UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

EVALUATION OF A BIOCIDE USED IN THE BIOLOGICAL ISOLATION AND CONTAINMENT UNIT OF THE VETERINARY TEACHING HOSPITAL OF THE FMV-ULISBOA

CATARINA DA SILVA GERALDES

ORIENTADORA:
Doutora Solange Judite Roque Coelho Alves Gil Neves
COORIENTADORA:
Doutora Maria Manuela Castilho Monteiro de Oliveira

2021

UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

EVALUATION OF A BIOCIDE USED IN THE BIOLOGICAL ISOLATION AND CONTAINMENT UNIT OF THE VETERINARY TEACHING HOSPITAL OF THE FMV-ULISBOA

CATARINA DA SILVA GERALDES

DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

JÚRI
PRESIDENTE:
Doutora Yolanda Maria Vaz
VOGAIS:
Doutora Berta Maria Fernandes Ferreira São Braz
Doutora Solange Judite Roque Coelho Alves Gil Neves

ORIENTADORA:
Doutora Solange Judite Roque Coelho Alves Gil Neves
COORIENTADORA:
Doutora Maria Manuela Castilho Monteiro de Oliveira

2021

DECLARAÇÃO RELATIVA ÀS CONDIÇÕES DE REPRODUÇÃO DA DISSERTAÇÃO

Nome: Catarina da Silva Geraldes

Título da Tese ou Dissertação: Evaluation of a Biocide Used in the Biological Isolation and Containment Unit of the Veterinary Teaching Hospital of the FMV-ULisboa

Ano de conclusão (indicar o da data da realização das provas públicas): 2021

Designação do curso de Mestrado ou de Doutoramento: Mestrado Integrado em Medicina Veterinária

Área científica em que melhor se enquadra (assinale uma):

☐ Clínica ☐ Produção Animal e Segurança Alimentar

☐ Morfologia e Função ☒ Sanidade Animal

Declaro sobre compromisso de honra que a tese ou dissertação agora entregue corresponde à que foi aprovada pelo júri constituído pela Faculdade de Medicina Veterinária da ULISBOA.

Declaro que concedo à Faculdade de Medicina Veterinária e aos seus agentes uma licença não-exclusiva para arquivar e tornar acessível, nomeadamente através do seu repositório institucional, nas condições abaixo indicadas, a minha tese ou dissertação, no todo ou em parte, em suporte digital.

Declaro que autorizo a Faculdade de Medicina Veterinária a arquivar mais de uma cópia da tese ou dissertação e a, sem alterar o seu conteúdo, converter o documento entregue, para qualquer formato de ficheiro, meio ou suporte, para efeitos de preservação e acesso.

Retenho todos os direitos de autor relativos à tese ou dissertação, e o direito de a usar em trabalhos futuros (como artigos ou livros).

Concordo que a minha tese ou dissertação seja colocada no repositório da Faculdade de Medicina Veterinária com o seguinte estatuto (assinale um):

1. ☐ Disponibilização imediata do conjunto do trabalho para acesso mundial;
2. ☒ Disponibilização do conjunto do trabalho para acesso exclusivo na Faculdade de Medicina Veterinária durante o período de ☐ 6 meses, ☒ 12 meses, sendo que após o tempo assinalado autorizo o acesso mundial*;

* Indique o motivo do embargo (OBRIGATÓRIO)

O trabalho apresentado nesta dissertação terá por base a formulação de um artigo científico.

Nos exemplares das dissertações de mestrado ou teses de doutoramento entregues para a prestação de provas na Universidade e dos quais é obrigatoriamente enviado um exemplar para depósito na Biblioteca da Faculdade de Medicina Veterinária da Universidade de Lisboa deve constar uma das seguintes declarações (incluir apenas uma das três):

1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.
2. É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE/TRABALHO (indicar, caso tal seja necessário, nº máximo de páginas, ilustrações, gráficos, etc.) APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.
3. DE ACORDO COM A LEGISLAÇÃO EM VIGOR, (indicar, caso tal seja necessário, nº máximo de páginas, ilustrações, gráficos, etc.) NÃO É PERMITIDA A REPRODUÇÃO DE QUALQUER PARTE DESTA TESE/TRABALHO.

Faculdade de Medicina Veterinária da Universidade de Lisboa, 26 de Fevereiro de 2021

(indicar aqui a data da realização das provas públicas)

Assinatura: _____

Agradecimentos

Gostaria de agradecer primeiramente à minha orientadora, a Professora Doutora Solange Gil Neves, por me ter dado esta oportunidade e todos os apoios fundamentais no desenvolvimento desta dissertação. Agradeço também pela paciência e disponibilidade constante para todos os problemas que foram surgindo aos longo deste processo.

À minha co-orientadora, a Professora Doutora Manuela Oliveira, por ter sido uma das maiores ajudas no desenvolvimento desta dissertação e pelo apoio, paciência e dedicação incondicional durante todo este processo.

Gostaria de agradecer à minha família, principalmente aos meus pais, por terem sido o meu maior apoio durantes todo este percurso, que teve início nos meus dias de criança nos quais eu sempre dizia que este seria o meu futuro. Aos meus avós que sempre tiveram orgulho em mim, com uma dedicatória especial ao meu avô José e avó Maria que infelizmente não conseguiram assistir à conclusão desta fase da minha vida. Um obrigada também ao Sammy e à Mel (que está precisamente no meu colo enquanto escrevo).

Agradeço ainda à minha família acrescentada, Rute, João e Mariana, por me aturarem há tanto anos e por estarem sempre disponíveis para me apoiar e distrair (o que também é importante). Margarida, ainda não tens tantos anos no bolso, mas foste, e espero que continues a ser, um dos meus maiores apoios. Sem ti esta dissertação não seria a mesma (literalmente).

A todas as pessoas que me acompanharam durante todo o meu percurso em Medicina Veterinária, mas especialmente à Inês, à Catarina e à Manuela por serem uma salvação nos momentos mais aborrecidos.

À Eva que me recebeu de braços aberto e que teve toda a paciência de me guiar e ensinar durante a minha fase de laboratório, tornando a minha experiência mil vezes mais divertida e menos complicada. À Cláudia por me proporcionar os melhores momentos de distração entre protocolos laboratoriais. Acho que sem ti tinha acabado a bater com a cabeça nas paredes. Finalmente agradeço também ao restante pessoal do Laboratório de Microbiologia que me acompanhou, Carolina, Miguel e Carla, por toda a companhia e momentos de ensino.

À Dra. Inês por me ter apoiado e ensinado durante a minha fase de estágio na Unidade de Isolamento e Contenção Biológica, mesmo durante uma pandemia.

Finalmente agradeço ao CIISA pelo apoio financeiro que permitiu o desenvolvimento desta dissertação.

Evaluation of a Biocide Used in the Biological Isolation and Containment Unit of the Veterinary Teaching Hospital of the FMV-ULisboa - Abstract

Hospital-acquired infections (HAIs) are a rising problem worldwide. The best way of coping with these HAIs is through infection tracking and surveillance systems combined with prevention strategies namely efficient disinfection protocols. These considerations, associated with increasing reports about reductions in biocide susceptibility and development of cross-resistance to antimicrobials, emphasise the need of identifying how different factors can influence a biocide's efficiency.

In this study, 29 bacterial isolates (*E. coli* n=3, *Pseudomonas* spp. n=2, *Enterococcus* spp. n=23 and *Staphylococcus pseudintermedius* n=1), obtained from environmental samples collected from the Biological Isolation and Containment Unit (BICU), of the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon, were characterized regarding their antimicrobial susceptibility profile. From these isolates, 13 were further selected to investigate their susceptibility to Virkon® S, with and without the presence of organic matter. After this determination, 7 isolates (all enterococci) were submitted to sub-lethal concentrations of this formulation and, subsequently, new antimicrobial susceptibility profiles were determined.

Fourteen of the 29 isolates (48.3%), all previously identified as enterococci, were classified as Multi-Drug Resistant. Concerning Virkon® S's susceptibility in the absence of organic matter, the Minimal Bactericidal Concentration (MBC) of this biocide regarding all isolates was lower than the concentration regularly used. However, when organic matter was added, MBC values experienced a significant rise. After submission to sub-lethal concentrations of Virkon® S, 4 enterococci presented a phenotypic change in their susceptibility towards gentamicin. Virkon® S also presented higher MBC values in the presence of low concentrations of organic matter, but no rise in these values was observed in assays without interfering substance.

Virkon® S proved to be an efficient formulation in eliminating the bacterial isolates collected from the BICU. However, organic matter may impair this efficiency, which reinforces the importance of proper sanitization before disinfection procedures. The changes observed in antimicrobial susceptibility profile may be due to a stress induced response, promoted by the sub-lethal concentration of Virkon® S and not necessarily due to the occurrence of mutations or other alterations in the isolates' genome.

Keywords: Hospital-acquired Infections; Biological Isolation and Containment Unit; Virkon® S, Susceptibility; Antibiotic

Evaluation of a Biocide Used in the Biological Isolation and Containment Unit of the Veterinary Teaching Hospital of the FMV-ULisboa - Resumo

As infeções adquiridas em ambiente hospitalar são um problema emergente a nível mundial. O combate a estas infeções tem sido feito principalmente através da aplicação de sistemas de rastreio de infeções e de sistemas de vigilância, conjuntamente com estratégias de prevenção eficazes, como são os protocolos de desinfeção. A associação destes fatores com o aumento de estudos referentes a diminuições de suscetibilidade a biocidas e também a uma possível resistência cruzada a vários antibióticos, enfatizam a necessidade de entender de que forma certos fatores podem influenciar a eficiência destes compostos.

Neste estudo, foram caracterizados 29 isolados bacterianos (*E. coli* n=3, *Pseudomonas* spp. n=2, *Enterococcus* spp. n=23 e *Staphylococcus pseudintermedius* n=1), obtidos de amostras ambientais de várias superfícies da Unidade de Isolamento e Contenção Biológica (UICB) pertencente ao Hospital Escolar da Faculdade de Medicina Veterinária, Universidade de Lisboa, relativamente ao seu perfil de suscetibilidade a antimicrobianos. Deste isolados, 13 foram selecionados de maneira a investigar a sua suscetibilidade ao Virkon® S, aquando a presença/ausência de matéria orgânica. Após esta determinação, 7 isolados (todos enterococos) foram incubados conjuntamente com uma concentração sub-letal de Virkon® S e foram determinados novos perfis de suscetibilidade.

Catorze dos 29 isolados (48,3%), todos identificados como enterococos, foram classificados como multirresistentes. Na ausência de matéria orgânica, os valores de Concentração Mínima Bactericida (CMB) do Virkon® S foram inferiores à concentração regularmente utilizada. No entanto, aquando a adição de matéria orgânica, estes valores sofreram um grande aumento. Após submissão das bactérias a concentrações sub-letais de Virkon® S, 4 enterococos apresentaram uma alteração fenotípica relativamente à sua suscetibilidade à gentamicina. Em ensaios realizados na presença de matéria orgânica, observou-se um aumento dos valores de CMB do Virkon® S, enquanto que, quando esta estava ausente, este mesmo aumento não foi observado.

O Virkon® S parece ser uma formulação eficiente, uma vez que conseguiu eliminar todos os isolados bacterianos testados. A matéria orgânica pode, no entanto, ser um entrave a esta eficiência, o que reforça a importância da limpeza pré-desinfeção. As alterações observadas relativamente ao perfil de suscetibilidade a antimicrobianos podem ser explicadas por uma adaptação ao stress químico incitado sobre as bactérias, devido à presença da concentração sub-letal de Virkon® S, e não necessariamente por mutações ou outras alterações genómicas.

Palavras-chave: Infeções Adquiridas em Hospitais; Unidade de Isolamento e Contenção Biológica; Virkon® S; Suscetibilidade; Antibiótico

INDEX

INTERNSHIP REPORT 1
CHAPTER I: INTRODUCTION 2
1. Hospital-Acquired Infections (HAIs) 2
1.1 Enterococci as Nosocomial Pathogens 3
1.2 Prevention 3
1.2.1 Infection Control Programs 4
1.2.2 Isolation Units 4
2. Biocides 5
2.1 Antiseptics, Sterilants, Disinfectants and Preservatives 5
2.2.1 Main Types of Disinfectants 6
2.2.1.1 Alcohols 7
2.2.1.2 Alkalis 7
2.2.1.3 Aldehydes 8
2.2.1.3.1 Glutaraldehyde (Pentanedial) 8
2.2.1.3.2 Formaldehyde (Methanal) 9
2.2.1.3.3 Ortho-phthalaldehyde 9
2.2.1.4 Oxidizing Agents 10
2.2.1.4.1 Halogens - Chlorine Compounds 10
2.2.1.4.2 Peroxygen Compounds 11
2.2.1.4.2.1 Hydrogen Peroxide 11
2.2.1.4.2.2 Potassium Peroxymonosulfate 12
2.2.1.4.2.2.1 Virkon® S 12
2.2.1.5 Phenolic Compounds 13
2.2.1.6 Quaternary Ammonium Compounds 14
2.2 Factors Influencing the Activity of Biocides 15
2.2.1 Factors Related to the Biocide 15
2.2.1.1 Concentration 15
2.2.1.2 pH 16
2.2.1.3 Formulation 16
2.2.1.4 Other Factors 17
2.2.2 Factors Related to the Treatment Conditions 17
2.2.2.1 Temperature 17
2.2.2.2 Organic Matter 17
2.2.2.3 Contact Time 17
2.2.2.4 Other Factors 18

2.2.3 Factors Related to the Microorganisms....18
2.2.3.1 Mechanisms of Resistance in Microorganisms....18
2.2.3.1.1 Reduced Biocide Penetration....18
2.2.3.1.2 Efflux Mechanisms....20
2.2.3.1.3 Enzymatic Degradation....21
2.2.3.1.4 Acquired Resistance....21
2.2.3.2 Biofilms....21
2.3 Association Between Biocide and Antibiotic Resistance....22
CHAPTER II: EVALUATION OF VIRKON® S' EFFICENCY....24
1. OBJECTIVES....24
2. MATERIALS AND METODS....25
2.1 Biological Isolation and Containment Unit of the Veterinary Teaching Hospital....25
2.1 Bacterial Isolates....25
2.2 Antimicrobial Susceptibility Testing....27
2.3 Determination of Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs)....28
2.3.1 Preparation of Virkon® S Solutions....28
2.3.2 Preparation and Controls of the Remaining Solutions....28
2.3.3 MIC Determination Protocol....30
2.3.4 MBC Determination Protocol....32
2.4 Influence of Virkon® S Sub-MIC Values on Isolates Biocide Resistance Ability....33
3. RESULTS....34
3.1 Antimicrobial Susceptibility Testing....34
3.2 Determination of Virkon® S's Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs)....36
3.3 Influence of Bacterial Exposure to Sub-lethal Concentrations of Virkon® S....38
4. DISCUSSION....41
4.1 Antimicrobial Susceptibility Testing....41
4.2 Determination of Virkon® S's Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs)....44
4.3 Influence of Bacterial Exposure to Sub-lethal Concentrations of Virkon® S....46
5. CONCLUSION....48
6. FUTURE PRESPECTIVES....48
CHAPTER III: BIBLIOGRAPHY....49
CHAPTER IV: APPENDICES....70
Appendix 1: Virkon® S's Information Page by Lanxess (2019)....70
Appendix 2: Isolates Identification....72

FIGURE INDEX

Figure 1: Hierarchy of infection control, with the top tiers being more effective than the bottom. Adapted from NIOSH (2015) and Stull et al. (2018). 4
Figure 2: Decreasing order of susceptibility of microorganisms to biocides, as well as a simplified representation of barrier differences between three microorganisms (Gram-positive and Gram-negative bacteria and mycobacteria). Adapted from McDonnell and Russell (1999), Lambert (2002) and Willey et al. (2008).....19
Figure 3: Example of a neutralizer control plaque after a 24 hours incubation at 37ºC (±1ºC). 29
Figure 4: Representation of a 96-well plaque with Virkon® S.....30
Figure 5: Representation of the wells after the addition of the bacterial suspension mixed with the interfering substance to the Virkon® S solution.....31
Figure 6: Representation of the final 96-well plaque (plate 3). 32
Figure 7: Example of a division of an MBC plate if the MIC obtained was 5 g/L. 32

TABLE INDEX

Table 1: Virkon® S's formulants and corresponding actions and quantities. Adapted from Lanxess (2019) and Nakama (2017) 12
Table 2: Antibiotics used in the characterization of the isolate susceptibility profile, their concentrations and susceptibility limits. 27
Table 3: Results from the antimicrobial susceptibility tests of all isolates under study (n=29), with the corresponding categorization (susceptible, intermedium or resistant) and diameter of the inhibition halo in millimetres. 35
Table 4: Virkon® S's medium MICs and MBCs values towards all isolates tested, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (grams per litre, g/L) 37
Table 5: Parallel between the antibiotic susceptibility tests performed before (A) and after (B) the induction of bacterial multiplication at sub-MIC levels of the 7 selected *Enterococcus* spp. isolates, with the corresponding categorization (susceptible, intermedium or resistant) and diameter of the inhibition halo in millimetres. 39
Table 6: Virkon® S's MICs and MBCs medium values towards the selected *Enterococcus* spp. isolates after induction of bacterial multiplication at sub-MIC levels, including assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (grams per litre, g/L) 40
Table 7: Comparison of antibiotic resistance rates presented by *Enterococcus* spp. isolated from animals (dogs or cats) or from VTH's surfaces, obtained in different studies. 43

GRAPHIC INDEX

Graphic 1: Virkon® S's of MBCs medium values obtained in the assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) towards the different groups of isolates (grams per litre). 38

ABBREVIATIONS

ɳ – Dilution Factor

ABC – ATP-Binding Cassette Super Family

API – Analytical Profile Index

BHI – Brain Heart Infusion

BICU – Biological Isolation and Containment Unit

CFU – Colony Forming Unit

CLSI – Clinical and Laboratory Standards Institute

CMC – Critical Micelle Concentration

DMT – Drug/Metabolite Transporter

DNA – Deoxyribonucleic Acid

EN – European Standard

EPA – Environmental Protection Agency

FeLV – Feline Leukaemia Virus

FIV – Feline Immunodeficiency Virus

GSP – Glutamate Starch Phenol Red Selective Agar

GTA – Glutaraldehyde

HAI – Hospital Acquired Infection

HEPA – High Efficiency Particulate Arrestance

HIS – High Interfering Substance

ICP – Infection Control Practitioner

LIS – Low Interfering Substance

LPS – Lipopolysaccharides

MATE – Multidrug and Toxic Compound Extrusion

MDR – Multidrug Resistant

MES – Multidrug Efflux Systems

MFS – Major Facilitator Super Family

MIC – Minimal Inhibitory Concentration

MRSA – Methicillin Resistant *Staphylococcus aureus*

MRSP – Methicillin Resistant *Staphylococcus pseudintermedius*

MSA – Mannitol Salt Agar

NIOSH – National Institute for Occupational Safety and Health

NIS – No Interfering Substance

OA – Oxidizing Agent

OPA – Ortho-Phthalaldehyde

PBP – Penicillin Binding Proteins

PPE – Personal Protective Equipment

QAC – Quaternary Ammonium Compound

RNA – Ribonucleic Acid

RND – Resistance-Nodulation Cell Division Superfamily

SBM – Slanetz Bartley Medium

SMR – Small Multidrug Resistance Family

SOP – Standard Operation Procedure

TSA – Trypticase Soy Agar

TSB – Trypticase Soy Broth

US – United States

VTH – Veterinary Teaching Hospital

INTERNSHIP REPORT

As part of the integrated master's degree in Veterinary Medicine of the Faculty of Veterinary Medicine of the University of Lisbon (FVM-UL), my curricular internship had a total of 950 hours and was divided between two locations: the Laboratory of Veterinary Bacteriology and Immunology and the Biological Isolation and Containment Unit (BICU) of the Veterinary Teaching Hospital (VTH), both located in the FMV-UL.

Laboratory of Veterinary Bacteriology and Immunology

The first part of the internship was carried out between September 2019 and March 2020, in a total of 800 hours, and consisted in the development of the project that resulted in my master thesis.

During this period of time I had the opportunity to perform various microbiological techniques, such as the preparation of different growth mediums for bacteria (both solid and liquid), completing different methods of identification of bacterial genus and species and developing protocols of antimicrobial susceptibility testing, minimal inhibitory concentrations and minimal bactericidal concentrations, among others.

Biological Isolation and Containment Unit (BICU)

The second part of the internship was carried out between June and July 2020 and was divided between 4 weeks, in a total of 150 hours.

In this period of time, I had the opportunity of participating in the unit’s day-to-day routine which implicated performing patient monitorization and regular physical examination, treatment elaboration and consequent administration, different medical procedures (e.g. catheterization, urinary catheterization and sample collection) and diagnostic tests such as imagiological examinations (abdominal echography and x-rays). Every Saturday I also had the opportunity of participating in several vaccination consultations.

Each case that passed through the unit was discussed in order to encourage the development of clinical reasoning.

CHAPTER I: INTRODUCTION

1. Hospital-Acquired Infections (HAIs)

Hospital-Acquired Infections (HAIs), also described sometimes as nosocomial infections, are a rising problem in veterinary hospitals, as also observed in human hospitals. A study by Benedict et al. (2008), in the United States (US), indicated that 82% of the veterinary teaching hospitals (VTHs) investigated reported outbreaks of nosocomial infections, 42% of which reported more than one outbreak. These infections can take many forms, but will most likely appear as urinary tract infections, pneumonias, surgical site infections, bloodstream infections and gastrointestinal diseases (Stull and Weese 2015).

The increment of HAIs in the veterinary field can be attributed especially to the rising number of invasive surgical procedures as well as antimicrobial and immunosuppressive therapies (Guardabassi 2012). Some studies indicate that there have also been positive associations between HAIs and some other factors such as prolonged time of hospitalization, surgeries (especially in patients who receive surgical implants), placement of urinary catheters and geriatric or chronically ill patients (Ruple-Czerniak et al. 2013; Wieler et al. 2014).

HAIs are frequently caused by opportunistic pathogens that can be found in either sick or healthy animals, which facilitates the dissemination of these agents in veterinary hospitals (Weese 2011; Stull and Weese 2015). Some veterinary hospital-associated pathogens include bacteria [e.g. methicillin-resistant staphylococci, such as *Staphylococcus aureus* (MRSA) and *S. pseudintermedius* (MRSP), *Escherichia coli*, *Enterococcus* spp., *Salmonella* spp. and *Pseudomonas* spp.], very often multidrug-resistant (MDR), viruses (e.g. Influenza, Parvovirus and Herpesvirus), fungus (mainly *Microsporum canis*) and also parasites (mainly *Cryptosporidium parvum*) (Benedict et al. 2008; Guardabassi 2012; Stull and Weese 2015). Several studies have proven the presence of these pathogens in various surfaces (namely the floor, hand contact surfaces and medical instruments) of veterinary hospitals (Weese et al. 2004; Burgess et al. 2004; Hamilton et al. 2012), making these places possible sources of contamination.

It is known that HAIs can lead to dire consequences not only for hospitalized animals, for being associated with increments in morbidity and mortality rates, but also to the hospitals hosting them (especially financially). Furthermore, considering the zoonotic potential of some of these pathogens, they can also pose a great risk to veterinary personnel and owners (Weese et al. 2006; Benedict et al. 2008; Guardabassi 2012). A study by Jordan et al. (2011) corroborates this risk, showing that veterinary personnel have a higher probability of becoming colonized by MRSA (between 5 and 23-fold higher depending on their area of work).

Although there are no conclusive studies that indicate the proportion of preventable HAIs in veterinary medicine, it is estimated that it is similar to what is established for human

medicine (Stull and Weese 2015), which is around 35-55% (Schreiber et al. 2018). This means that the implementation of adequate infection control programs could diminish the incidence of HAIs in veterinary medicine (CCAR 2008; Weese 2011; Guardabassi 2012; Stull and Weese 2015; Mount et al. 2016; Stull et al. 2018).

1.1 Enterococci as Nosocomial Pathogens

Enterococcus spp. are ubiquitous Gram-positive and facultative anaerobic bacteria, commensal of the intestinal tract of human and animals alike (Hollenbeck and Rice 2012; Torres et al. 2018). These microorganisms are largely characterized by their ability to tolerate high concentrations of salt (6.5% NaCl), but also a wide range of temperature (from 10ºC to 40ºC) and pH (from 4.4 to 9.6) values (Hollenbeck and Rice 2012).

Initially portrayed as organisms of little clinical importance, enterococci, particularly *E. faecalis* and *E. faecium*, have been progressively associated with an increasing number of HAIs in both human and veterinary medicine (Torres et al. 2018). Not being highly virulent organisms, their capacity to cause these types of infections mainly derives from:

(i) The ability to linger in the environment for long periods of time associated with their biofilm-forming capacity (García-Solache and Rice 2019);

(ii) The reduced susceptibility to many antimicrobial agents such as β-lactams, aminoglycosides and glycopeptides (Hollenbeck and Rice 2012; Kristich et al. 2014; Torres et al. 2018; García-Solache and Rice 2019);

(iii) The plasticity of their genome, which allows them to easily acquire, conserve and disseminate genetic traits among not only enterococci but also other Gram-positive bacteria (Weigel et al. 2003; de Niederhäusern et al. 2004; Zhu et al. 2008; Limbago et al. 2014; García-Solache and Rice 2019).

These characteristics make the prevention of the existence and maintenance of these microorganisms in the environment an essential practice in order to reduce the number of HAIs.

1.2 Prevention

An infectious disease has five main pathways of transmission between a source and a susceptible host (CCAR 2008): direct contact, fomites, aerosol (airborne), oral (ingestion) and vector-borne (Stull et al. 2018). The primary actions that need to be taken, in order to break this chain of infection, are minimizing host exposure; decreasing host susceptibility by rationalizing the use of antibiotics, proper nutrition, adequate pain control and management of other diseases that might be present; and increasing host resistance mainly by the administration of vaccines, when applicable (CCAR 2008; Guardabassi 2012).

These actions should also be associated with the four levels of infection control, adapted by Stull et al. (2018) from the hierarchy of controls of occupational hazards, created by the National Institute for Occupational Safety and Health (NIOSH) (2015) (Figure 1). Although the elimination of associated sources is the most efficient level, quite often the origin of infections in hospitals are the hospitalized animals, which makes the application of this measure, in these cases, impossible. However, if these sources are correctly isolated, they can impede the propagation of the infection to other hosts (Stull et al. 2018).

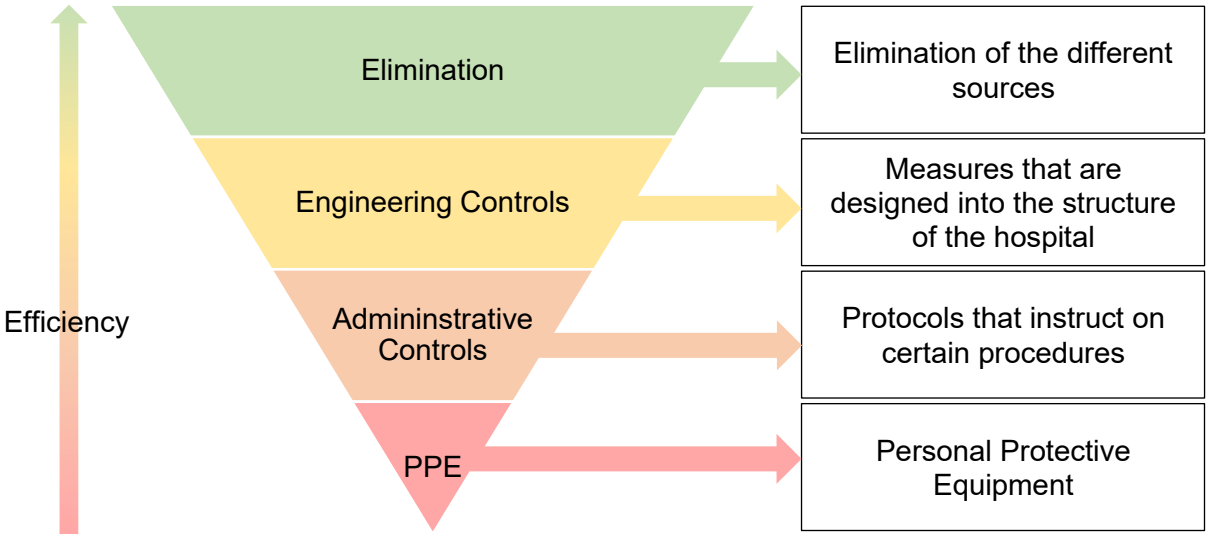


Figure 1: Hierarchy of infection control, with the top tiers being more effective than the bottom. Adapted from NIOSH (2015) and Stull et al. (2018).

1.2.1 Infection Control Programs

Every veterinary hospital should have a member appointed as Infection Control Practitioner (ICP), responsible for the formulation, implementation and management of an Infection Control Program and corresponding Standard Operation Procedures (SOPs), which should be based especially on the different methods of prevention (CCAR 2008; Stull et al. 2018).

These programs should be divided into two separate parts: surveillance (active and passive) and routine practices (CCAR 2008). These include hand hygiene, risk reduction [such as the utilization of personal protective equipment (PPE) and cleaning and disinfection], risk evaluation (including patient management, such as identifying high-risk patients) and risk communication (CCAR 2008; Guardabassi 2012; Stull and Weese 2015; Stull et al. 2018).

1.2.2 Isolation Units

As previously mentioned, although elimination of the sources is often not possible due to its correspondence to infected animals, they can be isolated in order to prevent the mitigation of the infection (Stull et al. 2018). To achieve this segregation, these animals are usually placed in an isolation unit, preferably located in a distinct section of the hospital, usually with a

separate entrance, properly identified and restricted only to essential staff for the treatment of the hospitalized animals. These areas should always have their own SOP, available to all personnel (AIDAP 2015). If possible, they should also be under negative pressure (< 2.5 Pa) with 6 to 12 air changes per hour, in order to contain all pathogenic organisms present in the air, exhausting them directly to the exterior; if not, an High Efficiency Particulate Arrestance (HEPA) filter should be installed (CDC and HICPAC 2003; Portner and Johnson 2010).

Other procedures that should be considered in these areas are:

(i) Owner visits should be short, with a duration of 10 minutes or less, and restricted only to animals in critical condition in which important decisions (such as euthanasia) need to be made (Guptill 2015);

(ii) The staff working in these units should only contact with the animals therein present with the adequate PPEs in order to protect themselves, the patient itself and other animals (AIDAP 2015);

(iii) The re-usable materials present in these units, such as stethoscopes and thermometers, should not be used elsewhere. If this is not a possibility, these objects should be properly cleaned and disinfected before being used again (Guptill 2015);

(iv) All areas that come in contact with animals present in these spaces should also be cleaned and disinfected (Guptill 2015).

2. Biocides

2.1 Antiseptics, Sterilants, Disinfectants and Preservatives

Biocides have been used for a long time with the intent of reducing the quantity of microorganisms present in different surfaces and are a helpful weapon for preventing the growing quantity of multi-resistant organisms, the spread of infections and, consequently, the amount of HAIs occurring in todays practice. Regulation (EU) nº 528/2012 of the European Parliament and the Council of 22 May 2012 defines “biocidal product” as a compound that contains in its composition (or that leads to the formation of) one or more active substances, utilized with the intent of "destroying, deterring or rendering harmless" microorganisms (by other means besides physical or mechanical ones), in order to attenuate or eliminate any detrimental action these agents may have towards host health. Beyond this definition, Maillard (2005a) and Quinn et al. (2011) determine that ideally, biocides should also:

(i) have a large spectre of activity (multiple bacteria including mycobacteria, spores, virus and fungi);

(ii) lead to the destruction of most microorganisms in low concentrations and in the shortest contact time possible, even if these microorganisms are organized in biofilms or in dry pellicles on surfaces;

(iii) not be easily neutralized and retain their activity in the presence of organic matter;

(iv) not have any kind of detrimental effect on human/animal health, including irritancy, toxicity, teratogenicity, mutagenicity and/or carcinogenicity;

(v) cause the minimum damage to the materials on which it is used;

(vi) be non-polluting and biodegradable;

(vii) have a reasonable price and be readily available.

According to its definition, the word “biocide” encompasses a great assortment of different compounds. To facilitate the distinction of these products pursuant to their typical characteristics and usages, they can be divided into four categories:

(i) Antiseptics: biocides applied to living tissues (e.g. skin), that act upon the multiplication of vegetative forms of multiple microorganisms either by inhibiting or killing them (Sheldon 2005; Hernández-Navarrete et al. 2014);

(ii) Sterilants: biocides applied to inanimate surfaces and/or objects, in order to remove all forms of micro-organisms (including fungus and spores) (Sheldon 2005);

(iii) Disinfectants: biocides applied to inanimate surfaces, objects and/or the environment, acting primarily upon vegetative forms (Sheldon 2005; Hernández-Navarrete et al. 2014). The process of disinfection can be divided into three levels, according to the range of micro-organisms destroyed (Rutala and Weber 2013):

- High-level disinfection destroys all forms of microorganisms, except when there is a high degree of contamination by bacterial spores;
- Intermediate-level disinfection destroys most organisms, including vegetative bacteria and most viruses and fungi, with the exception of bacterial spores;
- Low-level disinfection destroys most organisms, including vegetative bacteria and some viruses and fungi, with the exception of bacterial spores or mycobacteria.

(iv) Preservatives: biocides used to impede bacterial multiplication in order to prevent product deterioration (Sheldon 2005).

2.2.1 Main Types of Disinfectants

Virkon® S, the biocide evaluated in this study, is categorized as a disinfectant since it is mainly used with the purpose of decontaminating surfaces and objects. This is one of the disinfectants extensively used in the Biological Isolation and Containment Unit (BICU) of the VTH. However, it is not the only one available. There are six main types of surface disinfectants consistently used worldwide: alcohols, alkalis, aldehydes, oxidizing agents, phenolic compounds, and quaternary ammonium compounds (CCAR 2008; Stull et al. 2018).

2.2.1.1 Alcohols

Even though a lot of compounds are included in this group, in a clinical setting, the word ‘alcohol’ is usually used to denote either ethyl alcohol (ethanol) or isopropyl alcohol (isopropanol, 2-propanol, or propan-2-ol) (Rutala et al. 2008; Quinn et al. 2011; Al-Adham et al. 2013; Boyce 2018).

It is thought that alcohols act by denaturation of proteins, which is corroborated by the fact that water increases their bactericidal activity (Quinn et al. 2011), since protein denaturation occurs more rapidly in the presence of this element (Al-Adham et al. 2013). Dilution between concentrations of 60 and 90%, makes alcohols most efficient, however, in concentrations below 50% they lose most of their microbicidal action (McDonnell and Russell 1999; Şahiner et al. 2019). The presence of organic matter renders the action of alcohols ineffective (Rutala et al. 2008; Quinn et al. 2011).

These disinfectants are known for their rapid bactericidal activity against vegetative forms (Şahiner et al. 2019), but their spectre also includes mycobacteria (Rutala et al. 2008; Quinn et al. 2011), some fungi (Terleckyj and Axler 1987; Salzman et al. 1993; Bundgaard-Nielsen and Nielsen 1996; Şahiner et al. 2019) and some viruses (Sattar et al. 1989). Isopropyl alcohol is a more effective bactericide than ethyl alcohol (Al-Adham et al. 2013), with the latter being usually more effective against non-enveloped hydrophilic viruses (Chang et al. 2013; Rabenau et al. 2014; Uzuner et al. 2018). In their natural form, alcohols do not have any kind of sporicidal action (Russell 1990). However, a study led by Nerandzic et al. (2015) proved that, by a process of acidification, ethanol was efficient in reducing *Clostridium difficile* spores within a 30 second contact time. Further studies are still needed in order to determine if this altered alcohol is effective against other spores and if it is possible to apply it to all formulations.

These two compounds are mostly used for topical disinfection (Quinn et al. 2011) and for the disinfection of small items and surfaces (Boyce 2018).

2.2.1.2 Alkalis

Alkalis, such as sodium hydroxide and potassium hydroxide, are strong bases (Quinn et al. 2011) that lead to the hydrolysation and destruction of lipids and proteins (Greenwood et al. 2016), compromising cell wall and membrane structures and leading to leakage of cytoplasmic components (Fernandes et al. 2013).

They have a wide spectre of action, killing many viruses (including foot and mouth disease virus, adenovirus, and swine vesicular disease virus), bacterial endospores and even prions (Quinn et al. 2011). This efficiency, however, makes them extremely caustic and hazardous leading frequently to chemical burns in the operator, when not manipulated with the recommended PPEs (Greenwood et al. 2016).

2.2.1.3 Aldehydes

2.2.1.3.1 Glutaraldehyde (Pentanedial)

Glutaraldehyde (GTA) is a rapidly acting and highly effective microbicidal agent (Quinn et al. 2011). Although its maximum efficiency is dependent of its alkaline pH (of approximately 8), it is not very stable in this form due to the polymerization of aldehyde molecules, which block the active sites responsible for its activity (Karsa 2007; Rutala et al. 2008; Al-Adham et al. 2013). Taking this into account, this compound is usually sold at an acidic pH, more stable but with less microbicidal activity, and subsequently activated by alkalinising agents, such as sodium bicarbonate (Karsa 2007; Rutala et al. 2008; Fernandes et al. 2013). Some authors claim that acidic GTA is equally as microbiocidal as its alkaline form; however, it has been proven that this only happens at a superior contact time (10 hours) and/or temperature (37ºC) (Al-Adham et al. 2013).

This compound acts through alkylation of sulfhydryl, hydroxyl, carboxyl, and amino groups, altering RNA, DNA and protein synthesis (Rutala et al. 2008). It also causes cross-linkage of proteins and macromolecules of the cell envelope, disrupting cell functionality (Maillard, Sattar et al. 2013). Organic matter does not seem to interfere with the action of this compound (Al-Adham et al. 2013).

GTA's spectre of action encloses bacteria, spores (Quinn et al. 2011), fungi (Terleckyj and Axler 1987; Howie et al. 2008) and viruses (Sattar et al. 1989; Doultree et al. 1999; Howie et al. 2008). The effect upon *Mycobacteria* spp. varies between papers due to differences mainly in the concentration and formulation used, but also in contact time and temperature (Rutala 1991; Rikimaru et al. 2000; Hernández et al. 2003; Bello et al. 2006; Howie et al. 2008; Fisher et al. 2012; Burgess et al. 2017).

Due to being non-corrosive to metals and non-damaging to lenses, rubber or plastic (Rutala et al. 2008), concentrations above 2.4% are usually used in high-disinfection and sterilization processes of thermosensitive materials, such as endoscopes and surgical instruments (Zeiger et al. 2005; FDA 2019). Unfortunately, when not cleaned properly, this biocide has been associated with several acute but self-limiting colitis in humans (Caprilli et al. 1998; Stein et al. 2001; Ahishali et al. 2008; Wang et al. 2015). GTA can also cause irritation to the eyes, skin and mucous membranes, asthma, rhinitis and allergic contact dermatitis, reason why it should not be used to clean non-critical surfaces (Shaffer and Belsito 2000; Quinn et al. 2011). Its association with carcinogenesis has not been proven neither in animals (NTP 1999) nor in humans (Collins et al. 2006; EPA 2019).

2.2.1.3.2 Formaldehyde (Methanal)

Formaldehyde's mechanism and spectre of action are similar to those of glutaraldehyde, although it is not as quick to act and is less effective in the presence of organic matter (Quinn et al. 2011; Al-Adham et al. 2013).

Contact with this substance can cause skin irritation and asthma-like respiratory problems (Karsa 2007; Rutala et al. 2008; Quinn et al. 2011). Through an evaluation of different human and animal studies, IARC (2006) concluded that there is enough evidence to consider formaldehyde as a carcinogenic compound.

Due to being less effective than glutaraldehyde, and also due to the fact that its application poses a risk to human and animal health, the use of this compound should be kept to a minimum.

2.2.1.3.3 Ortho-phthalaldehyde

Ortho-phthalaldehyde (OPA) has been one of the main compounds used as a replacement for glutaraldehyde. This compound has an excellent stability in acidic and alkaline pH, consequently not needing any kind of activation pre-utilization (Hession 2003; Pala and Moscato 2013).

Although the degree of cross-linking by OPA is not as effective as by GTA, this loss is compensated by the lipophilic aromatic component that characterizes OPA, which most likely facilitates its passage through the lipid-rich cell walls present in Gram-negative bacteria and *Mycobacteria* (Simons et al. 2000).

A study by Akamatsu et al. (2005) showed that even at a low concentration (0.25%), OPA has a faster microbicidal activity than glutaraldehyde (at 2%) and like this last disinfectant, its action is not affected by the presence of organic matter. Another study by Fraud et al. (2001) also showed that OPA has a higher mycobactericidal activity even though the concentration of GTA used in this study was lower (0.5%) than the concentration usually recommended (2%). This allows for a shorter high-level disinfection time at room temperature (a 0.6% concentration takes 12 minutes to act at 20ºC) when compared to GTA (a 2.4% concentration takes 45 minutes to act at 20ºC) (Hession 2003; FDA 2019). However, it is important to refer that OPA is not as sporicidal as GTA, but that this activity increases with alkaline pH (8) (Walsh et al. 1999).

The usage of lower concentrations of OPA has been associated with a lower toxicity (Iwasawa et al. 2011). Even though a Health Hazard Evaluation Report (Chen et al. 2015) did not find any evidence of adverse health effects caused by this compound, a study by Catlin et al. (2017) in rats and mice showed that exposure to OPA not only led to respiratory, skin and ocular lesions, but also that these lesions were of higher severity than those observed with

GTA. In human medicine, OPA high-level disinfection has also been associated with multiple anaphylactic reactions following laryngoscopies (Suzukawa et al. 2006; Atiyeh et al. 2015) and cystoscopies (Sokol 2004; Cooper et al. 2008), chemical burns (Venticique et al. 2003; Horikiri et al. 2011) and perioral and face staining (Streckenback and Alston 2003; Abdulla and Adams 2007), when the material used was not properly washed after disinfection. In veterinary medicine no similar reports were found, which could be justified by the fact that endoscopy is not a process as frequently used.

2.2.1.4 Oxidizing Agents

2.2.1.4.1 Halogens - Chlorine Compounds

Chlorine-releasing compounds are divided into two main groups: hypochlorite and N-chloro compounds (or organic chlorine compounds) (Quinn et al. 2011; Al-Adham et al. 2013).

Hypochlorite can exist in liquid (e.g. sodium hypochlorite) or solid (e.g. calcium hypochlorite) form (Rutala et al. 2008). Being a fast-acting and non-staining compound, sodium hypochlorite, is a common constituent of household bleaches, extensively used for disinfection of various surfaces, even though it cannot be stored for long periods of time due to loss of efficacy (Quinn et al. 2011).

N-chloro compounds (e.g. chloramine-T, dichloramine -T, halozone, sodium dichloro-isocyanurate and potassium dichloroisocyanurate) appear to have a slower microbicidal activity when compared to hypoclorite (Quinn et al. 2011; Al-Adham et al. 2013). However, they can retain chlorine over longer periods of time, which grants them a lengthier microbicidal effect (Rutala et al. 2008). Some of them are also less susceptible to the inhibitory action of organic matter (Quinn et al. 2011).

The disinfection capacity of these compounds occurs mainly due to the formation of hypochlorous acid resulting of the interaction between free chlorine and water molecules (Gray 2014). If in alkaline pH, this acid rapidly dissociates and loses most of its effect (Gray 2014), but, when in a favourable environment, it can lead to the formation of an extremely strong oxidant – nascent oxygen (Ponzano 2007). This molecule acts through the breakdown of nucleic acids and essential enzymes which, consequently, leads to the destruction of the microorganisms (Sansebastiano et al. 2007).

Dependent on the amount of free chlorine released (Rutala et al. 1991; Doultree et al. 1999), these compounds can be highly effective against viruses (Bloomfield et al. 1990; Kahler et al. 2010; Garcia et al. 2010; Cavali et al. 2018), vegetative bacteria (Rutala et al. 1998) and spores (Wheeldon et al. 2008). Fungi (Hageskal et al. 2012; Ma and Bibby 2017) and mycobacteria (Taylor et al. 2000; Whan et al. 2001; Le Dantec et al. 2002) may not be as susceptible, depending on the genus/species tested.

Exposure to chlorine can lead to irritative respiratory symptoms, skin burns and corneal abrasions. These symptoms are quick to appear but also rapid to recede once exposure is ceased (Winder 2001).

2.2.1.4.2 Peroxygen Compounds

2.2.1.4.2.1 Hydrogen Peroxide

Hydrogen peroxide is a non-polluting compound (Quinn et al. 2011), used frequently as an environmental surface disinfectant (Rutala et al. 2008).

The mechanism of action of this compound is associated to the formation of hydroxyl free radicals that react with various cell components, such as DNA, membrane lipids and amino acids. This action can be neutralized by some enzymes, such as catalase, superoxide dismutase and alkyl hydroperoxidases, that decompose the radicals produced by this compound into water and oxygen (Demple 1996; Rutala et al. 2008; Quinn et al. 2011; Maillard 2013b). This, however, only happens when exceptionally low concentrations of this compound are present (McDonnell and Russell 1999). The presence of organic matter does not seem to interfere with its activity (Al-Adham et al. 2013).

When used on its own, this disinfectant is not stable, and it is somewhat slow acting, having a more limited antimicrobicidal activity (Rutala et al. 2012). With the intent of reducing these negative effects, stabilizers, surfactants and/or other excipients have been added to this compound, creating an “improved” or “accelerated” hydrogen peroxide (Omidbakhsh and Sattar 2006; Rutala et al. 2012). Omidbakhsh and Sattar (2006) showed that one of these new formulations not only required a reduced contact time (1-5 minutes at 20ºC) in order to kill a wide spectre of pathogenic agents (vegetative bacteria, spores, mycobacteria, viruses and fungi), but also fell into the lowest Environmental Protection Agency (EPA) toxicity category (i.e., category IV). Howie et al. 2008 also confirmed the wide antimicrobial spectrum of improved hydrogen peroxide.

This improved/accelerated hydrogen peroxide can be used in low-level disinfection of non-critical environmental surfaces and patient care (in concentrations of 0.5-1.4%), but can also be used in high-level disinfections of semi-critical medical devices (in concentrations ≥2%) (Rutala et al. 2012), such as endoscopes (Omidbakhsh 2006).

2.2.1.4.2.2 Peracetic Acid

Peracetic acid, also called peroxyacetic acid, is a strong oxidant and disinfection agent (more so than hydrogen peroxide), usually sold as a balanced solution that also contains acetic acid and hydrogen peroxide (Al-Adham et al. 2013). This solution can be unstable, especially when diluted (Kitis 2004) and can be corrosive to some types of metals (Quinn et al. 2011; Rutala et al. 2012; Al-Adham et al. 2013). Similarly to hydrogen peroxide, its final decomposing

products are water and oxygen, making it innocuous to the environment (Al-Adham et al. 2013).

This compound has a fast efficacy against mycobacteria (Hernández et al. 2003), but also against bacteria, spores, fungi (Baldry 1983) and viruses (Quinn et al. 2011; Al-Adham et al. 2013). Its oxidative power acts upon sulfhydryl and amino groups and denatures proteins, nucleic acids and other metabolites (Kitis 2004; Quinn et al. 2011).

Deemed by the FDA (2019) as an efficient high-level sterilant and chemosterilant, it is considered the most effective biocide for endoscopic disinfection, when compared to GTA and OPA (Chenjiao et al. 2016).

Contrary to hydrogen peroxide that presents practically no toxicity, peracetic acid can be irritating to the eyes and respiratory system (Quinn et al. 2011).

2.2.1.4.2.2 Potassium Peroxymonosulfate

2.2.1.4.2.2.1 Virkon® S

Virkon® S is a disinfectant frequently used in veterinary medicine. Its initial form is of a powder that, when diluted in water usually at a 1% concentration, turns into a pink solution with a lemony scent, active up to seven days (Lanxess 2019). According to the manufacturers, Virkon® S's contact time should vary according to the target-microorganism but is most frequently applied during a 10 minute period.

With a mainly oxidative activity, Virkon® S is a complex formulation, as presented in Table 1, which main constituent is a peroxygen compound named potassium peroxymonosulfate (Lanxess 2019).

Table 1: Virkon® S's formulants and corresponding actions and quantities. Adapted from Lanxess (2019) and Nakama (2017)

Components		Action	%
Oxidizing Agents	Potassium Peroxymonosulfate (Peroxygen)	Membrane and wall disruption through damage of protein's and enzyme's sulphur bonds	21.41%
	Sodium Chloride (Halogen)		1.50%
Organic Acids	-	Possible adjuvants to the antimicrobial action of the oxidizing agents	77.09%
Anionic Surfactant	Sodium Dodecylbenzenesulfonate	Through the formation of micelles, transforms non-soluble compounds, such as lipids, into soluble ones	
Inorganic Buffer	-	pH control	

Lanxess (2019) declared this formulation to be effective against a total of 31 bacterial strains, 58 viruses, and 6 fungi according to US EPA standards (see Appendix 1). Some recent studies testify to Virkon® S's high efficiency against a panoply of microorganisms (Saklou et al. 2016; Sylte et al. 2017; Gabbert et al. 2018; Skinner et al. 2018), but further research is still crucial to better understand the optimum conditions required to maximise its effectiveness, especially since some doubts have surfaced regarding its efficiency against higher loads of microorganisms (Dunowska et al. 2005); and against *Mycrosporum canis* (Marchetti et al. 2006). To the best of our knowledge, there are no studies reporting Virkon® S's efficacy against microorganisms present in biofilms.

Beyond its large spectrum, Virkon® S is also characterized for being non-corrosive to stainless steel, having a low ecotoxicity/high biodegradability and also low toxicity (McCormick and Maheshwari 2004). However, according to the safety data sheet emitted by Lanxess (2017), it can still cause severe eye damage, skin and respiratory irritation, which means safety precautions should always be taken when utilizing this compound.

2.2.1.5 Phenolic Compounds

Phenol was one of the first compounds ever used with the intent of reducing infection. To this day, this molecule has been modified several times in order to originate phenolic compounds, a vast group of chemicals with an improved antimicrobial activity. The two most used phenolics in a clinical setting are ortho-phenylphenol and ortho-benzyl-para-chlorophenol (Rutala et al. 2008).

The mechanism of action of these disinfectants is concentration-dependent. At lower concentrations, these compounds interact with cell wall synthesis by interfering with essential bacterial enzymes, leading to leakage of essential metabolites; at higher concentrations they act by lysis of intracellular components (Quinn et al. 2011).

Phenolic compounds are generally considered bactericidal (Quinn et al. 2011) but not sporicidal (Russell 1990). They are also considered virucidal against enveloped viruses, but this activity, as well as their mycobactericidal and fungicidal activities, are all formulation-dependent. Their action does not seem to vary in the presence of organic matter (Quinn et al. 2011).

These compounds are toxic to cats (Addie et al. 2015) and pigs (Quinn et al. 2011), reason why they should not be used in clinics or hospitals where these animals are present.

Triclosan, a compound inserted into the bisphenol category, has been one of the most studied biocides in recent literature, especially due to its proven induction of cross-resistance to antibiotics when present in lower concentrations (Carey and McNamara 2015; Carey et al. 2016; Lu et al. 2018).

2.2.1.6 Quaternary Ammonium Compounds

Quaternary Ammonium Compounds (QACs) are a group of cationic amphiphilic surfactants (Bureš 2019) that can be used on their own or added to other compounds in order to make them more effective against all types of microorganisms (Moore et al. 2008). Due to their high variable structure, their molecules have been modified throughout the years in order to lower associated resistances and toxicity and increase their efficiency (Gerba 2015; Bureš 2019).

Being cationic compounds, they are incompatible with a wide variety of chemical agents, such as other surfactants and phospholipids (Quinn et al. 2011; Al-Adham et al. 2013). Their activity is also affected by the presence of organic matter, pH (neutral to slightly alkaline pH is preferred) and temperature (more efficient at higher temperatures) (Hegstad et al. 2010; Quinn et al. 2011; Al-Adham et al. 2013).

These compounds are membrane-active agents that cause a disruption between the lipids and proteins in membrane structures, which leads to an alteration of cell permeability and leakage of intracellular components (Ioannou et al. 2007; Quinn et al. 2011; Al-Adham et al. 2013; Gerba 2015). At higher concentrations they can also lead to coagulation of these same components (Quinn et al. 2011). Usually, this mechanism of action makes them more effective against enveloped (lipid-containing) viruses (Gerba 2015).

Even though each compound has its own antimicrobial characteristics (Rutala et al. 2008), QACs generally have a higher efficiency against Gram-positive bacteria than Gram-negative (Tischer et al. 2012). For example *Pseudomonas aeruginosa* is known to be less susceptible to these disinfectants, which could be attributed to adaptative alterations of their membranes (Méchin et al. 1999; Guérin-Méchin et al 1999; Guérin-Méchin et al 2000; Tabata et al. 2003). A reduced susceptibility (either intrinsic or acquired) of some bacterial species to these compounds can also be associated with the activity of efflux pumps (Hegstad et al. 2010). Their fungicidal activity is variable (Terleckyj and Axler 1987; Bundgaard-Nielsen and Nielsen 1996) and they are generally effective against enveloped virus (Quinn et al. 2011; Gerba 2015), but not against non-enveloped virus (Mbithi et al. 1990; Doultree et al. 1999) nor mycobacteria (Best et al. 1990; Rutala et al. 1991; Best et al. 1994; Bello et al. 2006; Tischer et al. 2012). They also do not seem to be sporicidal, except when associated with other compounds (Russell 1990; Best et al. 1994).

2.2 Factors Influencing the Activity of Biocides

The ability to eradicate different classes of microorganisms presented by each biocide does not depend solemnly on the type of biocide, but also on other factors extrinsic to the biocide. Maillard (2005a, 2005b, 2013a) divides the factors that affect biocide activity in three different groups: (i) factors that depend on the biocide formulation; (ii) factors that depend on the conditions on which the biocide is applied; and (iii) type of microorganisms present at the site of application.

2.2.1 Factors Related to the Biocide

2.2.1.1 Concentration

Considered the most important factor inherent to the biocide (Russell and McDonnell 2000), concentration correlates not only with efficacy but also with toxicity, being essentially an equilibrium between these two components (Maillard 2005b).

The concentration present in formulation is one of the factors that dictates the amount of target sites affected and the degree of damage inflicted in these sites (Maillard 2002). Both components are directly proportional to the biocide's lethal effect (Maillard, Bloomfield et al. 2013). Taking this into consideration, a low (sub-lethal) concentration could cause a selective pressure on the different microorganisms present and, subsequently, lead to a decrease in susceptibility or even resistance to a biocide (Thomas et al. 2000; Walsh et al. 2003; Maillard, Bloomfield et al. 2013; Knapp et al. 2013; Wesgate et al. 2016).

The dilution factor (η), which denotes the effect of dilution on microbicidal efficacy (Russell and McDonnell 2000; Maillard 2002, 2005a, 2005b, 2013a; Maillard, Bloomfield et al. 2013), can serve as an indicator of the biocides most at risk of inducing this kind of resistance in micro-organisms (Maillard, Bloomfield et al. 2013). Biocides such as alcohols and phenolics have a high η, which means that, when diluted, they lose most (if not all) of their microbicidal activity, needing a more prolonged contact time in order to kill the same number of microorganisms. Other biocides, such as QACs and aldehydes, have a low η, which means that they are more tolerant to changes in concentration (Russell and McDonnell 2000; Maillard 2002, 2005b, 2013a; Maillard, Bloomfield et al. 2013).

This factor is also closely related to the interaction between the biocide and the target site. While the interaction of biocides with a low η (< 2) is based on chemical or ionic binding, those with higher η (> 4) have a weak physical interaction with the lipophilic components of the bacterial envelope, acting as membrane disruptors or proton conductors (Hugo and Denyer 1987; Denyer and Stewart 1998; Russell and McDonnell 2000; Maillard 2002).

2.2.1.2 pH

The pH present in a formulation can alter biocide efficiency by two different methods: either by altering the biocide itself or by altering the cell surface of the target micro-organisms (Maillard 2013a).

To some biocides, an increase in pH leads to an increase in dissociation, which, in turn leads to a decrease in efficiency (Maillard 2013a). Chlorine releasing compounds, such as hypochlorite, can serve as an example to this rule, since an alkaline pH equals a higher degree of dissociation of their active molecule, hypochlorous acid, and makes them less effective (Ponzano 2007; Maillard 2013a; Gray 2014). On the other hand, some biocides are more efficient in their ionized form, thus benefiting from an increase in pH (Maillard 2013a).

Some molecules' stability can depend on an environmental acidic or alkaline pH (Maillard 2013a). One previously mentioned example to this rule are aldehydes, but especially glutaraldehye. At an alkaline pH, this disinfectant is highly efficient but also highly unstable, due to polymerization of aldehyde molecules; at an acidic pH it presents less microbicidal activity but becomes more stable (Karsa 2007; Rutala et al. 2008; Al-Adham et al. 2013).

Finally, pH can also alter the biocide-microorganism interaction by modifying the charge properties of the cell surface. Therefore, an increase in pH equates to a more negatively charged cell thus making cationic molecules, such as QACs, more efficient (Bruinsma et al. 2006; Maillard 2013a).

2.2.1.3 Formulation

One of the problems raised by Maillard (2018) and Maillard, Bloomfield et al. (2013) in relation to *in vitro* studies about bacterial resistance to biocides is that most of them tested the biocide by itself, when *in vivo* these compounds were usually applied alongside other excipients (such as solvents, emulsifiers/surfactants, thickeners, chelating agents, alkalis or acids, buffering agents, corrosion inhibitors, colourants and flagrances), most of them added in order to influence the activity of the microbicide itself (Maillard 2013a; Maillard, Bloomfield et al. 2013). One example of this influence is the synergic/antagonistic effect of cationic disinfectants (QACs) and non-ionic surfactants, which has been reported multiple times (Gomi et al. 2012; Maillard 2013a; Tobe et al. 2015). It is thought that, if the non-ionic surfactant is present in a concentration below critical micelle concentration (CMC), this agent can increase the cell permeability of microorganisms. On the other hand, if the concentration is above CMC, micelles form complexes with the cationic parts of the biocide, reducing its uptake by the microorganisms (Maillard 2013a; Tobe et al. 2015).

2.2.1.4 Other Factors

Other factors, such as the presence of metal ions and the biocide's bioavailability in the formulation on which it is used (Maillard 2013a), may not be as impactful as those before mentioned but should be considered nonetheless.

2.2.2 Factors Related to the Treatment Conditions

2.2.2.1 Temperature

Almost all biocides' efficacy can benefit from a rise in temperature (SCENIHR 2009; Maillard 2013a). Knowledge of how microbicides react to an increment in temperature can be useful in applications such as combining a microbicide and heat in sterilization procedures but also to determine the efficacy of disinfection of certain surfaces at low temperatures (Maillard 2005b, 2013a). On the other hand, if this rise is too steep, it could lead to loss of efficiency due to degradation or evaporation of the biocide (Maillard 2013a).

2.2.2.2 Organic Matter

In a clinical setting, organic matter can be represented by a number of elements such as blood, urine and fecal matter. This factor has been studied thoroughly throughout the years, especially in bacteria and viruses' elimination. Almost all studies demonstrate a progressively reducing efficiency of biocides in the presence of rising concentrations of organic matter (Gélinas and Goulet 1983; Wang et al. 2005; Kawamura-Sato et al. 2008; Martin et al. 2008; Chandler-Bostock and Mellits 2015; Cavalli et al. 2018; Satto et al. 2019).

This factor can affect the efficiency of biocides in three different ways: (i) it can interfere with the biocide's molecule, reducing the quantity of this compound that is able to react with the microorganism; (ii) it can form a protective barrier around the microorganism; and/or (iii) it can lead to the aggregation of microorganisms (Maillard 2005a, 2013a; Cavalli et al. 2018).

With this in mind, the importance of cleaning before disinfectant use and the incorporation of this step in disinfecting protocols becomes clear.

2.2.2.3 Contact Time

The contact time, usually stipulated by the manufacturer, is often not respected in practice, which could lead to the survival of less susceptible microorganisms (Maillard 2005a; Williams et al. 2009; Cheeseman et al. 2009).

Although it is usually thought that low concentrations and high contact times are equivalent to high concentrations and low contact times (SCENIHR 2009), it has been proven that this is not always the case (Pinto et al. 2009).

2.2.2.4 Other Factors

Other factors such as oxygenation, type of surface, relative humidity and the usage of hard water should also be taken into consideration in the establishment of disinfecting protocols (Maillard 2013a).

2.2.3 Factors Related to the Microorganisms

Over the last years, there have been many definitions appointed to various related terms such as resistance, insusceptibility and tolerance (Cloete 2003; Russell 2003a; Maillard 2007, 2013b; Maillard, Bloomfield et al. 2013; Maillard 2018), which leads to a lot of uncertainty when trying to compare results from certain works (Maillard 2013b; Maillard, Bloomfield et al. 2013). This is also aggravated due to the lack of standardized laboratory methods that could be used to determine biocide susceptibility; and MIC/MBC breakpoints that define the line between biocide tolerance and susceptibility (Maillard 2013b, Roedel et al. 2019).

Taking this into account, Maillard (2018) defined resistance as the capability that a microorganism has of surviving in antibacterial products, independently of this product's concentration. Reduced susceptibility (sometimes characterized as tolerance) can be defined as an increase in the minimal inhibitory concentration (MIC) required to inhibit the microorganism, even if it means that at an in-use concentration of the biocide in question is still effective (Maillard 2013b; Maillard, Bloomfield et al. 2013; Maillard 2018).

Resistance can also be divided into two types: intrinsic (sometimes characterized as innate or insusceptibility) or acquired. While an intrinsic resistance denotes a resistance due to mechanisms innate to the microorganism, an acquired resistance denotes mechanisms that can be inherited through mutation(s) or the acquisition of resistance genes (Poole 2002; Maillard 2013b). This distinction, however, is not always possible (Maillard 2007). In this case, it is also important to consider the possibility of transient resistances, present only when the biocide is exerting its selective pressure on microorganisms and disappearing thereafter (Wesgate et al. 2016; Maillard 2018).

2.2.3.1 Mechanisms of Resistance in Microorganisms

2.2.3.1.1 Reduced Biocide Penetration

The first line of defence of every microorganism against any kind of antimicrobial agent is the cell wall (IFH, 2000), which means that a different wall composition should portray a different degree of resistance (see Figure 2) (Denyer and Maillard 2002; Lambert 2002; Maillard 2005b, 2007, 2013a, 2013b, 2018).

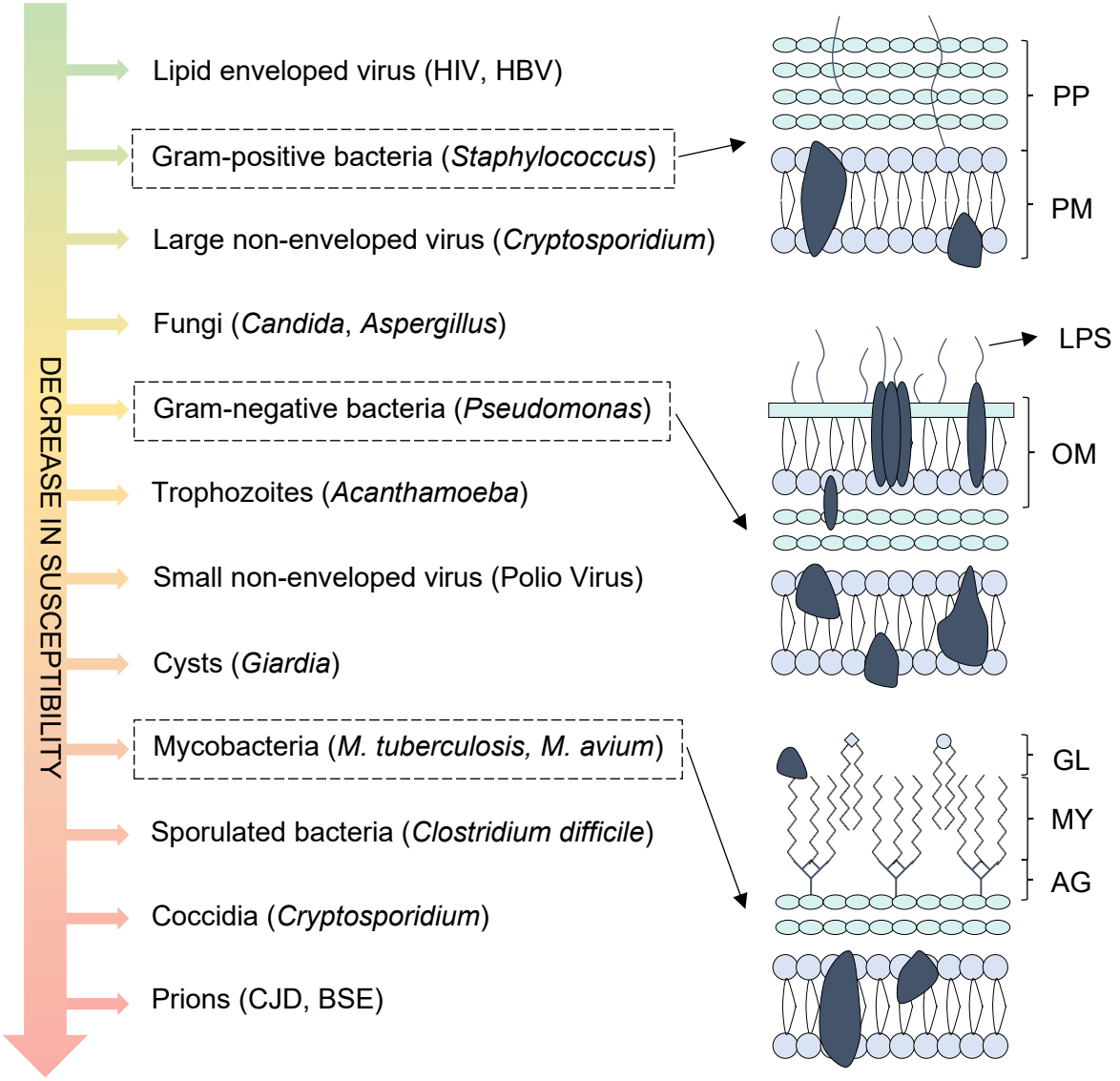


Figure 2: Decreasing order of susceptibility of microorganisms to biocides, as well as a simplified representation of barrier differences between three microorganisms (Gram-positive and Gram-negative bacteria and mycobacteria). Adapted from McDonnell and Russell (1999), Lambert (2002) and Willey et al. (2008).

PP: Peptidoglycan; PM: Plasma Membrane; LPS: Lipopolysaccharide; OM: Outer Membrane; GL: Glycolipids; MY: Mycolic Acid; AG: Arabinogalactan

This is seen especially in bacterial spores, which are composed by five to six (according to the absence/presence of the exosporium) different layers that coat the core. All of these layers, except for the outer membrane, play a role in the resistance presented by these structures (Setlow 2006, 2013). While some layers, such as the coat, are composed of proteins capable of detoxifying chemicals (Setlow 2006; Henriques and Moran Jr 2007), others, such as the inner membrane, act as a filter to different molecules (Cortezzo and Setlow 2005; Setlow 2006).

In mycobacteria, the lipid-rich cell wall comprised of important elements such as arabinogalactan/arabinomannan and the mycoylacylarabinogalactan layer, forms a waxy barrier impeding the penetration of various antimicrobials (Walsh et al. 2001; Lambert 2002). Similarly, in Gram-negative bacteria, the presence of lipopolysaccharides (LPSs) in the outer membrane is responsible for the reduced efficiency of certain biocides (Denyer and Maillard 2002; Maillard 2018).

Alterations to these barriers following biocide exposure can also contribute to an ever-growing decrease in susceptibility (Maillard 2018). Some of these alterations pass as changes in membrane protein composition (Winder et al. 2000; Tattawasart et al. 2000), quantity of fatty acid (Guérin-Méchin et al. 2000) and phospholipid present (Boeris et al. 2007), and also in membrane potential (Bruinsma et al. 2006). The loss/alteration of porins can also be responsible for a hinder in susceptibility (Tattawasart et al. 2000; Fernández-Cuenca et al. 2015).

2.2.3.1.2 Efflux Mechanisms

When a biocide is able to penetrate a bacterial cell and begins to accumulate intracellularly, efflux is one of the instruments responsible for impairing this same accumulation (Poole 2001, 2002; Piddock 2006, SCENIHR 2009, Maillard 2013b, 2018). This mechanism occurs due to the presence of membrane-associated proteins known as multidrug efflux systems (MES) that extrude different antimicrobials (Quinn et al. 2011; Tegos et al. 2011). In microorganisms, MES can be divided into five different classes: ATP-binding cassette super family (ABC), major facilitator super family (MFS), resistance-nodulation cell division super family (RND), small multidrug resistance family (SMR) [a member of the much larger drug/metabolite transporter (DMT) super family] and multidrug and toxic compound extrusion (MATE) (Poole 2001, 2005; Piddock 2006; Poole 2007; Tegos et al. 2011). In bacteria, the RND is the only family exclusive to Gram-negative microorganisms, with all the other families being present in either Gram-positive or negative species (Handzlik et al. 2013; Sun et al. 2014). These systems originate from the expression of genes located either in the bacterial chromosome or in plasmids (Poole 2005, 2007).

Efflux pumps associated with a decrease in disinfectant susceptibility have been identified in *Staphylococcus aureus* (Heir et al. 1998; Kazama et al. 1998; Heir et al. 1999; Kaatz et al. 2005; Liu et al. 2015; Conceição et al. 2016), *Pseudomonas aeruginosa* (Chuanchuen et al. 2001, 2003; He et al. 2004; Mima et al. 2007), *Escherichia coli* (Nishino and Yamaguchi 2001; Chung and Saier Jr 2002; Burgos et al. 2016), *Samonella enterica* serovar Typhimurium (Randall et al. 2007; Weber et al. 2008), *Enterococcus faecalis* (Kazama et al. 1998), among others. Most of these systems correspond to multidrug transporters (Poole 2005) but have been especially associated with decreased susceptibility in QACs and

phenolics (mainly triclosan) (Heir et al. 1998; Kazama et al. 1998; Heir et al. 1999; Chuanchuen et al. 2001; Nishino and Yamaguchi 2001; Chung and Saier Jr, 2002; Chuanchuen et al. 2003; He et al. 2004; Kaatz et al. 2005; Mima et al. 2007; Randall et al. 2007; Webber et al. 2008; Mc Cay et al. 2010).

It is concurred that, maybe excluding triclosan's case, efflux pumps alone cannot induce resistance, causing only a decrease in susceptibility, which means that resistance must originate from an overlapping of efflux pumps with other mechanisms (Maillard 2013b; SCENIHR 2009; Maillard 2018).

2.2.3.1.3 Enzymatic Degradation

As mentioned before, low concentrations of oxidative compounds can be inactivated due to the presence of enzymes such as catalase, superoxide dismutase and alkyl hydroperoxidases, which are able to transform the radicals formed by the biocide into molecules with no biocidal activity (water and oxygen) (Demple 1996; McDonnell and Russell 1999; Rutala et al. 2008; Quinn et al. 2011; Maillard 2013b). Aldehydes can also be inactivated by aldehyde dehydrogenases (Kümmerle et al. 1996).

Similarly to efflux pumps, it is thought that this mechanism alone cannot induce resistance in bacteria, leading however to a lower susceptibility (Maillard 2013b, 2018).

2.2.3.1.4 Acquired Resistance

Until this day, acquired resistance to biocides through mutation have been mostly reported regarding triclosan, and generally only when low concentrations of this biocide are used. This mutation occurs in the gene responsible for the formation of an enzyme called enoyl-acyl carrier protein reductase, one of triclosan's sites of action, reducing the bacteria susceptibility to this biocide (McMurry et al. 1999; Heath et al. 1999; Levy et al. 1999; Stewart et al. 1999; Zhu et al. 2010).

There are also reports of acquired resistance (especially associated with efflux pumps and enzymatic degradation) through transference of genetic elements related to the decrease in susceptibility to different biocides present in plasmids (Bjorland et al. 2001; Noguchi 2005; Hansen et al. 2005; Hansen et al. 2007).

2.2.3.2 Biofilms

A biofilm is characterized as a community of microorganisms connected by an extracellular matrix composed mainly of exopolysaccharides, proteins and nucleic acids. This cluster grants microbes a lower susceptibility to hostile environment conditions (Bridier et al. 2011; Roy et al. 2017).

When present in biofilm communities (in contrast with their planktonic forms), microorganisms usually present a lower susceptibility to biocides (Pajkos et al. 2004; Smith and Hunter 2008; Bressler et al. 2009; Wong et al. 2009; Luciano et al. 2016), which can vary according to the microorganism(s) present in the biofilm and the active substance/formulation used (Craveiro et al. 2015; Cai et al. 2018; Chowdhury et al. 2018; Lineback et al. 2018).

It is thought that this alteration in susceptibility is due to a multicomponent mechanism:

(i) Variations in oxygen and nutrient availability within the biofilm, leading to the presence of different metabolism rates, including quiescent bacteria in the inner layer of the biofilm (Huang et al. 1995; Maillard 2013a);

(ii) Reduced bioavailability of the biocide due to a hindered penetration or quenching, caused by the polymeric matrix (Pan et al. 2006; Maillard 2013a; Venkatesan et al. 2015);

(iii) Presence of enzymes in the extracellular matrix that lead to biocide inactivation (Huang et al. 1995; Maillard 2013a);

(iv) Increase in mutability and transference of resistance determinants between cells leading to an increased induction of efflux-pumps (Maira-Litrán et al. 2000; Maillard 2013a; Venkatesan et al. 2015).

2.3 Association Between Biocide and Antibiotic Resistance

Antibiotic resistance has been a rising global problem in both human and veterinary medicine, especially due to a drop in treatment efficiency, leading to prolonged illnesses and higher monetary costs (WHO, 2018).

Biocides, contrarily to antibiotics, are frequently used, either in households or hospitals, without any kind of control or restraint. This means that possible associations between biocide use and antibiotic resistance (or decrease susceptibility) are dangerous. The debate that exists of whether or not this connection is a reality has been a long one, with numerous reports pointing both ways, varying in both the biocides and microorganisms tested (Cole et al. 2003; Márquez et al. 2016; Wieland et. 2017; Alotaibi et al. 2017; Ghanem and Haddadin 2018; Humayoun et al. 2018; Roedel et al. 2019).

In reports that indeed conclude the existence of an association between biocide and antibiotic resistance development, common resistant mechanisms (to biocides and antibiotics) such as multidrug efflux pumps (Rakic-Martinez et al. 2011; Curiao et al. 2016; Buffet-Bataillon et al. 2016; Burgos et al. 2016) and changes in bacterial cell wall permeability (Tattawasart et al. 2000; Gadea et al. 2017; Kim et al. 2018) were reported. A study by Han et al. (2019) also indicated that biocides (in this case QACs) could enhance the transference of certain genetic elements related to antibiotic resistance between bacteria.

In conclusion, with the intention of reducing the quantity of microorganisms present in different surfaces of veterinary hospitals/clinics and consequently reducing the percentage of HAIs therein present, a better understanding of all the factors that can alter the efficiency of biocides is of utmost importance. Furthermore, there are still a lot of doubts about possible associations between biocide and antibiotic resistance development that need to be clarified, especially since biocides are used as a unsupervised weapon in preventing infections by MDR bacteria.

CHAPTER II: EVALUATION OF VIRKON® S' EFFICENCY

1. OBJECTIVES

Biocides correspond to a variety of products that have been used for a long time, mainly to reduce the quantity of microorganisms present in a certain environment. This usage has experienced a significant increase in these last years, and with its expansion, several doubts about the development of bacterial resistance associated with these compounds as well as a possible relation of their application with an increase in bacterial resistance to antibiotics have been raised (Maillard 2018). These concerns become even more important when we consider the several factors that can affect biocide efficacy (Maillard 2005a, 2005b, 2013a), which may not be of common knowledge to every user.

Considering these factors, the main objectives of this study are:

- To determine if there are any differences in biocide susceptibility between MDR and non-MDR isolates;
- To determine to which degree certain factors (concentration and presence of organic matter) can affect the efficiency of a biocide's formulation (Virkon® S) against certain bacteria collected from BICU surfaces (*E. coli*, *Pseudomonas* spp., *Enterococcus* spp., *Staphylococcus pseudintermedius*);
- To determine the influence of the presence of Virkon® S's sublethal concentrations in the resistance profile of selected enterococci.

2. MATERIALS AND METODS

2.1 Biological Isolation and Containment Unit of the Veterinary Teaching Hospital

The BICU of the Veterinary Teaching Hospital (VTH) (Veterinary Medicine Faculty, University of Lisbon) is located in a building separate from the hospital, with its own entrance and restricted access. It receives animals suspected or confirmed of having an infectious disease mainly: gastrointestinal diseases of unknown aetiology, gastroenteritis with suspected infectious aetiology, canine distemper, respiratory diseases of unknown aetiology, infectious canine tracheobronchitis, canine infectious hepatitis, diarrhoea by *Campylobacter* spp., leptospirosis, feline leukaemia, feline immunodeficiency, feline infectious peritonitis, panleukopenia, superior respiratory tract infections in felines, cutaneous diseases (such as dermatitis by multi-resistant bacteria, dermatophytosis and scabies) and any clinical suspicion of a disease caused by a multi-resistant bacteria. A study by Machado (2016), indicates that, between 2013 and 2016, the most common infectious diseases diagnosed in this unit were FeLV (26.5%), FIV (18.6%) and panleukopenia (14.2%) in cats and gastroenteritis (55.2%), mainly by canine parvovirus (24.1%), but also hemorrhagic gastroenteritis (21.6%) in dogs.

The space of the unit is divided into a total of four hospitalization rooms: one for cats, two for dogs and one for intermedium patients, i.e., those suspected but not confirmed of having an infectious disease due to lack of vaccination or testing. There is also a room that works as a reception for the animals that come in, a work room for medical personnel and students, a preparatory room and a storage room.

This unit has several measures appointed for infection control. These measures were implemented as engineering controls, such as the presence of a ventilation system equipped with an HEPA filter that maintains a stable temperature and negative pressure; as administrative controls, which include several SOPs that, amongst others, give indications on how to manipulate patients (including utilization of PPEs), on hand hygiene and also on cleaning and disinfecting different surfaces.

All hospitalization rooms are equipped with cages and examination tables made of stainless steel and a cabinet for material storage needed for patient examination (each room has its own materials).

2.1 Bacterial Isolates

In this study, a collection of 34 bacterial isolates, obtained by Verdial (2020) in her master thesis "*Preventing Nosocomial Bacterial Infections: Bacteriological Control of Hospital Surfaces of the Biological Isolation and Containment Unit of the Veterinary Teaching Hospital of the FMV-ULisboa*", was used (isolate original identification and origin presented in Appendix 2). These isolates were, presumptively identified through macroscopic observation in selective

media, Gram staining and coagulase/catalase tests, as *Enterococcus* spp. (n=23), *Escherichia coli* (n=3), *Pseudomonas* spp. (n=3) and *Staphylococcus* spp. (n=5) and then kept in cryopreservation tubes (VWR® International, Leuven, Belgium) filled with an association of buffered peptone water (VWR® International, Leuven, Belgium) and glycerol (VWR® International, Leuven, Belgium), at a temperature of -20ºC.

Before the beginning of this study, the presumptive identification made beforehand was confirmed by inoculation in Brain Heart Infusion (BHI) agar medium (VWR® International, Leuven, Belgium), for a period of 24 hours at 37ºC (±1ºC), followed by inoculation in selective media, with the purpose of evaluating morphology and discerning possible contaminations. Therefore, the enterococci were inoculated in Slanetz Bartley Medium (SBM) (AppliChem®, Darmstadt, Germany), *E. coli* in MacConkey (VWR® International, Leuven, Belgium), the *Pseudomonas* spp. in Glutamate Starch Phenol Red Selective Agar (GSP) (Merck KGaA®, Darmstadt, Germany) and the staphylococci in Mannitol Salt Agar (MSA) (VWR® International, Leuven, Belgium). After incubation, it was possible to corroborate the presumptive identification of all enterococci and *E. coli*, however, an additional step was performed in the identification of all staphylococci isolates and one *Pseudomonas* isolate.

The staphylococci isolates were further identified using the BBL™ Crystal™ Gram-Positive Identification System (BD®, Sparks, USA), allowing the identification of four isolates as *S. schleiferi* and one as *S. pseudintermedius*. From these five isolates, only the *S. pseudintermedius* was selected to be included in this study, considering the role of this bacterial species in ear and skin infections in dogs but also in HAIs (one of the main concerns in this study) and due to the increase of infections promoted by MRSPs, to which treatment is problematic (Weese and van Duijkeren 2010; Bannoehr and Guardabassi 2012).

One of the *Pseudomonas* spp. isolates presented a distinct optimal incubation temperature (of 25-30ºC instead of 37ºC). As such, this isolate was further identified using the Analytical Profile Index® (API) 20 NE Test System (bioMérieux®, France), which allowed its identification as *P. fluorescens*. As this species is not frequently associated with disease in dogs and cats, with only a few cases being described so far (Chapalain et al. 2008; Donnarumma et al. 2010), this isolate was not included in this study.

Lastly, all the selected isolates were inoculated once more in BHI agar and after 24 hours at a temperature of 37ºC (±1ºC) they were transferred to new cryopreservation tubes to be used throughout the study. The *Enterococcus* spp. isolates were re-labelled from “E1” to “E23”, the *E. coli* isolates as “C1”, “C2” and “C3”, the *Pseudomonas* spp. isolates as “P1” and “P3” and the *S. pseudintermedius* isolate as “S3”.

In this study, four control strains were used, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, *Enterococcus hirae* ATCC 10541 and *Escherichia coli* ATCC 10536, as recommended by the European Standard (EN) 1656 (CEN 2009).

2.2 Antimicrobial Susceptibility Testing

The characterization of the susceptibility profile of all isolates under study (n=29) was performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines VET01-A4, VET01-S2 and M100S (CLSI 2013a, 2013b, 2016). A control strain (*E. coli* ATCC 25922) was also tested, as recommended by CLSI.

The antibiotics to be tested were chosen according to their frequent use as a treatment option in the VTH: amoxicillin associated with clavulanic acid (AMC), ampicillin (AMP), cefalexin (CL), cefotaxime (CTX), ciprofloxacin (CIP), enrofloxacin (ENR), gentamicin (CN), sulfamethoxazole associated with trimethoprim (SXT) and tetracycline (TE) (Oxoid Limited®, Hampshire, United Kingdom) (Table 2).

Table 2: Antibiotics used in the characterization of the isolate susceptibility profile, their concentrations and susceptibility limits.

Antibiotic		Concentration	Limits			Isolates
			S	I	R	
Amoxicillin + Clavulanic Acid	AMC	20/10 µg	≥ 20	-	19 ≤	*Staphylococcus*
			≥ 18	14-17	13 ≤	Other isolates
Ampicillin	AMP	10 µg	≥ 29	-	≤ 28	*Staphylococcus*
			≥ 17	-	≤ 16	*Enterococcus*
			≥ 17	14-16	≤ 13	*E. coli*
Cefalexin	CL	30 µg	≥ 18	15-17	≤ 14	All isolates
Cefotaxime	CTX	30 µg	≥ 23	15-22	≤ 14	
Ciprofloxacin	CIP	5 µg	≥ 21	16-20	≤ 15	
Enrofloxacin	ENR	5 µg	≥ 23	17-22	≤ 16	
Gentamicin	CN	10 µg	≥ 15	13-14	≤ 12	
Sulfametoxazole + Trimethoprim	SXT	1.25/23.75 µg	≥ 16	11-15	≤ 10	
Tetracycline	TE	30 µg	≥ 19	15-18	≤ 14	

S: Susceptible; I: Intermedium; R: Resistant

Bacterial suspensions equivalent to 0.5 in the McFarland scale (1.5 x 10^8 CFU/mL) were prepared for all isolates. These suspensions were then inoculated using the lawn technique in Muller-Hinton agar (Oxoid Limited®, Hampshire, United Kingdom) plates, followed by placement of the antibiotics in the agar surface and incubation at 37°C (±1°C) for 24 hours. After incubation, the diameter of the inhibition halos formed around the antibiotic disks was measured. Results were interpreted according to the CLSI guidelines VET01-S2 (CLSI 2013b) and M100S (CLSI 2016).

2.3 Determination of Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs)

The MIC and MBC values of the biocide Virkon® S were determined regarding seven selected enterococci isolates, six obtained from samples collected from areas with higher contact with animals (i.e. examination tables and cages) (E2, E3, E5, E14, E16, E17) and one from a sponge located the preparatory of the BICU (E19); and also regarding the three *E. coli*, the two *Pseudomonas* spp. isolates and the *S. pseudintermedius* isolate (n=13).

2.3.1 Preparation of Virkon® S Solutions

Before each assay, a total of 9 solutions of Virkon® S (Lanxess®, Köln, Germany) were prepared using sterilized water. These volumes represented a range of initial concentrations that, when added into the mix, would represent the final concentrations of 15 g/L, 12.5 g/L, 10 g/L (equivalent to 1%, which is the concentration regularly used in de BICU), 7.5 g/L, 5 g/L, 2.5 g/L, 1 g/L, 0.5 g/L and 0.25 g/L.

2.3.2 Preparation and Controls of the Remaining Solutions

Firstly, a diluent solution was prepared as suggested in EN 1656 (CEN 2009) to be used in the formulation of the bacterial suspensions. This diluent solution was made by diluting tryptone (Liofilchem®, Abruzzi, Italy) (1 g) and sodium chloride (Merck KGaA®, Darmstadt, Germany) (8.5 g) in 1L of water, followed by sterilization by autoclave.

Unlike antibiotics, biocides usually have a stipulated contact time determined by the manufacturer (which in Virkon® S's case is of 10 minutes) after which they are removed. In order to replicate this time period, a neutralizing substance was prepared in order to be used in the assays to neutralize Virkon® S's effect by the end of the contact time. According to EN 1656 (CEN 2009), neutralizers composed by the following substances are recommended to be utilized against oxidative biocides:

(i) Sodium thiosulfate (Scharlau®, Barcelona, Spain): between 3 and 20 g/L;

(ii) Polysorbate 80 (AppliChem®, Darmstadt, Germany): 30 g/L;

(iii) Lecithin (Scharlau®, Barcelona, Spain): 3 g/L.

For this study, different neutralizer compositions (with sodium thiosulfate variations of 3, 5, 8, 10, 15, 20 g/L) were first tested using a control strain (*E. coli* ATCC 10536) in order to determine the most efficient concentration of sodium thiosulfate, which was established to be 3 g/L.

With the purpose of confirming the absence of toxicity by the neutralizer and its components towards the bacteria, a “neutralizer control” was performed first to test the best sodium thiosulfate concentration, and then prior to every assay. This control comprised of formulating a solution of 8 mL of neutralizer, 1 mL of sterile water and 1 mL of a bacterial suspension with 10^3 CFU/mL, that was then incubated for 5 minutes (± 10 seconds) at room temperature [20ºC (±1ºC)] (time period indicated for the neutralizer to produce its effect). After this incubation period, 1 mL of this solution was inoculated by incorporation in TSA (Trypticase Soy Agar) (VWR International®, Leuven, Belgium). Following another incubation at 37ºC (±1ºC) for 24 hours, inoculated plates were observed in order to detect any possible adverse effects (Figure 3).

Figure 3: Example of a neutralizer control plaque after 24 hours of incubation at 37ºC (±1ºC).

Secondly, in order to recreate the organic matter conceivably present in surfaces disinfected with Virkon® S, low-level and high-level interfering substances were prepared as described in EN 1656 (CEN 2009).

Solutions representative of a low-level interfering substance were prepared by dissolving 3 g of bovine albumin fraction V (AppliChem®, Darmstadt, Germany) in 100 mL of water followed by sterilization by membrane filtration using a sterile syringe filter of 0.2 µm (VWR International®, Leuven, Belgium).

The high-level interfering substance solutions were prepared by dissolving 50 g of yeast extract (Scharlau®, Barcelona, Spain) in 250 mL of water. The suspension was then sterilized by autoclave and cooled until 20ºC (± 1ºC). In a separate container, 5 g of albumin were dissolved in 25 mL of water and then sterilized by membrane filtration using a sterile syringe filter of 0.2 µm, after which 25 mL of yeast extract were added.

Both solutions were preserved at 8ºC ±1ºC and used until 1 month after being prepared.

To confirm that these solutions did not affect bacterial viability an “interfering substance control” was also performed before each assay. 1 mL of the interfering substance (low or high) was added to a test tube, along with 1 mL of the bacterial suspension with 10^3 CFU/mL. After a 2 minute (± 10 seconds) incubation at room temperature, 8 mL of sterile water (the same water used to prepare the different suspensions of Virkon® S) was added to the solution,

followed by a 10 minute (± 10 seconds) incubation at room temperature. Afterwards 1 mL of this solution was inoculated by incorporation in TSA, which was incubated at 37ºC (±1ºC) for 24 hours. These plates were then evaluated in order to detect any possible adverse effects of the interfering substances towards the bacteria.

Lastly, and in order to ensure that there were no problems associated with the method employed, a final control called “neutralization method validation” was performed, first along with the neutralizer control in order to test the best sodium thiosulfate concentration and then prior to every assay. 1 mL of interfering substance, 1 mL of diluent and 8 mL of the highest concentration of Virkon® S to be tested (15 g/L) were added to a test tube. After a 10 minute (± 10 seconds) incubation at room temperature, 1 mL of this solution was transferred to another test tube with 8 mL neutralizer and 1 mL of sterile water, followed by a 5 minute (± 10 seconds) incubation at room temperature. Post-incubation, 1 mL of bacterial suspension with 10^3 CFU/mL was added to the suspension, followed by another 10 minute (± 10 seconds) incubation at room temperature. 1 mL of the final solution was inoculated by incorporation in TSA at 37ºC (±1ºC) for 24 hours. In the end, plates were evaluated in order to detect any possible adverse effects.

2.3.3 MIC Determination Protocol

The protocol used was based on the EN 1656 (CEN 2009).

Before the beginning of each assay, three 96-well plates (VWR International®, Leuven, Belgium) were prepared.

In the first plate (labelled plate 1), each well between columns 4 and 12 was filled with 160 µL of Virkon® S in the different concentrations to be tested, while columns 1 to 3 were left empty (Figure 4).

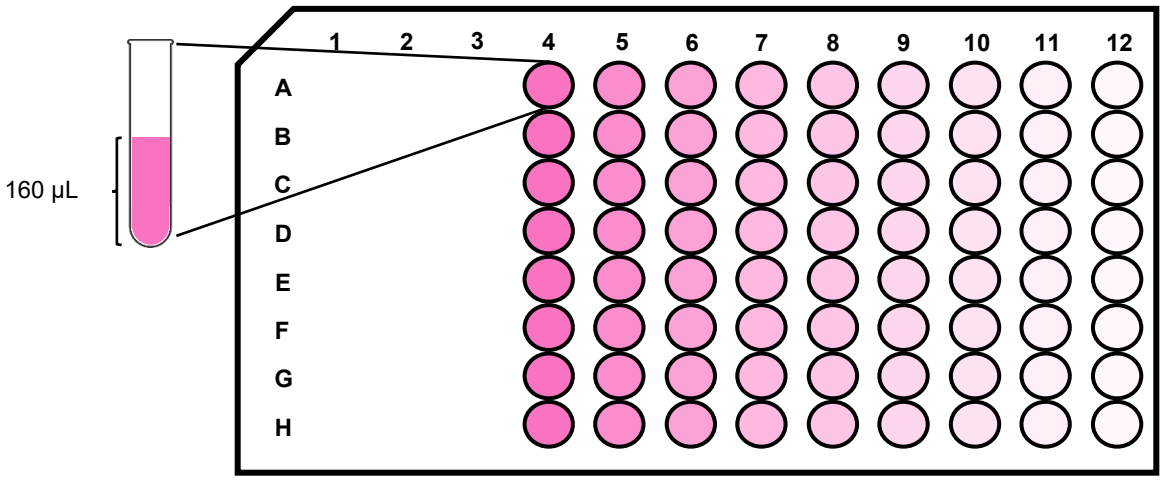


Figure 4: Representation of a 96-well plaque with Virkon® S.

O Virkon® S - concentrations: column 4 = 15.00 g/L; column 5 = 12.50 g/L; column 6 = 10.00 g/L; column 7 = 7.50 g/L; column 8 = 5.00 g/L; column 9 = 2.50 g/L; column 10 = 1.00 g/L; column 11 = 0.50 g/L; column 12 = 0.25 g/L

In the second plate (labelled plate 2), all wells between columns 4 and 12 were filled with 160 µL of neutralizer and 20 µL of sterile water. The remaining wells were left empty.

In the third plate (labelled plate 3), column 1 was used as a negative control in order to confirm the sterility of the liquid medium used [Trypticase Soy Broth (TSB) (VWR International®, Leuven, Belgium)] and filled with 200 µL, while column 3 was used as a positive control in order to confirm the presence of bacteria in the initial bacterial suspension and filled with 180 µL of TSB. Columns 4 to 12 (used for testing) were also filled with 180 µL of TSB, while column 2 was left empty.

After this preparation, and immediately prior to the start of each assay, bacterial suspensions with a concentration corresponding to 0.5 McFarland were prepared in the diluent solutions using 24 hour cultures in BHI agar.

All MIC determinations assays were performed at room temperature [20ºC (± 1ºC)].

First, all the positive control wells were filled with 20 µL of bacterial suspensions. In the assays preformed in the presence of interfering substances, 40 µL of a 1:1 mixed suspension of bacteria plus interfering substances (low or high) were added to two consecutive lines (A and B, C and D, E and F or G and H) of plate 1, containing different concentrations of Virkon® S (columns 4 to 12) (Figure 5).

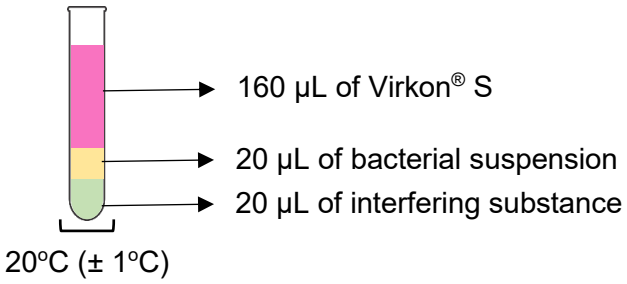


Figure 5: Representation of the wells after the addition of the bacterial suspension mixed with the interfering substance to the Virkon® S solution.

In assays performed without interfering substances, these were replaced by 20 µL of TSB, that were incorporated into the plate before the 20 µL of bacterial suspension.

After the distribution of all the solutions in plate 1, these were incubated and agitated (at approximately 500 rpm) for a period of 10 minutes (± 10 seconds) (contact time of Virkon® S given by the manufacturer) at room temperature.

Afterwards, 20 µL of the suspensions from the wells of plate 1 were transferred to plate 2 (with the 160 µL of neutralizer and 20 µL of sterile water) and this plate was incubated and agitated for 5 minutes (± 10 seconds) (time taken for the neutralizing substance to act), at room temperature.

Following this step, 20 µL of the suspensions from the wells of plate 2 were transferred to columns 4 to 12 of plate 3 (final plate), filled with 180 µL of TSB (Figure 6). This plate was then incubated at 37°C (±1°C) for 24 hours, after which it was visually evaluated to detect bacterial multiplication.

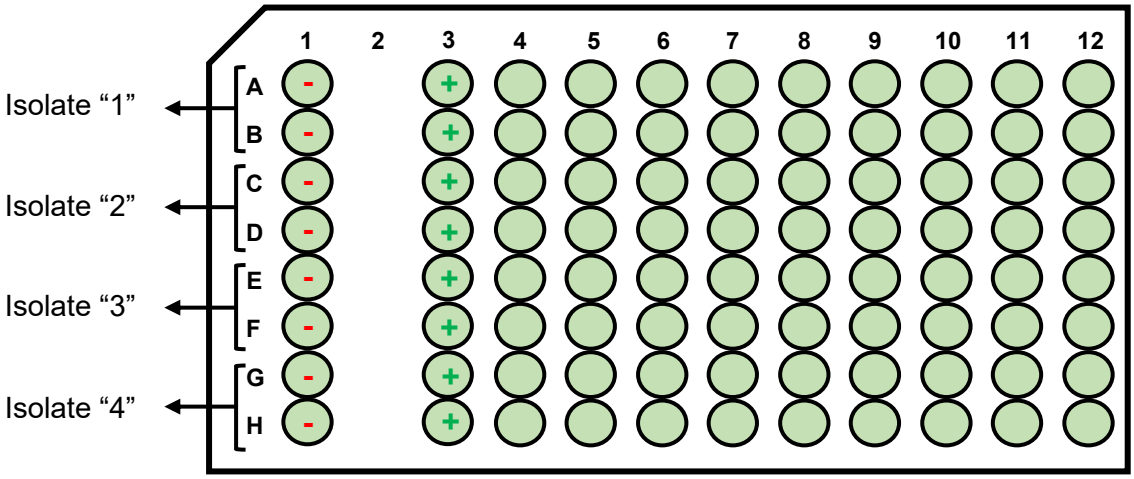


Figure 6: Representation of the final 96-well plaque (plate 3).

O 180 µL TSB and 20 µL of Virkon® S, neutralizing substance, interfering substance, sterile water and bacterial suspension

The MIC was considered to be the lowest concentration of Virkon® S that visually inhibited bacterial multiplication.

2.3.4 MBC Determination Protocol

In order to determine MBC values, 5 µL were taken from all the wells that showed no bacterial multiplication and inoculated by spot on lawn into a TSA plate (Figure 8). After incubation at 37°C (±1°C) for 24 hours, these agar plates were observed to detect bacterial colonies' formation.

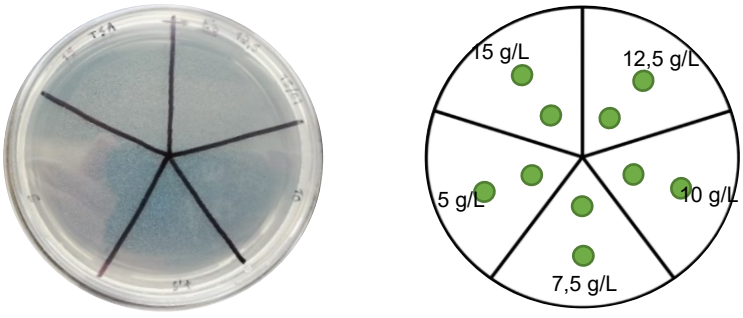


Figure 7: Example of a division of an MBC plate if the MIC obtained was 5 g/L.

O Inoculation spots for the MBCs

2.4 Influence of Virkon® S Sub-MIC Values on Isolates Biocide Resistance Ability

After determination of the MICs and MBCs of Virkon® S regarding the original isolates, 7 *Enterococcus* spp. were selected in order to perform this next task. This decision is predicated on the fact that this bacterial genus was the one detected in higher number in the BICU by Verdial (2020) but also on the fact that this was the only group of bacteria that presented MDR isolates.

Aiming to evaluate the influence of the presence of sub-MIC concentrations of Virkon® S in the isolates' resistance profile to this biocide, bacterial suspensions with 0.5 McFarland of the 7 selected enterococci were diluted (in a proportion of 1:10) in TSB associated with a sub-MIC concentration of Virkon® S and incubated at 37ºC (±1ºC) for 24 hours. After incubation, suspensions were inoculated in TSA, and the isolates obtained after a new incubation at 37ºC for 24 hours were used to determine the new MICs and MBCs of Virkon® S.

The antimicrobial resistance profile of the isolates was also determined towards the antibiotics previously tested using the disk diffusion method, and results were compared to the ones of the original isolates.

3. RESULTS

3.1 Antimicrobial Susceptibility Testing

Antibiotic susceptibility tests were performed to a total of 29 isolates and the results were as presented in Table 3.

The isolates from the *E. coli* group (C1, C2 and C3), presented a susceptibility rate of 83.3% and a resistance rate of 10%, regarding all the antibiotics tested. The two most remarkable results presented by this group were regarding ampicillin susceptibility, being observed that 2 out of 3 isolates were classified as resistant, representing the majority of resistances presented by this group; regarding tetracycline susceptibility, 1 isolate was classified as resistant and another as intermedium.

Regarding the *Pseudomonas* spp. isolates (P1 and P3), no breakpoints for ampicillin were found in CLSI (2013a, 2013b, 2016), which means no classification was attributed regarding isolates susceptibility to this antibiotic. These isolates present a lower susceptibility rate (of 33.3%) when compared with the previous group, and a higher resistance rate (of 55.6%).

The enterococci group (from E1 to E23) presented the lowest susceptibility rate (of only 5.9%). These bacteria also presented the highest resistance rate (of 74.5%), primarily regarding tetracycline (99.6%) and antibiotics belonging to the β-lactams group (amoxicillin associated with clavulanic acid, ampicillin, cefalexin and cefotaxime) (88%).

The staphylococci (S3) presented a susceptibility rate of 77.8% and a resistance rate of 22.2%. This isolate, along with isolate P1, were the only ones that conferred resistance towards sulfamethoxazole associated with trimethoprim.

When comparing all 29 isolates, the antibiotic responsible for the highest percentage of resistance was tetracycline (89.7%), followed by cefalexin (86.2%), cefotaxime (82.2%), ampicillin (75.9%), amoxicillin associated with clavulanic acid (65.5%), gentamicin (48.3%), enrofloxacin (44.8%) and ciprofloxacin (27.6%). Sulfamethoxazole associated with trimethoprim had a resistance rate of 33.3%, however, this rate excludes the enterococci group since in this genus, an apparent *in vitro* susceptibility profile obtained in a medium void of folic acid may not be equivalent to an *in vivo* susceptibility (Hollenbeck and Rice 2012).

According to the classification presented by Magiorakos et al. (2011), in which a multidrug-resistant (MDR) bacteria is defined as a microorganism resistant to one or more antibiotics from three or more antibiotic classes (that vary accordingly to the bacterial species), in this study, 14 isolates (E1, E3, E5, E6, E7, E8, E12, E13, E15, E16, E17, E19, E21 and E23) fall into this category, corresponding to 60.9% of the total of the enterococci tested and 48.3% of the total of isolates evaluated.

Table 3: Results from the antimicrobial susceptibility tests of all isolates under study (n=29), with the corresponding categorization (susceptible, intermedium or resistant) and diameter of the inhibition halo in millimetres.

Isolate Code	AMC	AMP	CL	CTX	CAZ†	IMP†	CIP	ENR	CN	SXT	TE	MDR
C1	S (30)	S (27)	S (24)	S (45)	S (31)	-	S (22)	I (20)	S (19)	S (21)	S (22)	-
C2	S (20)	R (6)	S (19)	S (31)	S (23)	-	S (23)	S (30)	S (19)	S (23)	R (6)	-
C3	S (21)	R (6)	S (20)	S (29)	S (30)	-	S (28)	S (28)	S (19)	S (23)	I (17)	-
P1	R (6)	-	R (6)	I (18)	-	S (31)	S (29)	I (20)	S (19)	R (6)	R (12)	-
P3	R (6)	-	R (6)	R (6)	-	R (6)	S (25)	R (14)	S (15)	S (29)	R (13)	-
E1	R (6)	R (6)	R (6)	R (6)	-	-	I (16)	R (14)	R (6)	-	R (7)	✓
E2	S (21)	R (16)	R (6)	R (6)	-	-	I (18)	I (20)	R (12)	-	R (11)	-
E3	R (6)	R (6)	R (6)	R (6)	-	-	I (17)	R (16)	R (6)	-	R (7)	✓
E4	S (24)	S (23)	R (6)	R (6)	-	-	I (18)	I (21)	R (11)	-	R (6)	-
E5	R (6)	R (6)	R (6)	R (6)	-	-	R (6)	R (6)	I (14)	-	R (12)	✓
E6	R (6)	R (6)	R (6)	R (6)	-	-	R (15)	I (17)	R (12)	-	R (11)	✓
E7	R (6)	R (6)	R (6)	R (6)	-	-	R (6)	R (6)	R (12)	-	R (11)	✓
E8	R (6)	R (6)	R (6)	R (6)	-	-	R (6)	R (6)	I (13)	-	R (12)	✓
E9	R (6)	R (6)	R (6)	R (6)	-	-	I (19)	I (18)	R (6)	-	R (7)	-
E10	R (6)	R (6)	R (6)	R (6)	-	-	I (19)	I (18)	I (13)	-	R (12)	-
E11	R (6)	R (6)	R (6)	R (6)	-	-	I (20)	I (17)	R (11)	-	R (12)	-
E12	R (6)	R (6)	R (6)	R (6)	-	-	I (19)	R (14)	R (6)	-	R (7)	✓
E13	R (6)	R (6)	R (6)	R (6)	-	-	I (17)	R (13)	R (6)	-	R (7)	✓
E14	S (19)	S (17)	R (6)	R (6)	-	-	I (20)	I (20)	R (10)	-	R (7)	-
E15	R (6)	R (6)	R (6)	R (6)	-	-	R (16)	I (17)	R (11)	-	R (11)	✓
E16	R (6)	R (6)	R (6)	R (6)	-	-	I (16)	R (15)	I (14)	-	R (7)	✓
E17	R (6)	R (6)	R (6)	R (6)	-	-	R (14)	R (10)	I (14)	-	R (6)	✓
E18	S (28)	S (25)	R (6)	R (6)	-	-	I (20)	I (19)	R (10)	-	I (18)	-
E19	R (6)	R (6)	R (6)	R (6)	-	-	R (6)	R (6)	I (14)	-	R (13)	✓
E20	S (21)	S (20)	R (6)	R (10)	-	-	I (20)	I (19)	R (12)	-	R (19)	-
E21	R (6)	R (6)	R (6)	R (6)	-	-	I (20)	R (19)	I (14)	-	R (13)	✓
E22	S (21)	S (17)	R (6)	R (6)	-	-	I (19)	I (18)	I (13)	-	R (12)	-
E23	R (6)	R (6)	R (6)	R (6)	-	-	R (6)	R (6)	I (14)	-	R (11)	✓
S3	S (21)	S (29)	S (31)	S (31)	-	-	S (30)	S (27)	S (20)	R (6)	R (10)	-
%R	65.5%	75.9%	86.2%	82.8%	-	-	27.6%	44.8%	48.3%	33.3%	89.7%	

AMC: Amoxicillin associated with clavulanic acid; AMP: Ampicillin; CL: Cefalexin; CTX: Cefotaxime; CAZ: Ceftazidime; IMP: Imipenem; CIP: Ciprofloxacin; ENR: Enrofloxacin; CN: Gentamicin; SXT: Sulfamethoxazole associated with trimethoprim; TE: Tetracycline

† Antibiotics tested by Verdial (2020)

MDR (✓): Multidrug-Resistant Isolates

C1-C3: *E. coli* isolates; P1 and P3: *Pseudomonas* spp. isolates; E1-E23: *Enterococcus* spp. isolates; S3: *Staphylococcus pseudintermedius* isolate

S: Susceptible; I: Intermediate; R: Resistant

%R: Percentage of isolates resistant to each antibiotic

3.2 Determination of Virkon® S's Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs)

Table 4 provides all the medium values obtained regarding Virkon® S's MICs and MBCs towards the selected isolates.

In assays preformed with no interfering substance (NIS), Virkon® S presented a higher MBC medium value towards the *E. coli* group (1.250 g/L), followed by the *Pseudomonas* spp. group (0.938 g/L), the enterococci group (0.750 g/L) and finally the staphylococci group (0.625 g/L). It is also noteworthy that these assays (with no interfering substance) were the ones that presented the most remarkable difference between MIC and MBC values, which was highest in the second group (1.4 times), followed closely by the first and third groups (both 1.1 times higher).

In assays preformed with a low interfering substance (LIS), Virkon® S's MBC medium value was highest in the enterococci group (4.911 g/L), followed by the *E. coli* group (2.542 g/L), then the staphylococci (2.500 g/L) and finally the *Pseudomonas* spp. group (1.000 g/L).

Lastly, in assays performed with a high interfering substance (HIS), MBC medium values were considered to be superior to 15.000 g/L towards the enterococci group, being important to refer that this was the highest concentration tested. Virkon® S's MBC medium value towards staphylococci was of 14.375 g/L, of 12.708 g/L towards the *E. coli* group and of 8.750 g/L towards the *Pseudomonas* spp. group.

When compared to MDR isolates, Virkon® S's MBC medium value towards non-MDR isolates were higher in NIS assays (0.775 g/L regarding MDR isolates and 1.063 g/L regarding non-MDR isolates) but lower in LIS assays (4.750 g/L towards MDR isolates and 4.375 g/L towards non-MDR isolates).

Table 4: Virkon® S's medium MICs and MBCs values towards all isolates tested, divided between assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (grams per litre, g/L)

Isolate Code	MDR	MIC			MBC		
		NIS	LIS	HIS	NIS	LIS	HIS
C1	-	1.000	1.375	11.250	1.000	1.375	11.250
C2	-	1.000	3.125	13.125	1.375	3.125	13.125
C3	-	1.375	3.125	13.750	1.375	3.125	13.750
$\bar{x}$		1.125	2.542	12.708	1.250	2.542	12.708
σ		0.177	0.825	1.062	0.177	0.825	1.062
P1	-	0.875	1.000	9.375	1.250	1.000	9.375
P3	-	0.500	1.000	8.125	0.625	1.000	8.125
$\bar{x}$		0.688	1.000	8.750	0.938	1.000	8.750
σ		0.188	0.000	0.625	0.313	0.000	0.625
E2	-	0.750	5.000	> 15.000	0.750	5.625	> 15.000
E3	✓	0.500	5.000	> 15.000	0.625	5.000	> 15.000
E5	✓	1.000	3.125	> 15.000	1.375	3.125	> 15.000
E14	-	0.625	5.000	> 15.000	0.625	5.000	> 15.000
E16	✓	0.625	5.000	> 15.000	0.750	5.000	> 15.000
E17	✓	0.625	5.000	> 15.000	0.625	5.000	> 15.000
E19	✓	0.500	5.625	> 15.000	0.500	5.625	> 15.000
$\bar{x}$		0.661	4.821	> 15.000	0.750	4.911	> 15.000
σ		0.160	0.725	-	0.267	0.778	-
S3	-	0.625	2.500	14.375	0.625	2.500	14.375
$\bar{x}$		0.625	2.500	14.375	0.625	2.500	14.375
σ		-	-	-	-	-	-

MDR (✓): Multidrug-Resistant Isolates

NIS: No Interfering Substance; LIS: Low Interfering Substance; HIS: High Interfering Substance

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration

MDR (✓): Multidrug-Resistant Isolates

C1-C3: *E. coli* isolates; P1 and P3: *Pseudomonas* spp. isolates; E1-E23: *Enterococcus* spp. isolates; S3: *Staphylococcus pseudintermedius* isolate

$\bar{x}$: Medium MIC and MBC value of each group; σ: Corresponding standard deviation of the population

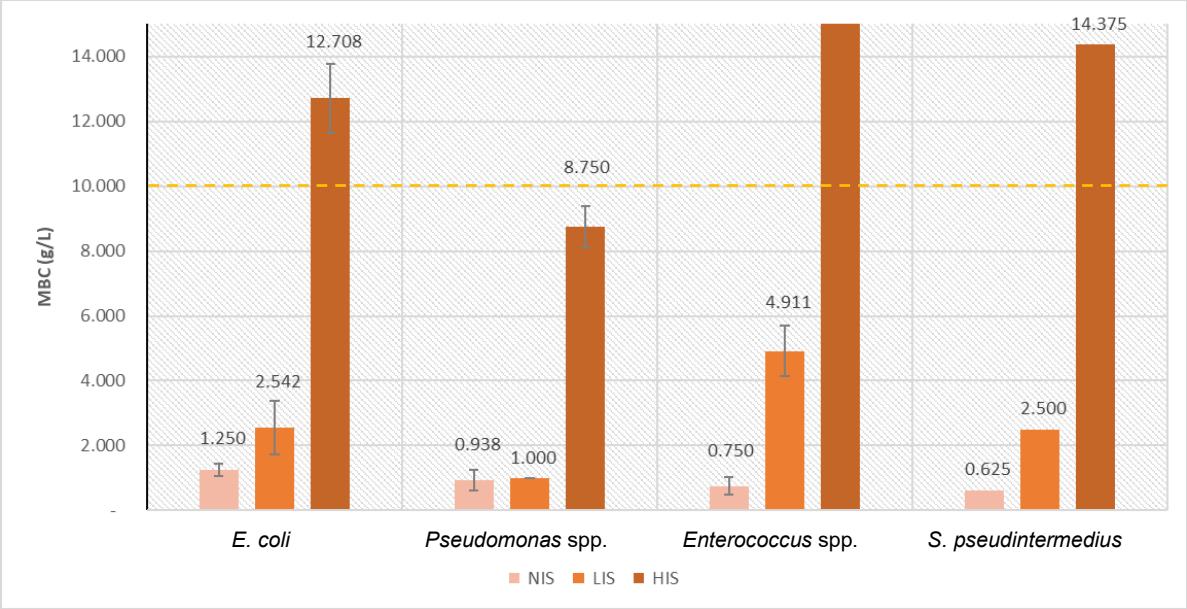
A proportional rise between MBC values and the amount of organic matter used was observed in all the isolates tested, best represented in Graphic 1.

Virkon® S's MBC medium values for the *E. coli* group were 2 times higher in the LIS assays and 10.2 times higher in the HIS assays, when compared with the NIS assays. For the *Pseudomonas* spp. group they were, respectively, 1.1 and 9.3 times higher; and for the staphylococci group, 4 and 23 times higher. Virkon® S's MBC medium values for the

enterococci was 6.5 times higher in the LIS assays, however, the rise regarding the HIS assays could not be determined as it was not possible to establish the exact MBC value (> 15.000 g/L). However it is possible to observe that it is, at least, 20 times higher than the NIS essay.

Of major concern are the results corresponding to the Virkon® S's MBCs obtained from the HIS assays of the *E.coli* group, the enterococci group and the staphylococci group, since the values obtained surpass the threshold of 10 g/L, which is the concentration regularly used in the BICU (also seen in Graphic 1).

Graphic 1: Virkon® S's of MBCs medium values obtained in the assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) towards the different groups of isolates (grams per litre).



NIS: No interfering substance; LIS: Low interfering Substance; HIS: High interfering substance
MBC: Minimal Bactericidal Concentration; --- Concentration used in the BICU

3.3 Influence of Bacterial Exposure to Sub-lethal Concentrations of Virkon® S

Comparing the susceptibility tests performed before and after the induction of bacterial multiplication at sub-MIC levels, the only alteration worth noting is the susceptibility pattern of four (E5, E16, E17, E19) of the seven isolates regarding gentamicin that changed from intermedium to resistant, as highlighted on Table 5.

Table 5: Parallel between the antibiotic susceptibility tests performed before (A) and after (B) the induction of bacterial multiplication at sub-MIC levels of the 7 selected *Enterococcus* spp. isolates, with the corresponding categorization (susceptible, intermedium or resistant) and diameter of the inhibition halo in millimetres.

Isolate Code	MDR	Assay	AMC	AMP	CL	CTX	CIP	ENR	CN	TE
E2	-	**A**	S (21)	R (16)	R (6)	R (6)	I (18)	I (20)	R (12)	R (11)
		B	S (19)	R (16)	R (6)	R (9)	I (20)	I (21)	R (12)	R (10)
E3	✓	**A**	R (6)	R (6)	R (6)	R (6)	I (17)	R (16)	R (6)	R (7)
		B	R (6)	R (6)	R (6)	R (6)	I (17)	R (15)	R (6)	R (7)
E5	✓	**A**	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	**I (14)**	R (12)
		B	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	**R (12)**	R (12)
E14	-	**A**	S (19)	S (17)	R (6)	R (6)	I (20)	I (20)	R (10)	R (7)
		B	S (21)	S (22)	R (7)	R (6)	I (19)	I (19)	R (9)	R (7)
E16	✓	**A**	R (6)	R (6)	R (6)	R (6)	I (16)	R (15)	**I (14)**	R (7)
		B	R (6)	R (6)	R (6)	R (6)	I (16)	R (14)	**R (11)**	R (6)
E17	✓	**A**	R (6)	R (6)	R (6)	R (6)	R (14)	R (10)	**I (15)**	R (6)
		B	R (6)	R (6)	R (6)	R (6)	R (13)	R (10)	**R (11)**	R (6)
E19	✓	**A**	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	**I (14)**	R (13)
		B	R (6)	R (6)	R (6)	R (6)	R (6)	R (6)	**R (11)**	R (11)

AMC: Amoxicillin associated with clavulanic acid; AMP: Ampicillin; CL: Cefalexin; CTX: Cefotaxime; CAZ: Ceftazidime; IMP: Imipenem; CIP: Ciprofloxacin; ENR: Enrofloxacin; CN: Gentamicin; TE: Tetracycline

MDR (✓): Multidrug-Resistant Isolates

E2, E3, E5, E14, E16, E17 and E19: *Enterococcus* spp. isolates;

A: Assay performed before the induction of bacterial multiplication at sub-MIC levels; B: Assay performed after the induction of bacterial multiplication at sub-MIC levels

S: Susceptible; I: Intermediate; R: Resistant

Finally, when comparing Virkon® S's MBCs values determined before and after the induction of bacterial multiplication at sub-MIC levels, a slight decrease in Virkon® S's MBCs medium values in the NIS assays (of 0.7 times) was noted and, contradictorily, a significant increase of these values was observed in the LIS assays (of 1.5), as can be seen in Table 6.

As presented before induction of bacterial multiplication at sub-MIC levels, when compared to Virkon® S's MBCs medium values for the MDR isolates, medium values presented towards non-MDR isolates were higher in NIS assays (0.525 g/L for MDR isolates and 0.563 g/L for non-MDR isolates) but lower in LIS assays (7.750 g/L for MDR isolates and 7.500 g/L for non-MDR isolates).

Table 6: Virkon® S's MICs and MBCs medium values towards the selected *Enterococcus* spp. isolates after induction of bacterial multiplication at sub-MIC levels, including assays with no interfering substance (NIS), low interfering substance (LIS) and high interfering substance (HIS) (grams per litre, g/L).

Isolate Code	MDR	Assay	MIC			MBC		
			NIS	LIS	HIS	NIS	LIS	HIS
E2	-	**A**	0.750	5.000	> 15.000	0.750	5.625	> 15.000
		B	0.625	7.500	> 15.000	0.625	7.500	> 15.000
E3	✓	**A**	0.500	5.000	> 15.000	0.650	5.000	> 15.000
		B	0.625	6.250	> 15.000	0.625	6.250	> 15.000
E5	✓	**A**	1.000	3.125	> 15.000	1.375	3.125	> 15.000
		B	0.500	6.875	> 15.000	0.500	7.500	> 15.000
E14	-	**A**	0.625	5.000	> 15.000	0.625	5.000	> 15.000
		B	0.500	6.875	14.125	0.500	6.875	14.125
E16	✓	**A**	0.625	5.000	> 15.000	0.750	5.000	> 15.000
		B	0.500	8.750	> 15.000	0.500	8.750	> 15.000
E17	✓	**A**	0.625	5.000	> 15.000	0.625	5.000	> 15.000
		B	0.500	8.750	> 15.000	0.500	8.750	> 15.000
E19	✓	**A**	0.500	5.625	> 15.000	0.500	5.625	> 15.000
		B	0.500	7.500	> 15.000	0.500	7.500	> 15.000
$\bar{x}$		**A**	0.661	4.821	> 15.000	0.750	4.911	> 15.000
		B	0.536	7.500	> 15.000	0.536	7.589	> 15.000
σ		**A**	0.160	0.725	-	0.267	0.778	-
		B	0.056	0.884	-	0.056	0.847	-

MDR (✓): Multidrug-Resistant Isolates

NIS: No interfering substance; LIS: Low interfering Substance; HIS: High interfering substance

MBC: Minimal Bactericidal Concentration

$\bar{x}$: Medium MIC and MBC values of all selected isolates; σ: Corresponding standard deviation of the population

A: Assay performed before the induction of bacterial multiplication at sub-MIC levels; B: Assay performed after the induction of bacterial multiplication at sub-MIC levels

4. DISCUSSION

4.1 Antimicrobial Susceptibility Testing

Biocides are an indispensable component in the process of reducing and/or eliminating various opportunistic pathogens that can be responsible for many negative consequences such as HAIs. Many of these pathogens are bacteria that have increasingly been classified as MDR, forcing a more prolonged treatment and hospital stay (Guardabassi 2012), but also rendering veterinary hospitals a possible source of transmission for these bacteria to professionals and also to the community (Hamilton et al. 2012). This is why the characterization of the bacterial species present in high-risk locations, such as isolation units, as well as the evaluation of their antimicrobial resistance profile is of utmost importance.

From all the isolates tested (Table 3), around 48.3% were classified as MDR, all belonging to the enterococci group. High levels of resistance presented by these bacteria are not considered a novelty (Hollenbeck and Rice 2012; Kristich et al. 2014; Torres et al. 2018; García-Solache and Rice 2019) and have been reported in many studies (Jackson et al. 2009; Iseppi et al. 2015), including in bacteria isolated from surfaces in other VTHs (Hamilton et al. 2012). Comparatively to this last study conducted by Hamilton et al. (2012), the rate of enterococci presenting resistance to at least one antimicrobial was similar (94.7% compared to the 100% obtained in this study), but the panresistance rate obtained for this same group in our study was much higher (19.7% compared to the 52.2% obtained in this study). However, there are some differences between studies that impair this comparison, such as the number of isolates tested (75 compared to 23), the fact that enrofloxacin was not one of the antibiotics tested and the different method used to determine isolates' antimicrobial susceptibility profile (microdilution system *vs* disk diffusion).

The categorization of the fourteen *Enterococcus* spp. as MDR was made according to the exhibition of a resistant phenotype towards penicillins (ampicillin), fluoroquinolones (ciprofloxacin and enrofloxacin) and tetracyclines (tetracycline), as suggested by Magiorakos et al. 2011.

Cephalosporins were not considered in this classification due to the fact that these microorganisms have a well characterized intrinsic resistance to this group of antimicrobial compounds, due to the expression of low-affinity penicillin binding proteins (PBPs), which are the main site of action of these antibiotics. These PBPs confer a loss of susceptibility not only to cephalosporins but to all β-lactam antibiotics (Sifaoui et al. 2001; Hollenbeck and Rice 2012; Kristich et al. 2014; Miller et al. 2014; Torres et al. 2018; García-Solache and Rice 2019). However, the consequences vary according to the antibiotic and enterococcal species, with penicillin usually being the β-lactam with the most activity (Kristich et al. 2014).

Likewise, gentamicin was also excluded from this classification due to a known intrinsic resistance that enterococci have against this antibiotic class, primarily caused by a lower membrane permeability (Hollenbeck and Rice 2012; Kristich et al. 2014; Torres et al. 2018).

Antimicrobial susceptibility testing of trimethoprim-sulfamethoxazole was not evaluated concerning enterococci due to a specific characteristic presented by this species. As is known, both molecules act through inhibition of folate synthesis, essential to the production of purines and protein in the microbial cell. *Enterococcus* spp., however have the ability of extracting this element (folic acid) from the environment, rendering this mechanism of action obsolete. This is why, as said before, an apparent *in vitro* susceptibility profile obtained in mediums void of folic acid may not be equivalent to an *in vivo* susceptibility (Hollenbeck and Rice 2012).

As seen in Table 7, tetracycline resistance rate was higher than the one observed in most of the selected studies, with the exception of the works presented by Rodrigues et al. (2002) and Iseppi et al. (2015), in which similar rates were found. This antibiotic is also frequently one of the antibiotics to which enterococci present the highest percentage of resistance, along with enrofloxacin (Rodrigues et al. 2002; Delgado et al. 2007; Ossiprandi et al. 2008; Ghosh et al. 2011; Hamilton et al. 2012; Kataoka et al. 2013; Leite-Martins et al. 2015; Iseppi et al. 2015; Awosile et al. 2018).

The percentage obtained concerning ampicillin resistance was the second highest, surpassed only by the values obtained by Ghosh et al. (2011) in the US. However, this study only focused on *E. faecium* isolates, which are known to usually present a higher level of resistance (i.e. higher MIC values) towards β-lactams than other enterococci species (Miller et al. 2014).

From all four antibiotics, the resistance rate related to ampicillin is the most worrisome, not only because results were the ones with greatest difference when compared to other studies, but also because β-lactams are still considered the first line of defence against enterococcal infections in both animals (to which amoxicillin is the predominant antibiotic used) (Weese et al. 2019) and humans (to which ampicillin is the predominant antibiotic used, sometimes associated with an aminoglycoside) (Hollenbeck and Rice 2012; Kristich et al. 2014; García-Solache and Rice 2019).

Table 7: Comparison of antibiotic resistance rates presented by *Enterococcus* spp. isolated from animals (dogs or cats) or from VTH's surfaces, obtained in different studies.

Study (Year)	Country	Antibiotic Resistance Rate				Species	Sample Origin
		AMP	CIP	ENR	TE		
Rodrigues et al. (2002)	PT	21.2 %	**73.1 %**	**76.9 %**	95.2 %	ND	Animals
Delgado et al. (2007)	PT	0.05 %	45.5 %	NT	67.3 %	ND	Clinical cases
Ossiprandi et al. (2008)	IT	6.2 %	16.9 %	52.3 %	84.6 %	*E. faecalis*	Healthy animals and clinical cases
		25 %	57.7 %	88.5 %	84.6 %	*E. faecium*	
Ghosh et al. (2011)	US	≈ 5 %	NT	31.9 %	59.6 %	*E. faecalis*	Animals with known AB therapy
		96.5 %	NT	97.3 %	84.1 %	*E. faecium*	
Hamilton et al. (2012)	US	39 %	25 %	NT	43%	ND	Surfaces
Kataoka et al. (2013)	JP	4.3 %	NT	12.1 %	NT	ND	Animals with no exposure to AB†
		6.6 %	NT	23.4 %	NT		Healthy animals
		37.5 %	NT	75 %	NT		Clinical cases
Iseppi et al. (2015)	IT	56.5 %	7.8 %	NT	97.5 %	ND	Clinical cases
Leite-Martins et al. (2015)	PT	0.3 %	5.4 %	NT	21 %	ND	Animals with no exposure to AB
		3.8 %	6.3 %	NT	22.9 %		Healthy animals
		8.3 %	23.2 %	NT	44.1 %		Clinical cases
		11.8 %	24.1 %	NT	46 %		Animals with known AB therapy
Awosile et al. (2018)	CA	4.3 %	NT	NT	NT	ND	Healthy animals
Present Study (2021)	PT	78.3 %	34.8 %	52.2 %	**99.6 %**	ND	Surfaces

PT: Portugal; US: United States; JP: Japan; IT: Italy; CA: Canada

AMP: Ampicillin; CIP: Ciprofloxacin; ENR: Enrofloxacin; TE: Tetracycline

NT (Not Tested): Antibiotic in question was not tested in this study; ND (Not Differentiated): There was no differentiation between *E. faecium* and *E. faecalis* isolates in terms of susceptibility to antimicrobials

AB: Antibiotic(s); †: Animals younger than 3.5 months

These higher resistance rates could be explained by contemplating isolate origin. As seen in both Kataoka et al. (2013) and Leite-Martins et al. (2015), isolates obtained from clinical samples and from animals under antibiotic selective pressure, which possibly represents the majority of cases admitted to the BICU, present a higher resistance rate than isolates collected from healthy animals. This indicates that antimicrobial treatments to different ailments can potentially exert antibiotic pressure on commensal microbiota, leading to selection of resistant microbial strains (Donskey et al. 1999, 2000, 2004; Donskey 2004; Brandl et al. 2008; Jakobsson et al. 2010; Ubeda et al. 2010). This could be especially true regarding enterococci since they present intrinsic resistance to many of the antibiotics regularly used in these treatments (Hollenbeck and Rice 2012; Kristich et al. 2014; Miller et al. 2014; Torres et al. 2018). Also considering that the majority of the animals admitted to the BICU present some kind of intestinal disease, as stated by Machado (2016), which commits to alterations in the gut microbiota, the results obtained become a little more comprehensible.

These results become even more worrisome when considering potential zoonotic transmissions of these MDR isolates (Damborg et al. 2009) and also the possibility of the transference of resistance determinants from these bacteria to other species, such as *Staphylococcus aureus* (Weigel et al. 2003; de Niederhäusern et al. 2004; Zhu et al. 2008; Limbago et al. 2014).

4.2 Determination of Virkon® S's Minimal Inhibitory Concentrations (MICs) and Minimal Bactericidal Concentrations (MBCs)

Determining MIC and MBC values of different biocide formulations, in different functional conditions, is an essential practice in order to ascertain how to efficiently eliminate possible HAIs' agents, such as the ones tested in this study. The establishment of these values not only enables a better understanding of the optimum conditions of application of the different compounds, consequently assisting in reducing the possible consequences of the interaction between sub-inhibitory/sub-lethal concentrations and different bacteria, but also allows for a monitorization of loss of susceptibility to these compounds by bacteria.

As seen in the first assays (Table 4 and Graphic 1), when Virkon® S's bactericidal concentrations are determined in the absence of organic matter, Gram-negative isolates present a lower susceptibility to this biocide than Gram-positive isolates, which is conveyed by the higher Virkon® S's MBC medium values regarding the first group. This corroborates the

theory that the lipopolysaccharide barrier that comprises the outer layer of Gram-negative bacteria is responsible for decreasing the activity of many biocides, including membrane active agents (Maillard 2018), as is Virkon® S's case. However, in the presence of organic matter, this tendency is no longer observed.

Beyond the inexistence of an established guideline to test the variability of biocides efficiency, no MIC nor MBC breakpoints are available for these compounds, which makes the categorization of bacteria as resistant or susceptible difficult (Humayoun et al. 2018). This becomes even harder in Virkon® S's case since, to the best of our knowledge, no other studies were published till date regarding MIC and MBC values of this biocide. Nevertheless, considering that the MBC values obtained are approximately 8 times or more lower than the concentration regularly used, it seems correct to affirm that, in the absence of organic matter, Virkon® S is an efficient biocidal agent against all the organisms tested. Nevertheless, it should be considered that these assays were performed using suspended planktonic bacteria, that are usually considered more susceptible to antimicrobials than bacteria present in biofilm form (Bridier et al. 2011; Roy et al. 2017).

However, the presence of organic matter is known to alter biocide susceptibility, especially of compounds constituted by oxidizers (Kalchayanand et al. 2016). Generally, this action seems to be due to three different mechanisms: (i) neutralization of the biocide molecule, reducing its availability; (ii) the formation of a protective barrier around the microorganism; and (iii) the formation of microbial aggregates; with (ii) and (iii) being normally associated with biofilm formation (Maillard 2005a, 2013a; Cavalli et al. 2018).

According to the results obtained in both LIS and HIS assays, organic matter does seem to reduce Virkon® S's bactericidal capacity, especially when applied in high concentrations. In HIS assays, this formulation's MBC values towards *Enterococcus* spp., *E. coli* and *Staphylococcus pseudintermedius* surpass the concentration regularly used in the BICU. Although this was not observed regarding the *Pseudomonas* spp. group, there is still a very significant rise in the bactericidal concentration (of 9 times the base concentration) towards those isolates. These results suggest that, in the presence of higher loads of organic matter, Virkon® S may not be effective in eliminating all targeted microorganisms.

The loss of efficiency presented by this disinfectant in both LIS and HIS assays has also been demonstrated by McCormick and Maheshwari (2004) regarding adenovirus, and also by Chandler-Bostock and Mellits (2015) regarding rotavirus. On the other hand, two studies, one by Wu et al. (2017) regarding rabies virus, and another by Skinner et al. (2018) regarding *E. coli* O157:H7, indicated that Virkon® S behaved effectively in the presence of organic matter. This disparity could be explained by the differences in the protocols used, by the different microorganisms tested and especially by the variations in types of organic matter used, since, as concluded before, this disinfectant could still be efficient in the presence of low

quantities of organic matter. No MIC nor MBC values were presented by Skinner et al. (2018) concerning Virkon® S, which makes comparison between results even more difficult.

Even though the number of isolates used was low, making it impossible to perform a statistical assessment, the results obtained seem to indicate that there are no associations between decreased biocide susceptibility and MDR isolates. No other studies were found that analysed this possible correlation concerning Virkon® S. Nevertheless, other authors performed this same analysis regarding other disinfectants such as QACs, triclosan and other phenolics, ethanol, sodium hypochlorite, hydrogen peroxide, among others, regarding different bacteria. While some authors concluded that there is indeed some degree of correlation between decreased susceptibility to biocides and resistance to antibiotics (Lerma et al. 2015; Márquez et al. 2016; Wieland et al. 2017; Alotaibi et al. 2017), others conclude the exact opposite (Cole et al. 2003; Ghanem and Haddadin 2018; Humayoun et al. 2018; Roedel et al. 2019), sometimes even regarding the same biocide (Cole et al. 2003; Márquez et al. 2016; Alotaibi et al. 2017; Roedel et al. 2019). These different results indicate that this correlation does not only depend upon the active substance tested, but also on the target bacteria. However, the significant variations in protocol should also be considered as a possible motive for these distinct conclusions.

4.3 Influence of Bacterial Exposure to Sub-lethal Concentrations of Virkon® S

Upon contact with sub-lethal concentrations of Virkon® S, a modification of the phenotypic susceptibility pattern from intermedium to resistant was observed in all the isolates that did not present a resistant phenotype concerning gentamicin (Table 5).

A stress-induced response translates as the capacity that some microorganisms exhibit that allows them to adapt to numerous forms of stress, including chemical stress (Russell 2003b). This response has been associated with alterations in gene expression and cell physiology which, consequently, can lead to decrease in antimicrobial susceptibility (Poole 2012). This adaptation mechanism could also be indicated as one of the causes of cross-resistance between biocides and antibiotics, especially since both are associated with overexpression of multidrug efflux pumps (Rakic-Martinez et al. 2011; Poole 2012; Curiao et al. 2016; Buffet-Bataillon et al. 2016; Burgos et al. 2016) and changes in cell wall permeability (Tattawasart et al. 2000; Poole 2012; Gadea et al. 2017; Kim et al. 2018).

Considering the well-known aminoglycoside resistance ability presented by *Enterococcus* strains, associated most commonly due to cell wall impermeability and enzyme modification (Hollenbeck and Rice, 2012; Kristich et al. 2014; Torres et al. 2018; García-Solache and Rice 2019), the change in susceptibility observed in this study could be due to an adaptative stress-induced response, caused by the subjection of these bacterial isolates to a

subinhibitory concentration of Virkon® S. However, this could also indicate that this response is not specific to Virkon® S and could be promoted by similar biocide formulations.

As no increase in Virkon® S's MIC and MBC values was observed in the absence of organic matter (Table 6), it may be an indication that the tested isolates were not able to develop any mechanisms that could lead to a decrease in susceptibility to Virkon® S.

If this hypothesis were to be true, however, it would not explain the rise observed in Virkon® S's MBC values when in contact with an interfering substance. The results obtained could mean that organic matter does not only act by neutralization of the biocide, but forms a protective layer around bacteria, promoting their aggregation and consequently reducing their susceptibility to Virkon® S. The increase seen regarding these MBC values also suggests that there could have been a slight modification to this interaction (between bacteria and organic matter), after bacterial subjection to a subinhibitory concentration of Virkon® S. As there seems to be few studies concerning the influence of organic matter in bacterial susceptibility to Virkon® S, further research is still needed in order to better understand this relationship.

5. CONCLUSION

Virkon® S seems to be an efficient compound in eliminating all bacteria tested, which were previously isolated from the BICU; however, further studies should be made in order to evaluate the ability of this compound to remove bacteria in biofilm form. This becomes an even more pressing matter considering the fact that the results obtained seem to indicate that organic matter appears to inactivate Virkon® S's activity. Results also highlight the importance of cleaning the surfaces thoroughly before disinfecting them with this biocide.

After exposure to sublethal concentrations of Virkon® S, slight alterations were observed in the antimicrobial susceptibility profile of four enterococci regarding gentamicin, possibly due to a chemical stress response caused by this exposure.

This contact with Virkon® S did not seem to decrease isolate susceptibility to this formulation in the absence of organic matter. However, an increase in MBC values was indeed seen when low levels of organic matter were used.

Finally, despise not being its main objective, this study emphasizes the rising importance that *Enterococcus* strains have as a nosocomial pathogen, since not only it was the most frequent bacteria isolated in the BICU by Verdial (2020), but it was also the isolate that presented a higher frequency of antimicrobial resistance, with a worrisome percentage of MDR isolates (60.9%).

6. FUTURE PRESPECTIVES

Beyond the need of testing Virkon® S against bacteria in biofilm form, as stated before, studies concerning the factors that could possibly influence this formulation's efficiency are still scarce. Considering that Virkon® S is a good substitute to a lot of other disinfectants used, not only because of its efficiency but also due to the fact that it presents no threat to the environment, these studies are very important in order to use this compound to the maximum of its efficiency and to proficiently reduce the degree of HAIs observer nowadays.

More studies should also be made in order to definitely determine the cause of the alteration in the antimicrobial susceptibility profile obtained after exposure of the enterococci to sub-lethal concentrations of Virkon® S. It is also important to establish the stability of this alteration, that is, to determine how many subcultures does it take to see a reverse in this phenotype, if such reverse is possible. These same questions should be studied relatively to the changes in MBC values when a low-level of organic matter is present.

CHAPTER III: BIBLIOGRAPHY

Abdulla FR, Adams BB. 2007. Ortho-phthalaldehyde causing facial stains after cystoscopy. Archives of Dermatology, 143(5): 659.

Addie DD, Boucraut-Baralon C, Egberink H, Frymus T, Gruffyd-Jone T, Hartmann K, Horzinek MC, Hosie MJ, Lloret A, Lutz H, et al. 2015. Disinfectant choices in veterinary practices, shelters and households. ABCD guidelines on safe and effective disinfection for feline environments. Journal of Feline Medicine and Surgery, 17(7): 594-605.

Ahishali E, Uygur-Bayramiçli O, Dolapçioğlu C, Dabak R, Mengi A, Işik A, Ermiş E. 2008. Chemical colitis due to glutaraldehyde: case series and review of the literature. Digestive Diseases and Sciences, 54(12): 2541–2545.

[AIDAP] Australasian Infectious Diseases Advisory Panel. 2015. Practical infection control guidelines [internet]. [Assessed 2020 Apr 09]. https://www.ava.com.au/siteassets/resources/fighting-antimicrobial-resistance/aidap-infection-control-guidelines.pdf

Akamatsu T, Minemoto M, Uyeda M. 2005. Evaluation of the antimicrobial activity and materials compatibility of orthophthalaldehyde as a high-level disinfectant. Journal of International Medical Research, 33(2): 178–187.

Al-Adham I, Haddadin R, Collier, P. 2013. Types of Microbicidal and Microbistatic Agents. In: Fraise AP, Lambert PA & Maillard J-Y, editors. Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. 5th edition. London (GB): Blackwell Publishing. Chapter 2, p. 5-70.

Alotaibi SMI, Ayibiekea A, Pedersen AF, Jakobsen L, Pinholt M, Gumpert H, Hammerum AM, Westh H, Ingmer H. 2017. Susceptibility of vancomycin-resistant and -sensitive *Enterococcus faecium* obtained from Danish hospitals to benzalkonium chloride, chlorhexidine and hydrogen peroxide biocides. Journal of Medical Microbiology, 66(12): 1744-1751.

Atiyeh K, Chitkara A, Achlatis S, Branski RC, Amin MR. 2015. Allergic reaction to ortho-phthalaldehyde following flexible laryngoscopy. The Laryngoscope, 125(10): 2349–2352.

Awosile BB, McClure JT, Saab ME, Heider LC. 2018. Antimicrobial resistance in bacteria isolated from cats and dogs from the Atlantic Provinces, Canada from 1994–2013. Canadian Veterinary Journal, 59(8): 885-893.

Baldry MGC. 1983. The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. Journal of Applied Bacteriology, 54(3): 417-423.

Bannoehr, J, Guardabassi, L. 2012. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Veterinary Dematology, 23(4): 253-266.

Bello T, Rivera-Olivero IS, de Waard JH. 2006. Inactivación de micobacterias con desinfectantes registrados como tuberculicidas. Enfermedades Infecciosas y Microbiología Clínica, 24(5): 319-321.

Benedict KM, Morley PS, Van Metre DC. 2008. Characteristics of biosecurity and infection control programs at veterinary teaching hospitals. Journal of the American Veterinary Medical Association, 233(5): 767–773.

Best M, Sattar SA, Springthorpe VS, Kennedy ME. 1990. Efficacies of selected disinfectants against *Mycobacterium tuberculosis*. Journal of Clinical Microbiology, 28(10): 2234-2239.

Best M, Springthorpe VS, Sattar SA. 1994. Feasibility of a combined carrier test for disinfectants: studies with a mixture of five types of microorganisms. American Journal of Infection Control, 22(3): 152–162.

Bjorland J, Sunde M, Waage S. 2001. Plasmid-borne *smr* gene causes resistance to Quaternary Ammonium Compounds in bovine *Staphylococcus aureus*. Journal of Clinical Microbiology, 39(11): 3999-4004.

Bloomfield SF, Smith-Burchnell CA, Dalgleish AG. 1990. Evaluation of hypochlorite-releasing disinfectants against the Human Immunodeficiency Virus (HIV). Journal of Hospital Infection, 15(3): 273–278.

Boeris PS, Domenech CE, Lucchesi GI. 2007. Modification of phospholipid composition in *Pseudomonas putida* ATCC 12633 induced by contact with tetradecyltrimethylammonium. Journal of Applied Microbiology, 103(4): 1048-1054.

Boyce, JM. 2018. Alcohols as surface disinfectants in healthcare settings. Infection Control & Hospital Epidemiology, 39(3): 323–328.

Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG. 2008. Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature, 455(7214): 804-807.

Bressler D, Balzer M, Dannehl A, Flemming H-C, Wingender J. 2009. Persistence of *Pseudomonas aeruginosa* in drinking-water biofilms on elastomeric material. Water Supply, 9(1): 81–87.

Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. 2011. Resistance of bacterial biofilms to disinfectants: a review. Biofouling, 27(9): 1017-1032.

Bruinsma GM, Rustema-Abbing M, van der Mei HC, Lakkis C, Busscher HJ. 2006. Resistance to a polyquaternium-1 lens care solution and isoelectric points of *Pseudomonas aeruginosa* strains. Journal of Antimicrobial Chemotherapy, 57(4): 764-766.

Buffet-Bataillon S, Tattevin P, Maillard J-Y, Bonnaure-Mallet M, Jolivet-Gougeon A. 2016. Efflux pump induction by quaternary ammonium compounds and fluoroquinolone resistance in bacteria. Future Microbiology, 11(1): 81-92.

Bundgaard-Nielsen K, Nielsen PV. 1995. Fungicidal effect of 15 disinfectants against 25 fungal contaminants commonly found in bread and cheese manufacturing. Journal of Food Protection, 59(3): 268-275.

Bureš F. 2019. Quaternary Ammonium Compounds: simple in structure, complex in application. Topics in Current Chemistry, 377(3): 1–21.

Burgess BA, Morley PS, Hyatt DR. 2004. Environmental surveillance for *Salmonella enterica* in a veterinary teaching hospital. Journal of the American Veterinary Medical Association, 225(9): 1344–1348.

Burgess W, Margolis A, Gibbs S, Duarte RS, Jackson M. 2017. Disinfectant susceptibility profiling of glutaraldehyde-resistant nontuberculous mycobacteria. Infection Control and Hospital Epidemiology, 38(7): 784-791.

Burgos MJG, Márquez MLF, Pulido RP, Gálvez A, López RL. 2016. Virulence factors and antimicrobial resistance in *Escherichia coli* strains isolated from hen egg-shells. International Journal of Food Microbiology, 238: 89-95.

Cai L, Wang H, Liang L, Wang G, Xu X, Wang H. 2018. Response of formed-biofilm of *Enterobacter cloacae*, *Klebsiella oxytoca*, and *Citrobacter freundii* to chlorite-based disinfectants. Journal of Food Science, 83(5): 1326-1332.

Caprilli R, Viscido A, Frieri G, Latella G. 1998. Acute colitis following colonoscopy. Endoscopy, 30(4): 428-431.

Carey DE, McNamara PJ. 2015. The impact of triclosan on the spread of antibiotic resistance in the environment. Frontiers in Microbiology, 5(780): 1-12.

Carey DE, Zitomer DH, Kappell AD, Choi MJ, Hristova KR, McNamara. 2016. Chronic exposure to triclosan sustains microbial community shifts and alters antibiotic resistance gene levels in anaerobic digesters. Environmental Science: Processes & Impacts, 18(8): 1060-1067.

Catlin NR, Willson CJ, Stout M, Kissling GE, Waidyanatha S, Baker GL, Hayden BK, Wyde M. 2017. Evaluation of the respiratory tract toxicity of ortho-phthalaldehyde, a proposed alternative for the chemical disinfectant glutaraldehyde. Inhalation Toxicology, 29(9): 414-427.

Cavalli A, Marinaro M, Desario C, Corrente M, Camero M, Buonavoglia C. 2018. *In vitro* virucidal activity of sodium hypochlorite against canine parvovirus type 2. Epidemiology and Infection, 1-4.

[CCAR] Canadian Committee on Antibiotic Resistance. 2008. Infection prevention and control best practices: for small animal veterinary clinics [internet]. [Accessed 2019 Oct 23]. https://www.wormsandgermsblog.com/files/2008/04/CCAR-Guidelines-Final2.pdf

[CDC] Centers for Disease Control and Prevention, [HICPAC] Healthcare Infection Control Practices Advisory Committee. 2003. Infection control in health-care facilities: recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee (HICPAC) [internet]. [Accessed 2019 Apr 09]. https://www.cdc.gov/infectioncontrol/pdf/guidelines/environmental-guidelines-P.pdf

[CEN] European Committee for Standardization. 2009. Chemical disinfection and antiseptics: quantitative suspension tests for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in the veterinary area – test method and requirements (phase 2, step 1). European standard (EN) document 1656. Brussels (BE): European Committee for Standardization.

Chandler-Bostock R, Mellits KH. 2015. Efficacy of disinfectants against porcine rotavirus in the presence and absence of organic matter. Letters in Applied Microbiology, 61(6): 538-543.

Chang S-C, Li W-C, Huang K-Y, Huang Y-C, Chiu C-H, Chen C-J, Hsieh Y-C, Kuo C-Y, Shih S-R, Lin T-Y. 2013. Efficacy of alcohols and alcohol-based hand disinfectants against human enterovirus 71. Journal of Hospital Infection, 83(4): 288–293.

Chapalain A, Rossignol G, Lesouhaitier O, Merieau A, Gruffaz C, Guerillon J, Meyer J-M, Orange N, Feuilloley MGJ. 2008. Comparative study of 7 fluorescent pseudomonad clinical isolates. Canadian Journal of Microbiology, 54(1): 19–27.

Cheeseman KE, Denyer SP, Hosein IK, Williams GJ, Maillard J-Y. 2009. Evaluation of the bactericidal efficacy of three different alcohol hand rubs against 57 clinical isolates of *S. aureus*. Journal of Hospital Infection, 72(4): 319-325.

Chen L, Eisenberg J, Mueller C, Burton NC. 2015. Health hazard evaluation report: evaluation of ortho-phthalaldehyde in eight healthcare facilities [internet]. Health Hazard Evaluation Report 2006-0238-3239. [Washington (WA)]: National Institute for Occupational Safety and Health, [accessed 2020 Apr 23]. https://www.cdc.gov/niosh/hhe/reports/pdfs/2006-0238-3239.pdf

Chenjiao W, Hongyan Z, Qing G, Xiaoqi Z, Liying G, Ying F. 2016. In-use evaluation of peracetic acid for high-level disinfection of endoscopes. Gastroenterology Nursing, 39(2): 116-120.

Chowdhury D, Rahman A, Hu H, Jensen SO, Deva AK, Vickery K. 2018. The effect of disinfectant formulation and organic soil on the efficacy of oxidising disinfectants against biofilms. Journal of Hospital Infection, 103(1): 33-41.

Chuanchuen R, Beinlich K, Hoang TT, Becher A, Karkhoff-Schweizer RR, Schweizer HP. 2001. Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. Antimicrobial Agents and Chemotherapy, 45(2): 428-432.

Chuanchuen R, Karkhoff-Schweizer RR, Schweizer HP. 2003. High-level triclosan resistance in *Pseudomonas aeruginosa* is solely a result of efflux. American Journal of Infection Control, 31(2): 124-127.

Chung YJ, Saier Jr MH. 2002. Overexpression of the *Escherichia coli sugE* gene confers resistance to a narrow range of Quaternary Ammonium Compounds. Journal of Bacteriology, 184(9): 2543-2545.

[CLSI] Clinical and Laboratory Standards Institute. 2013a. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. 4th edition. CLSI document VET01-A4. Wayne (PA): Clinical and Laboratory Standards Institute.

[CLSI] Clinical and Laboratory Standards Institute. 2013b. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. 4th edition. CLSI supplement VET01-S2. Wayne (PA): Clinical and Laboratory Standards Institute.

[CLSI] Clinical and Laboratory Standards Institute. 2016. Performance Standards for Antimicrobial Susceptibility Testing. 26th edition. CLSI supplement M100S. Wayne (PA): Clinical and Laboratory Standards Institute.

Cloete TE. 2003. Resistance mechanisms of bacteria to antimicrobial compounds. International Biodeterioration & Biodegradation, 51(4): 277-282.

Cole EC, Addison RM, Rubino JR, Leese KE, Dulaney PD, Newell MS, Wilkins J, Gaber DJ, Wineinger T, Criger DA. 2003. Investigation of antibiotic and antibacterial agent cross-

resistance in target bacteria from homes of antibacterial product users and nonusers. Journal of Applied Microbiology, 95(4): 664-676.

Collins JJ, Burns C, Spenser P, Bodnar CM, Calhoun T. 2006. Respiratory cancer risks among workers with glutaraldehyde exposure. Journal of Occupational and Environmental Medicine, 48(2): 199–203.

Conceição T, Coelho C, Lencastre H, Aires-de-Sousa M. 2016. High prevalence of biocide resistance determinants in *Staphylococcus aureus* isolates from three African countries. Antimicrobial Agents and Chemotherapy, 60(1): 678-681.

Cooper DE, White AA, Werkema AN, Auge BK. 2008. Anaphylaxis following cystoscopy with equipment sterilized with Cidex® OPA (ortho-phthalaldehyde): a review of two cases. Journal of Endourology, 22(9): 2181–2184.

Cortezzo DE, Setlow P. 2005. Analysis of factors that influence the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. Journal of Applied Microbiology, 98(3): 606-617.

Craveiro S, Alves-Barroco C, Crespo MTB, Barreto AS, Semedo-Lemsaddek T. 2015. *Aeromonas* biofilm on stainless steel: efficiency of commonly used disinfectants. International Journal of Food Science and Technology, 50(4): 851-856.

Curiao T, Marchi E, Grandgirard D, Léon-Sampedro R, Viti C, Leib SL, Baquero F, Oggioni MR, Martinez JS, Coque TM. 2016. Multiple adaptive routes of *Salmonella enterica* Typhimurium to biocide and antibiotic exposure. BMC Genomics, 17: 491.

Damborg P, Top J, Hendrickx APA, Dawson S, Willems RJL, Guardabassi L. 2009. Dogs are a reservoir of ampicillin-resistant *Enterococcus faecium* lineages associated with human infections. Applied and Environmental Microbiology, 75(8): 2360–2365.

de Niederhäusern S, Sabia C, Messi P, Guerrieri E, Manicardi G, Bondi M. 2004. Glycopeptide-resistance transferability from vancomycin-resistant enterococci of human and animal source to *Listeria* spp.. Letters in Applied Microbiology, 39(6): 483-489.

Delgado M, Neto I, Correia JHD, Pomba C. 2007. Antimicrobial resistance and evaluation of susceptibility testing among pathogenic enterococci isolated from dogs and cats. International Journal of Antimicrobial Agents, 30(1): 98-100.

Demple B. 1996. Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress regulon - a review. Gene, 179(1): 53-57.

Denyer SP, Maillard J-Y. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-negative bacteria. Journal of Applied Microbiology Symposium, 92(S1): 35-45.

Denyer SP, Stewart GSAB. 1998. Mechanisms of action of disinfectants. International Biodeterioration & Biodegradation, 41(3-4): 261-268.

Donnarumma G, Buommino E, Fusco A, Paoletti I, Auricchio L, Tufano MA. 2010. Effect of temperature on the shift of *Pseudomonas fluorescens* from an environmental microorganism to a potential human pathogen. International Journal of Immunopathology and Pharmacology, 23(1): 227–234.

Donskey CJ. 2004. The role of the intestinal tract as a reservoir and source for transmission of nosocomial pathogens. Clinical Infectious Diseases, 39(2): 219-226.

Donskey CJ, Helfand MS, Pultz NJ, Rice LB. 2004. Effect of parenteral fluoroquinolone administration on persistence of vancomycin-resistant *Enterococcus faecium* in the mouse gastrointestinal tract. Antimicrobial Agents and Chemotherapy, 48(1): 326-328.

Donskey CJ, Hanrahan JA, Hutton RA, Rice LB. 1999. Effect of parenteral antibiotic administration on persistence of vancomycin-resistant *Enterococcus faecium* in the mouse gastrointestinal tract. The Journal of Infectious Diseases, 180(2): 384-390.

Donskey CJ, Hanrahan JA, Hutton RA, Rice LB. 2000. Effect of parenteral antibiotic administration on the establishment of colonization with vancomycin-resistant *Enterococcus faecium* in the mouse gastrointestinal tract. The Journal of Infectious Diseases, 181(5): 1830-1833.

Doultree JC, Druce JD, Birch CJ, Bowden DS, Marshall JA. 1999. Inactivation of feline calicivirus, a Norwalk virus surrogate. Journal of Hospital Infection, 41: 51-57.

Dunowska M, Morley PS, Hyatt DR. 2005. The effect of Virkon® S fogging on survival of *Salmonella enterica* and *Staphylococcus aureus* on surfaces in a veterinary teaching hospital. Veterinary Microbiology, 105(3-4): 281–289.

[EPA] Environmental Protection Agency. 2019. Chemicals evaluated for carcinogenic potential annual cancer report [internet]. [Washington (WA)]: Environmental Protection Agency; [accessed 2020 Apr 20]. http://npic.orst.edu/chemicals_evaluated.pdf

[FDA] Food and Drug Administration. 2019. FDA-cleared sterilants and high-level disinfectants with general claims for processing reusable medical and dental devices [internet]. [Maryland (MD)]: Food and Drug Administration; [accessed 2020 Apr 23]. https://www.fda.gov/medical-devices/reprocessing-reusable-medical-devices-information-manufacturers/fda-cleared-sterilants-and-high-level-disinfectants-general-claims-processing-reusable-medical-and

Fernandes S, Simões M, Dias N, Santos C, Lima N. 2013. Fungicidal activity of microbicides. In: Fraise AP, Lambert PA & Maillard J-Y, editors. Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. 5th edition. London (GB): Blackwell Publishing. Chapter 7, p.142-154.

Fernández-Cuenca F, Tomás M, Caballero-Moyano F-J, Bou G, Martínez-Martínez L, Vila J, Pachón J, Cisneros J-M, Rodríguez-Baño J, Pascual A. 2015. Reduced susceptibility to biocides in *Acinetobacter baumannii*: association with resistance to antimicrobials, epidemiological behaviour, biological cost and effect on the expression of genes encoding porins and efflux pumps. Journal of Antimicrobial Chemotherapy, 70(12): 3222–3229.

Fisher CW, Fiorello A, Shaffer D, Jackson M, McDonnell GE. 2012. Aldehyde-resistant mycobacteria bacteria associated with the use of endoscope reprocessing systems. American Journal of Infection Control, 40(9): 880-882.

Fraud S, Maillard J-Y, Russell AD. 2001. Comparison of the mycobactericidal activity of *ortho*-phthalaldehyde, glutaraldehyde and other dialdehydes by a quantitative suspension test. Journal of Hospital Infection, 48(3): 214-221.

Gabbert LR, Smith JD, Neilan JG, Ferman GS, Rasmussen MV. 2018. Smart card decontamination in a high-containment laboratory. Health Security, 16(4): 244-251.

Gadea R, Glibota N, Pulido RP, Gálvez A, Ortega E. 2017. Adaptation to biocides cetrimide and chlorhexidine in bacteria from organic foods: association with tolerance to other antimicrobials and physical stresses. Journal of Agricultural and Food Chemistry, 65(8): 1758-1770.

Garcia EG, Peso GD, Celadilla O, Castro MJ, Martinez V, Muñoz I, Sanchez-Villanueva R, Guevara CL, Selgas R, Bajo MA. 2010. Efficacy of sodium hypochlorite in eradicating Hepatitis C Virus (HCV)-RNA from the peritoneal effluent of PD patients. Peritoneal Dialysis International, 30(6): 644-646.

García-Solache M, Rice LB. 2019. The Enterococcus: a model of adaptability to its environment. Clinical Microbiology Reviews, 32(2).

Gélinas P, Goulet J. 1983. Neutralization of the activity of eight disinfectants by organic matter. Journal of Applied Bacteriology, 54(2): 243-247

Gerba CP. 2015. Quaternary Ammonium biocides: efficacy in application. Applied and Environmental Microbiology, 81(2): 464-469.

Ghanem B, Haddadin RN. 2018. Multiple drug resistance and biocide resistance in *Escherichia coli* environmental isolates from hospital and household settings. Antimicrobial Resistance and Infection Control, 7:47.

Ghosh A, Dowd SE, Zurek L. 2011. Dogs leaving the ICU carry a very large multi-drug resistant enterococcal population with capacity for biofilm formation and horizontal gene transfer. PLoS ONE [internet]. [Accessed 2020 Nov 09]; 6(7): [13 p.]. doi: 10.1371/journal.pone.0022451

Gray N. 2014. Free and Combined Chlorine. In: Percival SL, Yates MV, Williams DD, Chalmers R, Gray N. Microbiology of waterborne diseases: microbiological aspects and risks. 2nd edition. London (GB): Academic Press. Chapter 31, p. 571-590.

Greenwood JE, Tan JL, Ming JCT, Abell AD. 2016. Alkalis and skin. Journal of Burn Care & Research, 37(2): 135–141.

Gomi M, Osaki Y, Mori M, Sakagami Y. 2012. Synergistic bactericidal effects of a sublethal concentration of didecyldimethylammonium chloride (DDAC) and low concentrations of nonionic surfactants against *Staphylococcus aureus*. Biocontrol Science, 17(4): 175-181.

Guardabassi L. 2012. Veterinary hospital-acquired infections: the challenge of MRSA and other multi-drug-resistant bacterial infections in veterinary medicine. The Veterinary Journal, 193(2): 307-308.

Guérin-Méchin L, Dubois-Brissonnet F, Heyd B, Leveau JY. 1999. Specific variations of fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by Quaternary Ammonium Compounds and relation with resistance to bactericidal activity. Journal of Applied Microbiology, 87(5): 735–742.

Guérin-Méchin L, Dubois-Brissonnet F, Heyd B, Leveau, JY. 2000. Quaternary ammonium compound stresses induce specific variations in fatty acid composition of *Pseudomonas aeruginosa*. International Journal of Food Microbiology, 55(1-3): 157–159.

Guptill L. 2015. Patient management. Veterinary Clinics of North America: Small Animal Practice, 45(2): 277–298.

Hageskal G, Tryland I, Liltved H, Skaar I. 2012. No simple solution to waterborne fungi: various responses to water disinfection methods. Water Science & Technology, 12(2): 220-226.

Hamilton E, Kaneene JB, May KJ, Kruger JM, Schall W, Beal MW, Hauptman JG, DeCamp CE. 2012. Prevalence and antimicrobial resistance of *Enterococcus* spp. and *Staphylococcus* spp. isolated from surfaces in a veterinary teaching hospital. Journal of the American Veterinary Medical Association, 240(12): 1463–1473.

Han Y, Zhou Z-C, Zhu L, Wei Y-Y, Feng W-Q, Xu L, Liu Y, Lin Z-J, Shuai X-Y, Zhang Z-J, et al. 2019. The impact and mechanism of quaternary ammonium compounds on the transmission of antibiotic resistance genes. Environmental Science and Pollution Research, 26(27): 28352-28360.

Handzlik J, Matys A, Kieć-Kononowicz K. 2013. Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria *S. aureus*. Antibiotics, 2(1): 28-45.

Hansen LH, Jensen LB, Sørensen HI, Sørensen SJ. 2007. Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria. Journal of Antimicrobial Chemotherapy, 60(1): 145-147.

Hansen LH, Sørensen SJ, Jørgensen HS, Jensen LB. 2005. The prevalence of the OqxAB multidrug efflux pump amongst olaquindox-resistant *Escherichia coli* in pigs. Microbial Drug Resistance, 11(4): 378-382.

He G-X, Kuroda T, Mima T, Morita Y, Mizushima T, Tsuchiya T. 2004. An H^+-coupled multidrug efflux pump, PmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. Journal of Bacteriology, 186(1): 262-265.

Heath RJ, Rubin JR, Holland DR, Zhang E, Snow ME, Rock CO. 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. The Journal of Biological Chemistry, 274(16): 11110-11114.

Heir E, Sundheim G, Holck AL. 1998. The *Staphylococcus qacH* gene product: a new member of the SMR family encoding multidrug resistance. FEMS Microbiology Letters, 163(1): 49-56.

Heir E, Sundheim G, Holck AL. 1999. The *qacG* gene on plasmid pST94 confers resistance to Quaternary Ammonium Compounds in staphylococci isolated from the food industry. Journal of Applied Microbiology, 86(3): 378-388.

Henriques AO, Moran Jr CP. 2007. Structure, assembly, and function of the spore surface layers. Annual Review of Microbiology, 61(1): 555-588.

Hernández A, Martró E, Matas L, Ausina V. 2003. In-vitro evaluation of Perasafe® compared with 2% alkaline glutaraldehyde against *Mycobacterium* spp.. Journal of Hospital Infection, 54(1): 52-56.

Hernández-Navarrete M-J, Celorrio-Pascual J-M, Morros CL, Bernad V-MS. 2014. Fundamentos de antisepsia, desinfección y esterilización. Enfermedades Infecciosas y Microbiología Clínica, 32(10): 681–688.

Hegstad K, Langsrud S, Lunestad BT, Scheie AA, Sunde M, Yazdankhah SP. 2010. Does the wide use of Quaternary Ammonium Compounds enhance the selection and spread of

antimicrobial resistance and thus threaten our health? Microbial Drug Resistance, 16(2): 91-104.

Hession SM. 2003. Endoscope disinfection by *ortho*-phthalaldehyde in a clinical setting: an evaluation of reprocessing time and costs compared with glutaraldehyde. Gastroenterology Nursing, 26(3): 110–114.

Hollenbeck BL, Rice LB. 2012. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence, 3(5): 421-569.

Horiki M, Park S, Matsui T, Suzuki K, Matsuoka T. 2011. Ortho-phthalaldehyde-induced skin mucous membrane damage from inadequate washing [internet]. BMJ Case Reports; [accessed 2020 Apr 23]: [3 p.]. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3062304/pdf/bcr.02.2010.2709.pdf

Howie R, Alfa MJ, Coombs K. 2008. Survival of enveloped and non-enveloped viruses on surfaces compared with other micro-organisms and impact of suboptimal disinfectant exposure. Journal of Hospital Infection 69(4): 368-376.

Huang C-T, Yu FP, McFeters GA, Stewart PS. 1995. Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. Applied and Environmental Microbiology, 61(6): 2252-2256.

Hugo WB, Denyer SP. 1987. The concentration exponent of disinfectants and preservatives. In: Board RG, Allwood MC, Banks JG, editors. Preservatives in the Food, Pharmaceutical and Environmental Industries. London (GB): Blackwell Scientific Publications, p. 281-292.

Humayoun SB, Hiott LM, Gupta SK, Barrett JB, Woodley TA, Johnston JJ, Jackson CR, Frye JG. 2018. An assay for determining the susceptibility of *Salmonella* isolates to commercial and household biocides. PLoS One [internet]. [Accessed 2020 Nov 09]; 13(12): [24 p.]. doi: 10.1371/journal.pone.0209072.

[IARC] International Agency for Research on Cancer. 2006. IARC monographs on the evaluation of carcinogenic risks to humans - Formaldehyde, 2-Butoxyethanol and 1-tert-Butoxypropan-2-ol [internet]. Volume 88. Lyon (FR): International Agency for Research on Cancer; [accessed 2020 Apr 23]. https://monographs.iarc.fr/wp-content/uploads/2018/06/mono88.pdf

[IFH] International Scientific Forum on Home Hygiene. 2000. Microbial resistance and biocides - a review by the International Scientific Forum on Home Hygiene (IFH) [internet]. [Accessed 2020 June 19]. https://www.ifh-homehygiene.org/sites/default/files/publications/antresFINAL.pdf

Ioannou CJ, Hanlon GW, Denyer SP. 2007. Action of disinfectant Quaternary Ammonium Compounds against *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy, 51(1): 296-306.

Iseppi R, Messi P, Anacarso I, Bondi M, Sabia C, Condò C, de Niederhausern S. 2015. Antimicrobial resistance and virulence traits in *Enterococcus* strains isolated from dogs and cats. New Microbiologica, 38(3): 369-378.

Iwasawa A, Niwano Y, Kohno M, Ayaki M. 2011. Bactericidal effects and cytotoxicity of new aromatic dialdehyde disinfectants (ortho-phthalaldehyde). Biocontrol Science, 16(4): 165-170.

Jackson CR, Fedorka-Cray PJ, Davis JA, Barrett JB, Frye JG. 2009. Prevalence, species distribution and antimicrobial resistance of enterococci isolated from dogs and cats in the United States. Journal of Applied Microbiology, 107(4): 1269-1278.

Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson, Jansson JK, Engstrand L. 2010. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. PLoS ONE [internet]. [Accessed 2020 Oct 10]; 5(3): [12 p.]. doi 10.1371/journal.pone.0009836

Jordan D, Simon J, Fury S, Moss S, Giffard P, Maiwald M, Southwell P, Barton MD, Axon JE, SG Morris, et al. 2011. Carriage of methicillin-resistant *Staphylococcus aureus* by veterinarians in Australia. Australian Veterinary Journal, 89(5): 152-159.

Kahler AM, Cromeans TL, Roberts JM, Hill VR. 2010. Effects of source water quality on chlorine inactivation of Adenovirus, Coxsackievirus, Echovirus, and murine Norovirus. Applied and Environmental Microbiology, 76(15): 5159-5164.

Kalchayanand N, Koohmaraie M, Wheeler TL. 2016. Effect of exposure time and organic matter on efficacy of antimicrobial compounds against Shiga Toxin–Producing *Escherichia coli* and *Salmonella*. Journal of Food Protection, 79(4): 561-568.

Karsa DR. 2007. Biocides. In: Johansson I, Somasundaran P, editors. Handbook for Cleaning/Decontamination of Surfaces. London (GB): Elsevier B.V.. Chapter F2, p. 593-623.

Kataoka Y, Ito C, Kawashima A, Ishii M, Yamashiro S, Harada K, Ochi H, Sawada T. 2013. Identification and antimicrobial susceptibility of enterococci isolated from dogs and cats subjected to differing antibiotic pressures. Journal of Veterinary Medicine Science, 75(6): 749-753.

Kaatz GW, McAleese F, Seo SM. 2005. Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel Multidrug and Toxin Extrusion (MATE) transport protein. Antimicrobial Agents and Chemotherapy, 49(5): 1857-1864.

Kawamura-Sato K, Wachino J, Kondo T, Ito H, Arakawa Y. 2008. Reduction of disinfectant bactericidal activities in clinically isolated *Acinetobacter* species in the presence of organic material. Journal of Antimicrobial Chemotherapy, 61(3): 568–576.

Kazama H, Hamashima H, Sasatsu M, Arai T. 1998. Distribution of the antiseptic-resistance gene *qacEΔ1* in Gram-positive bacteria. FEMS Microbiology Letters, 165(2): 295-299.

Kim M, Hatt JK, Weigand MR, Krishnan R, Pavlostathis SG, Konstantinidis KT. 2018. Genomic and transcriptomic insights into how bacteria withstand high concentrations of benzalkonium chloride biocides. Applied Environmental Microbiology [internet]. [Accessed 2020 Nov 09]; 84(12): [15 p.]. doi: 10.1128/AEM.00197-18

Kitis M. 2004. Disinfection of wastewater with peracetic acid: a review. Environment International, 30(1): 47-55.

Knapp L, Rushton L, Stapleton H, Sass A, Stewart S, Amezquita A, McClure P, Mahenthiralingam E, Maillard J-Y. 2013. The effect of cationic microbicide exposure against *Burkholderia cepacia* complex (Bcc); the use of *Burkholderia lata* strain 383 as a model bacterium. Journal of Applied Microbiology, 115(5): 1117-1126.

Kristich CJ, Rice LB, Arias CA. 2014. Enterococcal infection – treatment and antibiotic resistance. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. Enterococci: From

Commensals to Leading Causes of Drug Resistant Infection [internet]. Boston (MA): Eye and Ear Infirmary, [accessed 2020 Oct 28]. https://www.ncbi.nlm.nih.gov/books/NBK190420/

Kümmerle N, Feucht H-H, Kaulfers P-M. 1996. Plasmid-mediated formaldehyde resistance in *Escherichia coli*: characterization of resistance gene. Antimicrobial Agents and Chemotherapy, 40(10): 2276-2279.

Lambert PA. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. Journal of Applied Microbiology, 92(S1): 46-54.

Lanxess. 2017. Safety data sheet [internet]. [Pittsburgh (PA)]: LANXESS Corporation, [accessed 2020 Jul 08]. https://virkon.us/wp-content/uploads/sites/15/2017/11/VirkonTM-S-USA.pdf

Lanxess. 2019. Virkon® S – disinfectant and virucide [internet]. [Pittsburgh (PA)]: LANXESS Corporation, [accessed 2020 Jul 08]. https://syndel.com/wp-content/uploads/2019/01/Virkon-S-Information-Page-Lanxess.pdf

Le Dantec C, Duguet J-P, Montiel A, Dumoutier N, Dubrou S, Vincent V. 2002. Chlorine disinfection of atypical mycobacteria isolated from a water distribution system. Applied and Environmental Microbiology, 68(3): 1025-1032.

Leite-Martins L, Mahú MI, Costa AL, Bessa LJ, Vaz-Pires P, Loureiro L, Niza-Ribeiro J, Matos AJF, Martins da Costa P. 2015. Prevalence of antimicrobial resistance in faecal enterococci from vet-visiting pets and assessment of risk factors. Veterinary Record, 176(26): 674-681.

Lerma LL, Benomar N, Casado Muñoz MC, Gálvez A, Abriouel H. 2015. Correlation between antibiotic and biocide resistance in mesophilic and psychrotrophic *Pseudomonas* spp. isolated from slaughterhouse surfaces throughout meat chain production. Food Microbiology, 51: 33-44.

Levy CW, Roujeinikova A, Sedelnikova S, Baker PJ, Stuitje AR, Slabas AR, Rice DW, Rafferty JB. 1999. Molecular basis of triclosan activity. Nature, 398: 383-384.

Limbago BM, Kallen AJ, Zhu W, Eggers P, McDougal LK, Albrecht VS. 2014. Report of the 13th vancomycin-resistant *Staphylococcus aureus* isolate from the United States. Journal of Clinical Microbiology, 52(3): 998-1002.

Lineback CB, Nkemngong CA, Wu ST, Li X, Teska PJ, Oliver HF. 2018. Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms than quaternary ammonium compounds. Antimicrobial Resistance and Infection Control, 7: 154.

Liu Q, Zhao H, Han L, Shu W, Wu Q, Ni Y. 2015. Frequency of biocide-resistant genes and susceptibility to chlorhexidine in high-level mupirocin-resistant, methicillin-resistant *Staphylococcus aureus* (MuH MRSA). Diagnostic Microbiology and Infectious Disease, 82(4): 278-283.

Lu J, Jin M, Nguyen SH, Mao L, Li J, Coin LJM, Yuan Z, Guo J. 2018. Non-antibiotic antimicrobial triclosan induces multiple antibiotic resistance through genetic mutation. Environmental International, 118: 257-265.

Luciano CC, Olson N, Tipple AFV, Alfa M. 2016. Evaluation of the ability of different detergents and disinfectants to remove and kill organisms in traditional biofilm. American Journal of Infection Control [internet]. [Accessed 2020 Jul 15]; 44(11). doi: 10.1016/j.ajic.2016.03.040.

Ma X, Bibby K. 2017. Free chlorine and monochloramine inactivation kinetics of *Aspergillus* and *Penicillium* in drinking water. Water Research, 120: 265-271.

Machado ICT. 2016. Frequência de doenças infeciosas em carnívoros domésticos hospitalizados na unidade de isolamento do hospital escolar da faculdade de medicina veterinária da universidade de lisboa de outubro de 2013 a janeiro de 2016 outubro de 2013 a janeiro de 2016 [master's thesis]. Lisbon (PT): FMV-ULisboa.

Magiorakos AP, Srinivasan A, Carey, RB, Carmeli Y, Falagas ME, Giske, CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B et al. 2011. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection, 18(3), 268–281.

Maillard J-Y. 2002. Bacterial target sites for biocide action. Journal of Applied Microbiology Symposium Supplement, 92(S1): 16-27.

Maillard J-Y. 2005a. Biocides: health care applications. The Pharmaceutical Journal, 275: 639-642.

Maillard J-Y. 2005b. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. Therapeutics and Clinical Risk Management, 1(4) 307– 320.

Maillard J-Y. 2007. Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern? Journal of Hospital Infection, 6(S2): 60-72.

Maillard J-Y. 2013a. Factors affecting the activities of microbicides. In: Fraise AP, Lambert PA & Maillard J-Y, editors. Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. 5th edition. London (GB): Blackwell Publishing. Chapter 3, p. 71-86.

Maillard J-Y. 2013b. Bacterial sensitivity and resistance to microbicides. In: Fraise AP, Lambert PA & Maillard J-Y, editors. Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. 5th edition. London (GB): Blackwell Publishing. Chapter 6.1, p. 108-120.

Maillard J-Y. 2018. Resistance of bacteria to biocides. Microbiology Spectrum, 6(2): 1-17.

Maillard J-Y, Bloomfield S, Coelho JR, Collier P, Cookson B, Fanning S, Hill A, Hartemann P, Mcbain AJ, Oggioni M et al. 2013. Does microbicide use in consumer products promote antimicrobial resistance? A critical review and recommendations for a cohesive approach to risk assessment. Microbial Drug Resistance, 19(5): 344-354.

Maillard J-Y, Sattar SA, Pinto F. 2013. Virucidal activity of microbicides. In: Fraise AP, Lambert PA & Maillard J-Y, editors. Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. 5th edition. London (GB): Blackwell Publishing. Chapter 9, p. 178-207.

Maira-Litrán T, Alisson DG, Gilbert P. 2000. An evaluation of the potential of the multiple antibiotic resistance operon (*mar*) and the multidrug efflux pump *acrAB* to moderate

resistance towards ciprofloxacin in *Escherichia coli* biofilms. Journal of Antimicrobial Chemotherapy, 45(6): 789-95.

Marchetti V, Mancianti F, Cardini G, Luchetti E. 2006. Evaluation of fungicidal efficacy of benzalkonium chloride (Steramina G u.v.) and Virkon-S against *Microsporum canis* for environmental disinfection. Veterinary Research Communications, 30(3): 255-261.

Márquez MLF, Burgos MJG, Pulido RP, Gálvez A, López RL. 2016. Biocide tolerance and antibiotic resistance in *Salmonella* isolates from hen eggshells. Foodborne Pathogens and Disease, 14(2): 89-95.

Martin DJH, Denyer SP, McDonnell G, Maillard J-Y. 2008. Resistance and cross-resistance to oxidising agents of bacterial isolates from endoscope washer disinfectors. Journal of Hospital Infection, 69(4): 377-383.

Mbithi JN, Springthorpe VS, Sattar SA. 1990. Chemical disinfection of Hepatitis A virus on environmental surfaces. Applied and Environmental Microbiology, 56(11): 3601-3604.

Mc Cay PH, Ocampo-Sosa AA, Fleming GTA. 2010. Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture. Microbiology, 156(1): 30-38.

McCormick L and Maheshwari G. 2004. Inactivation of adenovirus types 5 and 6 by Virkon® S. Antiviral Research, 64(1): 27-33.

McDonnell G, Russell AD. 1999. Antiseptics and disinfectants: activity, action, and resistance. Clinical Microbiology Reviews, 12(1): 147–179.

McMurry LM, McDermott PF, Levy SB. 1999. Genetic evidence that InhA of *Mycobacterium smegmatis* is a target for triclosan. Antimicrobial Agents and Chemoterapy, 43(3): 711-713.

Méchin L, Dubois-Brissonnet F, Heyd B, Leveau JY. 1999. Adaptation of *Pseudomonas aeruginosa* ATCC 15442 to didecyldimethylammonium bromide induces changes in membrane fatty acid composition and in resistance of cells. Journal of Applied Microbiology, 86(5): 859-866.

Miller WR, Munita JM, Arias CA. 2014. Mechanisms of antibiotic resistance in enterococci. Expert Review of Anti-infective Therapy, 12(10): 1221-1236.

Mima T, Joshi S, Gomez-Escalada M, Schweizer HP. 2007. Identification and characterization of TriABC-OpmH, a triclosan efflux pump of *Pseudomonas aeruginosa* requiring two membrane fusion proteins. Journal of Bacteriology, 189(21): 7600-7609.

Moore LE, Ledder RG, Gilbert P, McBain AJ. 2008. In vitro study of the effect of cationic biocides on bacterial population dynamics and susceptibility. Applied and Environmental Microbiology, 74(15): 4825-4834.

Mount R, Schick AE, Lewis II TP, Newton HM. 2016. Evaluation of bacterial contamination of clipper blades in small animal private practice. Journal of the American Animal Hospital Association, 52(2): 95–101.

Nakama Y. 2017. Surfactants. In: Cosmetic Science and Technology. 1st edition. London (GB): Elsevier. Chapter 15, p. 231–244.

Nerandzic MM, Sunkesula VCK, Sankar C T, Setlow P, Donskey CJ. 2015. Unlocking the sporicidal potential of ethanol: induced sporicidal activity of ethanol against *Clostridium difficile* and *Bacillus* spores under altered physical and chemical conditions. PLoS ONE [internet]. [Accessed 2020 Mar 21]; 10(7): [15 p.]. doi: 10.1371/journal.pone.0132805

[NIOSH] The National Institute for Occupational Safety and Health. 2015. Hierarchy of Controls [Internet]. [Washington (WA)]: Centre for Disease Control and Prevention; [accessed 2020 Apr 10]. https://www.cdc.gov/niosh/topics/hierarchy/default.html

Nishino K, Yamaguchi A. 2001. Analysis of a complete library of putative drug transporter genes in *Escherichia coli*. Journal of Bacteriology, 183(20): 5803-5812.

Noguchi N, Suwa J, Narui K, Sasatsu M, Ito T, Hiramatsu K, Song J-H. 2005. Susceptibilities to antiseptic agents and distribution of antiseptic-resistance genes *qacA/B* and *smr* of methicillin-resistant *Staphylococcus aureus* isolated in Asia during 1998 and 1999. Journal of Medical Microbiology, 54(6): 557-565.

[NTP] National Toxicology Program. 1999. NTP technical report of the toxicology and carcinogenesis studies of glutaraldehyde (cas no. 111-30-8) in F344/N rats and B6C3F$_1$ mice (inhalation studies) [Internet]. Research Triangle Park (NC): National Toxicology Program (NTP); [accessed 2020 Apr 20]. https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr490.pdf?utm_source=direct&utm_medium=prod&utm_campaign=ntpgolinks&utm_term=tr490

Omidbakhsh N. 2006. A new peroxide-based flexible endoscope-compatible high-level disinfectant. American Journal of Infection Control, 34(9): 571–577.

Omidbakhsh N, Sattar SA. 2006. Broad-spectrum microbicidal activity, toxicologic assessment, and materials compatibility of a new generation of accelerated hydrogen peroxide-based environmental surface disinfectant. American Journal of Infection Control, 34(5): 251–257.

Ossiprandi MC, Bottarelli E, Cattabiani F, Bianchi E. 2008. Susceptibility to vancomycin and other antibiotics of 165 *Enterococcus* strains isolated from dogs in Italy. Comparative Immunology, Microbiology & Infectious Diseases 31(1): 1–9.

Pajkos A, Vickery K, Cossart Y. 2004. Is biofilm accumulation on endoscope tubing a contributor to the failure of cleaning and decontamination? Journal of Hospital Infection, 58(3): 224-229.

Pala G, Moscato G. 2013. Allergy to ortho-phthalaldehyde in the healthcare setting: advice for clinicians. Expert Review of Clinical Immunology, 9(3): 227-234.

Pan Y, Breidt F, Kathariou S. 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. Applied and Environmental Microbiology, 72(12): 7711-7717.

Piddok LJ. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical Microbiology Reviews, 19(2): 382-402.

Pinto F, Maillard J-Y, Denyer SP, McGeechan P. 2009. Polyhexamethylene biguanide exposure leads to viral aggregation. Journal of Applied Microbiology, 108: 1880-1888.

Ponzano GP. 2007. Sodium hypochlorite: history, properties, electrochemical production. In: Ronco C, Mishkin GJ, editors. Disinfection by sodium hypochlorite: dialysis applications. Basel (CH): Karger Publishers. Volume 154, p. 7-23.

Poole K. 2001. Multidrug resistance in Gram-negative bacteria. Current Opinion in Microbiology, 4(5): 500–508.

Poole K. 2002. Mechanisms of bacterial biocide and antibiotic resistance. Journal of Applied Microbiology, 92(S1): 55–64.

Poole K. 2005. Efflux-mediated antimicrobial resistance. Journal of Antimicrobial Chemoterapy, 56(1): 20-51.

Poole K. 2007. Efflux pumps as antimicrobial resistance mechanisms. Annals of Medicine, 39(3): 162-176.

Poole K. 2012. Similarities and differences in the responses of microorganisms to biocides. Journal of Antimicrobial Chemotherapy, 52 (5): 750–763.

Portner JA, Johnson, JA. 2010. Guidelines for reducing veterinary hospital pathogens. Compendium Continuing Education for Veterinarians, 32(5): E1-E11.

Quinn PJ, Markey BK, Leonard FC, FitzPatrick ES, Fanning S, Hartigan PJ. 2011. Disinfection, biosecurity and other aspects of disease control. In: Veterinary Microbiology and Microbial Disease. 2nd edition. West Sussex (GB): Willey Blackwell. p. 851-889.

Rabenau HF, Steinmann J, Rapp I, Schwebke I, Eggers M. 2014. Evaluation of a virucidal quantitative carrier test for surface disinfectants [internet]. [Accessed 2020 Jun 10]; 9(1): [10 p.]. doi:10.1371/journal.pone.0086128

Rakic-Martinez M, Drevets DA, Dutta V, Katic V, Kathariou S. 2011. *Listeria monocytogenes* strains selected on ciprofloxacin or the disinfectant benzalkonium chloride exhibit reduced susceptibility to ciprofloxacin, gentamicin, benzalkonium chloride, and other toxic compounds. Applied and Environmental Microbiology, 77(24): 8714-8721.

Randall LP, Cooles SW, Coldham NG, Penuela EG, Mott AC, Woodward MJ, Piddock LJV, Webber MA. 2007. Commonly used farm disinfectants can select for mutant *Salmonella enterica* serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. Journal of Antimicrobial Chemotherapy, 60(6): 1273–1280.

Regulation (UE) nº 528/2012 of 22 of May 2012. Official Journal of the European Union – L 167/1. European Parliament and Council.

Rikimaru T, Kondo M, Kondo S, Oizumi K. 2000. Efficacy of common antiseptics against mycobacteria. The International Journal of Tuberculosis and Lung Disease, 4(6): 570-576.

Roedel A, Dieckmann R, Brendebach H, Hammerl JA, Kleta S, Noll M, All Dahouk S, Vincze S. 2019. Biocide-tolerant *Listeria monocytogenes* isolates from german food production plants do not show cross-resistance to clinically relevant antibiotics. Applied and Environmental Microbiology [internet]. [Accessed 2020 Oct 09]; 86(20): [15 p.]. doi: 10.1128/AEM.01253-19

Rodrigues J, Poeta P, Martins A, Costa D. 2002. The importance of pets as reservoirs of resistant *Enterococcus* strains, with special reference to vancomycin. Journal of Veterinary Medicine, 49(6): 278-280.

Roy R, Tiwari M, Donelli G, Tiwari V. 2017. Strategies for combating bacterial biofilms: a focus on anti-biofilm agents and their mechanisms of action. Virulence, 9(1): 522-554.

Ruple-Czerniak A, Aceto HW, Bender JB, Paradis MR, Shaw SP, Van Metre DC, Weese JS, Wilson DA, Wilson JH, Morley PS. 2013. Using syndromic surveillance to estimate baseline rates for healthcare-associated infections in critical care units of small animal referral hospitals. Journal of Veterinary Internal Medicine, 27(6): 1392–1399.

Russell AD. 1990. Bacterial spores and chemical sporicidal agents. Clinical Microbiology Reviews, 3(2): 99-119.

Russell AD. 2003a. Biocide use and antibiotic resistance: the relevance of laboratory findings to clinical and environmental situations. The Lancet Infectious Diseases, 3(12): 794–803.

Russell AD. 2003b. Similarities and differences in the responses of microorganisms to biocides. Journal of Antimicrobial Chemotherapy. 52(5): 750–763.

Rutala WA, Cole EG, Thomann CA, Weber DJ. 1998. Stability and bactericidal activity of chlorine solutions. Infection Control and Hospital Epidemiology, 19(5): 323-327.

Rutala WA, Cole EC, Wannamaker NS, Weber DJ. 1991. Inactivation of *Mycobacterium tuberculosis* and *Mycobacterium bovis* by 14 hospital disinfectants. The American Journal of Medicine, 91(S3B): 267–271.

Rutala WA, Gergen MF, Weber DJ. 2012. Efficacy of improved hydrogen peroxide against important healthcare-associated pathogens. Infection Control and Hospital Epidemiology, 33(11): 1159-1161.

Russell AD, McDonnell G. 2000. Concentration: a major factor in studying biocidal action. Journal of Hospital Infection, 44(1): 1-3.

Rutala WA, Weber DJ. 2013. Disinfection and sterilization: an overview. American Journal of Infection Control, 41(S5): 2–5.

Rutala WA, Weber DJ, [HICPAC] Healthcare Infection Control Practices Advisory Committee. 2008. Guideline for Disinfection and Sterilization in Healthcare Facilities [internet]. [Washington (WA)]: Centre for Disease Control and Prevention; [accessed 2020 Apr 15]. https://www.cdc.gov/infectioncontrol/pdf/guidelines/disinfection-guidelines-H.pdf

Şahiner A, Halat E, Yapar EA. 2019. Comparison of bactericidal and fungicidal efficacy of antiseptic formulations according to EN 13727 and EN 13624 standards. Turkish Journal of Medical Science, 49: 1564-1567.

Saklou NT, Burgess BA, Van Metre DC, Hornig KJ, Morley PS, Byers SR. 2016. Comparison of disinfectant efficacy when using high-volume directed mist application of accelerated hydrogen peroxide and peroxymonosulfate disinfectants in a large animal hospital. Equine Veterinary Journal, 48(4): 485-489.

Salzman MB, Isenberg HD, Rubin LG. 1993. Use of disinfectants to reduce microbial contamination of hubs of vascular catheters. Journal of Clinical Microbiology, 31(3): 475-479.

Sansebastiano G, Zoni R, Bigliardi L. 2007. Cleaning and Disinfection Procedures in the Food Industry General Aspects and Practical Applications. In: McElhatton A, Marshall R, editors. Food Safety: A Practical and Case Study Approach. New York (NY): Springer. Chapter 13, p. 253-280.

Satto Y, Ishihara M, Nakamura S, Fukuda K, Kuwabara M, Takayama T, Hiruma S, Murakami K, Fujita M, Yokoe H. 2019. Comparison of various disinfectants on bactericidal activity under organic matter contaminated environments. Biocontrol Science, 24(2): 103-108.

Sattar SA, Springthorpe VS, Karim Y, Loro P. 1989. Chemical disinfection of non-porous inanimate surfaces experimentally contaminated with four human pathogenic viruses. Epidemiology and Infection, 102(3): 493-505.

[SCENIHR] Scientific Committee on Emerging and Newly Identified Health Risks. 2009. Assessment of the antibiotic resistance effects of biocides [internet]. [Brussells (BE)]: European Commission; [accessed 2020 May 23]. https://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf

Schreiber PW, Sax H, Wolfensberger A, Clack L, Kuster SP. 2018. The preventable proportion of healthcare-associated infections 2005–2016: Systematic review and meta-analysis. Infection Control & Hospital Epidemiology, 1–19.

Setlow P. 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. Journal of Applied Microbiology, 101(3): 514-525.

Setlow P. 2013. Resistance of bacterial spores to chemical agents. In: Fraise AP, Lambert PA & Maillard J-Y, editors. Russell, Hugo & Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization. 5th edition. London (GB): Blackwell Publishing. Chapter 6.2, p. 121-130.

Shaffer MP, Belsito DV. 2000. Allergic contact dermatitis from glutaraldehyde in health-care workers. Contact Dermatitis, 43(3): 150–156.

Sheldon AT. 2005. Antiseptic "resistance": real or perceived threat? Clinical Infectious Diseases, 40(11): 1650-1656.

Sifaoui F, Arthur M, Rice L, Gutmann L. 2001. Role of penicillin-binding protein 5 in expression of ampicillin resistance and peptidoglycan structure in *Enterococcus faecium*. Antimicrobial Agents and Chemotherapy, 45(9): 2594-2597.

Simons C, Walsh SE, Maillard J-Y, Russell AD. 2000. A note: *ortho*-phthalaldehyde: proposed mechanism of action of a new antimicrobial agent. Letters in Applied Microbiology, 31(4): 299-302.

Skinner BM, Rogers AT, Jacob ME. 2018. Susceptibility of *Escherichia coli* O157:H7 to disinfectants *in vitro* and in simulated footbaths amended with manure. Foodborne Pathogens and Disease, 15(11): 718-725.

Smith K, Hunter IS. 2008. Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. Journal of Medical Microbiology, 57(8): 966-973.

Sokol WN. 2004. Nine episodes of anaphylaxis following cystoscopy caused by Cidex OPA (ortho-phthalaldehyde) high-level disinfectant in 4 patients after cytoscopy. Journal of Allergy and Clinical Immunology, 114(2): 392–397.

Stein BL, Lamoureux E, Miller M, Vasilevsky C-A, Julien L, Gordon PH. 2001. Glutaraldehyde-induced colitis. Canadian Journal of Surgery, 44(2): 113-116.

Stewart MJ, Parikh S, Xiao G, Tonge PJ, Kisker C. 1999. Structural basis and mechanism of enoyl reductase inhibition by triclosan. Journal of Molecular Biology, 290(4): 859-865.

Streckenbach SC, Alston TA. 2003. Perioral stains after *ortho*-phthalaldehyde disinfection of echo probes. Anesthesiology, 99(4): 1032.

Stull JW, Bjorvik E, Bub J, Dvorak G, Petersen C, Troyer HL. 2018. 2018 AAHA infection control, prevention, and biosecurity guidelines. Journal of the American Animal Hospital Association, 54(6): 297–326.

Stull JW, Weese JS. 2015. Hospital-Associated Infections in Small Animal Practice. Veterinary Clinics of North America: Small Animal Practice, 45(2): 217–233.

Sun J, Deng Z, Yan A. 2014. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochemical and Biophysical Research Communications, 453(2), 254–267.

Suzukawa M, Yamaguchi M, Komiya A, Kimura M, Nito T, Yamamoto K. 2006. Ortho-phthalaldehyde–induced anaphylaxis after laryngoscopy. Journal of Allergy and Clinical Immunology, 117(6): 1500–1501.

Sylte MJ, Chandra LC, Looft T. 2017. Evaluation of disinfectants and antiseptics to eliminate bacteria from the surface of turkey eggs and hatch gnotobiotic poults. Poultry Science, 96(7): 2412–2420.

Tabata A, Nagamune H, Maeda T, Murakami K, Miyake Y, Kourai H. 2003. Correlation between resistance of *Pseudomonas aeruginosa* to Quaternary Ammonium Compounds and expression of outer membrane protein OprR. Antimicrobial Agents and Chemotherapy, 47(7): 2093-2099.

Tattawasart U, Maillard J-Y, Furr JR, Russell AD. 2000. Outer membrane changes in *Pseudomonas stutzeri* resistant to chlorhexidine diacetate and cetylpyridinium chloride. International Journal of Antimicrobial Agents, 16(3): 233-238.

Taylor RH, Falkinham III JO, Norton CD, LeChevallier MW. 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of *Mycobacterium avium*. Applied and Environmental Microbiology, 66(4): 1702-1705.

Tegos GP, Haynes M, Strouse JJ, Khan MMT, Bologa CG, Oprea TI, Sklar LA. 2011. Microbial efflux pump inhibition: tactics and strategies. Current Pharmaceutical Design, 17(13): 1291-1302.

Terleckyj B, Axler DA. 1987. Quantitative neutralization assay of fungicidal activity of disinfectants. Antimicrobial Agents and Chemotherapy, 31(5): 794-798.

Thomas L, Maillard J-Y, Lambert RJW, Russell AD. 2000. Development of resistance to chlorhexidine diacetate in *Pseudomonas aeruginosa* and the effect of a 'residual' concentration. Journal of Hospital Infection, 46(4): 297-303.

Tischer M, Pradel G, Ohlsen K, Holzgrabe U. 2012. Quaternary Ammonium Salts and their antimicrobial potential: targets or nonspecific interactions? ChemMedChem, 7(1): 22–31.

Tobe S, Majima T, Tadenuma H, Suekuni T, Sakai K, Sakai H, Abe M. 2015. Nonionic surfactants enhancing bactericidal activity at their critical micelle concentrations. Journal of Oleo Science, 64(1): 61-68.

Torres C, Alonso CA, Ruiz-Ripa L, León-Sampedro R, Del Campo R, Coque TM. 2018. Antimicrobial Resistance in *Enterococcus* spp. of animal origin. Microbiology Spectrum, 6(4): 1-41.

Ubeda C, Taur Y, Jenq R, Equinda MJ, Son T, Samstein M, Viale A, Socci ND, van den Brink MRM, Kamboj M, et al. 2010. Vancomycin-resistant *Enterococcus* domination of intestinal microbiota is enabled by antibiotic treatment in mice and precedes bloodstream invasion in humans. The Journal of Clinical Investigation, 120(12): 4332-4341.

Uzuner H, Karadenizli A, Er DK, Osmani A. 2018. Investigation of the efficacy of alcohol-based solutions on adenovirus serotypes 8, 19 and 37, common causes of epidemic keratoconjunctivitis, after an adenovirus outbreak in hospital. Journal of Hospital Infection, 100(3): 30-36.

Venkatesan N, Perumal G, Doble M. 2015. Bacterial resistance in biofilm-associated bacteria. Future Microbiology, 10(11): 1743-1750.

Venticinque SG, Kashyap V, O'Connell RJ. 2003. Chemical burn injury secondary to intraoperative transesophageal echocardiography. International Anesthesia Research Society, 97: 1260-1261.

Verdial CSS. 2020. Prevenção de Infeções Nosocomiais: Controlo Bacteriológico de Superfícies Hospitalares da Unidade de Isolamento e Contenção Biológica do Hospital Escolar da FMV-ULisboa [master's thesis]. Lisbon (PT): FMV-ULisboa.

Walsh SE, Maillard J-Y, Russell AD. 1999. *Ortho*-phthalaldehyde: a possible alternative to glutaraldehyde for high-level disinfection. Journal of Applied Microbiology, 86(6): 1039-1046.

Walsh SE, Maillard J-Y, Russell AD, Catrenich CE, Charbonneau DL, Bartolo RG. 2003. Development of bacterial resistance to several biocides and effects on antibiotic susceptibility. Journal of Hospital Infection, 55(2): 98-107.

Walsh SE, Maillard J-Y, Russell AD, Hann AC. 2001. Possible mechanisms for the relative efficacies of orthophthalaldehyde and glutaraldehyde against glutaraldehyde-resistant *Mycobacterium chelonae*. Journal of Applied Microbiology, 91(1): 80-92.

Wang X, Han Z, Li Y, Guo Y, Xu W, Liu S. 2015. A case of acute glutaraldehyde-induced colitis following polyps treated by EMR. Internal Journal of Colorectal Disease, 30(2): 277-278.

Wang X-W, Li J-S, Jin M, Zhen B, Kong Q-X, Song N, Xiao W-J, Yin J, Wei W, Wang G-J et al. 2005. Study on the resistance of severe acute respiratory syndrome-associated coronavirus. Journal of Virological Methods, 126(1-2): 171-177.

Webber MA, Randall LP, Cooles S, Woodward MJ, Piddock LJV. 2008. Triclosan resistance in *Salmonella enterica* serovar Typhimurium. Journal of Antimicrobial Chemotherapy, 62(1): 83-91.

Weese JS. 2011. Infection control in veterinary practice; the time is now. Journal of Small Animal Practice, 52(10): 507–508.

Weese JS, Blondeau J, Boothe D, Guardabassi LG, Gumley N, Papich M, Jessen LR, Lappin M, Rankin S, Westropp JL, et al. 2019. International Society for Companion Animal Infectious Diseases (ISCAID) guidelines for the diagnosis and management of bacterial urinary tract infections in dogs and cats. The Veterinary Journal, 247: 8-25.

Weese JS, DaCosta T, Button L, Goth K., Ethier M, Boehnke K. 2004. Isolation of methicillin-resistant *Staphylococcus aureus* from the environment in a veterinary teaching hospital. Journal of Veterinary Internal Medicine, 18(4): 468–470.

Weese JS, Dick H, Willey B, McGeer A, Kreiswirth B, Innis B, Low D. 2006. Suspected transmission of methicillin-resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. Veterinary Microbiology, 115(1-3): 148–155.

Weese JS, van Duijkeren E. 2010. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. Veterinary Microbiology, 140(3-4): 418-429.

Weigel LM, Clewell DB, Gill SR, Clark NC, McDougal LK, Flannagan SE, Kolonay JF, Shetty J, Killgore GE, Tenover FC. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science, 302(5650): 1569-1571.

Wesgate R, Grasha P, Maillard J-Y. 2016. Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage. American Journal of Infection Control, 44(4): 458-464.

Whan LB, Grant IR, Ball HJ, Scott R, Rowe MT. 2001. Bactericidal effect of chlorine on *Mycobacterium paratuberculosis* in drinking water. Letters in Applied Microbiology, 33(3): 227–231.

Wheeldon LJ, Worthington T, Hilton AC, Lambert PA, Elliott TSJ. 2008. Sporicidal activity of two disinfectants against *Clostridium difficile* spores. British Journal of Nursing, 17(5): 316-320.

[WHO] World Health Organization. 2018. Antibiotic resistance [internet]. [Accessed 09 Jul 2020] https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance

Wieland N, Boss J, Lettmann S, Fritz B, Schwaiger K, Bauer J, Hölzel CS. 2017. Susceptibility to disinfectants in antimicrobial-resistant and -susceptible isolates of *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus faecium* from poultry–ESBL/ AmpC-phenotype of *E. coli* is not associated with resistance to a Quaternary Ammonium Compound, DDAC. Journal of Applied Microbiology, 122(6): 1508-1517.

Wieler LH, Walther B, Vincze S, Guenther S, Luebke-Becker A. 2014. Infections with multidrug-resistant bacteria: has the post-antibiotic era arrived in companion animals? In: Sing A, editor. Zoonoses – Infections Affecting Humans and Animals: Focus on Public Health Aspects. Oberschleißheim (DE): Springer. Chapter 7, p. 433-452.

Willey JM, Sherwood LM, Woolverton CJ. 2008. Procaryotic Cell Structure and Function. In: Prescott, Harley and Klein's Microbiology. 7th edition. New York (NY): McGraw-Hill. Chapter 3, p. 39-78.

Williams GJ, Denyer SP, Hosein IK, Hill DW, Maillard J-Y. 2009. Use of sodium dichloroisocyanurate for floor disinfection. Journal of Hospital Infection, 72(3): 279–281.

Winder W. 2001. The toxicology of chlorine. Environmental Research, 85(2): 105-114.

Winder W, Al-Adham ISI, Abdel Malek SMA, Buultjens TEJ, Horrocks AJ, Collier PJ. 2000. Outer membrane protein shifts in biocide-resistant *Pseudomonas aeruginosa* PAO1. Journal of Applied Microbiology, 89(2): 289-295.

Wong HS, Townsend KM, Fenwick SG, Trengove RD, O'Handley RM. 2009. Comparative susceptibility of planktonic and 3-day-old *Salmonella* Typhimurium biofilms to disinfectants. Journal of Applied Microbiology, 108(6): 2222-2228.

Wu G, Selden D, Fooks AR, Banyard A. 2017. Inactivation of Rabies Virus. Journal of Virological Methods, 243: 109-112.

Zeiger E, Gollapudi B, Spencer P. 2005. Genetic toxicity and carcinogenicity studies of glutaraldehyde - a review. Mutation Research, 589(2): 136–151.

Zhu W, Clark NC, McDougal LK, Hageman J, McDonald LC, Patel JB. 2008. Vancomycin-resistant *Staphylococcus aureus* isolates associated with Inc18-Like *vanA* plasmids in Michigan. Antimicrobial Agents and Chemotherapy, 52(2): 452–457.

Zhu L, Lin J, Ma J, Cronan JE, Wang H. 2010. Triclosan resistance of *Pseudomonas aeruginosa* PAO1 is due to FabV, a triclosan-resistant enoyl-acyl carrier protein reductase. Antimicrobial Agents and Chemoteraphy, 54(2): 689-698.

Appendix 1: Virkon® S's Information Page by Lanxess (2019)

Virkon® S

DISINFECTANT AND VIRUCIDE

Virkon® S is the multipurpose virucidal disinfectant with the greatest numbers of EPA-registered claims against pathogens affecting domestic and companion animals.

Overview

Virkon® S is a balanced, stabilized blend of peroxygen compounds, surfactant, organic acids, and inorganic buffer. Virkon® S is recommended for use as a hard surface disinfectant in livestock production and transportation facilities. Virkon® S delivers 99.9999% kill of numerous pathogens including 31 bacterial strains, 58 viruses, and 6 fungi with no evidence of resistance, eliminating the need to rotate disinfectants to avoid resistance buildup.

Active Ingredients

Potassium peroxymonosulfate	21.41%
Sodium Chloride	1.50%
Other ingredients	77.09%
Total ingredient	100.00%

EPA Registration No. 71654-6

Mode of Action

Virkon® S oxidizes sulfur bonds in proteins and enzymes disrupting the function of the cell membrane causing rupturing of the cell wall.

Directions for Use

Use Disinfectants safely. For complete instructions on product use, handling and safety, always read and understand the product label and MSDS before use. It is a violation of Federal law to use this product in a manner inconsistent with its labeling. The information set forth herein is furnished free of charge and was prepared as a possible aid to use when considering the product. Anyone intending to use recommendations or information contained in this handout should first be satisfied that the information is suitable for their application and meets all safety and health standards appropriate for their intended use.

LANXESS
Energizing Chemistry

Effective Against Viruses, Bacteria and Fungi In Industrial and Agricultural/Animal Facilities.

VIRUSES

Adenovirus Pneumonia
African Horse Sickness Virus
African Swine Fever Virus
Avian Influenza Virus
Avian Laryngotracheitis Viruses
Bovine Adenoviruses Type 4
Bovine Polyoma Virus
Bovine Pseudocopox Virus
Bovine Viral Diarrrhea Virus
Calf Rotavirus
Canine Adenovirus (Canine Hepatitis)
Canine Coronavirus
Canine Parainfluenza Virus
Canine Parvovirus
Chicken Anemia Virus
Coital Exantherma Virus
Distemper Virus
Duck Adenovirus
Duck Enteritis Virus
Egg Drop Syndrome Adenovirus
Equine Arteritis Virus
Equine Contagious Abortion Virus
Equine Herpes Virus (Type 1)
Equine Herpes Virus (Type 3)
Equine Infectious Anemia Virus (Swamp Fever)
Equine Influenza Virus (Type A)
Equine Influenza Virus (The Cough)
Equine Papillomatosis Virus
Feline Calicivirus
Feline Herpes Virus
Feline Infectious Peritonitis Virus
Feline Panleukopenia Virus
Feline Parvovirus
Feline Rhinotracheitis Virus
Foot and Mouth Disease Virus
Helicobacter pylori
Hog Cholera Virus
Infectious Bronchitis Virus
Infectious Bursal Disease Virus
Infectious Canine Hepatitis Virus
Infective Bovine Rhinotracheitis Virus
Leptospira Canicola Virus
Maedi-Visna Virus
Marek's Disease Virus
Mouse Parvovirus
Newcastle Disease Virus
PCVS Virus (PMWS)
Porcine Parvovirus
Porcine Reproductive and Respiratory Syndrome Virus (PRRS)
Pseudorabies Virus (Aujesky's Disease)
Rotaviral Diarrhea Virus
Simian Virus (SV40 Virus)
Swine Influenza Virus
Swine Vesicular Disease Virus
Transmissible Gastroenteritis Virus (TGE)
Turkey Herpes Virus
Turkey Rhinotracheitis Virus
Vesicular Stomatitis

BACTERIA

Actinobacillus pleuropneumoniae
Bacillus cereus
Bordetella avium
Bordetella bronchiseptica
Brucella abortus
Campylobacter jejuni
Clostridium perfringens
Dermatophilus congolensis
Escherichia coli
Fistulous withers (Poll Evil)
Haemophilus somnus
Klebsiella pneumoniae
Listeria monocytogenes
Moraxella bovis (Pink Eye)
Mycoplasma gallisepticum
Mycoplasma hyopneumonia
Mycoplasma mycoides
Pasteurella multocida
Pseudomonas aeruginosa
Pseudomonas mallei (Glanders)
Pseudomonas vulgaris
Salmonella choleraesuis
Salmonella typhimurium
Shigella sonnei
Staphylococcus aureus
Staphylococcus epidermidis
Streptococcus equi (Strangles)
Streptococcus pyogenes
Streptococcus suis
Taylorella equigenitalis
Treponema hyodysenteriae

FUNGI

Aspergillus fumigatus
Fusarium moniliforme
Microsporum canis
Trichophyton mentagrophytes
Trichophyton spp. (Mud Fever)
Trichophyton spp. (Ringworm)

Physical Properties

State	Solid (powder or tablet)
Color	yellow
Odor	slight lemon
pH, 25°C in-use solution	2.2-2.6
Specific gravity	1.07
Stability	1% solution stable for 7 days 20% loss of activity after 14 days in 350ppm hard water
Boiling Point	decomposes on heating
Solubility	9.2 oz/gal @ 69°F
Density of 1% solution	9.3 lbs/gal.

Dilution Rates

Quantity of water	0.5% Solution	1.0% Solution	2.0% S olution
1 quart	0.15 oz	0.3 oz	0.7 oz
1 gallon	0.65 oz	1.3 oz	2.7 oz
10 gallons	6.7 oz	13.4 oz	26.7 oz
50 gallons	33.4 oz	66.8 oz	133.5 oz
1 scoop equals 1.3 oz or 1 scoop per gallon of water = 1%			

For 24-hour emergency information on the product, call 1-800-441-3637 (U.S. and Canada) or 1-302-774-1139 (all other areas)

biosecurity@lanxess.com

Not all products, applications, claims and/or uses are registered in all regions, countries or states. Greatest number of EPA-registered claims is based on a label search of major disinfectants for animal health, using the EPA Pesticide Product Label System at http://oaspub.epa.gov/pestlabl/ppls.home. The information set forth herein is furnished free of charge and is based on technical data that DuPont believes to be reliable. It is intended for use by persons having technical skills and should be used at their own discretion. Because conditions of use are outside our control, we make no warranties, expressed or implied, and assume no liability in connection with any use of this information. Nothing herein is to be taken as a license to operate under or a recommendation to infringe any patents.

LANXESS
Energizing Chemistry

Distributed by:

NEOGEN CORPORATION
944 Nandino Blvd., Lexington, KY 40511
Phone: 1-800-621-8829 • Fax: 1-800-255-1168
Email: bioprotection@neogen.com

©2018 LANXESS. Virkon™ and any associated logos are trademarks or copyrights of LANXESS Corporation. LANXESS™ and the LANXESS Logo are trademarks of LANXESS Deutschland GmbH. All trademarks are registered in many countries worldwide.

Appendix 2: Isolates Identification

The following table indicates the correspondence between the isolates initially obtained by Verdial (2020) in her master thesis (including the date and place they were collected) and the identification used in this work.

New Identification	Old Identification	Place of Collection	Date of Collection
C1	E4 27/3	Isolate collected from a sponge – room 4 (C)	27/02/19
C2	S2J2 5/3	Isolate collected from the cages – room 2 (D)	05/03/19
C3	S2J 21/5	Isolate collected form the cages – room 2 (D)	21/05/19
E1	S2MA31 5/6	Isolate collected from the first three cabinet handles – room 2 (D)	05/06/19
E2	S2J 21/5	Isolate collected form the cages – room 2 (D)	21/05/19
E3	S3M 12/6	Isolate collected form the examination table – room 3 (D)	12/06/19
E4	S2MA32 5/6	Isolate collected from the first three cabinet handles – room 2 (D)	05/06/19
E5	S1M 6/2	Isolate collected from the examination table – room 3 (D)	06/02/19
E6	S1MA2 20/5	Isolate collected from the last two cabinet handles – room 1 (C)	20/05/19
E7	S1MA31 6/2	Isolate collected from the first three cabinet handles – room 1 (C)	06/02/19
E8	S1MA32 6/2	Isolate collected from the first three cabinet handles – room 1 (C)	06/02/19
E9	S2T 5/6	Isolate collected from a faucet – room 2 (D)	05/06/19
E10	S2A 21/5	Isolate collected from a cabinet – room 2 (D)	21/05/19
E11	S2MA3 21/5	Isolate collected from the first three cabinet handles – room 2 (D)	21/05/19
E12	S2A 5/6	Isolate collected from a cabinet – room 2 (D)	05/06/19
E13	S2P 5/6	Isolate collected from the door handle – room 2 (D)	05/06/19
E14	S3J 28/5	Isolate collected from the cages – room 3 (D)	28/05/19

E15	S2T 21/5	Isolate collected from the faucet – room 2 (D)	21/05/19
E16	S2J 5/6	Isolate collected from the cages – room 2 (D)	05/06/19
E17	S3J 12/2	Isolate collected from the cages – room 3 (D)	12/02/19
E18	S2MA2 5/3	Isolate collected from the last two cabinet handles – room 2 (D)	05/03/19
E19	E 27/3	Isolate collected from a sponge – central room	27/03/19
E20	S4A 13/2	Isolate collected from the cabinet – room 4 (C)	13/02/19
E21	S2P 21/5	Isolate collected from the door handle – room 2 (D)	21/05/19
E22	S2T 5/3	Isolate collected from the faucet – room 2 (D)	05/03/19
E23	S1MA 6/2	Isolate collected from the last two cabinet handles – room 1 (C)	06/02/19
P1	S2T 5/6	Isolate collected from the faucet – room 2 (D)	05/06/19
P2	E2 5/6	Isolate collected from a sponge – room 2 (D)	05/06/19
P3	T 12/6	Isolate collected from a bowl of food	12/06/19
S1	S2MA21 5/3	Isolate collected from the last two cabinet handles – room 2 (D)	05/03/19
S2	S2MA22 5/3	Isolate collected from the last two cabinet handles – room 2 (D)	05/03/19
S3	S3MA2 28/5	Isolate collected from the first three cabinet handles – room 3 (D)	28/05/19
S4	S2J 28/5	Isolate collected from the cages – room 3 (D)	28/05/19
S5	S2T 5/3	Isolate collected from the faucet – room 2 (D)	05/03/19

D: Dogs; C: Cats