APPLICATIONS OF TRADITIONAL CHINESE MEDICINES: BACTERIAL BIOFILMS AS A TARGET

Xueqing Wu

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APPLICATIONS OF TRADITIONAL CHINESE MEDICINES: BACTERIAL BIOFILMS AS A TARGET

TOEPASSINGEN VAN TRADITIONELE CHINESE MEDICIJNEN: BACTERIËLE BIOFILMS ALS DOEL

(met samenvatting in het Nederlands en Chinees)

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Xueqing Wu

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Promotor:	Prof. dr. J. Fink-Gremmels
Copromotor:	Dr. R.R. Santos

What is of all
things most
yielding
Can overcome
that which is
most hard

-Lao Zi

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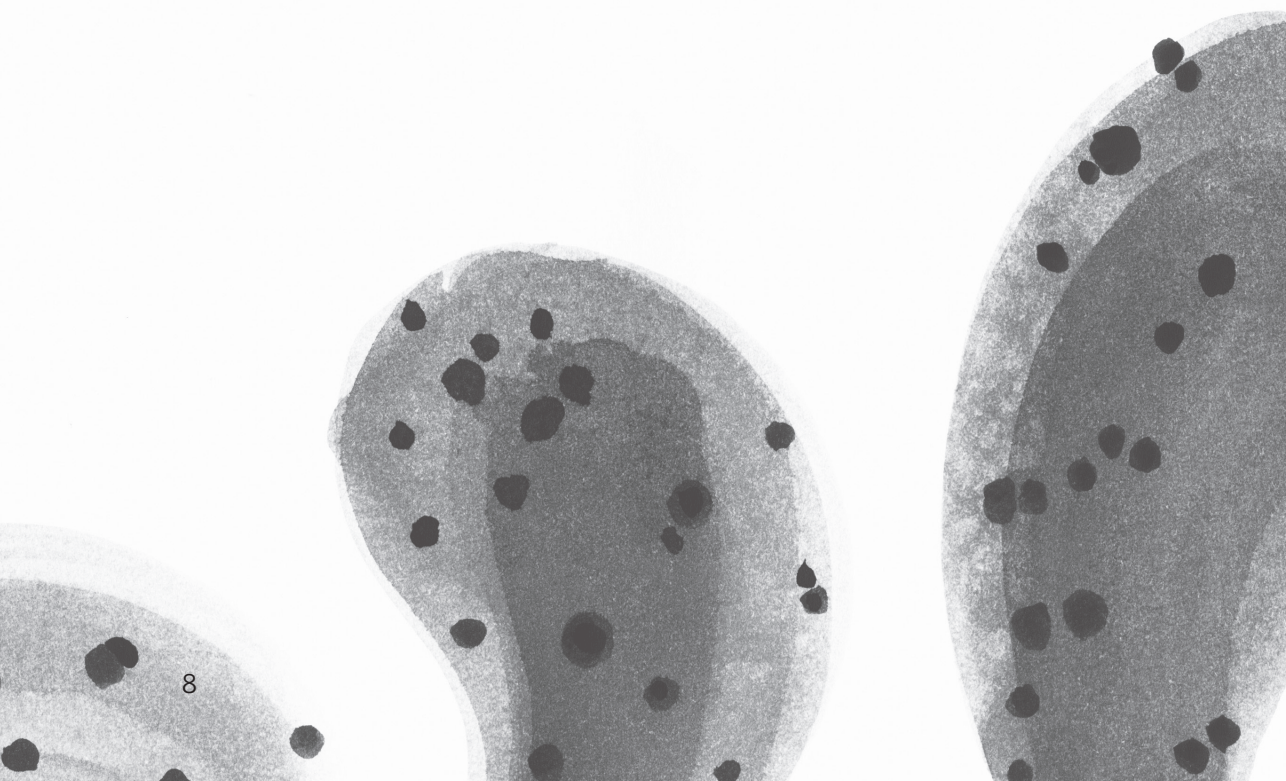
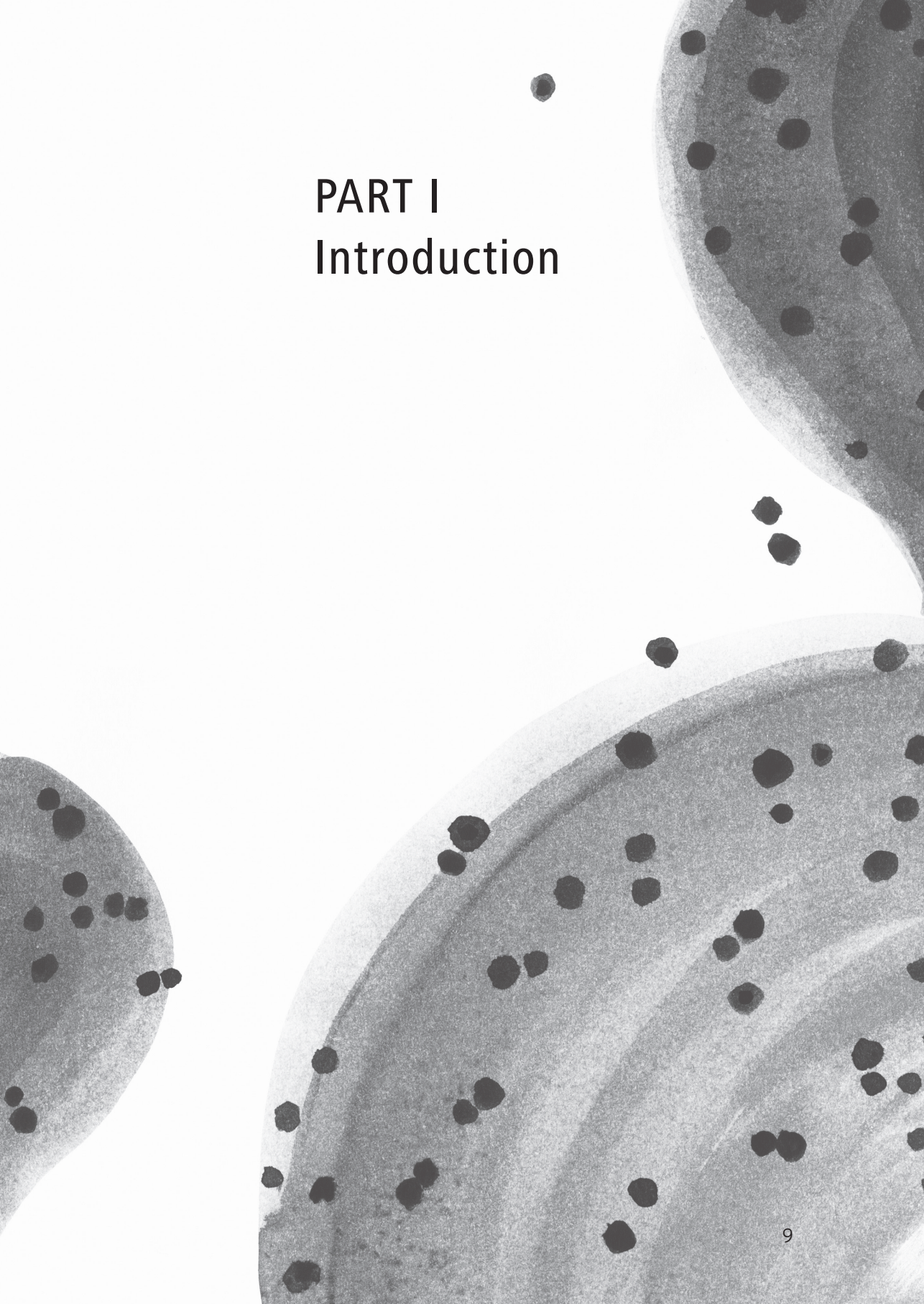
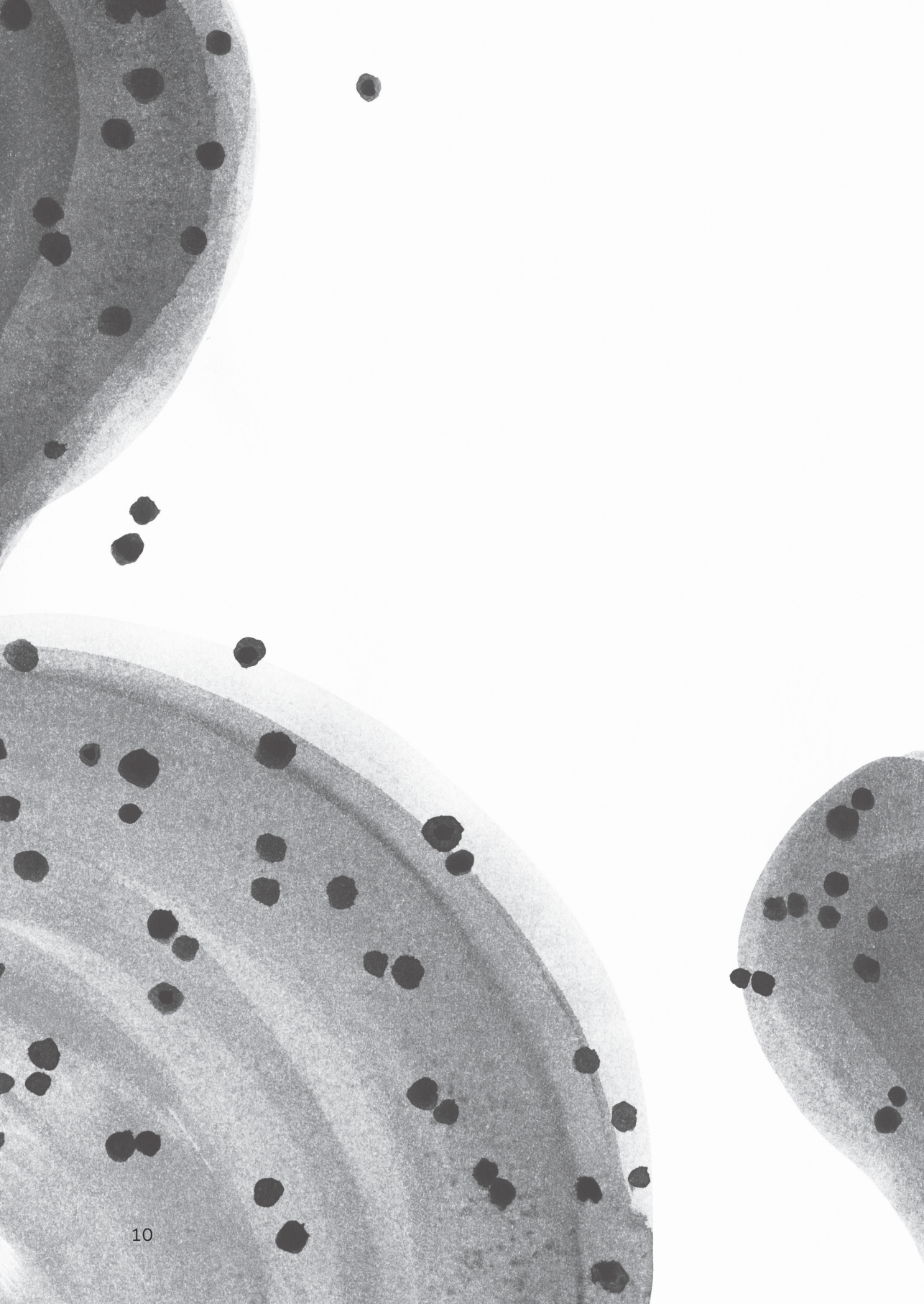
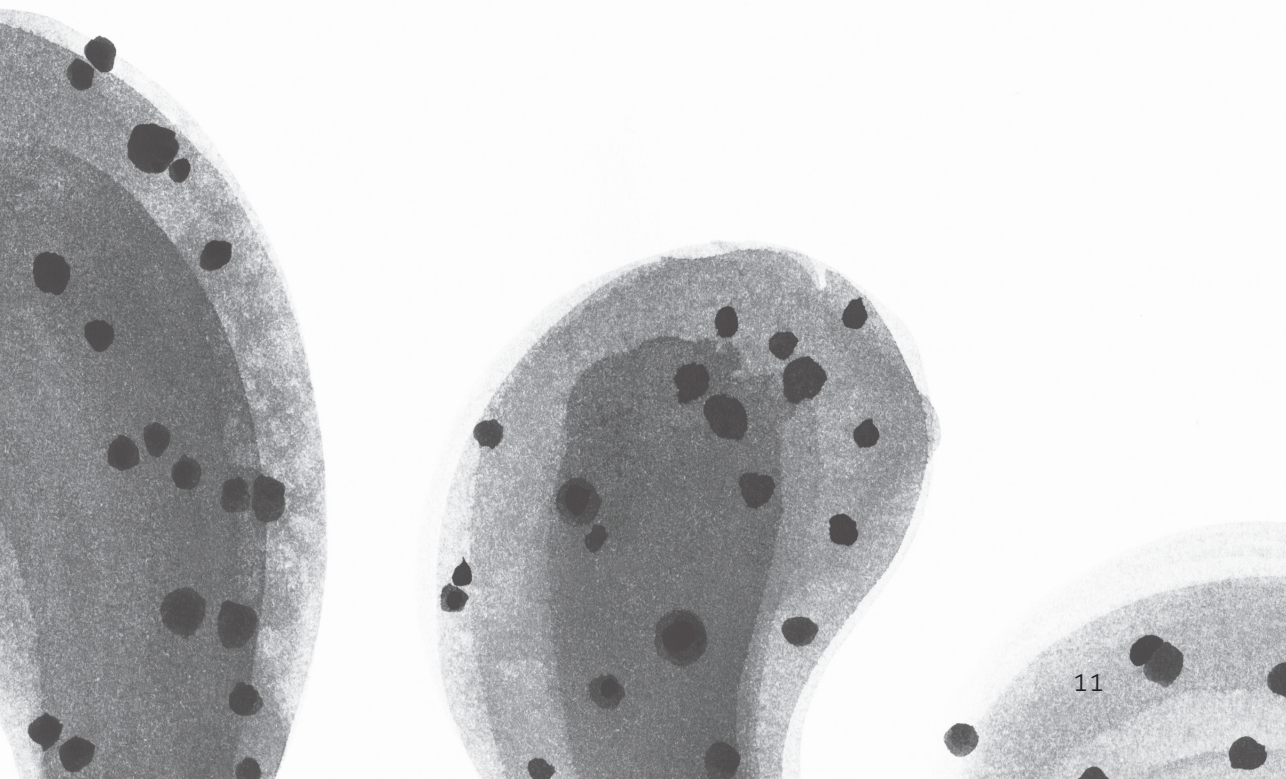


PART I
Introduction





1 Scope and aim of the thesis



Traditional Chinese medicine (TCM) covers different form of therapeutic interventions, and next to meditation, exercises, special diets, acupuncture and acupressure techniques, the use of herbal remedies (Chinese Herbal Medicine, CHM) is a major element of public health care.

The TCM theory is based on ancient spontaneous thoughts, and gradually transformed into a theoretical system based on long-term medical practice. TCM systems contain the *Yin-Yang* concept; the five-element theory; the *Qi*, blood and fluid theory; the visceral manifestation theory; the influence of meridians and the *Zheng* differentiation. CHM medication is guided by the TCM concepts and focusses on the meridian regulating effects of individual herbs. Among different categories, astringent and heat-clearing effects are often related to the potential antibacterial effects of CHMs. Heat-clearing CHMs are devoted to the treatment of heat-*Zheng*, which appear in the course of bacterial infections and correlate also with an inflammatory response to infections (for details see **Chapter 2**).

Considering the demand in the Western world for new active molecules, the interest in TCM grew during the last decennia. The Chinese Association of Integrated Chinese and Western Medicine Research was established in Beijing already in 1981 and was renamed the Chinese association of Integrated Medicine (CAIM) in 1990. From the 1980s onwards research identified many opportunities for TCM to be researched according to established methods for the evaluation of (Western) therapeutic concepts. Today the so-called Integrated Health Care clinics employ physicians from different backgrounds that work together to find the best treatment for the individual patient combining traditional and modern approaches.

One of the major challenges in modern health care is the emergence of antimicrobial resistance and the stagnating development of new classes of antibiotics. This situation has also fuelled the interest in resources for new active molecules, and resulted in an increased interest in experience-based ethno-medicine and the potential identification of new anti-infectives from traditional therapeutic approaches, including CHMs. Herbal remedies used in TCM treatments consist of a large variety of biological active substances (described in more detail in **Chapter 2**). Some of these compounds possess clear antibiotic activities, but their absorption and distribution in the body, and other kinetic characteristics are largely unknown and no exact dosing regimens (in mg/kg body weight) can be deduced from the descriptions of the medical use. Nevertheless, CHMs are still widely and successfully used in Chinese medical care, and their complex nature might be beneficial in the treatment of prevention of complex biological processes, including the formation of antimicrobial biofilms. Biofilm formation is a multi-stage process that cannot be addressed by a single (antibiotic) substance, and hence is considered as a potential target of CHMs. Biofilm formation is also one of the major reasons for therapy resistance and therapy failure in the case of chronic infections

(see **Chapter 3**). Biofilm embedded bacteria are not only protected by an extracellular polymeric matrix hampering the diffusion of antibiotics, but more importantly, biofilm bacteria exhibit a dormant stage, characterized by minimal synthetic processes. Hence, antibiotics targeting the de *novo* synthesis of bacterial cell wall components (penicillins and cephalosporins), bacterial protein synthesis (tetracyclines and macrolides) and bacterial DNA synthesis (sulphonamides) and DNA-replication (fluoroquinolones) become largely ineffective, and act even as danger molecules stimulating the formation of bacterial biofilm at sub-inhibitory concentrations. Only much higher concentrations of certain antibiotics, such as sulphonamides, ketolides and fluoroquinolones, seem to be effective in the treatment of biofilm bacteria. The difference between a MIC and MBIC (minimal biofilm inhibitory concentration), can reach a factor of 1000 and such high concentrations cannot be achieved under *in vivo* conditions.

A promising approach seems to combine common antibiotics with multi-component herbal remedies that interfere with bacterial quorum sensing (the first step of biofilm formation) or with other components of biofilm architecture. Hence, one of the main aims of this thesis was to investigate the anti-biofilm effect of selected CHMs. A summary of the current knowledge of the effects of CHMs on bacterial biofilms is incorporated in **Chapter 2**, in which the theoretical aspects of CHM applications are presented, which guided the selection of the substances used for the biofilm experiments.

Among the different biofilm forming bacteria, the Gram-positive coagulase-negative bacterium *Staphylococcus epidermidis* is often used as a model. *S. epidermidis* is a facultative pathogen and known to form biofilms on biological surfaces such as the skin, but also the alveolar surfaces, resulting in chronic infections that exhibit therapeutic resistance. For *S. epidermidis*, two largely isogenic type strains are available. The wild type rapidly forms biofilms, also under experimental conditions, while its counterpart lacks the ica gene thus being unable to rapidly form biofilms. Hence we used these two *S. epidermidis* bacterial strains as a model to study the effects of selected CHM extracts.

The first step was the establishment of the methodologies applied for the characterization of biofilm formation and inhibition. The standard biofilm assay and the potential effect of different solvents on biofilm formation are summarized in **Chapter 4**.

As a reference point and template for the investigations with herbal products, a broad study was conducted with garlic extracts and the main garlic-derived antimicrobial agent allicin (**Chapter 5**). Garlic is used in almost all parts of the world, including for more than 500 years as CHMs against infections of the gastro-intestinal tract as well as the respiratory tract. Recently the observation that garlic is also effective against methicillin-resistant Staphylococci (*S. aureus*, MRSA) revived the interest in garlic and its active ingredients. In addition, an anti-biofilm activity of garlic on *S. epidermidis* had been reported, but various details were lacking in these previously published data.

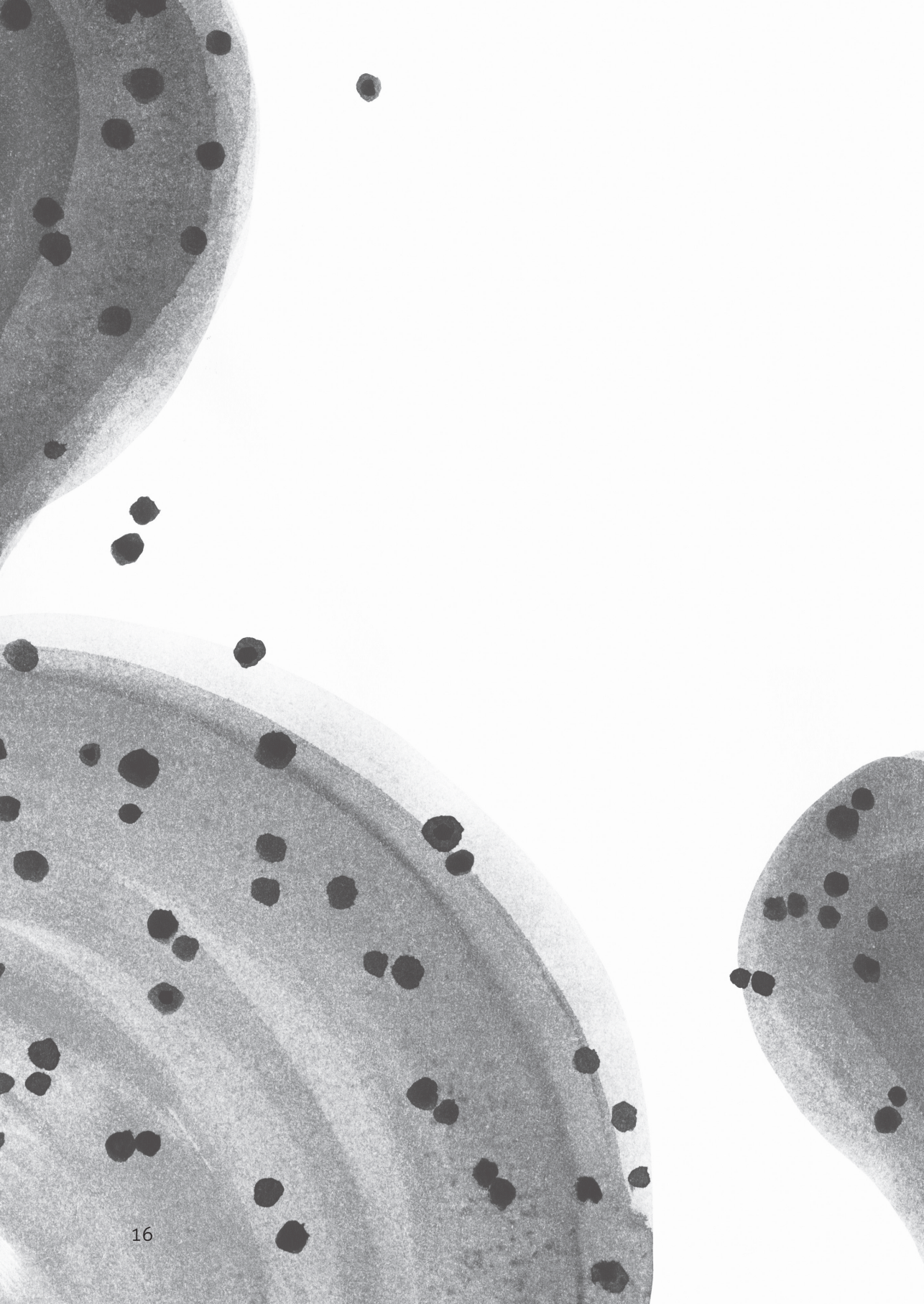
We therefore considered garlic extracts and allicin are a very appropriate model to establish the methodologies for further investigations. The study with garlic extracts and allicin described in **Chapter 5** comprises the measurement of MIC and MBIC values, as well as the morphological characterization of (affected) biofilms with confocal laser scanning microscopy to show the biofilm architecture. The study was completed with gene expression profiling addressing the bacterial genes characteristic of *S. epidermidis* biofilm formation.

In **Chapter 6**, comparative investigation of the CHM potency to affect bacterial biofilm formation is presented, using the above mentioned methodology. These herbal products were selected according to the principles of TCM. Heat-clearing CHMs are often used to treat a heat *Zheng* (pattern), which has similar symptoms as bacterial infections, such as redness, tissue edema, heat (including fever) and pain. Thus, heat-clearing CHMs were selected for our study with *S. epidermidis* biofilms, and the effects of the selected CHMs on *S. epidermidis* biofilm formation are presented.

In consideration of the inflammatory response observed during bacterial infections, a model was established with murine macrophages to assess the potential anti-inflammatory of the selected CHMs, and the first results of two heat-clearing CHMs are presented in **Chapter 7**.

The safety of Chinese Herbal Medicine has been critically evaluated, as many herbs are known for their ability to take up and accumulate heavy metals, including cadmium, which is a common soil contaminant in China. As a cadmium contamination of herbal products cannot be excluded entirely, we wanted to explore the effect of metals on biofilm formation in more detail. In an initial screening (**Chapter 8**) the effects of different bivalent metals on biofilm formation was investigated. In **Chapter 9**, the biofilm-stimulating effect of cadmium is described in more detail.

Considering again the potential toxicity of cadmium contaminated herbs and food supplies, in **Chapter 10**, the effect of selected CHMs on the direct cytotoxicity of cadmium to HepG2 cells is described.

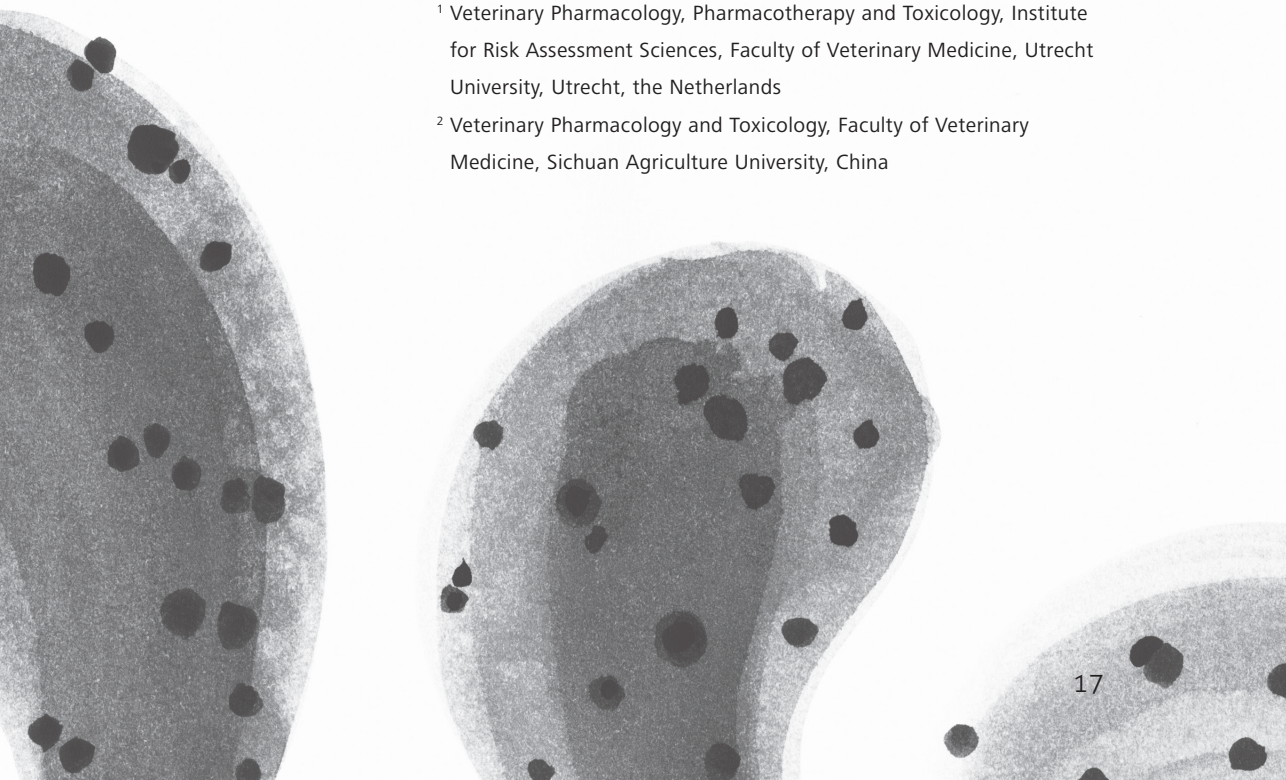


2 Traditional Chinese Medicine: Theory and possible applications of herbal extracts against bacterial biofilms

X. Wu[1], Y. Li[2]

[1] Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

[2] Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Sichuan Agriculture University, China



Traditional Chinese medicine (TCM) theory is based on ancient spontaneous thought, gradually formed into a theoretical system of medicine through long-term medical practice. The TCM systems contain different theories, amongst which are *Yin-Yang* theory, five-element theory, *Qi*, blood, fluid theory, visceral manifestation theory as well as meridians and *Zheng* differentiation. The traditional use of Chinese medicines (CHMs) is guided by the TCM theory, which focuses on the evaluation and regulation of meridians by individual herbs. Among different categories of CHM, astringent and heat-clearing effects are often related to their potential antibacterial effects. Moreover, heat-clearing CHMs are devoted to treat heat-*Zheng*, which appears in the course of bacterial infections and correlates with an inflammatory response. Hence, in the present study we summarized six main concepts of TCM and reviewed the antimicrobial and anti-biofilm effects of one astringent (*Galla chinensis)* and seven heat-clearing CHMs (*Coptis teeta, Forsythia suspense, Isatis indigotica, Lonicera japonica, Scutellaria baicalensis, Taraxacum mongolicum*, and *Viola Yedoensis*).

INTRODUCTION

The science behind traditional Chinese Medicine (TCM) has often been questioned when relating it to therapy concepts used in modern, Western medicine. The word *science* originates from Latin word *scientia*, meaning knowledge (Harper, 2014). For instance, science is used for observations and experiments to understand the physical and natural world (Science, 2014). TCM is a life science with Chinese characteristics (Yang and Wang, 2013) based on the knowledge of Chinese people after thousands of years' experience and experiments. In this sense, it definitely meets the criteria of science. However, different from Western natural science, the basis of TCM is traditional Chinese philosophy. The core of TCM theory considers the human body as a unitary system and it is far more than the simple application of herbs or herbal extracts. The application of Chinese Herbal Medicine (CHM) is under the guidance of the TCM theory systems. This system includes, but is not limited to: the *Ying-Yang* concept; the five-element theory; *Qi*, blood and body fluid theory; and the role of meridians and *Zheng* (pattern) differentiation (Song et al., 2013). Using these TCM theories, Chinese people have successfully treated diseases for a long time, including chronic infections caused by pathogenic or non-pathogenic bacteria.

Recent investigations consider the use of certain CHM as replacements for antibiotics. This approach is driven by the need for new active substances to counteract antimicrobial resistance. An alternative approach is to use such multi-component CHMs as adjuvants to common antibiotics, with the aim to improve their efficacy and to prevent the rapid development of resistance in the course of a long-term therapy. One of the most promising targets is the use of herbal extracts to prevent bacterial biofilm formation, a mechanism that results in therapy resistance. The ability to form biofilms has been observed in almost all bacterial species, including major pathogens, such as *S. aureus, S. epidermis, E.coli,* and *P. aeruginosa*. Biofilm embedded bacteria are resistant to common therapeutic concentrations of antibiotics as they are protected by a polymeric matrix and remain in a dormant stage. A major risk factor in biofilm formation is, however, the fact that within a biofilm generic information and mobile DNA elements are exchanged between the bacterial communities. In this way, the bacterial biofilm contributes significantly to the spread of resistance. It has been estimated that worldwide 65% of human bacterial infections involve biofilms worldwide (Potera, 1999).

In the present review, we first introduced the main ideas of TCM and its understanding of diseases, as this concept guided the selection of the CHMs used in this study. In the second part a description of the selected CHMs are given which includes, where appropriate, the current knowledge of their antimicrobial and anti-biofilm effects.

CONCEPTS APPLIED IN TRADITIONAL CHINESE MEDICINE

The basic of TCM theory can be divided into five main parts: *Yin-Yang* theory; five-element theory; *Qi*, blood, fluid theory; visceral manifestation theory; meridians and *Zheng* differentiation. CHM medication is under the guidance of such theories, focusing on the test and meridian regulation of individual herbs, *Guijing* (meridian tropism). In the following sections we summarized the main concepts of TCM and tried to bridge the gap between western and eastern medicine.

Ying-Yang

Traditional Chinese philosophy considers human and nature in integration; life is a holistic, dynamic, spiritual and functional unity; disease is a disorder of the human functional balance, a balance controlled by *Yin-Yang*, representing a unity of opposites (Sun et al., 2013).

The original description of *Yin-Yang* is two sides of the sun, *Yin* is back to the sun and *Yang* is facing the sun, the relationship between them is not absolute but relative (Figure 1). For better understanding the function of *Yin-Yang* in TCM, four aspects of their relationship are introduced as follows. Firstly, *Yin* and *Yang* are interdependently, they "rooted" into each other, and cannot exist alone. For example, there is no cold (*Yin*) without heat (*Yang*), no up (*Yang*) without down (*Yin*) and vice versa. Secondly, *Yin* and *Yang* are mutually restricted as they control each other to ensure that another party will not be too assertive. Thirdly, *Yin* and *Yang* trading off and taking turns: one aspect wanes, the other waxes. Seasons' changing is a good example to show this concept. For instance, from the winter to spring, weather is changing form cold (*Yin*) to heat (*Yang*), which is *Yin* declining when *Yang* growing; form summer to autumn, weather is changing from heat (*Yang*) to cold (*Yin*), which is *Yang* declining when *Yin* growing (Dang, 2002). Finally, the reciprocal transformation is also an important character of *Yin-Yang* that presents things will develop in the opposite direction when they become extreme. Just like Huang Di said in *"Su Wen": Zhong yin bi yang, zhong yang bi yin* meaning extreme *Yin* will turn in to *Yang* and vice versa.

Yin-Yang conception plays an important role in the TCM theory, and explains the physiological functions of the human body and disease development as well as serves as a guide towards a clinical diagnosis and treatment. In physiology, *Yin* represents the lower body, the interior, the chest, the abdomen, the Z and the nutrients while *Yang* represents the upper body, the exterior, the back, the bowel and the organ function (Liu and Xu, 2002). Pathologically, in TCM, disease is caused by an internal imbalance of *Yin* and *Yang*. Predominant *Yang* induces *Yin* disorder and vice versa; only when *Yin* is in peace and *Yang* is compact, the spirit can be normal (Li, 2008c). For example: heat shock represents *Yin* deficiency *Zheng* in TCM, with patients showing the following

clinical signs, sweating, weak and vomit caused by high temperature in the summer. This high temperature is considered as predominant *Yang*. On the disease diagnose and treatment, TCM uses the *Yin-Yang* theory to classify different symptoms, for example, red/yellow tongue indicates *Yang Zheng* while white/blue indicates *Yin Zheng*. The treatment of disease is following the principle of adjust *Yin* and *Yang* disharmony, which favors the idea of prevention first by maintaining health and wellness before disease occurs (Sun et al., 2013). CHMs also can be distinguished by their *Yin-Yang* character, such as the sour/bitter test herbs belong to *Yin* and the sweet/pungent test herbs belong to *Yang* (Liu and Xu, 2002).

Five-element

Ancient Chinese believes indicate that our world is composed of five materials: wood, fire, earth, metal and water. They are the origin of the TCM five-element theory: *Wu Xing*. *Wu* means five which represents the five elements and Xing means the dynamic changes and interactions between these five elements (Figure 2). The nature of wood, fire, earth, metal and water are growing, warm/upward, bearing/receiving, descending/restraining and cold/moist, respectively. They represent different tastes, colors, seasons, directions, Zang-viscera, *Fu*-viscera, facial features and emotions (Liu and *Xu*, 2002) (Table 1).

The five-element theory is used as a model to investigate the human body in TCM. Using *Zang*-viscera (parenchymatous organs with functions of metaplasia and storage, will be introduced in chapter *Visceral manifestation theory*) as an example, the liver belongs to wood because it is a metaplastic organ, fitting the nature of growing; the

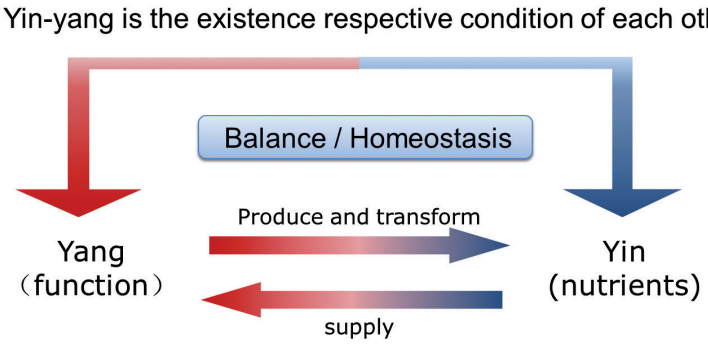


Figure 1. The relative relationship between *Yin* and *Yang*. Red part represents *Yang*, blue part represents *Yin*.

heart belongs to fire due to its pump function which can be seen as a pushing up character; the spleen belongs to earth because it stores red blood cells and lymphocytes; the lung belongs to metal because it's main work is purify the air and descend the oxygen to the blood; and the kidney belongs to water because it is a body liquid filter.

The main relationships between these five elements are engendering, restraining, inhibition/generation, over-restriction, counter-restriction and transaction between "mother" and "child" element (influence of one element exerted on another one that it engenders or that engenders it sequentially) (Li, 2008a) (Figure 2). Zhang Zhongjin said at the beginning of the third century: *"Jian gan zhi bing, zhi gan chuan pi, dang xian shi pi"*, which means when you see a liver disorder, you should know that liver restrains spleen, and that the treatment should start with spleen reinforcement. Understanding the relationships of these five elements is a crucial step during the TCM treatment of a certain disease.

Qi, blood and body fluid theory

In the early Qin Dynasty, the philosophy of "primordial qi theory" is the beginning of traditional Chinese sciences, considering the primitive world as a holistic unity of *Qi* (Sun et al., 2013). *Qi* means gas, anger or atmosphere in Chinese. In ancient China, physicians considered not only natural gases (inhaled air) but also an invisible energy/force capable of diffusing solid matter (blood and body liquid) within the body as *Qi*. The function on *Qi* includes the forces necessary to move the blood through our blood vessels, and at the same time *Qi* will warm our organs, muscles and skin; subsequently a defending *Qi* is built to prevent the invasion of external pathogenic factors. Moreover, *Qi* is also controlling the blood circulation within the vessels and the body liquid i.e. urine, sweat and saliva secretion under normal circumstance. *Qi* also has an effect on metabolism and is involved in transforming food into nutrients and waste. According to the *Yin-Yang* theory, *Qi* is mobile and in charge of moving and warming things which is attributed to *Yang*. Different from *Qi*, blood and body fluids are attributed to *yin*, because they are responsible for nourishing and moistening things (Reninger, 2014). Based on TCM, they are derived from the same sources, which are food and water. Overall, *Qi*, blood and body fluid is an indivisible unity: *Qi* drives blood and body fluid, while blood and body fluid bear *Qi* (support the *Qi* generation), hence *Qi* is the commander of blood and body fluid and lives with them.

Visceral manifestation theory

The term "visceral manifestation" has been described first in *Huangdi's Internal Classic* (*Huangdi Neijing*), which is the earliest existing TCM theory book. Ancient Chinese physicians divided viscera in three kinds: *Zang*-viscera, *Fu*-viscera and extraordinary organs (Li, 2008b). *Zang*-viscera refers to parenchymatous organs, which have functions

Table 1. *Five-element* classification of common things.

Five elements	wood	fire	earth	metal	water
taste	sour	bitter	sweet	pungent	salty
color	blue	red	yellow	white	black
season	spring	summer	late summer	autumn	winter
direction	east	south	center	west	north
***Zang*-viscera**	liver	heart	spleen	lung	kidney
***Fu*-viscera**	gallbladder	small intestine	stomach	large intestine	bladder
facial features	eye	tongue	mouth	nose	ear
emotion	angry	happy	miss	sad	fear

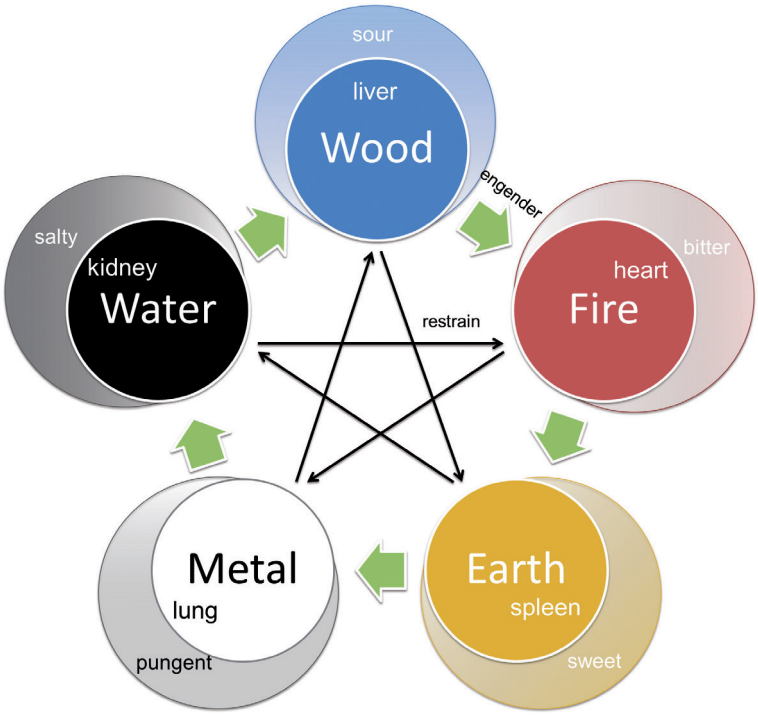


Figure 2. Interactions of five elements. Engender: the relationship in which each element and its associated phenomena give rise to or promote another sequential element. Restrain: the relationship in which each element and its associated phenomena restrict/control another element.

of metaplasia and storage, including the heart, liver, spleen, lung and kidney; *Fu*-viscera refers to luminal organs, which have functions of receiving and transforming, including the gallbladder, stomach, small intestine, large intestine, and bladder; extraordinary organs refer to those organs with the morphology of *Fu*-viscera and the function of *Zang*-viscera, including brain, marrow, bone, vessel and uterus (Liu and Xu, 2002).

The relationship between different *Zang*-viscera and *Fu*-viscera can be explained by the five-element theory (Ma et al., 2014). For example, *Zang*-viscera heart and kidney's characters fit with fire and water, respectively (Table 1). Under normal physiological conditions, fire (heart) is restrained by water (kidney), they are in a dynamic equilibrium. Once the kidney is injured, the water cannot control fire well, inducing hyperactive heart fire then causing morbid symptoms, e.g. cardiopalmus and insomnia.

Meridian tropism

The meridian system (channel network, *Jingluo*) is a definition of paths or channels used for *Qi* flows running through the body. This system is used for acupuncture, moxibustion and medication in TCM (Liu and Xu, 2002). The World Health Organization (WHO) indicated that in total there are fourteen main meridians and eight extra meridians in the meridian system (World Health Organization, 1991). There are only twelve main meridians in TCM and their naming principles are indicated in table 2. The WHO included the governor vessel and the conception vessel into to the main meridians system and this is an overlapping with the eight extra meridians (governor vessel, conception vessel, thoroughfare vessel, belt vessel, *Yin* heel vessel, *Yang* heel vessel, *Yin* link vessel and *Yang* link vessel). The existences of these vessels have been proven, but the anatomic structure of them remains unclear (Hempel et al., 2014). In TCM however, physiology is more important than anatomy and function is more important than structure (Chang, 2012).

The application of the meridian system in TCM refers to *Guijing* (meridian tropism) which means that CHMs often produce their therapeutic effects on portions of the body via their *Guijing* nature. *Guijing* is the bridge between TCM theory and clinical medication and is developed in the Ming dynasty (1368–1644), and is included in the Compendium of Materia Medica (*Ben Cao Gang Mu*) (Xu et al., 2012). It is the most important herbal description and clinical principle in TCM. For example, the heat-clearing CHMs *Forsythia Suspensa's Guijing* is heart, lung and small intestine meridian, which means that this CHM is mainly used to treat heat-*Zheng* from upper parts of the body.

Zheng differentiation

The effectiveness of TCM treatment (acupuncture or herbal formulae) dependent on the accuracy of *Zheng* (pattern or symptom) differentiation. The reason why different medicines and formulas are used to treat the same disease depends on the different

processions of a certain stage or on the individual's symptoms (Song et al., 2013). For this, four diagnostic methods (inspection, listening and smelling, inquiring, and palpation) are used to identify different *Zheng* (Ryu et al., 2010). The principals of *Zheng* consist four main pairs being *Yin* and *Yang*, exterior and interior, cold and heat and deficiency and excess. For example, *Yin* and *Yang* pattern identification categorizes syndromes according to *Yin-Yang* theory. A collective term for exterior, heat and excess patterns with excitatory, hyperfictional, restless or bright manifestations, or outward and upward symptoms, as well as morbid conditions caused by pathogenic factors of a *Yang* nature is *Yang Zheng*, opposed by *Yin Zheng* (Li, 2010). However, *Zheng* differentiation might be subjective because it mainly relies on the experiential verdict of TCM practitioners to estimate and read symptoms, tongue appearance, and pulse of patients (Hogeboom et al., 2001). Studies have been performed to standardize this TCM diagnose and treatment methods on different subjects, such as post-genomics medicine (Wang and Chen, 2013), coronary heart disease (Guo et al., 2013) and chronic hepatitis (Zhang et al., 2013).

TCM MEDICATION

CHMs are categorized into fifteen groups: diaphoretics (*Jiebiao*), heat-clearing (*Qingre*), purgatives (*Xiexia*), digestants (*Xiaodao*), relieve cough and reduce sputum (*Zhike-Huatan*), internal cold-dispelling (*Wenli*), dampness-dissolving (*Qushi*), regulating *Qi* (*Liqi*), blood-regulating (*Lixue*), astringent (*Shouse*), tonic (*Buxue*), liver-pacify*in*g (*Pinggan*), tranquilizers (*Anshen*), insect repellents (*Quchong*) and externally applied agents (*Waiyong*). According to the TCM theory, heat-*Zheng* should be distributed by cool/cold medicine. Bacterial infections cause erythema, swelling, heat and pain fitting with heat-*Zheng*. Astringent and heat-clearing CHMs are mostly cold in nature and are used to clear away heat, purge fire, dry dampness and cool blood (Muluye et al., 2014). Their main TCM characteristics are presented in Table 3.

Biofilms as a target for the use of selected CHMs

Heat-clearing CHMs have been investigated for their antimicrobial activity. Moreover, astringent and heat-clearing CHMs are considered to be potential anti-biofilm candidates. A biofilm is a sessile bacterial community composed of one or more bacterial species. They are visible as dental plaques, are important to the protective intestinal microbiome, but are detrimental when pathogens form biofilms on indwelling catheters, wounds, or on internal biological surfaces such as the alveolar space, the urinary bladder or the endocardium (for details see Chapter 3). Biofilm formation is induced by environmental stress factors, including shear-stress, but also by chemical substances, such as antibiotics or heavy metals. Here we describe the traditional use,

antimicrobial, and antibiofilm activities of the selected astringent (*Galla Chinensis*) and seven heat-clearing CHMs (*Coptis teeta, Forsythia suspense, Isatis indigotica, Lonicera japonica, Scutellaria baicalensis, Taraxacum mongolicum*, and *Viola Yedoensis*).

Galla chinensis

Galla chinensis is the gall produced by some parasitic aphids (family Pemphigidae) on the Rhus leaves of the family Anacardiaceae, mainly *Rhus chinensis* Mill., *Rhus potaninii* Maxim, and *Rhus punjabensis* var. (Tian et al., 2009). The gall needs to be boiled and broken into pieces before it can be used as a CHM. *G. chinensis's* TCM characters are sour and neutral (between warm and cool), its meridian tropism is lung, stomach and large intestine. Traditionally *G. chinensis* is often used to treat cough, diarrhea, bleeding and pus sores (Jiangsu New Medical College, 1986) .

G. chinensis is rich in gallotannins, and has nearly 20% gallic acid and 7% methyl gallate (Djakpo and Yao, 2010). The main organic composition of *G. chinensis* water extract is gallic acid (71.3±0.2% w/w) and its isomer, and, to a lesser extent, small molecule gallotannins (Huang et al., 2012). For quantitative control *G. chinensis* should contain gallic acid > 50% (Chinese Pharmacopoeia Commission, 2005). Antimicrobial activity of *G. chinensis* was reported against *Staphylococcus aureus* (1.25–1.75 mg/ml water extract), *Pseudomonas aeruginosa* (1mg/ml gallic acid) and *Escherichia coli* (5mg/ml gallic acid) (Borges et al., 2012; Huang et al., 2012; Tian et al., 2009). Anti-biofilm effect studies with *G. chinensis* mostly focused on dental bacteria, where an oral multispecies biofilm was inhibited by 4 mg/ml *G. chinensis* water extract (Xie et al., 2008). It was also reported that two compounds present in the extractions (methyl gallate and gallic acid) can inhibit the biofilm formation of *Streptococcus mutans* (Kang et al., 2008). Only limited studies reported 4 mg/ml gallic acid as an inhibitor of *S. aureus* biofilm formation (Luis et al., 2014), and at lower concentration (1 mg/ml) it was able to counteract *P. aeruginosa* and *E. coli* biofilm (Borges et al., 2012).

Coptis teeta

Coptis teeta Wall. is a flowering plant in the Buttercup family. Its root is used as CHM after being dried. *C. teeta's* TCM characteristics are bitter and cold, its meridian tropism is heart, liver, stomach, and large intestine. Traditionally *C. teeta* is often used to treat heat *Zheng* of the stomach and diarrhea (Jiangsu New Medical College, 1986) .

C. teeta contains different alkaloids, such as berberine, methyl coptisine, and palmatine. For quantitative control, *C. teeta* should contain berberine >3.6% (Chinese Pharmacopoeia Commission, 2005). *C. chinensis* (from the same genus of *C. teeta*) exhibited antibacterial effects on *S. aureus* (Feng et al., 2011) and a range of other organisms including *Salmonella typhi, Serratia marcescens, Vibrio cholerae, V. parahaemolyticus, P. aeruginosa,* and *E. faecalis* (Mekseepralard et al., 2010).

Table 2. Twelve main meridians in TCM

Operation site (*Yin* meridian runs inside; *Yang* meridian runs outside)		*Yin* meridian	*Yang* meridian
fore body	front edge	lung meridian	large intestine meridian
	middle	pericardium meridian	triple energizer meridian
	trailing edge	heart meridian	small intestine meridian
after the body	front edge	spleen meridian	stomach meridian
	middle	liver meridian	gallbladder meridian
	trailing edge	kidney meridian	bladder meridian

Table 3. The characteristics (nature, test and meridian) of candidate CHM and their main components

Species	Nature	Test	Meridian regulation
Coptis teeta Wall.	cold	bitter	heart, liver, stomach and large intestine meridian
Forsythia Suspensa (Thunb.) Vahl.	cold	bitter	heart, lung and small intestine meridian
Galla Chinensis	cold	sour	lung, kidney and large intestine meridian
Isatis indigotica Fort.	cold	bitter	heart and lung meridian
Lonicera japonica Thunb.	cold	sweet	lung, stomach and large intestine meridian
Scutellaria baicalensis Georgi.	cold	bitter	lung, gallbladder, spleen, large intestine and small intestine meridian
Taraxacum mongolicum Hand-Mazz.	cold	sweet	liver and stomach meridian
Viola yedoensis Makin.	cold	bitter	heart and liver meridian

For the anti-biofilm study, only berberine, the main compound of *C. teeta*, was found to inhibit *S. epidermidis* bacterial attachment and biofilm formation (Wang et al., 2009).

Forsythia Suspensa

Forsythia suspensa Thunb. Vahl is a striking yellow flowering plant originating from China and belongs to the Oleaceae family. Its fruit is used as CHM after been twisted open and dried. *F. suspensa's* TCM characters are bitter and cool, its meridian tropism is heart, lung and gallbladder. Traditionally *F. suspense* is often used to treat heat *Zheng*, swollen and erysipelas (Jiangsu New Medical College, 1986). Over 40 Chinese medicinal preparations containing *F. suspensa* are listed in Chinese Pharmacopoeia, such as *Shuanghuanglian* oral solution, *Yinqiao Jiedu* tablet and *Qinlian* tablet (Chinese Pharmacopoeia Commission, 2005).

Several compounds have been isolated from *F. suspensa* including caffeoyl glycosides, cyclohexylethanes, flavonoids, iridoid glycosides, lignans, and triterpenes (Chang et al., 2008). For quantitative control, *F. suspensa* should contain >0.15% forsythin (Chinese Pharmacopoeia Commission, 2005). *F. suspensa* has antibacterial activities against *S. aureus, E. coli* and *P. aeruginosa* (Nishibe et al., 1982) and its water extract was reported to inhibit *S. epidermidis* (1:40 diluted) (Li et al., 2000). No anti-biofilm study has been done on this CHM. However, a CHM injection, TanRe*Qi*ng (TRQ) was able to not only inhibit the formation of *S.aureus* biofilm but also killed the living cells embed in the biofilm matrix (Wang et al., 2011). This injection is the combination of water extracts from five CHMs (*S. baicalensis, Bear gall powder, Goral horn, L. japonica,* and *F. suspensa*). The main indications of this reagent are acute upper respiratory infections and early stages of pneumonia which are a typical biofilm related diseases.

Isatis indigotica

Isatis indigotica refers to the rhizome of a flowering plant in the family Brassicaceae. Its dried root is used as CHM. *I. indigotica's* TCM characters are bitter and cold, its meridian tropism is liver and stomach. Traditionally *I. indigotica* is often used to clear heat-toxicity, cool blood, and relieve sore throat (Jiangsu New Medical College, 1986).

Indigotin, indirubin, isatin, isaindigotidione, organic acids, and aminoacids were isolated from the *I. indigotica* root (Wu et al., 1997). In the aqueous extract, cytidine, hypoxanthine, uridine, xanthine and guanosine were identified (Liu et al., 2005). The extracts of this CHM show antibacterial activity against *S. aureus* (Zhao et al., 2006). These authors compared different Isatis root extracts on their antibacterial effects and indicated that Isatis root exerts its antibacterial effects by cooperation of different active fractions instead of a single compound. No data is available on *I. indigotica* activity against bacterial biofilms.

Lonicera Japonica

Lonicera japonica is a species of the honeysuckle. Its flower is used as CHM after being dried. *L. japonica's* TCM characters are sweet and cold, its meridian tropism is lung and stomach. Traditionally *L. japonica* is often used to treat heat *Zheng*, bleeding, sores and abscess due to heat-toxicity, swollen and sore throat, and dysentery (Jiangsu New Medical College, 1986).

Over 140 compounds have been isolated and characterized from *L. japonica* including essential oils, flavones, organic acids, triterpenoid saponins and iridoids. The main compounds in this CHM are linalool, hexadecanoic acid, octadecadienoic acid, ethyl palmitate and dihydrocarveol (Shang et al., 2011). For quantitative control, *L. japonic* should contain chlorogenic acid > 1.5% and galuteolin > 0.1% (Chinese Pharmacopoeia Commission, 2005). It was proven that this CHM has broad-spectrum antimicrobial

activity, for instance, against *S. aureus, S. hemolyticus, E. coli, P. aeruginosa* and *Bacillus* spp.. Flavonoids from *L. japonica* have antibacterial action against methicillin resistant *S. aureus* (Shang et al., 2011). No study has been done to its effect on bacterial biofilm formation. Only one report indicated that *L. japonica* is able to inhibit oral biofilm forming bacteria *Porphyromonas gingivalis* (Wong et al., 2010).

Scutellaria baicalensis

Scutellariae baicalensis Georgi is a labiatae plant. Its root is used as CHM after being dried or fired with wine. *S. baicalensis's* TCM characteristics are bitter and cold, its meridian tropism is lung, gallbladder and large intestine. Traditionally *S. baicalensis* is often used to treat heat *Zheng*, moistening aridity, detoxify*in*g toxicities, bleeding and preventing miscarriage diarrhea. It also can be used together with *F. suspense* and *L. japonica* to disperse pathogenic heat from upper parts of the body (Jiangsu New Medical College, 1986).

Up to now 295 different compounds have been isolated from the genus Scutellariae. Most of them are flavonoids and phenyletnoid glycosides, but also iridoid glycosides, diterpenes, triterpenoids, alkaloids, phytosterols and polysaccharides exist. The main pharmacologic active compounds are baicelein, baicalin, wogonin, wogonoside, oroxylin A, and oroxylin A-7-glucuronide (Shang et al., 2010). For quantitative control, *S. baicalensis* must contain baicalin > 9% (Chinese Pharmacopoeia Commission, 2005). *S. baicalensis* is believed to be beneficial in the treatment of bacterial infections of the respiratory and gastrointestinal tract (Li et al., 2004). Baicalin inhibits apoptosis and has protective effects on mammary gland tissues during *S. aureus*-induced mastitis via reducing TLR2 expression, p53 phosphorylation and regulating the apoptosis-related factors in mammary gland tissues (Guo et al., 2014). Synergies between baicalein & tetracycline and baicalein & Ð-lactams against methicillin-resistant *S. aureus* have been reported (Fujita et al. 2005). Baicalein was indicated to interfere with the quorum sensing system of P. *aeruginosa* (Zeng et al., 2008) and it is able to inhibit *Candida albicans* biofilms (Cao et al., 2008).

Taraxacum Mongolicum

Taraxacum Mongolicum HandMazz. is known as dandelion, it is from the genus of flowering plants in the family *Asteraceae*. The whole plant can be used as CHM after being dried. *T. Mongolicum's* TCM characteristics are bitter/sweet and cold, its meridian tropism is liver and stomach. Traditionally *T. mongolicum* is used to clearing heat, removing toxicity, treat sores and abscesses (both interior and exterior) caused by excessive heat-toxicity. It is an essential herb for treating mammary abscesses caused by heat-toxicity accumulation in liver and stomach (Jiangsu New Medical College, 1986).

The most active family of compounds contained in dandelion is reported to be the saponins. Sesquiterpene lactones, phenylpropanoids and polysaccharides are also found in this CHM. Major sesquiterpene lactones, generally occurring as glycosides, include taraxacosides, taraxacolides, dihydrolactucin, ixerin, taraxinic acids, and ainslioside. Phenylpropanoids include cichoric acid, monocaffeoyltartaric acid, 4-caffoeylquinic acid, chlorogenic acid and caffeic acid (Schutz et al., 2006a). *Taraxacum spp.* was also reported to contain 20.67% (w/w) polysaccharides. Inulin is also present in a large amount in dandelion root (Schutz et al., 2006b; Wang, 2014). For quantitative control, *T. mongolicum* should contain caffeic acid > 0.02% (Chinese Pharmacopoeia Commission, 2005). Dandelion water extract was able to help epithelial cells against *E. coli* infection (Luthje et al., 2011). Oligosaccharides from dandelion have antibacterial activity against *E.coli, B. subtilis* and *S. aureus* (Qian et al., 2014). Water-soluble polysaccharides from the *Taraxacum* specie (100 mg/ml) inhibited *S. aureus* growth (Wang, 2014). However, no bacterial biofilm inhibition effect for this CHM has been reported.

Viola Yedoensis

Viola yedoensis Makin. is a flowering plant in the violet family *Violaceae*. The whole plant can be used as CHM after being dried. *V. yedoensis's* TCM characteristic are bitter and cold, its meridian tropism is heart and liver. Traditionally *V. yedoensis* is used to clearing heat, removing toxicity, cool blood and relieve swelling. It is another commonly-used herb for treating boils, furuncles, sores and abscesses (Jiangsu New Medical College, 1986).

Six bioactive components including coumarins and flavonoids were found in *V. yedoensis* (Hong et al., 2011). Sulphonated carbohydrate polymer, dicoumarin, euphorbetin and esculetin were also isolated from its ethyl acetate extract (Zhou et al., 2009). Quantitative control of this CHM is not specified in the Chinese Pharmacopoeia. Aesculetin, 6,7-dimethoxycoumarin, scopoletin and 5-methoxy-7-hydroxymethylcoumarin, isolates from *V. yedoensis* all exhibited a broad antibacterial activity against *S. aureas, Streptococcus agalactiae, S. uberis, S. dysgalactiae, E. coli* and *Salmonella* spp. (Sun et al., 2011). The petroleum ether and ethyl acetate extracts of this CHM were also indicated having antimicrobial activity against *B. subtilis* and *Pseudomonas syringae* (Xie et al., 2004). No data is available on this CHM's effect on bacterial biofilm, but coumarins were reported to be able to reduce biofilm formation of *E. coli* O157:H7 (Lee et al., 2014) and flavonoids isolated from other plants (lingonberry and *Moringa oleifera* seed coat) also showed antibiofilm activities against *S. aureus, P. aeruginosa, C. albicans* and oral bacteria (Onsare and Arora, 2014; Riihinen et al., 2014).

CONCLUSIONS

TCM is an ancient Chinese medical science devoted to disease prevention and treatment. Different theory systems in TCM are used together to understand and modulate the reactions of the human body to disease conditions. The principles of TCM guide also the use of CHMs. Among the TCM concepts the meridian tropism and *Zheng* differentiation are the most important elements that determine the selection of individual CHM. Based on these considerations, we selected in total 8 CHMs and conducted a literature survey to identify their main properties and their antibacterial and anti-biofilm effects as determined by current standard techniques in bio-medical research. Subsequently, decoctions of these CHM were used in biofilm assays aswell as *in vitro* assays to elucidate their antibacterial, anti-biofilm and anti-inflammatory effects.

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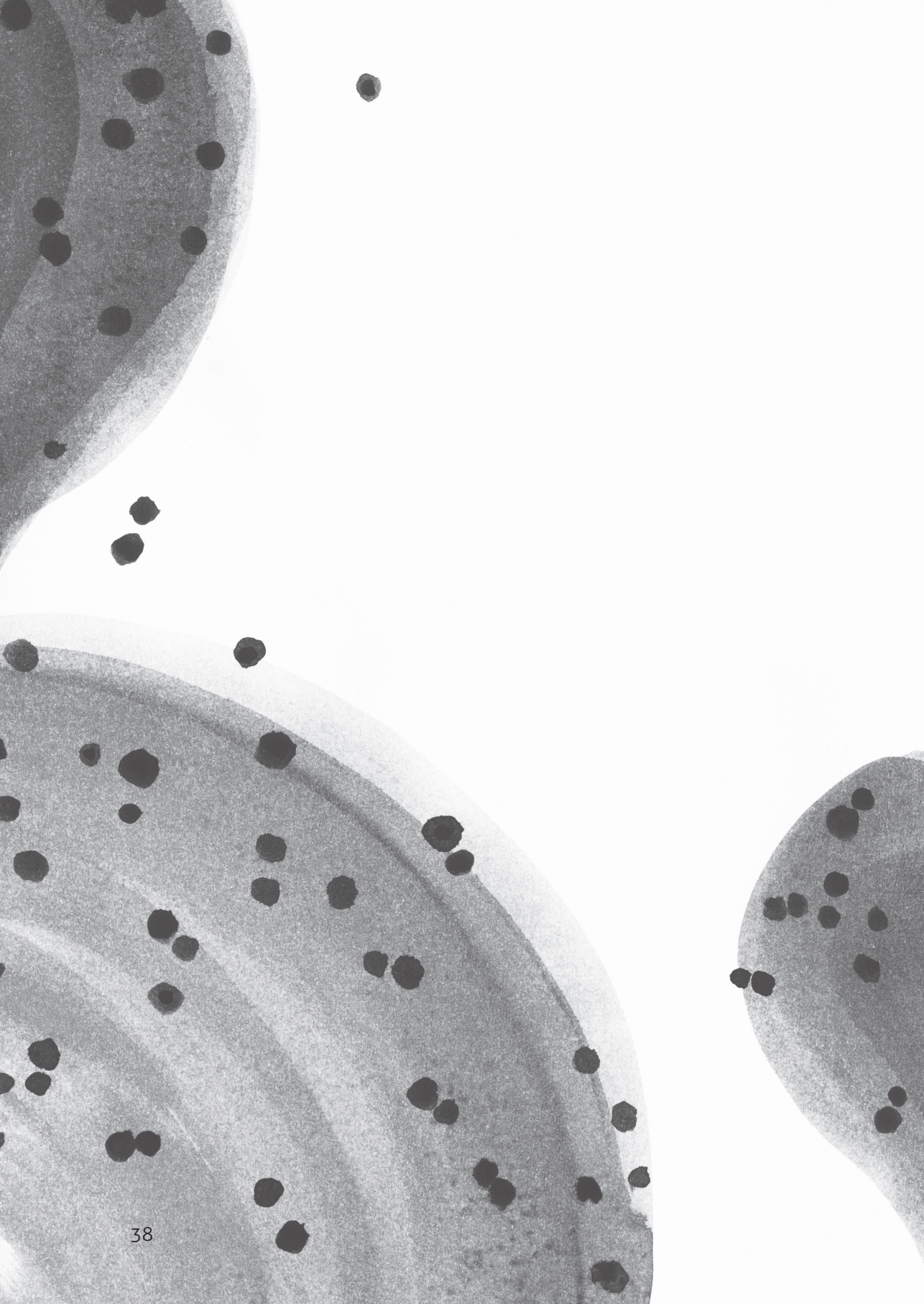

3 Bacterial biofilms: a neglected cause of therapy failure?

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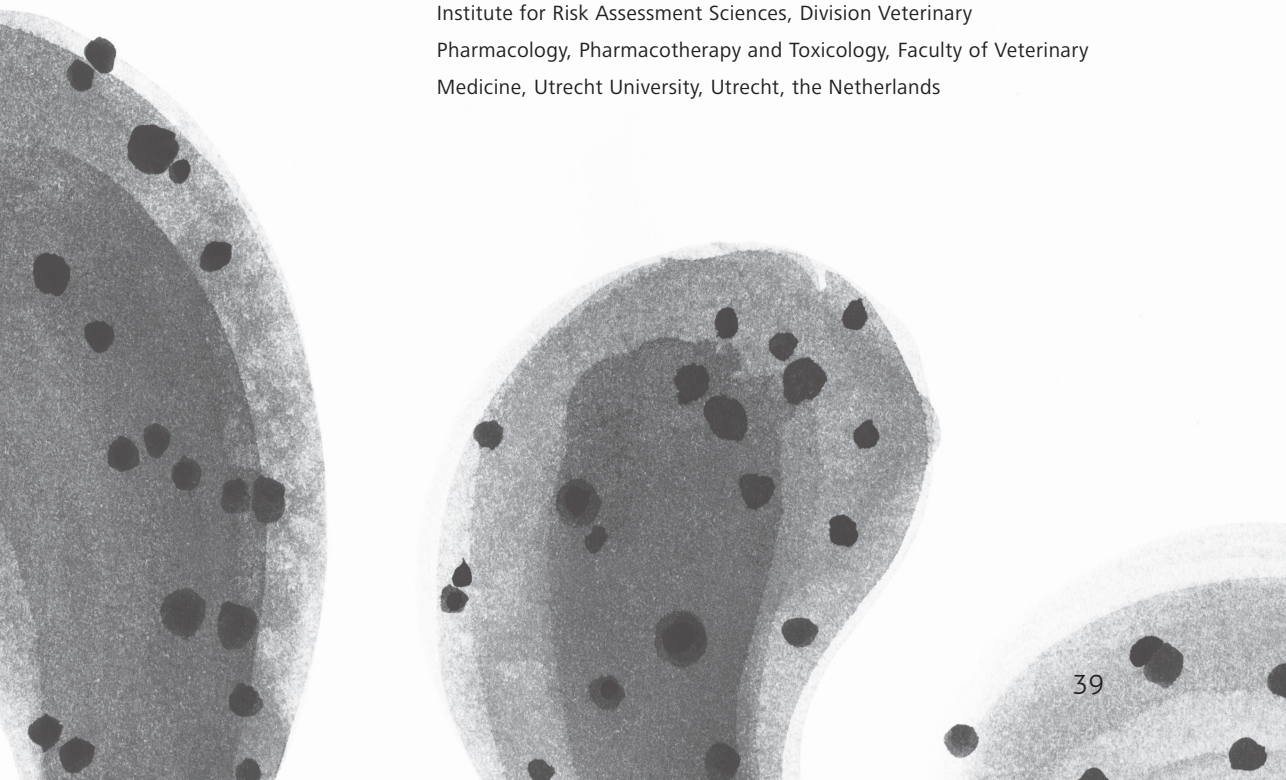
X. Wu, R. R. Santos, J. Fink-Gremmels

Institute for Risk Assessment Sciences, Division Veterinary Pharmacology, Pharmacotherapy and Toxicology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands



Bacterial biofilms are of increasing concern as they confer a barrier protection to pathogenic bacteria against host defence mechanisms and antibiotics. This barrier includes the formation of a polymeric extracellular matrix, as well as significant changes in gene expression during biofilm formation, transferring bacteria into a dormant stage, which makes several major classes of antibiotics interacting with cell membrane or protein synthesis ineffective. As the extracellular matrix is not recognized by the host immune system, the poor response to common antibiotic therapies and host defence mechanisms favours the development of chronic and recurrent infections. The present review aims to summarize the current knowledge on biofilm formation associated with common veterinary pathogens, providing also a summary of the genotypic changes that occur during biofilm formation that may be considered as biomarkers in clinical investigations. The most prominent example for recurrent infections associated with biofilm formation is *Staphylococcus aureus* infections, a major health concern in dairy cattle. In small ruminants, the ability of *Listeria* spp. to form biofilms is of animal and public health concern. Contagious pleuro-pneumonia and *Escherichia coli* infections are the major disease conditions associated with biofilm formation in pigs. Comparably, biofilm formation of avian pathogenic *E.coli* is an important risk factor in poultry health. A delay in wound healing in horses as well as in pet animals (dogs and cats) has been attributed to bacterial biofilms as well. Considering the obvious importance of bacterial biofilm, several approaches to prevent biofilm formation or dissolve existing biofilms are discussed, including among others enzymes derived from bacteriophages as well as plant-derived essential oils and related quorum sensing inhibitors.

INTRODUCTION

In veterinary practice, the failure of antibiotic therapy in chronic infections is of increasing concern. Therapy resistance can be caused by the inappropriate selection of the antibiotic agent, pathophysiological changes that influence the kinetics of the antibiotics and reduce the accessibility of target structures and the ability of bacteria to develop or acquire resistance genes (Anderson and O'Toole, 2008; Levy and Marshall, 2004; Marshall and Levy, 2011). An often neglected cause is the fact that most bacteria, pathogenic as well as commensal bacteria, form a biofilm under conditions of stress. Biofilm formation is a multi-stage process and is associated with the production of a polymeric extracellular matrix, which protects biofilm-embedded bacteria from environmental factors and host defence mechanisms (Anderson and O'Toole, 2008). More importantly, bacteria achieve a dormant stage within a biofilm, to reduce the expenditure of nutrients and oxygen. This transition is facilitated by distinct sets of biofilm-associated genes and makes biofilm-embedded bacteria insensitive to many antibiotics that interfere with metabolic processes such cell wall or protein synthesis.

Bacterial biofilms are generally formed on the interphase of fluid and solid surfaces and were initially found on indwelling catheters and implants. Moreover, dental biofilms (commonly noticed as plagues) have been recognized as a complex bacterial community embedded in an extracellular matrix. Mixed bacterial biofilms are also found in infected wounds (Westgate et al., 2011), surgical site infections (Dicicco et al., 2012), and otitis (Pye et al., 2013). Of considerable clinical importance are bacterial biofilms on internal biological surfaces such as the urinary bladder, the alveolar surface, the vascular system and on the endocardium, as well as in the mammary gland in cows with mastitis (Melchior et al., 2006a; Oliveira et al., 2007).

The current short review aims to present an overview over biofilm-associated infections in different animal species and the changes in bacterial gene expression during biofilm formation. These changes in gene expression may serve as biomarkers in the diagnosis of biofilm-associated infections and provide guidance for therapeutic intervention.

BACTERIAL BIOFILMS

A bacterial biofilm consists of a polymeric matrix enclosing a bacterial population of similar or different species. This extracellular matrix protects the embedded bacteria against conditions of environmental stress. The biofilm structure allows the necessary diffusion of nutrients and oxygen to the bacteria if they remain at a low metabolic rate. In turn, and as an adaptive mechanism, bacteria within a biofilm achieve a dormant stage, in which they do not proliferate and reduce many metabolic functions (Davies, 2003). In turn, biofilm related antibiotic resistance is not only caused by the protective

extracellular matrix that hinders the diffusion of antibiotics into and within the biofilm, but by the change in metabolic rate that reduces the sensitivity to antibiotics that target bacterial processes such as membrane of protein synthesis. In addition, the newly formed polymeric matrix is not recognized by host immune cells (macrophages, lymphocytes) and hence this matrix protects the embedded bacterial from innate defence mechanisms (Burmolle et al., 2010; Fey and Olson, 2010; Hall-Stoodley et al., 2004; Hoiby et al., 2010).

Biofilm formation and dispersion is a dynamic and complex process, regulated by various gene cassettes. In general, four phases in biofilm formation have been distinguished: an initial phase described as attachment or adherence phase, that includes the formation of a bacterial monolayer (micro-colonies), a maturation phase, in which the extracellular matrix is formed and the biofilm obtains a three-dimensional structure, a maintenance phase characterized by very low metabolic activity, and a dispersion or dissolution phase in which the persister cells are released from the biofilm (Clutterbuck et al., 2007). In consideration of these descriptions, we here depicted the biofilm life cycle as a four stage process (Figure 1). Within a biofilm, bacteria can communicate with each other and also exchange mobile elements (DNA, RNA). The latter may encore resistance mechanisms and hence biofilm formation is considered as an important mechanism for the spread of resistance within living organisms (Fey and Olson, 2010; O'Toole et al., 2000; Suh et al., 2010).

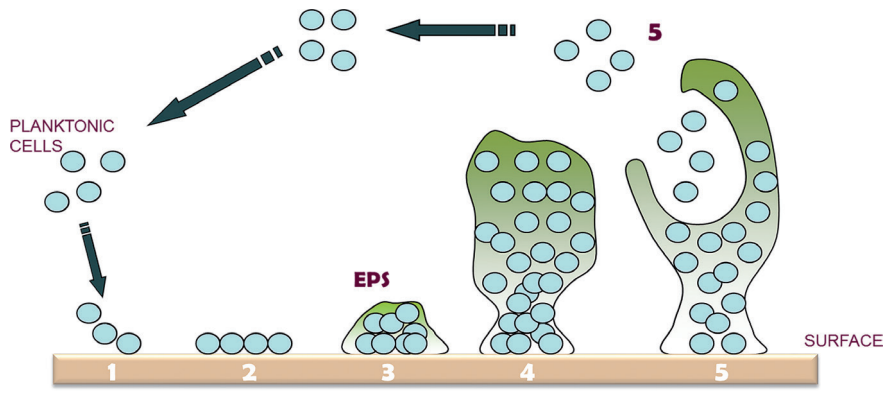


Figure 1. Five stages of biofilm development:
1. Initial attachment of planktonic cells to surface. 2. Permanent chemical attachment, single layer, bugs begin making slime. 3. Early vertical development of biofilm. 4. Multiple towers with channels between, maturing biofilm. 5. Mature biofilm with seeding/dispersal of more free swimming cells.

QUORUM SENSING - THE LANGUAGE OF BACTERIA

Biofilm forming bacteria use a cell-to-cell communication system called quorum sensing (QS) to control their biofilm formation or dispersion, as well as the expression of virulence factors. QS allows bacteria to detect the population density (quorum) and coordinates the expression of genes involved in adhesion, virulence and biofilm formation. Common signaling molecules are oligopeptides in Gram-positive bacteria, N-Acyl Homoserine Lactones (AHL) in Gram-negative bacteria, and a family of different autoinducers, for example autoinducer-2 (AI-2), which occurs in both Gram-negative and Gram-positive bacteria. Many secreted signaling molecules up-regulate their own synthesis (auto-activators) (Novick and Geisinger, 2008).

Most of the autoinducers in **Gram-positive** bacteria are peptides (AIPs) which bind to specific polytopic transmembrane receptors resulting in the activation of target gene transcription (Novick et al., 1995). In Gram-positive bacteria, a typical example is the accessory gene regulator (*agr*) QS system (Novick, 2003). This operon contains four genes: *agrB, D, C,* and *A*. Both, *agrA* and *C*, are constituting a classical two-component signaling module (TCS) and *agrB* and *D* are combined to generate the activating ligand. The methylthioadenosine/S-adenosyl-homocysteine nucleosidase (*LuxS*) system is another QS system found particularly in staphylococci. Unlike the agr system, the LuxS QS system is involved directly in the polysaccharide intercellular adhesion (PIA) regulating intercellular adhesion genes (*icaADBC*) that contribute to the final architecture of a biofilm (O'Gara, 2007). The complex gene profile that is involved in and coordinates the production of the extracellular matrix has been recently summarized for Staphylococci by Laverty and colleagues (Laverty et al., 2013).

Different from Gram-positive bacteria, the autoinducers in **Gram-negative** bacteria are N-acyl-homoserine lactones (HSLs), which diffuse freely into the bacterial cell and bind to specific intracellular receptor proteins (Fuqua et al., 2001). The acyl-HSL-based quorum-sensing system has been characterized in more than 70 Gram-negative species and the HSLs differ in the length of the side chains (C4-C16) (Jakobsen et al., 2013). For example, *Pseudomonas aeruginosa* has two acyl-HSL-based QS systems: *Las* and *Rhl*, both of which are *LuxI* homologs. *LasI* produces a freely diffusible N-3-oxo-dodecanoyl-L-homoserine lactone (3O-C12-HSL) that is recognized by the *LuxR* homolog cytoplasmic receptor *LasR. RhlI* synthesizes another acyl-HSL, N-butyryl-L-homoserine lactone (C4-HSL) that binds to the cytoplasmic receptor *RhlR*. Together they are controlling the activation of genes encoding extracellular matrix production (Holm and Vikstrom, 2014).

The quorum sensing system that occurs in Gram-positive as well as Gram-negative bacteria employs as auto-inducer a furanosyl borate ester that is formed by the LuxS protein and is described in more than 50 bacterial species (Chen et al., 2002). The

4th system facilitates inter-species and even inter-kingdom signaling, using mainly epinephrine and norepinephrine as signaling molecules, thus contributing also to the relation between the host and the invading pathogen (Boyen et al., 2009).

BACTERIAL BIOFILM IN VETERINARY MEDICINE

Many chronic infections in animals have been correlated with bacterial biofilms. Typical animal pathogens know to form biofilm *in vivo* are *Staphylococcus* spp. and *Streptococcus* spp. and the Gram-negative species *Actinobacillus pleuropneumoniar, Escherichia coli* and *Pseudomonas aeruginosa*. These bacteria are involved in diverse animal diseases, such as otitis in dogs (Silva et al., 2014), mastitis in cattle (Atulya et al., 2014), pleuropneumonia in swine (Grasteau et al., 2011), local and systemic infections in chickens (Han et al., 2013) and ducks (Tu et al., 2014), enteritis in cats (Ghosh et al., 2013), and wound infections and endometritis in horse (LeBlanc, 2010; Westgate et al., 2011) (Figure 2). The pioneering studies in bacterial biofilm identification focused on *Staphylococcus* spp. (Gram-positive) and *P. aeruginosa* (Gram-negative). Exposure to different environmental stresses (anaerobic and pH conditions) (Atulya et al., 2014; Li et al., 2014) and sub-therapeutic doses of antibiotics (enrofloxacin, gentamicin, cotrimoxazole and ampicillin) (Costa et al., 2012; Silva et al., 2014) were reported to induce bacterial biofilms.

MAIN ANIMAL DISEASES RELATED TO BACTERIAL BIOFILMS

Cattle

Since the isolation of an extracellular slime material from mastitis pathogens, bacterial biofilms have been related to bovine mastitis (Watson and Watson, 1989). Different bacteria with the ability to produce biofilms were isolated from mastitis milk or from the mammary gland, including *Staphylococcus aureus* (Dhanawade et al., 2010; Melchior et al., 2006b), *S. epidermidis* (Park et al., 2013), *Streptococcus. uberis* (Crowley et al., 2011) and *Enterococcus faecalis* (Elhadidy and Elsayyad, 2013) as well as the Gram-negative *E. coli* (Costa et al., 2012) and *P. aeruginosa* (Atulya et al., 2014). *Staphylococcus* spp. is considered as the most important etiological agent of recurrent clinical and subclinical bovine mastitis (Bardiau et al., 2014). The mechanism involved in *Staphylococcus* spp. biofilm formation is mainly related to the *ica* operon, and its intercellular adhesion locus, consisting of the *icaADBC* gene cluster. This operon regulates PIA production during biofilm maturation. Both lactose and milk can induce *S. aureus* biofilm formation by enhancing the PIA production (Xue et al., 2014). The genes *icaA, icaD, lbp* (laminin binding protein gene) and *bap* (biofilm associated protein gene) were confirmed to be present in bovine subclinical mastitis *Staphylococcus* spp. isolates (Darwish and Asfour, 2013). Therefore, *icaA* and *icaD* gene expression can be

used as markers to identify the biofilm producing potential of *S. aureus* from bovine mastitis (Dhanawade et al., 2010). Moreover, the *icaA* gene was also detected in 40.5% of the bovine mastitis induced by coagulase-negative staphylococci (CNS) (Rumi et al., 2013), indicating that the *ica* operon is widely distributed among Staphylococci. Intra-mammary infusion of antibiotics belongs to the standard therapies in mastitis treatment (du Preez, 2000). The therapy resistance to otherwise effective antibiotics such as ampicillin and enrofloxacin, seems be associated with the bacterial ability to form biofilms (Elhadidy and Elsayyad, 2013; Saini et al., 2012).

In cattle husbandry, infectious bovine keratoconjunctivitis (IBK) is also of considerable economic interest, as infections result in reduced weight gain of the animals and decreased milk production (Webber and Selby, 1981). The major etiologic agent of IBK is *Moraxella bovis* (Pugh et al., 1966). Only recently this bacterium was reported to be

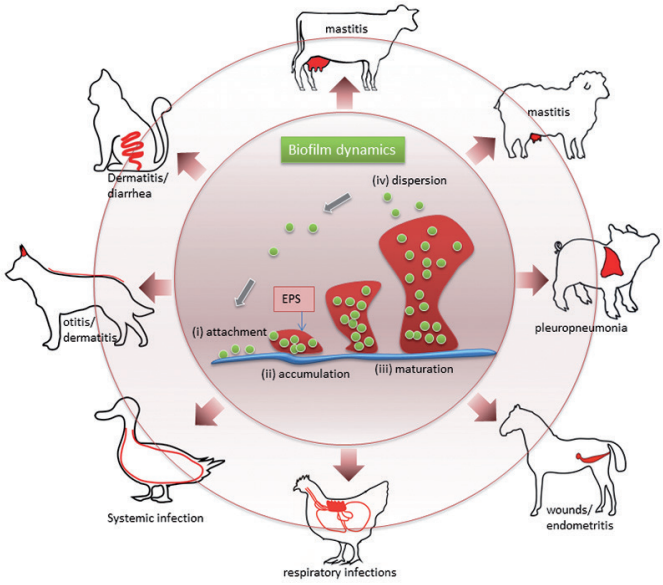


Figure 2. Biofilms and their occurrence in veterinary species:
Inner circle: Biofilm dynamics. From left to right the four main stages of biofilm development are shown: (i) attachment; (ii) accumulation; (iii) maturation; (iv) dispersion. After attachment to a surface, bacteria produce extracellular polymeric substances (EPS) to form a multilayer biofilm (maturation). At the dispersion stage, planktonic (free) bacteria are released from the biofilm into the environment and can become virulent and cause infection or start to build a new biofilm (chronic infections). Outer circle/red areas: Predilection sites in animals where species specific pathogens have shown to form biofilms.

a biofilm-forming organism (Prieto et al., 2013). Meanwhile, the same group of authors also demonstrated that $MgCl_2$ exposure would remove type IV pili from *M. bovis* cell surface, which results in biofilm inhibition and also biofilm dispersion and hence has suggested as adjuvant therapy to antibiotic treatments.

Mycobacterium avium subsp. paratuberculosis (Map) is considered to be the causative agent of Johne's disease, a chronic enteric infection in cattle. Studies on the epidemiology of Map-infections pointed towards the importance of mixed bacterial biofilms containing Map in the water circuit in cattle stables and feedlots (Click, 2011). Map biofilm formation is related to the synthesis of Glycopeptidolipids (GPLs) initiated by an operon containing two large genes encoding for non-ribosomal peptide synthetases (*pstA* and *pstB*) (Wu et al., 2009).

The biofilm formation ability of Map seems to explain the chronic nature of infections and the poor therapeutic response (Rowe and Grant, 2006). Recently elegant studies conducted with *Mycobacterium avium subsp. hominussuis*, causing airway infections in humans, showed the biofilm-induced TNF-α-driven hyper-stimulation and apoptosis of surveilling phagocytes that prevented clearance of the biofilm by cells of the innate immune system, hence allowing the biofilm-associated infection to persist (Rose and Bermudez, 2014).

Sheep and goats

Although mastitis is studied in a lesser extent in small ruminants than in cattle, *S. aureus* isolates of ewe gangrenous mastitis were demonstrated to produce biofilms (Tel et al., 2012). The involved *S. aureus* isolates carried the *icaA* and *icaD* genes, but none of them harboured bap, which distinguishes these strains from *Staphylococcus* spp. isolates of bovine mastitis (Vautor et al., 2008). The *bap* gene encodes the biofilm-associated protein, which contributes to biofilm formation in *S. aureus* promoting the primary attachments as well as the cell-to-cell aggregation during the biofilm formation process (Cucarella et al., 2001). *S. aureus* isolates of ewes lacking the *bap* gene, but which are fully capable to form biofilm, suggest that this gene is not the only regulator of the initial phase of biofilm formation in *S. aureus* strains.

In small ruminants, *Listeria monocytogenes* and *Listeria ivanovii* show strong biofilm formation ability when isolated from goat and sheep mastitis milk (Osman et al., 2014). Research on the molecular mechanism of biofilm formation was conducted on flagella-mediated motility that is involved in the initial cell surface attachment and subsequently in biofilm maturation (Lemon et al., 2007). A deletion of the *agrA* gene in *L. monocytogenes* resulted in a decrease in bacterial adherence without affecting the subsequent biofilm development (Rieu et al., 2008), again pointing towards the existence of alternative pathways in the initiating phase of biofilm formation. The gene *lmo1386*, encoding a putative DNA translocase,

was also described to be essential for *L. monocytogenes* biofilm formation, but it remains to be elucidated how this gene influences the biofilm formation process (Chang et al., 2012).

Swine

Contagious **pleuropneumonia** is a worldwide-occurring serious respiratory disease of swine, caused by *Actinobacillus pleuropneumoniae*. The prevalence of biofilm formation and its relevance for colonization, pathogenesis and transmission of porcine *A. pleuropneumoniae* isolates was reported already ten years ago (Kaplan and Mulks, 2005). *A. pleuropneumoniae* biofilms have Poly-β-1, 6-N-acetyl-D-glucosamine (PGA) as major matrix component (Izano et al., 2007). The histone-like protein (H-NS) acts as a regulator repressing the PGA operon (Bosse et al., 2010) and might present a target for intervention. Host factor Q-β gene (*hfq*) plays also a role in biofilm formation by regulating poly-β-1, 6-N-acetylglucosamine (PNAG) synthesis (Subashchandrabose et al., 2013). Under oxygen-deprived growth conditions *arcA* reduces the ability of *A. pleuropneumoniae* for autoaggregation and biofilm formation (Buettner et al., 2008) and has been considered as a target gene for intervention also. Methylthioadenosine/ S-adenosyl-homocysteine (MTA/SAH) nucleosidase (*LuxS*) act opposite to *arcA* (Li et al., 2008) on *A. pleuropneumoniae* biofilm and this effect is autoinducer-2 (AI-2) independent (Li et al., 2011). It is also worthwhile to mention that *A. pleuropneumoniae* serotype 5 capsular polysaccharide was reported to have a biofilm inhibitory effect on *S. aureus, S. epidermidis* and *Aggregatibacter actinomycetemcomitans* biofilms, but not on *A. pleuropneumoniae* biofilms (Karwacki et al., 2013).

Oedema disease and post weaning diarrhoea in pigs caused by *E. coli* is interrelated with biofilm formation. Recently, 53.3% of the EHEC (enterohaemorrhagic *E.coli*) isolates from animals, including those from pig faeces, were reported to have the ability to form biofilms (Wang et al., 2014). A subgroup of porcine EHEC was reported to express adhesin involved in diffuse adherence (AIDA-I) (Ngeleka et al., 2003) which is essential for intestinal colonization and *in vivo* bacterial autoaggregation and biofilm formation (Ravi et al., 2007). Moreover, the expression of flagella, which are also essential for adhesion of *E. coli* was found to be regulated by autoinducer-1 and the disruption of this QS pathway is considered as a new anti-biofilm strategy in the treatment of these infections (Yang et al., 2013).

Streptococcus suis is another important pathogen in swine husbandry associated with meningitis, septicaemia, arthritis and endocarditis (Bonifait et al., 2010). Different genes were reported to be involved in *S. suis* biofilm formation, such as *luxS* affecting cell adhesion, but its transcription is not correlated with AI-2 as also described for *A. pleuropneumoniae* biofilms (Wang et al., 2011b; Wang et al., 2013). In addition, the atl gene encoding autolysin, involved in *S. suis* cell autolysis, fibronectin-binding activity

and cell adhesion plays also an important role in biofilm formation (Ju et al., 2012). Collagen-binding protein 40 (*cbp40*) seems to be the major extracellular matrix (ECM) adhesion protein (Zhang et al., 2013).

Equines

In equine practice, trauma and **wounds**, and subsequently wound healing is of high clinical importance and hence biofilm studies were devoted to these clinical conditions. Common wound-contaminating bacteria such as *Enterococcus faecium, P. aeruginosa* and *Staphylococcus* spp. were described to form biofilms and subsequently hindering wound healing (Westgate et al., 2011). Additionally, the failure of antibiotic treatment in chronic and post–mating-induced **endometritis** in mares was been reported to be associated with biofilm formation by *Enterobacter cloacae, E. coli, P. aeruginosa* and *S. epidermidis* (LeBlanc, 2010).

Chickens

Avian pathogenic *E. coli* (**APEC**) causes serious systemic infections in chicken (Skyberg et al., 2007). Investigation of Pace et al. (2011) showed that *IcmF* (intracellular multiplication factor) enhances APEC biofilm formation, since an *icmF* mutant (with gene deletion) showed less adherence to model surfaces. Other investigations (Han et al., 2014) demonstrated that the lipid A-core ligase gene (*waaL*) is responsible for the lipopolysaccharide (LPS) synthesis in APEC, while at the same time inhibiting APEC biofilm formation.

Campylobacter jejuni is an important zoonotic pathogen frequently occurring in chickens. The biofilm formation ability of *C. jejuni* in poultry farm enhances its survival (Trachoo and Frank, 2002; Trachoo et al., 2002). CprRS (campylobacter planktonic growth regulator) allows *C. jejuni* to respond to nutrient availability via inducing or repressing genes related to biofilm formation (Svensson et al., 2009). More recently, a large series of individual genes including peptidoglycan peptidase gene (*pgp1*), exopolyphosphatases (PPX), polyphosphate kinase 2 (PPK2), glycocylation of major outer membrane protein (MOMP), *Campylobacter jejuni* transcriptional regulator Cj1556, DNA-binding protein (*dps*) gene, and carbon starvation regulator (*csrA*) gene were all reported to be involved in *C. jejuni* biofilm formation. Interestingly, *in vitro* experiments under micro-aerobic conditions with mixed cultures indicated that *C. jejuni* is also involved in the biofilm formation of other intestinal bacteria isolated from poultry chicks, such as *E. faecalis* and *S. simulans* under micro-aerobic conditions (Teh et al., 2010). This co-aggregation of different bacterial species in enteral biofilms might contribute to the high prevalence of *Campylobacter* in poultry.

Ducks

Ducks are a major domestic bird in China, which explains biofilm researches in duck were predominantly conducted by Chinese research groups. Like in chicken, avian pathogenic *E. coli* (**APEC**) causing neonatal meningitis in ducklings is a major clinical problem. Wang et al. (2011a) studied the effect of *ibeA* in APEC isolated from ducks, demonstrating that the expression of *ibeA* stimulates biofilm formation. The same group of authors (Zhuge et al., 2013) identified also a novel autotransporter adhesin gene, *aatB* from ducks, which promotes bacterial adherence to host tissues.

Another important pathogen for ducks is *Riemerella anatipestifer*, (**duck septicaemia**) an epizootic infectious disease (Zhong et al., 2009). Biofilm formation of *R. anatipestifer* prolongs the infection (Hu et al., 2010). The same group identified the importance of SIP (Siderophore-interacting protein) which is involved in *R. anatipestifer* iron acquisition in biofilm formation (Tu et al., 2014).

Dog and cats

Wound infections (Swanson et al., 2014), otitis (Figueredo et al., 2012), skin and ear infections (Bardiau et al., 2013; Osland et al., 2012) and surgical site infections (Singh et al., 2013) in dogs and cats are the most investigated disease conditions in relation to bacterial biofilm formation (Moyaert et AL., 2006). Dicicco et al., (2012) described that therapeutic doses of clarithromycin did not eradicate *S. pseudintermedius* biofilms. Subsequent investigations indicated that manuka oil (Song et al., 2013) and dispersin B (Turk et al., 2013) seem to be potent inhibitors for methicillin-resistant *S. pseudintermedius* biofilm formation. In contrast to many other findings with low concentrations of antibiotics, sub-MIC concentrations of imipenem, metronidazole and clindamycin were able to inhibit the *Bacteroides fragilis* biofilm formation (Silva et al., 2014).

P. aeruginosa biofilms are often found in dog otitis (Pye et al., 2013) and Tris-EDTA (Pye et al., 2014) was suggested as adjuvant to the common antibiotics used in canine practice such as gentamicin or neomycin. Moreover, urinary catheters coated with sustained-release varnish of chlorhexidine effectively decreased urinary catheter-associated mixed bacterial biofilm formation in model studies with dogs (Segev et al., 2013). Surprisingly, no data on chronic urinary tract infections and biofilm-forming pathogens are available and detailed studies on bacterial isolates from dogs or cats are also lacking, although various studies describe the impaired sensitivity of bacterial infections to common antibiotic treatments in such disease conditions. Empirically cranberry juice or extracts are also used in dogs for the treatment of chronic urinary infections, as they are recommended for the prevention and treatment of urinary tract infections in humans (Barnoiu et al., 2014; Fernandez-Puentes et al., 2014), but detailed clinical studies on recommendable dosing regimens are lacking.

FUTURE PERSPECTIVES

The presented data related to infections in domestic animal species support the hypothesis that the ability of pathogens to form biofilms is a common phenomenon. The identification of target genes involved in biofilm formation, which are included in this description, may guide further research activities to quantify the prevalence of biofilm infections in daily practice. Various domestic animal (goats and pigs) are used as models in biofilm research regarding vaginal infections (Joraholmen et al., 2014), urinary tract infections (Neheman et al., 2013), sinusitis (Drilling et al., 2014; Paramasivan et al., 2014), and wound infections caused by *P. aeruginosa* (Phillips et al., 2013) and *S. aureus* (Nusbaum et al., 2012). It is therefore surprising that only very few studies could be identified that explore strategies to overcome the potential therapeutic resistance caused by biofilm formation in daily veterinary practice.

Table 1. Examples for recently evaluated strategies to combat bacterial biofilm formation of important veterinary pathogens

Biofilm forming bacteria	Strategies	Target
Gram positive (G^+)		
Staphylococcus aureus	**Lemongrass oil** (citral, geraniol) affect the bacterial cell surface which might compromise the initial attachment of *S. aureus* cells to the surface[1]	Bacterial attachment
	Extracellular c-di-GMP inhibits cell-to-cell (intercellular) adhesive interactions in liquid medium[2]	Bacterial attachment
	Proteinase K addition inhibits biofilm formation when added at the initiation of biofilm growth and is able to disperse pre-established mature biofilms[3]	Bacterial attachment, biofilm maturation and preformed biofilm
	Bacteriophage-derived peptidase ($CHAP_k$) rapidly lyses sessile staphylococcal cells[4]	Bacterial attachment and preformed biofilm
	Wheat-bran extract inhibits different bacterial enzymatic processes or catalyse synthesized biofilm components; decreases quorum sensing (QS) signal AHL activity[5]	Biofilm maturation, preformed biofilm and QS system
Staphylococcus pseudintermedius	**DispersinB** catalyzes the hydrolysis of poly-N-acetylglucosamine (PNAG)[6]	Biofilm dispersion and preformed biofilm
	***Leptospermum scoparium* essential oil**[7]	Unknown
Streptococcus suis	**Zinc** interacts with components of the biofilm matrix[8]	Biofilm maturation
	Subinhibitory concentrations of **ciprofloxacin** and **ofloxacin**[9]	Unknown
	Licochalcone A[10]	Unknown
	Bacteriophage lysin (LySMP) acts to maximize dispersal of the biofilm and inactivate the released cells[11]	Preformed biofilm

The identification of substances that may be clinically used to prevent biofilm formation focusses on the interruption of bacterial quorum sensing, as this is the initial phase of biofilm formation. In human medicine, the use of cranberry products, as mentioned above, is already implemented widely in the prevention and treatment of urinary tract infections with the aim to prevent biofilm formation, particularly of *E.coli*. Moreover, other plant-derived extracts such as garlic extract (see this thesis and the related literature) have been identified as biofilm inhibiting agents. Current strategies consider also the use of essential oils or extracts from plants used in traditional medicine (this thesis) as to prevent biofilm formation (Aiemsaard et al., 2011; Meng et al., 2011; Nicholson et al., 2013; Prieto et al., 2013; Wu et al., 2013). Examples of these different strategies are given in Table 1. As biofilm formation is a complex process involving multiple regulatory processes in bacteria, plant extracts containing multiple active substances, remain one of the most promising short-term approaches to be used as supportive agents to veterinary antibiotic therapy.

Table 1. Continued.

Gram negative (G-)		
Escherichia coli	Zinc interacts with components of the biofilm matrix[8]	Biofilm maturation
Actinobacillus pleuropneumoniae	**Bovine apo-lactoferrin** can diminish bacterial adherence and protease activity[12]	Bacterial attachment and biofilm maturation
	Zinc interacts with components of the biofilm matrix[8]	Biofilm maturation
Campylobacter jejuni	**2(5H)-Furanone, epigallocatechin gallate,** and a **citric-based disinfectant** inhibite autoinducer-2 activity and biofilm motility[13]	QS system and biofilm motility
	Diallyl sulphide destroy the biofilm EPS structure[14]	
	Chlorine inactivates *C. jejuni* in the biofilms after treatment at 50 ppm for 45 s[15]	Biofilm maturation
	ZnO nanoparticles penetrate the biofilm extracellular polymeric substances (EPS) within 1 h without damaging it and interact directly with sessile cells in biofilms[16]	Bactericidal within biofilm Bactericidal within biofilm
Haemophilus parasuis	**Staphylococcal nuclease** prevents the bacterial adherence and induces cell death in the biofilms[17]	Bacterial attachment and bactericidal within biofilm
	Zinc interacts with components of the biofilm matrix[8]	Biofilm maturation
Moraxella bovis	**MgCl2** remove the type IV pili from cell surface[18]	Bacterial attachment and preformed biofilm

Reference: 1: Aiemsaard et al. (2011) 2: Karaolis et al. (2005); 3: Nicholson et al. (2013); 4: Fenton et al., (2013); 5: Gonzalez-Ortiz et al. (2014); 6: Turk et al. (2013); 7: Song et al. (2013); 8: Wu et al. (2013); 9: Dawei et al. (2012); 10: Hao et al. (2013); 11: Meng et al. (2011); 12: Luna-Castro et al. (2014); 13: Castillo et al. (2014); 14: Lu et al. (2012a); 15: Trachoo and Frank (2002); 16: Lu et al. (2012b); 17: Tang et al. (2011); 18: Prieto et al. (2013)

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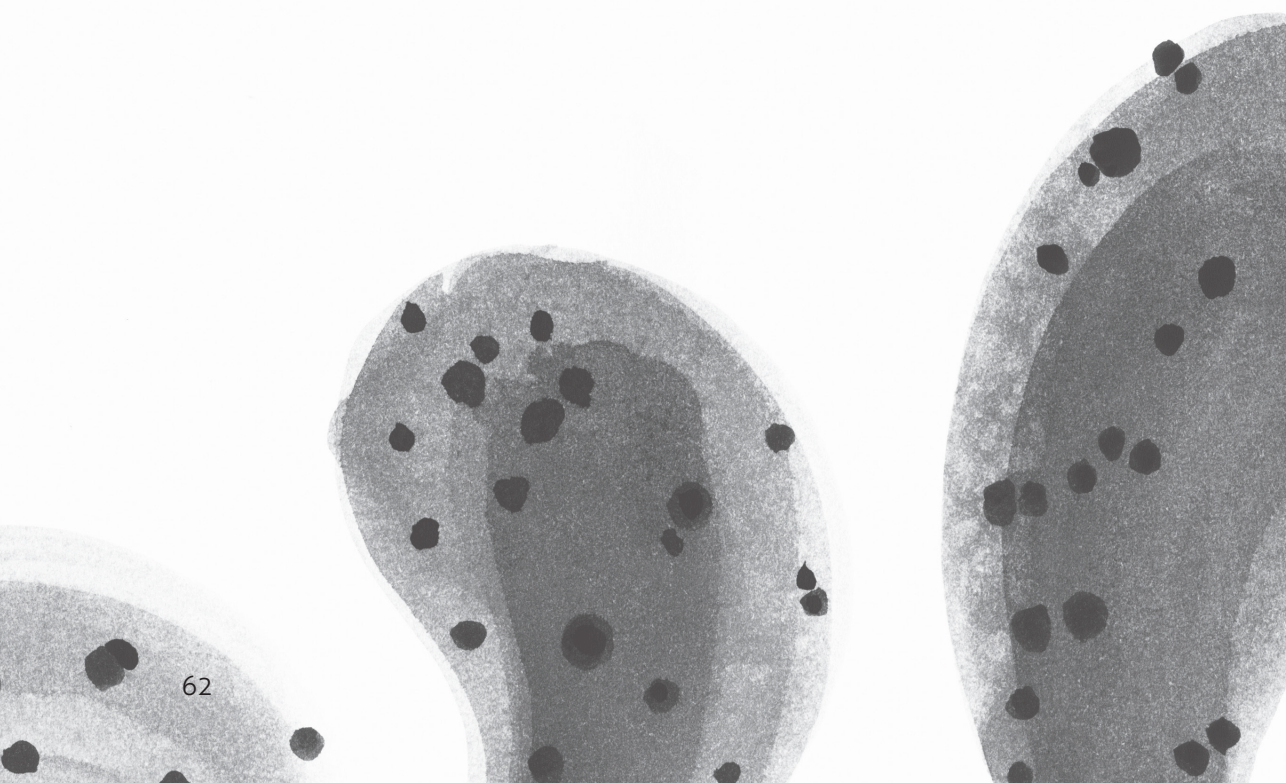
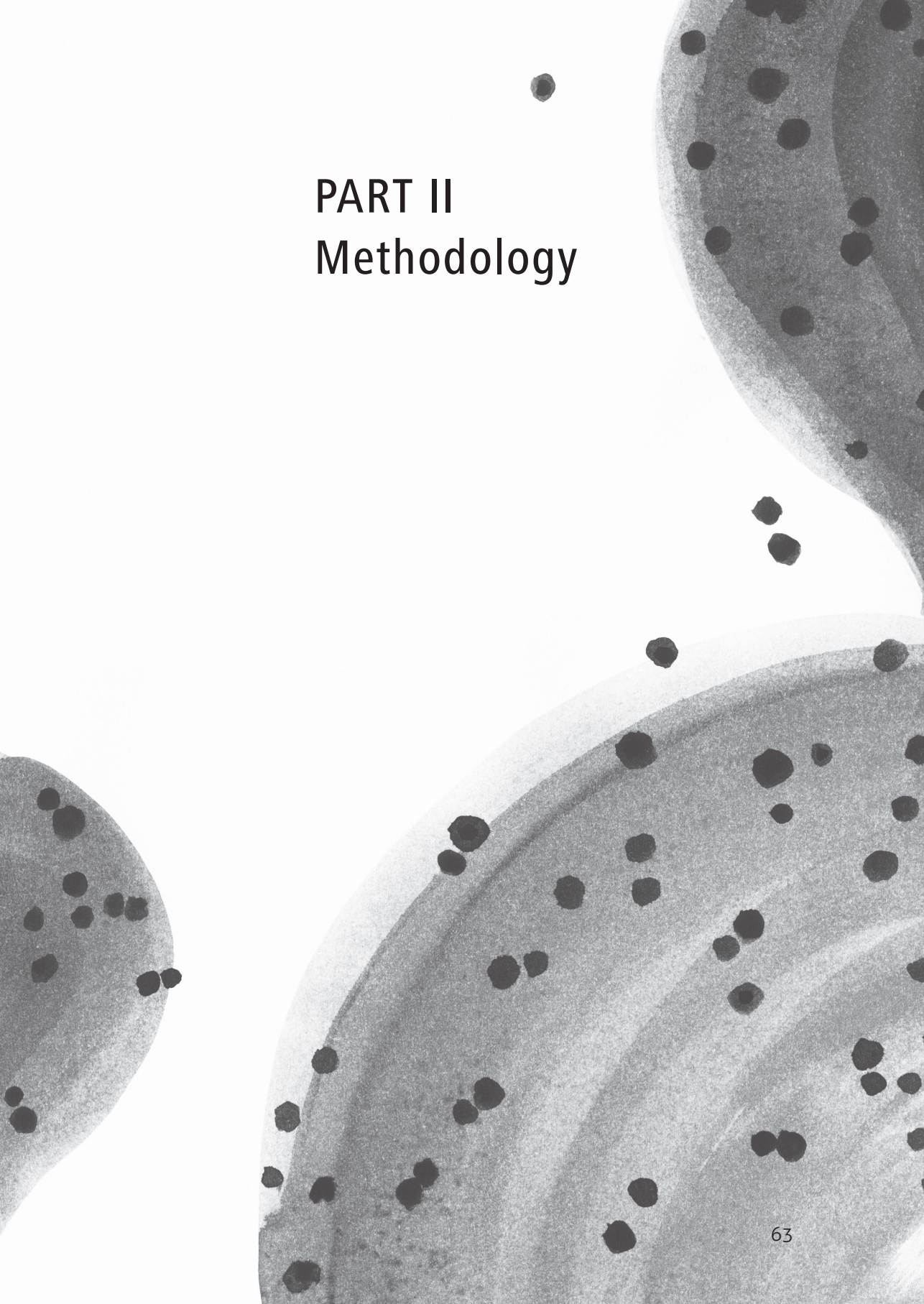
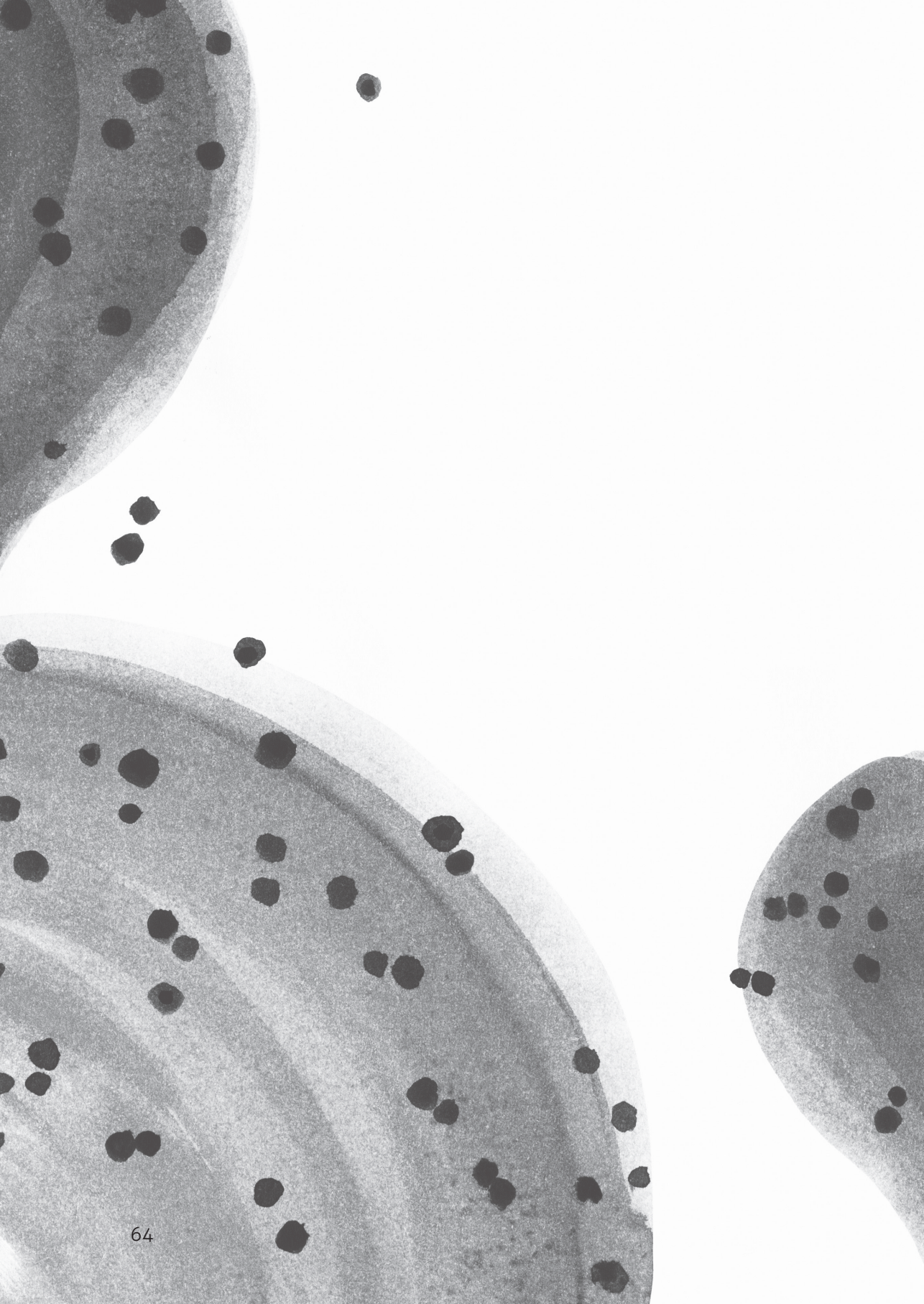


PART II
Methodology





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Staphylococcus epidermidis biofilm quantification: effect of different solvents and dyes

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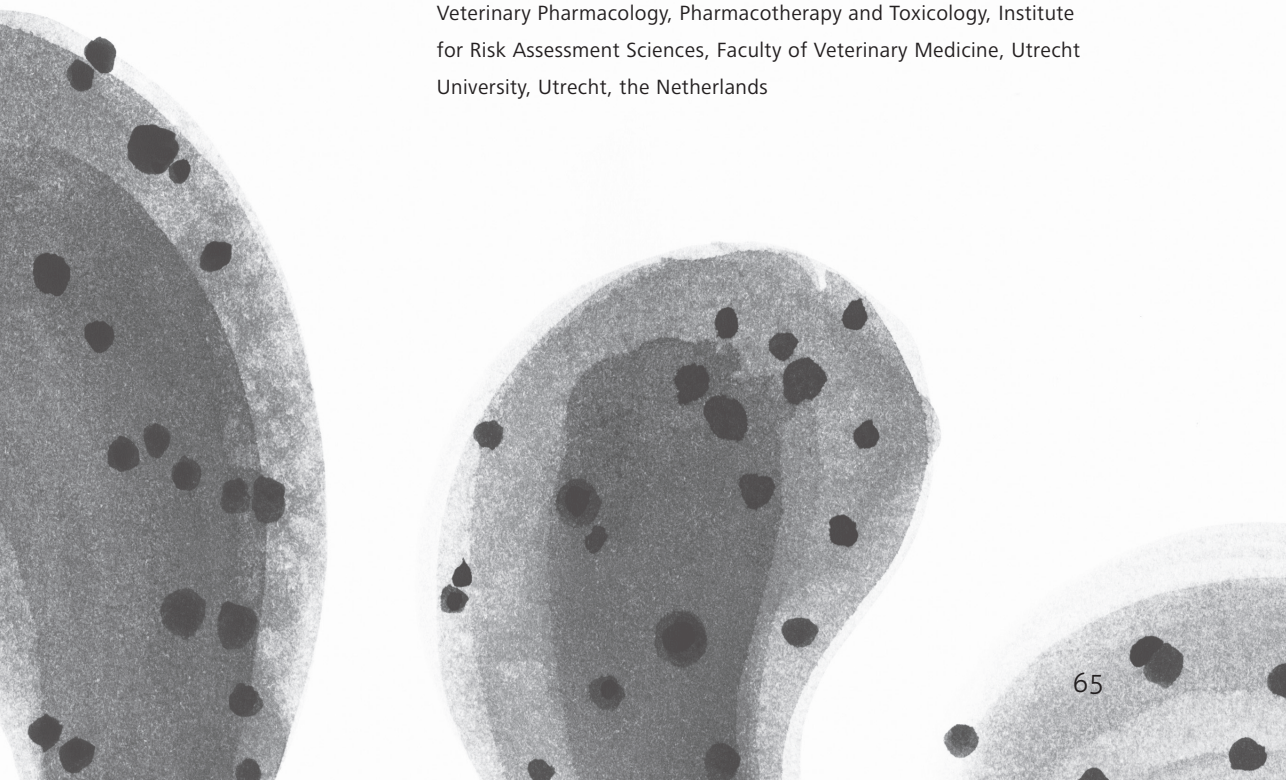
X. Wu, R. R. Santos, J. Fink-Gremmels

Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands



Staphylococcus epidermidis biofilm formed in the presence of the solvents DMSO, ethanol or methanol was quantified using safranin or crystal violet staining protocols. We found that biofilm quantification was the most accurate when safranin protocol was applied. Moreover, both DMSO and ethanol stimulated biofilm formation.

INTRODUCTION

Staphylococcus epidermidis is known to produce biofilm on indwelling medical devices causing chronic infections. Hence, approaches to inhibit biofilm formation or to disperse formed biofilm have been proposed (Kostakioti et al., 2013). Many of the tested anti-biofilm compounds, however, are not water-soluble and must be dissolved with the help of solvents for *in vitro* tests. Dimethyl sulfoxide (DMSO) is commonly the solvent of choice because it is able to dissolve polar and apolar compounds. This solvent has been used when testing candidate compounds to inhibit biofilm formation by *Pseudomonas aeruginosa* (Bijtenhoorn et al., 2011), *Escherichia coli* (Begde et al., 2012), *S. aureus* (Fallarero et al., 2013) and *S. epidermidis* (Panmanee et al., 2013). DMSO has been used at concentrations ranging of 0.1% (to dissolve thiazolidione derivatives; (Huang et al., 2012) to 5% (to dissolve tea tree oil; Karpanen et al., 2008) in tests against *S. epidermidis* biofilm. Panmanee et al. (2013) reported that 0.2% DMSO did not affect *S. epidermidis* biofilm analysis when applying crystal violet staining. Using this same dye, (Lim et al., 2012) showed that *E. coli* biofilm formation was stimulated (increase in 140%) in response to an exposure to 4% DMSO. Ethanol is also applied as solvent in bacterial biofilm tests because of its ability to dissolve polar and apolar substances (Vestby et al., 2014). Chaieb et al. (2007) indicated that 2% ethanol induced *S. epidermidis* biofilm formation, and this stimulation was strain dependent, where increase in biofilm ranged of 10–75%. Only in few cases, methanol is used as solvent (Reck et al., 2011), and no reports showing the interference of this solvent on bacterial biofilm formation are available. A simple assay is often used to quantify bacterial biofilm formation (Christensen et al., 1985) applying specific protocols for the dyes safranin or crystal violet. It is remarkable that most biofilm reports are based on crystal violet staining (Croes et al., 2009; Kaplan et al., 2004; Waldrop et al., 2014). Ayed et al. (2010) reported *S. epidermidis* ability to degrade crystal violet after 12 hours incubation with this dye. However, it is unknown if crystal violet can be decolorized by *S. epidermidis* after a short-term incubation. Safranin staining is also used to quantify bacterial biofilm, but in a less extent (McKenney et al., 1998; Melchior et al., 2006). In the present study, we used a biofilm formation assay to compare the effect of different solvents (DMSO, ethanol and methanol) on *S. epidermidis* biofilm formation. Moreover, both crystal violet and safranin staining protocols were tested. To estimate the direct effect of *S. epidermidis* on both dyes, a direct decolorization test was performed.

MATERIALS AND METHODS

S. epidermidis (ATCC 35984) was cultured in tryptic soy broth + 0.25% glucose (pH 7.0) (TSB+) under aerobic conditions at 37 °C for 24 hours before exposure to DMSO (≥

99.5%), absolute ethanol or methanol (≥ 99.8%) (Merck KGaA, Darmstadt, Germany). Each solvent was separately diluted in TSB+ to a work concentration of 2% before use. Safranin and crystal violet (Sigma-Aldrich, St. Louis, MO) were individually diluted with de-ionized water to a working concentration of 0.1%. For exposure test, 100 µl of bacterial suspension (10^6 CFU/ml) was transferred to a U-bottomed 96-well microtiter polystyrene plate (Costar, Corning, NY, USA) containing different concentrations of DMSO, ethanol or methanol (0.0078–2%). Hence, the final tested concentrations of DMSO, ethanol or methanol were 0.0039–1%. There were six replicate wells per treatment group and wells with sterile TSB+ alone served as blanks. The plates were incubated on a microplate shaker (Heidolph titramax 100) at 600 rpm, 37°C for 24 hours. Biofilm formation was quantified by safranin or crystal violet staining protocols. Regarding safranin, the supernatant from all wells was discarded and the biofilms adhering to the bottom of the wells were washed with de-ionized water three times, and then incubated with 0.1 M HCl for 1 hour at room temperature (RT). After which, HCl was replaced by safranin (0.1% in water) and incubation was performed for 45 minutes at RT. Non-bound safranin was removed by rinsing the stained biofilm three times with de-ionized water, and thereafter incubation was performed in 125 µl 0.2 M NaOH at 57 °C for 1 hour. At the end of incubation, 100 µl of solution from each well was pipetted to a new flat-bottom 96-well microtiter polystyrene plate. The absorbance of each sample was determined at a wavelength of 540 nm using a microplate reader (3550-UV, Bio-Rad, Hercules, CA). Crystal violet staining was carried out as O'Toole et al. (2000) reported before, with slight modifications. In brief, after washed the adhering biofilms with de-ionized water three times, and incubated with crystal violet (0.1% in water) for 15 minutes (RT). Non-bound crystal violet was removed by rinsing the biofilm three times with de-ionized water and dried out for 2 hours (RT). Thereafter incubation was performed in 125 µl acetic acid (30% in water) (RT) for 15 minutes. At the end of incubation absorbance was measured at a wavelength of 540 nm. As described above, protocols used for safranin and crystal violet staining present some notable differences, e.g. time of incubation with dye (45 minutes for safranin vs. 15 minutes for crystal violet), elution (0.2M NaOH for 1 h at 57 °C for safranin vs. 30% acetic acid for 15 minutes at RT for crystal violet) and presence (safranin) or not (crystal violet) of inactivation step before staining. Therefore, to determine if the variation in approaches used for the two methods were responsible for discrepancy in sensitivities between the two dyes, we switched safranin and crystal violet staining protocols. Each test was repeated four times. Finally, to elucidate the possible decolorization effect of *S. epidermidis* (biofilm and planktonic) on crystal violet during short-term exposure, we performed a decolorization test as described by Parshetti et al. (2011). For biofilm, 200 µl of bacterial suspension (10^6 CFU/ml) was cultured in a 96-well plate, under aerobic conditions at 37 °C for 24 hours. The supernatant from all wells was discarded

and the biofilms adhering to the bottom of the wells were washed with de-ionized water three times, and then incubated with safranin (0.01% in water) or crystal violet (0.01% in water), both for 15 or 45 minutes (RT). Incubation was performed, either or not with previous inactivation with 0.1 M HCl (safranin) or 100% ethanol (crystal violet). We used 100% ethanol of inactivation (Christensen et al., 1985) because HCl was not suitable for crystal violet; neither ethanol was suitable for safranin. At the end of incubation, 100 µl of dye solution from each well was pipetted to a new flat-bottom 96-well microtiter polystyrene plate. Absorbance was measured at a wavelength of 540 nm. Stock dye solutions of 0.01% safranin or crystal violet served as control. For planktonic bacteria, one colony of *S. epidermidis* bacteria was culture in 10 ml TSB+ under aerobic conditions at 37 °C for 24 hours. Supernatant (1 ml) was taken into a new Eppendorf tube and incubated with safranin and crystal violet at a final concentration of 0.01%. Incubation, with both dyes, was performed for 15 and 45 minutes (RT), TSB+ alone with dyes served as control. At the end of incubation, bacterial cells were separated by centrifugation at 5000 rpm for 20 min. 100 µl of dye supernatant from each tube was pipetted carefully to a new flat-bottom 96-well microtiter polystyrene plate. Absorbance was measured at a wavelength of 540 nm. Each test was repeated three times. Biofilm formation data were analyzed using Kruskal-Wallis test followed by post hoc Dunn's multiple comparisons. Degradation data was evaluated using one-way ANOVA followed by Tukey multiple comparisons post hoc test. Differences were considered significant at P values < 0.05. For all statistical analyses, GraphPad Prism version 6.04 was used.

RESULTS AND DISCUSSION

S. epidermidis biofilm formation was stimulated ($P < 0.05$) when bacteria was exposed to 0.0078–1% DMSO (increase in 13.28–42.2%, respectively) according safranin staining and to 0.125–1% DMSO (increase in 12.87–24.35%, respectively) according crystal violet staining (Figure 1, panel A). Likewise, based on both safranin and crystal violet staining, *S. epidermidis* exposure to 0.0625–1% ethanol boosted ($P < 0.05$) biofilm formation in 20.22–60.68% for safranin and 12.92–30.21% for crystal violet staining (Figure 1, panel B). Lim et al. (2012) reported that much higher concentrations of DMSO (4%) and ethanol (2%) were necessary to stimulate biofilm formation (increase in 140% and 10%) by *E. coli*. According these authors, *E. coli* is capable to adapt to DMSO and ethanol exposure by increasing their biofilm matrix via amyloid production, which is involved in bacterial attachment (Otzen and Nielsen, 2008). In the present study, using the same staining procedure, 0.125% DMSO was sufficient to stimulate *S. epidermidis* biofilm formation in 21.7%, and quantification of biofilm was more sensitive by safranin staining when compared to crystal violet. Although this increase in biofilm might not

be biologically relevant, there is still a concern that choosing inaccurate solvents and staining techniques may provide misleading results. There was no significant change of *S. epidermidis* biofilm formation in any tested concentrations of methanol when compared with control (Figure 1, panel C). Detailed information on the percentages of biofilm stimulation is given in supplementary Table 1. As reported by Ayed et al. (2010), crystal violet can be degraded by *S. epidermidis* after incubation for 12 hours at 30 °C. Therefore, to assume if degradation was involved or not with our less sensitive crystal violet data, we have followed crystal violet degradation test protocol (Parshetti et al., 2011); safranin degradation was also tested. Time of degradation was accordingly staining protocols used in the present study, i.e. 15 or 45 minutes. Safranin was decolorized only if no previous inactivation was performed and incubation interval was of 45 minutes (Figure 2, panel A). In the present study, safranin staining was performed with previous inactivation as indicated by Melchior et al. (2006). Independently on the inactivation step before staining and time of incubation, crystal violet was significantly decolorized after exposure to *S. epidermidis* biofilm (Figure 2, panel B). However, this data were not robust enough to state that degradation was occurring during such short-term exposure at RT. Hence, we tested the color degradation ability of planktonic bacteria as well, and no decolorization occurred after 15 and 45 minutes incubation with both dyes (Figure 2, panels C and D). We suggest, therefore, that crystal violet adsorption by *S. epidermidis* biofilm leads to an over staining, affecting the sensitivity of the test. Intriguingly, when *S. epidermidis* was exposed to 0.0625% ethanol, biofilm detection was similar for both dyes. Unfortunately, we cannot find a coherent explanation for this result. Knobloch et al. (2001) proposed that ethanol induces *S. epidermidis* biofilm formation as a stress factor that leads to induction of PIA (polysaccharide intercellular adhesin) synthesis and rsbU (putative ribose uptake protein) up-regulation. The switched protocol test (Supplementary Figure 1) gave unstable results with great deviations, which supports the need to use specific staining procedures for each dye. To our knowledge, no stimulation or inhibition effect of methanol on bacterial biofilm has been reported up to now. Wadhwani et al. (2008) showed that the growth percentage of *S. epidermidis* during exposure to 1% DMSO, 1% ethanol and 1% methanol are 98%, 91% and 100%, respectively, but no data on biofilm formation was given.

In conclusion, we recommend the use of up to 1% methanol as solvent followed by safranin protocol staining when biofilm formation by *S. epidermidis* (ATCC 35984) must be quantified.

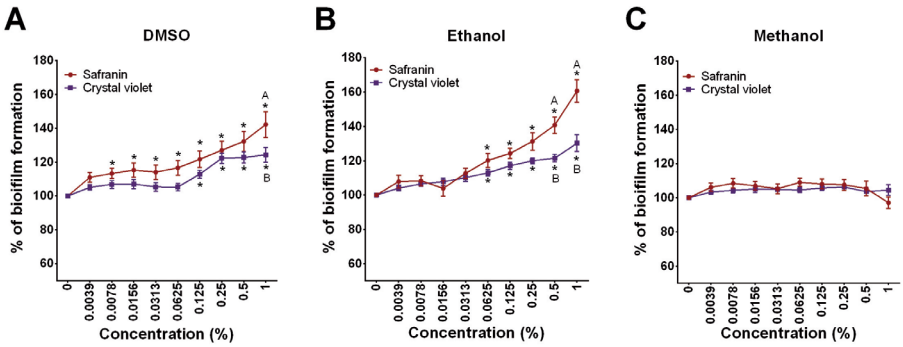


Figure 1. Mean percentage (± SEM) of *S. epidermidis* (ATCC35984) biofilm formation after exposure to different concentrations (0.0039–1%) of DMSO (A), ethanol (B) and methanol (C). * indicates significant ($P < 0.05$) difference between treatments and control (0%).

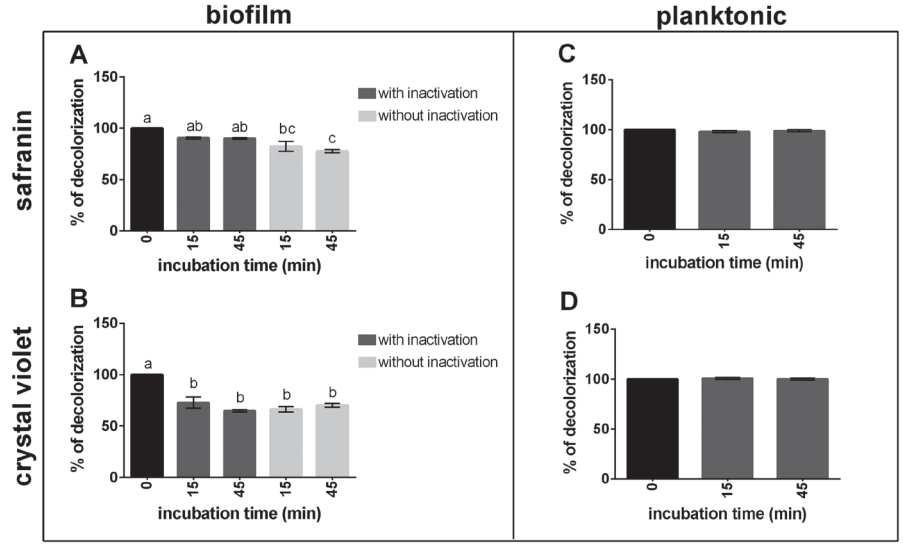


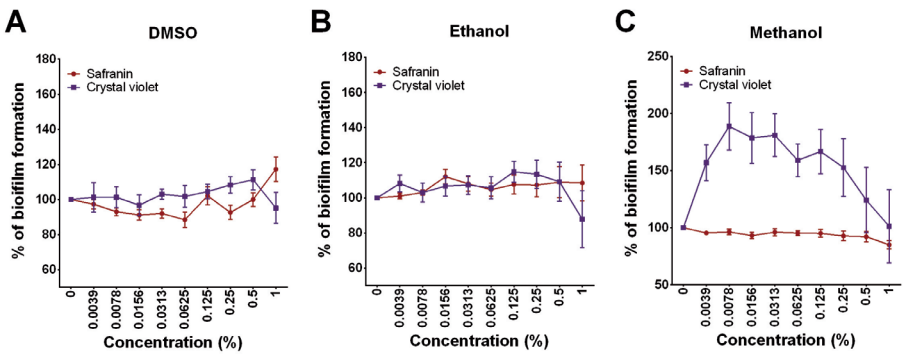
Figure 2. Mean percentage (± SEM) of safranin (A, C) and crystal violet (B, D) decolorization after incubation (15 or 45 min) with biofilm (A, B) or planktonic (C, D) *S. epidermidis* (ATCC35984). Biofilm decolorization was performed with (dark gray) or without (light gray) a previous inactivation step. Different letters (a–c) indicate significant ($P < 0.05$) difference between groups within the same staining protocol (safranin or crystal violet).

SUPPLEMENTARY DATA

Table S1. Mean percentage (± SEM) of increase in *S. epidermidis* biofilm formation after exposure to the solvents DMSO or ethanol at different concentrations (0.0039–1%). Data were collected after safranin or crystal violet staining.

Concentration (%)	DMSO		Ethanol	
	safranin	crystal violet	safranin	crystal violet
0.0039	10.98 ± 2.89	5.05 ± 1.64	7.86 ± 3.75	4.01 ± 1.64
0.0078	13.28 ± 3.09*	6.86 ± 2.32	8.32 ± 2.97	6.51 ± 1.45
0.0156	15.30 ± 4.19*	7.03 ± 2.72	3.91 ± 4.59	7.77 ± 2.16
0.0313	14.11 ± 4.30*	5.31 ± 2.65	12.86 ± 3.01	10.15 ± 2.32
0.0625	16.62 ± 4.31*	5.27 ± 2.2	20.22 ± 4.11*	12.92 ± 1.99*
0.125	21.70 ± 4.93*	12.87 ± 2.33*	24.28 ± 2.93*	17.07 ± 2.23*
0.25	27.01 ± 5.33*	22.32 ± 3.1*	31.32 ± 5.20*	20.00 ± 1.65*
0.5	32.24 ± 5.98*	22.66 ± 3.31*	40.76 ± 4.84*	21.54 ± 2.17*
1	42.20 ± 7.64*	24.35 ± 4.35*	60.68 ± 6.68*	30.21 ± 4.92*

* indicates significant ($P < 0.05$) increase of biofilm formation compared with untreated biofilm



Supplementary Figure 1. Mean percentage (± SEM) of *S. epidermidis* (ATCC35984) biofilm formation after exposure to different concentrations (0.0039–1%) of DMSO (A), ethanol (B) and methanol (C) after switching the protocols of safranin and crystal violet staining.

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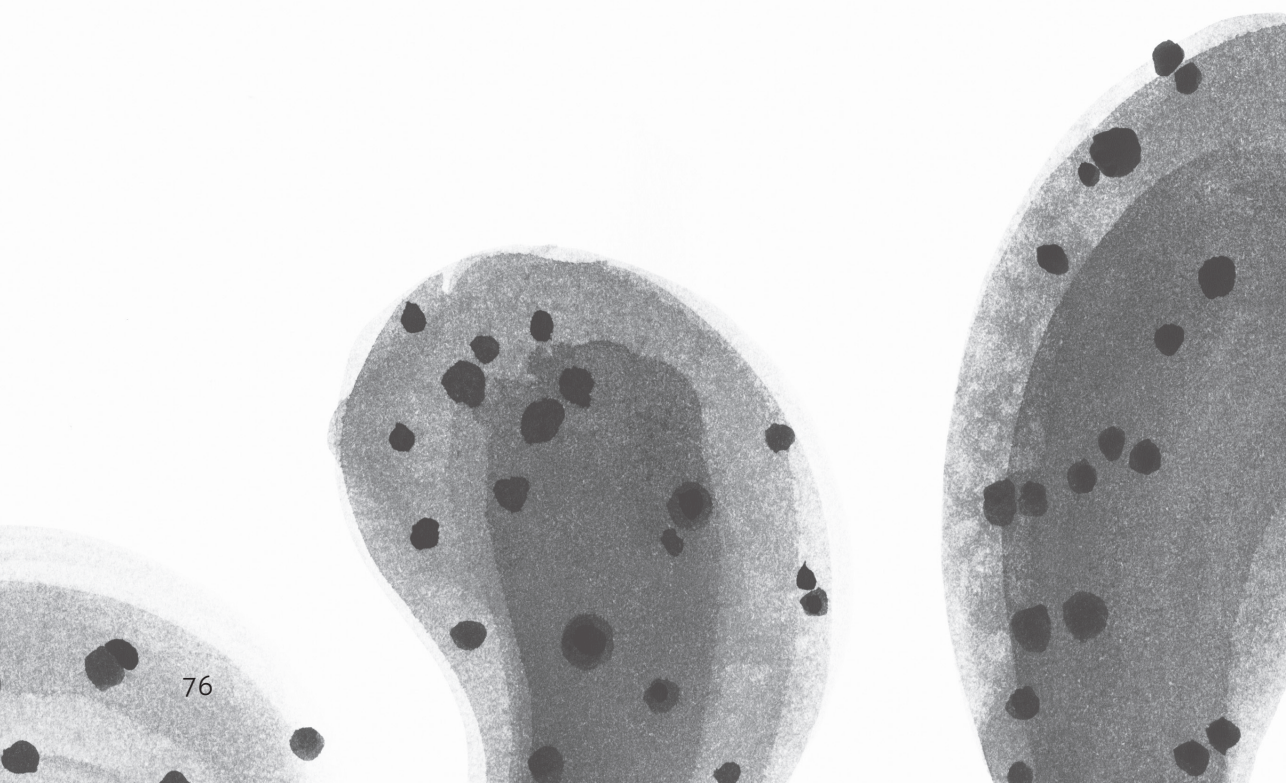
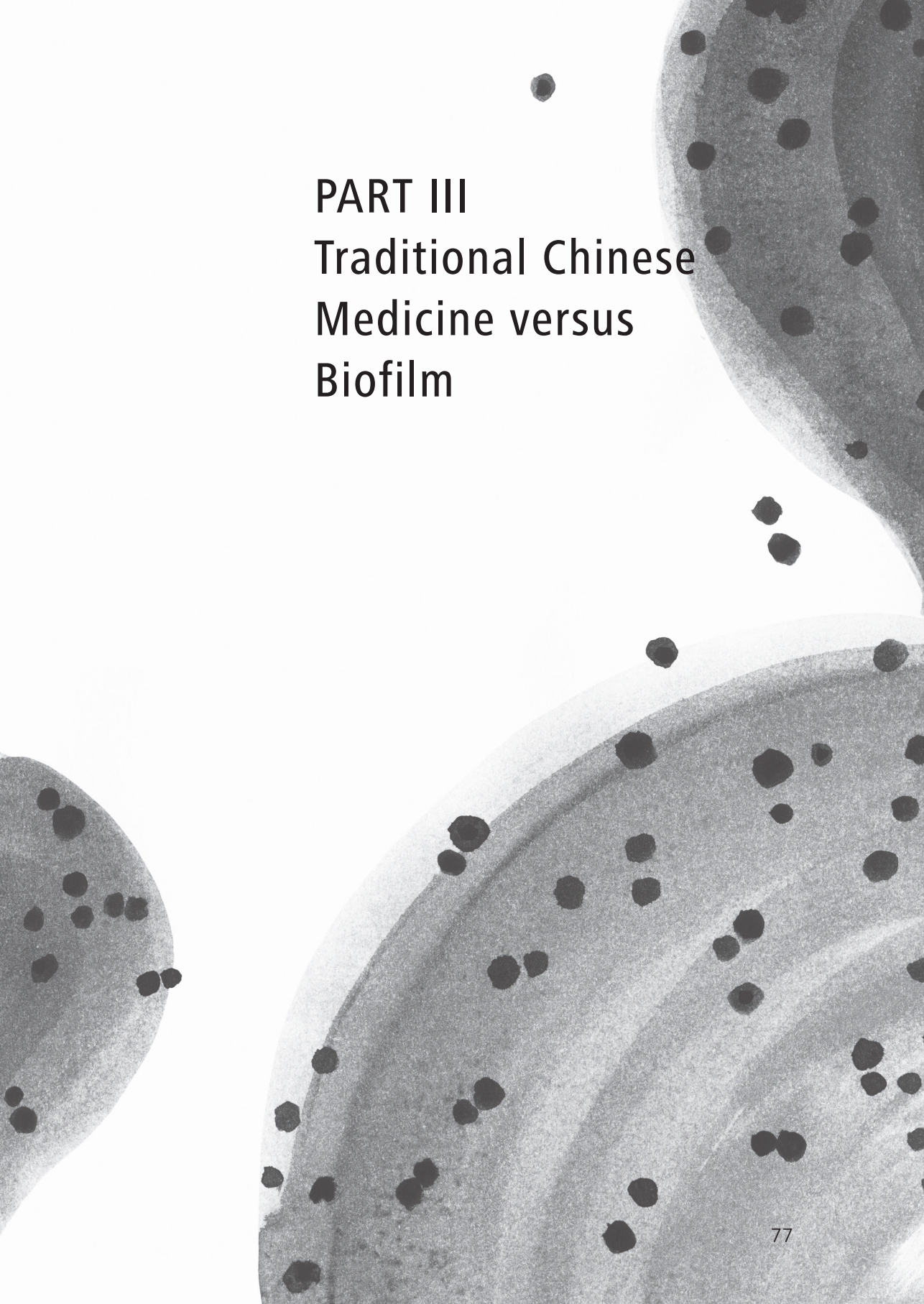
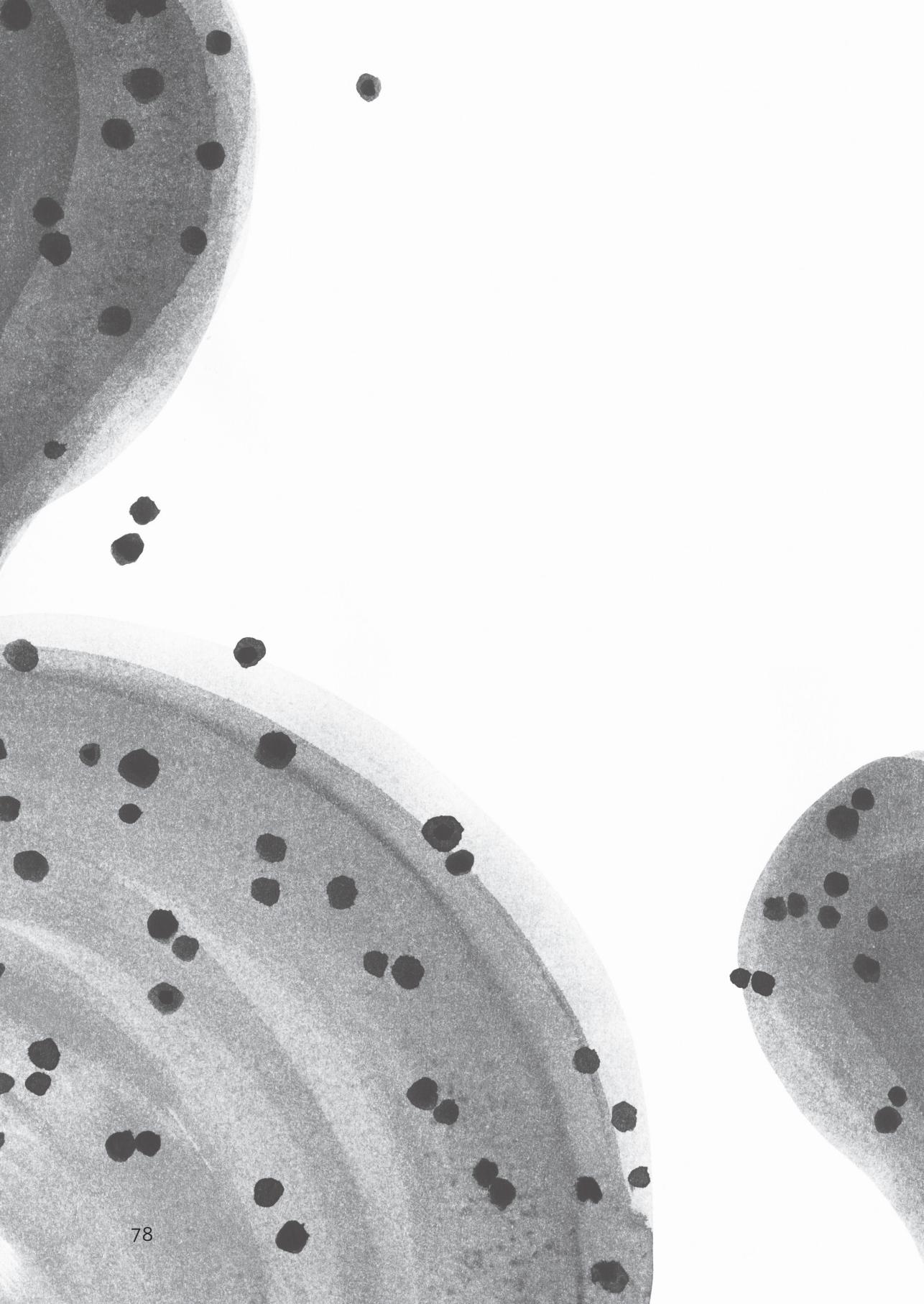


PART III
Traditional Chinese Medicine versus Biofilm





5 Analysing the antibacterial effects of food ingredients: model experiments with allicin and garlic extracts on biofilm formation and viability of *Staphylococcus epidermidis*

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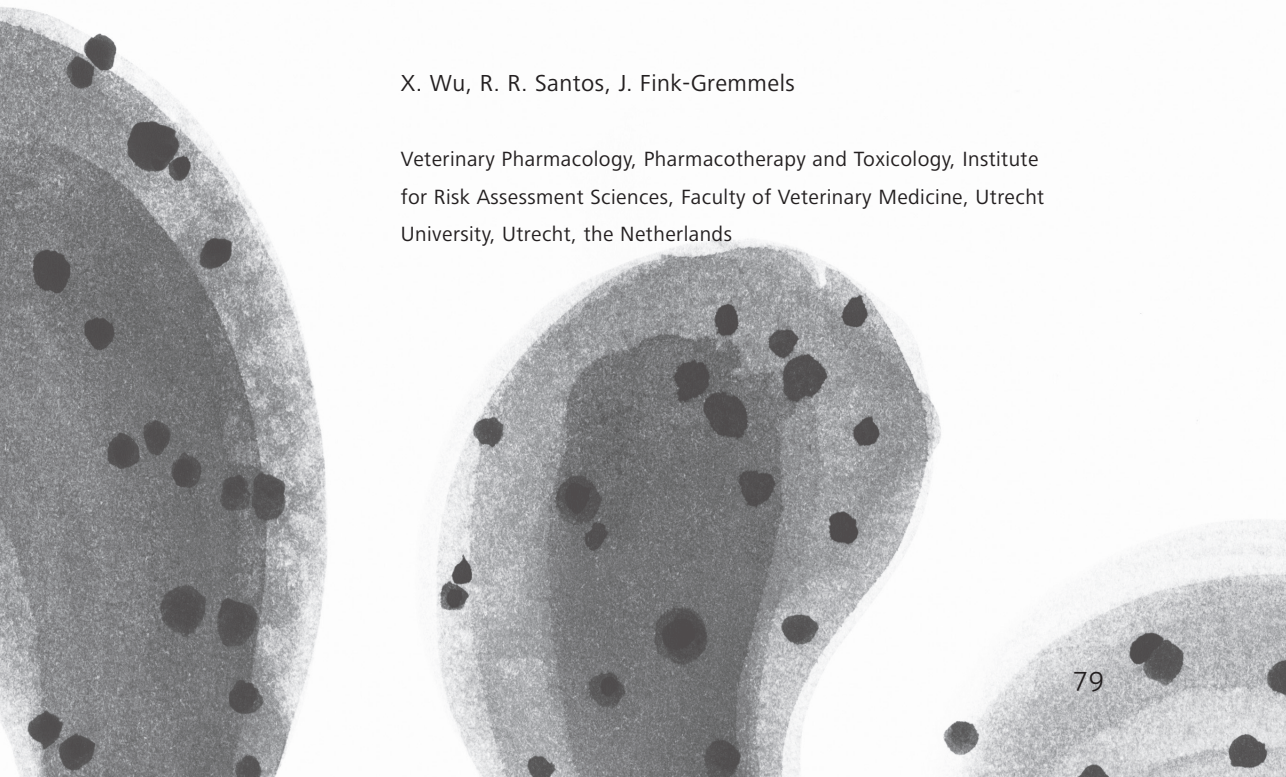
X. Wu, R. R. Santos, J. Fink-Gremmels

Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands



Scope: To demonstrate different effects of garlic extracts and their main antibiotic substance allicin, as a template for investigations on the antibacterial activity of food ingredients. Methods and Results: *Staphylococcus epidermidis* ATCC 12228 and the isogenic biofilm-forming strain ATCC 35984 were used to compare the activity of allicin against planktonic bacteria and bacterial biofilms. The minimal inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC) for pure allicin were identical and reached at a concentration of 12.5 µg/ml. MBICs for standardized garlic extracts were significantly lower, with 1.56 and 0.78 µg/ml allicin for garlic water and ethanol extract, respectively. Biofilm density was impaired significantly at a concentration of 0.78 µg/ml allicin. Viability staining followed by confocal laser scanning microscopy showed, however, a 100% bactericidal effect on biofilm embedded bacteria at a concentration of 3.13 µg/ml allicin. qRT-PCR analysis provided no convincing evidence for specific effects of allicin on biofilm-associated genes. Conclusions: Extracts of fresh garlic are more potent inhibitors of *S. epidermidis* biofilms than pure allicin, but allicin exerts a unique bactericidal effect on biofilm embedded bacteria. The current experimental protocol has proven to be a valid approach to characterize the antimicrobial activity of traditional food ingredients.

INTRODUCTION

Garlic (*Allium sativum*) is recognized as a medicinal herb for more than 5000 years and truly deserves to be described as functional food. Its technical and potential medical use are described in more than 4400 scientific articles and cover medical applications as an antibiotic described already by Louis Pasteur, and more recently the use of stabilized garlic extracts in the prevention of cardio-vascular diseases, diabetes, hyperlipidaemia, chronic inflammation and as anti-aging and anti-cancer remedy (Amagase, 2006; Borlinghaus et al., 2014). In food production, garlic has been used not only as flavouring agent, but particularly as preservative for meat and meat-products (Aguirrezabal et al., 2000; Cao et al., 2013; De Moura Oliveira et al., 2005; Mariutti et al., 2008; Yin and Cheng, 2003). Due to its antioxidant capacity garlic prevents rancidity and exerts antimicrobial activity prolonging the shelf-life of meat products and increasing food safety due to its effect against zoonotic pathogens such as Staphylococci, *Salmonella spp*. and *Escherichia coli* (Ankri and Mirelman, 1999). The antimicrobial activity of garlic extract, particularly the water-soluble ingredients such as allicin, have gained recently increasing attention as they are active against emerging pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) (Cutler and Wilson, 2004) and plaque-forming organisms in the oral cavity, which may be associated with garlic consumption (Bachrach et al., 2011). The dental plaques contain bacteria in a biofilm stage. A biofilm can be described as sessile community of bacteria, surrounded by a self-produced extracellular matrix that adhere the cells to biological and artificial surfaces. Within a biofilm, bacteria are temporary in a dormant stage, which conveys resistant to antibiotics as in dormancy the molecular targets for antibiotics such as cell wall synthesis, DNA and protein-synthesis are largely suppressed (Burmolle et al., 2010). The ability of bacteria, including almost all common pathogens, particularly those residing on the skin or mucosal surfaces, to form biofilms has revived the interest in herbs, as plants are known to express substances with antibiofilm/antifouling properties.

Here we describe a series of experiments conducted with allicin, the major antimicrobial compound in garlic, and allicin-containing aqueous and ethanol extracts resembling the ingestion of garlic as food additive and herb in a normal diet. As a model organism to study the antibacterial and anti-biofilm effects, we chose *Staphylococcus epidermidis*, from which two well characterized isogenic type-strains are available denoted ATCC 12228, a strain growing in planktonic cultures, and ATCC 35984 (also described as RP62A) that rapidly forms biofilms under in vitro culture conditions (Fey and Olson, 2010). Both type strains were used to test the effects of allicin and allicin containing garlic extracts on bacterial viability, biofilm formation and the survival of bacteria within a biofilm. The ultimate aim of this study was to provide a template for the assessment of commonly used herbs and related plant products in human (and animal) diets.

MATERIALS AND METHODS

Chemicals

Allicin was purchased from LKT (LKT Laboratories Inc., St. Paul, USA) at a concentration of 10.2 mg/ml in methanol/water (40/60) with 0.1% formic acid and stored at -20Ð. This original solution was diluted 200 times in tryptone soya broth (TSB) + 0.25% glucose (TSB+) to obtain the working solution of 50 µg/ml allicin. Therefore, the final concentrations of methanol and formic acid were 0.1 and 0.00025%, respectively. At these concentrations the solvents did not affect biofilm formation or bacterial viability (Wu et al., 2014). All other chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest available purity.

Preparation of garlic extracts

Extracts of fresh garlic obtained in a normal grocery were prepared with water (garlic water extract; gWE) or with pure ethanol (garlic ethanol extracts; gEE). The gWE mixture consisted of 15 g garlic bulbs, which were fragmented and milled in 0.2 M phosphate buffer (PBS) (pH 6.5) for 10 min, mixed for 1 hour after adding 60 ml de-ionized water at room temperature and centrifuged for 10 min at 3076 x *g* at room temperature (Sigma 3-16K). The precipitate was discarded and the supernatant was kept at 4 °C before use. gEE was also obtained from commercially available garlic bulbs as described before (Yang et al., 2009) with minor modifications. In brief, 15 g of the garlic bulbs were fragmented and milled in an aliquot 0.2 M PBS (PH6.5) for 10 min at room temperature, and then 60 ml of 95% ethanol (fresh garlic: ethanol/1:4 w/w) was added to this mixture. This suspension was kept in a thermostatic water bath for 1.5 hours at 30 °C and was then filtered through Watman paper filters and evaporated to absolute dryness to exclude any effects of ethanol residues. In the obtained extracts, allicin was quantified by HPLC according to the method of Ilic et al. (2012). Intra- and inter-day precision of the method was within the acceptable limits with relative standard deviation (RSD) < 5%. The limit of quantification was 0.1µg/ml. Working solutions of these extracts in the microbiological assays were standardized for the allicin content.

Bacterial strains and culture conditions

Two strains of *S. epidermidis* were purchased from the American Type Culture Collection (ATCC). The ATCC 35984 is known for its ability to form biofilms, while the strain ATCC 12228 is unable to form biofilm under experimental conditions (Fey and Olson, 2010) (Figure S1A, B). Both strains were maintained on tryptone soya agar (TSA) (Oxoid CM 129) slants at 4 °C. One colony of bacteria was cultured in 10 ml TSB+ (pH 7.0) under aerobic conditions at 37 °C for different times according to different experiments.

Minimum inhibitory concentration of allicin on *S. epidermidis* in planktonic cultures

To identify the concentration range for the following biofilm experiments, initially the minimum inhibitory concentration (MIC) of allicin was determined using the non-biofilm forming ATCC 12228 strain, following the Clinical and Laboratory Standards Institute (CLSI) Standard Broth Micro-dilution Method with minor modifications. Briefly, serial two-fold dilutions of 50 µg/ml allicin in TSB^+ down to a final concentration of 0.195 µg/ml allicin were prepared in a U-bottom 96-well plate (100 µl per well). To each well, 100 µl of 10^6 CFU/ml of the bacterial suspension was added, resulting in a final volume of 200 µl and final concentrations of allicin of 0.098, 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 or 25 µg/ml, respectively. Wells with sterile TSB^+ alone served as blanks. Plates were incubated at 37°C for 24 hours. Thereafter, OD values were measured at 655 nm wavelength after transferring 100 µl of the incubated suspension to a new sterile flat bottom 96-well plate. MIC was defined as the lowest allicin concentration resulting in OD value similar to blank (TSB^+). To allow a comparison with data from the bacterial viability in the biofilm, next to this standard broth dilution assay, a viability staining was conducted with SYTO® 9 green (green staining of total bacteria) and propidium iodide (red staining of bacteria with membrane damage; non-viable) (Molecular Probes Europe, Leiden, the Netherlands).

Bacterial inoculum and time-dependent biofilm formation

To establish the experimental conditions for the biofilm assays, a series of experiments were conducted to determine the optimal inoculum size as well as the incubations times required for the formation of a stable biofilm. Results of these assays are presented as supplementary data (Figure S1A, Figure S2) and revealed that an inoculum size of 10^6 CFU/ml is requested to achieve a dense biofilm of ATCC 35984 and that maximum biofilm density could be measured after 24 hours.

Determination of the minimum biofilm inhibitory concentration (MBIC)

Biofilm formation was assessed by the standard safranin colorimetric assay as described earlier (Melchior et al., 2006; Wu et al., 2014) using the strain ATCC 35984. In brief, 100 µl of the bacterial suspension (10^6 CFU/ml) was transferred into each well of a U-bottom 96-well microtiter polystyrene plates (Costar, Corning, NY, USA). To this suspension gWE and gEE or pure allicin was added dissolved in broth to reach allicin concentrations of 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25 or 50 µg/ml resulting in final tested concentration in the samples of 0.098–25 µg/ml allicin. Wells with sterile TSB^+ alone served as blanks. The plates were incubated on a microplate shaker (Heidolph titramax 100) at 37 °C for 24 hours. At the end of culture period, the supernatant from all wells were discarded and

the biofilms adhered to the bottom of the wells were incubated with 200 µl 0.1 M HCl for 1 hour at room temperature. Thereafter, HCl was replaced by safranin (0.1% in water) and the plates incubated for 45 minutes at room temperature. Non-bound safranin was removed by rinsing the wells 3 times with de-ionized water, and thereafter plates were incubated with 125 µl 0.2 M NaOH per well at 57 °C for 1 hour. At the end of incubation, 100 µl from the stained dissolved biofilm in each well was pipetted to a new flat-bottom 96-well microtiter polystyrene plate and its intensity was measured at a wavelength of 540 nm in a microplate reader. The minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration that inhibited at least 90% biofilm formation. Each test was performed in quadruplicate with 3 independent repetitions.

Bacterial viability within biofilms determined by confocal laser scanning microscopy

Biofilms of ATCC 35984 formed on coverslips, which were inserted into tubes containing either control medium or medium supplemented with gWE and gEE or allicin at the standard range of test concentrations (0.098–1.56 µg/ml allicin) were evaluated. After culture, coverslips carrying bacterial biofilms were incubated for 15 minute in the dark at 37°C with 1 nM SYTO® green fluorescent nucleic acid dye and 6 nM propidium iodide. After being labelled, biofilms were washed three times in PBS and finally examined using confocal laser scanning microscopy (CLSM) (Leica TCS SPE-II, Mannheim, Germany). Bacteria in the biofilm were classified as non-viable if stained positively by propidium iodide (red). Image generation was achieved using the 488 and 543 nm wavelengths for SYTO® green and propidium iodide, respectively. To estimate the percentages of non-viable bacteria, the program Image J 1.4.7 was used to count the propidium stained cells (given as cell area).

Quantitative RT-PCR

Along with the biofilm formation assay, samples were taken at the end of incubation and submitted to RNA isolation. For this, 1 ml of bacterial suspension was centrifuged for 10 minute at 4 °C 15000 x *g*. The supernatant was removed and 1 ml Trizol reagent was added, mixed and then the suspension was transferred to a Lysing Matrix E tubes (MP Biomedicals Germany GmbH, 37269 Eschwege Germany) and homogenized in the FP120 Cell Disrupter (Thermo Savant, Qbiogene, Inc. Cedex, France) for 45 seconds at speed of 6.5 m/s two times. Subsequently, the samples were centrifuged for 5 minute at15000 x *g* 4 °C, and the supernatants were separately transferred to 1.5 ml Eppendorff tubes and subjected to the phenol-chloroform RNA extraction protocol. The concentration and purity of total RNA was spectrophotometrically assessed using a NanoDrop 1000™ (Thermo Scientific, Waltham, MA, US), and 1 µg of extracted total RNA from each sample was reverse transcribed with the iScriptTM cDNA Synthesis

kit (BIO-RAD, Hercules, CA, USA) according to the instructions of the manufacturer. The obtained cDNA was diluted to a final concentration of 30 ng/ml. Primers (Table S1) complementary to *S. epidermidis* were designed according to literature, and were commercially produced (Eurogentec, the Netherlands). The primers were selected based on specificity and efficiency by qPCR analysis of a dilution series of pooled cDNA at a temperature gradient (55 °C to 65 °C) for primer-annealing and subsequent melting curve analysis. The reaction mixture for the qPCR contained of 10 µL diluted cDNA, 12.5 µL iQSYBR Green Supermix (Bio Rad Laboratories Inc., USA), forward and reverse primers (final concentration of 0.4 pmol/µL for each primer) and sterile water according to the manufacturer's instructions. qPCR was performed using the MyiQ single-colour real-time PCR detection system (Bio-rad, Hercules, CA) and MyiQ System Software Version 1.0.410 (Bio Rad Laboratories Inc., USA). The mRNA quantity was calculated relative to the expression of two reference genes, hsp60 and tpi (Table S1).

Statistical analysis

For the biofilm formation assay and bacterial viability test, data was evaluated using one-way analysis of variance (ANOVA) by Prism 6.04. For qRT-PCR test, down- and up-regulation were considered significant when the relative expression was decreased or increased ≥ 4 folds. All experiments were repeated at least three times.

RESULTS

Minimum inhibitory concentration (MIC) of allicin on S. epidermidis ATCC 12228 in planktonic cultures

Using the standard broth micro-dilution protocol, we could determine a MIC value of 12.5 µg/ml for *S. epidermidis* ATCC 12228, despite the observed a slight reduction of OD values already at a concentration of 6.25 µg/ml allicin (Figure 1A). This value was confirmed using a live/dead staining with SYTO® green and propidium iodide and a quantitative evaluation of confocal microscopy images (Figure 1B).

Time- and concentration-dependent effects of allicin on biofilm formation

Using the safranin staining protocol to assess biofilm formation by (ATCC 35984) after 12, 24, and 48 hours, respectively, it could be shown that allicin inhibits biofilm formation in a concentration-dependent manner (Figure 2). In the tested concentration range varying from 0.78–25 µg/ml, significant differences in comparison to the controls were observed already during the initial 12 hours of culture. Biofilm biomass was increasing during the following 12 h significantly, whereas between 24 h and 48 h only a slight further increase was observed. Based on these results, 24h biofilms were used for the forthcoming experiments on biofilm sensitivity and architecture.

Minimum biofilm inhibitory concentration and bacterial viability in the biofilm

S. epidermidis (ATCC 35984) was cultured in the presence of different concentrations of allicin for 24 hours to determine the MBIC. The MBIC is defined as the concentration at which biofilm density is reduced by > 90% when compared with OD values of controls (here at an OD value < 0.06). Using this definition, it could be shown that the MBIC of allicin was 12.5 µg/ml, but biofilm formation was inhibited significantly already after exposure to 0.78 µg/ml allicin (Figure 3A). The percentage of viable bacteria in the biofilm showed a rapid and significant decrease ($p < 0.0001$) to 60% of controls already after exposure to 0.195 µg/ml allicin, and exposure to 3.13 µg/ml allicin resulted in a complete loss of viability (100%) of bacteria within the biofilm (Figure 3B).

Effects of garlic extracts on biofilm formation and bacterial viability

S. epidermidis ATCC 35984 biofilm was exposed to either an aqueous (water) extract (gWE) or and ethanol extract (gEE) standardized for their content of allicin. MBIC was 1.56 and 0.78 µg/ml allicin for gWE and gEE, respectively (Figure 4A1, B1), when using the safranin staining method. Confocal laser scanning microscopy was used to estimate the percentages of non-viable *S. epidermidis* in the controls as well as after treatments with gWE and gEE. It was observed that incubations with gEE containing 1.56 µg/ml allicin resulted in 100% loss of bacterial viability (Figure 4A2, B2). Differently, bacteria treated with gWE containing 1.56 µg/ml allicin, rendered ~ 70 and 60% of non-viable bacteria, and a 100% loss of viability was observed at a concentration of 3.13 µg/ml of allicin in the extract (Figure 4A3, B3).

Biofilm architecture determined by confocal laser scanning microscopy

To obtain further insight in the biofilm structure and density CLSM was used to obtain surface plot images showing *S. epidermidis* (ATCC 35984) encapsulated in biofilm matrix when bacteria were treated with different concentrations of pure allicin for 24 hours (Figure 5). Control samples presented a compact and dense biofilm with a thickness of 10.7 µm. Exposure to allicin resulted in biofilm remodelling, and biofilm thickness decreased rapidly from 10.7 µm (controls) to 8.6 µm after exposure to 0.098 µg/ml allicin, and gradually with increasing allicin concentrations to 4.7 µm after exposure to the highest allicin concentration (1.56 µg/ml). The reduction in biofilm mass was accompanied by a loss a viability of biofilm bacteria. These results support the findings of the MBIC determination.

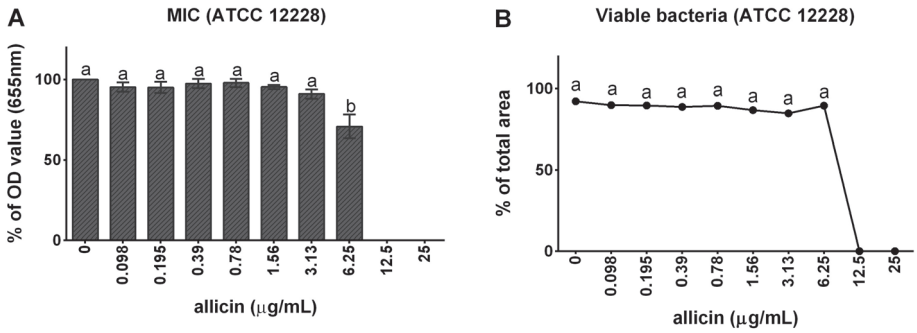


Figure 1: Minimum inhibitory concentration (Mean ± SEM) of allicin determined in *S. epidermidis* ATCC 12228 growing in planktonic cultures. A: results obtained with the standard CLSI broth dilution protocol; B: Results (Mean ± SEM) obtained with a live/dead staining and quantification of dead and live bacteria with confocal microscopy. Different lower-case letters (a, b) indicate significant differences ($p < 0.001$) between allicin concentrations.

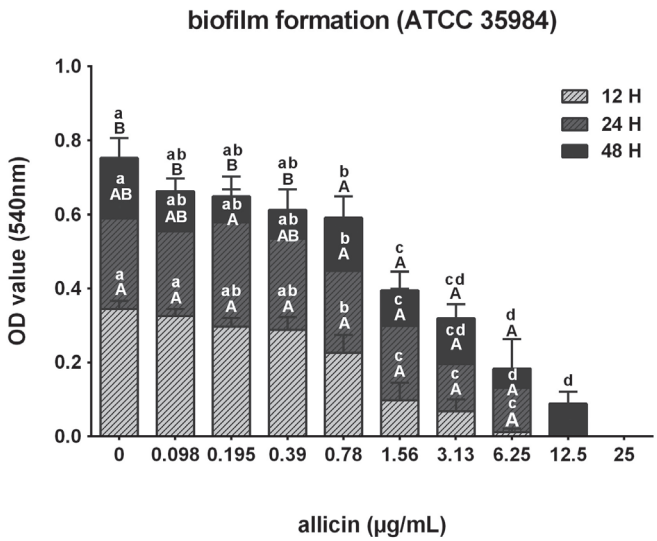


Figure 2: *S. epidermidis* (ATCC 35984) biofilm formation within 48 hours and after exposure to different concentrations (0.098–25 µg/ml) of allicin. Different upper-case letters (A, B) indicate significant ($p < 0.001$) differences between time points within the same allicin concentration. Different lower-case letters (a–d) indicate significant ($p < 0.0001$) differences between allicin treated groups within the same time point.

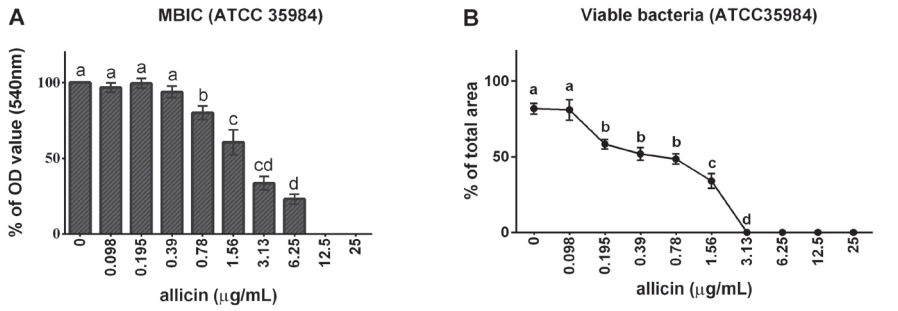


Figure 3: Minimum biofilm inhibitory concentration (MBIC) and viability of *S. epidermidis* (ATCC 35984) within a biofilm exposed to different concentrations of allicin. A: Results of the safranin staining method (% OD value at 540 nm wavelength, Mean ± SEM). B: Results of live/dead staining with SYTO® green and propidium iodide and quantitative evaluation with CLSM (Mean ± SEM). Different letters (a–d) indicate significant ($p < 0.0001$) differences.

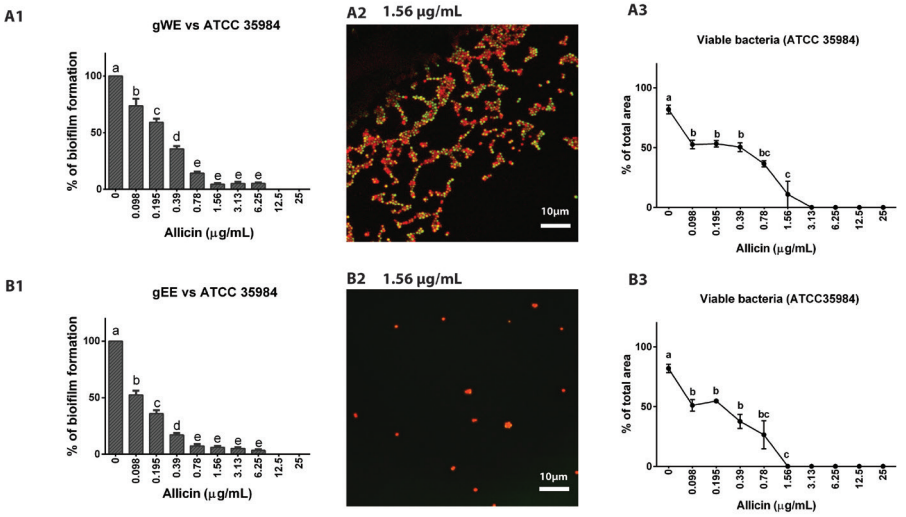


Figure 4: Comparison of the effect of aqueous (gWE) and ethanol (gEE) extracts of fresh garlic standardized for their allicin concentration on *S. epidermidis* biofilm formation and bacterial viability in the biofilm. The left panel (A1, B1) show the results of the safranin staining methods as mean percentage (± SEM) of biofilm formation. The middle panel (A2, B2) give examples of this viability staining of the two extracts with the same allicin concentration (1.56 µg/ml allicin). The right panel (A3, B3) show the result (Mean ± SEM) obtained with quantitative evaluation of bacterial viability determined by differential viability staining with SYTO® green and propidium iodide and confocal microscopy.

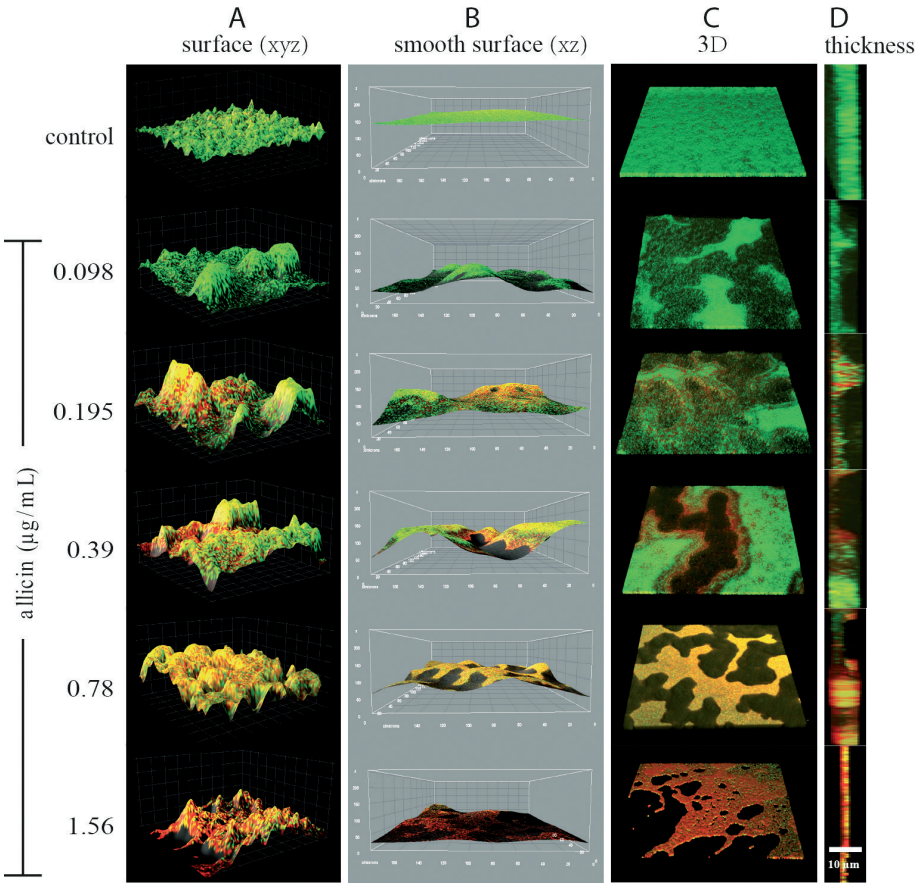


Figure 5: *S. epidermidis* (ATCC 35984) biofilm architecture after exposure to increasing concentrations of allicin. CSLM images of *S. epidermidis* biofilm surface and smooth surface from (xz) (A, B); biofilm 3D and thickness images (C, D) after exposure to different allicin concentrations (0.098–1.56 µg/ml). The biofilm thickness decreased from 10.7 (controls) concentration-dependently to 8.6, 7.7, 8.0, 7.9 and 4.7 µm (1.56 µg/ml allicin) with increasing allicin concentrations. Cells were distinguished by staining total bacterial cells with SYTO® green (green) and non-viable bacterial cells with propidium iodide (red).

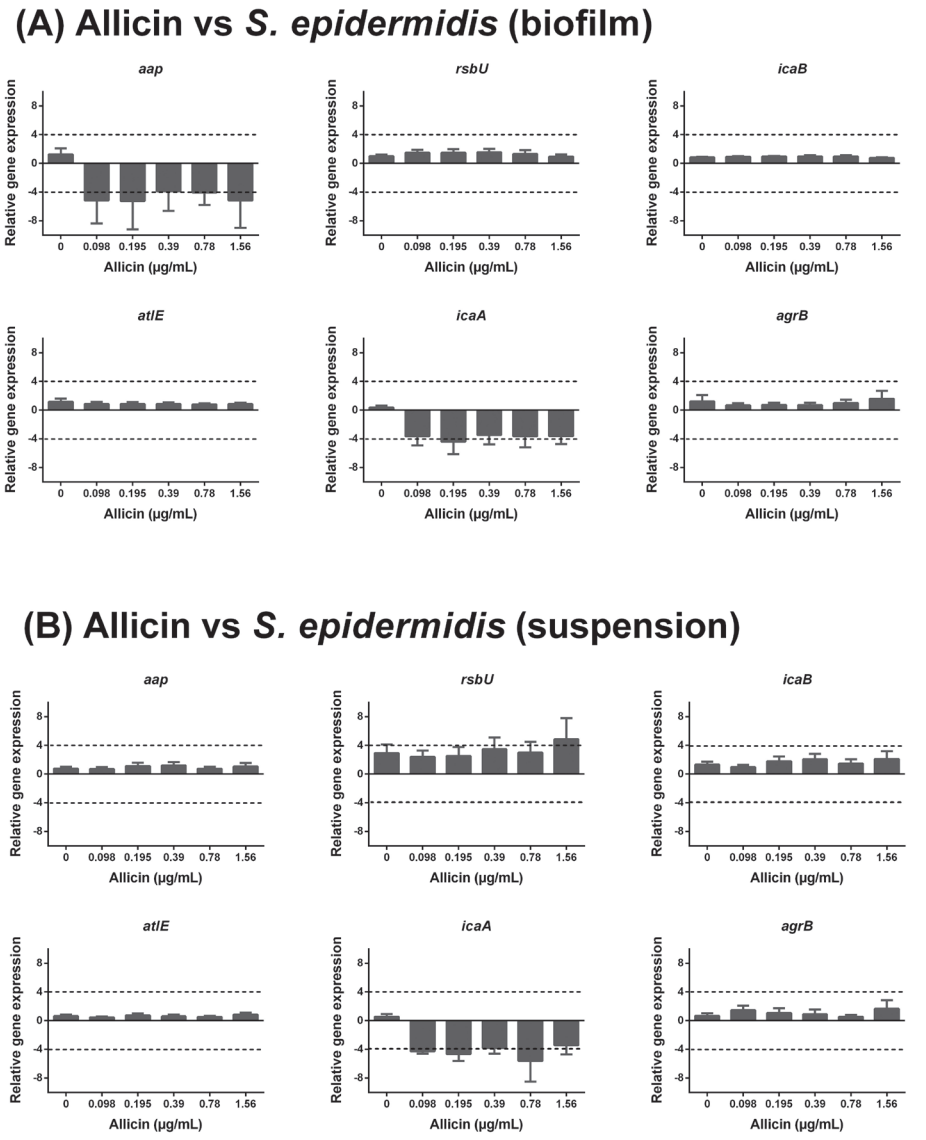


Figure 6: Relative mRNA expression (Mean ± SEM) of selected biofilm-related genes in biofilm bacteria (A) and persister cells in suspension (B). Down- and up-regulation of gene expression (marked by dashed lines) were considered significant when the relative expression was decreased or increased ≥ 4 folds.

Concentration and time-dependent effects of allicin on biofilm-related gene expression

To gain further inside into the effects of allicin on *S. epidermidis* ATCC 35984 biofilm, the expression of selected genes that mark different phases of biofilm formation were analysed in parallel in biofilm-adhered bacteria, as well as in non-adhered, persister cells present in the supernatant above the biofilm. In the biofilm-embedded bacteria (Figure 6A), allicin exposure resulted in a down-regulation of *aap* and *icaA*, which are associated with the adhesion and bacterial accumulation in a biofilm. In persister cells (Figure 6B) *aap* expression was not influenced by allicin, but *icaA* was down-regulated and a concentration-dependent increase in *rsbU,* a bacterial stress factor, was visible.

DISCUSSION

Garlic is one of the most widely used herbal products and garlic and its extracts are traditionally added to food not only for their taste but also for their food preserving properties. In this study we aimed to characterize the anti-biofilm properties of allicin, the major ingredient of garlic extracts in more detail and to compare these effects with gross aqueous and organic extracts of fresh garlic bulbs. Allicin is not present in fresh intact garlic cloves, but is enzymatically formed from its precursor alliin by alliinase within seconds upon crushing garlic cloves by mechanic means (garlic press) or by chewing (Ankri and Mirelman, 1999). The concentrations of alliin and subsequently allicin may vary between individual garlic plants, depending on the culture conditions, but an average concentration of 3.4–4.6 mg/allicin per gram fresh garlic can be assumed (Rybak et al., 2004). As model strain for these investigations two type strains of *S. epidermidis* were selected, as these stains are isogenic and well characterized. ATCC 12228 grows in planktonic cultures, and ATCC 35984 (also described as RP62A) rapidly forms biofilms under in vitro culture conditions. *S. epidermidis* may be considered as a relevant model strain also as it is a facultative pathogen residing on human skin from which is distributed to other individuals directly or transferred to food (Fey and Olson, 2010).

As a first step, the MIC of allicin determined in planktonic *S. epidermidis* (strain ATCC 12228) in the broth-dilution assay was determined to be 12.5 µg/ml, and confirmed by live-dead staining of bacteria in planktonic growth. These findings are in line with previous investigations (Perez-Giraldo et al., 2003) and were used to establish the concentration range used in the biofilm experiments. Subsequently, we measured the effect of allicin on ATCC 35984 biofilm formation. In non-treated cultures, *S. epidermidis* biofilm mass increases up to 24 hours, which is in agreement with previous studies by Vuong et al. (2003) and Qin et al. (2007) describing also that approximately 24 hours are necessary for the stable formation of a *S. epidermidis* biofilm. Therefore we selected

this time point to establish the MBIC. The MBIC value is defined as the concentration of an anti-biofilm agent that inhibits biofilm formation by > 90%. Applying this criterion, a MBIC of 12.5 µg/ml was established when the standard safranin protocol was applied, a value that seems not to differ from the MIC value. This indicate already the relative potency of allicin against biofilms as MBIC values of modern antibiotics, such as levofloxacin and vancomycin, show MBIC values of 25 µg/ml and 50 µg/ml respectively (Shapiro et al., 2011). To further investigate the effect of garlic on biofilms we applied a live/dead differential staining and could show that the decrease in biofilm formation was accompanied by a strong bactericidal effect towards biofilm-embedded bacteria, with a 100% loss of viability observed already at a concentration of 3.13 µg/ml allicin. This observation could be confirmed by CLSM analysis of the biofilm architecture, which again showed not only a decrease in biofilm thickness, but also an allicin concentration-dependent bactericidal effect on bacteria within the biofilm. This finding is unique, as biofilm embedded bacteria are generally insensitive to common antibiotics and MIC and MBC values may differ by a factor of 1000 (Taylor, 2013).

S. epidermidis is in the stationary phase after 24 hours culture. Lewis (Lewis, 2007) reported that in stationary cultures with biofilm formation, persister cells will appear in the supernatant. Persisters are dormant, non-dividing cells, which seen to be responsible for most biofilm-associated antibiotic tolerance. Shapiro et al. (Shapiro et al., 2011) reported the same phenomenon in *S. epidermidis* RP62A (denoted here ATCC 35984), and showed the reduced antibiotic sensitivity of stationary planktonic bacteria. This is of relevance for biofilm sensitivity on non-viable materials such as food of food processing aids, whereas under *in vivo* conditions, the phagocytes may be able to opsonise and kills persisters (Lewis, 2007). These previous results support our observation that planktonic stationary *S. epidermidis* bacteria were less sensitive to allicin than biofilm bacteria.

CLSM analysis of bacterial biofilm was primarily conducted to assess the effect of allicin on *S. epidermidis* biofilm architecture. Previously Cruz-Villalon and Perez-Giraldo (Cruz-Villalon and Perez-Giraldo, 2011)reported that 4 µg/ml allicin is able to decrease polysaccharide intercellular adhesion (PIA) production in 24 hours cultures. CLSM analysis showed a typical tower formation in *S. epidermidis* biofilms already at the lowest concentration of allicin (0.098 µg/ml) and the tower structure remained visible also at the higher concentrations when an increasing percentage of non-viable cells were found. Tower formation is associated with the production of extracellular matrix and the PIA system, requiring viable cells. The fact that this tower formation was observed at all allicin concentrations, support the hypothesis that the decline in biofilm density exerted by allicin is correlated with its concentration–dependent bactericidal effect against biofilm-embedded bacteria, rather than the solely inhibition of biofilm formation via interference with quorum sensing molecules. PIA production is catalysed by various glucuronyltransferases regulated by *icaA* in conjucntion with *icaD* and other

genes, such as *icaB* involved in the processing of PIA (Gerke et al., 1998; Spiliopoulou et al., 2012). These glucuronyltransferases contain cytein-rich moieties that may be inhibited by allicin (Cruz-Villalon and Perez-Giraldo, 2011; Rohde et al., 2005).
This hypothesis is in agreement with our results of the relative gene expression, where one of the main effects was the down-regulation of *icaA.* The concomitantly observed down-regulation of *aap* (accumulation-associated protein mediating the intercellular adhesion between bacteria) (Rohde et al., 2005) in the biofilm, that was not observed in the persister cells, suggest the involvement of PIA-independent mechanism in the inhibition of biofilm formation by *S. epidermidis*, but the function and regulation of *aap* is still incompletely understood (Fey and Olson, 2010). *AtlE* (autolysin E) mediates the initial attachment of bacteria (Vandecasteele et al., 2003) and generally acts in concert with *agrB* that was not affected by the allicin treatment. The *RsbU* gene is a positive regulator of the activity of sigma factor B, which is the general stress-response factor of Gram+ microorganisms and involved in PIA production as well (Delumeau et al., 2004; Knobloch et al., 2001). *RsbU* was upregulated in persister cells, but not in biofilm-attached cells. *Agr* (accessory gene regulator) is a marker of biofilm dispersion and was not affected by allicin treatment (Dai et al., 2012; Vuong et al., 2003). Taken together, these results demonstrate that allicin affects various stages of biofilm formation in *S. epidermidis*. This is in line with findings in *Pseudomonas aeruginosa* biofilms, indicating that allicin reduced biofilm mass by reducing the adhesion ratio as well as the production of extracellular matrix and the expression of virulence factors (Lihua et al., 2013). In contrast to our finding with allicin in *S. epidermidis*, in *P. aeruginosa* no significant reduction of bacterial growth or loss of bacterial viability was observed and allicin mainly acted as a quorum sensing inhibitor.

Summarizing the presented results obtained with allicin, it can be concluded that the most prominent effect of allicin is its unprecedented bactericidal effect on *S. epidermidis* embedded in biofilms. The antibacterial activity of allicin has been attributed to an interaction with cellular thiol groups (Fujisawa et al., 2009), a mechanism comparable to the effects of allicin in eukaryotic cells, where allicin interacts with the GSH/GSSG redox system. In gram-positive bacteria the redox homeostasis is mainly achieved by means of the bacillithiol (BSH) system (Helmann, 2011) and further investigations should be devoted to the inhibitory effect on allicin on BSH, which might explain its strong bactericidal effect in biofilms.

Our experimental studies included also an assessment of aqueous (water) (gWE) and organic ethanol (gEE) extract. These extracts were standardized for their allicin content, but contain a variety of other substances present in fresh garlic. A decrease in biofilm formation was observed already at a concentration equal to 0.098 µg/ml allicin, and CLSM analysis showed 100% of non-viable bacteria already at a concentration of 1.56 µg/ml in the gEE group which was lower when compared with gWE and pure allicin (3.13 µg/ml)

exposed biofilms. Such a difference was expected and suggests that other components, including ajoene, another sulphuric component and known quorum-sensing inhibitor in garlic, may contribute to the inhibition of biofilm formation (Jakobsen et al., 2012).
In conclusion, our data show a unique bactericidal effect of allicin on *S. epidermidis* biofilms. This effect could only be noted as different assays assessing the antibacterial effects of allicin were combined with differential viability staining followed by confocal microscopy and software aided analysis of the images, which allows the quantification of results. This stepwise assessment can be recommended in general for the assessment of small plant molecules that gain interest as supportive treatment of biofilm forming bacteria. The presented results underline also the opportunities to use garlic or garlic derived extracts for medical indications such as wound infections, which are often complicated by resistant bacteria like MRSA (Ankri and Mirelman, 1999; Borlinghaus et al., 2014; Cutler and Wilson, 2004) and in dental medicine where biofilm formation is common (Bachrach et al., 2011). At the same time, garlic and its extracts remain important additives in food preservation increasing the self-life and the safety of food due to their unique antimicrobial and bactericidal effects.

ACKNOWLEDGEMENTS

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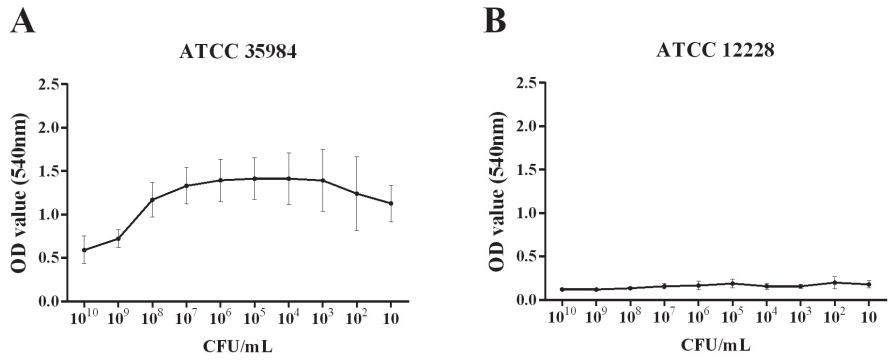
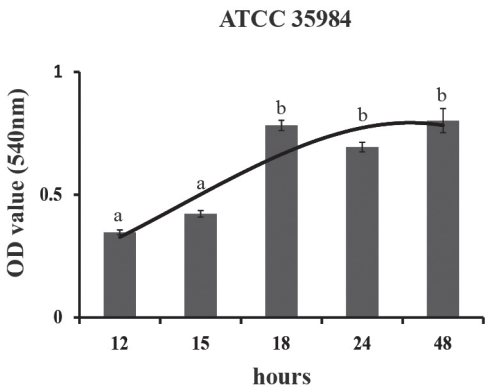
SUPPLEMENTARY DATA



Supplementary Figure 1. Biofilm formation by *S. epidermidis* strains ATCC 35984 (A) and ATCC 12228 (B). Data are expressed as mean OD value (± SD) of untreated biofilms from different inoculum sizes measured in three independent experiments using each time six replicates. CFU/ml: Colony-forming unit/ml.



Supplementary Figure 2. Time-dependent biofilm formation of *S. epidermidis* (ATCC 35984). Biofilm formation was quantified by safranin staining. Mean OD values (± SEM) were measured at 540 nm wavelength. Different lower-case letters (a, b) indicate significant ($p < 0.05$) differences between time.

Supplementary table 1: Primers for biofilm-associated genes used in this study.

Gene	GenBank	Primers sequence (5'-3')	Product size (pb)	Reference
hsp60	AF029245	F: GTTTTAGCACAATCAATGATTCAG	491	Wang et al. (2003)
		R: GCATCGCCTTCTACTTCATCC		
tpi	AF269838	F: CATCTGATAAACCTTCGACAGCTTT	128	Vandecasteele et al. (2001)
		R: GTAGCCGTCCAAGTTTTACCAG		
agrB	AF012132	F: TTCGTTTAGGGATGCAGGTA	141	Patel et al. (2012)
		R: ATGGCACACGTACAGAGGAT		
atlE	U71377	F: TGTCCTGCTTTCACGTATGA	139	Patel et al. (2012)
		R: AGAAACCTTAACCACGTAAA		
icaA	U43366	F: AACAAGTTGAAGGCATCTCC	166	Tormo et al. (2005)
		R: GATGCTTGTTTGATTCCCT		
icaB	U43366	F: AATGGCTTAAAGCACACGAC	144	Patel et al. (2012)
		R:TTTGTCCTTTCCGTAACAGT		
rsbU	NC002976	F: TCTCTTCATACAGTCCAT	172	Knobloch et al. (2001)
		R: ATAGGTTCAGGTATTCCA		

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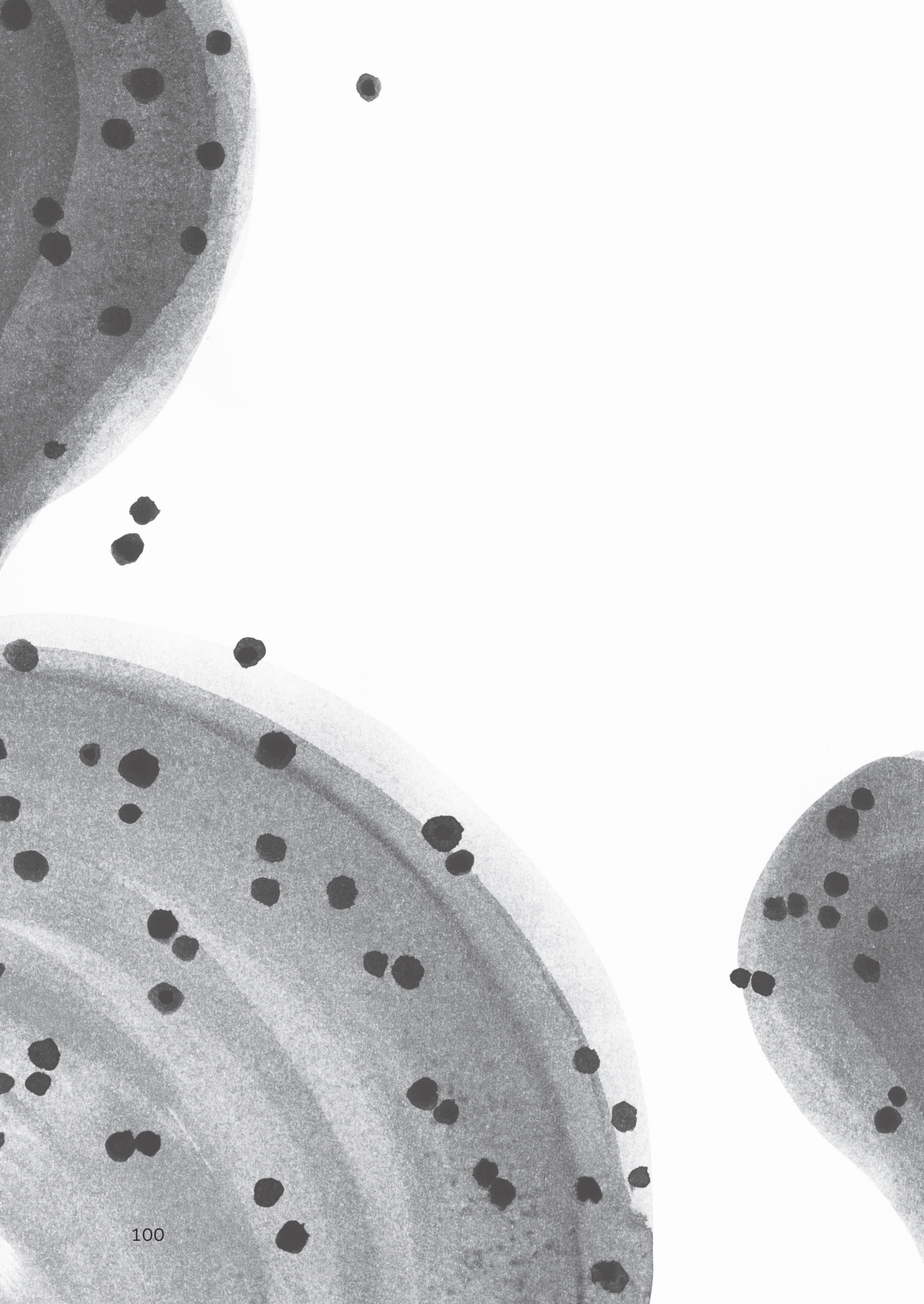


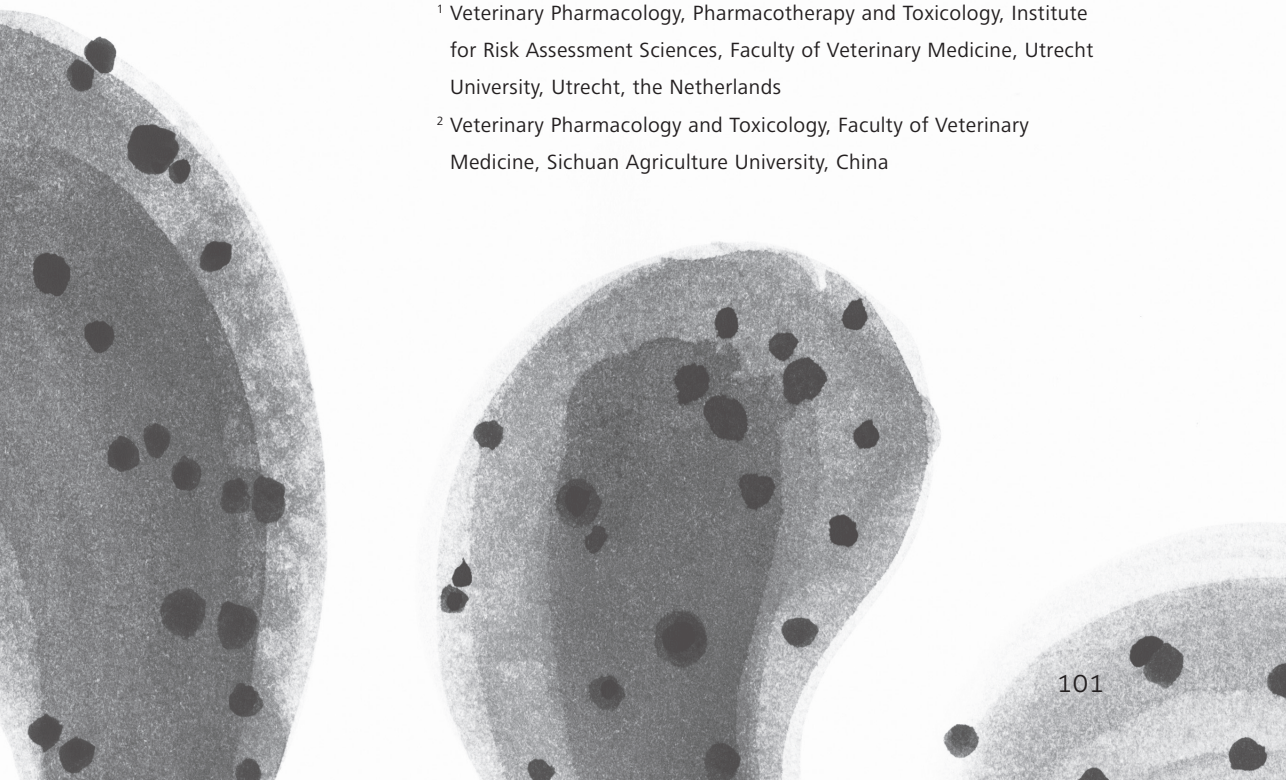
6 The effect of selected heat-clearing Chinese Herbal Medicines on *Staphylococcus epidermidis* biofilm formation

Submitted to Planta Medica

X. Wu[1,2], Y. Li[2], R. R. Santos[1], J. Fink-Gremmels[1]

[1] Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

[2] Veterinary Pharmacology and Toxicology, Faculty of Veterinary Medicine, Sichuan Agriculture University, China



Astringent and so call heat-clearing Chinese Herbal Medicines (CHMs), selected according to the paradigms of heat-*Zheng* are often used to treat bacterial infections. The common route of application is in the form a tea (decoction). The aim of this study was to evaluate the antibacterial and antibiofilm properties of the astringent (*Galla Chinensis*) decoctions and compare this effect with the results of comparable extracts of seven heat-clearing herbs using two type strains of *Staphylococcus epidermidis* as a model organism. Water extracts were prepared according to the prescribed methods, involving the preparation of serial decoctions. Bacterial susceptibility and biofilm quantification after exposure to different concentrations of these CHM water extracts were measured by broth, the micro-dilution method and biofilm formation, quantified by safranin staining. The standardized water extract of *G. chinensis* showed the lowest MIC value (0.078 mg/ml) against *S. epidermidis*, but inhibited biofilm formation already significantly at a concentration of 0.039 mg/ml. Comparative MIC values for the other CHM extracts were: *Coptis teeta* (MIC: 0.313 mg/ml); *Scutellaria baicalensis* (MIC: 12.5 mg/ml); *Forsythia suspensa* and *Viola yedoensis* (MIC: 25 mg/ml) and *Isatis indigotica, Lonicera japonica* and *Taraxacum mongolicum* (MIC: 100 mg/ml). *S. epidermidis* biofilm formation was inhibited already at sub-MIC concentrations of the *G. chinensis* extract. In addition, *C. teeta, F. suspensa, I. indigotica, L. japonica,* and *S. baicalensis* presented *S. epidermidis* biofilm inhibitory properties, albeit it a higher concentrations. Remarkably, *T. mongolicum* and *V. yedoensis* were also able to inhibit biofilm at high concentrations (12.5–100 mg/ml) but at lower concentrations these herb extracts promoted significantly the biofilm formation. In conclusion, the presented results show the differences in MIC, MBC and MBIC of in total 8 (traditionally prepared) decoctions of CHMs, belonging to the group of heat-clearing remedies. These results warrant further investigation on the interaction of at least five of the selected CHMs with common antibiotics in the treatment of chronic infections.

INTRODUCTION

Traditional Chinese Medicine (TCM) theory is applied when so-called heat-clearing decoctions, selected according the heat-*Zheng*, are prescribed for the treatment of bacterial infections (Ding et al., 2014; Feng et al., 2011). According to TCM theory, heat-clearing means control of a febrile response of local inflammatory processes including and astringent effect , preventing the loss of body fluid though mucosal surfaces for example during diarrhea, and combatting capillary bleeding (Table 1). Moreover, it is assumed that these herbal extracts contain substances that exert antimicrobial activity.

During the last decades, research on anti-infective agents considered also the effect of drug candidates on biofilm formation, as it is well established that common antibiotics are largely ineffective against biofilm-embedded bacteria. Biofilms, the sessile communities formed by bacteria under conditions of stress, are involved in at least 65% of human bacterial infections (Potera, 1999). The biofilm structure consists of the self-produced extra-cellular polymeric matrix that protects the enveloped bacterial community against external chemical and physical stressors. More importantly, the extracellular matrix is not detected by the host's immune system, which recognized only individual pathogens (planktonic bacteria) Within a biofilm, dormancy is achieved by a significant switch in the expression of multiple genes and the metabolic activity of bacteria is significantly reduces. In this stage they are insensitive to antibiotics that target bacterial metabolic process such as cell wall synthesis or protein synthesis. The biofilm matrix allows the necessary diffusion of nutrients and oxygen to the bacteria, corresponding to the low metabolic rate of biofilm embedded bacteria. The ultimate shortage of nutrients, however, forces the resolution of a biofilm after a certain time, resulting in a population of sequester cells, that regain virulence causing infection and tissue damage and/or are able to form new biofilms.

It is generally recognized that CHMs cannot replace an antibiotic therapy, but their mild antibiotic effects and their potential inhibitory effect on bacterial quorum sensing (the first step in biofilm formation) make them interesting drug candidates, particularly in the treatment of chronic infections that are largely depend on biofilm dynamics. The effect on quorum sensing is not achieved by a single compound, but is typically generated by non-purified plant extracts, composed of multiple biologically active substances. CHMs are commonly orally administered as a water extract obtained by decoction. Decoction in this context means mashing of the herb (leaves, seeds or roots) and thereafter sequential boiling it in water to get an extract, which can be freeze dried, stored and used to prepare a standardized stock solution for testing (Gao, 2012).

In this study such defined water extract prepared by decoctions were used. As a model organism *Staphylococcus epidermidis* was selected. *S. epidermidis* is an important

facultative pathogen, causing acute and chronic infection. Moreover, the availability of two closely related type strains, expressing or not expressing the *ica* operon that plays a pivotal role in biofilm formation, are available at the American type strain culture collection (ATCC) (Figure 1).

MATERIAL AND METHODS

Chemicals

Unless mentioned otherwise, all chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest available purity.

Preparation of CHM water extracts

The seven selected CHMs from the heat-clearing group and a positive control CHM (*Galla Chinensis*) were purchased (Table 1) form CMC Tasly Group BV (1011 EK Amsterdam, The Netherlands). The herbal water extracts were prepared according to a traditional protocol describe in detail by (Gao, 2012) with minor modifications. In brief, CHMs were dried in the oven for 1.5 hour at 60ºC before being submitted to extraction. For each dried CHM, 50 grams was weighed, 350 ml de-ionized water was added in and incubated one hour at room temperature (RT). After incubation, the CHM was extracted firstly by boiling them in water for 25 minutes. The clear supernatant of this mixture (approximately 100 ml) was collected. To the remaining mixture, 150 ml de-ionized water was added, the boiling process repeated for 20 minutes. Thereafter, extracts were filtered and the combined extracts boiled until a volume of 50 ml was reached by evaporation. Subsequently, the extract was freeze dried and the powder stored at 4ºC for further experiments. Prior to the biofilm formation assay, the powder extracts were weighted and re-suspended in tryptic soy broth (TSB) + 0.25% glucose (TSB+) for testing.

Bacterial strains and culture conditions

Two strains of *S. epidermidis* were purchased from the American Type Culture Collection (ATCC). The ATCC 35984 is known for its ability to form biofilms, while the strain ATCC 12228 is unable to form biofilm under experimental conditions (Fey and Olson, 2010) (Figure 1). Both strains were maintained on tryptone soya agar (TSA) (Oxoid CM 129) slants at 4 ºC. One colony of bacteria was cultured in 10 ml TSB^{+} (pH 7.0) under aerobic conditions at 37 ºC for different times according to different experiments.

Bacterial susceptibility to CHM extracts

Bacteriostatic and bactericidal concentrations of CHM extracts were determined by using the non-biofilm forming ATCC 12228 strain, following the Clinical and Laboratory Standards Institute's standard broth micro-dilution method with slight modifications.

Table 1. CHM used in present study and their TCM characteristics.

CHM	character	Test	Used part
Positive control			
Galla Chinensis	cold	sour	gall
Heat-clearing			
Coptis teeta Wall.	cold	bitter	root
Forsythia Suspensa (Thunb.) Vahl.	cold	bitter	fruit
Isatis indigotica Fort.	cold	bitter	root
Lonicera japonica Thunb.	cold	sweet	flower
Scutellaria baicalensis Georgi.	cold	bitter	root
Taraxacum mongolicum Hand-Mazz.	cold	sweet	whole plant
Viola yedoensis Makin.	cold	bitter	whole plant

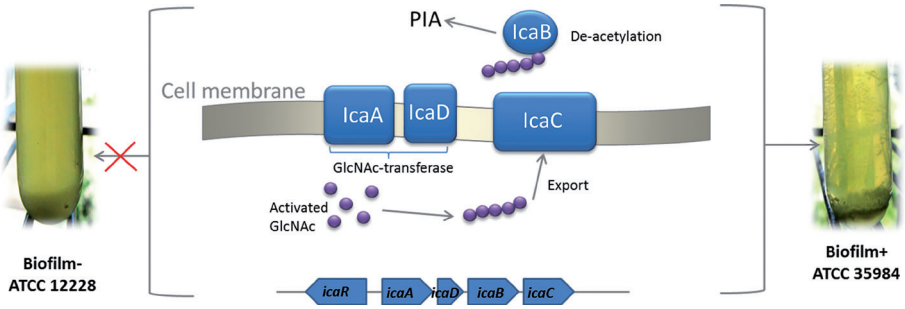


Figure 1. The *ica*ADBC-operon controls the polysaccharide intercellular adhesin (PIA) production: Deactylated beta1-6-linked N-acetylglucosamine (GlcNAc) is synthesized by GlcNAc transferase IcaA/D and exported by the IcaC protein. Thereafter IcaB is de-acetylating GlcNAc to allow the synthesis of PIA, which is important for biofilm formation. The Ica proteins are encoded by the icaADBC operon and the icaR gene. Biofilm formation positive strain (right side photo: ATCC 35984) contains the icaADBC-operon while the biofilm negative strain (left side: ATCC 12228) lacks of this operon.

Briefly, serial two-fold dilutions of CHM extracts in TSB^+ were prepared in a U-bottom 96-well plate (100 µl/well). To each well, 100 µl of 10^6 CFU/ml bacterial suspension was added, resulting in a final volume of 200 µl. Wells with sterile TSB^+ alone served as blanks (negative control). Plates were incubated at 37°C for 24 hours. Thereafter, the minimal inhibitory concentration (MIC) was defined as the lowest CHM concentration that inhibited visible growth after 24 hours of culture. After these 24 hours incubation at 37°C, aliquots of 10 µl from each well were spotted on to tryptic soy agar (TSA) plates containing no CHM. The minimal bactericidal concentration (MBC) was read as the lowest CHM concentration with no growth after 24 hours culture. All MIC/MBC experiments were carried out in triplicate with three independent repetitions.

Quantitative biofilm formation assay

Biofilm formation, in the presence or absence of CHM was assessed by the standard safranin colorimetric assay, as described previously (Wu et al., 2014), using the strain ATCC 35984. In brief, 100 µl of bacterial suspension (10^6 CFU/ml) was transferred into a U-bottom 96-well microtiter polystyrene plate. To this suspension, TSB^+ containing different concentrations of CHM extract was added. Wells with sterile TSB^+ alone served as blanks. The plates were incubated on a microplate shaker (Heidolph titramax 100) with 600 rpm at 37°C for 24 hours. At the end of the culture period, the supernatants from all wells were discarded and the biofilms adhered to the bottom of the wells were incubated with 0.1 M HCl for one hour at room temperature. Thereafter, HCl was replaced by safranin (0.1% in water) and the plates were incubated for 45 minutes at RT (Figure 2). Non-bound safranin was removed by rinsing the wells three times with de-ionized water, and thereafter the plates were incubated with 125 µl 0.2 M NaOH per well at 57°C for one hour. At the end of incubation, 100 µl from the stained dissolved biofilm in each well was pipetted into a new flat-bottom 96-well microtiter polystyrene plate and its OD was measured at a wavelength of 540 nm using a microplate reader model 3550. Minimal biofilm inhibition concentration (MBIC) was defined as the lowest CHM concentration that significantly decreased biofilm formation.

Statistical analysis

Data was evaluated with original one-way analysis of variance (ANOVA) using GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

Bacteriostatic and bactericidal effects of CHMs on *S. epidermidis*

The bacteriostatic (MIC) and bactericidal (MBC) concentration of the CHM water extracts on *S. epidermidis* were determined (Table 2). *G. chinensis* presented the lowest MIC (0.078 mg/ml) against planktonic *S. epidermidis* and its MBC was determined to

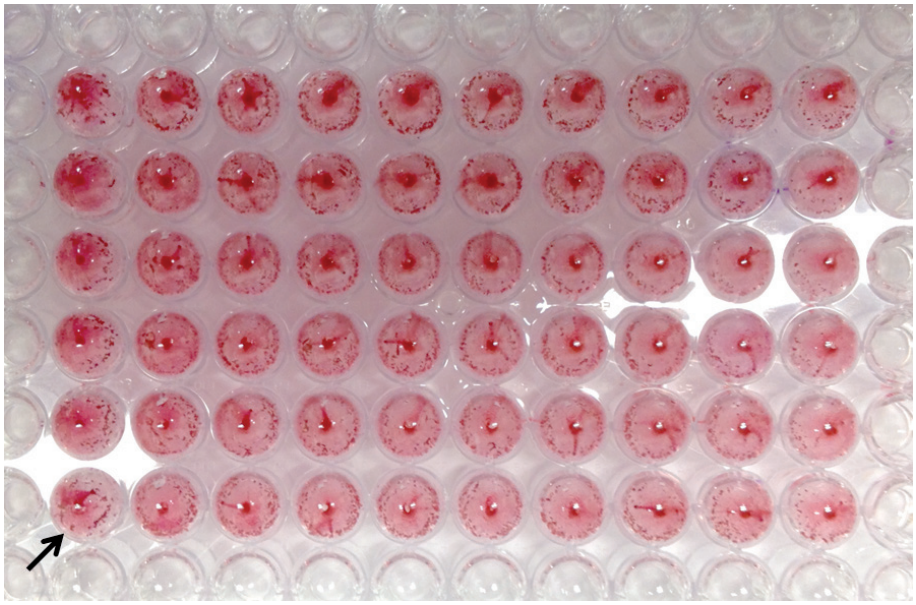


Figure 2. Rapid biofilm formation in agitated 96-well plates. Biofilms can be stained with safranin for spectrophotometric quantification.

Table 2. MIC, MBC and MBIC value of selected CHM on *S. epidermidis*.

TCMs	MIC (mg/ml)	MBC (mg/ml)	MBIC (mg/ml)
Astringents			
G. chinensis	0.078	0.625	0.039
Heat-cleaning			
C. teeta	0.313	0.625	0.313
F. suspensa	25	25	12.5
I. indigotica	100	200	100
L. japonica	100	200	100
S. baicalensis	12.5	12.5	12.5
T. mongolicum	100	200	100
V. yedoensis	25	N/D	12.5

N/D: not determined

be 0.625 mg/ml. None of other tested heat-clearing CHMs presented antimicrobial activity unless higher concentrations (> 0.1 mg/ml) were applied. *C. teeta* showed a MIC of 0.313 mg/ml on planktonic *S. epidermidis*, followed by *S. baicalensis* (MIC: 12.5 mg/ml); *F. suspensa* and *V. yedoensis* (MIC: 25 mg/ml); *I. indigotica, L. japonica* and *T. mongolicum* (MIC: 100 mg/ml). No bactericidal effect was observed even at the highest tested concentration (100 mg/ml) of *V. yedoensis*.

Effect selected CHM water extracts on *S. epidermidis* biofilm formation

Results of the biofilm assay are summarized in Figure 2, and MBIC values are added to table 2. *S. epidermidis* biofilm formation was inhibited after exposure to a concentration of 0.039 mg/ml *G. chinensis*, which is the lowest effective concentration among all CHMs tested. It should be noted that also for *G. chinensis*, at very low concentrations, (< 0.02 mg/ml) a slight increase in biofilm density was observed, although these findings were not considered as significant. The same pattern of a biofilm inhibitory effect was observed for 5 of the tested CHMs including *C. teeta, F. suspensa, I. indigotica, L. japonica,* and *S. baicalensis*, which presented a significant *S. epidermidis* biofilm inhibitory effect at concentrations of 0.313, 12.5, 100, 100, and 12.5 mg/ml, respectively. However, *T. mongolicum* and *V. yedoensis* shown at lower concentrations significant biofilm-stimulation effect and only at the highest concentrations of 100 mg/ml and 12.5 mg/ml, respectively, also a significant inhibitory effect.

DISCUSSION

In the present study, *S. epidermidis* susceptibility to *G. chinensis* water extract and standardized extracts of seven heat-clearing CHMs were tested. We have used decoctions, which extract predominantly water-soluble substances and which differ considerably from the ethanol-soluble compounds tested previously. This choice was made in consideration of the traditional and successful use of the selected CHMs, although such water extracts are not chemically characterized yet. Moreover, plant extracts are known to inhibit biofilm formation by interruption of bacterial quorum sensing more successfully than single compounds.

As expected, *G. chinensis* water extract presented antibacterial activity with a low MIC value of 0.078 mg/ml on planktonic *S. epidermidis*. This concentration is even lower than the previously reported MIC of 0.288 mg/ml of *G. chinensis* ethanol extract and 2 mg/ml gallic acid, the main component of *G. chinensis* (Li et al., 2007; Moran et al., 2014). The antibacterial effect of *G. chinensis* extracts and gallic acid was also demonstrated for *Staphylococcus aureus* (MIC 1.25–1.75 mg/ml) (Huang et al., 2012; Tian et al., 2009), for *Pseudomonas aeruginosa* (MIC 1mg/ml) and for *Escherichia coli* (MIC 5mg/ml) (Borges et al., 2012). Anti-biofilm studies with *G. chinensis* focused

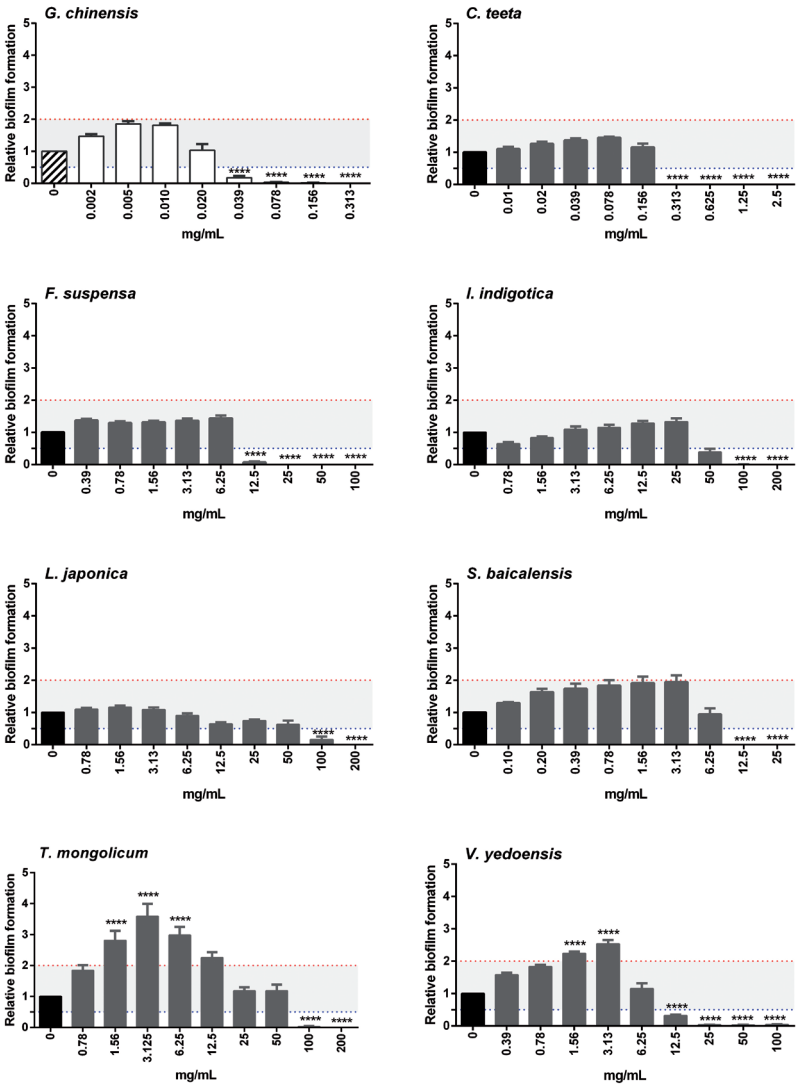


Figure 3 Fold change (± SEM) of *S. epidermidis* (ATCC 35984) biofilm formation after exposure to different concentrations of CHM. **** indicate the fold changes ≥ 2 and the related statistically different ($P < 0.0001$) differences when compare to control (0 mg/ml).

on dental plaque bacteria, one of the most common and prominent multi-species biofilm in the human body. An inhibition of such a biofilm could be achieved at a concentration of 4 mg/ml *G. chinensis* water extract (Xie et al., 2008) and comparable results were reported for pure gallic acid on *S. aureus* biofilms (Luis et al., 2014). At a lower concentration (1 mg/ml) gallic acid was able to counteract *P. aeruginosa* and *E. coli* biofilms (Borges et al., 2012). In our model with *S. epidermidis* biofilms already a concentration of 0.039 mg/ml was effective in inhibiting biofilm formation, while the MIC was 0.078 mg/ml. These values are lower than most MIC and MBC values described in literature, and supported our choice to use *G. chinensis* as a reference in this *S. epidermidis* bioassay.

Comparing the heat-clearing CHM water extracts, none of them exhibited a potent antimicrobial effect (all MICs were > 0.1 mg/ml) against *S. epidermidis*. Previous investigations with *F. suspensa* water extract reported an inhibition of *S. epidermidis* when a 1:40 dilution of the water extract was used (Li et al., 2000), but MIC values were not reported. *C. teeta* (0.093 mg/ml) and *S. baicalensis* (0.4 mg/ml) inhibited *S. aureus* growth (Feng et al., 2011; Franzblau and Cross, 1986) and *F. Suspensa* (12.5 mg/ml) prevented *E. coli* growth (Kuo et al., 2014); Water-soluble polysaccharides from other *Taraxacum* species (100 mg/ml) also inhibited *S. aureus* (Wang, 2014). All above reported concentrations were lower or equal (*T. mongolicum*) to the MICs determined in the current study, indicating that *S. epidermidis* is less sensitive to these CHMs extracts than other bacterial species.

As we had hypothesized that heat-clearing CHMs act differently on *S. epidermidis* biofilm formation we tested *C. teeta, F. suspensa, I. indigotica, L. japonica* and *S. baicalensis* over a broad concentration range. *C. teeta* water extract appeared to be the second most effective (0.313 mg/ml) CHM in the prevention of *S. epidermidis* biofilm formation. Its main compound, berberine, was already reported to inhibit *S. epidermidis* biofilm formation at 0.045 mg/ml (Wang et al., 2009). The concentration of berberine in the decoction of *C. teeta* has not been determined.

F. suspensa, L. japonica and *S. baicalensis* are the three main herbs of an approved CHM water extract injection named TanReQing (TRQ) (Approval No. Z20030054). Previous investigations showed that CHM injection was able to inhibit *S. aureus* biofilm formation at a concentration of 0.129 mg/ml (Wang et al., 2011). According to our results, the MBIC of individual extracts of *F. suspensa, L. japonica* and *S. baicalensis* are higher than that of the combined TRQ extract on *S. aureus*. Therefore additive effects of these three components can be assumed.

Finally, *T. mongolicum* and *V. yedoensis* were found to be strong biofilm inducers at lower concentrations, while inhibiting biofilm formation at high concentrations. This inverse pattern of biofilm stimulation at low concentration was observed in all tested herbal extracts, albeit to a different extent. Similarly to our observation with

T. mongolicum, sub-MIC concentrations of *Taraxacum* water extracts were reported to stimulate *S. aureus* biofilms (Lau and Plotkin, 2013). Plant polysaccharides (0.05%) from *Arabidopsis thaliana* stimulated *Bacillus subtilis* biofilm formation by serving as a carbon source to produce the extracellular matrix (Beauregard et al., 2013). *Taraxacum spp.* was reported to contain 20.67% (w/w) polysaccharides (Wang, 2014). These findings are in line with the effect of poly- and oligosaccharides on the intestinal flora, one of the few places where biofilm formation, often composed of different bacterial species, is considered beneficial to maintain a balanced and protective intestinal flora. At higher concentrations (12.5–100 mg/ml), however, both herbal extracts were able to inhibit biofilm formation, but such effect was related to very high concentrations.

In conclusion, sub-MIC of *G. chinensis* water extract was found to effectively suppress *S. epidermidis* biofilm formation, an effect that was observed also for extracts of other CHMs, albeit at much higher concentrations. The mechanisms behind such an inhibitory effect remain to be elucidated, bur likely to involve several steps in biofilm-associated quorum sensing. A full chemical analysis of the decoctions would be a prerequisite for further investigations and could address the differences between water and ethanol extracts, and the CHM-induced modifications of the expression of biofilm-associated genes.

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Conflicts of Interest

The authors declare no conflict of interest.

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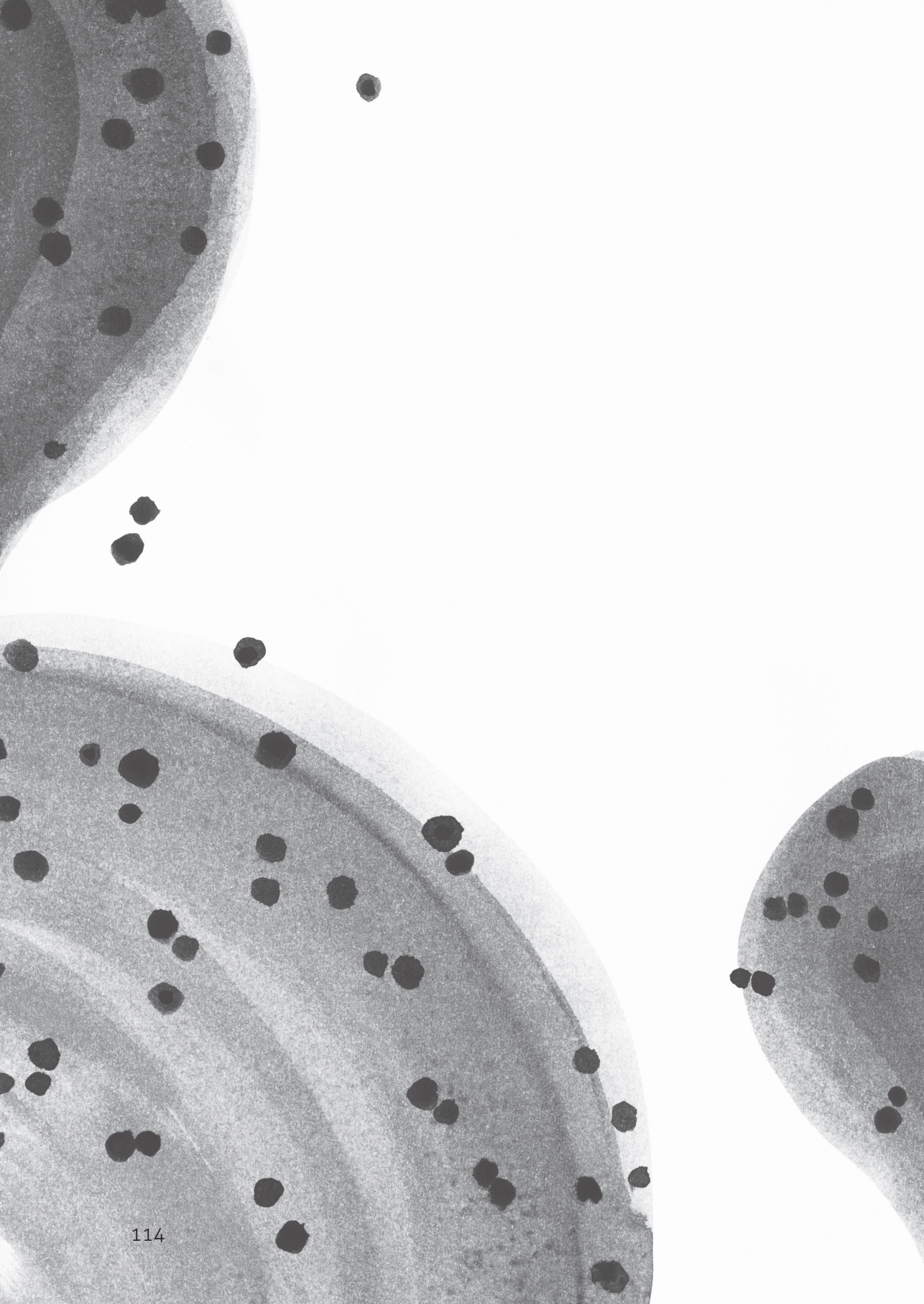

7 Anti-inflammatory activities of Chinese Herbal Medicines: effects of aqueous extracts of *Forsythia suspensa* and *Scutellaria baicalensis* on LPS- stimulated murine macrophages

Preliminary data

X. Wu[1], Y. Jacobs[1], J. Fink-Gremmels

Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

[1] Both authors contributed equally

The Chinese Herbal Medicines (CHMs) *Forsythia suspensa* (Thunb.) Vahl and *Scutellaria baicalensis* Georgi represent two so-called heat-clearing traditional herbs used to treat inflammatory and infectious conditions. In the current study, traditionally prepared decoctions (water extracts) of *F. suspensa* (fruits) and *S. baicalensis* (roots) were tested for their anti-inflammatory effects by measuring the production of pro-inflammatory mediators (NO, TNF-α, IL-1β, and IL-6) and the anti-inflammatory cytokine IL-10 in LPS-stimulated macrophages (RAW 264.7 cells). Cytokines production was measured by ELISA, and NO production was determined by the Griess reaction assay. In parallel, the inducible NO-synthetase (iNOS) gene expression was determined by qRT-PCR. *F. suspensa* decoctions decreased significantly NO and IL-1β production at concentrations of 10 and 100 µg/ml, respectively. *S. baicalensis* water extracts decreased NO production at a concentration range between 100–1000 µg/ml, but did not affect significantly the production of pro-inflammatory cytokines. Both, *F. suspensa* and *S. baicalensis* decoctions were able to decreases dose-dependently the IL-6/IL-10 ratio significantly as they stimulated the IL-10 production (0.1–100 µg/ml). In conclusion, *F. suspensa* and *S. baicalensis* water extracts show an anti-inflammatory effect on LPS stimulated macrophages by inhibiting the production of the pro-inflammatory nitric oxide and by inducing the production of the anti-inflammatory cytokine IL-10. These findings are in line with the traditional use of these herbal remedies as so-called heat-clearing agents.

INTRODUCTION

Traditional Chinese Medicine (TCM) is the most ancient documented medical health care practice existing in human civilization, dating back from several thousands of years. Especially the use of herbal products is deeply rooted in the Chinese culture. Safety and effectiveness of Chinese Herbal Medicine (CHM) is based upon ancient knowledge, experience and the passing-on of this valuable information (Chen and Xu, 2003). The interests for CHMs arose in the scientific community because of the demand for alternative, additional and new therapies, particularly for chronic diseases. To allow CHMs to be accepted and officially admitted to the therapeutic arsenal and health care market, these traditional remedies should be subjected to standardized testing for safety and effectiveness (Foster et al., 2005; Ioannides, 2002).

Testing on CHMs should be performed under the guidance of TCM theory. The so-called *Zheng* (pattern) differentiation theory is the basic for CHM medication. *Zheng* refers to the progress of a disease at a certain phase, being dependent on the cause, nature, location, manifestation, and prognosis of a disease condition (Li, 2010). Taking the heat/cold Zheng as an example, the description of heat *Zheng* refers to a red mouth, high body temperature and a rapid pulse; the cold *Zheng* refers to a white mouth, low body temperature and a weak pulse (Liu and Xu, 2002). To control the heat-*Zheng*, the heat-clearing CHMs are considered as appropriate treatment. Examples of such heat-clearing CHMs are *Forsythia, Scutellaria decoctions. Forsythia suspensa* (Thunb.) Vahl, is a yellow flowering plant belonging to the family Oleaceae. Traditionally, its dry fruits are used as medicines to treat carbuncle, disperse lumps, and stagnation, and to expel the heat at early stage (Chinese Medicine Dictionary, 1986). *Scutellaria baicalensis* Georgi is another important heat-clearing CHMs belonging to the Lamiaceae family. Its dry roots are often used together with *F. suspensa* to expel lung heat and to treat the diarrhea caused by pathogenic heat (Liu and Xu, 2002). The classical signs of inflammation are pain, heat, redness, and swelling, which fit largely into the signs attributed to heat-*Zheng* (Wang et al., 2013).

Some main compounds of CHMs have been tested already for their anti-inflammatory effect. For example, forsythin isolated from *F. suspensa* was reported to act as an anti-inflammatory agent as it inhibited (50 μg/ml) IL-1β, IL-6, and TNF-α production and down-regulated (200 μg/ml) *iNOS* gene expression (Pan et al., 2014). Moreover, major flavones from *S. baicalensis* (3–5 μM) exhibited inhibitory effects on NO, IL-6, and IL-1β production in LPS-stimulated RAW 264.7 macrophages (Lee et al., 2014). These reports, however, focus on individual biologically active substances isolated from TCM, but did not investigate the original decoctions used in clinical therapy. Therefore it is recommended to use CHMs prepared according to the traditional way of use, which comprises often decoctions, prepared by mashing of the

herbal product and boiling it in water, to explore their principle therapeutic benefits (Gao, 2012).

In the screening for anti-inflammatory effects, isolated macrophages are commonly used as *in vitro*-model. Macrophages act as the first line of defense against invading microbial pathogens by recognizing pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS), a part of the outer membrane of gram-negative bacteria. When LPS binds the CD14 receptor on a macrophage a complex is formed with Toll-like Receptor 4 (TLR4), which activates the NF-κB signal transduction pathway leading to the activation of pro-inflammatory genes encoding for the transcription of iNOS (NO-production) and the cytokines TNF-α, IL-1β, IL-6 and IL-10 (Murphy et al., 2008; Jin Lee et al., 2014).

In consideration of the different aspects, the current study was designed to explore the potential anti-inflammatory effects of traditionally prepared decoctions of *F. suspensa* (fruits) and *S. baicalensis* (root) in a model with LPS-stimulated murine macrophage-derived cells (RAW 264.7), a frequently used model to study inflammatory reactions.

MATERIALS AND METHODS

Chemicals

Unless mentioned otherwise, all chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest available purity.

Cell culture reagents and phosphate buffered saline (PBS) were purchased from Life Technologies Europe BV (Bleiswijk, The Netherlands). Lipopolysaccharide (LPS) from *Escherichia coli* was diluted in PBS to achieve a concentration of 10 mg/ml stock solution, stored at -20°C before use.

Preparation of CHM water extracts

The selected CHMs (*Forsythia Suspensa* Vahl. fruits, and *Scutellaria baicalensis* Georgi roots) were purchased form CMC Tasly Group BV (Amsterdam, The Netherlands). The water extracts were prepared according to Gao (2012) with minor modifications. In brief, CHMs were dried in the oven for 1.5 hour at 60°C before being submitted to extraction. For each dried CHM, 50 grams was weighed, 350 mL de-ionized water was added and the mixture incubated 1 hour at room temperature. Thereafter, the CHM was extracted by boiling it for 25 minutes, after which the first liquid extract was collected (~ 100 ml). To the remaining drug suspension, 150 ml de-ionized water was added and boiled again for 20 minutes. Thereafter, both water extracts were filtered, combined and boiled until a volume of 50 ml was reached by water evaporation. Subsequently, the condensed extract was freeze-dried and the obtained powder stored at -20°C until being used. The powder was used to establish a stock solution with 20 mg/ml CHM extract per ml of cell culture medium.

Cell cultures

RAW 264.7 (ATCC TIB-71) murine macrophages were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 µg/ml), 2 mM glutamine, and 1 mM sodium pyruvate in a humidified atmosphere containing 5% CO2/95% air at 37°C. For sub-culturing, cells were scraped from the bottom of the flask and sub-cultured twice a week with a seeding density of 0.8×10^6 cells/25 ml medium in a 75 cm^2 cell culture flask. For the experiments, cells were seeded at a density of 25×10^4 cells/ml into 96-well sterile flat bottom Costar tissue culture plates (Corning, NY, USA) and 4×10^5 cells/ml into 24-well sterile flat bottom Costar tissue culture plates (Corning NY, USA), respectively, and kept for 24 hours under 5% CO2/95% air at 37°C in humidified cell culture incubators before being exposed to the test substances.

Cell viability assay

Cell viability was determined by the standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay. After pre-incubation for 24 hours as described above, RAW 264.7 macrophages were exposed to absence or presence of different concentrations of CHM extracts (0.1, 1, 10, 100, and 1000 µg/ml) or LPS (0.001, 0.01, 0.1, 1, 10, and 100 µg/ml in 0.02% DMSO) for 24 hours. The untreated group (culture medium with 0.02% DMSO) served as control. After incubation, 0.5 mg/ml MTT (prepared in PBS) was added to each well, and the cells were incubated for another 60 minutes. Thereafter, the supernatant was discarded and cells were lysed with 200 µl 99.5% DMSO. The absorbance of the formed tetrazolium product was measured by using a microplate reader (Easy Reader 400 AT, SLT, Salzburg, Austria) at 595 nm. Cell viability was calculated relative to the optical density of MTT formazan formed in untreated cells, which was set at 100%.

NO- assay

The Griess-reaction was performed in order to measure the amount of nitrite in the culture medium, which is an indicator of NO synthesis. RAW 264.7 cells were exposed to different concentrations of CHM extracts (10, 100, and 1000 µg/ml) in the presence of 1 µg/ml LPS for 24 hours. At the end of incubation period, an aliquot (100 µl) of the supernatant from each well was transferred to a new 96-wells flat bottom plate and mixed with an equal volume of Griess reagent [5% (v/v) phosphoric acid (Merck KGaA, Darmstadt, Germany), 40 mg sulphanilamide and 4 mg N-(1-naphthyl) ethylenediamine.2HCl], and then incubated at room temperature for 5 min. Subsequently, the absorbance was measured at 540 nm with the microplate reader. Untreated cells and cells treated with 1 µg/ml LPS served as negative and positive controls in all experiments.

Expression of inducible nitric oxide synthase (*iNOS*)

RNA was isolated from CHM extracts (0.1, 1, 10, 100, and 1000 µg/ml) with 1 µg/ml LPS exposed RAW 264.7 cells using Promega SV total RNA isolation system (Madison, USA), according to manufacturer's protocol. Briefly, samples were lysed by 175 µl lysisbuffer (2% beta mercaptoethanol added) and mixed well with 350 µl RNA dilution buffer. Then samples were incubated at 70°C for 3 minutes and centrifuged (15,682 × g) for 5 minutes at 20°C. The supernatants were transferred to 1.5 ml Eppendorf tubes and 600 µl 95% ethanol was added. Thereafter, the solution (around 800 µl) was placed onto the spin column and centrifuged (15,682 × g) for 10 minutes at 20°C. Then, the total RNA (attached on the membrane) was washed once by 500 µl RNA washing solution and then incubated with 50 µl DNase-mix (40 µl yellow core buffer, 5 µl 0.09M $MnCl_2$, and 5 µl DNase) 15 minutes at room temperature. Subsequently, 200 µl DNase stop solution was added and then the RNA was washed twice with RNA washing solution. At the end of isolation, 100 µl RNase free water was added to the column in order to dissolve the RNA on the membrane, and the total RNA was collected by centrifuge (15,682 × g) 1 minute at 4°C. The concentration and purity of total RNA was spectrophotometrically assessed using a NanoDrop 1000TM (Thermo Scientific, Waltham, MA, USA).

Quantitative real time-PCR

1 µg of extracted total RNA from each sample was reverse-transcribed with the iScriptTM cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The obtained cDNA was diluted to a final concentration of 30 ng ml/1. Primers (Table 1) complementary to RAW 264.7 macrophages were designed according to the literature. The primers used were commercially produced (Eurogentec, the Netherlands) and selected based on specificity and efficiency by qPCR analysis of a dilution series of pooled cDNA at a temperature gradient (55–65°C) for primer-annealing and subsequent melting curve analysis.

The reaction mixture for qPCR contained 6 µl of diluted cDNA, 9 µl iQSYBR Green Supermix (Bio Rad Laboratories Inc., USA), forward and reverse primers (final concentration of 0.4 pmol µl-1 for each primer), and sterile water according to the manufacturer's instructions. qPCR was performed using the CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA) and CFX System software version 3.0 (Bio-Rad, Hercules, CA, USA). The relative mRNA expression was calculated from comparison with the expression levels of two reference genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta actin (*β-actin*) (Table 1).

Cytokine assays

RAW 264.7 cells in 24-well tissue culture plates were exposed for 24 hours to different concentrations CHM extracts (0.1, 1, 10, and 100 µg/ml) with or without 1 µg/ml LPS. Supernatants from treated cells were collected and the quantification of IL-1β, IL-6,

IL-10 and TNFÐ release were measured by ELISA MAXTM Standard Sets (BioLegend, CA, USA), according to manufacturer's instruction. Briefly, standards and samples were incubated on capture antibody coated plates at 4 °C, overnight. Detection antibody was incubated for one hour and Avidin-HRP bound to the detection antibody. To visualize, substrate solution was added to each well, and then the reaction was stopped by stop solution (1M NH_2SO_4). Absorbance was measured by a microplate reader at 450 and 570 nm wavelengths. Untreated cells and the ones with only 1 µg/ml LPS served as negative and positive control in all experiments, respectively.

Statistical analysis

Data was evaluated with one-way analysis of variance (ANOVA) for cell viability assay, Nitrite assay, and ELISA using GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, California, USA). For qRT-PCR test, down- and up-regulation were considered significant when the relative expression was decreased or increased ≥ 4 folds.

Table 1. Primers for RAW 264.7 macrophages genes used in this study

Gene	GenBank	Primers sequence (5' to 3')	Product size (pb)	Reference
GAPDH	NM_008084.3	F: GAACATCATCCCTGCATCC	109	This study
		R: CACATTGGGGGTAGGAACAC		
β-actin	NM_007393.3	F: TGTTACCAACTGGGACGACA	573	Gao et al. (2014)
		R: AAGGAAGGCTGGAAAAGAGC		
iNOS	NM_010927.3	F: CCCTTCCGAAGTTTCTGGCAGCAGC	497	Chiou et al. (2000)
		R: GGCTGTCAGAGCCTCGTGGCTTTGG		

RESULTS

Identification of the LPS concentration for the induction of an inflammatory response

RAW 264.7 macrophages were tested with different concentrations (0.01, 0.1, 1, 10, and 100 µg/ml) of LPS dissolved in DMSO and NO production was measured. According to the results 1 µg/ml LPS was chosen for further RAW 264.7 cell stimulation experiments. This LPS concentration did not affect the cell viability (data not shown).

Concentration range-finding experiments: Effect of CHM extracts in cell viability

MTT assay was performed to measure the RAW 264.7 macrophages cell viability after exposure to different concentrations (0.01, 0.1, 1, 10, 100, 1000, and 2000 µg/ml) of

F. suspensa and *S. baicalensis* water extracts (Figure 1). *F. suspensa* extract did not affect cell viability up to a concentration of 1000 µg/ml and only at 2000 µg/ml extract showed a mild cytotoxicity effect. *S. baicalensis* extracts decreased cell viability significantly at a concentration of 1000 µg/ml, but the cell viability did not decrease below 65%. Based on these results a concentration range of 0.01–100 µg/ml of both CHM water extracts was selected for all further experiments.

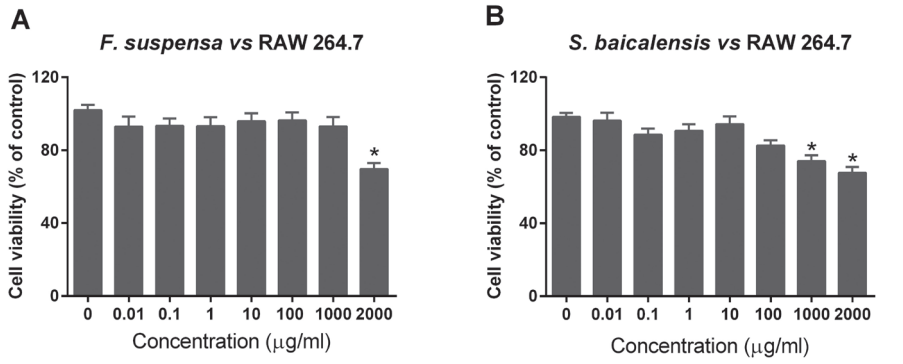

Figure 1. The mean (± SEM) percentage of cell viability (% of control) after exposure to different concentrations (0.01, 0.1, 1, 10, 100, 1000, and 2000 µg/ml) of *F. suspensa* (A) and *S. baicalensis* (B) water extracts. * indicate the significant ($P < 0.0001$) difference when compare to control (0 µg/ml).

NO production

NO Griess reaction was applied to determine the NO production of LPS (1 µg/ml) stimulated RAW 264.7 cells after exposure to different concentrations (10, 100, and 1000 µg/ml) of *F. suspensa* and *S. baicalensis* water extracts (Figure 2). *F. suspensa* decreased the LPS stimulated RAW 264.7 cells NO production only at the highest concentration of 1000 µg/ml. In contrast, the *S. baicalensis* decoction prevented the LPS induced NO-production in a concentration-depended manner in RAW 264.7 cells.

Gene expression of *iNOS* in LPS-simulated RAW 264.7 cells treated with CHM decoctions

The qPCR results indicated that the relative mRNA expression levels of *iNOS* decreased after exposure to 1000 µg/ml *F. suspensa*, in parallel to the measured NO concentrations. Similarly, a concentration-dependent decrease in the expression of *iNOS* was observed after exposure of LPS-stimulated RAW cells to *S. baicalensis* with significant differences at 100–1000 µg/ml (figure 3).

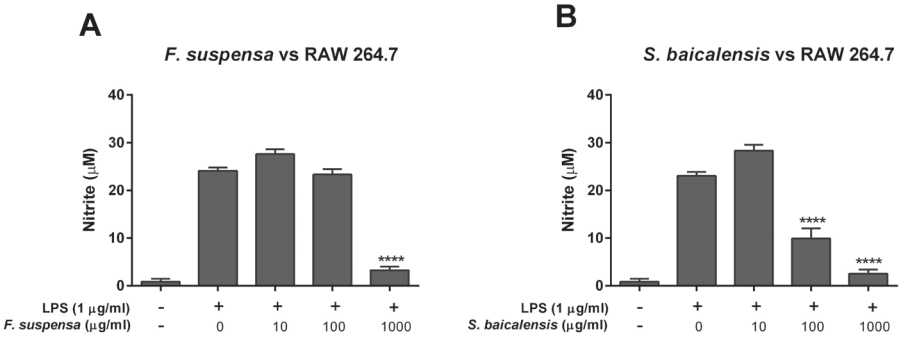

Figure 2. The mean (± SEM) value of NO production (μM) after exposure to different concentrations (10, 100 and 1000 μg/ml) of *F. suspensa* (A) and *S. baicalensis* (B) water extracts. **** indicate the significant ($P < 0.0001$) difference when compare to LPS (1 μg/ml) stimulated group.

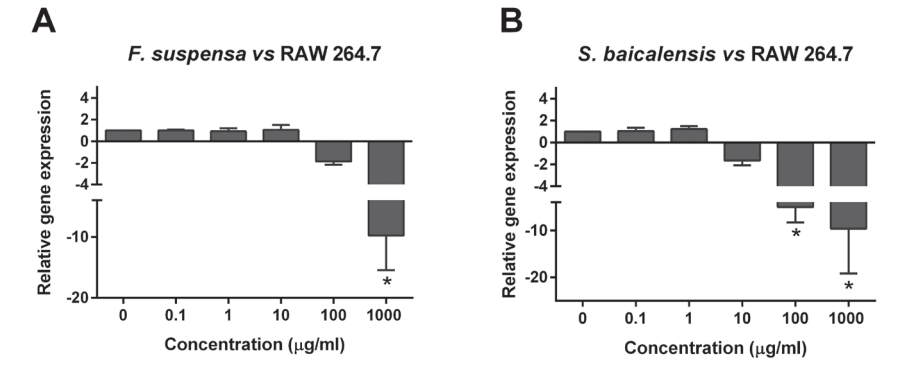

Figure 3. Mean (± SEM) relative mRNA expression of a gene involved in NO production (*iNOS*) of the LPS (1 μg/ml) stimulated RAW 264.7 cells after exposure to different concentrations (0.1, 1, 10, 100, and 1000 μg/ml) of *F. suspensa* and *S. baicalensis* water extracts. * indicate significant relative down-regulation (over 4-fold) of gene expression when compared with the untreated group.

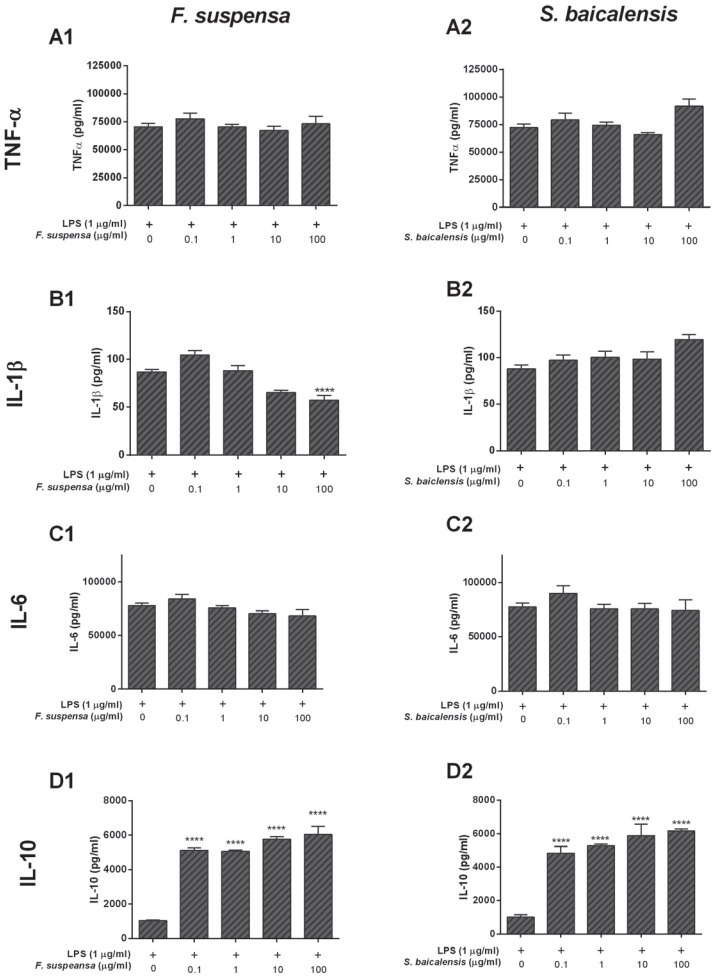

Figure 4. The mean (± SEM) values of LPS (1 μg/ml) stimulated RAW 264.7 cell cytokines: TNF-α (row A), IL-1β (row B), IL-6 (row C), IL-10 (row D), concentration (pg/ml) in the cell supernatant after exposure to different concentrations (0.1, 1, 10, and 100 μg/ml) of *F. suspensa* (A1, B1, C1, and D1) and *S. baicalensis* (A2, B2, C2, and D2) water extracts. **** indicate the significant ($P < 0.0001$) difference when compare to control (0 μg/ml).

Cytokine assays

Different concentrations (0.1–1000 µg/ml) of CHM water extracts were tested also for their effect on cytokine production of naïve (non-LPS stimulated) cells. In all cases cytokine production remained below detection levels (data not shown).

In a second series of experiments, the effect of CHM water extracts were tested on LPS-stimulated RAW 264.7 cells (LPS: 1 µg/ml). Figure 4 shows the secretion of the cytokines TNF-α, IL-1β, IL-6, and IL-10. Following exposure of LPS-stimulated RAW 264.7 to the *F. suspensa* extract TNF-Ð remained unchanged and no significant difference was noted in comparison of CHM-treated cells and the LPS-treated reference. The IL-1β expression was concentration-dependently decreased after exposure *F. suspensa* water extracts. Such a concentration-dependent decrease was also observed in the IL-6 production, although these results were not significant. More importantly, the IL-10 production was significantly increased after exposure to all tested concentrations of *F. suspensa*.

Exposure of LPS-stimulated RAW 264.7 cells to *S. baicalensis* water extracts resulted in a slight increase in TNF-α concentration at a concentration 100 µg/ml and the same trend was observed for the IL-1β production, but these changes were not significant (Figure 4). In addition, no significant changes in the secretion of IL-6 could be observed. However, in parallel to the results observed with *F. suspensa* extracts, the production and release of the anti-inflammatory IL-10 was concentration-depend and significantly increased. In turn, the ratio of IL-6/IL-10 was calculated and showed the significant changes in a more pronounced way (Figure 5).

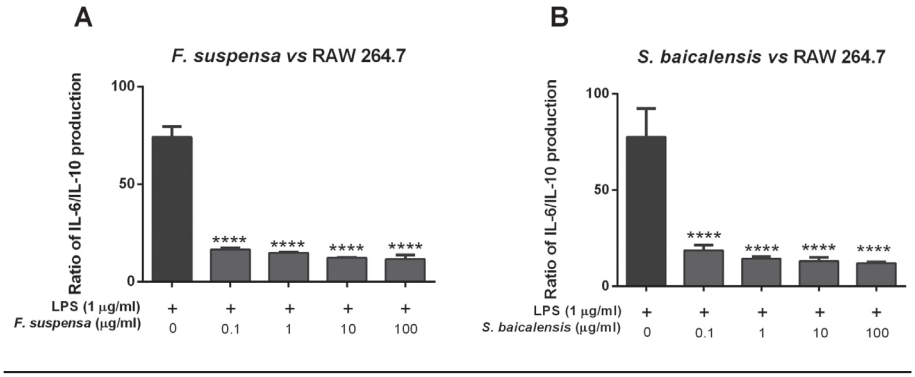

Figure 5. The ratio of IL-6/IL-10 production by stimulated RAW 264.7 cells after exposure to different concentrations (0.1, 1, 10, and 100 µg/ml) of *F. suspensa* (A) and *S. baicalensis* (B) water extracts. **** indicate the significant ($P < 0.0001$) difference when compare to control (0 µg/ml).

DISCUSSION

The CHMs *F. suspensa* (fruits) and *S. baicalensis* (roots) are used in Chinese medicine for thousands of years as treatment for inflammatory and infectious diseases. To understand the rationale for their use, as a first step *in vitro* experiments were conducted in isolated murine macrophages (RAW 264.7 cells) to identify any anti-inflammatory activity. In this study, non-cytotoxic concentrations of traditionally prepared decoctions of these two CHMs were tested for their effects on the expression and production of NO and *iNOS* and the inflammatory markers TNFĐ, IL-1β, IL-6, IL-10, in LPS activated RAW 264.7 cells.

In the present study, *F. suspensa* water extract affected both the synthesis (reduction of *iNOS* expression) and the release of NO (measured in the Griess reaction in the cell supernatant). The effect of *F. suspensa* water decoction on NO production has not been reported before. However, the ethanol extract (100 µg/ml) of *F. suspensa* was reported to inhibit NO production in LPS stimulated RAW 264.7 macrophages (Chen and Zhang, 2014), but *iNOS* expression was not measured in that study. However, as both the assay design and the concentrations tested were comparable, it can be concluded that *F. suspensa* contains active substances that interfere with NO synthesis. Additionally, when measuring the effect of *F. suspensa* water extracts on the production of pro-inflammatory cytokine production, initially no significant effects could be found for the production of TNF-α, which is often the first cytokine released after tissue injury or infection (Murphy et al., 2008). No other data is available on this CHM water extract, but arctiin, a lipophilic component isolated from *F. suspensa* was reported to decrease IL-6 and TNF-α production (12.5 µg/ml) (Lee et al., 2011). Due to its poor water solubility, it is unlikely that the significant amount of arctiin, which occurs also in other plants of the genus *Centaurus*, is present in the water extracts used in our study. In contrast, IL-1β, which elicits an innate immune response (Murphy et al., 2008), was significantly inhibited by decoctions from *F. suspensa*. This effect is in line with the recommendation to use *F. suspensa* decoction as heat-clearing agent. Surprisingly, in the current study no significant effect on IL-6 expression could be demonstrated, but it should be considered that only one measurement was conducted after 24 hours of exposure. IL-6 is described as pro- and anti-inflammatory cytokine as it induces the acute phase protein production in the liver and increases in antibody production in B-lymphocytes. However, IL-10, an anti-inflammatory cytokine that terminates an inflammatory response and blocks NF-κB activity, was induced immediately after exposure to *F. suspensa* water extract. IL-10 inhibits the synthesis of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α (Fiorentino et al., 1991) and this function may explain partly the negative findings for TNF-α and Il-6, described above. The calculated ratio of IL-6/IL-10 is an important parameter used in clinical settings, as a high ratio is correlated with poor outcomes in patients with a systemic inflammatory response syndrome, while a low ratio indicates

regeneration (Kilic et al., 2006; Taniguchi et al., 1999). *F. suspensa* showed a significant decrease (from 80 to 20) in the IL-6/IL-10 ratio at all concentrations tested when compared to untreated controls, confirming the anti-inflammatory effect of this CHM water extract on LPS stimulated macrophages. Whether these *in vitro* data can be translated into a clinical relevant effect *in vivo* still remains to be elucidated. Previous *in vivo* investigations suggested that a *F. suspensa* water fraction has no significant anti-inflammatory and pain-releasing effects compare to methanol extract (Ozaki et al., 1997) in an acute model of inflammation, but they might be effective at the local level and in chronic inflammatory diseases.

Similar to *F. suspensa, S. baicalensis* water extract also affected both pro- and anti-inflammatory cytokine productions in LPS stimulated macrophages. It deceased NO production and induced IL-10 expression. The decrease in NO production correlated with an inhibitory effect on *iNOS* expression. Choi et al., (2014) reported *S. baicalensis* water extract presented a NO inhibitory effect at a higher concentration (500 µg/ml) on LPS stimulated macrophages, whereas Yoon et al., (2009) described that *S. baicalensis* water extracts inhibited the production of NO, IL-6 and IL-10 in LPS-induced RAW 264.7 cells at the concentrations of 25, 50, 100, 200 µg/ml. In the current experiments, *S. baicalensis* extracts did not affect any of the tested pro-inflammatory cytokines. We demonstrated here, that *S. baicalensis* water extract has an anti-inflammatory effect on LPS stimulated macrophages as next to the inhibition of the NO synthesis it induced the release of the anti-inflammatory cytokine IL-10. In turn, the ratio IL-6/IL-10 decreased significantly, as IL-6 was not induced.

In conclusion, the heat-clearing CHMs *F. suspensa* and *S. baicalensis* water decoctions exhibited an anti-inflammatory effect on LPS activated macrophages, characterized predominantly by an inhibition of NO production and a stimulation of the anti-inflammatory cytokine IL-10. These effects were achieved at concentrations that can be considered as realistic dosing levels following oral application. The partial difference between the presented results and earlier studies reflect the origin of the herbs, the age and storage condition, which are common variables in herbal remedies that influence the outcome and comparability of individual test results.

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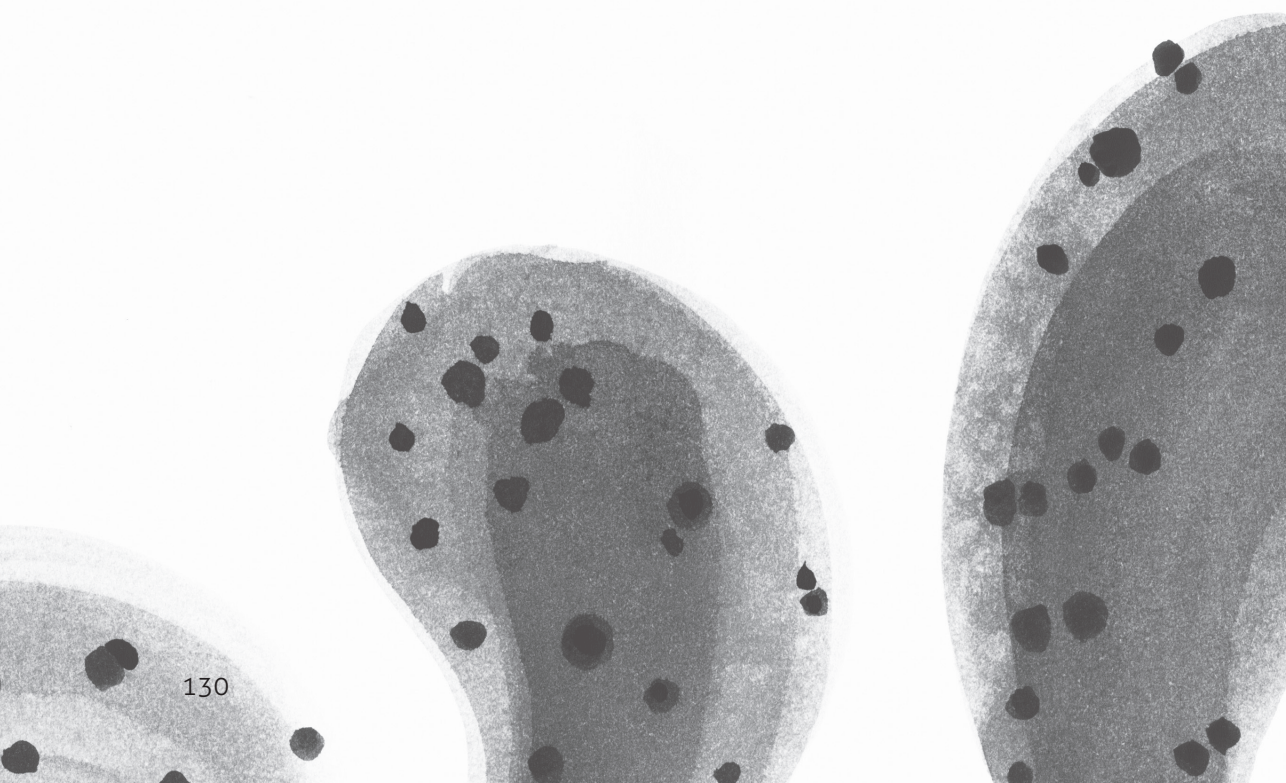
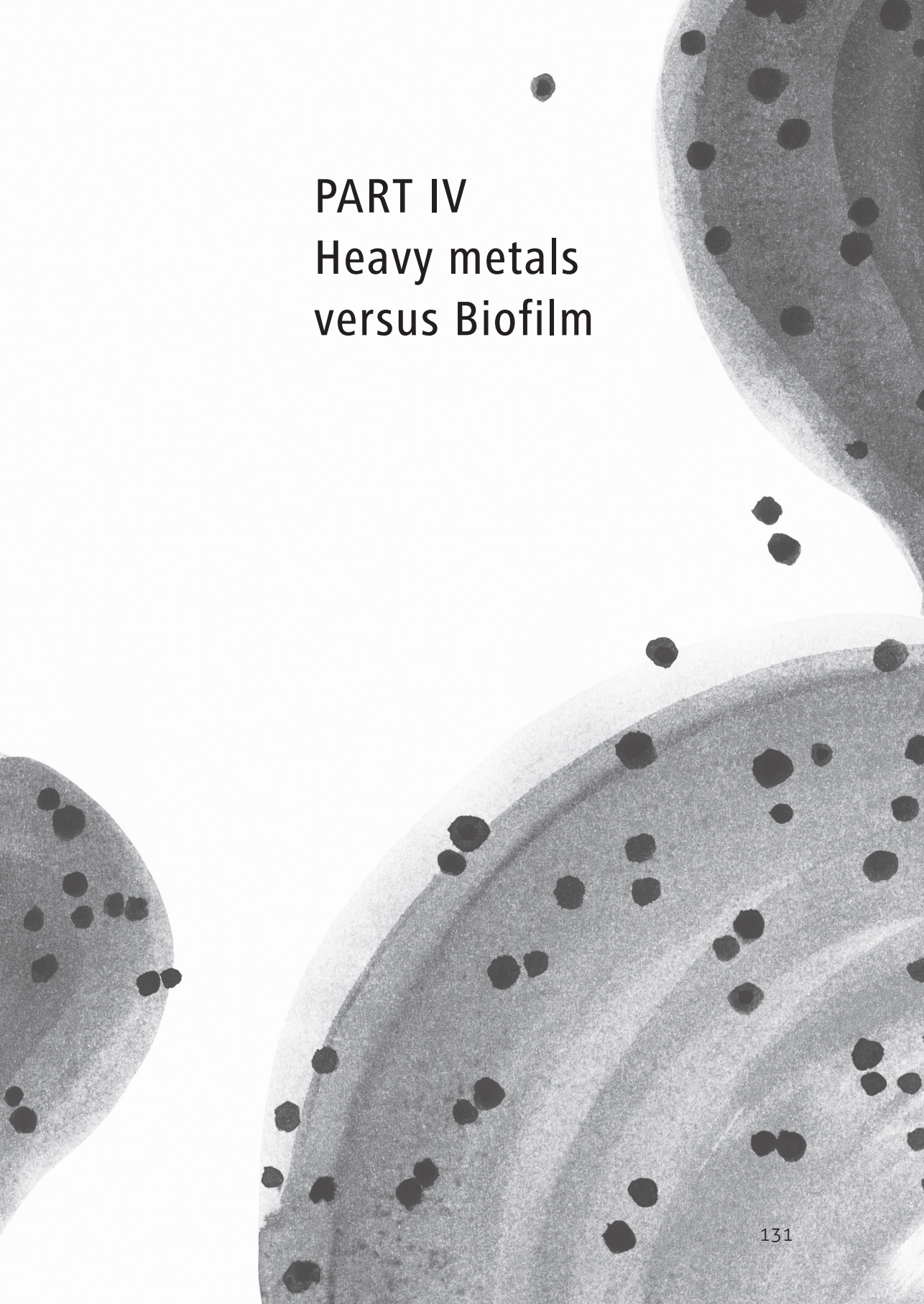
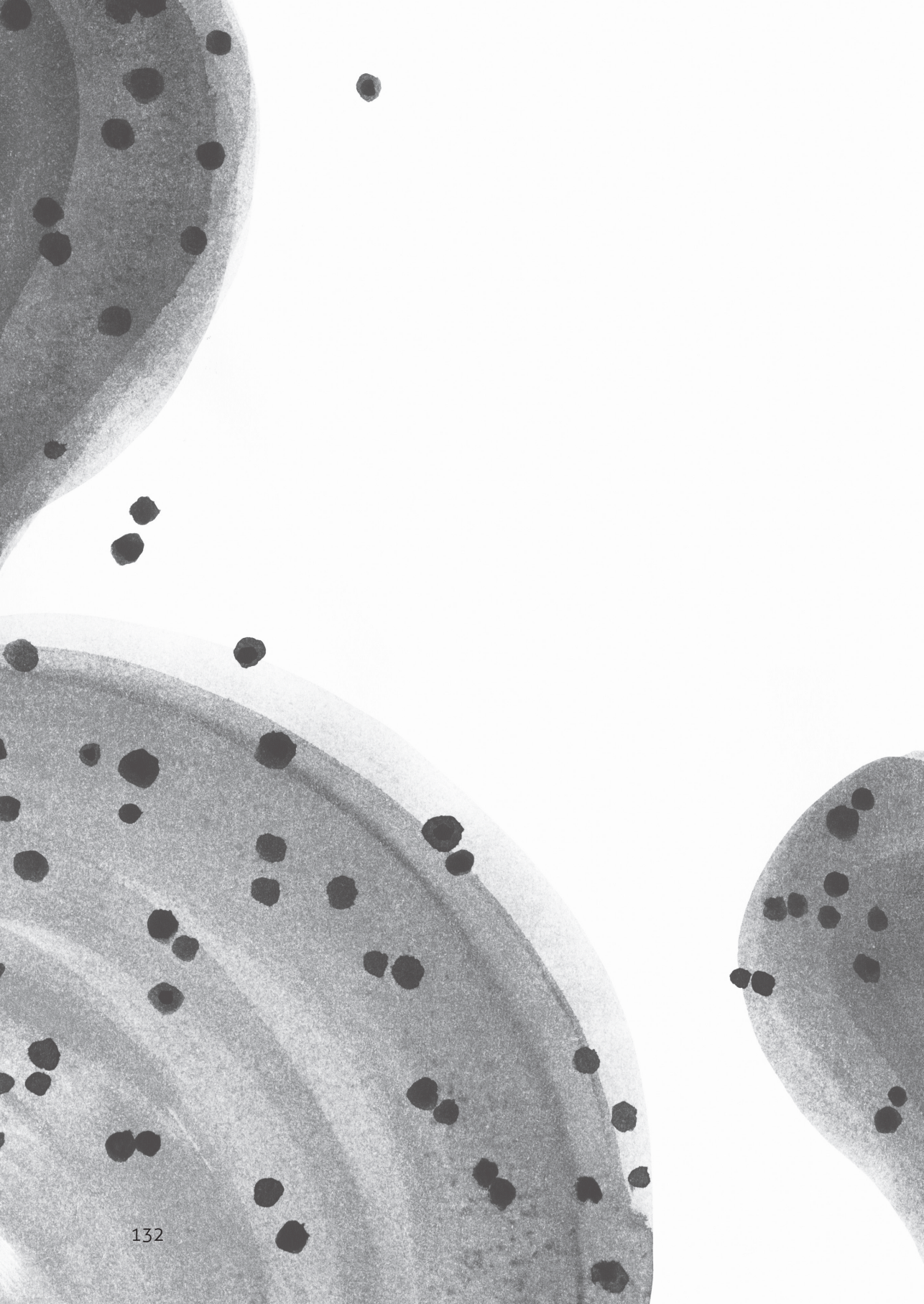


PART IV
Heavy metals versus Biofilm





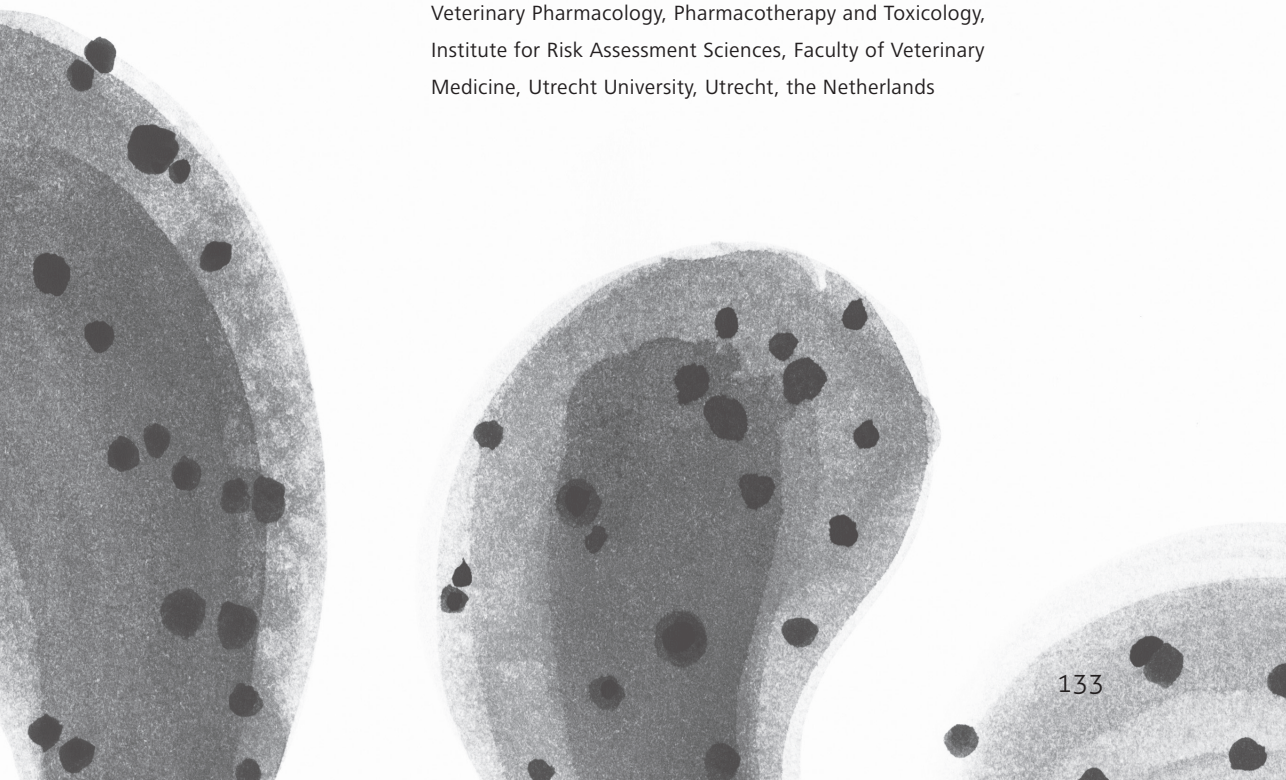
8

Do heavy metals stimulate *Staphylococcus epidermidis* biofilm formation?

Preliminary data

X. Wu, R. R. Santos, J. Fink-Gremmels

Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands



We evaluated the effect of four different heavy metals (lead, manganese, mercury and nickel) exposure on *Staphylococcus epidermidis* (ATCC 35984) biofilm formation. Bacteria were cultured in the absence or presence of these compounds at different concentrations: lead, manganese and nickel (1.56–200 μM), mercury (0.39–50 μM). *S. epidermidis* biofilm formation was inhibited ($P < 0.001$) by 200 μM lead, 100 μM manganese and 12.5 μM mercury, and the MBIC of mercury on this bacterial biofilm was determined to be 25 μM. *S. epidermidis* biofilm formation was not effected by any of the tested nickel concentrations. Taken together, our findings show that *S. epidermidis* could form biofilms even in the presence of these toxic metals, but that neither lead or mercury, nor manganese or nickel stimulated biofilm formation. The relevance of the findings for the safety of feed and medicinal plants and for the use of *S. epidermidis* as bio-sorbent in the cleaning of water supplies is discussed. Further investigations should include cadmium, the most toxic heavy metal, of its effect on *S. epidermidis* biofilm (see Chapter 9).

INTRODUCTION

One of the worldwide risks for human and animal health is the contamination of soil and water and subsequently plants with toxic heavy metals. Plants can uptake heavy metals from soil and even accumulate these in their edible parts resulting in an undesirable human and animal exposure (Liu et al., 2014b; Singh et al., 2013; Stasinos and Zabetakis, 2013). Of particular interest are spices and herbal products known as heavy-metal accumulating plants (Gomez et al., 2007). Cadmium and lead concentrations in herbal drugs were even beyond the permissible limits established by the WHO (Rai et al., 2008). Next to the direct toxicity of food products and herbal products with heavy metals, their potential ability to stimulate bacterial biofilms is of concern. A biofilm is a sessile community of bacteria, surrounded by a self-produced extracellular matrix that adheres to the surfaces (Flemming and Wingender, 2010). It has been stated that bacterial resistance is associated with biofilm formation, because bacteria are protected within this matrix against antimicrobial agents and host defense mechanisms (Coenye and Nelis, 2010; Fey and Olson, 2010). Perrin et al., (2009) reported that sub-inhibitory concentrations of nickel promotes *E. coli* K-12 biofilm formation and Schue et al., (2011) showed that cadmium triggers *Rhizobium alamii* biofilm formation and these observation supported the hypothesis that other heavy metals stimulate biofilm formation as well.

In contrast to the concerns associated with biofilm formation of pathogenic bacteria and the resulting consequences for the therapy of infectious diseases, bacterial biofilms are intentionally used as bio-sorbents in water supplies and the cleaning of waste water. For example, *Staphylococcus aureus* biofilms have been used to bind cadmium (Sochor et al., 2011); *Micrococcus luteus* has been used to absorb lead (Puyen et al., 2012); lactic acid bacteria are able to bind mercury (Kinoshita et al., 2013) and living *Bacillus* spp. were shown to absorb manganese (Hasan et al., 2012) and nickel (Pan et al., 2011). *S. epidermidis* was also indicated as a potential biodegrading agent for the decolourization of triphenylmethane dyes from textile wastewater (Ayed et al., 2010).

Although *S. epidermidis* occurs commonly in the soil (Rabah et al., 2010) and in wastewater (Ayed et al., 2010), the use of organisms to clean water would mean that large amounts of this facultative pathogens are introduced into the environment, which entails the risk of the selection of resistant bacteria. For instance, nickel-ampicillin double resistance was detected in bacterial isolates of sea water (Sabry et al., 1997). Resistant bacteria in wastewater and soil presented also multiple tolerances to cadmium, copper, chrome and nickel, concomitantly with the resistance to multiple antibiotics (Krishna et al., 2012; Yamina et al., 2012). The aim of the current study was to assess the effects of heavy metals (lead, manganese, mercury and nickel) on *S. epidermidis* biofilm formation.

MATERIALS AND METHODS

Unless mentioned otherwise, chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bacterial strains and culture conditions

Two strains of *S. epidermidis* were purchased from the American Type Culture Collection (ATCC). The strain ATCC 35984 forms a thick biofilm (Fey and Olson, 2010) on the bottom of a 96-well plate, which was used for standard biofilm formation assay. ATCC 12228 is commonly included in experiments as a quality control and as a test of reproducibility of the method in general (Skovgaard et al., 2013). Bacteria were cultured in tryptic soy broth + 0.25% glucose (TSB^+) (pH 7.0) under aerobic conditions at 37°C for 24 hours. For all experiments, biofilm formation was performed in TSB+ in the presence or absence of different heavy metals; final concentrations ranged from 0.39 to 50 µM for mercury, 1.56 to 200 µM for lead, manganese and nickel.

Heavy Metals

A 0.2 M solution of lead (Pb^{2+}), manganese (Mn^{2+}), mercury (Hg^{2+}) and nickel (Ni^{2+}), was prepared and stored at 4°C, respectively. Before use, the stock solution was diluted in TSB^+ to a concentration of 100 µM for mercury, 400 µM for lead, manganese and nickel.

Quantitative Biofilm formation assay

Biofilm formation was assessed by the standard safranin colorimetric assay as described earlier (Wu et al., 2014) using the strain ATCC 35984. In brief, 100 µl of bacterial suspension (10^6 CFU/ml) was transferred into a U-bottom 96-well microtiter polystyrene plate (Costar, Corning, NY, USA). To this suspension heavy metals were dissolved in broth at concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50 or 100 µM for mercury; 3.13, 6.25, 12.5, 25, 50, 100, 200 or 400 µM for lead, manganese and nickel were added, resulting in final concentrations of cadmium and mercury of 0.39–50 µM; lead, manganese and nickel are 1.56–200 µM in the tested samples. Wells with sterile TSB^+ alone served as blanks. The plates were incubated on a microplate shaker (Heidolph titramax 100) at 37°C for 24 hours. At the end of the culture period, the supernatant from all wells were discarded and the biofilms adhered to the bottom of the wells were incubated with 0.1 M HCl for 1 hour at room temperature. Thereafter, HCl was replaced by safranin (0.1% in water) and the plates incubated for 45 minutes at room temperature. Non-bound safranin was removed by rinsing the wells 3 times with de-ionized water, and thereafter plates were incubated with 125 µl 0.2 M NaOH per well at 57°C for 1 hour. At the end of incubation, 100 µl from the stained dissolved biofilm in each well was pipetted to a new flat-bottom 96-well microtiter polystyrene plate

and its intensity was measured at a wavelength of 540 nm in a microplate reader model 3550 (Bio-Rad, CA, USA). The minimum biofilm inhibitory concentration (MBIC) of these heavy metals on *S. epidermidis* biofilm defined as the lowest concentration that inhibited at least 90% biofilm formation.

Statistical analysis

Data was evaluated with one-way analysis of variance (ANOVA) by Prism 6.04. All incubations were conducted in quadruplicate and experiments were repeated at least three times.

RESULTS

To evaluate the effects of different heavy metals on *S. epidermidis* ATCC 35984 biofilm formation, a quantitative biofilm formation assay was performed. Biofilm formation was inhibited ($P < 0.001$) by 200 µM lead as shown in Figure 1, panel A. Similar result was observed when *S. epidermidis* was cultured in the presence of 100 and 200 µM

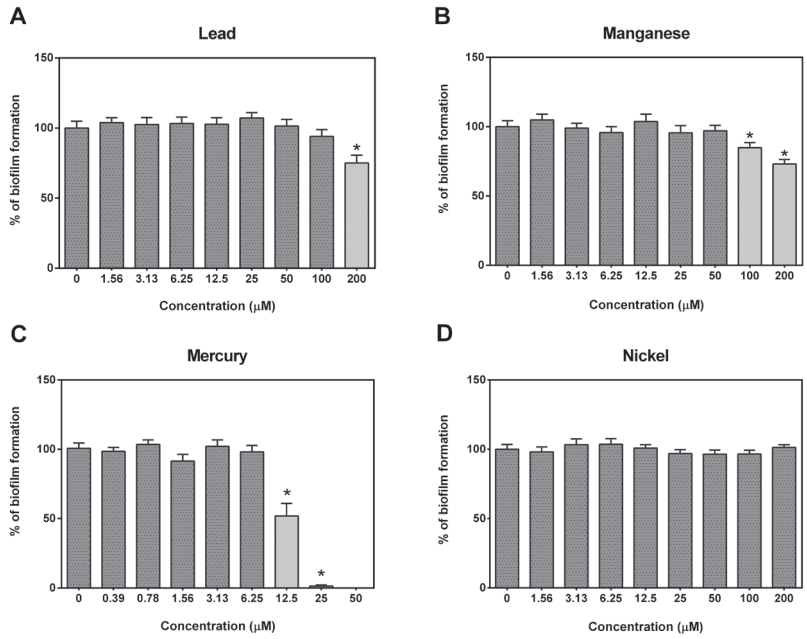


Figure 1. Mean percentage (± SEM) of *S. epidermidis* (ATCC35984) biofilm formation after exposure to 1.56–200 µM lead (A), manganese (B) and nickel (D); 0.39–50 mercury (C). * indicates significant ($P < 0.01$) difference between treatments and control (0 µM).

manganese (Fig. 1, panel B). Biofilm formation was also observed to be inhibited after exposure to 12.5–50 μM mercury (Fig. 1, panel C). Differently, nickel did not affect *S. epidermidis* ATCC 35984 biofilm formation in any of the tested concentrations (Fig. 1, panel D). MBIC values of lead, manganese and nickel on *S. epidermidis* biofilm were above 200 μM (Fig. 1, panel A, B and C). The MBIC of mercury on *S. epidermidis* biofilm was 25 μM (Fig. 1, panel D).

DISCUSSION

In this study, the effects of different heavy metals (lead, manganese, mercury and nickel) on *S. epidermidis* biofilm were investigated. Most studies involving heavy metals and bacterial biofilms are focusing on the biofilm bio-sorption abilities, i.e. the binding of environmental pollutants to natural biofilms in surface water. In medicine, however, biofilm formation by pathogens is of concern, as this conveys therapy resistance and increases the risk for chronic infections.

Our results show, that *S. epidermidis* biofilm inhibition was observed after exposure to 200 μM lead. The minimal inhibitory concentration (MIC) of lead for *S. aureus* was reported to be 1000 μg/mL (~4826 μM) (Radhi, 2012), which is much higher than the concentration that inhibited *S. epidermidis* biofilm formation. *S. aureus* could precipitates lead inside the cell as lead phosphate ($Pb_3(PO_4)_2$), and this mechanism conveyed a high tolerance to a 600-fold higher dose of lead compared to a sensitive strain (Levinson and Mahler, 1998). This difference between *S. aureus* and *S. epidermidis* may indicate that *S. epidermidis* is lacking this kind of strategy to resist lead.

Manganese is an essential cofactor for processes such as photosynthesis, lipid biosynthesis and oxidative stress in plants (Socha and Guerinot, 2014). It belongs to the less toxic metals in mammalians, but high levels can exert neurotoxic effects in children (Bouchard et al., 2011). Some medical herbals are known to accumulate manganese such as *Celosia argentea* Linn., which is also used to clean manganese contaminated soil (Liu et al., 2014a). In the present study, *S. epidermidis* biofilm inhibition was observed after exposure to 100 μM manganese, and no biofilm stimulation effect was measurable.

Mercury is a well-known toxic heavy metal, which has been linked to neurotoxicity, immunotoxicity, genotoxicity, reproductive toxicity, teratogenicity, and nephrotoxicity (Kim et al., 2012). It is known that the MIC of mercury is about 44 ppm (~162 μM) on gram positive cocci (Moghbeli et al., 2011). The measured biofilm-inhibiting concentration MBIC for *S. epidermidis* was 25 μM, which is much lower than the reported MIC value. Bio-sorption in the extracellular polymeric matrix of a bacterial biofilm is reported as one of the bacterial resistance mechanisms and different mercury species can be transformed within each biofilm, resulting in turn in the selection of

resistant bacteria (Kovac Virsek et al., 2013). It can be assumed that similar mechanisms can be observed also in *S. epidermidis* biofilms exposed to mercury.

Human and animal are exposed to nickel by dietary ingestion, inhalation and dermal contact and it may causes diseases of the respiratory tract and allergic contact dermatitis (Schaumloffel, 2012). Perrin et al., (2009) reported that *E. coli* exposure to 100 µM nickel promoted biofilm formation, but according to our results, nickel had no effect on *S. epidermidis* biofilm formation up to a concentration of 200 µM. Similarly, Rajbanshi (2008) showed that *Staphylococcus* spp. were tolerant to 150 µg/ml (~1.16 mM) nickel. Furthermore, Shaivastave et al., (2013) showed that the MIC of nickel on *S. aureus* is 450 µg/ml (~3.47 mM), which is higher than the maximal concentration tested in the current study.

In conclusion, the presented experiments show that the four tested metals mercury, lead, nickel and manganese do not stimulate biofilm formation of the facultative pathogen *S. epidermidis*. Considering the fact the *S. epidermidis* was also proposed as agent to clean contaminated wastewater, it is important to consider, that *S. epidermidis* was found to be sensitive to lead, mercury and manganese at higher concentrations. Thus at higher concentrations no *S. epidermidis* biofilm will be produced that could absorb environmental pollutants. Considering also the fact that *S. epidermidis* is a facultative pathogen, its use as bio-absorbent is strongly discouraged. Further studies on the potential effects of heavy metals on biofilm formation should include cadmium (see Chapter 9).

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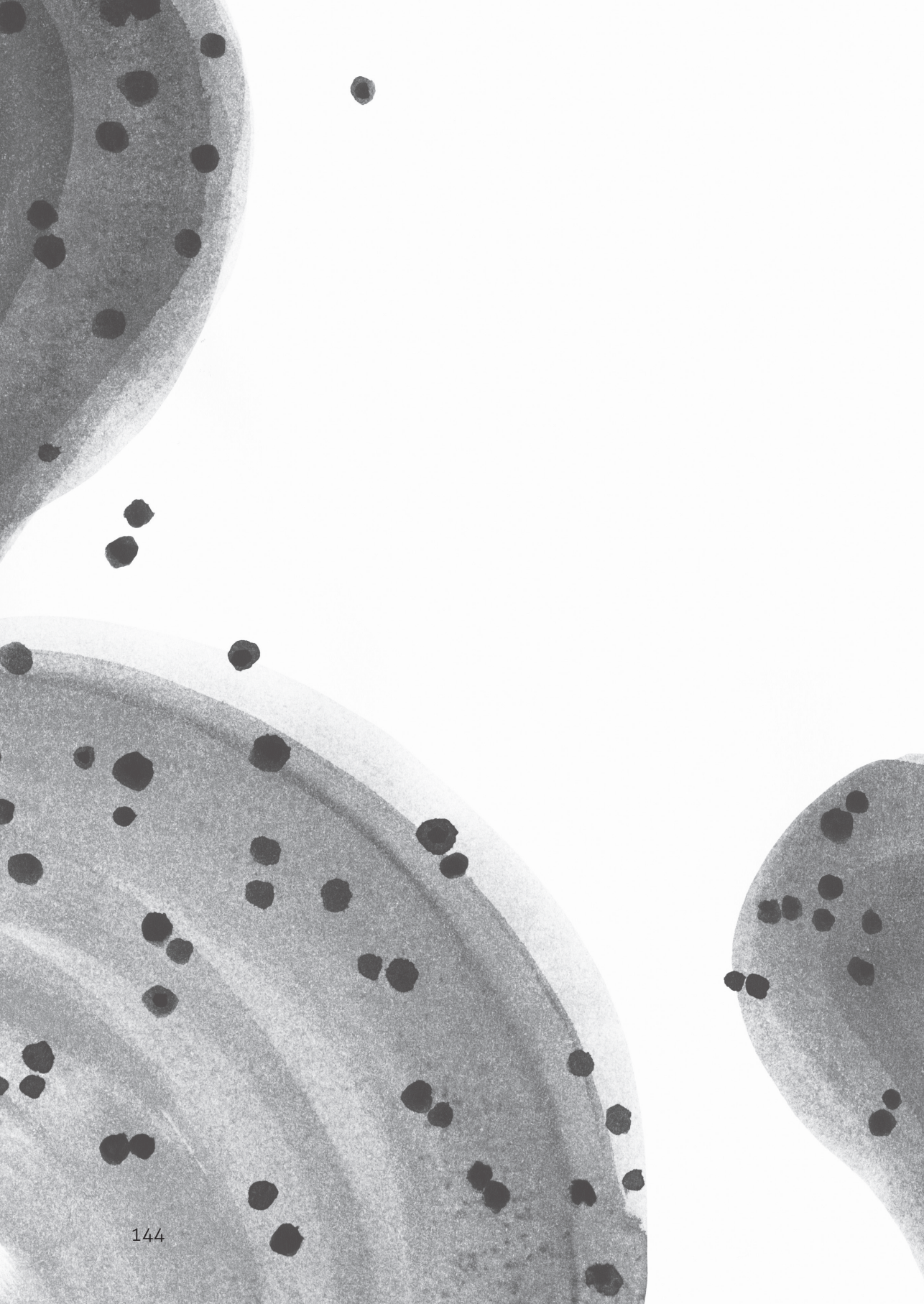

9 Cadmium modulates biofilm formation by *Staphylococcus epidermidis*

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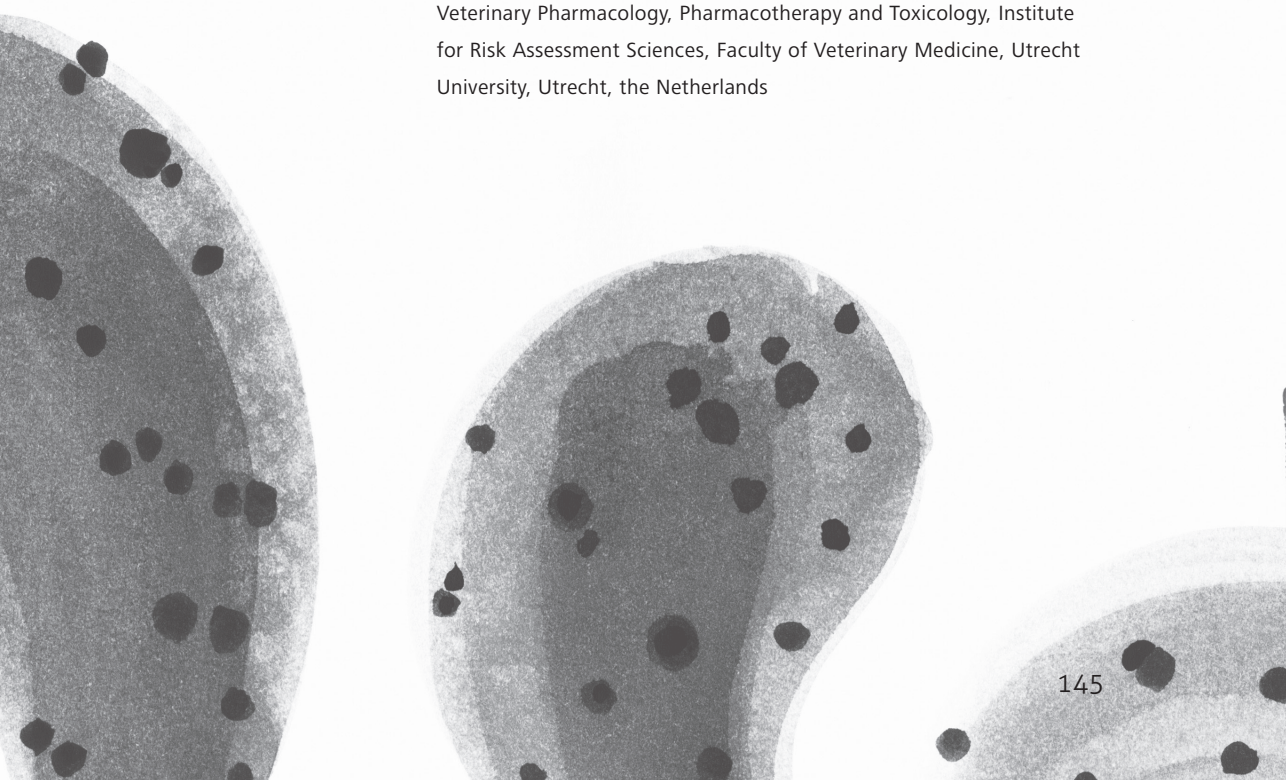
X. Wu, R. R. Santos, J. Fink-Gremmels

Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands



The aim of the study was to evaluate the effect of cadmiumexposure on *Staphylococcus epidermidis* (ATCC 35984) biofilm formation. Bacteria were cultured in the absence or presence of cadmium at different concentrations (0¬¬–50 μM). Biofilm formation and bacterial viability were assessed. Quantitative Real Time-PCR (qRT-PCR) was used to determine the mRNA expression of molecular markers of *S. epidermidis* biofilm formation and dispersion. *S. epidermidis* biofilm formation was stimulated ($P< 0.001$) by 1.56 and 3.13 μM cadmium. Confocal laser scanning microscopy (CLSM) analysis confirmed an increase in biofilm thickness (23 and 22 μm, versus 17.8 μm in the controls) after exposure to1.56 or 3.13 μM cadmium, respectively. qRT-PCR was performed showing the up-regulation of *atlE, embp, aap, icaA* and *icaB* after exposure to 3.13 μM cadmium. Taken together, these findings show that cadmium at low, sub-toxic concentrations acts as inducer of *S. epidermidis* biofilm formation.

INTRODUCTION

Heavy metal contamination of soil and water is a worldwide risk affecting food and feed safety (Jarup, 2003; Zhao et al., 2012). Among various heavy metals, cadmium is considered to be the most toxic element predominantly due to its long biological half-life resulting in an accumulation in the body, particularly the kidneys (Wang and Du, 2013). Plants can take up cadmium from soil and hence cadmium is a regular contaminant of food and animal feeds (Wang et al., 2014). The hazard of cadmium was well studied in past decades, and has been recently summarized in a broad exposure analysis by the World Health organisation (JECFA, 2013).

Sub-toxic concentrations of heavy metal have been described as potential inducers of biofilm formation (Perrin et al., 2009). This effect might be desirable, when microorganisms are used to decontaminate soil or water in industrial areas, but is undesirable in pathogenic bacteria, as biofilm formation confers resistance to most commonly used antibiotics and protects pathogens from host defense mechanisms (Coenye and Nelis, 2010; Fey and Olson, 2010). One of the organisms known for its rapid biofilm formation is *Staphylococcus epidermidis*. *S. epidermidis* can be isolated from soil (Rabah et al., 2010) and wastewater (Ayed et al., 2010), but is also a common commensal organism on the human skin and a facultative pathogen (Fey and Olson, 2010).

Bacterial biofilm formation can be stimulated by diverse stress factors, such as drugs (Cargill and Upton, 2009) or heavy metals from the environment. For example, Perrin et al. (2009) reported that sub-inhibitory concentration of nickel promotes *Escherichia coli* K-12 biofilm formation. Schue et al. (2011) showed that cadmium triggers *Rhizobium alamii* biofilm formation. The *S. epidermidis* strain ATCC contains putative cadmium resistance genes and it has been suggested that these are associated with its ability to readily form biofilms (Gill et al., 2005).

The aim of the current study was to assess the effect of cadmium on *S. epidermidis* viability and its ability to form biofilms. As an experimental model, two ATCC type strains of *S. epidermidis* were compared. The ATCC 12228 strain does not produce biofilms, as it lacks the ica operon, and was used to establish the MIC values for cadmium chloride. In contrast, ATCC 35984, forms biofilms (Fey and Olson, 2010). Recent investigations by Mertens and Chebremedhin (2013) indicated that approximately 50% of all clinical isolates of *S. epidermidis* are genetically positive for this ica operon. To demonstrate the association of cadmium tolerance and the capacity of ATCC 35984 to form biofilms, a detailed analysis of the induction of biofilm formation, biofilm architecture and the expression of genes encoding key regulators of biofilm formation is presented.

MATERIALS AND METHODS

Unless mentioned otherwise, chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bacterial strains and culture conditions

Two strains of *S. epidermidis* were purchased from the American Type Culture Collection (ATCC). The strain ATCC 35984 (RP62a-corresponding to ST10) forms a thick biofilm on the bottom of a 96-well plate, affecting optical density (OD) measurement required for a quantitative MIC determination. Therefore, we used the non-biofilm-forming strain (ATCC 12228-corresponding to ST8) for the measurement of MIC and minimal bactericidal concentration (MBC). Both strains were maintained on tryptic soy broth (TSA) (Oxoid CM 129) slants at 4°C. Before use, one colony of bacteria was cultured in 10 mL tryptic soy broth (TSB) + 0.25% glucose (TSB+) (pH 7.0) under aerobic conditions at 37°C for 24 hours.

Cadmium

A 0.2 M solution of cadmium (Cd^{2+}) (cadmium chloride) was prepared and stored at 4°C. Before use, the stock solution was diluted in TSB^{+} to a final concentration of 100 µM.

Bacterial susceptibility to cadmium

The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the non-biofilm forming strain (ATCC 12228), following Clinical and Laboratory Standards Institute standard broth micro-dilution method with slight modifications. Briefly, serial two-fold dilutions of 100 µM cadmium in TSB^{+} were prepared, down to a final concentration of 0.78 µM cadmium, in a U-bottom 96-well plate (100 µl/well). To each well, 100 µl of 10^6 CFU (colony-forming unit)/ ml bacterial suspension was added, resulting in a final volume of 200 µl and final concentrations of cadmium of 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 µM. Wells with sterile TSB^{+} alone served as blanks. Plates were incubated at 37°C for 24 hours. Thereafter, 100 µl of the incubated suspension was transferred into a new sterilized flat bottom 96-well plate and its OD was measured at 655 nm wavelength using a microplate reader (model 3550, Bio-Rad, CA, USA). MIC was defined as the lowest cadmium concentration that inhibited visible growth after 24 hours of culture and resulted in an OD value similar to the blank (TSB^{+} only). After these 24 hours incubation at 37°C, aliquots of 10 µl from each well were spotted on to tryptic soy agar (TSA) plates containing no cadmium. The MBC was read as the lowest cadmium concentration with no growth after 24 hours culture. All MIC/MBC experiments were carried out in triplicate with three independent repetitions.

Quantification of biofilm formation

Biofilm formation was assessed by the standard safranin colorimetric assay as described previously (Melchior et al., 2006; Wu et al., 2014) using the strain ATCC 35984. In brief, 100 µl of bacterial suspension (10^6 CFU/ml) was transferred into a U-bottom 96-well microtiter polystyrene plate (Costar, Corning, NY, USA). To this suspension, a solution of TSB^+ containing cadmium at concentrations of 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, or 100 µM was added, resulting in final concentrations of cadmium are 0.39–50 µM in the tested samples. Wells with sterile TSB^+ alone served as blanks. The plates were incubated on a microplate shaker (Heidolph titramax 100) at 600 rpm, 37Ð for 24 hours. At the end of the culture period, the supernatants from all wells were discarded and the biofilms adhered to the bottom of the wells were incubated with 0.1 M HCl for 1 hour at room temperature. Thereafter, HCl was replaced by safranin (0.1% in water) and the plates were incubated for 45 minutes at room temperature. Non-bound safranin was removed by rinsing the wells three times with de-ionized water, and thereafter plates were incubated with 125 µl 0.2 M NaOH per well at 57°C for 1 hour. At the end of incubation, 100 µl from the stained dissolved biofilm in each well was pipetted into a new flat-bottom 96-well microtiter polystyrene plate and its intensity was measured at a wavelength of 540 nm in a microplate reader. Minimum biofilm inhibitory concentration (MBIC) was defined as the lowest concentration that inhibited at least 90% biofilm formation. Each test was performed in quadruplicate with three independent repetitions.

Determination of bacterial viability in suspension and in the biofilm

To quantify the number of culturable (i.e. actively growing) bacterial cells in suspension and in the biofilm after exposure to different concentrations of cadmium (0.39–50 µM), a plating method was conducted as described by Cabal et al. (2012), with some modifications. Briefly, for counting the bacteria in suspension (planktonic bacteria), 200 µl of supernatant was separately taken from each well, without disturbing the biofilm, and subjected to 10-fold serial dilutions in TSB^+. Subsequently 10 drops of 10 µl supernatant samples from each well were plated onto TSA and cultured for 24 hours at 37°C. After culture, drops that had 3–30 counts were chosen to calculate the number of viable bacteria in suspension by the formula: N (mean value of colony number from 10 drops) × 100 × 2 = CFU/well (200 µl) (Figure S1). For counting the bacteria in biofilm, plates with biofilm attached on the bottom were carefully washed three times with sterile saline solution to remove free cells before adding 200 µl TSB+. Biofilms from each well were scraped and then the dispersed by using a 0.5 × 16 mm needle until no visible flocculent was observed in the suspension. Bacteria obtained from biofilms were diluted, plated, and the total number of viable culturable cells in the biofilm was calculated and expressed as CFU/well by following the same methods used

for the bacteria in the suspension. Experiments were carried out in triplicate with three independent repetitions.

Bacterial viability and biofilm thickness determined by confocal laser scanning microscopy

Biofilms of ATCC 35984 were formed after culture at 37°C for 24 hours on coverslips inserted into tubes containing either control medium or medium supplemented with cadmium at a range of test concentrations (0.39–50 µM). After culture, coverslips carrying biofilms were incubated for 15 minutes in the dark at 37°C with 1 nM SYTO® green fluorescent nucleic acid dye and 6 nM propidium iodide from propidium/SYTO green viability staining kit (Life Technologies Europe BV, Bleiswijk, Netherlands). Biofilms were washed three times in PBS and then examined using confocal laser scanning microscopy (CLSM) (Leica TCS SPE-II, Mannheim, Germany). Bacteria in biofilms were classified as viable if not stained positively by propidium (red). The used excitation/emission wavelength for these dyes were 480/500 nm for SYTO® green and 490/635 nm for propidium iodide. To estimate the percentages of dead bacteria, the program Image J 1.47 was used to count the propidium stained cells (given as cell area).

Biofilms were analysed by a series of images in the z-axis, followed by digitized images in selected optical planes with a Leica CLSM (Leica TCS SPE-II, Mannheim, Germany). Automated on-line collection of confocal two-dimensional cross-sectional images was used to determine biofilm architecture from a three-dimensional reconstruction by Image J 1.47.

Quantitative RT-PCR

RNA was isolated from planktonic bacteria and those embedded in biofilm (the strain ATCC 35984 was used) using the Trizol reagent and FastPrep® Disrupter (Thermo Savant, Qbiogene, Inc. Cedex, France). Firstly, 1 ml of bacterial suspension or biofilm in TSB^{+} was centrifuged (15,000 × g) for 10 minutes at 4°C. The supernatant was removed and 1ml Trizol reagent was added and pipetted several times. The suspension was transferred to a Lysing Matrix E tube (MP Biomedicals Germany GmbH, 37269 Eschwege Germany) and homogenized in the FastPrep® FP120 Cell Distrputer (Thermo Savant, Qbiogene, Inc. Cedex, France) for two periods of 45 seconds at a speed of 6.5 m/s. Subsequently, the samples were centrifuged (15,000 × g) for 5 minutes at 4°C, and the supernatants were separately transferred to 1.5 ml eppendorf tubes and subjected to the phenol-chloroform RNA extraction protocol. The concentration and purity of total RNA was spectrophotometrically assessed using a NanoDrop 1000™ (Thermo Scientific, Waltham, MA, US), and 1 µg of extracted total RNA from each sample was reverse transcribed with the iScript™ cDNA Synthesis kit (BIO-RAD, Hercules, CA, USA). The obtained cDNA was diluted to a final concentration of 30 ng/ml. Primers (Table

1) complementary to *S. epidermidis* were designed according to literature, and were commercially produced (Eurogentec, the Netherlands). The primers used were selected based on specificity and efficiency by qPCR analysis of a dilution series of pooled cDNA at a temperature gradient (55–65°C) for primer-annealing and subsequent melting curve analysis. The reaction mixture for qPCR contained 10 µl of diluted cDNA, 12.5 µl iQSYBR Green Supermix (Bio-Rad Laboratories Inc., USA), forward and reverse primers (final concentration of 0.4 pmol/µl for each primer) and sterile water according to the manufacturer's instructions. qPCR was performed using the MyiQ single-colour real-time PCR detection system (Bio-rad, Hercules, CA) and MyiQ System Software Version 1.0.410 (Bio-Rad Laboratories Inc., USA). The relative mRNA expression was calculated from the comparison with the expression levels of two reference genes, heat shock protein 60 (hsp60) and triosephosphate isomerase (*tpi*) (Table 1).

Table 1. Primer sequences used in this study

Gene	GenBank	Sequence (5'-3')	Product size (pb)	Reference
hsp60	AF029245	F: GTTTTAGCACAATCAATGATTCAG	491	Wang et al., (2003)
		R: GCATCGCCTTCTACTTCATCC		
tpi	AF269838	F: CATCTGATAAACCTTCGACAGCTTT	128	Vandecasteele et al. (2001)
		R: TGCTATCTTCAATCACGGTATGACA		
aap	AJ249487	F: ATACAACTGGTGCAGATGGTTG	400	Vandecasteele et al. (2003)
		R: GTAGCCGTCCAAGTTTTACCAG		
agrB	AF012132	F: TTCGTTTAGGGATGCAGGTA	141	Patel et al. (2012)
		R: ATGGCACACGTACAGAGGAT		
atlE	U71377	F: TGTCCTGCTTTCACGTATGA	139	Patel et al. (2012)
		R: AGAAACCTTAACCACGTAAA		
embP	AY101364.1	F:AGCGGTACAAATGTCAATATC	455	Mekni et al. (2012)
		R:AGAAGTGCTCTAGCATCATCC		
icaA	U43366	F: AACAAGTTGAAGGCATCTCC	166	Tormo et al. (2005)
		R: GATGCTTGTTTGATTCCCT		
icaB	U43366	F: AATGGCTTAAAGCACACGAC	144	Patel et al. (2012)
		R: TTTGTCCTTTCCGTAACAGT		
sarA	NC002976.3	F: TGGTCACTTATGCTGACAGATT	313	Frebourg et al. (2000)
		R: TTTGCTTCTGTGATACGGTG		
sepA	NC002976.3	F: CGCACCAGACAACGCTGTA	170	Christner et al. (2012)
		R: TCAATCGCACATGTAAATAACTTCC		
rsbU	NC002976	F: TCTCTTCATACAGTCCAT	172	Knobloch et al.(2001)
		R: ATAGGTTCAGGTATTCCA		

Statistical analysis

Data were evaluated with one-way analysis of variance (ANOVA) using Graph Pad Prism version 6.04 for windows (Graph Pad Software, San Diego, California, USA).

RESULTS

Bacteriostatic and bactericidal effects of cadmium on *S. epidermidis*

MIC and MBC of cadmium were determined. The MIC of cadmium on *S. epidermidis* (ATCC 12228) was determined as 25 µM (Figure 1), as there was no difference between the OD values obtained after bacterial exposure to 25 µM cadmium and the negative control. Thereafter, by plating the cadmium-exposed (12.5, 25, and 50 µM) bacteria, we found that the MBC of cadmium on *S. epidermidis* (ATCC 12228) was 50 µM, as no bacterial colonies were present at this concentration.

Cadmium increases *S. epidermidis* biomass

S. epidermidis biofilm formation and inhibition were quantified. *S. epidermidis* (ATCC 35984) biofilm formation was increased ($P < 0.001$) by cadmium at concentrations of 1.56 and 3.13 µM, but biofilm formation was inhibited at a cadmium concentration of 6.25 µM (Figure 2). The MBIC of cadmium on *S. epidermidis* (ATCC 35984) biofilm was 25 µM, at which concentration biofilm formation was inhibited by 90%.

Viable counts in suspension and in the biofilm

To evaluate the viability of the bacterial population in suspension and biofilm after exposure to cadmium, growth of the biofilm-positive strain *S. epidermidis* (ATCC 35984) was measured using the drop plate method. The results (Figure 3) showed a cadmium concentration-dependent decrease of bacterial counts in both the suspension and the biofilm. Bacterial counts in suspension were decreased ($P< 0.05$) after exposure to 3.13 µM cadmium and no culturable counts were observed after exposure to 12.5 µM cadmium. When enclosed in the biofilm, bacterial counts were diminished ($P< 0.05$) immediately after exposure to 0.78 µM cadmium. However, in contrast to bacterial suspension, 10^5 CFU/well of bacterial counts in the biofilm were culturable even after exposure to 12.5 µM cadmium.

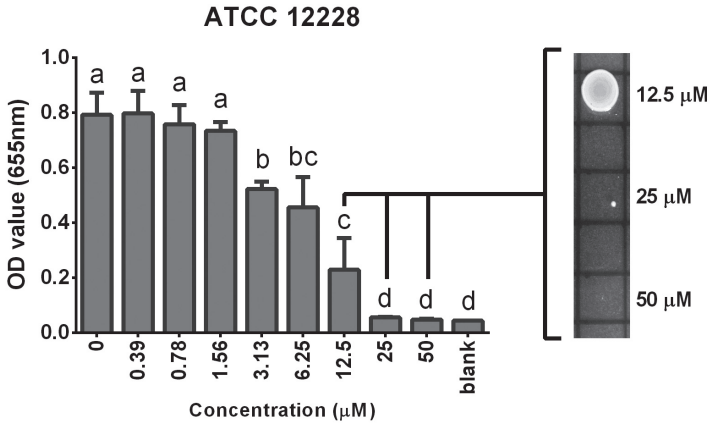


Figure 1. Mean (± SEM) OD values of *S. epidermidis* (ATCC 12228) after exposure to cadmium (0.39–50 μM); measured at 655 nm wavelength. Different letters (a–d) indicate significant difference between compared groups. 12.5, 25, and 50 μM cadmium treatment groups were chosen for drop plating (right side image).

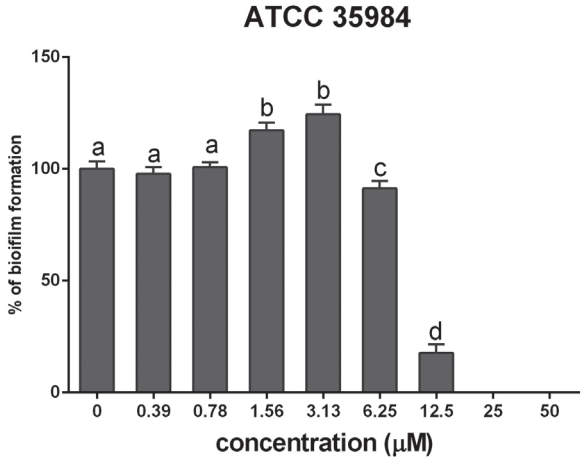


Figure 2. Mean (± SEM) percentage of biofilm formation by *S. epidermidis* (ATCC 35984) after exposure to different concentrations of cadmium. Different letters (a–d) indicate significant differences between compared treatment groups. MBIC was defined as the lowest concentration that inhibited at least 90% biofilm formation.

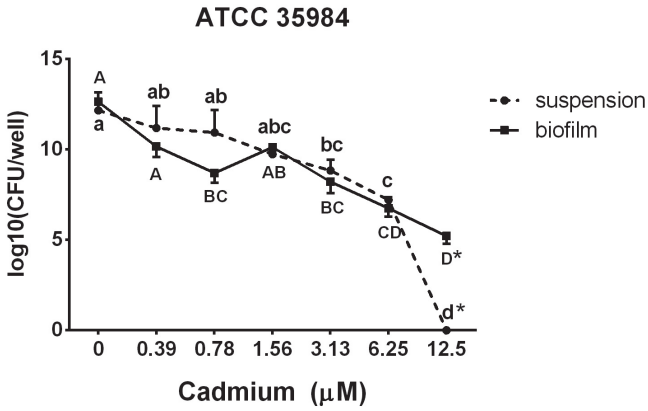


Figure 3. The bacterial population *S. epidermidis* (ATCC 35984) in suspension and biofilm after exposure to different concentrations (0.39–12.5 μM) of cadmium. Different letters indicate significant differences between cadmium concentrations in each suspension (lower case letters: a–d) or biofilm (upper case letters: A–D). * indicates significant difference between biofilm and suspension in same cadmium treatment group.

Bacterial viability and biofilm architecture

Figure 4 illustrates our findings when fluorescent markers (propidium/SYTO green viability staining kit) were used to quantify the percentage of viable bacteria (ATCC 35984) encapsulated in matrix biofilms. Exposure to cadmium resulted in a decrease in viable bacteria after exposure to 1.56 (41.4 ± 7.0%) or 3.13μM (39.6 ± 5.3%) cadmium, whereas the viability in control biofilms exceeded 60% (67.1 ± 5.2%). Similar results had been obtained with viability counts as presented in Figure 3. Noteworthy, non-viable bacteria were mostly found in the outer layers of the biofilm.

When a 3D reconstruction was performed (Figure 5), it was observed that control samples presented a compact and dense biofilm with a thickness of 17.8 μm. Biofilms became thicker with increasing cadmium concentrations, reaching the highest values after exposure to 1.56 μM (23 μm) and 3.13 μM (22 μm) cadmium. However, when the cadmium concentration was increased to 6.25 μM, biofilm thickness (17.3 μm) decreased significantly with a notable alteration in the biofilm architecture.

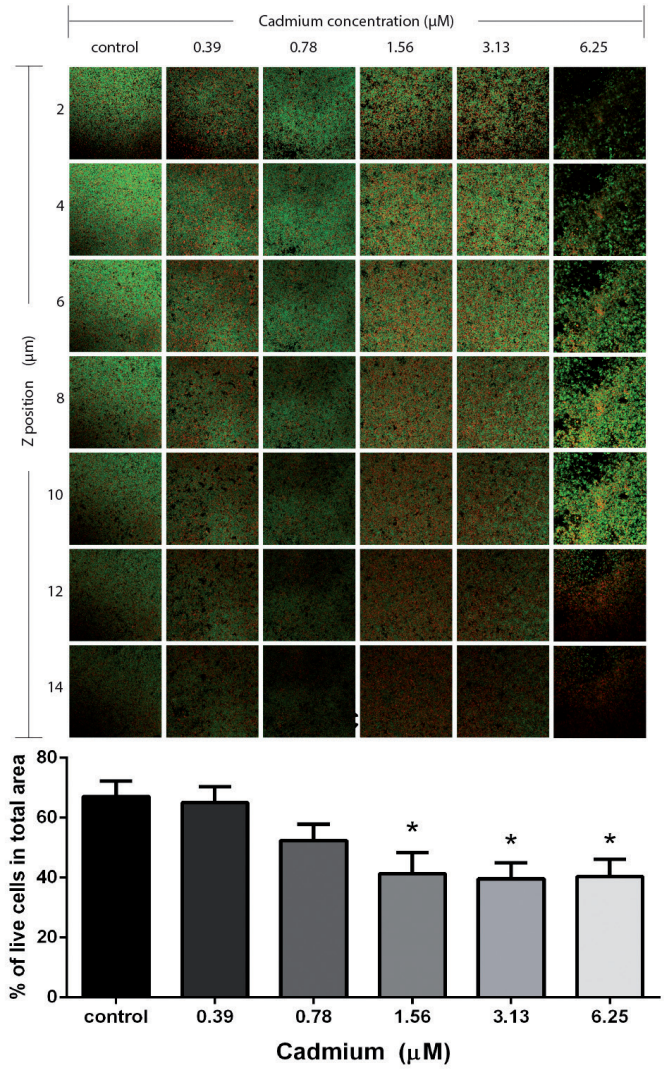


Figure 4. Bacterial viability in *S. epidermidis* (ATCC 35984) biofilms measured by confocal scanning laser microscopy. Non-viable bacteria were stained red, and total bacteria were stained green by using the propidium/SYTO green viability staining kit. Bacteria not stained in red were thus considered viable. Z-position images of controls and different cadmium concentrations (0.39–6.25 µM) were acquired in every 2 µm section. The graphic under the images shows the percentage of viable bacteria determined from all biofilm layers by imageJ 1.47.

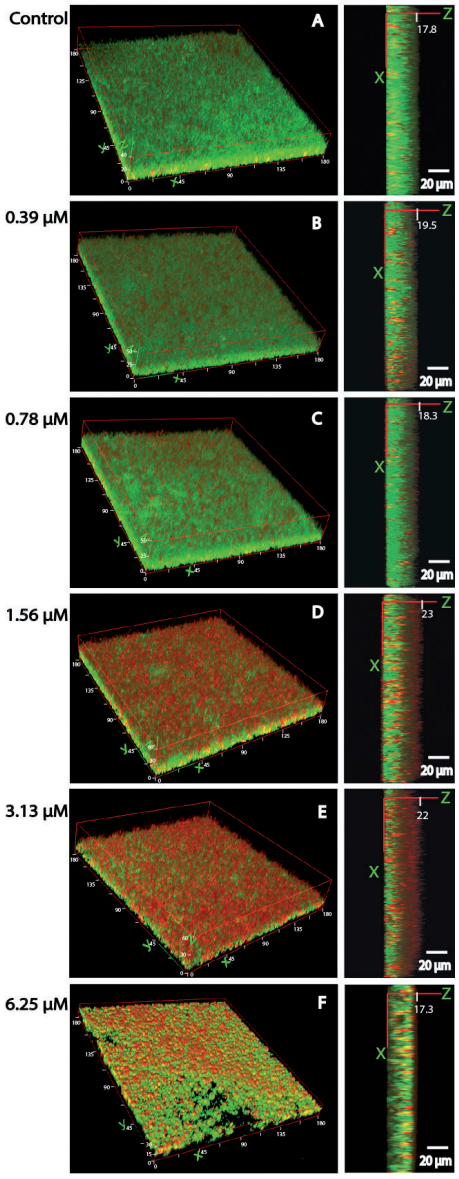


Figure 5. The thickness (right panel) and 3D (left panel) pictures of *S. epidermidis* (ATCC35984) measured by CLSM. Non-exposed (control) (A) or exposed to different concentrations (0.39–6.25 µM) (B–F) of cadmium. The thicknesses of biofilms from control to 6.25 µM treatment group were 17.8, 19.5, 18.3, 23, 22, and 17.3 µm, respectively. Non-viable bacteria were stained red, and viable bacteria were stained green by using the propidium/SYTO green viability staining kit.

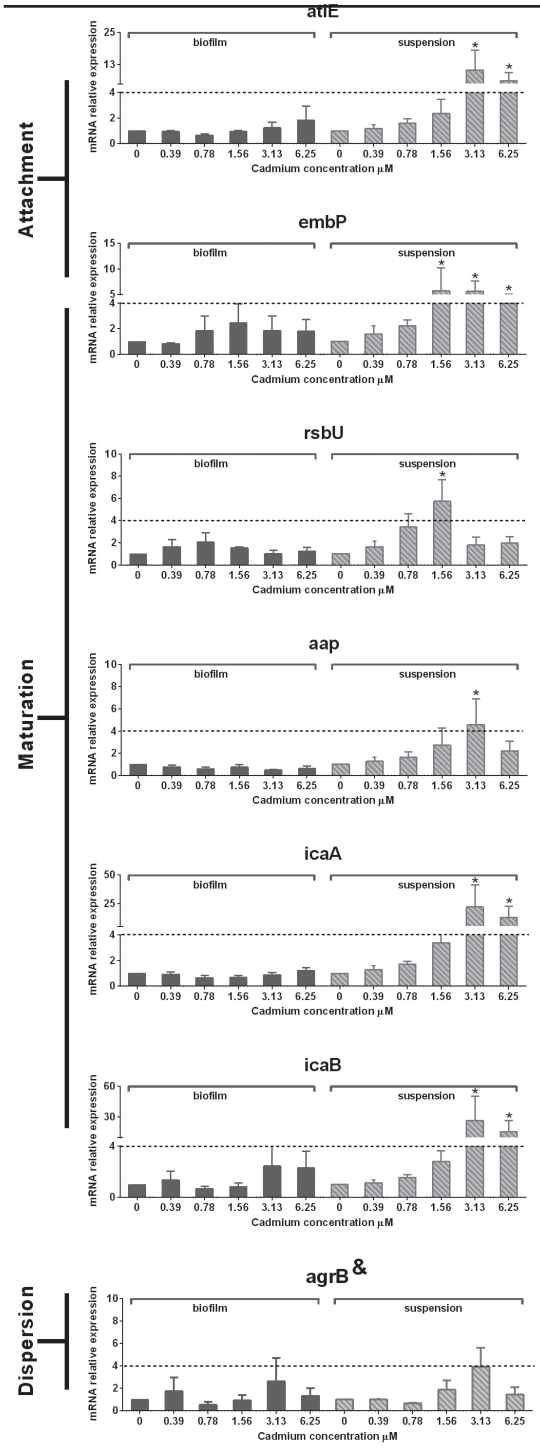


Figure 6. Mean (±SEM) relative expression of mRNA encoding genes involved in biofilm formation and quorum sensing. *indicate significant relative down- or up-regulation of genes when compared with untreated controls. & indicate *agrB* is considered to be involved in bacterial attachment and biofilm dispersion.

Gene expression in planktonic cells and mature biofilms

The relative mRNA expression levels of *sarA* and *sepA* in suspension and biofilm were unaltered compared to the untreated controls after treatment of *S. epidermidis* with different concentrations of cadmium (0.39–6.25μM) (data not shown). The relative mRNA expression levels of *atlE, embp, rsbU, icaA, icaB*, and *agrB* in planktonic bacteria and biofilm-embedded bacteria measured 24 hours after culture are depicted in Figure 6. *AltE, embp, icaA* and *icaB* were up-regulated (≥ 4 fold) in suspension when bacteria were treated with 3.13 and 6.25 μM cadmium, but none of them were altered in biofilm-embedded bacteria. In planktonic bacteria, *RsbU* mRNA expression was up-regulated when exposed to 0.78 and 1.56 μM cadmium. The relative mRNA expression of *aap* was up-regulated in suspension when bacteria were treated with 3.13 μM cadmium.

DISCUSSION

As yet, most studies involving the effect of heavy metals on bacteria focused on the bacterial bio-sorption ability, including binding to biofilms. Only occasionally studies emphasize the effect of heavy metals on bacterial biofilm formation in commensal and potential pathogenic bacteria, such as clinical isolates of *S. epidermidis*, although biofilm formation is one of the most common causes of antimicrobial resistance (Arciola et al., 2005; Mertens and Ghebremedhin, 2013). Investigations with 300 clinical isolates of blood cultures and indwelling devices indicated that are indeed 32% of these clinical isolates are biofilm producers (measured on Congo Red Agar). However, as also 3 *icaA/D*-negative strains were found to produce phenotypically biofilms, the authors suggested that other factors such as sub-inhibitory concentrations of antibiotics and stress factors might have a significant role in biofilm formation.

Perrin et al. (2009) reported that *E. coli* (K-12) exposure to 100 μM nickel promoted biofilm formation, but data on the effect of cadmium are lacking. It is therefore of interest that we could show that cadmium indeed stimulated *S. epidermidis* (ATCC 35984) biofilm formation at concentrations of 1.56 and 3.13 μM, whereas pilot experiments with other metals, such as lead, mercury, nickel and manganese did not indicate that these metals affected biofilm formation in *S. epidermidis* (Chapter 8).

At higher concentrations, cadmium exerted an antibacterial effect. This inhibition was observed at a concentration range from 6.25 to 50 μM. Shaivastave et al. (Shaivastave et al., 2013) reported that the MIC of cadmium on *S. aureus* isolates from industry wastewater was 450 μg/mL (~2.45 mM), which is much higher than the concentration that was effective against *S. epidermidis* (ATCC 12228) in our *in vitro* study. AbubAkr et al. (2010) showed *in vitro* that the *S. sciuri* (18 strains) MIC values range between 10–300 μg/mL (54–1633 μM). These values are also much higher than

the MIC values reported here indicating the specific sensitivity of *S.epidermidis*. The MBIC, MIC and MBC concentrations were found to be in the same range, amounting for *S. epidermidis* (ATCC 35984 and ATCC 12228) to 25, 25 and 50 µM, respectively. These minor differences between a biofilm producer and a non-biofilm producing strain suggest that sensitivity and resistance to cadmium are not only mediated by the ability of *S. epidermidis* (ATCC 35984) to form readily biofilms. This is also suggested by Gill et al. (2005), who identified a plasmid denoted vSe1. This plasmid has prophage integrase genes in the biofilm forming strain RP62A (ATCC 35984), but direct evidence for its involvement in cadmium tolerance was not shown.

Of medical and also eco-toxicological importance is the biofilm stimulation that was observed after exposure to very low concentrations of cadmium, such as 1.56 or 3.13 µM. These results were confirmed by our CLSM analysis, which indicated the increase in biofilm thickness after exposure to cadmium at concentrations of 1.56 µM (22 µm) and 3.13 µM (23 µm) together with a loss of bacterial viability at the same concentrations. This loss of viability was mainly observed in the outer layer of the biofilms. Similar results were obtained with *Pseudomonas aeruginosa* exposed to copper, where the highest percentage of dead biomass was found in the outer layer of the biofilm (Teitzel and Parsek, 2003). It was suggested that this phenomenon is related to the fact that bacteria in the outer layers of a biofilm are exposed to the highest concentrations of a toxic agent, whereas bacteria in the central parts of a biofilm are protected by the biofilm matrix.

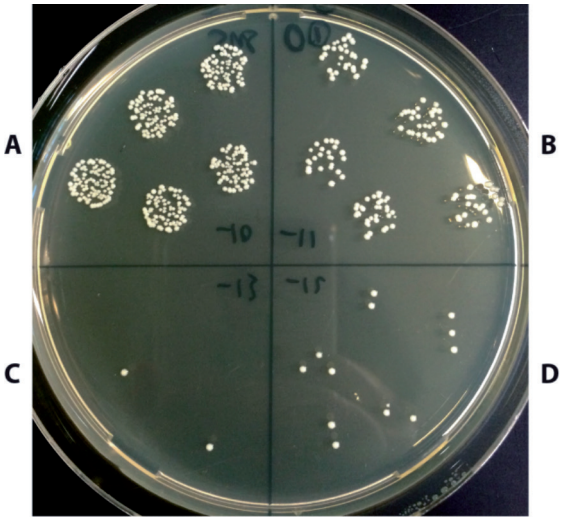
For a better understanding of the mechanism involved in biofilm formation and maturation, the relative mRNA expression during *S. epidermidis* biofilm formation was determined. No significant changes were noted in bacteria embedded in a biofilm. In contrast, concentration-dependent changes following cadmium exposure were observed in planktonic persister cells. For example, in persisters, *rsbU*, a stress marker (Delumeau et al., 2004), was already up-regulated at low concentrations (0.78 and 1.56 µM Cd), and subsequent up-regulation of biofilm related genes was expected. Genes related to attachment such as *atlE* and *embp* were up-regulated, comparable to previous findings where this upregulation was associated with the attachment to a polystyrene device (Vandecasteele et al., 2003) and the intercellular adhesion during bacterial attachment (Williams et al., 2002). Concomitantly, *icaA* and *icaB* were up-regulated not only as a stimulus to intercellular adhesion, but also because they regulate PIA synthesis, a main agglutination agent in the biofilm formation of *S. epidermidis* (Fey and Olson, 2010; Gerke et al., 1998). Transcription of *aap* was increased in planktonic persister bacteria during exposure to 3.13 µM cadmium. This gene supports bacterial accumulation (Gerke et al., 1998), and is one of the most important proteins involved in *S. epidermidis* biofilm formation (Hu et al., 2011). *agrB* is a biomarker of agr quorum-sensing system (Dai et al., 2012). This gene was slightly (non-significant) up-regulated

after exposure to 3.13 µM cadmium in both planktonic and biofilm bacteria, indicating that cadmium did not affect the quorum-sensing system in the present study. The gene regulation in persisters may reflect the stimulatory effect of cadmium on *S. epidermidis*. This is in line with the hypothesis that as a commensal opportunistic pathogen, *S. epidermidis* can adapt to various environmental stress conditions. A typical example is the metal inducible TCS (two component system) allowing an adaptive response of bacteria to changing environmental conditions as it regulates the expression of various genes determining virulence factors and biofilm formation (Dieppois et al., 2012). In the present study, cadmium was found to stimulate *S. epidermidis* biofilm formation by positively influencing bacterial attachment and biofilm maturation, and this may indeed be related to the two-component system (TCS).

As mentioned above, gene expression within the biofilm was not affected by cadmium. This phenomenon may be explained by the fact that bacterial cells in a biofilm are in dormant state. Hu et al. (2011) also showed that *aap* transcription in planktonic *S. epidermidis* was increased, while it was decreased in biofilm-embedded bacteria. Pintens et al. (2008) demonstrated that in the initial 4–7 hours during biofilm formation, *aap* expression was transiently higher in sessile than in planktonic bacteria, after which it progressively declined in sessile bacteria. Most changes in bacterial biofilm gene expression were observed within the first 8 hours of culture in *S. epidermidis* biofilm-embedded bacteria (Hu et al., 2011). As in the current experiments gene expression was measured only after a 24 hours culture period, i.e. at a time point when the biofilm was already mature, such as stable gene expression could be expected. Comparably, it has been reported that gene expression in *Pseudomonas aeruginosa* remains relatively stable in mature (after 14 hours) biofilms (Waite et al., 2005).

In conclusion, it could be demonstrated that low concentrations of cadmium, which do not affect bacterial viability, act as stressors that stimulate biofilm formation of *S. epidermidis*. This is of clinical interest, as previous investigations have shown that approximately 50% of all clinical isolated of *S. epidermidisa* produce biofilms and that this biofilm formation does not correlate in all cases with the presence of the *ica*A/D operon. Hence further investigations should clarify if cadmium is one of the stress factors provoking biofilm formation also in strains that do not form biofilms under stress-free conditions. Considering that human exposure to cadmium occurs often by inhalation (occupational exposure or smoking), its biofilm-stimulating effect may be an important factor in the pathogenesis of chronic bacterial airway infections and the resistance of these infections to therapeutic approaches.

SUPPLEMENTARY DATA
Drop plate method



Supplementary Figure 1. Example of a drop plate assay. Five 10 µl drops of 10^{10}, 10^{11}, 10^{12} and 10^{13} times diluted bacterial suspension were dropped on areas A, B, C and D, respectively. Bacterial colonies from each drop can be counted after culture the plate 24 h at 37 °C.

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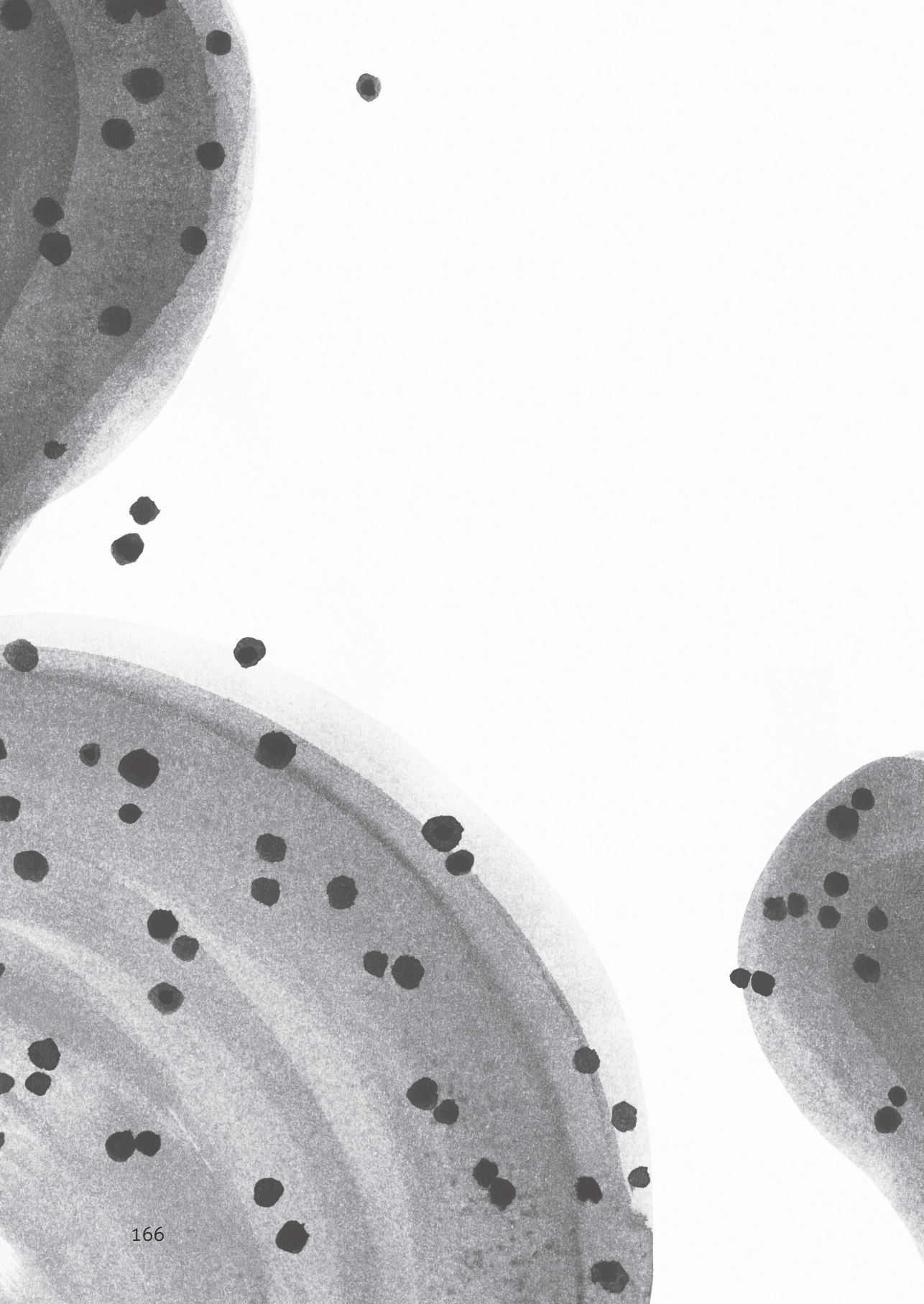
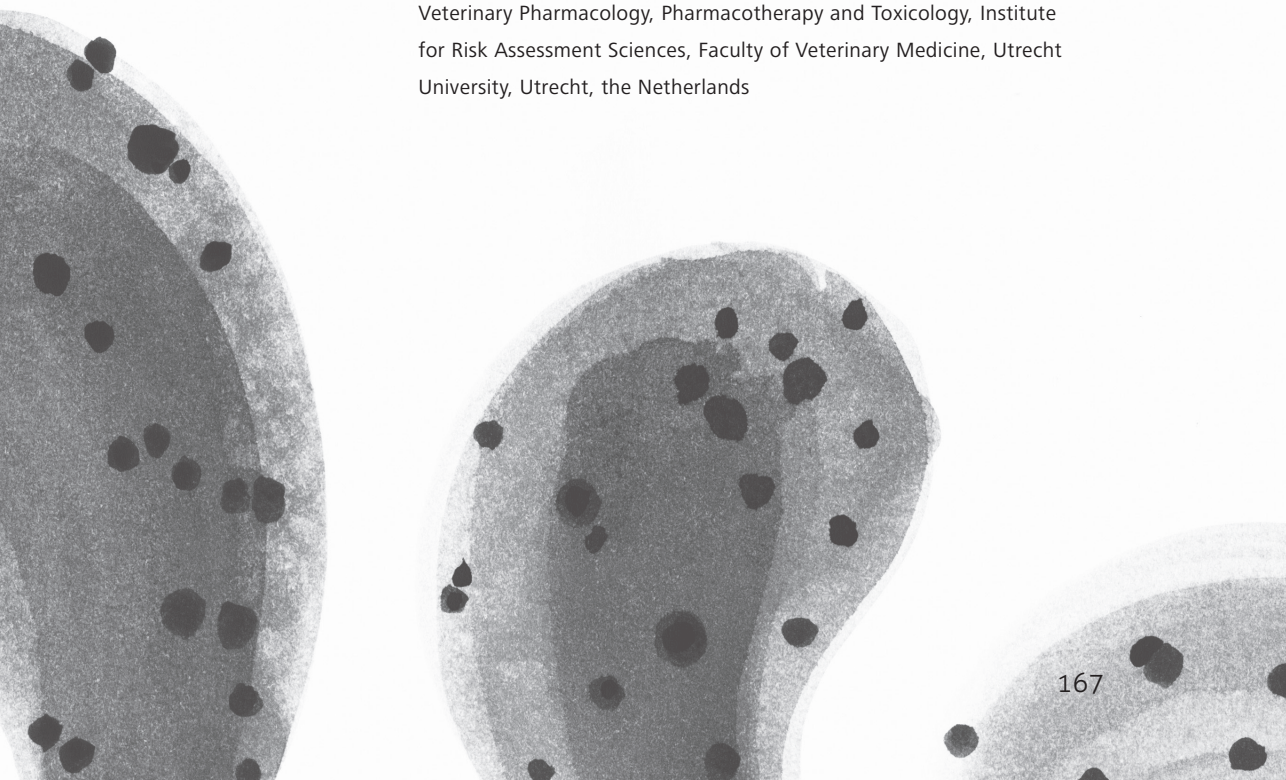


10 Modulation of cadmium cytotoxicity by aqueous extracts of Chinese Herbal Medicines

Preliminary data

X. Wu, A. Fumagalli, R.R. dos Santos, J.Fink-Gremmels

Veterinary Pharmacology, Pharmacotherapy and Toxicology, Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands



Chinese Herbal Medicines (CHMs) have been used to prevent and treat different diseases. One of the common features of CHMs is their antioxidant activity that is considered as a general beneficial health effect. In the present study, the water extract of *F. suspensa* (fruits) and *S. baicalensis* (roots) were tested for their preventive effect on cadmium-induced cytotoxicity on HepG2 cells. For this, a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay was applied. Both *F. suspensa* and *S. baicalensis* water extracts were able to restore cell viability after a pre-treatment. This preventive effect might be related to the antioxidant properties of both CHMs, as well as their potential activity to upregulated membrane efflux transporters such as P-gp hence reducing the intracellular concentration of cadmium. In conclusion, *F. suspensa* and *S. baicalensis* water extracts show protective effects against cadmium induced HepG2 cell injury, which is of clinical relevance, as cadmium can also contaminate herbal plants.

INTRODUCTION

For many thousands of years, Chinese Herbal Medicines (CHMs) have been used for the treatment and prevention of various diseases. Among fifteen categories (Liu and Xu, 2002), heat-clearing CHMs are known to possess antioxidant activity (Shan et al., 2014). For example, the ethanol extract of the CHM *Forsythia suspensa* (Thunb.) Vahl. or its main compound forsythiaside (10 µg/ml) are able to scavenge superoxide radicals (Qu et al., 2008; Schinella et al., 2002). Another heat clearing herb *Scutellaria baicalensis* Georgi also presents antioxidant properties. Its water extract showed protective effect against the toxic unsaturated aldehyde acrolein induced oxidative stress in human umbilical vein endothelial cells *in vitro* (Zhang et al., 2011). *S. baicalensis* root water extract prepared as a tea is applied as functional food for the prevention of oxidant radical-related diseases (Chan et al., 2010), and its antioxidant properties are mainly linked to the flavone compound baicalein (Wozniak et al., 2014).

Oxidative stress occurs when there is an imbalance between reactive oxygen species (free radicals) production and the antioxidant machinery (Betteridge, 2000). This imbalance is associated with infectious diseases, but also with many undesirable contaminants of foods, including heavy metals (Kaur et al., 2014). Among the group of heavy metals, cadmium is considered to be of high risk to human health, due to its long persistence in the human body and accumulation in the kidneys. Human exposure to cadmium has been associated with nephrotoxic, hepatotoxic and potential carcinogenic effects (Rani et al., 2014).

In the present study, we investigated the potential protective effect of two heat-clearing CHMs against cadmium induced damage in HepG2 cells. As the first line of defense of orally ingested food contaminants, the liver is an important target organ exposed to dietary cadmium (Cichoz-Lach and Michalak, 2014). HepG2 (human hepatocellular carcinoma cells) cells are used commonly as a model for the assessment of liver injury due their high degree of morphological and functional differentiation *in vitro*, which closely resembles that of primary hepatocytes (Mersch-Sundermann et al., 2004). Using HepG2 cells in a standard cytotoxicity assay with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT assay), we investigated the possibility of *F. suspensa* and *S. baicalensis* to modulate cadmium cytotoxicity. This was considered as the first step to assess the potential protective effects of CHMs on chemically induced cell injury.

MATERIAL AND METHODS

Unless mentioned otherwise, all chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) at the highest available purity.

Preparation of *F. suspensa* and *S. baicalensis* water extracts

Two heat-clearing CHMs (*F. suspensa* and *S. baicalensis*) were purchased from CMC Tasly Group BV (Amsterdam, The Netherlands). The water extracts were prepared according Gao (2012) with minor modifications. In brief, CHMs were dried in an oven for 1.5 hour at 60°C before being submitted to extraction. For each dried CHM, 50 grams were weighed and added to 350 ml de-ionized water, followed by one hour incubation at room temperature. After incubation, the CHM was boiled for 25 minutes, after which liquid extract (~ 100 ml) was collected. Then, 150 ml de-ionized water were added to the remaining dregs and boiled for 20 minutes. Thereafter, this two times extractions were merged, filtered and boiled to evaporate until reach a volume of 50 ml. Extracts were freeze-dried to obtain a powder sample, which was dissolved in Dulbecco's Mimimal Essential Medium (GIBCO 31095-029) supplemented with 10% of fetal bovine serum (Gibco, 10106-169), 1% penicillin (100 U/ml)/streptomycin (100 µg/ml) (Biocambrex, DE17-602E), 1% glutamine 2mM (Biocambrex 17-605E), 1% 100mM Sodium pyruvate (Gibco, 11360-039) and 1% Gibco Minimum Essential Medium Non-Essential Amino Acids (REF 11140-035) in a concentration of 20 mg/ml. This solution was stored at -20°C for further experiments.

Cell preparation for culture

The HepG2 (ATCC HB-8065) cells were purchased from American Type Culture Collection (Wesel, Germany) and maintained in complete Dulbecco's Minimal Essential Medium (DMEM) supplemented as described above. Cells were sub-cultured twice a week in 1:2 sub-culture ratios and incubated at a humidified atmosphere containing 5% CO_2 in air at 37°C. For sub-culturing, cells were scraped off from the flask, and seeded at a density of 0.8×10^6 cells/25 ml medium in a 75 cm^2 cell culture flask. Cells with 3 to 20 passages were used. Before the experiments, cells were seeded at a density of 25×10^4 cells/ml in a 96-well sterile flat bottom Costar tissue culture plates (Corning, NY, USA) and kept for 24 hours under 5% CO_2 in air at 37°C before exposure to the test substances.

Cytotoxicity test for CHMs or Cadmium

To evaluate the concentration-dependent cytotoxicity of CHMs or cadmium, HepG2 cells were cultured for 24 hours under 5% CO_2 in air at 37°C in the absence or presence of different concentrations of CHM water extracts (0.01, 0.1, 1, 10, 100, 1000 and 2000 µg/ml), cadmium (cadmium chloride) (0.625, 1.25, 2.5, 5, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 200 µM) (0.02% DMSO). The untreated group (culture medium with 0.02% DMSO) served as control. After culture, cell viability was determined by the standard MTT assay. In brief, 20µl of MTT solution (5 mg/ml) (prepared in PBS) was added to each well, and the cells were incubated for another hour at 37Ð. Thereafter,

the supernatant was discarded and cells were lysed with 200 µl 99.5% DMSO. The absorbance was measured in each well by using a microplate reader (Easy Reader 400 AT, SLT, Salzburg, Austria) at 595 nm. Cell viability was calculated relative to the optical density of MTT formazan formed in untreated cells, which was set to 100%. Nonlinear regression (curve fit), log (agonist) vs response–variable slope (GraphPad Prism 6.04 for Windows) was used to calculate the cadmium cytotoxicity. Each experiment was repeated three times.

HepG2 cells co-exposure to CHMs and cadmium

To determine the cell viability after co-exposure to cadmium and CHMs, cells were cultured for 24 hours under 5% CO_2 in air at 37°C in the absence or presence of different concentrations of CHM water extracts (0.01, 0.1, 1, 10, 100, 1000 and 2000 µg/ml) together with 5, 10 or 30 µM of cadmium (0.02% DMSO), respectively. The untreated group served as control. Thereafter, the same MTT assay was applied according to the protocol described above. Experiment was performed in duplicate.

HepG2 cells co-exposure to CHMs and cadmium after pre-incubation with CHMs

To test the protective effect of CHMs on cell viability, cells were cultured for 24 hours under 5% CO_2 in air at 37°C in the absence or presence of different concentrations CHM water extracts (0.01, 0.1, 1, 10 and 100 µg/ml). Subsequently, the medium was removed and cells were co-exposed to different concentrations CHM water extracts (0, 0.01, 0.1, 1, 10 and 100 µg/ml) together with 5, 10 or 30 µM of cadmium, for another 24 hours. The untreated group (culture medium with 0.02% DMSO) served as control. At the end, the same MTT assay was carried out. Experiment was performed in duplicate.

Statistical analysis

Data was evaluated with one-way analysis of variance (ANOVA) for cell viability assay; Two-way ANOVA for CHM and cadmium co-exposure/CHM pre-exposure test. GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, California, USA).

RESULTS

Cytotoxicity test for CHMs

The results of cell viability after exposure to *F. suspensa* and *S. baicalensis* water extracts are presented in Figure 1. Both CHMs (*F. suspensa* and *S. baicalensis*) decreased HepG2 cell viability significantly at a concentration range of 1000–2000 µg/ml. Hence, the concentration range of 0.001–100 µg/ml for both CHMs was selected for further experiments.

Cytotoxicity test for cadmium

The results of cell viability after exposure to cadmium are depicted in Figure 2. The percentage of cell viability started to decrease after exposure to 5 µM of cadmium and reached 20% after exposure to 40 µM of cadmium. The concentration of cadmium causing a loss of 50% of cell viability (EC50) was determined to be 13 µM. To assess the restoring, improving or protective effect of CHMs on cadmium exposed HepG2 cells, different concentrations of cadmium were selected for further experiments: 5, 10 and 30 µM for low, middle and high levels of cadmium exposure, respectively.

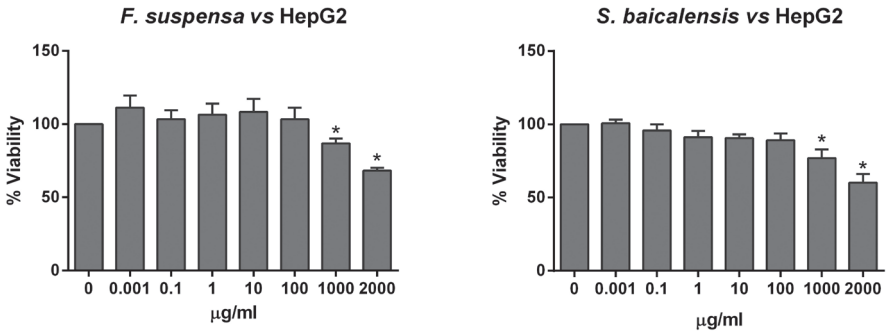


Figure 1. Mean (± SEM) percentage of cell viability after exposure to different concentrations (0.001, 0.1, 1, 10, 100, 1000 and 2000 µg/ml) of *F. suspensa* (panel A) and *S. baicalensis* (panel B). * indicates significant ($P < 0.05$) difference when compared to control (0 µg/ml).

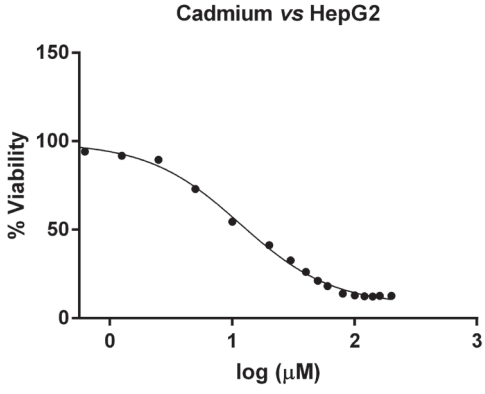


Figure 2. Cadmium cytotoxicity to HepG2 cells: Concentration – response curve established from increasing concentration of $CdCl_2$ (0.625, 1.25, 2.5, 5, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 200 µM) of $CdCl_2$. The X axis represents the log values of the tested cadmium concentrations. Results represent the mean values of 3 different experiments in which each concentration was tested in *duplo*.

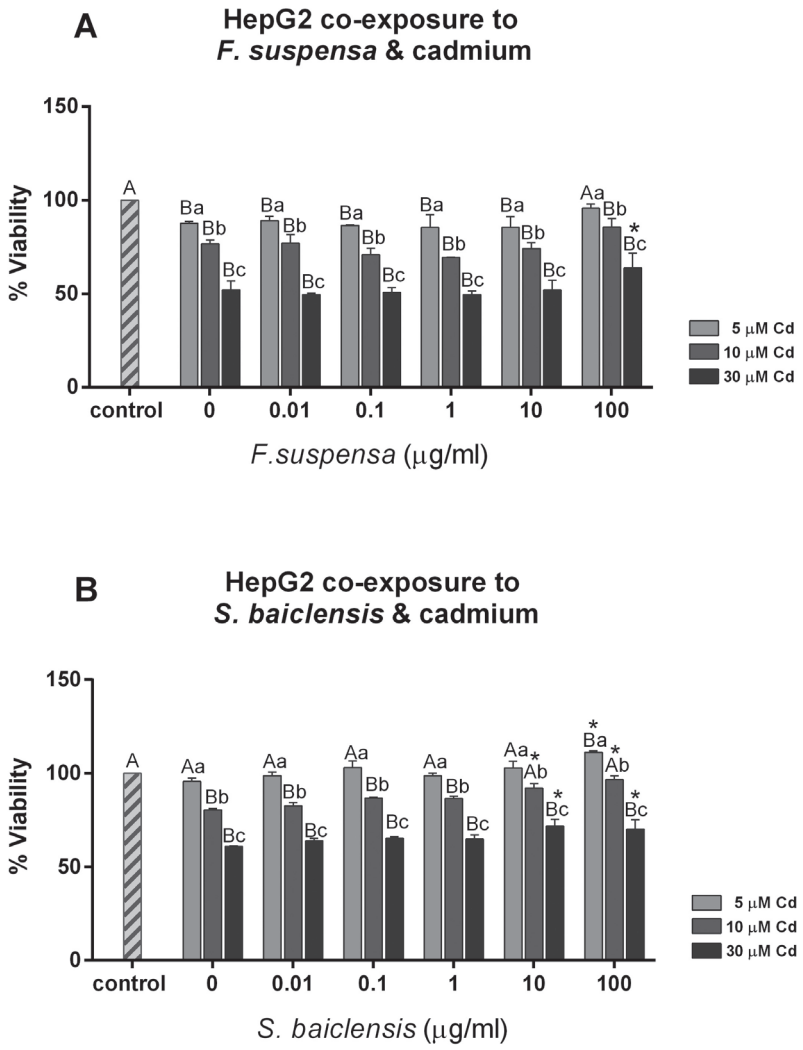


Figure 3. Mean (± SEM) percentage of cell viability after co-exposure to different concentrations (0, 0.01, 0.1, 1, 10 and 100 µg/ml) of *F. suspensa* and cadmium (5, 10 and 30 µM) (Figure A) or S. baicalensis (0, 0.01, 0.1, 1, 10 and 100 µg/ml) and cadmium (5, 10 and 30 µM) (Figure B). Different capital letters (A and B) on each bar indicate significant ($P < 0.05$) differences between all treated groups and the control. Different small letters (a - c) on each bar indicate significant ($P < 0.05$) difference between different cadmium treatment groups within each CHM concentration. * indicates significant ($P < 0.05$) difference when comparing different CHM treatment groups with only cadmium exposed groups (0 µg/ml CHM).

HepG2 cells co-exposure to CHMs and cadmium

F. suspensa (100 µg/ml) restored the HepG2 viability to control levels when these cells were exposed to 5 µM cadmium. When compared to cadmium treated cells, the same concentration of *F. suspensa* increased the cell viability after exposure to 30 µM cadmium (Figure 3, panel A). The decreasing cell viability caused by 10 µM cadmium recovered to the control levels by 10 and 100 µg/ml *S. baicalensis* extract. Compared to the cadmium treated groups, 10 µg/ml *S. baicalensis* also enhanced cell viability in 30 µM cadmium treated cells, and 100 µg/ml *S. baicalensis* was able to increase cell viability at all three concentrations (5, 10 and 30 µM) of cadmium exposed HepG2 cells (Figure 3, panel B).

HepG2 cells co-exposure to CHMs and cadmium after pre-treatment of cells with CHMs

All three tested cadmium concentrations (5, 10 and 30 µM) did affect HepG2 viability after the pre-exposure treatment by *F. suspensa* and *S. baicalensis* (0.01–100 µg/ml) to some extent. (Figure 4; panel A and B). The *F. suspensa* extract (10 and 100 µg/ml), exerted a significant improvement only against the highest cadmium concentration. *S. baicalensis* extract improved at a concentration of 100 µg/ml the cell viability of cadmium-exposed cells significantly, while at lower concentrations (5, 10 and 30 µM) a protective effect was visible only against the highest cadmium concentration.

DISCUSSION

Cell viability using the standardize MTT assay (Fotakis and Timbrell, 2006), which measures the activity of the mitochondrial enzyme succinate-dehydrogenase as a marker, was assessed in HepG2 cells following the exposure to the toxic heavy metal cadmium in the absence and presence of two selected CHMs. For the assay water extracts prepared in the traditional way recommended for heat-clearing CHMs of *F. suspensa* and *S. baicalensis* were used. Both CHM extracts showed a protective effect against cadmium induced loss of cell viability at higher concentrations. As a first step, the cytotoxicity of the CHM extracts was measured, as a concentration-range finding approach. It could be demonstrated that up to a concentration of 100 µg/ml no cytotoxicity could be observed. These findings are different from that previously reported result with S. baicalensis water extract (100 µg/ml) in which a decrease in HepG2 cell viability to less than 20% was observed (Ye et al., 2002). In a comparable experiment with CdCl2 only, a significant loss of cell viability at concentrations exceeding 5 µg was observed, and the EC50 was calculated as 13 µM of cadmium, which is in line with previous findings (Dehn et al., 2004; Fotakis and Timbrell, 2006; Oh and Lim, 2006). Subsequently, the concentrations of 5, 10 and 30 µM of cadmium were selected for the further studies.

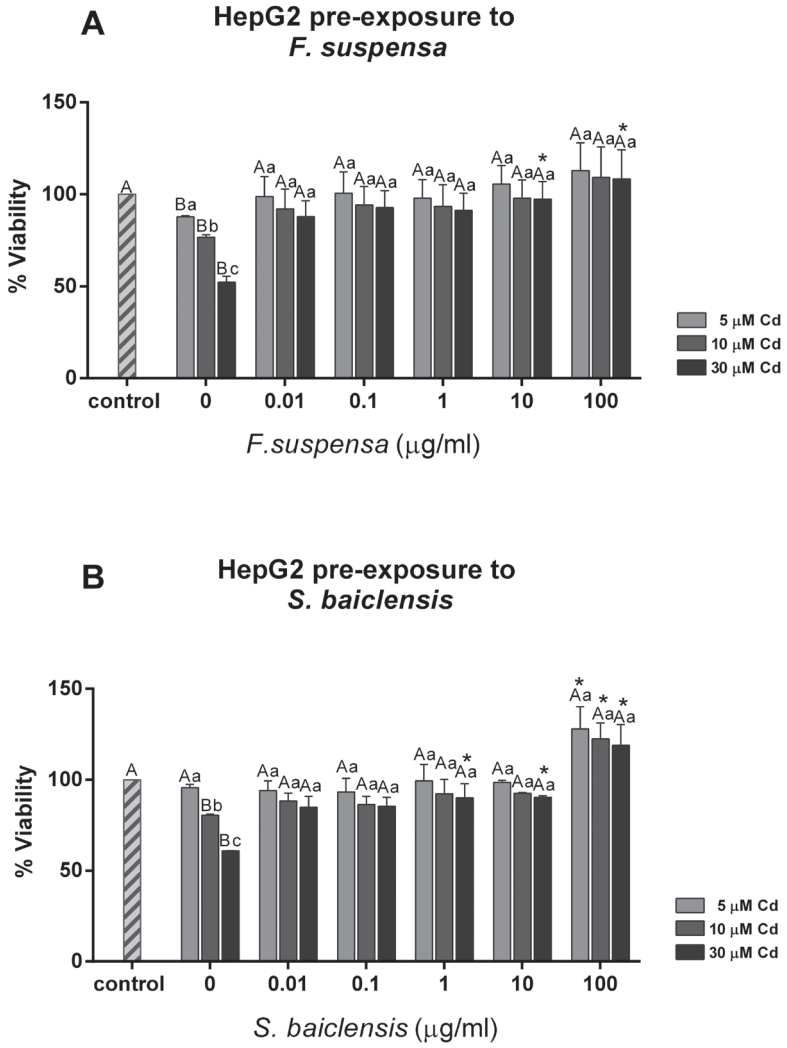


Figure 4. Mean (± SEM) relative cell viability after pre-exposure to different concentrations of CHM (0, 0.01, 0.1, 1, 10 and 100 µg/ml) followed by a co-exposure period of 24 hours to different concentrations of cadmium (5, 10 and 30 µM) (B). Different capital letters (A and B) on each bar indicate significant ($P < 0.05$) difference between all treatment groups and the untreated control. Different small letters (a-c) in each bar indicate significant ($P < 0.05$) difference between different cadmium treatment groups within each CHM concentration. * indicates significant ($P < 0.05$) difference when comparing different CHM treatment groups with only cadmium exposed groups (0 µg/ml CHM).

To investigate the protective effect of CHMs on cadmium stressed HepG2 cells, we performed two sets of experiments: co-exposure HepG2 cells to cadmium with CHM extracts and pre-treatment of cells with CHM extracts followed by exposure to cadmium in the presence of CHMs. *F. suspensa* was able to improve cell viability only at highest tested concentration (100 µg/ml) after exposure to 30 µM cadmium but did not restore it to control levels. *S. baicalensis* presented a better cell viability restoring effect; already at 10 µg/ml it was able to increase cell viability reaching control levels even after exposure to 10 µM cadmium. Even though these effective CHM concentrations (10 or 100 µg/ml) are relatively high when considering clinic applications, they were able to reduce the effects of high cadmium concentrations as well. The direct mechanism of this protective action is unknown, but it may be explained by the antioxidant activity of the tested CHM extracts. The main mechanism of cadmium-induced cytotoxicity on HepG2 cells is indeed oxidative stress via the induction of reactive oxygen species (ROS) (Gebhardt, 2009). Although no data are available showing the direct effect of *F. suspensa* extract, previous data suggest that *F. suspensa* extracts exert an antioxidant activity (Schinella et al., 2002). The main compound of *F. suspensa*, the forsythiaside possess antioxidant activity by scavenging free radicals at a concentration of 10 µg/ml (Qu et al., 2008).

S. baicalensis was investigated more intensively than *F. suspensa* on its antioxidant activities. Three flavonoids baicalein, baicalin and wogonin from *S. baicalensis* were all reported to inhibit oxidation and nitrogen radical fomentation in HepG2 cells at a concentration range between 5–25 µM (~1.35–6.75 µg/ml) (Zhao et al., 2006). A gene regulation study on baicalein-treated HepG2 cells revealed that baicalein (~2.7 µg/ml) activates Nrf2 (nuclear factor-erythroid 2 p45-related factor 2)-mediated activation of ARE (antioxidant response element) signaling pathway, hence providing an effective cellular protection against a variety of toxicants as well as ROS in general (Qin et al., 2012). These reported concentrations were higher than our observations for both F. *suspensa* and *S. baicalensis* water extract protective concentration (0.01 µg/ml) on HepG2 cells, indicating that for the protective use, a lower CHM concentration is efficient enough to increase the anti-oxidant reserve of HepG2 cells.

Additionally, the CHM pre-treatment may also reduce the cadmium uptake by the cells via up-regulation of efflux transporters such as multidrug resistance P-glycoprotein (P-gP, ABCB1). P-gP is a broad-spectrum multidrug efflux pump located in the cell membrane of different cells including intestinal epithelium, kidney proximal tubule cells, liver cells and cancer cells (Gottesman et al., 2002). This transporter is assumed to protect cells by reducing the intracellular cadmium concentration (Lee et al., 2011). Baicalein has the potential to up-regulate P-gp (MDR1) through the direct activation of the corresponding constitutive androstane receptor and pregnane X receptor pathways (Li et al., 2010). This action may reduce the cadmium absorption by HepG2 cells and hence contributed to the protective activity of CHM extracts.

In conclusion, we demonstrate that pre-treatment of *F. suspensa* and *S. baicalensis* water extracts already at a low concentration (0.01 μg/ml) exhibited a protective effect against cadmium cytotoxicity in HepG2 cells. The direct mechanism of such protection is still not clear, but it is suggested that this protective effect is attributed to both the CHM antioxidant activity and the reduction of cadmium uptake via P-gp transporter up-regulation.

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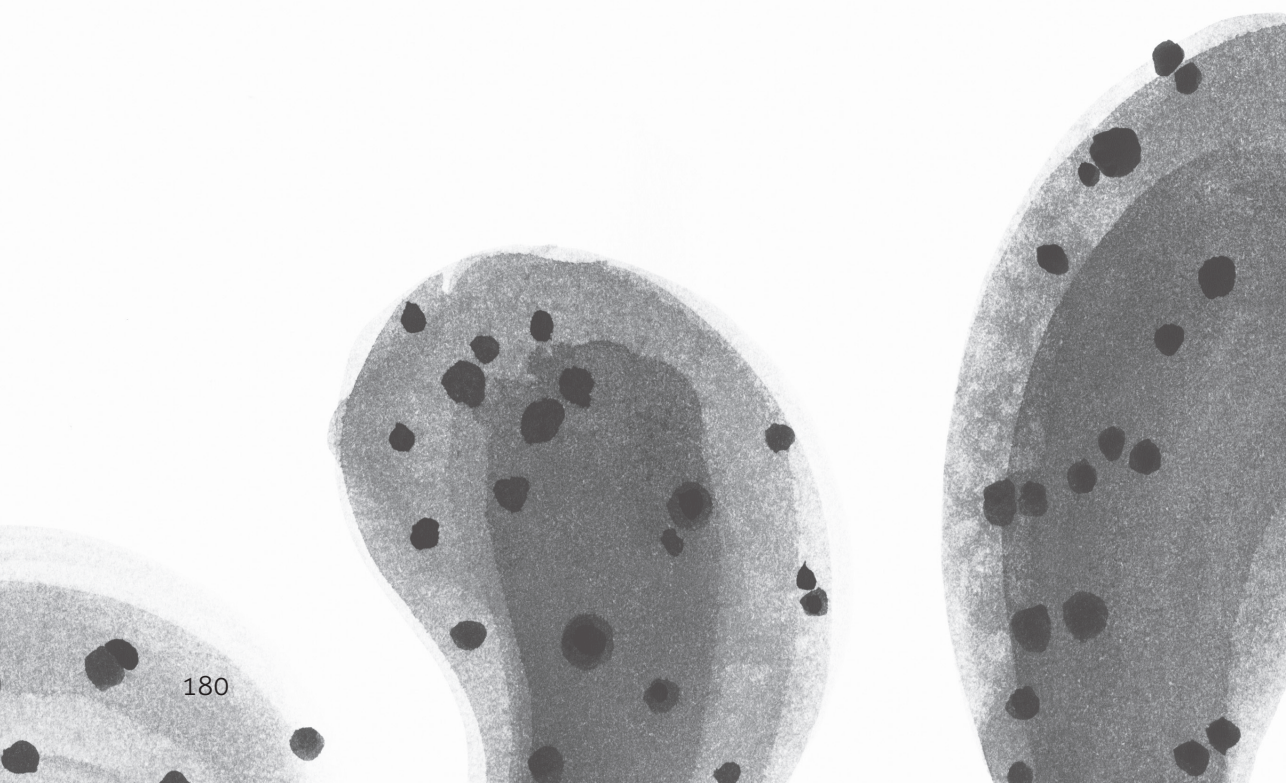
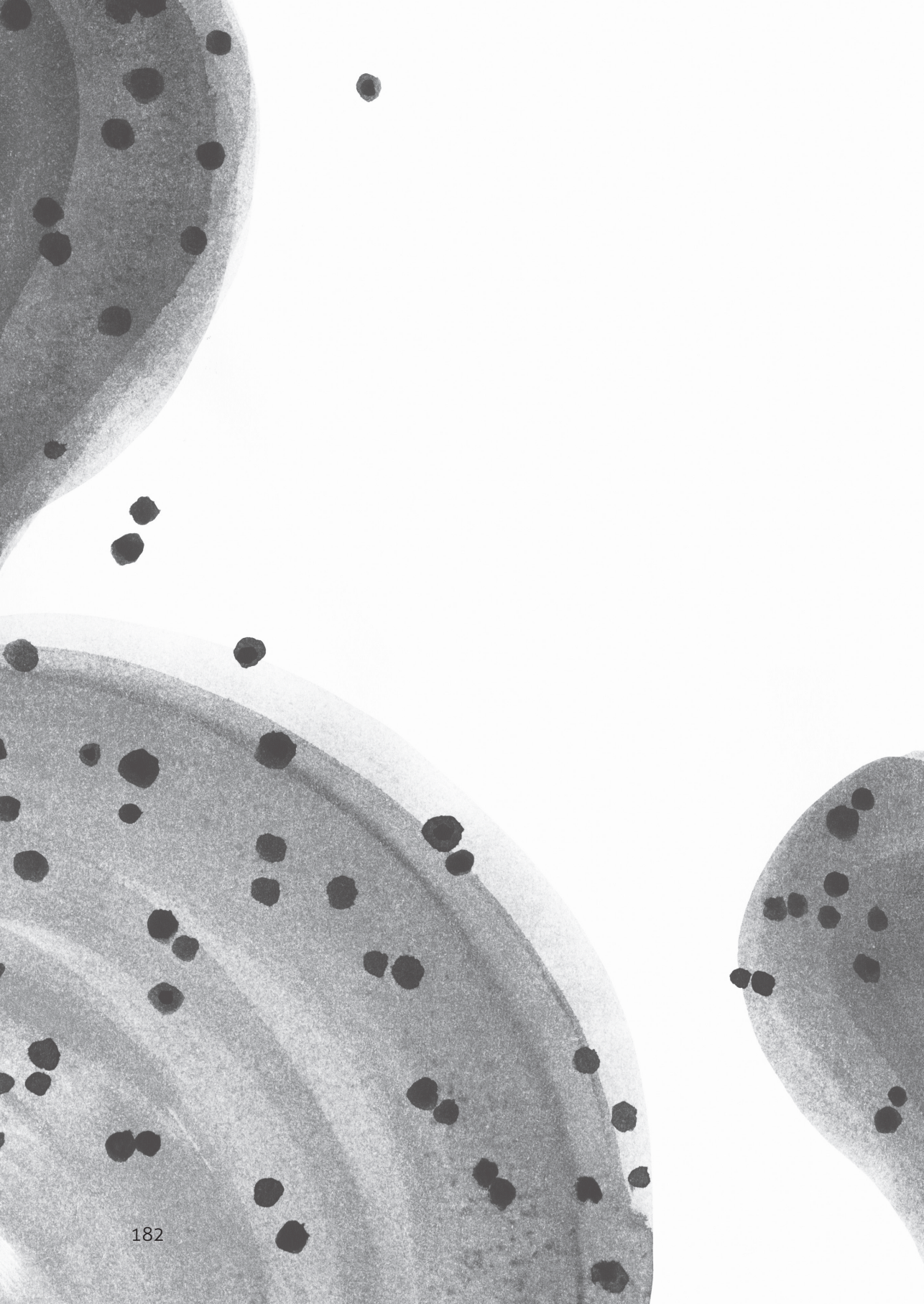
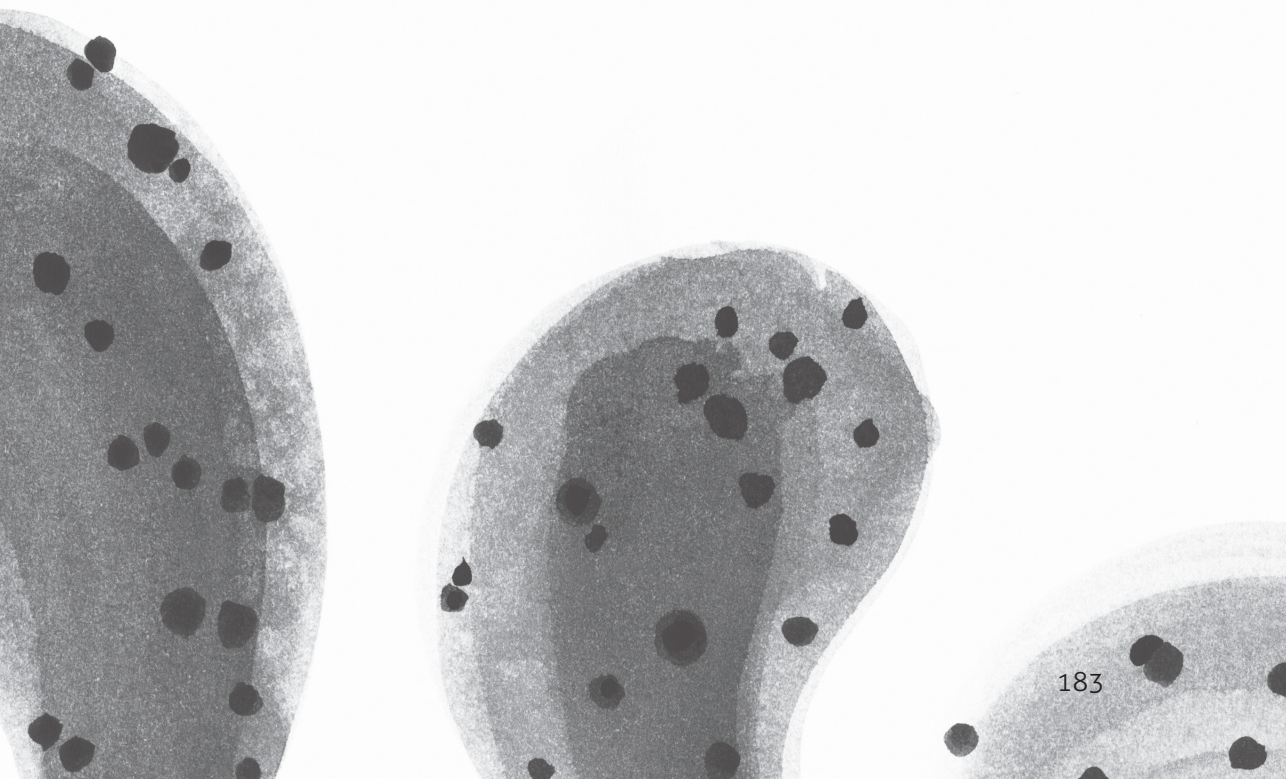


PART V
Summarizing Discussion & Conclusions



11 General Discussion



The use of herbal remedies (Chinese Herbal Medicine, CHM) is a major element in Traditional Chinese Medicine (TCM). The interpretation of clinical symptoms in a patient and the rationale for treatment options are based on the TCM philosophy that differs from Western diagnostic and therapeutic approaches. An example of such differences are bacterial infections, which are treated in modern medicine with selective antibiotics, which successfully destroy pathogens, but rarely support the repair of tissue injury caused by the infection. This monolithic approach has resulted in an increasing emergence of antimicrobial resistance and the need to investigate new concepts. In TCM the heat-*Zheng* closely resembles the diagnosis of bacterial infections and CHMs with anti-infective properties are selected for the treatment. The rationale is that herbal products consisting of multiple compounds, reduce the bacterial load but more importantly are able to restore the homeostasis of all body functions and support tissue repair.

The aim of this study was to investigate antimicrobial properties of selected CHMs that are used in the treatment of the heat-*Zheng*. In particular their anti-biofilm properties were investigated, as bacteria in a biofilm are almost non-responsive to common antibiotics. Considering that CHMs are applied in the form of a traditionally prepared tea, such decoctions were prepared in a standardized way and used as test products in a series of *in vitro* experiments addressing their antimicrobial and antibiofilm activity as well as potential anti-inflammatory and antioxidant effects. Additionally, as many herbs are known for their ability to absorb and accumulate heavy metals, including cadmium, which is a common soil contaminant in China, the effect of metals on biofilm formation was studied as well.

UNDERSTANDING TCM AND ITS POSSIBLE APPLICATION TO PREVENT BACTERIAL BIOFILMS

As introduced in **Chapter 2**, philosophy-based concept seems to replace anatomical and physiological considerations in TCM, but both concepts have considerable overlaps. Different theory systems in TCM are used together to understand the human body and disease conditions and guide CHM medication. The main concepts of TCM encompasses the *Ying-Yang* concept, which corresponds to the co-regulation or organ functions by the sympathic and parasympathic system, the five-elements theory, which shows characteristics of circadian rhythms and seasonal changes described in comparative physiology, *Qi* blood and body fluid theory (body homeostasis), the role of meridians, reflecting many elements from the developmental physiology and *Zheng* (pattern) differentiation, which contains elements of immunomodulation (Song et al., 2013). Among them, meridian tropism and *Zheng* differentiation are the most important principles guiding CHM application. The meridian tropism is the bridge between TCM

theory and clinical medication, once CHM may be of therapeutic use in some diseases (Xu et al., 2012). *Zheng* differentiation, on the other hand, is characterized by the application of different medicines and formulas to treat the same disease; it will depend on the disease stage of development and the individual symptoms (Song et al., 2013). For example, heat-clearing CHMs are often used to treat heat *Zheng*, and different herbs from this herbal group have different meridian tropisms as they are considered to work on the various parts of the human/animal body. This property of CHM appears as an ideal strategy to counteract complex processes related to infections. Their multi-target activity and their origin from plants that essentially need to prevent biofilm formation on their small roots without inhibiting bacterial growth entirely, makes CHM a source for potential bacterial biofilm formation inhibiting substances.

According their different functions, CHMs can be divided into fifteen categories (Liu and Xu, 2002): heat-clearing (*Qingre*), diaphoretics (*Jiebiao*), purgatives (*Xiexia*), digestants (*Xiaodao*), relieve cough and reduce sputum reducing herbs (*Zhike-Huatan*), internal cold-dispelling herbs (*Wenli*), dampness-dissolving agents (*Qushi*), regulating remedies *Qi* (*Liqi*), blood-regulating substances (*Lixue*), astringents (*Shouse*), tonics (*Buxue*), liver-pacifying substances (*Pinggan*), tranquilizers (*Anshen*), insect repellents (*Quchong*) and externally applied agent (*Waiyong*). Heat-clearing CHMs are herbs of great interest due to their antimicrobial activity (Muluye et al., 2014). Furthermore, they are CHMs used in the treatment of heat-*Zheng*, which is related to bacterial infections and inflammatory responses. However, most of the studies involving heat-clearing CHMs focus on single compounds isolated from the herbal extracts and neglect that the complex nature of total extracts might be beneficial in the prevention or treatment of complex biological processes, including inflammation, oxidative stress, and also the formation of antimicrobial biofilms. Biofilm formation is a multi-stage process that cannot be addressed by a single (antibiotic) substance, and hence is considered as a potential target of CHMs. Thus, a screening study of heat-clearing CHMs anti-biofilm activity was suggested.

BACTERIAL BIOFILM IN VETERINARY MEDICINE

For a better understanding of the importance to counteract bacterial biofilm formation, a review is presented in **Chapter 3** summarizing the current studies on bacterial biofilms focusing on pathogens that are of relevance to Veterinary Medicine. Bacterial biofilm formation is a multi-stage process regulated by quorum sensing and characterized by different phases as follows: (i) bacterial attachment, (ii) bacterial accumulation, (iii) biofilm maturation and (iv) biofilm dispersion. During the biofilm formation, the wide differences in gene regulation in individual bacterial species complicates the application of a general strategy to combat its formation (Aiemsaard et al., 2011; Meng et al., 2011;

Nicholson et al., 2013; Prieto et al., 2013; Wu et al., 2013). Also, bacteria embedded in the biofilm exhibit a dormant stage, characterized by minimal synthetic processes. As most antibiotics are effective during bacterial replication, therapy failure is expected and recurrent chronic infections due to biofilm are often observed (Lewis, 2007). Moreover, exposure to different environmental stresses (anaerobic and pH conditions) (Atulya et al., 2014; Li et al., 2014) and antibiotics as clearly demonstrated among others for enrofloxacin, gentamicin, cotrimoxazole and ampicillin (Costa et al., 2012; Silva et al., 2014) induces bacterial biofilms formation.

Both gram-positive (*Staphylococcus* spp. and *Streptococcus* spp.) and gram-negative (*Actinobacillus pleuropneumoniae, Escherichia coli* and *Pseudomonas aeruginosa*) bacterial species may form biofilms and the pioneering studies in bacterial biofilm formation focused on *Staphylococcus* spp. and *P. aeruginosa.* Bacteria forming biofilm are involved in diverse animal diseases, such as mastitis in cattle (Atulya et al., 2014; Melchior et al., 2006a; Melchior et al., 2006b; Melchior et al., 2009), pleuropneumonia in swine (Grasteau et al., 2011), systemic infectious diseases in chicken (Han et al., 2013) and ducks (Tu et al., 2014), otitis in dogs (Silva et al., 2014), enteritis in cats (Ghosh et al., 2013) and wound infections and endometritis in horses (LeBlanc, 2010; Westgate et al., 2011). *S. epidermidis* is known to form biofilms and to cause chronic infections characterized by therapeutic resistance (Fey and Olson, 2010), and was used as a bacterial formation model in the current thesis as suitable type strains are available.

QUANTIFICATION OF BIOFILMS

To develop efficient strategies to inhibit bacterial biofilm, the use of standard and reliable assays able to properly quantify biofilm formation is needed. A common approach for the quantification of a biofilm is staining with safranin or crystal violet followed by a spectrophotometric quantification. As solvents, which are used during bacterial exposure to herbal extracts may interact with these measurements, their concentration dependent effects need to be assessed. Therefore, **Chapter 4** focused on biofilm quantification methods. The most commonly used solvents used to dissolve different substances are dimethyl sulphoxide (DMSO), ethanol and methanol. Therefore, biofilm quantification is performed with different concentrations of these solvents using the safranin or crystal violet dyes. In the presented chapter, it is reported that *S. epidermidis* biofilm formation is stimulated when bacteria are exposed to DMSO (0.0078–1%) and ethanol (0.0625–1%) according safranin staining. Lim et al. (2012) reported as well that 4% DMSO and 2% ethanol are able to stimulate biofilm formation by *E. coli* via its ability to adapt to DMSO and ethanol exposure and subsequently increase the biofilm matrix via amyloid production (Otzen and Nielsen, 2008). Moreover, the staining technique is crucial when quantifying biofilm as it was found that safranin staining is

more sensitive than crystal violet, which can be absorbed by the bacteria leading to over staining. There was no significant change of *S. epidermidis* biofilm formation in any tested concentrations of methanol when compared with control. In conclusion, it is recommended the use of up to 1% methanol as solvent followed by safranin staining for the quantification of biofilm formation by *S. epidermidis* (ATCC 35984).

THE EFFECT OF CHINESE HERBAL MEDICINES ON BACTERIAL BIOFILMS

At **Chapter 5** of this thesis, a template was developed for the investigation of CHMs on *S. epidermidis* biofilms. For this, *Allium sativum* (garlic) extracts (water and ethanol) and its main effect compound allicin were used, as a model substance. Garlic is an acrid-warm herb relieving exterior syndrome in TCM, and its meridian tropisms are the stomach and lung. It is traditionally used to treat gastric dilations (gastrectasia) and for detoxification (Jiangsu New Medical College, 1986). As garlic and garlic-derived substances have been found to be effective against methicillin-resistant Staphylococci (*S. aureus*, MRSA) (Cutler and Wilson, 2004) its antibacterial properties and the anti-biofilm activity of garlic on *S. epidermidis* has gained considerable interest (Nidadavolu et al., 2012). In a series of experiments the effect of garlic extracts and allicin on *S. epidermidis* biofilm formation were compared. These experiments did not only evaluate bacterial viability and biofilm formation, but includes also the analysis of biofilm architecture by 3D analyses with confocal images and the monitoring of genes that regulate biofilm formation. It was found that allicin exerts a bactericidal effect on *S. epidermidis* embedded in biofilms, but allicin, as a single compound, was less potent than the extracts of fresh garlic to combat biofilm formation of *S. epidermidis.*

As mentioned above, to substantiate the effect of individual substances and herbal extracts, different assays can be considered to evaluate both anti-bacterial and anti-biofilm effects. Firstly, the minimal inhibitory concentration (MIC) was determined and combined with differential viability staining followed by confocal microscopy measurement, allowing the quantification of cell death. Subsequently, the standard biofilm formation assay was applied in combination with confocal images at different *z* positions allowing the construction of a 3D image of the biofilm to understand its architecture before and after exposure to the test compounds. Finally, gene regulation was evaluated to understand the mechanisms behind the anti-bacterial and anti-biofilm effect of the tested substances or extracts. For *S. epidermidis* biofilm, several genes are considered as markers of different biofilm formation stages: *atlE* (autolysin E) mediates the initial bacterial attachment (Vandecasteele et al., 2003); *aap* (accumulation-associated protein) mediates the intercellular adhesion between bacteria (Rohde et al., 2005) resulting in bacterial accumulation; *ica* operon is a suitable candidate for checking

the PIA pathway, which is involved in biofilm maturation; *rsbU* positively regulates sigma factor B, which is the general stress-response factor of Gram+ microorganisms and is involved in PIA production as well (Delumeau et al., 2004; Knobloch et al., 2001); Agr (accessory gene regulator) is a marker of quorum sensing involved in biofilm attachment and dispersion (Dai et al., 2012; Vuong et al., 2003). It was demonstrated that allicin has a bactericidal effect on biofilm embedded bacteria and it affects the bacterial accumulation and biofilm maturation of *S. epidermidis* via down-regulation of *icaA* and *aap*. The biofilm architecture was also disturbed by low concentration (0.098 µg/ml) of allicin. Garlic water extract inhibited *S. epidermidis* biofilm formation more effectively, but its mechanism of action remained less clear.

In **Chapter 6**, the optimized biofilm formation assay was used for a comparative investigation of the CHMs regarding their potency to affect bacterial biofilm formation. These herbal products were selected according to the principles of TCM. Heat-clearing CHMs are often used to treat a heat *Zheng* (pattern), which has similar symptoms as bacterial infections, such as redness, tissue edema, heat (including fever) and pain. The astringent CHM *Galla chinensis* was used as a reference for biofilm inhibition since its anti-bacterial and anti-biofilm effects have been reported on *P. aeruginosa, E. coli* and *S. aureus* (Borges et al., 2012; Luis et al., 2014; Xie et al., 2008). Water extracts of *G. chinensis* (0.078 mg/ml) showed anti-bacterial activity against planktonic *S. epidermidis*. This concentration was lower than that previously reported for *G. chinensis* and its main compound gallic acid (0.288 and 2 mg/ml, respectively) for the same bacteria (Li et al., 2007; Moran et al., 2014). More importantly, 0.039 mg/ml *G. chinensis* water extract was effective in inhibiting biofilm formation by *S. epidermidis*. This concentration was again lower than the reported data for the decoction (Xie et al., 2008) and for gallic acid (Borges et al., 2012; Luis et al., 2014) against other bacterial (*S. aureus, P. aeruginosa* and *E. coli*) biofilms. These results indicate that *S. epidermidis* is more sensitive to *G. chinensis* when compared to other bacterial species. This positive finding supported the decision to use *G. chinensis* extract as internal reference. The tested heat-clearing CHMs acted differently on *S. epidermidis* biofilm, leading to inhibition or stimulation of bacterial biofilms. *C. teeta* water extract appeared to be the second most effective (0.313 mg/ml) herb against *S. epidermidis* biofilm formation. Its main compound, berberine, was already reported to inhibit *S. epidermidis* biofilm formation at 0.045 mg/ml (Wang et al., 2009). *F. suspensa, L. japonica* and *S. baicalensis* are the three main herbs of an approved CHM water extract injection named TanReQing (TRQ), which inhibited *S. aureus* biofilm formation at a concentration of 0.129 mg/ml (Wang et al., 2011). In the present study, the individual water extract of *F. suspensa, L. japonica* and *S. baicalensis* inhibited *S. epidermdis* biofilm at higher concentration indicating that together, these herbs become more potent against *S. epidermidis biofilm. T. mongolicum* and *V. yedoensis* water extracts

strongly induced *S. epidermidis* biofilm formation at lower concentrations (1.56–6.25 and 1.56–3.13 mg/ml), while inhibiting biofilms at high concentrations (100–200 and 12.5–100 mg/ml). The effect of *T. mongolicum* and *V. yedoensis* on *S. epidermidis* biofilm formation was not reported before. Similarly to the present observation with *T. mongolicum*, sub-MIC concentrations of *Taraxacum* water extracts stimulated *S. aureus* biofilms (Lau and Plotkin, 2013). Actually, the general inverse pattern of biofilm stimulation at low concentrations and biofilm inhibition at high concentrations was observable in all tested herbal extracts. *Taraxacum* spp. contains 20.67% (w/w) of polysaccharides in its composition (Wang, 2014) and plant polysaccharides can stimulate biofilm formation by serving as a carbon source to produce the extracellular matrix (Beauregard et al., 2013). Therefore, it can be concluded that heat-clearing CHMs will behave differently on bacterial biofilm and their use as supportive therapy must be based on previous knowledge of the herb and not only its TCM group.

THE EFFECT OF HEAVY METALS ON BACTERIAL BIOFILM

Many herbs are known for their ability to take up and accumulate heavy metals, including cadmium. In fact, contamination of herbal products cannot be excluded entirely; hence the effects of different heavy metals on biofilm formation were tested as well. In an initial screening (**Chapter 8**) the effects of different bivalent metals on biofilm formation was investigated. None of the tested metals (lead, nickel, manganese and mercury) stimulated *S. epidermidis* biofilm. While nickel did not affect biofilm at all, lead, manganese and mercury were able to inhibit bacterial biofilms when used at high concentrations, over the limit set up for public health (Jaroslawiecka and Piotrowska-Seget, 2014; Khan et al., 2012). In this first screening, cadmium was identified as inducer of bacterial biofilms and further studies were initiated (**Chapter 9**).

Cadmium is the most toxic heavy metal predominantly due to its long biological half-life and ability to accumulate in the body (Wang and Du, 2013). Plants can take up cadmium from soil (Wang et al., 2014), which increase the risks of contamination of feed and food. In **Chapter 9**, the biofilm-stimulating effect of cadmium is described in more detail. Using the similar investigation method established for the garlic experiment (**Chapter 5**), it was shown that cadmium indeed stimulates *S. epidermidis* biofilm formation at concentrations of 1.56 and 3.13 μM; at higher concentrations (6.25–50 μM) cadmium exerted an anti-biofilm effect. These results were confirmed by CLSM analysis, which indicated the increase in biofilm thickness after exposure to cadmium at concentrations of 1.56 μM (22 μm) and 3.13 μM (23 μm) together with a loss of bacterial viability at the same concentrations. The increase in biofilm formation can be triggered by a reduced bacterial population by agents that regulate quorum sensing (Vuong et al., 2003). Gene expression analysis showed concentration-dependent

changes following cadmium exposure in planktonic persister cells: the stress marker *rsbU* was up-regulated at low concentrations (0.78 and 1.56 μM), and subsequent an up-regulation of *atlE* and *embp* (associated with bacterial attachment and accumulation) was observed. Concomitantly, *icaA, icaB* and *aap* (related to biofilm maturation) were also up-regulated. Interestingly, the gene expression within the biofilm was not affected by cadmium. This phenomenon may be explained by the fact that bacterial cells in a biofilm are in dormant state already. Hu et al. (2011) also showed the similar situation of aap transcription in *S. epidermidis* biofilm. Most changes in bacterial biofilm gene expression were observed within the first 8 hours of culture in *S. epidermidis* biofilm-embedded bacteria. In the current experiments, gene expression was measured only after a 24 hours culture period, i.e. when a biofilm is already mature, and hence this stable gene expression was expected. Taken together, low concentrations of cadmium, which do not affect bacterial viability, act as stressors that stimulate biofilm formation by positively influence bacterial attachment and biofilm maturation.

In the assessment of the effects of heavy metals on biofilm formation, it should be considered that on one hand, this property of *S. epidermidis* biofilm generates concerns about the possible impact of heavy metals on biofilm-associated bacterial resistance to common antibiotics, but that at the other hand, biofilm formation is favorable when bacterial biofilms are intentionally used to bind environmental harmful heavy metals for example in surface water (Seiler and Berendonk, 2012). In the discussion of **Chapter 8** and **Chapter 9** this feature of bacterial (and other microbial) biofilms in eco-systems is included in the discussion.

CHINESE HERBAL MEDICINE ANTI-INFLAMOTORY AND ANTI-OXIDANT ACTIVITIES

In consideration to the inflammatory response observed during bacterial infections, it was aimed to develop also a simple screening model to compare the anti-inflammatory effects of heat-clearing CHMs (**Chapter 7**). To this end, the murine macrophage cell line RAW 264.7, which are known to react to the stimulation with LPS with an increase production of cytokines and an overall inflammatory response, was used. In this assay, non-cytotoxic concentrations of *F. suspensa* and *S. baicalensis* decoctions were tested for their effects on the production and expression of NO and *iNOS* and the inflammatory markers TNFα, IL-1β, IL-6, IL-10, in LPS (1 μg/ml) activated RAW 264.7 cells. Both *F. suspensa* and *S. baicalensis* water extracts inhibited the synthesis (reduction of *iNOS* expression) and the release of NO. The pro-inflammatory cytokines production was not affected by any tested CHM concentrations, but IL-10, an anti-inflammatory cytokine, was significantly induced immediately after exposure to both water extracts at a low concentration (0.1 μg/ml). Subsequently, the ratio of IL-6/IL-10 was also significantly

decreased (from 80 to 20) at all concentrations tested when compared to untreated controls, confirming the anti-inflammatory effect of these CHM water extracts on LPS stimulated macrophages. These findings are in agreement with other studies devoted to the anti-inflammatory properties on these two heat-clearing CHMs (Chen and Zhang, 2014; Choi et al., 2014). However, an *in vivo* study failed to confirm that a *F. suspensa* water fraction has significant anti-inflammatory and pain-releasing effects, while these effects could be clearly measured for the methanol extract of *F. suspensa* in an acute model of inflammation (Ozaki et al., 1997). This points towards limitations in the absorption and distribution of active components in the water extract, but does not exclude local beneficial effects in chronic inflammatory diseases.

In **Chapter 10**, a second *in vitro* model was used to study the effect CHMs on the cellular toxicity of cadmium, which is known to exert cellular oxidative stress on all cell types tested so far. Hence the effect of *F. suspensa* and *S. baiclaensis* on the direct cytotoxicity of cadmium to HepG2 cells is presented, as a first step. Results show that these two CHMs did not restore the cell viability significantly when they were used together with cadmium. However, after a pre-treatment of cells with both CHMs cadmium did not decrease cell viability at any tested concentration, suggesting that these CHM extracts have indeed a protective effect on HepG2 cell exposure to cadmium. The main mechanism of cadmium-induced cytotoxicity on HepG2 cells is the oxidative stress (Gebhardt, 2009). *F. suspensa* ethanol extract and its main compound forsythiaside have been shown to possess antioxidant activity as they prevented lipid peroxidation and act as radical scavengers (Qu et al., 2008; Schinella et al., 2002). Flavonoids from *S. baicalensis* also inhibited the production of reactive oxygen and nitrogen species in HepG2 cells (Zhao et al., 2006). Hence these results seem to indicate that the selected CHM water extracts have also antioxidant activities. Moreover, CHM pre-treatment may also reduce the cadmium uptake by the cells via up-regulation of efflux transporters such as multidrug resistance P-glycoprotein (P-gp, ABCB1), which protects against cell apoptosis caused by cadmium exposure (Lee et al., 2011). It has already been demonstrated that baicalein is able to up-regulate P-gp (Li et al., 2010). It is evident that **Chapter 10** presents only preliminary results, but it confirmed that the chosen approach may allow details studies on the properties of CHMs to reduce cellular oxidative stress.

CONCLUSIONS

In conclusion, to improve the acceptance and explore indications for the use of CHMs, generally applied as decoctions (a special form to prepare water extracts) in health care, a series of model experiments were conducted. As the selection of the applied CHM was based on their traditional use as heat-clearing remedies, which resembles the

treatment of chronic bacterial infections, emphasis was given to the potential effects of these compounds on biofilm formation. As biofilm formation is a multistage process that cannot be combatted with common antibiotics (antibiotics at low concentrations even stimulate biofilm formation acting as danger signals) a multi-component approach as applied with the use of herbal extracts may offer the possibility to prevent biofilm formation. In the different Chapters of this thesis, methodological expects first results with selected CHMs regarding their ability to present bacterial biofilm formation are presented. These findings were completed with studies on the effect of heavy metals on biofilm formation, which is considered to be detrimental in infectious diseases. Finally the first results obtained with the same CHM extracts, tested in two different *in vitro* models to assess anti-inflammatory and anti-oxidant activity, are presented.

RECOMEDATIONS FOR FUTURE RESEARCH

CHM formula research and pharmacological testing

As shown in **Chapter 6**, the individual water extracts of *F. suspensa* and *S. baicalensis* are not as effective as a TRQ injection, which is a mixture of water extracts from *F. suspensa, S, baicalensis* and *L. japonica*. Although the formula (*Pei Wu*) application is a major traditional way of disease treatment in TCM, it is crucial to assess isolated herbs to understand their mode of action and to confirm any synergism. As different herbs have their own meridian tropism, they might work together to behave as a multi-target strategy to adjust body balance. This idea can be applied also in biofilm related research, in consideration of the multiple processes involved in biofilm formation.

At the same time, traditional remedies, and not only the potentially active individual substances, should be evaluated in standardizes models. To this end the first results with two common *in vitro* assays devoted to the assessment of anti-inflammatory and anti-oxidant properties were presented. The preliminary findings show that these assays can be successfully applied to study complex extracts. In further investigations more specific endpoints of an inflammatory response and antioxidant activity should be addressed.

CHM improve bacterial sensitivity to antibiotics

CHMs show antibacterial effects only at high concentrations, which might not be achieved during clinical applications. However, the general properties to prevent biofilm formation seems to be a promising approach to maintain bacterial sensitivity to common antibiotics, by preventing the therapy resistance related to biofilm formation that increases MIC values of bacteria (the increase in MIC can amount to a factor of 1000). Hence, further research should address the interactions between CHMs and antibiotics regarding the bactericidal and anti-biofilm properties of such combinations.

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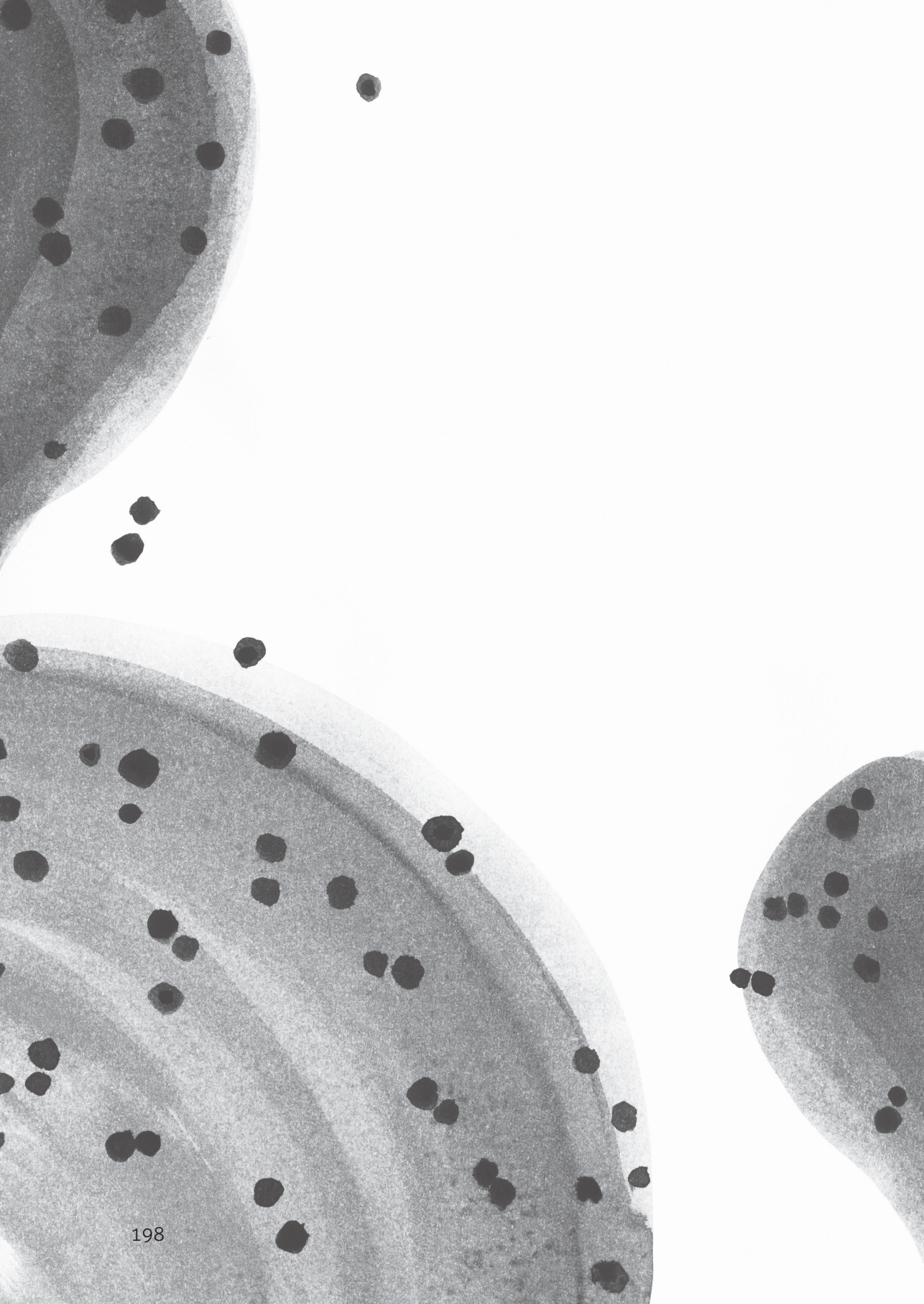
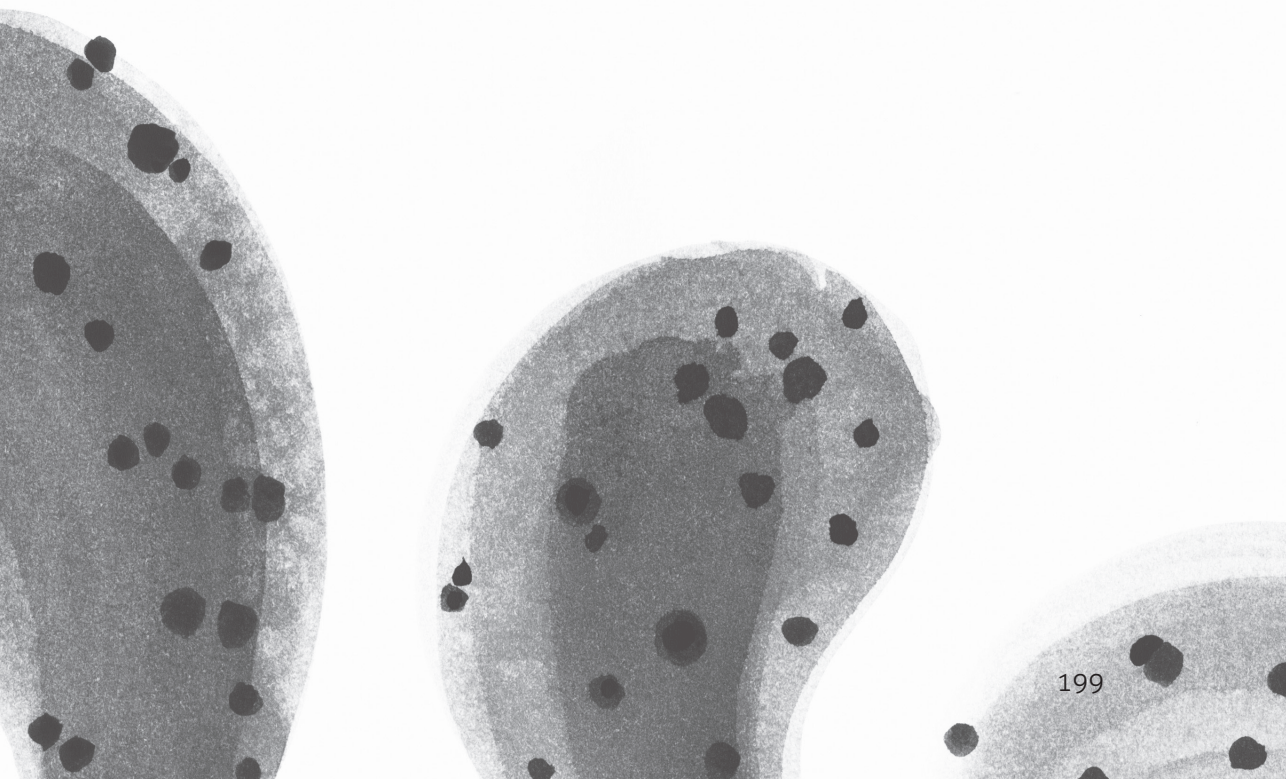


12 Summary



Chinese Herbal Medicines (CHMs) are of significant importance for human health care in China and have a profound cultural and philosophical background described in the Traditional Chinese Medicine (TCM) theories. The main objective of TCM is to maintain or restore the balance of the body as a whole (homeostasis), rather than to treat an individual symptom or functional defect. These different objectives create significant differences in the understanding and interpretation of disease conditions. For example, TCM does not describe bacteria as individual pathogens but treats bacterial infections with herbs, selected for their so-called heat-clearing properties (heat, i.e. elevated temperature is the common symptom of local as well as systemic infections (fever). Heat-clearing properties (the heat-*Zheng*) encompass antimicrobial, anti-inflammatory and tissue repairing effects and herbal extract combining all these properties are selected for the treatment of patients with bacterial infections. This is in contrast to the Western medicine applying single antibiotics as primary remedies to destroy the invading bacteria, but which do generally not contribute to tissue repair.

Recently the use of antibiotics is challenged by the world-wide emergence of antimicrobial resistance, resulting in apparent therapy resistance and an increase in chronic diseases. One of the mechanisms that is involved in therapy resistance, is the formation of biofilms, which can be described as a sessile community of bacteria surrounded by a self-produced extracellular matrix. In the biofilm mode, bacteria are rather insensitive to most antibiotics, as they are not only protected by the polysaccharide extracellular matrix, but also as bacteria achieve a dormant stage within in the biofilm, which makes many antibiotics ineffective as they work predominantly on dividing bacteria. Moreover, biofilms are not detected by the immune cells of the host and this phenomenon also favours the development of chronic infections. Previous investigations had indicated already that various herbal products might have antibacterial as well as anti-biofilm properties by interfering with bacterial signalling (quorum sensing) and/or by suppressing bacterial genes encoding factors that determine biofilm formation and maturation.

Hence it was the aim of the experimental work described in this thesis to identify out of the group of heat-clearing CHMs, with a proven beneficial effect in the management of bacterial infections, those plant extracts that have antibacterial and anti-biofilm properties. For this approach, *Staphylococcus epidermidis* was used as model for Gram-positive bacteria that commonly forms biofilms. In line with the heat-*Zheng* theory, the anti-inflammatory and anti-oxidant properties of CHMs were tested as well. Moreover, to assess the effect of environmental stress factors on biofilm formation, the same bacterial strains of *S. epidermidis* were used to assess the effect of heavy metals, including cadmium, lead, nickel, manganese and mercury on biofilm formation.

MAIN FINDINGS

Methodology: Assessing *Staphylococcus epidermidis* biofilm formation

- The two ATCC type strains (ATCC 3598 and ATCC 12228), representing a biofilm-forming and non-biofilm-forming bacterial strain, were found to be an unique model that can be easily applied in fundamental research to test drugs and herbal products for their ability to interfere with biofilm formation in Gram-positive organisms.
- *S. epidermidis* biofilm quantification was the most accurate, when the safranin staining protocol was applied, allowing a quantification of biofilm formation in a semi- automated 96 well plate format.
- The regularly used solvents DMSO and ethanol can stimulate biofilm formation. Comparative experiments could show, however, that the use of up to 1% methanol as solvent followed by safranin staining protocol allows a reproducible quantitative analysis of *S. epidermidis* (ATCC 35984) biofilms.
- Biofilm maturation and architecture, as well as viability of bacterial cells within the biofilm can be assessed by confocal laser scanning microscopy (CLSM) following a dual staining of bacteria with SYTO® green and propidium iodide.
- Insight into the changes in gene expression profiles during biofilm formation and maturation exerted by herbal extracts (or other agents) can be obtained by qRT-PCR analysis of specific biofilm genes that are well described for *S. epidermidis*.

The effect of Chinese Herbal Medicines on bacterial biofilms

- Testing of Chinese herbal extracts should be in line with their traditional use, which is mainly as decoction (tea). The traditional preparation of these decoctions is well described in the Chinese literature and the obtained water extracts can be easily lyophilized and stored.
- Among the tested CHMs, allicin (the main compound of garlic) was found to exert an unique bactericidal effect on *S. epidermidis* bacteria embedded in biofilms.
- Allicin also affected the bacterial accumulation and biofilm maturation of *S. epidermidis* as demonstrated by gene expression profiles of biofilm bacteria.
- An aqueous garlic extract inhibited *S. epidermidis* biofilm formation even more effectively than allicin, but the composition of this extract is not entirely elucidated yet.
- Galla chinensis water extract was found to effectively suppress *S. epidermidis* biofilm formation already at sub-MIC (minimal inhibitory concentration) levels.
- The selected commonly-used heat-clearing CHMs acted differently on *S. epidermidis* biofilm formation leading either to an inhibition (*Coptis teeta, Forsythia suspensa, Isatis indigotica, Lonicera japonica* and *Scutellaria baicalensis*) or a stimulation (*Taraxacum mongolicum* and *Viola yedoensis*) of biofilm formation.

From these investigations it could be concluded that various CHMs are promising candidate products to suppress biofilm formation of Gram-positive bacteria, while at the same time the variable results indicate the need that all different remedies have to be tested individually.

The effect of heavy metals on bacterial biofilm

- In contrast to lead, nickel, manganese and mercury, cadmium at concentrations between 1.56 and 3.13 µM clearly stimulated *S. epidermidis* biofilm formation, while only at higher concentrations a loss of bacterial viability within the biofilm was observed.

It needs to be concluded from these experiment that cadmium can act as a stressor stimulating biofilm formation. Exposure even to low cadmium concentrations (by inhalation or via food) needs therefore to be considered as undesirable confounding factor in bacterial infections as it stimulates biofilm formation thereby increasing the risk for development of chronic infections.

Chinese Herbal Medicines exert anti-inflammatory and antioxidant activities

Using traditional tea preparations, resulting in a watery extract of the selected CHMs, pilot experiments could demonstrate that:

- Both *Forsythia suspensa* and *Scutellaria baicalensis* exhibit an anti-inflammatory effect by inhibiting the synthesis and release of NO and by inducing the expression of the anti-inflammatory cytokine IL-10.
- Forsythia suspensa and *Scutellaria baicalensis* extracts partly protect HepG2 cell against cadmium toxicity most likely by their antioxidant effects.

These preliminary results indicated that some of the traditionally prepared aqueous extracts of heat-clearing herbs have measurable anti-inflammatory properties.

CONCLUSIONS

The ATCC type strains of *S. epidermidis* are an interesting and easy to standardize model for studies on the effects of new drugs or herbal products on biofilm formation. Combining a colorimetric quantification of the formed biofilms with qRT-PCR analysis and CLS-microscopy, allows an exact analysis of anti-biofilm properties. Using these assays, different CHMs can be rapidly compared. The finding that even within this small group of selected CHMs, both an inhibition and a stimulation of bacterial biofilms was observed, underlines the need to investigate the effects of CHM individually.

Biofilm formation is triggered by various environmental stress factors, and cadmium was identified as one of these triggers. This finding is of clinical relevance as occupational and dietary exposure to cadmium is common and cadmium is accumulating in various tissues.

Further research should focus on the characterization of the identified CHMs with antibacterial and anti-biofilm properties and on the assessment of combinations of these CHMs with common antibiotics used in the treatment of infections with Gram-positive bacteria.

SAMENVATTING IN HET NEDERLANDS

Chinese kruidengeneesmiddelen (Chinese Herbal Medicine, CHM) zijn erg belangrijk in de Chinese humane gezondheidszorg en maken onderdeel uit van de theorieën en filosofische overwegingen die in de traditionele Chinese geneeskunde (TCM) toegepast worden. De belangrijkste doelstelling van TCM is het behouden of herstellen van de totale lichaamsbalans (homeostase), en niet het behandelen van één symptoom of één orgaan-specifieke dysfunctie. Deze verschillende uitgangspunten leiden tot grote verschillen in de evaluatie en interpretatie van ziekteverschijnselen. Zo omschrijft de TCM bijvoorbeeld bacteriën niet als individuele pathogenen, maar worden bacteriële infecties behandeld met kruiden die worden gekozen vanwege hun zogeheten warmte-verwijderende werking (warmte is het gemeenschappelijk symptoom van lokale systemische infecties de vorm van koorts). Warmte-verwijderende eigenschappen (beschreven in de warmte-*Zheng*) omvatten antimicrobiële, anti-inflammatoire en weefselherstellende effecten. Plantenextracten die al deze eigenschappen combineren worden gekozen voor de behandeling van patiënten met bacteriële infecties. Dit in tegenstelling tot de Westerse geneeskunde waarin infecties primair worden behandeld met antibiotica, die de bacterieel pathogene onschadelijk kunnen maken, maar niet of nauwelijks bijdragen aan het herstel van weefselschade.

Het gebruik van antibiotica staat wereldwijd steeds meer onder druk vanwege de toename in antimicrobiële resistentie, hetgeen leidt tot therapieresistentie en een toename van chronische ziekten. Een van de mechanismen die leidt tot therapieresistentie is de vorming van bacteriële biofilms. Biofilms kunnen worden omschreven als een vastzittende groep bacteriën die omgeven zijn door een zelfgeproduceerde extracellulaire matrix. Bacteriën die in een biofilm zitten zijn ongevoelig voor de meeste antibiotica, niet alleen vanwege het beschermde effect van de extracellulaire matrix bestaande uit polysachariden, maar vooral omdat de bacteriën in de biofilm in een 'sluimer toestand' verkeren. Dit zorgt ervoor dat de meeste antibiotica, die werken door remming van de bacteriële celwand- of eiwitsynthese, geen effect hebben. Bovendien worden biofilms niet gedetecteerd door cellen van het immuunsysteem wat bijdraagt aan de ontwikkeling van chronische ziekten. Eerder onderzoek toonde al aan dat verschillende kruidenproducten niet alleen een antimicrobiële werking maar ook anti-biofilm-eigenschappen vertonen doordat ze de communicatie tussen bacteriën (aangeduid als *quorum sensing*) remmen en/of de expressie van genen die de biofilmvorming aansturen onderdrukken.

Het doel van het onderzoek beschreven in dit proefschrift was het identificeren van plantenextracten, behorende tot de groep Chinese kruidengeneesmiddelen met warmte-verwijderende eigenschappen, die een bewezen positief effect hebben op het beheersen van bacteriële infecties, en dus zowel antibacteriële alsmede 'anti-biofilm'-

eigenschappen hebben. Ten behoeve hiervan is gebruik gemaakt van *Staphylococcus epidermidis* als model voor infecties met grampositieve bacteriën die veelal biofilms vormen. In overeenstemming met de warmte-*Zheng* theorie werden de anti-inflammatoire en de anti-oxidatieve eigenschappen van deze Chinese kruidengeneesmiddelen eveneens onderzocht. Daarnaast werden, om het effect van omgevingsstressoren op de vorming van biofilms te testen, dezelfde bacteriestammen van *S. epidermidis* gebruikt voor onderzoek naar de effecten van zware metalen (waaronder cadmium, lood, nikkel, mangaan en kwik) op de biofilmvorming.

BELANGRIJKSTE CONCLUSIES:

Methoden: onderzoek naar *Staphylococcus epidermidis* biofilmvorming

- De twee geselecteerde ATCC stammen (ATCC 3598 en ATCC 12228) zijn representatief voor een biofilm-vormende en een niet-biofilm-vormende stam en bleken een geschikt model te zijn dat eenvoudig kan worden ingezet voor fundamenteel onderzoek naar de invloed van medicijnen en kruidenproducten op de biofilmvorming door grampositieve bacteriën.
- De nauwkeurige kwantificatie van *S. epidermidis* biofilms is mogelijk met behulp van een safranine-kleuring. Zodoende konden kwantitatieve biofilmbepalingen halfautomatisch in 96-wells-platen worden uitgevoerd.
- De regelmatig gebruikte oplosmiddelen, DSMO en ethanol, kunnen biofilmvorming stimuleren. Vergelijkende metingen toonden aan dat het gebruik van tot en met 1% methanol als oplosmiddel, gevolgd door het safranine kleuringsprotocol, een reproduceerbare kwantitatieve analyse van *S. epidermidis* (ATCC 35984) biofilms mogelijk maakt.
- De ontwikkeling en architectuur van een biofilm alsmede de levensvatbaarheid van de bacteriën in de biofilm, kan worden onderzocht door middel van confocale laserscan microscopie (CLSM) na een dubbele kleuring van de bacteriën met SYTO® groen en propidiumjodide.
- Kennis over de veranderingen in de genprofielen gedurende de biofilmvorming en –rijping die door plantenextracten (of andere agentia) te weeg gebracht worden kan worden verkregen door een qRT-PCR analyse van specifieke biofilm genen, die voor *S. epidermidis* reeds beschreven zijn.

De effecten van Chinese kruidengeneesmiddelen op bacteriële biofilms

- Het testen van Chinese kruidengeneesmiddelen zou in overeenstemming moeten zijn met de traditionele bereidingswijzen. In de meeste gevallen worden deze kruiden als thee (decoctie) gebruikt. De traditionele bereiding van deze afkooksels is nauwgezet omschreven in de Chinese literatuur en de verkregen waterige extracten kunnen eenvoudig gevriesdroogd en bewaard worden.
- Een van de geteste Chinese kruidengeneesmiddelen, allicine (het belangrijkste bestanddeel van knoflook), oefent een uniek bacteriedodend effect uit op *S. epidermidis* bacteriën ingebed in een biofilm.
- Allicine beïnvloedt de adhesie van bacteriën en de biofilmrijping van *S. epidermidis* hetgeen bleek uit de analyse van de genexpressieprofielen van biofilm bacteriën.
- Een waterig knoflookextract remt de *S. epidermidis* biofilmvorming zelfs effectiever dan allicine, maar de samenstelling van dit extract is tot nu toe niet geheel opgehelderd.
- Voor water-extracten van *Galla chinensis* kon aangetoond worden dat deze de *S. epidermidis* biofilmvorming reeds remden bij concentraties die duidelijk lager zijn dan MIC-waarden.
- De verschillende veel gebruikte warmte-verwijderende Chinese kruidengenees-middelen beïnvloeden op verschillend wijze de *S. epidermidis* biofilms; een aantal van deze kruiden (*Coptis teeta, Forsythia suspensa, Isatis indigotica, Lonicera japonica* en *Scutellaria baicalensis*) leidt tot remming en een aantal anderen (*Taraxacum mongolicum* en *Viola yedoensis*) leidt tot stimulatie van de biofilmvorming.

Uit de resultanten van dit onderzoek kan worden geconcludeerd, dat verschillende Chinese kruidengeneesmiddelen veelbelovende zijn om ter onderdrukking van een biofilmvorming door grampositieve bacteriën gebruikt te worden. De variatie in de gemeten effecten geeft echter duidelijk aan dat alle traditionele producten individueel onderzocht moeten worden.

Het effect van zware metalen op bacteriële biofilms

- In tegenstelling tot lood, nikkel, mangaan en kwik leiden cadmiumconcentraties van 1,56 tot 3,13 µM duidelijk tot een inductie van de biofilmvorming door *S. epidermidis*; pas bij hogere concentraties is er sprake van een vermindering van de levensvatbaarheid van bacteriën in de biofilm.

Uit deze experimenten kan worden geconcludeerd dat cadmium als een stimulator voor de biofilmvorming gezien moet worden. Dit betekent dat een blootstelling zelfs aan lage cadmiumconcentraties (door inademing of via het voedsel) gezien moet worden als ongewenste risicofactor tijdens bacteriële infecties, omdat het de biofilmvorming stimuleert en hierdoor een bijdrage levert aan het ontstaan van chronische infecties.

Anti-inflammatoire en anti-oxidatieve werking van Chinese kruidengeneesmiddelen

Door gebruik te maken van traditionele theebereidingsmethoden, leidend tot waterige extracten van de geselecteerde Chinese kruidengeneesmiddelen, kon worden aangetoond dat:

- Zowel *Forsythia suspensa* en *Scutellaria baicalensis* een anti-inflammatoire werking hebben door remming van de vorming en het vrijkomen van NO en de inductie van de anti-inflammatoire cytokine IL-10.
- *Forsythia suspensa* en *Scutellaria baicalensis* extracten beschermen HepG2 cellen gedeeltelijk tegen cadmiumtoxiciteit, waarschijnlijk door hun anti-oxidatieve werking.

Deze eerste resultaten tonen aan dat traditioneel bereide waterige extracten van bepaalde warmte-verwijderende kruiden een anti-inflammatoir effect lijken te vertonen.

CONCLUSIES

De ATCC bacteriestammen van *S. epidermidis* zijn een interessant en eenvoudig te standaardiseren model voor onderzoek naar de effecten van nieuwe medicijnen of kruidenproducten op de bacteriële biofilmvorming. Een combinatie van de colorimetrische kwantificatie van de gevormde biofilms, qRT-PCR analyses van biofilmgenen en CLS-microscopie maakt een precieze analyse van de effecten van medicijnen of kruidenproducten op de bacteriële biofilmvorming mogelijk. Door gebruik te maken van deze onderzoeksmethoden kunnen verschillende Chinese kruidengeneesmiddelen betreffende hun 'anti-biofilm' eigenschappen vergeleken worden. Het feit dat in dit klein aantal onderzochte traditionele Chinese medicijnen zowel remmers als stimulatoren van bacteriële biofilmvorming werd gevonden benadrukt de noodzaak van onderzoek naar de individuele eigenschappen van Chinese kruidengeneesmiddelen.

Biofilmvorming wordt beïnvloed door verschillende omgevingsfactoren en cadmium is een van deze factoren. Dit is van klinisch belang omdat zowel beroepsmatige blootstelling als blootstelling via voeding veelvuldig voorkomen en cadmium in verschillende weefsels wordt opgeslagen.

Nader onderzoek zou moeten worden gedaan naar de samenstelling van de onderzochte Chinese kruidengeneesmiddelen met antibacteriële en 'anti-biofilm' eigenschappen, en naar de effecten van combinaties van deze Chinese kruidengeneesmiddelen met antibiotica die veelvuldig ter behandeling van infecties met grampositieve bacteriën gebruikt worden.

简介

中草药在中国人的健康保健中有着极其重要的作用，其倚赖的是中医深厚的文化和哲学背景。中医治疗疾病的主要原则是将机体作为一个整体来调节其内外各部间的平衡，而不只专注于疾病发展过程中出现病变的某一器官。在此原则下，中医对疾病的理解便与西医有着极大的差异。例如，在中医中并没有将细菌作为病原体的描述，但用相关中药却可治疗细菌感染，比如清热解毒类中药。清热药在中医中主要治疗对象是中医中所述热症，比如感染局部的温度或体温升高便是热症的其中一项判断标准。清热药同时具有抗菌，消炎和组织修复功效。因此，这类中草药的提取物常被用作治疗细菌感染的良药，类似应用却与西医讲究精确的治疗措施大有不同，如西医常应用单个抗生素对特定细菌入侵引起的感染作为补救措施，在普遍情况下并不具有组织修复功能。

全球性的细菌耐药性问题对抗生素的使用提出了严峻的挑战，并造成明显的治疗困难以及使临床感染发展成慢性疾病的几率大大增加，而细菌生物膜的形成毫无疑问地在其原因中占有一席之地。细菌生物膜是黏附于生物或人工材料表面的由细菌自产的细胞外基质包围的细菌群落。在生物膜模式中，细菌对抗生素表现不敏感状态，不仅因为它们有多糖胞外基质的保护，更重要的是它们长期处于一个休眠状态，这使得许多抗生素与细胞壁生物合成或细菌蛋白质合成无效，从而造成抗生素治疗失败。另外，生物膜不被机体免疫细胞识别的现象也有利于相关细菌慢性感染的发展。此前已有研究表明许多中草药可作为群体感应抑制剂和编码生物膜成熟以及结构基因的抑制剂来拮抗生物膜的形成。

因此，我们在该论文中所述研究的目的之一便是鉴定对抗细菌及细菌生物膜感染有效的清热中草药。为此，作为细菌生物膜形成代表的革兰氏阳性菌，表皮葡萄球菌，被用作研究模型。同时，针对清热药主要治疗热症的中医理论，我们同时对所选药物进行了抗炎和抗氧化功效的试验研究。此外，为了评估环境压力因素对生物膜形成的影响，同菌株表皮葡萄球菌也被用于测试重金属，包括镉，铅，镍，锰，汞对生物膜形成作用。

主要研究结果：

表皮葡萄球菌生物膜的形成评估

- 生物膜形成典型菌株 （ATCC 35984）和非生物膜形成菌株（ATCC 12228）被作为测试药物对细菌生物膜形成作用的一对独特的模型，该模型可以便利地应用到对革兰氏阳性菌细菌生物膜基础研究中。
- 在对表皮葡萄球菌生物膜定量试验中，番红染色-96孔板半自动试验被证明是可得出相对最精确试验结果的方案。
- 在植物提取物相关试验中常用溶剂二甲基亚砜和乙醇能刺激生物膜的形成。但是，试验证明采用1％的甲醇作为溶剂，并以番红作为染色剂的方案可用作表皮葡萄球菌（ATCC 35984）生物膜定量试验的可重复性优选试验方案。

- 生物膜成熟和构造以及生物膜内的细菌细胞的存活率可以通过对生物膜进行SYTO®绿和碘化丙啶双染色后共聚焦激光扫描显微镜进行评估。
- 中药提取物或其他药物细菌生物膜的形成和成熟基因表达的影响可通过对表皮葡萄球菌生物膜形成过程中特定的已被较好研究的基因的定量RT-PCR分析来获得。

中草药对细菌生物膜的作用研究

- 对中草药的研究应该于中医传统用药方法一致，即为煎煮法。中药的传统煎煮法（汤剂论）在中文文献中有详细记载。煎煮后所获得的水提取物也可以很容易地冻干保存。
- 在所有被检测的中草药中，大蒜素（大蒜的主要化合物）表现对表皮葡萄球菌生物膜内细菌的杀菌作用。
- 生物膜细菌基因表达检测表明大蒜素对细菌生物膜的形成（细菌聚集阶段）和成熟有干预作用。
- 大蒜水提物表现比大蒜素更有效地抑制表皮葡萄球菌生物膜形成，但本水提物的组成成分有待进一步鉴定。
- 亚抑菌浓度（MIC）的五倍子水提取物可有效抑制表皮葡萄球菌生物膜的形成。
- 本研究所测试清热中草药中对表皮葡萄球菌生物膜的影响表现不同，黄连，连翘，板蓝根和黄芩表现为生物膜抑制作用， 蒲公茵和紫花地丁则表现生物膜形成刺激作用。

根据以上研究结果，可得出结论，清热中草药是有效抑制革兰氏阳性菌表皮葡萄球菌生物膜形成的候选药物，同时这些中草药对生物膜的不同作用也表明其作用原理还需要进一步研究。

重金属对细菌生物膜的作用研究

- 与铅，镍，锰和汞不同，1.56 和3.13 μM镉对表皮葡萄球菌生物膜形成具有明显的刺激作用，而在较高浓度下通过对生物摸内细菌产生杀灭作用。

试验结果表明镉可作为对表皮葡萄球菌形成压力的环境因素而刺激其生长。人体可通过呼吸或食物而暴露于低浓度的镉，并由于细菌生物膜的形成而增加感染发展为慢性感染的风险。

中草药抗炎和抗氧化活性研究

通过对中药传统煎煮所得中草药水提物的试验研究， 结果表明：

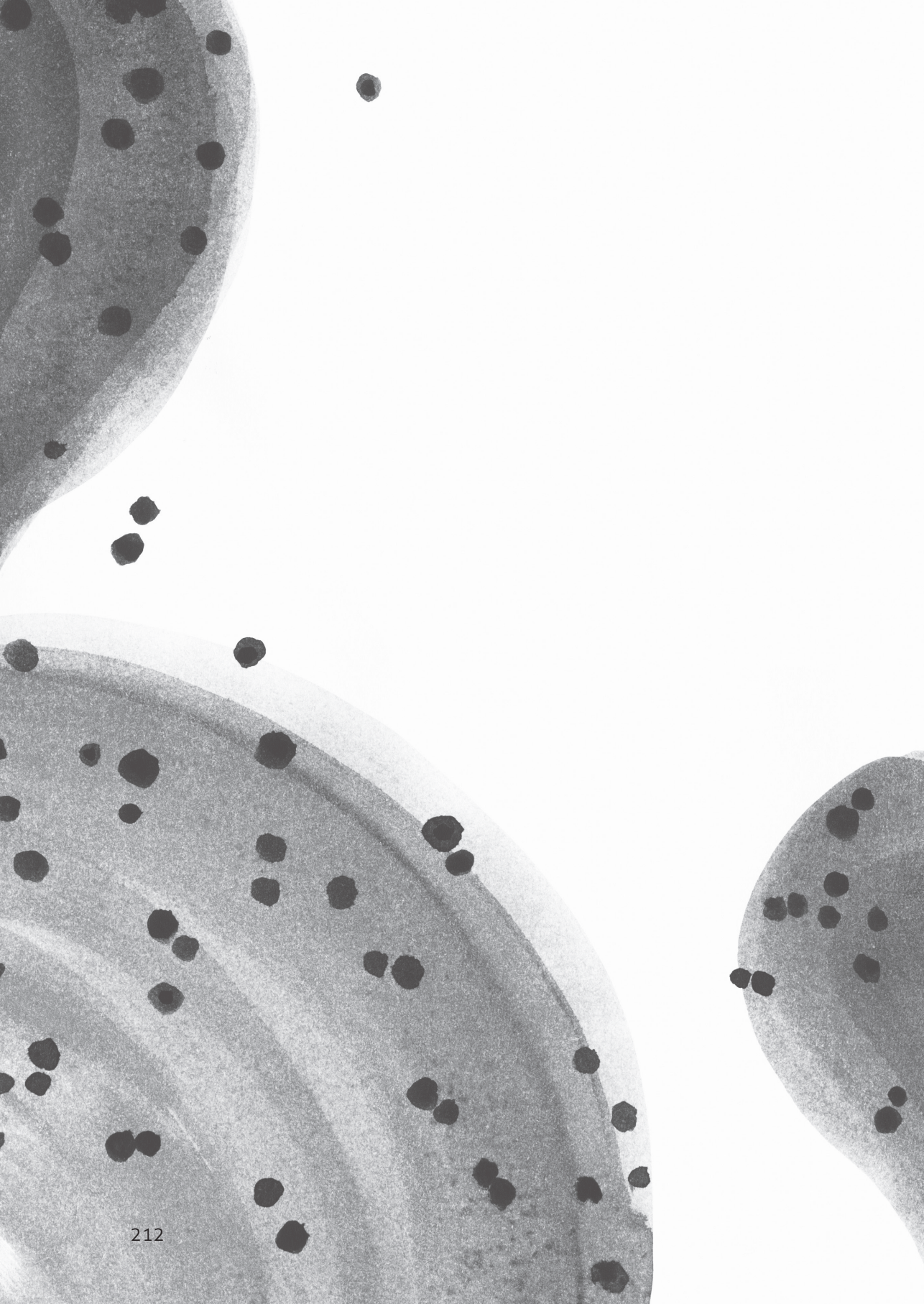
- 连翘和黄芩水提物可通过抑制NO的合成和释放以及诱导抗炎细胞因子IL-10的表达而对所测细胞模型表现消炎作用。
- 连翘和黄芩提取部对镉毒性的抑制很可能是由它们对HepG2细胞的抗氧化作用而起效的。

这些初步结果表明，传统制备的清热药水提物具有有抗炎抗氧化的特性。

总结：

表皮葡萄球菌，ATCC型菌株，对新药或中草药研究来说是一个且易于标准化的细菌生物膜模型。在试验方法上，生物膜定量显色试验结合RT-PCR和共聚焦扫描显微镜的应用对药物作用于细菌生物膜的结果提供一个较准确全面的判断。通过应用以上试验方法，不同中草药对细菌生膜的作用特性得以展示。试验结果表明，尽管同样是清热药，却表现出对细菌生物膜不同作用（抑制或刺激），因此需要对所研究药物进行进一步深入研究。

细菌生物膜的形成与多种环境应激因素相关，重金属镉被鉴定为其中之一。鉴于镉是摄入食物以及特殊职业中的常见污染物，这一发现对临床治疗相关疾病有重要意义。未来研究重点将是对具有抗菌和抗生物膜的中草药性能的进一步评估以及在对抗革兰氏阳性菌生物摸形成研究中将中药与抗生素结合应用。



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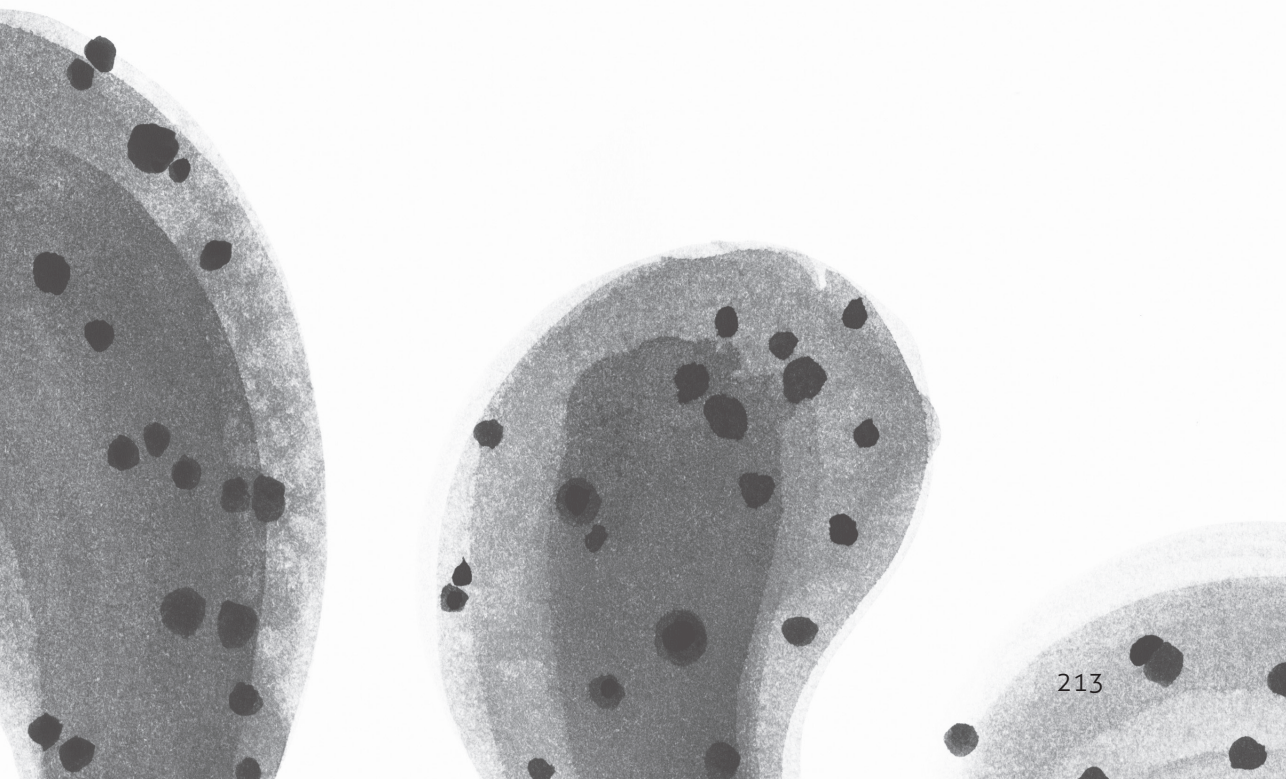

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"A true friend is one soul in two bodies"

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"No matter what you decide to do, we all support you!" – Yaning An

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亲爱的爸爸妈妈，我爱你们！　用我的全心全意爱你们！在这里，我想要首先表达对你们深深的歉意：　对不起，我不是一个好女儿可以常伴你们左右；对不起，我有时候还是太脆弱，学不会在你们面前掩饰自己的情绪而让你们担心。但是，有你们的支持和理解我得以顺利地在荷兰完成了我的博士学业，　这不仅仅是我一个人的成就，这也是你们的成就！妈妈，你很久以前说过：“雪晴，　你要接受你自己，相信你自己。生命可以有很多种不同的形式，你终会找到只属你的那一种！”谢谢你妈妈！我想我已经开始了这趟寻找的旅程！

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Xueqing Wu
25-2-2015

CURRICULUM VITAE

Xueqing Wu was born in Kangding, Sichuan, P. R. China on February 9, 1982. In 2000 she graduated from secondary school at Yaan High School and started a one year pre-college study in Sichuan Agriculture University. Thereafter she continued with a bachelor of animal biotechnology in Veterinary Medicine Faculty of Sichuan Agriculture University. In 2005, she started her one year internship at the Companion Animal Hospital of Sichuan Agriculture University by performing a clinic survey on canine distemper, as well as working as a clinician. At the same year she graduated in Veterinary Medicine. In 2006 she started her master study at the Pharmacology and Toxicology Department of Veterinary Medicine Faculty of Sichuan Agriculture University. The main research line during her master was the development of a Traditional Chinese Medicine injection (YAJIANGXIN) against *Riemerella anatipestifer* infections on ducklings, focusing on the pharmacodynamics of such injection. In 2011 she had the opportunity to undertake a Ph.D research program at the Veterinary Pharmacology, Pharmacotherapy and Toxicology department in the Institute for Risk Assessment Sciences of Faculty of Veterinary Medicine of Utrecht University. Under the supervision of Prof. Dr. J. Fink-Gremmels and Dr. R. R. Santos she investigated the effects of Chinese Herbal Medicines on *Staphylococcus epidermidis* biofilm formation. The work and the results from this research are presented in this thesis.

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