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Assessment of sustainable antimicrobial methods with regard to their ability to reduce airborne and surface bacteria in the food supply chain

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Meinen Eltern

What we know
is a drop,
what we don't know
is an ocean

Sir Isaac Newton
(Physicist, mathematician, astronomer, natural philosopher,
alchemist and theologian 1643 – 1727)

Abstract

Assessment of sustainable antimicrobial methods with regard to their ability to reduce airborne and surface bacteria in the food supply chain

The objective of this thesis was the assessment of antimicrobial methods with regard to their ability to reduce airborne and surface bacteria during processing and storage of perishable food. In particular, possible influencing factors on the rate of antimicrobial activity were investigated, such as temperature, food residuals and microflora. Based on the results, an evaluation scheme was developed for the assessment of antimicrobial techniques with regard to the prevailing requirement profiles for the respectively application area.

The study focuses on the application of materials containing silver for reduction of surface bacteria and on the use of ionization for the reduction of surface and airborne bacteria.

For the determination of the influence of selected factors on the antimicrobial activity of these both techniques (materials containing silver and ionization) the standard test procedure JIS Z 2801 (2000) was used in a modified form. By comparing colony forming units under the influence of the antimicrobial technique with the colony forming units at reference conditions, the rate of antimicrobial activity was determined.

It became apparent, that both of the techniques examined were generally able to reduce surface and airborne bacteria. However, the rate of reduction was strongly dependent on the influencing factors of time, temperature, and the sensitivity of test organisms used as well as on the effect of possible food residuals on the surface. Thus, the application of the described techniques in contact with perishable food is only beneficial, if the available microbiological, material and environmental factors fit to the activity profile of the antimicrobial technique. Based on the results, an evaluation scheme was developed for the testing of antimicrobial methods as to their applicability for use in the food chain. The scheme gives priority to the integration of the influencing factors - material, microbiology and environment.

Kurzbeschreibung

Bewertung dauerhaft antimikrobieller Verfahren im Hinblick auf deren Fähigkeit, Luft- und Oberflächenkeimgehalte im Bereich leicht verderblicher Lebensmittel zu reduzieren

Ziel der vorliegenden Arbeit war es, antimikrobiell wirkende Methoden im Hinblick auf deren Eignung zur Reduzierung des Luft- und Oberflächenkeimgehaltes während der Verarbeitung und Lagerung von leichtverderblichen Lebensmitteln zu bewerten. Dabei galt es, insbesondere mögliche Einflussfaktoren auf die antimikrobielle Wirksamkeit, wie Temperatur, Lebensmittelrückstände und Keimflora, zu erforschen. Basierend auf den Ergebnissen wurde ein Prüfschema entwickelt für die Bewertung dieser Technologien in Hinblick auf das jeweilige Einsatzgebiet und den damit verbundenen, spezifischen Anforderungsprofilen.

Im Fokus der Betrachtung lag die Anwendung von antimikrobiell wirkenden Silberadditiven in Werkstoffen zur Reduzierung von Oberflächenkeimgehalten sowie die Anwendung von Ionisatoren zur Reduktion von Oberflächen- und Luftkeimgehalten.

Zur Ermittlung des Einflusses ausgewählter Faktoren auf die antimikrobielle Aktivität von Werkstoffen und der Ionisation wurde das Standardprüfverfahren JIS 2801 (2000) entsprechend modifiziert. Über den Vergleich des Keimgehaltes unter Einfluss der zu prüfenden Methode und des Keimgehaltes unter Referenzbedingungen wurde die Höhe der antimikrobiellen Aktivität bestimmt.

Es konnte gezeigt werden, dass die analysierten, antimikrobiellen Verfahren grundsätzlich in der Lage waren, den Oberflächen- und Luftkeimgehalt zu reduzieren. Jedoch hing die Aktivität signifikant von den Faktoren Zeit, Temperatur, Sensitivität des verwendeten Testorganismus sowie möglichen Lebensmittelrückständen auf den Oberflächen ab. Der Einsatz der vorgestellten Methoden in Kontakt mit kühlpflichtigen Lebensmitteln ist somit nur vorteilhaft, wenn die mikrobiologischen, materialtechnischen und Umweltbedingungen während der Anwendung dem Aktivitätsprofil der antimikrobiellen Methode entsprechen. Basierend auf den Ergebnissen wurde ein Prüfschema zur Bewertung der Anwendbarkeit von antimikrobiellen Methoden im Lebensmittelbereich entwickelt. Das Schema stellt die Integration der relevanten Einflussfaktoren Material, Mikrobiologie und Umwelt entsprechend des Anwendungsprofils der antimikrobiellen Methode in den Vordergrund.

CONTENTS

CHAPTER 1

Introduction

1.1 DESCRIPTION OF HYGIENE WEAK POINTS IN THE FOOD PROCESSING CHAIN

The quality, safety and shelf life of perishable food is mainly influenced by environmental conditions during production, processing, transport and storage in private households. Next to temperature, as the most important influencing factor, the hygienic conditions in the food chain are of high relevance. Cross-contamination via surface or air is a key aspect for bacterial spread. Planktonic cells in food residuals on surfaces, like cutting tables, conveyer belts, tube systems and refrigerators or in atmospheric aerosols can lead to contamination of fresh food. Furthermore, sessile bacterial cells in biofilms have a high potential for cross-contamination as parts of the biofilm, combined with extracellular polymer substances (EPS) and organic substrates, are continuously released leading to bacterial spread (Joseph et al. 2001; Jessen and Lammert 2003; Thomas et al. 2009).

Cross-contamination of food by microaerophilic and psychotrophic pathogens via surfaces or the air increases the risk of the propagation of infectious diseases (Zottola and Sasahara 1994; Quintavalla and Vicini 2002; Skandamis and Nychas 2002; Chaititemwong et al. 2008). In addition to this, cross-contamination of spoilage organisms on food products increases the bacterial starting concentration thus the spoilage process of a product is enhanced. Figure 1.1 shows the influence of the starting concentration on the spoilage process respectively the shelf life of pork, exemplified by the growth of *Pseudomonas* spp. as the main spoilage organism in pork. It becomes evident, that the starting concentration of *Ps.* spp. correlates with the shelf life: If the starting concentration is $\log_{10}$ 1.0 CFU g^{-1}, the end of shelf life is reached after 170 hours of storage, whereas if the starting concentration is $\log_{10}$ 2.0 respectively $\log_{10}$ 3.6 CFU g^{-1}, end of shelf life is reached nearly one respectively two days earlier.

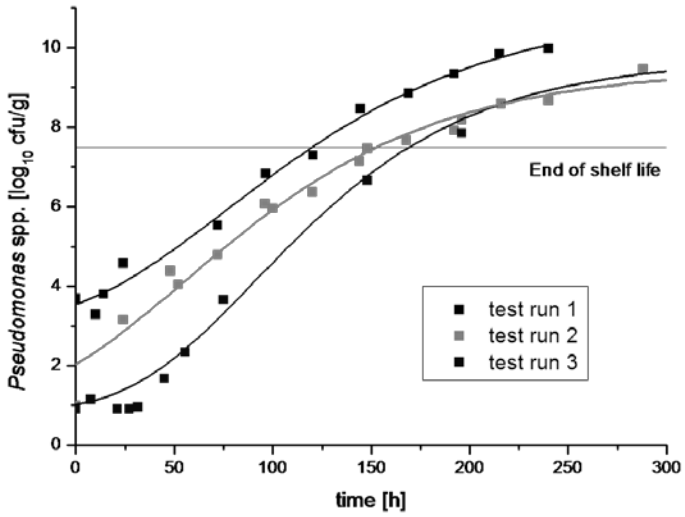

Figure 1.1 Growth of *Pseudomonas* spp. in pork when stored at 5°C, as correlated with initial bacterial count (Bruckner, not published)

Classical cleaning and disinfection methods in the food industry and in private households are often not sufficient to remove all bacteria from surfaces. In particular, biofilms are hard to remove, because bacteria in biofilms have a strong connection to surfaces (Joseph et al. 2001; Jessen and Lammert 2003; Thomas et al. 2009). Moreover, cleaning and disinfection does not proceed continuously.

For the prolonged reduction of bacterial counts on surfaces and in the air within food processing companies and in private households, long term antimicrobial agents can provide an additional benefit (Leung et al. 2009). Long term antimicrobial agents can reduce microorganism counts on surfaces and in the air or suppress their growth even during the times between cleaning operations (Sandmeier and Kensbock 2004). Not only by the improvement of hygienic conditions while processing is underway, but also while food is in storage, antimicrobial agents can reduce the growth of microorganisms, thus prolonging shelf life as well as improving food safety. Lee et al. (2004) reported a significant decrease in bacterial growth in milk by adding nisin and/or chitosan to the inner layer of the package. Also other authors report extended levels of shelf life due to the integration of antimicrobial agents into the contact surface of packaging materials. Brody et al. (2001) describe how crepe paper treated with sorbic acid was effective against the growth of fungi in bread.

1.2 MODE OF ACTION OF SELECTED AGENTS

During recent years, various proposals for the long term reduction of surface and airborne bacteria in the food chain have been investigated and developed. Most of these agents are based on surface release. In addition, also antimicrobial methods exist that rely on the propagation through the atmosphere, such as ionization, UV-light and chlorine dioxide gas.

The following chapter gives an overview on the most common surface acting antimicrobial agents.

1.2.1 Surface-acting antimicrobial agents

Among the surface-acting antimicrobial agents, two basic principles can be defined (Cooksey 2001; Brody et al. 2001; Aymerich 2008) - repelling of bacteria and killing of bacteria (Tiller 2006, see Figure 1.2).

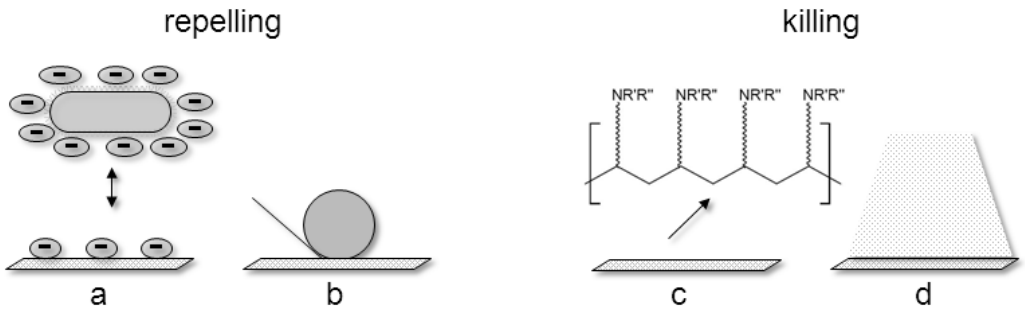

Figure 1.2 General principles of antimicrobial coatings: repelling of bacteria by a) the same electrostatic charge of surface and bacteria b) ultrahydrophobic surfaces; killing of bacteria by c) antimicrobial surfaces d) releasing of biocides (mod. from Thölmann et al. 2003; Tiller 2006)

Repelling of bacteria is related to the surface composition. In this approach, bacteria are not killed by the surface, but they are prevented from attaching to the surface. This effect occurs, for example, if surface and bacterial load have the same electrostatic charge (a). Another approach to repelling bacteria involves the use of ultrahydrophobic surfaces, where the contact angle is > 150° (b).

The second category of surfaces which leads to cell death of bacteria can be subdivided into two basic approaches. One approach is effective against food surface bacteria without migration of the active agent out of the surface (c). The

other approach is based on migration of antimicrobial agents (d). For both methods, direct contact between the surface and the food is necessary (Tiller 2006). Table 1.1 gives an overview on different antibacterial agents for potential applications within the food chain. Most of these agents belong to the migration group (d), except the bioactive polymers.

Table 1.1 Examples of antimicrobial agents for potential application in the food industry (mod. from Appendini and Hotchkiss 2002; Suppakul et al. 2003)

Class (principal)	Examples	References
Bioactive polymers (c)	chitosan, SAM-polymers	Cooksey 2001; Thölmann et al. 2003; Li et al. 2008
Bacteriocins (d)	nisin, pediocin, lactacin	Henning et al. 1986; Breukink and Kruijff 1999
Enzymes (d)	peroxidase, lysozyme	Appendini and Hotchkiss 1997; Gill and Holly 2000
Organic acids (d)	bezoic acid, sorbic acid, lactic acid	Brody et al. 2001; Jay et al. 2005
Plant extracts (d)	mustard oil, grapefruit seed, rosemary oil, wasabi derivates	Brody et al. 2001
Metals (d)	silver, copper, steel	Ranjit and Viswanathan 1997; Quintavalla and Vinci 2002

Bioactive polymers, such as chitosan, are effective against microorganisms without migration out of the surface (see Figure 1.2, c). As chitosan is polycationic, its amino groups interfere with the negatively charged cell surfaces and molecules (Cooksey 2001; Möller 2004). A further reason for the antimicrobial activity of chitosan is the chelating of essential metals, such as zinc, which are necessary for growth of bacteria and fungi (Cuero et al. 1991). Chitosan has been approved as GRAS (Generally Recognized As Safe) by the FDA in 2001. Various surfaces containing chitosan have already been developed for contact with food, like pure chitosan and chitosan-silver nanoparticle films (Jay et al. 2005; Thomas et al. 2009). Overall, chitosan seems to be more effective against Gram-positive rather than Gram-negative bacteria (Jay et al. 2005).

A totally new class of bioactive polymers are Sustainable Active Microbicidal Polymers (SAM) (Kossmann und Ottersbach 2009). At present, little is known about the precise mode of action. Thölmann et al. (2003) describe that their bactericidal activity is based on functional amino acids on the surface. The authors assume that the protonation of these amino acids leads to an increase of

the local pH value as well as to a charge separation of the surface. Buranasombop (2005) states that the toxicity of SAM-Polymers to mammalian cells is low, as the acute toxic level is higher than 2000 mg kg^{-1} and the polymers do not lead to irritation of skin. Because of its low toxicological level towards mammals, SAM-Polymers are in principle suitable for the use in the food industry. However, the mechanical and technical properties of these polymers need further development for their successful application as bactericidal food contact surfaces (Thölmann et al. 2003; Kreyenschmidt et al. 2008).

Bacteriocins are proteins that are derived from microorganisms (Brody et al. 2001). An example of such an antimicrobial peptide type compound is nisin. Nisin is generally recognized as safe for humans (GRAS; FDA). Its mode of action is based on destroying target cells by interacting with the bacterial plasma membrane. Hereby, the membrane's semi permeable function is destroyed. This leads to a collapse of the vital ion gradient and influences the pH level causing cell lyses (Breukink and Kruijff 1999; Brody et al. 2001). There are already a few approaches in existence for the integration of nisn into packaging materials. Ercolini et al. (2010) found out, that nisin-activated packages were able to reduce the number of spoilage organisms in beef.

Another bio-preservative group of antimicrobial active agents are enzymes. Antimicrobial enzymes are ubiquitous in nature, protecting living organisms, including humans, against microbiological infection (Fuglsang et al. 1995). The most common antimicrobial enzyme is lysozyme which is isolated from egg white. It is a single peptide protein which splits the bonds between the disaccharides of peptidoglycan in the cell wall of bacteria leading to cell death (Gill and Holley 2000; Coma 2008). Because of its mode of action, lysozyme is mainly active against gram positive bacteria. The integration of lysozyme into surfaces is described by Padgett et al. (1998). They succeeded in the integration of lysozyme into soy protein and corn zein films, resulting in a good antimicrobial activity of the films containing lysozyme against *Lactobacillus* and *Escherichia*. However, the activity of enzymes is strongly dependent on temperature and pH level (Han 2005). Also the integration of enzymes into plastic surfaces may be difficult, as enzymes are denatured at high temperatures.

A further class of antimicrobial agents are organic acids. Some of these acids are available from plants. A pre-stage of sorbic acid can, for example, be isolated from rowan berries. Sorbic acid leads to cell death of bacteria by interfering the

bacteria's metabolism. It bonds to enzymes, interacts with the cell wall of bacteria or it leads to stress on intracellular pH homeostasis (Brul and Coote 1999). There is already some use of organic acids as a component integrated into packaging materials. Brody et al. (2001) relate that sorbic acid treated crepe paper was effective against fungal growth in bread. As preserving agent, sorbic acid is mainly used as a fungicide e.g. in bread or in meat products (Brody et al. 2001).

The class of antimicrobial agents extracted from plants includes a variety of compounds isolated from e.g. spices like cinnamon, thyme, and rosemary. Also extracts from garlic and mustard oil belong to this group. The mode of action of these compounds is based on chelating key chemicals or oxidative splitting of disulphide bonds. Most compounds act by interruption of the metabolic pathway as they interfere to the cell membrane (Brul and Coote 1999; Brody et al. 2001). In comparison to several other antimicrobial agents, organic extracts are characterized by their low toxicity regarding mammalian cells. On the other side, high concentrations are necessary for the antimicrobial activity of plant extracts to be effective. As most of these extracts have a strong or irritating odor and taste, the concentrations needed for leading to cell death are way above the tolerable odor and taste threshold (Brul and Coote 1999; Brody et al 2001). Moreover, most organic substances are not stable at high temperatures. Thus they are not processable with standard methods in polymer or plastic industries (Meyer 2010). However, a Japanese company (Sekisui Jushi, Osaka, Japan) succeeded in the integration of plant extracts into packaging materials. The researchers developed a polyethylene film containing extracts from horseradish (wasabi).

Antimicrobial metals are the most common antimicrobial additives in food industry due to their temperature and mechanical stability. Silver can be used as an additive in several food contact materials based on plastics, glass or metal. As active silver ions are only built while humidity is present, materials containing silver are comparatively long lasting (Ovington 2004). The mode of action of antimicrobial metals is multifaceted. Metals, such as copper and silver, lead to structural changes in the cell wall of bacteria, interact with thiol groups in proteins and enzymes and interrupt replication by damaging the DNA (Gupta and Silver 1998; Morones et al. 2005). In particular, silver compounds or nano silver are already used in many applications with actual or potential food contact, e.g. for refrigerator inner liners (Schierholz et al. 2002; Quintavalla and Vicini

2002; Fries et al. 2009). Packaging materials containing silver inhibit bacterial growth and thus slow down the spoilage processes in perishable food (Simon et al. 2008). Different glasses containing silver, as well as defined silver substituted zeolite, are approved in the EU for application in articles for daily use (17. BedGgstVÄndV). Also in the USA, a silver substituted zeolite (AgIon Technologies, Inc.) is approved by the FDA (FCN No. 773) "as an antimicrobial agent used to preserve food-contact polymers". The EFSA specifies a maximum level of 0.05 mg silver per kg food (EFSA 2004).

1.2.2 Non-surface contact antimicrobial agents

As well as agents that are related to direct surface contact, there are techniques that are also able to reduce airborne bacteria. UV light, ionization as well as chlorine dioxide belong to the non-surface contact antimicrobial agents (Appendini and Hotchkiss 2002; Jay et al. 2005; Comi et al. 2006). These methods may provide additional benefits especially for reduction of airborne bacteria in enclosed spaces, such as refrigerators or dust-free rooms.

UV light is characterized by having a strong antimicrobial activity. Particularly light with a wavelength below 260 nm leads to cell damages as it is absorbed of proteins and especially nucleic acids. It causes lethal mutations within the DNA leading to cell death of bacteria (Jay et al. 2005). UV light can be used for the reduction of airborne bacteria levels in food processing plants (German law: §1 V LMBestrV), disinfection of packaging materials and surfaces like convey belts. The antimicrobial activity of UV-light is decreased by the adsorbent effect of dust particles in the air. A further limitation is due to the decline of effectiveness with the increasing distance from the UV lamp (Krämer 2002). It has to be considered, that UV irradiation accelerates the aging of plastic materials and increases the rate of gradual photo bleaching (Kerry et al 2006). Treatment of food surfaces with UV light can cause oxidative changes within the food, such as discolorations, rancidity or splitting of vitamins (Krämer 2002; Jay et al. 2005). Thus, the direct UV irradiation of food is only permitted for three applications: disinfection of hard cheese while storage, surface disinfection of fruits or vegetables, and drinking water disinfection (German law: §1 IV LMBestrV). Also for the user of UV light for decontamination procedures, hazards exist if they do not use correct procedures and protective eye wear (Cazzuli and Giroletti 2002).

The antimicrobial activity of UV light can be increased by catalysts, such as titanium dioxide (TiO_2). TiO_2 can be integrated into surfaces or coatings. It is the most commonly used semiconductor photocatalyst (Li et al. 2008). It is able to decompose organic molecules like bacteria or dust in the presence of UV light. Its antimicrobial activity is based on oxidative and reductive pathways by the production of hydroxyl radicals under UV irradiation. These strong oxidizers lead to the death of organic molecules (Lee et al. 1993). A disadvantage of photocatalysts is that they can also accelerate the aging of plastic, as plastics are made from organic substrates (Brody et al. 2001). Besides that, the antimicrobial effect of TiO_2 strongly depends on the presence of UV-light. Moreover, charge carrier recombination can occur very quickly (Ranjit and Viswanathan 1997).

Chlorine dioxide (ClO_2) is another bactericidal agent that can be spread via the air. The antimicrobial activity of this agent is based on oxidation. It attacks multiple cellular components like membranes and fundamental microbial cellular processes. The FDA has approved ClO_2 for applications in contact with food. In a study of Cooksey (2001), counts of *S. typhimurium* were significant reduced by direct contact to fresh chicken. On the negative side, the use of ClO_2 led to an adverse effect on the color of chicken breasts. Furthermore, beginning from 10 ppm, a typical ClO_2 -odor can be detected (Wellinghoff 1995). The integration of ClO_2 within a package's interior is possible using solid state technology. The ClO_2 within the foil is activated by humidity and turns out to be effective in reducing *Escherichia coli* in ground beef (Wellinghoff 1995).

Also some natural compounds, (such as the already mentioned allyl isothiocyanate (AIT) extracted from mustard oil) have been used to develop a sachet for the reduction of airborne bacteria, named WasaOuro®. WasaOuro® releases the volatile fractions of AIT, thus producing an antimicrobial effect using the atmosphere as a transmission medium (Brody et al. 2001). The application of this is limited due to the intensive odor of AIT.

The final antimicrobial method for reduction of airborne bacteria is ionization. Ionization is a non-selective method affecting a wide spectrum of air pollutants and biological contaminants (e.g. microorganisms, pollen, and olfactory molecules) by the combined effect of ozone and active ions (Krueger and Reed 1976; Comi et al. 2006). The mode of action is based on the production of reactive positive and negative ions as well as ozone. This leads to an oxidation of components within the bacterial cell (Forney et al. 2001; Boub 2005). Also the by

product, ozone, is a potent oxidizer of cellular components, such as proteins, and thus has an antimicrobial activity itself (Guzel-Seydim et al. 2004). The technology is already used in various industries, e.g. in dust-free rooms, in the food industry and in medicine. However, studies on the activity of ionization in contact with food are rare.

It is clear that a wide range of antimicrobial agents for possible application in contact with food are available. Most of the agents described, such as bacteriocins, enzymes or plant extracts, have a restricted range of applications and low durability. Thus they are primarily usable for packaging materials, but not for long term applications. In contrast to these agents, some metals, like silver, can also be used for long term applications, like cutting tables, conveyor belts or refrigerators. Also ionization can be used in long term applications to reduce surface and airborne bacteria, as the active agents are continuously produced.

Both methods are already used in applications involving contact with perishable food. Their general antimicrobial activity at standard conditions (e.g. JIS Z 2801) is described in several publications, especially those published for the medical sector. However, the specific conditions encountered in the perishable food supply chain, such as low temperatures or prevalent microorganisms are not considered in these tests. Furthermore, studies on the influence of food residuals on the rate of bactericidal activity are rare.

1.3 RESEARCH OBJECTIVE AND OUTLINE OF THE THESIS

The objective of this thesis is the assessment of sustainable long term antimicrobial agents with regard to their ability to reduce airborne and surface bacteria in the food processing chain and in private households. For this purpose, three research questions are posed:

- What is the antimicrobial rate of activity of selected antimicrobial agents against prevalent surface and airborne bacteria in the supply chain of perishable food?
- How is this antimicrobial rate of activity influenced by environmental factors such as low temperature and food residuals?

- What extensions to standard test procedures are necessary for the assessment of antimicrobial agents with regard to their applicability for the food industry?

For this purpose, two methods have been chosen, that have been used in contact with perishable foods: silver coating and ionization. These methods are analyzed with regard to their ability to reduce surface and airborne bacteria in the perishable food chain in the stages production, storage, transport and in private households. In particular, the possible application within refrigerators will be examined, as refrigerator inner liners illustrate a typical example of surfaces that are potentially in long term contact with a range of differing perishable foods. Furthermore, within refrigerators, the low temperature conditions and typical microflora are comparable to conditions within the whole food chain of perishable food.

The first part of this dissertation (chapter 2.1 and 2.2) deals with key aspects of using silver ions in food contact surfaces. Thereby, chapter 2.1 focuses on the antimicrobial effect of silver in the special application of refrigerator inner liners. This section includes environmental influence factors towards the rate of antimicrobial activity like temperature and the sensibility of microorganisms that are typical for perishable foods. Chapter 2.2 comprises the application of silver in the entire food chain of perishable food, while focusing on the possible inhibition of the antimicrobial activity of silver by food components.

Chapter 3 illustrates the use of ionization for reduction of surface and airborne bacteria within refrigerators. In this chapter such aspects as the composition of surface and the sensitivity of test organism are considered. Regarding the activity of ionization on airborne bacteria, the influence of time, and temperature are analyzed, as well as the characteristics of ionizers on the reduction rate.

In the last Chapter, an evaluation scheme for testing antimicrobial agents for the application in contact with perishable foods is presented. This scheme is based on the results originated in chapter 2 and 3. Moreover, model approaches are discussed for supporting laboratory tests in the determination of environmental and microbiological influence factors on the antimicrobial activity.

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CHAPTER 2

The use of materials containing silver in contact with food

CHAPTER 2.1

Study on the antimicrobial effect of silver-containing inner liners in refrigerators[1]

[1]Published in the Journal of Applied Microbiology - see list of publications (Kampmann et al. 2008)

ABSTRACT

Aims To investigate the effect of silver-based antimicrobial material incorporated in the inner liners of refrigerators on food safety and quality.

Methods and Results In the first stage, the bactericidal effect was tested in the laboratory. Silver-containing samples and control plates were inoculated with different bacterial suspensions and stored at various temperatures. After defined storage periods the bacterial reduction was calculated by comparing viable cell count on reference plates and on silver-containing plates. The reduction caused by the silver-containing material varied between 1.0 and 5.9 $\log_{10}$ units, depending on bacterial strain, incubation time and temperature. In the second stage, food storage experiments have been carried out. Thus, perishable foods were stored in coated and untreated refrigerators. After certain time periods the products were analyzed for their sensorial and microbiological characteristics. A clear drop in viable counts both on the refrigerator wall and on the food was demonstrated using the silver-based antimicrobial material.

Conclusions Silver prevents refrigerators from being a hot spot for contaminants that could be transferred upon contact with food.

Significance and Impact of the Study This study provides original results regarding the antimicrobial activity of silver-containing refrigerator surfaces.

2.1.1 INTRODUCTION

Cross-contamination through surfaces that get in touch with food, like work tables, cutting boards and refrigerators has been shown to be an area of great concern (Sneed et al. 2004). Despite low temperatures, hygienic designs and cleaning recommendations, refrigerators can be hot spots for bacteria (Bielecki 2003). Studies in Ireland have shown that the concentration of viable cells in refrigerators is around $\log_{10}$ 7.1 CFU cm^{-2} in average. In addition, more than half of the inspected refrigerators contained pathogenic microorganisms (Kennedy et al. 2005). Spills, inefficient cleaning or contaminated foods trigger accumulation of these contaminants. Bacteria tend to concentrate on these surfaces, thereby increasing the risk of biofilm formation (Lindsay and Holy 2006). As a consequence, refrigerator walls can act as a contamination source for materials and foods inside a refrigerator. *Staphylococcus*, *Pseudomonas, Bacillus, Enterobacteriacea* and fungi are primarily found in household refrigerators (Timm 1993; Ojima et al. 2002; Kennedy et al. 2005). These spoilage-causing or pathogenic organisms are often able to grow at low temperatures and lead to reduction of shelf life or even affect consumer's health (Krämer 2002; Kreyenschmidt 2003).

Treating the internal walls of refrigerators with an antimicrobial additive is a possible way to control this type of cross-contamination better. The antibacterial activity of silver is well documented. Both laboratory and clinical testing have demonstrated the effectiveness and safety of a range of silver-based antimicrobial additives (e.g. Brady et al. 2003; Ip et al. 2006).

The cellular effects of silver on several bacterial species were demonstrated by Feng et al. (2000). Silver ions react with electron-donating groups, such as those containing sulfur, oxygen or phosphorus. This means target sites in bacteria are abundant, like proteins in the cell wall or cell membrane, enzymes and DNA (Lansdown 2004). Binding of silver to bacterial compounds results in protein inactivation, cell wall detachment, DNA condensation and will finally lead to cell destruction. The rate of bacterial inhibition is dependent on silver concentration and individual sensibility of microorganisms to silver ions. In general, Gram-positive bacteria are less sensible to antibacterial compounds, because their peptidoglycan layer may protect them better from incoming silver ions (Schlegel 1992; Feng et al. 2000). There are various external factors that influence the antimicrobial action of silver. Liau et al. (1997) investigated the possibility that

some amino acids, e.g. those with thiol groups like cysteine, bind to silver ions, thus lowering the amount of available silver for antimicrobial purposes. Temperature is another factor that may influence the antibacterial action of silver (MacKeen et al. 1987; Slawson et al. 1992; Russel and Hugo 1994). Because of kinetic reasons, at low temperatures the release of silver from its carrier material decreases (Quintavalla and Vicini 2002).

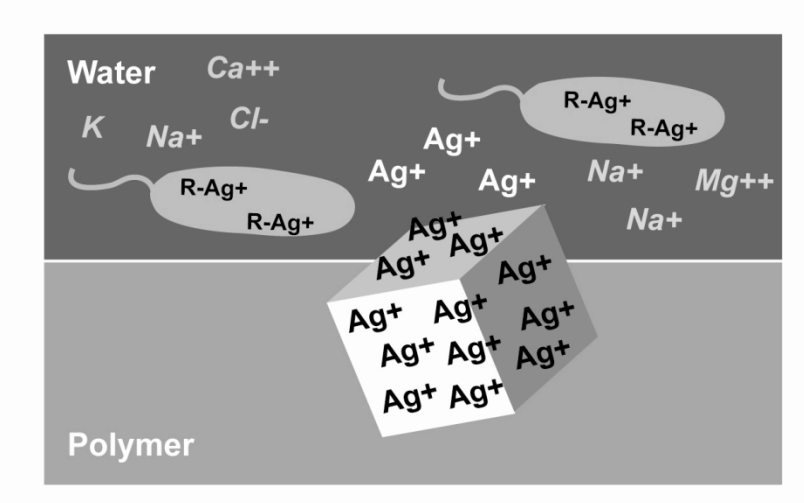

Figure 2.1.1 Schematic representation of the mode of action of silver release from AlphaSan® incorporated in a polymer

In this study, the antibacterial action of silver-containing High Impact Polystyrene (HIPS) materials (Alpha- San, Milliken, Gent, Belgium) for the use as inner liners of refrigerators were tested. AlphaSan is a zirconium phosphate-based ceramic ion-exchange resin containing silver. The applied antimicrobial additive technology is based on controlled release of silver ions and was developed for safe use in a variety of polymeric materials. The release mechanism is activated under humid conditions and is based on ion exchange of cations from the environment with silver from the insoluble inorganic zirconium phosphate carrier, located at the surface of the polymer matrix (Figure 2.1.1). Once the silver is released, it has a detrimental effect on bacteria, thus controlling their proliferation.

2.1.2 MATERIALS AND METHODS

Efficacy of the material was tested in a two-stage process. To investigate the bactericide effect of different materials, laboratory tests have been performed

according to JIS Z 2801 (2000). In the second stage, food storage experiments have been carried out to measure the influence of silver on food quality.

Laboratory tests according JIS Z 2801

The laboratory tests were based on JIS Z 2801 (2000) standard method for efficacy testing of plastics (JIS Z 2801). To test the antimicrobial activity of silver, sheets (50 · 50 mm) of HIPS with a thin coating of silver-containing HIPS were used (AlphaSan®, Milliken). Uncoated test pieces without silver in the same size were used as a reference. The described test-sheets and reference plates were disinfected with ethanol impregnated bandages and dried in a sterile atmosphere. Twenty-four silver-containing plates and six reference sheets without silver were inoculated with 0.4 ml of a 10^5 CFU ml^{-1} concentrated *Staphylococcus aureus* suspension (DSM No. 346). To prevent evaporation and to standardize the contact area, the inocula were covered loosely by sterile PE films (45 · 45 mm). The bacterial concentrations on three untreated test pieces were determined immediately after inoculation to determine the starting concentration. These sheets were placed in sterile stomacher bags and doused with 10 ml of soybean-casein digest broth with lecithin polysorbate (Roth, Karlsruhe, Germany) each. The other reference sheets and all test sheets were incubated at 35°C (humidity 90%) for 24 h and washed out after incubation in a similar manner.

Viable counts were determined by plate counting in plate count agar (Roth) of appropriate decimal dilutions, made in sterile phosphate-buffered saline (0.9%). Agar plates were incubated at 37°C for 48 h before counting. Antibacterial activity was calculated by subtracting the logarithmic value of viable counts on coated material from untreated material after inoculation and incubation:

$$\log_{10} \text{Reduction} = \log_{10} (T_{x,Re} / T_{x,Pr})$$

where $T_{x,Re}$ = bacterial concentration on reference material, x hours after inoculation and $T_{x,Pr}$ = bacterial concentration on coated material, x hours after inoculation.

For materials to account as antibacterial, the calculated value of antimicrobial activity should not be <2.0.

In addition, the examination method was adapted to refrigerating conditions. For this reason, psychotropic microorganisms were applied and test temperatures

were lowered. *Lactobacillus delbrueckii*, subspecies lactis (DSM No. 20072) and *Pseudomonas fluorescens* (DSM No. 304) were used as inoculums in a concentration of 10^5 CFU ml^{-1}, each. For long-term performance testing *Lactobacillus monocytogenes* (LMG 13305) were used as inoculum in a concentration of 10^3 CFU ml^{-1}. The incubation temperature was decreased to either 4 or 5°C. The incubation time was extended up to 4 weeks. The samples (for each trial: three silver containing and six untreated plates) were prepared, incubated, washed out and plate counted in the way as described in JIS Z2801 (2000).

Food storage experiments

The second part of testing aimed at visualizing the antimicrobial effect in a refrigerator, in contact with food and materials. Therefore, refrigerators identical in construction were used. One refrigerator was treated with AlphaSan®, whereas the reference refrigerator had no antimicrobial coating. The described AlphaSan® treated, and the control refrigerators were inoculated with *L. monocytogenes* (LMG 13305) inocula (10^3 CFU ml^{-1}) to simulate a contaminated area, e.g. caused by a spill. After incubation, food was brought into contact with the contaminated spot. Apple slices and lettuce were used for this purpose as these are typically foods that are not wrapped when put in a refrigerator, nor cooked before consumption. Contamination of the refrigerator wall, a cover film and the food was visualized using an indicator growth medium, based on Tryptic Soy Agar (Oxoid, Hampshire, UK) supplemented with 0.01% triphenyl-tetrazolium chloride. Each of these experiments was performed in duplicate.

Additionally, the antimicrobial effect of sterile silver coated refrigerators in contact with food was analyzed. Foods like meat, cheese and vegetables were stored in silver-coated and control refrigerators without silver for different time intervals and different temperatures (room temperature and 5°C). The food products were packed pork loins, sliced cooked ham, sliced cheese, sliced pork sausage and sliced salami as well as lose cucumbers, oranges and lettuce. All foodstuffs were unwrapped and equally distributed in the two refrigerators. The cucumber, orange and lettuce were divided in two equal parts so that the bacterial concentration on food in both refrigerators was the same in the beginning of storage. After the storage period, the products were evaluated from a sensory point of view. A panel of 10 persons estimated food quality in a blind test using a hedonic scale from 1 to 10, where 1 was equal to good quality and 10

to bad quality. Color, texture and odor were used as quality parameters. The microbiological contaminations of refrigerator walls were determined exemplarily underneath some stored food products using surface swabbing according to DIN 10113-1 1997.

Statistical analysis

Differences in bacterial growth on AlphaSan® and reference materials were analyzed for significance by the t-test for independent samples. Significance was defined as $P < 0.05$, and all evaluations were carried out with SPSS 12.0 for Windows©.

2.1.3 RESULTS

Laboratory tests according to JIS Z2801

The results, illustrated in Figure 2.1.2 for tests according to JIS Z2801 (2000), indicate a clear drop of *Staph. aureus* concentration on silver-containing materials.

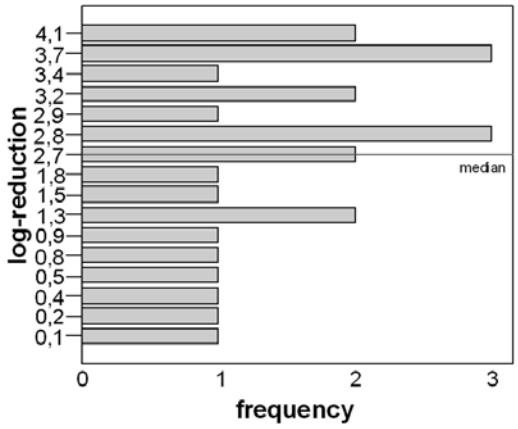

Figure 2.1.2 Frequency of $\log_{10}$ – reduction of *Staph. aureus* on 24 spot samples of silver-containing High Impact Polystyrene materials

Compared to bacterial concentration on the reference materials after incubation, the coated samples showed a reduction of 0.1–4.1 $\log_{10}$ units. Six of 24 tested samples demonstrated a reduction of <1.0 $\log_{10}$ units. On the other hand, 14 samples showed a reduction of more than 2.0 $\log_{10}$ units. The median of reduction was 2.7 $\log_{10}$ units.

The reduction of *Lact. delbrueckii* is illustrated in Table 2.1.1.

Table 2.1.1 Log_{10} - reduction of *Lact. delbrueckii* on silver containing HIPS materials at different incubation temperatures and incubation times

Incubation-temperature (°C)	Incubation-time (h)	Starting-concentration (log_{10} CFU ml^{-1})	Concentration after incubation on reference materials (log_{10} CFU ml^{-1})	Concentration after incubation on silver containing materials (log_{10} CFU ml^{-1})	Log_{10} – reduction
35	24	5.3	3.9 ± 0.15	2.9 ± 0.28	1.0
5	24	5.3	5.4 ± 0.15	5.6 ± 0.19	0*
5	72	5.3	4.9 ± 0.12	3.4 ± 0.14	1.4
5	144	5.3	4.9 ± 0.23	3.8 ± 0.23	1.1

* no significant difference

At 35°C an antibacterial decrease of 1.0 log_{10} units has been proven after 24 h. At 5°C there was no decline of bacterial concentration in the same time interval, but a bactericidal effect (reduction of 1.4 log_{10} units) was achieved after 72 h.

At 35°C a strong reduction of *Ps. fluorescens* by silver became apparent after only 24 h (reduction of 5.1 log_{10} units), while at 5°C – as already shown for *Lact. delbrueckii* – a similar reduction could only be achieved by longer storage periods (up to a reduction of 6.7 log_{10} units, see Table 2.1.2). Compared to decline of *Lact. delbrueckii* by silver-coated surfaces, the reduction of *Ps. fluorescens* is much higher.

Table 2.1.2 Log_{10} - reduction of *Ps. fluorescens* on silver containing HIPS materials at different incubation temperatures and incubation times

Incubation-temperature (°C)	Incubation-time (h)	Starting-concentration (log_{10} CFU ml^{-1})	Concentration after incubation on reference materials (log_{10} CFU ml^{-1})	Concentration after incubation on silver containing materials (log_{10} CFU ml^{-1})	Log_{10} – reduction
35	24	5.9	6.1 ± 0.14	< 1.0	5.1
5	24	5.9	6.1 ± 0.06	5.9 ± 0.12	0.2*
5	72	5.9	6.4 ± 0.28	< 1.0	5.4
5	144	5.9	7.7 ± 0.03	< 1.0	6.7

* no significant difference

Figure 2.1.3 demonstrates the long-term performance (over a time interval of 4 weeks) of AlphaSan® against *L. monocytogenes* at refrigeration temperatures. The testing was also based on the JIS Z 2801 (2000) standard. A clear reduction of cell counts was observed on AlphaSan® treated samples. After 2 weeks, the bacterial concentration on the silver-containing surface was constantly lower

than $\log_{10}$ 2 CFU ml^{-1} while bacterial concentration on untreated surface increased up to $\log_{10}$ 6 CFU ml^{-1} within the 4 weeks of testing.

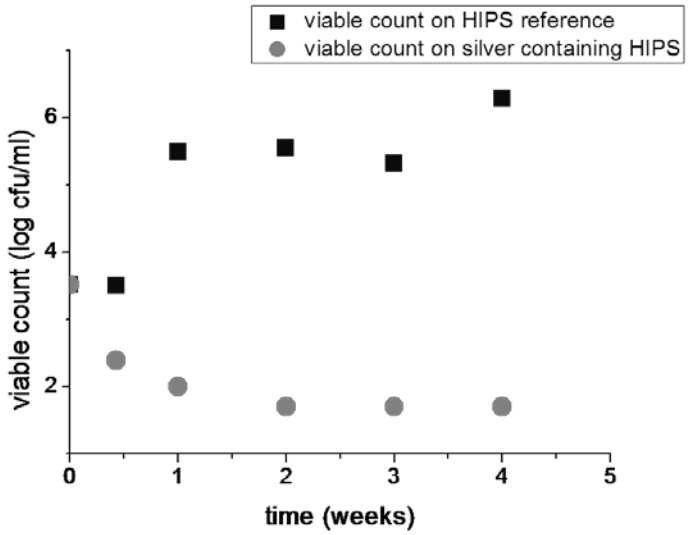

Figure 2.1.3 Antibacterial effect of AlphaSan® in refrigerator inner liners test samples. Samples were inoculated with *L. monocytogenes* and tested based on a modification of the JIS Z 2801 standard method

Food storage experiments

A severe reduction in contamination both on an AlphaSan®-treated refrigerator walls and on food that was brought in contact with this was demonstrated in Figure 2.1.4. The apple slices as well as the lettuce had a clearly decreased level of bacterial contamination. This contamination was not measured in a quantitative way, as in previous JIS Z 2801 (2000) experiments, but in a visual way using an indicator growth medium. The red color clearly indicates higher cell numbers when handling non-AlphaSan®-treated surfaces.

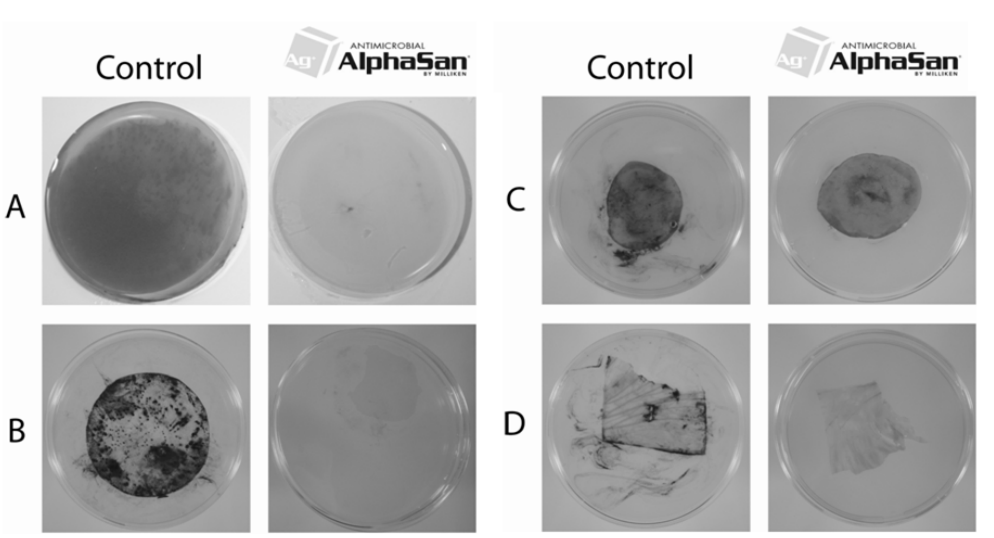

Figure 2.1.4 Visualization of the antimicrobial effect of AlphaSan® using an indicator growth medium directly on the refrigerator inner wall (A) and on a cover film (B), an apple slice (C) and lettuce (D) that were brought in contact with the contaminated area.

The food storage experiments made evident that the silver-coated inner liners provide a better quality of stored products. In all three test repetitions all food stored in silver-coated refrigerators tends to have higher sensory quality than food stored in untreated refrigerators, except for the cucumber in the second trial (Table 2.1.3).

Table 2.1.3 Sensory estimation of stored food in coated and uncoated refrigerators

Product	Sensory estimation after storage in uncoated refrigerator	Sensory estimation after storage in coated refrigerator	Significance (2-sided)	Difference
[1]Pork cutlet	4.0 ± 0.00	3.2 ± 0.44	0.00	0.8
[1]Cooked ham	4.0 ± 0.00	1.5 ± 0.79	0.00	2.5
[1]Pork sausage	3.9 ± 0.33	2.9 ± 0.60	0.00	1.0
[1]Salami	2.1 ± 0.81	1.9 ± 1.13	0.81	0.2*
[2]Pork cutlet	9.9 ± 0.32	9.4 ± 0.97	0.15	0.4*
[2]Cooked ham	9.0 ± 1.05	7.3 ± 1.95	0.03	1.7
[2]Sliced Cheese	9.6 ± 0.70	8.3 ± 1.77	0.04	1.3
[2]Cucumber	5.0 ± 2.36	5.7 ± 1.70	0.46	0*
[3]Pork cutlet	7.9 ± 1.87	7.7 ± 1.90	0.82	0.2*
[3]Pork sausage	6.9 ± 1.70	6.5 ± 2.02	0.65	0.4*
[3]Sliced cheese	6.5 ± 1.92	5.8 ± 2.32	0.43	0.7*
[3]Orange	6.5 ± 2.25	6.5 ± 2.33	1.00	0*
[3]Lettuce	7.1 ± 2.30	6.6 ± 2.73	0.68	0.5*

[1]n = 9; without cooling, storage period: 6 days; [2]n = 10; without cooling, storage period: 8 days; [3]n = 11; with cooling, storage period: 11 days; *no significant difference

But only in five cases, the difference was significant. Particularly the quality of cooked ham stored in silver-coating refrigerator was regarded as being better. In the first trial, the quality of ham stored in the coated refrigerator was judged in average 2.5 points better than ham stored in the uncoated refrigerator and in the second trial, the difference was 1.7 points. This observation could be validated by the viable cell count on the refrigerator surface underneath the food (spot sampling). The viable cell count was at least 1.0 $\log_{10}$ scale lower in the silver-containing refrigerator compared to the untreated one (Table 2.1.4).

Table 2.1.4 The bacterial concentration under different products on the refrigerator ground without and with silver coating

Test conditions	Sampling position	Uncoated refrigerator (CFU cm^{-2})	Coated refrigerator (CFU cm^{-2})
without cooling, storage period: 6 days	Under pork cutlet	$> 10^9$	$3.3 * 10^8$
	Under cooked ham	$1.2 * 10^8$	$1.3 * 10^7$
without cooling, storage period: 8 days	Under cooked ham	$3.4 * 10^8$	$3.1 * 10^7$
	Under sliced cheese	$7.3 * 10^5$	$3.0 * 10^4$
with cooling to 4°C, storage period: 11 days	Under sliced pork sausage	$6.5 * 10^2$	< 10

2.1.4 DISCUSSION

The conducted studies demonstrate the general possibility to reduce contamination of refrigerator surfaces using silver compounds. Several studies described the antimicrobial effect of silver against a wide spectrum of micro-organisms (Carr et al. 1973; Russel and Hugo 1994; Hipler et al. 2006). This study confirm these findings. Both, testing in the laboratory, and food storage trials, demonstrated lower bacterial counts on the coated material compared to the untreated material. Two types of Gram-positive and one type of Gram-negative bacterium have been used for laboratory investigations. The silver-coated material produced approximately a reduction of 1.0–5.9 $\log_{10}$ units of *Staphylococcus*, *Lactobacillus* and *Pseudomonas* within a 24- to 144 h period at 5–35°C, depending on the bacterial strain. The higher reduction of *Ps. fluorescens* compared to *Staph. aureus* and *Lact. delbrueckii* may be due to the cell wall construction. Gram-positive bacteria have a bigger layer of polyglycan. Therefore, it is more difficult for antimicrobial substance to pass the cell wall (Jansen et al. 1995; Krämer 2002).

Tests at 5 and 35°C demonstrated that temperature has a distinct influence on the antimicrobial effect of silver-coated materials. This is caused by two different factors: on one hand, silver release from materials is reduced at low temperatures. On the other hand, absorption of silver into microorganisms is slowed down, because this process is energy dependent (MacKeen et al. 1987; Slawson et al. 1992; Russel and Hugo 1994; Quintavalla and Vicini 2002).

Food storage tests proved a clear influence of the silver-containing surface on food quality when it comes directly in contact with the surface. Even though meat, pork products and cheeses are not typically stored unwrapped on the ground of refrigerators, these tests indicate, that the antibacterial effect is not suppressed by proteins, as mainly described in the literature (Liau et al. 1997; Matsumura et al. 2003).

Recapitulatory, this study demonstrates the antibacterial activity of AlphaSan® under both laboratory and real life conditions. This indicates that the silver-based antimicrobial AlphaSan® material used in refrigerators produced by Bosch and Siemens Hausgeräte GmbH (Giengen, Germany) protects the inner walls of the refrigerator and helps preventing them being a hot spot for contaminants that could be transferred upon contact with food. The antimicrobial-treated inner liners have been proven to be an additional safety barrier that contributes to the overall hygienic concept within a refrigerator. Nevertheless, additional tests are necessary to investigate interactions between food and silver compounds.

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CHAPTER 2.2

Effects of food components on the antibacterial activity of silver ions (Ag^+)

ABSTRACT

Aims Investigation of the influence of food components on the antimicrobial effect of silver ions

Methods and Results In the first part of investigations, the antimicrobial activity of a polypropylene compound with a zirconium phosphate based ceramic ion exchange resin containing silver was investigated by comparing the growth of *Pseudomonas fluorescens* on sample and reference materials. A marked reduction of bacterial counts on silver samples of 7.4 $\log_{10}$-units was determined. In the second part of the investigations, different food isolates and complex foods from the carbohydrate, protein and fat group were added to the inocula to investigate their influence on the antimicrobial activity of silver. The influence of food components on the antimicrobial attributes of silver varied. Apparently, the addition of protein-rich food, starch and honey reduced the antimicrobial activity of silver, while all other tested components did not affect the antimicrobial activity of silver.

Conclusions The effect of materials containing silver needs to be verified individually for the respective food components in contact with the surface.

Significance and Impact of the Study The conclusions divided from this study will allow a reasonable implementation of the use of surfaces containing silver in the food industry.

2.2.1 INTRODUCTION

During the recent years, the application of silver as an additive in several applications including food storage, packaging and processing has steadily increased (Quintavalla and Vicini 2002; Hogan and Kerry 2008; Yang et al. 2009). The antimicrobial activity of silver is detailed by several research groups. Most publications describe the effect of silver as related to silver components, silver concentrations, particle size, and individual sensitivity of microorganisms (e.g. Furr et al. 1994; Russel and Hugo 1994; Ip et al. 2006). For use as food contact surface, reactions between silver ions and food are also of high relevance. Gadd et al. (1989) and Liau et al. (1997) assume that food residuals (such as carbohydrates, proteins and fat) on surfaces (e.g. conveyor belts, or cutting tables) influence the antimicrobial effect of silver.

Generally, each one of the three macro nutrients is a potential reaction partner for silver ions. Gomes et al. (2002) and Hold and Bard (2005) have shown that silver reactivity is inhibited in the presence of glucose. As a reason they give a reduced intake and thus a short term protection against silver in *E. coli* by glucose. The authors suppose that efflux pathways which require ATP by glucose are responsible for this effect (Hold and Bard 2005). Furthermore, Tajmir-Riahi (1986) and Gyurcsik and Nagy (2000) conclude that carbohydrates react via carbonyl oxygen atom, hydrogen or nitrate group with Ag^+ and thus bind silver molecules. Contrary to this, Gomes et al. (2002) found out that the afflux of silver complexes with glucose into yeast cells is increased. The authors ascribe this to an enhanced membrane transport mechanism in microorganism cells caused by the presence of glucose (Gomes et al. 2002; Gottschaldt et al. 2006). Also Tan et al. (2002) and Thomas et al. (2009) observed that carbohydrates can be used for technical improvements of silver components. In such a way starch, glucose and dextrose can be applied as a stabilizing agent in order to form silver nanostructures.

As nonspecific protein absorption occurs by surface contact, the interactions between silver and proteins are of high relevance (Schierholz et al. 2002). Gruen (1975) found out that some amino acids inhibit the antimicrobial effect of silver. They demonstrated a high binding potential of Ag^+ to cysteine, methionin, lysin and arginine in aqueous solutions, whereas aspartic and glutamic acids does not have this effect. Tilton and Rosenberg (1978) remark that proteins and products with high protein contents are able to reverse the inhibition of bacteria by silver.

In a study by Kampf et al. (1998), the antimicrobial activity of silver was completely inhibited by 5% of horse serum. The authors explain this effect by the great affinity of silver to proteins. Sulfur-hydrogen containing amino acids are, in particular, known to inhibit the antimicrobial effect of silver (Tilton and Rosenberg 1978; Li et al. 1997; Stewart and Fredericks 1999). This effect can be explained by the mechanism of action of silver cations (Ag^+). Prime molecular targets for Ag^+ are electron donor groups including nitrogen, oxygen, and sulfur (Antolini et al. 1980; Lansdown 2004). These components are abundant in biological molecules - in bacteria as well as in food residuals. In addition it is known that silver initially reacts with abundant thiol groups in a protein solution (Madsen 1963). Thus, e.g. silver substituted zeolites are not active if lysine, sulfates, sulfides and other sulfur containing amino acids are present (Appendini and Hotchkiss 2002). Bounded Ag^+ has no antimicrobial potential (Schierholz et al. 1998). However, any remaining Ag^+ will directly start associating with other targets (Madsen 1963). Kampmann et al. (2008) have confirmed that the residual silver ions are still active. They investigated an inhibitory effect of silver on the spoilage of protein rich cooked ham.

Fat is also a potential bonding partner for silver. Morris (1966) and Nikolova-Damyanova et al. (1992) showed that both double bonds and carboxyl bonds of fat molecules react with silver. Landau (2006) describes a reduced sensitivity of bacteria towards Ag^+ with increasing lipoid fraction. For the formation of silver ions, sufficient humidity is needed (Ovington 2004).

Most of the described studies have been conducted in liquid media with added silver ions. However, information on the activity of silver containing surfaces in the presence of food still remains scant, although silver is already used in many applications in contact with food.

Thus, the present work is aimed at the investigation of interactions between surfaces containing silver and food to determine the effect of silver use in food contact surfaces.

2.2.2 MATERIALS AND METHODS

Investigations were subdivided into two parts. In the first part, the general antimicrobial activity of polypropylene (PP) samples containing silver was investigated. In the second part, the influence of different food isolates and complex foods on the antimicrobial action of silver was investigated. Appropriate carbohydrate-, protein- or lipid- sources were used as test substances.

The method for testing of antimicrobial activity is based on the Japanese Industrial Standard JIS Z 2801 (2000). Details of the microbiological method have already been described in chapter 2.1. Therefore, only a general description will be provided here. The antimicrobial activity is determined by comparing growth rates on samples containing silver with growth rates on reference sheets. For part II of the investigations, different food isolates and complex foods were added to the inocula to define the influence of these components on the antimicrobial activity of silver.

Test materials

Square PP sheets (70 · 50 · 1 mm) containing 1% silver in a zirconium phosphate based ceramic ion exchange resin (Alpha San®, Milliken, Gent, Belgium) were used as samples. PP sheets without silver were used as references. Sets of 25 individual samples and reference sheets each were utilized several times in the trials. For decontamination after every experimental run, the samples were rinsed with distilled water and immersed in ethanol (70%) for 10 minutes. Afterwards, the sheets were towel dried, dried of in the air and stored in plastic bags until the next experiment commenced.

Preparation of inocula

As exemplified test organism *Ps. fluorescens* (DSM No 304) was used. The inocula were prepared by transferring a frozen culture (-20°C) in 10 ml nutrient broth (Roth, Karlsruhe, D) followed by incubation for 24 h at 30°C. At the beginning of each experimental series, the suspension was diluted in saline solution (Oxoid, Hampshire, UK) up to 10^5 – 10^6 CFU ml^{-1}. For testing the influence of food components (part II of investigations), different food isolates or complex foods were added to the inocula (each 0.1 - 1 g per 10 ml inoculum, depending on solubility of substance). The components used are described in Table 2.2.1.

Table 2.2.1 Summary of the investigations with different food components added to the inocula

Part	Group	Food component added to inocula	Amount added to the inocula	Total number of inoculated reference materials*	Total number of inoculated materials containing silver *
I	Control	None	-	21	40
II	Carbo-hydrates	d(+)glucose, Merck, Darmstadt, D	1 g	6	6
		d(-)fructose, Merck, Darmstadt; D	1 g	6	6
		cyclodextrin, Roquette, Lestrem, F	1 g	6	6
		starch flour	1 g	9	9
		saccharose	1 g	18	18
		honey	1 g	6	6
	Proteins	l-argenine, Gebru, Gaiberg; D	0.1 g	15	21
		l-cysteine, Gebru, Gaiberg, D	0.1 g	6	12
		l-prolin, Gebru, Gaiberg, D	0.1 g	5	11
		turkey meat	1 g**	6	6
		egg white	1 g	6	6
		yogurt	1 g	6	6
	Fat	palmitic acid, Merck, Darmstadt, D	0.1 g	6	6
		oleic acid, Merck, Darmstadt, D	0.1 g	6	6
		thistle oil	1 g	6	5
		soy oil	1 g	6	5

*All investigations were repeated twice in minimum

**As meat cannot be diluted in the inoculum, 1 g turkey meat were applied on 0.4 ml of *Ps. fluorescens* suspension

Food isolates were chosen that are typical for numerous foodstuffs. The complex foods were purchased from a local retailer. To ensure homogeneous dispersion in the inoculum even if the added food component is not soluble in water (fatty acids and oils), 0.4 ml tween 80 (Roth, Karlsruhe, D) were added to the inocula. In addition, the palmitic acid had to be heated in NaCl solution to get an emulsion. Before inoculation with *Ps. fluorescens*, the emulsion was cooled to less than 40°C.

Test performance

For each experimental run, a minimum of three samples and six reference sheets were used. At the beginning of each trial, the appropriate samples and reference sheets were disinfected with ethanol impregnated pads. The sheets were dried

within a sterile atmosphere and placed in Petri dishes without nocks. For testing the general antimicrobial activity of silver samples (part I of investigations), silver and reference sheets were inoculated with 0.4 ml of the prepared pure *Ps. fluorescens* inocula. All sheets were covered with a foil to prevent evaporation of inoculum. Three reference sheets were directly washed out with 10 ml soybean-casein digest broth with lecithin polysorbate (Roth, Karlsruhe, D) to determine the starting concentration. The test sheets and the remaining reference sheets were washed out after 24 h incubation at 35°C and humidity > 90%.

Determination of colony forming units (CFU) was raised using the pour plate method with plate count agar (Roth, Karlsruhe, D) followed by incubation at 30°C for 48 hours.

Antibacterial activity was calculated by subtracting the medians of the logarithmic values of viable counts on coated materials from the median on untreated materials after inoculation and incubation:

$$\log_{10} \text{Reduction} = \log_{10} (T_{x,Re} / T_{x,Pr})$$

where $T_{x,Re}$ = median of bacterial concentration on reference material, x hours after inoculation and $T_{x,Pr}$ = median of bacterial concentration on coated material, x hours after inoculation.

For materials to be regarded as antibacterial, the calculated reduction value of antimicrobial activity should not be <2.0 $\log_{10}$ units (JIS Z 2801).

In the second part of investigations, different food isolates and complex foods were added to the inocula (see Table 2.2.1). As turkey meat cannot be dissolved in the inocula, the meat was separated into 1 g slices and applied additionally to the 0.4 ml *Ps. fluorescens* inocula on test and reference sheets. Determination of starting concentration as well as plate counts after 24 h incubation were carried out in the same way as described for part I of investigations.

Statistical analysis

All plate count data were transformed into $\log_{10}$ values before statistical analysis. Differences in bacterial growth on silver containing and reference materials were analyzed for significance using the Mann-Whitney U test for independent samples. Significance levels were defined as $p < 0.05$, resp. $p < 0.001$ (as highly significant). For description of results, box plots were used. The lower and upper quartiles of the boxes are the 25th and 75th percentile, and the ends of the

whiskers represent a maximum 1.5 of the interquartile range (IQR). Outliers are defined as minimum 1.5 IQR of the lower or upper quartile. All evaluations were carried out using SPSS 17.0 for Windows©.

2.2.3 RESULTS

The starting concentration evaluated on reference sheets was in average $\log_{10}$ 5.4 ± 0.63 CFU ml^{-1} (n=152). Except for investigations with cysteine, the plate counts at time t=0 were within the required concentration. By adding cysteine to the inoculum, the starting concentration was reduced to $\log_{10}$ 3.6 CFU ml^{-1} on average.

The antimicrobial activity of silver samples under control terms without any food components added to the inoculum was clearly proven (see Figure 2.2.1). The median of surface bacteria on reference sheets was $\log_{10}$ 8.4 CFU ml^{-1} compared to $\log_{10}$ 1.0 CFU ml^{-1} on silver sheets. This demonstrated a highly significant reduction of *Ps. fluorescens* by the tested silver sheets. However, in the silver sample group, plate counts on eight of 40 samples were identified as outliers, which is equal to 20% of the total data. Nevertheless, plate counts on every of the 40 silver samples were lower than plate counts on the reference sheets. Only in two of 40 cases, differences in bacterial counts on silver and reference materials were less than three $\log_{10}$-units respectively less than the reduction of 2 $\log_{10}$ units as required by the JIS.

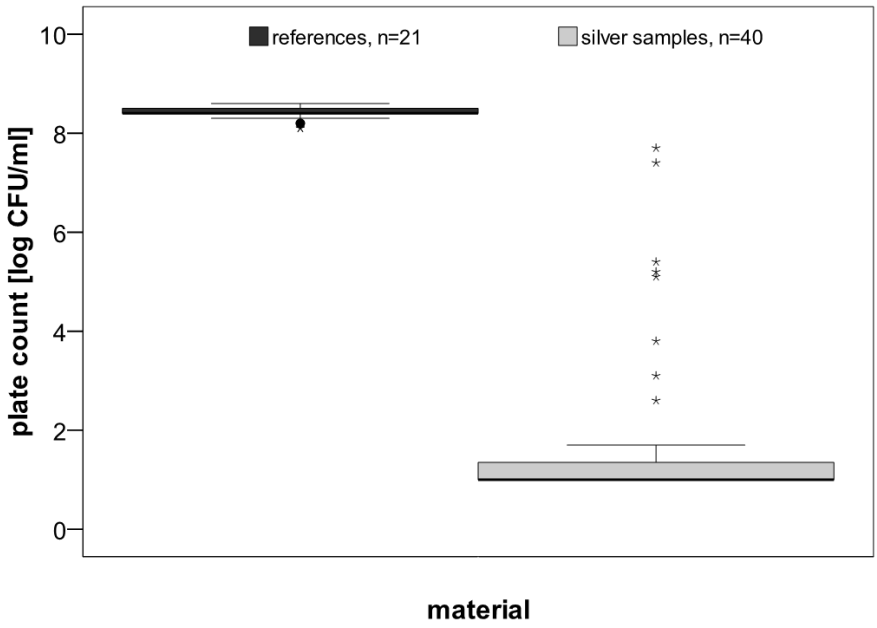

Figure 2.2.1 Boxplots of viable counts on reference PP sheets (n=21) compared to PP samples containing silver (n=40), after 24 h incubation at 35°C

Figure 2.2.2 shows the influence of carbohydrates on the antimicrobial activity of silver. In investigations with glucose, fructose, cyclodextrin, and saccharose containing inocula, differences between growth on reference and silver sheets were still significant. With all four test conditions, the median of surface bacteria on silver containing sheets was $\log_{10}$ 1 CFU ml^{-1}, which equates to the detection limit. Contrary to this, the median of surface bacteria on reference sheets was between $\log_{10}$ 4.6 and 8.5 CFU ml^{-1}. Hence, the reduction rate was between 3.6 (saccharose) and 7.5 (fructose) $\log_{10}$ units. After adding complex foods like starch or honey to the inocula, plate counts on reference and silver materials did not differ significantly. Furthermore, the spreading of surface bacteria concentration on silver sheets was, with an IQR of nearly 4 $\log_{10}$ units, noticeably high when starch is present.

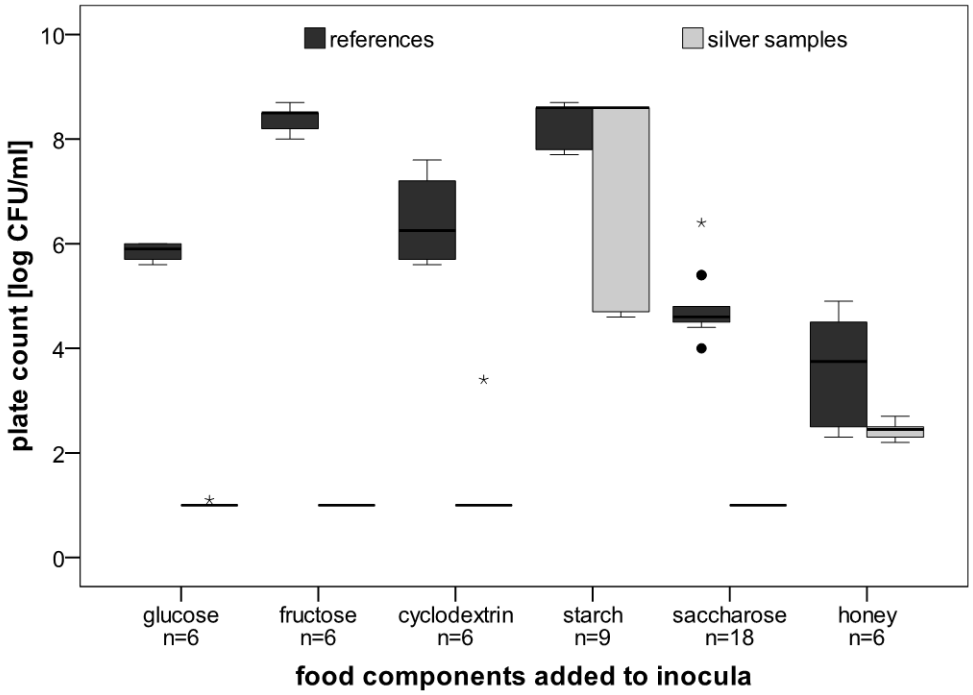

Figure 2.2.2 Boxplots of viable counts on reference PP sheets compared to PP samples containing silver with different supplements from the carbohydrate group added to the inocula, after 24 h incubation at 35°C

Figure 2.2.3 shows the total viable counts on reference and silver sheets after incubation with six different inocula containing amino acids or foods rich in proteins. The difference between growths on reference sheets and silver samples in the presence of arginine and prolin was with 6.6 resp. 7.5 $\log_{10}$-units significant. However, the IQR in the arginine investigation was 6.6, and in the prolin investigation 3.1 $\log_{10}$-units. Certainly, individual results with the silver samples with arginine and prolin in the inoculum were relatively wide spread. Results of test runs with cysteine differ from tests with other amino acids. Differences in plate counts on reference and silver materials were not significant. On both materials, viable counts on surfaces were close to the detection limit of $\log_{10}$ 1 CFU ml^{-1} except for one outlier each.

While adding complex foods rich in proteins to the inocula, bacterial concentration on silver samples and reference sheets did not differ significantly. In all three cases, growth levels were almost the same on both materials.

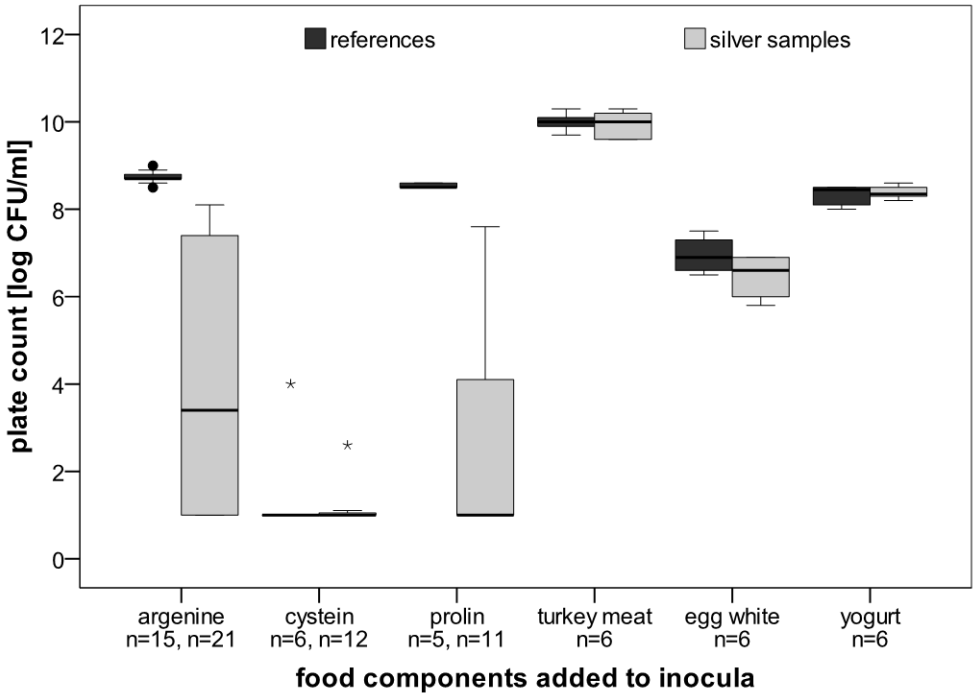

Figure 2.2.3 Boxplots of viable counts on reference PP sheets compared to PP samples containing silver with different supplements from the protein group added to the inocula, after 24 h incubation at 35°C

Investigations with fatty acids and vegetable fat resulted in only a marginal influence of these substrates being evidenced on the antimicrobial activity of silver (see Figure 2.2.4). The differences in growth on the silver and reference materials were still significant. The median of plate counts on the surfaces containing silver was between 6.2 – 7.8 $\log_{10}$-units lower than the plate counts on reference materials.

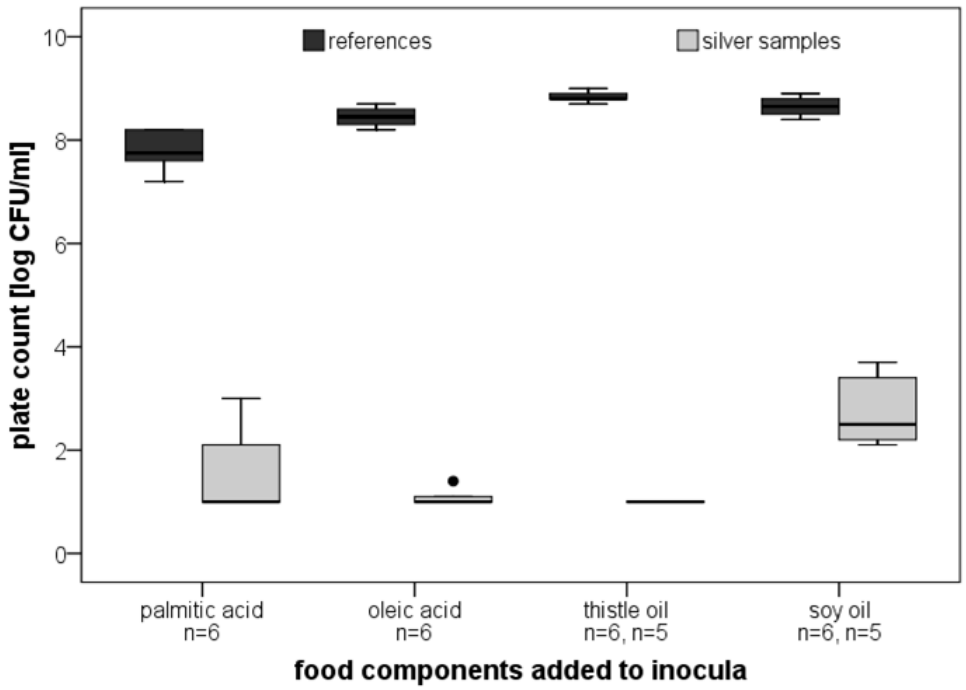

Figure 2.2.4 Boxplots of viable counts on reference PP sheets compared to PP samples containing silver with different supplements from the fat group added to the inocula, after 24 h incubation at 35°C

2.2.4 DISCUSSION

The general antimicrobial activity of silver containing materials was with 7.4 $\log_{10}$ units clearly proven in the part I investigations. The outliers on the samples containing silver may be explained by irregularities of the material itself or environmental influencing factors not regulated by the experimental design. For instance interactions between H_2S in the ambient air and silver surfaces while in storage could be a reason for irregularities on samples containing silver (Hund-Rinke et al. 2008).

The influence of food components on the antimicrobial attributes of silver varied. Apparently, the addition of protein-rich food, starch and honey reduced the antimicrobial activity of silver, while all other tested components did not inhibit the activity.

Although differences between bacterial counts on silver and reference sheets in the presence of arginine and prolin were still significant, the large spread of plate counts on silver sheets indicates an influence by these amino acids on the activity of silver. The inhibition of silver activity by arginine, but not of the nonpolar

amino acid prolin is also described by Gruen (1975). Cysteine overlaps the antibacterial effect of silver as it reduces bacterial counts by itself. The antimicrobial activity of cysteine from 0.1% w/v on was also shown by Liau et al. (1997). Thus, no conclusion on the effect of cysteine on the antimicrobial activity of silver can be drawn as a result of this investigation.

All complex foods from the protein group added to the inocula clearly neutralized silver activity. Inhibition of antimicrobial activity may be explained by reactions between silver and thiol-groups in food proteins. Egg white and yogurt both contains nearly all amino acids including the sulfur containing amino acids cysteine and methionin. Turkey meat has a high content of aspartic acid, glutamic acid, leucine, and lysine (Berlitz et al. 2008). Gruen (1975) showed that silver bonds to cysteine, methionin, arginine and lysine. This bonding potential could be an explanation for the reduction of antimicrobial activity by the addition of products rich in these amino acids. Tilton and Rosenberg (1978) and Schierholz et al. (2002) describe the inhibition of silver by protein rich residuals, too. They explain this effect as being caused by functional groups of food proteins that act as bonding partners for Ag^+ thus being not free for reactions with microorganisms. This is confirmed by Williams and Williams (1988). They showed by micro autoradiography that silver ions bound albumin at a rate of 3:1.

Similar to these results, activity of other antimicrobial substances like ozone, copper and chlorine dioxide are decreased in the presence of amino acids or protein rich products (Güzel-Seydim et al. 2004; Abushelaibi 2005; Vandekinderen et al 2009). Vandekinderen et al. (2009) explain the reduced antimicrobial activity of chlorine dioxide initiated by proteins being caused by a reduction in free ClO_2 volumes as ClO_2 reacts with aromatic amino acids, sulfur containing amino acids and with glutathione.

Investigations with carbohydrate sources added to the inocula made evident the fact that saccharose, fructose, glucose and cyclodextrin do not reduce the antimicrobial activity of silver.

The effectiveness of silver is only negatively influenced by two carbohydrate-constellations - starch and honey. It is possible that other starch ingredients than amylose or amylopectin are responsible for inhibition of silver, as cyclodextrin has a similar structure but does not affect the antimicrobial activity of silver. Starch, as well as honey has a protein content of 0.4%. This equates to 1.6 mg protein per 400 µl inoculum which may be a potential bonding partner for silver

ions. Furthermore, starch was visible less soluble than cyclodextrin. Thus it is possible that starch grains build a layer on surfaces containing silver and thereby separates the active silver ions from bacteria. This could also explain the strong variation in plate counts on silver sheets in the presence of starch. Vandekinderen et al. (2009) also assert that plate counts after exposure to chlorine dioxide subjected to starch varied strongly, but they give no explanation for that.

The medians of bacterial counts in the presence of honey on silver (log_{10} 2.5 CFU ml^{-1}) and reference sheets (log_{10} 3.7 CFU ml^{-1}) did not significantly differ. The reduced bacterial concentration also on the reference material compared to the starting concentration can be explained by the antibacterial effect of honey itself (Wahdan 1998).

Fatty acids and oils showed no inhibitory effect on the antimicrobial activity of silver as differences between plate counts on reference and silver sheets were with minimum 6.2 log_{10}-units still significant in all investigations. These results are contrary to the investigations of Gomez-Lopez et al. (2005) and Vandekinderen et al. (2009) in the context of other bactericides. They describe an inhibition of antimicrobial activity of intense pulsed light resp. chlorine dioxide caused by oil, assumedly caused by antioxidants or reactions with unsaturated lipids.

The presence of food components, especially protein rich food, apparently interferes with the antimicrobial activity of silver. This has consequences for the practical applications of silver as an additive in food contact surfaces. According to the results of this study, application of silver additives for contact materials where protein is involved (e.g. the diary or meat industry) or where starch rich foods are processed (e.g. in bakeries) delivers no additional benefit in the presence of these products. However, after cleaning and disinfection, a silver additive in food contact surfaces contributes to the killing of residual bacteria. Thus, silver provides an additional beneficial characteristic that is complementary to classical decontamination methods. In order to ensure antimicrobial activity also while protein or starch contact, either free Ag^+ concentration or Ag^+ intake into the bacterial cell has to be increased. The maximum amount of silver in food contact surfaces is limited by law. According to this limitation, the amount of free silver ions cannot be significantly increased by a change of the silver content. In contrast to this, the first approaches to

increase silver intake into prokaryote cells have already been developed by Loher et al. (2008). They strongly improved the antimicrobial activity of silver by the application of biodegradable silver carriers. The effect of the carriers is based on the bacteria's need for mineral uptake. This approach could also be a strategy to circumvent reversal of biocides activity by food.

As monosaccharides and fats apparently have no effect on the antimicrobial activity of the tested silver material, silver is able to significantly reduce bacterial load both after cleaning and disinfection as well as during production and storage. However, factors like long time stability, temperatures at which it is effective and contact time need to be further experimented before practical implementation of silver additives to food contact surfaces.

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CHAPTER 3

The application of ionizers in domestic refrigerators for reduction of airborne and surface bacteria[2]

[2]Published in the Journal of Applied Microbiology - see list of publications (Kampmann et al. 2009)

ABSTRACT

Aim To investigate the antimicrobial effect of ionization on bacteria in household refrigerators.

Methods and Results Ionizer prototypes were tested with respect to their technical requirements and their ability to reduce surface and airborne contamination in household refrigerators. Ion and ozone production of the tested prototypes were measured online by an ion meter and an ozone analyzer. The produced negative air ion (NAI) and ozone amounts were between 1.2 and $3.7 \cdot 10^6$ NAI cm^{-3} and 11 and 19 ppb O_3, respectively. To test the influence of ionization on surface contamination, different materials like plastic, glass and nutrient agar for simulation of food were inoculated with bacterial suspensions. The reduction rate was dependent on surface properties. The effect on airborne bacteria was tested by nebulization of *Bacillus subtilis* – suspension (containing spores) aerosols in refrigerators with and without an ionizer. A clear reduction in air contamination because of ionization was measured. The antimicrobial effect is dependent on several factors, such as surface construction and airflow patterns within the refrigerator.

Conclusions Ionization seems to be an effective method for reduction in surface and airborne bacteria.

Significance and Impact of the Study This study is an initiation for a new consumer tool to decontaminate domestic refrigerators.

3.1 INTRODUCTION

The importance of food safety and quality is steadily increasing. Food manufacturers are attempting to satisfy consumer requirements by the implementation of stage overlapping quality management systems. Over all stages, from primary production to point-of-sale, appropriate systems are implemented to ensure food safety and quality. Within the home, at virtually the very last stage of the food chain, the consumer alone bears the responsibility for proper food storage and safety (Lettmann 2007). Food handling and storage parameters are of high relevance in homes. The prevalent microclimate, mainly defined by storage temperature and storage humidity, has a huge impact on air and surface contamination that occurs within refrigerators. Excessive numbers of microorganisms increase the risk of cross-contamination and thus accelerate spoilage (Kreyenschmidt 2003; de Jong et al. 2008; Kampmann et al. 2008).

Refrigerator manufacturers actively try to support consumers with respect to storage hygiene and food safety by the integration of innovative methods like silver-containing inner liners for reduction in bacterial counts on interior surfaces (Kampmann et al. 2008). Another method, which is already used to reduce airborne bacteria in food industries, is ionization. Ionization is a nonselective method affecting a wide spectrum of air pollutants (e.g. dust particles) and biological contaminants (e.g. microorganisms, pollen and olfactory molecules (Krueger and Reed 1976; Comi et al. 2006). The functioning principle is based on the separation of outer electrons by single air molecule and their attachment to neutral molecules. Thus, positive and negative ions are formed (Forney et al. 2001; Fan et al. 2002). Mostly, the following primary ions are formed: H^+, H_3O^+, O^+, N^+, CO_4^-, O^-, OH^-, H_2O^- and O_2^-. The superoxide (O_2^-) represents around 95% of the negatively charged ions and is more stable than the other primary negatively charged ions (Forney et al. 2001; Wu et al. 2006a). Cluster ions accumulate on airborne pollutants, aerosols and microorganisms in the air and give them a positive or negative charge. Ions are discharged and air contaminants are oxidized. Microorganisms are killed or inhibited in their growth (Marin et al. 1989; Daniels 2001). Ions act as nucleophiles, thus supporting the hydrolysis of phospholipids in the cell wall (Belitz et al. 2001).

Along with the release of negatively charged air ions, the ionization process is accompanied by the release of ozone (Boub 2005). Ozone is a potent oxidizer and thus has an antimicrobial activity itself. Ozone attacks vital cellular components

like sulfhydryl groups, amino acids of enzymes, peptides and proteins, thus consequently destroying microorganisms (Guzel-Seydim et al. 2004). It has an even greater degree of antimicrobial activity than the superoxide and attaches to the surfaces of bacterial cells (Guzel-Seydim et al. 2004; Jay et al. 2005). In combination with ions, synergistic effects appear (Forney et al. 2001; Fan et al. 2002).

At higher concentrations, ozone is harmful to humans and also to food products. The threshold limit value (TLV) for ozone is between 50 and 100 ppb, depending on the intensity of work (NIOSH 1993). Analyses by Goldstein et al. (1992) have shown that the ozone level produced by ionizers does not exceed the level of normal ambient conditions (30–50 ppb) if the corona discharge does not exceed 3 ± 0.1 kV. In contrast to these results, Song et al. (2000) measured a concentration up to 250 ppb at a voltage of around 2.5 kV.

Along with the application of ionization in food industry, it is already used in a wide range of other industries, e.g. in dust-free rooms and in various medical technologies. Ionization is particularly important where both dust reduction and bactericidal properties are required (Forney et al. 2001; Arnold and Mitchell 2002; Boub 2005; Comi et al. 2006). However, up to the present time, studies on the application of this method to the field of domestic refrigeration are rare.

In this study, ionizers (Xi'an KongHong Information Technology Co., Xi'an, Shaanxi, China) were tested for their ability to reduce surface contamination and airborne bacteria and for the feasibility of their being used in household refrigerators. The power supply of the tested ionizer converts the mains AC voltage into a smoothed DC voltage and provides the ionizer with the necessary input voltage of 12 V DC. The negative high voltage between the electrodes was 4.5 ± 0.5 kV DC.

3.2 MATERIALS AND METHODS

To investigate the antimicrobial effect of ionization processes and to test its application in household refrigerators, a variety of different experiments were performed. In an initial stage, the technical performance parameters of the prototypes (single module and within a plastic housing) were tested. In the next stage, the effect of ionization on bacterial counts on different surfaces was analyzed in static cooled refrigerators. In the third stage, the antimicrobial effect

of ionization on airborne bacteria was investigated in refrigerators, with special focus on the impact of static and dynamic cooling conditions. For use in household refrigerators, the ionizers were tested as a single module and integrated in a plastic housing prototype (Figure 3.1). The analysis of an ionizer module within a plastic housing was additionally applied to simulate final application conditions within refrigerators, providing a protection for the ionizer module and the consumer at the same time.

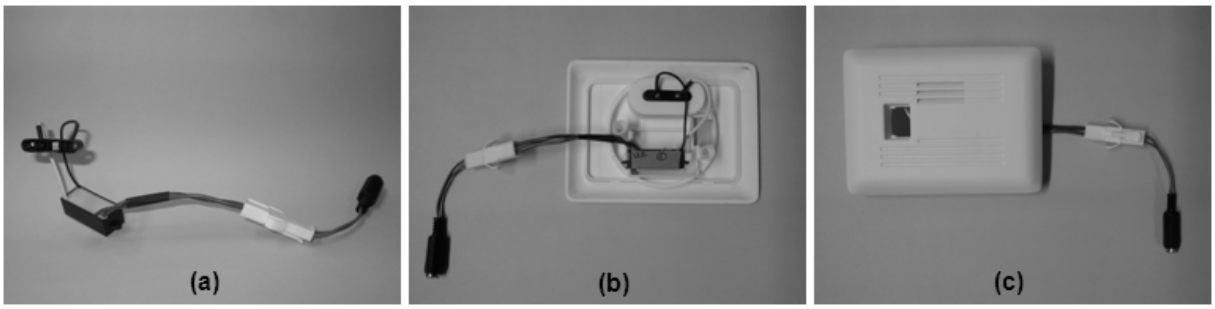

Figure 3.1 Picture of the Kong Hong ionizer as a single module (a), and integrated in a plastic housing (rear view: b, frontal view: c)

Measurement of ion and ozone production during ionization

In order to determine the negative air ion (NAI) concentration, an ion meter was used (Air Ion Counter 2000; AlphaLab, Salt Lake City, UT, USA). The ion meter transports air samples at a rate of 800 cm^3 s^{-1} to a collector plate with negative polarity, and calculates the number of elementary charges per second, which hit the collector plate. The air ion meter has a measuring range of $8 \cdot 10^6$ NAI cm^{-3}, with a sensitivity of 10 NAI cm^{-3}. The sampling is performed within a distance of 5 cm from the ionizer electrode for 5 min.

Along with the ion concentration, the ozone concentration accumulating within the refrigerator atmosphere was measured. The evolution of the ozone level was monitored online by the use of the ozone analyzer O341M (Environment S.A., Paris, France). The measurement principle is based on ultraviolet absorption of ozone molecules at 254 nm. The ozone concentration is determined by the calculation of the deviation of the gas sample itself and the gas sample from which ozone has been filtered out by a catalytic converter. The sampling gas is directed to the measuring bulb via the ozone filter every 10 s to determine the transmission rate of the ozone-adjusted sample, which is then compared with the transmission rate of the ozone containing sampling gas. The ozone concentration is calculated by the application of the Beer–Lambert law, adjusted to standard

conditions by integrated temperature and pressure sensors. The ozone analyzer provides a lower detectable limit of 0.1 ppb O_3 and a linearity of ±1% of F.S. (functional specification). The sample flow rate is set to 60 l h^{-1} with a response time of 30 s. The gas samples were directly taken from the refrigerator atmosphere. A 5-mm diameter Teflon tube was fed from the sampling port of the ozone analyzer into the refrigerator compartment via a hole bored in the refrigerator sidewall. The sampling tube was placed opposite the ionizer module with a separation distance of 20 cm. The development of the ozone concentration was analyzed for c. 2–3 h per ionizer. In order to determine the effects of the storage atmosphere on the ozone concentration, both temperature and relative humidity (RH) were logged in parallel using temperature and humidity loggers (EBI-2TH-611; Ebro, Ingolstadt, Germany) with a measuring accuracy of ±2% RH and ±0.3°C.

Antimicrobial activity of ionization on surface bacteria

As it became evident in pretests that there was no antimicrobial activity apparent on surfaces resulting from ionization in refrigerators with a volume of 287 l (KSR30425; Bosch, Giengen, Germany), the test volume was reduced. Investigations were conducted in two vegetable drawers (volume 16 l) of two refrigerators. One of the drawers was equipped with the single ionizer module (Xi'an KongHong Information Technology Co.), the other drawer served as a reference. The ionizer was fixed to the inner liner of the disinfected vegetable drawer. To ensure a closed test space, the tops of the drawers were covered with glass plates. The experiment was conducted at 8°C, as bad storage conditions should be simulated. To test the antimicrobial effect of ionization on the surface area, three different bacterial suspensions were prepared from *Bacillus subtilis* (DSM no. 704), *Lactobacillus brevis* and *Pseudomonas fluorescens* each. The *Lactobacillus* and *Pseudomonas* strains were descended from the stock culture of the Institute of Animal Science, Bonn, Germany.

One loop of the described strains each was taken from the culture plate and transferred to a separate tube for every culture with 9 ml of saline solution and 1 ml of nutrient broth. After incubation (24 h / 25°C), the subcultures were diluted in saline solution up to 10^3 CFU ml^{-1} (*B. subtilis*, *Lact. brevis*) and 10^4 CFU ml^{-1} (*Ps. fluorescens*).

The antimicrobial effect was investigated on three different materials: sterile plastic material (to simulate refrigerator inner liners), sterile glass plates (for

simulating of glass shelves) and selective agar plates (to simulate food products). The plastic material and glass plates (placed in sterile Petri dishes) were inoculated with 500 µl of three bacterial suspensions each (*B. subtilis*, *Lact. brevis*, and *Ps. fluorescens*). The selective agar plates (depending on inocula, for *B. subtilis*: CASO (casein-soya-peptone) agar; Roth, Karlsruhe, Germany; for *Lact. brevis*: de Mann Rogosa Sharpe or MRS agar; Merck, Darmstadt, Germany, and for *Ps. fluorescens*: CFC (cephaloridine fucidin cetrimide) agar plus CFC Supplement; Oxoid, Hampshire, GB) were inoculated with 100 µl of the appropriate bacterial suspensions. The Petri dishes with the three different surfaces (in duplicate each with three different bacterial suspensions) were placed in the previously described test chambers both with and without the ionizer. All samples were stored at 8°C without lids to ensure that the ions have contact with the surface without prior drying of inoculums. After a 72 h storage period, the Petri dishes with the inoculated plastic and glass plates were treated according to the pour plate method with the appropriate selective agars. The inoculated agar plates were incubated without further treatment. Colony forming units of *B. subtilis* (aerobic) and *Lact. brevis* (anaerobic) were enumerated after 72 h incubation at 30 C, and *Ps. fluorescens* after 48 h at 25°C (aerobic). The viable counts on surfaces stored in reference test chamber were compared with the viable counts on surfaces stored in the test chamber with the ionizer.

Antimicrobial activity of ionization on airborne bacterial concentration

For testing the antimicrobial effect of ionization to airborne bacteria, two refrigerators, identical in construction were used (KSR30425; Bosch, Giengen, Germany; volume 287 l). A drawer with a holder for Petri dishes was installed to define an exact positioning and to allow the changing of Petri dishes at predetermined time intervals without opening the refrigerator door and to prevent contamination from outside. A lockable opening was installed in the door to facilitate the spraying of microorganisms. A ventilator including an on/off switch was integrated in both refrigerators to simulate dynamic and static cooling conditions.

One of the refrigerators was equipped with the previously described electronic ionizer (Xi'an KongHong Information Technology Co.). The ionizer was tested as a single module and integrated in a plastic housing with slits (Figure 3.1). The second refrigerator served as a reference. During the experiments, the shelves were taken out of the refrigerators to allow an equal distribution of bacteria and

spores within the refrigerators. In one of the refrigerators, the ionizer was fixed on the left side of the inner liner, close to the slab and the ventilator, so that the negative ions were transported with the air flow of the refrigerator. For the static experiments, the ventilators were switched off. Disinfection was conducted in two steps: (i) the evening before a new experiment started, both refrigerators were cleaned and disinfected with Melisepthol rapid (B. Braun, Melsongen, Germany); (ii) proximately before the start of the experiment, the refrigerators were wiped out with ethanol (80%). After volatilization of ethanol (30 min), the ionizer and refrigerators (adjusted to 8°C) were switched on.

Preparation of inoculum

The freeze-dried *B. subtilis* (DSM no. 704) culture was cultivated according to the instructions of DSMZ and incubated for 24 h in a 10-ml nutrient broth (Roth, Karlsruhe, Germany) at 30°C. One loop of this *B. subtilis* suspension was used to inoculate 500 ml of fresh, sterile nutrient broth. To assure generation of endospores, the suspension was incubated for not <5 days at 30°C. Development of spores was verified by gram staining and successional microscopy. The viable cell count was each time determined immediately before use and was constantly around 10^6 CFU ml^{-1}. After four weeks, a new suspension was prepared in the same way.

For homogeneous dispersion of *B. subtilis* suspension (containing spores), 4 ml of the described suspension was nebulized using an airbrush pistol (Beginner ESB 100; Revell GmbH and Co. KG, Bünde, Germany) in both refrigerators. At defined time intervals (0.5, 1, 1.5, 2 and 2.5 h), the airborne bacterial concentration was determined by the use of the sedimentation method with four CASO agar plates (Roth) each for 30 min. Counting of CFU followed after 72 h incubation at 30°C.

Reduction was calculated by the following formula:

$$\text{Reduction} = \frac{T_{x,\text{Re}} - T_{x.Ion}}{T_{x,\text{Re}}} \times 100 \qquad (1)$$

where $T_{x.ion}$ is the Ø (average) airborne bacteria, x h after incubation in refrigerator with ionization and $T_{x,Re}$ is the Ø airborne bacteria, x h after incubation in refrigerator without ionization.

Statistical analysis

Kolmogorow–Smirnow test was performed to verify the normality of results. Differences in ion and ozone production between the single module and the housing prototype were analyzed using the two-sample t-test in Minitab Software (Minitab Inc., State College, PA, USA). Differences in bacterial growth were analyzed for significance by the t-test for independent samples. Analyses were performed using SPSS 17.0 for Windows© (SPSS Inc., Chicago, IL). The level of significance was defined as follows: (i) $p > 0.05$ indicating no significance (n.s.) and (ii) $p \leq 0.05$ indicating significant difference (*).

3.3 RESULTS

Production of ozone and ion during ionization *(technical performance parameters)*

The single ionizer module shows an average ion release of $3.7 \cdot 10^6$ ions cm^{-3}. The placement of the ionizer into the prototype housing shows a significant decrease in the ion release to $1.2 \cdot 10^6$ ions cm^{-3}.

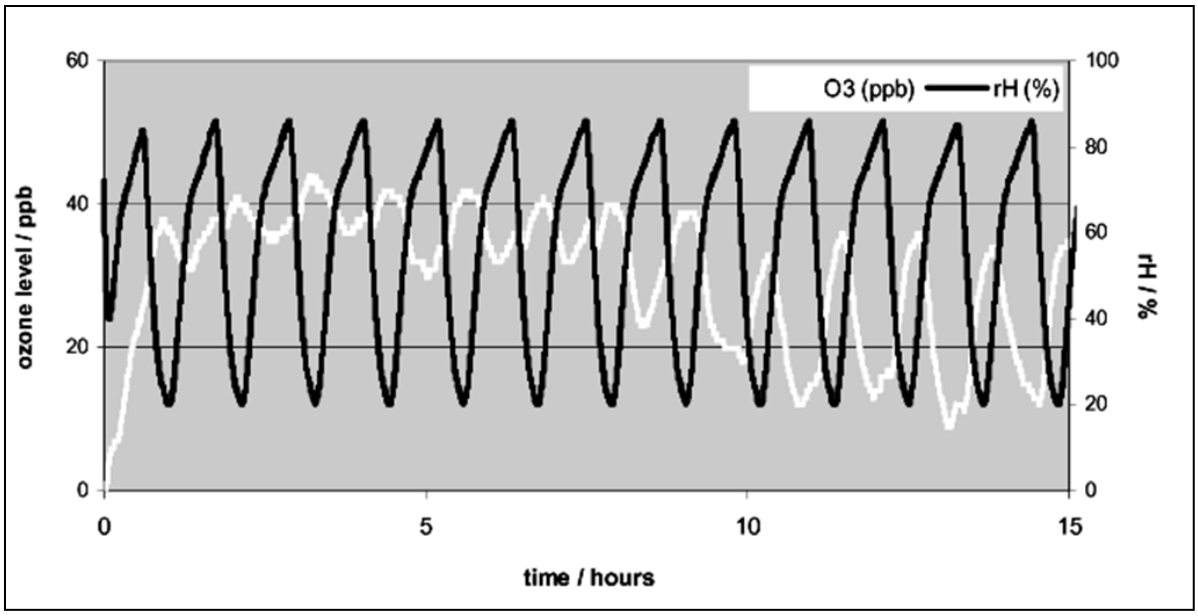

Figure 3.2 Impact of relative humidity on the ozone concentration within the refrigerator atmosphere

The ozone level within the refrigerator reaches a stable level after 20 min of ionizer run-time. Because of the high dependence on the RH within the refrigerator atmosphere, the ozone concentration fluctuates with an average amplitude of 10 ppb O_3 (Figure 3.2). At high levels of RH, the ozone concentration is low and vice versa. The ozone concentration within the analyzed appliances shows a mean of 19 ppb O_3 for single modules, with maximum concentration of 28 ppb O_3. The ionizer housing prototype induces a significant decrease in the ozone concentration within the refrigerator atmosphere, showing a mean of 11 ppb O_3.

Antimicrobial activity of ionization on bacterial count in surfaces

Tables 3.1 - 3.3 show the bactericidal effect of ionization on different surfaces. It becomes obvious that reduction rate is dependent on the material used and the bacterial suspension used. Table 3.1 shows the effect of ionization on plastic surfaces. For *B. subtilis*, an average reduction of 0.7–0.9 $\log_{10}$ units was calculated while the viable cell counts of *Lact. brevis* was reduced between 1.0 and 2.1 $\log_{10}$ units on average. The first test run with *Ps. fluorescens* (P1) could not be determined, because surfaces of plates stored in both test chambers were overgrown with bacteria. However, visually colony numbers on the plates stored in the chamber with ionizer were clearly reduced. In the second test run, the reduction rate was higher than 2.5 $\log_{10}$ units.

Table 3.1 Log_{10} reduction of bacteria on inoculated plastics in a defined test chamber by ionization

Test	Bacterial strain and agar used	Duration (days)	Average CFU after storage in reference refrigerator ($\log_{10}$ CFU/dish)	Average CFU after storage in refrigerator with ionizer ($\log_{10}$ CFU/dish)	$\log_{10}$ reduction
P1	*Bacillus subtilis* (PC)	3	2.2	1.3	0.9
	Lactobacillus brevis (MRS)		2.1	1.1	1.0
	Pseudomonas fluorescens (CFC)		> 2.5	> 2.5	n.e.
P2	*Bacillus subtilis* (PC)	3	1.8	1.1	0,7
	Lactobacillus brevis (MRS)		2.1	0	2.1
	Pseudomonas fluorescens (CFC)		> 2.5	0	> 2.5

n.e.: not evaluable

In the reference chamber, *Ps. fluorescens* growth was well above 300 CFU per plate (≈ 2.5 $\log_{10}$ CFU per petri dish). In contrast to this, in the ionizer-containing chamber, no bacteria were determined after storage period. Table 3.2 shows the results of testing the effect of ionization on incubated glass surfaces. Viable count of *B. subtilis* on glass plates stored in the reference test chamber is between 1.0 and 1.5 $\log_{10}$ CFU per dish on average compared to 0–0.5 $\log_{10}$ CFU per dish on average in the chamber with ionizer. This is equal to a $\log_{10}$ reduction of 1.0 each. Using *Lact. brevis* as inoculum, in the reference chamber, 1.9 $\log_{10}$ CFU per dish on average was detected, while in both test runs no bacteria were determined on

the glass plates after storage in the test chamber with ionizer. This is equal to a reduction rate of 1.9 $\log_{10}$ units. A similar effect was determined with *Ps. fluorescens*. In the reference test chamber, glass plates were overgrown with *Pseudomonas* in both test runs, while in the test chamber with ionizer, nearly all bacteria were killed. This implies a reduction rate higher than 2.2 $\log_{10}$ units. Table 3.3 shows the effect of ionization on nutrient agar for simulation of food products. It becomes obvious that in test runs A1–A3 no antimicrobial activity was measured.

Table 3.2 Log_{10} reduction of bacteria on inoculated glass slides in a defined test chamber by ionization

Test	Bacterial strain and agar used	Duration (days)	Average CFU after storage in reference refrigerator ($\log_{10}$ CFU/dish)	Average CFU after storage in refrigerator with ionizer ($\log_{10}$ CFU/dish)	Log_{10} reduction
G1	*Bacillus subtilis* (PC)	3	1.0	0	1.0
	Lactobacillus brevis (MRS)		1.9	0	1.9
	Pseudomonas fluorescens (CFC)		> 2.5	0.3	> 2.2
G2	*Bacillus subtilis* (PC)	3	1.5	0.5	1.0
	Lactobacillus brevis (MRS)		1.9	0	1.9
	Pseudomonas fluorescens (CFC)		> 2.5	0	> 2.5

Table 3.3 Log_{10} reduction of bacteria on incubated agar plates in a defined test chamber by Ionization

Test	Bacterial strain and agar used	Duration (days)	Average CFU after storage in reference refrigerator (log_{10} CFU/dish)	Average CFU after storage in refrigerator with ionizer (log_{10} CFU/dish)	log_{10} reduction
A1	*Bacillus subtilis* (CASO)	3	2.2	2.2	-
	Lactobacillus brevis (MRS)		> 2.5	> 2.5	-
	Pseudomonas fluorescens (CFC)		> 2.5	> 2.5	-
A2	*Bacillus subtilis* (CASO)	3	2.3	2.3	
A3	*Bacillus subtilis* (PC)	3	1.7	1.7	-
	Lactobacillus brevis (MRS)		> 2.5	> 2.5	-
	Pseudomonas fluorescens (CFC)		> 2.5	> 2.5	-
A4	*Bacillus subtilis* (CASO)	3	1.2	0.7	0.5

- no antimicrobial activity

However, the morphology was changed by ionization, as the sizes of colonies were much smaller because of the influence of ionization compared to reference colonies. Only in the last test run (A4), a slight reduction in *B. subtilis* was measured. The average CFU on reference plates was 1.2 log_{10} CFU per plate compared to 0.7 log_{10} CFU per plate with ionization. This is equal to a reduction of 0.5 log_{10} units by ionization.

Antimicrobial activity of ionization on airborne bacterial concentration

The investigations show that ionization is an appropriate and effective method for reducing airborne bacteria in domestic refrigerators. Figure 3.3 shows the development of airborne bacteria in static refrigerators (ventilator switched off) with and without an ionizer single module. It becomes evident that after 0.5 h total viable count (TVC) is 2.3 log_{10} CFU per plate in average in the refrigerator with the ionizer and 2.5 log_{10} CFU per plate in the reference refrigerator. This is equal to a moderately significant reduction of 0.2 log_{10} units. At all other measurement points, the rate of reduction is highly significant with 0.5–0.7 log_{10} units. Figure 3.4 shows the development of airborne bacteria in dynamic refrigerators (with ventilator) with and without a single module ionizer. The difference between bacterial count in the reference and ionizer refrigerators is

significant at all measurement points except from the starting concentration. After 0.5 h, the difference in average is 0.2.

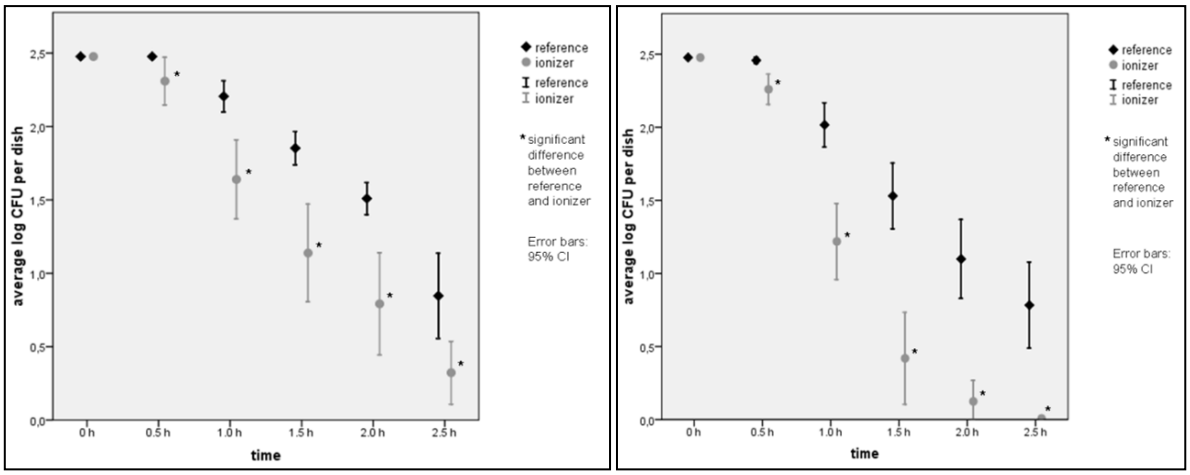

Figure 3.3, 3.4 (3.3) Average airborne bacteria (*B. subtilis*) in $\log_{10}$ CFU per dish in static refrigerators with and without ionizer (single module) at defined points in time (n = 16) (3.4) Average airborne bacteria (*B. subtilis*) in $\log_{10}$ CFU per dish in dynamic refrigerators with and without ionizer (single module) at defined points in time (n = 12)

At the other measurement points, differences are between 0.8 and 1.1 $\log_{10}$ units in average. The development of airborne bacteria in static refrigerators with and without ionizer within a plastic housing is shown in Figure 3.5. Comparing the average of airborne bacteria in the reference and the ionizer-containing refrigerator in variations of time, at all measurement points the calculated reduction by ionization is not significant. The difference between the average airborne bacteria per dish in the reference refrigerator and in the refrigerator with ionizer is highest 0.14 $\log_{10}$ units 0.5 h after the starting point.

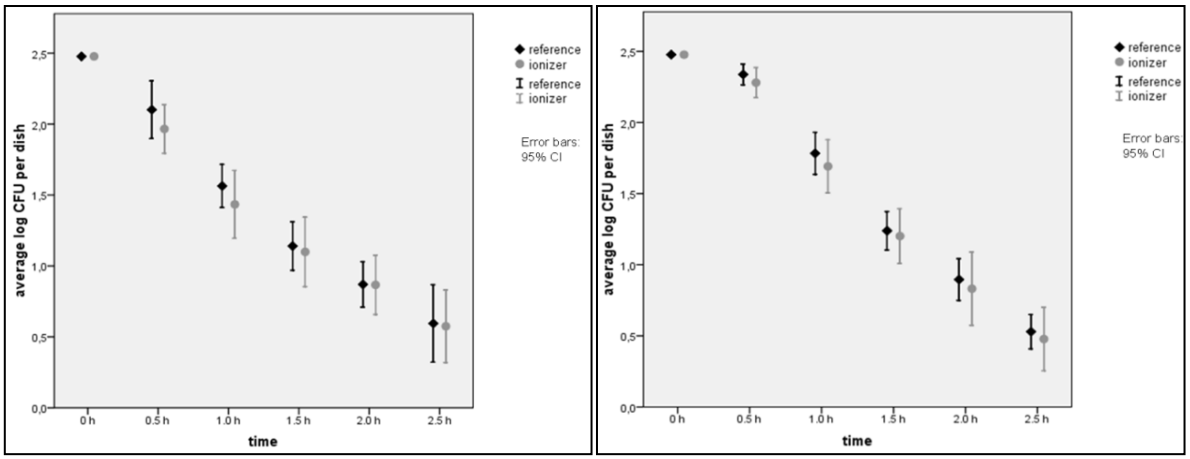

Figure 3.5, 3.6 (3.5) Average airborne bacteria (*B. subtilis*) in $\log_{10}$ CFU per dish in static refrigerators with and without ionizer, integrated in plastic housing, at defined points in time (n = 10) (3.6) Average airborne bacteria (*B. subtilis*) in $\log_{10}$ CFU per dish in dynamic refrigerators with and without ionizer, integrated in plastic housing, at defined points in time (n = 12)

Also in the refrigerators with ventilators, the differences between the average air contaminants at different measurement points are not statistically significant (Figure 3.6). The average reduction by the ionizer in the prototype housing rate varies between 0.04 and 0.09 $\log_{10}$ units. Comparing the antimicrobial activity of ionizers as a single module and as integral part of a plastic housing in refrigerators with and without ventilator, bacterial reduction is much higher when individual ionizer modules are used.

3.4 DISCUSSION

For the reduction in surface and airborne bacteria, ion amounts of c. $5 \cdot 10^4$ - $5 \cdot 10^6$ ions cm^{-3} are necessary. The tested ionizer prototype produces an ion amount of $1.2–3.7 \cdot 10^6$ ions cm^{-3}, which is within the target zone. The by-product ozone supports the antimicrobial effect of ionization. If no ozone is present, the antimicrobial effect is much lower than the combined effect of ions and ozone (Song et al. 2000; Forney et al. 2001; Palou et al. 2001; Fan et al. 2002). Nevertheless, it has to be considered that excess ozone concentrations can lead to decolorization of food surfaces as well as off-flavors resulting from oxidizing processes (Fan et al. 2002). Hundred parts per billion should therefore not be exceeded (NIOSH 1993). Ozone concentrations produced by the prototype tested varied between 10 and 45 ppb, which are still within the limit values. For both, ion and ozone concentrations, the RH has a reductive effect on their activity, as

water molecules in the air are potential reaction partners for the ions (Forney et al. 2001; Wu et al. 2006a). The effect of humidity on ozone production also became visible in our investigations (see Figure 3.2).

The single module ionizer tested provides a good degree of antimicrobial activity as to surface and airborne bacteria in refrigerators. There are several studies that describe the antimicrobial activity of ionizers in other industries than the food industry. Mitchell et al. (1998, 2000), Gast et al. (1999), and Holt et al. (1999) showed the general possibility of reducing surface and airborne bacteria by ionization. Also, Seo et al. (2001) proved the antimicrobial activity of ionization against *Salmonella enteritidis* with a reduction rate of 95–99%. The investigations made evident that the surface material and the structure influence the antimicrobial activity. A decreased level of reduction in inoculated nutrient agar compared to inoculated plastic or glass was measured. This could be because of the different surface contribution as described by Lajcikova et al. (1999) and Yao et al. (2005). In contrast to plastic and glass, the surface of agar is more porous. Furthermore, inactivation of ions is favored by the water content of the agar. This implies for the integration of ionizers in refrigerators that the bacterial concentration on refrigerator materials like refrigerator interior surfaces and glass plates can be decreased by this method. On the contrary, first investigations with inoculated agar plates for simulation of food products indicate no or a low reduction in bacterial count on food resulting from ionization. Moreover, the used bacterial strain influences the rate of reduction in plastic and glass surfaces. In three of four test runs, the reduction in the gram-negative strain *Ps. fluorescens* by ionization was higher than 2.2 $\log_{10}$ units.

Using the Gram-positive strains *B. subtilis* or *Lact. brevis* as inocula, reduction rate was between 1.0 and 2.1, respectively between 0.7 and 1.0 $\log_{10}$ units. This indicates a lower sensibility of Gram-positive strains caused by differences in the cell wall.

Next to the antimicrobial effect on surfaces, ionization has an inhibitory effect on airborne bacteria. The rate of reduction is strongly decreased by the tested prototype plastic housing. The tested single module ionizer leads to reduction up to 1.1 $\log_{10}$ units, while the ionizer integrated in a plastic housing reaches a maximum reduction level of 0.14 $\log_{10}$ units. A possible reason for this observation is that isolating materials like the plastic housing can be electrostatically loaded and thus obstruct the passage of ions (Anon. 1990). In

addition, measurements indicate that the rate of ozone emission is reduced from 19 to 11 ppb by the plastic housings. As ozone has an antimicrobial effect by itself, the reduction in ozone concentration leads to a reduction in antimicrobial activity. Therefore, the tested prototype is not suitable for the use as ionizer housing. It has a surface impressed with whorl shapes, and this fosters the discharge of ions and probably avoids ozone emission (Lajcikova et al. 1999). However, for the integration of ionizers into refrigerators, a plastic housing is unavoidable, as it protects the ionizer emitters from dust, humidity, and mechanical damage. Certainly, the housing should have a rounded, smooth surface to avoid the discharge of ions and large slits to allow the emission of ions and ozone. Complementary to the ionizer type, air circulation in the refrigerator has a great influence on antimicrobial activity on airborne bacteria. Investigations showed that the reduction rate in dynamic refrigerators is much higher than that in static refrigerators. This could be because of the increased mobility through the use of the ventilator.

Moreover, the positioning of the ionizer within the refrigerator is of crucial importance. The size of the test chamber compared to the distance of the ionizer from the inoculated surface has a great influence on the antimicrobial activity level (Wu et al. 2006a, b). Our investigations on surface contamination also showed that the antimicrobial effect of ionizers decreases with the increase in the distance of the ionizer to the bacteria. No bactericidal ionization effect was detected on inoculated surfaces in a normal refrigerator (287 l) but in the smaller test chamber within the refrigerator (16 l).

To recapitulate, investigations made evident that the reduction rate achieved by ionization depends on several factors, these being: the constructional layout and material properties of the ionizer housing, air circulation within the refrigerator and the interior volume.

Apart from all of the factors influencing antimicrobial activity because of ionization, the olfactory aspects have to be considered and analyzed. Contrary to the view expressed by Daniels (2001) that ionization can neutralize odor, random sampling in this study detected an ‘unnatural’ smell or odor (such as is emitted from some electronic devices) in the refrigerators with the ionizer. One explanation for this effect could be that the by-product, ozone, has a pungent characteristic odor (Guzel-Seydim et al. 2004).

Beyond these results, investigations are required concerning the influence of reactive species to refrigerator materials as well as to food products. All in all, ionizing is a possible method for reduction in surface and airborne bacteria. However, before the integration in domestic refrigerators, further studies are required to assure homogenous distribution of ions and ozone within the refrigerator.

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CHAPTER 4

Evaluation scheme for the testing of antimicrobial methods for their applicability in the food industry

In recent years, several antimicrobial agents and methods have been examined and evaluated concerning improving hygiene in the food chain. They range from biocides, organic acids, and bioactive polymers up to plant extracts. Silver usage and ionization as two examples of antimicrobial methods have already been described. Both methods are generally capable of reducing the number of surface and airborne bacteria. With standard measuring procedures (e.g. JIS Z 2801), reduction of surface bacteria > 2 $\log_{10}$-steps were detected after 24 h at 35°C. However, investigations made evident, that the reduction rate is strongly influenced by environmental conditions, such as temperature or nutritional content. Thus, for the successful implementation of these technologies into the food supply chains, the special conditions obtaining there have to be considered. Normally, temperatures in perishable food chains during processing are rarely above 10°C resp. above 7°C during storage. Furthermore, most perishable products, like meat and meat products, fish or dairy products, are rich in nutritionals. Components of these foods can interfere with the antimicrobial activity of antimicrobial agents. Moreover, food residuals on contact surfaces, such as conveyor belts in the food industry or inner liners in domestic refrigerators provide an optimal nutritional basis for the growth of microorganisms. Hence, it is of vital importance to adapt the testing procedure to the actual environmental conditions which are found in applications in the food supply chain. Figure 4.1 gives an overview on different influencing factors on the rate of antimicrobial activity. The factors can be subdivided into three groups: antimicrobial agents, microorganisms, and the ambient environment. Each single factor influences the rate of antimicrobial activity. Besides that, the factors also affect each other. The environmental factor of temperature, for example, has an impact on the antimicrobial agents as the release rate of the agents increases with increasing temperature. On the other hand, temperature is one of the most important influencing factors on the growth rate of microorganisms, thus having a direct effect on the bacterial count. Due to these interactions, making predictions about the precise antimicrobial activity under certain practical conditions is a very complex challenge. For a better understanding of the influencing factors on the rate of antimicrobial activity, the effect of the single factors will be described first.

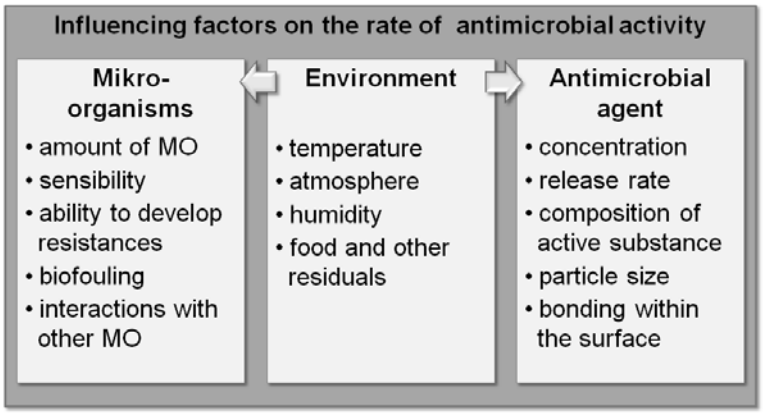

Figure 4.1 Influencing factors on the rate of activity of antimicrobial technologies to reduce surface and airborne bacteria

4.1 EFFECT OF SINGLE INFLUENCING FACTORS ON THE ANTIMICROBIAL ACTIVITY

Since silver is the most widely used antimicrobial additive for applications within the food supply chain (Brody et al. 2001; Appendini and Hotchkiss 2002) the parameters related to the rate of antimicrobial activity will be subsequently illustrated by materials containing silver.

The rate of antimicrobial activity is strongly dependent on the *properties of the antimicrobial agent*. With an increasing concentration of silver ions, the antimicrobial effect of silver increases (Sondi & Salopek-Sondi 2004; Shrivastava et al. 2007). Thereby, it is not the concentration within the surface, but the rate of silver release from the surface that is of paramount importance (Ovington 2004). Monteiro et al. (2009) state that the characteristics of silver release depend on the silver compound as well as on the polymer matrix were it is bond. For the integration of silver into polymers, appropriate carriers are needed. Examples of carriers are glass particles, glass foam, polymer foils, or hydro gel. They are based on zeolite, boraic, phosphate, or borosilicate glass, sodium-zirconium - glass or calcium phosphate. These carriers enhance the ion release out of the material by extension of the reaction area (Ovington 2004; Loher et al. 2008). Each compound has its specific characteristics relating to ion release and antimicrobial activity. For instance, silver nitrate is easily soluble and ionizes rapidly, while silver chloride is mainly insoluble in aqueous solutions and thus releases fewer ions (Lansdown 2004). Silver sulfadiazine has a special role as both components - silver and sulfadiazine - have an antimicrobial effect (Russel and Hugo 1994).

Also the size of silver molecules has a direct influence on silver activity, respectively the release, as the attributes of silver changes by nano-scaling. Nano-sized silver particles are much more reactive than macro sized silver ions and thus ionize more rapidly (Jang et al. 2007; Loher et al. 2008). Furthermore, the surface area of nano sized silver based on its mass is bigger than those of macro sized silver (Fries et al. 2009). Thus, more ions can be released to the environment (Lok et al. 2007). Moreover, nano sized silver is able to react directly with cell membranes or enter bacterial cells, because of their size (Morones et al. 2005). Choi and Hu (2008) found out that the 5 nm sized Ag nano-particles show the largest inhibition rate.

On the one hand, the level of silver release is important for the antimicrobial activity. For the threshold limit level of silver, different limits exist. Shrivastava et al. (2007) state that the threshold limit value of silver toxicity is, depending on bacterial strain, between 8 and 70 mg l^{-1}. Similar inhibitory concentrations of 1-10 mg l^{-1} are indicated by Jansen (2002). The summary of the UNO study describes an even lower threshold limit of silver toxicology against micro-organisms from 1-5 µg l^{-1} (IPCS 2002). On the other hand, silver migration into food products must be within proscribed limits to avoid a risk to the consumer of excess silver ingestion.

Further influencing factors on the rate of activity are the *microorganisms* itself. The bacterial concentration influences the rate of activity. A higher bacterial count on the surface leads to a decrease of the antimicrobial effect (Kampf et al. 1998; Zhao et al. 1999; Bidlas et al. 2008). Also the sensitivity of each microorganism has an important contribution on the rate of antimicrobial activity. For example Shrivastava et al. (2007) showed that *Escherichia* and *Salmonella* are much more sensitive to silver than *Staphylococci*. Similar results are described in chapter 2.1. The decrease of the *Pseudomonas* count was far higher compared to the decrease of *Lactobacillus* and *Staphylococcus* on silver containing surfaces. The differing sensitivity of microorganisms is a particular problem, where spoilage organisms are influenced as to their growth but pathogens are growing without hindrance. One reason for the differing sensitivities of gram positive and gram negative microorganisms against biocides is the different contribution of the cell wall (Jansen et al. 1994; Shrivastava et al. 2007).

Also other factors influence the sensitivity of bacteria to biocides, since within the group of gram positive or gram negative bacteria, strong variations in sensitivity to antimicrobial compounds are possible. For example, in chapter 2.1, a reduction rate against *Lactobacillus* of $\log_{10}$ 1.0 CFU ml^{-1} was described whereas reduction rate against *Staphylococcus* reached $\log_{10}$ 2.7 CFU ml^{-1}, both at standard conditions. Additionally, the development of resistances against antimicrobial compounds leads to a decreased sensitivity of bacteria. Even though resistance to silver is rare, the continuous presence of silver concentrations below the toxic level for microorganisms enhances the development of resistances (Fries et al. 2009).

A further aspect that reduces the rate of activity is biofouling. Organisms living in biofilms are considerably less sensitive to biocides than planctonic cells (Doyle 1999). Kim et al. (2008) describe how many antimicrobial substances which are effective against planctonic cells turn out to be ineffective against the same bacteria living in biofilms.
The given examples show, that the composition and the number of microorganisms strongly influences the rate of antimicrobial activity. For the appraisal of the rate of activity during a practical application in the food chain, it is necessary to use the typical microorganisms in realistic concentrations for test purposes.

Furthermore, the *environmental conditions* have a strong impact on the degree of antimicrobial activity of silver. The rate of activity is clearly dependent on temperature. In a range of 5°C to 35°C, the antimicrobial activity enhances with increasing temperature (MacKeen et al. 1987; Slawson et al. 1992; Russel and Hugo 1994; Quintavalla and Vicini 2002; Kampmann et al. 2008).

The presence of food compounds may disrupt the antimicrobial activity of silver. Especially protein residuals on surfaces strongly inhibit the bactericidal effectiveness, as described in chapter 2.2.

Also the condition of the ambient atmosphere has an influence on the rate of antimicrobial activity. Qi et al. (2010) found out by using an energy dispersive x-ray method, that silver nano particles are oxidized in air. This process is dependent on the size of the particles, as the oxidation rate of nano-sized silver increases with the decreasing particle size (Yang et al. 2005). Furthermore,

humidity influences the rate of activity, as silver in its metallic form is inert. Only in the presence of moisture, such as water (e.g. in the atmosphere), active silver ions are formed by oxidation to Ag^+ (Ovington 2004). Thus, the humidity in the air influences the antimicrobial activity.

For the implementation of antimicrobial agents in the food chain, it is not only the rate of antimicrobial activity that has to be considered, but also influencing factors on the material itself as well as toxicological aspects. For toxicology, migration in particular is important. As explained before, a steady and prolonged ion release is necessary for the effectiveness of this method, as only silver ions (Ag^+) on the surface act in an antimicrobial way. However, migration of silver ions into food may imply a health risk. Till now, data concerning migration rates from surfaces containing silver are rare (Simon et al. 2008). Kumar and Münstedt (2005) found out that the silver release from silver composites increases with time and the degree of concentration of the silver particles. Thus, the migration rate is related to the silver concentration and bonding within the surface as well as contact time and temperature while in storage (Brien et al. 1999; Kumar and Münstedt 2004). The critical amount for human health is dependent on the actual amount within a food product. In general, the WHO classifies silver as a toxic substance. Small amounts of silver in humans are eliminated in feces, with the result that only 10% of silver intake in food is reabsorbed by humans (Lansdown 2004; Landau 2006). However, the "EPA established in 1991 an oral Reference Dose (RfD), or daily intake limit, of 0,005 mg kg^{-1} day^{-1}" (EPA 1993). In contrast to this relatively low level, the "Guidelines for drinking-water quality", state that "a total lifetime oral intake of about 10 g of silver (equal to 0.39 mg $person^{-1}$ day^{-1}) can be considered on the basis of epidemiological and pharmacokinetic knowledge as the human NOAEL" (No Observed Adverse Effect Level) (EFSA 2004). For exposure via air, Threshold Limit Values (TLV) between 0.01 mg m^{-3} and 0.1 mg m^{-3} are defined, depending on the type of silver compound (Drake and Hazelwood 2005). Ailments caused by silver ingestion are mainly of a cosmetic nature. The so called "Argyria" is characterized with skin coloration from blue to grey as well as an accumulation of silver in the eyes (Drake and Hazelwood 2005). Serious changes due to exposure to silver are described by Drake and Hazelwood (2005). They refer to changes in blood cells as well as degenerative processes in the liver and kidneys. Also Wan et al. (1991) describe silver accumulation in the skin, cornea, liver, gingival area,

and kidneys. In particular nano-sized silver particles may cause damage to biological cells as they are able to penetrate cellular barriers (Simon et al. 2008).

4.2 EVALUATION SCHEME

The multifarious influencing factors and the special regulations in the food chain make it evident, that the definition of a requirement profile for the planned application is an important prerequisite for the successful implementation of an antimicrobial method. Thus it is necessary to evaluate materials with regard to their specific area of application. The common test methods for testing antimicrobial activity of surfaces, like JIS Z 2801, ASTM E 2180-07 or ISO 22196 2007, do not take account of realistic application conditions. Only the rate of activity against the standard test organisms *Staphylococcus aureus* and *Escherichia coli* in nutrient broth at 35°C and high humidity are determined by these methods. During processing, transport and storage of perishable food, environmental and microbiological conditions differ from standard test conditions. Thus, by using these standard tests, no information on the rate of activity at specific practical application conditions will be ascertained. Therefore the test procedure has to be adapted to the requirements of the practical application.

As a result, Figure 4.2 shows an evaluation scheme for the assessment and testing of antimicrobial methods. The scheme shows that the definition of a requirement profile with regard to the application area provides the basis for the further evaluation of an antimicrobial method. Relating to the defined requirement profile, prototypes have to be developed. The testing itself has to cover several aspects, which can be subdivided into “physical and chemical” and “antimicrobial” aspects. Both kinds of testing are essential to guarantee the fulfillment of mechanical-technical requirements on the one side as well as the requirements concerning antimicrobial activity on the other side. Furthermore it is absolutely necessary to perform testing that takes account of special application conditions. Only those factors, that are important for the special purpose, have to be considered while testing. Hence, application of antimicrobial agents in environments, where they are not active, can be avoided.

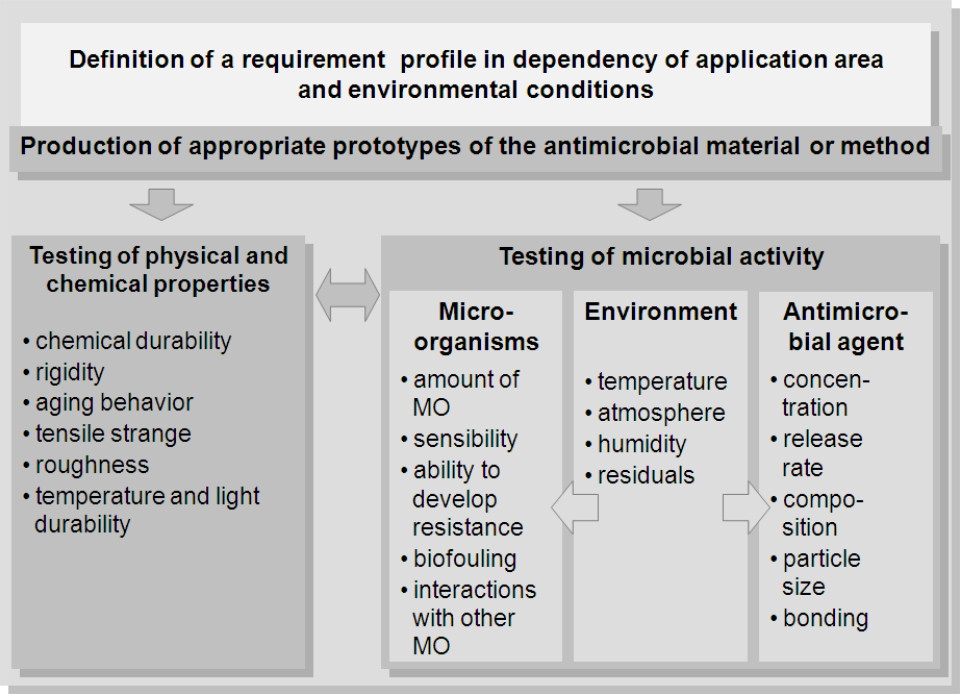

Figure 4.2 Evaluation scheme for the assessment and testing of antimicrobial methods with regard to their special application (mod. from Kreyenschmidt 2010)

Tests concerning the *physical and chemical* properties of materials are necessary, as the addition of antimicrobial additives can lead to changes within the material. As an example, the addition of silver nitrate can result in discolorations of plastic materials as silver oxides react in the presence of UV light causing color changes. This possibly leads to limitations in the rate of activity (Landsdown 2004; Meyer 2010). Furthermore, micro or macro scaled silver in some cases results in an increasing fragility of plastic materials. This implicates the development of virtually predetermined breaking points, whereby the material is less resilient (Meyer 2010). The use of nano scaled silver involves risks, too. The bigger surface to mass ratio implies a higher surface tension. This problem can be reduced by covalent surface modifications, the use of adhesion promoter or encapsulation (Meyer 2010). As described before, the application of methods spread via air can influence surfaces they are in contact with, as well. For example, UV-light accelerates the aging of many materials, particularly polymers.

On the other hand, tests concerning the *rate of microbial activity,* depending on the requirement profile are necessary, as the rate of activity is affected by the antimicrobial technique used, microbiology and the environment. Besides that, the single influencing factors also influence each other. Thus, the testing or

determination of the rate of activity is time and cost intensive, depending on the application area. To reduce the number of tests and to obtain a better understanding about the interactions of the different influencing factors, mathematical models can be used. For application in the area of antimicrobial methods, especially reduction models, growth models and migration models can be applied. Reduction models and growth models have already been used for several years in the field of shelf life prediction of perishable products. Their principles can also be applied in the area of antimicrobial methods.

For the calculation of antimicrobial activity depending on additive concentration, a reduction model can be used. This model is based on an equation that describes bacterial growth as an exponential function related to time. The parameters used "logarithmic initial cell count" and the "growth rate constant" are derived in a linear relationship to the additive concentration. This includes different parameters which are determined in experimental measurements for the specific polymer and respective bacteria (Shrivastava et al. 2007).

Beside this kind of model, growth models can predict the growth rates of microorganisms in contact with antimicrobial agents in comparison to growth rates on reference materials. Typical growth models are the modified Gompertz model and the Baranyi model. From these models, growth parameters like lag phase, and growth rate, can be calculated, which delivers a better understanding of the effect of the antimicrobial agents regarding the bacterial growth (Baranyi et al. 1995; Gospavic et al. 2008). This is especially important for surfaces, which are continuously in contact to food, such as packaging materials, transport boxes or, in some cases, refrigerators. Also differences in sensitivity of microorganisms to an antimicrobial method or material can be determined by comparison of growth parameters calculated for different microorganisms (Gibson et al. 1987; Kleer and Hildebrandt 2002; Lee et al. 2004).

Temperature models can be applied for the evaluation of the reaction rate of an antimicrobial agent in relation to temperature. The most frequently used temperature model is the Arrhenius model (Lee et al. 2008). This model can be used for the determination of temperatures influence on additive release as well as for the appraisal of bactericidal activity as related to temperature. The Arrhenius model can also be applied to predict the shelf life under different temperature conditions while storage on surfaces containing antimicrobial agents.

For the calculation of migration of additives from antimicrobial surfaces into food, several approaches are available. Some of these approaches are already used for the calculation of migration from packaging materials. For defined plastic materials, the appraisal of diffusion by migration models is even defined by law (European guideline RL 2002/72/EG). Migration is described mathematically in order to predict the degree and rate of the migrated amount in specific conditions. The available models differ from each other as they are based on different assumptions and use other parameters. The most often used modeling approach is based on the Fick's Second Law, which describes the diffusion process as a change in concentration related to time and location (Lee et al. 2008). Thus, migration in this sense is a time dependent parameter describing the particle velocity. This approach is extended by Piringer (O'Brien et al. 1999; Begley et al. 2005). According to the Piringer model, migration is not only time dependent, but also dependent on the relative mole mass of the migrant as well as on a material specific constant and a material specific temperature constant.

It becomes apparent that for the assessment of antimicrobial agents for use in contact with food, several aspects have to be considered. This is necessary for a realistic evaluation of a long term antimicrobial agent for a special approach. Mathematical models can assist in estimating the effect of different influencing factors on the relevant antimicrobial activities. As these models are always based on assumptions, validations of the data under practical condition are necessary.

Overall, it should be considered, that even after a positive appraisal with regard to antimicrobial activity, the use of antimicrobial agents can never substitute for classical hygienic methods, but serve as an additional hurdle in helping to prevent harmful bacterial growth (Appendini and Hotchkiss 2002; Cooksey 2001).

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CHAPTER 5

Summary

Cross-contamination of microorganisms via surfaces or air in contact with perishable products influences the food quality, safety and shelf life of these products. The application of long term antimicrobial methods, such as intelligent surfaces or ionization in addition to classical hygienic methods can support a reduction of surface and airborne bacteria. Several publications are available about long term antimicrobial methods. Most of these studies describe the effect on selected bacteria, like *E. coli* or *Staph. aureus* under standard conditions, which means at 35 °C. Studies about the use of these methods in the food industry as well as the effects on different food specific microorganisms and influencing factors like temperature and food residuals are rare.

Hence, the objective of this thesis was the assessment of sustainable antimicrobial agents with regard to their ability to reduce airborne and surface bacteria in the food processing chain. As a result, three research questions were identified.

The first question was aimed at the determination of the rate of antimicrobial activity of selected antimicrobial agents against food specific microorganisms. Therefore, two different methods for long term applications to reduce airborne and surface bacteria were examined in detail: silver coating and ionization. The antimicrobial activity of these methods was investigated by comparing surface and airborne bacterial counts with silver coating or ionization being used, and with reference conditions after storage for a defined time interval under defined conditions.

The tested plastic surfaces with 0.5% silver zeolite showed a rate of activity between $\log_{10}$ 1.0 und 5.9 CFU ml^{-1} after storage at 35°C for 24 h, depending on the sensitivity of test organisms. The highest rate of activity was achieved against *Pseudomonas* spp. which is a main spoilage organism in fresh meat and fresh fish, followed by *Staph. aureus*. The same effect was attained at while testing silver samples with higher concentrations.

The tested ionizer showed a good antimicrobial activity against surface counts on glass and plastic. As with silver, the highest reduction rates were measured by investigations using *Pseudomonas* spp. as a test organism followed by *Lactobacillus* spp. To investigate the activity of ionization on airborne bacteria, a bacterial suspension was nebulized in refrigerators. Measurement of airborne bacteria using the sedimentation method indicated a significant reduction of these bacteria caused by ionization.

The second research question focused on the influence of environmental conditions on the rate of activity of antimicrobial methods. In particular, the influence of low temperature, time and the presence of food residuals were analyzed.

Temperature had a strong influence on the rate of activity of samples containing silver. For example, with *Ps. fluorescens* as a test organism and polystyrol samples containing 0.5% AlphaSan, a reduction of $\log_{10}$ 5.1 CFU ml^{-1} was achieved after 24 h at 35°C, whereas the reduction of 0.2 $\log_{10}$-steps was not significant after 24 h at 5°C. However, the reduction rate increased with the incubation time also at low temperatures. Food components inhibited the antimicrobial activity of silver. Especially protein rich food totally neutralized the activity of silver, whereas the addition of honey and starch reduced the antimicrobial activity significantly. All other tested substances, such as fatty acids, cyclodextrin or monosaccharides did not negatively affect the rate of antibacterial activity.

Also investigations with ionization indicated a clear temperature dependency, as ionization reduced the surface count on agar plates only at room temperature but not at 8°C (results shown only for 8°C). In contrast, ionization applied at 8°C reduced surface count on glass and plastic surfaces.

The investigations reveal that the antimicrobial activity is strongly influenced by environmental, microbiological factors and factors relating to the methodology. Thus, a realistic appraisal of the rate of antimicrobial activity of an agent is only possible, if the test conditions are similar to realistic application conditions with regard to microbiology and environment.

Therefore the final research question was aimed at the development of an evaluation scheme for the assessment of the antimicrobial methods with regard to their application area and the special requirement profile occurring in the food industry. The developed scheme includes the most important influencing factors material, environment and microbiology, on the antimicrobial activity. Since the testing of the different influencing factors is time-consuming and expensive, approaches are presented that demonstrate how predictive models can facilitate the assessment of different antimicrobial methods for their use in different application areas.

In general, silver coating and ionization have a high capability to improve food quality and safety by the reduction of airborne and surface bacteria in selected application areas within the food supply chain. However the implementation of long term antimicrobial methods in food chains is a complex issue, since the rate of antimicrobial activity is strongly influenced by the application conditions. Both antimicrobial technologies are able to reduce typical microorganisms in the perishable food chain. In contrast, the investigations indicate a strong reduction in the antimicrobial activity at low temperatures. This means when applications involve contact with chilled food, the antimicrobial activity is reduced. However, the reduced activity at low temperatures can partly be compensated by increasing the amount of silver ions; but, the migration of silver particles with regard to their possible toxic levels has to be considered. The application of surfaces containing silver during processing or storage of products with a high protein content like meat or fish, reduces or totally inhibits the antimicrobial activity. However, after cleaning and disinfection, silver coated surfaces can also in this area act as an additional hurdle to the growth of remaining bacteria. Furthermore, the application of silver coatings in the area of vegetables and fruits can provide an additional benefit. Also in the beverage industry, including such areas as breweries or soft drink companies, silver coated surfaces can help to prevent cross-contamination.

With regard to the implementation of ionization in food supply chains, the lower antimicrobial activity at temperatures commonly met with chilled foods has to be considered. Besides that, the long term effect on food products, especially on those products with high fat contents, should be investigated in the future, since ionization can lead to fat rancidity.

Overall, a careful choice of long term antimicrobial technologies could reduce the degree of risk of cross-contamination in industrial, commercial, and domestic environments.

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