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An den Vorsitzenden des

Diplom-Prüfungsausschusses

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Freiburg, 2013-10-11

Referat zur Diplomarbeit von Herrn Rüdiger Trojok

Towards the utilization of lactic acid bacteria as contraceptive agents

Die Dimplomarbeit von Herrn Trojok ist von der Idee getragen, ein gemeinnützig verfügbares, nicht invasives alternatives Verhütungsmittel zu entwickeln. Die Zunahme der Weltbevölkerung beruhe - neben den bekannten sozioökonomischen Rahmenbedingungen - auch auf der schlechten Zugänglichkeit zu preiswerten Kontrazeptiva.

Die grundlegende Idee, die Herr Trojok im Vorfeld der Arbeit mit Fachleuten diskutierte, beruht auf der Chemotaxis Spermien, die auf Gradienten von Chemo-Attraktantien mit Veränderungen ihres Bewegungsmusters antworten. Beispiele sind Progesteron und gewisse Peptide, wie ANP, RANTES und NGF-ß. Die Expression solcher Peptide durch genetisch veränderte symbiontische Milchsäurebakterien sollte Spermien fehlleiten und somit die Wahrscheinlichkeit einer Befruchtung stark herabsetzen.

Daraus ergibt sich zur Entwicklung eines Prototypen die Notwendigkeit, (i) die Chemokinese von menschlichen Spermien in Abhängigkeit von wirksamen Peptiden untersuchen zu können, (ii) ein geeignetes transformierbares Bakterien-System zu identifizieren, (iii) ein definiertes, das Vaginalsekret simulierendes Medium zu optimieren und (iiii) das identifizierte Peptid in ausgewählten Bakterien zu exprimieren.

Herr Trojok entwickelte ein Computer-gestütztes Video-Verfahren, um die Chemokinese zu verfolgen. Um die für menschliche Spermien optimierten Eingangsparameter zu finden, verwendete Herr Trojok einen Algorithmus, der mittels

„Directed Evolution“ zu aussagefähigen Daten über die Spermien-Trajektorien in Abhängigkeit von unterschiedlichen Konzentrationsbereichen von vier Chemo-Attraktantien und einer Positivkontrolle (Progesteron) führte. Zwei der verwendeten Peptide wurden als geeignete Kandidaten identifiziert. Herr Trojok untersuchte weiterhin fünf symbiontische Milchsäurebakterien hinsichtlich ihrer Kultivierbarkeit und optimierte das Medium auf der Grundlage von publizierten Daten in Abhängigkeit von zahlreichen Additiven. Auch hier entwickelte er ein automatisiertes Hochdurchsatz-Verfahren, das die Erstellung einer großen Anzahl an Wachstumskurven mittels eines selbstentwickelten Computerskripts automatisierte. Diese Untersuchungen wiesen auf *L. vaginalis* und *L. jesenii* als geeignete Kandidaten. Jedoch zeigte sich *L. lactis* als ebenfalls geeignet und hat den Vorteil, als gut untersuchter und transformierbarer Modellorganismus über eine gute molekulare Infrastruktur zu verfügen. Herr Trojok kann somit die Punkte (i) – (iii) mit aussagefähigen Daten bedienen. Die Herstellung eines Expressionsplasmids und Transformationen konnten im zeitlichen Rahmen der Arbeit nicht durchgeführt werden; dieser Punkt ist daher in der Diskussion ausgeführt.

Die Diplomarbeit von Herrn Trojok ist in mehrerer Hinsicht außergewöhnlich und hebt sich deutlich von anderen ab. Hervorzuheben ist die kompromisslose Selbständigkeit in Planung und Durchführung. So ist dieses recht unkonventionelle Thema selbständig gewählt, gründlich recherchiert und detailliert geplant worden, wie auch das betreuende Labor am Department of Systems Biology an der Denmark Technical University (das aber leider diesbezüglich sehr enttäuschte) von ihm ausgewählt wurde. Weiterhin wurde die Finanzierung des Projektes von Herrn Trojok bei der EU selbst eingeworben und über das „Katapult“-Office dieser Universität verwaltet. Diese sehr limitierten Mittel (wie auch leider nicht funktionierendes Equipment des gastgebenden Labors) zwangen Herrn Trojok zu erheblichem Erfindungsreichtum. So ist das komplette Video-Setup Eigenbau – das dann auch wirklich funktionierte. Dank der Systembiologie-orientierten Ausrichtung von Herrn Trojok und seines (in unserer Fakultät bekannten) Engagements in der biotechnologischen „Open-Source“-Debatte, sind für ihn monetäre Limitationen eher Herausforderung denn Hemmnis. Hervorzuheben ist auch seine Programmier-Kompetenz, die ebenfalls unter Verwendung von kostengünstigen open-source Plattformen diese Arbeiten erst ermöglichten. Sicher – dem Konzept sind früher oder später Grenzen gesetzt und in verschiedenen Diskussionen habe ich den Eindruck gewonnen, dass Herr Trojok diesen Umstand auch erkennen musste.

Die Diplomarbeit ist in flüssigem Englisch abgefasst und gut lesbar. Es herrscht kein Mangel an Wissenschaftlichkeit, zielgerichteter Planung und statistisch haltbarer Ergebnisverwertung. Einleitung und Diskussion sind zielführend, kritisch und zeugen von einer gründlichen Literaturkenntnis. Vor Allem zeugt Herrn Trojoks Arbeit beispielgebend von originärem eigenständigen Forschergeist in jungen Jahren, gepaart mit wissenschaftlich kompetenter Herangehensweise. Obwohl die hochgesteckten Ziele (z.T. auch krankheitsbedingt) nicht ganz erreicht werden konnten, zögere ich deshalb nicht, seine Arbeit mit 1,0 zu bewerten.

(Prof. P. Beyer)

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Freiburg, 09.10.2013

Gutachten Diplomarbeit Rüdiger Trojok

Herr Rüdiger Trojok hat unter Anleitung von Prof. Peter Beyer eine Diplomarbeit mit dem Titel „Towards the utilization of lactic acid bacteria as contraceptive agents“ angefertigt. Hervorzuheben ist hierbei die Tatsache, dass Herr Trojok sowohl die Themenwahl, die Finanzierung der Versuchsreihen als auch die Wahl des Labors (Denmark Technical University) weitestgehend selbständig vornahm und dabei von Peter Beyer nur beraten wurde.

Die grundlegende Idee für diese Arbeit entwickelte Herr Trojok im Rahmen seiner Beteiligung an iGEM. Ausdrücklich versteht er seine Versuche als Basis für weitere Verbesserungen im Rahmen von open-source Versuchen. Diese Arbeit sticht in vielerlei Hinsicht aus den bei uns üblichen Arbeiten positiv heraus. Neben der kompromisslosen Eigenständigkeit ist besonders die außergewöhnliche Breite des Methodenspektrums zu erwähnen.
Herr Trojok führt kompetent und konzis in das Thema ein, beschreibt seine Ergebnisse klar und anschaulich und diskutiert sie ebenso kompetent und umfassend wie realistisch. Kleinere Fehler bei Grammatik und Rechtschreibung scheinen dem Termindruck beim Verfassen der Arbeit und beim Ausfall des dänischen Anleiters geschuldet.
Die vorliegende Diplomarbeit kann ohne weiteres als beispielgebend für eine ganze Generation Freiburger Studierender gesehen werden und ist jedes Preises würdig, den unsere Fakultät zu vergeben hat.
Daher bewerte ich die vorliegende Arbeit mit

sehr gut (1,0)

Prof. Dr. Ralf Reski

- Lehrstuhl für Pflanzenbiotechnologie der Fakultät für Biologie der Albert-Ludwigs-Universität Freiburg, Prof. Dr. Ralf Reski
- Senior Fellow, Freiburg Institute for Advanced Studies (FRIAS)
- Ordentliches Mitglied, Heidelberger Akademie der Wissenschaften (HAW)
- Gründungsmitglied von BIOSS, SGBM und FRISYS der Albert-Ludwigs-Universität Freiburg
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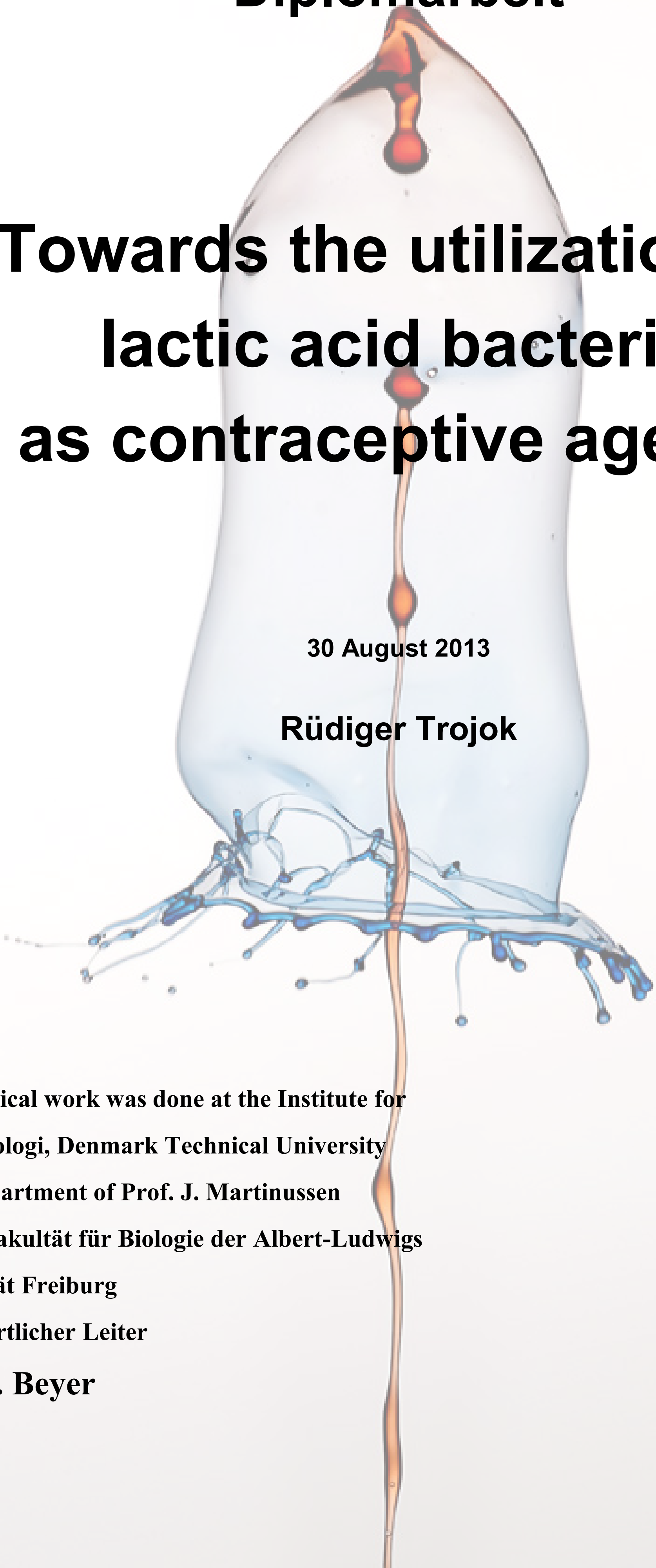

Diplomarbeit

Towards the utilization of lactic acid bacteria as contraceptive agents

30 August 2013

Rüdiger Trojok

The practical work was done at the Institute for
Systembiologi, Denmark Technical University
in the department of Prof. J. Martinussen
Für die Fakultät für Biologie der Albert-Ludwigs
Universität Freiburg
verantwortlicher Leiter
Prof. P. Beyer

Diese Arbeit ist meinen Eltern gewidmet, die mich lange Jahre trotz ungewissen Ausgangs und aller Widrigkeiten mit viel Geduld in meiner Studienzeit unterstützt haben.

This work is dedicated to my parents who patiently supported me despite all odds and unknowns for the many years of my studies.

Hereby I declare that all presented work was entirely done by myself. No other auxiliary means besides the ones mentioned in the text were used.

For questions and more data please send an email to **trojok@openbioprojects.net** If you want to use my work further, please let me know. This project is meant to become an international open source drug discovery cooperation.

The cover picture "The Liquid Condom" is a courtesy of Markus Reugels

Preface

I developed the concept to use symbiotic bacteria as a contraceptive agent during the iGEM jamboree 2009 at the MIT. After discussing it with several scientists, I gained confidence that the idea can be realized. With the wish to develop an independent research topic, I decided to settle on it for my diploma thesis to use the 12 months of laboratory research to kickstart this new topic. To be able to work with *Lactobacilli* within a laboratory experienced in this field, I chose to relocate to the Department of Systems Biology at the Denmark Technical University near Copenhagen. To fund such an unconventional research idea, I applied and received a scholarship for materials of 28500 Danish Krona (around 4000€) from the European Union. This fund was granted for research projects with marketing potential and was issued by the Katapult office of the Copenhagen University. Since the project required methods of very diverse areas of biological research, it was a big challenge to work without tutoring for large parts of the project. I had to develop and conduct almost all experiments on my own. Another obstacle was to overcome limited equipment availability. To make optimal use of the given budget it was necessary to make adjustments to some experiments and to improvise methods. Also, ethical questions arose concerning the future application of envisioned product which I do not feel entitled to give answers to alone. Despite these difficulties, I believe to have gathered sufficient data to lay a foundation for future research on the idea of bacteria-mediated contraception. The development of a functional prototype requires a long term commitment and infrastructure which exceeds the frame of this Diploma thesis. This will require significant financial investments which would most likely have to come from the private sector. The classical way to achieve the long term goal - to develop the idea into an applicable contraceptive - would be to patent it. However, contraception is of major importance for people worldwide. Using the classical way of proprietary research and development combined with the possibilities of misuse of technology such as overpricing did not seem justifiable to me. New ways of development and distribution need to be found to allow any person in demand for this technology to profit from it as soon as possible. Given the idea finds broader interest in the health and science community, I can envision a potential for public sector translational research towards prototype development, maybe even including clinical trials. The work could be done in a collaborative manner involving scientists and companies worldwide contributing their ideas and results free of charge and under open-access licenses. The more people are involved in creating this system, the better the self-optimization and adaptation to local needs will be. Good examples of the effectiveness of such crowd-sourced developments can be seen in the field of open software development. Using low cost and high output methods, the development will also allow less well funded laboratories to contribute. Therefore, I have published the work under a creative commons license 3.0.

I would like to thank the Katapult office team for providing the funding, Prof. Jan Martinussen for letting me use his laboratory, Prof. Karin Hammer for valuable suggestions regarding the growth conditions *Lactobacilli*, Phillip Meißner and Leander Damme for advice on the bioinformatics part of the work as well as Prof. Peter Beyer, Dr. Sarah Chareza and Dr. Jessica Bernds for reviewing the work and all my friends who cheered me up and motivated me when a experiment failed.

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Results

Discussion

Towards the utilization of lactic acid bacteria as contraceptive agents

Zusammenfassung

Die exponentielle Zunahme der Weltbevölkerung erfordert eine Verbesserung der Verhütungstechnologie. Durch gentechnische Veränderung von symbiotischen Bakterien, die das Lumen der Vagina besiedeln, erscheint es möglich, ein nicht-spürbares, nicht-invasives und lokal wirksames Verhütungsmittel zu entwickeln. Das Ziel dieser Diplomarbeit ist es die experimentellen Grundlagen für die Entwicklung bakterieller Verhütungsmittel zu legen. Es wurde ein programmatischer Leitfaden entworfen, der die Entwicklung eines Prototyps eines transgenen Bakteriums, das die Fruchtbarkeit von Spermien beeinträchtigt, skizziert, sowie Testgerät um dieses zu evaluieren. Die anfängliche Entwicklung wurde in drei Segmente gegliedert: Die Auswahl geeigneter symbiotischer Bakterien aus dem vaginal Mikrobiom, Tests zur Identifizierung von chemotaktisch Wirkstoffen und deren Auswertung sowie das Design eines genetischen Konstrukts welches alle notwendigen Komponenten für einen funktionierenden Prototyps beinhaltet. *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus vaginalis, Lactobacillus jensenii u*nd *Lactococus lactis* wurden als die am häufigsten vorkommenden symbiotischen Bakterienstämme bei Frauen kaukasischer Abstammung festgestellt und die Möglichkeit diese zu kultivieren untersucht. Es wurde ein definiertes Medium zur Simulation von Vaginalsekret auf Grundlage der Arbeit von Liu et al. 2011 entwickelt um in zukünftigen Studien ein Expressionssystems für chemotaktische Wirkstoffe unter realistischen Umständen testen zu können. 13 Inhaltsstoffe bzw. Gemische wurden dem Medium hinzugefügt und deren Auswirkung auf die Verdopplungszeit der Bakterienstämme mit einem Plate-reader gemessen. Die Auswertung der Wachstumskurven wurde mittels eines neu entwickelten Computerskripts automatisiert. Alle Bakterienstämme können im Medium von Liu et al. kultiviert werden. Die Zugabe von Spurenelementen ($(NH_4)_6(MO_7)_{24}$, H_3BO_3, $CoCl_2$, $CuSO_4$, $MnCl_2$, $ZnSO_4$), $CaCl_2$, den Deoxynucleosiden Adenine, Guanine, Cytosine und Thymine, das Ersetzen des Kao & Michayluk Vitamin Mix sowie Vitamin K_1 durch den MEM Vitamin Mix, die Erhöhung der Glukosekonzentration auf 20 g/l sowie die Glykogenkonzentration auf 10 g/l verkürzte jeweils die Verdopplungszeit mindestens eines der Bakterienstämme. Die Kulturen von *L. vaginalis* und *L. jensenii* wuchsen besser als von *L. gasseri* und *L. crispatus.* Daher wird empfohlen erstere für die Entwicklung eines bakteriellen Verhütungsmittels in Betracht zu ziehen. *L. lactis* wuchs zwar verhältnismäßig langsam, die Ergebnisse waren jedoch verlässlich reproduzierbar. Zudem ist es eine gut erforschte Spezies dessen Codon Usage dem von Lactobazillen ähnelt und häufig in Transformationexperimenten verwendet wurde und sich daher als Modellorganismus anbietet. Für die Analyse der chemotaktischen Wirkstoffe wurden frische Spermaproben gesammelt und schwimmfähige Zellen mit dem Swim up Assay extrahiert. Die Spermien wurden auf die Möglichkeit der Beeinflussung ihrer chemokinetischen Eigenschaften hin untersucht, mit dem Ziel sie letztendlich unfruchtbar machen zu können. Um die Bewegungsmuster einzelner Zellen analysieren zu können wurde ein neues kostengünstiges Mikroskopie-Setup entwickelt und die aufgenommenen Videos mithilfe eines Computer Assistes Semen Analysis (CASA) Plugin für ImageJ ausgewertet. Um für menschliche Spermien geeignete Eingangsparameter für das CASA Plugin zu finden wurde ein Algorithmus entwickelt, der mittels eines “directed Evolution” Ansatzes die Idealwerte approximiert. Das Bewegungsmuster von Spermien wurde unter Einfluss fünf verschiedener Konzentrationen der chemotaktischen Wirkstoffe Atrial Natiuretic Peptide (ANP), RANTES, Neural Growth Factor-ß (NGF-ß) und des Stickstoffmonoxidradikale emittierende S- Nitrosogluthation (SNOG) gemessen. Progesteron diente als Positivkontrolle und dazu, die Funktionalität des Setups zu evaluieren. Die Sensitivität des Setups war präzise genug um die Linearität (LIN) und die Durchschnittsgeschwindigkeit der Spermientrajektorien zu bestimmen. ANP und SNOG hatten den stärksten Einfluss auf die Bewegungsmuster und könnten für einen Prototypen für ein baktierielles Verhütungsmittel verwendet werden. Der Effekt von NGF-ß hingegen erschien die Vitalität von Spermien zu erhöhen und könnte daher einen Empfängnis fördernden Effekt erzielen.

Abstract

World's exponentially growing population calls for better contraception methods. In this diploma thesis a novel method of contraception is suggested. By genetically reprogramming symbiotic bacteria which populate the vaginal vault, creating an insensible, non invasive and non systemic biological contraceptive for women appears feasible. This thesis aims at establishing experimental foundations for the development of bacterial-based contraception. A programmatic outline was developed towards the prototype of a transgenic bacterium that targets spermatozoa together with the construction of a testing environment. The work flow was structured to encompass three initial topics: First, the selection and culture of suitable vaginal symbiotic bacteria, second, the observation of spermatozoa in the presence of chemoattractants including data collection and their evaluation and third, the design of the genetic construct providing the necessary genetic components to develop a functional prototype.

Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus vaginalis, Lactobacillus jensenii and *Lactococus lactis* were identified as common symbionts of Caucasian women and evaluated for their growth in culture. To allow for future tests of the expression of chemoattractans by genetically manipulated symbiotic vaginal bacteria under realistic circumstances, a defined media for vaginal fluid based on the work of Liu et al. 2011 was experimentally evaluated and components for its optimization identified. 13 new compounds or mixes of compounds were introduced to the medium and changes of the doubling times of the symbiotic bacteria assessed with a plate reader. The analysis of the growth curves was automated with a newly developed computer script. All strains can be grown in the media designed by Liu et al. Altering the medium by adding micronutrients ($(NH_4)_6(MO_7)_{24}$, H_3BO_3, $CoCl_2$, $CuSO_4$, $MnCl_2$, $ZnSO_4$), $CaCl_2$, the deoxynucleosides Adenine, Guanine, Cytosine and Thymine, replacing Kao & Michayluk Vitamins and Vitamin K_1 with MEM vitamins, increasing the glucose concentration to 20 g/l and glycogen to 10 g/l significantly shortened the doubling times of at least one of the observed strains, each. Cultures of *L. vaginalis* and *L. jensenii* grew better than *L. gasseri* and *L. crispatus* and are therefore deemed suitable candidates for bacterial-based contraception. *L. lactis* grew comparably slowly but stably in all experiments. As the codon usage is similar to *Lactobacilli* and since it is a readily transformable species with a large body of available literature, *L. lactis* was found to be an adequate model organism for the development and testing of a prototype. Fresh human sperm samples were collected and healthy cells isolated using the swim up technique. Spermatozoa were examined to elucidate the possibility of interfering with their chemokinetic properties to eventually render them infertile. To extract the movement pattern of single cells from recorded sperm samples, computer assisted sperm analysis (CASA) was used. A cheap and simple imaging setup was developed to allow video tracking of individual sperm cells. A macro was scripted for the ImageJ CASA plugin to interpret the recorded videos. Using a computational "directed evolution" approach, parameters for the CASA plugin to analyze human spermatozoa were identified. The chemoattractants Atrial Natiuretic Peptide (ANP), RANTES, Neural Growth Factor-ß (NGF-ß) and the nitric oxide radicals emitting S- nitrosogluthation (SNOG) were tested, each at five different concentrations. Progesterone was used as a positive control. The developed imaging setup and analysis software was demonstrated to be sufficiently sensitive to detect changes in the linearity (LIN) and curvilinear velocity of the spermatozoa induced by chemoattractants. It was found that spermatozoa can be chemotactically irritated with ANP and SNOG. Contrarily, it was found that NGF-ß could be used to promote fertility by improving spermatozoa vitality.

Abbreviations

A.A.	Amino Acid
ANP	Atrial Natiuretic Peptide
CASA	Computer Assisted Semen Analysis
EPS	Exopolysaccharide
HIV	Human Immunodeficiency Virus
fps	frames per second
L.	*Lactobacillus* or *Lactoccus* - context dependent
LA	Lactic Acid
LIN	Linearity of a spermatozoon path
MRS	broth Man, Rogosa, Sharpes broth (a bacterial growth media)
NGF-ß	Neural Growth Factor Beta
OD	Optical Density
ORF	Open Reading Frame
PROG	Progesterone
RBS	Ribosome Binding Site
ROS	Reactive Oxygen Species
SA medium	Synthetic Amino Acid medium
SNOG S-	Nitrosoglutathione
STD	Sexually Transmitted Disease
UN	United Nations
VAP	Velocity Average Path of a spermatozoon
VCL	Velocity CurviLinear of a spermatozoon path
VSL	Velocity Straight Line of a spermatozoon path

Introduction

The need for a new contraception method

World's exponentially growing population is becoming a threat to mankind's sustainable life on the planet. The United Nations Organization reports that if fertility remains constant in each country at the level of the 2005-2010 period, the world population could reach nearly 27 billion by 2100, whereby the population of the least developed countries is projected to triple by the end of the century according to the medium variant (United Nations 2011). This would be an outcome unlikely to be sustainable (Dietz et al. 1994). However, if fertility remains just half a child below that of the medium variant (Fig. 1), the world population in 2100 could be 6,2 billion. This is the same size as during the start of the 21st century (United Nations 2011). To encompass a reduced world population growth several programs were launched by international public and private actors such as the Bill and Melinda Gates foundation, various national governments, the WHO and others. Amongst educational programs about family planning and the distribution of condoms, research for new methods of contraception is ongoing. State of the art contraception methods are often unaffordable for poor families in less developed countries and unintended pregnancy comes along with significant personal and financial costs (Trussell et al. 1995). This diploma thesis suggests a new method of contraception to mitigate those problems. Edible probiotic bacteria which naturally populate the vagina could be utilized to act as a insensible, non invasive and non systemic biological contraceptive for women. This could be achieved by genetically reprogramming those bacteria to biologically immobilize, disorient and kill spermatozoa before they reach the uterus Such a method could overcome practical problems like the distribution of contraceptives, as living bacteria can be reproduced on a local basis. This contraceptive method might also help to overcome cultural barriers which often prevent the use of physical contraceptives like condoms, since an edible contraception does not require special attention during sexual intercourse. This study paves the way towards a practical and theoretical exploration addressing this idea.

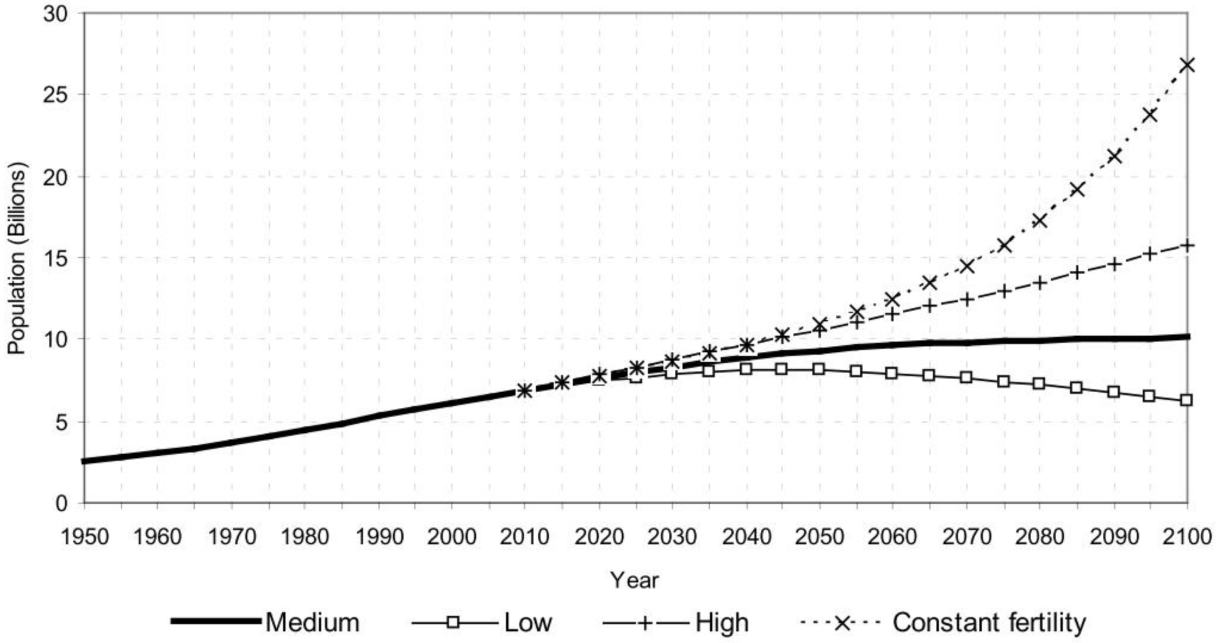

Figure 1: *Population of the world 1950-2100, according to different projections and variants. (Picture taken from United Nations, 2011)*

The development of a bacterial contraceptive method for women requires a detailed understanding of the anatomy and physiology of the female reproductive tract, especially of the vagina and cervix and its secretions. The following section provides a brief overview of human reproduction physiology in the female reproductive tract which could be targeted by bacterial contraception. In addition, the section provides a description of the conceptual idea and an outline of how such a hypothesized contraception method could be realized and how the work of this thesis approaches this question.

The female reproductive tract

Anatomy and physiology

The reproductive tract of women starts as a single pathway through the vagina and cervix, splitting in the uterus into two the fallopian tubes, both of which end at the ovaries (Fig. 2). The ovaries produce a fertile oocyte on average every 28 days, controlled by varying hormonal concentrations in the body. The oocyte then travels within a few days within the 7-11 cm long fallopian tubes towards the uterus. This is the site where fertilization happens. In case of successful fertilization, the ovum is implanted in the endometrium of the uterus. If no fertilization happened, menstruation begins. The uterus has only a volume of 100 µl at the middle of the menstruation cycle (termed midcycle) and is filled with cervical mucus. It is connected with the vaginal vault by the cervix, a 3 – 4 cm long muscular tube that shields of pathogens from reaching into the uterus with a mucus plug. The plug is produced by glands in crypts of the cervical epithelia and seals the uterus for most of the time of the period, except during the menses and the 4 - 5 fertile days. The pH is about one magnitude less acidic than the vaginal vault and does not change over the menstrual cycle as much as in the vagina (Murta et al. 2005).

The vaginal mould is 7 - 9 cm long and has a ruffled texture making up a surface of about 65 to 107 cm^2 . It is filled with up to 10 ml of acidic and anaerobe vaginal fluid, which has a flow-through of approximately 5 ml/day (probably due to evaporation and physical activity) and is inhabited by a plethora of bacteria (Lai et al. 2009; Owen & Katz 1999).Vaginal fluid consists of vaginal transudite, Bartholin’s and Skenes’s glands secretions, exfoliated epithelial cells, residual urine, endometrial and tubal fluids and mucus, which is secreted by glands in the crypts of the cervical canal (Murta et al. 2005; Owen & Katz 1999). During the menses, the pH shifts to neutral and the blood from the uterus enriches the secretions with oxygen.

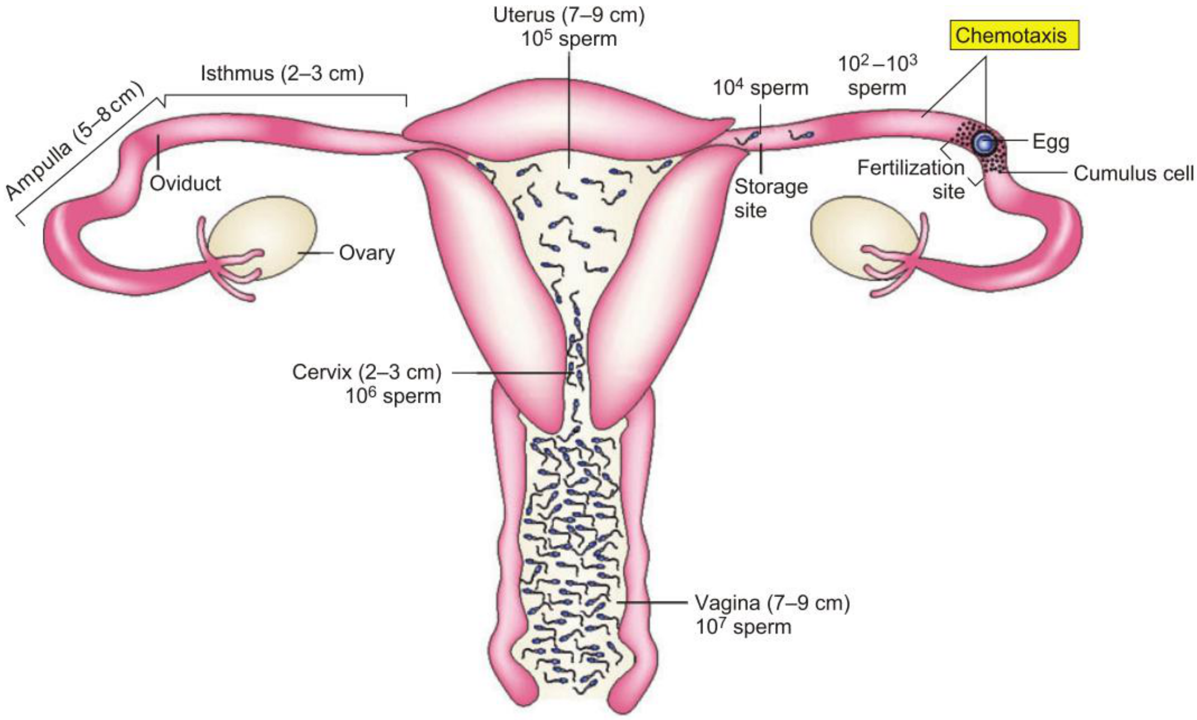

Figure 2: *The human female reproductive tract and its dimensions. Sperm concentrations in the different organs are shown symbolically. Of around 50 million spermatozoa present in the vagina after ejaculation, only a few hundred arrive in the fallopian tubes (here divided into the sections Ampulla and Isthmus). There the oocyte travels from the ovary towards the uterus. In the Ampulla part of the tubes, chemotaxis towards the oocyte and its fertilization take place. (Picture taken from Kaupp et al.,2008)*

Symbiotic bacteria in the vaginal environment

The vaginal microbiome varies among different populations, with the age and monthly over the course of the menstruation cycle and this change is correlated to a different pH level (Bélec et al. 1997) Pre-menopausal fertile women for example have a different vaginal pH than postmenopausal or girls before puberty. The pH during the proliferative phase of the menstrual cycle tends to be higher than the value measured during the secretory phase and its change is strongly correlated to estrogen levels in the blood. Low levels of estradiol and high levels of follicle-stimulating hormone are associated with high vaginal pH values (Gorodeski et al. 2005). During menses, fewer *Lactobacilli* are present, aerophilic bacteria tend to grow better and the pH rises to neutral (Santiago et al. 2011).

The vaginal epithelial cells actively secrete protons to regulate the pH inside the vagina. In vitro studies showed an estrogen dependent reduction from pH 7.4 to 6 in a co-culture of cervico-vaginal-ephithelial cells and vaginal fibroblasts (Murta et al. 2005). However the acidification of the vaginal environment through microbiomes lactic acid production seem to have the bigger impact on the pH level, adjusting it around an average of pH 4 (Boskey et al. 1999; Graver & Wade 2010). However, it is not entirely clear whether the vaginal epithelium or the symbiotic bacteria are the main producers of acids. The pH is a gradient inside the vagina, with a minimum in the mid-portion of the vagina, and a higher pH towards the cervix. Acidification from pH 6 to 4 takes about 30 – 40 h. It is estimated that around 10^8 to 10^9 bacteria populate the vagina (Boskey et al. 1999).

Asian and Caucasian woman have a bacterial flora which is mostly dominated by *Lactobacilli* species and the pH goes down to 4. In Latin and African woman, the pH often ranges around 5 and different sets of bacteria have been observed (Ravel et al. 2011). The predominant *Lactobacilli* in Caucasians are decreasing in frequency by the following order: *Lactobacillus iners, Lactobacillus crispatus, Lactobacillus jensenii, Lactobacillus gasseri, Lactobacillus vaginalis*. *Lactococcus lactis* is also present in most of the analyzed microbiomes, although only in small proportions (Hyman et al. 2005; Ravel et al. 2011).

It was thought that *L. lactis* is well suited as a model organism for vaginal symbiotic organisms to test new genetic constructs. It is fully sequenced, has almost the same codon usage as *Lactobacilli* and is a readily transformable species (Gao et al. 2011). In addition, an in vitro co-culture of vaginal epithelial cells demonstrated *L. lactis* to be a good production host for proteins and thus makes it an interesting candidate for protein delivery into the vaginal vault (Kaushal et al. 2006).

Immune system activity in the vagina and cervix

The vagina is open to the exterior and thus to infection, especially at the time of coitus. To prevent infections, the vagina is equipped with antimicrobial defenses, an acidic pH and immunological responses. Pro-inflammatory chemokine and cytokine expression by vaginal epithelial cells exerts a permanent influence on the constitution of cervical mucus by activating the immune system (Steele & Fidel 2002). A tight control of the vaginal microbiome by the body is supported by the fact that a significant amount of proteins found in the vaginal fluid are related to the immune system (Ravel et al. 2011). Studies show that the vaginal fluid has selective antimicrobial properties against non-resident bacteria such as *E. coli* species (Eggert-Kruse et al. 2000), while the low pH of the vaginal fluid protects against the spread of viruses like human immunodeficiency virus (HIV) (Lai et al. 2009). Furthermore, the self-defense mechanisms of the probiotic bacteria populating the vagina stabilize a community of beneficial commensals. They produce a range of substances, from lactic acid and other organic acids, hydrogen peroxide and antimicrobial polypeptides, especially against *E. coli* (Valore et al. 2002). It was found that lactic acid (LA) bacteria adhere to human epithelial cells (Todorov et al. 2007) and that *L. iners* and *L. crispatus* interact with the hosts innate immune system (Strömbeck 2008). During the non-fertile phase of the menses, the mucus, as a physical barrier, is important in shielding of the inner reproductive organs from infection. It is also tightly controlled by the immune system (Willits & Saltzman 2001). Vaginal insemination stimulates the migration of reactive oxygen species (ROS) emitting leukocytes, particularly neutrophils and macrophages, into the cervix as well as into the vagina. They migrate through midcycle human cervical mucus to protect the cervix against infections (Suarez & Pacey 2006).

Effects of the menstruation cycle on the cervix

The change of the hormone concentrations induces a change in the physiological state of the tissues in the whole reproductive tract. In the vagina, the constitution of symbiotic bacteria changes dramatically and also the properties of the mucus resented in the endocervical canal vary during the ovulatory cycle (Sherman & Korenman 1975). The hydration of the gel increases at midcycle due to elevated progesterone and estrogen levels and the mucus becomes less visco-elastic (Murta et al. 2005). The mucus grid at midcycle for example already affects the diffusion of particles as small as than 100nm in diameter. Even smaller particles (as most viruses are) diffuse freely (Olmsted et al. 2001). Mucus ultrastructure changes during ovulation cycle are accomplished by variations of the splicing patterns of mucin gene transcipts (Gipson et al. 1997; Gipson et al. 2001)and enzymatic modification of the resulting proteins. It is thought that the mucus grid size is determined through exogenous enzyme activity which is controlled by the cervical epithelium (Rutllant et al. 2005). Over the course of the menstruation cycle mucus hydration changes from around 93 % in the mucus before ovulation to 98,5 % between six to four days before midcycle, remaining at a high level for the following days. This is the only time when spermatozoa are able to pass the cervical canal (Katz et al. 1997). Cervical mucus and the possibility to alter its ultrastructure to block spermatozoa has already been a subject of contraception studies (Tang et al. 1999).

Spermatozoa in the female reproductive tract

Vagina

During coitus, about 50 million spermatozoa in a volume usually between 3 to 5 ml per ejaculation are deposited in the anterior part of the vagina (Fig. 2). The flow back of ejaculate out of the vagina was found to happen in 94 % of all studied cases with a median loss of 35 % of volume of the ejaculate (R. Robin Baker 1993). Directly after ejaculation, sperm is a liquid coagulate. It takes 30 to 60 minutes of enzymatic degradation to dissolve the coagulant and turn it into a viscous liquid. This process is called liquefaction The acidic and anaerobic environment of the vagina is temporarily neutralized by buffers in the ejaculate. To prevent immune responses, seminal plasma contains immune inhibitors and protective components that coat spermatozoa (Bélec et al. 1997; Suarez & Pacey 2006).

Cervix

Avoiding the harsh conditions and immune responses in the vagina, the first spermatozoa swim out of the seminal plasma into the cervical canal within minutes after ejaculation (Fig. 2). There, the spermatozoa have to squeeze through the mesh of the cervical mucus. The pore size of the mucus is just big enough during the fertile days of the menstruation cycle to allow some spermatozoa to pass. Through this mechanism, cervical mucus filters out spermatozoa with abnormal morphology and motility and thus only a minority of ejaculated spermatozoa enters the uterus (Suarez & Pacey 2006).

Uterus

Once in the uterus, hormone induced muscular contractions actively transport spermatozoa towards the fallopian tube containing the oocyte. Theoretically the uterine cavity could be traversed by spermatozoa within 10 min, at a swimming speed of about 5 mm/min. Observation of spermatozoa distribution in the uterus and fallopian tubes after surgical excision of the organs after intercourse showed that cells arrive in the fallopian tubes already within 10 min after insemination and can remain there for up to 16 days. Spermatozoa were discovered all over the fallopian tubes, however, in those studies the motility of the cells was not assessed (Suarez & Pacey 2006). Of a number ranging in the magnitude of 10^5 cells in the uterus, only a few thousand spermatozoa swim through the uterotubal junctions to reach the Fallopian tubes (Fig. 2)(Kaupp et al. 2008).

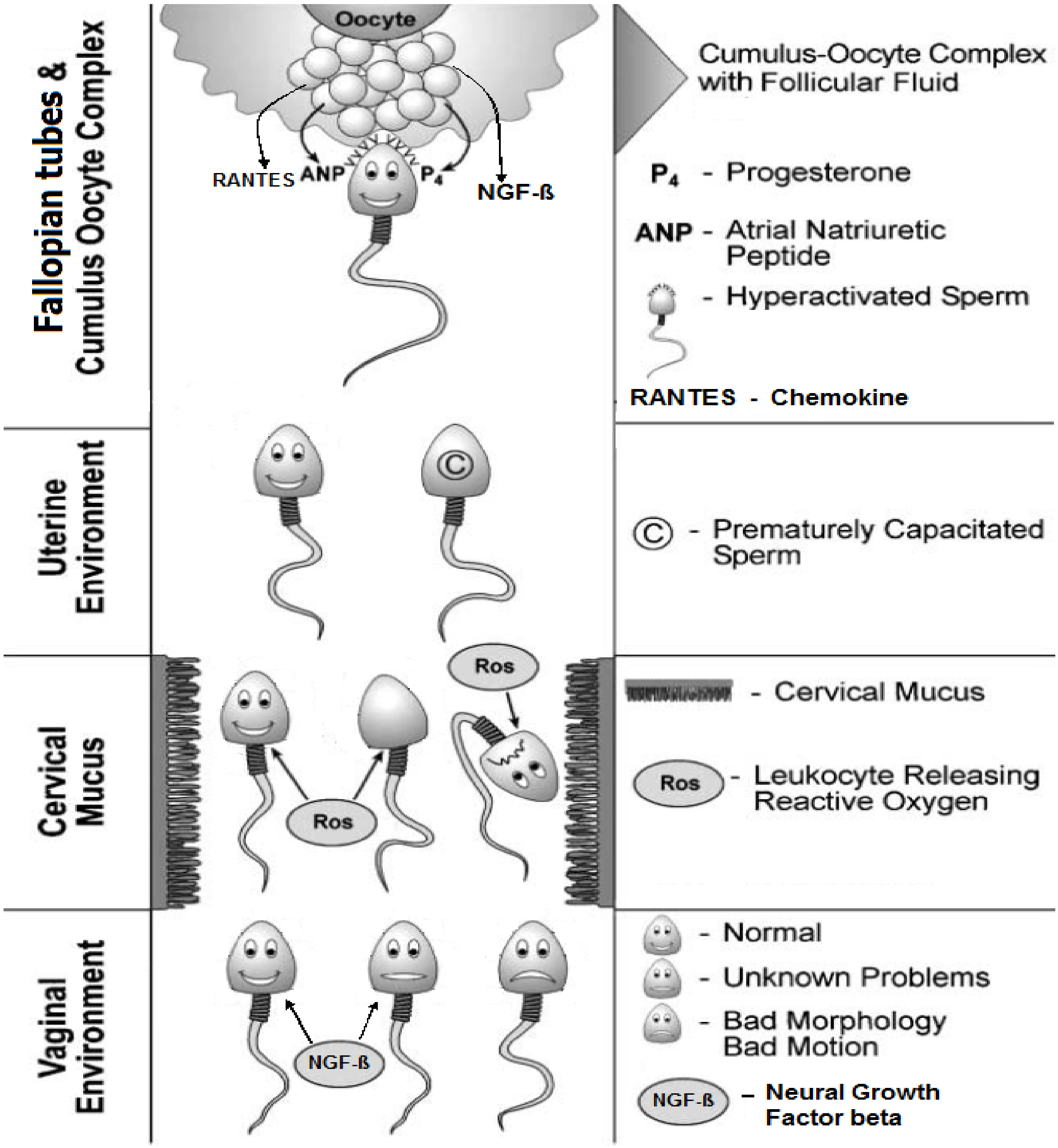

Figure 3: *Progression of spermatozoa through the female reproductive tract. In the vaginal environment spermatozoa are surrounded by seminal plasma which contains substances protecting the cells from the acidic vaginal fluid. Among the substances, NGF-ß is found to influence spermatozoa. Only a fraction of the cells have a normal motility and are able to swim out of the seminal plasma into the cervix. Some have a bad morphology, motion or unknown problems and thus cannot progress further into the reproductive tract. In the* ***cervix****, cells travel through the cervical mucus and are exposed to ROS emitted by cells of the immune system that have a pro-capacitating influence on normally functioning spermatozoa and a deleterious influence on dysfunctional spermatozoa. The passage time through the* ***uterus*** *is likely to be short due to muscle contraction transporting the cells towards the fallopian tube containing the fertile oocyte. The influence of the uterine environment causes dysfunctional cells to capacitate prematurely, further homogenizing the spermatozoa population. In the* ***fallopian tubes****, spermatozoa capacitate and become hyperactivated. The oocyte and cumulus complex emits hormones, among them Progesterone, RANTES, ANP and NGF-ß. Spermatozoa are chemotactically guided towards increasing gradients of the hormones. Upon contact with the zona pellucida they undergo the acrosome reaction and ultimately one spermatozoon fertilizes the oocyte. (Figure modified after De Jonge, 2005*

Fallopian tube and oocyte-fertilization

Attached to the tubal epithelium (Fig. 2), spermatozoa are maintained in a beneficial chemical environment and are able to survive for a up to five days waiting for the oocyte to hatch and travel past. After receiving hormonal signals emitted by the oocyte and the surrounding cumulus cells, spermatozoa undergo capacitation. Capacitation is a gradual and irreversible physiological maturation process necessary to render spermatozoa able to travel towards the tubal ampulla and thus to fertilize the oocyte (Fig. 2). It is assumed that spermatozoa detach from the epithelia and swim through the viscous tubal mucus by a mechanism called hyperactivation (Suarez & Pacey 2006).

Hyperactivation is a change in flagellar beating that typically involves an increase in the flagellar bend amplitude. In studies with artificial mucus containing viscoelastic solutions of long-chain polyacrylamide or methylcellulose, hyperactivated spermatozoa progressed far more effectively than non-hyperactivated. Hyperactivation as it is observed under the microscope displays a rather erratic and circular movement pattern. However, the swimming behaviour of spermatozoa in aqueous solutions could be quite different from the more viscous fluids inside the female reproductive tract. Therefore, the in vitro observations might be an artifact, or, at least not the natural mode of movement. The swimming pattern itself might be modulated by chemotactic signals so that spermatozoa can swim towards the oocyte more effectively. However, little is known about what the exact trigger of hyperactivation is, how and to what extent it is linked to chemotaxis and capacitation, its timing in the course of travel within the female reproductive tract and its individual physiological role in the fertilization process (Kaupp et al. 2008; Suarez 2008). The literature is not always clear whether hyperactivation and the ability to respond to chemoattractants are part of the capacitation process or not. There are indicators that hyperactivation and capacitation processes are independend from each other, or at least are activated by a diverging pathway (Suarez 2008). However it seems to be current state of knowledge, that the capacitation process leads to chemoattranct receptibility (Armon & Eisenbach 2011; Suarez & Pacey 2006).

Chemotaxis and Chemokinesis

The last few centimeters the spermatozoa use chemotaxis to swim towards the hormone emitting cumulus cells. After reaching the zona pellucida of the oocyte, spermatozoa undergo the acrosome reaction and finally fuse with the oocyte plasma membrane (Suarez & Pacey 2006). Spermatozoa chemotaxis is the spatially directed movement towards an increasing gradient of chemoattracts emitted by the cumulus cells and/or the contained oocyte (Fig. 3). Chemokinesis is the change of the spermatozoon movement pattern in response to chemoattractants which does not necessarily involve a spatially directed movement. Several chemoattractants have been found for human spermatozoa and the utilization of those compounds for interfering in spermatozoa motility is a promising approach for future contraceptives (Spehr et al. 2004). To date, several small organic substances, short peptides and proteins are known to induce chemotaxis. The best known example is the hormone progesterone. At very low concentrations it chemotactically guides mammalian spermatozoa towards the cumulus oophorus (Teves et al. 2006).

Peptide chemoattractants bind to surface receptors on spermatozoa and steroid hormones act as second messengers to induce intracellular signalling cascades resulting in a periodic change of cytoplasmatic Ca^{2+} concentration (Yoshida & Yoshida 2011). However, the relationship between intracellular spatiotemporal Ca^{2+} concentrations and the chemotactic response of a spermatozoon flagellum is poorly understood. Intracellular processes are not considered in this study. Extensive literature research screening for proteinous chemoattractants which can be artificially produced in prokaryotic cells revealed the following chemotactically active proteins (Fig. 3): Atrial natiuretic peptide (ANP), RANTES, neural growth factor beta (NGF-ß) and nitric-oxide (NO) – an inorganic substance toxic to spermatozoa, which can be produced by nitric-oxide-synthases and therefore was taken into consideration. ANP is present in follicular fluids and ANP receptors have been found on spermatozoa (Armon & Eisenbach 2011). It enhances spermatozoa swimming speed and acts as a chemoattractant (Zamir et al. 1993). RANTES, a chemokine also known as CCL5, is present in male and female genital tract fluids and increased levels are correlated to infertility, making it an interesting target (Isobe et al. 2002). Freshly ejaculated human spermatozoa express RANTES receptors (CCR3 and CCR5) and a chemoattractive effect for RANTES has been found (Barbonetti et al. 2008). Exogenous reactive oxygen species such as nitric-oxide can cause human spermatozoa to capacitate prematurely. They are emitted in the cervix by cells of the immune system as means of defense against pathogens and can damage spermatozoa significantly by oxidising the membrane. ROS play a major factor in male infertility (Villegas et al. 2003). NGF-ß is a highly conserved protein in mammals (Ratto et al. 2012) and a component of the seminal plasma (Heinrich & Meyer 1988) It stimulates spermatozoa motility and the acrosome reaction in a time- and dose-dependent manner (Jin et al. 2010) and has been shown to influence viability and apoptosis of bovine spermatozoa. It therefore could play an important role in male fertility (Li et al. 2010).

Scientific approach

This chapter places the endeavor of creating a new contraception method in a wider perspective. To move into the field of applied development it seemed reasonable to use the latest life science concepts such as an approach of system biology and the engineering idea of synthetic biology.

The concept of synthetic biology is to turn biological science into biological engineering by implementing product development procedures. To develop a concrete product prototype, an idea is required at first, followed by a concept of how to approach the idea. Lastly a concrete plan of actions and experiments has to be developed which results in a functional prototype that can be tested, evaluated and optimized. Among the considerations about the concept, questions why the product is needed, how it should work, whom to address with it, how it can be realized, who is going to construct it and what is the budget and time line to realize it need to be considered (Wozniak 2011). This thesis aims to address the first two steps in this development by describing the idea of the bacterial contraception and by working out a methodological approach towards the realization of a prototype. To develop an functioning device is beyond the scope of a one year project and therefore the idea of bacterial contraception remains a fiction. However, the fiction sets the direction for present actions. While this teleological engineering approach was to determine the direction of the experiments, a systems biology concept was used in the design of experiments. Systems biology uses high throughput methods and computational analysis to generate and understand an amount and diversity of data impossible to manually process or interpret. The approach promises to achieve a broader, more detailed and thus more realistic overview of biological processes than it was possible in the past, where a strict reductionism was the only applicable logic due to technical limitations. In itself, the method in system biology is still reductionist, since it has physical and rational limitations due to a non-perfect machinery, systematic errors and a limited understanding of biological processes. Nevertheless, the strict reductionism can be opened up towards an approach that tries to incorporate as much evidence as practically possible to collect and thus allow to draw a more realistic picture of the biological reality. I tried to implement that thinking in the design of my experiments. In practice, the development of the idea was a vast and divergent literature research, trying to identify all relevant and known aspects of molecular processes of conception, which could be influenced on a cellular and subcellular level. Regarding the experimental setups a trial and error development was combined with the attempt to reduce the number of type of experiments to keep costs down, while at the same time maximizing the data output of those methods by creating numerous combinations and variations within a type of experiment. To process and interpret this large amount of data, novel computational tools had to be developed.

Working hypothesis

Principally, it seemed possible to use bacteria to prevent spermatozoa from progressing into the uterus and / or to render them incapable of fertilization. The natural vaginal symbiotic bacteria could be genetically engineered to express and secrete proteins to interfere with spermatozoa chemotaxis to disorient, alter chemokinesis and eventually kill the cells. The chemoattractant proteins could create a gradient with a high concentration in the vaginal environment, decreasing towards the uterus. Chemotactically active spermatozoa would be directed away from the uterus, capacitate prematurely or die off and thus decrease the chance of fertilization of the oocyte. Other mechanisms, such as secreted proteins altering the mucus consistency in the cervical canal to block the passage of spermatozoa, as well as medical functionality, e.g. expression of antibodies against diseases are also thinkable. To test bacterial protein expression and physiological effects on spermatozoa, a media simulation to grow the symbiotic bacteria under realistic conditions was necessary. Two working hypothesis were defined. Fist: It is possible to formulate a realistic growth media based on data available in literature. Second: Spermatozoa can be significantly disturbed in their physiological functionality by creating an artificial source of chemoattractants mimicking the effects of the oocyte. Based on these assumption, a methodological approach was worked out and experiments designed to verify the hypothesis.

Methodological approach

To find out, whether it is possible to create such a hypothesized bacteria-mediated contraceptive, the idea was dissected into two phases of prototype development (Fig. 4). The goal in Phase I is the development of a testing environment and the identification and preparation of all relevant elements for the prototype construction. To achieve this goal, Phase I was again split into three subtopics which needed to be addressed separately, before a prototype can be build. The Phase II would then contain the assembly of the single elements into one prototype, followed by detailed analysis of the functionality and effectiveness in the in vitro system. Only a fully assembled prototype, which consists of pre-tested parts in a clearly defined test-setup can shed a light on whether bacteria-mediated contraception is possible or not.

For Phase I the following threads were developed and worked out as a logical guideline for this thesis work. A detailed description of the practical procedure is depicted in figure 4:

1. How can spermatozoa be chemotactically irritated, rendered physiologically inactive and physically blocked? How should a testing environment for spermatozoa look like and what parameters need to be observed?
2. Which of the symbiotic bacteria are good candidates for genetic manipulation, protein expression, secretion and cultivation? How should a testing environment for manipulated bacteria look like and what parameters need to be observed?
3. How should a genetic construct that provides the desired features look like? How can the sperm-test system be combined with a bacterial test system to assess the effect of genetically manipulated bacteria on spermatozoa?

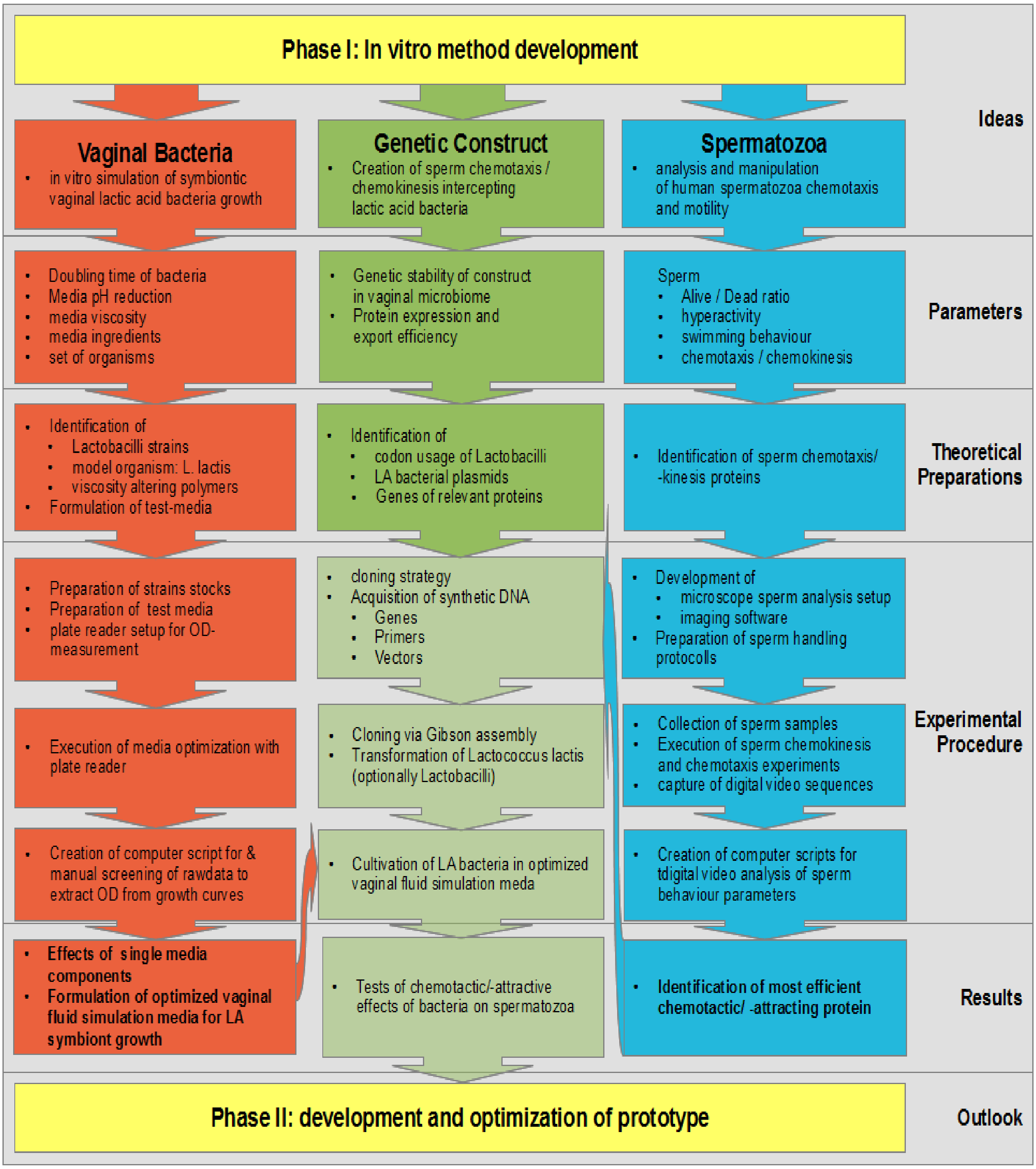

Figure 4: *Procedure during Phase I of the project development. Phase I is divided into three main topics: the development of a growth media for symbiotic vaginal bacteria, the description and construction of a plasmid encoding the contraceptive functions and the development of a test system to evaluate the effect of substances on the migration pattern of spermatozoa. Each topic is compartmentalized into a subset of steps shown in the grey boxes. The results of the vaginal and spermatozoa test systems influence the choices of sequences for the genetic construct (indicated by the arrows pointing towards the middle column). A successful construction and testing of the construct leads to Phase II of the development, entailing the assembly of the first prototype for bacterial contraception, its evaluation and optimization.*

During the development of the in vitro tests the maxim was to work with as simple and affordable equipment as possible, in order to provide easy access for future studies elaborating upon the idea. To find out if and how strong spermatozoa react to a certain chemoattractant, a microscopy setup was designed with an automated digital video analysis software to test for chemotactic behavior. To allow for tests of the expression of chemoattractants by genetically manipulated symbiotic vaginal bacteria under circumstances simulated as realistic as possible, a defined media for vaginal fluid had to be experimentally evaluated. For the in vitro study, bacterial strains and growth conditions as well as chemotactically relevant proteins had to be identified and tested for effectiveness in the developed test setup. Subsequently, a testing system for spermatozoa chemotaxis and chemokinesis and the genetic construct to make the bacteria emit chemoattractants needed to be designed. The phase II will consist of the assembly of the single elements into one prototype followed by detailed analysis of the functionality and effectiveness in the in vitro system. The final goal of the phase II would be to develop and optimize a fully functional prototype of symbiotic lactic acid bacteria which are able to intercept the chemotaxis of a significant percentage of spermatozoa.

Material

Vaginal Bacteria

Microorganisms

Lactobacillus iners	DSM No.: 13335	DSMZ, Germany
Lactobacillus crispatus	DSM No.: 20584	DSMZ, Germany
Lactobacillus gasseri	DSM No.: 20604	DSMZ, Germany
Lactobacillus vaginalis	DSM No.: 5837	DSMZ, Germany
Lactobacillus jensenii	DSM No.: 20557	DSMZ, Germany
Lactococus Lactis MG1363	from Christian Solem	CSM, DTU

***Table 1**: List of symbiotic LA bacteria commonly found in the vaginal vault*

Media Compounds	**Product No.**	**Company**
Inorganic		
NaCl	A4661	AppliChem
KCl	P9541	Sigma-Aldrich
$MnCl_2$	203734	Sigma-Aldrich
$MgSO_4$	230391	Sigma-Aldrich
$NaHCO_3$	401676	Sigma-Aldrich
K_2HPO_4	P9666	Sigma-Aldrich
KH_2PO_4	Y1251	Sigma-Aldrich
$CaCl_2 * 2 H_2O$	C1016	Sigma-Aldrich
$MnSO_4 x H_2O$	M7634	Sigma-Aldrich
Micronutrients Mix		All Sigma-Aldrich
Organic		
$(NH_4)_2$ citrate	25102	Sigma-Aldrich
deoxynucleosides A G T C		Sigma-Aldrich
Dextrose	A1349	AppliChem
Hemin	H9039	Sigma-Aldrich
Inosine	I4125	Sigma-Aldrich
Lactic acid	W261114	Sigma-Aldrich
Oleic acid	O1008	Sigma-Aldrich
Resazurin	R7017	Sigma-Aldrich
Sodium acetate	S2889	Sigma-Aldrich
Sodium pyruvate	P2256	Sigma-Aldrich
Tween 20	P1379	Sigma-Aldrich
TWEEN 80	P1379	Sigma-Aldrich
Uracil	U0750	Sigma-Aldrich
Urea	U5378	Sigma-Aldrich

(continues on next page)

Vitamins		
Kao and Michayluk Vitamin Solution	K3129	Sigma-Aldrich
Vitamin K_1	V3501	Sigma-Aldrich
Vitamin SA mix		All Sigma-Aldrich
MEM Vitamin Solution (100X)	11120-037	Life technologies
Proteins		
Glycogen (bovine)	G0885	Sigma-Aldrich
Mucin (porcine stomach Type II)	M2378	Sigma-Aldrich
Bovine serum albumin, fraction V	A4503	Sigma-Aldrich
Rich Media Mixes		
Casein peptone, tryptic digest	1.07213	Merck KGaA
Difco Beef Heart for Infusion	213210	Becton, Dickinson and Company
M.R.S. broth	CM0359	Oxoid
Yeast extract	1.3753	Merck KGaA
Amino Acids (A.A.)		
Alanine	1.01007	Merck KGaA
Arginine	1.01542	Merck KGaA
Asparagine	A8381	Sigma-Aldrich
Aspartic Acid	1.00126	Merck KGaA
Glutamic Acid	G1626	Sigma-Aldrich
Glutamine	1.00289	Merck KGaA
Glycine	G7126	Sigma-Aldrich
Histidine	1.04351	Merck KGaA
Isoleucine	1.05362	Merck KGaA
Leucine	L8000	Sigma-Aldrich
Lysine	1.05700	Merck KGaA
Methionine	1.05707	Merck KGaA
Phenylalanine	1.07256	Merck KGaA
Proline	P0380	Sigma-Aldrich
Serine	S4500	Sigma-Aldrich
Taurine	T8691	Sigma-Aldrich
Threonine	1.08411	Merck KGaA
Tryptophan	T0254	Sigma-Aldrich
Tyrosine	T3754	Sigma-Aldrich
Valine	1.08495	Merck KGaA
Cystein	1.02838	Merck KGaA

Table 2: *Compounds used in various media for symbiotic lactic acid bacteria.*

Spermatozoa

7 human sperm samples		Nordic Cryobank ApS
Atrial Natriuretic Factor	H-2095	Bachem, Swiss
Eosin Y	E4009	Sigma-Aldrich
Glass capillaries	3520-050	CM Scientific
NGF-ß, human, recombinant (*E. coli*)	C-66129	PromoKine
Nigrosin	43925	Sigma-Aldrich
Nutrient Mixture F-10 Ham	N6908	Sigma-Aldrich
Progesteron	A0677	Applichem
RANTES protein	CHM-328	ProSpec,Germany
S-Nitrosoglutathione	N4148	Sigma-Aldrich

***Table 3:** Compounds used in the spermatozoa chemokinesis experiments*

Methods

Vaginal bacteria

Plate reader analysis

For analysis of the growth rates, prepared media were distributed to a 96 well plate with a flat transparent bottom. The bacterial cultures in the exponential phase, which were grown overnight from -80 °C glycerol stocks in reduced MRS, were adjusted to an optical density (OD) of 0,2 at 630 nm and then inoculated by adding 5 µl of the culture into each well. A breathable foil (Breathe-Easy gas permeable sealing membrane for microtiter plates, Diversified Biotech) was used to seal the 96 well plate and maintained it sterile during the measurement in the plate reader. The reader Biotec elx 808 was programmed to maintain 37 °C and to make an OD measurement at 630 nm every 5 min for a total duration of 20 h. Before each recording, the plate was shaken vigorously for 10 s to create a homogenous solution. The resulting OD values of all measurements were saved in csv files for further analysis.

Bacteria cultures

DSMZ bacteria cultures were obtained either directly from DSMZ (*L. iners*) or regrown from prepared glycerol stocks, which were available at the DTU CSM institute. *L.iners* freeze dried culture was cultured in PYG-Media (Tab. 7) under a low oxygen atmosphere without shaking at roomtemperature. *L. crispatus* was cultured in PYG-Media. *L. vaginalis*, *L. jensenii* and *L. gasseri* were grown in reduced MRS media (Tab. 7). Cystein-HCl was always added to the media and then sterile filtered right before start of the cultures. *L. lactis* GM1363 was cultured in GM17 media. All cultures except *L. iners* were grown overnight in sealed falcon tubes at 37 °C without stirring. Liquid cultures were streaked out on agar-plates of the corresponding media and incubated overnight. New glycerol stocks were prepared from single colonies and grown in reduced MRS media at 37 °C overnight. When the cultures were in their exponential growth phase, the OD was adjusted to 0,2 for all cultures and glycerol added to a total of 20 % volume. Glycerol cultures were kept as stocks at -80 °C for further experimentation.

Vaginal fluid simulation preparation

The basis for the media optimization experiment was the defined growth media for *L. plantarum* after Liu et al. 2011 (Liu et al. 2011) further referred to as Hart mix. A 25-fold overconcentrated solution of the basic Hart-media ingredients (Basic Hart mix, see Tab. 5) was prepared in bidest water and then autoclaved. Single components, which were to be tested in different concentrations (see Supplementary Information), were dissolved in water as separate, overconcentrated stocks. Cystein, Tyrosin, Glucose, TWEEN 20, Vitamins and Glycogen were sterile filtered and Mucin autoclaved. Amino Acid mixes, without Cystein and Tyrosin, were prepared as 10X overconcentrated stocks. All components were mixed under sterile conditions. Subsequently, the required volumes of all single component stocks (except for Cystein and Tyrosin) and a fraction of the Basic Hart mix were combined to to meet the final concentrations as indicated in Tab. 1 in the supplementary information. Autoclaved bidest water was added to reach the calculated final dilution of all components. The pH was adjusted by taking small media samples out of the flask in a sterile manner, measuring them with a pH-meter and then step by step adding small amounts of sterile filtered HCl or NaOH until a pH of 6,5 was reached. The created media stocks were kept in the fridge for up to 3 weeks. Sterile Cystein and Tyrosin solution was added to the Hart mixes at the day of the experiment.

Basic Hart mix	**final concentration**	
NaCl	3,5	g/l
KCl	1,5	g/l
$MnCl_2$	0,05	g/l
$MgSO_4$	0,3	g/l
$NaHCO_3$	0,04	g/l
Hemin	0,0025	g/l
K_2HPO_4	1,7	g/l
KH_2PO_4	1,4	g/l
BSA	2	g/l
Urea	0,5	g/l
Sodium pyruvate	0,1	g/l
Sodium acetate	1	g/l
Uracil	0,5	g/l
Inosine	0,2	g/l
Single Components		
Cystein	0,5	g/l
Tyrosin	0,2	g/l
Glucose	5	g/l
Tween 20	0,222	ml/l
Amino Acid Hart-mix	1	factor
Kao & Michaylak Vitamins	0,5	factor
Vitamin K_1	0,005	g/l
Glycogen	1	g/l
Mucin	3	g/l

Table 5: *A recipe for a defined vaginal fluid simulation by Hart (further referred to as Hart media). For clarity, the ingredients were grouped into two categories: "Basic Hart mix", consisting of a set of ingredients which were not altered in the media optimization tests, and "single components", which were varied individually in contrentration or replaced by an alternative.*

Vitamin solutions	**Kao & Michaylak Vitamins**	**SA Vitamins**	**MEM Vitamins**
final concentration	**mg/l**	**mg/l**	**mg/l**
p-Aminobenzoic Acid	0,01	-	-
L-Ascorbic Acid	1	-	-
D-Biotin	0,005	0,00001	-
D-Calcium Pantothenate	0,5	0,0001	1
Choline Chloride	0,5	-	1
Cyancobalamin	0,01	-	-
Folic Acid	0,2	0,0001	1
Myo-Inositol	50	-	2
Nicotinamide	0,5	-	1
Pyridozal	-	0,0002	1
Niacinamide	-	0,0001	-
Riboflavin	-	0,0001	0,1
Thiamin	-	0,01	1
Vitamin K_1	5	-	-

Table 4: *A comparison of the three vitamin mixes which were tested in the media optimization experiments.*

Micronutrients	**final concentration [M]**
$(NH_4)_6(MO_7)_{24}$	3 * 10^{-6}
H_3BO_3	4 * 10^{-4}
$CoCl_2$	3 * 10^{-5}
$CuSO_4$	10^{-5}
$MnCl_2$	8 * 10^{-5}
$ZnSO_4$	10^{-5}

Table 6: *Micronutrient mix ingredients for Lactobacilli cultivation.*

	PYG	reduced MRS
Final concentration	**g/l**	**g/l**
$(NH_4)_2$ citrate	-	2
Beef extract	5	-
$CaCl_2$	0,01	-
Casein peptone	5	10
Dextrose	5	20
Hemin*	0,1	-
K_2HPO_4	2,04	2
KH_2PO_4	0,04	-
L-cystein*	0,5	0,5
Meat extract	-	10
$MgSO_4$	0,02	0,2
$MnSO_4$	-	0,05
NaCl	0,08	-
$NaHCO_3$	0,4	-
Peptone	5	-
Resazurin*	0,001	-
Sodium acetate	-	5
TWEEN 80	1	1
Vitamin C*	-	-
Vitamin K_1*	0,2	-
Yeast extract	10	5

***Table** 7: PYG and reduced MRS media ingredients. Substances marked with * were prepared as separate stocks, sterile filtered and added after autoclavation. Each media was adjusted to 1l with bidest water and a pH 6.5 and autoclaved.*

Amino Acids (A.A.)	**Hart A.A. mix**	**SA A.A. mix**
Final concentration	**g/l**	**g/l**
L-Alanine	0,32	0,3
L-Arginine	0,08	0,2
L-Asparagine	0,4	0,1
L-Aspartate	0,76	-
L-Cysteine	0,5	0,2
L-Glutamate	0,36	0,3
L-Glutamine	0,4	0,1
Glycine	0,2	0,2
L-Histidine	0,16	0,5
L-Isoleucine	0,12	0,1
L-Leucine	0,2	0,1
L-Lysine	0,2	0,2
L-Methionine	0,04	0,1
L-Phenylalanine	0,04	0,2
L-Proline	0,28	0,3
L-Serine	0,12	0,3
Taurine	0,1	-
L-Threonine	0,12	0,2
L-Tryptophan	0,04	0,1
L-Tyrosine	0,2	-
L-Valine	0,68	0,1
L-Cystine	0,5	-

***Table 8:** Amino acid mixes and concentrations for Lactobacilli cultivation*

Spermatozoa

Collection of sperm samples and initial screening

Seven sperm samples were obtained from the company European Sperm Bank in Copenhagen. To recruit donors, men who approached the company the first time were asked by the technician to take part in a scientific study. The donors signed an informed consent informing them about the participation in an anonymous study about sperm fertility at the Denmark Technical University, that no one but the scientist had access to the sample and that the sample was going to be destroyed after the experiment. Further, the following information was requested in written form from the donor: age, hours of sexual abstinence before donation, date and time of collection, completeness of the sample. No other personal data from the donor was taken, so that the donors stayed anonymous. The signed forms were collected by the company and later handed over to the Institut für Ethik und Geschichte der Medizin, University of Freiburg, for archivation. The samples were produced through masturbation and collected by the donor in a 50 ml falcon tube. After collection, the technician of the company assessed the following information of the samples directly: Volume, average cell count, average motility, progression, viscosity. The volume was assessed by weighing the sample and dividing it by 1 g/ml. The amount of cells was determined by manually counting a diluted sample under the microscope. The motility and progression was intuitively determined by visual examination of the spermatozoa under the microscope. The viscosity was assessed by measuring the length of the thread formed by a drop of semen liquid when dispensing it out of a pipette.

Transportation and liquefaction of sperm samples

To prevent cell damage through a dropping pH and temperature changes, the collection of the samples were kept in an insulated and airtight container during transportation from the company to the laboratory. After 60 min of transportation, the samples were liquefied and ready for further processing. Liquefaction is a natural process caused by enzymatic degradation of semen liquid starting immediately after ejaculation. The enzymes are constituents of the seminal plasma.

Eosin-nigrosin test

To test sperm samples for the ratio of dead and alive cells the Eosin-Nigrosin staining technique was employed. Preparation of the stain: 0,67 g of Eosin Y and 0,9 g of NaCl were dissolved in 100 ml bidest water and heated for 2 h to approximately 80 °C. Then 2,5 g of Nigrosin (colour index 50420) were added and the suspension shortly boiled, cooled to room temperature and filtered through a filter paper to remove coarse and gelatinous precipitates. The suspension was stored at room temperature in a sealed and light protected glass bottle. To examine a sperm sample, a drop of liquified semen was mixed on a glass slide with a drop of Eosin-Nigrosin suspension and then evenly spread over the entire slide by dragging a second glass slide over the surface. Using the adhesion force of the liquid to the glass slide made sure that the spermatozoa were not destroyed in the process. The smear was then air dried and analyzed with a brightfield microscope using a 100X objective with oil immersion. The numbers of stained (dead) and unstained (alive) spermatozoa were counted to a total of two times 200 cells. The average of live cells of each count was calculated and compared with the second count. The acceptability of difference between the two counts as well as the indicators for the liveliness of a cell are according to the WHO standards (World Health Organisation 2010).

Direct sperm swim up procedure

To separate spermatozoa from semen liquid and other cells as immune cells, the swim-up procedure was used (World Health Organisation 2010). Fresh semen samples at room temperature were distributed into 50 ml falcon tubes in 1 ml fractions. 1,2 ml of supplemented F-Ham media was carefully layered over the semen liquid. 50 ml of Ham's F-10 media was supplemented with 300 mg highly purified Bovine Serum Albumine (BSA), 1,5 mg of Sodium Pyruvate, 0,18 ml of Sodium Lactate (60% (v/v) syrup), 100 mg of Sodium Bicarbonate and subsequently sterile filtered and stored in a fridge. The tubes were then positioned in a 30 ° angle to allow for a large surface between the two liquid layers and put into an incubator with 37 °C and 4 % CO_2 for 60 min. Motile spermatozoa swam up into the F-Ham media layer, while immotile cells collected at the bottom of the tubes. After the process, the tubes were slowly brought into upright position and 1 ml of the F-Ham layer was carefully sucked up with a cut tip of a 1 ml pipette and transferred to a new tube. 50 µl of the solution was then mixed in a separate tube with 50 µl of 1 M NaCl solution to immobilize the spermatozoa with an osmotic shock. The concentration of the extracted cells was assessed with a Neubauer-Türk counting chamber and the collected cells adjusted to 10^5 cells/ml. To prevent cell damage and stress a further washing step with centrifugation was omitted. The tubes were then put back into the incubator for 60 min more to allow them to capacitate (Schwane 2006).

Imaging and computational analysis

For imaging the spermatozoa a Olympus Microscope CKX41, inverted bright field with an Altra 20 CCD camera mounted on a trinocular without rotatable prism was used. A computer with Windows XP and the software CellSens Standard was used to capture the sequences and operate the camera. Swim-up purified semen samples were put on the newly developed imaging slide and after 5 min placed on the microscope table to allow bulk flow of the fluid to cease. The light source was a halogen lamp, the light intensity set to maximum and the condensor wide open. A phase contrast filter was inserted into the light path. Greyscale videos were recorded on the computer with 8 Gb RAM and 500 GB harddisk space at a rate of 33 frames per second (fps) with a resolution of 400x300 pixels. After recording they were saved as tiff stacks. The CASA plugin (Wilson-Leedy & Ingermann 2007) for ImageJ http://rsb.info.nih.gov/ij/index.html was used to analyze the acquired videos of the sperm samples. The plugin was obtained from http://rsbweb.nih.gov/ij/plugins/casa.html and installed according to the online description. The videos loaded into the software ImageJ and the threshold adjusted to turn them monochrome image stacks with a white background and spermatozoa heads appearing as black spots. Then the CASA plugin was operated using a newly developed macro for ImageJ.

Results

Vaginal Bacteria

In vitro vaginal fluid formulation

Most of the existing formulations of vaginal fluid simulations focused on chemical or physical properties of the liquid (Marques et al. 2011; Owen & Katz 1999; Geshnizgani & Onderdonk 1992; Chervaux et al. 2000; Dorr et al. 1982). To test the growth of the most prevalent vaginal symbiotic bacteria strains *L. iners*, *L. crispatus*, *L. gasseri*, *L. vaginalis*, *L. jensenii* and *L. Lactis* realistically, a vaginal fluid simulation had to be designed. The first attempt to simulate bacteria growth was done by Silvina et. al. 2007. Liu et. al., 2011 formulated the most recent vaginal fluid simulation to cultivate genetically modified *Lactobacillus plantarum* and detect its protein secretion (further referred to as "Hart mix", see Tab. 5). Based on previous attempts further substances related to improved growth of the common vaginal symbiotic strains were identified from literature. As the most complete formulation, the Hart mix was chosen as a basic list of ingredients to which new substances were added, the concentrations of proposed ingredients varied, or in part replaced by substances which seemed to be more likely to be found in vivo. All five strains were grown in each new media formulation and all experiments were independently repeated four times, whereby the results that yielded three consistent replicates were selected to calculate average doubling times. The experiments were done to find out which of the bacteria strains are suitable for in vitro simulation of vaginal fluid symbionts growth and in the same time identify ingredients to adapt the Hart mix formulation to those strains.

Data processing with R script to calculate doubling times

To asses and compare the efficiency of bacterial growth in the formulated media, OD measurements were done with a plate reader (see Methods). In bacterial growth, the parameter which can be reproduced most reliably is the doubling time. To cope with the big amount of OD data of a total of 95 experiments, it was necessary to automate the data analysis.

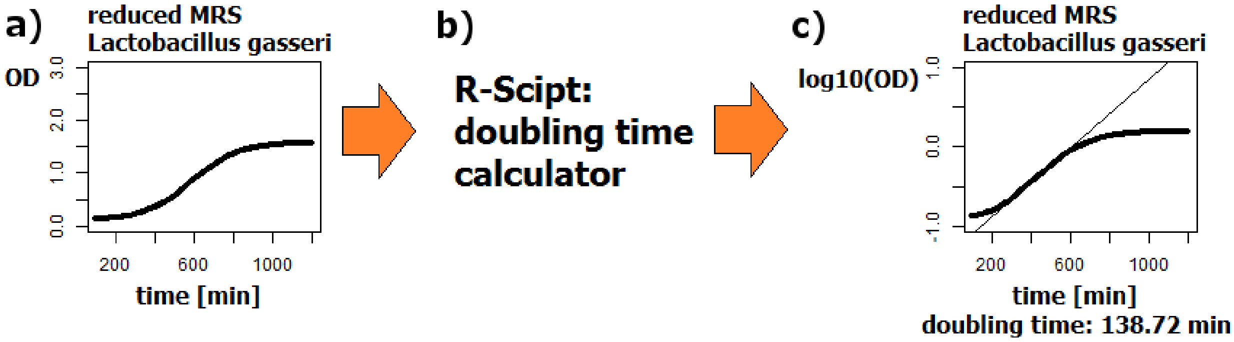

Figure 5: *Procedure to calculate doubling times of symbiotic bacteria in various vaginal fluid simulations* ***a)*** *example of a plate reader recording: The OD values of Lactobacillus gasseri growing in reduced MRS medium plotted against time.* ***b)*** *R script: The growth curve datapoints were loaded and the period of exponential growth identified by searching for the shortest doubling time (see supplementary information).* ***c)*** *output of the R-script: A semi-logarithmic display of the growth curve with the best fit abline of the steepest exponential phase with the smallest doubling time. R-Script code and all results see supplementary information.*

A script in the programming language R was written to readout the csv files containing the OD data (see supplementary information). For each experiment, a floating frame of 14 points, equaling an observation time of 2 h, was iterated through the dataset with an increment of 1 per iteration. Of each set of the 14 values, the $\log_{10}$ of the OD values was calculated and the linear fit function of the R-language applied to find the slope of the best fit straight line (p-value > 0,95 and R-squared > e^{-10}). The doubling time was calculated by dividing the slope by $\log_{10}(e)$. After all possible doubling times for one growth curve were calculated, the smallest is determined and a logarithmic chart generated, displaying the OD values over time, the straight line and doubling time for the smallest slope (Fig. 5). The average doubling of the three independent experiments and the standard deviation was calculated in Excel.

Vaginal fluid optimization results

Initial tests showed that the growth curves for *Lactobacilli* and *L. lactis* in the media suggested by the literature (see supplementary Fig. 1) showed a lag time of several hours, so the total time of data acquisition with the plate reader was set to 20 h. It also became evident, that all strains except *L. iners* can be grown in reduced MRS medium. (see Methods) *L.iners* was dropped from the choice of strains, as it was prafctically not feasible to use a strictly anaerobic strain (Jakobsson 2008) for experimentation. *L. lactis* grows very slow in both media, as a normal doubling time would be around 30 minutes. All other strains can be grown at reasonable speed in reduced MRS and Hart media (Fig. 6), while *L. gasseri* and *L. jensenii* replicate about 40 % slower in reduced MRS than in the Hart mix.

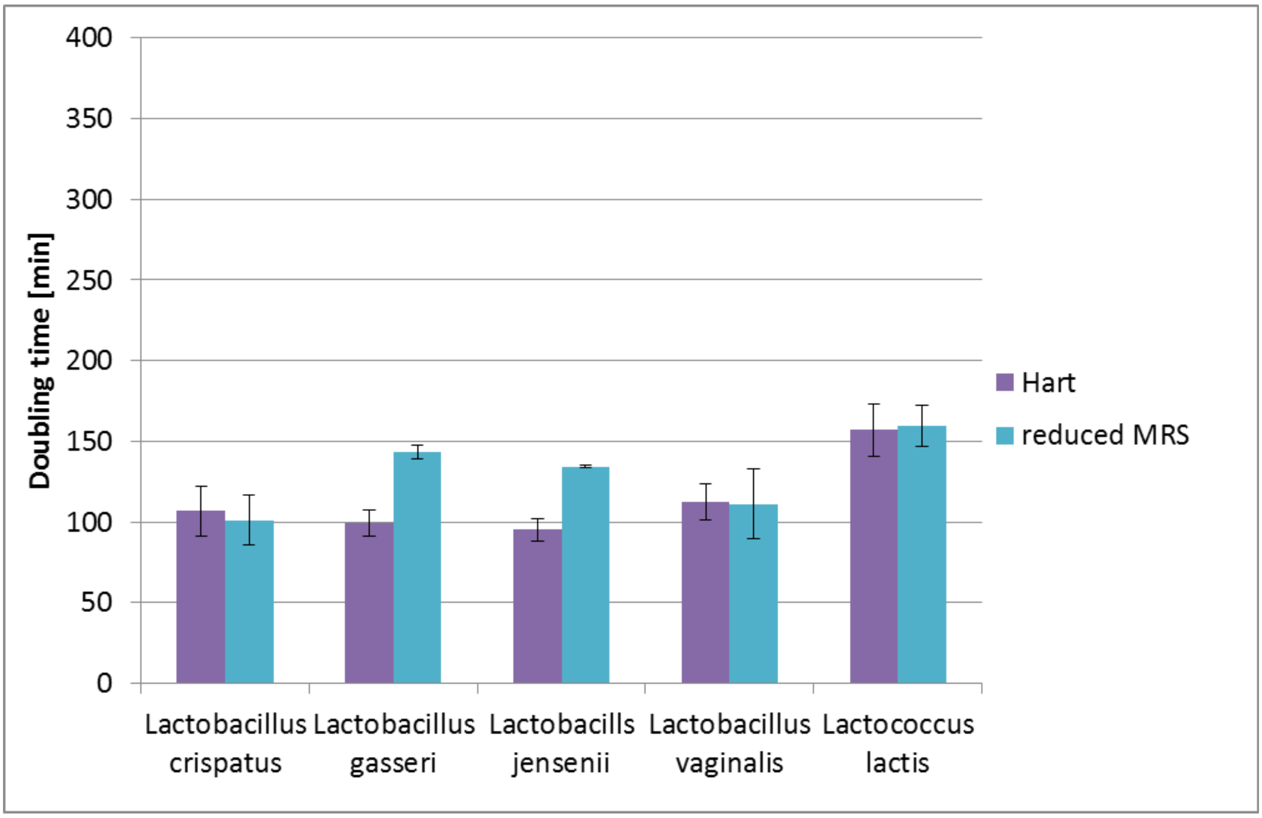

Figure 6: *Doubling time with standard deviation of L.crispatus, L. gasseri, L. jensenii, L. vaginalis and L. lactis in reduced MRS rich media (Tab. 7) and defined Hart vaginal fluid simulation (Tab. 5).*

To further approximate the vaginal fluid simulation to reality, substances with the highest portions in the fluid were varied in concentration, since these could affect osmotic properties and viscosity of the media and be used as an energy source for bacteria and thus influence their growth rates (Graver & Wade 2010). Older formulations for vaginal fluid simulations reported higher concentrations of Glucose (Geshnizgani & Onderdonk 1992), Glycogen, Mucin (Silvina et al. 2007) and lower concentrations of water (varying from 93 % to 98,5 %) compared to solid constituents in the fluid (Katz et al. 1997). Three different concentrations of Glycogen and Glucose were tested on the growth of the selected strains. It was not possible to monitor growth rates of higher concentrations of Mucin and lower concentrations of water with the automated plate reader reliably due to the intransparency and stickyness of the resulting media. *L. jensenii* grew fastest at a glucose concentration of 20 g/l, while there was no obvious improvement for the other strains (Fig. 7). *L. gasseri* and *L. lactis* did grow slower at a concentration of 10 g/l. *L. crispatus* did not yield

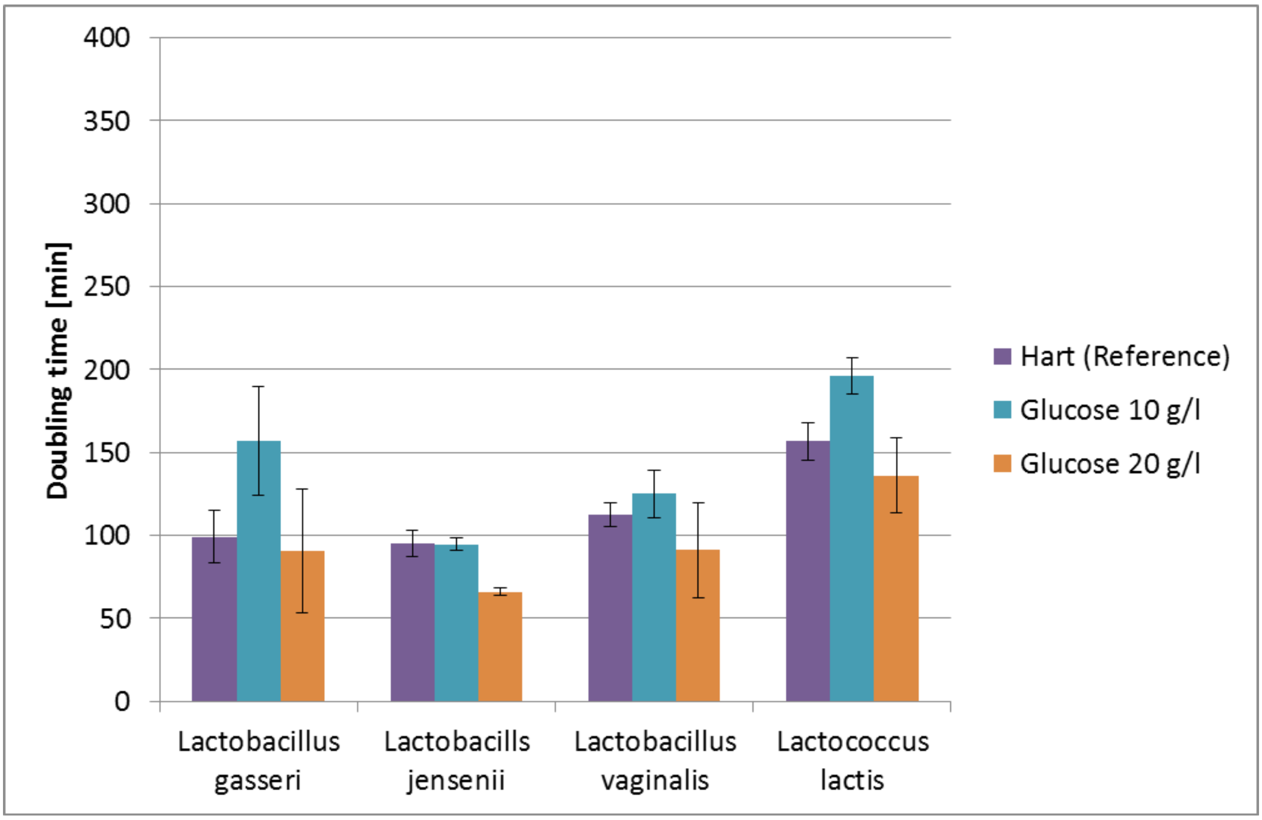

Figure 8: *Doubling times of L. gasseri, L. jensenii, L. vaginalis and L. lactis for Glucose concentration 5 (Hart, Tab. 5), 10 and 20 g/l. All other ingredients of the media as in Hart mix.*

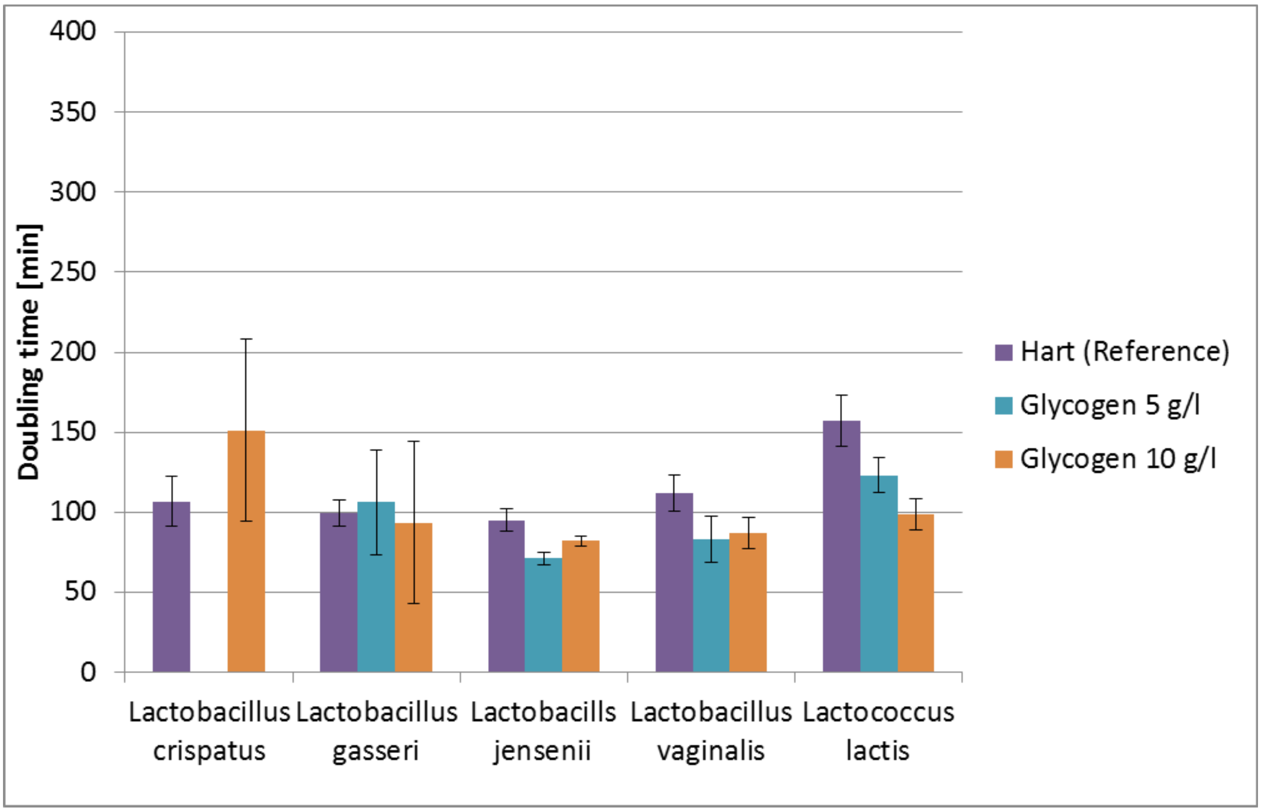

Figure 7: *Doubling times of L.crispatus, L. gasseri, L. jensenii, L. vaginalis and L. lactis for Glyogen concentrations 1 (Hart, Tab. 5), 5 and 10 g/l. All other ingredients of the media as in Hart mix.*

reproducible results. Glycogen improved the growth of *L.jensenii*, *L. vaginalis* and *L. lactis* at 5 and 10 g/l For *L. crispatus* and *L. gasseri* there was no clear effect detectable (Fig. 8).

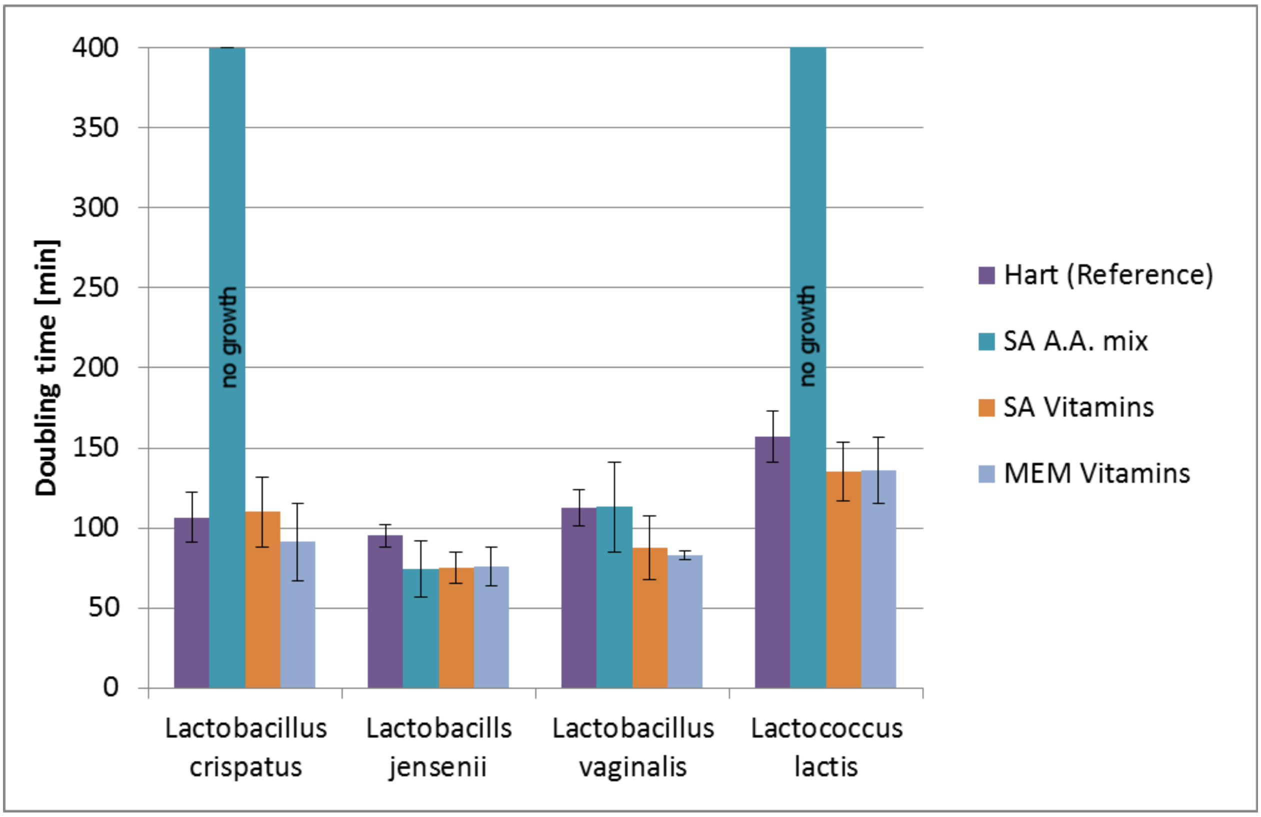

Figure 9: *Doubling times of L.crispatus, L. jensenii, L. vaginalis and L. lactis of experiments in which the Hart vitamin or amino acid mixes were replaced by a different set of amino acids (SA A.A. Mix, Tab. 8) or two different sets of vitamins (SA Vitamins and MEM Vitamins) respectively. All other ingredients of the media as in Hart mix. See Tab. 4 for a detailed list of ingredients of the mixes.*

To find out if the amino acid mix proposed by Liu et. al. was adequate for symbiotic *Lactobacilli*, the Hart amino acid mix was replaced by a prepared mix of amino acids designed for lactic acid bacteria cultivation (Tab. 8) (Jensen & Hammer 1993). The same test was done for the vitamins proposed for the Hart mix (Kao & Michaylak Vitamins and Vitamin K_1), which is a composition originally designed for plant tissue cultures. Vitamins were replaced by a mix designed for lactic acid bacteria cultivation (Jensen & Hammer 1993) and in a second experiment by a mix for in vitro cultivation of mammalian cells (ingredients see Tab. 4, results Fig. 9). Growing *L. crispatus* and *L. lactis* on the SA amino acid mix media resulted in a doubling time calculated by the R-script of more than 250 min. This was considered to be too long to make a reasonable judgement within an evaluation frame of 120 min and thus labeled as non growing. *L. jensenii* and *L. vaginalis* showed no significant change in their doubling times on SA amino acid mix media. SA. vitamins were about as fast as the Kao & Michaylak vitamin mix in the Hart formulation, except for *L. jensenii* where slightly faster growth rates compared to the Hart mix were measurable. MEM vitamins significantly shortened the doubling times of *L. jensenii* and *L. vaginalis*. *L. gasseri* experiments did not produce reproducible results and was therefore not included in the results.

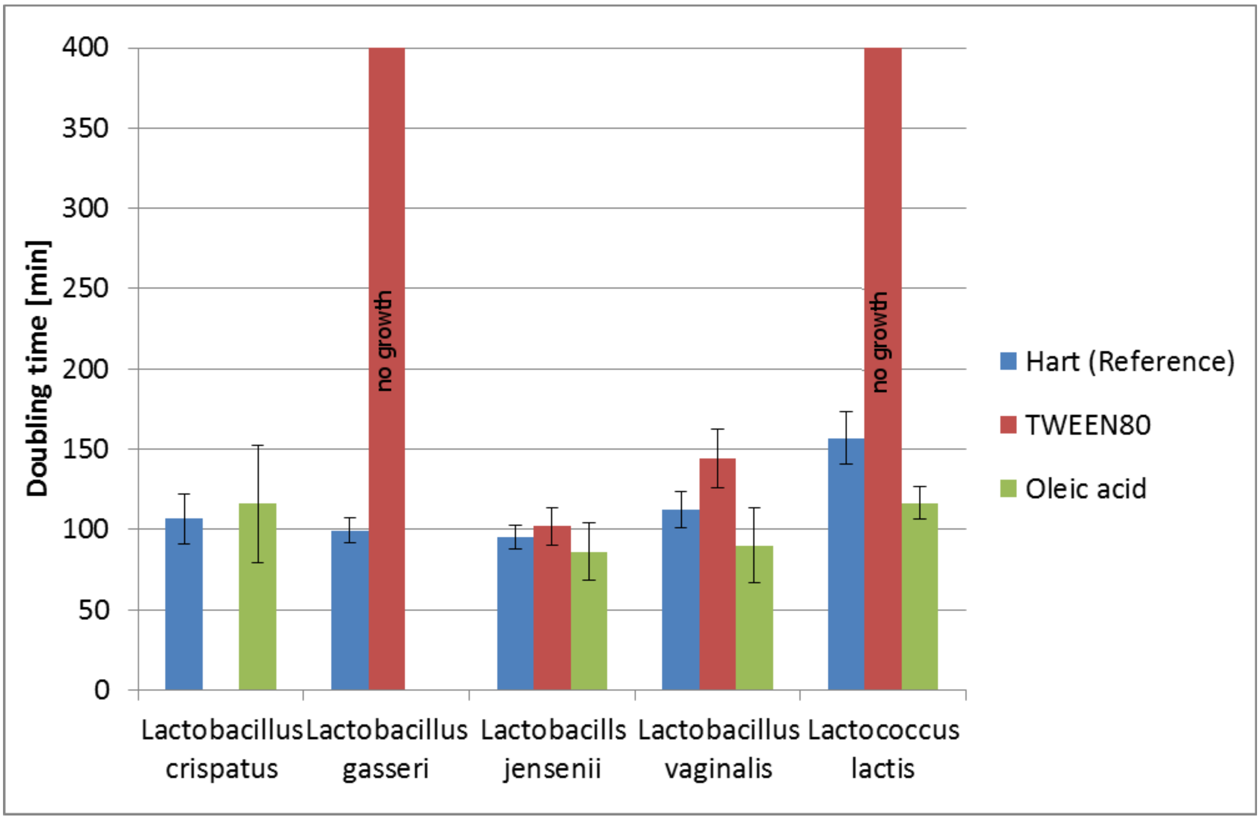

Figure 10: *Doubling times of L.crispatus, L. gasseri, L. jensenii, L. vaginalis and Lactococcus lactis for TWEEN 20 0,222 g/l (Hart), TWEEN 80 1,064g/l and oleic acid 0,214 g/l. All other ingredients of the media as in Hart mix (Tab. 5).*

TWEEN 20, the commercial name for Polysorbate 20, which is listed in the Hart formulation, is a surfactant and mostly used as an emulsifier. Silvina et al., 2007 suggested TWEEN 80 instead as it contains oleic acid , while studies showed the positive effect of TWEEN 80 compared to other trans fatty acids (Endo et al. 2006). Among those, Oleic acid was found to promote *L. gasseri* considerably, too. Fatty acid concentrations vary over the ovulation cycle (Chan & Black 1974) and since TWEEN 80 and TWEEN 20 are purely synthetic substances it was questionable which of the three was most realistic and beneficial for *Lactobacilli* growth. Two media were designed in which TWEEN 80 was replaced by Oleic acid and TWEEN 20 respectively with concentrations described in literature.

TWEEN 80 did not allow *L. gasseri* and *L. lactis* to grow and slowed down the growth in *L. vaginalis* compared to TWEEN 20 (Fig. 10). Oleic acid only affected the growth of *L. lactis* positively, while it had no significant effect on the other strains. Data for *L. crispatus* and *L. gasseri* was incomplete because results were not fully reproducible.

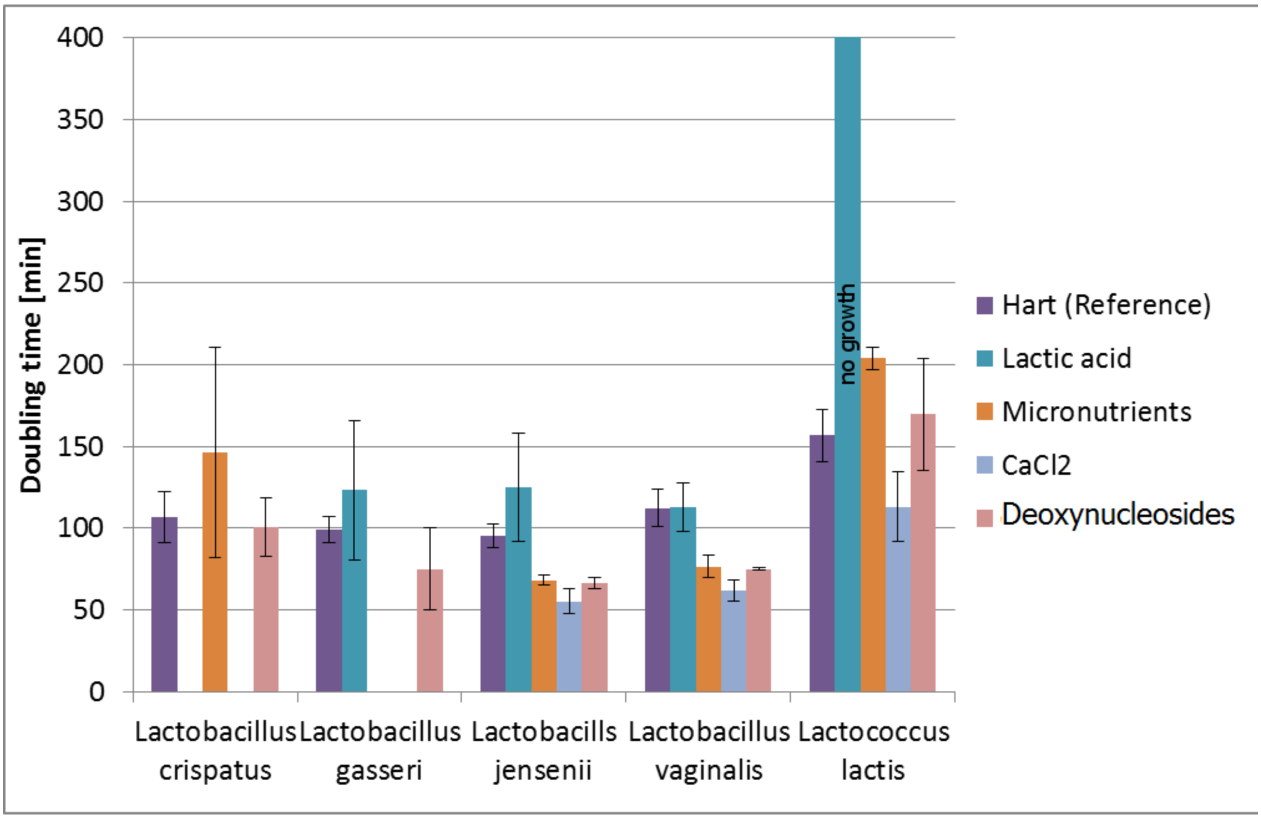

Figure 11: *Doubling times of L.crispatus, L. gasseri, L. jensenii, L. vaginalis and Lactococcus lactis for Hart media (Tab. 5) with the additives lactic acid 2 g/l, micronutrients (Tab. 6), $CaCl_2$ 0,36 g/l and deoxynucleosides.*

The Hart formulation contains a few metals and inorganic salts often found in defined media for bacteria, however the list seemed incomplete. Older formulations for vaginal fluid simulations contained the ingredients lactic acid, $CaCl_2$ (Geshnizgani & Onderdonk 1992; Owen & Katz 1999), deoxynucleosides and a set of rare metals referred to as Micronutrients (Tab. 6) (Jensen & Hammer 1993). These substances were added in reported concentrations to the Hart mix. Lactic acid did not have a positive effect on the growth speed of the strains. The results for *L. crispatus* and *L. gasseri* were incomplete and did not reveal clear indications. *L. lactis* grew slower with micronutrients and faster with $CaCl_2$. Micronutrients, $CaCl_2$ and deoxynucleosides however shortened the doubling times of *L. jensenii* and *L. vaginalis* significantly and also resulted in a more reliable doubling time, as it can be seen by the small standard deviation (Fig. 11).

Spermatozoa

Spermatozoa analysis setup

To detect spermatozoa response to chemoattractant substances it is necessary to take video recordings of a representative amount of single spermatozoa, to analyze their movement and condition. In the recent years several machines were developed for this purpose. However the availability of these machines is very limited and together with the time and organizational restrains of sperm analysis, it is indispensable to have a system for sperm analysis within the laboratory. Due to the high price and lack of appropriate equipment, a new and cheap setup was developed for semi automated microscopic computer assisted sperm analysis. Based on ealier works of Adler 1973 and Overman 2005 the system was built of a simple self constructed carrier slide to position a sperm sample in the right position on top of an inverted phase contrast microscope with a digital camera (see material) with a 10X objective. Sperm samples were collected from the donors by a company and directly after donation brought to the laboratory. There, spermatozoa for the analysis are extracted from the liquified semen samples by swim up procedure, adjusted in concentration to 10^5 cells/ml, allowed to capacitate and then kept in an incubator until the measurement started . Before the measurement started, the samples were taken out of the incubator, allowed to adapt to room temperature for 30 mins (see methods). The slide for chemotaxis measurements was made of a Neubauer-Türk cell counting chamber as a carrier. A spacer made from cut glass slides is glued on one side of the chamber with silicone grease (Fig 12 a). On the other side of the chamber a rectangular glass capillary with a size of 2 mm * 0,2 mm * 5 cm was positioned. The capillary was filled beforehand with F-Ham media in which the chemoattractant substance was diluted. After taking up the liquid into the capillary, it was sealed with silicone grease on one end to avoid currents within the liquid due to evaporation or capillary forces. The open end was pointed towards the platform in the center of the chamber. Above the spacer and the capillary a cover glass was placed and aligned with the corner of the platform of the chamber. When in position, approximately 60 µl of spermatozoa in F-Ham media are carefully placed between the chamber and the cover glass. The slide for chemokinesis measurements was constructed similarly, but without the capillary. Instead, a second spacer was glued on the other side of the Neubauer-Türk chamber (Fig. 12 b) and a cover glass placed on top. Here, the chemoattractant was added to the spermatozoa from the swim up sample and then the 60 µl were placed under the cover glass on the platform. Before the acquisition with the camera started, the cells were allowed to settle and the current in the liquid to cease. The filming took place between minute 5 and 10 after the sample had been placed on the chamber. Acquisition after 10 min was not possible, because the effects of evaporation became too strong. For the chemotaxis measurements the waiting time was also necessary to allow the chemoattractant to diffuse from the capillary into the semen sample. (Fig. 12 c and d). The field of view of the camera covered an area of 400 * 400 µm. The contrast was set so that the heads of spermatozoa appeared as white spots while the background was black. Cells settling on the bottom at the surface of the Neubauer-Türk chamber were unnaturally slowed down through contact with the glass. This effect was not observed for the cells at the cover glass. Cells swimming freely in the center of the media droplet were not long enough within the focal plane to gather enough data. Thus, the focus was adjusted on the cells swimming along the cover glass (Fig. 12 c). Greyscale videos of 3000 frames recorded at a frame rate of 33 fps with a resolution of 400 * 400 pixels and saved as tiff stacks.

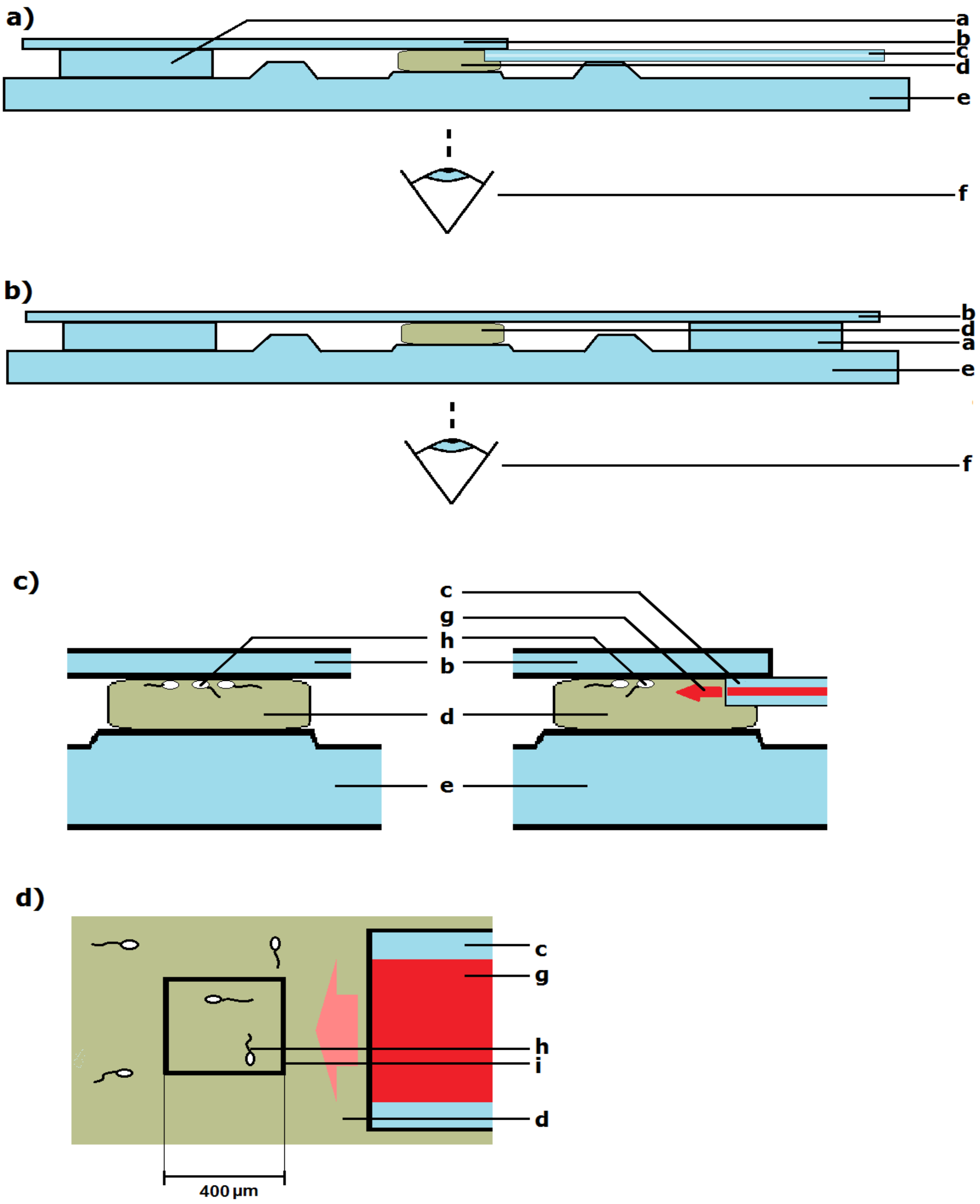

Figure 12: *Schematics of sperm analysis chamber.* ***a)*** *side view of chamber with capillary for chemotaxis measurements* ***b)*** *side view of chamber without capillary for chemokinesis measurements* ***c)*** *side view close up and position of tracked cells left: chemokinesis setup right: chemotaxis setup* ***d)*** *top view with region of interest, which is monitored by the camera. Drawn capillary and diffusing chemoattractant (red arrow) are not present in the chemokinesis assay.*

Description of objects*:* ***a****: spacer made from cut glass slide;* ***b****: thin long cover glass;* ***c****: rectangular capillary;* ***d:*** *semen-specimen;* ***e****: Neubauer-Türk counting chamber;* ***f:*** *direction of view;* ***g****: media with chemoattractant;* ***h****: spermatozoa;* ***i****: field of view for microscope camera*

Computer assisted semen analysis (CASA)

To extract the movement pattern of singles cells from the recorded sperm samples, computer assisted semen analysis was necessary. An open source computer program available from pubmed, which was originally developed to analyze fish spermatozoa was adapted to human cells for this purpose. The program is a plugin for the ImageJ software and with newly defined input parameters was able to track human spermatozoa.

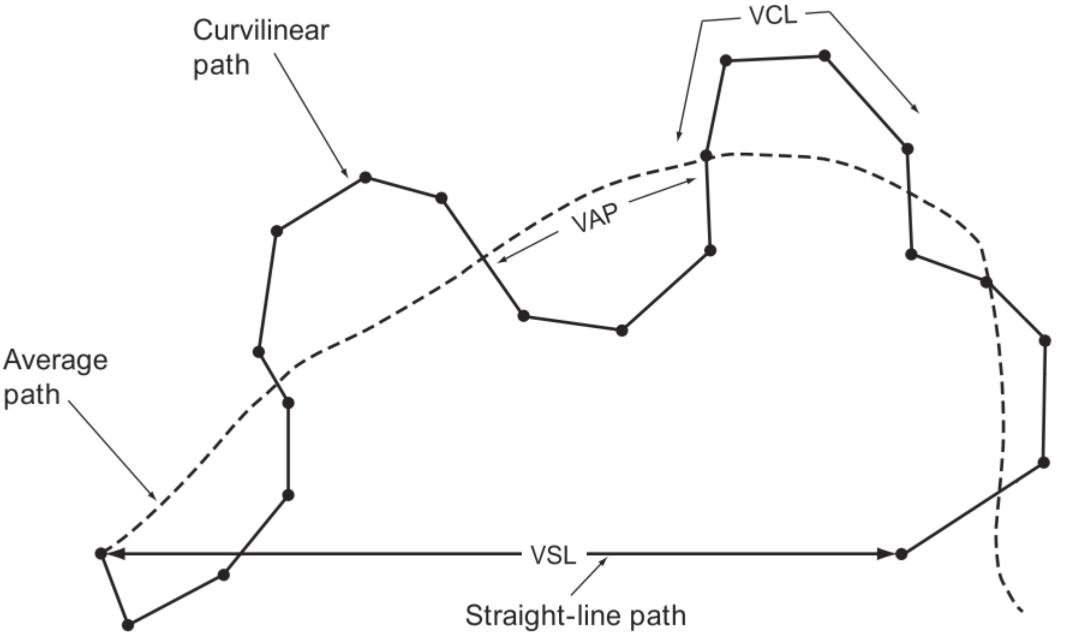

Figure 13: *Schematic of a spermatozoa path as it is recognized by the CASA program from a recorded sequence (see setup Fig. 12. Each point symbolizes the position of the head of the sperm per frame as it is detected by the software. The curvilinear path is reconstructed and based on this, the curvilinear velocity (VCL), the average path velocity (VAP) and the straight line velocity are calculated. (Figure modified after World Health Organisation, 2010)*

There are three basic parameters which had to be determined by the software to describe the spermatozoa movement on a two dimensional plane. All are measured in µm/s: The curvilinear velocity (VCL) - the time-averaged velocity of a sperm head along its actual curvilinear path and indicates the vigour of a cell. The straight-line velocity (VSL) - the time-averaged velocity of a sperm head along the straight line between its first detected position and its last. And the average path velocity (VAP) – the time-averaged velocity of a sperm head along its average path. The path is computed by the CASA plugin by smoothing the curvilinear trajectory according. Further pattern indicators are fractions of those values describing the linearity (LIN) and wobble (WOB), a measure of oscillation of the actual path about the average path (World Health Organisation, 2010). LIN is defined as VSL / VCL × 100, with 100 % representing an absolutely straight track. WOB is defined as VAP / VC*L*.

The CASA plugin required monochrome tiff stacks to run the analysis on. Since the recorded videos were saved in greyscale it was at first necessary to convert them to monochrome pictures. To automate the threshold finding for the tiff stack, a macro function was developed, which autonomously loads all files of one folder and then approximates the best threshold value until an maximum number of tracks was found.

The threshold values were linearly decreased and after each decrement the CASA plugin is run on the resulting monochrome tiff stack. To evaluate the success of the analysis the number of found tracks was counted and compared with the results of the before results. If the number did not increase anymore, the treshold values of the last experiment were used for further analysis. (compare supplemtary code: “treshold macro”). The CASA results using the optimal treshold and an image of all identified particles and sperm tracks were saved.

The initial parameters for the CASA plugin were developed to analyze fish sperm (See table 9) and were barely applicable on human spermatozoa. It was required to change 16 interdependent parameters (a-p, Tab. 9) to find adequate values for human spermatozoa. To automate that, an algorithm using a directed evolution optimization approach was developed (see supplementaries: Directed evolution script). To approximate the ideal values for the identification of moving spermatozoa and to distinguish them from dead cells and drifting debris, several subsequent rounds (further called “generations”) of dependent random changes (“mutations”) of every parameter were run. The mutation was dependent to the value of each parameter and the number generations in the following relation:

$$mp = p \pm x * 2 * (p \pm 1) / g$$

mp....	mutated parameter to be passed on to CASA plugin and next generation
x....	a random 0 or 1
p....	parameter variable of the last generation
g...	number of the generation

The mutations were calculated for all parameters from a to p (Tab. 9) and the resulting values then checked against predefined boundaries. Parameters q and r were variables which are determined by the used setup. The framerate q was 33 fps, the resolution r was 1000 µm per 1000 pixels. For the chemokinesis study (see below), x and y coordinates and the median values of all spermatozoa were not relevant and thus parameter s and u set to 0. The results relevant for chemokinesis were the calculated VCL and LIN values for each sperm track. To harvest these parameter t was set to 1. The script automatically saved the results for each tiff stack in a text file after the analysis. In case a value went outrange, it was set to the closest boundary. For each generation, five sets of mutated parameters were created (“mutants”). The parameters of all mutants were then passed on to the CASA plugin and applied on a tiff stack. The “fitness” of the mutants was determined by the user through visual evaluation of the resulting output images of the CASA plugin. The the best mutant of the five was selected for each generation. Selection criteria were: A minimum of detected debris together with a maximum of correctly detected and complete sperm tracks. All mutants of a new generation started their random mutations based on the parameter set of the fittest mutant of the last generation. After the first five generations were finished, a new tiff stack was loaded. For each tiff stack five generations with each five mutants were run. In total up to ten different tiff stacks were iterated. The count of generations restarted with a new tiff stack, but continued with the values of the chosen mutant of the last generation of the previous tiff stack. The first stack started with the parameters for fish, as they were reported by Leedy et. al. (Wilson-Leedy & Ingermann 2007) and then continued through all ten tiff stacks. The whole experiment was repeated three times, each time with different selection of tiff stacks from different donors.

The resulting parameters of the best mutant of the last generation of the last tiff stack are shown in table 9. To compare the efficiency of the three resulting sets of parameters, they were applied on a choice of six tiff stacks with different characteristics, such as a different focal plane, an over-concentration of cells in the sample, or a lot of debris (Fig. 14).

	Parameter	**Fish (Ref.)**	**1.Exp.**	**2. Exp.**	**3. Exp.**
a	Minimum Sperm size (pixel)	0	2	1	1
b	Maximum Sperm size (pixel)	40	30	25	25
c	Minimum Track length (frames)	97	35	31	25
d	Maximum Sperm velocity between pixels (µm/s)	8	3	6	4
e	Minimum VSL for motile (µm/s)	3	2	2	5
f	Minimum VAP for motile (µm/s)	20	1	1	3
g	Minimum VCL for motile (µm/s)	25	8	7	38
h	Low VAP Speed (µm/s)	5	1	1	1
i	Maximum percentage of path with zero VAP	1	7	2	2
j	Maximum percentage of path with low VAP	25	100	2	9
k	Low VAP speed 2 (µm/s)	25	1	11	7
l	Low VCL speed (µm/s)	35	1	8	6
m	High WOB (percent VAP/VCL)	80	19	53	1
n	High LIN (percent VSL/VAP)	80	1	45	17
o	High WOB two (percent VAP/VCL)	50	63	147	19
p	High LIN two (percent VSL/VAP)	60	145	1	74

Table 9: *Fish parameters are the starting parameters which were found in. Parameters for Experiments 1, 2 and 3 were different results of the "Directed evolution algorithm" (see Supplementary Information) after 50 generations of mutation and selection.*

The fish parameters showed a poor performance in detecting valid human spermatozoa tracks (black tracks Fig. 14 column 1). Only in the tiff stacks c), d), and e) tracks were detected, however, they were recognized as not valid. In b) and f) only debris (in blue circles) was detected and mostly not defined as invalid (red) pixels. Experiment 1 did result in a good number of detected tracks, although the analysis of tiff stack e) was incomplete. Additionally, much debris be was wrongly detected as cell movement. Experiment 2 and 3 showed a very similar performance. Many sperm tracks were correctly detected and only a small amount of debris was not sorted out (f). Experiment three was a little better in recognizing valid sperm tracks compared to Experiment 2 in tiff stack b), c) and e). Experiment 3 parameters were therefore used in further analysis of the video recordings of chemokinesis experiments.

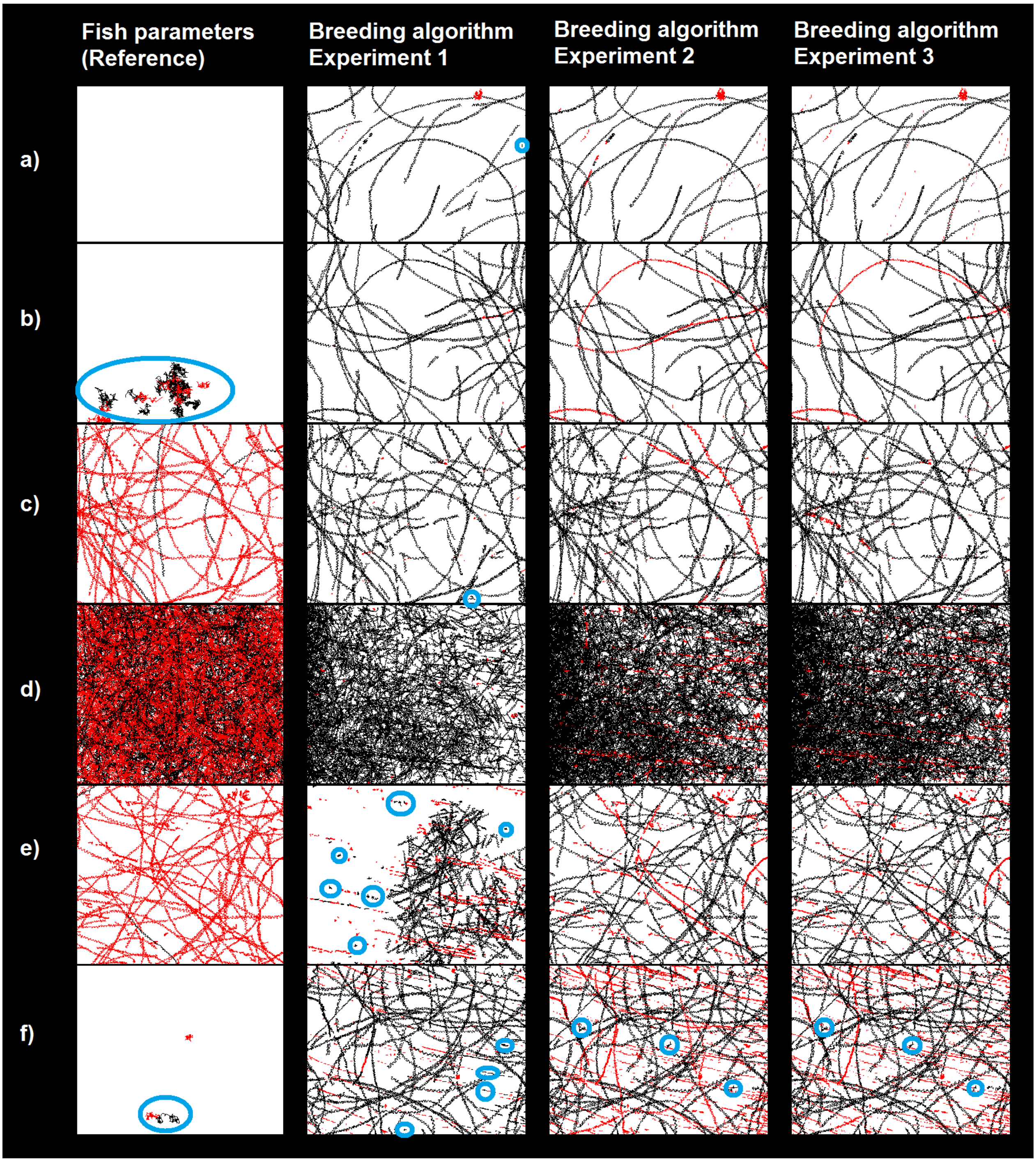

Figure 14: *CASA output graphs from four different sets of input parameters (arranged in columns) applied on six representative video recordings of sperm movement (a – f). Fish-sperm parameters found in the literature (Tab. 9) were used as a reference to three sets of parameters derived from the "directed evolution" script. (Paramerers see Tab. 9, for the script see Supplementary Information). Red lines indicate sperm tracks that the program identified, but sorted because they did not match the requirements defined by parameters e-p (Tab. 9). Blue circles indicate debris wrongly detected by the script as live spermatozoa.*

Chemokinesis experiment

Sperm chemotaxis towards the oocyte in the follicular fluid has been demonstrated (see introduction). Among the found substances, two protein-based chemoattractants were identified, namely ANP (Isobe et al. 2002) and RANTES(Zamir et al. 1993). Recently, NGF-ß was debated as a possible chemoattractant in humans (Isobe et al. 2002; Yoshida & Yoshida 2011) and thus included into this study. Besides these, nitric oxide radicals were shown to affect spermatozoa (Miraglia et al. 2007). Since nitric oxide can also be produced by the bacterial enzymes like nitric oxide synthases (Ratto et al. 2012) S- Nitrosogluthation (SNOG) was tested as an artificial donor of nitric oxide radicals (Gusarov et al. 2008).

To determine and directly compare the effects of the selected chemoattratants found in the literature, the substances were obtained from commercial suppliers (see Materials) and analyzed with the newly developed CASA system for chemokinesis (Fig. 12 b) using the parameters of experiment three (Tab. 9). Literature reports very small concentrations of the chemoattractants to cause a physiological response in spermatozoa. To map the effects in detail and to create a reference for future studies of biological expression systems for chemoattractants, five concentrations were measured. The physiologically active concentrations reported by literature was increased by factor 5 and 25 and reduced by 1/5 and 1/25. To verify the ability of the developed CASA system, a dilution series of progesterone (PROG) was tested as a positive control in parallel (Teves et al. 2006). The experiments were performed with ejaculates from different individuals, each carried out in three repetitions. To assure that only sperm samples of healthy donors were used in the study, each sample was stained with Eosin-Nigrosin stain and the percentage of dead and deformed cells was evaluated by visual inspection of 400 cells (see methods). The three samples contained 15 %, 16 % and 9,25 % of dead cells and 11,5 %, 18,75 % and 24 % of misshaped cells, which is within a healthy range according to the WHO standards (World Health Organisation 2010). For the chemokinesis assay the minimal number of analyzed spermatozoa per concentration of chemoattractant and repetition was 800 cells (total numbers of tracked cells see description texts in figures 15 to 19). LIN and VCL values of the CASA plugin output files of the three sperm samples were each pooled, assuming the same physiological responsiveness to the chemoattractants in all men. The data was read out and plotted with the help of an R-script (see Supplementary Information). The distribution of VCL (left columns) and LIN (right columns) values as a percentage of all tracked cells is plotted for each tested concentration of all chemoattractants. For a better comparison of the results, a threshold for VCL was set to 30 µm/sec and for LIN to 60 % VSL/VCL.

The figures 15 to 19 show the four tested chemoattractants and figure 15 progesterone in red and a negative control in black. The graphs in c) show the response to the concentration of chemoattractant reported to have a physiological effect. A Progesterone level of 78,5 pg/ml (Fig. 15 e) displayed the strongest increase of VCL with 25 % above threshold (13 % in blank measurement). Spermatozoa at the progesterone concentration of 3,125 pg/ml (Fig. 15 c) had 23 % of cells above LIN threshold compared to the other concentrations. However, comparing LIN values to the blank distribution, the overall observed effect of progesterone is a decrease of LIN from 28 % above LIN threshold in the blank measurement to as low as 14 % at a concentration of 0,625 pg/ml. (Fig. 15 b). In all experiments a narrowing of the basis of the LIN distribution and a higher peak can be observed.

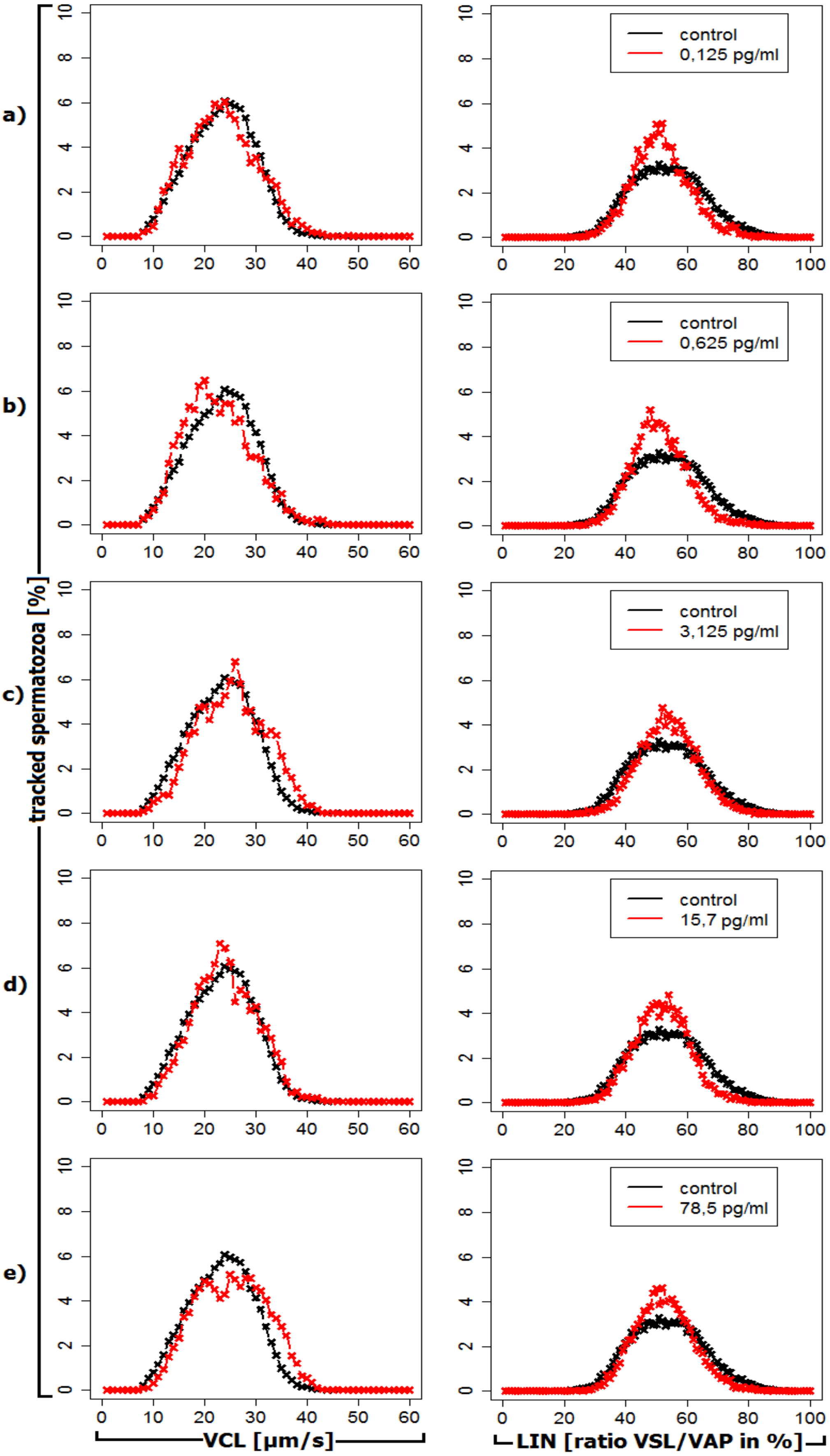

Figure 15: *Five Progesterone concentrations (red indicator in left column legend) increased in multiples of 5 compared to one negative control (black) and their effect on the movement pattern of individual human sperm. In the left column the x-axes indicate the curvilinear velocity of the spermatozoa, in the right column it is the linearity as the percentage of the straight line velocity of the average path velocity. The y-axis is a relative scale in percent of the total amount of analyzed cells. A Threshold for VCL was set to 30 µm/s and for LIN to 60 % for better comparison. Numbers in the following order:*

Total amount of analyzed cells per experiment, percent of cells with VCL above threshold, percent of cells with LIN above threshold.

Control (all graphs) 18812, 13 %, 28%
a) 4008, 16 %, 17 %
b) 5480, 12 %, 14 %
c) 5698, 24 %, 23 %
d) 5243, 16 %, 15 %
e) 7834, 25 %, 19 %

The data is the output of the CASA imageJ plugin and derived from microscopy videos of three indipendend experiments.

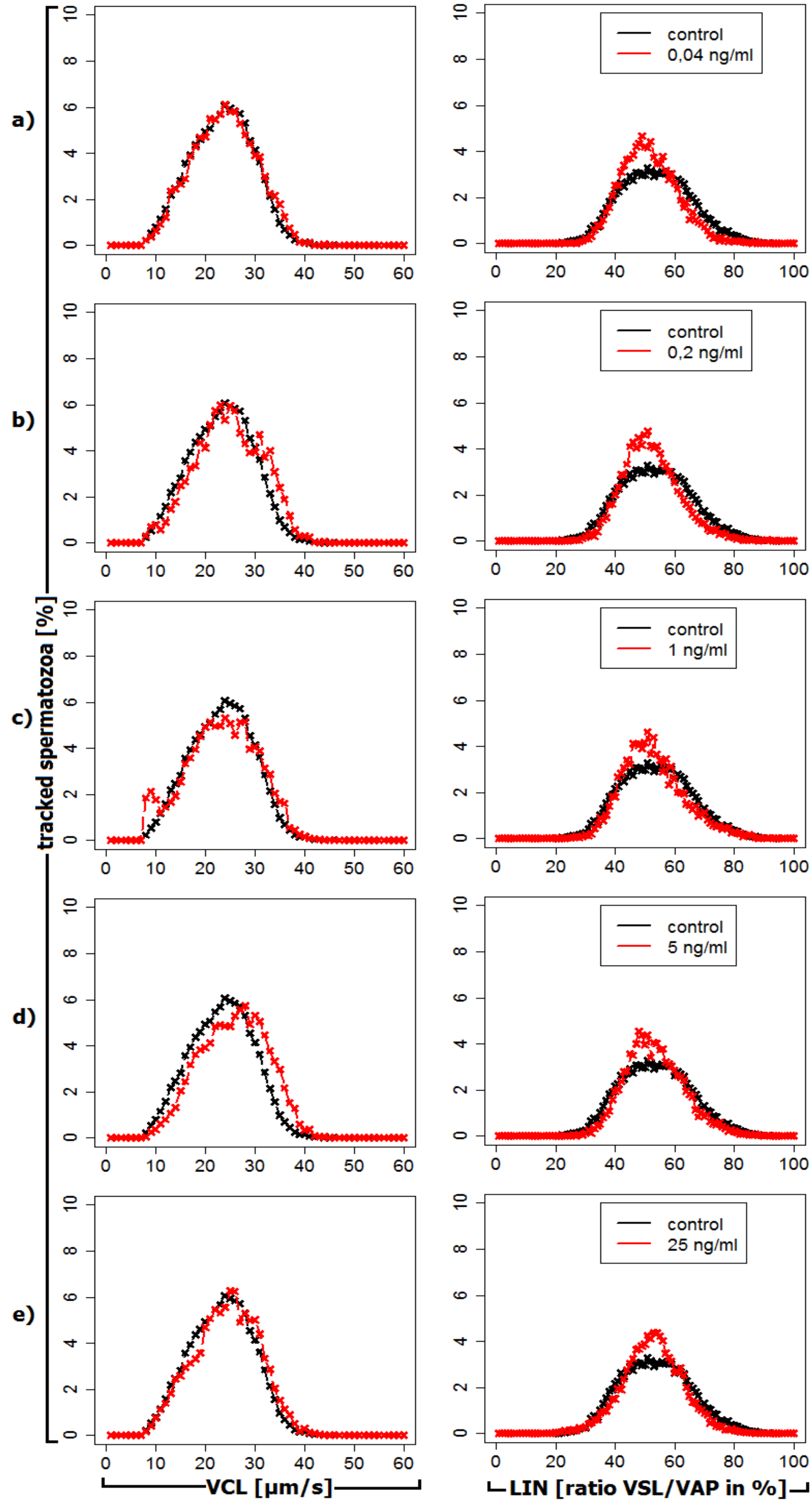

Figure 16: *Five RANTES concentrations (red indicator in left column legend) increased in multiples of 5 compared to one negative control (black) and their effect on the movement pattern of individual human sperm. In the left column the x-axes indicate the curvilinear velocity of the spermatozoa, in the right column it is the linearity as the percentage of the straight line velocity of the average path velocity. The y-axis is a relative scale in percent of the total amount of analyzed cells. A Threshold for VCL was set to 30 µm/s and for LIN to 60 % for better comparison. Numbers inthe following order: Total amount of analyzed cells per experiment, percent of cells with VCL above threshold, percent of cells with LIN above threshold.*

Control (all graphs) 18812, 13 %, 28 %
a) 6312, 16 %, 16 %
b) 6122, 22 %, 18 %
c) 6513, 17 %, 21 %
d) 7420, 26 %, 22 %
e) 8094, 17 %, 21 %
The data is the output of the CASA imageJ plugin and derived from microscopy videos of three indipendend experiments.

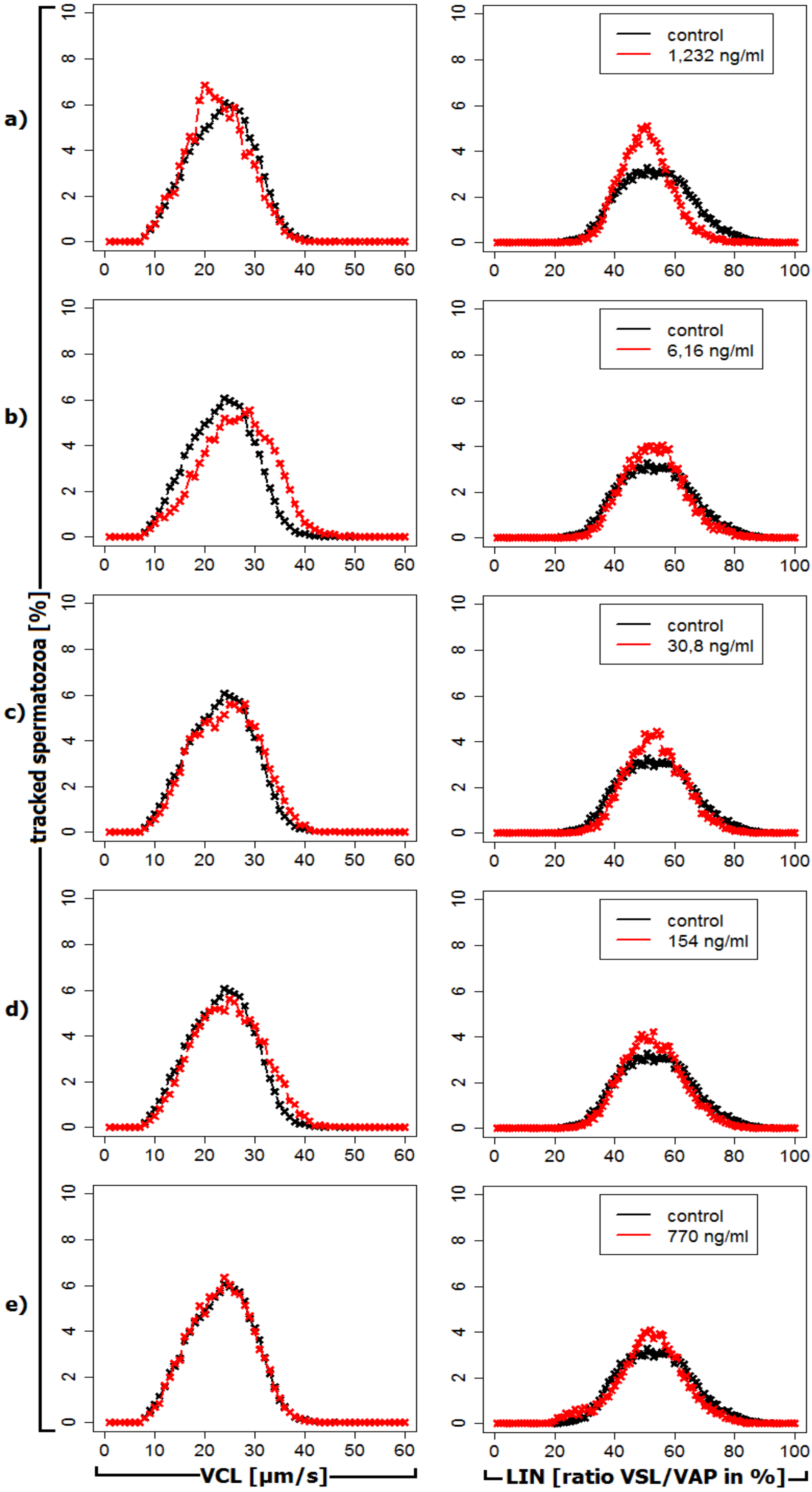

Figure 17: *Five ANP concentrations (red indicator in left column legend) increased in multiples of 5 compared to one negative control (black) and their effect on the movement pattern of individual human sperm. In the left column the x-axes indicate the curvilinear velocity of the spermatozoa, in the right column it is the linearity as the percentage of the straight line velocity of the average path velocity. The y-axis is a relative scale in percent of the total amount of analyzed cells. A Threshold for VCL was set to 30 µm/s and for LIN to 60 % for better comparison. Numbers in the following order:*
Total amount of analyzed cells per experiment, percent of cells with VCL above threshold, percent of cells with LIN above threshold.
Control (all graphs): 18812, 13 %, 28 %
a) 6798, 9 %, 11 %
b) 8398, 29 %, 22 %
c) 8845, 18 %, 23 %
d) 11601, 21 %, 21 %
e) 9963, 13 %, 21 %
The data is the output of the CASA imageJ plugin and derived from microscopy videos of three indipendend experiments.

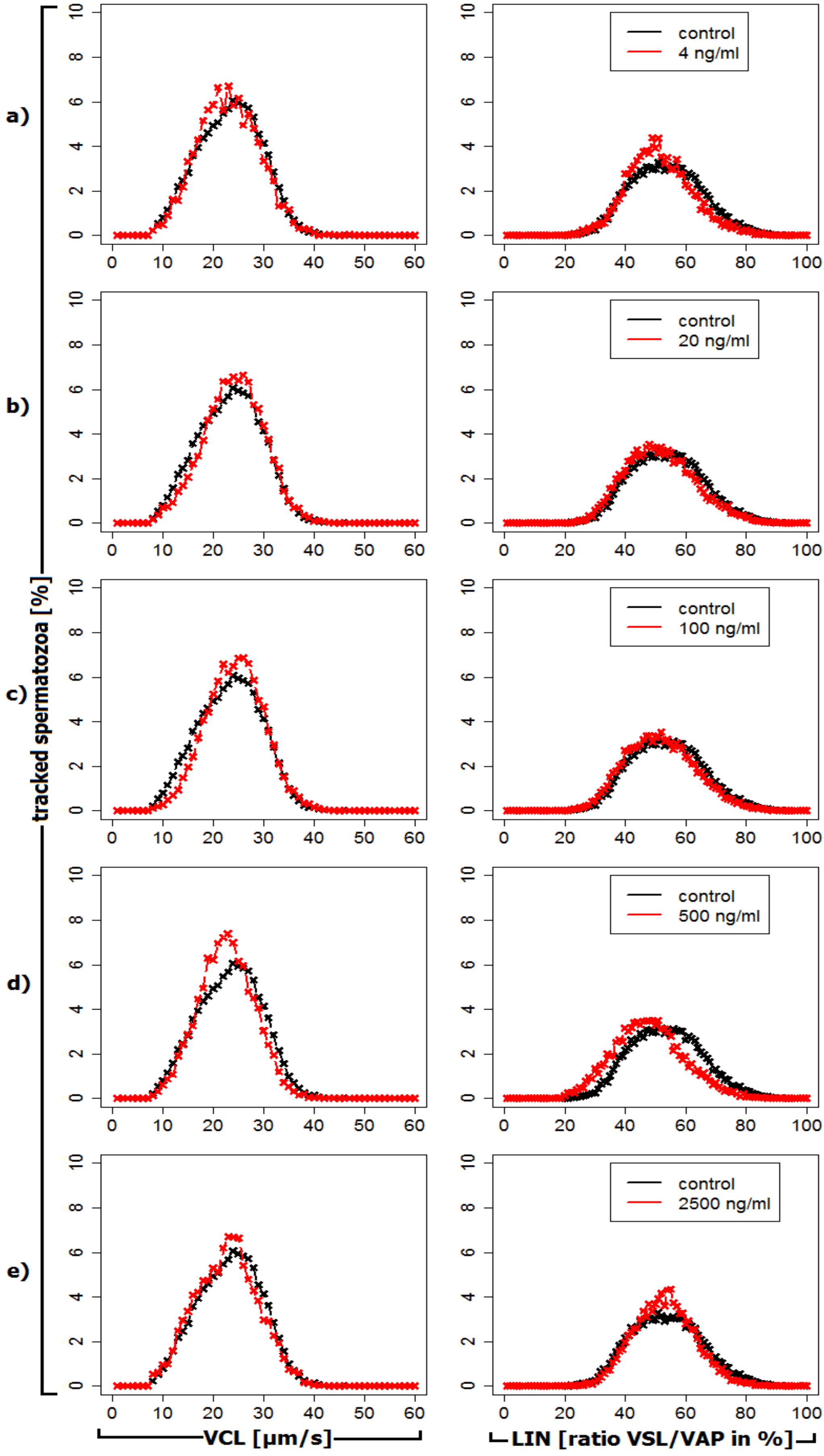

Figure 18:
Five NGF-ß concentrations (red indicator in left column legend) increased in multiples of 5 compared to one negative control (black) and their effect on the movement pattern of individual human sperm. In the left column the x-axes indicate the curvilinear velocity of the spermatozoa, in the right column it is the linearity as the percentage of the straight line velocity of the average path velocity. The y-axis is a relative scale in percent of the total amount of analyzed cells. A Threshold for VCL was set to 30 µm/s and for LIN to 60 % for better comparison. Numbers in the following order: Total amount of analyzed cells per experiment, percent of cells with VCL above threshold, percent of cells with LIN above threshold.
Control (all graphs) 18812, 13 %, 28 %
a) 4836, 11 %, 19 %
b) 12678, 14 %, 22 %
c) 14785, 14 %, 22 %
d) 11709, 8 %, 14 %
e) 5797, 11 %, 22 %
The data is the output of the CASA imageJ plugin and derived from microscopy videos of three independend experiments.

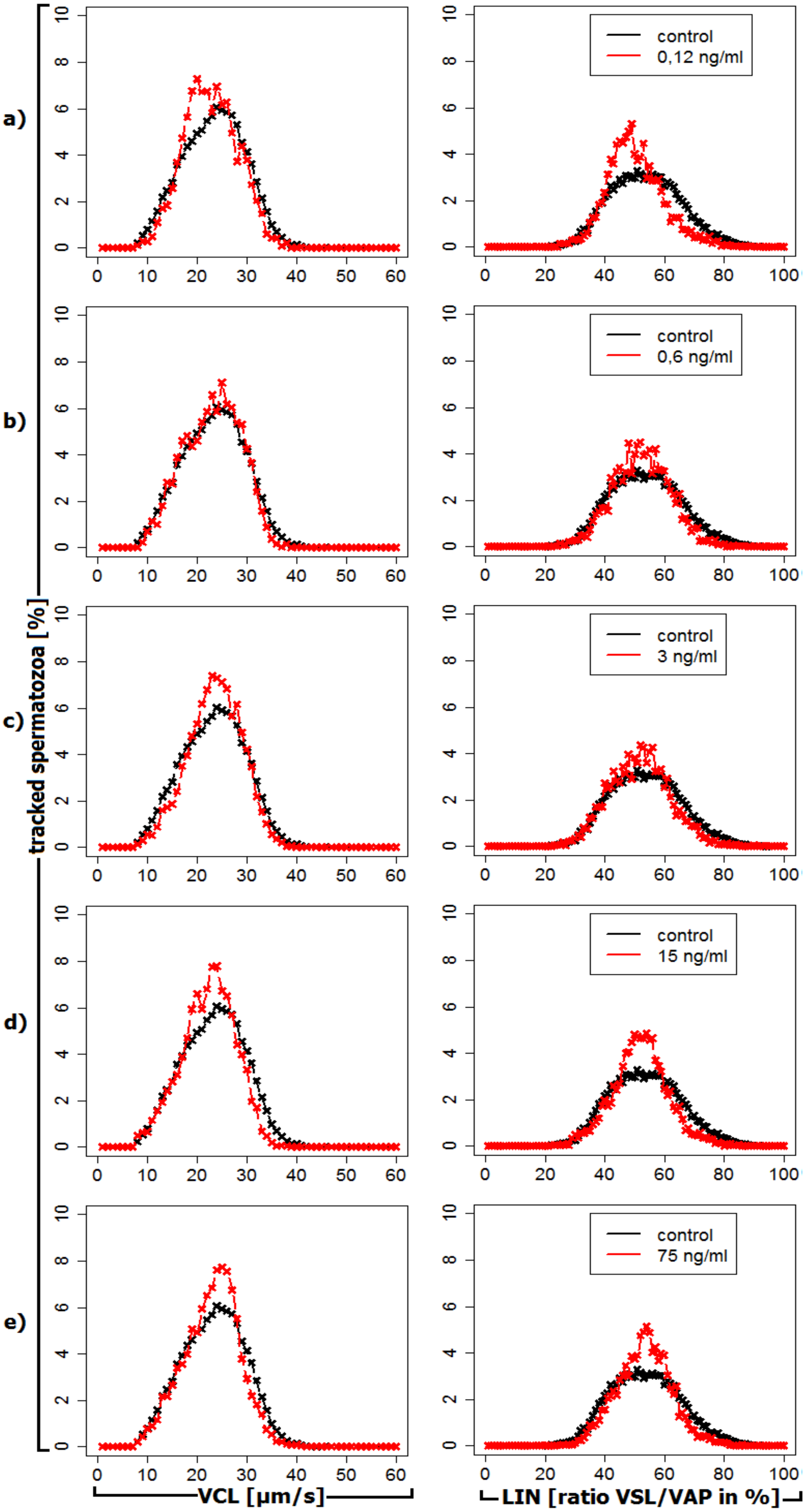

Figure 19: *Five SNOG concentrations (red indicator in left column legend) increased in multiples of 5 compared to one negative control (black) and their effect on the movement pattern of individual human sperm. In the left column the x-axes indicate the curvilinear velocity of the spermatozoa, in the right column it is the linearity as the percentage of the straight line velocity of the average path velocity. The y-axis is a relative scale in percent of the total amount of analyzed cells. A Threshold for VCL was set to 30 µm/s and for LIN to 60 % for better comparison. Numbers in the following order: Total amount of analyzed cells per experiment, percent of cells with VCL above threshold, percent of cells with LIN above threshold.*

Control (all graphs) 18812, 13 %, 28 %
a) 4227, 8 %, 13 %
b) 2730, 9 %, 19 %
c) 4451, 9 %, 18 %
d) 4244, 5 %, 16 %
e) 4382, 7 %, 19 %

The data is the output of the CASA imageJ plugin and derived from microscopy videos of three indipendend experiments.

NGF-ß at a concentration of 500 ng/ml slowed down the spermatozoa and the VCL above threshold was reduced to 8 % (Fig. 18 d).. For the other concentration no obvious change was visible. The distribution of LIN was slightly shifted to a smaller ratio in all measured concentration, except for 500 ng/ml, where LIN above threshhold was reduced to 14 % (Fig. 18 d). ANP increased VCL above threshold at concentrations of 6,16 ng/ml, 30,8 ng/ml and 154 ng/ml compared to blank with the strongest effect of 29 % at 6,16 ng/ml (Fig. 17 b). LIN above threshold was reduced at all concentration and with a minimum of 11 % at 6,16 ng/ml (Fig. 17 a). SNOG reduced VCL above threshold at all concentrations compared to blank and the most at 15 ng/ml with 5 % (Fig. 19 d). LIN above threshold was also reduced with a minimum of 13 % at 0,12 ng/ml (Fig. 15 a). In all experiments a strong narrowing of the basis of the LIN distribution and a higher peak can be observed. RANTES increased VCL above threshold at all concentrations with the strongest effect of 26 % at a concentration of 5 ng/ml (Fig. 16). The second strongest effect with 22 % was at a concentration of 0,2 ng/ml. The first is a five fold increased concentration compared the one reported in the literature, while the latter is a five fold reduction. LIN above thresshold was moderately decreased at all concentration, with a minimum of 16 % at 0,04 ng/ml (Fig. 16 a). In all experiments a slight narrowing of the basis of the LIN distribution and a higher peak can be observed.

Discussion

To to pave the way towards a functional bacterial contraception prototype an experimental schedule with defined aims was laid out. The work was arranged into two phases. For Phase I the following three questions were considered for this thesis: How can spermatozoa be chemotactically irritated, rendered physiologically inactive and physically blocked, what should a testing environment for spermatozoa be composed of and which parameters need to be observed? Which of the symbiotic bacteria are suitable candidates for genetic manipulation, protein expression, secretion and cultivation? Which parameters need to be observed? What should a genetic construct that provides the desired features look like and how can the sperm- and the bacterial test system be combined to assess the effect of genetically manipulated bacteria on spermatozoa? The general approach to these questions is shortly addressed in the following section and in more detail in the Discussion chapter ”Spermatozoa, Vaginal Bacteria and Genetic Construct”.

Scientific Approach

To ensure a goal-oriented work-flow and the evaluation of progress made, the conducted work was structured in accordance with an engineering approach. The experimental design was inspired by Systems Biology as witnessed, for instance, by the use of semi-automated data acquisition and computational analysis. Emphasis was put on a use of simple and low cost material and equipment, while in the same time maximizing the data output by creating as many combinations and variations as possible within each experiment. To process and interpret the large amount of resulting data, computational tools were developed. This yielded large datasets of valuable information with comparably little effort and would not have been possible by using classical approaches relying on manual serial work. In addition, automation of data collection and computational analysis led to a strong reduction of material costs.

To elucidate whether and how strongly spermatozoa react towards a set of four peptide chemoattractants plus a negative and a positive control, a microscopy setup equipped with an automated digital video analysis software had to be designed. To allow for tests of the expression of chemoattractans by genetically manipulated symbiotic vaginal bacteria under realistic circumstances, a defined medium for vaginal fluid was experimentally evaluated and its components optimized. Some problems arose that led to deviations from the predefined goals. This relates to several experiments that were planned but could not be carried out. Practical difficulties stood in the way such as dysfunctional equipment, the underestimation of the time required for each step and other unexpected problems. Initially, the plan was to get as far as to having the first genetic construct cloned and funds were allocated to ordering synthesized DNA. However, constructing and establishing a test system for sperm chemoattractants and formulating the growth media for symbiotic vaginal bacteria required more time than estimated so that considerations for a genetic construct remained theoretical. Chemotaxis measurements of all five chemoattractants at five different concentration and a control were done with the newly developed setup (Fig. 12 a). Due to a lack of time the resulting data could not be evaluated. With respect to media optimization, the final experiment combining all identified beneficial components in one formulation still has to be carried out. Thus, not all of the predefined goals could be reached in the given time frame.

Vaginal Bacteria

Vaginal Fluid Media Simulation

Media optimization experiments were carried out using the Hart formulation (Liu et al. 2011)as a basis. This was varied by 13 compounds or mixes, some in several concentrations resulting in a total of 17 new media formulations to be tested. The resulting media were tested on five strains which were reported as the predominant symbiotic bacteria in the vaginal vault of women of Caucasian ethnicity (Ravel et al. 2011). The bacteria were used as indicators for the effects of the compounds. Aiming at better cross-comparability several bacterial species were investigated at a time rather than investigating them in subsequent order. To be able to do a media optimization of 18 media plus the Hart mix as a reference, the experiments were done in small volumes in 96 well plates and automatically monitored by a temperature controlled plate reader with a shaking table. Four repetitions were made for all media with all 5 strains, resulting in a total of 360 single experiments, each with 240 measured OD data points collected over a 20 h time course. To analyze such an amount of data it was necessary to automate the analysis using a computer program, which was written for this purpose in the R-language using the freeware program R-studio. The output of the program was the shortest doubling time calculated from a 120 min observation time frame. Visual inspection of the output semi-logarithmic growth curve plots together with the steepest abline found by the program showed it was not always able to detect the exponential phase reliably. The program was not able to detect experimental problems caused by contamination or debris in the well disturbing the OD signal. In several of the experiments, only two of four repetitions yielded results and thus had to be discarded. This was especially the case for *L. gasseri* and *L. crispatus*. A reason might be that the cells did not survive the inoculation from the reduced MRS into the newly formulated Hart media variants. Due to the high frequency with which this problem occurred with these strains, pipetting errors can certainly be ruled out. Very problematic for detecting a reasonable exponential phase was the fact that mucin and to a lesser extent also glycogen increased the basal absorption of the media. This can be explained by the mucus and glycogen constituents which tend to form slime threads which settled on the bottom and corner of the wells, obstructing the light path. Although the shaking of the 96 well plate was vigorous for 10 seconds before every OD measurement, this turned out in some cases to be insufficient to disperse the solids and bacteria evenly. For these reasons all mucin measurements and the experiment with a reduced water content could not be evaluated and the data obtained could not be considered. Of the remaining results, three repetitions for each of the 90 experiments were manually selected to calculate the average doubling time and the according standard deviation. The fact that the OD measurements above OD 0,4 are not linear to the actual concentrations of cells is not of concern, although the Hart media had baseline OD values of around 0,35. This is a systemic effect and affected all samples and data points with the same intensity. As long as the measured growth curves are considered as relative and compared among each other, the deducted results whether a substance increased the doubling time or not can be considered as being reliable.

The increased doubling times resulting from the replacement of MEM Vitamins appear to be the effect of the compounds Pyridozal, Riboflavin, Thiamin, which are not included in the Hart vitamin mix. Vitamin K_1, p-Aminobenzoic Acid, L-Ascorbic Acid, D-Biotin and Cyancobalamin which are included in the Hart but not the MEM vitamins do not seem to be relevant for the growth of the tested strains. Previous studies reported higher concentrations of the carbon sources glycogen and glucose (Silvina et al. 2007; Owen & Katz 1999). To evaluate the relevance of their concentrations glycogen was increased from 1 to 5 and 10 g/l and glucose from 5 to 10 and 20 g/l (Fig. 8 and 7). For glycogen and glucose no obvious dose response effect could be observed. Some strains displayed a shorter doubling time for the higher concentration of glucose, but not for the lower. 10 g/l was even detrimental for two of four tested strains. The reason for this response remains elusive. An increased glycogen concentration was beneficial for three out of five strains. A stronger effect of 10 g/l than of 5 g/l could only be seen for *L. lactis*. A higher glycogen secretion by vaginal epithelial cells has been correlated with increased estrogen levels in the blood and increased lactic acid production in the vaginal vault (Jakobsson 2008; Silvina et al. 2007). The results of this study are in agreement with these published findings and clearly show that glycogen has a growth stimulating effect on lactic acid bacteria. The replacement of the amino acid mixes of the Hart mix with the SA mix inhibited the growth of *L. crispatus* and *L. lactis* (Fig. 9). The ingredients of both amino acid mixes differ in total quantity. The Hart A.A. mix contains a total weight of 5,82 g/l, while the SA A.A. mix sums up to 3,6 g/l. The SA A.A. mix also lacks L-Aspartate and Taurine. Taurine, although not an amino acid, was found to be present in vaginal fluid (G.Neumann, J. Gartzke 1984; Liu et al. 2011). It was suggested by , although it is not known if and how it affects the vaginal microbiome. Therefore it can be assumed, that the two strains which did not grow have an auxotrophic dependency on at least one of these amino acids. Therefore, the use Hart A.A. mix is recommended for media mimicking the chemical environment of vaginal fluid. The Hart Mix suggested TWEEN 20 as an ingredient, which is a non-ionic surfactant. The inclusion of TWEEN 80, a longer chain polyoxethylen surfactant, was suggested by Silvina et. al. 2007. TWEEN 80 is known to contain Oleic acid, which in turn was shown to influence the growth of *Lactobacilli* positively (Endo et al. 2006). However the replacement of TWEEN 20 by TWEEN 80 had no positive effects on the strains. Oleic acid shortened the doubling time of *L. lactis* significantly. However, the handling of Oleic acid is problematic, because it is unstable at room temperature, needs to be added freshly to the culture right before the experiments and is problematic to store. As it did not show a positive effect, it appears justified to use TWEEN 20 in future experiments. The growth stimulating effect of the Deoxyadenosine, Deoxyguanosine, Deoxyacytidine, and Thymidine is beneficial to bacterial strains with a deficiency of ribonucleotide reductases. It was assumed that symbiotic strains have might be auxotrophic in this respect. This could apply for *L. jensenii* and *L. vaginalis*, as these strains showed increased growth speed as a repsonse to the added deoxynucelosides. Jensen and Hammer reported a positive effect of a mix of micronutrients on the growth of *L. lactis (Jensen & Hammer 1993; Neidhardt et al. 1974)*. This finding could not be reproduced. Mineral micronutrients, in fact, improved the doubling time of *L. jensenii* and *L. vaginalis* considerably. They are thought to be required as cofactors in various enzymatic reactions . A similar effect was also for expected for $CaCl_2$ and could be demonstrated for *L. jensenii*, *L. vaginalis* and *L. lactis.* Lactic acid was suggested for a vaginal media simulation by Silvina et. al. 2007, however no positive effect on the growth of the tested strains was found.

Taken together, the plate reader experiments showed that the symbiotic vaginal bacteria strains *L. jensenii, L. crispatus, L. gasseri, L. vaginalis* and *L. lactis* can be grown in reduced MRS medium and in the Hart vaginal fluid simulation. Further, it could be demonstrated that adding the mineral micronutrients ($(NH_4)_6(MO_7)_{24}$, H_3BO_3, $CoCl_2$, $CuSO_4$, $MnCl_2$, $ZnSO_4$), $CaCl_2$, the deoxynucleosides Adenine, Guanine, Cytosine and Thymine and replacing Kao & Michayluk Vitamins and Vitamin K_1 with MEM vitamins and increasing the glucose concentration to 20 g/l and glycogen to 10 g/l each significantly shortened the doubling times of at least one of the observed strains. For future studies using artificial vaginal fluid it is suggested to include these named compounds. However, it remains to be tested whether all these compounds have an increased positive effect on the bacterial growth, when used in combination.

In most of the experiments *L. gasseri* and *L. crispatus* did not grow very well or their growth was very inconsistent making it difficult to use them as indicators for media efficiency. Thus it is not recomended to rely on these strains in future studies. *L. lactis* grew comparably slowly in all experiments and often displayed different reactions than the *Lactobacilli* strains to newly introduced compounds of media. However its growth was consistent throughout all experiments, justifying its use as a model organism for bacterial contraception.

Spermatozoa

To extract the movement pattern of singles cells from recorded sperm samples, computer assisted sperm analysis was necessary. Since commercially available CASA setups were not available and too costly to acquire, a new microscopy setup was developed and the digitally recorded data analyzed with an open source computer program available from pubmed. The program is a plugin for the ImageJ software and was originally developed to analyze fish sperm but could be successfully adapted to human sperm cells. To define input parameters to track human spermaotoza, a script using a directed evolution mechanism was developed. With these tools, four chemoattracants reported in the literature and progesterone were compared against a negative control and their effects on spermatozoa chemokinetic parameters analyzed.

Spermatozoa imaging setup

The preparation of the Neubauer Türk chamber to position the sperm cell in a focal plane to record videos had to be done manually. The spermatozoa in the F-Ham media had to be transferred with a pipette onto the platform in the center of the chamber, the coverglass and – for the chemokinesis experiment also the capillary – had to be carefully placed on top of the droplet, supported by the spacer on the sides. Keeping the coverglass in the appropriate position was not always successful due to a certain instability of the construction. Before imaging, samples had to be observed for internal currents. Evaporation of the liquid itself also created a current, however this was thought to be slow enough to not disturb the movement of the spermatozoa. If, and to what extent an internal current would affect the diffusion of the chemoattracant out of the capillary was not analyzed.

The self-made chamber allowed to analyze spermatozoa movement at very low costs and with little effort. All analysis could be done in house and therefore a precise timing of the experiments was possible. This was absolutely necessary due to the short life span of spermatozoa after ejaculation. However, the chamber design can be improved. Especially the chemotaxis setup was fragile and its handling required patience and practice. A modified design to stabilize the single parts of the setup on top of each other would be useful.

Computer Assisted Semen Analysis

The CASA plugin parameters were created with a newly developed program. The program was designed to automatically load greyscale tiff stacks of recorded spermatozoa. Automatically optimizing the threshold parameters was crucial to detect sperm tracks, since the focus for the recordings of the samples was manually set and not fully reproducible. Doing the procedure manually would have required too much time, since hundreds of recordings had to be processed. The “threshold function” of the “directed evolution” script (see Supplementary Information) searches for the optimal threshold parameters to transform the greyscale tiff stack into monochrome. The upper boundary of the threshold was therefore slowly decreased and after each decrement the CASA plugin executed. The total number of detected sperm tracks was evaluated and compared to the number of tracks detected in the preceding the analysis. In some tiff stacks the sperm heads appeared as big white spots after the transformation to monochrome, while in others, pixels around the spot were wrongly detected. This was a result of light interference and reflections on the coverglass and depended on the focal adjustment. In tiff stacks with such settings, the CASA plugin had problems to detect the spermatozoa tracks clearly (compare Fig. 14 experiment 1e). Another problem in identifying the optimal threshold parameters related to the problem that the number of detected spermatozoa tracks did not only depend on the clarity of the monochrome picture, but also on the input parameters for the CASA plugin. Debris, erroneously detected as sperm tracks caused the threshold function to continue decreasing the threshold parameters, although no correct tracks were found anymore. To overcome this problem, before running the directed evolution macro, a best guess for input parameters was made which was good enough to detect human spermatozoa to allow the threshold function to work reliably. After the directed evolution macro resulted in the three presented sets of parameters (Tab. 9), the input parameters for the threshold function were replaced by those capable in generating the results shown in figure 14 column 2 to 4. These results of the "directed evolution macro" were compared against the parameters suggested by Leedy et. al. for fish semen (Fig. 14 column 1). The fish parameters showed a poor performance in finding valid spermatozoa tracks. Some tracks were detected, however, they were recognized as not valid. A lot of debris was detected and mostly not defined as invalid. Experiment 1 resulted in a good number of detected tracks, although the parameters failed to define a proper threshold in all tested cases and thus was not applicable to stacks with diverging focal planes. Additionally, much debris was wrongly detected as cell movement. Experiment 2 and 3 showed a very similar performance. Experiment 3 was a little better in recognizing valid sperm tracks compared to experiment 2 and was therefore used in further analysis of the video recordings of chemokinesis experiments. Among the results in the three experiments, several parameters still varied significantly after 21 rounds of mutant selection. This indicates that they are either not of importance to asses human spermatozoa, or influence parameters which the setup cannot properly detect. The results might be biased to some extent by the order of analyzed tiff stacks. Each stack changed the selection of parameters in a distinct way, depending on the specifics of the recording, most notably by the varying focal plane. Therefore, the last generation of the last tiff stack had the strongest influence on the parameters relative to the generations before. Further, the entire range of possible values of all parameters can not be represented by comparing three results only. However, this inaccuracy did not seem to have a too strong influence. The overall outcome was a considerable improvement compared to the fish parameters which were used to initiate the optimization. The parameters of experiment 3 enabled the threshold function to create monochrome tiff stacks of good quality and the CASA plugin correctly detected many spermatozoa tracks and properly sorted out debris pixels. The procedure could not be repeated more often, due to a calculation time of several days per experiment. The selected results are therefore a rough but sufficient approximation to the optimal values for human spermatozoa.

Besides its use in research, the developed CASA setup and the software tools in combination with the CASA plugin of Leedy et. al. together with ImageJ could serve as a cheap and simple way for medical doctors to study fertility problems in men. It would be especially useful in places where specialized laboratories and equipment are not available.

Chemokinesis experiment

The chemokinetic behaviour of human spermatozoa towards five substances which were reported to affect spermatozoa was tested in this study (Isobe et al. 2002; Teves et al. 2006; Jin et al. 2010; Nadav Zamir, Rivka Riven-Kreitman, Mira Manor, Amnon Makler, Shmaryahu Blumberg, Dina

Ralt 1993; Miraglia et al. 2007). The two protein-based chemoattractants ANP at 30,8 ng/ml (≈ 10 nM) and RANTES at 1 ng/ml (≈ 0,13 nM), NGF-ß as a putative chemoattractant at 100 ng/ml (≈ 7,35 nM), the nitric oxide radicals emitting SNOG at 3 ng/ml (≈ 100 nM) and - as a positive control - PROG at 3,125 pg/ml (≈ 10 pM). By demonstrating a responsiveness of the spermatozoa to PROG, the newly developed setup would be proven to be functional. A direct comparison of different concentrations of the four other substances should provide evidence on their suitability for bacterial contraception. To create a reference for future studies on bacterial expression systems for chemoattractants, five concentrations were measured. The concentrations which were tested and reported in literature were in most studies tested in exponential intervals to the base of 10. Chemokinetic response however usually showed a clear peak at a distinct concentration. Therefore, smaller intervals around the concentration with the strongest effect were chosen to map the effects in detail. The concentrations of all chemoattractants tested were increased by a factor of 5 and 25 as well as decreased to 1/5 and 1/25 relative to the published recommendations.

The analysis was made with the newly developed CASA system using the parameters of experiment 3 of the directed evolution script (Tab. 9). The three spermatozoa samples were donated by different individuals, but were processed and recorded in exactly the same way (see Spermatozoa Results and the Methods section). All concentrations of all chemoattractants were tested on each sperm sample. The CASA video analysis of each repetition of all experiments yielded valid tracks for at least 800 cells. The data of the repetitions were pooled and the relative percentage of the total amount of cells used to analyze the results. The difficulty with detecting chemotactic effects is that only about 8 % of the cells of an ejaculate are responsive to the chemoattractant (Armon & Eisenbach 2011). The chemokinetic effect was observed by comparing VCL and LIN values of the spermatozoa tracks. A LIN percentage of 100% indicates a straight line and the lower the percentage, the stronger the curvature of the track. A reduction and narrowing of the LIN value distribution thus indicates that the cells tend to swim in increasingly narrowing spirals.

For both observed parameters, it can be noticed that the measurement of the control has a more even distribution and only one clear peak, while the results of the other experiments are less clear. This can be explained by the lower number of tracked cells for each concentration, ranging from 2730 to 12678 cells, while the blank distribution is calculated from 18812 cells. The higher the number of analyzed cells, the smoother is the distribution of the measured values. It can be expected that the results of the chemoattractant experiments will show clearer distributions when the database is adequately increased. The number of analyzed spermatozoa is high enough though to see distinct patterns in the graphs. The fact that the data from three donors per experiment was pooled automatically leads to an averaging of the distribution. Since the data is displayed as the percentage of the the total amount of analyzed cells per experiment, the distributions of VCL and LIN can be compared between the experiments.

The strongest increase of spermatozoa speed caused by PROG was at a concentration of 78,5 pg/ml and the second highest with almost the same intensity at 3,14 pg/ml. The second highest increased matches the concentration which was reported in previous studies. Its 25 fold increase as well as its decrease to 1/5th and 1/25th are also in agreement with the results reported literature (Teves et al. 2006). A dilution of 1/25 of this concentration resulted in the strongest effect on LIN. However LIN values were reduced at all concentrations, although the effect was stronger for the concnetration lower than the literature value. This means, that spermatozoa swim smaller circles under the influence of PROG and swim faster at 78,5 pg/ml than normal. NGF-ß had the opposite effect on VCL. All cells were slowed down, the most at a concentration of 500 ng/ml. The effect on LIN however was also reduced and comparable to the effect of PROG. These observations are in concordance with the literature, where it was speculated that NGF-ß might have a vitality promoting effect on spermatozoa through regulating intracellular leptin production and counteracting effects of stress through ROS (Sharma & Agarwal 1996; Li et al. 2010). As it is also present in relatively high concentrations in the ejaculate, NGF-ß probably does not help preventing fertilization, but might be have the opposite effect, shedding light on a possible use of vaginal symbiotic bacteria to promote fertility (Ratto et al. 2012). SNOG also reduced the VCL at all measured concentrations and caused the distribution of LIN values to narrow around 50 %. The effect on VCL and LIN was most prominent at 15 ng/ml, which is five fold higher compared to the literature data (Miraglia et al. 2007). The actual concentration of nitric-oxide radicals in solution however is difficult to estimate, because SNOG constantly releases nitric-oxide at a temperature dependent rate. Therefore, it cannot be excluded that the concentration prepared actually results in a lower level of nitric-oxide, which is closer to the literature data. The reduction in speed could be due to a oxidizing damage of the spermatozoa membrane, like known for cells of the immune system that emit nitric-oxide radicals to kill unwanted cells intruding the cervical canal. The effect on LIN might be related to chemotaxis, but a different analysis is required to validate this assumption. Both proteins ANP and RANTES increased the VCL and reduced LIN values. The strongest effect of ANP on VCL was at a concentration of 6,16 ng/ml, and thereby 1/5 of the concentration as reported in previous studies (Zamir et al. 1993). For RANTES it was 5 ng/ml and thus five times higher than the literature value (Isobe et al. 2002). LIN values of both substances showed the strongest decrease at concentrations which were lower than the ones affecting VCL. For ANP this concentration was 5 fold lower and 25 fold for RANTES. Comparing both substances, ANP had the stronger effects on LIN and VCL than RANTES.

A general observation is that the clearest reduction of LIN values was at lower concentration than the strongest effect (increase or decreased values) on VCL, except for NGF-ß, where the greatest effect on VCL and LIN was found at the same concentration. Since ANP showed the strongest effect on spermatozoa movement, it appears to be the best choice to construct the prototype plasmid (see below). Nitric-oxide showed a very strong effect on VCL. Under the microscope, it could be visually observed that the spermatozoa died off much faster compared to the other experiments. Thus an enzyme producing nitric oxide could also be considered for bacterial-based contraception. The results obtained also indicate, that the effects of the chemoattractants on the parameters LIN and VCL are not dependent on each other. This means some concentrations and substances induce a change in the curvature of the tracks, while others are affecting the speed of the cell. Generally, a response to all tested substances was noted, mainly due to a narrowing of the distribution of LIN values compared to the control. Assuming that spermatozoa react in the same way to the presence of hormones emitted by the oocyte despite of a higher viscosity of the tubal fluid, spermatozoa would increase their chance to encounter the oocyte. The more distant from the oocyte, the more often the spermatozoa change the direction of their path to increase the chance to hit the large and slowly traveling oocyte. The closer to the oocyte cell, the higher is the chemoattractant concentration and the straighter and faster is the trajectory of the spermatozoa. These mechanisms could be disturbed by bacteria in the vaginal fault emitting these substances thereby reducing the likelihood of spermatozoa capable of fertilization reaching the oocyte.

Overall, VCL values ranged between velocities of as low as 7 µm/s and as high as 50 µm/s. This is significantly lower than most studies reported. Hyperactivated sperm cells, for example, move at least at 60 µm/s (Armon & Eisenbach 2011). This difference can be explained by the low frame rate of 33 fps and the low resolution of 400 by 400 pixels. Only for fast moving cells, a distinct track with the spermatozoon head oscillating along the average path was visible. Slower moving cells could not be resolved clearly impairing the measurement of VCL. The VAP values, however, are not affected. Therefore, LIN values are not changed, as the program should not have problems in detecting the average path and its start and end points. For future studies it is recommended to repeat the measurements at a higher frame rate and resolution. To ensure comparability of videos of higher quality, the videos could be digitally resized and the framerate sampled down. It would also be beneficial to rework the code of the CASA plugin. If the starting and endpoints of all positively selected tracks could be printed out, chemotaxis could be easily measured. Furthermore, a calculation of the average lateral head displacement of a track would enable determining hyperactivation of spermatozoa (Castellini et al. 2011). The code developed in this study for the ImageJ macros (see supplementary information: “directed evolution” and “threshold” functions) could also be incorporated into the CASA plugin to allow easier use.

Taken together, the results of the spermatozoa experiments proved that the newly developed CASA setup is sufficiently sensitive to detect small changes in the spermatozoa chemokinesis parameters LIN and VCL. A large amount of cells can be analyzed with the developed software tools from one donor sample. A finding that was contrary to expectations was, that NGF-ß could be used to promote fertility by improving spermatozoa vitality. To chemotactically irritate spermatozoa and to reduce their vitality, ANP and nitric-oxide turned out to be most effective.

Genetic construct

To address how a genetic construct that provides the desired features should look like, a draft is introduced here. *L. lactis* growth was tested in the vaginal fluid simulation experiments and found to be principally usable as model organism. *L. lactis* and the tested symbiotic *Lactobacilli* live in the same habitat and show similar characteristics. Both are gram positive facultative aerobic, lactic acid producing bacteria. They also have a very similar codon usage. The website http://www.kazusa.or.jp, for example, states that the mean difference between the sequence of codons between *Lactobacillus gasseri* and *Lactococcus lactis* is only 9,08 %. This would allow designing vectors with the same sequence and transform them into symbiotic *Lactobacillus* and *Lactococcus* species. Further, there is information available on how to successfully transform *Lactococci* (Hugenholtz et al. 2000; Ravn 2003; Le Loir et al. 2005) which have been intensely studied during the last decades. There is a plethora of commercial strains and methods available, mostly developed for dairy industries. This pool of knowledge can be expected to considerably accelerate the development of a prototype for bacterial-based contraception in future studies. The spermatozoa chemokinesis experiments revealed that the prototype should at first be built using the gene coding for ANP. As this protein is very short, it might be possible to even place a second gene for another chemoattractant next to it, under the control of the same promotor. RANTES could be tested here, because its physiologically active concentration is only a little lower than that of ANP. Another promising option could be to use the vector to express a bacterial nitric oxide synthase-oxygenase. This enzyme produces nitric oxide from L-arginine, NADPH, protons and oxygen. As it catalyzes the production of a substance which is influencing spermatozoa chemokinesis, it does not need to be expressed in high concentrations, thereby reducing the energetic costs for the transgenic cell. However, no research was done whether the catalyzed reaction is actually possible or happening at a high enough rate in vaginal fluid.

The bacteria could be kept alive in the vagina with resistance genes of food grade lantibiotics, which they can produce themselves. This would reduce growth of competing naturally occurring strains and give the genetically altered ones a selective advantage. To counteract a too high energy cost for the expression of the chemoattractant proteins, the expression of lantibiotic and of chemoattractants could be done in two different strains, while both also express the resistance genes. A commercially available food grade and well known lantibiotic resistance construct is the NICE vector (Hu et al. 2009). The NICE system was developed in the early 1990ies and the patent will expire soon. The expression of the lantibiotic nisin is stably integrated into the genome of the lactic acid bacteria and a stable concentration of nisin is established in the culture. A widely used strain is *L.lactis* NZ9000, a modified MG1363 strain, which was subject of this study. The strain can be transformed with a designed plasmid – for example the commercially available pNZ8123, which contains the nisin inducible promotor Pnis. The chemoattractant to distort the sperm chemotaxis and/or chemokinesis could be cloned into the open reading frame (ORF) under the control of the Pnis promotor (Fig. 20). It was developed for *L. lactis* but has also successfully been used in *Lactobacilli* (Pavan et al. 2000; Hazebrouck et al. 2007). Other lantibiotics such as lactocin (Dover et al. 2007) or bacteriocin HV219 (Todorov et al. 2006) might be considered as alternatives to nisin.

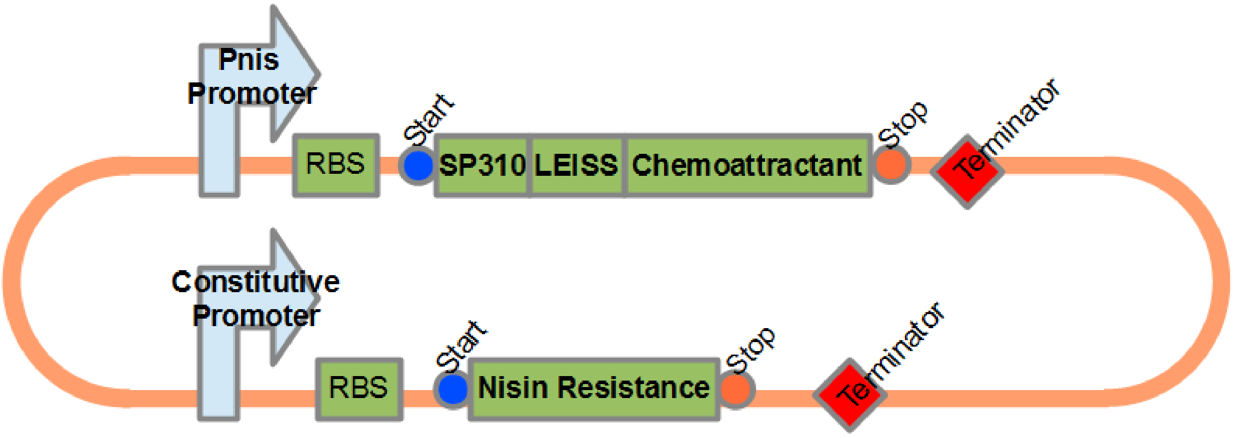

Figure 20: *hypothetical bacterial contraception plasmid for Lactococcus lactis or vaginal symbiotic Lactobacilli. Pnis Promoter: A nisin inducible promotor. RBS: Ribosome Binding Site. Start and Stop → codons defining the open reading frame. SP310: export leader sequence. LEISS: artificial peptide linker. Chemoattractant: the genetic sequence coding for a protein for sperm chemoattractant. Nisin Resistance: Gene coding for a protein which provides resistance against the lantibiotic nisin. Terminator: m-RNA sequence forming a secondary structure to terminate translation.(Protein sequences see Supplementary Information)*

To get the bacterium to secrete the chemoattractant, an export leader peptide and a short peptide linker needs to be fused to the N-terminus of the protein. I suggest to use the recently published SP310mut2 leader in combination with the LEISS linker (amino acid codes see Supplementary Information) (Le Loir et al. 2005; Ravn 2003). On the same vector, an ORF under the control of a constitutive promotor contains the nisin resistance gene. The genes are flanked by start and stop codons and have a ribosome binding site (RBS) upstream and a terminator downstream of the gene. The RBS could be used in future studies to adjust the expression strength and thus to stabilize the concentration of the expressed chemoattractant. The Pnis controlled expression of the chemoattractant would only allow transgenic bacteria to start stronger protein expression when there is a certain concentration of nisin in the vaginal fluid diminishing competing non-resistant bacteria. This would help establishing a stable population of the strain, making it easier to adjust the concentration of chemoattractants in the vaginal fluid.

Once a vector has been established in *L. lactis*, it needs to be transferred into one or more *Lactobacilli* strains. There have been reports about successful transformation of *L. gasseri*, *L. crispatus* and *L. jensenii* (Beasley et al. 2004; Wegkamp et al. 2004; Vangelista et al. 2010) . Interestingly, a research group recently tested the heterologous expression of RANTES in *L. jensenii* as a means to protect women from HIV infections (Vangelista et al. 2010). To avoid the difficulties with stably transforming the chromosome of wild type *Lactobacilli* strains, it may be recommended to include a cassette for the expression of nisin into the same vector.

Outlook

To finish phase I of the prototype development, the vaginal fluid simulation has to be tested with the new compounds which were found to improve growth of the symbiotic *Lactobacilli* strains. To verify the detected chemokinetic effects on sperm of the tested chemoattractants, the data which was recorded with the chemotaxis setup should be analyzed. If the setup works and the detected effects of nitric-oxide, ANP and RANTES can also be found to influence sperm chemotaxis, the genetic construct can be made.

Altering viscoelastic properties of vaginal fluid to improve bacterial contraception

Besides finishing these phase I projects, another interesting option would be to use the bacteria to influence their extracellular matrix and thus alter the viscoelastic properties of the vaginal fluid to affect spermatozoa progression. Research into this idea was omitted in this study due to a lack of adequate equipment and time constraints. Altering the vaginal fluid ultrastructure to influence sperm progression was first hypothesized in the review of Rutllant et. al., 200. A mucus plug in the cervix occurs naturally during the non-fertile phase and only needed to be artificially maintained during the four to six fertile days of a womens period. It might be possible to make the bacteria secrete enzymes to alter the mucus ultrastructure within the cervix in a similar way hormonal contraception does. Lactic acid bacteria could secrete polysaccharides or build them extracellularly to form an artifical mucus plug that prevents sperm from entering the cervical canal (Tang et al. 1999). Exopolysaccharides (EPS) can be built by glycosyltransferases (Lairson et al. 2008; Péant et al. 2005; Taylor & Draget 2011) for example as in kefiran which is produced by *L. kefiranofaciens* (Maeda et al. 2004; Lebeer et al. 2008). Recent studies already demonstrated the feasilbility of altering EPS secretion by genetic manipulation of *Lactobacilli* chromosomes (Claesson et al. 2006). By varying the expression of genes responsible for the branching of EPS, the density of the resulting mucus grid structure can be increased resulting in significant change of macro and microscopic rheology.. Altering viscoelastic properties of the vaginal envirnment to block sperm and also STD has been demonstrated in vitro (Willits & Saltzman 2001) . For in vitro testing the viscoelastic properties of vaginal fluid can be easily quantified by tracking the random walk of fluorescent beads (Hugenholtz et al. 2000; Lai et al. 2009). Although this seems to be a promising approach for improved bacterial contraception, yet very little is known about the molecular mechanisms through which bacteria and the human body affect extracellular matrix viscosity and density.

Phase II: Assembly and testing of the bacterial contraception prototype

After the optimized vaginal fluid simulation is finalized, the best chemoattractant identified and a vector synthesized, *L. lactis* and *Lactobacilli* can be transformed. Then, the protein secretion efficiency of the transformed strains need to be determined under near-natural conditions. Also, the growth rates of the transgenic strains competing with a natural vaginal microbiome have to be assessed.. If phase I will be extended into research for a viscosity altering construct, the sperm chemotaxis setup can be used to test spermatozoa penetration efficiency by filling the capillary with the mucus media and observe how far sperm protrude into it in a given time. Similarly, by filling the capillary with chemoattractant emitting bacteria the chemotactic effects on spermatozoa can be tested. The sampled and yet to analyze chemotaxis videos could serve as a reference. Different genetic layouts (e.g. varying promotor or RBS strengths) need to be evaluated as well as tested for reliability under various conditions. The greater goal in these tests will be to determine if and to what extent sperm survival under near-natural situation can be effected by the transgenic bacteria.

It has to be noted that the suggested bacterial strains are not found in genital tracts of all women. Instead, vaginal microbiomes vary considerably. It will be necessary to identify more strains from different types of microbiomes and to find ways for their transformation. A major question that also remains to be answered is how reliable bacterial contraception would be. Also, the possibility of unwanted proliferation of the strain and lateral gene transfer have to be taken into consideration. Ways to switch off the contraceptive function and to prevent unintended side effects such as the use of auxotrophic strains, genetic indicators and kill switches will have to be elaborated. If a working prototype can be developed, it will be necessary to prove the safety of the method.

Hypothetical mode of application of the bacterial contraception

Ideally, the final product could consist of a normal yoghurt, which women ingest in regular intervals, or a cream or pill to be applied directly in the vagina. In case of ingestion, the bacteria would travel through the intestinal system and eventually reach from the anus into the vagina – a naturally occurring phenomenon. In the vaginal environment they would then start expressing the relevant genes which help preventing pregnancy (Fig. 21). Contrary to the original idea and as indicated by the findings about NGF-ß (Li et al. 2010), a conception promoting strain could also be developed.

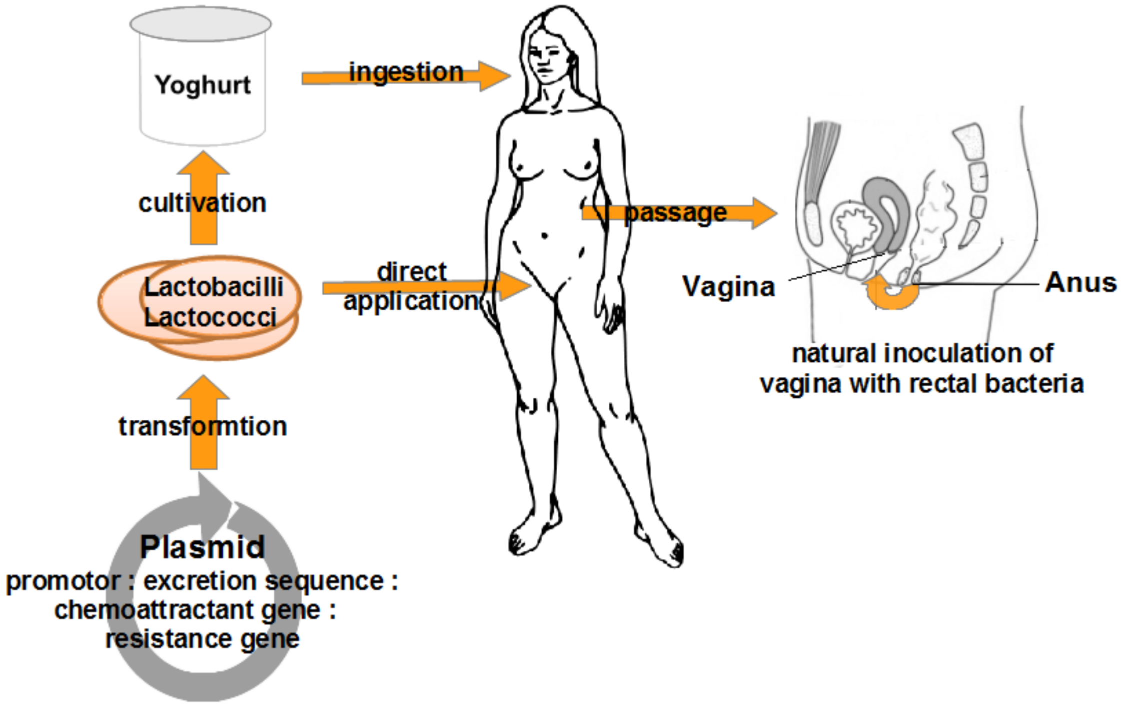

Figure 21: *Hypothetical bacterial contraception product:Symbiotic Lactobacilli or Lactococci are genetically modified with a plasmid including a resistance gene for increased fitness to survive in a natural environment and an expression cassette for production and secretion of chemoattractant proteins to intercept spermatozoa in the female reprodocive tract. The bacteria could be directly applied in the vaginal environment or, for example enriched in yoghurt, be ingested by women. The natural passage through the digestive tract into the vagina could be used as a mode of enriching the modified bacteria in the vaginal environment.*

The bacteria would then emit substances to promote spermatozoa movement and cell vitality in case of a deficiency of the man, or for example anti inflammatory substances to prevent problems such as immune responses to sperm. Besides the effect on contraception, a very promising use of transgenic vaginal symbiotic bacteria will be immunity promoting mechanisms to fight sexually transmitted diseases (STD). To my knowledge there is already at least one ongoing clinical study in trial in phase I using transgenic *Lactobacilli*. In future, the various described applications for symbiotic bacteria could be combined in different products. Women could chose from the variety of functions depending on personal needs. Apart from the practical considerations discussed above, how the product could work and how to realize it, there is need to address questions like who is going to use it, how will the idea of transgenic bacteria for contraception be perceived, what is the societal impact of the method and how can an effective distribution to any woman in need of such contraception be guaranteed. Furthermore, legal questions concerning the use of genetically modified organisms need to be considered.

These questions however will have to be answered in future studies. Ideally, the development of the contraceptive will be conducted in a worldwide, non-profit cooperation between scientists, state and private actors. Thus, the concept could be developed quicker and in greater detail, accustomed to local needs. A safe and fair use of the method would be easier to realize and any person in demand could benefit from it.

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Supplementary Information

Content Supplementary Information

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Growth curves

The shown graphs in supplementary Fig. 3 to Fig.15 are the output of the R-script (see table 1 for information which supplementary graphic corresponds to which results figure), based on the data acquired from five different symbiotic lactic acid bacteria strains grown on variations of the Hart media mix (see methods and Suppl. Fig 1) and reduced MRS (Suppl. Fig.2). They show the logarithmic OD values over time and the steepest abline detected by the script. From the slope of the abline, the doubling time was calculated.

Dose-response experiments	**Concentration**	**Supplmentary information**
Dextrose	10 g/l	Fig.6
Dextrose	20 g/l	Fig.7
Glycogen	5 g/l	Fig.8
Glycogen	10 g/l	Fig.9
Additives		
Lactic acid	2 g/l	Fig.11
deoxynuceloside A G T C	100.00%	Fig.12
Micronutrients	100.00%	Fig.10
CaCl2 x 2 H2O	0,36 g/l	Fig.13
Replacements - substance in brackets is left out of the Hart mix		
Tween 80 (- Tween 20)	1,064 g/l	Fig.15
Oleic acid (- Tween 20)	0,214 g/l	Fig.14
SA amino acid mix (Hart amino acid mix)	100.00%	Fig.3
MEM Vitamin mix (- Kao&Michaylak, Vitamin $K_{1)}$	100.00%	Fig.5
SA Vitamin mix (- Kao&Michaylak, Vitamin $K_{1)}$	100.00%	Fig.4

Table 1: *Variations of Hart mix formulation for media optimization experiments. Dose-response experiments: Concentrations of components of Hart media were increased. Additives: New components were added to the media. Replacements: Components of the Hart mix were exchanged. Removed components in brackets. All concentrations in g/l – for the mixes 100% indicates the recommended final concentration.. Ingredients of Micronutrients, amino acid mixes and vitamin mixes see Methods.*

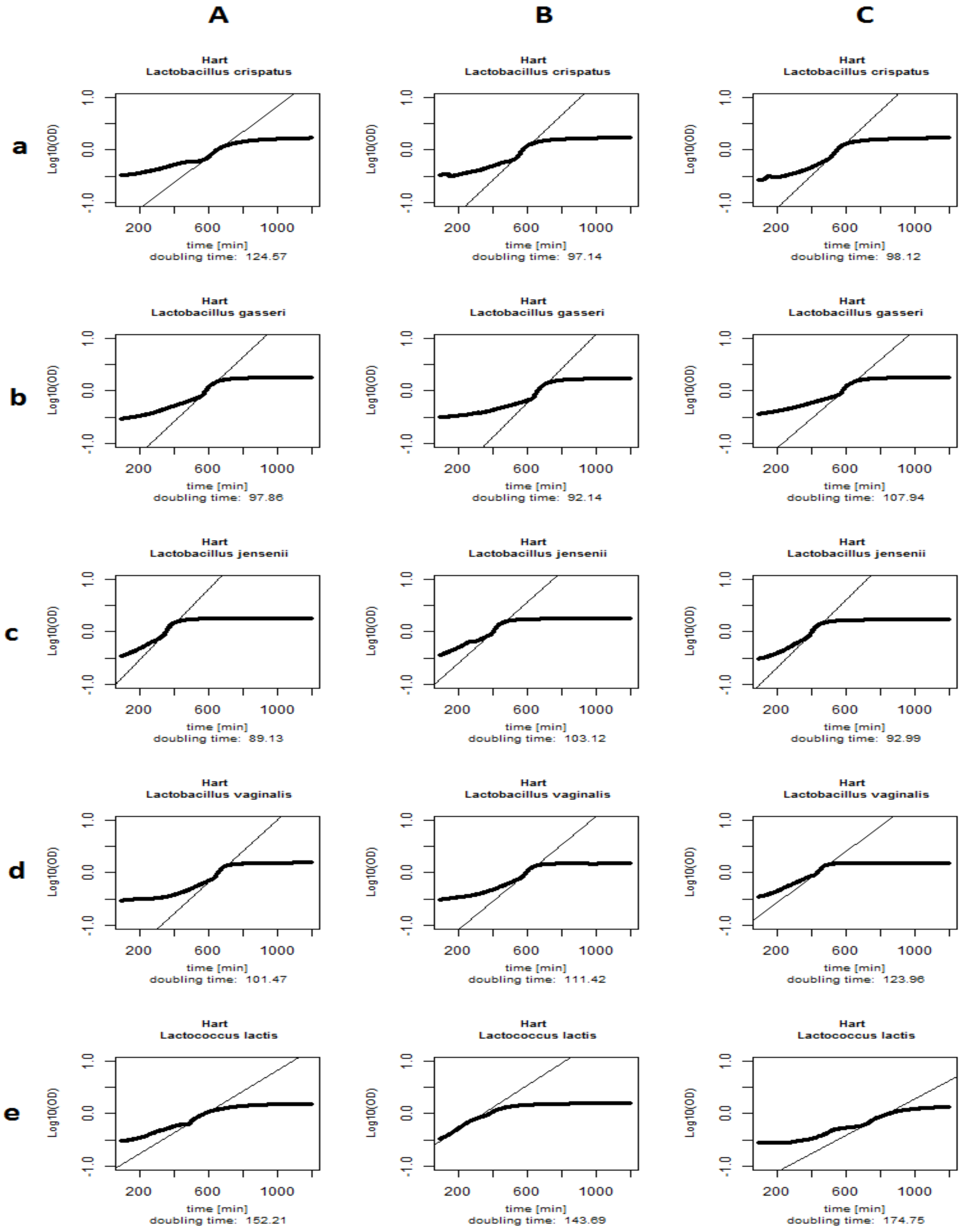

Figure 1: *Hart media growth curves for Lactobacillus crispatus (a), Lactobacillus gasseri (b), Lactobacillus jensenii (c), Lactobacillus vaginalis (d), Lactococcus lactis (e). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

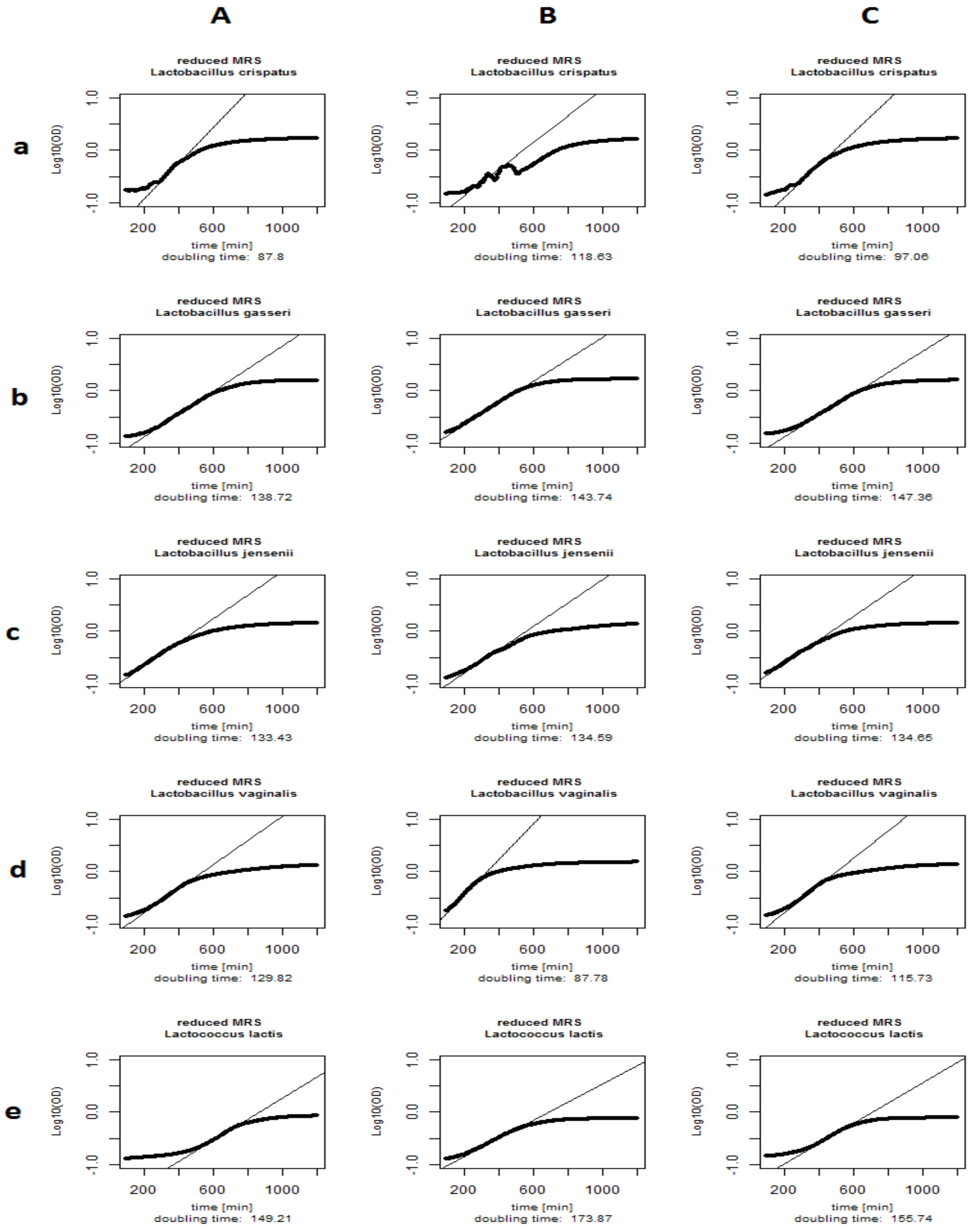

Figure 2: *MRS media growth curves for Lactobacillus crispatus (a), Lactobacillus gasseri (b), Lactobacillus jensenii (c), Lactobacillus vaginalis (d), Lactococcus lactis (e). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

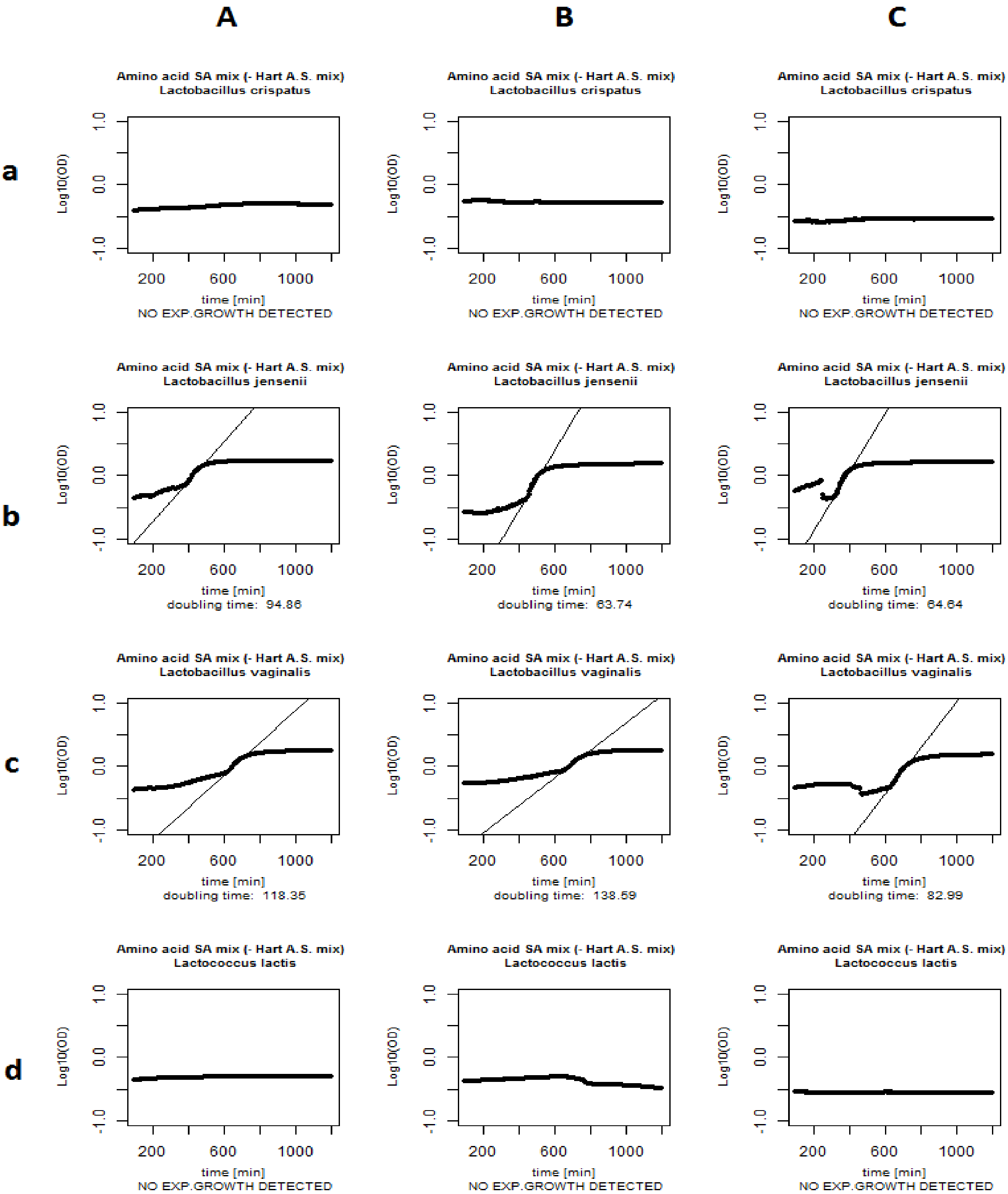

Figure 3: *SA amino acid media (Hart mix derivative) growth curves for Lactobacillus crispatus (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

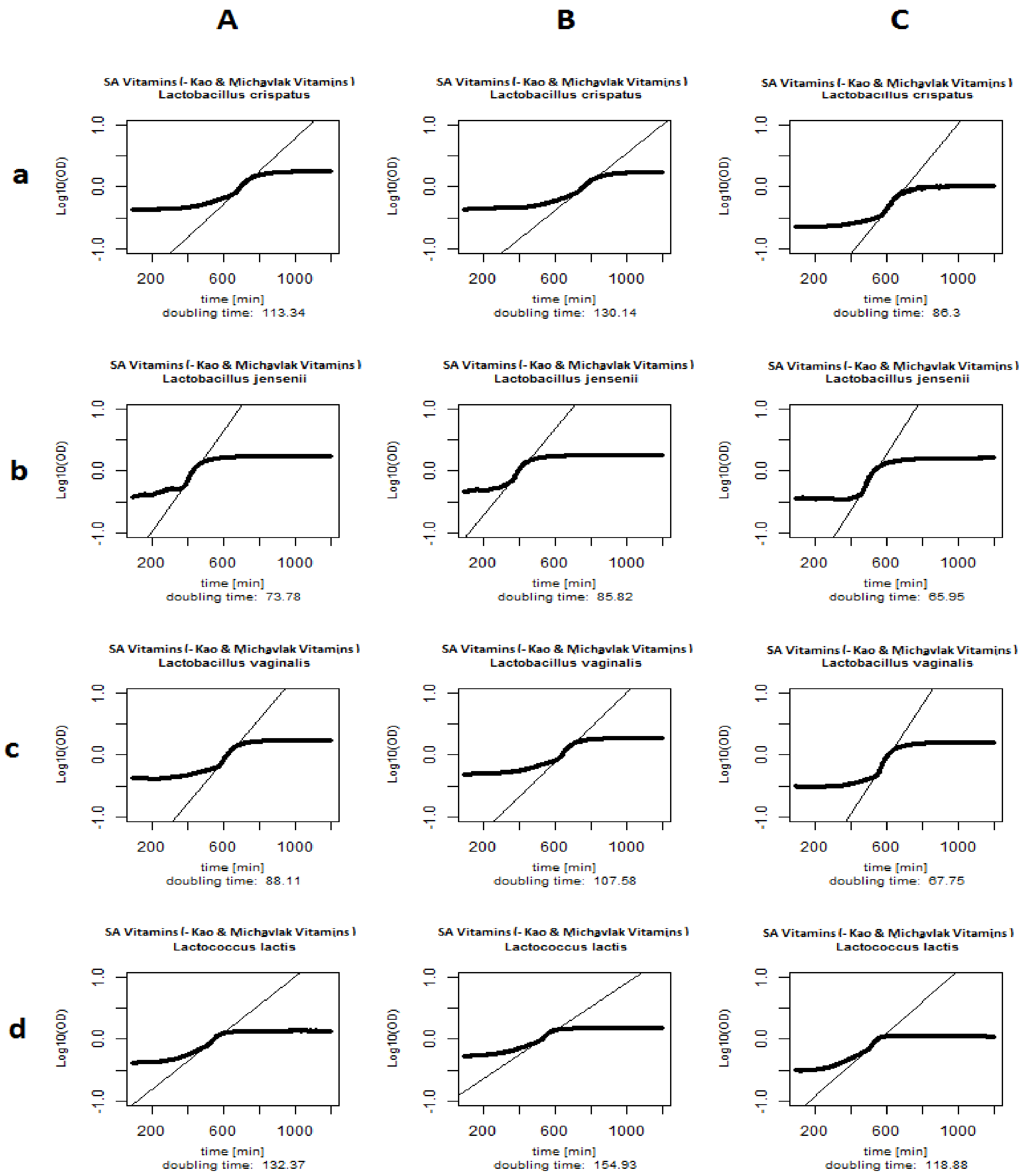

Figure 4: *SA vitamin mix media (Hart mix derivative) growth curves for Lactobacillus crispatus (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

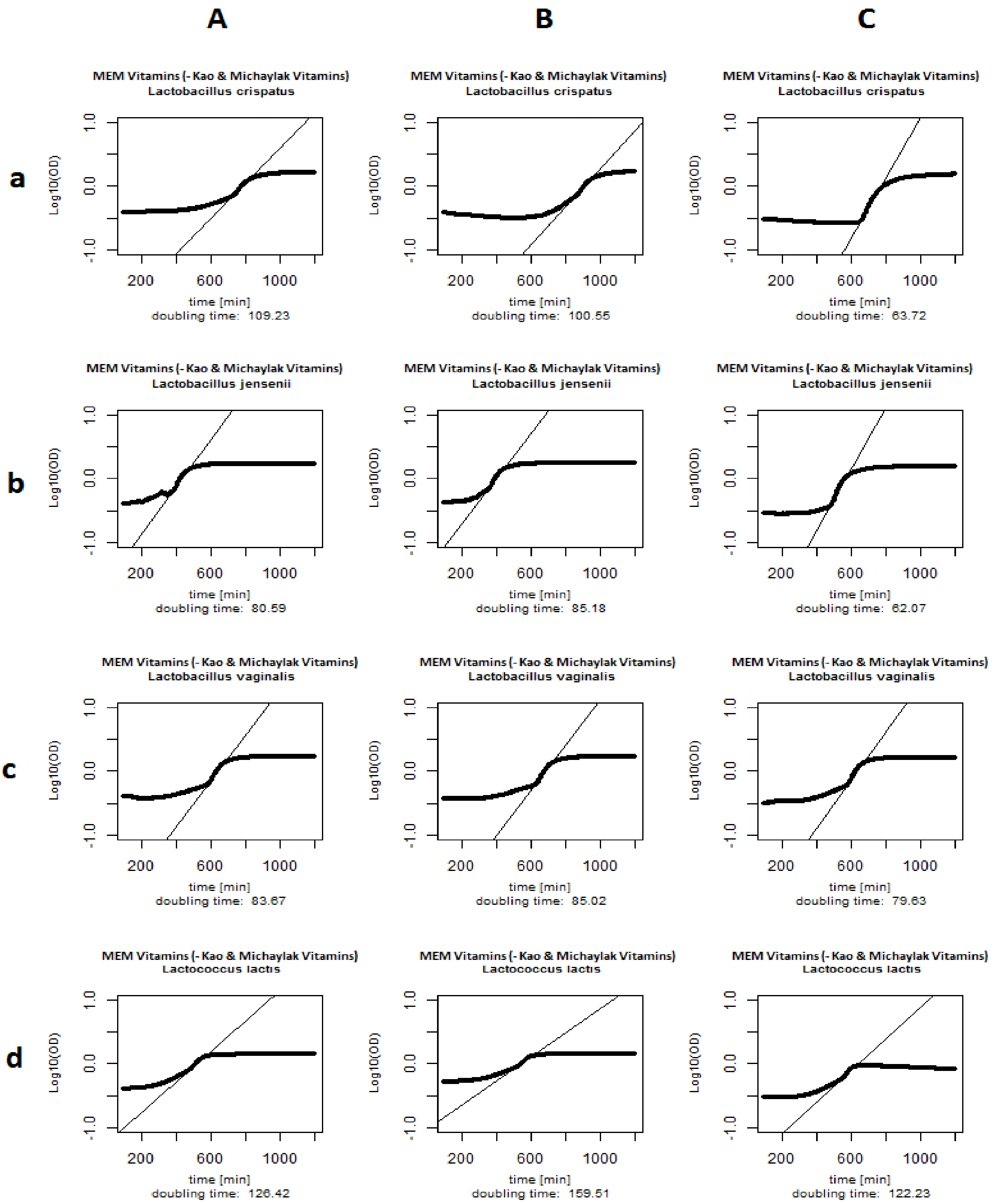

Figure 5: *MEM vitamin mix media (Hart mix derivative) growth curves for Lactobacillus crispatus (a), Lactobacillus gasseri (b), Lactobacillus jensenii (c), Lactobacillus vaginalis (d), Lactococcus lactis (e). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

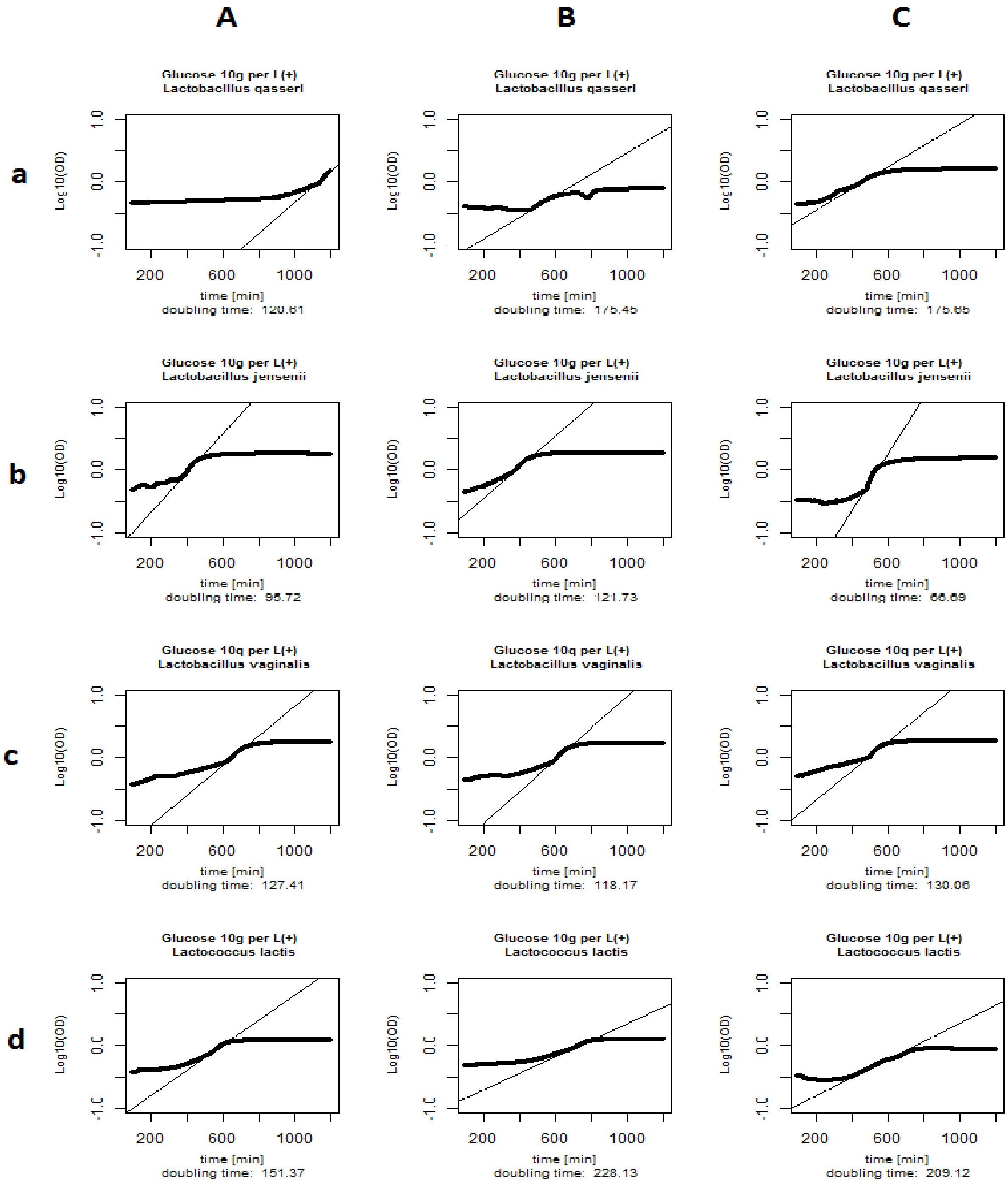

Figure 6: *Glucose 10g/l media (Hart mix derivative) growth curves for , Lactobacillus gasseri (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (e). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

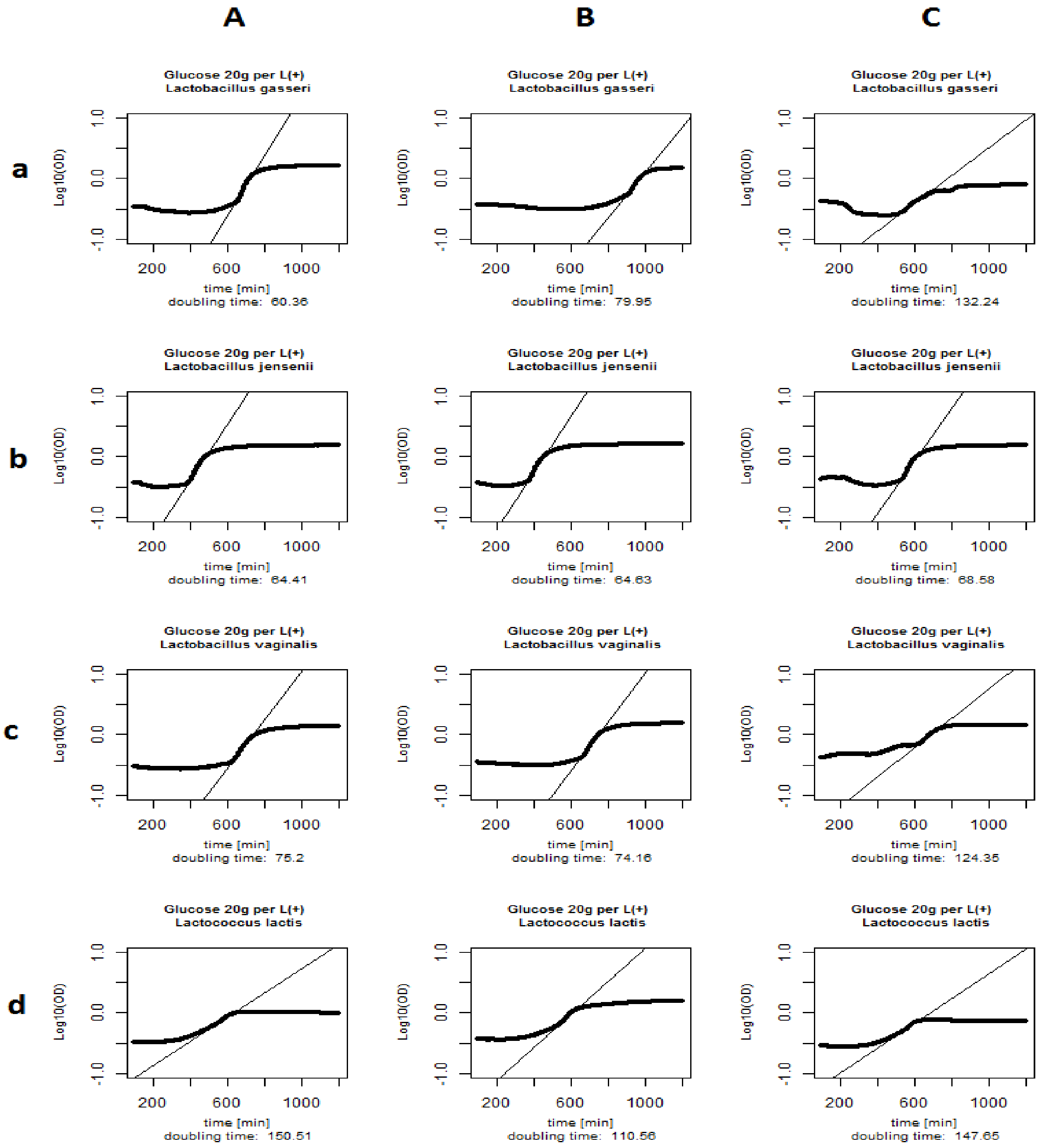

Figure 7: *Glucose 20g/l media (Hart mix derivative) growth curves for Lactobacillus gasseri (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (e), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

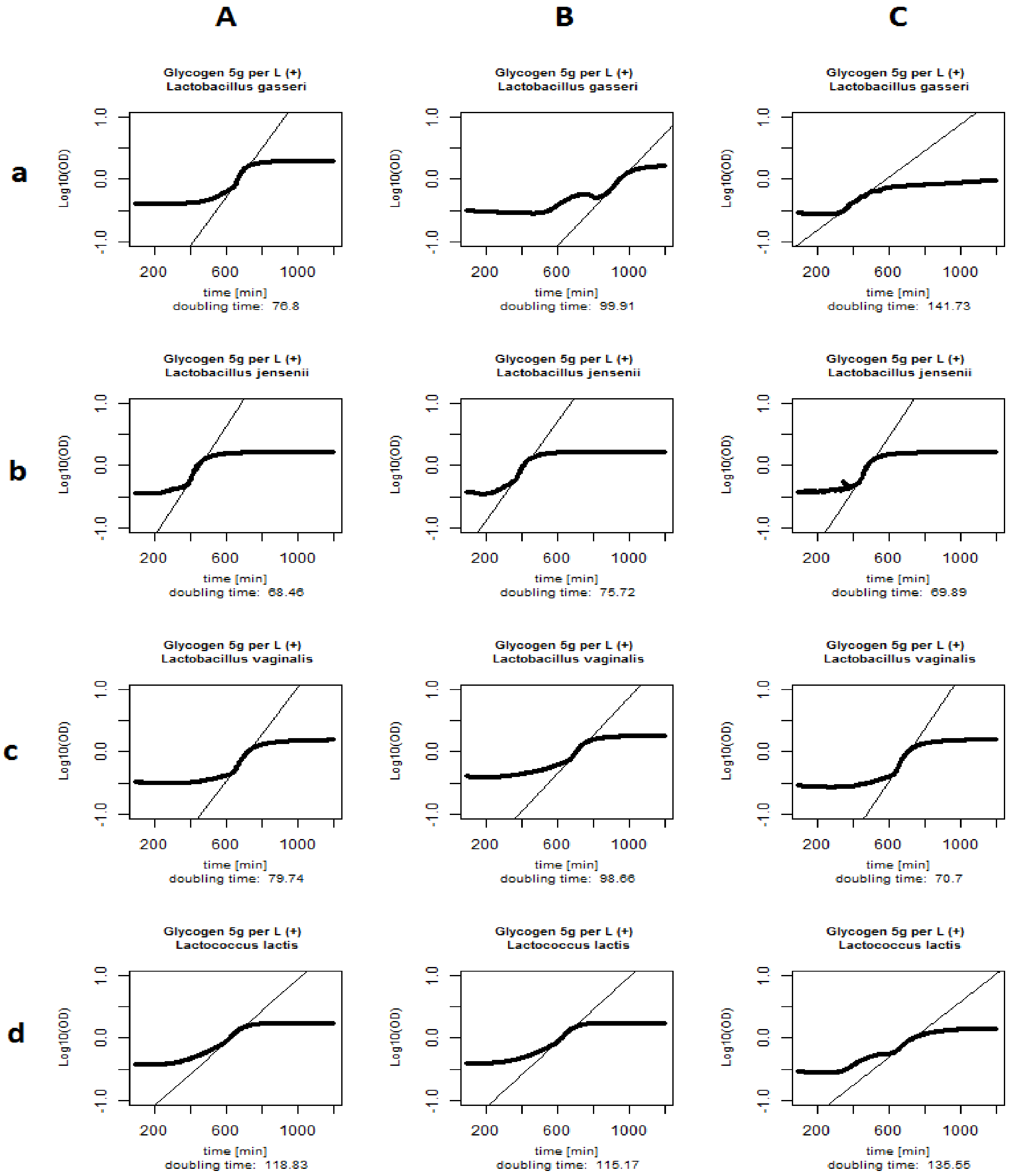

Figure 8: *Glycogen 5g/l media (Hart mix derivative) growth curves for Lactobacillus gasseri (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

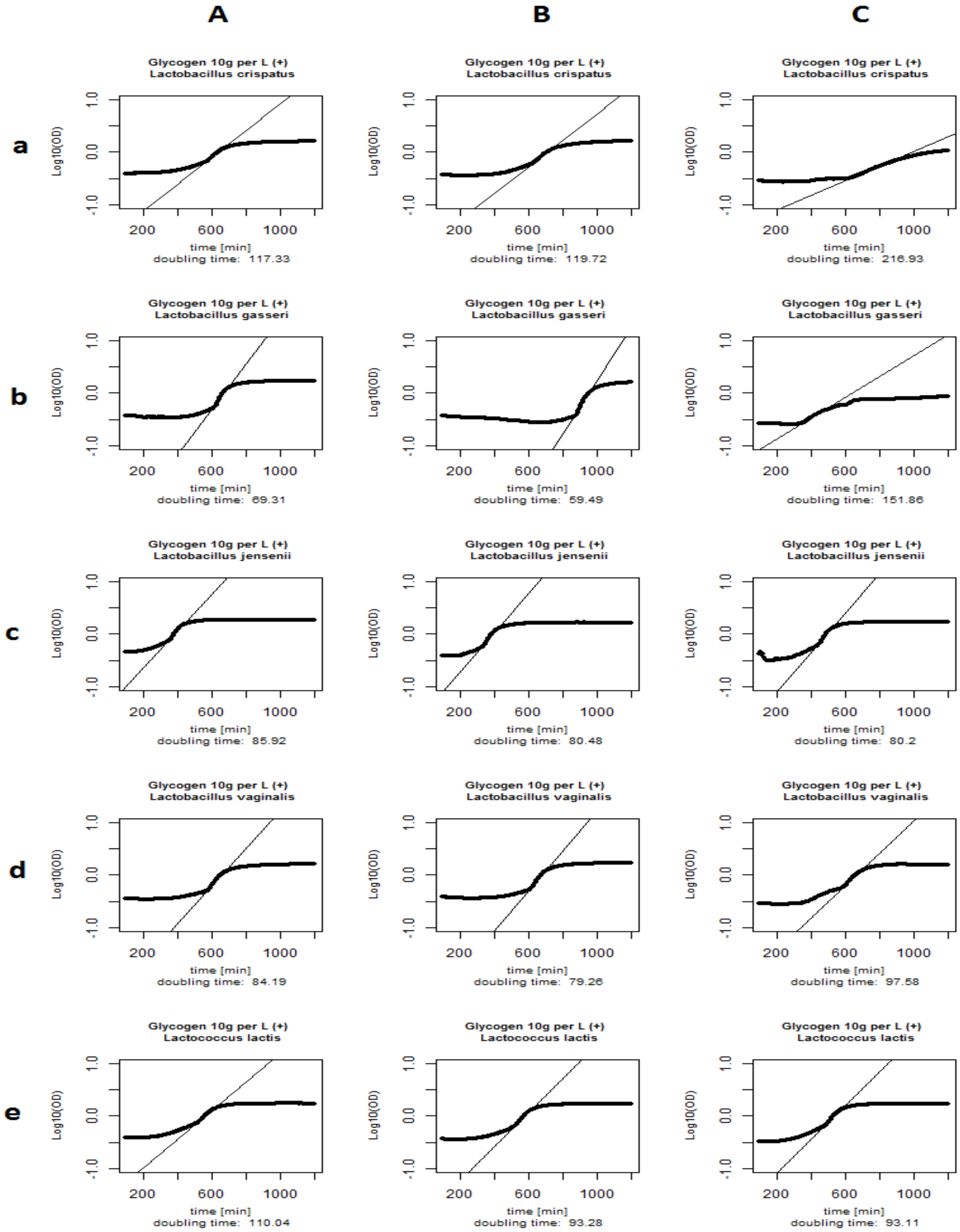

Figure 9: *Glycogen 10 g/l media (Hart mix derivative) growth curves for Lactobacillus crispatus (a), Lactobacillus gasseri (b), Lactobacillus jensenii (c), Lactobacillus vaginalis (d), Lactococcus lactis (e). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

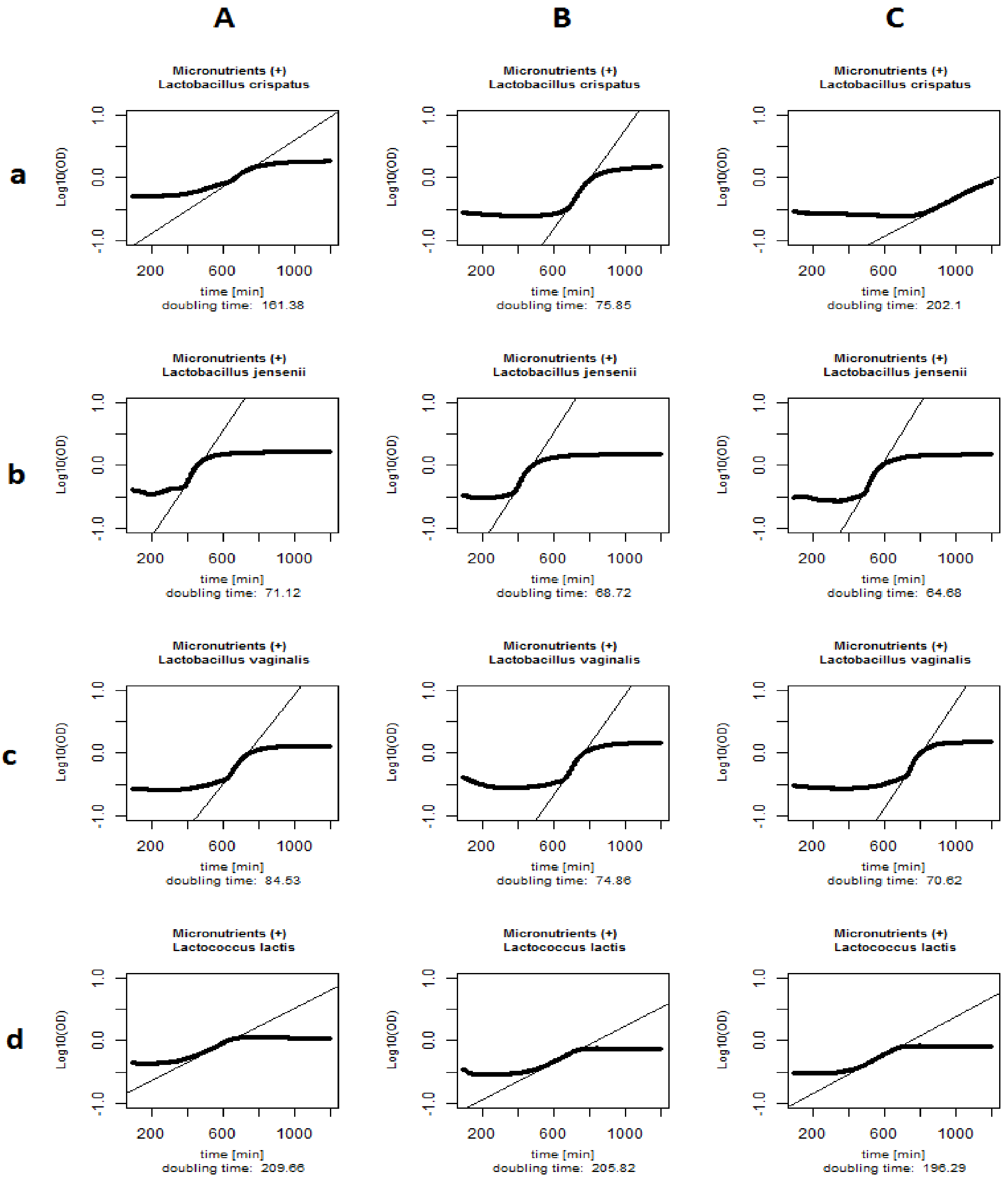

Figure 10: *Micronutrients media (Hart mix derivative) growth curves for Lactobacillus crispatus (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

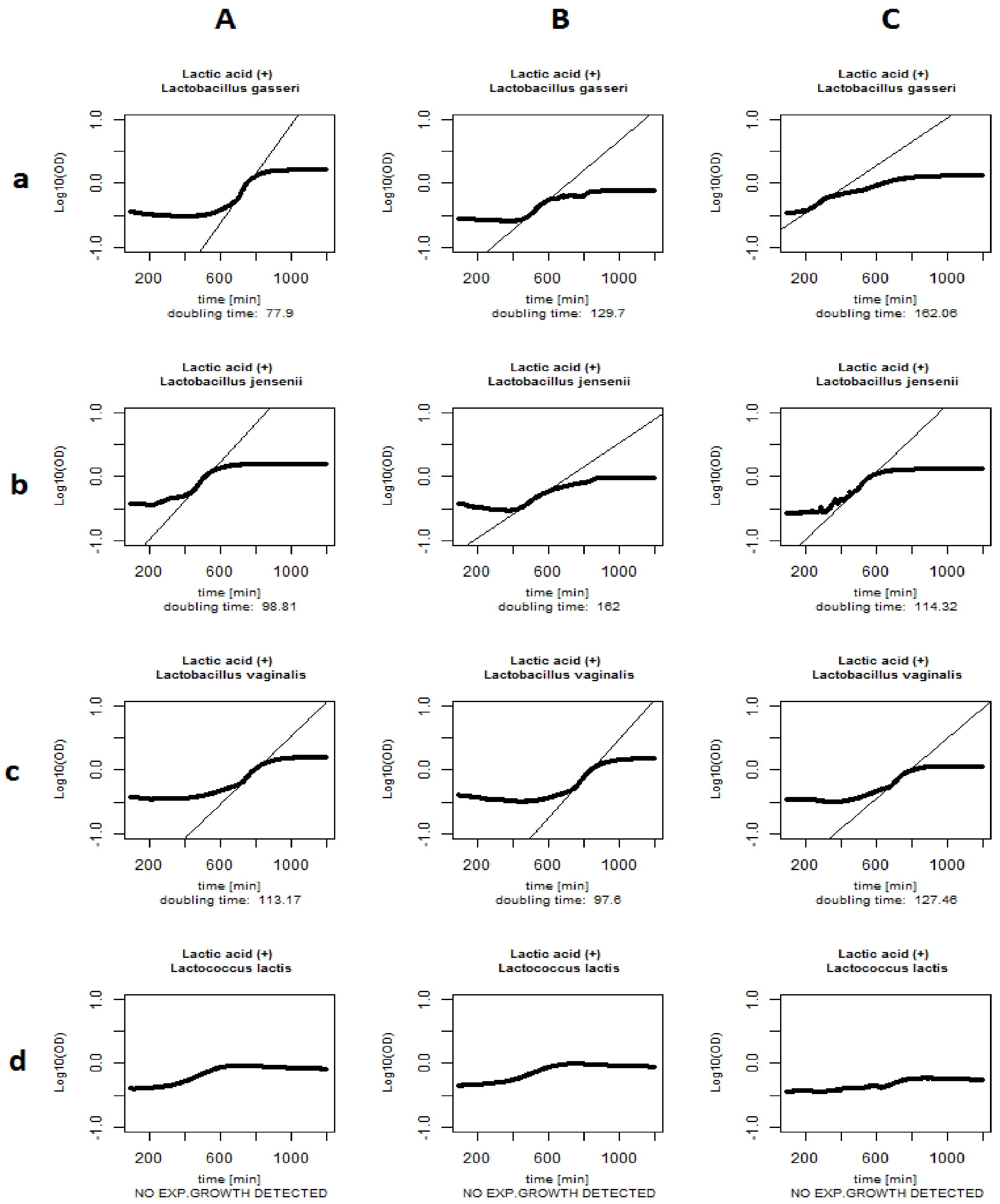

***Figure 11:** Lactic acid media (Hart mix derivative) growth curves for Lactobacillus gasseri (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

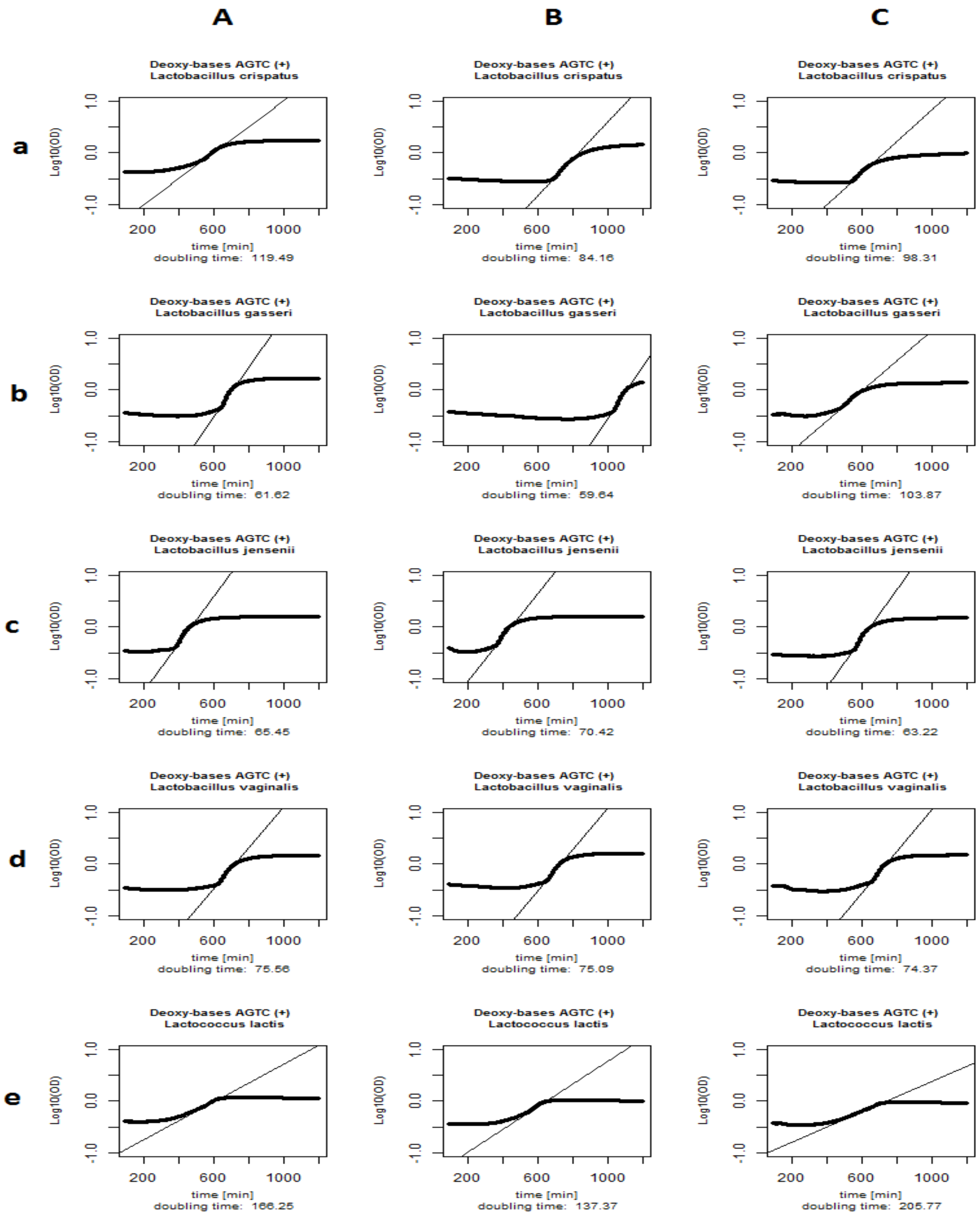

Figure 12: *Deoxynucleosides AGTC media (Hart mix derivative) growth curves for Lactobacillus crispatus (a), Lactobacillus gasseri (b), Lactobacillus jensenii (c), Lactobacillus vaginalis (d), Lactococcus lactis (e). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

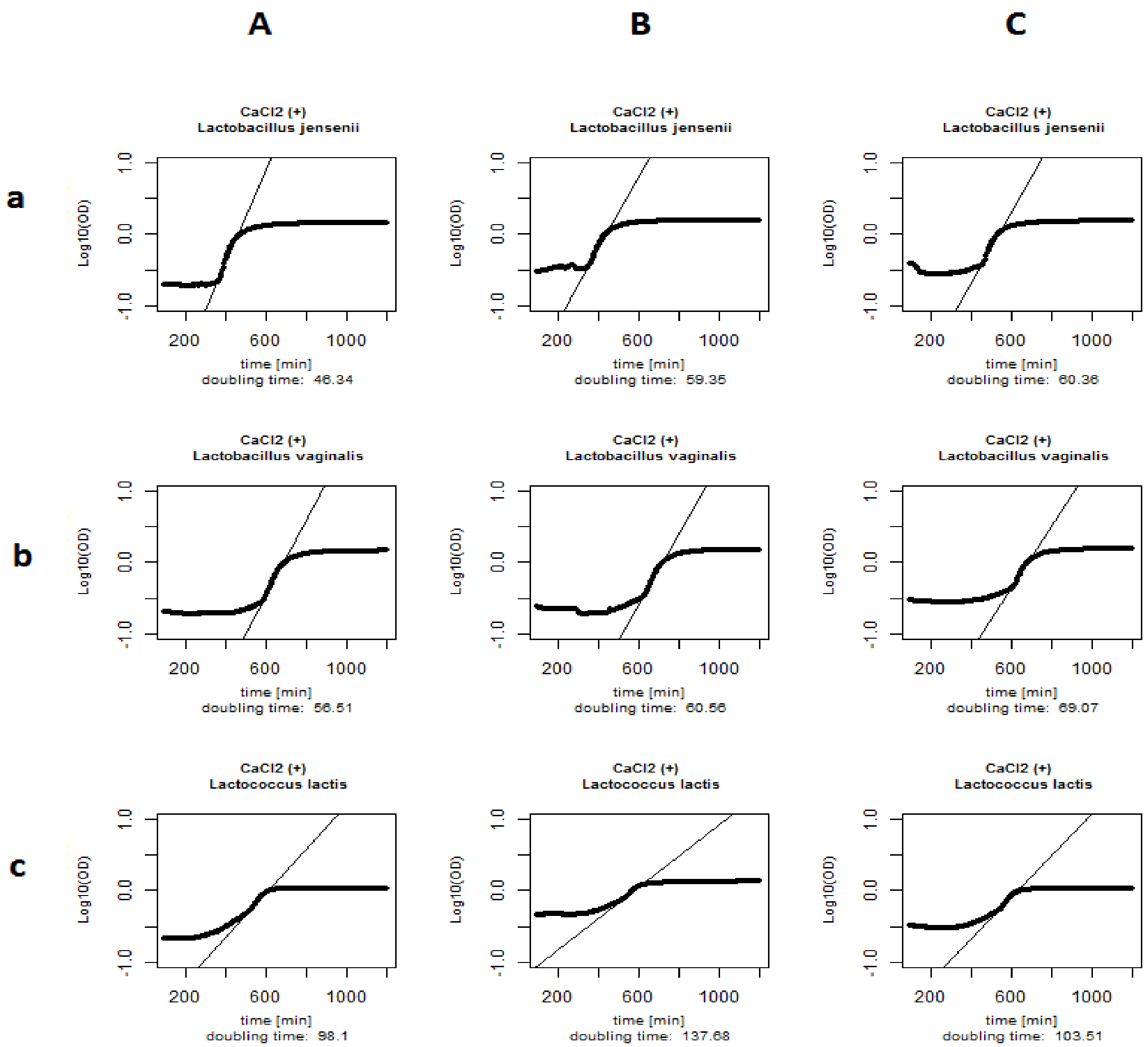

Figure 13: *CaCl$_2$ media (Hart mix derivative) growth curves for Lactobacillus jensenii (a), Lactobacillus vaginalis (b), Lactococcus lactis (c). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

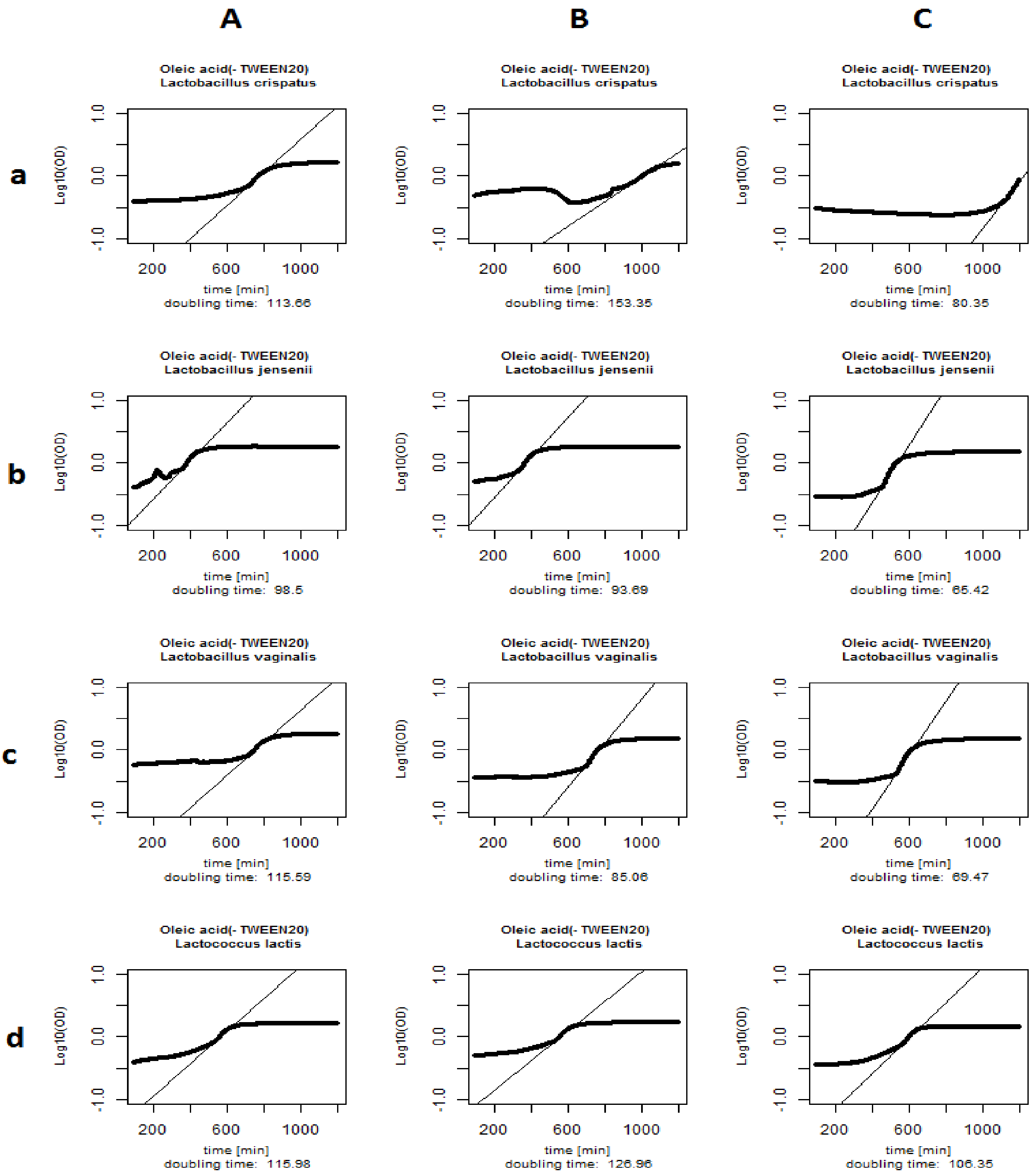

Figure 14: *Oleic acid (Hart mix derivative) media growth curves for Lactobacillus crispatus (a),Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

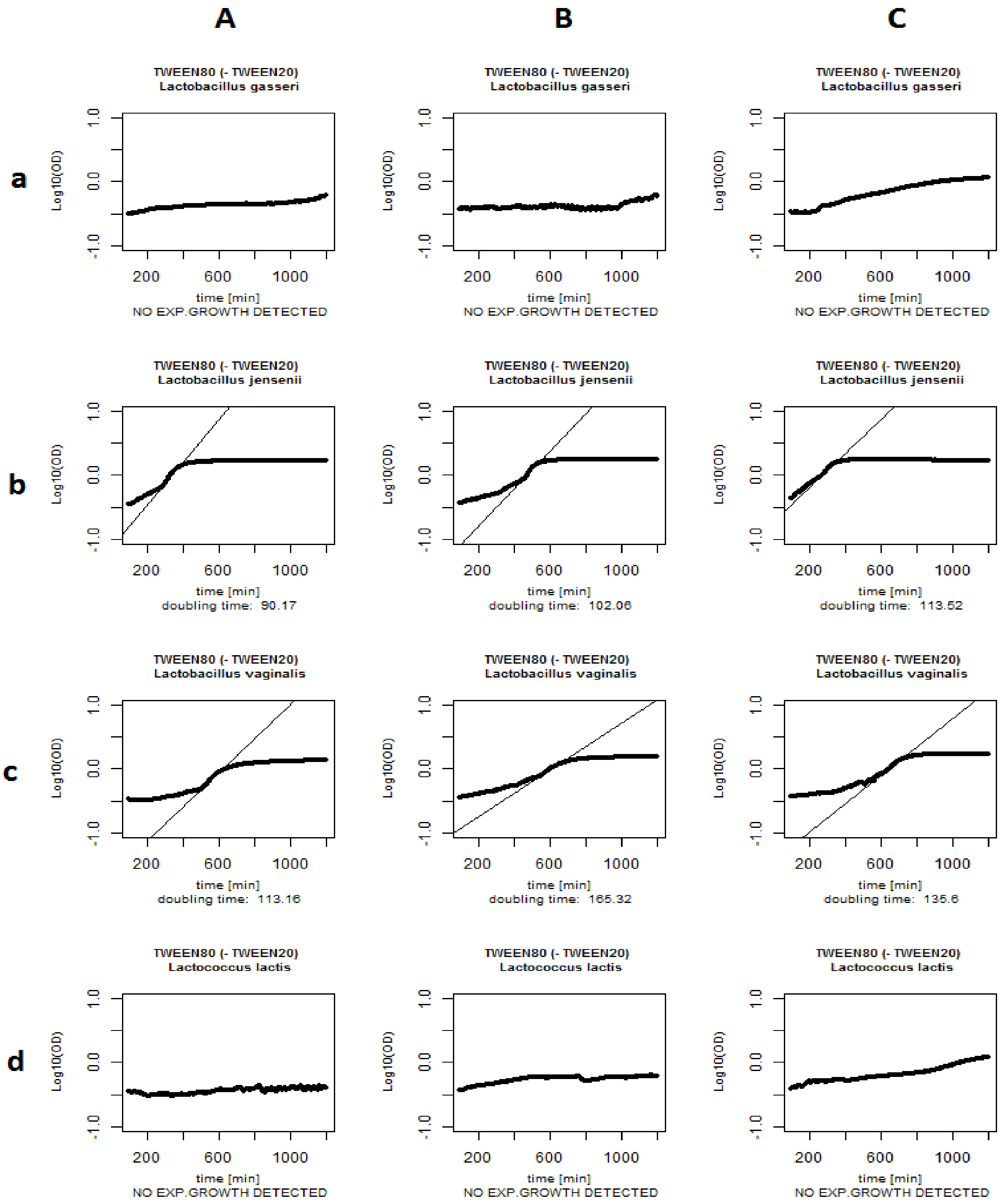

Figure 15: *TWEEN 80 media (Hart mix derivative) growth curves for Lactobacillus gasseri (a), Lactobacillus jensenii (b), Lactobacillus vaginalis (c), Lactococcus lactis (d). Y-axis: Logarithm to the base of 10 of the OD values measured by the plate reader. X-axis: time in minutes. The straight line is the steepest abline detected by the doubling time calculator script. The corresponding doubling time is printed below each graph. Columns A, B, C show three replicates of a strain in the media.*

Protein sequences

Chemoattractant	Open reading frame	Reference	Uniprot Accession Number
NGF-ß **(Neural growth factor beta)**	SSSHPIFHRGEFSVCDSVSVWVGDKTTATDIKGKEVMVLGEVNINNSVFKQYFFETKCRDPNPVDSGCRGIDSKHWNSYCTTTHTFVKALTMDGKQAAWRFIRIDTACVCVLSRKAVRRA	http://www.rcsb.org/pdb/explore.do?structureId=3ij2	P01138|122-241
ANP **(Atrial Natiuretic Peptide)**	SLRRSSCFGGRMDRIGAQSGLGCNSFRY	http://www.uniprot.org/blast/?about=P01160[124-151]	P01160|124-151
BNOS – example sequence **(bacterial nitric oxide synthase oxygenase)**	MEEKEILWNEAKAFIAACYQELGKEEEVKDRLADIKSEIDLTGSYVHTKEELEHGAKMAWRNSNRCIGRLFWNSLNVIDRRDVRTKEEVRDALFHHIETATNNGKIRPTITIFPPEEKGEKQVEIWNHQLIRYAGYESDGERIGDPASCSLTAACEELGWRGERTDFDLLPLIFRMKGDEQPVWYELPRSLVIEVPITHPDIEAFSDLELKWYGVPIISDMKLEVGGIHYNAAPFNGWYMGTEIGARNLADEKRYDKLKKVASVIGIAADYNTDLWKDQALVELNKAVLHSYKKQGVSIVDHHTAASQFKRFEEQEEEAGRKLTGDWTWLIPPISPAATHIFHRSYDNSIVKPNYFYQDKPYE	http://www.uniprot.org/uniprot/O34453 http://www.rcsb.org/pdb/explore/explore.do?structureId=2FC2	O34453
RANTES	YSSDTTPCCFAYIARPLPRAHIKEYFYTSGKCSNPAVVFVTRKNRQVCANPEKKWVREYINSLEMS	http://www.uniprot.org/uniprot/P13501 http://www.rcsb.org/pdb/explore/remediatedSequence.do?structureId=2L9H	P13501
LEISS **(Peptide linker)**	LEISSTCDA	(Le Loir et al., 2005)	-
SP310mut2 **(N-terminal Export sequence for lactic acid bacteria)**	MKFNKKRVAIATFIALIFVSFFTISSIQDAQAAERS	(Ravn, 2003)	-

Table 2: *Protein sequences of suggested chemoattractants*

Computer Scripts

R- scripts were run in the program R-studio http://www.rstudio.com/. ImageJ macros were used in the ImageJ program http://rsb.info.nih.gov/ij/ and require the CASA plugin to be operated http://rsb.info.nih.gov/ij/plugins/casa.html.

Chemokinesis analyser (R - script)

The chemokinesis analyser R-script collects and analyzes files from CASA plugin output and gives out graphs which display the percentages of active cells determined by tresholds of LIN and VCL (see results).

```
#initialize list for working directories of all used chemoattractants containing the text files with sperm parameters calculated by the CASA imagej plugin

ANP <- c(
  "D:/denmark imaging/results/chemokinesis/blank/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/ANP/0,04/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/ANP/0,2/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/ANP/1/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/ANP/5/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/ANP/25/Analysis/final")

NGF <- c(
  "D:/denmark imaging/results/chemokinesis/blank/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NGF/0,04/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NGF/0,2/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NGF/1/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NGF/5/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NGF/25/Analysis/final")

NO <- c(
  "D:/denmark imaging/results/chemokinesis/blank/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NO/0,04/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NO/0,2/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NO/1/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NO/5/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/NO/25/Analysis/final")

PROG <- c(
  "D:/denmark imaging/results/chemokinesis/blank/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/PROG/0,04/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/PROG/0,2/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/PROG/1/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/PROG/5/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/PROG/25/Analysis/final")

RANTES <- c(
  "D:/denmark imaging/results/chemokinesis/blank/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/RANTES/0,04/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/RANTES/0,2/Analysis/final",
```

```
  "D:/denmark imaging/results/chemokinesis/RANTES/1/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/RANTES/5/Analysis/final",
  "D:/denmark imaging/results/chemokinesis/RANTES/25/Analysis/final")


#concentrations used in chemokinesis experiments. Dilutions are a multiplicator of 5
conc_ANP <- c("Control","1,232 ng/ml","6,16 ng/ml","30,8 ng/ml","154 ng/ml","770 ng/ml")
conc_NGF <- c("Control","4 ng/ml","20 ng/ml","100 ng/ml","500 ng/ml","2500 ng/ml")
conc_RANTES  <- c("Control","0,04 ng/ml","0,2 ng/ml","1 ng/ml","5 ng/ml","25 ng/ml")
conc_NO  <- c("Control","0,12 ng/ml","0,6 ng/ml","3 ng/ml","15 ng/ml","75 ng/ml")
conc_PROG <- c("Control","0,125 pg/ml","0,625 pg/ml","3,125 pg/ml","15,7 ng/ml","78,5 ng/ml")

name <- c("ANP", "NGF", "SNOG", "PROG", "RANTES")

resultsdir <- cbind(ANP, NGF, NO, PROG, RANTES)
resultsVCL <- cbind(ANP, NGF, NO, PROG, RANTES)
resultsLIN <- cbind(ANP, NGF, NO, PROG, RANTES)
results_totalsperm<- cbind(ANP, NGF, NO, PROG, RANTES)
directories <- cbind(ANP, NGF, NO, PROG, RANTES)

#collect CASA script output files from directories
for (ee in 1:length(directories[1,])){
#  ee<-1

  for (dd in 1: length(directories[,1])){
#dd <-1
    setwd(directories[dd,ee])
    print(getwd())

    allfiles <- list.files(path = ".", pattern = NULL, all.files = FALSE,
                       full.names = FALSE, recursive = FALSE,
                       ignore.case = FALSE, include.dirs = FALSE)
    allfiles <- allfiles[4:length(allfiles)]

    #extract values from files and truncate first and last line from datasets,
    #then merge all datasets of one experiment into one matrix (allvals)
    allvals <- {}
    allvals <-matrix()

    for (ff in 1:length(allfiles)){
      if(grepl("txt", allfiles[ff])==TRUE){
        file <- allfiles[ff]
        vals = read.table(file, sep = " ", dec = ".", skip = 1, fill=TRUE)
        cutvals <- vals[,2]
        vals <- vals[1:(length(cutvals)-2),2:8]
        allvals <- merge(allvals, vals, all = TRUE)
        }
    }
    print(allfiles)
    allvals <- allvals[,1:7]

    #extract VCL and LIN values from dataset amd sort them in two groups defined by threshold values
    VCL = allvals[,2]
```

```
LIN = allvals[,5]

#threshholds for spermatozoa types
VCL_threshhold <- 30#40
LIN_threshhold <- 0.6#0.6

#calculate  x axis to draw different grades relative to total number of analyzed cells. total number of cells = 100%
VCL <- sort(VCL, decreasing=FALSE)
LIN <- sort(LIN, decreasing=FALSE)

#calculating the probability distribution of VCL values
VCL_dev <- 0
VCL_stud <- {}
VCL_dist <- {}
VCL_dist_x <- {}
VCL_dist_std <- {}

for (bb in 1:60){
  counter <- 0
  for (vv in 1:length(VCL)){
    if(VCL[vv]<=bb){if(VCL[vv]>=bb-1){counter <- counter+1}}
  }
  VCL_dist <-c(VCL_dist, counter)
  VCL_dist_x <- {c(VCL_dist_x, bb)}
}

for (vv in 1:length(VCL_dist)){
  VCL_dist_std <- c(VCL_dist_std,  VCL_dist[vv]*100/sum(VCL_dist))
}
sum(VCL_dist_std)
if (dd==1){VCL_dist_str_blank <- VCL_dist_std}
else{
  setwd("C:/Users/ruediger/denmark/work in progress/video capture/video analysis/chemokinesis results")
  png(filename = paste("chemokinesis VCL",name[ee], dd , "png", sep="."), width = 400, height = 400)
  plot(-10,-10, xlim=c(0,60),ylim=c(0,10), #sets size of the plot
       cex= 1.5, cex.lab=1.5, cex.main=1.5, cex.axis=1.5, main=name[ee],
        xlab="VCL [µm/s]", ylab="tracked spermatozoa [in %]")

#  png(filename = paste("chemokinesis VCL",concentrations[ee], name[dd], "png", sep="."), width = 400, height = 400)
  lines(VCL_dist_x,VCL_dist_str_blank, col="black",pch=20,lty=5,lwd=2)
  points(VCL_dist_x,VCL_dist_str_blank, col="black",pch=4,lwd=3)
  lines(VCL_dist_x,VCL_dist_std, col="red",pch=20,lty=5,lwd=2)
  points(VCL_dist_x,VCL_dist_std, col="red",pch=4,lwd=3)

  legend(35,10, # places a legend at the appropriate place
        c("control",concentrations[dd,ee]), # puts text in the legend
        lty=c(1,1), # gives the legend appropriate symbols (lines)
        lwd=c(2,2,2,2,2), col=c("black", "red"), cex=1.5) # gives the legend lines the correct color and width

  dev.off()
}

#calculating the probability distribution of LIN values
```

```
LIN_dev <- 0
LIN_stud <- {}
LIN_dist <- {}
LIN_dist_x <- {}
LIN_dist_std <- {}

for (bb in 1:100){
  counter <- 0
  for (vv in 1:length(LIN)){
    if((LIN[vv]*100)<=bb){if((LIN[vv]*100)>=bb-1){counter <- counter+1}}
  }
  LIN_dist <-c(LIN_dist, counter)
  LIN_dist_x <- {c(LIN_dist_x, bb)}
}

for (vv in 1:length(LIN_dist)){
  LIN_dist_std <- c(LIN_dist_std,  LIN_dist[vv]*100/sum(LIN_dist))
}
sum(LIN_dist_std)
if (dd==1){LIN_dist_str_blank <- LIN_dist_std}
else{

  setwd("C:/Users/ruediger/denmark/work in progress/video capture/video analysis/chemokinesis results")
  png(filename = paste("chemokinesis LIN",name[ee], dd , "png", sep="."), width = 400, height = 400)
  plot(-10,-10, xlim=c(0,100),ylim=c(0,10), #sets size of the plot
       cex= 1.5, cex.lab=1.5, cex.main=1.5, cex.axis=1.5, main=name[ee],
       xlab="LIN [ratio VSL/VAP in %]", ylab="tracked spermatozoa [in %]")

  #  png(filename = paste("chemokinesis LIN",concentrations[ee], name[dd], "png", sep="."), width = 400, height = 400)
  lines(LIN_dist_x,LIN_dist_str_blank, col="black",pch=20,lty=5,lwd=2)
  points(LIN_dist_x,LIN_dist_str_blank, col="black",pch=4,lwd=3)
  lines(LIN_dist_x,LIN_dist_std, col="red",pch=20,lty=5,lwd=2)
  points(LIN_dist_x,LIN_dist_std, col="red",pch=4,lwd=3)

  legend(35,10, # places a legend at the appropriate place
        c("control",concentrations[dd,ee]), # puts text in the legend
        lty=c(1,1), # gives the legend appropriate symbols (lines)
        lwd=c(2,2,2,2,2), col=c("black", "red"), cex=1.5) # gives the legend lines the correct color and width

  dev.off()
}

#set threshholds for spermatozoa types to compare effects of chemokinesis substances to blank
VCL_threshhold <- 30
LIN_threshhold <- 0.6
VCL_high <- {}
LIN_high <- {}

#count spermatozoa with VCL>trheshold and assign x value as percentage relative to total amount of cells.
for (xx in 1:(length(VCL))){
  if (VCL[xx]>VCL_threshhold){
    VCL_high <- c(VCL_high, VCL[xx])
  }
```

```
     if (LIN[xx]>LIN_threshhold){
       LIN_high <- c(LIN_high, VCL[xx])
     }
   }

   #calculate percentage and store in matrix - these are printed into table files at the end
   VCL_high_per <- length(VCL_high)*100/length(VCL)
   LIN_high_per <- length(LIN_high)*100/length(VCL)

   resultsdir[dd,ee] <- directories[dd,ee]
   resultsVCL[dd,ee] <- VCL_high_per
   resultsLIN[dd,ee] <- LIN_high_per
   results_totalsperm[dd,ee] <- length(VCL)
 }
}

setwd("C:/Users/ruediger/denmark/work in progress/video capture/video analysis/chemokinesis results")

write.table(resultsdir, file = "Chemokinesis resultsdir.txt",sep = ";")
write.table(resultsVCL, file = "Chemokinesis resultsVCL.txt",sep = ";")
write.table(resultsLIN, file = "Chemokinesis resultsLIN.txt",sep = ";")
write.table( results_totalsperm, file = "Chemokinesis totalsperm.txt",sep = ";")
```

Doubling time calculator (R - script)

The doubling time calculator is a script in the R-language and can for example be run in the R-studio program. It reads out csv files with stored OD values from the plate reader experiments, calculates the logarithm of it and searches for the steepest slope of the exponential phase. The output are graphs for all experiments with the $\log_{10}$ OD against the time, the steepest abline and the doubling time derived from the slope of the abline.

```
#set working directory
setwd("C:/Users/ruediger/denmark/work in progress/media/results media optimisation/neuer versuch/final")
#getwd()



#create list of experiments
ingredients <- c("Hart", "TWEEN80 (- TWEEN20)","Oleic acid(- TWEEN20)","Amino acid SA mix (- Hart A.S. mix)","DTU Vitamin mix (- Sigma K3129)","MEM Vitamin solution (- Sigma K3129)","Lactic acid (+)","Micronutrients (+)","CaCl2 (+)","Deoxy-bases AGTC (+)","Glycogen 5g per L (+)","Glycogen 10g per L (+)","Glucose 10g per L(+)","Glucose 20g per L(+)","Mucin 6.5g per L(+)","Mucin 10g per L(+)","Dilution 3X","Dilution 0.33X"," reduced MRS")
strains <- c("Lactobacillus crispatus","Lactobacillus gasseri","Lactobacillus jensenii","Lactobacillus vaginalis","Lactococcus lactis")
experiments <- {}
name <-{}

for (f in 1:19){
  for (g in 1:5){
    experiments <- c(experiments, paste(ingredients[f], strains[g]))
    name <- c(name, paste(ingredients[f],"\n", strains[g]))
  }
```

```
}

# define input file here - had to be prepared in excel manually to define area of exponential growth in the boundaries
OD[630]0 to 0,4. Blanks had to be substracted before.
input <- "1.csv"

allslopes <- {}
sortslopes <- {}
slope <- {}
position <-{}


# read csv file with OD values and extract columns with od values and time points

odall = read.csv2(input, sep = ";", dec = ",")[1:242 ,1:96]
time = odall[1:241 ,1]

#kick out 0 values and replace with 10 (to avoid log(0) error)
for (q in 2:length(odall)) {
  for (w in 1:(length(odall[,q])-1)){
    if (odall[w,q]==0){odall[w,q] <- 10
    }}}

#iterate csv file readout to analyze every column
for(i in 1:95) {
  od <- odall[0:241 ,i+1]

  #iterate sets of 14 values for each experiment to determine slopes and then select the lowest
  allslopes <- {}
  for (a in 0:(length(od)-24))  {
    y <- od[a:(a+24)]
    x= time[a:(a+24)]

    #calculate slopes for a frame of 10 values
    m1 <- lm(log10(y) ~ x)
    summary(m1)
    append <- log10(2)/m1$coefficients[2]

    #sort out empty and bad p-values smaller than 0.90 and bad R-squared values bigger than e-10 and keep good ones for
further analysis
    if (m1$coefficients[2]==0){allslopes[a]<- -1}
    else{
      if (summary(m1)$r.squared>0.90){
           if(anova(m1)$'Pr(>F)'[1]<0.5e-10){
                allslopes[a]<- c(append)
            }
            else {allslopes[a]<- -1}
        }
        else {allslopes[a]<- -1}
    }
  }

  #extract lowest positive slope from results
```

```
  sortslopes <- {}

    for (o in 1:length(allslopes)) {
      if (allslopes[o]>0){
        sortslopes[o] <- c(allslopes[o])
      }
    }

  sortslopes <- sort(sortslopes, decreasing=FALSE)

  if (length(sortslopes)>0){slope[i] <- sortslopes[1]}
  else {slope[i] <- 0}

    #filter doubling times within a reasonable range, plot and save as png. if not within range, dont plot abline and doubling
time
   if (slope[i]<251){
    if(slope[i]>0){

    #get timespan and corresponding y values for minimal slope
      position[i] <- (1:length(allslopes))[allslopes==sortslopes[1]]
      yplot <- od[20:240]
      xplot= time[20:240]

      yplotabline <- od[(position[i]):(position[i]+24)]
      xplotabline= time[(position[i]):(position[i]+24)]

    #plot the curve of minimal slope and save as png
      png(filename = paste(i, input,experiments[i], round(slope[i]), "png", sep="."), width = 250, height = 250)
      plot (xplot,log10(yplot), main=paste(name[i]), xlab=paste("time [min]\n","doubling time: ", round(slope[i], digits=2)),
ylab="Log10(OD)",ylim=c(-1,1), cex.lab=0.8, cex.main=0.8, pch=20)
      m1 <- lm(log10(yplotabline) ~ xplotabline)
      abline(m1)
      dev.off()
      }
    else {

      yplot <- od[20:240]
      xplot= time[20:240]


      png(filename = paste(i, input, experiments[i], round(slope[i]), "png", sep="."), width = 250, height = 250)
      plot(xplot,log10(yplot),main=paste(name[i]), xlab=paste("time [min]\n","NO EXP.GROWTH DETECTED"),
ylab="Log10(OD)",ylim=c(-1,1), cex.lab=0.8, cex.main=0.8, pch=20)
      dev.off()
    }
   }
  else {

      yplot <- od[20:240]
      xplot= time[20:240]

      png(filename = paste(i, input, experiments[i], round(slope[i]), "png", sep="."), width = 250, height = 250)
      plot(xplot,log10(yplot),main=paste(name[i]), xlab=paste("time [min]\n","NO EXP.GROWTH DETECTED"),
```

```
ylab="Log10(OD)",ylim=c(-1,1), cex.lab=0.8, cex.main=0.8, pch=20)
    dev.off()
   }
}

slope

#write results into txt file
write(name, file = "all slopes.txt",
    ncolumns = 1,
    append = TRUE, sep = " ")
write(slope, file = "all slopes.txt",
    ncolumns = 1,
    append = TRUE, sep = " ")

dev.off()
```

threshold macro (ImageJ macro)

A macro for ImageJ, which executes the CASA plugin to find the threshold in which the most sperm tracks are detectable. The script runs through all folders – in the case for this work five chemoattractants at five different concentrations plus one folder with blanks. The folders are loaded individually (see the end of the script). All files in the loaded folder are analyzed. All track image results of the best threshold parameters and all calculated parameters for each sperm track are saved in the Analysis folder for each selected mutant (the Analysis folder is created automatically).

```
function treshold(FolderString, filelist,subFolderString) {
        print("select the folder in which your sperm tiff stacks are saved. min 33fps");
                //parameters which came out of each 21 rounds of breeding algorithm script:
                //1,25,25,4,5,3,38,1,2,9,7,6,1,17,19,74                       //exp3 -evolution algorithm results
                //1,25,31,6,2,1,7,1,2,2,11,8,53,45,147,1             //exp2 -evolution algorithm results
                //2,30,35,3,2,1,8,1,7,100,1,1,19,1,63,145            //exp1 -evolution algorithm results
                //4,80,99,10,4,0,0,100,100,100,0,0,100,1,200,200 //best guess
                //0,40,97,8,3,20,25,5,1,25,25,35,80,80,50,60         //fish - reference

                newparameters = newArray(1,25,25,4,5,3,38,1,2,9,7,6,1,17,19,74);                         //initialize array
                parameters        =        newArray(1,25,25,4,5,3,38,1,2,9,7,6,1,17,19,74);
        //starting parameters.
                NameString = 'Process_';
                path = 'path_';
                Ending = '.txt';

                //create general variables to pass them on into CASA plugin
                a=newparameters[(newparameters.length-16)];
                b=newparameters[(newparameters.length-15)];
                c=newparameters[(newparameters.length-14)];
                d=newparameters[(newparameters.length-13)];
                e=newparameters[(newparameters.length-12)];
                f=newparameters[(newparameters.length-11)];
                g=newparameters[(newparameters.length-10)];
                h=newparameters[(newparameters.length-9)];
                i=newparameters[(newparameters.length-8)];
```

```
        j=newparameters[(newparameters.length-7)];
        k=newparameters[(newparameters.length-6)];
        l=newparameters[(newparameters.length-5)];
        m=newparameters[(newparameters.length-4)];
        n=newparameters[(newparameters.length-3)];
        o=newparameters[(newparameters.length-2)];
        p=newparameters[(newparameters.length-1)];

        //create argument to pass on to CASA
        myParamString = "a,=" +a+ " b,="+b+ " c,="+c+ " d,="+d+ " e,="+e+ " f,="+f+ " g,="+f+ " h,="+h+ " i,="+i+ "
j,="+j+ " k,="+k+ " l,="+l+ " m,="+m+ " n,="+n+ " o,="+o+ " p,="+p+ " q,=33.000000000 r,=1000.000000000 s,=0 t,=1 u,=0";


        //FolderString = getDirectory("Choose Destination Directory ");
        //filelist = getFileList(FolderString);
        //File.makeDirectory(FolderString+"\\Analysis");
        //subFolderString = getDirectory("Select Analysis directory in the folder you choose");

    print(subFolderString);

    for (i=0; i<lengthOf(filelist); i++) {
        print("current file being processed:");
        print(filelist[i]);
        open(FolderString+filelist[i]);
        ID1 = getImageID();

        //now iterating the threshold to find the level where CASA plugin detects the most spermatozoa.
        //CASA plugin runs through and the results are saved in a txt file with the same name of the tiff
stack+threshold value.
        //a tiff of the tracked path is saved in the end.

        for (oo=60; oo>15; oo--) {

            selectImage(ID1);
            run("Make Substack...", "  slices=1-1000");
            ID2 = getImageID();
            selectImage(ID2);
            setAutoThreshold("Default");
            //run("Threshold...");
            setThreshold(oo, 255);
            setOption("BlackBackground", false);
            run("Convert to Mask", "method=Default background=Light");

            //call CASA with standard parameters and evaluate how many tracks have been found
            run("CASA ", myParamString);

            //record results in a txt file in the Analysis folder
            if(isOpen("Results")==1){
                selectWindow("Results");
                saveAs("Results",subFolderString+filelist[i]+oo+Ending);
                IJ.deleteRows(0, 10000);
                };
```

```
            x = oo+1;

            print ("current treshold: "+oo);
            if(File.exists(subFolderString+filelist[i]+oo+Ending)==1&&File.exists(subFolderString+filelist[i]
+x+Ending)==1){

                if(isOpen("Paths")==1){
                    selectWindow("Paths");
                    saveAs("Tiff", subFolderString+filelist[i]+oo+"path");
                    close();
                    };


                //finding the optimal treshold by comparing this oo and last run's (x) amount of
detected tracks
                lines_oo=split(File.openAsString(subFolderString+filelist[i]+oo+Ending),"\n");
                lines_x=split(File.openAsString(subFolderString+filelist[i]+x+Ending),"\n");

                print("now compare if there is more sperm in this analysis than in the one before:");
                print(lengthOf(lines_oo));
                print("than the one before:");
                print(lengthOf(lines_x));

                //check if best treshold value has been found by comparing number of tracks of this
with the last iteration
                if (lengthOf(lines_oo)<lengthOf(lines_x)){
                    File.delete(subFolderString+filelist[i]+oo+Ending);              //clear
up folder from unused txt files
                    File.delete(subFolderString+filelist[i]+oo+"path");              //clear
up folder from unused path files

                    //close Paths window to not confuse evolve function
                    if(isOpen("Paths")==1){
                        selectWindow("Paths");
                        close();  //close current path window
                        };
                    if(isOpen(ID2)==1){
                        selectImage(ID2);
                        newpathID = getImageID();
                        close();
                        };

                    //call evolutionary optimization function to find best parameters and give
function the last and best treshold setting and current filename
                    currentfile = filelist[i];
                    besttreshold = oo-1;

                    //reset treshold of current file (full stack) with last best value (besttreshold)
again and run CASA
                    selectWindow(currentfile);
                    setAutoThreshold("Default");
                    setThreshold(besttreshold, 255);
                    setOption("BlackBackground", false);
```

```
                    run("Convert to Mask", "method=Default background=Light");
                    run("CASA ", myParamString);

                    if(isOpen("Results")==1){
                        selectWindow("Results");

    saveAs("Results",subFolderString+"final"+currentfile+"t"+besttreshold+".txt");
                        IJ.deleteRows(0, 20000);
                        };
                        ID3 = getImageID();
                        selectImage(ID3);
                        rename(FolderString+currentfile+"t"+besttreshold);
                        saveAs("Tiff", subFolderString+"final "+filelist[i]+oo+"path");
                        close();

                    oo = 0;         //to quit for-loop
                    };

                else {
                    File.delete(subFolderString+filelist[i]+x+"path");          //clear up folder
from unused files
                    File.delete(subFolderString+filelist[i]+x+Ending);          //clear up folder
from unused files
                    };
                };
            if(isOpen(ID2)==1){
                selectImage(ID2);
                close();        //close last threshhold window
                };
            };

        if(isOpen("Paths")==1){
            selectWindow("Paths");
            close();    //close current path window
            };
        if(isOpen(filelist[i])==1){
            selectWindow(filelist[i]);
            close();    //close current monochrome video sequence
            };
        if(isOpen(ID1)==1){
            selectImage(ID1);
            close();    //close current video sequence
            };
        };
    };


setBatchMode(true);


//calculate BLANK
//FolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\";
//filelist = getFileList(FolderString);
```

```
//File.makeDirectory(FolderString+"\\Analysis");
//subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\Analysis\\";
//treshold(FolderString, filelist,subFolderString);


//now analyse ANP videos
FolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\0,04\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\0,04\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\0,2\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\0,2\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\1\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\1\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\5\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\5\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\25\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\ANP\\25\\Analysis\\";
treshold(FolderString, filelist,subFolderString);



//now analyse NGF videos
print("now NGF videos");
FolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\0,04\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\0,04\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\0,2\\";
```

```
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\0,2\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\1\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\1\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\5\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\5\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\25\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NGF\\25\\Analysis\\";
treshold(FolderString, filelist,subFolderString);



//now analyse NO videos
print("now NO videos");
FolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\0,04\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\0,04\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\0,2\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\0,2\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\1\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\1\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\5\\";
```

```
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\5\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\25\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\NO\\25\\Analysis\\";
treshold(FolderString, filelist,subFolderString);


//Now analyse RANTES videos
print("Now RANTES videos");
FolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\0,04\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\0,04\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\0,2\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\0,2\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\1\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\1\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\5\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\5\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\25\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\RANTES\\25\\Analysis\\";
treshold(FolderString, filelist,subFolderString);



//Now analyse PROG videos
```

```
print("Now PROG videos");
FolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\blank\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\0,04\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\0,04\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\0,2\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\0,2\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\1\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\1\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\5\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\5\\Analysis\\";
treshold(FolderString, filelist,subFolderString);

FolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\25\\";
filelist = getFileList(FolderString);
File.makeDirectory(FolderString+"\\Analysis");
subFolderString = "D:\\denmark imaging\\results\\chemokinesis\\PROG\\25\\Analysis\\";
treshold(FolderString, filelist,subFolderString);



setBatchMode("exit & display");
```

directed evolution macro (ImageJ macro)

A macro for ImageJ which executes the the CASA plugin, detects the best threshold and then runs the CASA plugin again time with a sets of parameters which are mutated by the evolution function. The CASA output for all mutants are displayed and the best mutant selected by the user. The script iterates through several generation of mutants. All track image results and all calculated parameters for each sperm track are saved in the “Analysis” folder for each selected mutant. The script asks you to chose the folder where the tiff stacks of the sperm videos are saved and creates a new folder (“Analysis”) within this. This folder has to be selected manually.

```
//macro "Macro 1 [a]" {

function evolve(currentfile, besttreshhold, inputparameters, subFolderString) {
        //initialize directed evolution parameters, read in current file (zz) with last best treshhold value (oo)
        print("now running evolve function:\nGenerating Mutant Offsprings");
        FolderString = subFolderString;

        oo= besttreshhold;
        zz= currentfile;

        mutants = 5;                                                            //set number of mutants:
        generations= 5;                                                         //set number of generations
        print("No. of generation"+generations+"\nNo. of mutants per generation:"+mutants);
        bestmutant = newArray();
        mutanthalloffame = newArray();
        survival= newArray();
        mutant = 1;
        parameters = inputparameters;

        //initialize start parameters  min und max Values for CASA-evolution
        //                                              (a,             b,              c,              d,
        e,              f,              g,              h,              i,              j,              k,
        l,              m,              n,              o,              p       );
        //variable names to be passed on to CASA plugin
        maxVals =       newArray(60,    80,             300,    30,             10,             60,
        60,             100,    100,    100,    100,    100,    100,    100,    200,    200     );
        //Max Values for all parameters
        minVals  =      newArray(1,             10,             20,             1,              1,
        1,              1,              1,              1,              1,              1,              1,
        1,              1,              1,              1       );                      //Max Values for all
parameters



        evolution =  Array.concat(evolution, parameters); //records evolution of all mutants

        //reset treshhold of current file (zz) with last best value (oo) again
        selectWindow(zz);
        setAutoThreshold("Default");
        setThreshold(oo, 255);
        setOption("BlackBackground", false);
        run("Convert to Mask", "method=Default background=Light");
        title = getTitle();


        // iterate generations and create 10 new mutants per generation.
        for (gg=0; gg<generations; gg++){
                print("\n NEW GENERATION\n");

                mutationrate = 2/(gg+1);                        // Each generation mutates slower than the one
before(mutationrate)
```

```
        generationparameters =  newArray();             // saves all mutations in a generation temporarily
        pathID = newArray();

        evolution = Array.concat(evolution, "file", currentfile, "Generation", gg, "\n", parameters, "\n");
                //safe parent mutant parameters of this generation for evolutionary record
        print("initial parameters for this generation:");
        Array.print(parameters);

        //create new mutants
        for (mm=0; mm<mutants; mm++){
            newparameters = newArray(4,         80,         99,         10,
    4,          1,          1,          100,    100,    100,    1,          1,
    100,    1,          200,    200     );          //to generate a new array with the correct length
            // create variables from fittest mutant of last generation
            for(i = 0; i < parameters.length; i++){

                newparameters[i] = parameters[i]+(round(round(random()-
0.5)+parameters[i]*(random()-0.5)*mutationrate));           // mutate variables with current mutation rate

                //trim parameters to min and max values in case it mutated outside the boundaries
                if(newparameters[i] > maxVals[i]){newparameters[i] = maxVals[i];};
                if(newparameters[i] < minVals[i]){newparameters[i] = minVals[i];};
            };


            generationparameters = Array.concat(generationparameters,newparameters);  //create list of all
mutants of this generation for evaluation

            print ("\n generation: "+gg+"\n current mutant: "+mm+"\n mutated parameters:");
            Array.print(newparameters);

            //create general variables to pass them on into CASA plugin
            a=newparameters[(newparameters.length-16)];
            b=newparameters[(newparameters.length-15)];
            c=newparameters[(newparameters.length-14)];
            d=newparameters[(newparameters.length-13)];
            e=newparameters[(newparameters.length-12)];
            f=newparameters[(newparameters.length-11)];
            g=newparameters[(newparameters.length-10)];
            h=newparameters[(newparameters.length-9)];
            i=newparameters[(newparameters.length-8)];
            j=newparameters[(newparameters.length-7)];
            k=newparameters[(newparameters.length-6)];
            l=newparameters[(newparameters.length-5)];
            m=newparameters[(newparameters.length-4)];
            n=newparameters[(newparameters.length-3)];
            o=newparameters[(newparameters.length-2)];
            p=newparameters[(newparameters.length-1)];

            //create argument to pass on to CASA
            myParamString = "a,=" +a+ " b,="+b+ " c,="+c+ " d,="+d+ " e,="+e+ " f,="+f+ " g,="+f+ " h,="+h+ "
i,="+i+ " j,="+j+ " k,="+k+ " l,="+l+ " m,="+m+ " n,="+n+ " o,="+o+ " p,="+p+ " q,=33.000000000 r,=1000.000000000 s,=1
t,=1 u,=1";
```

```
            //select current monochrome sperm tiff stack and run CASA with current mutant parameters
            selectWindow(title);
            run("CASA ", myParamString);

            if(isOpen("Results")==1){
                selectWindow("Results");
                saveAs("Results",FolderString+zz+"t"+oo+"g-"+gg+" m-"+mm+".txt");
                IJ.deleteRows(0, 20000);
                };


            //save window ID for later display and evaluation. store results
            newpathID = getImageID();
            rename(" Generation "+gg+" Mutant "+mm);
            pathID = Array.concat(pathID, newpathID);
        };

        print("generationparameters");
        Array.print(generationparameters);
        print("\n finished mutating this generation\n");
        survival = newArray();


        //now manually screen optimized CASA results and evaluate the mutants efficiency and quality by
assinging grades
        //create Dialog box and present path results
        run("Tile", "");
        Dialog.create("click if you finished comparing the results");
        Dialog.show();

        for ( nn=0; nn<mutants; nn++) {
            //create Dialog box and ask for grade
            run("Cascade", "");
            selectImage(pathID[nn]);
            Dialog.create("Directed Evolution Mutant Survey");
            Dialog.addMessage("Generation: "+gg+"\nEvaluate Mutant: "+nn+" in active window\n give
your favourite mutant the highest number\n");
            Dialog.addNumber(nn, 1);
            Dialog.show();
            deathparameter = Dialog.getNumber();

            survival= Array.concat(survival, deathparameter);           //record the mutants and their
fitness

            if(isOpen(pathID[nn])==1){
                selectImage(pathID[nn]);
                saveAs("Tiff", FolderString+"generation-"+gg+" mutant-"+nn+".tiff");
                close();
                };

        };
```

```
        //determine fittest mutant of last generation by ranking grades decreasingly and then mapping it on the
bestmutantlist
        rank = Array.rankPositions(survival);
        last = rank.length-1;
        bestmutant =  rank[last]+1;

        if(survival[rank[last]]==1){
            print("all mutants died. New generation starts with parent parameters again.");
            mutanthalloffame = Array.concat(mutanthalloffame, 0);
        };
        else {
            print("and the winner with the best grade of "+survival[rank[last]]+" is mutant number:
"+bestmutant);
            mutanthalloffame = Array.concat(mutanthalloffame, bestmutant);
            parameterstart = bestmutant*16-16;
            parameterend = bestmutant*16;
            parameters = Array.slice(generationparameters,parameterstart,parameterend);
            print("\n best mutants parameters:\n");
            Array.print(parameters);
        };
    };

return parameters;              //returns best parameters after evolution is done
print("statistics\nwinning mutants in each generation:");
Array.print(mutanthalloffame);
print("all parameters of all parent mutants:");
Array.print(evolution);
print("\n EVOLUTION FINISHED\n");
};



    function treshhold() {
        print("select the folder in which your sperm tiff stacks are saved. min 33fps");


            parameters      =       newArray(1,         41,         21,         7,
    1,      6,      34,         1,          1,          29,         7,
    10,     1,      12,         9,          184     );
    //starting parameters.
            FolderString = getDirectory("Choose Destination Directory ");
            NameString = 'Process_';
            path = 'path_';
            Ending = '.txt';
            filelist = getFileList(FolderString);
            File.makeDirectory(FolderString+"analysis");
            subFolderString = getDirectory("Select Analysis directory in the folder you choose");
            //subFolderString = FolderString+File.separator+"analysis";
            //setBatchMode(true);


        for (i=0; i<lengthOf(filelist); i++) {
```

```
print("current file being processed:");
print(filelist[i]);
open(FolderString+filelist[i]);
title1 = getTitle();

//now iterating the threshold to find the level where CASA plugin detects the most spermatozoa.
//CASA plugin runs through and the results are saved in a txt file with the same name of the tiff
stack+threshold value.
//a tiff of the tracked path is saved in the end.

for (oo=50; oo>15; oo--) {

    selectWindow(title1);
    run("Make Substack...", "  slices=1-1000");
    title = getTitle();
    selectWindow(title);


    setAutoThreshold("Default");
    //run("Threshold...");
    setThreshold(oo, 255);
    setOption("BlackBackground", false);
    run("Convert to Mask", "method=Default background=Light");

    //call CASA with standard parameters and evaluate how many tracks have been found
    //7,69,97,12,4,1,1,71,80,71,1,1,100,1,165,200
    //2,35,36,3,2,1,8,1,7,92,1,1,21,1,70,134
    run("CASA ", "a,=2 b,=35 c,=36 d,=3 e,=2 f,=1 g,=8 h,=1 i,=7 j,=92 k,=1 l,=1 m,=21 n,=1
o,=70 p,=134 q,=33.000000000 r,=1000.000000000 s,=0 t,=1");

    //record results in a txt file in the Analysis folder
    if(isOpen("Results")==1){
        selectWindow("Results");
        saveAs("Results",subFolderString+filelist[i]+oo+Ending);
        IJ.deleteRows(0, 10000);
        };

    x = oo+1;

    print ("current treshhold: "+oo);
    if(File.exists(subFolderString+filelist[i]
+oo+Ending)==1&&File.exists(subFolderString+filelist[i]+x+Ending)==1){

        if(isOpen("Paths")==1){
            selectWindow("Paths");
            saveAs("Tiff", subFolderString+filelist[i]+oo+"path");
            close();
            };


        //finding the optimal treshold by comparing this oo and last run's (x) amount
of detected tracks
        lines_oo=split(File.openAsString(subFolderString+filelist[i]+oo+Ending),"\n");
```

```
                    lines_x=split(File.openAsString(subFolderString+filelist[i]+x+Ending),"\n");

                    print("now compare if there is more sperm in this analysis than in the one
before:");
                    print(lengthOf(lines_oo));
                    print("than the one before:");
                    print(lengthOf(lines_x));

                    //check if best treshhold value has been found by comparing number of tracks
of this with the last iteration
                    if (lengthOf(lines_oo)<lengthOf(lines_x)){
                        File.delete(subFolderString+filelist[i]+oo+Ending);
    //clear up folder from unused txt files
                        File.delete(subFolderString+filelist[i]+oo+"path");
    //clear up folder from unused path files

                        //close Paths window to not confuse evolve function
                        if(isOpen("Paths")==1){
                            selectWindow("Paths");
                            close();    //close current path window
                            };
                        if(isOpen(title)==1){
                            selectWindow(title);
                            close();
                            };


                        //call evolutionary optimization function to find best parameters and
give function the last and best treshhold setting and current filename
                        currentfile = filelist[i];
                        besttreshhold = oo-1;
                        parameters = evolve(currentfile, besttreshhold, parameters,
subFolderString);

                        oo = 0;                 //to quit for-loop
                        };
                    else {
                        File.delete(subFolderString+filelist[i]+x+"path");                  //clear
up folder from unused files
                        File.delete(subFolderString+filelist[i]+x+Ending);                  //clear
up folder from unused files
                        };
                    };
                if(isOpen(title)==1){
                    selectWindow(title);
                    close();                //close last threshhold window
                    };
                };

            if(isOpen("Paths")==1){
                selectWindow("Paths");
                close();    //close current path window
                };
```

```
				if(isOpen(filelist[i])==1){
					selectWindow(filelist[i]);
					close();   //close current monochrome video sequence
					};
				if(isOpen(title1)==1){
					selectWindow(title1);
					close();   //close current video sequence
					};

				};
			};
//};
treshhold();
```