Assessment of Microbial Behavior in Incontinence Products using a Laboratory Scale Model

Degree Project in MSc Biotechnology Engineering Programme

Astrid Sundström

Division of Applied Microbiology

Department of Chemistry

FACULTY OF ENGINEERING

LUND
UNIVERSITY

Assessment of Microbial Behavior in Incontinence Products using a Laboratory Scale Model

Astrid Sundström

LUND UNIVERSITY

Supervisors:

Sebastian Jankowski, Ed van Niel & Jenny Schelin
Division of Applied Microbiology, Department of Chemistry
Faculty of Engineering, Lund University

Examiner:

Peter Rådström
Division of Applied Microbiology, Department of Chemistry
Faculty of Engineering, Lund University

Abstract

Urinary Incontinence is a problem for many people which can give rise to both psychological and social issues. To their aid there are a range of incontinence products available in retail today. A customer concern is however possible malodor formation. Studies have shown that both urine and the bacterial flora can contribute to malodor but the impact of microbial behavior in incontinence products on possible malodor formation has not yet been explored. Incontinence products consist of cellulosic fibers and partly neutralized super-absorbent polymers (SAP) which may influence microbial growth and metabolism. The behavior of *Escherichia coli* and *Staphylococcus epidermidis*, in incontinence product and SAP, has been investigated using flow cytometry, viable count, HPLC and microcalorimetry. Localization of bacteria in product was also explored using confocal microcopy.

The results showed that the growth rate in product saturated to 50% (5x the weight of the product) with urine was 0.45 ± 0.05 for *E. coli* and 0.018 ± 0.04 for *S. epidermidis*. The product furthermore always buffered the environment to around pH 6.2 for both bacterial species and irrespective of saturation level. The percentage of damaged/dead cells was low during the first 3-6 h followed by an increase at 22 h of incubation at 35°C. A significant difference between the saturation levels for both species was observed where 5x saturation showed lower growth and survival compared to 10x saturation. On superabsorbent polymers (SAP) alone, one of the components of the product, the microbial activity was found to be higher for *E. coli* than *S. epidermidis*. The growth rate was higher for *E. coli* on SAP compared to in product and considerably lower for *S. epidermidis*. Confocal microscopy revealed that the majority of bacteria were localized on the surface of cellulosic fiber and SAP.

In conclusion; initial pH did not impact bacterial growth; both bacteria showed capacity to grow in this environment and also remained alive in the product for at least one day. Furthermore the level of liquid saturation of the product had an impact on bacterial survival over time indicating that this in an important factor to consider when studying metabolic activity and survival of cells in product.

Popular Science Summary

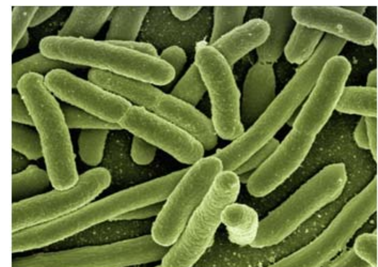
Utveckling av kunskap om bakteriers beteende i inkontinensprodukter

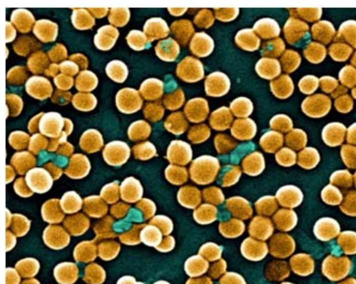
Av Astrid Sundström

Lukten av urin kan nog många känna igen men för vissa, de som använder sig av inkontinensskydd, kan det vara kopplat till en oro att det kommer från en själv. Bakterier som vi har naturligt på kroppen kan ha en roll i möjlig luktutveckling när man använder sig av inkontinensskydd. Frågan är då om och i sådana fall hur denna lukt utvecklas från bakterier i inkontinensskydd? För att ta reda på det så behöver man veta hur bakterierna beter sig i miljön som uppstår av urin i inkontinensskydd. Överlever de? Växer de? Vilka ämnen tillverkas av bakterierna? För att besvara dessa frågor har beteendet av de två olika bakterierna *Escherichia coli* och *Staphylococcus epidermidis*, undersökts.

Att inte kunna hålla sig helt när man är kissnödig eller att det ”läcker lite” när man skrattar t.ex. är något som flera människor upplever och detta är olika exempel på inkontinens. Inkontinens känns ofta pinsamt och många oroar sig för att det ska märkas. Det finns vuxenblöjor och andra så kallade inkontinensskydd för hjälpa till med problemet men många oroar sig för att det ska börja lukta.

Urin kan lukta av sig självt särskilt om man ätit något speciellt så som sparris men det finns också bakterier som i kontakt med urin kan tillverka illaluktande ämnen. Alla människor har bakterier på kroppen även om vi inte märker av dem. Det kan även ibland finnas bakterier i urin som när man har en urinvägsinfektion till exempel. Därför kan det finnas bakterier dels från huden och dels från urin i inkontinensskydd tillsammans med urin. Två olika bakterier som kan förekomma i urin och på huden, *E. coli* och *S. epidermidis,* har blivit analyserade i provbitar av inkontinensskydd. De beteenden som undersökts är framförallt tillväxt, aktivitet och lokalisering med hjälp av olika metoder däribland flödescytometri, mikrokalorimetri och mikroskopi.





Många ämnen kan tillverkas av bakterier när de växer, alltså ökar i antal, men också när de bara försöker överleva. Det visade sig att de två bakterierna som undersöktes i denna studie både överlever och växer i inkontinensskydd med urin. Det betyder att båda arter av bakterier kan bidra med eventuellt illaluktande ämnen. Mättnadsgraden, dvs. mängden urin i

förhållande till material, påverkade också graden av överlevnad och en högre mättnadsgrad, alltså en större volym urin som absorberats av inkontinensskyddet, visade på en högre överlevnad. Den ena bakterien*, E. coli*, växte mer och var mer aktiv än den andra, *S. epidermidis*. Det visar på att olika bakterier kan ge olika resultat och det är därför intressant att forska vidare inom detta område med fler arter av bakterier som kan finnas i den här miljön.

De två bakterier som undersökts har olika enzymer som kan tillverka olika illaluktande ämnen som t ex ammoniak. Ett enzym är ett typ av protein som gör att en reaktion, alltså bildandet av ett ämne, går snabbare än vanligt, som att ta hissen istället för trapporna till högsta våningen i ett hus. Med kunskap om hur reaktionerna går till kan man följa vissa ämnen, t ex urea som det finns mycket av i urin. Med hjälp av enzymet ureas bildas ammoniak från urea. Dessa ämnen, t ex urea och ammoniak, som spåras kallas biomarkörer och genom att undersöka hur de försvinner eller bildas kan man få en uppfattning om bakteriers tillväxt och aktivitet. Tre olika biomarkörer följdes i denna studie. De biomarkörer som testades visade dock tyvärr inga förändringar vilket skulle kunna bero på att bakterierna helt enkelt inte växer eller har mycket låg aktivitet. Dock kan detta inte vara anledningen i denna studie eftersom båda bakterierna visade att de både kunde överleva – alltså vara aktiva – och växa. Dessa resultat beror därför istället med stor sannolikhet på att metoden för att mäta dessa biomarkörer inte var känslig nog och måste förbättras samt att även andra ämnen också skulle kunna prövas som möjliga biomarkörer. Att hitta sådana ämnen skulle vara ett stort hjälpmedel för att visa om illaluktande ämnen bildas.

Inkontinensskydd består av flera olika material som ligger i olika lager. Ett ingående material är så kallade Super Absorberande Polymerer (SAP). SAP är ett material som har en otrolig förmåga att suga upp vätska, upp till 300 gånger sin vikt för vatten. Det betyder att 100 g av det materialet kan suga upp 30 liter vatten vilket är imponerande. Materialet påverkar också pH vilket har inflytande på överlevnad och tillväxt av bakterier. Det visade sig att materialet i inkontinensskydd justerade pH till runt 6.2 och att detta värde sedan var stabilt över tiden. pH-värden mellan 6-7 är också just de som bakterier mår allra bäst i vilket leder till att det är mycket gynnsamma förhållanden för att växa snabbt och mycket. Preliminära resultat visade vidare också att bakterierna verkade hamna och stanna närmare ytan, i de övre lagren, av inkontinensskyddet snarare än att fortsätta med urinen ned i de nedre lagren där SAP blandat med cellulosafibrer finns och suger upp vätskan.

Den kunskap och de metoder som utvecklats i detta projekt kan leda till en ökad förståelse och lägga grunden för mer forskning om och hur närvaro av bakterier kan vara en orsak till att olika luktar bildas i en viss miljö som t ex en inkontinensprodukt med absorberat urin. Detta kan vidare användas i utvecklingen av ännu mer effektiva inkontinensskydd som t ex aktivt kan hämma bakterier och således öka livskvalitén för de som lever med inkontinens – vilket är det primära och mycket angelägna målet i dessa studier.

Preface

This master's thesis is part of the project "Assessing microbial colonization and growth in incontinence products" funded by Bo Rydins Stiftelse. The thesis has been carried out at the Division of Applied Microbiology, Department of Chemistry at Lund University. It is registered as the course "KMB820" and is the last step towards acquiring an MSc in Engineering, Biotechnology, from the Faculty of Engineering (LTH), Lund University.

I would like to thank and acknowledge the project group that has worked with me on this; Sebastian Jankowski who has helped me immensely in laboratory work, Christer Larsson for all the support with the HPLC, Ed van Niel for helping with the theory behind the metabolic activity and my main supervisor Jenny Schelin who has helped me see the big picture. They have all put in so much work on developing the methods for me to use and been an essential support during my thesis. I would also like to thank my examiner Peter Rådström.

I would also like to give a special thanks to Ulla Forsgren-Brusk, Torun Wall and Frida Ryttsén at Essity (former SCA Hygiene Products AB) for valuable insight in the industry.

Front cover:

Confocal microscopy image of the surface of a super-absorbent polymer granule (SAP) at a magnification of 330x.

Table of Contents

Abstract **2**

Popular Science Summary **3**

Preface **5**

List of Abbreviations **8**

1 Introduction **9**
- **1.1 Human urine** **9**
- **1.2 Microbial skin flora and metabolism** **10**
- **1.3 Materials in incontinence products** **12**
  - 1.3.1 SAP 12
- **1.4 Analytical tool box** **13**
  - 1.4.1 Microbial Growth 13
  - 1.4.2 Metabolic activity 16

2 Scope **17**

3 Materials and method **18**
- **3.1 Bacterial strains and growth conditions** **18**
  - 3.1.1 Pre-culture for growth in urine 18
  - 3.1.2 Pre-culture for growth in bacterial growth medium 18
- **3.2 Urine** **18**
- **3.3 Product material** **19**
  - 3.3.1 Sample of complete product 19
  - 3.3.2 SAP 19
- **3.4 Growth measurements** **19**
  - 3.4.1 Viable Count and Optical Density 20
  - 3.4.2 Flow Cytometry 20
  - 3.4.3 Determining the relation between optical density, viable count and flow cytometry 21
- **3.5 Measurement of metabolic activity** **21**
  - 3.5.1 Microcalorimetry 21
  - 3.5.2 HPLC 22
- **3.6 Microbial behavior in product** **22**
  - 3.6.1 pH setting of urine and product inoculation 24
  - 3.6.2 Extraction of bacteria from product 24
- **3.7 Microbial behavior on SAP** **24**
  - 3.7.1 Plate set-up for microcalorimetry 25
  - 3.7.2 Extraction of bacteria from SAP 26
- **3.8 Enumeration of plate counts and statistical analysis** **27**
- **3.9 Microscopy** **27**

4 Results **29**
- **4.1 Correlation between methods for microbial growth measurements** **29**
- **4.2 Growth in product** **29**
  - 4.2.1 Cell recovery from product and SAP 29
  - 4.2.2 pH in product 31
  - 4.2.3 Growth of *E. coli* in product 32
  - 4.2.4 Growth of *S. epidermidis* in product 34
- **4.3 Total metabolic activity and growth of *E. coli* and *S. epidermidis* on SAP** **35**
- **4.4 Specific metabolic activity** **36**
- **4.5 Mean growth rate** **37**

4.6 Microscopy 38
5 Discussion 39
6 Conclusion 46
7 Future perspectives........ 46
References........ 47
Appendix 1. List of compounds tested in the HPLC 52
Appendix 2. Confocal microscopy images........ 53

List of Abbreviations

ATCC – American type culture collection
ATP – Adenosine triphosphate
BHI – Brain-heart infusion
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
EDTA - Ethylenediaminetetraacetic acid
FC – Flow cytometry
HPLC - High-performance liquid chromatography
LB – Luria-Bertani
MC – Microcalorimetry
MRD – Maximum recovery diluent
OD – Optical density
PBS – Phosphate-buffered saline
PCR – Polymerase chain reaction
SAP – Superabsorbent polymer
VC – Viable count
VOC – Volatile organic compound

1 Introduction

A serious but unfortunately usually hidden problem that is rarely brought to attention or discussed due to embarrassment is involuntary urine leakage, also known as urinary incontinence (UI) (Broome, 2003; Pandey et al., 2013). Many people experience UI for different reasons such as obesity or during the postpartum period for women but both men and women of various ages can be suffering of UI (Frawley et al., 2017; Wilson, 2015; de Viñaspre Hernández et al., 2013; Burgin et al., 1991). The prevalence is however high among elderly where some estimations report up to 60%, and most affected are elderly women (Emmons and Robinson, 2014). Stress incontinence, the involuntary leakage due to sudden pressure on the bladder, is reported for around 50% of all incontinent women, while for elderly women it is more common with urge incontinence – involuntary leakage when having a strong need to urinate (Pandey et al., 2013; Emmons and Robinson, 2014). UI is a problem that not only can cause physical issues such as dermatitis, fungus and skin infections due to repeated exposure to urine. It can also cause psychosocial issues such as depression, anxiety and social isolation as the condition becomes a barrier for enjoying recreational activities in fear of accidents and embarrassment (Emmons and Robinson, 2014). There are various absorbent products, i.e. incontinence products, available to facilitate with the issue but a concern for possible malodor formation during usage of the product can have a negative impact on the quality of life for those who suffer from UI (Broome, 2003; Emmons and Robinson, 2014). Malodor may arise from urine itself but also from the involvement of microorganisms (Troccaz et al., 2013). The impact of microorganisms in incontinence products on possible malodor formation has yet to be explored and therefore this study has been carried out to initiate the investigation of this area.

1.1 Human urine

Urine consists of high concentrations of urea from the metabolism of amino acids in the body, inorganic salts, ammonia, hippuric acid, organic acids, water-soluble toxins, and pigmented products among many other metabolites (Bouatra et al., 2013). Uric acid is also present from degradation of nucleic acids as well as creatinine as the break down product of creatine, which is utilized in the muscle tissues (Widmaier, 2014). A comprehensive list of a typical urine composition was made by Putnam, (1971) and includes in detail the impressive amount of possible constituents in urine (Figure 1). The normal range of pH of urine is 5.5 to 7.0 with an average of 6.2 according to Rose et al. 2015, but can be between pH 4.8 to 8.0 according to Martin, 2015. The pH and composition of urine can differ depending on age, sex, diet and health (Rose et al., 2015; Hesse et al., 1986). Urine is often thought to be sterile but it encounters a microbial flora in the distal urethra and outside the body (Hilt et al., 2014). For people with urinary tract infection (UTI), uropathogenic bacteria can be present earlier in the urinary system such as in the bladder, kidney and urethra (Lee and Neild, 2007). The study of Hilt et al., (2014) also suggests that there is a microbiota in the bladder of healthy adult women. The variation in composition and pH of urine becomes a challenge when investigation malodour formation in incontinence products as well as the impact of presence of microorganisms. The odor originates from volatile organic compounds (VOCs) and unpleasant odor from fresh urine is thought to be caused by malodorous compounds already

present in the urine which can come from diet (like asparagus) or otherwise it is developed from bacterial metabolism or thermal reactions (Troccaz et al., 2013).

ITEM	FORMULA	FORMULA WEIGHT	AMOUNT mg/ℓ
INORGANIC SALTS			14,157
Sodium Chloride	$NaCl$	58.4	8,001
Potassium Chloride	KCl	74.6	1,641
Potassium Sulfate	K_2SO_4	174.3	2,632
Magnesium Sulfate	$MgSO_4$	120.4	783
Magnesium Carbonate	$MgCO_3$	84.3	143
Potassium Bicarbonate	$KHCO_3$	100.1	661
Potassium Phosphate	K_3PO_4	212.3	234
Calcium Phosphate	$Ca_3(PO_4)_2$	310.2	62
UREA	H_2NCONH_2	60.1	13,400
ORGANIC COMPOUNDS			5,369
Creatinine	$C_4H_7N_3O$	113.1	1,504
Uropepsin (as Tyrosine)	$HO{\cdot}C_6H_4{\cdot}C_2H_3(NH_2){\cdot}CO_2H$	181.2	381
Creatine	$HN{:}C(NH_2)N(CH_3){\cdot}CH_2{\cdot}CO_2H{\cdot}H_2O$	149.2	373
Glycine	$NH_2{\cdot}CH_2{\cdot}CO_2H$	75.1	315
Phenol	$C_6H_5{\cdot}OH$	94.1	292
Histidine	$C_3H_3N_2{\cdot}CH_2{\cdot}CH{\cdot}(NH_2){\cdot}CO_2H$	155.2	233
Androsterone	$C_{19}H_{30}O_2$	290.5	174
1-Methylhistidine	$C_3H_3N_2CH_2CH(NH{\cdot}CH_3){\cdot}COOH$	169.2	173
Imidazole	$C_3H_4N_2$	68.1	143
Glucose	$C_6H_7O_6(COCH_3)_5$	390.4	156
Taurine	$NH_2{\cdot}CH_2{\cdot}CH_2{\cdot}SO_3H$	125.2	138
Cystine	$[HO_2C{\cdot}CH(NH_2){\cdot}CH_2S{\cdot}]_2$	240.3	96
Citrulline	$NH_2CONH(CH_2)_3{\cdot}CH{\cdot}(NH_2){\cdot}CO_2H$	175.2	88
Aminoisobutyric acid	$H_2N{\cdot}CH_2, CH_3 > CH{\cdot}COOH$	103.1	84
Threonine	$C_4H_9O_3N$	119.1	83
Lysine	$(NH_2)_2C_5H_9{\cdot}CO_2H$	146.2	73
Incloxysulfuric acid	$C_8H_7ON{\cdot}H_2SO_4$	231.2	77
m-Hydroxyhippuric acid	$C_4H_4COHC(CONH{\cdot}CH_2COOH)$	195.2	70
p-Hydroxyphenyl – hydrocrylic acid			70
Inositol	$C_6H_{12}O_6$	180.2	70
Urobilin	$C_{33}H_{40}O_6N_4$	588.7	63
Tyrosine	$HO{\cdot}C_6H_4{\cdot}C_2H_3(NH_2){\cdot}CO_2H$	181.2	54
Asparagine	$HO_2C{\cdot}CH(NH_2){\cdot}CH_2{\cdot}CONH_2$	132.1	53
Organics less than 50 mg/ℓ			606
ORGANIC AMMONIUM SALTS			4,131
Ammonium:			
Hippurate	$NH_4C_6H_5CO{\cdot}NHCH_2{\cdot}CO_2$	196.2	1,250
Citrate	$(NH_4)_2HC_6H_5O_7$	226.2	756
Glucuronate	$NH_4C_6H_9O_7$	211.1	663
Urate	$NH_4C_5H_3O_3N_4$	185.1	518
Lactate	$(NH_4)_2C_3H_5O_3$	127.1	394
L-Glutamate	$NH_4HO_2C{\cdot}CHNH_2{\cdot}(CH_2)_2{\cdot}CO_2$	164.1	246
Asparate	$NH_4C_4H_6O_4N$	150.1	135
Formate	NH_4HCO_2	63.1	88
Pyruvate	$NH_4CH_3{\cdot}CO{\cdot}CO_2$	88.1	44
Oxalate	$(NH_4)_2C_2O_4$	124.0	37
Total Solutes			37,057

Figure 1. **Comprehensive list of an analog for a typical human urine composition.** (Putnam, 1971, p.40)

1.2 Microbial skin flora and metabolism

The microbial flora in urine has been found to include both gram-positive bacteria like *Staphylococcus* and *Enterococcus* and gram-negative bacteria such as *Escherichia*, *Klebsiella* and *Proteus* (Troccaz et al., 2013). The bacteria have different metabolism and produce different VOCs such as ammonia, methyl mercaptan, trimethylamine, hydrogen sulfide among others, which are known to be malodorous (Storer et al., 2011; Troccaz et al., 2013;

Pandey et al., 2013). The bacteria in the flora are known uropathogens and can be causing UTI, especially *Escherichia* (*E.*) *coli* which is the most common pathogen for UTI (Lee and Neild, 2007; Storer et al., 2011). VOCs have also been investigated for bacteria associated with sepsis, a systemic inflammation caused by bacterial infection, and found to produce malodorous compounds (Bos et al., 2013). These bacteria can end up in the urinary tract so there might be a difference in the possible malodour formation depending on disease. Other bacteria that have been found are *Lactobacillus*, *Corynebacteruim* and *Streptococcus* (Hilt et al., 2014). *Lactobacillus* is also the most predominant species in the vaginal flora (Bartlett et al., 1977) but there is an individual variation and it is also changes over time (Priestley et al., 1997). For women, the vaginal flora is a factor to consider due to the vicinity of the urinary tract.

In this study, the focus has been on the *E. coli* and *Staphylococcus* (*S.*) *epidermidis.* The extra-intestinal strain *E. coli* ATCC 700928, an uropathological isolate, is a bacterium that could be present in urine and was therefore chosen for this study (Welch et al., 2002). The other bacterial strain used in this study is the non-biofilm forming *S. epidermidis* ATCC 12228, and is not linked to infection. *S. epidermidis* occurs naturally on skin as well as the distal portion of the urethra and in the vaginal flora but rarely cause infection in healthy persons (Willey et al., 2011; Zhang et al., 2003; Priestley et al., 1997). Most of the bacteria found in the microbial flora, including *E. coli* and *S. epidermidis*, have the capacity to produce volatile malodorous compounds as a result from urease and tryptophanase activity (Bos et al., 2013; Troccaz et al., 2013). *E. coli* produces indole, which can contribute to malodor and is one of the products when tryptophanase cleaves tryptophan (Bos et al., 2013; Troccaz et al., 2013). Another product formed from the enzymatic activity of tryptophanase, is ammonium (Wood et al., 1947) which can be transformed by deprotonation to the malodorous VOC ammonia.

S. epidermidis is known to form VOCs in urine, primarily ammonia, due to the presence of urease which degrades urea to ammonia (Storer et al., 2011; Podstawka, 2017a). Without urease as a catalyst, the reaction is 10^{14} times slower (Karplus et al., 1997) thus urease activity is an important factor in the development of ammonia. Urea can also serve as an indirect energy source as the urease reaction gives an increase in electrochemical potential that in turn lead to ATP synthesis (Smith et al., 1993). Urea is therefore a possible biomarker for both metabolic activity linking to possible malodor formation and to growth. Two other possible biomarkers are creatinine and creatine as it is possible that bacteria can convert creatinine, which is present in urine, into creatine again with the help of creatinine amidohydrolase. This have been observed for *Pseudomonads* as well as *Arthrobacter* and *Alcaligenes* strains (Hermann et al.,1992) but there is little information found if this is the case for *E. coli* and *S. epidermidis*. The pathway is also connected to formation of urea as a by-product when creatine is hydrolysed by creatine amidinohyrdolase to sarcosine as well as ammonia in a later step in the metabolism (Hermann et al., 1992). Changes in creatinine and creatine could then indicate that the bacteria utilize this pathway for bacterial degradation of creatinine and also indicate changes in ammonia production.

1.3 Materials in incontinence products

There are several different kinds of incontinence products to meet the needs of various groups of people suffering from UI. In general, they are a layered product with an absorbent core consisting of fiberized pulp of cellulosic fibers and superabsorbent polymers (SAP). The surface layer consists of either polyethylene or polypropylene or a mix of both and the bottom layer often consists of polyethylene (EDANA, 2008). A laboratory scale model was used in this study to simulate real-life conditions during usage of an incontinence product (Figure 2). The sample of the product had the same composition as the whole product, thus representative, but in a size more suitable for laboratory investigations (Figure 3).

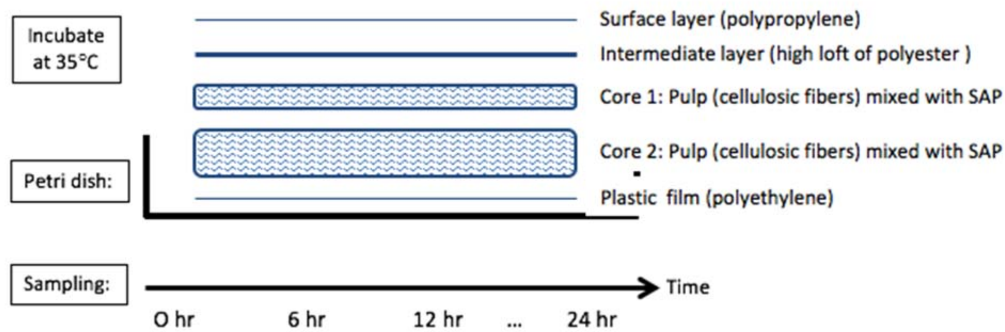


***Figure 2*. Schematic image of the laboratory scale model which mimics the real-life conditions for someone wearing an incontinence product.** Composition of product sample is the same as whole product, incubation temperature the same as the real-life environment and sampling points are made throughout possible usage time.

***Figure 3*. Sample of product used in the study that is representative of the whole incontinence product.**

1.3.1 SAP

Superabsorbent polymers or SAP have the ability to absorb a great amount of liquid and are therefore a material that is suitable for applications such as diapers, incontinence products and other absorbent products like absorbent medical dressings. SAP has the greatest absorbing and retention capacity for deionized water which can be up to 99wt%. The polymers are cross-linked sodium carboxylate that swell up to form a gel-like structure that is not water-soluble (Figure 4). The cross-linking of the polymers allows for a network to be formed and prevent the polymers from dissolving in the water. The polymers are neutralized by the attraction sodium and the carboxylate group of the polymer. The polymer is hydrophilic and in water the sodium carboxylate groups are dissociated which increases the osmotic pressure in the

network as the sodium ions move away from the carboxylate group. However, the dissociated sodium is trapped in the network of polymers by attraction of the negative carboxylate groups and maintains the electrical neutrality (Elliott, 2004). If the SAP is only partially neutralized it means there are negatively charged carboxylate groups free to bind cations, such as ammonium. The swelling is therefore driven by the difference of osmotic pressure inside and outside the gel. Testing for bodily fluids such as urine is usually made with saline solution to simulate the concentration of ions that are present in those types of fluids (Elliott, 2004).

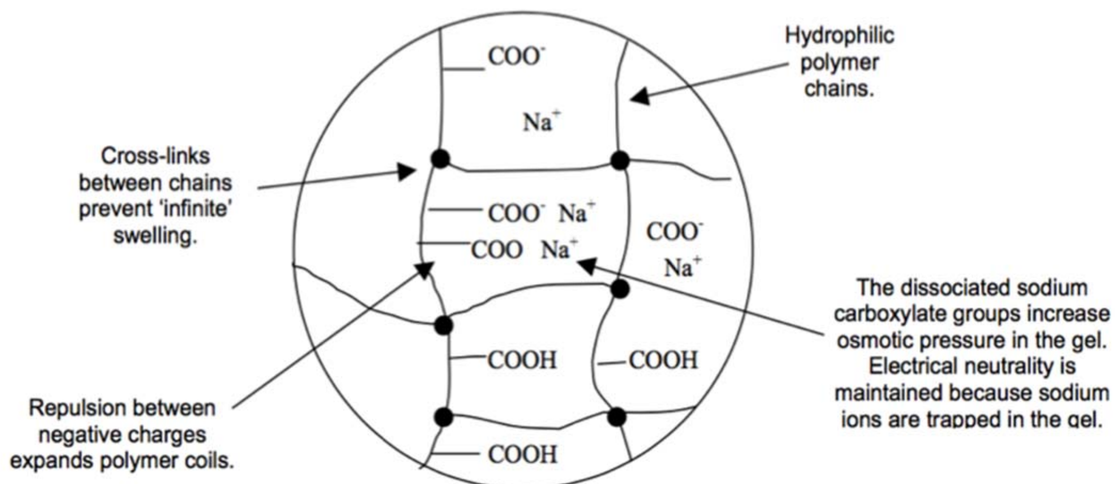


***Figure 4*. Illustrative figure of the crosslinking and absorption mechanism in SAP.** The backbone is made of sodium carboxylate polymers and is crosslinked to prevent water-solubility. The negative charge of carboxylate repulses each other which cause the polymer coil to expand. The dissociated sodium is trapped in the network of polymers and keeps electrical neutrality while also increasing osmotic pressure inside the gel. (Elliott, 2004)

1.4 Analytical tool box

1.4.1 Microbial Growth

The traditional way of measuring bacterial cell concentration is by plating, also known as viable count. The method includes dispersing a diluted sample of microorganisms on or within agar so that one cell generates one colony. The colonies are counted and after considering the dilution factor, a concentration can be calculated in colony forming units (CFU) per milliliter. Another technique for following growth is by measuring the optical density (OD) of a culture with a spectrophotometer. The microbial cells and other solid particles scatter light that is transmitted and the absorbance (optical density) is measured by the spectrophotometer. The relation between OD and cell concentration is linear up to a level of absorbance of 0.5 (Willey et al., 2011).

A more modern way of quantitative analysis of cell concentration is the molecular methods that have been developed over the years. One method is to measure the concentration of DNA in a bacterial culture with quantitative polymerase chain reaction (qPCR) and estimate the number of cells based on expected genome content per cell (Brown, 2013). PCR can also differentiate between bacteria in a mixed population or community with the use of specific primers (Nadkarni et al., 2002). Another method that is used for direct enumeration of cells is

flow cytometry (FC). Flow cytometry collects data as a stream of suspended particles or cells flow through a laser beam. The light from the laser is scattered and fluorescence is emitted which is collected by optical detectors and transferred electronically to a computer for analysis. In this way, each cell is detected independently and a cell count is obtained. Flow cytometry also provides information about size and shape of the cells as well as autofluorescence of cells, or other characteristics, such as indication of viability, with the use of fluorescent dyes (Gatza et al., 2013; Willey et al., 2011). Flow cytometry therefore has many applications such as in water quality, food safety and diagnostics (Diaz et al., 2009).

1.4.1.1 Flow cytometry – Viability Stain

Flow cytometry can be used to differentiate between particles and cell types with the use of fluorescent labelling. Staining the cells with different fluorescent dyes can discriminate cells from debris, but there are also stains that can show viability (Paparella et al., 2012). Assessing viability with flow cytometry has the advantage of being able to detect cells in a viable but non-culturable (VBNC) state. Fluorescent labeling can target many different parts of the cell to measure viability such as membrane potential, membrane integrity, enzymatic activity and pump activity (Figure 5).

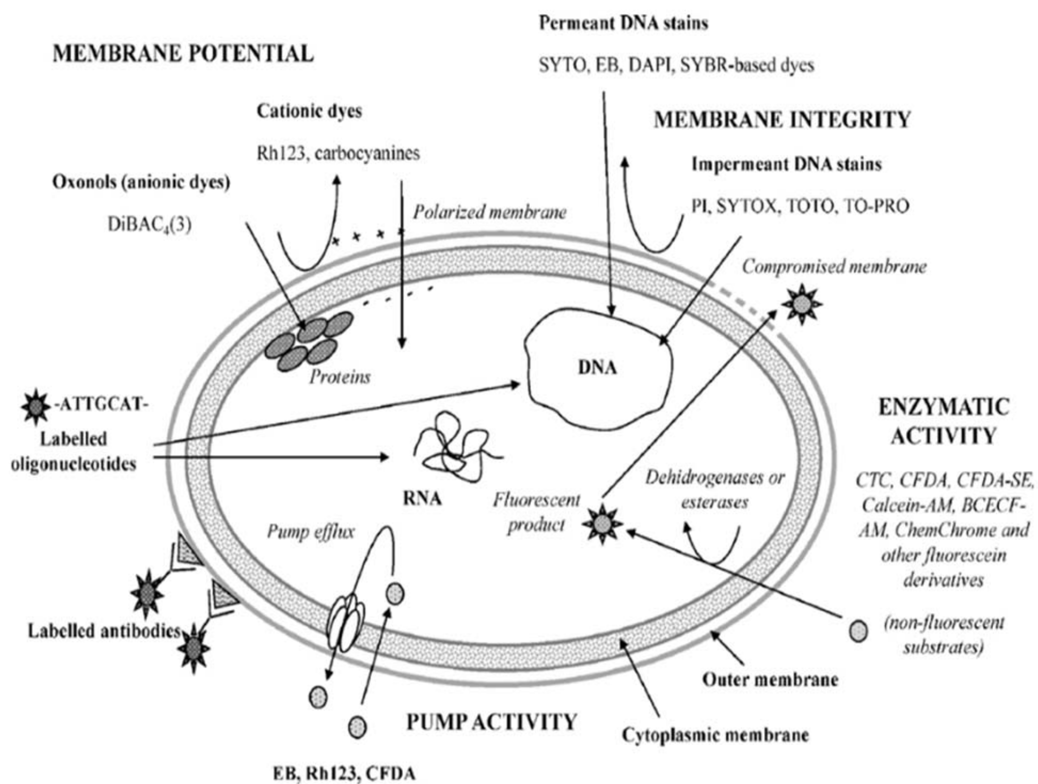


***Figure 5*. Schematic figure of different fluorescent labelling targets for viability in flow cytometry.** Fluorescent dyes can target membrane potential, membrane integrity, enzymatic activity and pump activity to measure viability of the cell. (Diaz et al., 2009, p.389)

To measure membrane integrity, it is common to use a combination of a cell-permeant and a cell-impermeant nucleic acid stains with the latter being a viability-dependent stain as it cannot pass through an intact cell membrane. Nucleic acids stains can bind to the DNA in

different positions such as in between the base pair (intercalating) or on the outside of the DNA on the minor or major groove (Figure 6) (Thermofisher, 2017).

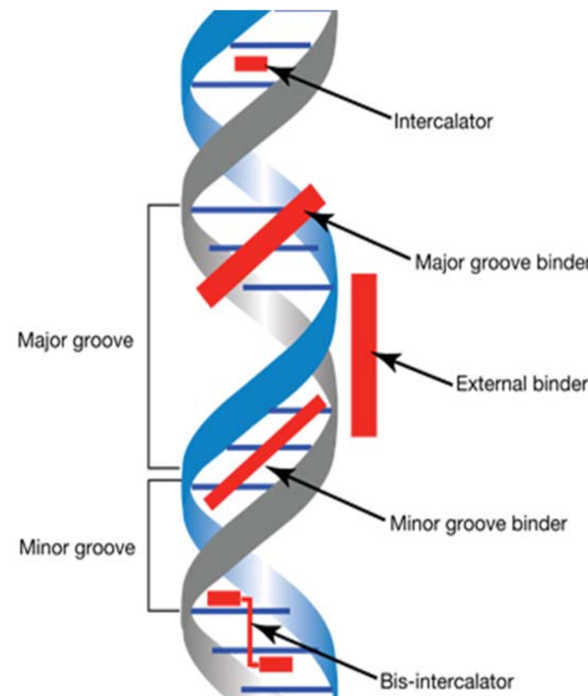


***Figure 6*. Schematic figure of how different nucleic acid dyes bind to the DNA.** Nucleic acid dyes can bind intercalating, bis-intercalating, externally or over the major or minor groove of the DNA strain. (Thermofisher, 2017).

Common nucleic acid dyes that are cell-permeant are DAPI, SYBR Green I and SYTO 9. Examples of cell-impermeant nucleic acid stains are Propidium Iodide (PI), SYTOX, the TOTO and TO-PRO dyes (Buysschaert et al., 2016). These two different types can be combined in a multicolor stain and an example is the combination of SYBR Green I and PI which are both intercalating dyes (Gatza et al., 2013; Thermofisher, 2017).

SYBR Green I is a cyanine dye that binds into double stranded DNA. PI binds to DNA and RNA of cells lacking an intact cell membrane. It is not able to bind to DNA of intact cells (Figure 7A). In this way, PI will only stain the severely damaged (indicating dead cells) or dead cells while SYBR Green I stains all cells, regardless of intact or disrupted membrane. In the flow cytometer, SYBR Green I is detected by FL1 and FL3 detectors while PI will primarily be detected by the FL3 detector and thus the cells stained with PI will shift out from the cluster of cells that have only been stained with SYBR Green I when co-stained (Figure 7B) (Gatza et al., 2013).

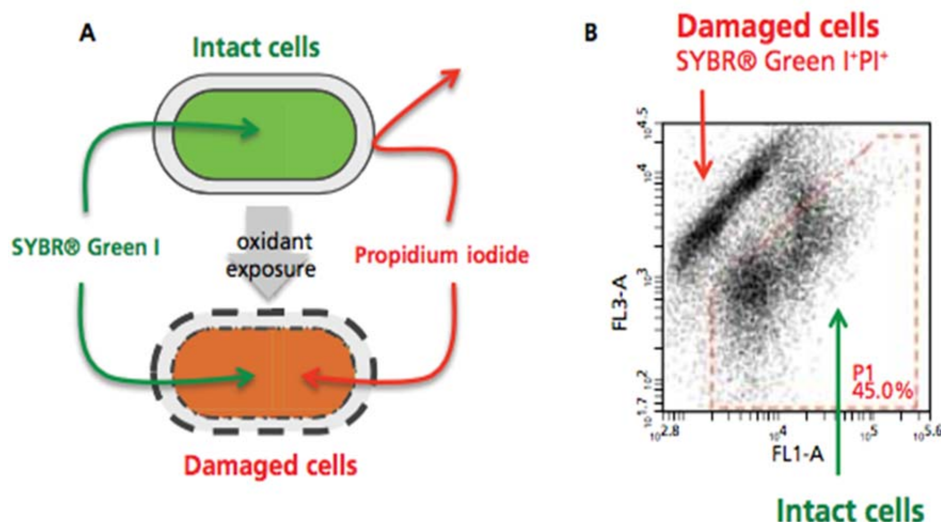


***Figure 7*. The mechanisms of SYBR Green I and PI for membrane integrity measurements** (Gatza et al., 2013, p.8)**.** (A) Schematic image of how SYBR Green I can permeate intact cell membranes while PI only can enter cells with a damaged cell membrane. (B) Differentiation of damaged and intact cells is achieved in the flow cytometer as SYBR Green I and PI are detected with different detectors.

1.4.2 Metabolic activity

Metabolic activity includes all processes that occur in a microorganism both to support cell division (growth) but also for maintenance of viability in resting (non-dividing) cells. Measurement of metabolic activity therefore presents an alternative way of detecting the presence of viable cells in an environment in the absence of cell growth. In addition, it is of interest to identify, e.g. using high performance liquid chromatography (HPLC), what compounds are produced and metabolized in microbial metabolism for other reasons such as the identification of bacteria (Storer et al., 2011). Biomarkers are compounds that are often used to measure metabolic processes. Usually the compounds used as biomarkers are in high concentration and easily detectable. A natural biomarker is NADH since NADH is a coenzyme in many reactions and thus gives a measure of the metabolic activity. NADH is also autofluorescent which makes it easily detectable with e.g. a spectrophotometer (Heikal, 2010). Another natural biomarker is ATP which is the energy molecule in cells and can be used as a measure of activity (Venkateswaran et al., 2003). Depending on the purpose of measuring metabolic activity, different biomarkers and different methods can be employed. Examples of methods that can be used to analyze the metabolic profile of urine is HPLC, nuclear magnetic resolution (NMR) and high performance liquid chromatography- tandem - mass spectrometry (HPLC-MS), to name a few (Bouatra et al., 2013).

1.4.2.1 Microcalorimetry

One way of measuring metabolic activity is with isothermal microcalorimetry. In recent years, the use of microcalorimetry for microbial processes have become increasingly popular and can be used in different applications such as detection of infection, food microbiology and material testing (Braissant et al., 2015). The method measures the heat flow generated by the microorganisms and is related to the total metabolic activity of the cell. Heat produced or consumed in the calorimetric ampoule is registered by the detecting sensor, the thermopile.

The sensor is attached to a heat-sink with a large mass compared to the cell-culture cups or vials. All heat produced is transferred to the heat-sink giving rise to a signal in the thermopile sensor proportional to the heat-flow (Figure 8). The method cannot however distinguish the difference between certain metabolic processes in the heat flow but is rather a sum of the processes (Braissant et al., 2009).

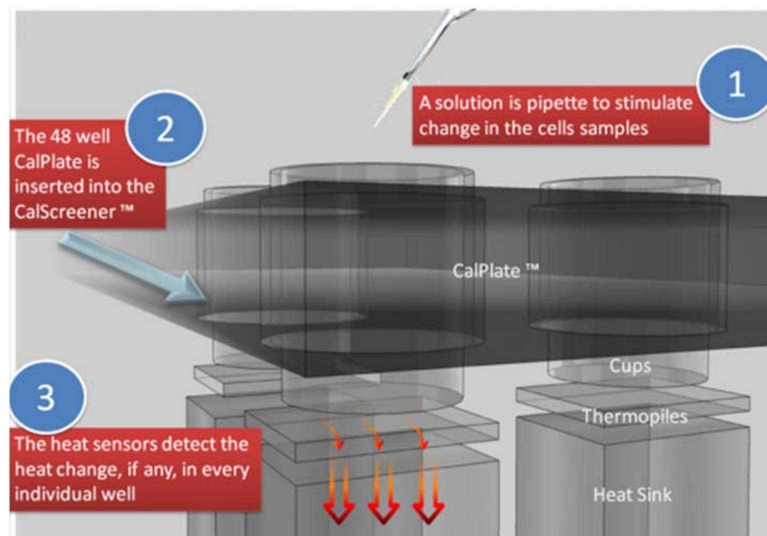


***Figure 8*. Diagram of measurement of heat flow with a microcalorimeter.** (1). A solution that will generate heat (for example bacteria culture) is added in cups.(2). Well-plate with cups is inserted in the microcalorimeter. (3). Heat is transferred to the heat sink where it is detected by thermophile sensors.

2 Scope

The scope of this master thesis project is to assess microbial growth and metabolic activity of *S. epidermidis* and *E. coli* in an incontinence product by using a laboratory scale model system. Visualization and localization of bacteria in the material was also studied by confocal electron microscopy.

The overall aim of this project is to investigate how incontinence product materials impact microbial growth, colonization and metabolic activity. Specifically, the following questions were addressed: (i) How do the material affect the microbial activity? (ii) Where in the material do the microbes colonize? This master thesis is part of a larger project entitled "Assessing microbial colonization and growth in incontinence products" funded by Bo Rydins Stiftelse.

3 Materials and method

Prior to this study, the project group worked on method development for the methods used in this study. The results from the characterization of bacteria as well as extraction method and flow cytometry protocol were all used as a foundation for this study.

3.1 Bacterial strains and growth conditions

In this project, *Escherichia coli* ATCC 700928, an uro-pathological isolate, and *Staphylococcus epidermidis* ATCC 12228, a non-clinical isolate, was used. The isolates were stored in cryotubes with 20% glycerol and 80% bacterial growth medium at -80°C. Difco LB-Miller Broth/Agar (Luria-Bertani) (BD, Franklin Lakes, NJ) was used as bacterial growth medium for *E. coli* and Bacto BHI Broth/Agar (BD, Franklin Lakes, NJ) for *S. epidermidis*.

Four days prior to each set of experiments bacteria were transferred from a glycerol stock at -80°C and cultivated on an agar plate overnight at 37°C using the appropriate medium described above. After incubation, the plates were stored in a refrigerator at 5-8°C until use.

3.1.1 Pre-culture for growth in urine

A three-step pre-adaptation to growth in urine was made during the pre-culturing step. First, a pre-culture of the bacteria was prepared in Falcon Tube (50 ml) by inoculating 10 ml of 50% sterile urine and 50% medium with a colony from the prepared agar plate of bacteria. Three biological replicates were prepared by inoculating an independent colony to each of the three separate pre-cultures. The pre-cultures were incubated overnight at 35°C and rotation at 200 rpm (Innova® 40, Incubator Shaker Series, New Brunswick Scientific, Hamburg, Germany). The next day, the pre-cultures were centrifuged at 3220 x g (Centrifuge 5810 R, Eppendorf, Hamburg, Germany) for 10 min at room temperature and the supernatant was discarded. Each pellet was re-suspended with 10 ml of 90% sterile urine and 10% medium. The new pre-culture was incubated overnight at 35°C and rotation at 200 rpm. The following day, the pre-cultures were centrifuged like previously described, the supernatant discarded and the pellet was re-suspended in 10 ml of 100% sterile urine.

3.1.2 Pre-culture for growth in bacterial growth medium

A pre-culture was prepared in Falcon Tube (50 ml) by inoculating 10 ml medium with one colony from the prepared agar plate of bacteria. One biological replicate was made. The pre-culture was incubated overnight at 35°C and rotation at 200 rpm 200 rpm.

3.2 Urine

For this project, a stock of pooled urine was generated from urine donated by female employees and students, age range from 20-60 years, at the Division of Applied Microbiology. During the collection period, the urine was stored at -18°C until approximately 15 litres was obtained. All urine was then thawed at 4°C, pooled and centrifuged at 17 700 x g (Beckman Coulter Avanti J-25 I)). The supernatant was separated from pellet and then stored in aliquots of 50ml and 80ml at -80°C. Samples of the urine were sent to the University Hospital in Malmö (SUS) for analysis of glucose, urea, creatinine and amino acids when it

was collected in June 2016 and then again in March 2017 to ensure that the storage conditions did not affect the composition. No change in composition was detected over time. An appropriate volume of urine was thawed and filter sterilized using Filtropur S 0.2 (Sarstedt, Nümbrecht, Germany) before being used in each experiment.

3.3 Product material

The product materials were provided by SCA Hygiene AB (Mölndal, Sweden). Both samples of complete product in various shapes and sized as well as separate components such as super-absorbent polymer granules (SAP) (Figure 9A) and cellulosic pulp (Figure 9B) were kindly donated (Figure 9).

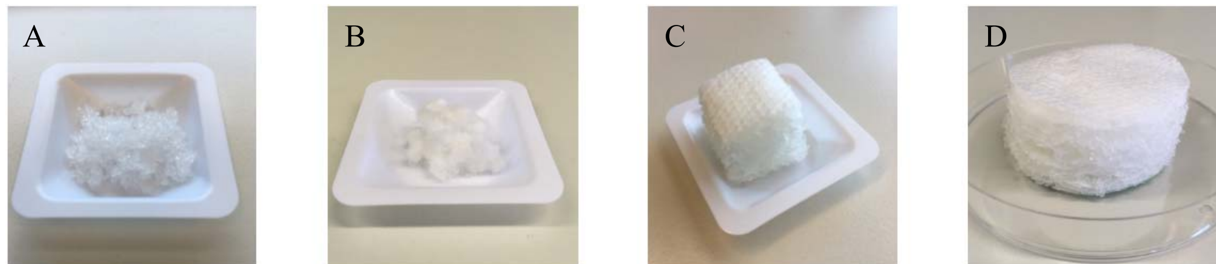


***Figure 9*. Materials included in incontinence product.** (A) SAP, (B) cellulosic fibers, (C) Complete product sample adapted for confocal microscopy and (D) Complete product sample adapted for growth experiments.

3.3.1 Sample of complete product

A complete product material consists of several layers; a polypropylene surface layer, an intermediate layer of polyester, a 2-part core of cellulosic fibers mixed with SAP and a plastic film of polyethylene in the bottom (Figure 2). The product sample used for all growth experiments was circular in the shape (punched out from the true consumer product) and had a weight of 1.62 – 1.89 g (Figure 3, Figure 9D). For microscopy analysis, the shape of the complete product was instead squared in order to better be adapted for sample preparation for confocal microscopy (Figure 9C).

3.3.2 SAP

SAP granules, neutralized to 75%, were also provided by SCA.. The maximum absorption capacity of SAP was determined by weighing 15 mg of SAP in a Falcon 40 µm cell strainer and sinking it down in a petri dish with 20 ml of urine. The SAP/cell strainer was submersed in urine for60 minutes and then weighed again. Based on the assumption that maximum absorption of urine was achieved after 60 minutes, the maximum absorbing capacity was calculated from the difference in weight. According to the obtained results maximum absorption capacity (100 % saturation of SAP) corresponded to about 50x the weight of SAP.

3.4 Growth measurements

Both the traditional growth measurement methods viable count (VC) and optical density (OD) was used as well as the newer method flow cytometry (FC).

3.4.1 Viable Count and Optical Density

For VC, samples were diluted in MRD (Oxoid, Thermo Fisher Scientific, Hampshire, UK) in a 10-foldserial dilution. A volume of 0.1 ml of two different dilutions was plated on agar plates and three technical replicates (three agar plates) were made for each dilution. The plates were then incubated at 37°C overnight, counted and CFU/g product was calculated. Cell concentration is calculated using the dry weight of the product. For OD measurements, appropriate dilutions of the bacterial culture were prepared, according to the linear range (0.05-0.3) of the spectrophotometer (Ultrospec 2100 pro spectrophotometer Amersham Biosciences, Little Chalfont, UK) and measured at 620 nm (OD_{620nm}).

3.4.2 Flow Cytometry

The flow cytometry protocol is based on the standard staining method of Swiss Federal Institute of Aquatic Science and Technology, Eawag, Switzerland for analyzing quality of drinking water. It works for cell concentrations between 10^2-10^7 cells/ml and makes use of the two different dyes SYBR Green I and Propidiun Iodide (PI) for staining of bacterial cells.

Samples were diluted with phosphate-buffered saline (PBS) (137 mM NaCl [Merck KGaA, Darmstadt, Germany], 2.7 mM KCl [Merck KGaA, Darmstadt, Germany], 10mM Na_2HPO_4 [Merck KGaA, Darmstadt, Germany], 1.8mM KH_2PO_4 [Merck KGaA, Darmstadt, Germany]) with the addition of 1mmol/L EDTA (Sigma-Aldrich, Saint Louis, MO) and 0.01% Tween 20 (Sigma-Aldrich, Saint Louis, MO), to achieve a cell concentration of 1000 – $5x10^6$ cells/ml. The SYBR Green I (Life Technologies, Eugene, OR) was diluted in DMSO (Sigma-Aldrich, Saint Louis, MO) from a 10000X concentrate stock solution to SYBR Green I 100X. For each technical replicate two samples were prepared and analyzed; one was stained with 5 µl SYBR Green I 100x and one with 6 µl of a mix of 105 µl of 100X SYBR Green I and 21 µl 1 mg/ml PI (Sigma-Aldrich, Saint Louis, MO). The final volume of each sample was 500 µl. The samples were vortexed and then incubated for 10 min at 37°C. After incubation, the samples were vortexed again and analyzed on the BD Accuri C6 plus flow cytometer with a BD C sampler (BD Biosciences, Franklin Lakes, NJ) with the fast fluid speed (flow rate of 66 µl/min), an uptake limit of 50µl, with one agitation cycle and using a FL1-H threshold of 5000. There were tubes with milli-Q-water in between samples with SYBR Green I and the mix as well as between biological replicates. For the water, the settings were fast fluid speed, 2 min and the default settings (80000 on FSC-H) for the detector threshold. The blue 488 nm laser (λmax = 497 nm) was used and the optical filters FL1 (533± 30 nm) and FL3 (≥ 670nm). The data was collected and analyzed with the help of the BD Accuri C6 Plus software. A log scale density plot of FL1-H vs FL3-H was obtained to visualize the fluorescence of the dyes. The cells were first separated from possible background by making a histogram of FL1-H and gating at a threshold of around 10^4 cells/ml. The gate filtered out the background in the FL1-H vs FL3-H plot and a new gate was made to capture all the cells in the SYBR Green I samples. The same gates were then kept so when viewing the samples with the mix of both dyes, the cells with PI were outside the gate. The data was then viewed in the statistical analysis tab to obtain the cell number per µl as well as the percentage of viable cells and percentage of cells vs background. A cut-off of at least 50% cells was applied.

3.4.3 Determining the relation between optical density, viable count and flow cytometry

A pre-culture was prepared as described in section 3.1.2 – *Preculture for growth in bacterial medium.* One biological replicate was made. OD of the pre-culture was measured at 620 nm (Ultrospec 2100 pro spectrophotometer Amersham Biosciences, Little Chalfont, UK). The volume of culture needed to obtain $OD_{620\ nm}$ 0.1, 0.2 and 0.3 in 5 ml bacterial growth medium was calculated. Three cultures were then prepared from pre-culture in medium corresponding to $OD_{620\ nm}$ 0.1, 0.2 and 0.3. and $OD_{620\ nm}$ was measured to ensure the cultures had the expected OD. Cell concentration was measured according to previously described methods for viable count and flow cytometry. The information about relationship between OD, VC and FC in bacterial growth medium was used to estimate for the corresponding relationship in urine when preparing the inoculation urine culture in the experimental set-up for growth in product and in SAP.

3.5 Measurement of metabolic activity

Total metabolic activity was measured by monitoring heat flow in a microcalorimeter instrument and specific metabolic activity was followed by selected biomarkers analyzed using HPLC.

3.5.1 Microcalorimetry

The microcalorimeter used was a 48-channel isothermal microcalorimeter (Symcel Sverige AB, Kista Sweden). Primarily a 48-well microtiter sample plate (Cal Plate) is prepared by inserting different combinations of bacterial cells, media/urine and incontinence product components into the different wells (cups). Control samples such as negative controls, pure medium or urine were also included. The isothermal microcalorimeter was set to 37°C and, as per recommendation from the manufacturer, a three-step procedure was applied for thermal equilibration of the Cal Plate. The sample plate was inserted to the first mark for 10 minutes and then pushed into the second mark and left for 20 min (Figure 10). It was then finally pushed all the way into the measurement position in the instrument and recording of heat generation was started.

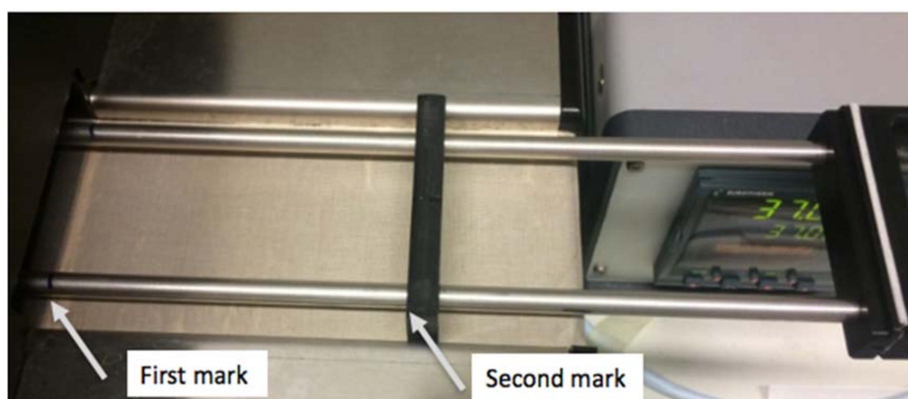


***Figure 10.* Image of the marks used for insertion of the well-plate into the microcalorimeter for the equilibration procedure.** First mark shows when the plate is just inserted and stays for 10 min. The second mark shows to which point the plate is pushed in for the second step of the equilibration and where it stays for 20 min.

It takes another 30 minutes at least to get the proper equilibration which is accounted for in the analysis of the results. Data was collected by the calView software (SymCel Sverige AB, Kista, Sweden) and was later exported to a spreadsheet program.

3.5.2 HPLC

For measurements of specific metabolic activities of *E. coli* and *S. epidermidis* in the various growth experiments in this project, the compounds urea, creatinine and creatine in urine were selected as possible biomarkers. Samples were collected during the growth experiments and stored at -20°C awaiting HPLC analyses. Prior to HPLC analysis the samples were thawed.Samples that were not diluted in saline solution during extraction, e.g. the samples from the inoculating urine culture, were diluted 1:10 in saline solution (0.9% NaCl). 1 ml of each sample was then transferred to HPLC vials (VWR, Radnor, PA) and placed in the HPLC autosampler. The HPLC used was the Waters HPLC system (1515 Isocratic HPLC Pump, UV/Visible detector 2489, Autosampler 2707) with a C18, XSelect HSS, T3 Column (4.6 × 150 mm, 3.5 µm) (Waters, Milford, MA). The HPLC were run for 23 min per sample at 22°C, with a flow rate of 1.4 ml/min, an injection volume of 20 µl and detection at 195 nm. The eluent was 20 mM phosphate buffer made with 0.54 g/l $NaH_2PO_4*H_2O$ (Merck KGaA, Darmstadt, Germany) and 1.09 g/l $Na_2HPO_4*2H_2O$, pH 7 (VWR, Radnor, PA). The data was recorded and analyzed with the Empower Chromatography Software (Waters, Milford, MA). A set of amino acids and compounds found in urine, chosen from a report by Putnam (1971), was primarily tested prior to the samples. The full list of tested compounds can be found in Appendix 1.

3.6 Microbial behavior in product

Microbial behavior in product was investigated by applying a defined volume of urine inoculated with a known concentration of either *E. coli* or *S. epidermidis* onto a product sample followed by incubation and sampling over a period of time. Primarily the bacteria were adapted to urine as a medium during the pre-culturing step as described above. The final experimental culture, in 100 % urine, was used to inoculate the product by slowly dripping the liquid onto the top of the product, kept in a Petri dish, to simulate real-life conditions. A number of identical samples were prepared, the lid of the Petri dish added to prevent drying and then incubated at 35°C. At several time points samples were collected and the bacteria were extracted by homogenization with stomacher. Samples for measuring pH, growth by VC and FC, and metabolic activity analyses by HPLC were collected (Figure 11). Detailed description of the procedure will follow below.

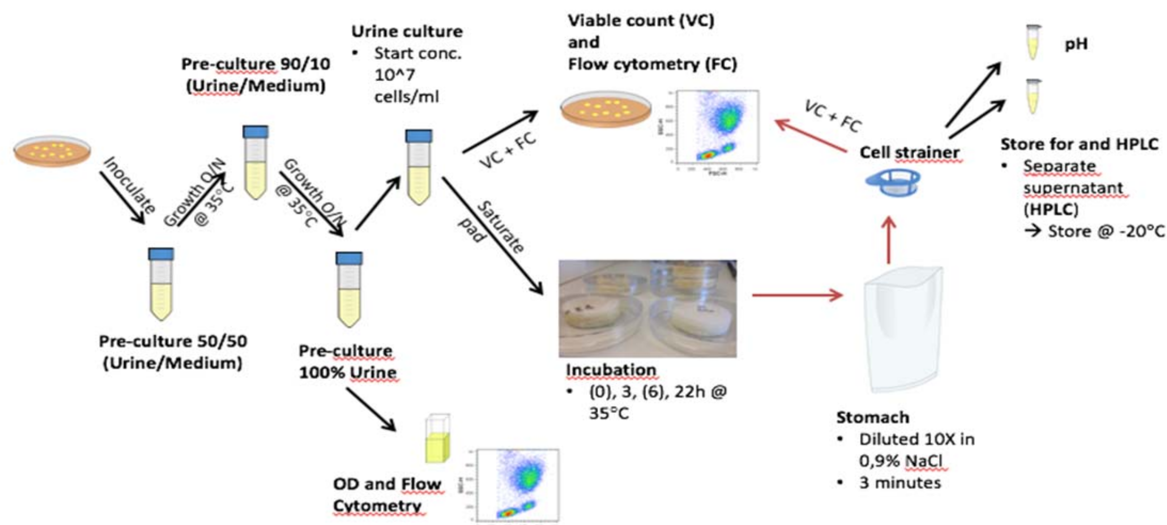


***Figure 11*. A schematic figure of the experimental set up for assessing microbial behavior in product.** Bacteria was cultivated in a three-step pre-culture with increasing ratio of urine to medium, first 50% urine and 50% medium then 90% urine and 10% medium and lastly 100% urine. Cell concentration was measured with OD and flow cytometry on the 100% urine pre-culture. An inoculation urine culture with a set cell concentration was made and viable count and flow cytometry was performed to obtain cell concentration. Samples of product were inoculated, incubated and cells were extracted at sampling points. pH was measured, samples for HPLC was taken and cell concentration was then measured with flow cytometry and viable count.

Two different levels of liquid (urine) saturation of the product were investigated: 5x saturation (product 50% saturated) corresponding to a volume of liquid of 5 times the weight of the product (e.g. product weight of 1.6 g + 8 ml of urine) and 10x saturation (product 100 % saturated) corresponding to a volume of liquid of 10 times the weight of the product (e.g. product weight of 1.6 g + 16 ml of urine). Urine was assumed to have the same density as water (1g/ml). An overview of the experiments can be seen in Table 1.

***Table 1*. Overview of the different experiments of microbial behavior in product.**

Organism	Saturation	Biological replicate	Initial pH
E. coli	10x	#1-3	high
E. coli	10x	#4-6	low
E. coli	5x	#1-3	Not modified
E. coli	5x	#4-6	Not modified
S. epidermidis	10x	n=3	Not modified
S. epidermidis	5x	n=3	Not modified

Each experiment contained three biological replicates and there were in total six separate experiments. For *E. coli*, two experiments were made for each saturation. To differentiate between the two experiments within the saturation level, the first experiment is called biological replicates #1 to #3 and the second is called biological replicates #4 to #6.

3.6.1 pH setting of urine and product inoculation

For experiment *E coli,* 10x, #1-3, the initial pH of urine was set to 7.6 by adding 1 M NaOH in drops and measuring pH. In experiment *E coli,* 10x, #4-6, the initial pH of urine was set to 5.6 by adding 1M HCl and measuring pH. The pH of urine was set before sterile filtration. pH measurements were performed during preparation of the experiment and at at each sampling point by transferring 1 ml from urine culture or homogenate to an Eppendorf tube and the pH meter FE20 (Mettler Toledo, Columbus, OH) was used.

OD_{620nm} of the last pre-cultures described in section 3.1.1. – *culture conditions in urine*, of 100% urine was measured. FC was performed on the pre-cultures according to the section 3.4.2 – *flow cytometry*. From the OD measurements and data from the FC, the amount of pre-culture necessary to obtain the desired cell concentration of 10^7 cells/ml in 40 ml of urine culture was determined and the experimental urine cultures was then prepared.

Samples of product were inoculated with different amount of urine culture based on the weight of the product sample and chosen level of saturation. One saturation was done per experiment and one sample of product for each sampling point was prepared. The inoculated samples of product were incubated in a Cooled Incubator ES 110 (Nüve, Ankara, Turkey) at 35°C for 3 and 22 h. For two experiments, samples of product for 0 h and 6 h time point analysis were included as well.

3.6.2 Extraction of bacteria from product

At each sampling point, the samples of product were placed into BagLight Polysilk blender bags, size L, (Interscience, Saint Nom, France) containing a volume of 0.9% NaCl solution (Merck KGaA, Darmstadt, Germany) that corresponds to a 10-fold dilution of the inoculation volume. The bags were placed in a Stomacher Lab Blender (Model BA6021, Seward. Laboratory UAC House, London, UK) and the samples were homogenized for 3 min. A volume of 10-15 ml of the homogenate was subsequently decanted through a Falcon 40µm cell strainer (Corning, New York, USA) into a 50 ml Falcon tube.

Samples of the homogenates were analyzed with both FC and VC as described in section 3.4 – *Growth measurements*. Samples were also collected for metabolic analyses using HPLC by transferring 1.1 ml of urine-culture or homogenate to an Eppendorf tube and centrifuged at 15 000 x g (Centrifuge 5424, Eppendorf, Hamburg, Germany) for 5 min. 1 ml of the supernatant was removed to a clean tube and stored in the freezer at -20°C until analysis in the HPLC, see section 3.5.2 - *HPLC*. The pellet was also stored at -20°C.

3.7 Microbial behavior on SAP

Microbial behavior on SAP alone was investigated by using both microcalorimetry to measure total metabolic activity through heat generation as well as conducting a parallel set-up that enabled analyzes of growth and specific metabolic activity using selected biomarker (Figure 12).

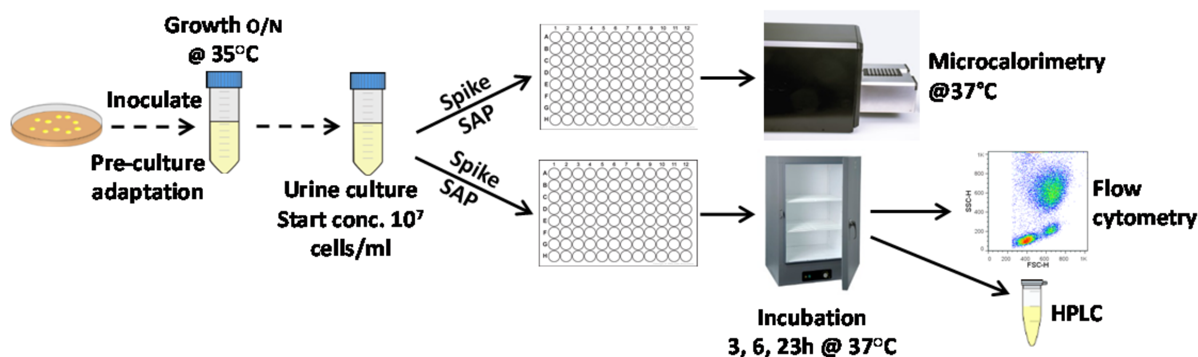


***Figure 12*. A schematic figure of the experimental set-up for assessing microbial behavior on SAP**. Bacteria was cultivated in a three-step pre-culture with increasing ratio of urine to medium, first 50% urine and 50% medium then 90% urine and 10% medium and lastly 100% urine. Cell concentration was measured with OD and flow cytometry on the 100% urine pre-culture. An inoculation urine culture with a set cell concentration was made and viable count and flow cytometry was performed to obtain cell concentration. Microcalorimtry vials with SAP were inoculated and one plate of vials was put in the microcalorimeter where heat flow was measured. The other plate was placed in the incubator and cells were extracted at sampling points. Cell concentration was then measured with flow cytometry and samples for HPLC were taken.

3.7.1 Plate set-up for microcalorimetry

Two different levels of saturation were investigated; 50% and 100%. To obtain a 50% saturation of SAP with a volume of 300 µl (the specified volume for the cups in the Cal Plate), 12 mg SAP was used and 6 mg SAP was used in 300 µl to obtain 100% saturation based on a maximum absorption capacity of 50x the weight of SAP (see section 3.3.2). Pre-cultures were prepared in the same way as in section 3.4.1 – *pre-culture condition for growth in urine*. The inoculum concentration of the urine culture was aimed to be 10^7 cells/ml. SAP was weighed in the plastic microcalorimetry cup (Figure 13).

***Figure 13*. The microcalorimetry stainless steel vial and the sample cup.**

Three biological replicates were made with two technical replicates. The references for each sample were made with urine without bacteria and the amount of SAP corresponding to sample it was reference to. The cups were placed in the microcalorimetry stainless steel vials and 300 µl of culture were added to each sample and 300 µl of sterile urine was added to each reference cell. The vials were placed in a plastic 48-well plate in a diagonal pattern to minimize possible heat overspill between cells (Table 2).

***Table 2*. Overview of placement of samples in the 48 well plate in the microcalorimeter.**

	1	2	3	4	5	6	7	8
A	Ref	Ref	Ref	Ref	Ref	Ref		
B	Sample 1.1		Sample 2.1		Sample 3.1			
C		Sample 1.2		Sample 2.2		Sample 3.2		
D		Sample 1.2		Sample 2.2		Sample 3.2		
E	Sample 1.1		Sample 2.1		Sample 3.1			
F	Ref	Ref	Ref	Ref	Ref	Ref		

The top half of the plate (row A-C) had 6 mg SAP, 100% saturation, and the bottom half (row D-F) had 12 mg, 50% saturation. Once set up, the plate was inserted into the microcalorimeter and measurements were made according to section 3.5.1 – *Microcalorimetry*.

To investigate the relationship between heat generation, growth and metabolic activity, a parallel experiment was conducted where an identical set up was prepared. Samples were prepared in the same way and from the same inoculum urine culture as used for the experiments in the microcalorimeter, but with one technical replicate. The vials were placed diagonally in a plastic 48-well plate (Table 3). The top half of the plate (row A-C) had 6 mg SAP, 100% saturation, and the bottom half (row D-F) had 12 mg, 50% saturation. The samples were placed in an incubator (Termaks, Bergen, Norway) at 37°C and analyzed three times, at 3 h, 6 h and 23 h for growth and specific metabolic activity.

***Table 3*. Overview of placement order of samples in the 48 well plate in the micro calorimeter.**

	1	2	3	4	5	6	7	8
A	Sample 1, 3h		Sample 1, 6h		Sample 1, 23h			
B		Sample 2, 3h		Sample 2, 6h		Sample 2, 23h		
C			Sample 3, 3h		Sample 3, 6h		Sample 3, 23h	
D	Sample 1, 3h		Sample 1, 6h		Sample 1, 23h			
E		Sample 2, 3h		Sample 2, 6h		Sample 2, 23h		
F			Sample 3, 3h		Sample 3, 6h		Sample 3, 23h	

3.7.2 Extraction of bacteria from SAP

Once taken out of the incubator, the vial inserts with the samples were placed upside down in a 15 ml falcon tube containing 2.7 ml of 0.9% NaCl solution, a 10-fold dilution of the initial

volume (300 µl). The tube was vortexed to force the SAP-culture mix out from the vial insert into the Falcon tube. The insert was removed using a tweezer. The Falcon tube was then placed and fastened with tape on top of a Digital Heating Shaking Drybath (Thermo Fisher Scientific, Waltham, MA), which was set to room temperature to avoid heating. The tubes were shaken at 1400 rpm for 2 min. The samples were taken off the shaker and quickly vortexed before filtered through a Falcon 40 µm cell strainer into a 50 ml Falcon tube. Flow cytometry was performed on the extracted solution according to Section 3.4.2. – *flow cytometry*. Samples were taken for HPLC by transferring 1.1 ml to an Eppendorf tube and centrifuged at 15 000 x g for 5 min in the Centrifuge 5424 (Eppendorf, Hamburg, Germany). 1 ml of the supernatant was removed to an empty tube and stored in the freezer at -20°C until analysis in the HPLC, see section 3.5.2 - *HPLC*. The pellet was also stored at -20°C.

3.8 Enumeration of plate counts and statistical analysis

A protocol was developed to determine acceptable plate count results obtained from VC. Based on guidelines from Jensen (2009) and Niemelä (1983) the following criteria were used to ensure accuracy and consistency of plate counts. The plates were first checked if the count was between 25-250 colonies. The dilution with most or all plates in that range was chosen. If any plate was below 25, the mean of the dilution was compared to the other dilution. If the two dilutions did not follow a decimal dilution trend, then the chosen dilution was compared with the FC data as well as the other biological replicates to decide which one is most likely to be accurate. The mean of the three technical replicates (3 plates) in one dilution was used for each biological replicate. If a majority of the plates were outside the range of 25-250 CFU it was marked as an estimation. For each experiment, a mean of the three biological replicates were made and if a majority of the biological replicates were estimations they were marked in the results with an asterisk. Statistical analysis of significance was performed in MS Office Excel using the Student t-test at a significance level of 0.05 (p value).

3.9 Microscopy

Pre-culture was prepared according to Section 3.1.2 - *Pre-culture for growth in bacterial medium.* $OD_{620\ nm}$ was measured and the theoretical cells/ml of the pre-culture was calculated using the results from 3.4.3 - *Determining the relation between optical density, viable count and flow cytometry.* 1 ml aliquot of the pre-culture was prepared and washed twice in 0.9% NaCl by repeated centrifugation at 10 000 x g for 2 min at 20°C (Centrifuge 5424, Eppendorf, Hamburg Germany) and resuspended in 1 ml PBS. The washed aliquot was then stained with 5 µl of either 10 mg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) or 5 mM DRAQ5 (Pierce Biotechnologies, Rockford, IL), vortexed and incubated 30 minutes in dark at room temperature. After incubation, the aliquot was washed twice and then diluted in PBS to a cell concentration of 10^7 CFU/ml. Samples of product were inoculated 10 times its weight while SAP was inoculated with 10 or 20 times its weight. Bacterial migration and attachment to the material was then allowed during a 30-minute incubation at room temperature in dark. When investigating the distribution of bacteria in samples of product, the samples were stored overnight at -80°C, embedded with cryo matrix and sliced with cryo microtome in 100 µm cuts. Cuts of the sample of product were placed on a glass slide and fixed with coverslip.

When investigation cellulosic fibres and/or SAP, the material was collected in the middle layer of the samples of product and single placed on an uncoated 35 mm glass bottom dish (MatTec, Ashland, MA). The same procedure was conducted with separately inoculated SAP. All visualization and localization of bacteria in the material was performed with confocal microscopy (Nikon Confocal A1+) at Lund University Bioimaging Center (LBIC). For each material investigated two blanks was prepared and analysed with the same microscopy configuration; one inoculated with PBS, and one inoculated with unstained bacteria.

4 Results

4.1 Correlation between methods for microbial growth measurements

Flow cytometry (FC) and viable count (VC) were used to measure cell concentration (i.e. growth) in all experiments. In order to determine if the methods give corresponding results dilution series of bacterial cells were made to obtain samples with OD_{620} of 0.1 to 0.3. Both FC and VC were performed and the obtained results were correlated to each other (Figure 14).

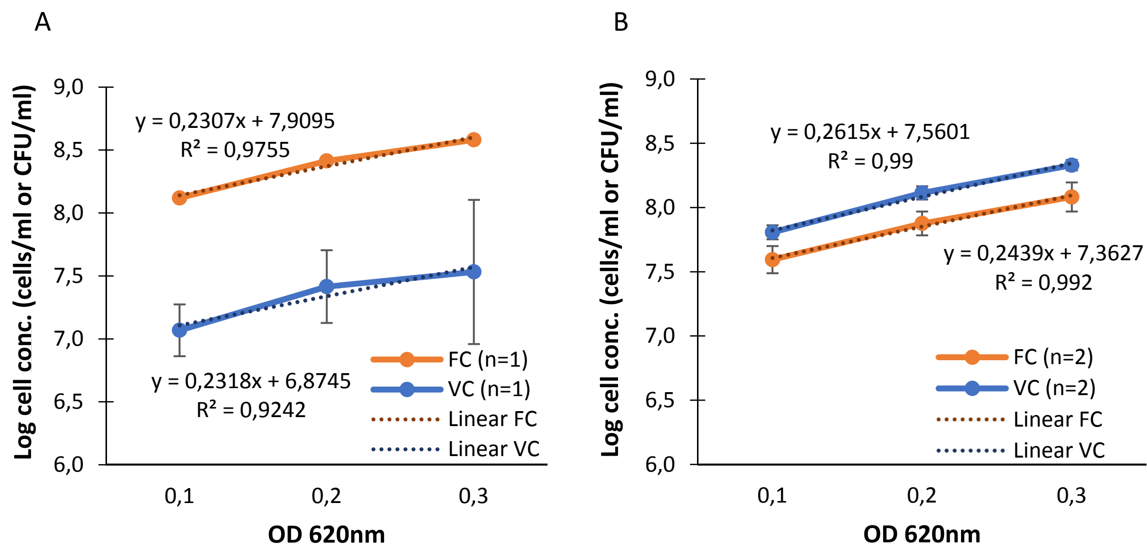


Figure 14*. Growth measurement data obtained by flow cytometry and viable count in relation to OD for *E. coli* and *S. epidermidis. (A) Cell concentration obtained by viable count and flow cytometry for $OD_{620\ nm}$ values of 0.1, 0.2 and 0.3 for *E. coli.* (B) Cell concentration obtained by viable count and flow cytometry for $OD_{620\ nm}$ values of 0.1, 0.2 and 0.3 for *S. epidermidis.*

The cell concentration increased with increased OD for both FC and VC. The correlation between FC and OD was linear, as was it for VC and OD, for both *E. coli* and *S. epidermidis*. For *E. coli*, there was about a log unit difference between FC and VC. OD 0.1 corresponded to about 7.1 log CFU/ml in VC and 8.1 cells/ml in FC for *E. coli*. For *S. epidermidis*, VC showed a higher cell concentration compared to FC, however the difference was not statistically significant (t-test, p=0.05). OD 0.1 corresponds to about 7.8 log CFU/ml in VC and 7.6 log cells/ml in FC.

4.2 Growth in product

The results from assessing microbial growth and pH change in product are presented in the following section.

4.2.1 Cell recovery from product and SAP

To investigate the efficiency of extraction of cells from the incontinence product samples a series of cell recovery experiments were performed for both *E. coli* and *S. epidermidis*. Primarily initial cell concentration of the culture used for inoculation of the product was measured (start). A defined volume of cells was then inoculated onto the product to reach either 5x saturation (5 times the weight of the product) or 10x saturation (10 times the weight

of the product) followed by a short incubation to allow for cell migration through the product and attachment of cells to the material. Cells were then immediately extracted from the product and cell concentration measurements (0 h) were performed. The results are presented in Tables 4 and 5. One biological replicate is displayed for *E. coli* and only the results from flow cytometry due to unreliable results for the other two biological replicates as well as for viable count for all three replicates.

***Table 4*. Cell concentration obtained by flow cytometry (FC) of inoculation culture (start) and after extraction of cells (0 h) as well as the recovery of cells in % for *E. coli*.** The saturation level of the product was 5 times the weight of the product (5x).

E. coli	FC		
	5x saturation		
Biological replicate	**Start (cells/ml)**	**0h (cells/ml)**	**Recovery (%)**
1	$1.1*10^8$	$1.4*10^8$	129

***Table 5*. Cell concentration obtained by (flow cytometry) FC and viable count (VC) of inoculation culture (start) and after extraction of cells (0 h) as well as the recovery of cells in % for *S. epidermidis*.** The saturation level of the product was 5 times the weight of the product (5x).

S. epidermidis	FC			VC		
	5x saturation			5x saturation		
Biological replicates	**Start (cells/ml)**	**0h (cells/ml)**	**Recovery (%)**	**Start (cfu/ml)**	**0h (cfu/ml)**	**Recovery (%)**
1	$7.4*10^7$	$9.7*10^7$	131	$5.4*10^7$	$6.8*10^7$	126
2	$9.1*10^7$	$1.2*10^8$	127	$6.8*10^7$	$7.6*10^7$	111
3	$9.0*10^7$	$1.1*10^8$	126	$6.8*10^7$	$8.6*10^7$	126

The recovery of cells is over 100% and similar for both bacteria indicating that the cells are extracted from the product and not lost during sample preparation.

To investigate the efficiency of extraction of cells from SAP alone a series of cell recovery experiments were performed for both *E. coli* and *S. epidermidis* as described above. Cell concentration was measured with flow cytometry in the inoculation culture (start) and then again after extraction from SAP. Both 50 % and 100 % saturation levels were included (Tables 6 and 7).

***Table 6*. Cell concentration obtained by flow cytometry (FC) for *E. coli* of inoculation culture (start) and after extraction of cells (0 h) from SAP as well as the recovery in % of cells.** The saturation level of SAP was either 50% or 100%

E. coli	FC					
	50% saturation			100% saturation		
Biological replicate	**Start (cells/ml)**	**0h (cells/ml)**	**Recovery (%)**	**Start (cells/ml)**	**0h (cells/ml)**	**Recovery (%)**
1	$7.4*10^7$	$7.1*10^7$	96%	$2.7*10^7$	$2.8*10^7$	106%

***Table 7*. Cell concentration obtained by flow cytometry (FC) for *S. epidermidis* of inoculation culture (start) and after extraction of cells (0 h) from SAP as well as the recovery in % of cells.** The saturation level of SAP was either 50% or 100%

S. epidermidis	FC					
	50% saturation			**100% saturation**		
Biological replicates	**Start (cells/ml)**	**0h (cells/ml)**	**Recovery (%)**	**Start (cells/ml)**	**0h (cells/ml)**	**Recovery (%)**
1	$1.3*10^7$	$1.8*10^7$	140%	$1.3*10^7$	$1.5*10^7$	113%
2	$1.6*10^7$	$2.1*10^7$	131%	$1.6*10^7$	$1.7*10^7$	109%
3	$1.5*10^7$	$2.0*10^7$	132%	$1.5*10^7$	$1.5*10^7$	104%

The recovery of cells from extraction was slightly higher than 100% for both bacteria at 100% saturation and for 50% saturation it was higher for *S. epidermidis* and lower for *E. coli*. The lowest recovery was 96%.

4.2.2 pH in product

The behavior of pH in product over time in the presence of different bacteria and varying starting pH values of urine was investigated. Primarily experiments were performed with urine inoculated with *E. coli* where the initial pH was set to a high and a low value (Figure 15 A). In biological replicates #1 to #3 the initial pH of urine was set to 7.56 using 1M of NaOH and in biological replicates #4 to #6 the initial pH was set to 5.69 with 1M HCl. Samples where the original pH of urine was not modified was also included (blue line in Figure 15 A). The concentration of *E. coli* was around 10^7 CFU/ml in all experiments. The product was inoculated with a volume to reach 10x saturation level of product. The pH was primarily controlled in the inoculation culture (start) as well as of the extracted samples at 3 h and at 22 h for all experiments. The samples with high initial pH decreased from 7.63 ± 0.006 to 6.40 ± 0.02 at 3 h and then increased to 6.47 ± 0.01 at 22 h. The experiment with low initial pH increased from 5.69 ± 0.06 to 6.16 ± 0.02 during the first 3 hours and remained stable until 22h with a pH of 6.15 ± 0.03. The samples with the original, non-modified pH of urine decreased from 6.67 ± 0.01 to 6.12 ± 0.05 during the first 3 hours and then increased to 6.18 ± 0.06 at 22 hours.

For two experiments, *E. coli* with 5x saturation, biological replicates #4 to #6, and *S. epidermidis* 5x saturation, an additional pH measurement was made at 0 h. In these experiments the original pH of the urine was not modified. An immediate decrease in pH between the start and 0 h samples was observed for (Figure 15 B). The pH did not change significantly (t-test, p=0.05) between 3 h and 22 h for *E. coli*, 10x saturation, with low initial pH or for the experiments with non-modified pH. For the experiment with high initial pH, the increase from 6.40 ± 0.02 at 3 h and to 6.47 ± 0.01 at 22 h was statistically significant.

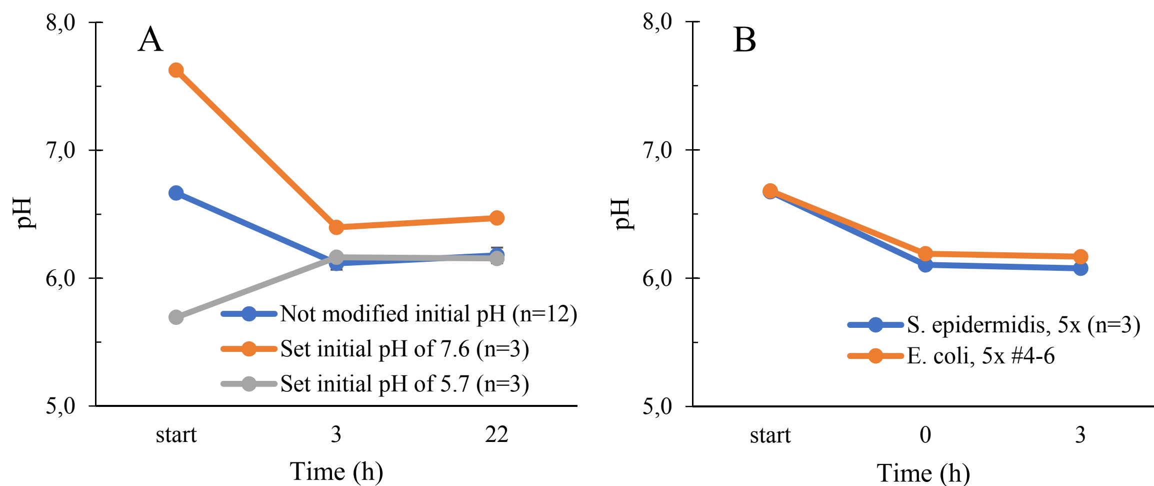


Figure 15. **pH change in product with different initial pH of urine and bacteria.** (A) Experiments with *E. coli* and 10x saturation of the product. Blue line = initial pH of urine not modified, orange line = initial pH set to 7.6 and grey line = initial pH set to 5.6.. (B) Experiments with *E. coli* and, 5x saturation of the product and non-modified starting pH of urine. Blue line = *S. epidermidis* and orange line = *E. coli.*

4.2.3 Growth of *E. coli* in product

To study the growth behavior of *E.coli* in the environment of an incontinence product, representative product samples were used and different volumes of urine with a defined amount of *E. coli* cells added were applied onto the product samples and incubated for a period of time. Two different volumes of contaminated urine was used to achieve either 5x or 10x saturation of the product. Cell concentration, *i.e.* growth, was measured with FC and VC after 3 h and 22 h of incubation at 35°C for all experiments and also at 0 h and 6 h for the 5x saturation experiment, biological replicates #4 to #6 (Figure 16).

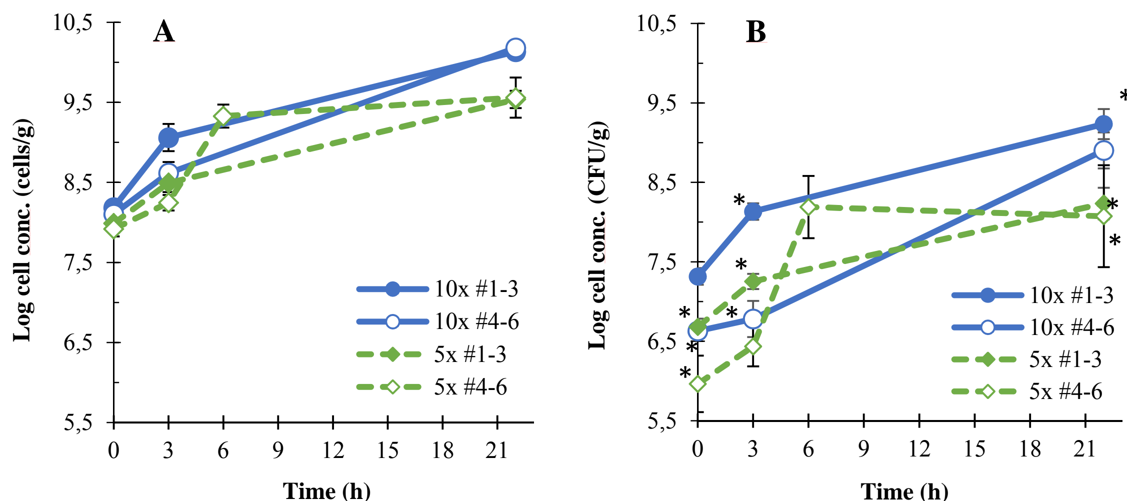


Figure 16. **Growth of *E. coli* in incontinence product samples with two different levels of saturation.** (A) Growth determined by measuring cell concentration using flow cytometry for 10x saturation experiment, biological replicate #1 to #3 (blue full line, blue mark) and #4 to #6 (blue full line, white mark), and 5x saturation experiment, biological replicate #1 to #3 (green dashed line, green mark) and #4 to #6 (green dashed line, white mark). (B) Growth determined by measuring cell concentration using viable count for 10x saturation experiment, biological replicate #1 to #3 (blue full line, blue mark) and #4 to #6 (blue full line, white mark), and 5x saturation experiment, biological replicate #1 to #3 (green dashed line, green mark) and #4 to #6 (green dashed line, white mark). *estimated values

The results showed an increase in cell concentration over time. There was a discrepancy in the cell concentration obtained from viable count compared to flow cytometry of about a 1-2 log units. The results from viable count had, in general, a higher standard deviation than flow cytometry. The results from flow cytometry showed that the cell concentration at 22 h is less for 5x saturation than for 10x saturation. The same trend can be seen for most of the experiments in VC but it is more inconsistent. The results from flow cytometry showed a significant difference in cell concentration between 5x and 10x saturation levels at 22 h (t-test, $p<0.05$). Exponential growth was observed already from the beginning; however, due to the limited amount of time points in the exponential phase for both 10x 5x saturation experiments, biological replicate #1 to #3, a reliable growth rate could not be established for these experiments. For the 5x saturation experiment, biological replicates #4 to #6, there were three samplings within the exponential phase and a mean growth rate was calculated to 0.45 ± 0.05 for 0 h to 6 h.

A distinction between viable cells and cells with severely damaged cell membrane (which indicates cell death) was obtained in the FC analyses. This provided information regarding damaged/dead cells in the product during growth (Figure 17).

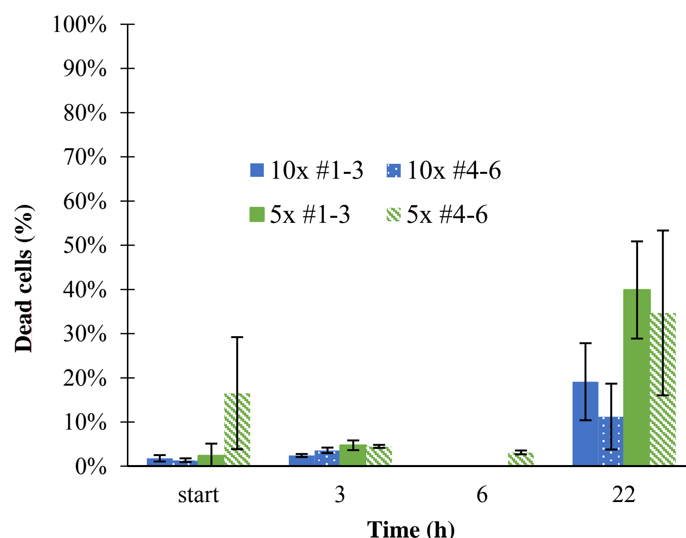


***Figure 17*. Percentage of damaged/dead *E. coli* cells during growth in incontinence product samples with two different levels of saturation.** Percent of dead cells during growth in product determined from flow cytometry for 10x saturation experiment, biological replicates #1 to #3 (blue) and #4 to #6 (blue, dotted), and 5x saturation experiment, biological replicates #1 to #3 (green) and #4 to #6 (green, striped).

The results showed that cell damage/death increased over time in general. The cell damage/death was low during the first 3 h to 6 h, not exceeding 5.9%. The cells in the starting culture of the 5x saturation experiment, biological replicates #4 to #6, had a higher mean percentage of damaged cells and a higher standard deviation than the other experiments. There was a higher percentage of cell damage/death at 22 h compared to earlier time points and the difference between the two saturations was again significant (t-test, $p<0.05$).

4.2.4 Growth of *S. epidermidis* in product

To study the growth behavior of *S. epidermidis* in the environment of an incontinence product, representative product samples were used and different volumes of urine with a defined amount of *S. epidermidis* cells added were applied onto the product samples and incubated for a period of time. Two different volumes of contaminated urine were used to achieve either 5x or 10x saturation of the product. Cell concentration, *i.e.* growth, was measured with FC and VC after 3 h and 22 h for both experiments with 10x and 5x saturation levels and also at 0 h and 6 h for the 5x saturation experiment (Figure 18).

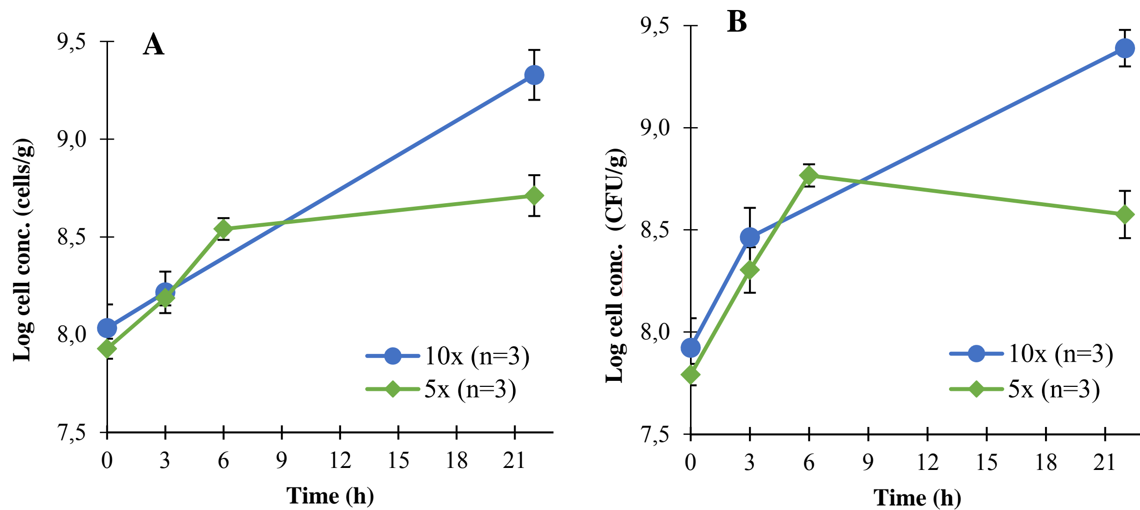


***Figure 18*. Growth of *S. epidermidis* in incontinence product samples with two different levels of saturation.** (A) Growth determined by measuring cell concentration using flow cytometry for 10x (blue) and 5x saturation (green). (B) Growth determined by measuring cell concentration using viable count for 10x (blue) and 5x (green) saturation.

The results from flow cytometry showed that there was a significant difference (t-test, $p<0.05$) in cell concentration between the two saturation levels at 22 h. The same trend was observed for both FC and VC. Exponential growth was observed already from the beginning; however, due to the limited number of samplings in the exponential phase for the 10x saturation experiment, a reliable growth rate could not be established for this experiment. For the 5x saturation experiment, there were three samplings within the exponential phase and a mean growth rate based on flow cytometry data was calculated to 0.18 ± 0.04 for 0 h to 6 h which was 2.5 times slower than *E. coli*.

The percentage of damaged/dead cells was determined from the data obtained by FC (Figure 19).

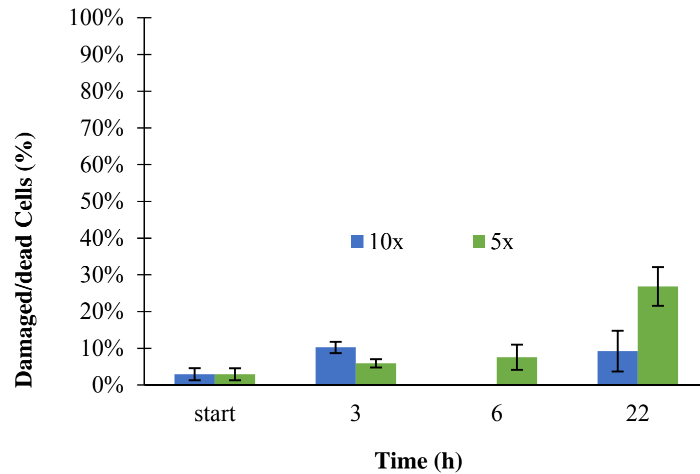
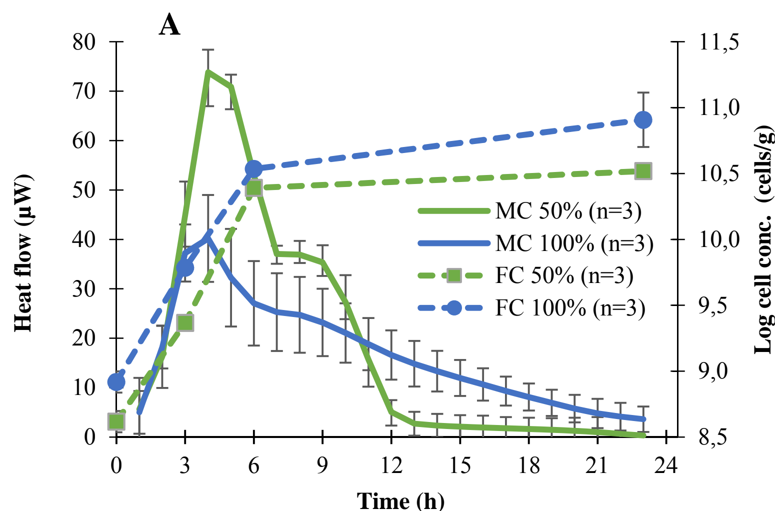


Figure 19. **Percentage of damaged/dead *S. epidermidis* cells during growth in incontinence product samples with two different levels of saturation for.** Percent of damage/dead cells determined from flow cytometry for 10x saturation (blue) and 5x saturation (green) experiments.

The percentage of damaged/dead cells did not exceed 11.5% during the first three hours but increased for 5x saturation at 22h. There was a significant difference between saturations at 22h (t-test, $p<0.05$).

4.3 Total metabolic activity and growth of *E. coli* and *S. epidermidis* on SAP

Microcalorimetry was used to investigate the total metabolic activity of bacteria on SAP. To differentiate if the activity mainly originated due to growth or from other metabolic activities ongoing in the absence of growth, an identical set up was prepared and placed in an incubator to enable sampling for measurements of cell concentration in parallel with measurements of heat generation using microcalorimetry (Figure 20).



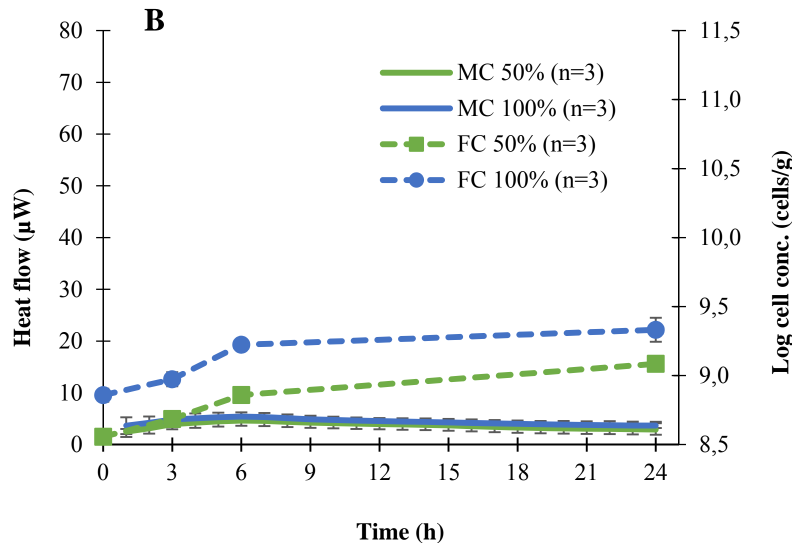


***Figure 20*. Heat generation and growth for *E. coli* and *S. epidermidis* on SAP at two different levels of saturation.** (A) *E. coli* heat flow determined by microcalorimetry (MC) for 50% saturation (green, full line) and 100% saturation (blue, full line) as well as cell concentration from flow cytometry (FC) for 50% saturation (green, dashed line) and 100% saturation (blue, dashed line). (B) *S. epidermidis* heat flow determined by microcalorimetry (MC) for 50% saturation (green, full line) and 100% saturation (blue, full line) as well as cell concentration from flow cytometry (FC) for 50% saturation (green, dashed line) and 100% saturation (blue, dashed line).

Both the increase in heat flow and cell concentration was higher for *E. coli* in comparison to *S. epidermidis*. The highest increase in heat flow was measured during the first six hours before it declined. The highest increase of cell concentration was in the first six hours as well. Mean growth rate of *E. coli* during the first six hours was 0.68 h^{-1} ± 0.03 for 50% saturation and 0.62 h^{-1} ± 0.03 for 100% saturation. The mean growth rate for *S. epidermidis* was 0.058 h^{-1} ± 0.004 for 50% and 0.12 h^{-1} ± 0.01 for 100% saturation. The generated heat production with *E. coli* reached a higher level for the 50% saturation than for the 100%. The total heat flow was also calculated for *E. coli* by integration of the two curves obtained from microcalorimetry. The total heat flow was estimated to be 445 µW in 24 hours for 50% saturation and 410 µW for 100% saturation.

4.4 Specific metabolic activity

Samples were collected from both experiments performed in product as well as the experiments made in parallel to the microcalorimetry. The specific metabolic activity using a set of selected biomarkers was measured with HPLC of the samples after extraction in saline solution as well as samples from the urine culture. The result showed peaks that were identified as urea, creatine, uric acid, creatinine, and hippuric acid (Figure 21). In addition, other small, unknown peaks were detected.

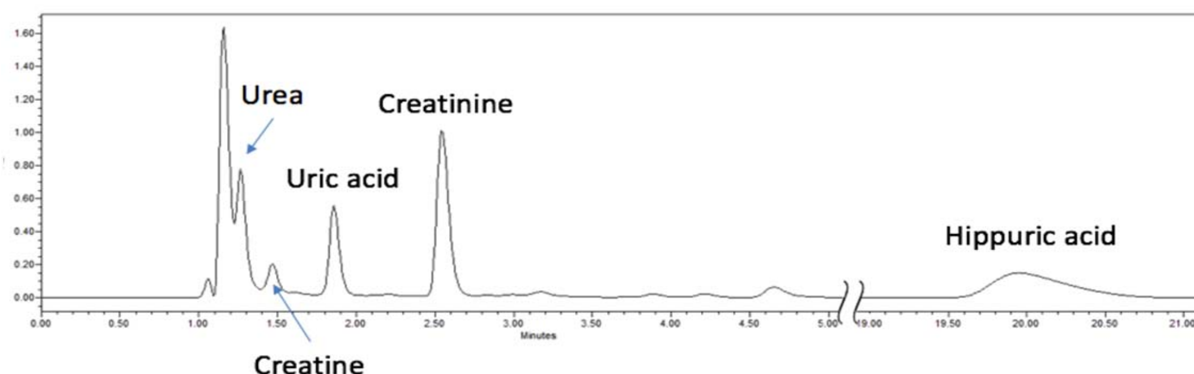


***Figure 21*. Representative chromatogram from HPLC measurements for growth of *E. coli* and *S. epidermidis* in product and on SAP.** Identified peaks were urea, creatine, uric acid, creatinine and hippuric acid.

The observed peaks were urea at 1.3 min, creatine at 1.5 min, uric acid at 1.9 min, creatinine at 2.6 min and hippuric acid at 20 min. The peak at 1.1 min, before urea, includes NaCl from the saline solution used in extraction among other compounds that were not distinguishable. There was no change between *E. coli* and *S. epidermidis* or between growth in product or on SAP. Therefore, the following results were in general for all samples. There were no observed changes in level over time for the identified compounds indicating that the metabolic activity related to these compounds were low or non-detectable. Unknown peaks appeared around 0.7 min, 4.6 min and 6.0 min. One of the unknown peaks which appeared at around 0.72 (not depicted in figure), only showed up on the chromatogram for the samples that had been in contact with either the samples of product or SAP. The unknown peak at around 6 min disappeared in the samples for 22 h.

4.5 Mean growth rate

Growth rates calculated from all experiments are summarized in Table 8. Data from previous experiments (not part of this thesis) that characterized bacterial growth in rich medium and urine are also included for comparison.

***Table 8*. Summary of mean growth rate for *E. coli* and *S. epidermidis* in different liquid or solid matrices.**

E. coli		*S. epidermidis*	
Matrix	**Mean μ_{max} (h^{-1})**	**Matrix**	**Mean μ_{max} (h^{-1})**
NB	0.93 ± 0.02	BHI	0.94 ± 0.04
Urine	0.72 ± 0.04	Urine	0.19 ± 0.01
Urine in product, 5x saturation	0.45 ± 0.05	Urine in product, 5x saturation	0.18 ± 0.04
Urine in SAP, 50% saturation	0.68 ± 0.03	Urine in SAP, 50% saturation	0.058 ± 0.004
Urine in SAP, 100% saturation	0.62 ± 0.03	Urine in SAP, 100% saturation	0.12 ± 0.01

The growth rate for *E. coli* decreased in urine compared to rich medium. It also decreased in in product with urine compared to pure urine. The difference between the growth rate in pure urine and urine in SAP was not significant for 50% saturation but significant for 100% saturation (t-test, $p<0.05$).There was however no statistical difference between the two growth rates in SAP. The growth rate was the lowest for *E. coli* in complete product with urine.

The growth rate for *S. epidermidis* decreased in urine compared to rich medium. In rich medium the growth rate was approximately the same for *E. coli* and *S. epidermidis* but in urine it was almost four times lower for *S. epidermidis* compared to *E. coli*. The growth rate was the same for *S. epidermidis* in product with urine compared to pure urine. The growth rate decreased however noticeably for urine in SAP and especially in the 50 % saturation which had the lowest growth rate. There was a significant difference (t-test, $p<0.05$) between saturations in SAP. The results from the growth rate of *S. epidermidis* were almost 4 times slower in urine than for *E. coli* and 2.5 times slower in product.

4.6 Microscopy

Confocal microscopy of bacteria in the product and on SAP was performed in parallel to the growth experiments with the aim to visualize the bacteria on the different materials and to localize the bacteria in the complete product. The results showed that the bacteria are localized on the surface of SAP and cellulosic fiber. When looking at the whole product, the results showed that most bacteria were localized in the top layer and then distributed evenly in the rest of the layers. Microscopy images are presented in Appendix 2.

5 Discussion

For this project, the focus of the investigation of microbial behavior has been on *E. coli* and *S. epidermidis* as they are both present in the microbial flora in contact with urine. The bacteria are interesting to compare as they provide a range of different characteristics such as the origin of isolation, metabolism and cell structure which can affect the behavior. The strains used in this project is a uropathological strain of *E. coli* ATCC 700928 (Welch et al., 2002) and a strain of *S. epidermidis* that does not form biofilm and is not associated with infection (Zhang et al, 2003). *S. epidermidis* non-infectious characteristic could be a reason that it is not as resistant in urine as the infectious strain of *E. coli*. The species of *S. epidermidis* is known to form biofilm (Otto, 2009), but the strain used in this study does not, which may also influence their ability to adapt to the environment as biofilm-forming bacteria are more tolerant to environmental stress (Fux et al., 2005). The *S. epidermidis strain* is not adapted to the urine environment which may also impact the behavior and survival. The physiological difference in cell wall structure can affect the bacteria's resistance for water activity. Gram-negative bacteria like *E. coli*, need a water activity of 0.97 while gram-positive bacteria, like *S. epidermidis*, can handle a lower water activity of 0.90 (Adams and Moss, 2008). This would suggest a higher tolerance for a dryer environment for *S. epidermidis* which might be advantageous in the product as the liquid is absorbed by SAP. The difference in cell wall structure can also impact the staining for FC (Buysschaert et al. 2016).

The bacterial production of ammonia from urea is a thought to be contributing factor to the urine smell (Norberg et al., 1984). The presence of bacteria with urease activity such as *S. epidermidis* could then indicate possible malodor formation from ammonia. Urease activity for *S. epidermidis* may also be related to increase of growth as urea is an indirect source of energy (Smith et al., 1993). *E. coli* ATCC 700928, on the other hand is likely to be urease negative as the type strain *E. coli* ATCC 11775, which is isolated from urine, is urease negative (Podstawka, 2017b). However, ammonia could be produced by *E. coli* through degradation of indole but since pH in the product is around 6.2, the reaction between water and ammonium should be shifted towards ammonium to stay in its ion form and not form ammonia which is the more volatile, malodorous form. The SAP also has a neutralizing capacity (Elliot, 2004) that can capture the ammonium and shift the reaction further towards forming ammonium ions rather than ammonia.

Confocal microscopy was used to visualize the bacteria in the product and to simulate how the bacteria enter the product the application of culture to the product was done by dripping it onto the product sample from the top. The microscopy showed that the bacteria are localized on the surface of the cellulosic fiber. The fiber showed auto-fluorescence which required a modification of DNA stain used in the method to properly visualize and differentiate the bacteria and the material. It also showed that they were localized on the surface of the SAP rather than entering the SAP granule. This is advantageous for the extraction of cells from the product since the bacteria are not trapped in the system of polymers that the SAP consists of. For the distribution of bacteria in product, it was theorized that the majority of bacteria would end up in the core part of the product with the cellulosic fiber and SAP as it is the part that

absorbs the liquid. However, the results showed that the bacteria were gathered mostly in the top layer and then evenly distributed in the rest of the product. The application of culture may have an impact on distribution and localization of the bacteria so different applications methods such as using another pressure, stream or saturation can be explored in future studies. One experiment was performed for the distribution of bacteria in product due to time limitations and more experiments need to be made to verify the results. The slice of product that was examined for distribution of bacteria is cut from top to bottom so there may be a risk that bacteria were spread over the rest of the product after going through the layer with the most dense bacterial load. Repeating the experiment and cutting it from the other way may give different results. The product is also porous and it is difficult to distinguish what might be an air pocket and what is SAP which makes it difficult to visualize a possible preference in material. The bacteria were also fixated in this method but it would be interesting to in the future also look at viable cells, to make the localization closer to representing real life.

In order to make the assessment of microbial behavior in incontinence products, new techniques and methods were developed. A laboratory scale model system was used as it is impractical to use the entire product. The smaller samples of product are more suitable for a laboratory setting and they are representative since they have the same composition as the whole product. Since the product is a solid matrix, it was important to develop a method to extract and recover the cells for an accurate measurement of growth. The extraction of bacteria from product was established by method-development by the project group prior to this study and was based on the standard for extraction from a solid matrix in food microbiology. The recovery of cells was found to be over 100% which is most likely due to some growth occurring due to the short period of time from preparation of the sample to extraction. The recovery for *E. coli* is a more uncertain as the VC did not give reliable results and for FC, there was one biological replicate that gave reliable results. Due to high background and differences between technical replicates indicating that the staining was not properly executed, the other two biological replicates were not included. However, the results gave an indication of the recovery for *E. coli* and were consistent with the recovery for *S. epidermidis*. The high recovery allows for the assumption that all cells can be extracted from the product. A possible source of error to the cell concentration is that the recovery is not consistent throughout the experimental time. There might for example be biofilm formation or other mechanisms that cause the bacteria to adhere to the product more securely towards later stages of the experiment and hence more difficult to extract, thus giving a lower cell concentration than the true value. The strain of *S. epidermidis* is non-biofilm forming so it is not likely to be the case for *S. epidermidis*. However, it might be true for *E. coli* as the strain used in these experiments can create biofilms (Yang et al., 2016).

During the methodology development of the FC protocol viable count was performed to correlate the two methods to each other. The cells were cultured in rich medium and cell concentration was measured with OD, VC and FC. There was a linear correlation for both FC and VC to OD which means that they follow the concentration increase in a linear fashion as expected since OD has a linear relationship with cell concentration within a limited range. Since both methods follow the same linear increase in cell concentration, the experiment

verified that the FC method can be used instead of the traditional VC method. The difference in obtained cell concentration between the two methods was not significant for *S. epidermidis* but it was about a log unit difference for *E. coli*. The difference could be caused by *E. coli* entering a state of viable but non-culturable (VBNC) which have been reported for *E. coli* (Oliver, 2005). The state of VBNC would mean that there are cells having an intact membrane that would be stained as viable in the FC but not capable of forming a visible colony when cultured on a plate. *S. epidermidis* has also been reported to be able to enter a state of VBNC; however this was on excess of glucose and in a biofilm-forming strain (Cerca et al., 2011). It is unlikely that the cells would enter VNBC in the conditions of this experiment, which is also apparent in the results of VC and FC for *S. epidermidis.* Due to time limitations, one biological replicate was made for the relationship between OD, FC and VC which decreases the certainty of the data. However, it gives an indication of the relationship. The experiment was executed three times for *E. coli* but only one replicate was displayed in the results as there were technical issues such as a time delay between analysis by FC and VC. The time difference between analyses is especially a concern since the bacteria was cultivated in rich medium which could cause a growth during that time delay and affect the results.

There was also a difference of about 1-2 log units in cell concentration between FC and VC for *E. coli* growth in product, higher than in rich medium. The increase in discrepancy between FC and VC is unlikely to be caused by the medium as urine supports growth of bacteria (Asscher et al., 1966) but it might be caused by the product environment. Due to the variation and difference in VC, FC is regarded as providing more reliable results compared to VC, especially in the case of strains with high biological variation such as is evident for *E. coli* ATCC70028. The FC results are therefore what will be discussed in the forthcoming parts.

Flow cytometry has many advantages as it can give a lot of information simultaneously, and in this study both cell concentration and viability was measured. The protocol used in this master thesis was established, optimized and tested earlier in the project and was found to be suitable for the bacteria and also the bacterial stains were stable. However, there are limitations to the method and one limitation that became evident was the absorption of dye by the product materials. There are many dyes to choose from for viability measurements and according to Buysschaert et al. (2016), there is no general stain and techniques that is suitable to every strain and condition. The staining protocol is very important as the concentration, temperature and time are essential factors for the method to work properly. There was considerably less background in the analysis of the samples with only SAP which indicates that the background signal mainly originates from the cellulose in the material. The method can in the future be improved by optimizing the flow cytometry staining protocol for both the bacteria and this product by trying different types of dyes such as DNA stain that utilizes an external binder rather than an intercalating one. In this way, the dye might not be able to attach in between the matrix of material and be stable enough to emit fluorescence. However, it might still be able to attach to the side instead and therefore another way to measure viability may be a better alternative such as using a dye for membrane potential to measure viability instead of a nucleic acid one for membrane integrity. Membrane potential might also

be a better measure of viability instead of membrane integrity as the loss of membrane integrity for PI to enter the cell does not necessarily mean cell death for sure, but rather mean severely damaged as an indication of cell death.

To establish a method for the microcalorimetry for different levels of saturation of the product, the absorptions capacity of SAP had to be determined. The absorption capacity of SAP is up to 300 times their weight in water (EDANA, 2017) but increasing the sodium ions in the water would decrease the swelling capacity because of the reduction of osmotic pressure difference (Elliott, 2004). Since urine consists of many different compounds, including sodium, the absorbing capacity of SAP was tested. The method used in the experimental set-up was based on the method of using microcalorimetry for detection of bacterial growth described by Braissant et al. (2015). The authors use an inoculating volume of 300 µl, which is what was also used in these experiments. According to Braissant et al. (2015), this allows for a head space of 200 µl in the sealed vials and should contain about 1.9 µmoles of oxygen. However, since SAP swells there might be less head space and oxygen. The volume of culture was used to calculate the amount of SAP required for the different saturations. SAP is a challenging material to work with in small amounts as the granules vary in size and weight. The SAP granules are also electrostatic which provides a difficulty when working with them in plastic containers such as the cups of the microcalorimetry vials. Therefore, the amount of SAP in each vial is exposed to a certain error as the SAP tended to move out of the cups. It is also a limitation that very small amounts are necessary to use for SAP to fit in the microcalorimetry vials and small amounts gives a higher error in relation to the amount if some of the SAP is lost by electrostatic force. Different saturations are also difficult to try out in the microcalorimetry method since the extraction is based on a 10-fold dilution of the inoculation volume. This is not always enough liquid to efficiently over-saturate the SAP to the point where it is more liquid and not gel-like, which makes it more difficult to extract the cells from SAP for analysis.

When looking at the behavior in product, it is evident that both bacteria grow in this matrix of product and urine. For both *E. coli* and *S. epidermidis* there was an increase in cell concentration over time in the product with no apparent lag phase in the beginning of the experiment. *E. coli* has a higher growth rate in product compared to *S. epidermidis*. Looking at the growth rate in urine and in product, it seems as if *E. coli* is better to adapt to urine as a medium compared to *S. epidermidis.* It was expected that *S. epidermidis* would have a poor ability to adapt to urine because *S. epidermidis* did not survive cultivation directly in urine, in contrast to *E. coli*. Therefore, the adaption steps in the pre-culture were implemented. It appears that urine is the factor that has the highest impact on growth of *S. epidermidis* in product since there is no significant difference between growth rate in urine and in product. The cells entered stationary phase after around 6 h, as the cell concentration of *E. coli* 5x saturation experiment, biological replicates #4 to #6, at 6 h and 22 h was very close which was also evident in the shape of the curve. This was also strengthened by the activity measurements from the microcalorimetry which decreased after around 6 hours. The initial cell concentration was high so it was not surprising that growth would enter stationary phase. However, the bacterial load when using these products in real life might be less than the

inoculation concentration in these experiments. A study by Norberg et al. (1984) showed a bacterial growth of about 10^4 to 10^8 CFU/g in incontinence products and according to Troccaz et al. (2013) a normal bacterial load is less than 10^4 cells/ml. This suggests that the initial bacterial concentration can be lower than that was tested in this study. A lower initial concentration could affect the behavior of the bacteria as they would have more room for growth and remain in exponential phase the entire time as competition for the nutrients is lower. That would mean a higher activity and can possibly cause more odor, but nutrients might also run out sooner and thus promote entering the stationary phase. A lower initial concentration was of interest to investigate but due to the background signal from the product in the FC, it was not possible to try a lower initial concentration with the established method.

pH was investigated as it is a factor that can have an impact on growth of bacteria. In both cases of adjusted initial pH, the pH decreased or increased to around the same pH value, of 6.2-6.4, when in product. The pH stayed at around that value for the entirety of the time span tested and did not change significantly for the majority of the experiments. However, there was a statistically significant difference in the pH at 3 h compared to 22 h for the experiment with high initial pH. The pH increased with 0.07 pH units and even though it was statistically significant, there was not significant impact in growth and thus is not significant in practice. The pH measurements show that the product buffers the pH and that an initial pH, within pH 5.7 to 7.6, does not affect the pH in the product. Due to these results the rest of the experiments, did not have a modified initial pH of the urine. The results for the non-modified initial pH followed the same trend with the pH decreasing to the same level as the other experiments. With the addition of the 0 h analysis for *E. coli* 5x saturation experiment, biological replicates # 4 to #6, and *S. epidermidis* 5x saturation experiments, it also became evident that the buffering of pH occurs immediately after inoculation. When looking at the mean values of all sets of experiments, *E. coli* 10x saturation, biological replicates #1 to #3, with high initial pH, did have slightly higher pH in the product than the rest, 6.4 compared to 6.2, which suggest that the buffering capacity has an upper limit. In general, the pH in the product is slightly acidic but it is optimal pH for both *E. coli* and *S. epidermidis* (Willey et al., 2011; Korting et al., 1992; Asscher et al., 1966) so there should not be a negative impact on growth, on the contrary, the pH is beneficial. There can be an effect in *E. coli* as the growth rate decreased in product compared to urine but it was not possible to distinguish if the pH is what caused it from the measurements in this study. The pH does not have an impact on growth for *S. epidermidis* as the rates are similar.

The saturation level of the product is important to consider as the product is rarely saturated to the same level each time it is used and it could influence the behavior of bacteria as well as the product characteristics. For both bacteria, there was a significant difference in cell concentration between the two saturation levels at 22 hours. This could be due to cell damage or death due to a dryer environment for the lower saturation. A lower saturation could make water less available and decrease the water activity which is vital for survival of bacteria as it maintains the osmotic pressure in the cytoplasm (Adams and Moss, 2008). Since 5x saturation is dryer, it is thought to have lower water activity than 10x and thus it would not be surprising if the bacteria in the 5x saturation experiments had a higher percent cell damage/death than

10x. However, the water activity in these experiments was not known so it is not possible to confirm. Other explanations to the lower cell concentration for 5x saturation at 22 h could be a combination of a lower bacterial load and lower growth rate. Due to the limited samplings in the exponential phase for 10x saturation experiments, a growth rate was not possible to determine and it is therefore not possible to establish any differences on growth between saturations. The collection of samples was limited by the length of the analytic procedure. The time used for analysis could be decreased with the use of only FC as a method for cell concentration. Another improvement of the experimental set-up would be to have the same initial concentration in the product as it would be easier to compare changes.

Flow cytometry allows for distinction of viability of cells during growth. The survival of cells was high in the first 3 to 6 h, as cell damage/death did not exceed 5.9% for *E. coli* and 11.5% for *S. epidermidis*. At 22h, the cell damage/death was significantly different between the two saturations for both bacteria which strengthen the theory that difference in cell concentration between saturations was due to cell damage/death. At 22 h the cell damage/death is higher for *E. coli* than *S. epidermidis* which may be due to *E. coli* being gram-negative and thus less resistant to lower water activity than gram-positive *S. epidermidis*. Even though the survival is lower at 22 h, the majority of cells are still alive which mean that even if they are not growing, or growing slowly, they can still metabolically active and possibly cause malodor formation.

It is of interest to understand the impact on microbial growth by the different components of the material and therefore growth on SAP alone was examined. It also provided an opportunity to try alternative ways of measuring microbial activity with microcalorimetry and a parallel experiment was conducted to relate the heat generation to growth and metabolic activity. For both bacteria, the highest activity was detected during the first 6 h which corresponds to the growth in product that also showed the highest growth in the first 6 h. The heat generation decreased after 6 h, but there was still activity during the whole 22 h for both bacteria which suggests that there is growth in the first 6 h and then as the bacteria reach stationary phase they remained metabolically active. Both the heat generation as well as the growth was higher for *E. coli* compared to *S. epidermidis*. For *E. coli*, the growth rate was also higher in SAP than in product, closer to growth rate of urine. For *S. epidermidis,* the growth rate is much lower in SAP than in product. This again shows how *S. epidermidis* is less resistant to environmental stress that this matrix provides than *E. coli*. There was also a large difference between the two saturations for *S. epidermidis*. The growth rate for 50% saturation is almost half of the rate of 100% saturation. A reason for the low growth rate for *S. epidermidis* in SAP could be that the initial concentration is already high and close to maximum possible cell concentration. Another reason for *S. epidermidis* low growth rate in the microcalorimetry vials could be the lack of oxygen as the vials were sealed. *S. epidermidis* is facultative anaerobe and can grow in both aerobic and anaerobic conditions (Bartlett et al, 1977) however; in a study by Cramton et al. (2001) growth was slower in anaerobic conditions. This might also explain the lower growth rate for 50% saturation, as the SAP is less expanded when not fully saturated and the structure that SAP creates when fully expanded might be more beneficial for oxygen distribution. The lack of oxygen is also a

limitation for microcalorimetry as a method and in this case, can show results farther from the real-life situation as oxygen will be present in the product. Although it can be argued that there can be an oxygen limitation when wearing the product.

The heat generation for *E. coli* is higher for 50% saturation than for 100% saturation which was surprising as it was thought that there would be a lower water activity for 50% and thus less activity. There was no significant difference in growth rate between the saturations even though it was slightly higher for 50% saturation than 100% saturation. It could be that the small difference in growth rate is due to the sensitivity of the microcalorimetry system. Another interesting observation is that the integral of the two curves for heat generation i.e. the total heat flow, (Figure 19A), is overall very similar due to the difference in the shapes of the curve. So, the bacteria seem to behave differently in the two saturations but in the end, amount to the same activity and thus give similar growth. Based on the results for *E. coli*, one hypothesis is that SAP, in its gel-like consistency, is absorbing some of the nutrients. The 50% saturation would have a less swollen SAP formation than 100% saturation and thus the diffusion distance is shorter and diffusion rate higher. In this way, the bacteria in the 50% saturation would have faster nutrient availability than 100% and therefore have a higher activity in the beginning but then the nutrients would be depleted faster which explains the faster decline in activity compared to 100%.

Specific metabolic activity was measured by monitoring changes in the potential biomarkers urea, creatine and creatinine. Uric acid and hippuric acid were also detected in the analysis but were not of primary interest. There were no observed differences in any of the identified compounds during growth for either bacteria or the different materials. There were some unknown peaks in the chromatogram but they were very minor. There were small changes to these peaks but it is difficult to establish any remarks about it since the substances are unknown and there are at least 2651 metabolites in urine (Bouatra et al., 2013). However, the unknown peaks were most likely compounds consumed or produced by the bacteria since they changed slightly over time. Considering the high concentration of urea in urine (Bouatra et al., 2013) and since *S. epidermidis* is urease-positive it was expected to be a difference in urea between the species as well as a decrease in urea for *S. epidermidis*. The non-detectable changes in metabolism could be caused by the dilution of the samples. Furthermore, the dilution step makes the impact of human error larger as the concentrations are so low and a smallest change, such as the human error when pipetting, can impact the result. However, the dilution is difficult to change since it is needed for the extraction method. To improve these metabolic activity analyses another method could be used such as NMR or mass spectrometry (MS). Since the metabolic activity is related to volatile compounds, gas chromatography could also be a good option.

6 Conclusion

This project has brought new insights to how *E. coli* and *S. epidermidis* grow in incontinence products and also established a set of methods as well as a laboratory scale model system that are appropriate for analyses of microbial behavior in this type of solid and complex matrix.as.

- The bacteria are localized on the surface of cellulose and SAP
- Initial pH does not impact growth due to buffering capacity of the product
- Both bacteria grow in this matrix of product and urine
- Cells stay alive and can thus be metabolically active
- Saturation-level of product makes a difference on survival of cells over time
- *E. coli* has a higher activity than *S. epidermidis*
- *E. coli* is less affected in urine compared to *S. epidermidis*
- *E. coli* is more affected by the product compared to *S. epidermidis*
- No observed differences in biomarkers (e.g. urea, creatine, creatinine)

7 Future perspectives

The work in this project is a step on the way to determine microbial behavior in incontinence products and how it can relate to formation of malodor. There are however still a lot that can be explored further in this area of research. Examining the behavior of different strains of *Staphylococcus* would be interesting to see if there are different results depending on origin of strain. Assessing other bacterial species relevant in this environment such as *Proteus* or *Lactobacillus* would be of interest. Head space analysis with gas chromatography coupled with mass spectrometry could be a way to determine generation of VOCs responsible for malodors and also to measure ammonia production. Other possible biomarkers could be tested and the HPLC method optimized. If more data is acquired, a mathematical model could be developed and implemented as a support for predicting possible malodor formation. It could also be of importance to consider a mix of bacteria as that would simulate real life situation better as well as determine if the bacteria affect each other's metabolism and growth. qPCR could be used as a method to determine the cell concentration of different species when investigating mixed cultures. Testing urine from people with disease could also be interesting as disease impact urine composition as well as the microflora. Further development and optimization of the microscopy method by using GFP-strains and viable cells instead of fixed cells would also be interesting and valuable to explore.

References

Adams, M. and Moss, M., 2008. *Food microbiology*. 3rd ed. Cambridge, UK: RSC Publishing.

Asscher, A.W., Sussman, M., Waters, W.E., Davis, R.H. and Chick, S., 1966. Urine as a medium for bacterial growth. *The Lancet*. **288**(7472), pp.1037-1041.

Bartlett, J.G., Onderdonk, A.B., Drude, E., Goldstein, C., Anderka, M., Alpert, S. and McCormack, W.M., 1977. Quantitative bacteriology of the vaginal flora. *Journal of Infectious Diseases*. **136**(2), pp.271-277.

Bos, L.D., Sterk, P.J. and Schultz, M.J., 2013. Volatile metabolites of pathogens: a systematic review. *PLOS Pathogens*. **9** (5)

Bouatra, S., Aziat, F., Mandal, R., Guo, A.C., Wilson, M.R., Knox, C., Bjorndahl, T.C., Krishnamurthy, R., Saleem, F., Liu, P. and Dame, Z.T., 2013. The human urine metabolome. *PLOS one*. **8**(9)

Braissant, O., Wirz, D., Göpfert, B. and Daniels, A., 2009. Use of isothermal microcalorimetry to monitor microbial activities. *FEMS Microbiology Letters*. **303**(1), pp.1-8.

Braissant, O., Keiser, J., Meister, I., Bachmann, A., Wirz, D., Göpfert, B., Bonkat, G. and Wadsö, I., 2015. Isothermal microcalorimetry accurately detects bacteria, tumorous microtissues, and parasitic worms in a label-free well-plate assay. *Biotechnology Journal*. **10**(3), pp.460-468.

Broome, B.A.S., 2003. The impact of urinary incontinence on self-efficacy and quality of life. *Health and Quality of Life Outcomes*. **1**(35)

Buysschaert, B., Byloos, B., Leys, N., Van Houdt, R. and Boon, N., 2016. Reevaluating multicolor flow cytometry to assess microbial viability. *Applied Microbiology and Biotechnology*. **100**(21), pp.9037-9051.

Cerca, F., Andrade, F., França, Â., Andrade, E.B., Ribeiro, A., Almeida, A.A., Cerca, N., Pier, G., Azeredo, J. and Vilanova, M., 2011. Staphylococcus epidermidis biofilms with higher proportions of dormant bacteria induce a lower activation of murine macrophages. *Journal of medical microbiology*. **60**(12), pp.1717-1724.

Cramton, S., Ulrich, M., Gotz, F. and Doring, G., 2001. Anaerobic Conditions Induce Expression of Polysaccharide Intercellular Adhesin in Staphylococcus aureus and Staphylococcus epidermidis. *Infection and Immunity*. **69**(6), pp.4079-4085.

de Viñaspre Hernández, R.R., Aranda, E.R. and Aznar, C.T., 2013. Urinary incontinence and weight changes during pregnancy and post partum: A pending challenge. *Midwifery*. **29**(12)

EDANA, 2008. *Fact Sheet Incontinence Products.* [online] Available at: http://www.right4hygiene.eu/docs/librariesprovider3/fact-sheets/fact_sheet_incontinence_products_ext2_7-2008_r4h_logo-2008-00407-01.pdf?sfvrsn=4 [Accessed 8 Jun. 2017].

EDANA. 2017. *Superabsorbents*. [online] Available at: http://www.edana.org/discover-nonwovens/how-they're-made/superabsorbents [Accessed 5 Jun. 2017].

Elliott, M., 2004. "Superabsorbent polymers." *Product development scentist for SAP. BASF Aktiengesellschaft ss* 13.

Emmons, KR. and Robinson, JP., 2014. The impact of urinary incontinence on older adults and their caregivers. *Journal of Aging Life Care*. Spring 2014 Issue

Frawley, J., Sibbritt, D., Steel, A., Chang, S. and Adams, J., 2017. Complementary and Conventional Health-care Utilization Among Young Australian Women with Urinary Incontinence. *Urology*. *99*, pp.92-99.

Fux, C.A., Costerton, J.W., Stewart, P.S. and Stoodley, P., 2005. Survival strategies of infectious biofilms. *Trends in microbiology*. **13**(1), pp.34-40.

Gatza, E., Hammes, F. and Prest, E., 2013. Assessing water quality with the BD Accuri™ C6 flow cytometer. *White paper. BD Biosciences*.

Heikal, A., 2010. Intracellular coenzymes as natural biomarkers for metabolic activities and mitochondrial anomalies. *Biomarkers in Medicine*. **4**(2), pp.241-263.

Hermann, M., Knerr, H., Mai, N., Groß, A. and Kaltwasser, H., 1992. Creatinine and N-methylhydantoin degradation in two newly isolated Clostridium species. *Archives of Microbiology*. **157**(5), pp.395-401.

Hesse, A., Classen, A., Knoll, M., Timmermann, F. and Vahlensieck, W., 1986. Dependence of urine composition on the age and sex of healthy subjects. *Clinica Chimica Acta*. **160**(2), pp.79-86.

Hilt, E.E., McKinley, K., Pearce, M.M., Rosenfeld, A.B., Zilliox, M.J., Mueller, E.R., Brubaker, L., Gai, X., Wolfe, A.J. and Schreckenberger, P.C., 2014. Urine is not sterile: use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. *Journal of clinical microbiology*. **52**(3), pp.871-876.

Jensen, O. B., 2009. *NMKL Prodecure 23 (2008) - Guide on Quality Assurance for Microbiological Laboratories* , Oslo: NMKL.

Karplus, P.A., Pearson, M.A. and Hausinger, R.P., 1997. 70 years of crystalline urease: what have we learned?. *Accounts of chemical research*. **30** (8), pp.330-337.

Korting, H.C., Lukacs, A., Vogt, N., Urban, J., Ehret, W. and Ruckdeschel, G., 1992. Influence of the pH-value on the growth of Staphylococcus epidermidis, Staphylococcus aureus and Propionibacterium acnes in continuous culture. *Zentralblatt fur Hygiene und Umweltmedizin= International journal of hygiene and environmental medicine*. **193**(1), pp.78-90.

Lee, J.B. and Neild, G.H., 2007. Urinary tract infection. *Medicine*. **35**(8), pp.423-428.

Martin, L. J., 2015. *Urine pH test.* [Online]
Available at: https://medlineplus.gov/ency/article/003583.htm
[Accessed 03 06 2017].

Nadkarni, M.A., Martin, F.E., Jacques, N.A. and Hunter, N., 2002. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology*. **148**(1), pp.257-266.

Niemelä, S., 1983. *Report no. 1 - Statistical Evaluation of Results from Quantitative Microbiological examinations,* Uppsala: Nordic Commitee on Food Analysis.

Norberg, A., Sandström, S., Norberg, B., Eriksson, S. and Sandman, P.O., 1984. The urine smell around patients with urinary incontinence. *Gerontology*. **30**(4), pp.261-266.

Oliver, J.D., 2005. The viable but nonculturable state in bacteria. *J Microbiol*. **43**(1), pp.93-100.

Otto, M., 2009. Staphylococcus epidermidis—the'accidental'pathogen. *Nature Reviews Microbiology*. **7**(8), pp.555-567.

Pandey, S.K., Kim, K.H., Choi, S.O., Sa, I.Y. and Oh, S.Y., 2013. Major Odorants Released as Urinary Volatiles by Urinary Incontinent Patients. *Sensors*. **13**(7), pp.8523-8533.

Paparella, A., Serio, A. & López, C. C., 2012. Flow Cytometry Applications in Food Safety Studies. In: I. Schmid, ed. *Flow Cytometry - Recent Perspectives.* InTech, pp. 69-93.

Priestley, C.J., Jones, B.M., Dhar, J. and Goodwin, L., 1997. What is normal vaginal flora? *Genitourinary medicine*, **73**(1), pp.23-28.

Podstawka, A., 2017a. *BacDive - The Bacterial Diversity Metadatabase: Staphylococcus epidermidis (Winslow and Winslow 1908) Evans 1916*. [online] Bacdive.com. Available at: https://www.bacdive.com/index.php?site=search&rd=14522 [Accessed 1 Jun. 2017]. doi:10.13145/bacdive14522.20170425.1

Podstawka, A., 2017b. *BacDive - The Bacterial Diversity Metadatabase: Escherichia coli (Migula 1895) Castellani and Chalmers 1919*. [online] Bacdive.com. Available at: https://www.bacdive.com/index.php?site=search&rd=4907 [Accessed 13 Jun. 2017].

Putnam, D.F., 1971. Composition and concentrative properties of human urine. NASA [online] Available at: https://ntrs.nasa.gov/archive/nasa/casi.ntrs.nasa.gov/19710023044.pdf [Accessed 14 Jun. 2017].

Rose, C., Parker, A., Jefferson, B. and Cartmell, E., 2015. The Characterization of Feces and Urine: A Review of the Literature to Inform Advanced Treatment Technology. *Critical Reviews in Environmental Science and Technology*, **45**(17), pp.1827-1879.

Smith, D., Russell, W., Ingledew, W. and Thirkell, D., 1993. Hydrolysis of urea by Ureaplasma urealyticum generates a transmembrane potential with resultant ATP synthesis. *Journal of Bacteriology*, **175**(11), pp.3253-3258.

Storer, M.K., Hibbard-Melles, K., Davis, B. and Scotter, J., 2011. Detection of volatile compounds produced by microbial growth in urine by selected ion flow tube mass spectrometry (SIFT-MS). *Journal of microbiological methods*. **87**(1), pp.111-113.

Thermofisher (2017). *Nucleic Acid Stains—Section 8.1 | Thermo Fisher Scientific*. [online] Available at: https://www.thermofisher.com/se/en/home/references/molecular-probes-the-handbook/nucleic-acid-detection-and-genomics-technology/nucleic-acid-stains.html [Accessed 8 Jun. 2017].

Troccaz, M., Niclass, Y., Anziani, P. and Starkenmann, C., 2013. The influence of thermal reaction and microbial transformation on the odour of human urine. *Flavour and Fragrance Journal*. **28**(4), pp.200-211.

Venkateswaran, K., Hattori, N., La Duc, M.T. and Kern, R., 2003. ATP as a biomarker of viable microorganisms in clean-room facilities. *Journal of Microbiological Methods*. **52**(3), pp.367-377.

Welch, R.A., Burland, V., Plunkett, G.I.I.I., Redford, P., Roesch, P., Rasko, D., Buckles, E.L., Liou, S.R., Boutin, A., Hackett, J. and Stroud, D., 2002. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic Escherichia coli. *Proceedings of the National Academy of Sciences*. **99**(26), pp.17020-17024.

Widmaier., E. (2014). *Vander's Human Physiology*. 13th ed. McGraw-Hill.

Willey, J., Sherwood, L., Woolverton, C. and Prescott, L., 2011. *Prescott's microbiology*. 7th ed. New York: McGraw-Hill.

Wilson, M., 2015. Assessing and treating urinary incontinence in men, *British Journal of Community Nursing*, **20**(6) pp. 268-270,

Wood, W.A., Gunsalus, I.C. and UMBEEIT, W., 1947. Function of pyridoxal phosphate: resolution and purification of the tryptophanase enzyme of Escherichia coli. *Journal of Biological Chemistry*. **170**, pp.313-321.

Yang, X., Sha, K., Xu, G., Tian, H., Wang, X., Chen, S., Wang, Y., Li, J., Chen, J. and Huang, N., 2016. Subinhibitory Concentrations of Allicin Decrease Uropathogenic Escherichia coli (UPEC) Biofilm Formation, Adhesion Ability, and Swimming Motility. *International Journal of Molecular Sciences*, **17**(7), pp.979.

Appendix 1. List of compounds tested in the HPLC

Urea
Creatine
Hippuric acid
Citric acid
Glucuronic acid
Ammonia
Uric Acid
Tyrosine
Bicarbonate
Creatine
Glycine
Phenol
Lactic acid
Histidine
Glutamic acid
Aspartic acid
Cysteine
Threonine
Lysine
Glucose
SAP in water
Other amino acids

Appendix 2. Confocal microscopy images

Microscope images 1-2: *E. coli* stained with Hoechst on cellulosic fibers.

- Image 1: Laser; filter (405 nm; 450±50) + transmission light.
- Image 2: Laser; filter (405 nm; 450±50).

Microscope image 3: *E. coli* stained with DRAQ5 on cellulosic fibers and SAP.

- Laser; filter (640 nm; 700±75) + transmission light.

Microscope images 4-5: *E. coli* stained with DRAQ5 on cellulosic fibers.

- Image 4: Laser; filter (405 nm; 450±50) and (640 nm; 700±75) + transmission light.
- Image 5: Laser; filter (405 nm; 450±50) and (640 nm; 700±75).

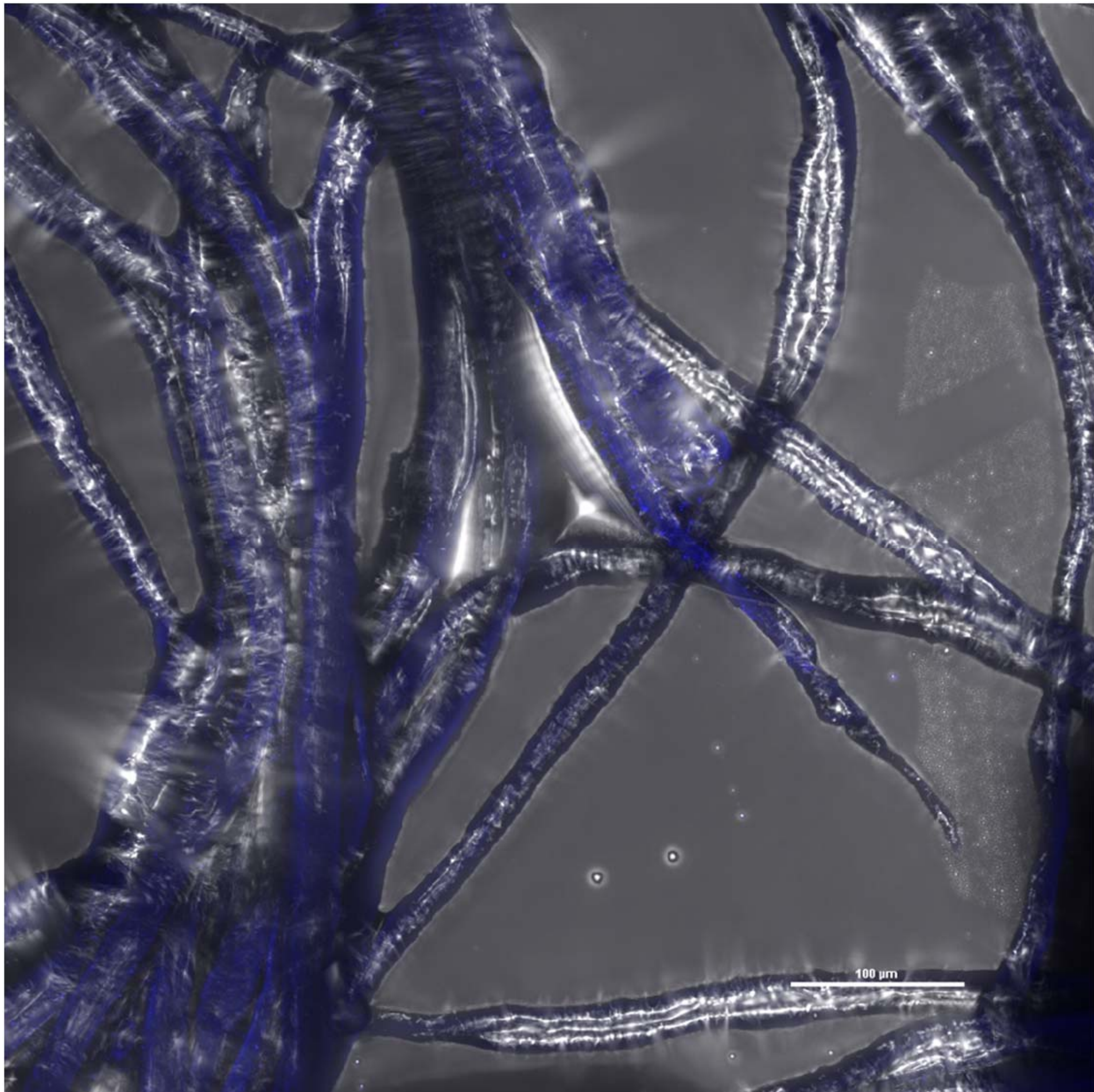
Microscope image 6: *E. coli* stained with DRAQ5 on cellulosic fibers and SAP.

- Laser; filter (405 nm; 450±50) and (640 nm; 700±75) + transmission light.

Microscope image 7 (montage of three images): *E. coli* stained with DRAQ5, distribution in product.

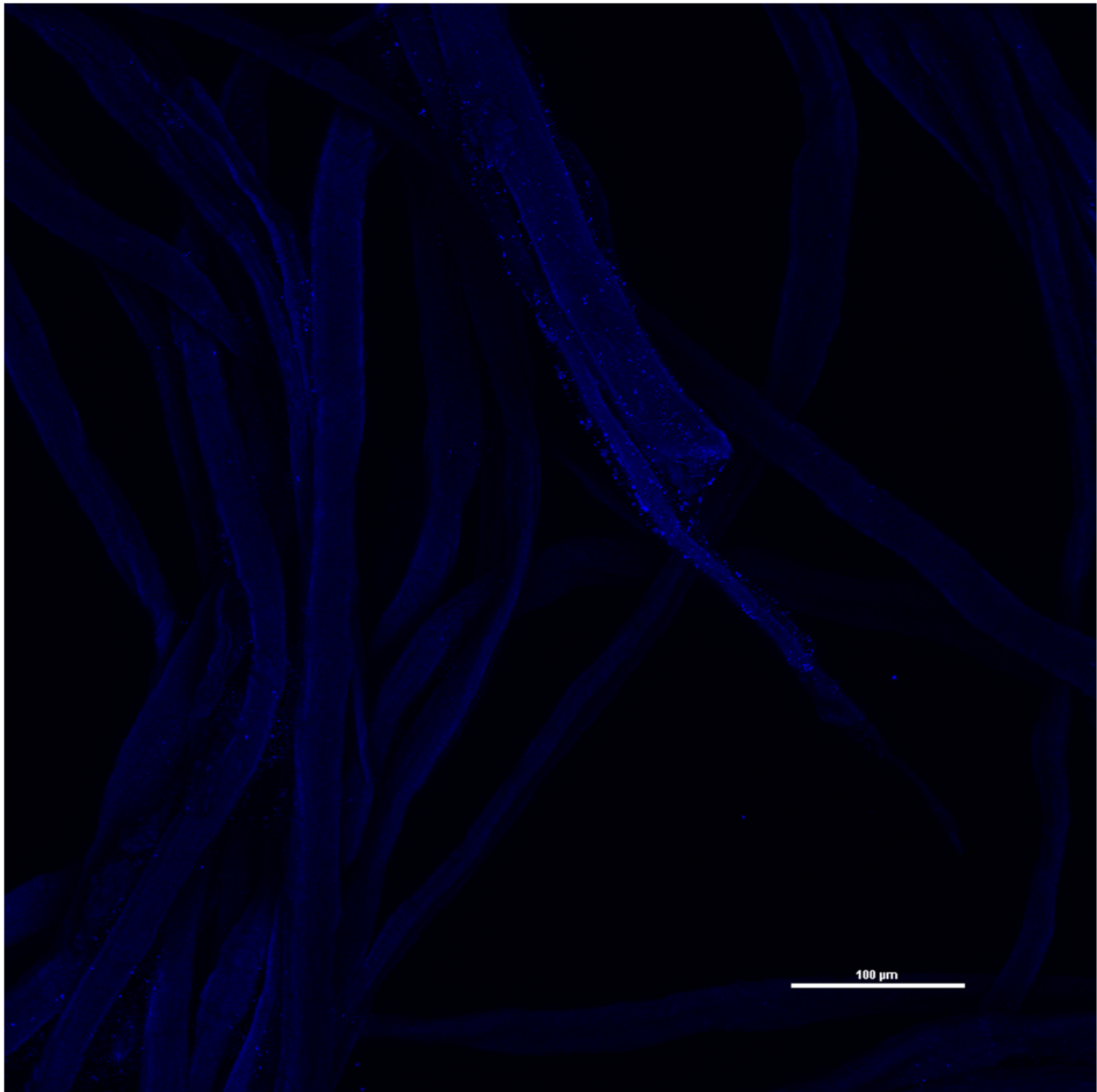
- Top image: Transmission light.
- Middle image: Laser; filter (640 nm; 700±75).
- Bottom image: Fluorescence intensity along the yellow arrow in middle image measured with laser; filter (640 nm; 700±75).

Image 1. *E. coli* stained with Hoechst on cellulosic fibers.



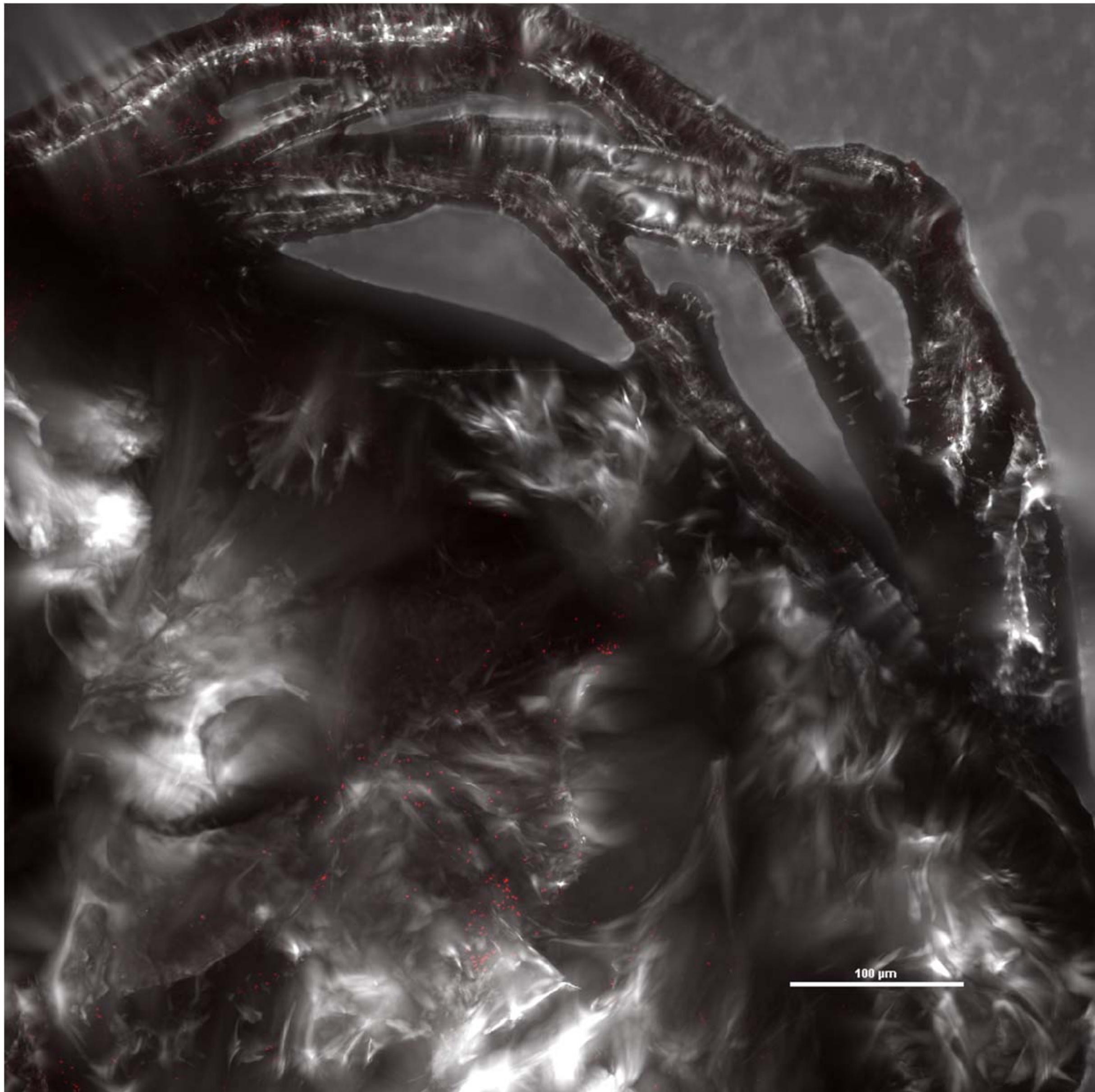
Comment:
Laser; filter (405 nm; 450±50) + transmission light.
Demonstrating the presence of auto fluorescence from cellulosic material interfering with the detection of bacteria stained with Hoechst.
(Auto fluorescence caused by laser [excitation] at 405 nm.)
(Band filter [detection of emission]: 450±50)

Image 2. *E. coli* stained with Hoechst on cellulosic fibers.



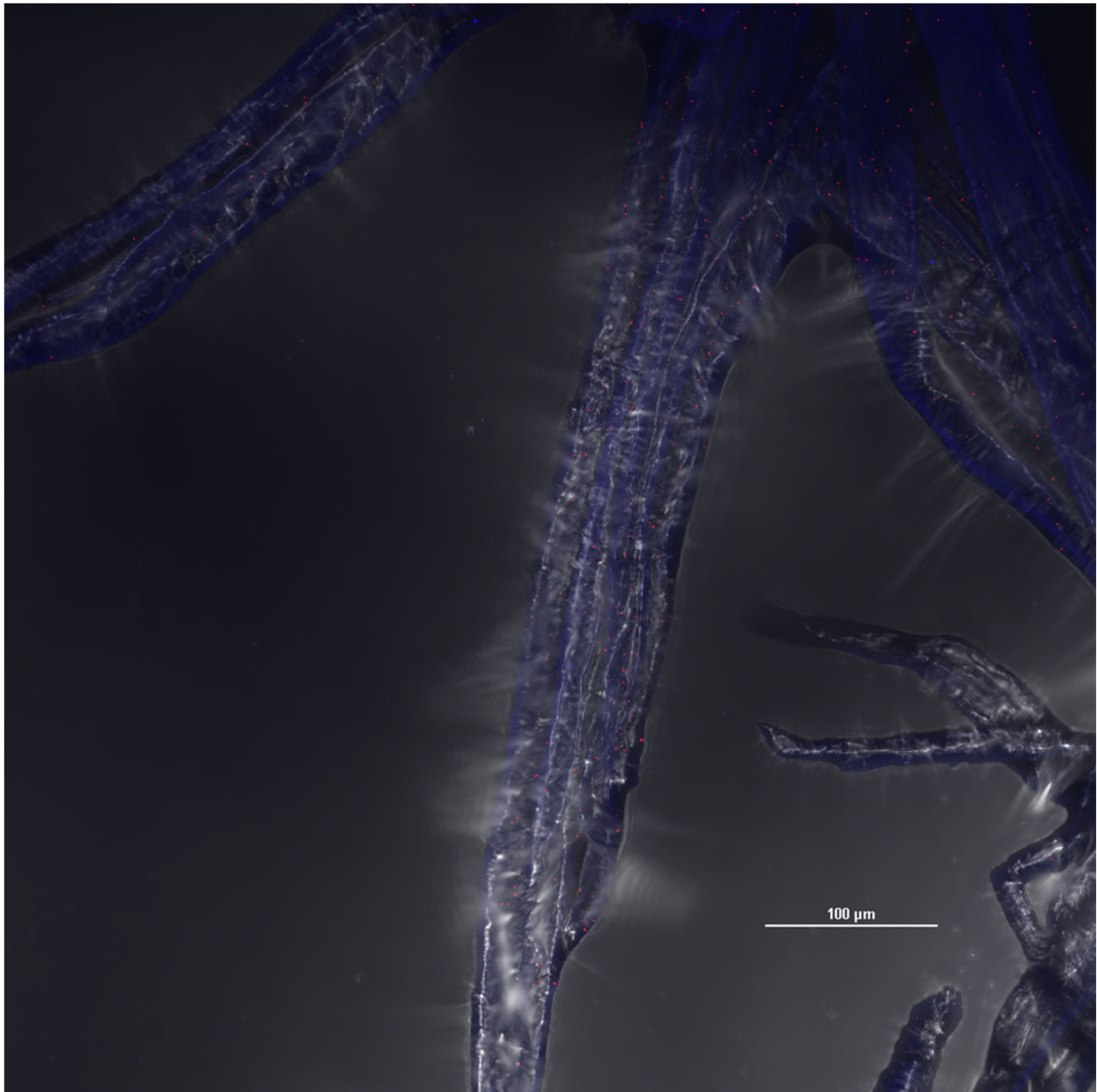
Comment:
Same image as “Image 1” but without transmission light.
(Laser; filter (405 nm; 450±50))
Demonstrating the presence of auto fluorescence from cellulosic material interfering with the detection of bacteria stained with Hoechst. However, with only fluorescence light the bacteria are more visible.
(Auto fluorescence caused by laser [excitation] at 405 nm.)
(Band filter [detection of emission]: 450±50)

Image 3. *E. coli* stained with DRAQ5 on cellulosic fibers and SAP.



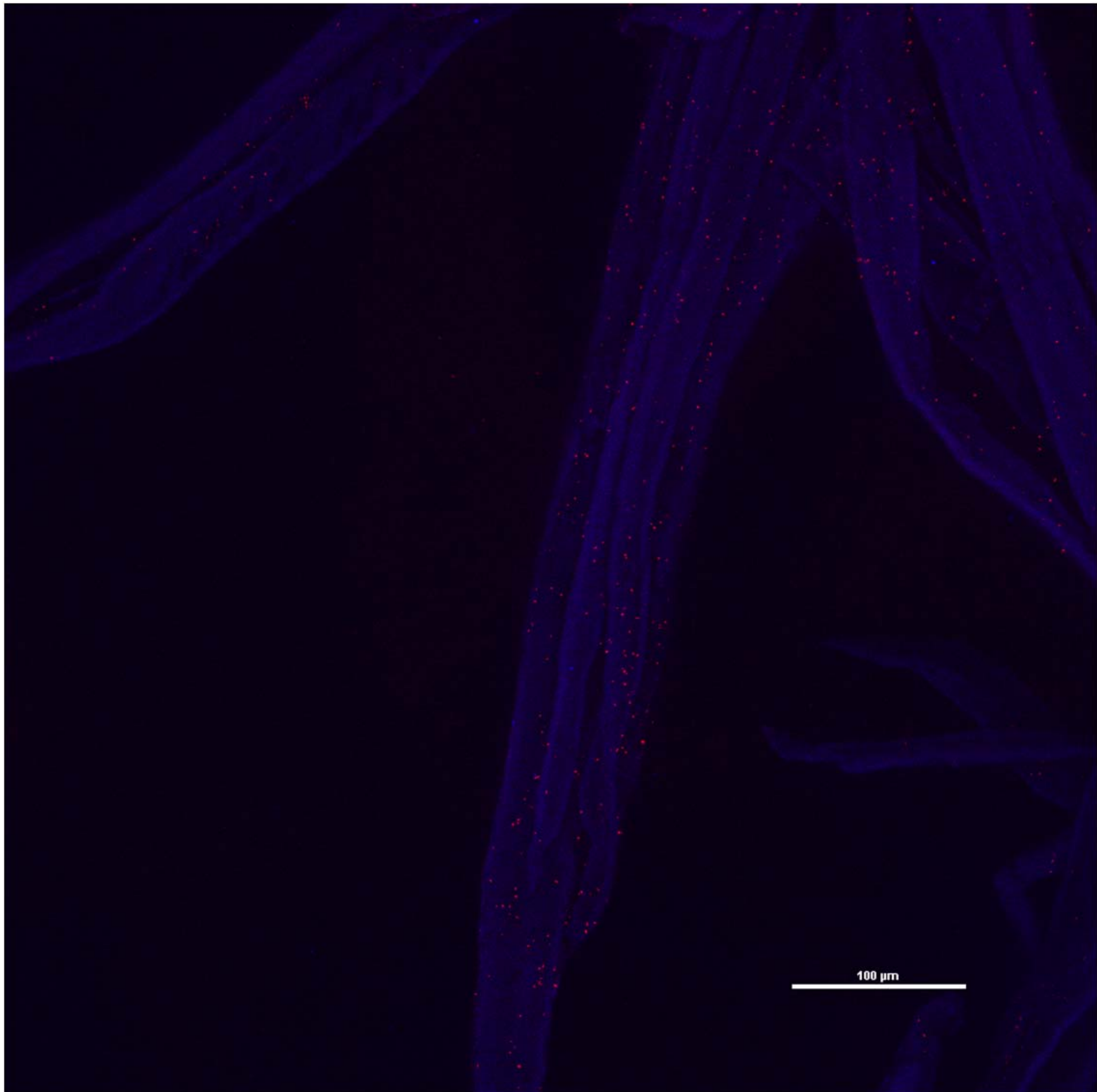
Comment:
Laser; filter (640 nm; 700±75) + transmission light.
The bacteria (red dots) are more easily visible using DRAQ5.
Auto fluorescence not present when using laser and detector for DRAQ5. This makes it difficult to distinguish between cellulose and SAP.
(Laser [excitation] at 640 nm.)
(Band filter: 450±50)

Image 4. *E. coli* stained with DRAQ5 on cellulosic fibers.



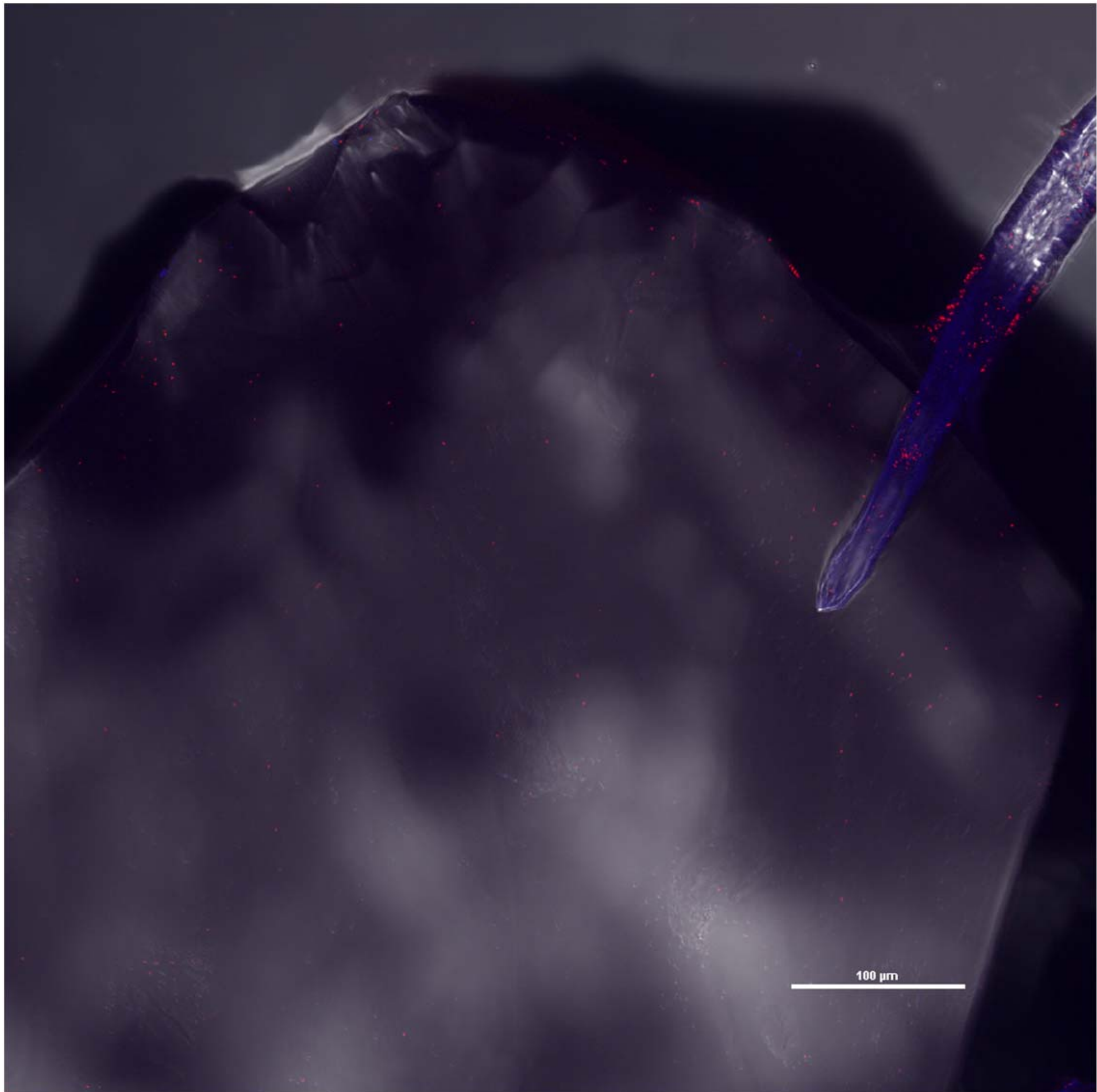
Comment:
Laser; filter (405 nm; 450±50) and (640 nm; 700±75) + transmission light.

Image 5. *E. coli* stained with DRAQ5 on cellulosic fibers.



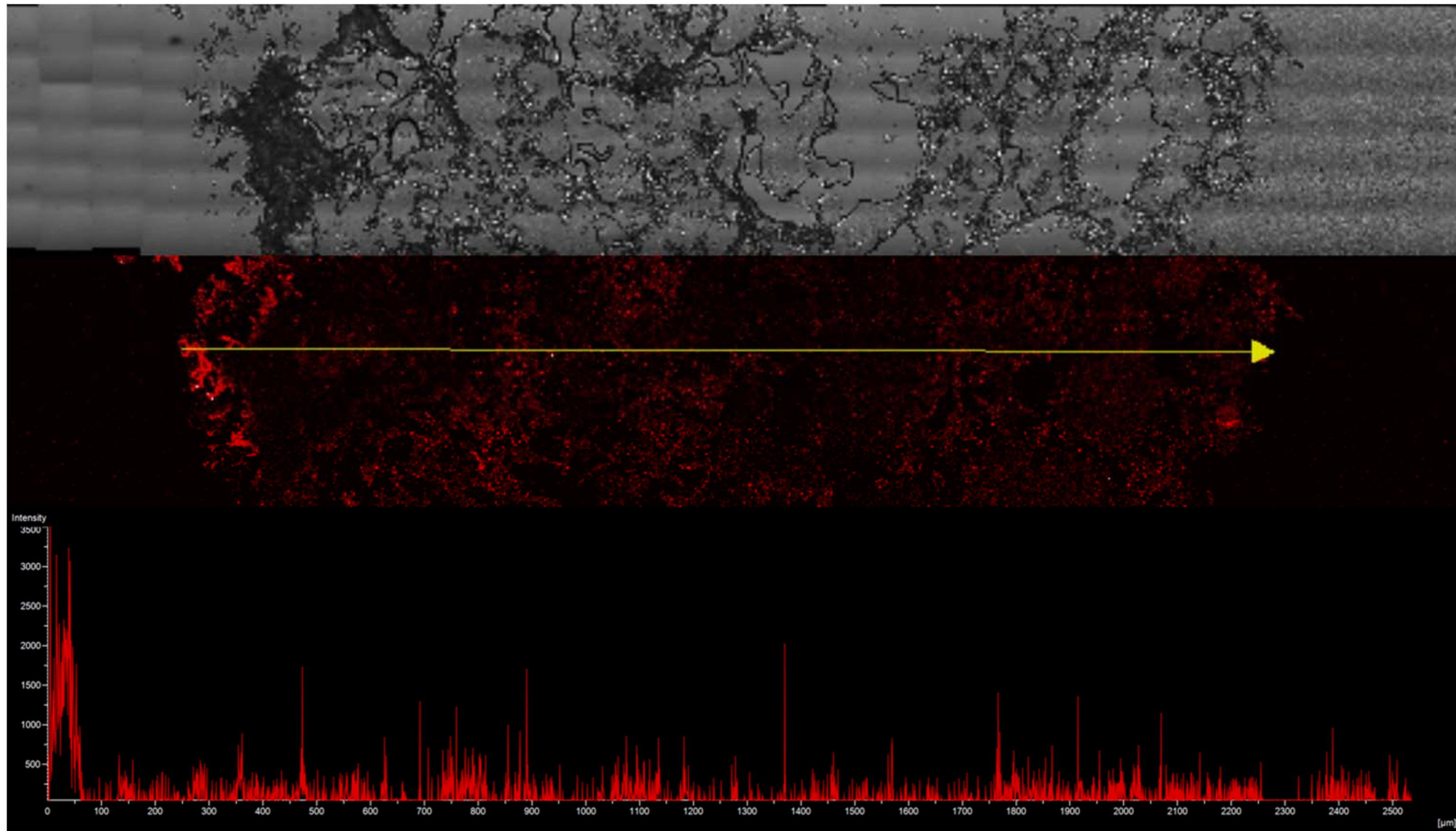
Comment:
Same image as "Image 4" but without transmission light.
Laser; filter (405 nm; 450±50) and (640 nm; 700±75).

Image 6. *E. coli* stained with DRAQ5 on cellulosic fibers and SAP.



Comment:
Laser; filter (405 nm; 450±50) and (640 nm; 700±75) + transmission light.
Cellulose fiber (right edge in image) and SAP granule in the background.

Image 7. *E. coli* stained with DRAQ5, distribution in product (montage of three images).



Comment:
Top image: Transmission light. Middle image: Laser; filter (640 nm; 700±75). Bottom image: Fluorescence intensity along the yellow arrow in middle image measured with laser; filter (640 nm; 700±75).

LUNDS UNIVERSITET

Lunds Tekniska Högskola