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Method development for determining antimicrobial effect of silver-containing materials and dressings with varying level of moisture saturation

Bachelor's Thesis in Chemical Engineering

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Abstract

The concept of treating infected wounds has been addressed for centuries, long before the knowledge of microbial was known. Historically a wide range of attributes have been used for this purpose such as spider web, seaweed, honey, leaches etc. Also, noble metals have been used to fight various pathogens of wounds. Although silver has been used in the field for centuries, it gained ground as an antiseptic in the 1960's and during the past decade a wide range of silver wound dressings have been introduced.

Silver dressings function through the release of silver ions (Ag^+) from elemental silver or silver compounds incorporated into the dressing. Ag^+ then acts as an antimicrobial agent by binding to walls and DNA of cells, thus interfering with cell division and replication. The presence of moisture is a prerequisite for silver ions to be released. For this reason most test methods in the field involve completely wetting of products prior to measuring antimicrobial effect. The aim of this project work is to develop a method that enables determination of microbial effect using products that are pre-wetted at various saturation degrees and thereby investigate if there is a correlation between degree of pre-wetting and antimicrobial effect. This would be an important complement to methods in use today.

The method involves application of a test piece (pre-wetted at various degrees of saturation) onto inoculated agar which after incubation is dissolved and spread onto TSA plates. The antibacterial effect is then determined by determining the number of viable counts. Tests were also performed using test pieces with different silicone layers to investigate whether properties of the silicone film have an impact on the antibacterial effect. Test pieces in use were punched out from prototypes and existing products in the Mölnlycke assortment.

Results show that even test pieces which are applied without any pre-wetting exert antibacterial effect. This is explained by the fact that due to the absorbing foam of the dressings in use, test pieces absorb liquid from the agar and are thus able to release silver ions. This phenomenon could be avoided by using a firmer agar composition which disables liquid absorption of the test piece. However, a prerequisite in order for the method to function was the possibility to dissolve the agar prior to plating and this was only possible for less firm agar concentrations. Moreover, it seems complete pre-wetting (100% saturation degree) is coupled with a greater antibacterial effect.

Test pieces with different silicone layers all displayed viable counts below the detection limit in performed tests. This corresponds to a significantly greater antibacterial effect compared to viable counts for the control test piece, in contradiction with the results of previous tests. However, for this test the test pieces in use were prototypes of existing products and a poor quality of the silicone film may be a possible explanation to these results. If this is the case, results can be interpreted as though the silicone properties of the product may be a significant factor in exerted antibacterial effect.

Abbreviations

CFU	Colony Forming Unit
O/N	Over night
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
SWF	Simulated Wound Fluid
log	Logarithmic

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1. Introduction

Wound healing has always been one of the physician's most important tasks. Papyrus from 1700 BC show how the healing of wounds were treated, this in spite of the fact that knowledge of anatomy was minimal at the time. Historically, attributes used for wound healing purposes include spider web, seaweed, honey and leeches (Lindholm 2007).

Today, the prevalence of skin and wound infections worldwide is high, causing great patient suffering. Recent reports suggest that approximately 1 % of all wound patients die from microbial infections (Percival, 2011).

Silver has been used as an antimicrobial agent for centuries and has been proven effective against a wide range of pathogens (Benbow, 2005). The antimicrobial effect is exerted by silver in the form of Ag^+ which binds to the wall and DNA of cells, thus interfering with cell division and replication. Over the past decade, silver wound dressings have gained popularity and today there is a large range of silver dressings that exert microbial action (White, 2011).

1.1 Aim

The aim of this project work was to develop a method for quantitative determination of antimicrobial effect of dry and partly wetted wound care products. In the literature of wound healing, quantitative test methods in use for determining antimicrobial effect involve completely wetting of the product or material prior to testing. For this reason it was desirable to develop an *in vitro* method using products and materials that are not totally soaked prior to or during testing. This would better correlate to the *in vivo* situation as products are dry when applied to the wound and then gradually wetted by wound fluid. Furthermore, the aim was to see if it is possible to correlate degree of product liquid saturation to antimicrobial effect.

2. Background

2.1 Wound Healing

Wound healing is a dynamical process consisting of a number of well-balanced and precisely regulated activities, sequentially arranged for optimum healing result. The interaction between cytokines, growth factors and proteases is crucial in the process to repair and rebuild new tissue. If the process is disturbed for any reason and wound healing is interrupted, the consequence is lengthened healing time or even the development of a non-healing wound, i.e. a hard-to-heal wound (Gilmore, 1991). Hard-to-heal wounds have been reported to comprise 60-80 % of all human infectious diseases (Percival, 2011). Various definitions of what constitutes an infected wound are available in the literature. Usually an infected wound is defined as a wound of 10^5 (Lindholm, 2007) or 10^6 (Bishara, 2011; Vermeulen, 2009) bacteria per gram of tissue or greater. Furthermore, Bishara (2011) considers prevention of wound infection to be the most important factor in order to prevent the development of hard-to-heal wounds.

The wound healing process is divided into three overlapping phases. The *inflammation phase* appears somewhere around 3-4 days after the injury occurred and is identified by symptoms such as redness, temperature increase and pain in the wound. For hard-to-heal wounds this phase is active during most of the healing process (Lindholm, 2007).

Immediately after the injury occurs, an intense contraction of surrounding blood vessels takes place which promotes hemostasis and thus healing. At this point capillaries are extended, resulting in increased permeability (Lindholm, 2007). This is followed by a process in which thrombocytes form a coagel by adhesion. As a consequence, thrombocyte formation is modified and granulation occurs, i.e. growth factors and vasoactive substances are released. These constitute an essential contribution to the wound healing process, for example by attracting leukocytes to the wound area (Toshikazu, 2006). Initially, the wound is protected against infection by polymorphonuclear neutrophils which are eventually replaced by lymphocytes and finally by macrophages (monocytes). The latter perform phagocytosis on microbes and other particles as well as initiate the development of granulation tissue. Moreover, they are also presumably involved in attracting fibroblasts to the wound area (Lindholm, 2007).

The *proliferative phase* lasts for approximately 3-4 weeks in acute wounds. Fibroblasts produce collagen, which constitute 70-80 % of the dermis, along with extracellular matrix. These are essential in the formation of new tissue (Lindholm, 2007). Further, elastin fibers are also produced which provide skin elasticity and make it resilient. Compositions of collagen and elastin fibers migrate toward the wound center and gradually replace the granulation tissue formed in the previous phase (Toshikazu, 2006). In addition to this, new epithelial cells are produced. Migrating from wound edges, sweat glands and hair follicles,

epithelia cells will initiate wound contraction and the last phase of wound healing is entered (Lindholm, 2007).

The *remodeling phase* occurs 1-3 weeks and may continue for years (Lindholm, 2007). Tissue is now rebuilt by synthesis of collagen. The transition from granulation tissue to scar tissue is controlled by a perfectly balanced process of synthesis and degradation of collagen in which macrophages, epidermal cells and endothelial cells along with fibroblasts cooperate in the degradation of collagen (Toshikazu, 2006). Connective tissue cells constituted of collagen form bundles of fibrils which are rotated to provide the newly formed tissue with maximum strength. However, new tissue will at most attain 80 % of the original capacity (Lindholm, 2007).

As in previous phases, the processes of the remodeling phase are primarily controlled by growth factors. The emergence of a chronic wound is, at a cellular level, considered be due to interference of the well-controlled and precisely coordinated activities that constitute wound healing (Lindholm, 2007).

2.2 Composition, function and microflora of the skin

The skin is the largest organ in the human body. It constitutes an effective barrier between the organism and the environment, preventing invasion of pathogens and chemicals. Its composition also protects internal organs by decreasing mechanical and physical stress as well as prevents diffusion of ions and nutrients from the body (Ehrhardt, 2008). Another important property of skin is as a barrier against water loss. This is essential for most terrestrial organisms which would otherwise become desiccated and die. A characteristic of skin is its prominent topographical differences (Montagna, 1998). Examples of this feature are the striking dissimilarity between the palms and the backs of the hands as well as the difference in skin properties of the eye lid compared to the much thicker and hairy counterpart of the brow.

The skin is composed of complementary layers, which contributes to desirable skin properties such as strength and elasticity (Montagna, 1998). The skin is usually divided into two layers of tissue, the outermost layer constituted by the *epidermis* (Montagna, 1998).

The surface of human skin is colonized by indigenous micro-organisms which are more or less constant in number throughout the lifetime of an individual. There is also a so called transient micro flora which inhabits the skin temporarily and likely influences the infection life cycle (Percival 2011). According to Percival, some of the most frequently encountered bacterial species in skin infections are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter* spp., and coagulase-negative staphylococci. The author also stresses the fact that wound infections are polymicrobial which adds to the difficulty of studying wound healing by in vitro studies. Fairly recent studies have shown that many of these various types of bacteria are sustained in communities known as biofilms, which are highly resistant to cleansing by irrigation and by treatment with antibiotics (Atiyeh, 2009).

Furthermore, it is important to keep in mind that the microbiology of the skin is affected by various factors such as age, sex, skin site and level of hygiene. Temperature and humidity can also have a great impact on microbial numbers and composition. As an example, bacteria seem to survive longer in a moist environment compared to a dryer area (Percival, 2011).

In the lower region of epidermis, the *stratum germinativum*, rapid cell division (mitosis) pushes older cells towards the surface of the skin. During the process cell deformation occurs resulting in destruction of nuclei and other organelles, ultimately leading to cell death. Cell migration builds up the outermost layer of the epidermis, the *stratum corneum* (SC), during keratin formation (Bennett, 2008). The SC is composed of dead cells which are continuously peeled off due to abrasion of the skin. The high proportion of keratin combined with lamellae of lipid makes the epidermis an effective barrier against water loss (Montagna, 1998). As cells are flaked off from the skin surface the production of new cells will increase. A perfect balance between production and loss of cells is a prerequisite for the skin to function optimally. Interference of the cell cycle may lead to skin conditions such as *psoriasis* (Montagna, 1998). The high keratin content of SC makes it poor in nutrition which limits bacterial density. In addition to this, flaking of skin also contributes to decreasing bacterial levels (Percival, 2011).

The second layer is the *dermis* which is a fibrous layer composed mainly of collagen. The fiber composition enables restoration of the skin after being stretched and keeps the skin hydrated due to its ability to hold large amounts of water (Montagna, 1998). Sweat glands and hair follicles are supported structurally as well as nutrition wise by the surrounding dermis. The dermis also plays a supportive and nutritive role for the epidermis which has no blood vessels of its own. This is provided through blood vessels of the dermis extending in small projections into the lower margin of the dermis as can be seen in Figure 1 (Bennett, 2008).

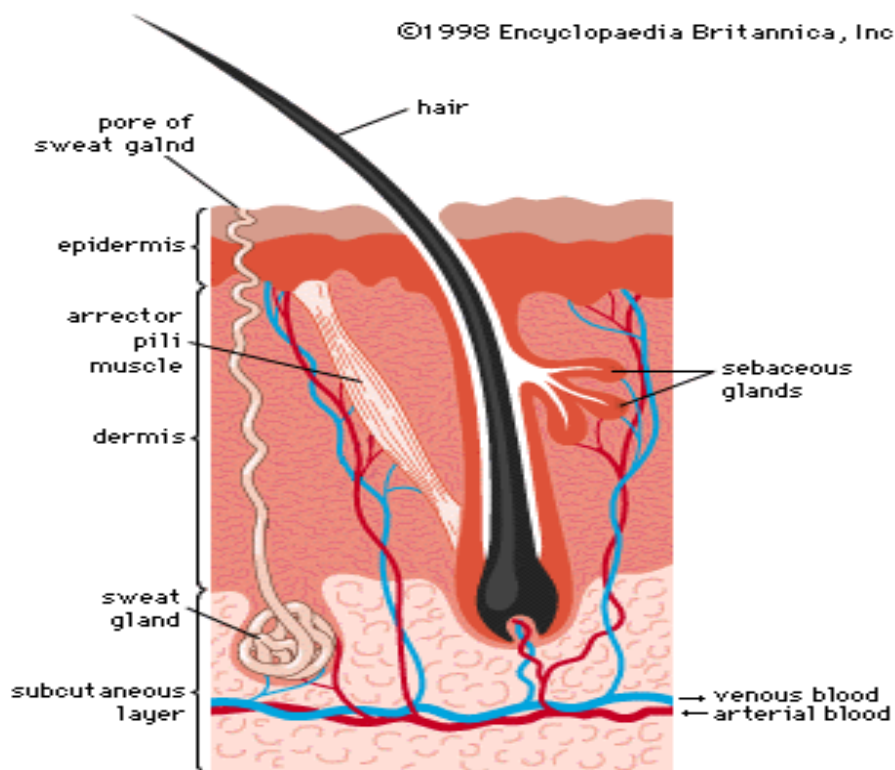


Figure 1. Layers of the skin (Montagna, 2012).

Beneath the dermis a *subcutaneous layer* is situated. It is basically constituted of loose connective tissue and fat cells and serves as a nutrient supplier to the layers above. It also provides the body with insulation (Montagna, 1998).

2.3 Topical treatment of wound infections

It has been shown that cleansing with uncontaminated saline or even regular tap water during dressing change promotes wound healing. Yet, the use of a topical antimicrobial is logical when treating infected wounds (Leaper, 2011). There is a wide range of topical agents used with dressings including antibiotics, antiseptics and disinfectants. Due to concerns of antibiotic resistance, alternative treatment of infected wounds has gained ground (Benbow, 2005). Topical antiseptics are antimicrobial agents that kill, inhibit or reduce the number of microorganisms and are thought to be essential for wound infection control (Atiyeh, 2009). While antibiotics act on a specific target, antiseptics target a broad spectrum of microbial including bacteria, fungi, viruses etc. According to Atiyeh (2009), advances in understanding the complex wound healing process have made it clear that in addition to merely controlling the surface bacterial contamination, the major objective in promoting wound healing is to minimize the risk of opportunistic infection while promoting the development of healthy granulation tissue as a part of the healing process. Topical antiseptic agents are flexible using a variety of mechanisms, acting at various rates and persistence intervals and show various levels of toxicity. Alas, when applied at the appropriate times and concentrations, antiseptics may be a valuable contribution in the process of reducing microbial numbers (Atiyeh, 2009).

Although antiseptics do not display many of the adverse effects associated with systemic medications (i.e. antibiotics), concerns have been raised regarding other negative effects (Helmke, 2004). The usefulness of antiseptics on intact skin is well established and broadly accepted, but repeated and excessive treatment of open wounds with antiseptics may promote a microenvironment similar to those found in hard-to-heal wounds. This has been an area of intense controversy for several years (Atiyeh, 2009).

Among available antiseptics some of the most commonly used are providine-iodine compounds, chlorhexidine, silver compounds, peroxygens, polyhexamethylene biguanide (PHMB) and honey (Leaper, 2011; Vermeulen, 2009).

2.4 Silver in wound healing

Silver has been used as an antimicrobial agent for centuries, long before the concept of microorganisms was known. The first use of metallic silver was in Mesopotamia and dates back to 3500-5000 B.C. (BOK). Silver is effective against a wide range of pathogens including bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and fungi such as *Candida albicans* (Benbow, 2005).

Silver nitrate has been the main compound of antiseptics used in wound care for more than 150 years, and is still used in burns clinics today. At higher concentrations (>10%) silver nitrate is severely caustic and forms soluble salts of silver albuminate and silver chloride in the tissue (Lansdown, 2004). Furthermore, silver nitrate is known for causing staining of the patient and the environment. During the 1960's, silver nitrate was replaced by safer antiseptics in many medical areas. Silver sulfadiazine (SSD) is for example used in silver coated catheters to stem biofilm formation and prevent infection (Brett, 2006). Over the past decade, silver wound dressings have gained popularity and today there is a large range of silver dressings that exert microbial action (White, 2011).

Silver can be present in four oxidation states; Ag^0 , Ag^+ , Ag^{2+} and Ag^{3+} , the most common being the monovalent form. Metallic silver (Ag^0) is inherently stable but can produce silver ions by oxidation in the presence of moisture. First, silver oxide (Ag_2O) is formed which may then release silver ions. The oxidation of elemental silver is a slow reaction and the rate is determined by the surface area of the exposed metal (Thomas, 2010). The antimicrobial effect is exerted by silver in the form of Ag^+ . The exact mechanism is not fully determined but silver ions are thought to bind to the bacterial cell membrane which causes disruption of the membrane and finally the death of the bacteria. In addition to this, Ag^+ is believed to bind to and interfere with enzyme activity as well as bind to bacterial cell DNA and thus interfere with cell division and replication (Vermeulen, 2009).

The silver ion is extremely reactive and reacts with a variety of anions found in biological fluids to form relatively insoluble complexes and precipitates. Evidently, Ag^+ does not only bind to cell membrane and DNA of bacteria but also to proteins and nucleic acids available in wound fluid. In vitro studies show that the choice of test media can have a significant impact

on antimicrobial effect. Inorganic and organic material in a given test media will bind and deactivate free Ag^+ , negating its antimicrobial attributes (Brett, 2006). As Ag^+ is deactivated, additional ions will be released in accordance with Le Chatelier's principle. Hence, if the object of interest is determining antimicrobial effect of a silver dressing, a test media mimicking wound fluid is the adequate choice (Brett, 2006).

The use of antiseptics and silver in particular is still controversial. Although the value of silver as an antimicrobial agent has been established, much of the evidence is experimental and there is still a lack of clinical evidence (Leaper, 2011). As previously mentioned, antiseptics are multifaceted and consequently, the risk of resistance is low. Historically, very high levels of silver have been released in wound care products thus additionally reducing the risk of resistance. Yet, the development of more advanced dressings introduced release of severely lower levels of silver, raising the discussion of possible development of silver resistance (Brett, 2006). Brett describes how Li et al. by *in vitro* studies were able to develop pathogens resistant to silver of a concentration greater than 1.000 ppm by subjecting them to sublethal doses of silver. Also, Vermeulen (2009) describes how studies performed in burn wounds show that bacteria may become resistant to SSD and silver nitrate.

Another concern is the potential cytotoxicity of silver as it does not act specifically on bacteria but on any alien protein (Vermeulen, 2009). Lansdown (2009) stresses the difficulty of retrieving accurate information as many articles on the subject are fragmentary, misleading or incompletely evaluated. As previously mentioned, the silver ion is extremely unstable and reacts with cell membranes and proteins in the wound during the formation of silver complexes. These may be deposited in any organ in the body such as the skin, liver or bones and cause transitory changes. However, the risk of permanent damage is considered very low (Lansdown, 2009; White, 2006). Moreover, although antiseptics have been shown to be cytotoxic *in vitro*, the relevance of this *in vivo* is probably limited (Cutting 2011).

Despite these claims against the use of silver (or any disinfectant) as an antimicrobial agent, Leaper (2011) argues that silver, due to its recorded antimicrobial properties, holds an important place in wound care. He stresses that there is a need for alternative topical agents in order to avoid antibiotic misuse and overuse which clearly *does* lead to the emergence of resistance among human pathogens. Finally, the author concludes that the emergence of silver resistance can be minimized by high and rapid levels of silver release.

3. Material and Methods

3.1 Bacterial strain

Since the purpose was to compare the effect of dry and wet products, it was sufficient to use only one kind of bacterial strain in this project. The bacterium in use was *Pseudomonas aeruginosa* (*P. aeruginosa*); ATCC 15442.

P. aeruginosa is a Gram-negative opportunistic pathogen that frequently persists in an innocuous state on human skin. It has the capacity to infect a wide range of tissues and is a common pathogen in wound infections (Percival, 2011).

3.2 Test Media

All media and chemicals used are presented below:

- Deionized water used for pre-wetting of test products.
- Simulated Wound Fluid (SWF; Fetal calf serum and Peptone water in proportions 1:1). It contains salts, proteins, carbohydrates, amino acids and vitamins along with other trace elements to simulate a wound like environment and is here used for pre-wetting of test products.
- Peptone water (PW; 0.85 % NaCl and 0.1 % peptone) used for dilution of overnight culture (O/N culture) and dilution of samples of dissolved, inoculated TSA with 0.6 % agar.
- Tryptic Soy Broth (TSB; 17 g Pancreatic digest of casein, 5 g Sodium chloride, 3 g Papaic digest of soybean meal, 2.5 g Dibasic potassium phosphate, 3.5 g Glucose mixed in 1 L deionized water) used for O/N culturing of *P. aeruginosa*.
- D/E Neutralization Broth (10 g Dextrose, 7g Lecithin, 6g Sodium Thiosulfate , 5g Enzymatic Digest of Casein, 5 g Polysorbate 80, 2.5 g Yeast extract, 2.5 g Sodium Bisulphite, 1 g Sodium Thioglycollate, 0.02 g Bromcresol Purpole). The solution neutralizes a broad spectrum of disinfectants and preservative antimicrobial chemicals, used here for neutralizing silver in dissolved, inoculated TSA with 0.6 % agar.
- Tryptic Soy Agar (TSA; 15g Tryptone, 15 g agar, 5 g Soya Peptone, 5 g Sodium chloride mixed in 1 L deionized water) used for determining viable counts by spreading dissolved inoculated samples onto TSA plates.
- TSA with various percentages agar (0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 g agar/ml) used for pouring 55 mm TSA plates.

3.3 Culture conditions

An overnight culture of *P. aeruginosa* was prepared as follows. One colony of the bacterium was picked from an agar plate and added to a test tube containing 3 ml TSB. The colonies originated from cryopreserved bacteria stored at -70°C. The test tube was vortexed and then incubated overnight at 35 ± 2 °C. The concentration after incubation was approximately 10^9 CFU/ml.

3.4 Products used

For the initial method development two Mölnlycke Health Care products were used. These are referred to as dressings A and B respectively, the former being used as a control. The dressings are briefly described below:

- *A*: foam dressing designed for a wide range of low to medium exuding wounds and maintains a moist environment. The wound contact layer of the product consists of a silicone adhesive. It is used as a negative control sample.
- *B*: antimicrobial foam dressing designed for the management of low to moderately exuding wounds. The foam contains silver sulfate (concentration 1.2 mg silver/cm²) and activated carbon. The silicone layer of the product is identical to that of dressing A.

Additional products used were:

- *C*: upgrade prototype of dressing B. It includes holes made in the silicon and foam in order to improve absorption of thick wound exudate.
- *D*: identical to dressing B but does not include a silicon adhesive. It is used to evaluate the effect of the silicon layer on antimicrobial effect.
- *E*: identical to dressing B but is not a proper product, i.e. it is not produced to be sold but to use in laboratory tests. It is used as a negative control sample in the microbiological tests.

Dressings C, D and E are all manufactured of the same foam, thus minimizing variations in material properties which could affect the result.

3.5 Development of method

3.5.1 Method Principle

The principle of the method is to subject inoculated agar to test pieces of varying degrees of saturation as illustrated in figure 2. It is critical that the agar and product are in contact throughout the test. The agar and product set-up was incubated for a certain time, followed by dissolution of the agar in an inactivation solution. The solution was then diluted and an aliquot was spread onto agar plates. These were incubated and the number of colonies was counted, representing the number of colony forming units (CFU), i.e. the number of microorganisms which have multiplied and formed a colony. All viable counts are reported in CFU/cm².

While developing the method, a number of critical parameters were taken into account. These are accounted for below by describing the process and experimental set-up in steps.

3.5.2 Determination of absorption capacity

In order to determine the impact of product moisture saturation on the antimicrobial effect the maximum absorption capacity of the test piece was determined. The dry weight of the test piece (*dressing A*) was noted and then placed in a bowl of excess liquid (deionized water). The test piece was left to swell for one hour. It was then removed from the bowl and allowed to drain for ten seconds after which the wet weight was noted. The difference in weight was considered as the volume of liquid absorbed (1 g of liquid approximated as 1 ml) and the maximum absorption capacity was calculated as the difference in weight divided by the test piece area. Five specimens were used to calculate a mean value. In further testing, percentages of the maximum absorption were calculated based on this value. The calculated value was considered to be valid for all dressings tested as they are all constituted of the same foam.

3.5.3 Pouring agar plates and determination of agar concentration

As the method required contact between the agar and the product, premade TSA plates could not be used as these produce a gap between product and substrate. Instead, agar plates were poured by hand under aseptic conditions. TSA was heated in a microwave, allowed to cool down to 44 °C in a water bath and then poured into empty 55 mm Petri dishes, filling them completely in order to ensure contact between the test piece and the agar (see Figure 2). The 55 mm Petri dish filled with TSA with 0.6 % agar content will from now on be referred to as *55 mm TSA plate*. It was then placed in a 140 mm Petri dish with a lid and left to rest until the next day to ensure proper solidification.

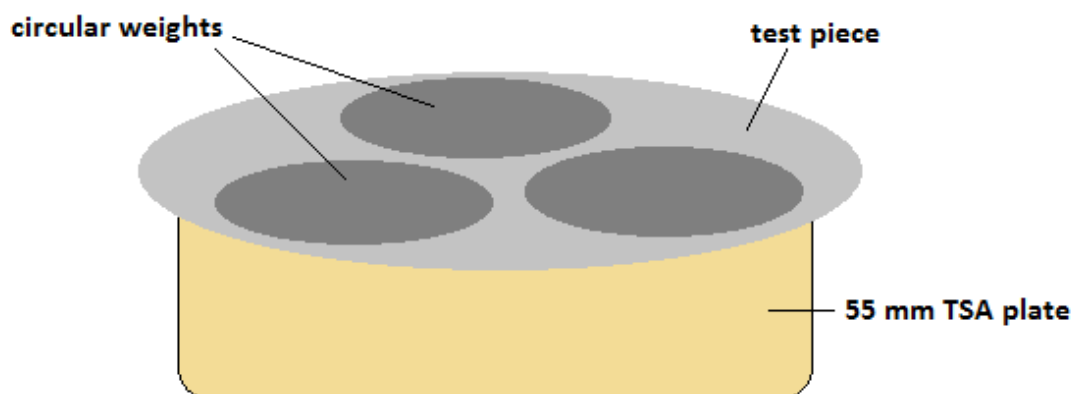


Figure 2. Method set-up demonstrating a test piece with circular metallic weights applied onto a 55 mm TSA plate.

When the composition of the agar was to be determined (i.e. the agar percentage), two criteria were taken into consideration. Firstly, the objective was to use an agar which test pieces would not absorb large amounts of liquid from. Since the aim of the method

development was to determine how varying saturation degree of products correlates to antimicrobial effect, the optimum situation would be to use an agar composition from which no liquid was absorbed by the test piece. Secondly, it must be possible to disperse chosen agar composition into a liquid when it is diluted in order to be able to determine the number of viable counts by plating and counting the number of CFU.

3.5.4 Inoculation of agar plates

Overnight culture (O/N) of approximately 10^9 CFU/ml was diluted in steps by transferring 500 μ l O/N culture into a test tube with 4.5 ml PW; i.e. diluting 10 times. This procedure was repeated onwards in the dilution series, each step corresponding to a tenfold dilution. Test tubes were vortexed between each step.

Aliquots of 50 μ l from adequate dilutions were spread onto the 55 mm TSA plates.

3.5.5 Incubation of inoculated test pieces and determination of viable counts

Test pieces (\varnothing 80 mm) were punched out and pre-wetted under aseptic conditions. Test liquid (deionized water or SWF) was added to the wound contact layer of the test piece using a pipette. The liquid was distributed as evenly as possible over the test piece surface.

The test piece was then centered on top of the inoculated 55 mm TSA plate using sterile tweezers, silicone layer facing down and in contact with the agar. Furthermore, a light pressure was desirable to ensure contact between the inoculated agar and the test piece. In order to attain an evenly distributed pressure three stainless steel weights were placed on top of the test piece. The 55 mm TSA plate with test piece and weights was then placed in a 140 mm plate, provided with a lid and incubated for 24 h at $35 \pm 2^\circ\text{C}$.

A sample (\varnothing 12 mm; area 1.1 cm^2) was removed from the inoculated agar by a single-use punch and placed in a sterile Stomacher bag (*BA0640 standard bag*). 10 ml of D/E Neutralization buffer was added to the Stomacher bag to inactivate antibacterial effect of silver. It was then placed in a Stomacher device (*Seward; Stomacher 80 lab blender*) at level *high* for 120 seconds. The Stomacher is an apparatus where two plates knead the sample and disperse its content. The purpose is to dissolve the agar-buffer solution into a smooth liquid and thereby enable dilution.

500 μ l of the Stomacher treated agar-buffer solution was transferred into a sterile test tube with 4.5 ml PW and a dilution series was carried out in the same way as described under 3.5.4. Thereafter, aliquots of 100 μ l from adequate dilutions were spread on duplicated TSA plates and incubated for another 24 h at $35 \pm 2^\circ\text{C}$.

After incubation, the colonies on the plates with appropriate number of colonies (20-200) were counted. The number of colonies represents the number of CFU/cm².

4. Experiments

4.1 Selecting appropriate agar concentration

Possible TSA-agar mixes available ranged from 0.4 % to 1.0 % in agar content, greater agar proportion corresponding to a firmer gel, i.e. a gel less likely to transfer liquid into the product. Initially, a test trial was set up to investigate the relation between agar content and amount liquid absorbed by the test piece.

The test included TSA-agar of 0.4, 0.6, 0.8 and 1.0 percentages agar respectively. Test pieces with saturation degrees of 0 % and 50 % were used. Test media was deionized water, all samples were performed in triplicates.

The weight of the specimen was noted and it was then placed onto a 55 mm TSA plate, wound contact layer facing down, with weights centered on top of the test piece as previously described. After incubation (24 h, $35 \pm 2^\circ\text{C}$), the weight of the specimen was recorded. The change in liquid absorption, i.e. the amount liquid absorbed or released by the product, was estimated as the difference between these two weights.

4.2 Settlement of appropriate bacterial start concentration

The purpose of these experiments was to determine an appropriate bacterial content which would make it possible to study a difference in antimicrobial effect between dry and wet products. An infected wound is defined as a wound with a bacterial content of 10^5 to 10^6 or more, for this reason the test piece should be subjected to values within this range. The relation between level of inoculation and antimicrobial effect was studied in experiments described below.

4.2.1 Experiment 1

O/N culture of approximately 10^9 CFU/ml was diluted with PW and aliquots of 50 μl corresponding to a bacterial content of 0.5×10^6 , 0.5×10^5 and 0.5×10^4 CFU/ml respectively were spread onto 55 mm TSA plates.

Deionized water corresponding to 100 % saturation degree (30 ml) was added to test pieces of *dressing A* and *dressing B*, all samples performed in triplicates. The test was then performed as described under 3.5.4 - 3.5.5

4.2.2 Experiment 2

O/N culture of approximately 10^9 CFU/ml was diluted with PW and aliquots of 50 μl corresponding to a bacterial content of 0.5×10^5 CFU/ml were spread onto 55 mm TSA plates. Three different samples were included: *dressing A*, *dressing B* (both added deionized water corresponding to 100 % saturation degree) and one 55 mm TSA plate without a test piece. The latter was used to be able to better observe the bacterial growth by visible inspection. The experiment was then carried out in the same way as in experiment 1, all samples performed in triplicates.

4.3 Determination of antibacterial effect of dressings

4.3.1 Test of varying test piece saturation degrees

The purpose of this test was to see if there is a correlation between test piece saturation degree and antimicrobial effect. The test procedure was the same as for experiments 1 and 2, all samples performed in triplicates. Samples used were *dressings A* and *dressings B* saturated with SWF to saturation degrees of 0, 50 and 100 % respectively.

4.4 Test of varying silicone layers

In this test prototypes of *dressings B* were used, all with different silicone layer surfaces. The purpose was to investigate whether silicone properties affect antibacterial effect. Test pieces used consisted of dressings *C, D, E* (described in 3.4) along with *dressings A* which was used as a control for antibacterial effect. All samples were pre-wetted with SWF and tested at saturation degrees of 0, 50 and 100 % respectively. Test procedure and pre-wetting was carried out in the same way as for previous experiments, all samples performed in triplicate with duplicate plating.

5. Results and Discussion

5.1 Initial method parameters

Initially, some factors affecting the practical implementation of the method will be discussed. These tests were of screening character. The section will then continue with the results of experiments described in 4.1 - 4.3 along with a general discussion of the method development and the use of silver as an antibacterial.

As previously described, an important factor in order for the method to function was to ensure contact between inoculated agar and test piece throughout the experiment. After creating 55 mm TSA plates which would make this possible it became clear that some kind of pressure which forced the test piece into the agar was needed as wetted test pieces tend to adopt a concave configuration, thus leaving a gap between agar and test piece. An idea was to pour the agar into the lid of the Petri dish and then place the bottom part of the dish (with slightly smaller area) on top of the test piece. This would provide an evenly distributed pressure. However, the attempt was soon discarded as the Petri dish lid was far too lightweight to have any impact on the quite rigid product material. After a screening experiment using weights of various types it was decided to use circular metallic weights (\varnothing 25 mm, weight: 16.5 g, height: 4 mm) for this purpose. By using three weights per test piece almost the entire surface of the 55 mm TSA plate was covered by weights which provided an evenly distributed pressure as desired.

The test pieces used in all experiments hold 80 mm in diameter. The main criterion was that the test piece (in both dry and wet state) should completely cover the 55 mm TSA plate to avoid leakage of silver onto the agar from the edges of the punched product. Cavanagh (2010) claims that the hydrophobic silicone film of the dressing wound contact layer of the dressing prevents fluid from entering or silver from being released. Since the edges of punched test pieces are not covered by the silicone film silver may be released to a higher extent through this surface. In order to mimic an in vivo situation it is therefore important to avoid contact between punched edges of the test piece and the inoculated TSA agar. The choice of test piece size was due to the fact that this was the smallest punch available which would guarantee coverage. However, since the Petri dishes in use only hold 55 mm in diameter, a slightly smaller test piece size of approximately 65 mm in diameter might be preferable in future tests.

5.2 Determination of absorption capacity

The maximum absorption capacity, calculated as the difference in weight between dry product weight and product weight after absorption in excess liquid, was calculated to be approximately 30 ml (referred to as 100 % saturation degree). The relative standard deviation for the test was 4%.

5.3 Settlement of agar concentration

Results are presented in figure 3. As expected, a lower agar concentration corresponds to a greater product liquid absorption.

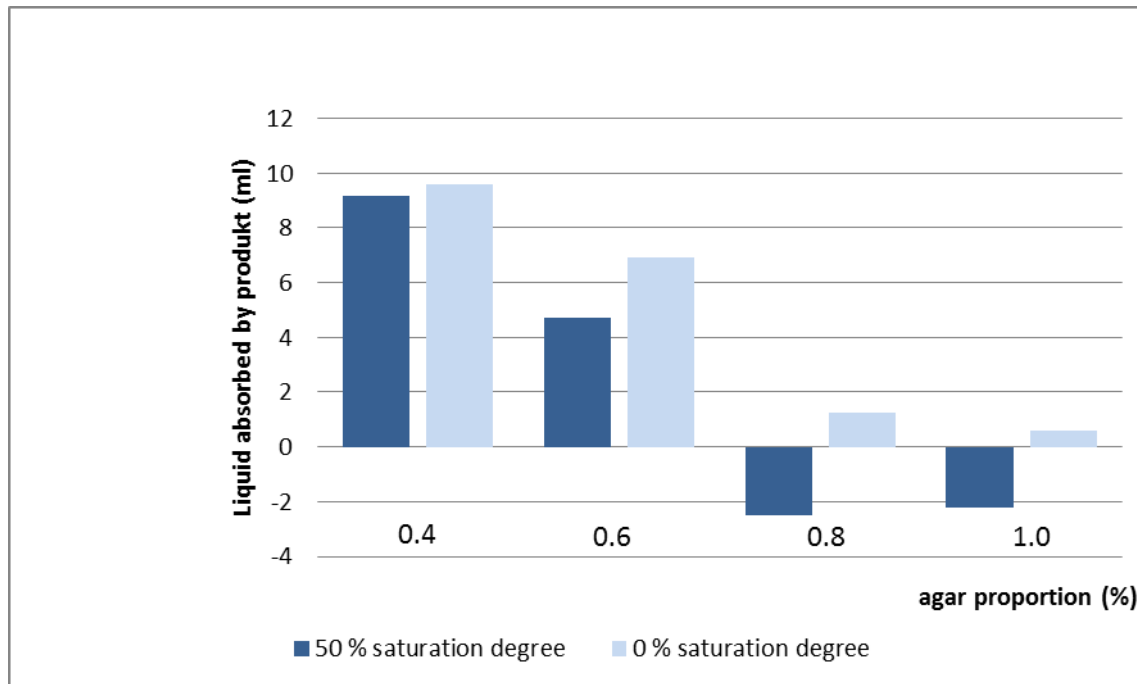


Figure 3. Variation in product liquid absorption for different agar concentrations. Lowest agar concentration (0.4 %) generates greatest liquid absorption.

The aim was to use an agar concentration which resulted in minimum product liquid absorption. This was a prerequisite for possibly coupling level of product pre-wetting with antimicrobial effect. On this basis, agar concentration of 1.0 % should be used as it yields the lowest liquid absorption. Moreover, an agar concentration of 0.8 % and higher generates a decline in saturation degree for pre-wetted products, i.e. liquid has been transported from the product in some way. This may be due to evaporation in part, but it is probable that also liquid has been transported from the test piece into the agar. The latter will most likely increase the amount of silver released from the product and thereby induce a greater antimicrobial effect. Whether or not agar concentrations of 0.4 % and 0.6 % also involve release of liquid is difficult to determine. Mentioned agar concentrations do however absorb liquid which most likely inhibits the release of silver. Accordingly, the choice of either 0.8 % or 1.0 % agar would probably result in a greater antimicrobial effect. This is also in agreement with Thomas (2010) who concluded that an absorbing dressing will withdraw moisture from the agar and thus inhibit the ability of silver ions to migrate in the reverse direction. However, it could also be argued that a higher level of product liquid absorption

should generate a greater antimicrobial effect since the presence of moisture is a prerequisite for release of silver ions.

Nevertheless, in this method selected agar concentration must also be possible to disperse into a smooth liquid when diluted in order for the number of viable counts to be determined by plating. An attempt to achieve this was by using sterile glass beads which were placed in a test tube along with the agar in question and then vortexed until the agar was dissolved. The result was a fairly smooth solution but as the procedure was quite time consuming it was decided to use a Stomacher device for this purpose. Nevertheless, none of these approaches were able to dissolve agar concentrations greater than 0.6 %. For this reason it was decided to use this agar concentration in the method.

After the decision to proceed with 0.6 % agar in future testing, the experiment for mentioned agar concentration was repeated, leaving a total of six specimens tested for each saturation degree.

5.4 Determination of appropriate bacterial content and antimicrobial effect

5.4.1 Experiment 1

Results for experiment 1 are presented in Figure 4. Three different start inoculation levels were used corresponding to bacterial contents ranging from 1.4×10^4 to 1.4×10^6 CFU/plate. Deionized water was used in this test since it is reported to be the most favorable test media for detecting antibacterial effect and the purpose was to map the extremes in antimicrobial effect. According to Thomas (2010), the use of deionized water as test media is associated with a greater antimicrobial effect than the use of serum, i.e. SWF. The use of SWF as test media combined with pre-wetting of test pieces to a saturation degree of 100% enabled observation of the maximum antibacterial effect.

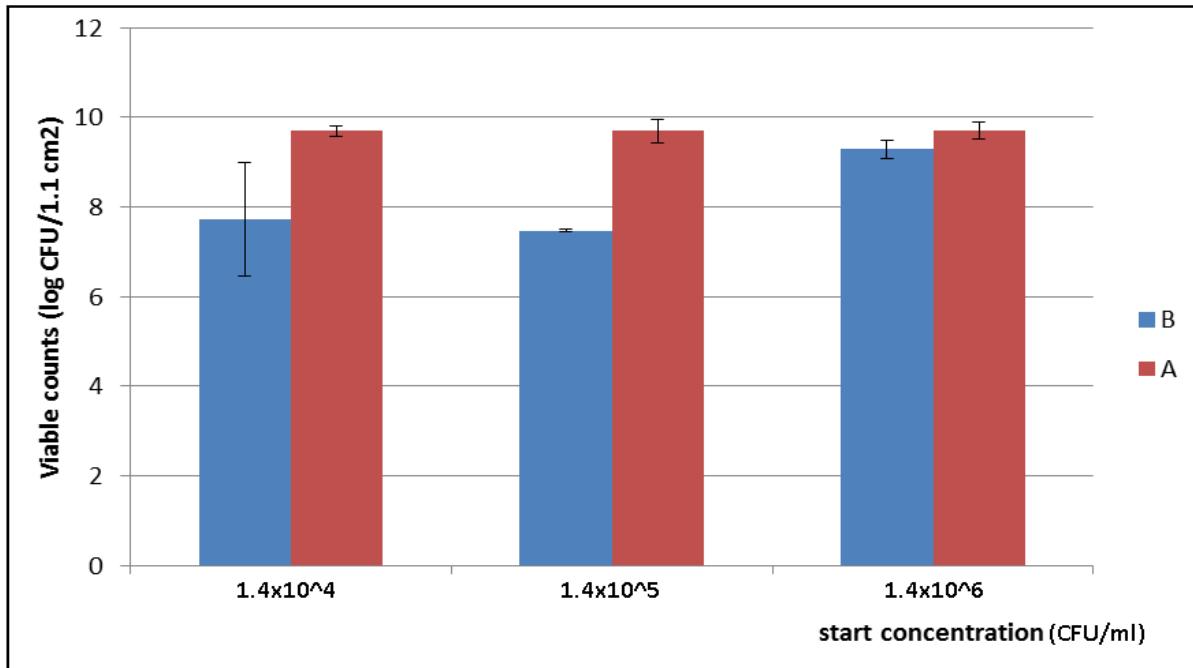


Figure 4. Viable counts for dressings A and B respectively using different start concentrations. Start concentration of approximately 10^6 corresponds to the bacterial level of infected wounds.

For a start plate concentration of approximately 10^6 CFU the difference in viable count for dressings A and B was less than one logarithmic unit, i.e. the antibacterial effect of dressing B was negligible. For lower levels of start concentration the difference in viable count increased to approximately two logarithmic units. Since an infected wound involves a bacterial content of approximately 10^6 bacteria/ml or higher, it is preferred to use a start concentration at this level. However, the small difference in viable counts at mentioned start concentration prevents the ability to clearly distinguish between dressings A and B. For this reason start concentrations of approximately 10^5 CFU were used in future tests of varying product saturation degree.

As test pieces were removed from the 55 mm TSA plates after incubation, a clear distinction in bacterial growth for dressings B and A was visible. The control test piece displayed bacterial growth as yellow, slimy goo. No visible growth was observed for dressing B. For this reason a distinct difference in viable counts was expected, yet this was not the case as displayed in Figure 4.

5.4.2 Experiment 2

In order to determine if the reported difference in viable count for dressings A and B in experiment 1 was correct, an experiment including a 55 mm TSA plate without a product applied on top was set up. It was suspected that part of the bacterial growth of dressing A remained on the product surface rather than in the agar as the difference in viable counts did not correspond to the visibly observed difference between dressings A and B. The 55 mm TSA plate without product made it possible to determine the level of bacterial growth in the

agar alone and thus define if mentioned suspicion was correct. Results of experiment 2 are presented in Figure 5. Start inoculation level was 0.5×10^5 CFU/plate.

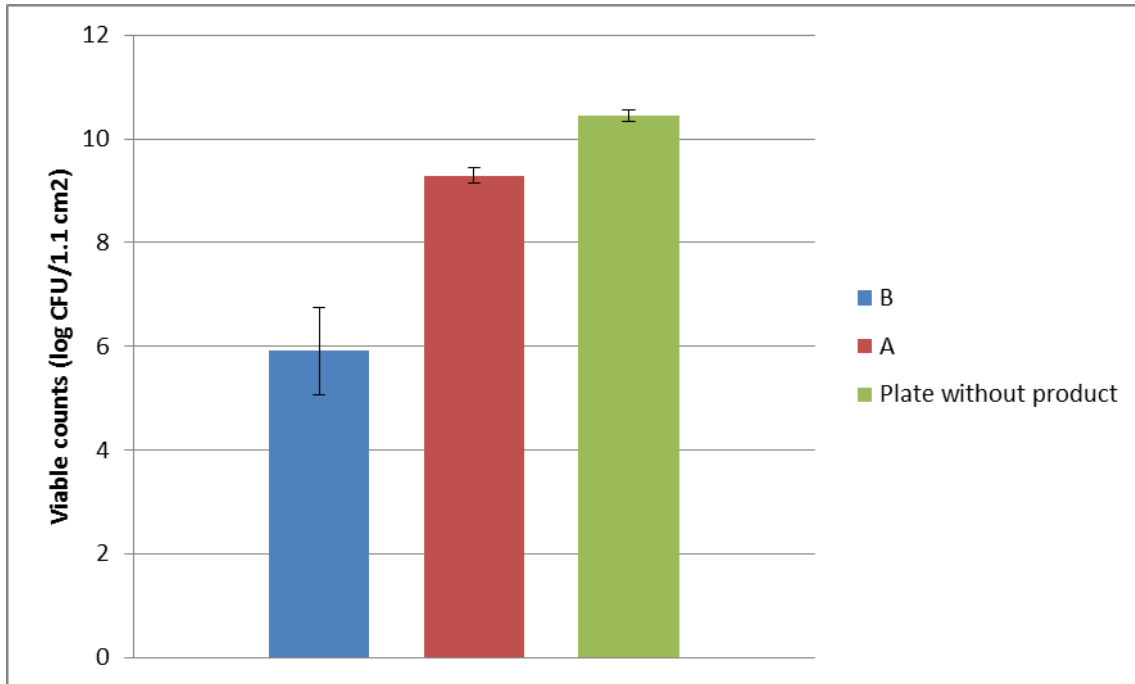


Figure 5. Experiment set-up enabling determination of bacterial growth in the agar alone without a dressing applied. Figure displays viable counts for dressings A and B compared with a plate without product.

Comparison of viable counts for plate with dressing A and plate without product indicates approximately one logarithmic unit is “lost” into dressing A. It is reasonable to assume that the number of viable counts for dressing B is also in fact one logarithmic unit greater than reported. However, since the fault is the same for both products, it is eliminated in comparison.

The difference in antibacterial effect of dressing B for experiments 1 and 2 may to some extent be due to the lower starting concentration of experiment 2. In microbiological tests it is not possible to ensure identical starting concentrations, but by repeating these experiments a more informed conclusion could be made regarding the dissimilarities in results. The varying antibacterial effect may also be explained by natural deviation.

5.5 Test of varying test piece saturation degrees

The purpose was to investigate if the degree of product saturation affects the antibacterial effect. Start inoculation level was 10^5 CFU/plate. Results are presented in Figure 6. The number of viable counts was below the detection limit for dressing B with 100 % saturation degree.

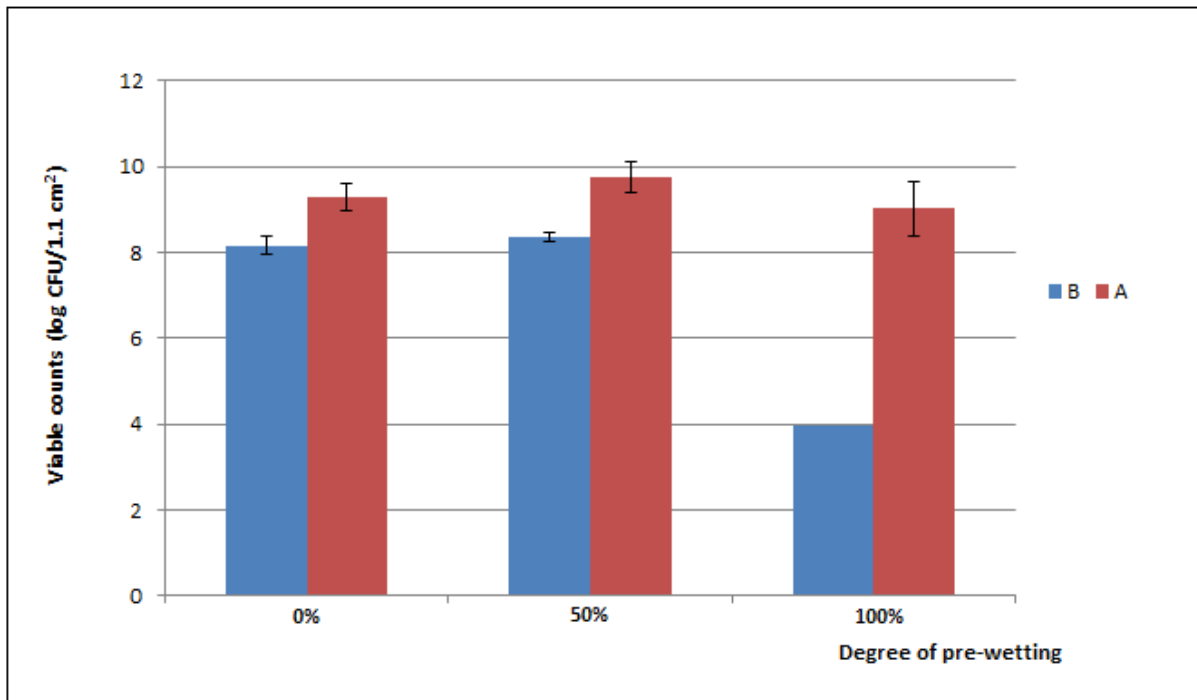


Figure 6. Test of varying product saturation degrees. Diagram displays viable counts for dressings A and B respectively.

As displayed in figure 6, it seems the level of pre-wetting does affect the antibacterial effect. Even the samples that were not pre-wetted display antibacterial effect, although it is marginal in comparison. However, as discussed in 5.3, dry test pieces absorb approximately 6 ml liquid (corresponding to approximately 20 % of the maximum absorption capacity) which probably explains the observed antibacterial effect observed for test pieces with no pre-wetting. According to these results, a fully saturated product yields severely greater antibacterial effect than a product that is not fully saturated. This could be interpreted as not only is a higher level of product saturation coupled with a greater release of silver, but rather that a fully saturated product is a prerequisite for significant antibacterial effect.

Furthermore, it is unexpected that the use of SWF as test media shows a greater antimicrobial effect in comparison with experiments 1 and 2 in which ionized water was used. According to the literature SWF should yield a lower antimicrobial effect due to the fact that silver ions bind to proteins and other components of SWF. The phenomenon of SWF yielding a greater antimicrobial effect than deionized water has however been observed in previous in vitro test at Mölnlycke (K. Hamberg, personal communication).

5.6 Evaluation of different silicone layers

Results are presented in Figure 7.

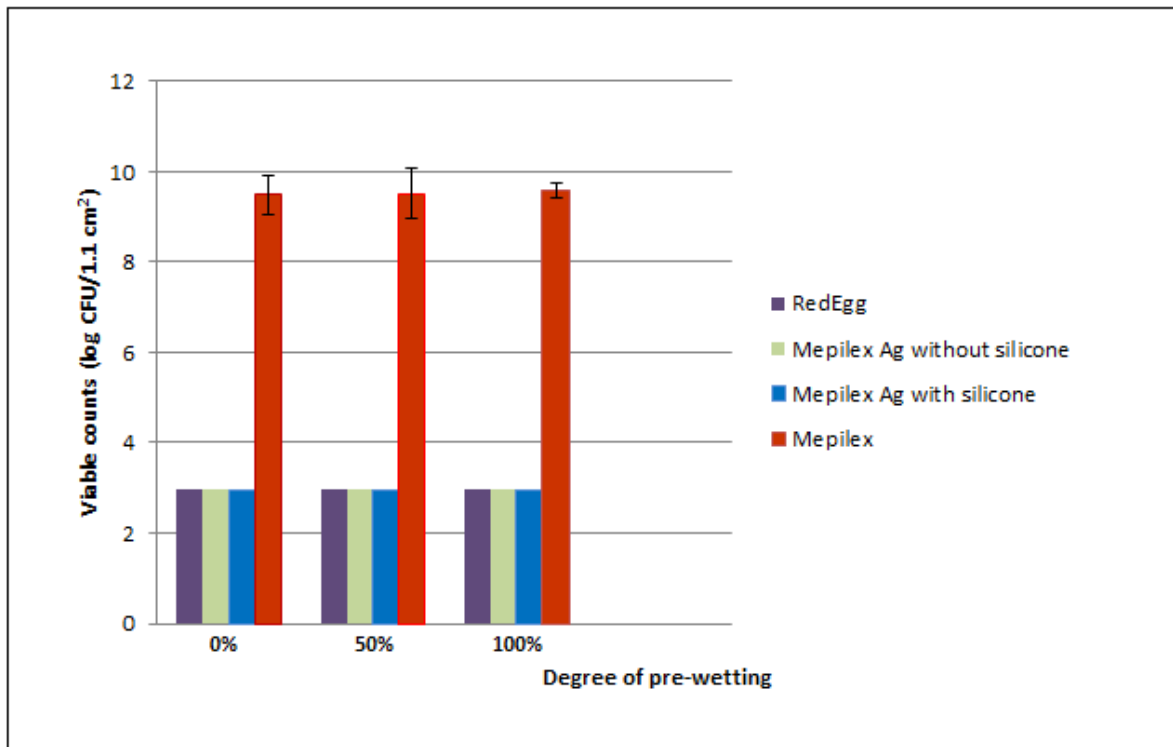


Figure 7. Viable counts for products of different silicone layers. Dressing A is the same dressing which has been used as a control in all previous tests. All other dressings show viable counts under the detection limit of 10^2 viable counts.

Because of the extent of the experiment it was divided into three sets, each product saturation degree carried out separately. For this reason the start concentrations varied for each saturation degree (0%: 4×10^5 ; 50%: 1.5×10^5 ; 100%: 1.5×10^5). All samples except for dressing A showed viable counts below the detection limit. These results were somewhat surprising in light of results for previous tests. The fact that samples with pre-wetting corresponding to 0% and 50% respectively yield viable counts below the detection limit contradicts the results presented in 5.5 where mentioned saturation levels showed significantly greater numbers of viable counts. Especially for dressing E, which is a prototype of dressing B and therefore should exhibit the same properties as its manufactured counterpart, the results of this experiment should more or less agree with reported results in 5.5.

However, since dressings used in this experiment were prototypes, the product quality of dressing E may not be comparable with that of a manufactured dressing B. A possible explanation of the displayed antimicrobial effect for all silver dressings in the test is that the silicone film of these products might be of questionable quality. Since all prototypes derive from the same silver foam batch, a poor silicone quality may have an effect on all silver dressing samples in use.

The intention of this test was to investigate whether or not the properties of the silicone film affect the antibacterial effect. If the case is that the silicone process of these products is poor, results may be interpreted as though properties of the silicone film might be a significant factor for the amount of silver released as the antibacterial effect significantly greater for these test products. In conclusion, it seems that properties of the silicone film may affect the amount of silver released from the product just as stated by Cavanagh (2010). In order to fully determine if this claim is correct, additional testing is needed. It would be interesting to repeat the test of different silicone layers including an additional test sample of a manufactured dressing B. By including this dressing in the same test as prototype dressing E an accurate comparison of mentioned dressings would be possible.

Furthermore, since no differentiation between test pieces of different silicone layers was possible a greater starting concentration would have been preferable. This is also applicable to the test of varying test piece saturation degrees presented in 5.5 in which results under the detection limit was noted as well. An adequate starting concentration would have produced viable counts above the detection limit, thus enabling differentiation between samples if there is one. The settlement of appropriate bacterial content was based on experiments using deionized water since this was expected to be the most favorable test media for silver release. However, since the use of SWF as test media resulted in a greater antimicrobial effect than the use of deionized water in these experiments, it is recommended to repeat experiments regarding settlement of bacterial content using SWF instead.

Because the method for determining silver release is based on microbiological tests in this project work, results may vary to a greater extent compared to that of a chemical measuring technique. Measuring silver release with inductively coupled plasma (ICP) was discussed as a possible additional method to be included in this diploma work. This would have provided an interesting additional dimension to the project as the level of silver release could then be coupled with corresponding antimicrobial effect. However, the main focus of this project work was to develop a method in which the *antimicrobial effect* (not amount of silver release) of dry and partly pre-wetted products and due to time constraints measurement using an ICP-device was excluded.

For all experiments, results are based on the assumption that bacterial growth only takes place on the surface of the agar and not in its interior. Also, it is assumed that bacteria are spread evenly onto the 55 mm TSA plates. Since only a portion of the agar is dispersed and spread onto TSA plates for determination of viable counts, an uneven spreading of bacteria would produce an error in the results. Finally, it should be stressed that all experiment included in this project work need to be repeated in order to ensure the accuracy of presented results.

6. Conclusion

The method developed in this diploma work enables quantitative determination of antimicrobial effect of dry and partly pre-wetted products as intended. Moreover, the release of silver through edges of punched products has been an issue in other in vitro methods but this phenomenon is avoided in the developed method. The method could be improved by using a firmer agar composition which prevents transfer of liquid from agar into test piece. This would enable a possible correlation between degree of product saturation and antimicrobial effect. This is not accomplishable in the current execution of the method since dispersion of firmer agar concentrations is not possible. Alternative approaches of dispersion which enable dissolution of firmer agar concentrations would be a valuable addition to the method configuration.

No clear correlation between product saturation degree and antimicrobial effect was noted in experiments carried out in this project work. However, results indicate that a fully saturated product seems to provide a significantly greater antimicrobial effect compared to products that are only partly wetted. This is most likely due to the fact that moisture is a prerequisite for the release of silver ions; hence a higher level of liquid saturation yields a higher release of silver ions. Also, results show that the silicone layer of the products in use may possibly prevent antimicrobial effect.

Finally, experiments in this project work need to be repeated in order to determine the accuracy of presented results.

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