sperm recovery was 96%, whereas epithelial cells (86%), white blood cells (approximately 95%) and erythrocytes (approximately 75%) were removed[43]. The reported sperm recovery with PFF and the virus removal rate are similar to the ones reported with Dean flow fractionation. Channel dimensions, particle size and flow characteristics are key design parameters for inertial microfluidics. Small deviations from optimal flow characteristics can have an impact on particle separation. Another proposed spermatozoa purification method is acoustic trapping. Spermatozoa were trapped by an acoustic standing wave, while other biological components originating from the female victim, such as free DNA, pass the acoustic field[44,45]. Spermatozoa were successfully purified from a 40-fold excess of female epithelial cells over spermatozoa[45]. Limitations of acoustic trapping are that it is not a continuous separation technique and an external field is required. Inertial microfluidics and acoustic trapping can also be applied to purify spermatozoa from virus containing semen. In contrast to inertial microfluidics and acoustic trapping, PFF has several benefit such as the simple design and no need for an outer field. The most important parameters of PFF are the width of the pinched segment and its ratio with the broadened segment. Key separation parameters such as flow rate ratio and fluid removal ratio can be easily optimized during testing, as previously reported by Berendsen *et al.*[30] and shown in this study.

The reported sperm recovery (86 ± 6%) is twice as high as the sperm recovery reported by other virus separation techniques (≈45%), which have used combinations of "swim-up" and density gradient centrifugation[22,23]. Nevertheless, the spermatozoa recovery is slightly lower than the sperm recovery of similar PFF sperma separation techniques as proposed by Berendsen *et al.*, who have achieved a sperm recovery of up to 95%[30]. Moreover, it has been shown that the effect of microfluidic processing, including PFF, on the sperm viability is low (Chapter 3)[37]. The used chip dimensions and flow rates used in the viability study[37] were similar and in the same range as the ones used in this study. There were differences in our proposed separation technique and the previously proposed one by Berendsen *et al.*, which can cause the difference in sperm recovery such as the chip design, flow rate ratio and sample composition. Berendsen *et al.* have used a sample which consisted mainly of erythrocytes and was spiked with spermatozoa, whereas in this study, a spermatozoa sample was spiked with smaller CCMVs and the particle density was lower. The particle density may influence the separation efficiency.

The asymmetrical shape of spermatozoa improves the separation. Berendsen *et al.* have reported that the tumbling effect influences the sperm behavior in PFF; the average

spermatozoa position in the broadened segment is further away from the channel wall and the distribution is broader than when considering the spermatozoa head size[30]. Therefore, in PFF spermatozoa can be associated with an average particle size of 15 µm, instead of a 4 µm one. Without the tumbling effect most spermatozoa would not be separated from the virus containing fluid, since the width of the sample flow in the pinched segment is equal or lower than 4 µm. However, due to the tumbling and as evidenced by the results, spermatozoa can be purified from viruses with our set-up. Another sperm characteristic is the motility and its rheotactic behavior; spermatozoa tend to swim against the flow stream with a stream velocity of 100 µm/s[46]. The flow velocity in the broadened segment of the PFF device is with approximately 9000 µm/s higher than the flow velocity needed for rheotaxis. Additionally, separation was performed at room temperature to prevent sperm movement and subsequent sperm fatigue, so both effects did not influence the separation. Moreover, it has been shown that the effect of microfluidic processing, including PFF, on the sperm viability is low[37]. The used chip dimensions and flow rates used in the viability study[37] were similar and in the same range as the ones used in this study. There were differences in our proposed separation technique and the previously proposed one by Berendsen *et al.*, which can cause the difference in sperm recovery such as the chip design, flow rate ratio and sample composition. Berendsen *et al.* have used a sample which consisted mainly of erythrocytes and was spiked with spermatozoa, whereas in this study, a sperm sample was spiked with smaller CCMVs and the particle density was lower. The particle density may influence the separation efficiency.

For analysis and quantification of the CCMV separation, two techniques based on the fluorescence signal have been used. Fluorescence microscopy visualizes the CCMV fluid width in the broadened segment of the microfluidic chip and determines its width of the channel wall, whereas with a fluorescence imaging plate reader the concentration of the viruses after the separation was determined. Fluorescence microscopy implies that all viruses are sorted from the spermatozoa, because the width of the fluid width is below 45 µm and the viruses exit the chip at outlet 1. However, the technique determining the CCMV concentration after the separation reveals that CCMVs are also found in outlet 2. As is shown in *Supporting Information* 4.6.6, both CCMV and the Atto 488 dye adhere to spermatozoa, which increase the fluorescence intensity and therewith the CCMV concentration in outlet 2. For several virus types, it has been shown that most virus particles are free in the seminal plasma instead of penetrating or attaching to spermatozoa[18–20]. Taking both CCMV analysis techniques into account, the CCMV removal is at least 84 ± 4%. Similar to other virus removal techniques from semen[22,23], not a complete virus elimination was achieved. To further eliminate CCMVs from the sample, it is an option to process the sample with the PFF device multiple times. A PFF

device with two cascading devices has been proposed to remove both larger particles and smaller particles from spermatozoa[47]. To improve the virus removal of our application, cascading two PFF chips may increase the virus removal 87% to 98%, assuming that for every device the efficiency is the same. However, simultaneously, this would decrease the sperm recovery to 81%.

PFF is based on the typical laminar flow behavior in microfluidic systems. Although the flow is laminar, particles experience Brownian motion and diffuse across streamlines. For our application, it is interesting to investigate the diffusion distance of viruses between entering the broadened segment and the separation channel. The diffusion coefficient for spherical viruses with a size of 10-400 nm ranges from 30 $\mu m^2/s$ to 0.8 $\mu m^2/s$[48]. For one-dimensional diffusion the average distance x travelled by a particle can be calculated with $x = \sqrt{2Dt}$, which D the diffusion coefficient and t the time[49]. Assuming the time critical for the diffusion is 0.15 s, which is the calculated time a particle needs to move between the pinched segment and the branch-off of the separation channel, the average distance is 3 μm to 0.5 μm for viruses with a size of 10 nm to 400 nm, respectively. With a separation channel width of 45 μm and a virus containing fluid width in the broadened segment of smaller than 40 μm, the virus separation is not affected.

Due to the regulations in our laboratory, we have chosen to use CCMVs as a model virus, because viruses found in semen are potential transmitters of diseases and require higher safety regulations in the laboratory. CCMVs with a size of 28 nm belong to the small viruses, when comparing it to the size range of viruses found in semen (10-400 nm[5]). Examples with a comparable size to CCMVs are the foot-and-mouth disease (FMD) virus (25-30 nm[50]) and CSF virus (40-60 nm). Another important virus type, which can be present in semen, is the African swine fever (AFS) virus (200 nm[51]). With the current use of PFF, spermatozoa are removed from the liquid containing sample based on the sperm size. As large viruses are more than twenty times smaller than spermatozoa, it is expected that spermatozoa can be separated from all virus types found in semen. As viruses can have a diameter of up to 400 nm, we performed PFF separation of semen spiked with 500 nm polystyrene beads. The results show that 98% of polystyrene beads were removed from semen while recovering 93% of the spermatozoa (*Supporting Information* 4.6.8). The PFF chip was similar to chip design I, but the chip was made out of cyclic olefin copolymer (COC). The semen sample was spiked with 74 ng/ml CCMVs, which is approximately 9.5 x 10^9 particles/ml. The high virus concentration was used to enable the analysis based on fluorescence intensity. CCMV analysis techniques have the limitation of a fluorescence signal threshold. If the signal is lower than the threshold, the intensity and therewith the CCMV will not be detected. In outlet 2 the sample is diluted with the sheath buffer, which is needed to pinch the particles. This dilution also decreases

the measured fluorescence signal. Other virus separation techniques have used virus concentration of a few 1 x 10^3 to 2 x 10^5 plaque forming units/ml[22,23]. Assuming that each plaque forming unit is one virus particle, in this study high virus concentrations were used. In the future, the separation of spermatozoa from viruses can be confirmed by using samples spiked with viruses found in semen, such as the CSF and AFS virus, at realistic concentrations.

The present study is a proof-of-principle for the separation of viruses from semen used by the veterinary industry. Before implementing this in routine semen processing, many steps must be performed, such as using a more realistic sample as mentioned previously. The only necessary pretreatment step is sample dilution, which is also currently part of routine semen processing. Another important aspect of the separation is the sample throughput, as the total sperm count of a boar ejaculate ranges between 75 x 10^9 – 100 x 10^9 cells[32]. The throughput of the proposed separation techniques is only a few µl/min, which would take too long to process a whole boar ejaculate. To minimize sample pretreatment and to increase the sample throughput, it is suggested to investigate PFF with higher sperm concentrations. There is a need to increase the throughput to become of more interest for the veterinary industry. Furthermore, the higher the sperm recovery the more attractive the separation technique will be, as every individual spermatozoon represents a fertilization opportunity.

4.4. Conclusion

The presented microfluidic chip based on PFF separates spermatozoa from virus spiked semen. With the optimized flow rate ratio and fluid removal fraction, a sperm recovery of 86 ± 6% and removal of at least 84 ± 4% of a model virus were achieved. The sperm recovery of this technique is twice as high as the sperm recovery of other virus separation techniques. By removing potential viruses from porcine semen before its distribution to recipient farms, the transmittance of diseases by artificial insemination is further reduced.

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4.6. Supporting Information

4.6.1. Analysis flow profile CCMVs

To analyze the width of the CCMVs in the broadened segment of the microfluidic chip, a matlab script was used. In figure 4.5, an example of the original images and processed images of the intermediate steps are shown. The starting point was the fluorescent image (figure 4.5B). The images were processed with Gaussian noise removal (low-pass Wiener filter, figure 4.5C) and the image intensity values were saturated (figure 4.5D). The image intensity values of a line orthogonal to the outer channel wall (red arrow in figure 4.5D) were plotted and the width of the fluid flow containing CCMVs was determined (figure 4.5E).

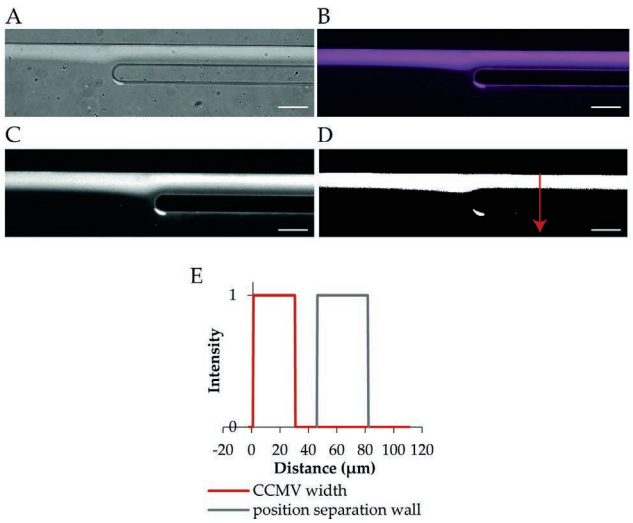


Figure 4.5: Images analysis to obtain the intensity profile of CCMV in the microfluidic chip: A) Merged brightfield and fluorescent image. B) Fluorescent image. C) Image after Gaussian noise removal (low-pass Wiener filter) D) binary image based on a threshold (red arrow: line of intensity plot) E) Intensity profile of CCMVs in microfluidic chip. (scale bar = 50 μm)

4.6.2. Calibration Curves Plate Reader

The concentration of CCMVs was determined using a fluorescence imaging plate reader. For relating the fluorescence intensity to a concentration, calibration curves with the applicable sample volumes and CCMV concentrations are needed. CCMVs were diluted in Solusem Bio+ to the desired concentrations and with a linear fit the calibration curves were obtained (figure 4.6).

In outlet 1, the CCMV concentration was higher than the CCMV concentration in outlet 2. Therefore, two calibration curves were for the expected CCMV range were

determined. For a CCMV concentration between 2 - 120 ng/ml ($2.6x10^{11}$ – 1,6 x 10^{13} CCMVs/ml) the calibration curve shown in figure 4.6A can be used. For CCMV concentrations of 0.01 ng/ml - 1 ng/ml (1.3 x 10^{9} – 1.3 x 10^{11} CCMVs/ml) the calibration curve shown in figure 4.6B can be used.

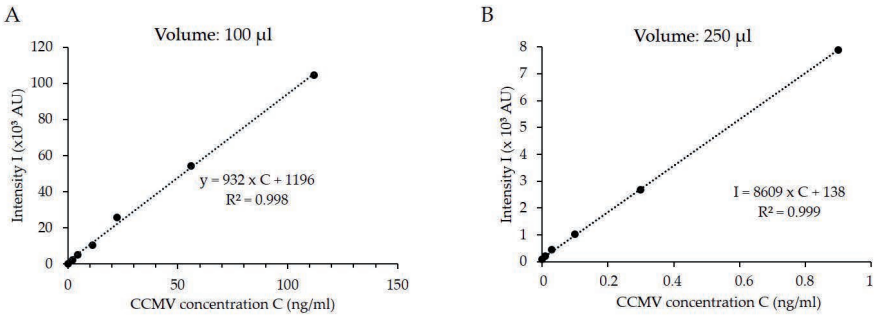


Figure 4.6: Calibration curves for the determination of the CCMV concentration for volumes of 100 µl (A) and 250 µl (B) with a fluorescence imaging plate reader.

4.6.3. CCMV characterization with dynamic light scattering (DLS)

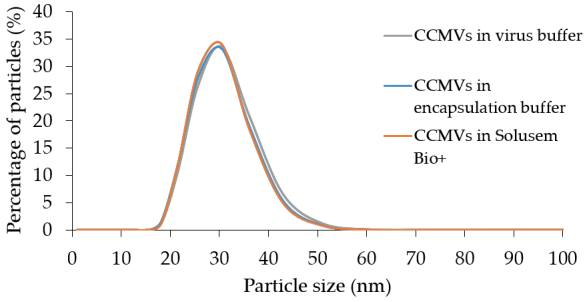


Figure 4.7: The CCMV size distribution as measured with DLS of CCMVs in virus buffer, encapsulation buffer and Solusem Bio+.

4.6.4. Chip design optimization

A CCMV spiked semen sample was processed with chip designs I and II to compare the separation efficiency of both designs. Chip design I had a broadened segment width of 1100 µm, whereas chip design II had a broadened segment width of 2200 µm. With a wider broadened segment width, it is expected that the effluent position of the different sized particles is further apart from each other than for a narrower broadened segment. This effect is thought to improve the separation efficiency. Because of the enormous size difference between viruses and spermatozoa, it was examined whether the separation efficiency with chip design I was sufficient. To be able to compare chip design I and II, for both chip designs the flow to outlet 1 (waste) was 2.8% of the total flow. The flow to

outlet 1 depends on the channel resistances of both outlets. Therefore, the resistances of outlet 2 were so chosen, that for both chip designs 2.8% of the flow exited at outlet 1. The fluid stream containing the CCMVs was optically investigated during the experiment (figure 4.8B), whereas the sperm recovery was determined after the separation (figure 4.8A). With both chip designs, CCMVs were collected in outlet 1, whereas most spermatozoa were collected in outlet 2. The fluid stream containing CCMVs in chip design I (41 μm) was almost twice as wide as the fluid stream in chip design II (23 μm). The sperm recovery was higher in chip design II (72%) compared to the sperm recovery in chip design I (63%). This suggests that the separation with chip design II is more effective than the separation with chip design I.

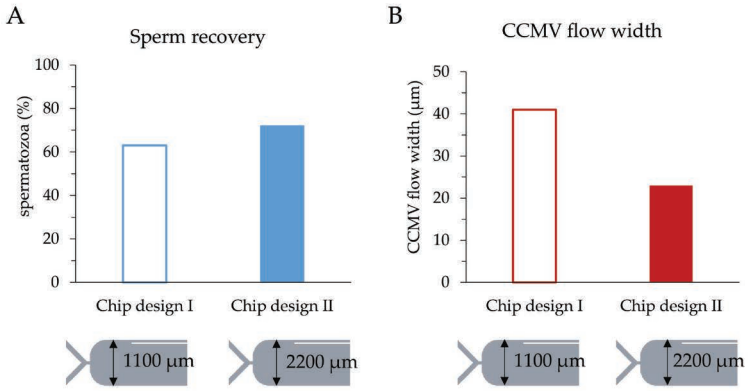


Figure 4.8: The sperm recovery and CCMV flow width with broadened segment width of 1100 μm (Chip design I) and 2200 μm (Chip design II). The separation efficiency increases when the broadened segment width is wider. (Outlet 1 volume: 2.8%; sheath pressure 400 mbar; sample pressure: 26 mbar (Chip design I) & 28 mbar (Chip design II); N=1)

4.6.5. CCMV loss during microfluidic processing

The CCMV loss during microfluidic processing has been investigated. Two sets of experiments were performed to study in which part of the microfluidic setup the CCMVs are lost; (1) the tubing which is connected to the inlets consisting of silicon capillary and Tygon tubing and (2) the setup with the microfluidic PFF chip. Three pressures were applied to induce the sample flow (50, 100, 200 mbar). For the PFF chip, the sample and sheath buffer were the same for this set of experiments. The concentrations before and after the processing are shown in figure 4.9A and B for the tubing and chip, respectively. In figure 4.9C the percentage of CCMV loss was determined with respect to the inlet concentration. The CCMV loss in the tubing is between 5 and 10%, whereas CCMV loss approximately 20% after being processed with the PFF chip. This implies that approximately 10% of CCMVs sticks or is absorbed by PDMS. From these measurements, no effect of the applied pressure/flow rate on the CCMV loss is observed.

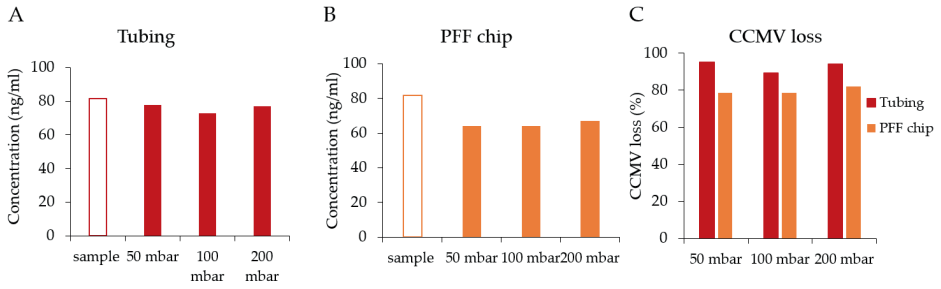


Figure 4.9: The concentration of CCMV loss during processing with tubing (A) and the microfluidic PFF chip (B) and the percentage of remaining CCMV with respect to the sample (C).

4.6.6. CCMV and dye adhere to spermatozoa

During the separation experiments with the CCMVs and spermatozoa, the separation has also been visualized with fluorescence microscopy. Thereby it has been observed, that spermatozoa were visible with the FITC filter when CCMVs were spiked to the semen, whereas the spermatozoa were not visible in a sample without CCMVs. Therefore, we have hypothesized that CCMV adhere to spermatozoa. This could downgrade the results of virus separation, because viruses attached to spermatozoa are detected in outlet 2 of the proposed separation technique.

A semen sample with a concentration of $10x10^6$ cells/ml was spiked with CCMV at a concentration of 30 ng/ml. One control sample contained only CCMVs to show that after centrifugation CCMVs free in buffer were eliminated from the sample. Another control sample containing only spermatozoa was used to confirm that spermatozoa are not autofluorescent. The control samples contain CCMVs at a concentration of 30 ng/ml and spermatozoa at a concentration of $10x10^6$ cells/ml. All samples were centrifuged five times at 1500xg for 15 min using the Minispin Plus (Eppendorf, Hamburg, Germany) to eliminate as many free CCMVs in the solution as possible. The samples were re-suspended to a volume of 250 µl and the fluorescence intensity at 488 nm was measured with a fluorescence imaging plate reader. From the fluorescence intensity the concentration was determined with the calibration curve (*Supporting Information* 4.6.2.). The microscopic images and CCMV concentrations of the CCMV spiked sperm sample and the control samples are shown in figure 4.10. Spermatozoa imaged with a fluorescence microscope (overlay brightfield and FITC channel) do not show any autofluorescence signal in contrast to spermatozoa which have been spiked with CCMVs (figure 4.10A and B). To further support this result, the CCMV concentration was determined using a fluorescence plater reader imaging system (figure 4.10C). The control samples containing only CCMVs or only spermatozoa have both a CCMV concentration

below 0.01 ng/ml. Concentrations below 0.01 ng/ml are lower than the calibration curve and the respective intensity value is similar to the background/noise measurement, meaning that these concentrations cannot be determined. From that, we conclude that with the centrifugation steps performed the CCMV concentration below the detection limit and there is no autofluorescence signal from the spermatozoa. The fluorescence signal measured in the CCMV spiked sperm sample corresponds to a CCMV concentration of 0.05 ng/ml, which is according to the calibration curve, above the detection limit. This implies that in a CCMV spiked spermatozoa sample, the CCMVs are not only free in the solution, but also adhere to the spermatozoa.

The CCMVs were labelled with the fluorescence dye ATTO 488 NHS, which can also be the cause of CCMV attachment to spermatozoa. Therefore, it was investigated whether the dye attaches to spermatozoa. A semen sample with a concentration of $10x10^6$ cells/ml was spiked with 10 μg/ml of ATTO 488 NHS dye. One control sample contained only the dye to show that after centrifugation the dye was eliminated from the sample. Another control sample contained only containing spermatozoa to confirm that spermatozoa are not autofluorescent. The samples contain ATTO 488 dye at a concentration of 10 μg/ml and spermatozoa at a concentration of 10×10^6 cells/ml. All samples were centrifuged five times at 1500 x g for 15 min to eliminate the dye as much as possible. The samples were re-suspended to a volume of 250 μl and the fluorescence intensity at 488 nm was measured with a fluorescence imaging plate reader. From the fluorescence intensity the concentration was determined with a calibration curve (figure 4.11A).

The concentrations of the dye present in the sample and controls after the centrifugation steps is presented in figure 4.11B. The control samples consisting of only the dye contains almost no dye after centrifugation, suggesting that the dye was removed by centrifugation. The control sample for the spermatozoa show, that no fluorescence signal is emitted from the spermatozoa. The sample containing both spermatozoa and the dye contains the dye at a concentration 30.6 ng/ml, which is higher than the control groups. This suggests that the dye attaches to spermatozoa.

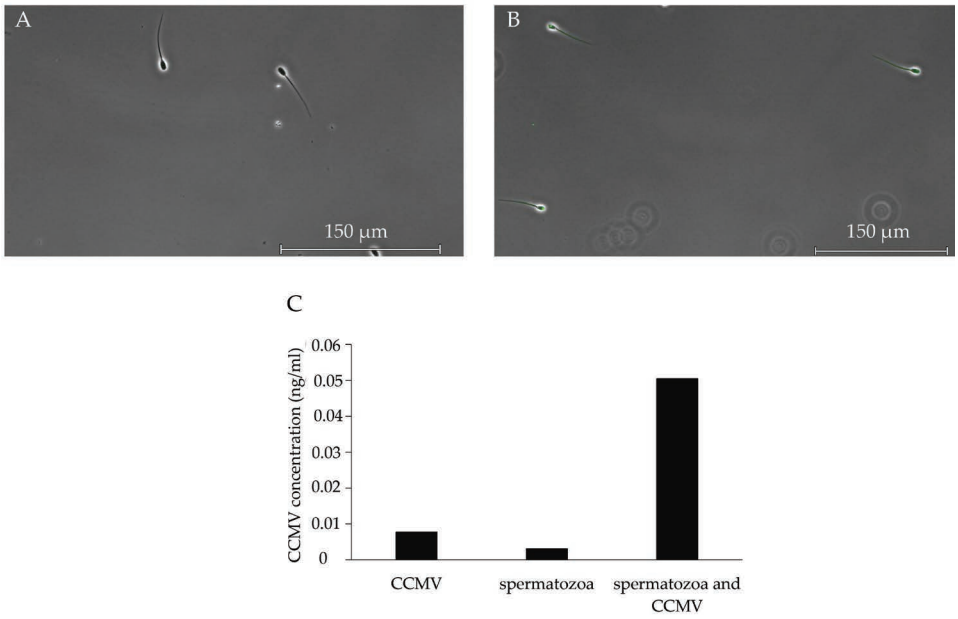


Figure 4.10: Brightfield and fluorescent image of FITC filter merged of the control sample containing spermatozoa (A) and the centrifuged sperm sample which has been spiked with CCMV (B). C) The measured concentration of CCMV as determined with the fluorescence intensity after several centrifugation steps. CCMV adhere to spermatozoa.

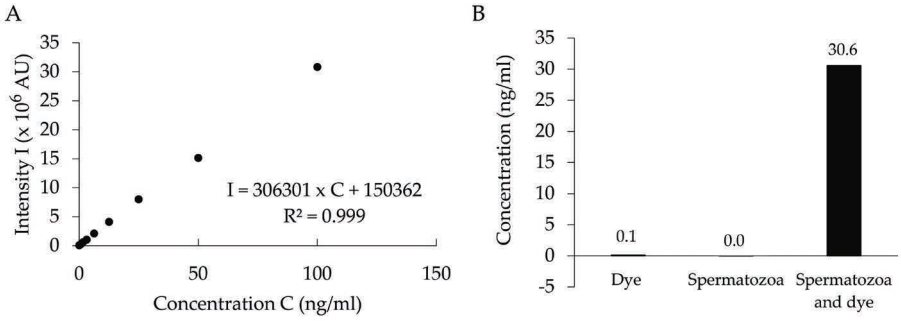
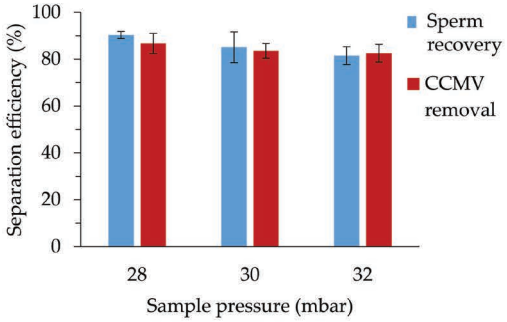


Figure 4.11: A) Calibration curve of Atto 488 NHS dye in Solusem Bio+. B) The amount of dye present in a sperm sample compared to the control groups. The concentration of dye present in the sample containing spermatozoa and the dye is higher than the control groups, suggesting that the dye attaches to the spermatozoa.

4.6.7. Separation with chip design I and 2.7% flow removal

A



B

Sample pressure (mbar)	CCMV flow width (μm)
28	30 ± 1
30	33 ± 3
32	33 ± 1

Figure 4.12: Separation efficiency for different sample pressures with chip design II and 2.7% fluid removal ratio A) Sperm recovery and CCMV removal. B) The CCMV flow width from the outer wall in the broadened segment. With increasing sample pressure, the separation efficiency decreases slightly. The CCMV flow width is below 45 μm, which indicates high CCMV removal. (Error bars=1 SD, N=3, sheath pressure 400 mbar; N=3)

Table 4.1: Results of independent t-tests

T-tests	**p-value**		
Sample pressures	Sperm recovery	CCMV removal	CCMV flow width
28 – 30	0.38	0.45	0.28
28 – 32	0.067	0.36	0.14

4.6.8. Separation of beads (500 nm) from semen

Reproduced from:

T. Hamacher, J.T.W. Berendsen, M.L.W.J. Broekhuijse, L.I. Segerink. Separation of viruses from spermatozoa using a microfluidic chip to achieve pinched flow fractionation; NanoBioTech-Montreux Conference; Switzerland, November 18-20, 2019

Separation experiments with polystyrene beads (diameter: 500 nm) were performed in a PFF chip fabricated out of cyclic olefin copolymer (COC) (Micronit, Enschede, the Netherlands). This PFF chips design is similar to chip design I used in the main article; the pinched segment width was 45 μm, the broadened segment width 1100 μm and the separation channel width 45 μm (figure 4.13).

Spermatozoa were spiked with polystyrene beads with a size of 500 nm. The sample was processed with the COC chip. The number of spermatozoa and beads found in outlet 1 and outlet 2 were manually enumerated using a Neubauer counting chamber. The collection efficiency of spermatozoa was 93%, whereas 98% of the 500 nm beads were depleted (figure 4.14).

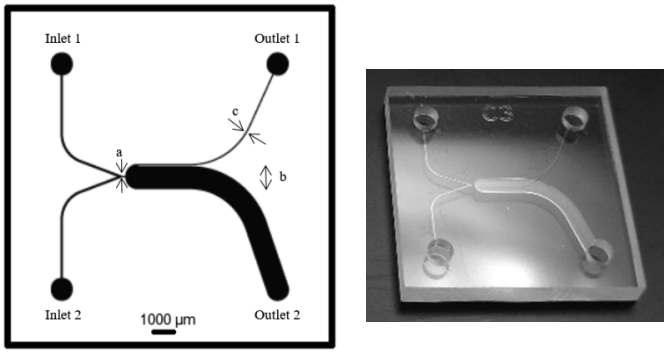


Figure 4.13: Schematic representation and photograph of the chip. The broadened segment width (b) is 1100 µm and separation channel width (c) is 45 µm.

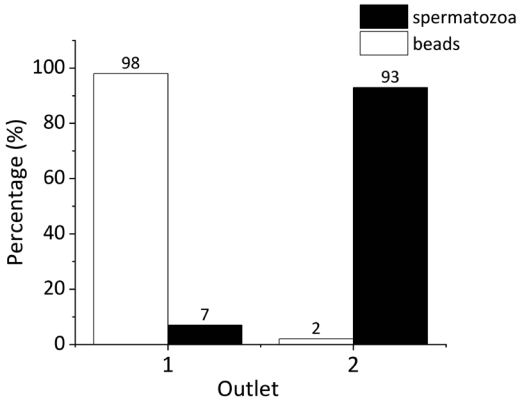
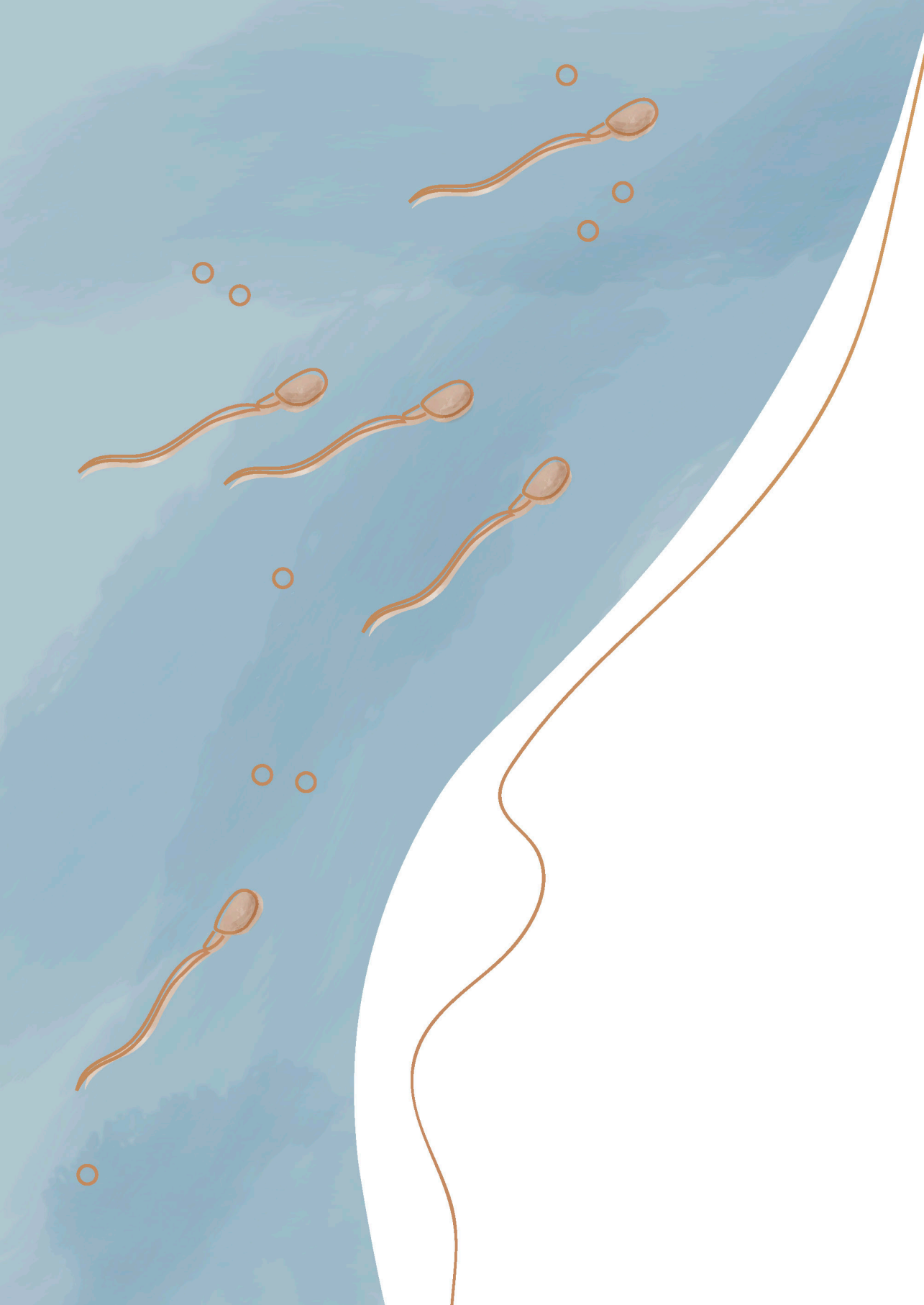
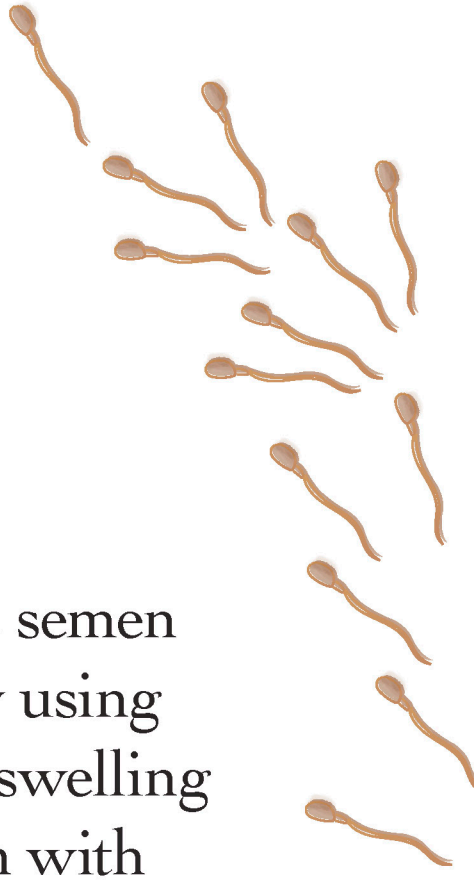


Figure 4.14: Percentage of particles found in both outlet 1 and 2. Most of the virus-sized 500 nm beads were collected in outlet 1, whereas most of the spermatozoa were collected in outlet 2. Approximately 6000 spermatozoa and 190 000 beads were processed.





5.

Improving the semen sample quality using hypo-osmotic swelling in combination with pinched flow fractionation

Abstract

Artificial insemination centers examine semen quality based on sperm concentration, morphology and motility. In contrast to these external characteristics, the hypo-osmotic swelling (HOS) test provides additional information about the membrane integrity. In response to a hypo-osmotic solution, intact spermatozoa swell and their tail curls up, thereby increasing their size. These swollen spermatozoa may be separated from unchanged spermatozoa with the size-based separation technique pinched flow fractionation. First, the effect of the HOS solution on the bovine sperm viability was examined and did not affect it. After that, the position of spermatozoa in a HOS solution in the broadened segment of the pinched flow fractionation device was compared to the position of spermatozoa in an isotonic solution. A significant difference in position between spermatozoa in a HOS solution was found for bovine spermatozoa, but not for porcine spermatozoa. However, the separation of swollen spermatozoa from unchanged spermatozoa with pinched flow fractionation was not feasible. This might be caused by the lower tumbling effect of curled up spermatozoa.

5.1. Introduction

In the veterinary industry, artificial insemination (AI) is the main breeding technique. The success of AI is highly determined by the semen quality and, therefore, the semen quality is examined prior its distribution to the farms. Parameters used to quantify semen quality in AI centers are sperm count, motility and morphology[1,2]. Although these parameters provide some insights into semen quality, no information about the cellular integrity is given.

In the 1860s it was reported, that seminal filaments curled up and formed loops after the addition of a few droplets of water to semen[3]. About a century later, this effect has been further investigated and related to the process of osmosis[4,5]. A vital cell membrane counteracts differences of solute concentrations outside and inside the cell. At a lower solute concentration outside the cell, the cell takes up water and swells, which is referred to as hypo-osmotic swelling (HOS). In the case of spermatozoa, HOS is not only expressed by an increase in cell volume, but also in bending and curling of the tail, whose fibers are surrounded by the cell membrane[6]. A cell with a malfunctioning membrane does not react to hypo-osmotic stress and therefore does not change its morphology. Hence, a spermatozoon reacting to hypo-osmotic stress with swelling is considered intact[5,6].

In contrast to the external visible sperm characteristics, a HOS test provides information about sperm membrane functionality[5]. Results obtained by the HOS test correlate with male fertility parameters such as motility[5,7–10], viability[7], morphology[9,10], DNA fragmentation[11,12] and *in vitro* fertilization capacity[5,13]. Due to its simplicity and useful insights about the functionality of spermatozoa, the HOS test has been suggested by the World Health Organization (WHO)[14] to aid in assessing male fertility and has been shown to be useable in routing semen processing for amongst others porcine[15] and horse[10].

Here, we combined the HOS test with a subsequent separation step to improve semen quality. By improving semen quality, AI success rates can be improved and this will be more (economical) beneficial for both AI centers and the recipient farms. A possible separation technique is the microfluidic and size-based principle pinched flow fractionation (PFF). Previously, we have shown that PFF removes viruses from semen (Chapter 4)[16]. Moreover, PFF was used to remove erythrocytes and epithelial cells from spermatozoa[17,18]. PFF is based on the characteristic laminar flow profile that often exist in microfluidic chips. In the pinched segment, particles are pushed against the channel wall and are forced to follow the streamlines based on the particle's size[19]. Due to laminar flow, the particle position in the broadened segment is amplified; smaller particles end

up closer to the channel wall, whereas larger particles are further away from the channel wall. In the case of spermatozoa in a HOS solution, it is expected, that in the broadened segment intact (swollen) cells emerge further away from the channel wall, whereas the dysfunctional (unchanged) cells end up closer to the channel wall. In this chapter, we will investigate, whether the separation of spermatozoa incubated in HOS solution can be combined with PFF to purify intact spermatozoa to improve semen quality.

5.2. Materials and methods

5.2.1. Sample preparation

Fresh porcine semen at a concentration of 20 x 10^6 cells/ml was obtained from a local AI center (Varkens KI Twenthe, Fleringen, the Netherlands). The porcine semen was stored at 16 °C and used within three days. Solusem Bio+ extender (AIM Worldwide, Vught, the Netherlands) was used as porcine sperm diluent.

Fresh and cryopreserved bovine semen at a concentration of 60 x 10^6 cells/ml were obtained from a local AI center (CRV, Arnhem, the Netherlands). Fresh bovine semen was stored at 4 °C and used within five days. Cryopreserved bovine semen was thawed shortly before use and kept at room temperature. Optixcell® extender (IMV technologies, L'Aigle, France) was used as bovine sperm diluent.

5.2.2. Hypo-osmotic swelling

The HOS test was performed as recommended by the guidelines of human semen processing of the WHO[14]. For the HOS solution 0.735 g sodium citrate dehydrate (Sigma-Aldrich, Saint Louis, MO, USA) and 1.35 g D-fructose (Sigma-Aldrich, Saint Louis, MO, USA) were dissolved in 100 ml purified water (150 mOsm/l). The solution was stored at -20 °C until use. Before adding the semen sample, the swelling solution was preheated to 37 °C. The semen sample was added to the swelling solution in a ratio of 1:10 v/v. The semen solution was kept at 37 °C until use. For a 75 mOsm/l swelling solution, the swelling solution was diluted with purified water in a ratio of 1:1 v/v prior use.

For the enumeration of swollen and unchanged spermatozoa, a sample was deposited under a microscope and examined with phase-contrast microscopy (Nikon TE2000-U microscope, Nikon, Tokyo, Japan). At least 100 cells were counted. The number of swollen and unchanged spermatozoa was manually counted, and the percentage of swollen spermatozoa was determined by dividing the number of swollen spermatozoa by the total number of counted spermatozoa.

5.2.3. Sperm viability

To investigate the effect of the HOS test on the sperma viability, cryopreserved bovine semen was exposed to the hypo-osmotic solution for 5, 15 and 30 min. Simultaneously, two control samples were diluted in Optixcell® extender 1:10 v/v. The first control sample was directly stained to determine the initial sperm viability. The other control sample was incubated at 37 °C for 35 minutes. The spermatozoa were stained with the live/dead sperm viability kit (L-7011, Molecular Probes, Eugene, OR, USA). Spermatozoa were incubated with 1 μM SYBR 14 (ex/em 488/518 nm) for 10 minutes and 240 μM PI (ex/em 535/617 nm) for 5 minutes at room temperature. A small volume was deposited onto a glass slide and visualized with the EVOS M5000 microscope (ThermoFisher Scientific, Waltham, MA, USA). The number of live and dead cells was manually counted, and the percentage of live cells was determined by dividing the number of live cells by the total number of cells. The sperm viability was normalized by dividing it by the percentage of viable spermatozoa of the initial control sample.

5.2.4. Chip design, fabrication & set up

Chip design, fabrication and processing are described in more detail in 4.2.1.[16]. Here, the method is summarized and changes with respect to the previous stated method are mentioned.

The microfluidic chip has a typical PFF design (figure 5.1). The pinched segment has a width of 20 μm (Chip design I) or 50 μm (Chip design II). For both chip designs, the broadened segment width was 2200 μm. In the broadened segment, the separation channel with a width of 45 μm branches off to outlet 1, whereas the broadened segment ends in outlet 2. The device height was designed to be 50 μm.

Master molds were produced using standard photolithography. The polydimethyl siloxane (PDMS) chips were fabricated in a 10:1 v/v ratio of base versus curing agent (Sylgard 184, Dow Corning, Midland, MI, USA). After curing, microfluidic inlets and outlets were punched and the chips were bonded to microscope glass slides.

A schematic representation of the microfluidic set-up is shown in figure 5.1. Both chip inlets were connected to the sample and sheath fluid, respectively, with tubing consisting of fused silica capillaries (Polymicro Technologies, inner diameter (ID) 100 μm, outer diameter (OD) 360 μm, length (L) 9 cm, Molex, Surrey, UK) and Tygon tubing (ND 100-80, ID 250 μm, OD 760 μm, L 20 cm, Saint-Gobain Performance Plastics, Akron, OH, USA). The flow in the inlets was controlled with a pressure pump consisting of two Flow-EZ modules (LineUp Series, Fluigent, Le Kremlin-Bicêtre, France).

Shortly before use, the chips were hydrophilized and the chips were rinsed and incubated with poly(L-lysine)-grafted-poly (ethylene glycol) (PLL-g-PEG, SuSoS, Dübendorf, Switzerland) at a concentration of 100 µg/ml in deionized (DI) water for at least 15 minutes. After coating, the chip was connected to the pressure pump. Sample and buffer solution were introduced via inlets 1 and 2, respectively. Flow was induced by applying the desired pressures to the sample and buffer inlet. At the outlets, the processed samples were collected in Eppendorf tubes.

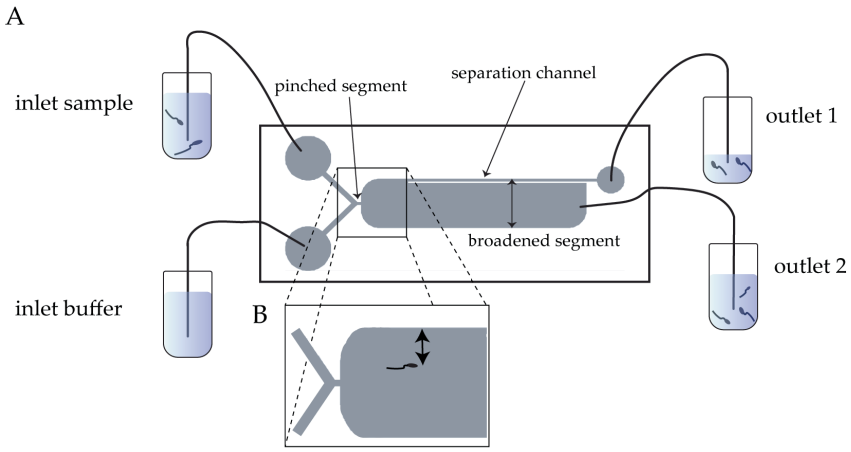


Figure 5.1: Schematic setup of PFF chip. A) Overview of the PFF separation chip connected to the containers B) The measured distance between the spermatozoa and outer channel wall in the broadened segment. (adapted from [16])

5.2.5. Spermatozoa distribution in the broadened segment

PFF chips of both chip designs punched with one outlet were used for this series of experiments. Experiments were performed with porcine and bovine spermatozoa. Two sample solutions were used: 1) a sperm solution diluted in its respective sperm diluent (control) and 2) a sperm dilution incubated in the HOS solution (150 mOsm/l) for at least 5 minutes. To investigate the effect of the osmolarity, bovine spermatozoa were also incubated in a 75 mOsm/l HOS solution. The used sheath buffers were the respective sperm diluents. The applied sheath pressure was 200 mbar for chip design I and 250 mbar for chip design II. Different sample pressures for each chip design and species were applied to investigate its influence on the spermatozoa distribution. Experiments were visualized with phase-contrast microscopy and a high-speed camera (Photron SA-3, West Wycombe, United Kingdom). With a Matlab script (Matlab R2017b, The MathWorks) the spermatozoa distances from the wall in the broadened segment were determined (figure 5.1B). To compare the mean distances of the swollen spermatozoa

with the spermatozoa in the isotonic solution, independent samples t-tests were performed ($p<0.05$).

5.2.6. Separation experiments

Chip design I with two outlets was used for the separation experiments. Outlet 1 was connected to a container using fused silica capillaries (L=11 cm) and Tygon tubing (L=15 cm). Outlet 2 was connected to a container with Tygon tubing (L=25-35 cm). Bovine spermatozoa were incubated in the 150 mOsm/l HOS solution for approximately 5 minutes. The sperm sample and the sheath solution were loaded into the PFF setup. The applied sheath pressure was 200 mbar, whereas the applied sample pressure was varied between 25-30 mbar. Experiments were visualized with phase-contrast microscopy and a high-speed camera. With the videos, the number of all and swollen spermatozoa ending up in outlet 1 and 2 were enumerated. From that, the percentages of swollen spermatozoa in outlet 1 and outlet 2 were calculated by dividing the number of swollen spermatozoa by the total number of spermatozoa exiting at that respective outlet. Similarly, the percentage of spermatozoa in outlet 1 was calculated by dividing the spermatozoa count in outlet 1 by the number of spermatozoa exiting both outlets. The percentage of swollen spermatozoa of an unprocessed sample in the HOS solution (150 mOsm/l, 5 min) was determined.

5.3. Results and discussion

5.3.1. Spermatozoa in HOS solution

To investigate the number of swollen spermatozoa of a semen sample, microscopic images were taken. In figure 5.2, two exemplary images are shown for both porcine and bovine spermatozoa. Blue arrows indicate swollen (intact) spermatozoa, whereas red arrows indicate unchanged spermatozoa. The sperm swelling is mostly visible in the curling or bending of the tail. The degree of tail curling differs from small curls at the tip of the tail to totally bend up tails. The difference in curling has been correlated with for example DNA damage and fertility. Human spermatozoa with distal curling have also been associated with lower DNA damage than total curled and non-curled spermatozoa[11,20]. In a study with bovine semen, spermatozoa with a high curling degree were correlated with fertility rates of high fertile bulls, whereas spermatozoa with a low degree of curling were correlated with fertility rates of low fertile bulls[21].

The percentage of swollen spermatozoa in cryopreserved semen sample was determined by counting the number of swollen and unchanged spermatozoa. The percentages of swollen spermatozoa were 31%, 29% and 66%. These percentages are comparable to previously reported sperm percentage; 32 ± 10% (N=11) and 42 ± 7% (N=11) for low and

high fertile bulls, respectively[21]. The percentage of swollen spermatozoa in fresh semen samples were not quantitatively determined in this study. As fresh semen samples usually have higher motility values than cryopreserved semen samples, it is expected that the percentage of swollen spermatozoa of fresh semen is higher. In literature, the percentage of swollen porcine spermatozoa reported were for example 62 ± 5% (N=4, 5 replicates)[22], 17 ± 13% (N=10)[15] and 52 ± 4% (N=32)[23].

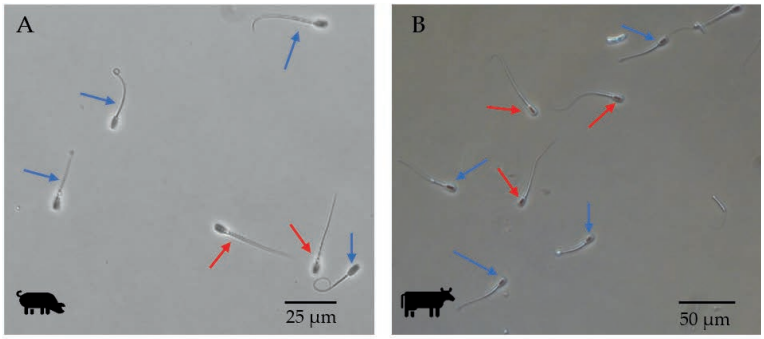


Figure 5.2: Phase contrast microscopy images of fresh porcine (A) and cryopreserved bovine (B) spermatozoa in HOS solution (150 mOsm/l). (blue arrow: swollen spermatozoa, red arrow: unchanged spermatozoa)

5.3.2. HOS test and sperm viability

The effect of the HOS test (150 mOsm/l) on the bovine sperm viability was investigated for incubation times of 5, 10 and 15 minutes. Simultaneously, the viabilities of two control sample were determined; one initial control to normalize the viabilities, and one control sample diluted in semen extender and incubated at the same environmental conditions (35 minutes) as the HOS samples.

Figure 5.3A shows a representative microscopic image of live (green) and dead (red) spermatozoa in the HOS solution. Cryopreserved bovine semen has usually a viability below 50%; reported percentages in literature are for example 39±8% (N=34)[24] and 43±9% (N=8)[25]. The lower viability of cryopreserved semen explains the higher number of dead spermatozoa in figure 5.3A. The normalized sperm viabilities with respect to the initial control were determined for the quantitative analysis. As shown in figure 5.3B, the normalized sperm viabilities in the HOS solution were with 89%, 98% and 95% similar to the control viability (88%). The differences in average sperm viability for the various incubation times were small. However, the standard deviations of the sperm viability after 15 minutes and the control sample were large. More repetitions can improve the reproducibility of this experiment. From the results, it can be concluded, that the effect of the HOS solution and its incubation time on the bovine sperm viability is similar to the control.

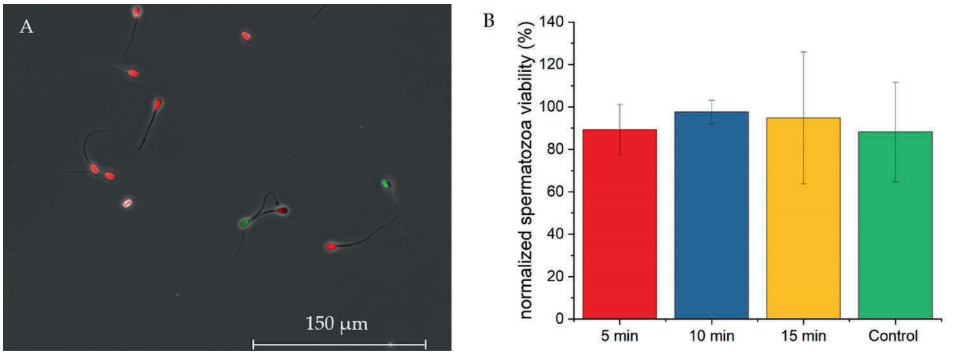


Figure 5.3: A) Overlaid phase contrast and fluorescence microscopy images of bovine spermatozoa in HOS solution and treated with live/dead staining. Live and dead cells are represented in green and red, respectively. B) The percentage of normalized spermatozoa viability after incubated in the HOS solution (150 mOsm/l) for 5, 10 and 15 minutes. The control sample was incubated in an isotonic solution at the same environmental conditions. (N=3, error bars=1 SD)

The main factors influencing the sperm viability are the exposure time to the HOS solution (short term), the time until the sample is used (long term), the HOS medium, and the animal of origin. In a study with human spermatozoa, the effect of HOS on sperm vitality (assessed with eosin Y) was investigated on short and long term[26]. On a short term (5-30 minutes), the sperm vitality did not changed compared to the control, which is similar to the results presented here with bovine spermatozoa[26]. However, on a long term (2 and 24 hours), after a 15 min exposure to the HOS solution, the sperm vitality and motility decreased[26]. The other factor, the HOS medium, may also have an effect on the sperm viability. The most often used HOS medium containing sodium citrate and fructose, first introduced by Jeyendran[5], is also recommended by the WHO[14]. An alternative solution consisting of sodium chloride (NaCl) has shown higher human sperm vitality (89.3%) compared to the solution consisting of citrate and fructose (54%)[27]. Also the descent of the semen sample might influence the viability after incubation in a HOS solution. For fresh bovine semen, similar results to the ones reported for cryopreserved semen are expected. Porcine spermatozoa may be more susceptible to the HOS solution. For example, it was reported in Chapter 3, that the effect of microfluidic processing on the porcine sperm viability is higher than on the bovine sperm viability[28]. Also, porcine spermatozoa are more affected by cryopreservation than bovine spermatozoa[29,30]. In the future, it is recommended to find the HOS solution with the least impact on semen quality, to investigate the long term effects. Also, the response of semen from various animal (types) to the HOS solution should be investigated.

5.3.3. Spermatozoa distribution in the broadened segment

The spermatozoa distributions in the broadened segment in a hypo-osmotic solution were determined and compared to a spermatozoa in an isotonic solution (control). Fresh porcine and fresh bovine spermatozoa were diluted in the HOS solution for at least five minutes. Then the control and HOS sample were processed with the PFF devices. The sperm distance from the outer channel wall in the broadened segment was determined for both chip designs and various pressure ratios. It is important to keep in mind that spermatozoa in the HOS sample contained both swollen and unchanged spermatozoa.

The porcine spermatozoa distribution of both spermatozoa in HOS solution and spermatozoa in an isotonic solution are shown in figure 5.4. Independent t-tests were performed to compare the mean distance of the spermatozoa in the HOS solution with the spermatozoa of the control solution. The p-values of the t-tests can be found in the *Supporting Information* 5.6.1. There were no significant differences in distances for three of the five experiments. A significant difference has been found for the experiments with chip design II (pressure ratio 200:18; figure 5.4A) and chip design I (pressure ratio 250:35; figure 5.4E). Although a significant difference in mean distance has been obtained for two experiments, the distribution of the HOS spermatozoa and control spermatozoa still overlap. Another option may be that spermatozoa had not reacted to the HOS solution

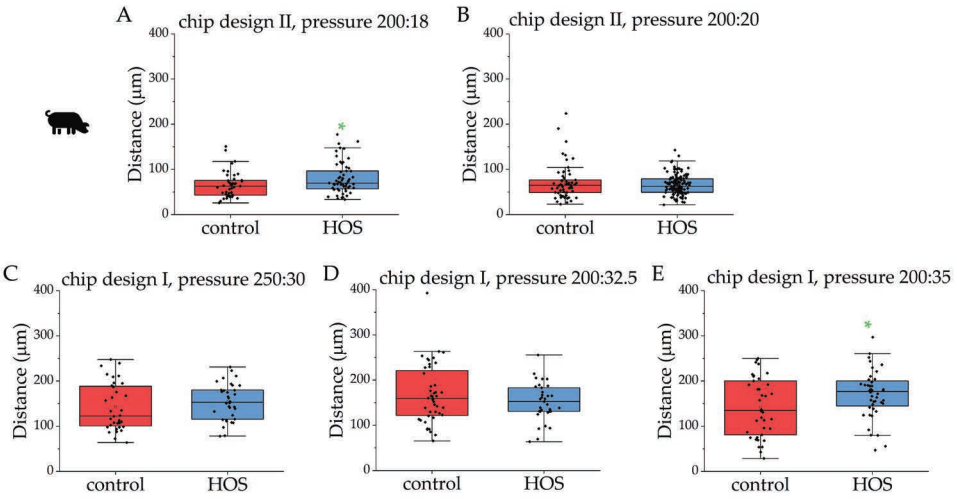


Figure 5.4: Porcine sperm distance and distribution in the broadened segment of the PFF device with chip design II (pinched segment width: 50 µm; A-B) and chip design I (pinched segment width: 20 µm; C-E) for various pressures (sheath pressure : sample pressure). The distribution of spermatozoa in the HOS solution (150 mOsm/l) were compared to spermatozoa in an isotonic (control) solution. (* indicates $p<0.05$ with respect to the control group)

when they reached the separation spot. It was reported, that the maximum volume response of porcine spermatozoa to a HOS solution is achieved within 5 minutes[31], indicating that spermatozoa had reacted to the HOS solution until they reached the separation spot.

Experiments were carried out with two chip designs of which the width of the pinched segment differs. The pinched segment of chip design I is with a width of 20 µm smaller than the pinched segment of chip design II (50 µm). It was expected, that the higher ratio of pinched segment to broadened segment increase the sperm distance from the wall, which can lead to a better separation of swollen spermatozoa from unchanged spermatozoa. The mean sperm distances of chip design I were approximately twice as high as the mean distances of chip design II. Similarly, the distribution increased with a smaller pinched segment width. In the case of porcine spermatozoa, this did not improve difference in mean distance and distribution of swollen spermatozoa from unchanged spermatozoa.

Petrunkina *et al.* have investigated the volume response of porcine and bovine spermatozoa to a hypo-osmotic solution[31]. The volume response of porcine spermatozoa to a hypo-osmotic solution reaches its maximum after five minutes with a cell volume of approximately 1.4 times the isotonic cell volume[31]. The small change in cell volume in response to the HOS solution explains that there is no or only a small difference in mean distance and distribution between porcine spermatozoa in the HOS and the isotonic solution in the PFF device. However, the volume of bovine spermatozoa in a HOS solution is twice the volume of bovine spermatozoa in an isotonic solution[31]. Since the volume response of bovine spermatozoa to a HOS solution is larger than the volume response of porcine spermatozoa, this may lead to a mean distance difference of the swollen spermatozoa and the unchanged spermatozoa in PFF. The difference of volume response between porcine and bovine spermatozoa may be caused by the difference in membrane permeability for water[32,33].

The next step was to see if this volume increase for bovine spermatozoa in a hypo-osmotic condition, affects the separation in the PFF device. Figure 5.5 shows the mean distance and distribution of bovine spermatozoa in the HOS and isotonic solution with both chip designs. For most cases, the mean distance of spermatozoa in the HOS solution is larger than the mean distance of spermatozoa in the isotonic solution. With independent t-tests the mean distances were compared with the controls, resulting in a significant differences between the spermatozoa in the HOS solution and spermatozoa in the isotonic solution. Only chip design I (pressure 250:30) did not show a significant difference between the spermatozoa in the HOS solution and isotonic solution. These

results suggest, that swollen bovine spermatozoa may be separated from unchanged spermatozoa using PFF.

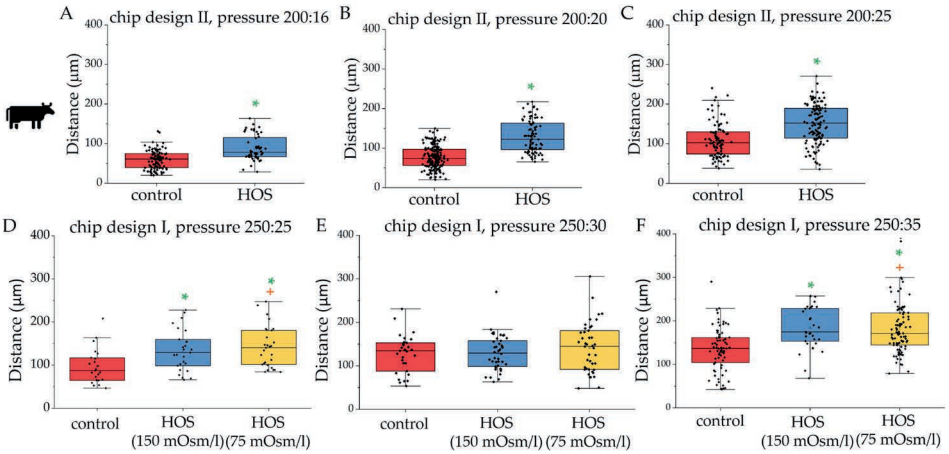


Figure 5.5: Bovine sperm distance and distribution in the broadened segment of the PFF device with chip design II (pinched segment width: 50 µm; A-B) and chip design I (pinched segment width: 20 µm; C-E) for various pressures (sheath pressure : sample pressure). The distributions of spermatozoa in the HOS solution (150 mOsm/l & 75 mOsm/l) were compared to spermatozoa in an isotonic (control) solution. The distribution of swollen bovine spermatozoa is similar to the distribution of the control group. (* indicates $p<0.05$ (significant difference) with respect to the control group, + indicates $p>0.05$ (no significant difference) with respect to HOS (150 mOsm/l))

The effect of a higher difference in osmolarity (75 mOsm/l) on the spermatozoa distribution in the broadened segment has been compared to the previous used HOS (150 mOsm/l) and isotonic solution. The results are shown in figure 5.5D-F. The mean distances of the 75 mOsm/l solution and distributions are similar to the ones obtained for the 150 mOsm/l solution. This has been confirmed by independent t-tests (*Supporting Information* 5.6.1.), which showed no significant differences between both HOS solutions. Spermatozoa from different species acquire best swelling at different osmolarities[34]. For bull semen, best swelling is achieved at 150 mOsm/l[35,36], which is in agreement with the presented results.

5.3.3. Separation experiments

PFF chips with two outlets were used to investigate the separation of HOS spermatozoa from unchanged spermatozoa. Chip design I and cryopreserved bovine spermatozoa were used. The effect of two parameters, the tubing length (outlet 2) and the sample pressure, on the separation was investigated. The percentage of spermatozoa exiting at outlet 1 and the percentage of swollen cells in both outlets 1 and 2, with respect to all spermatozoa exiting at that respective outlet, were determined.

In figure 5.6 and 5.7 the percentage of cells to outlet 1 was the variable, whereas in figures 5.4 and 5.5 this variable was the sperm distance from the wall. The median lines of the results presented in figures 5.4 and 5.5 correspond to 50% of the cells exiting the chip at outlet 1 (figure 5.6 and 5.7). When considering figure 5.5, one would expect to find more swollen spermatozoa in outlet 2 than in outlet 1.

The tubing lengths defines the resistance of both outlets and for this reason the tubing length determines the amount of flow and eventually spermatozoa exiting at outlet 1 and 2. The flow to outlet 1 and the amount of cells exiting to outlet 1 are both linearly related the channel resistance and tubing lengths. This relationship was also obtained with the used microfluidic chip (figure 5.6A). In figure 5.6B, the separation results are presented with the percentage of swollen cells in both outlets. The percentage of swollen spermatozoa in the outlets varied between 52% and 63%, which are similar to the amount of swollen cells in the control sample (68%). For the three measurements, the differences of swollen cells in outlet 1 and 2 were below 5%, indicating that swollen spermatozoa were not separated from unchanged spermatozoa.

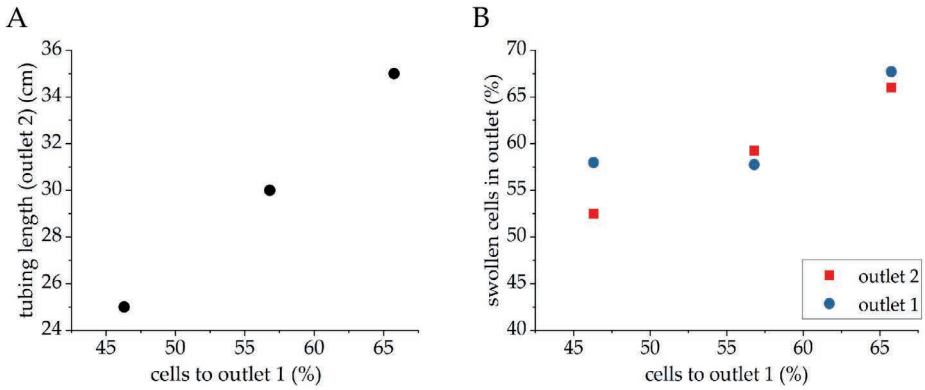


Figure 5.6: A) Relation between tubing length (outlet 2) and percentage of total spermatozoa to outlet 1: With increasing tubing length (outlet 2), the resistance outlet 2 increases and the percentage of spermatozoa ending up in outlet 1 linearly increased. B) The percentage of swollen spermatozoa, with respect to all spermatozoa flowing to that respective outlet, versus the percentage of spermatozoa ending up in outlet 1. The percentages of swollen spermatozoa in both outlets were similar to each other, indicating that swollen spermatozoa were not purified. (sample pressures: 200:25 (tubing length 25 cm), 200:30 (30 cm), 200:28 (35 cm), swollen spermatozoa (control): 68%)

By varying the sample pressure, the separation quality is directly influenced. With constant sheath pressure, a higher sample pressure induces a higher sample flow rate and increases particle containing fluid width in the pinched segment. The particle containing fluid width determines the streamlines the particles follow. Particles larger than the particle containing fluid width deviate from their original stream lines and

appear further away from the channel wall in the broadened segment. An increase in sample pressure not only increases the particle containing fluid width, but also decreases the percentage of cells exiting at outlet 1 (figure 5.7A). Figure 5.7B presents the separation results with the percentage of swollen cells in both outlets with respect to the spermatozoa exiting at outlet 1. Also here, the difference between the swollen cells in outlet 2 and 1 is below 10% for the three measurements. Increasing the sample pressure did not improve the purification of swollen spermatozoa.

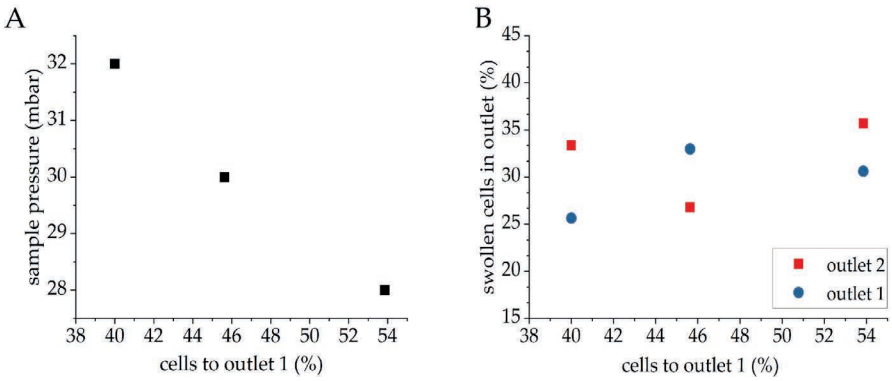


Figure 5.7: A) Relation between sample pressure (constant sheath pressure: 200 mbar) and percentage of total spermatozoa to outlet 1: With increasing sample pressure, the percentage of spermatozoa ending up in outlet 1 linearly decreased. B) The percentage of swollen spermatozoa, with respect to all spermatozoa flowing to that respective outlet, versus the percentage of spermatozoa ending up in outlet 1. The percentages of swollen spermatozoa in both outlets were similar to each other, indicating that swollen spermatozoa were not purified. (tubing length: 30 cm, swollen spermatozoa (control): 36%)

The combination of HOS and the subsequent PFF with two outlets did not improve the semen quality collected in one of the outlets. Based on the results presented of the (swollen) sperm position in the broadened segment, it was expected, that swollen spermatozoa could be purified in outlet 2. The results presented for the separation (figure 5.6B and 5.7B) did not show a higher percentage of spermatozoa in either one of the outlets. The sperm tumbling after the pinched segment in PFF influences the sperm position in the broadened segment and causes a wider distribution in contrast to circular particles[17]. Spermatozoa curl to different extents in reaction to a hypo-osmotic solution. Spermatozoa with a curling at the tip of their tail are still affected by tumbling (figure 5.8A and B). Totally curled spermatozoa are not as much affected by tumbling, because their tail is curled up, but due to their larger size they appear at the same distance as unchanged spermatozoa (figure 5.8A and C). This effect might be an explanation for the similar percentages of swollen spermatozoa found in both outlets. More research is

needed to investigate if there is a difference in behavior of swollen and unchanged spermatozoa in PFF.

The separation experiments were quantified by enumerating cells recorded in a video (length: 2-4 seconds). The number of spermatozoa enumerated is limited by the length of the video. At least 100 spermatozoa were recorded, but a larger sample size may increase the results. A more important aspect than the number of counted cells, was the discrimination between swollen and unchanged spermatozoa, which was not always feasible. A high curling degree was visible, whereas a small curling, such as a curling at the tip of the tail, could not always be discriminated from an unchanged spermatozoon. Therefore, this analysis is inaccurate due to human error. Enumerating the swollen spermatozoa in the fluid collected at the outlets was also not feasible, because spermatozoa counteract the swelling. During reproduction, spermatozoa are exposed to hypo-osmotic stress in the female tract[37]. Spermatozoa counteracting the experienced stress are thought to be more fertile[38,39]. Therefore, it might occur that spermatozoa, which are swollen during separation, appear unchanged when enumerated at the outlet. For future research, it is suggested to investigate other parameters such as the spermatozoa motility, DNA fragmentation, and acrosome integrity of the spermatozoa collected from the outlets.

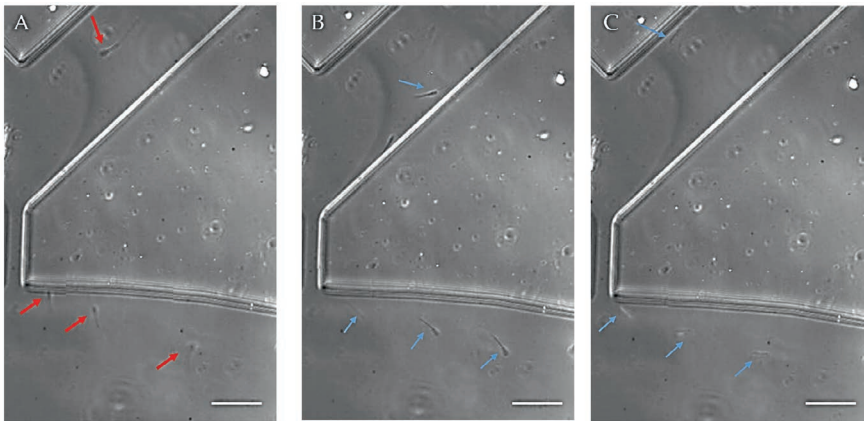


Figure 5.8: Behavior of unchanged (A), distal tail curled (B), and totally curled (C) spermatozoa at transition between the pinched and broadened segment in the PFF device. Swollen spermatozoa are less effected by the tumbling behavior than unchanged spermatozoa. (scale bar = 50 μm)

5.4. Conclusion

This chapter investigated the combination of HOS with the subsequent separation technique PFF. It is important that a processed semen sample incubated in the HOS solution maintains its quality. The short term sperm viability of bovine spermatozoa is

maintained when incubating a semen sample in the HOS solution. Regarding the separation with PFF, it was shown that bovine spermatozoa in a HOS solution appear significantly further away from the channel wall in the broadened segment than spermatozoa in an isotonic solution. However, this effect was not observed for porcine spermatozoa. Nevertheless, the presented combination of spermatozoa in a HOS solution and subsequent separation with PFF did not improve the semen quality of a semen sample.

5.5. References

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5.6. Supporting Information

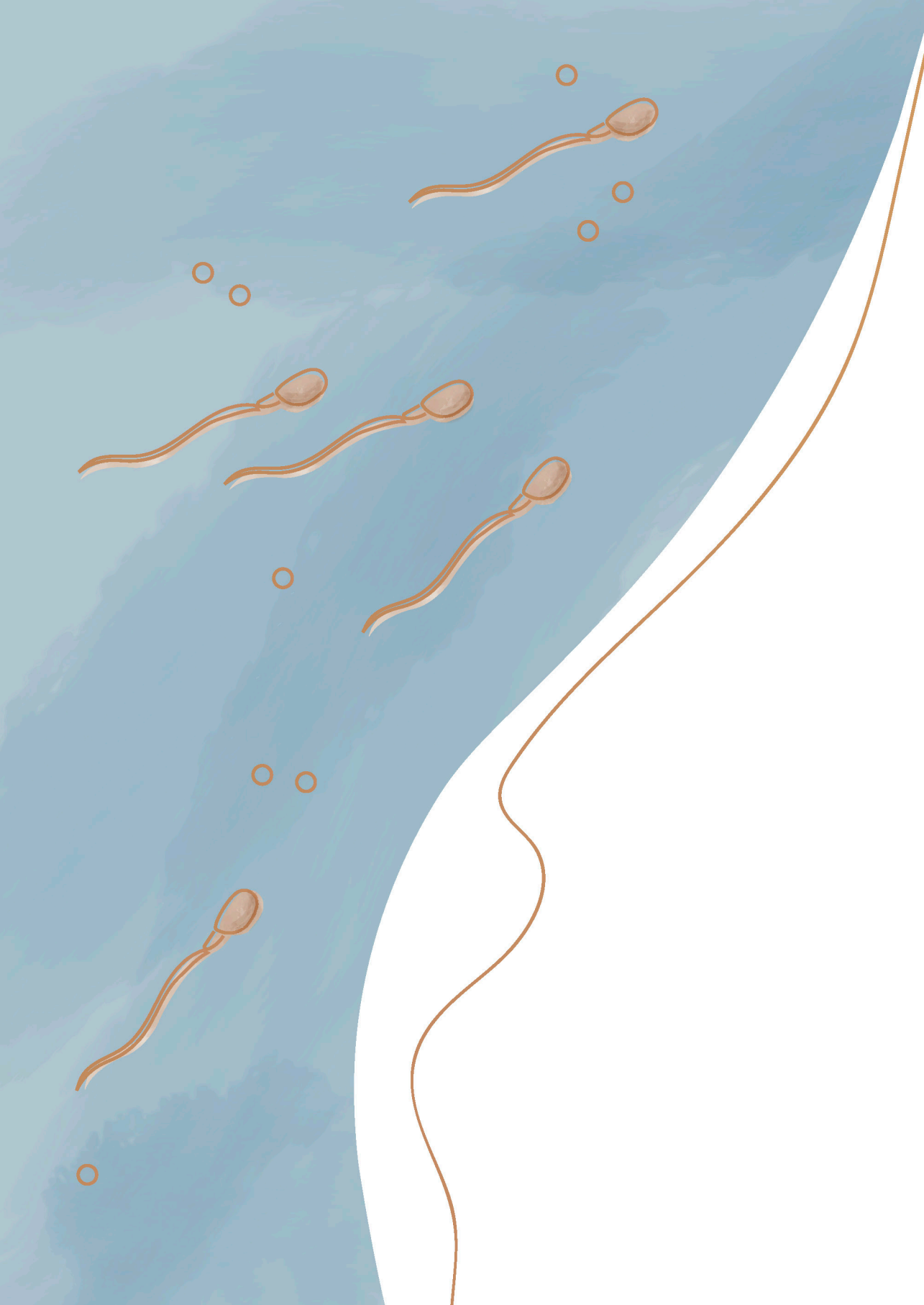
5.6.1. Spermatozoa distribution and statistics results

Table 5.1: Porcine spermatozoa average distance and standard deviation (Std) in the broadened segment of the PFF device with chip design II (pinched segment width: 50 μm; A-B) and chip design I (pinched segment width: 20 μm; C-E) for various pressures (sheath pressure : sample pressure). The distribution of spermatozoa in the HOS solution (150 mOsm/l) were compared to spermatozoa in an isotonic (control) solution. A p-value below 0.05 was a significant difference.

Chip design	Pressure (sheath:sample) [mbar]	Distance (Control) [μm]		Distance (HOS) [μm]		p-value
		Average	Std	Average	Std	
II	250:30	143	52	154	40	0.32
II	250:32.5	168	65	152	42	0.19
II	250:35	142	65	175	64	0.02
I	200:18	65	28	80	34	0.02
I	200:20	38	38	66	22	0.33

Table 5.2: Bovine spermatozoa average distance and standard deviation (Std) in the broadened segment of the PFF device with chip design II (pinched segment width: 50 μm; A-B) and chip design I (pinched segment width: 20 μm; C-E) for various pressures (sheath pressure : sample pressure). The distribution of spermatozoa in the HOS solution (150 mOsm/l and 75 mOsm/l) were compared to spermatozoa in an isotonic (control) solution. Additionally, the distribution in 150 mOsm/l and 75 mOsm/l were compared to each other. A p-value below 0.05 was a significant difference.

Chip design	Pressure (sheath:sample) [mbar]	Distance (Control) [μm]		Distance (HOS 150 mOsm/l) [μm]		p-value
		Average	Std	Average	Std	
II	250:16	59	23	89	32	0.00
II	250:20	77	29	129	41	0.00
II	250:25	109	44	151	49	0.00
I	250:25	97	40	134	47	0.00
I	250:30	127	43	129	41	0.80
I	250:35	135	50	184	49	0.00
		Distance (Control) [μm]		**Distance (HOS 75 mOsm/l) [μm]**		
I	250:25	97	40	142	47	0.00
I	250:30	127	43	142	57	0.21
I	250:35	135	50	182	58	0.00
		Distance (HOS 150 mOsm/l) [μm]		**Distance (HOS 75 mOsm/l) [μm]**		
I	250:25	134	47	142	47	0.53
I	250:30	129	41	142	57	0.26
I	250:35	184	49	182	58	0.82



6.

Micro-organism removal from porcine semen with acoustophoresis

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Bacteria removal from porcine semen with acoustophoresis
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Abstract

Micro-organisms present in semen are not only a risk for disease transmission, but also reduce semen quality and therewith fertilization rates. It is obligatory that antibiotics are added to semen to prevent the negative effects of bacteria during artificial insemination (AI). The worldwide overuse of antibiotics has led to the emergence of antibiotic resistant bacterial strains, which hampers the treatment of bacterial infections. Acoustophoresis is a continuous microfluidic separation technique which separates particles based on their size. In this study, we have applied acoustophoresis to sort spermatozoa from *Escherichia coli (E.coli*) spiked porcine semen in a microfluidic chip. After optimizing separation parameters, such as the sample flow rate and the buffer solution, sperm recovery of 90 ± 4% and bacteria removal of at least 88 ± 7% were achieved. Similar separation efficiencies were obtained by varying sperm and bacteria concentrations. Additionally, acoustophoresis has been applied to remove a model virus from semen. Furthermore, no effect on sperm viability by acoustophoresis was observed. The removal of bacteria from semen by acoustophoresis ensures high sperm recovery and viability rates. The implementation of a physical pathogen removal step during semen processing can reduce the use of antibiotics in semen and increases the biosecurity of AI.

6.1. Introduction

Antibiotic resistance is one of the "biggest threats to global health, food security, and development"[1] warns the World Health Organization (WHO) and states that the overuse of antibiotics in the livestock industry is one of the causes[2,3]. This not only restricts the use of antibiotics to treat diseases in animals, but also the use of antibiotics in breeding programs. In a third of extended semen doses, bacterial contaminants were found[4–6]. Although artificial insemination centers have taken many precautions of hygienic control during semen collection and handling, most bacterial contaminations occur during semen processing instead of originating from the ejaculate itself[6]. To reduce bacterial contamination of semen, it is legally obligated to add antibiotics to semen extenders, which in turn is used in the daily processing of porcine semen[7]. For porcine and bovine semen, the following combination of antibiotics must be added to every milliliter diluted semen: 500 µg streptomycin, 500 IU penicillin, 150 µg lincomycin and 300 µg spectinomycin[7]. An alternative antibiotic mixture is the combination of 75 µg amikacin and 25 µg divekacin[7]. Antibiotic resistant bacterial strains have been also identified in semen[8,9]. As antibiotics are used to treat bacterial infections in both humans and animals, the treatment of infections caused by antibiotic resistant bacterial strains becomes harder and less effective[10]. Therefore, reducing the use of preventive antibiotics is of importance.

The veterinary industry favors the reduction of bacteria in semen. Not only leads this to less transmission of possible diseases, but the presence of bacterial contaminants also reduces semen quality and fertilization rates. It has been shown that bacteria contaminated semen have reduced sperm motility rates compared to uncontaminated semen[11]. Also, the sperm motility can be reduced by 50% depending on the bacteria type and concentration. This has been studied for various bacteria types[12–14] like *Escherichia coli* (*E.coli*)[14] and *Clostridium perfringens*[14]. Additional negative effects on the spermatozoa caused by bacteria are sperm agglutination, reduced acrosome reaction, alterations in cell morphology, as well as the production of cytotoxic factors[15,16]. Studies have shown that the storage time and amount of bacteria present in semen reduce fertilization rates[17,18]. Consequently, the presence of bacteria in semen should be avoided.

Instead of reducing the bacterial growth with antibiotics, the physical reduction of bacteria from semen with a separation technique is a promising alternative. Previous studies have shown to remove bacteria from semen samples using single-layer centrifugation (SLC)[19,20]. SLC is able to reduce the bacterial contamination considerably in porcine semen contaminated with diverse bacteria types without a negative effect on sperm motility[19]. In many samples, no bacterial growth was detected, whereas in other samples the bacterial growth was much lower than the unprocessed samples[19]. In a similar study with stallion semen, SLC reduced the bacterial contamination between 68%

to 100% for various bacterial species, whereas most bacterial reductions were achieved between 80 to 90%[20]. For both studies, no information about the sperm yield was provided. For similar applications of SLC, for example the separation of live and dead spermatozoa or the removal of viral pathogens from semen, sperm yields were 80 to 86%[21] and 43 ± 15%[22], respectively. Another limit of using SLC in daily semen processing is that during the manually performed steps, such as removal of the supernatant and cell pellet, the semen can be recontaminated as equipment for a sterile working environment, such as laminar air flow benches, are not available in every AI center.

Semen samples are screened for the presence of viruses in semen with enzyme-linked immunosorbent assays (ELISA) or polymerase chain reaction (PCR)[23], which are expensive and time-consuming techniques (MLWJ Broekhuijse, Topigs Norsvin and CRV BV, personal communication, 2020). As semen quality of fresh porcine semen decreases with time[24,25], usually the screening results are not available at the moment of insemination. The physical removal of viral pathogens from semen may increase the biosecurity of AI.

In this study, we present a proof-of-principle for the continuous separation of pathogen from porcine semen using bulk acoustophoresis in a microfluidic chip. The continuous separation with bulk acoustophoresis has been reviewed by Lenshof *et al.*[26,27]. Most applications are for diagnostic purposes in human medicine such as enriching bacteria[28,29], platelets[30,31], white blood cells[30,32] or circulating tumor cells (CTC)[33,34] from blood. In acoustophoresis, the actuation of a half standing acoustic wave transversal to the sample flow induces an acoustic radiation force, which is dependent on the particle size, density, and compressibility in relation to the surrounding medium[26,35]. The velocity induced by the acoustic radiation force scales with the square of the particle radius[26,35]. Therefore, the spermatozoa experience a larger force and therefore move faster to the sample-free medium than the smaller bacteria.

We performed acoustophoretic separation to enrich spermatozoa from bacterial and viral pathogen contaminated semen. We show that spermatozoa are affected by the acoustic radiation force and are therewith separated from pathogens present in semen. The separation efficiency has been improved by optimizing the used flow rates and buffer density of the sample-free medium. At last, the effect of the acoustic radiation force on the sperm viability has been investigated.

6.2. Materials and methods

6.2.1. Semen sample preparation

Four fresh porcine semen doses at a concentration of 20 x 10^6 cells/ml were obtained from an AI center (Svenska Köttförtagen, Skövde, Sweden). The semen was stored at 16 °C

and used within five days. The samples were diluted with the sperm diluent Solusem Bio+ (AIM Extender, AIM Worldwide, Vught, the Netherlands) to a concentration of 2 x 10^6 cells/ml unless otherwise stated.

6.2.2. Bacteria preparation

E.coli DH5α competent cells (Thermo Fisher Scientific, Waltham, MA, USA) were cultured in Luria-Bertani (LB) broth medium (Lennox, Thermo Fisher Scientific) overnight at 225 rpm in a shaker incubator. Bacteria were washed with centrifugation (4000 x g, 5 minutes) and resuspended with the fixation solution consisting of 4% formaldehyde in phosphate buffer saline (PBS) for 30 minutes. Afterwards bacteria were washed twice using PBS and a centrifugation step (4000 x g, 5 minutes). With a cell counting chamber, the *E.coli* concentration was approximated to be 1 x 10^9 bacteria/ml. The fixed bacteria were stored at 4 °C until use. Shortly before use, E. coli were stained with SYTO 9 green fluorescent nucleic acid stain (Thermo Fisher Scientific) at a final concentration of 1 μM. The stained bacteria were diluted in Solusem Bio+ or added to the semen sample at a concentration of approximately 10 x 10^6 bacteria/ml unless otherwise stated.

6.2.3. CCMV preparation

The cowpea chlorotic mottle virus (CCMV), which served as a model virus, were prepared and fluorescently labeled with Atto 488 NHS ester (Sigma-Aldrich, Zwijndrecht, the Netherlands) as previously described in chapter 4 (Materials and Methods). CCMV were spiked to semen samples with a concentration of 120 ng/ml.

6.2.4. Experimental setup

Acoustic separation was performed with the AcouWash (AcouSort AB, Lund, Sweden), a benchtop research instrument for automated particle separation. The AcouWash contains an automated internal precision flow regulation via air pressure controllers and liquid flow sensors, an integrated thermistor for temperature monitoring and a Peltier element for temperature control.

The AcouWash instrument has a microfluidic glass chip with a channel structure consisting of a sample inlet, a V-shaped flow splitter around the central buffer inlet, a main separation channel in meander shape (2.5 cm x 420 μm x 150 μm) and a trifurcation with a target outlet and a common side outlet for the two lateral branches (figure 6.1). The standing wave field for acoustic separation was created using a 2 MHz piezoceramic transducer glued underneath the separation channel. The actuation frequency of the used chip was tuned by visual inspection using 5 μm polystyrene beads (Sigma-Aldrich, St.Louis, MO, USA) and set to an optimal frequency of 2.05 MHz.

With the provided software of the AcouWash instrument the flow rates for the inlets and outlets, the temperature, the frequency as well as power of the acoustic wave were controlled.

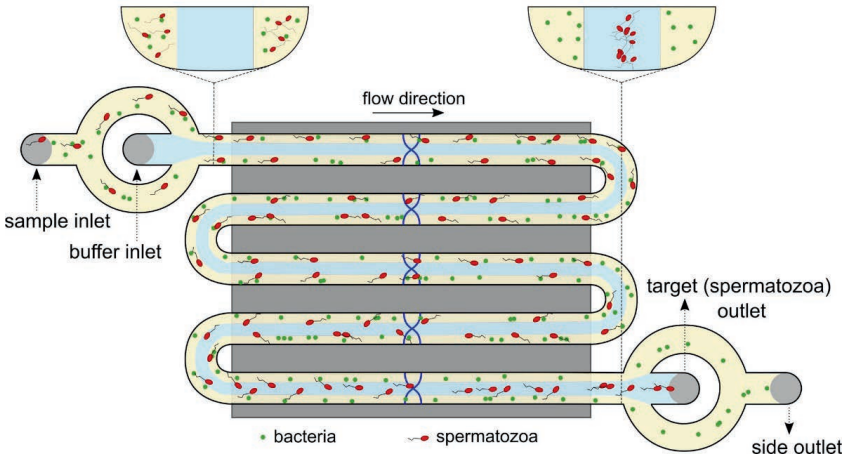

Figure 6.1: Schematic representation of the acoustic separation principle: The sample (spermatozoa and bacteria) is injected through the sample inlet, bifurcated around the center buffer inlet, and enters the separation channel laminated close to the side walls. An acoustic standing wave field created by a 2 MHz transducer attached underneath the separation channel forces larger particles (spermatozoa) to move towards the pressure node in the center of the channel and are collected at the target outlet. The smaller particles (bacteria/beads) remain along the side walls of the channel and can be collected at the side outlet.

6.2.5. Experimental procedure

Before sample processing, the AcouWash was primed with Solusem Bio+ to remove air in the fluidic system. For all experiments, the flow rate ratio of the sample to buffer inlet was 1:2 and the flow rate ratio for the target to side outlet was 1:2. The semen sample and buffer solution were connected to the respective inlets. The buffer solution consisted of Solusem Bio+ with 1% Ficoll-Paque (1.084, Sigma-Aldrich, St. Louis, MI, USA) unless otherwise stated. After inserting the desired flow rates and acoustic parameters, the separation process was performed, and the target and side fluids were collected at the outlets.

6.2.6. Analysis

The cells present in the fluids collected at both outlets were counted with flow cytometry (FACSCanto II cytometer and FACSDiva software, BD Biosciences, Franklin Lakes, NJ, USA). The sperm population was initially gated using the forward scatter (FSC) and side scatter (SSC). The bacteria population was gated based on the SSC and the fluorescence

signal (FITC) of the stained bacteria. Exemplary plots obtained from analysis can be found in *Supporting Information* 6.7.1.

The sperm separation efficiency is defined as the percentage of spermatozoa present in the target outlet relative to the total number of spermatozoa counted in all outlets. Similarly, the percentage of bacteria in the target outlet was determined by dividing the number of bacteria in the target outlet by the number of bacteria in all outlet. The bacteria removal is estimated as the percentage of bacteria present in the side outlet relative to the bacteria in all outlets.

The CCMV concentrations in both outlets were determined with the fluorescent microplate reader GENios FL (TECAN, Männendorf, Switzerland). Samples were pipetted in black 96-well-plates and measured in fluorescence mode (λ_{ex}=485 nM, λ_{em}=535 nm). With a calibration curve, the fluorescence intensities were transcribed to the CCMV concentrations (*Supporting Information* 6.7.2.).

6.2.7. Sperm viability

The influence of the acoustic force on the viability of the spermatozoa was assessed with Propidium Iodide (PI) staining, which stains dead spermatozoa. Semen samples with concentrations of 2 x 10^6 cells/ml from four different boars were processed with the AcouWash instrument at sample flow rates of 30, 40 and 50 µl/min. The collected fluid at the target outlet was stained with PI (24 µM, ex/em 535/617 nm, Life Technologies, Eugene, OR, USA) for 5 minutes at room temperature. The sperm population was enumerated with flow cytometry. The stained dead spermatozoa were gated based on their fluorescence signal whereas unstained spermatozoa were considered viable. The percentage of dead spermatozoa is defined by dividing the dead spermatozoa count by the total spermatozoa count from the target outlet. The percentage of viable spermatozoa is determined by subtracting the percentage of dead spermatozoa from 100%.

The Wilcoxon Signed Ranks Test was applied to investigate whether spermatozoa viability is affected by acoustophoresis. This test determines with ranks whether there is a statistical difference between two time points[36]. Here, one time point is before the separation procedure (control) and the other time point is after the separation procedure. Samples from four different boars were subjected to three different sample flow rates, which results in 12 subjects. The significance level of the two-tailed test was chosen to be 0.05.

6.3. Results

6.3.1. Separation optimization

The acoustophoretic separation of bacteria from semen was modelled by spiking porcine semen with *E.coli* bacteria in a spermatozoa to bacteria ratio of 1:5. The semen concentration was 2 x 10^6 spermatozoa/ml and fluorescently labelled *E.coli* bacteria were spiked at a concentration of approximately 10 x 10^6 bacteria/ml. After the separation, the number of spermatozoa and bacteria in both the target and waste outlet were counted with flow cytometry, from which the percentages of spermatozoa and bacteria in the target outlet were determined. The effect of the density of the central buffer solution and the effect of the sample flow rate on the separation quality were investigated.

To find the optimal central buffer solution for the separation of bacteria from semen, the effect of Ficoll concentration on the separation quality was tested for sample flow rates of 50 μl/min and 30 μl/min with two bacteria spiked samples (figure 6.2A). The acoustic impedance is a physical characteristic dependent on the density and speed of sound in the medium. The addition of Ficoll to the central buffer solution increases the density and therewith the acoustic impedance of the buffer solution, which prevents particles from crossing the fluid barrier. Without the use of an increased impedance of the central buffer solution, the number of bacteria (green) in the target outlet was at least 20% for both sample flow rates. For all Ficoll concentrations the number of bacteria in the target outlet was reduced to approximately 10%. The sperm recoveries with a sample flow rate of 50 μl/min were lower than the spermatozoa recoveries with a sample flow rate of 30 μl/min. No difference between the sperm recovery of the pure buffer and the buffer with 1% Ficoll was observed. For a sample flow rate of 30 μl/min, the sperm recovery was at least 80% for all Ficoll concentrations in the central buffer solution. Considering both the sperm recovery and bacteria present in the target outlet, a Ficoll concentration of 1% has been chosen to be optimal and has been used for the ensuing experiments.

The effect of sample flow rate on the separation efficiency was investigated by varying the sample flow rate between 30 and 100 μl/min. Sample flow rates below 30 μl/min could not be validated as the flow regulation was not stable. The percentage of spermatozoa (red) and bacteria (green) in the target outlet are presented in figure 6.2B. The sperm recovery decreased linearly with an increase in sample flow rate until a plateau was reached at approximately 40%. The best sperm recovery (90%) was reached at a sample flow rate of 30 μl/min. For all sample flow rates, the bacteria present in the target outlet were below 15%. At flow rates above 50 μl/min, the percentages of bacteria in the target outlet were below 10%, which was caused by the decreased effect of streaming at higher flow rates. At a sample flow rate of 30 μl/min the separation

efficiency was acceptable, because a sperm recovery of 90% was achieved with approximately 10% of the bacteria present in the target outlet.

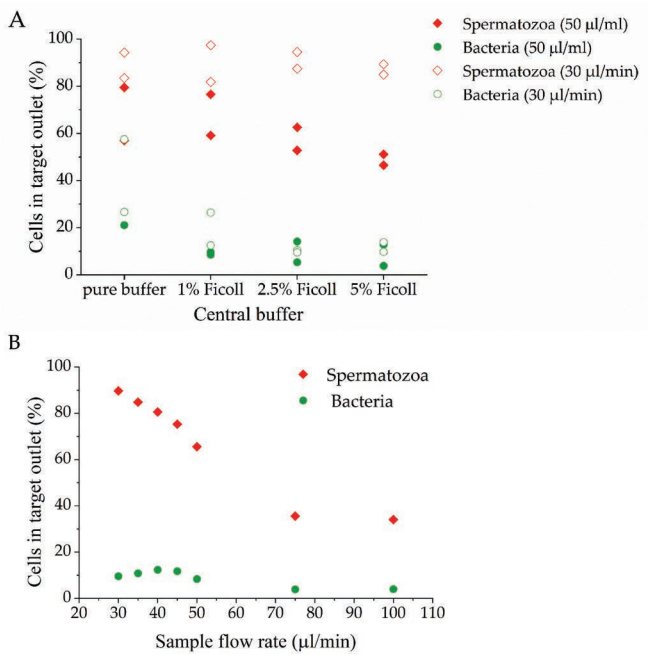

Figure 6.2: A) Effect of medium buffer with increasing Ficoll concentration on the separation quality in the target outlet for sample flow rates of 30 and 50 µl/min. An increase in Ficoll concentration decreases the number of bacteria and spermatozoa in the target outlet. For a sample flow rate of 30 µl/min the sperm recovery remains almost unchanged. (N=2) B) Effect of sample flow rate on the percentages of cells present in the target outlet. The percentage of spermatozoa (red) decreases with increasing sample flow rate, whereas the percentage of bacteria (green) decreases slightly and is for all flow rates below 20%. The central buffer solution contained 1% Ficoll. (N=1)

6.3.2. Sperm and bacteria concentration

The possibility to use higher sperm concentrations increases the sample throughput of the separation. The effect of the sperm concentration between 1 x 10^6 to 8 x 10^6 cells/ml on the separation efficiency was investigated at constant bacterial contamination (10 x 10^6 bacteria/ml) (figure 6.3A). For all concentrations, the sperm recovery was at least 90% and the standard deviation was below 4%. The percentage of bacteria present in the target outlet increased slightly with an increase in sperm concentration. When the spermatozoa move towards the pressure node, they also drag some of the surrounding liquid with them. The higher the sperm concentration, the higher the volume of the sperm surrounding liquid and the more bacteria are moved towards the sperm outlet.

The bacteria present in the target outlet was with 9 ± 5% the lowest for a sperm concentration of 1 x 10^6 cells/ml, but with 19 ± 6% acceptable at a sperm concentration of 8 x 10^6 cells/ml.

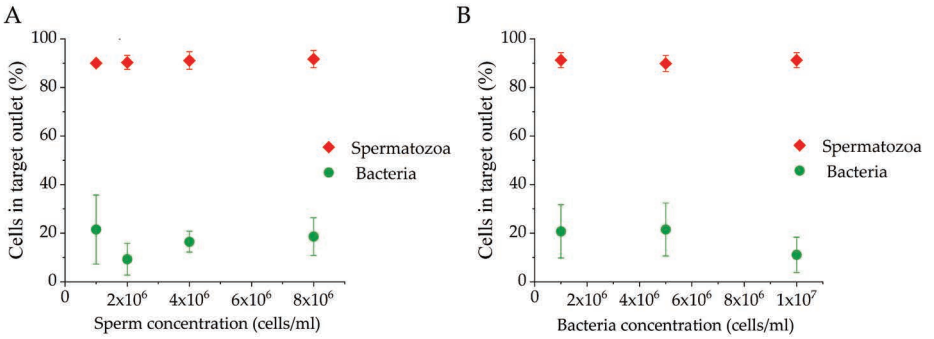

Figure 6.3: The effect of sperm (A) and bacteria (B) concentration on the separation quality at a sample flow rate of 30 µl/min and a central buffer solution with 1% Ficoll. (Error bars represent standard deviation, N=3)

In semen samples, the degree of bacterial contamination varies. To investigate whether the amount of bacterial contamination influences the separation efficiency, semen samples (2 x 10^6 spermatozoa/ml) were contaminated with bacteria concentrations between 1 x 10^6 to 10 x 10^6 bacteria/ml. The results of the bacteria concentration on the separation efficiency are shown in figure 6.3B. The sperm recovery was at least 85% and increased slightly with an increase in bacteria concentration up to 91 ± 3%. The bacteria concentrations in the target outlet were for all tested bacteria concentration approximately 20%.

6.3.3. Bacteria removal from porcine semen

Measurements performed during the experiments with the optimal buffer solution (1% Ficoll) and the optimal sample flow rate (30 µl/min) were summarized to present the separation efficiency. All samples contained a spermatozoa to bacteria ratio of 1:10 (sperm concentration: 1 x 10^6 cells/ml; bacteria concentration: 10 x 10^6 cells/ml). The average sperm recovery, average bacteria removal and their respective standard deviation are shown in figure 6.4. The obtained sperm recovery and bacteria removal were 90 ± 4% and 88 ± 7%, respectively.

6.3.4. Virus removal from porcine semen

Virus contaminated semen was modelled by spiking porcine semen with fluorescent labelled CCMVs at a concentration of 120 ng/ml. The virus spiked semen sample was processed with acoustophoresis at a sample flow rate of 30 µl/min. As a buffer solution,

pure sperm diluent and sperm diluent with 1% and 5% Ficoll were used. The separation was investigated by determining the virus concentration in both outlets. In figure 6.5, the virus concentrations in both outlets for the three buffer solutions are presented. For all three buffer solutions used, the virus exited the chip at the waste outlet and not at the target outlet. The difference between the three buffer solutions was small.

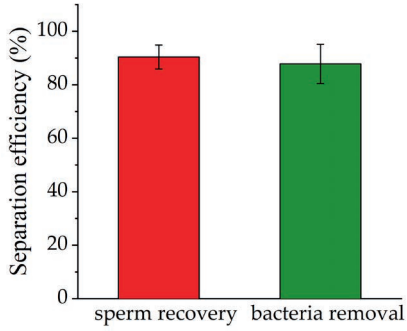

Figure 6.4: Separation efficiency at a sample flow rate of 30 µl/min and with a central buffer solution with 1% Ficoll. (Error bars represent standard deviation, N=9)

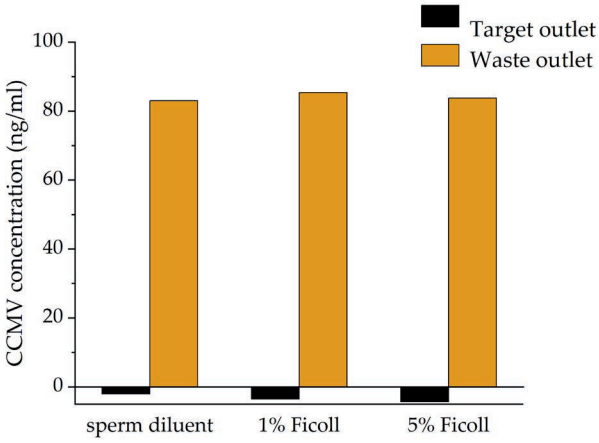

Figure 6.5: Virus concentration in both outlets after separation with three buffer solution (sample flow rate: 30 µl/min). CCMVs were detected in the waste outlet and not in the target outlet. (N=1)

6.3.5. Viability study of spermatozoa

After removing the bacteria from semen, the semen will be used for AI. It is important that spermatozoa retain their quality to assure successful fertilization. Therefore, it was investigated whether the sperm viability decreases after acoustophoretic separation. Semen samples from four boars were processed with the AcouWash at sample flow rates of 30, 40 and 50 µl/min. After processing, the spermatozoa exiting at the target outlet

were stained, and the percentage of live spermatozoa was determined. The percentage of live spermatozoa after acoustophoresis and the control measurements are shown in figure 6.6. No difference in sperm viability before and after processing was observed. Also, the standard deviation, which represents the different flow rates of the same semen sample, was small and varied between 0.5 and 2.5%. The lower viability of sample I for both the control and the separation compared to the viabilities of the other samples was due to the longer storage time before usage. A statistical analysis using the Wilcoxon Signed Ranks Test was applied to determine whether there is a statistical difference of the viability before and after the acoustophoretic separation. The value of the signed rank test statistic is 28, which is higher than the critical value (17; two-tailed significance level: 0.05; sample size: 12) and therefore, there is no significant difference in viability before and after acoustophoretic separation.

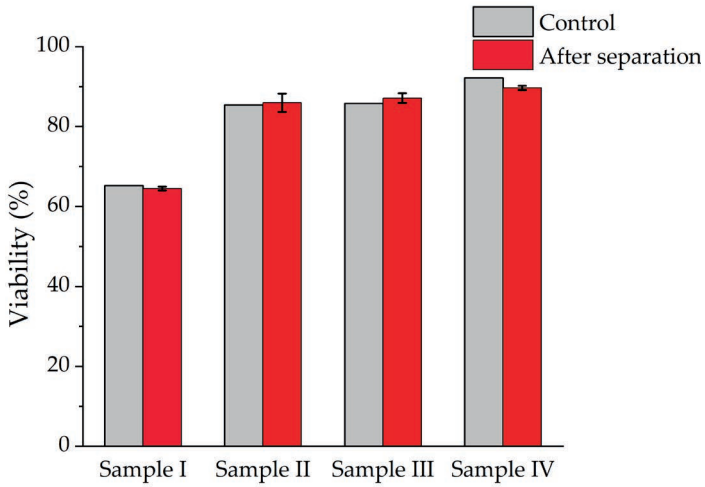

Figure 6.6: The effect of acoustophoresis on the viability of spermatozoa compared to an unprocessed sample for semen samples obtained from four different boars. No effect of sperm viability between the processed and unprocessed was observed. (Error bars represent standard deviation, N=3)

6.4. Discussion

The formation of antibiotic resistant bacteria is caused by an overuse of antibiotics and hampers the treatment of bacterial infections because antibiotics become ineffective. Therefore, the reduction of using preventive antibiotics is of worldwide interest. In the veterinary industry, antibiotics are added to semen extenders to prevent the spread of diseases and to maintain semen quality. As an alternative to adding antibiotics to semen, we present a continuous and physical bacteria removal technique based on acoustophoresis to reduce the number of bacteria in semen samples. Additionally, we have shown that also viruses can be removed from semen with acoustophoresis.

With acoustophoresis of sub-micron particles, such as pathogens, the effect of acoustic streaming can undesirably relocate the smaller particles across the streamlines to the target outlet[37,38]. This acoustic streaming can be prevented by increasing the acoustic impedance of the central buffer solution[28], which functions like a barrier preventing particles to cross the streamline. The acoustic impedance of the buffer solution was increased by adding Ficoll. The bacteria removal increased with a higher acoustic impedance of the central buffer solution. Additionally, an increase in impedance of the buffer solution hampers the spermatozoa to cross the streamline to reach the sperm outlet. Therefore, a Ficoll concentration of 1% was chosen to be optimal considering both sperm recovery and micro-organism removal.

Viable bacteria can spread and contaminate when they are not correctly handled. Therefore, many laboratories prefer handling of fixed bacteria for proof-of-principles studies. After fixation, the cell does not undergo any biochemical process and is preserved in its current state. The most important cell characteristic for acoustophoresis is the cell size. After fixation, the size of bacteria slightly decreases with 4 – 7%[39]. A small difference in size does not influence the separation process, especially when the cells are not be deflected by the acoustic field. Some other physical characteristics such as membrane permeability and rigidity can change as well, but their influence on the separation process is small when comparing it to the difference in cell size. It is suggested to investigate acoustophoresis with viable bacteria. For example, the separation of semen samples which have not been treated with antibiotics can be studied.

The effect of Ficoll on sperm viability and motility, and thus fertilizing capabilities, should be considered. Ficoll is a highly-branched polysaccharide with good solubility in aqueous solutions. Ficoll does not contain ionized groups, which may harm spermatozoa, and the osmotic pressure of Ficoll is isotonic to spermatozoa[40]. Several studies have used Ficoll and spermatozoa, for example to centrifuge[40,41] or cryopreserve[42,43] semen samples. The optimal Ficoll concentration used in this study is only 1% and the separation process lasts a few minutes. After the separation process the spermatozoa can be washed with the respective sperm diluent.

The sample flow rate is an essential parameter of the separation process. On the one hand, a high sample flow rate is desired to process large sample volumes within a short amount of time. On the other hand, the sample flow rate determines the separation efficiency, especially the sperm recovery. The smaller the sample flow rate, the longer the spermatozoa are exposed to the acoustic radiation forces, which moves the particles from the sample containing fluid to the central buffer solution and, thus, the higher the sperm removal. In a similar application for sepsis diagnostics, bacteria have been separated from blood with acoustophoresis at sample flow rates between 80 and 400

µl/min while achieving high red blood cell recovery (> 98%) and acceptable bacteria removal (> 90%)[28,44]. The sample flow rate for the removal of bacteria from semen is with 30 µl/min much lower than the achieved sample flow rate for the bacteria separation from blood. The results suggest that spermatozoa are harder to focus by the acoustic radiation force than red blood cells. Red blood cells have a disc-like shape with a diameter of approximately 8 µm and height of 2.5 µm[45], and a cell volume of 80 to 100 µm^3 [46]. The oval-shaped head of a spermatozoon has a length of 7 µm, width of 3.7 µm and thickness of 0.4 µm[47]. Sperm cell volume was measured to be around 26 µm^3 [48], explaining the lower acoustic radiation force on the spermatozoa with a nearly five-times lower volume as compared to red blood cells. It is unknown, whether the tail of a spermatozoon has an effect on acoustic focusing. The particle velocity due to the acoustic force depends not only on the particle size, but also on the acoustic contrast factor[26]. The acoustic contrast factor takes into account the density and compressibility of the particle in relation to the surrounding medium[27,35]. A higher acoustic contrast factor focuses particles faster towards the pressure node in the acoustic field. The density of red blood cells (1.11 g/ml[49]) and spermatozoa (1.10 g/ml[50]) and the compressibility of the surrounding media, which can be approximated as water for both applications, is very similar. The higher density of Solusem Bio+ (1.05 g/ml) decreases the acoustic contrast factor and therewith the sperm velocity towards the pressure node when comparing it to the red blood cell separation in PBS (1.01 g/ml). To increase the sample flow rate while maintaining a high sperm recovery, the chip length and the applied acoustic energy can be increased.

The separation efficiency, subdivided in bacteria removal and sperm recovery, is the most important aspect of the device. The achieved bacteria removal of 88 ± 7% with acoustophoresis is comparable to the previously reported bacterial removal rates of SLC of bacterial contaminated porcine and stallion semen. For stallion semen, bacteria removal rates varied between 68% to 100%[20], whereas for porcine semen removal rates were given in bacterial growth ranging from no growth to highly reduced growth rates compared to the unprocessed samples[19]. Apart from bacteria removal, it is also essential to consider the sperm recovery. The higher the sperm recovery, the less spermatozoa are lost during the separation process. Each spermatozoon is potentially high fertilizable, meaning that with each lost spermatozoon, the fertilization of that ejaculate is reduced. Therefore, it is important to have high sperm recovery rates. Although, no sperm recovery rates were reported for bacterial removal from semen, other SLC studies reported sperm yields of 80 to 86%[21] and 43 ± 15%[22]. In this study, a sperm recovery of 90 ± 4% was achieved, which is therewith higher than sperm yield achieved with SLC. Apart from the sperm recovery, the sperm fertility after acoustophoresis must be investigated. Acoustophoresis is known to be a gentle cell separation technique with low impact on

cell viability [51]. Böhm et al. have reported that plant cells, which are more fragile than mammalian cells, that are focused in the nodal plane stay intact and viable in contrast to cells in a propagating wave field[52]. In this study, we have investigated that the sperm viability was not affected by separation with acoustophoresis. Other sperm quality parameters such as motility and morphology, followed by field fertility experiments must reveal whether acoustophoresis is applicable to process semen for AI.

Examining the bacteria type and concentration is essential for future considerations and both influence sperm quality. *E.coli* have been identified in many semen samples[4,53,54] and their negative effect on semen quality, such as a decrease in motility[14], ultrastructural damage[55], and sperm agglutination[56], has been widely investigated. Therefore, *E.coli.* are a representative model for bacterial pathogens found in semen. Many other bacteria types have been isolated from porcine semen[4,53,54], among others *Pseudomonas* species and *Citrobacter* species. As the size of bacteria is in general smaller than the size of spermatozoa, it is expected that other bacteria can be removed from semen using acoustophoresis, which must be confirmed by future experiments. The decrease in sperm viability and motility depends not only on the bacteria type, but also on the degree of bacterial contamination. High bacterial contaminations (10^7 cfu/ml) cause lower sperm viability and motility than lower bacterial contaminations (10^1 cfu/ml) [14]. In a study by Morrell *et al.* the amount of bacteria in porcine semen varied between a few hundred colony forming units (cfu) per milliliter to a few thousand cfu/ml[19]. For stallion semen, it has been reported that bacterial loads vary between $0.7 \times 10^6 \pm 0.7\ 10^6$ to $3.7 \times 10^7 \pm 6.3 \times 10^7$ cfu/ml[57]. These concentrations are like the bacteria concentrations of 1×10^7 bacteria/ml mainly used in this study.

The separation efficiency of our method was determined with flow cytometry, which is commonly applied to count and characterize cells. Background or noise signal in the bacteria population was obtained from Solusem Bio+ and the staining solution (*Supporting Information* 6.7.3.). In a semen sample cellular debris or dead bacteria may be present, which cause the background or noise signal. Nevertheless, the results of the bacteria removal give a good indication that acoustophoresis can be used to remove bacteria from semen samples. To support the findings of this study, it is suggested to compare the bacterial growth before and after the separation process.

Bacterial contamination occurs mostly during the manual handling, although strict hygienic rules are applied[6]. It tells its own tale, that a bacterial removal step must be performed in a sterile manner and must not lead to recontamination. This is not guaranteed for the bacteria removal with SLC, in which manual handling steps, such as aspiration, must be performed and not every insemination center possesses sterile working environments such as laminar flow cabinets. In contrast to SLC, separation with

acoustophoresis can be automated as shown with the AcouWash instrument. Tubes containing the semen and buffer solution as well as tubes collecting the processed sample are screwed on the instrument. The whole separation process is automated and no further manual processing steps are needed. After the separation, the sample is collected in a tube and can be used for further processing or insemination.

In a proof-of-principle experiment with a model virus, we have shown that also viruses can be removed from semen with acoustophoresis. No information about the sperm recovery could be obtained. As the buffer and flow rate (30 µl/min) were the same as the one previously used for the bacteria separation, it is expected that similar sperm recoveries can be achieved. For future research, it is recommended to investigate and optimize the virus removal from semen with acoustophoresis in more detail similar to the experiments presented for the bacteria removal.

The presented results show that viral and bacterial pathogens can be removed by acoustophoresis with similar settings. In the future, it can be investigated if acoustophoresis is feasible of removing both pathogens types from semen. The removal of both viral and bacterial pathogens from semen in one separation step will simplify separation in contrast to having one separation step for each pathogen type.

6.5. Conclusion

Bacterial and viral pathogens can be continuously removed from semen samples using acoustophoresis. With the optimized buffer solution and sample flow rate a bacteria removal of 88 ± 7% and a sperm recovery of 90 ± 4% was achieved. Viruses were mainly detected in the waste outlet. With the implementation of a physical pathogen removal step during semen processing for AI, the addition of antibiotics and the risk of disease spread can be reduced, while maintaining semen quality. Reducing the use of antibiotics in the livestock industry is an important step towards the defeat of antibiotic resistant bacteria.

6.6. References

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6.7. Supporting Information

6.7.1. Sperm and bacteria gating with flow cytometry

To analyze the cell populations with flow cytometry, the cell populations were gated using the individual populations. The final populations and their controls are shown in figure 6.7. Spermatozoa (red) were gated based on forward scatter (FSC-A) and side scatter (SSC-A) (figure 6.7A & B), whereas bacteria (green) were gated based on SSC-A and fluorescence signal (FITC-A) (figure 6.7C & D). As a control an unstained bacteria sample was measured (figure 6.7E & F). In figure 6.7G & H the populations of a bacteria spiked semen sample are shown.

Exemplary plots obtained from analysis with flow cytometry after separation with acoustophoresis are presented in figure 6.8. The sample, containing both spermatozoa (2 x 10^6 cells/ml) and bacteria (1 x 10^7 /ml) was separated with acoustophoresis at a sample flow rate of 30 μl/min and a buffer flow rate of 60 μl/min. After processing, fluids from both the target and side outlet were analyzed with flow cytometry. The measured volume of the target outlet contained 6 183 spermatozoa and 8 026 bacteria, whereas the measured volume of the side outlet contained 357 spermatozoa and 38 028 bacteria. The volume obtained in the target outlet was half of the volume obtained in the side outlet. The number of cells in the target outlet has been doubled to correct for the difference in obtained volumes. Approximately 90% of the spermatozoa were recovered, while removing 90.5% of the bacteria.

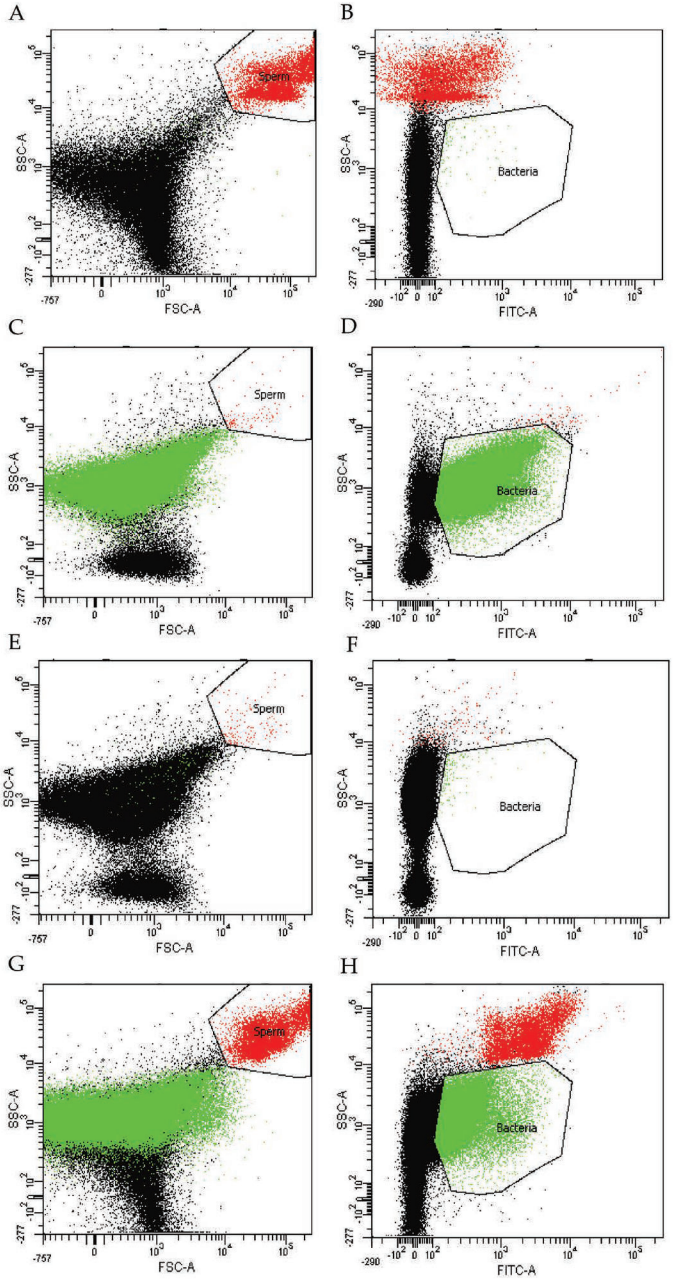


Figure 6.7: Gate setting of cell populations for flow cytometry for sample consisting of spermatozoa and bacteria using forward scatter (FSC-A) versus side scatter (SSC-A) plots (A,C,E,G) and fluorescence (FITC-A) versus SSC plots (B,D,F,H). A and B) semen sample. C and D) stained bacteria. E and F) unstained bacteria. G and H) bacteria spiked semen sample. Spermatozoa (red) are gated based on forward scatter (FSC-A) and side scatter (SSC-A), whereas bacteria (green) are gated based on fluorescence signal (FITC-A).

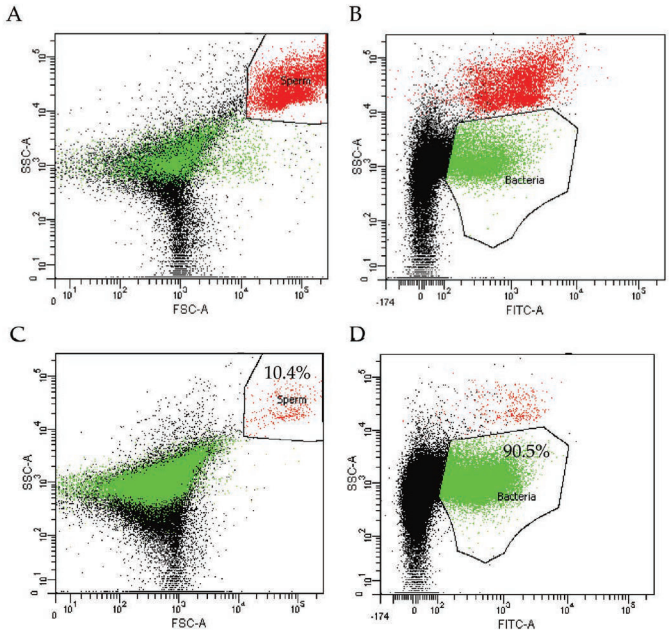


Figure 6.8: Sample analysis by flow cytometry for both target (A & B) and side (C & D) outlets. Spermatozoa (red) are gated based on forward scatter (FSC-A) and side scatter (SSC-A), whereas bacteria (green) are gated based on SSC-A and fluorescence signal (FITC-A).

6.7.2. Calibration curve: CCMV concentration

The concentration of CCMVs was determined using a fluorescence imaging plate reader. For relating the fluorescence intensity to a concentration, calibration curves with the applicable sample volumes and CCMV concentrations are needed. CCMVs were diluted in Solusem Bio+ to the desired concentrations and with a linear fit the calibration curves were obtained (figure 6.9).

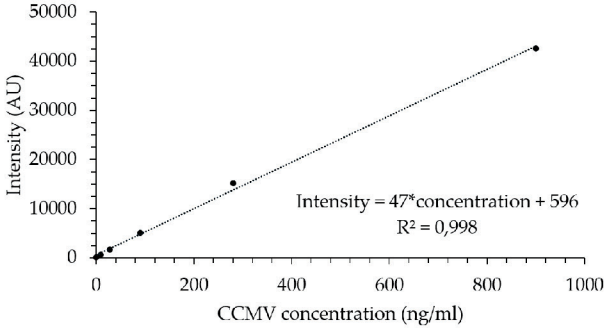


Figure 6.9: Calibration curves for the determination of the CCMV concentration for volumes of 100 µl with a fluorescence imaging plate reader.

6.7.3. Background noise of staining solution in analysis with flow cytometry

It has been investigated whether the staining solution Syto 9 caused background noise in analysis with flow cytometry. Solutions consisting of only Solusem Bio+, the buffer used to dilute porcine semen samples, and Solusem Bio+ with 1% Ficoll, which was used as the central buffer solution for acoustophoresis, were measured with flow cytometry (figure 6.10A & B). Syto 9 with a final concentration of 10 nM were added to both buffers and the spiked buffers were likewise measured with flow cytometry (figure 6.10C & D). When no staining solution was added to the buffers, the background noise in the bacteria solution approximately 100 events. When the staining solution was added, approximately 1000 events were detected in the bacteria solution. Therefore, the background noise of the staining solution is not negligible. We have chosen to not correct for the background noise, as we have obtained results with various degrees in background noise. In a semen sample, more background particles such as other cellular debris and dead bacteria may be present, which increases further the background noise.

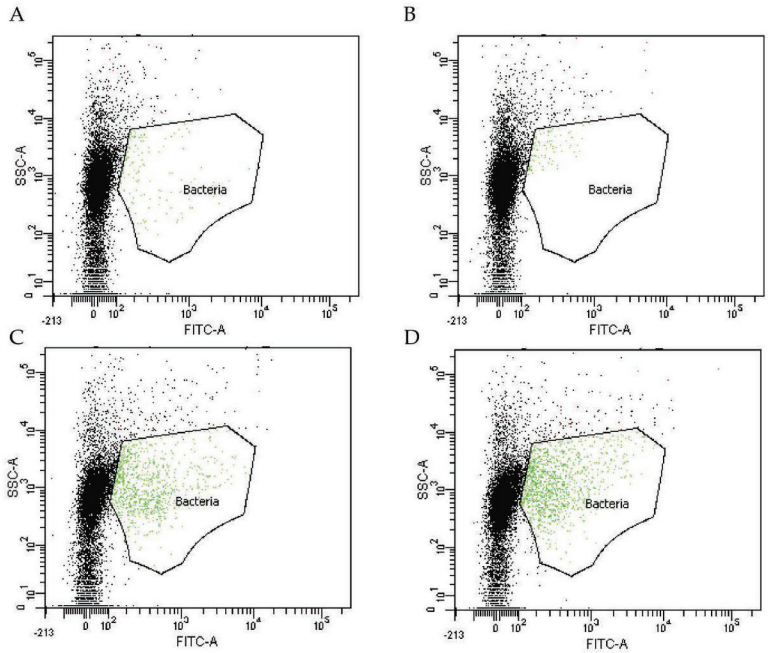
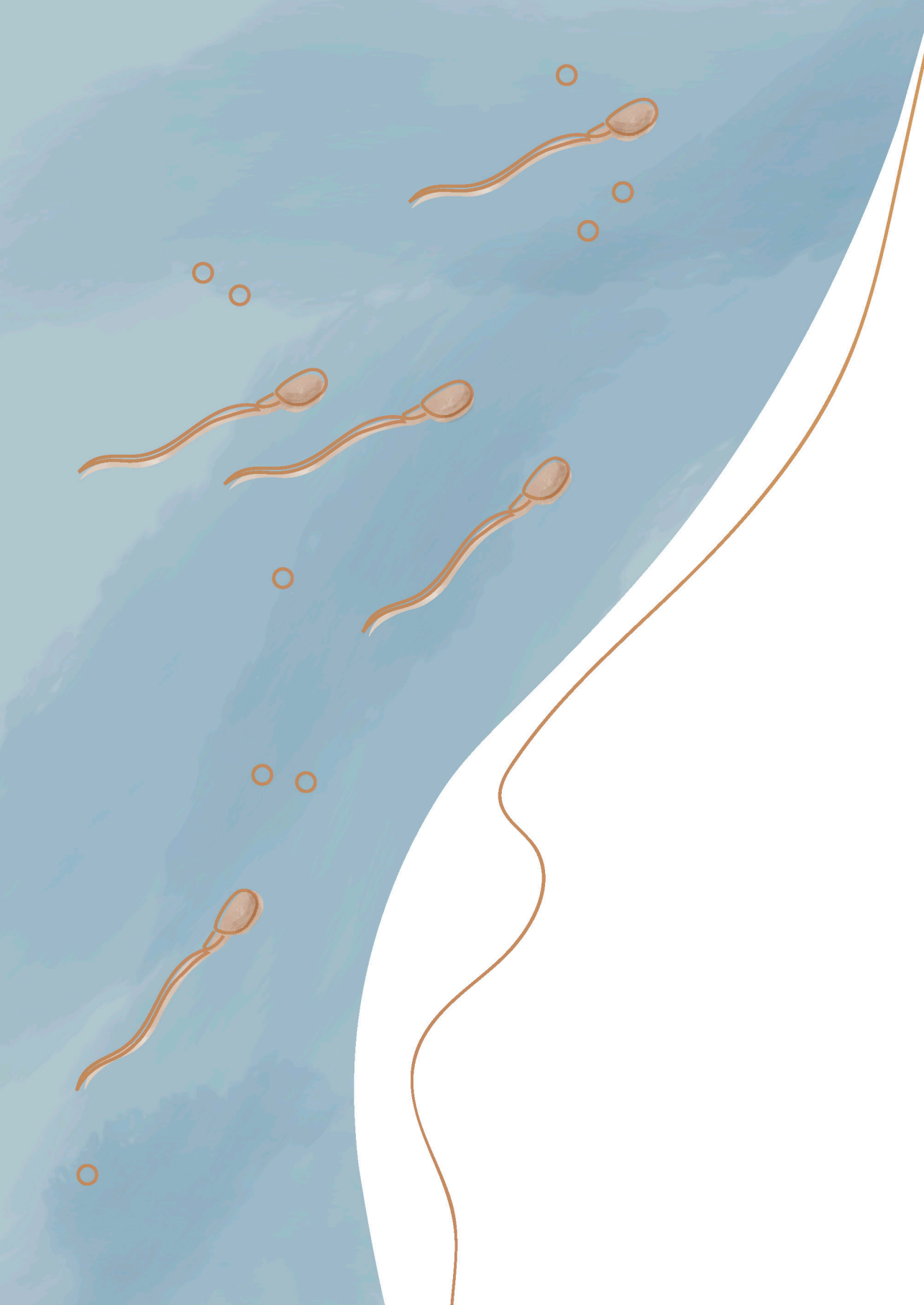


Figure 6.10: Analysis by flow cytometry for both Solusem Bio+ (A & C) and Solusem Bio+ with 1% Ficoll (B & D). A & B) plain buffer C & D) with the addition of the staining solution Syto 9. By adding the staining solution to the buffer, particles were detected in the bacteria solution although no bacteria should be present.



7.

Gap Analysis - From a proof-of-principle to a commercial device

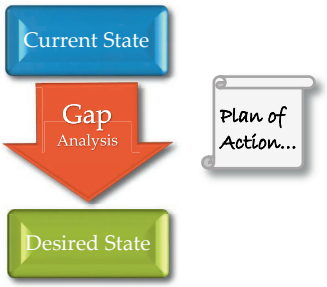
Abstract

Micro-organisms can be removed from semen with an automated cell separation device developed by AcouSort. The processing time of one boar ejaculate with the current device takes several days. The long processing time inhibits the implementation of a micro-organism removal step in routine semen processing. To investigate whether a boar ejaculate can be processed within the acceptable time (ten minutes), a gap analysis will offer valuable clues to decide on this project's continuation. The analysis includes a comparison of the current state with the desired state and several suggestions to improve the current system. Suggestions to decrease the processing time of an ejaculate are increasing (1) the sample throughput and (2) flow rate, (3) adjusting the chip design, (4) increasing the cell volume and (5) parallelizing several devices. To achieve the desired processing time several of the proposed suggestions must be optimized and implemented.

7.1. Introduction

A gap analysis can be divided in two main parts: (1) investigating the differences between the current state and the desired state and (2) suggesting ideas and possible solutions on how to achieve the desired state[1,2]. To approach a gap analysis in a structured manner, a step wise approach as shown in figure 7.1 is recommended.

A gap analysis has a broad range of applications in almost every type of economical oriented business, but also in science and technology. This type of analysis is especially popular for business managements to improve the performance of companies and teams, increase the production of created goods, or analyze the market size of a product[2]. A common example nowadays is the process of a yield gap analysis, which aims at reaching the increased need of agricultural products for the increase in world population in the upcoming decades[3]. In the field of science and technology a gap analysis can be used to improve the performance of a device or machine towards the desired needs.



Steps of a Gap Analysis

1. Identify area and goals
2. Identify desired state
3. State the current state
4. Compare the desired and current state
5. Describe the gap
6. Create Plan of Action

Figure 7.1: Schematic representation of a gap analysis and its chronological steps[1,2].

7.2. Gap Analysis

This gap analysis focuses on the implementation of an automated cell separation device in routine semen processing in the veterinary industry. The purpose of the device is to remove micro-organisms from semen.

7.2.1. Identify area and goals

In Chapter 6, the removal of micro-organisms from porcine semen with the AcouWash device, an automatic cell separation system developed by AcouSort (Lund, Sweden), is presented. The AcouWash device separates particles based on a standing acoustic wave and the particles' physical properties, such as size and acoustic density[4]. The standing acoustic wave induces an acoustic radiation force, whose magnitude and direction are affected by the particles' properties. In the *Supporting Information* 7.5.1. several important equations involved in the separation process are summarized. In general, larger particles are stronger affected by the acoustic radiation force than smaller particles[5]. The

AcouWash device has been previously applied to isolate for example platelets, mononuclear cells, and bacteria from blood[6]. As it is a label-free and gentle separation technique, it is hypothesized that spermatozoa maintain their motility and fertility characteristics. Moreover, sample throughput of several milliliter per minute have been achieved[7], suggesting that the sample throughput is higher than the sample throughput of other separation techniques based on microfluidics. The proof-of-principle experiments presented in Chapter 6 show that micro-organisms can be separated from spermatozoa, however, the sample throughput was lower than expected. Micro-organism removal from semen can become of high interest for the veterinary industry if the removal process can be implemented in routine semen processing. Therefore, an inexpensive and automated process with a short duration time is desired. The AcouWash device has already been automated, which could decrease development time and costs compared to other techniques, which have not been automated yet.

For these reasons, a gap analysis has been performed to investigate whether the AcouWash device can be adapted in such a way that it becomes implementable in routine semen processing. The gap analysis focuses on answering the following question:

Can the desired sample throughput be achieved with the AcouWash device while maintaining the separation quality?

7.2.2. Identify desired state

The desired state of micro-organism removal from semen was set after consultation with Marleen Broekhuijse (Topigs Norsvin & CRV B.V.), Maarten Moleman (CRV B.V.) and Peter Berkvens (Topigs Norsvin). It was indicated that the desired state of the processing time of one ejaculate is 10 minutes. Moreover, the desired sperm recovery and the desired bacteria removal are both at least 90%. The specifications of an ejaculate with respect to total cell count, cell concentrations and volume (table 7.1, Chapter 1 (sections 1.1-1.2)) were used to determine the required sample throughput, which is necessary to achieve the desired state. The desired throughputs and separation efficiencies for boar and bull ejaculates are shown in table 7.2. For an average boar ejaculate, a volume of 300 ml, which corresponds to 90 billion cells, needs to be processed within ten minutes. The total cell count of an average bovine ejaculate is about a ninth part of an average boar ejaculate, such that at least 1 ml or 1 billion cells per minute need to be separated.

It is important to notice that the removal of micro-organisms is more important for the porcine industry than for the cattle industry. In the porcine industry, fresh semen is used for artificial insemination (AI), because this leads to higher fertilization rates[8,9]. In contrast, bull semen is usually cryopreserved before it is used for insemination. The short amount of time, usually three to five days, between porcine semen collection and actual

insemination does not allow the practice of extended screening techniques, which are time consuming. Therefore, this analysis focuses on the processing of a boar ejaculate. Moreover, if the desired processing time of a boar ejaculate is achieved, it is transferable to bull ejaculates, because both the total cell count and volume of a bull ejaculate are less than the ones of a boar ejaculate.

Table 7.1: Background information of (unprocessed) average boar and bull ejaculate used for artificial insemination.

	Boar ejaculate 	**Bull ejaculate** 
Volume (ml)	300	10
Cell concentration (cells/ml)	3×10^8	1×10^9
Total cell count	9×10^{10}	1×10^{10}

Table 7.2: The desired throughput and separation efficiency with the current AcouWash device.

	Boar ejaculate 	**Bull ejaculate** 
Throughput (µl/min)	30 000	1 000
Throughput (cells/min)	9×10^9	1×10^9
Time (min)	10	10
Sperm recovery (%)	>90%	
Bacteria removal (%)	>90%	

7.2.3. State the current state

The current state is based on the results obtained in Chapter 6, where a proof-of-principle for the separation of bacteria from porcine semen is presented. With the AcouWash device experiments were performed to examine separation efficiencies. Acceptable separation efficiencies were obtained with a sample flow rate of 30 µl/min. Most experiments were performed with a cell concentration of 2 million cells/ml (current state I), but also higher cell concentrations up to 8 million cells/ml (current state II) were tested. The throughput and time needed for one boar ejaculate with the current settings are shown in table 7.3. With this system, it would take 25 000 hours or 6 250 hours to process a whole boar ejaculate with the settings of current state I and II, respectively.

7.2.4. Compare the desired and current state

When comparing the desired state (table 7.2) and the current state (table 7.3), there is a difference between the separation efficiency (sperm recovery and bacterial removal) and

sample throughput. With current state I, the desired sperm recovery and bacteria removal were achieved. For current state II, the bacteria removal is with approximately 80% lower than the desired 90%. Only three experiments with current state II were performed (Chapter 6), so it is recommended to test for reproducibility with this state. Furthermore, the sample throughput (volume) of the current system is with 30 µl/min smaller than the desired throughput of 30 ml/min. To make the throughput even worse, in this situation, the semen was diluted, which means that also the particle throughput (cells/min) is four or five orders of magnitude lower for current state I and II than is desired.

Table 7.3: The throughput and time needed with the current state compared to flow cytometry.

	Current state I	**Current state II**	**Flow cytometry**	
			BD Facsaria™ III [10]	**Imec cytometer [11]**
Cell concentration (cells/ml)	2×10^6	8×10^6		
Throughput (cells/min)	6.0×10^4	2.4×10^5	1.5×10^6	3.00×10^7
Throughput (µl/min)	30	30	4.4	100
Time (min)	1.5×10^6	3.75×10^5	6.0×10^4	3.00×10^3
Time (h)	25 000	6 250	1 000	50
Sperm recovery (%)	>90%	>90%		
Bacteria removal (%)	>90%	≈80%		
Factor to desired state	150 000	37 500	6 000	300

7.2.5. Describe the gap

There is a huge gap between the current state and the desired state regarding the sample throughput of porcine semen with the current AcouWash device. The factor of current state I and II to the desired state is 150 000 and 37 500, respectively.

To put the desired and current state in context, they are compared to the throughput achieved with flow cytometry. Flow cytometry is a widely applied cell sorting technique for research, clinical, and veterinary purposes. Two widely used fluorescence-automated cell sorting systems (FACSs) are the BD Facsaria III (BD Biosciences, San Jose, CA, USA) and Imec cytometer (Imec, Leuven, Belgium), which are used as examples for this analysis (table 7.3). Their throughput is in the range of 1.3-30 million cells/min[10,11]. It is important to note, that the throughput range was obtained from product data sheets and therefore it was not specified for spermatozoa. The throughput (cells/min) of the current state I and II is two to three orders of magnitude smaller compared to the throughput of the FACSs. Surprisingly, the throughput of the FACSs examples are two to three orders of magnitude smaller than the desired state, which highlights the challenge of this

project. The factors between the BD Facsaria or Imec cytometer and the desired state are 6 000 and 300, respectively.

7.2.6. Create *Plan of Action*

To close the gap between the current and the desired state, it was examined which conditions of the current system can be improved. The suggestions include not only improvements of the AcouWash device itself, such as the chip length or acoustic power, but also the sample composition, such as its concentration. The *Plan of Action* was developed in collaboration with Anke Urbansky from AcouSort. An overview of the suggestions, which will be presented and discussed in the following sections, is shown in figure 7.2.

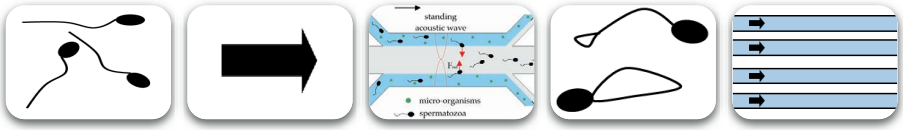


Figure 7.2: An overview of the *Plan of Action* to improve the throughput of the AcouWash device to remove micro-organisms from semen.

7.2.6.1. Sample concentration

The first idea is to investigate which sample concentration can be used for the separation process. The sample concentration is a relatively simple changeable parameter which also has an enormous impact on the throughput of the separation process. In figure 7.3, the effect of various sample concentrations on the total processing time of a boar ejaculate with respect to the desired processing time is shown. Current states I and II have low sample concentrations and therefore, need about four to five orders of magnitude more time to process an ejaculate than the required time of the desired state. When the concentration of a dose, (the concentration of the spermatozoa in tubes which is delivered to the receiver farm) is used, processing time is still too long. Shorter processing times than with the previously mentioned concentrations, can be achieved by using the concentrations of a pure boar ejaculate (3×10^8 cells/ml) or by using even higher concentrated semen (1×10^9 cells/ml). The latter concentration is based on bacteria separation from blood[12]. Furthermore, a concentrated sample means that an extra processing step such as centrifugation, which may also have negative effects on the semen quality, has to be added to the processing line to achieve the needed cell concentration.

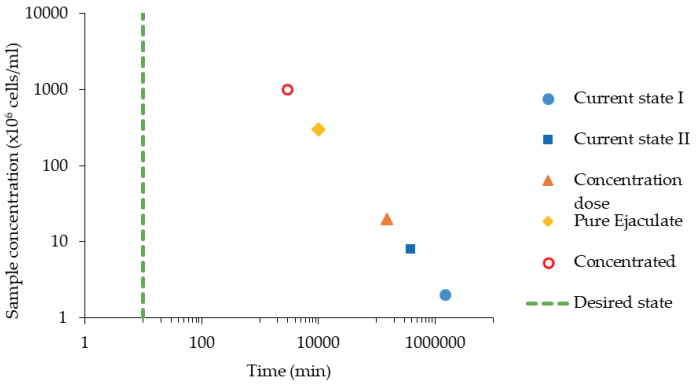


Figure 7.3: The time needed to process an average boar ejaculate with respect to the sample concentration with a sample flow rate of 30 µl/min with respect to the desired state.

A higher sample concentration increases the hydrodynamic interaction of particles which may decrease the separation quality. In acoustophoresis, the hydrodynamic interaction of both particles types has to be taken into account[13]. For this application, the interaction between the spermatozoa and micro-organisms is important. The focused spermatozoa also drag some of the surrounding liquid with them to the sample outlet. To achieve high separation quality, it is desirable that no micro-organism is dragged with the spermatozoa to the sample outlet. In acoustophoresis, it has been recommended to consider hydrodynamic interactions when the particle volume fraction is greater than 0.01[13]. The linear relation of the particle volume fraction and the sample concentration is shown in figure 7.4. The particle volume fraction was based on a total sperm volume of 67 μm^3 (6.7 x 10^{-8} µl); the sum of the volume of the head (43 μm^3) and volume of the tail (24 μm^3)[14]. For concentrations higher than 1.5 x 10^8 cells/ml, the particle volume fraction is larger than 0.01, which means that hydrodynamic interactions may influence the separation quality. Note that separation with semen concentrations of higher than 1.5 x 10^8 cells/ml can still be a possibility, since other factors such as acoustic force and flow direction can reduce the effect of hydrodynamic interactions[13]. For example, it has been shown that bacteria separation from blood was possible with a red blood cell concentration of 1 x 10^9 cells/ml[12], which corresponds to a particle volume fraction of almost 0.1.

Further experiments must be performed to determine the upper limit of the sperm concentration that can be processed, while maintaining the current separation quality. It is expected that the hydrodynamic interactions do not play a role up to a concentration of 1 x 10^9 cells/ml.

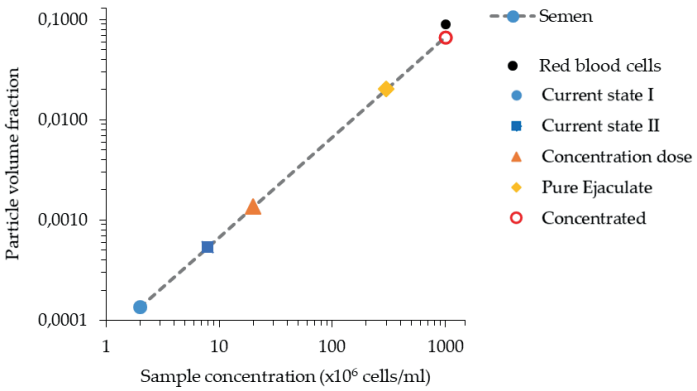


Figure 7.4: The particle volume fraction increases with the sample concentration.

7.2.6.2. Sample flow rate

Another important suggestion to improve the throughput of the separation process is the sample flow rate. With the current AcouWash device, the highest sample flow rate, at which an acceptable separation quality was achieved, was 30 µl/min. This sample flow rate is low when comparing it to the desired sample flow rate of 30 ml/min. For blood separation, sample flow rates of 400-500 µl/min have been used[15,16], which are much higher than the sample flow rate for semen. This implies that spermatozoa are harder to focus with an acoustic force than blood cells.

In figure 7.5 the effect of the sample flow rate on the total processing times of a boar ejaculate for various flow rates with respect to the desired state are shown. With the current concentrations, the desired state cannot be achieved by only increasing the sample flow rate. When increasing the sample concentration, the desired state becomes more feasible. For example, with a concentrated sample, a sample flow rate of 9 ml/min is needed to process a boar ejaculate within 10 minutes.

In acoustophoresis, increasing the sample flow rate while maintaining the separation quality could be accomplished by increasing the chip length and/or increasing the applied acoustic power.

When increasing the length of the chip, the spermatozoa are exposed to the acoustic force for a longer time. When the chip is longer, the sample flow rate can be increased as such that the total time, in which the spermatozoa are exposed to the acoustic field, remains the same. Therefore, the sample flow rate increases proportional to the chip length. By increasing the chip length, the flow resistance increases which also increases the required pressure to induce the flow. The limits of the flow regulation in the microfluidic chip

should be considered. The length of the chip can be increased by a factor of three, which then, in turn, increases the flow rate by a factor of three.

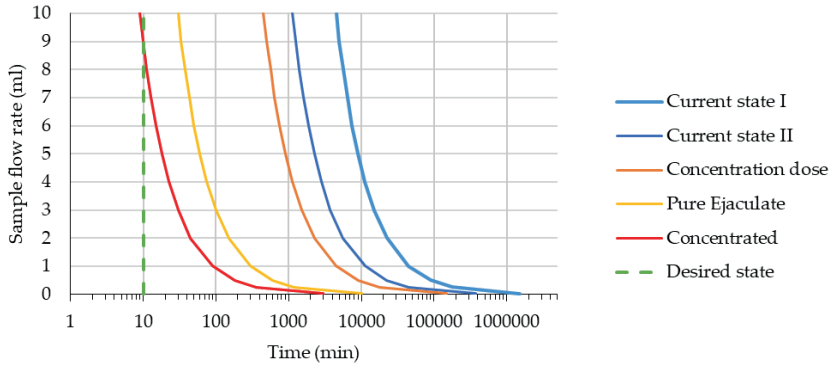


Figure 7.5: The influence of the sample flow rate on the total processing time of an average boar ejaculate for various sperm concentrations.

An additional option to increase the sample flow rate is to increase the applied acoustic power. By increasing the acoustic power, the spermatozoa are exposed to a higher acoustic radiation force, which increases the sperm velocity to the center of the acoustic node. The acoustic radiation force is proportional to the applied acoustic energy (*Supporting Information*, 7.5.1., equation 3)[5]. The piezoelectric transducer incorporated in the system is applied by an electric potential. The acoustic energy, in turn, is proportional to the electric potential squared. Increasing the applied acoustic power also raises the temperature in the system. The current AcouWash device has a Peltier cooling device implemented, which counteracts heating. Additionally, it has to be investigated whether spermatozoa are not negatively affected by a higher acoustic radiation force. In cell manipulation with a pressure node, the cells are exposed to ultrasound for a short amount of time, which is known to be a rather gentle processing technique and no harmful effects have been reported[17]. By increasing the applied electric potential (E_{ac}), the flow rate (Q) can be increased with the following relation, where X is the scaling factor:

$$XE_{ac} \sim X\sqrt{Q}.$$

7.2.6.3. Chip design

Alterations to the current chip design can further increase the throughput of the AcouWash device. An example is a device proposed by Chen *et al.* for the separation of platelets from whole blood[7]. This device achieved a throughput of up to 10 ml/min with a red and white blood cell removal as well as platelet recovery of more than 80%. This was achieved by modifying the transducer direction to the vertical plane in contrast to the common horizontal alignment of the transducer (figure 7.6). With this change in

transducer direction, the chip width can be broadened as a fixed chip width is not needed to form a standing wave. Therefore, Chen *et al.* have used a chip width of 17 mm. Furthermore, the device is based on a half standing wave, which resulted in a total chamber height of 525 µm and inner chip height of 375 µm with an applied acoustic frequency of 225 kHz. Note that currently implemented chip in the AcouWash device is 375 µm wide and 150 µm high resulting in an acoustic wave w a frequency of approximately 2 MHz.

By altering the chip design for example from a horizontal to a vertical configuration, the sample flow rate of the system can be increased to a few milliliters per minute. As spermatozoa are more difficult to focus with acoustic forces, the 10 ml/min for the blood separation will probably not be achieved by changing the chip design.

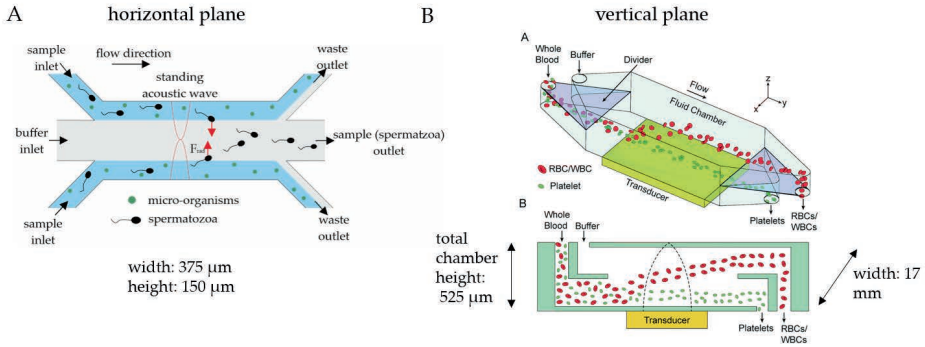


Figure 7.6: Comparison of the working mechanism with the acoustic wave applied in the horizontal (A) or vertical direction (B) and the chip dimensions for both chip designs. (Adapted from the original work by Chen *et al.*[7], Copyright 2016, with permission from the Royal Society of Chemistry)

7.2.6.4. Hypo-osmotic swelling (HOS)

In Chapter 5, the combination of HOS and pinched-flow fractionation is proposed. The HOS test gives information about the sperm membrane integrity. When having a lower solute concentration outside the cell membrane, the cell membrane transports water inside the cell to reach an equilibrium condition on both sides of the cell membrane. This process in known as hypo-osmotic swelling. In the case of a spermatozoon, the tail swells and curls at loose sides[18]. The water transport across the cell membrane is associated with functional cells. Therefore, spermatozoa which do not react to a change in osmolarity are considered defective. To support this claim, several studies have shown a significant correlation between hypo-osmotic swelling and among others motility[19–21], total intact acrosome[20] as well as fertility[18,22] for several types of animals including boars[21–23] and bulls[20,24].

It is suggested to investigate whether HOS improves the acoustic separation of spermatozoa. On the one hand, the volume of inctact spermatozoa increases when exposed to a hypo-osmotic solution (see figure 7.7). The volume of a porcine spermatozoon increases to almost 1.5, whereas the volume of a bovine spermatozoon increases by a factor of 2.0 when comparing them to their iso-osmotic volume[25]. The particle velocity due to an acoustic force scales with the square of the particle size (*Supporting Information* 7.5.1, equation 3). Therefore, it is suggested that a swollen spermatozoon moves faster to the pressure node than a spermatozoon of normal size. On the other hand, a swollen spermatozoon has taken up water to reach the equilibrium in solute concentrations in- and outside the cell membrane, which can decrease the acoustic contrast factor (ACF) of the spermatozoon. The ACF determines the magnitude and direction of the acoustic force (*Supporting Information* 7.5.1., equations 1 & 3) and depends on the particle's density as well as compressibility in relation to the medium. Future experiments can reveal whether the net effect of hypo-osmotic swollen spermatozoa increase the velocity of spermatozoa to the acoustic node. An improvement in particle velocity towards the acoustic node implies an increase in sample flow rate.

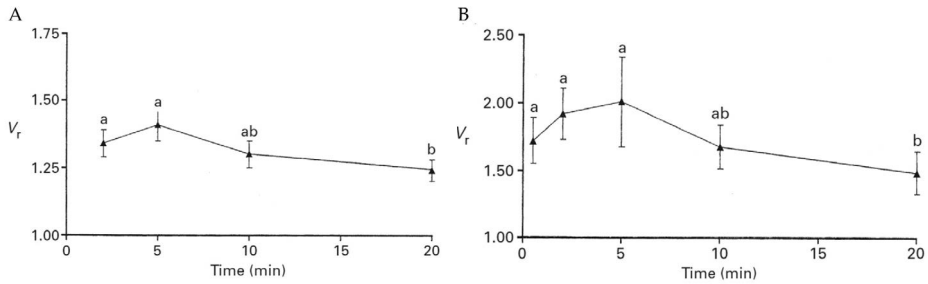


Figure 7.7: Volume response of porcine (A) and bovine (B) spermatozoa to hypo-osmotic stress for a time interval of 20 minutes. V_r is the hypo-osmotic volume relative to the iso-osmotic volume (error bars represent standard error of the mean (SEM)). Values without a common letter are significantly different. For both species, the sperm volume is the largest after 5 minutes of incubation time in the hypo-osmotic solution. Afterwards the sperm volume decreases slowly. (Reprinted from the original work by Petrunkina *et al.*[25], Copyright 2001, with permission from Society of Reproduction and Fertility.)

Additionally, separation with hypo-osmotic swollen cells can be of added value in sorting semen on quality as intact spermatozoa response to a HOS solution with swelling in contrast to defective cells.

7.2.6.5. Parallelization

The last step to achieve the desired processing time is to parallelize several microfluidic channels. An acoustophoretic device with twelve parallel channels has been proposed to purify lymphocytes with a sample flow rate of at least 1 ml/min[26] (figure 7.8). There are several challenges involved in parallelizing several channels in an acoustophoretic device. It is important that channels have the same height and distance along the wave-propagating direction as this determines the working frequency. Slight variances of the channel dimensions can lead to a less effective separation, because not in all channels the best separation quality can be achieved. It is also important to design the channels in such a way that the flow rate is the same in all channels because the flow rate also determines how long a particle is exposed to the acoustic force influencing the separation quality. The chip may also have some characteristics, which are not important for a design with a single microfluidic channel. For example, the previously mentioned parallelized device has holes in the chip between the parallel channels (figure 7.8A), which have shown to narrow the range of the optimal frequency across the parallel channels and improved the separation quality[26]. With parallelization, the sample flow rate can be scaled up with a factor of 10-20 times.

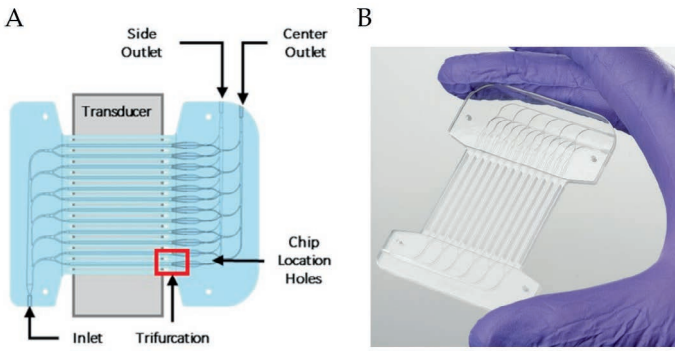


Figure 7.8: Schematic top view (A) and photograph (B) of 12-channel acoustophoresis device to purify lymphocytes. Between the channels are gaps, which improved the separation quality. (Reprinted from the original work by Dubay *et al.*[26], Copyright 2019, with permission from AIP Publishing)

7.2.6.6. Overview: *Plan of Action*

In figure 7.9 proposed suggestions with respect to their principles, challenges and estimated factors are summarized. Not one of the suggestions can achieve the desired throughput on its own. Therefore, it is important to test and optimize all suggestions individually. Subsequently, the successful suggestions can be assembled in one device.

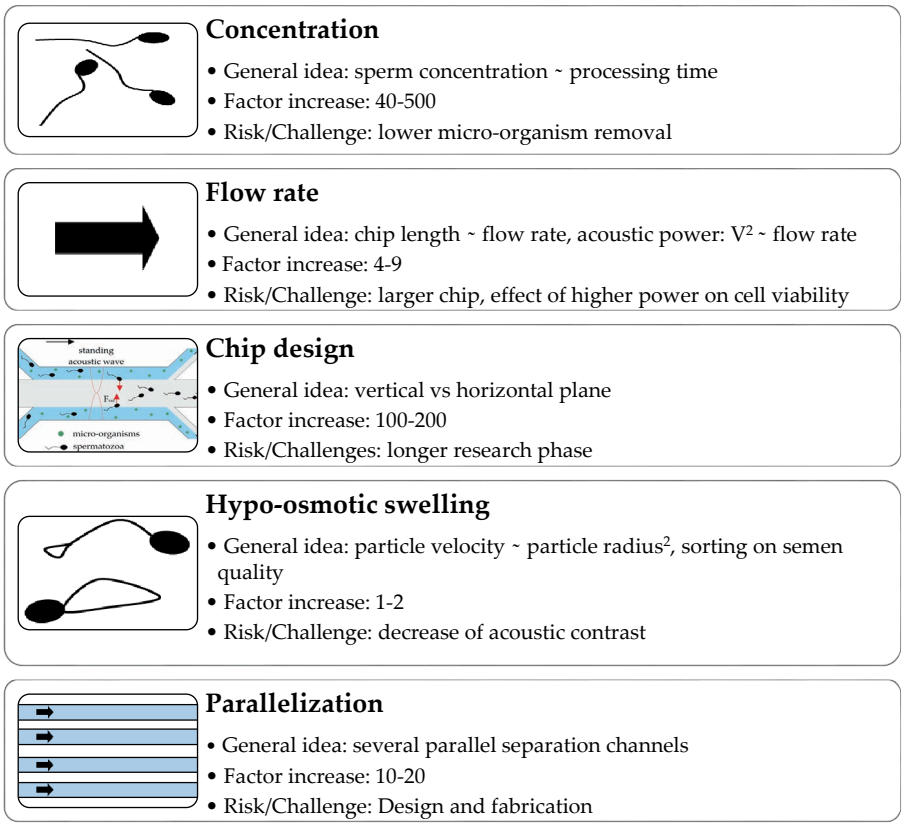


Figure 7.9: *Plan of Action*. Overview and summary of all possible suggestions including challenges and estimated factor increase of the current state towards the desired state.

7.2.6.7. Scenarios

With the previously proposed suggestions described in the *Plan of Action*, it became unambiguous that improving one single task is not sufficient to achieve the desired throughput. It is rather important to combine several suggestions. With the current knowledge it is not possible to propose the solution to solve the gap analysis, but several steps have been proposed, which can be systematically tested. Obtained results can be used to answer whether it is feasible in achieving the desired state.

To put the *Plan of Action* into context, two scenarios, which both lead to the desired state, will be shortly described. The scenarios combine different combinations of the previously described suggestions and provide a feeling whether the desired state can be achieved by optimizing the throughput of the AcouWash device.

In the first scenario (figure 7.10) it is assumed, that the boar ejaculate is concentrated to a spermatozoa concentration of 1 x 10^9 cells/ml. The microfluidic chip has the conventional horizontal design and is three times longer than the microfluidic chip used in the current state, which increases the sample flow rate by a factor of three. Similarly, the acoustic power was increased four times, which increases the sample flow rate by a factor of two. Therefore, the total flow rate could be improved from 30 µl/min to 180 µl/min. Assuming that hypo-osmotic swelling increases the focusing of spermatozoa by a factor of two, the sample flow rate can be increased to 360 µl/min. With these optimizations it is still necessary to parallelize 25 channels to process a boar ejaculate within ten minutes.

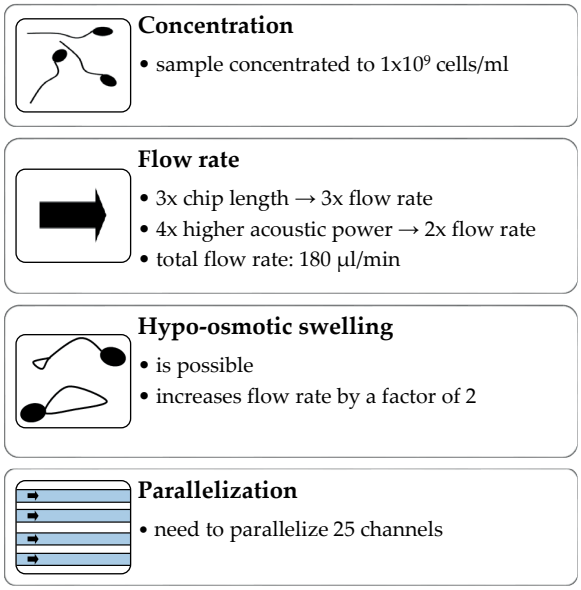


Figure 7.10: Exemplary scenario I to improve the processing time of the AcouWash device for micro-organism removal from semen.

The second scenario (figure 7.11) is based on a change of chip design, namely a chip design with the transducer in the vertical plane. With this chip design and configuration, it may be possible to process semen at a sample flow rate of 5 ml/min when using a concentration of a pure ejaculate (3 x 10^8 cells/min). To process a whole boar ejaculate within 10 minutes, six channels need to be parallelized.

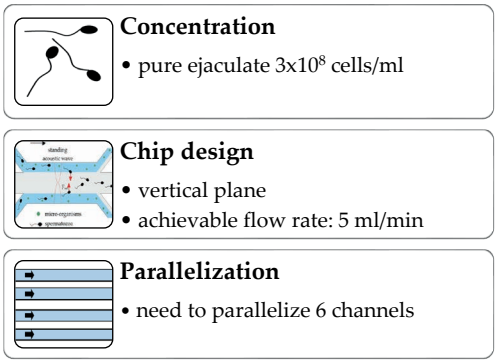


Figure 7.11: Exemplary scenario II to improve the processing time of the AcouWash device for micro-organism removal from semen.

7.3. Conclusion

With the current AcouWash device it is not possible to process a boar ejaculate within ten minutes. The gap between the current and the desired state is a factor of more than 30 000. To close the gap between the current state system and the desired state, a *Plan of Action* has been proposed, which discusses the possibilities, but also challenges, involved in increasing the throughput of the AcouWash device. The sample concentration and an alteration in chip design can increase the sample throughput by a factor of a few hundred. Other ideas to increase the sample flow rate are increasing the chip length and acoustic power, as well as increasing the focusing ability of spermatozoa with hypo-osmotic swelling. The last step towards the desired state, is parallelizing several channels. It became clear, that not one of the proposed suggestions is sufficient to achieve the desired state, but rather a combination of several suggestions must be combined to achieve the desired sample throughput to process one boar ejaculate within ten minutes with the AcouWash device.

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7.5. Supporting Information

7.5.1. Primary acoustic radiation force

The derivation and theory of acoustofluidics and the primary radiation force has been summarized in the series of *Acoustofluidics tutorial No. 7* by Henrik Bruus[27]. The most important equations are briefly summarized to support the suggestions of the *Plan of Action*.

The primary axial radiation force within a 1-dimensional half-standing wave field is given by the following equation:

$$F_{rad} = 4\pi\phi k a^3 E_{ac} \sin(2kz), \qquad (1)$$

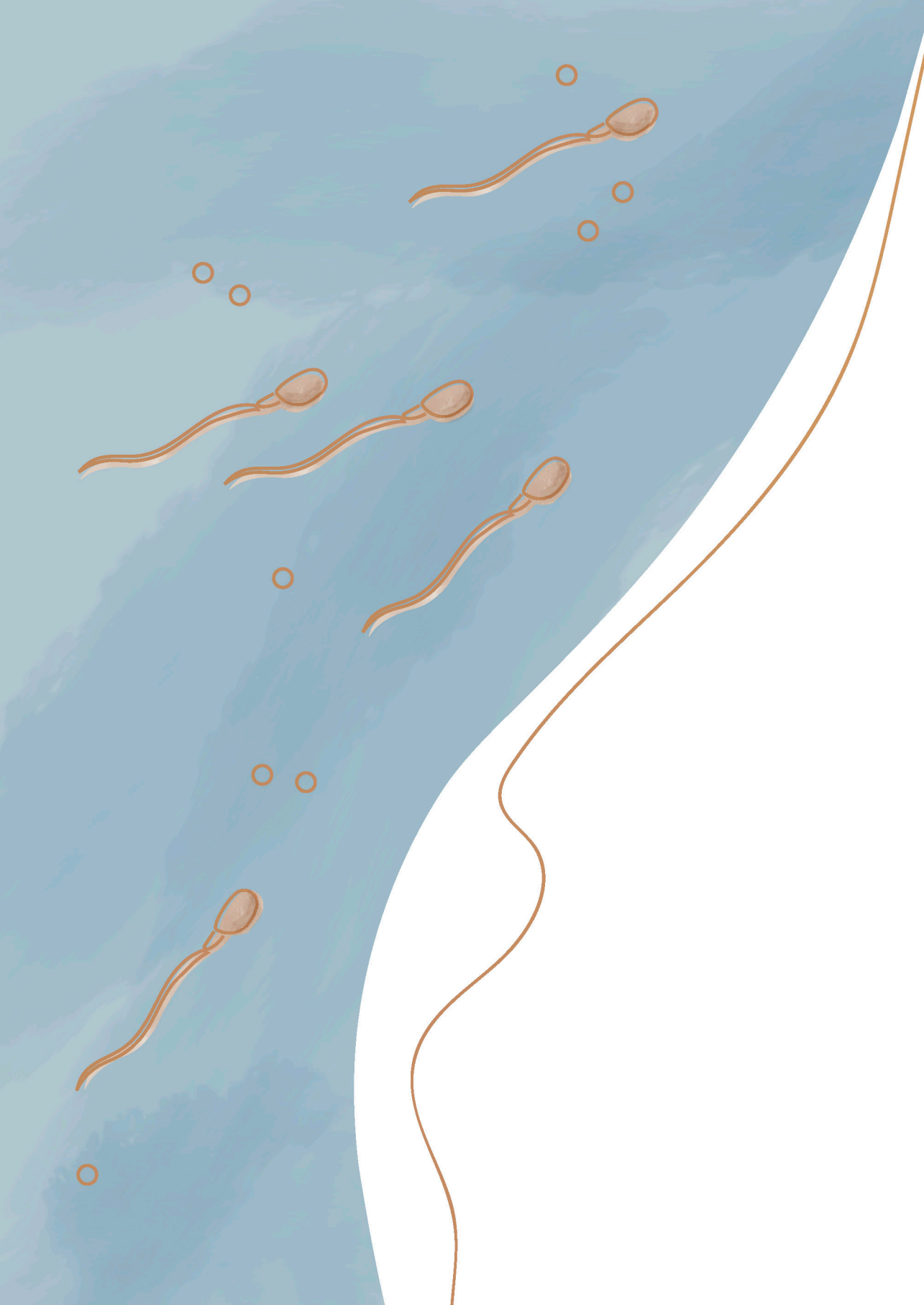
where F_{rad} is the acoustic radiation force, φ is the acoustic contrast factor, k is the wavenumber, E_{ac} is the acoustic energy density, a is the particle radius and z is the distance from the pressure anti-node of the wave axis.

A particle is not only exposed to the primary radiation force, but also to the counter-working Strokes' drag force F_d.

$$F_d = 6\pi\eta a v \qquad (2),$$

which is dependent on the fluid viscosity η and particle velocity v. From balancing the radiation and drag force, the particle velocity can be derived:

$$v = \frac{2}{3\eta}\phi a^2 k E_{ac} \sin(2kz). \qquad (3)$$



8.

Discussion, outlook and conclusion

Abstract

In this thesis, two microfluidic separation techniques are explored to refine semen for the veterinary industry. The presented results obtained from the proof-of-principle experiments are promising. In this chapter, the potentials of both techniques are discussed and compared to each other. Before these techniques can be implemented in routine semen processing more research is needed. Therefore, ideas and suggestions for additional experiments, optimizations, and improvements for both separation techniques are provided. The chapter closes with an overall conclusion.

8.1. Discussion and Outlook

8.1.1. Pinched flow fractionation (PFF)

The first experimental part of this thesis (Chapter 3-5) focuses on applying the size-based separation technique pinched flow fractionation (PFF) on the refinement of semen. In the following section, preliminary results and recommendations for further research with respect to PFF are presented.

8.1.1.1. Porcine and bovine spermatozoa

The proof-of-principle experiments with PFF were performed with porcine spermatozoa. Whether similar separation results can be obtained with bovine spermatozoa, must be investigated. Bovine spermatozoa with a total length of 74 μm are longer than porcine spermatozoa (43-45 μm)[1,2]. Also, the head of a bovine spermatozoon (length: 8.8 μm, width: 5 μm) is slightly larger than the head of a porcine spermatozoon (length: 7 μm, width: 3.7 μm)[1,2]. As PFF is based on the particle size, it can be hypothesized that due to the tumbling behavior at the pinched segment[3], bovine spermatozoa are further away from the outer channel wall in the broadened segment than porcine spermatozoa. A preliminary experiment has been performed to investigate the position of both porcine and bovine spermatozoa in the broadened segment of PFF device for three different pressure ratios. The results are shown in figure 8.1. The average position of spermatozoa in the broadened segment was similar for all pressure ratios and both sperm types. The distribution of porcine spermatozoa was, however, broader than the distribution of bovine spermatozoa. Previously, it has been shown that the sperm position in the broadened segment is affected by the sperm tumbling at the passing from the pinched to the broadened segment[3]. This tumbling behavior is caused by the elongated shape of the spermatozoa. It seems that the length of the tail does not affect the sperm position from the channel wall, since there is no difference observed between bovine and porcine spermatozoa. Also, the difference in head size seems not to influence the separation; a bovine sperm head is on average 2 μm longer and 1 μm wider than a porcine spermatozoon. An explanation for the broader distribution of porcine spermatozoa may be that more porcine spermatozoa are affected by the tumbling mechanism than bovine spermatozoa. Other intrinsic differences between porcine and bovine spermatozoa may play a secondary role as PFF is mainly based on particle size. It is therefore suggested to perform more separation experiments with bovine spermatozoa to investigate the behavior and separation properties of bovine spermatozoa in PFF.

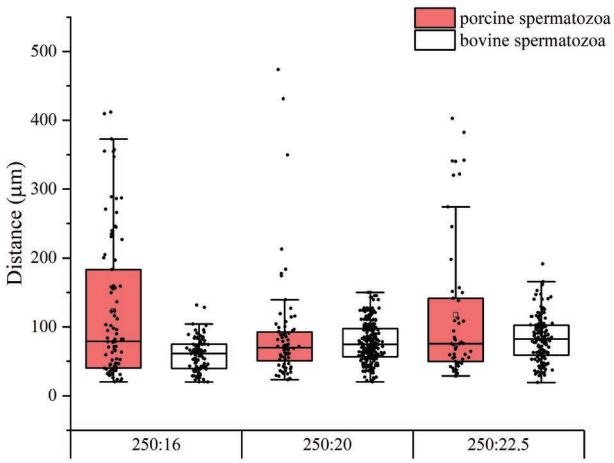


Figure 8.1: Position of porcine and bovine spermatozoa in the broadened segment of a PFF device (pinched segment: 50 µm; broadened segment: 2200 µm) for three pressure ratios (sheath pressure : sample pressure).

8.1.1.2. Sample throughput

For the implementation of a separation technique in routine semen processing, the processing time of an ejaculate, and thus the throughput of the system, should be acceptable. The desired processing time of one ejaculate was previously stated to be 10 minutes. With the current sample throughput of PFF (approximately 2 µl/min, 20 x 10^3 spermatozoa/min), the total processing time of a boar ejaculate will be too long (table 8.1). Three parameters, which have an influence on the throughput of PFF processing, were varied to test their effect on sperm position in the broadened segment: sperm concentration, Reynolds number and the particle containing fluid width in the pinched segment (w_P). Up to a sample concentration of 20 x 10^6 cells/ml, the sperm distances and standard deviations did not differ significantly (analysis of variance (ANOVA), $p>0.05$; figure 8.2A). With increasing Reynolds number, which is proportionally related to the total flow rate, the average sperm distance from the broadened segment wall increased linearly (figure 8.2B). The Reynolds number had no effect on the standard deviation of the sperm distance. The particle containing fluid width w_P in the pinched segment is another important parameter for the effective and efficient separation of particles in PFF and can be described by

$$w_p = r_{p1} + r_{p2}, \qquad (1)$$

with $r_{p1/p2}$ the radii of the two types of particles to be separated[4]. For the purification of spermatozoa, the particle containing fluid width calculated with formula (1) is approximately 2 µm, the effective size of a spermatozoon. However, this relation may

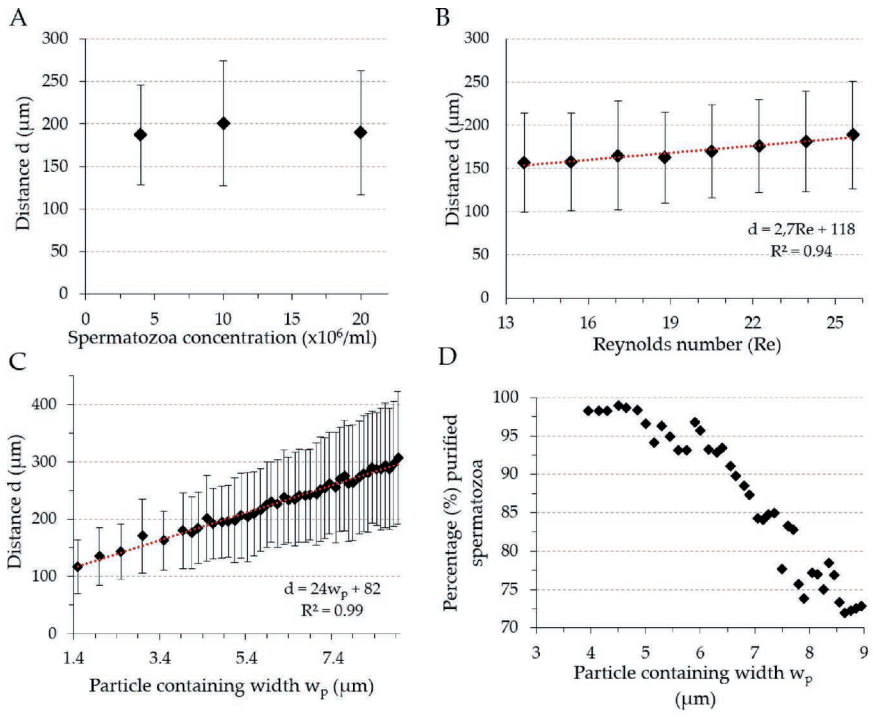


Figure 8.2: Three different parameters were investigated to increase sample throughput of PFF. While one parameter was varied, the others were kept constant. (A) The mean distance of spermatozoa in the broadened segment does not depend on sperm concentrations up to 20 x 10^6 cells/ml. The tested concentrations showed no significant difference. (B) The mean distance d of spermatozoa in the broadened segment increases with increasing Reynolds number (Re). (C) The mean distance d in the broadened segment increases linearly with increasing particle containing fluid width w_P. (D) For the purification of spermatozoa (>90% purification) a pinched segment width of 6.7 um is required. (Dimensions of microfluidic chip: pinched segment width: 50 µm, broadened segment width: 2500 µm, chip height: 50 µm; error bars indicate one standard deviation.)

not hold for separation of spermatozoa from other particles, as spermatozoa end up at positions further from the chip wall than one would expect[3]. To test this, the particle containing fluid width was decreased by decreasing the sample flow rate. The results of figure 8.2C show that the mean sperm distance and standard deviation increased with increasing particle containing fluid width. In figure 8.2D, the percentage of purified spermatozoa with respect to particle containing fluid width is presented. The particle containing fluid width was varied by changing the sample flow rate with constant sheath flow rate. With a particle containing fluid width of smaller than 6.7 µm at least 90% of the spermatozoa can be retained while the smaller particles are removed. The experimentally determined particle containing fluid width (6.7 µm) is larger than the calculated fluid width (2 µm).

Table 8.1: Throughput overview of the PFF presented in this thesis compared to the desired state. The throughput of the current PFF device can be increased by increasing the sperm concentration, increasing the sample flow rate and several PFF device can be parallelized. (* based on the preliminary results presented in figure 8.2)

	PFF (Chapter 3)	PFF after optimization			Desired state
		I	II	III	
Sperm concentration (spz/ml)	10×10^6	20×10^6 *	5×10^9 [3]		9×10^9
Particle containing fluid width (µm)	1.5	6.7*			
Reynolds number (in pinched segment)	15	25*			
Total flow rate (µl/min)	66.6	115			
Sample flow rate (µl/min)	2.1	15.3			30 000
Flow rate ratio (Total flow/sample flow)	32	6.5			
Parallel devices	1	1		100	
Processing time of one boar ejaculate	71 000 hours	4 900 hours	20 hours	12 min	10 min
Processing time of one bull ejaculate	790 hours	45 hours	0.2 (13 min)	< 1 minute	

The sample throughput of PFF as used in chapter 3 can be improved by increasing the sperm concentration, total flow rate and decreasing the particle containing fluid width to 6.7 µm. In table 8.1, the results of throughput calculations are presented to show the effect of the proposed throughput optimizations. The current processing time of a boar ejaculate of more than 70 000 hours is not realistic. With the previous presented throughput optimizations, the total processing can be decreased to less than 5 000 hours, which is still too long. With further increase of the sample concentration and the parallelization, the desired processing time of a boar ejaculate becomes more technically feasible. Parallelizing 100 devices is , however, challenging, so it is suggested to investigate if other parameters, such as the chip height, can also increase the sample throughput.

8.1.1.3. Microfluidic chip and benchtop device

The microfluidic chip is the key point of the separation technique. A change of material is necessary so that the microfluidic chip can be used for routine processing. The material used to fabricate the PFF devices, namely the elastomer polydimethylsiloxane (PDMS), is a popular material for research purposes as it holds many advantages: it is optically transparent, easily and low-priced fabricated, and elastic[5]. For a large-scale production and commercial use, however, thermoplastic materials, such as polystyrene[6] and cyclic olefin copolymer[7], are more suitable. In collaboration with Micronit (Enschede, the Netherlands) we have designed and tested several pinched flow fractionation chips. By

using thermoplastic materials, fabrication methods such as injection molding and embossing can produce a considerable number of devices[8].

Another step before implementing PFF in routine semen processing is the development of a benchtop device. Currently, the microfluidic chip is manually connected to pressure pumps, the tubing lengths are altered and the separation is visually observed with the help of a microscope. To my knowledge, no commercial benchtop device based on the separation technique PFF exists. Several development steps are necessary before a commercial benchtop device can be produced. The benchtop device should fulfill several requirements, of which some of the most important ones are: control of the necessary operations (e.g. sample flow rate), easily accessible microfluidic chip, manual and easy connection of necessary fluids and sample, simple operation by trained laboratory personnel, and sterile sample handling.

8.1.2. Acoustophoresis

In the second part of this thesis, pathogen removal from semen with acoustophoresis is confirmed (Chapter 6) and recommendations to increase the sample throughput of acoustophoresis for semen processing are provided (Chapter 7). For a detailed outlook on acoustophoresis and sample throughput, it is suggested to read chapter 7.

8.1.2.1. Virus removal

The removal of bacteria from semen with acoustophoresis was successfully examined along with reproducibility and variations in sample concentrations (Chapter 6). However, the removal of viruses from semen was solely shown with one experiment. It is recommended to further investigate virus removal in more detail. For a more extended and detailed proof-of-principle, a model virus can be spiked to semen samples and the separation quality can be investigated with similar experimental settings and variations as presented for the bacteria removal. In case the virus removal experiments are confirmed to be successful, it can be investigated whether both pathogens, i.e. bacteria and viruses, spiked to semen samples can be removed with acoustophoresis. For routine semen processing, a separation technique removing viral and bacterial pathogens from semen is preferred in contrast to using two differing separation steps. One joint separation technique decreases the amount of manual performed steps and reduces the risk of recontamination. It is also assumed that the processing time of an ejaculate is reduced.

8.1.2.2. Microfluidic chip and benchtop device

The microfluidic chip and the benchtop device used for acoustophoretic separation have been designed and fabricated for commercial use by the company AcouSort (Lund,

Sweden). The material of the microfluidic chip is glass and a piezoceramic transducer is glued underneath the chip. The chip material plays a significant role in acoustophoresis as the acoustic wave is reflected in such a way, that a standing wave occurs in the channel. Therefore, the material requires, in addition to possible large-scale production, good acoustic reflective capabilities. Glass and silicon, the most applied materials for acoustophoretic chips, have good acoustic reflective capabilities and the desired channel structures can be fabricated[9]. A few limitations of both silicon and glass are that bonding is not as straightforward as with polymers and fabrication is more expensive and complex than for polymers. Polymers, however, have low acoustic reflective properties and are by this means less suitable for acoustophoresis[9]. In the last years, the use of polymers, such as polystyrene, for acoustophoresis has become more popular. To overcome the limitations of polymers, a standing acoustic wave can be induced by two opposing transducers[10]. Moreover, acoustic parameters, such as the operating frequency must be optimized for the used polymer[11]. A huge advantage of acoustophoresis is the available benchtop device AcouWash (AcouSort, Lund, Sweden). Samples and fluids can be easily connected and parameters, such as the flow rates, actuation frequency and power, can be manually altered in the software. Although the device needs further improvements with respect to the sample throughput of semen (Chapter 7), the first development steps have been proposed.

8.1.3. General outlook

Recommendations for future studies, which can be associated with both presented separation techniques, will be discussed in this section.

8.1.3.1. Boar and bull semen: Separation and biocompatibility

The semen used for the experiments in this thesis were obtained from local artificial insemination (AI) centers. The semen samples were obtained in the same form as they are delivered to the recipient farms; tubes with porcine semen and straws with bovine semen. The semen must comply the quality parameters to be used for AI. Therefore, the semen used within this thesis had high fertility recordings. Although, boars and bulls and their semen must fulfill specific requirements, sperm inter- and/or intra species variances can influence the separation quality. Despite semen samples from different male animals/species were used, it was not investigated whether this influenced the separation. Spermatozoa have huge interspecies variances, for example when considering cell morphology[12] and fertility rates after cryopreservation[13,14]. Although the differences on the intra-species level are smaller, sperm variances among males from the same species were reported[12,15]. For these reasons, it is recommended to examine the

separation techniques with respect to inter and intraspecies variances for boar and bull semen.

The implementation of a microfluidic processing tool in routine semen processing is unique in the sense that the processed semen sample will be used for AI. As microfluidic processing tools are mainly used for diagnostic purposes[8,16], the sample condition after processing is of non-importance. However, for this application, the semen quality should not decrease, so that the success rate of AI is maintained. In this thesis, it is proven that the effect of microfluidic processing on the sperm viability is negligible (Chapter 3). Similarly, but in a lesser extent investigated, the spermatozoa viability after acoustophoretic processing is unaltered (Chapter 6). The manipulation of cells with standing acoustic waves is known to be biocompatible and gentle compared to alternative techniques[17]. Among others, it has been revealed that (plant and yeast) cells in the pressure node of a standing wave do not experience a decrease in viability in contrast to cells in an acoustic field gradient[18,19]. The sperm viability is one of the determinants representing the status of the cell. The special characteristic of intact spermatozoa is the motility, because only motile spermatozoa can swim to an oocyte. Therefore, it is suggested to investigate whether the sperm motility is not affected by microfluidic/acoustophoretic processing. The motility before and after processing can be studied by computer-assisted sperm analysis (CASA). Additionally, the acrosome integrity after microfluidic processing can be determined. An intact acrosome is required so that the spermatozoon can fuse with the oocyte. The acrosome integrity can be for example examined with Pisum Sativum Agglutinin (PSA), which binds to intact acrosomes, conjugated to a fluorescence marker[20]. Another key parameter representing the status of a spermatozoon is deoxyribonucleic acid (DNA) integrity. Although small DNA damages can be repaired by the oocytes and embryos[21], high degrees of DNA damage can cause mutations[22] and often lead to failed reproduction. The prognostics value of DNA integrity was proven by correlating it to fertility[23–26]. One well-known DNA integrity assays based on a staining is the use of acridine orange (AO)[27–29]. AO binds to DNA and emits a fluorescent signal; green light is emitted from double stranded DNA and red light is emitted from single stranded DNA. After investigating the effect of the separation techniques on the spermatozoa, the effect on the fertility must be directly determined. Field studies must reveal whether fertility rates, such as pregnancy rate, birth rate and off-spring rate, of microfluidic processed semen do not decrease when comparing them to conventional processed semen.

Another point of attention is the effect of cryopreservation on the separation quality and sperm fertility. Bull semen is cryopreserved prior its use for AI in contrast to fresh used boar semen. The removal of micro-organisms before cryopreservation is beneficial as

sperm quality is not affected by the presence of potential micro-organisms. Also, cryopreserved semen may be more vulnerable to the separation process than fresh semen. In the case of processing the semen before cryopreservation, recontamination may occur after thawing. Therefore, it should be considered whether the separation step is preferably implemented before cryopreservation or after thawing.

8.1.3.2. Micro-organisms

The cowpea chlorotic model virus (CCMV) and *Escherichia coli* (*E.coli*) were used as a virus and bacteria model, respectively. Safety regulations in a biological laboratory environment protect likewise biological samples and people working in the laboratory. For the usage of hazardous micro-organisms special regulations are required. The usage of model micro-organisms for proof-of-principles is a suitable alternative. A first set of continuative experiments could include semen samples with the addition of authentic micro-organisms. For example, the classical swine fever (CSF) virus and/or foot-and-mouth disease (FMD) viruses can be added to porcine semen, as porcine semen must not contain these virus types[30,31]. After the separation, (commercially available and quantitative) polymerase chain reactions (PCR) of the refined semen samples can reveal whether semen samples are free of viruses. *E.coli* is already an authentic bacteria type, as these bacteria are often present in semen. As the size and shape of bacteria is similar to the one of spermatozoa, it is recommended to investigate bacteria types with varying shape. In addition to the rod-shaped *E.coli*, spiral-shaped *Leptospirosa* and round *Chlamydia* can be spiked to semen. After the separation, the initial spiked semen sample and both the target and waste fluid can be cultured on an agar plate to determine the separation efficiency. Additionally, to investigate the removal of bacteria, semen samples, which were not treated with antibiotics, can be used for separation experiments, because about 25-30% of the (porcine) semen doses are contaminated with bacteria[32–34].

8.1.3.3. Implementation in routine semen processing

Until a physical removal technique can replace the current precautions to limit the presence micro-organisms in semen, it must be proven that the new techniques are more beneficial than the conventional techniques. Currently, it is legally obliged to add antibiotics to all semen samples used for AI[35]. The occurrence of antibiotic resistant bacteria strains due to the overuse of antibiotics has also gained attention in the veterinary industry. However, changing EU regulations is a rather difficult procedure and results must show that a separation technique is not risking biosecurity. Nevertheless, by proposing an alternative to the use of antibiotics, the veterinary industry projects the will of reducing the use of antibiotics. Furthermore, it must be justified that semen quality is retained without the addition of antibiotics. In the case of

viruses, screening techniques are obliged for semen import and export[30,35]. Virus screening test results of fresh porcine semen are due to time pressure usually not available prior its use for AI. In the latter case, the physical removal of viruses in routine semen processing is of interest and will increase biosecurity. Additionally, in contrast to a screening technique, a physical removal allows the use of virus contaminated semen samples, which are currently eliminated. Therefore, after removing the virus, current screening techniques must confirm that the semen samples are certainly free of viruses.

8.1.4. Comparison of both separation techniques

The potentials of both applied separation techniques for the removal of micro-organisms from semen are summarized in table 8.2 and compared to the desired state, which is represented by processing a boar ejaculate. The obtained separation qualities, sperm recovery and virus/bacteria removal, of both techniques meet the desired outcomes. It is important to notice that both separation techniques require more research into either virus or bacteria removal; PFF has not been applied for bacteria removal, whereas a preliminary experiment for virus removal with acoustophoresis already showed its promising potential. When considering the processing time of one boar ejaculate of both separation techniques, higher sample throughputs are needed. With acoustophoresis it is more likely to achieve shorter processing times, but parallelization of several devices is unavoidable. For the large-scale production, the use of thermoplastics, as suggested for PFF, can meet the desired needs. The current used glass chips for acoustophoresis are more expensive and harder to fabricate than thermoplastic devices. In the future, it may be possible to also use thermoplastics for acoustophoresis. For both thermoplastics and glass chips, it is possible to reuse the chips after a cleaning step. The availability of a benchtop device for acoustophoresis, decreases the amount of mandatory developmental steps compared to PFF, for which a new benchtop device must be developed. When considering all discussed parameters, acoustophoresis holds greater potential for the removal of micro-organisms in routine semen processing.

Table 8.2: Comparison of PFF and acoustophoresis for the removal of micro-organisms from semen. (Indication of being beneficial with respect to the other technique.)

		Desired state	Pinched flow fractionation (PFF)	Acoustophoresis
Sperm recovery (%)		>90%	≈ 86%	> 90%
Virus removal (%)		100%	≈ 84%	Possible (preliminary)
Bacteria removal (%)		>90%	(not tested)	≈ 80%
Processing time (min)	Presented in this thesis	10 min	71 000 hours	6000 – 25 000 hours
	Achievable after improvements		20 hours (without parallelization)	1 - 4 hours (without parallelization)
			10 min (with 100 parallel channels)	10 min (with 6 - 25 parallel channels)
Chip		Large-scale production	Polystyrene, cyclic olefin copolymer	Glass chip, polymer (future?)
Benchtop device		Demanded	To be developed	Available, needs improvements

8.2. Conclusion

The aim of this thesis was to develop a microfluidic separation device for the veterinary industry to remove potential viruses and bacteria from collected semen. Two separation techniques, PFF and acoustophoresis, were applied for the removal of viruses or bacteria from semen, respectively. Both techniques achieved sperm recoveries of approximately 90%, which are higher than the sperm recoveries reported with for example centrifugation and column filtration. High-throughput sample processing, a common challenge in microfluidics, was addressed for both presented separation techniques. Although, the desired throughput was not achieved, several improvements are suggested for future research. Especially, acoustophoresis holds high potential in achieving the desired separation requirements. Besides that, an existing benchtop device based on acoustophoresis minimizes future development steps. Overall, a physical removal step, such as acoustophoresis, implemented in routine semen processing will reduce the use of antibiotics and will increase the biosecurity of AI.

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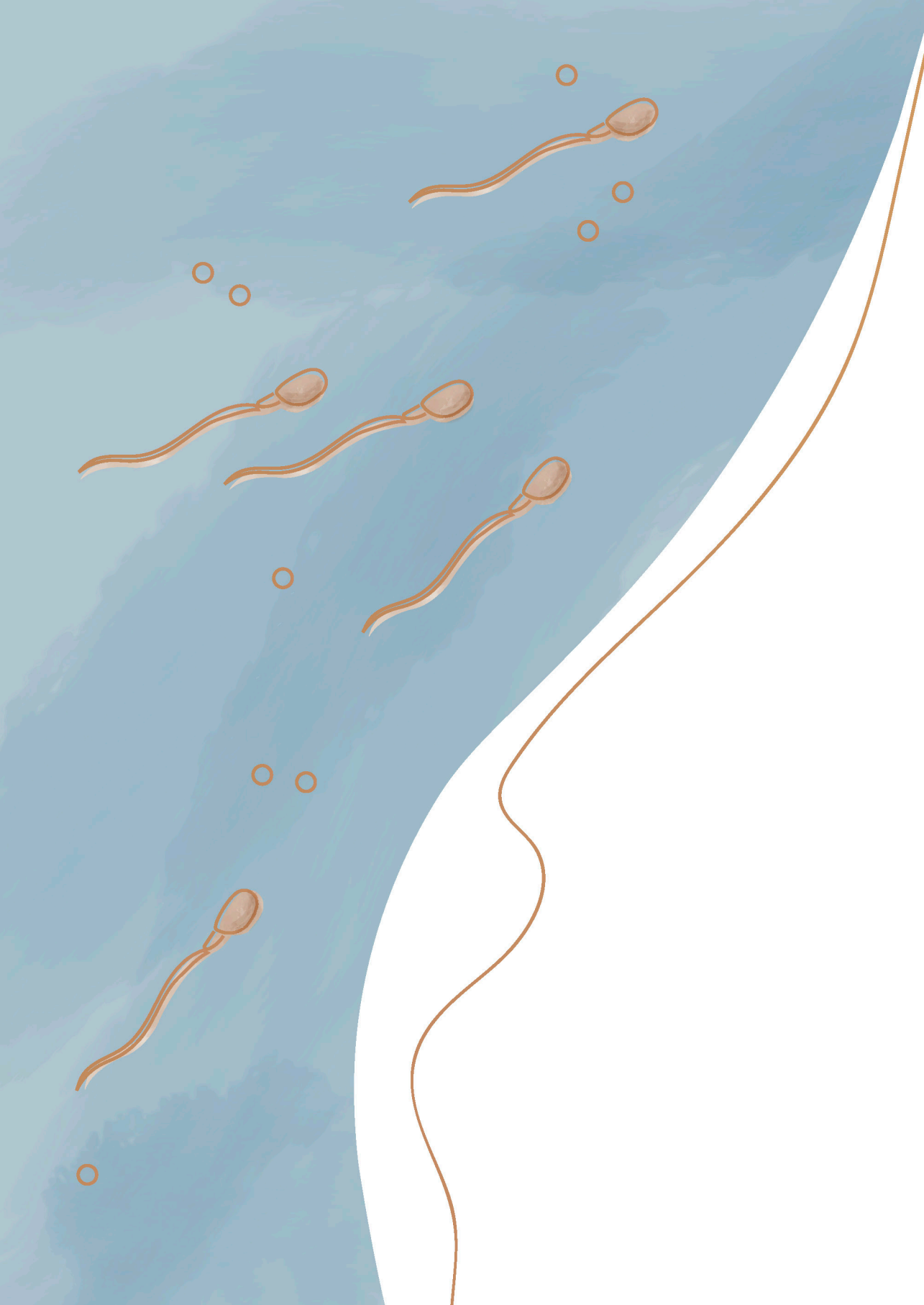

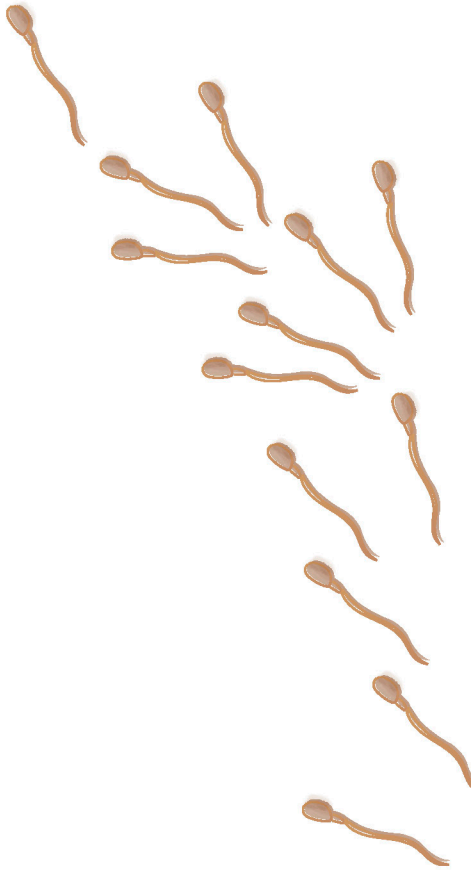

Appendices

Summary

Artificial insemination (AI) is the most widely used breeding technique in the porcine and bovine industry. The presence of micro-organisms in semen used for AI, is a potential risk for disease transmittance and decreases semen quality. To minimize the hazards caused by micro-organisms, many precautionary measures are in place. For example, antibiotics are mandatorily added to semen extenders to kill bacteria and semen is regularly screened for the presence of viruses. Although these practices are known to be beneficial, they hold limitations. The overuse of antibiotics leads to the emergence of antibiotic-resistant bacteria strains. Since antibiotics are the only treatment for bacterial infections, infections caused by antibiotic-resistant bacteria strains are a global threat. The transmission of viruses between animals can also spread to humans causing global pandemics. Two well-known examples are the swine flu and Covid-19 pandemics. To prevent the spread of viruses, semen is regularly screened but this is labor-intensive, expensive and time-consuming. Additionally, test results are usually not available prior insemination with fresh semen. The implementation of a step that removes micro-organisms during routine semen processing, increases the biosecurity of AI, eliminates the use of antibiotics, makes contaminated semen usable and can be economically beneficial.

The physical removal of micro-organisms from semen is challenging on several fronts. High micro-organism removal rates are desired, but sperm recovery and semen quality must be ensured, while processing an ejaculate within an acceptable amount of time. The differences in size between micro-organisms and spermatozoa allow for size-based separation techniques. Macroscale separation techniques achieved high micro-organism removal rates, but sperm recovery rates of approximately 50% were not acceptable. Microfluidic separation techniques, an emerging field with working mechanisms at the cellular size range, may be more proficient than macroscale separation techniques. Many microfluidic devices have been proposed to improve semen samples. Moreover, microfluidic devices were extensively suggested for the removal of micro-organisms from blood, and could also be applied for the removal of micro-organisms from semen. A well-known challenge of microfluidics, especially for routine semen processing in the veterinary industry, is high throughput processing; a porcine ejaculate, consisting of 90 billion (90×10^9) spermatozoa and 300 ml ejaculate volume, is desirably processed within ten minutes.

The aim of this project is to remove micro-organisms from semen with microfluidic separation techniques by considering not only the micro-organism removal rates, but also sperm recovery, semen quality and processing time. Hence, this thesis explores the

potential of two microfluidic separation techniques for the removal of micro-organisms from semen for the veterinary industry.

First, the effect of microfluidic processing on the sperm viability, i.e. the percentage of spermatozoa surviving the procedure, was investigated. Since the processed semen is subsequently used for AI, semen quality must be maintained. During microfluidic processing, spermatozoa are exposed to shear stresses. These shear stresses were compared to the shear stresses that spermatozoa experience during natural ejaculation. The sperm viabilities after microfluidic processing were also compared to conventional microfluidic processing techniques such as centrifugation and flow cytometry. The viability decrease due to microfluidic processing on porcine spermatozoa of 6% was small, but significant. Microfluidic processing did not affect the viability of bovine spermatozoa. The effect of microfluidic processing on sperm viability was comparable to the viabilities obtained after centrifugation and reported after flow cytometry. With respect to sperm viability, microfluidics holds potential of being implemented in routine semen processing.

The first microfluidic separation technique explored is pinched flow fractionation (PFF), in which hydrodynamic forces are used to separate different particles by size. The removal of viruses was modelled by spiking cowpea chlorotic mottle viruses (CCMVs) to porcine semen. After optimizing the chip design and flow parameters, virus removal rates of at least 84 ± 4% and sperm recovery of 86 ± 6% were achieved. The current processing time of one ejaculate with PFF is 72 000 hours (3 000 days), which is not feasible. With several optimizations, the processing time can be decreased to 20 hours, but parallelization of many devices is unavoidable to achieve the desired processing time of 10 minutes.

For semen quality improvements, the separation technique PFF was combined with the hypo-osmotic swelling (HOS) test. Intact spermatozoa incubated in a hypo-osmotic solution swell and their tails curl up. The effect of the HOS solution on the spermatozoa emergence in the separation device was investigated. In contrast to porcine spermatozoa, bovine spermatozoa in the HOS solution emerged at another position in the broadened segment of the PFF device than the control group. This can be explained by the higher water permeability of bovine spermatozoa compared to porcine spermatozoa. For this reason, separation experiments were performed with bovine semen samples. After all, by combining HOS test and subsequent separation with PFF, bovine semen quality was not improved. The unsuccessful separation might be caused by the lower tumbling effect of curled up spermatozoa.

The second explored microfluidic separation technique is acoustophoresis, in which particles are manipulated by an acoustic force. The removal of micro-organisms from semen was explored with the benchtop instrument AcouWash (AcouSort, Lund, Sweden). With optimized flow parameters, bacteria removal rates of at least 88 ± 7% and sperm recovery rates of 90 ± 4% were achieved. Also, virus removal from semen was successful. No significant effect of the acoustic forces on sperm viability was found.

With the current AcouWash instrument, the processing time of one (porcine) ejaculate is 6 000 – 25 000 hours (250 – 1 000 days). In a gap analysis, the difference between the current and desired state was examined. Several ideas to increase the sample throughput were suggested with respect to their impacts on separation quality and processing time. These suggestions were: (1) increasing the sample concentration and (2) sample flow rate, (3) optimizing the chip design for high throughput processing, (4) investigating the use of spermatozoa incubated in a HOS solution, and (5) parallelizing several devices. It was concluded that a combination of several improvements is necessary to achieve the desired processing time.

PFF was compared to acoustophoresis. Both applied separation techniques achieved similar micro-organism removal and sperm recovery rates. However, achieving the desired processing time of an ejaculate is more feasible with acoustophoresis. Additional, a commercial benchtop device using acoustophoresis is already available. For these reasons, acoustophoresis outperforms PFF.

In conclusion, the microfluidic separation techniques PFF and acoustophoresis removed (a high percentage of) micro-organisms from semen. In contrast to PFF, acoustophoresis holds more promise for removing both types of micro-organisms in one separation step and in reaching the desired processing time. The advantage of implementing a physical micro-organism removal step in routine semen processing can eliminate the use of antibiotics and will increase the biosecurity of AI in the veterinary industry.

Samenvatting

Kunstmatige inseminatie (KI) is de meest gebruikte foktechniek in de varkens- en runderindustrie. De aanwezigheid van micro-organismen in sperma dat voor KI wordt gebruikt, is een potentieel risico voor ziekteoverdracht en vermindert de kwaliteit van het sperma. Om de gevaren van micro-organismen tot een minimum te beperken, zijn er voorzorgsmaatregelen genomen. Zo worden antibiotica verplicht aan sperma toegevoegd om bacteriën te doden en wordt sperma regelmatig gescreend op de aanwezigheid van virussen. Hoewel bekend is dat deze praktijken voordelig zijn, hebben ze ook beperkingen. Het overmatig gebruik van antibiotica leidt tot het ontstaan van antibioticaresistente bacteriestammen. Omdat antibiotica de enige behandelingsmethode zijn voor bacteriële infecties, vormen infecties veroorzaakt door antibioticaresistente bacteriestammen een wereldwijde bedreiging. Virusscreeningtechnieken zijn arbeidsintensief, duur en tijdrovend, en testresultaten zijn meestal niet beschikbaar voorafgaand aan inseminatie met vers sperma. De implementatie van een stap die micro-organismen verwijdert tijdens routinematige spermaverwerking, verhoogt de bioveiligheid van KI, elimineert het gebruik van antibiotica en kan economisch voordelig zijn.

De fysieke verwijdering van micro-organismen uit sperma is een uitdaging. Hoge verwijderingspercentages van micro-organismen zijn gewenst, maar ook hoge terugwinningspercentages van zaadcellen en de kwaliteit van het sperma moeten worden gegarandeerd, terwijl een ejaculaat binnen een acceptabele tijd wordt verwerkt. De verschillen in grootte tussen micro-organismen en zaadcellen maken scheidingstechnieken op basis van grootte aantrekkelijk. Scheidingstechnieken op macroschaal bereikten hoge verwijderingspercentages van micro-organismen, maar terugwinningspercentages van zaadcellen van ongeveer 50% zijn niet acceptabel. Microfluïdische scheidingstechnieken, een opkomend veld met werkmechanismen op het cellulaire groottebereik, zijn mogelijk voordeliger dan scheidingstechnieken op macroschaal. Er zijn veel microfluïdische technieken voorgesteld om spermamonsters te verbeteren. Bovendien werden microfluïdische technieken uitgebreid gesuggereerd voor het verwijderen van micro-organismen uit bloed, en zouden ze ook kunnen worden toegepast voor het verwijderen van micro-organismen uit sperma. Een bekende uitdaging van microfluïdica, vooral voor routinematige spermaverwerking in de veterinaire industrie, is de verwerking met hoge doorvoer; een varkensejaculaat, bestaande uit 90 miljard (90×10^9) zaadcellen en 300 ml ejaculaatvolume, wordt bij voorkeur binnen tien minuten verwerkt.

Het doel van dit project is om micro-organismen uit sperma te verwijderen met microfluïdische scheidingstechnieken door niet alleen de verwijderingspercentage van

micro-organismen te beschouwen, maar ook de terugwinningspercentage van zaadcellen, de spermakwaliteit en de verwerkingstijd. Daarom onderzoekt dit proefschrift het potentieel van twee microfluïdische scheidingstechnieken voor het verwijderen van micro-organismen uit sperma voor de veterinaire industrie.

Eerst werd het effect van microfluïdische verwerking op de levensvatbaarheid van zaadcellen onderzocht, d.w.z. het percentage zaadcellen dat de procedure overleeft. Aangezien het verwerkte sperma vervolgens wordt gebruikt voor KI, is spermaverwerking geen eindpuntmeting en moet de spermakwaliteit worden behouden. Tijdens microfluïdische verwerking worden zaadcellen blootgesteld aan schuifspanningen. Deze schuifspanningen werden vergeleken met de schuifspanningen die ze tijdens natuurlijke ejaculatie ervaren. De levensvatbaarheid van spermatozoa na microfluïdische verwerking werd vergeleken met conventionele verwerkingstechnieken zoals centrifugatie en flowcytometrie. De afname van de levensvatbaarheid als gevolg van microfluïdische verwerking op varkens zaadcellen was met 6% klein, maar significant. Microfluïdische verwerking had geen invloed op de levensvatbaarheid van runder zaadcellen. Het effect van microfluïdische verwerking op de levensvatbaarheid van zaadcellen is vergelijkbaar met de levensvatbaarheid verkregen na centrifugatie en gerapporteerd na flowcytometrie. Door rekening te houden met de levensvatbaarheid van zaadcellen, heeft microfluïdica de potentie om te worden geïmplementeerd in routinematige spermaverwerking.

De eerste onderzochte microfluïdische scheidingstechniek is pinched flow fractionation (PFF), waarbij hydrodynamische krachten worden gebruikt om verschillende deeltjes op basis van hun grootte te scheiden (Hoofdstuk 4). De verwijdering van virussen werd gemodelleerd door cowpea chlorotic mottle-virussen (CCMV's) aan varkenssperma toe te voegen. Na optimalisatie van het chipontwerp en de stroomparameters werden virusverwijderingspercentages van ten minste 84 ± 4% en terugwinningspercentages van zaadcellen van 86 ± 6% bereikt. De huidige verwerkingstijd van één ejaculaat met PFF is 72 000 uur (3 000 dagen) en dus niet realistisch. Met verschillende optimalisaties kan de verwerkingstijd worden teruggebracht tot 20 uur, maar parallellisatie van veel apparaten is onvermijdelijk om de gewenste verwerkingstijd van 10 minuten te bereiken.

Voor verbetering van de spermakwaliteit werd de scheidingstechniek PFF gecombineerd met de hypo-osmotische zwelling (HOZ) test (Hoofdstuk 5). Intacte spermatozoa geïncubeerd in een hypo-osmotische oplossing zwellen op en hun staarten krullen. Vervolgens werd het effect van de HOZ-oplossing op de gedrag van zaadcellen in het scheidingsapparaat onderzocht. In tegenstelling tot varkens zaadcellen komen runder zaadcellen in de HOZ-oplossing op een andere positie in het verbrede segment van het PFF-apparaat naar voren dan de controlegroep. Dit kan worden verklaard door de

hogere waterdoorlaatbaarheid van runder zaadcellen in vergelijking met varkens zaadcellen. Om deze reden werden scheidingsexperimenten uitgevoerd met runder zaadcellen. Door de HOZ-test met de scheidingstechniek PFF te combineren werd de spermakwaliteit van runderen immers niet verbeterd. De mislukte scheiding kan worden veroorzaakt door het lagere tuimeleffect van opgerolde spermatozoa.

De tweede onderzochte microfluïdische scheidingstechniek is acoustoforese, waarbij deeltjes worden gemanipuleerd door een akoestische kracht (Hoofdstuk 6). De verwijdering van micro-organismen uit sperma is onderzocht met het tafelmodel AcouWash (AcouSort, Lund, Zweden). Met geoptimaliseerde stroomparameters werden bacterieverwijderingspercentages van ten minste 88 ± 7% en terugwinningspercentages van zaadcellen van 90 ± 4% bereikt. Ook was het verwijderen van virussen uit sperma succesvol. Er werd geen significant effect van de akoestische krachten op de levensvatbaarheid van zaadcellen gevonden.

Met het huidige AcouWash tafelmodel is de verwerkingstijd van één (varkens)ejaculaat 6 000 – 25 000 uur (250 – 1 000 dagen). In een gap-analyse (hoofdstuk 7) is het verschil tussen de huidige en de gewenste toestand onderzocht. Er worden verschillende ideeën geopperd om de doorvoer te verhogen met betrekking tot hun gevolg op de scheidingskwaliteit en verwerkingstijd. Deze suggesties zijn: (1) verhoging van de monsterconcentratie en (2) monsterstroomsnelheid, (3) optimalisatie van het chipontwerp voor verwerking met hoge doorvoer, (4) onderzoek naar het gebruik van zaadcellen die zijn geïncubeerd in een HOZ-oplossing, en (5) parallellisering meerdere kanalen. Geconcludeerd werd dat een combinatie van meerdere verbeteringen nodig is om de gewenste doorlooptijd te realiseren.

In Hoofdstuk 8 werd PFF vergeleken met acoustoforese. Beide toegepaste scheidingstechnieken behaalden vergelijkbare verwijderingspercentages van micro-organismen en terugwinningspercentages van zaadcellen. Het bereiken van de gewenste verwerkingstijd van een ejaculaat is echter beter haalbaar met acoustoforese. Bovendien is er al een commercieel tafelmodel dat gebruik maakt van acoustoforesis beschikbaar. Om deze redenen presteert acoustoforese beter dan PFF.

De microfluïdische scheidingstechnieken PFF en acoustophorese verwijderen micro-organismen uit sperma. In tegenstelling tot PFF is acoustoforese veelbelovend voor het verwijderen van beide typen micro-organismen in één scheidingsstap en het bereiken van de gewenste verwerkingstijd. Het implementeren van een fysieke verwijderingsstap van micro-organismen in routinematige spermaverwerking kan het gebruik van antibiotica elimineren en de bioveiligheid van KI in de veterinaire industrie vergroten.

Zusammenfassung

Künstliche Besamung (KB) ist die am weitesten verbreitete Zuchtmethode in der Schweine- und Rinderindustrie. Die Präsenz von Mikroorganismen in der Samenflüssigkeit, welche für die KB verwendet wird, stellt ein potenzielles Risiko für die Übertragung von Krankheiten dar und verringert die Qualität der Spermien. Um die Gefährdung durch Mikroorganismen zu minimieren, gibt es zahlreiche Vorkehrungen. Standardgemäß werden die Samenverdünner mit Antibiotika versetzt und der Samen wird regelmäßig auf Vieren untersucht. Obwohl diese Verfahren sich in der Praxis etabliert haben, bringen sie auch einige Nachteile mit sich. Der übermäßige Gebrauch von Antibiotika führt zur Entstehung antibiotikaresistenter Bakterienstämme. Da Antibiotika die einzige Behandlung für bakterielle Infektionen sind, stellen antibiotikaresistente Bakterienstämme eine globale Bedrohung dar. Die Übertragung von Viren zwischen Tieren kann sich auch auf den Menschen ausbreiten und weltweite Pandemien verursachen. Zwei bekannte Beispiele sind die Schweinegrippe und Covid-19-Pandemien. Um die Verbreitung von Viren zu verhindern, wird das Sperma regelmäßig untersucht, was jedoch arbeitsintensiv, teuer und zeitaufwändig ist. Darüber hinaus sind Testergebnisse vor einer Besamung mit Frischsamen in der Regel nicht verfügbar. Die Einführung eines einzelnen Arbeitsschrittes, der Mikroorganismen während der routinemäßigen Samenverarbeitung entfernt, erhöht die Biosicherheit der KB, macht den Einsatz von Antibiotika überflüssig, macht kontaminierten Samen nutzbar und kann wirtschaftlich vorteilhaft sein.

Die physikalische Beseitigung von Mikroorganismen aus der Samenflüssigkeit ist in vielerlei Hinsicht eine große Herausforderung. Hohe Entfernungsraten der Mikro-organismen sind erwünscht, gleichzeitig sollen auch die Spermienrückgewinnung und die Samenqualität sichergestellt werden. Zusätzlich soll ein Ejakulat innerhalb einer akzeptablen Zeit verarbeitet sein. Prinzipiell ermöglichen die Größen-unterschiede zwischen Mikroorganismen und Spermien größenbasierte Trenn-techniken. Hohe Entfernungsraten von Mikroorganismen konnten durch Trenntechniken im Makromaßstab erzielten werden, aber die Rückgewinnungsraten der Spermien mit etwa 50% sind nicht akzeptabel. Trenntechniken basierend auf Mikrofluidik, ein aufstrebendes Gebiet mit speziellen Mechanismen im Zellgrößenbereich, stellen eine attraktive und effiziente Alternative dar. Viele Methoden basierend auf der Mikrofluidik wurden bereits zur Verbesserung von Samenproben vorgeschlagen. Darüber hinaus wurden viele Methoden ausgiebig zur Entfernung von Mikroorganismen aus Blut angewandt und können auch potentiell auf Samenproben übertragen werden. Eine bekannte Herausforderung der Mikrofluidik, insbesondere für die routinemäßige Samenverarbeitung in der Veterinärindustrie, ist die Verarbeitung mit hoher

Durchflussleistung. Ein Schweineejakulat, bestehend aus 90 Milliarden (90×10^9) Spermien und 300 ml Ejakulat Volumen, soll wünschenswerterweise innerhalb von zehn Minuten verarbeitet werden.

Ziel dieses Projektes ist es, Mikroorganismen mit Trenntechniken aus der Mikrofluidika aus dem Samen zu entfernen. Hierbei sollen nicht nur die Mikroorganismen-Entfernungsraten, sondern auch die Spermienrückgewinnung, die Samenqualität und die Verarbeitungszeit berücksichtigt werden. Daher untersucht diese Dissertation das Potenzial zweier Trenntechniken um Mikroorganismen aus dem Samen zu entfernen.

Zunächst wurden die Auswirkungen der mikrofluidischen Verarbeitung auf die Lebensfähigkeit der Spermien untersucht, d. h. der Prozentsatz der Spermien, welche die Verarbeitung überleben. Da in der Industrie der aufbereitete Samen anschließend für die KB verwendet wird, sollte die Samenqualität aufrechterhalten bleiben. Während der mikrofluidischen Verarbeitung sind Spermien der mechanischen Schubspannungen ausgesetzt. Diese Schubspannungen wurden mit den natürlichen Schubspannungen, die Spermien während der natürlichen Ejakulation erfahren, verglichen. Die Lebensfähigkeit der Spermien nach mikrofluidischer Verarbeitung wurde mit herkömmlichen Verarbeitungstechniken wie Zentrifugation und Durchflusszytometrie verglichen. Die Abnahme der Lebensfähigkeit von 6% bei Schweinespermien aufgrund der mikrofluidischen Verarbeitung war gering, aber signifikant. Bei Rinderspermien konnte hingegen kein Einfluss auf die Lebensfähigkeit beobachtet werden. Die Wirkung der mikrofluidischen Verarbeitung auf die Lebensfähigkeit der Spermien war vergleichbar mit der Lebensfähigkeit, die nach Zentrifugation erhalten und nach Durchflusszytometrie berichtet wurde. Bezüglich der Lebensfähigkeit der Spermien hat die Mikrofluidik das Potenzial, um in der routinemäßige Samenverarbeitung eingesetzt zu werden.

Die erste untersuchte mikrofluidische Trenntechnik ist die *Pinched Flow* Fraktionierung (PFF), in welcher hydrodynamische Kräfte verwendet werden um Teilchen basierend auf ihrer Größe zu trennen. Die Entfernung von Viren aus der Samenflüssigkeit wurde mit *Cowpea Chlorotic Mottle* Viren (CCMV) modelliert. CCMV ist ein Virus, das die Kuherbsenpflanze oder schwarz-äugige Erbse infiziert. Nach der Optimierung des Chipdesigns und der Flussparameter wurden Virusentfernungsraten von mindestens 84 ± 4% und Spermienrückgewinnungsraten von 86 ± 6% erreicht. Die aktuelle Verarbeitungszeit eines Ejakulats mit PFF beträgt 72 000 Stunden (3 000 Tage) und ist somit nicht im industriellen Maßstab realisierbar. Mit mehreren Optimierungen ließe sich die Bearbeitungszeit auf 20 Stunden verkürzen, jedoch ist eine Parallelisierung vieler Kanäle unumgänglich, um die gewünschte Bearbeitungszeit von zehn Minuten zu erreichen.

Zur Verbesserung der Samenqualität wurde die Trenntechnik PFF mit dem hypoosmotischen Schwellungstest (HOS) kombiniert. Intakte Spermien in einer hypoosmotischen Lösung schwellen an und ihre Schwänze kräuseln sich. Der Einfluss der HOS-Lösung auf das Verhalten der Spermien in der Trenntechnik wurde untersucht. Im Gegensatz zu Schweinespermien erschienen Rinderspermien in der HOS-Lösung an einer anderen Position im verbreiterten Segment des PFF-Chips als die Kontrollgruppe. Dies kann durch die höhere Wasserdurchlässigkeit der Rinderspermien im Vergleich zu Schweinespermien erklärt werden. Aus diesem Grund wurden Trennversuche mit Rindersamenproben durchgeführt. Allerdings wurde durch die Kombination des HOS-Tests mit der anschließenden Trennung durch PFF die Qualität des Rindersamens nicht verbessert. Die erfolglose Trennung könnte durch den geringeren Taumeleffekt von zusammengerollten Spermien verursacht werden.

Die zweite erforschte mikrofluidische Trenntechnik ist Akustophorese, bei der Teilchen durch eine akustische Kraft manipuliert werden. Die Entfernung von Mikroorganismen aus Samen wurde mit dem Tischgerät AcouWash (AcouSort, Lund, Schweden) untersucht. Mit optimierten Strömungsparametern wurden Bakterienentfernungsraten von mindestens 88 ± 7% und Spermienrückgewinnungs-raten von 90 ± 4% erreicht. Auch die Virusentfernung aus dem Sperma war erfolgreich. Es wurde keine signifikante Wirkung der akustischen Kräfte auf die Lebensfähigkeit der Spermien gefunden.

Mit dem aktuellen AcouWash-Instrument beträgt die Bearbeitungszeit eines (Schweine)Ejakulats 6 000 – 25 000 Stunden (250 – 1 000 Tage). In einer Gap-Analyse, auch bekannt als Lückenanalyse, wurde der Unterschied zwischen Ist- und Soll-Zustand untersucht. Es wurden verschiedene Ideen zur Erhöhung der Durchflussleistung hinsichtlich ihrer Auswirkungen auf die Trennqualität und Verarbeitungszeit vorgeschlagen. Diese Vorschläge waren: (1) Erhöhung der Probenkonzentration und (2) Durchflussrate, (3) Optimierung des Chipdesigns für eine höhere Durchflussleistung, (4) Untersuchung der Verwendung von Spermien, die in einer HOS-Lösung inkubiert wurden, und (5) Parallelisierung mehrerer Kanäle. Eine Kombination mehrerer Vorschläge ist unumgänglich um die gewünschte Verarbeitungszeit zu erreichen.

PFF wurde mit Akustophorese verglichen. Beide angewandten Trenntechniken erzielten ähnliche Entfernungsraten von Mikroorganismen und Spermienrückgewinnungsraten. Das Erreichen der gewünschten Verarbeitungszeit eines Ejakulats ist jedoch mit Akustophorese realistischer. Darüber hinaus ist bereits ein kommerzielles Tischgerät mit Akustophorese erhältlich. Aus diesen Gründen ist die Verwendung von Akustophorese zu bevorzugen.

Zusammenfassend lässt sich sagen, dass die mikrofluidischen Trenntechniken PFF und Akustophorese (einen hohen Prozentsatz an) Mikroorganismen aus dem Samen entfernen. Im Gegensatz zur PFF ist Akustophorese vielversprechender um beide Arten der Mikroorganismen in einem Trennschritt innerhalb der gewünschten Verarbeitungszeit zu entfernen. Der entscheidende Vorteil der Einführung eines physikalischen Trennverfahrens von Mikroorganismen ist, dass der Einsatz von Antibiotika verringert wird und die Biosicherheit der KB in der Veterinärindustrie erhöht werden.

Scientific output

Peer-reviewed articles

T. Hamacher*, J.T.W. Berendsen*, S.A. Kruit*, M.L.W.J. Broekhuijse, L.I. Segerink. Effect of microfluidic processing on the viability of boar and bull spermatozoa. *Biomicrofluidics* 14, 044111 (2020), DOI: 10.1063/5.0013919, *Editor's pick*

T. Hamacher, J.T.W. Berendsen, J.E. van Dongen, R.M. van der Hee, J.J.L.M. Cornelissen, M.L.W.J. Broekhuijse, L.I. Segerink. Virus removal from semen with a pinched flow fractionation microfluidic chip, *Lab on a Chip* (2021) DOI: 10.1039/D1LC00643F

T. Hamacher, A. Urbansky, M.L.W.J. Broekhuijse, L.I. Segerink. Bacteria removal from porcine semen with acoustophoresis, *in review*

Conference abstracts

T. Hamacher, J.T.W. Berendsen, M.L.W.J. Broekhuijse, L.I. Segerink. Separation of viruses from spermatozoa using a microfluidic chip to achieve pinched flow fractionation; NanoBioTech-Montreux Conference; Switzerland, November 18-20, 2019

T. Hamacher*, J.T.W. Berendsen*, S.A. Kruit*, M.L.W.J. Broekhuijse, L.I. Segerink. Effect of microfluidic processing on the viability of boar and bull spermatozoa Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS); October 4-9, 2020

T. Hamacher, J.T.W. Berendsen, J.E. van Dongen, R.M. van der Hee, J.J.L.M. Cornelissen, M.L.W.J. Broekhuijse, L.I. Segerink. Virus removal from semen with a pinched flow fractionation microfluidic chip, Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS), Palm Springs, CA, USA; October 10-14, 2021

T. Hamacher, A. Urbansky, M.L.W.J. Broekhuijse, L.I. Segerink. Microbial pathogen removal from porcine semen with acoustophoresis, Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS), Palm Springs, CA, USA; October 10-14, 2021

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Contribution

The work presented in this thesis was supported by a number of people. The research was performed in close consultation with the breeding organizations Topigs Norsvin and CRV B.V., both represented by Marleen Broekhuijse. Marleen Broekhuijse also organized the semen samples. Loes Segerink and Marleen Broekhuijse were consistently involved in the project. Contributions from collaborators for each chapter are described below. Collaborators are from BIOS Lab on a Chip group (University of Twente (UT)) unless otherwise stated.

Chapter 1 & 2

These chapters were written by Tanja Hamacher with critical input from Loes Segerink and Marleen Broekhuijse.

Chapter 3

Tanja Hamacher, Johanna Berendsen and Stella Kruit equally contributed by designing the study, carrying out the experiments, performing calculations and writing the manuscript. Marleen Broekhuijse and Loes Segerink supervised the project. All collaborators involved in this chapter discussed the results and commented on the manuscript.

Chapter 4

Tanja Hamacher designed, as well as carried out the experiments and wrote the manuscript. Johanna Berendsen helped with designing and setting up the microfluidic chip, as well as assisted in semen handling. Jeanne van Dongen supported the experimental design and analysis. Regine van der Hee prepared the CCMVs under the supervision of Jeroen Cornelissen (both Department of Molecules & Materials, UT). Marleen Broekhuijse and Loes Segerink supervised the project. All collaborators involved in this chapter gave critical input on the manuscript. Johan de Bomer is acknowledged for the support in the cleanroom.

Chapter 5

Tanja Hamacher designed, carried out the experiments, and wrote the chapter. Jeanne van Dongen is acknowledged for the discussions on the experimental design. Johanna Berendsen is acknowledged for matlab script, which was used for analysis. Marleen Broekhuijse and Loes Segerink gave valuable input on the experiments and written draft.

Chapter 6

Albert van den Berg, Hiemke Knijn (CRV B.V., Semen Refinement B.V., the Netherlands) and Torsten Freltoft (AcouSort, Sweden) organized the collaboration. Tanja Hamacher designed and carried out the experiments. Anke Urbansky (AcouSort, Lund, Sweden) profoundly supported the experimental design. The company AcouSort (Lund, Sweden), under the direction of Torsten Freltoft, provided the AcouSort instrument. The artificial insemination center Svenska Köttförtagen (Skövde, Sweden) supplied the semen samples. Thomas Laurell and the Acoustofluidics group from Lund University are acknowledged for providing their laboratory facilities. Narges Baghi (Bioelectric Signaling and Engineering, UT) and Regine van der Hee prepared the bacteria and CCMVS, respectively. Tanja Hamacher wrote the manuscript with valuable input from Anke Urbansky, Marleen Broekhuijse and Loes Segerink.

Chapter 7

The gap analysis was the result of a progress meeting with collaborators from Semen Refinement B.V., BIOS Lab on a Chip group and AcouSort. The *desired state* was set in consultation with Semen Refinement B.V.. The *plan of action* was developed in collaboration with Anke Urbansky. Tanja Hamacher wrote the chapter with valuable input from Marleen Broekhuijse and Loes Segerink.

Chapter 8

This chapter was written by Tanja Hamacher with input from Loes Segerink and Marleen Broekhuijse. Siem van de Wetering (Bachelor Electrical Engineering, UT) designed and performed the experiments to increase the throughput of pinched flow fractionation.

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