UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS E TECNOLOGIAS PARA A SUSTENTABILIDADE (CCTS)

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA E MONITORAMENTO AMBIENTAL (PPGBMA)

AMANDA STEFANIE JABUR DE ASSIS

STUDY OF ANTIMICROBIAL ACTION OF $Ga(NO_3)_3$ ON BACTERIAL STRAINS

Sorocaba, 2022

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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia e Monitoramento Ambiental para obtenção do título de Mestre em Biotecnologia e Monitoramento Ambiental.

Orientação: Prof.ª Dr.ª Iolanda Cristina Silveira Duarte

Sorocaba, 2022

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Study of Antimicrobial Action of Ga(NO3)3 on Bacterial Strains / Amanda Stefanie Jabur de Assis -- 2022.
75f.

Dissertação (Mestrado) - Universidade Federal de São Carlos, campus Sorocaba, Sorocaba
Orientador (a): Iolanda Cristina Silveira Duarte
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Bibliografia

1. Gallium Nitrate. 2. Antimicrobial. 3. Microorganisms. I. Assis, Amanda Stefanie Jabur de. II. Título.

Ficha catalográfica desenvolvida pela Secretaria Geral de Informática (SIn)

DADOS FORNECIDOS PELO AUTOR

Bibliotecário responsável: Maria Aparecida de Lourdes Mariano - CRB/8 6979

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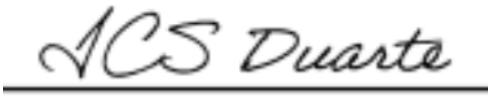
Orientadora



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À memória do meu avô Jorge Jabur, que não pôde presenciar a finalização desta etapa, mas que sempre me motivou a continuar estudando.

AGRADECIMENTOS

Agradeço à Deus por ter me guiado até aqui.

Aos meus pais, Hélio e Selma, por todo o apoio para tornar esse sonho possível.

À minha irmã, Juliana, por me direcionar e me motivar a ser melhor, pessoal e profissionalmente.

Aos meus avôs que me iluminam lá do céu e às minhas avós pelo carinho e suporte.

Ao meu namorado, Guilherme, que me acompanha neste caminho acadêmico desde os tempos de graduação e que têm sido meu porto seguro.

À minha orientadora Iolanda, que me acolheu em seu grupo de pesquisa e que me inspira por ser exemplo de simplicidade, sabedoria e docente inigualável.

Às minhas amigas de profissão, Mariana, Natália, Akemi e a todos do grupo LMA por me auxiliarem no desenvolvimento deste trabalho.

Às minhas amigas, Mariana e Bianca por todo o aprendizado, carinho e momentos inigualáveis que passamos juntas.

À Profª. Drª. Cleoni por abrir seu laboratório, prontamente, para realizar as leituras.

À Heidi por me auxiliar e guiar no decorrer das leituras.

Ao Prof. Dr. Natan por auxiliar no planejamento do experimento e por todas as dúvidas sanadas.

À CAPES pela concessão da bolsa deste mestrado.

ABSTRACT

According to the World Health Organization in 2020, from all hospitalized patients, a share of cases are infections acquired in a hospital environment due to invasive procedures. For decades, *Escherichia coli* and *Staphylococcus aureus* strains have been reported as the main cause of hospital infections. In this way, these microorganisms have developed resistance to various medications over time, reducing the diversity of means to contain their action, highlighting the importance of developing new alternatives with antimicrobial properties. Thus, the use of gallium (Ga^{3+}), which causes harmful effects to bacterial activities due to its characteristics and physicochemical properties similar to those of iron (Fe^{3+}), leads to the possibility to regulate the growth of both planktonic forms and biofilms. Therefore, due to the antimicrobial potential of Ga^{3+} compounds, this work has performed serial microdilution tests in order to identify the minimum inhibitory concentration (MIC) capable of reducing the growth of representative strains of *E. coli* and *S. aureus*. It was possible to verify a reduction in the growth of these microorganisms at 11.25 μM and 1.40 μM of gallium nitrate ($Ga(NO_3)_3$), respectively. Thus, the concentrations obtained in this work were able to reduce 50% of the growth of tested microorganisms. This result showed that minor concentrations of $Ga(NO_3)_3$ were sufficient against nosocomial pathogens cultures when compared to the literature data.

Key-words: Gallium nitrate; antimicrobial; microorganisms; nosocomial infections.

RESUMO

De acordo com a Organização Mundial da Saúde em 2020, de todos os pacientes hospitalizados, uma parte dos casos de infecções adquiridas em ambientes hospitalares ocorre devido a procedimentos invasivos. Por décadas, *Escherichia coli* e *Staphylococcus aureus* tem sido reportados como a causa principal de infecções hospitalares. Desta forma, esses microrganismos desenvolveram resistência a vários medicamentos ao longo do tempo, reduzindo a diversidade de meios para conter sua ação, destacando a importância de desenvolver novas alternativas com efeito antimicrobiano. Assim, Gálio (Ga^{3+}), pode causar efeitos nocivos às atividades bacterianas devido às suas características e propriedades físico-químicas semelhantes às do propriedades do ferro (Fe^{3+}) inibindo o crescimento de formas planctônicas e de biofilmes. Portanto, devido ao potencial antimicrobiano de compostos de Ga^{3+}, este trabalho realizou testes de microdiluição seriada, a fim de identificar a concentração mínima inibitória (MIC) capaz de reduzir o crescimento de *Escherichia coli* e *Staphylococcus aureus*. Redução no crescimento desses microrganismos foi observada nas concentrações de 11,25 μM e 1,40 μM de Nitrato de Gálio ($Ga(NO_3)_3$), para *E.coli* e *S. aureus*, respectivamente. Assim, as concentrações obtidas nesse trabalho foram capazes de reduzir 50% do crescimento dos microrganismos testados. Além disso, esses resultados mostraram que foram necessárias concentrações menores de $Ga(NO_3)_3$ contra culturas de patógenos nosocomiais quando comparados aos dados da literatura.

Palavras-chave: Nitrato de Gálio; antimicrobiano; microrganismos; infecções nosocomiais.

CONTENTS

DISSERTATION OUTLINE

This work is composed by 4 chapters. The content in each of them is mentioned as follows:

- **Chapter 1:** Gallium: a decisive “Trojan Horse” against microorganisms
- **Chapter 2:** Evolution of gallium research and applications in microbiology: a timeline
- **Chapter 3:** Determination of the Minimum Inhibitory concentration (MIC) of Gallium Nitrate against bacteria encountered in nosocomial infections.
- **Chapter 4:** Conclusion

Introduction

Context and Motivation

Characterized as diseases acquired under clinical care in a hospital setting, "nosocomial" or "medical care-associated" infections have increased in recent decades. According to the World Health Organization (2020), about 23.6% of all hospitalized patients are affected by at least one type of infection. The most frequent infections are those caused by gram-negative (34 to 64%) and gram-positive (16 to 40%) bacteria, whose most common representatives found in hospitals are *Escherichia coli* and *Staphylococcus aureus*, respectively (WHO, 2020; Markwart *et al*., 2020).

Led by Alexander Fleming, in 1928, the accidental discovery of penicillin opened the way for new research involving the control of pathogenic microorganisms (Fleming, 1941). After its advent, several antimicrobials were developed and nowadays are extensively used to cure diseases. In the United States (US), according to data from the Center for Disease Control and Prevention (CDC), in recent years, antibiotics were erroneously prescribed in 28% of unnecessary cases resulting in patient tolerance development to certain microorganisms, in other words, they developed resistance to the effects of drugs, invalidating its administration (CDC, 2020). Thus, the arrival of new instruments capable of effectively inhibiting bacterial infections has become an imminent obstacle.

New media with antimicrobial effects, such as the application of non-essential metals, has developed the rich history of using this alternative resource in medicine. There are several chemical elements with antimicrobial potential: whether for your biochemical intervention or through molecular mimicry (Lemire *et al*., 2013). A semi-metal that has been highlighted in the literature is the Gallium (Ga^{3+}): a chemical element with various favorable physicochemical characteristics that make it a microbial growth inhibitory agent for most cells (Kaneko *et al*., 2007). This is due to its similarity with Fe^{3+}, which is widely required for the activity of various cellular activities. Among them are the atomic, ionic and covalent radius, crystalline structure, ionization potential, electronegativity, electronic affinity and tendency to ionic bonding (Chitambar, 2017; Hegge *et al*., 1977). These characteristics facilitate their entry into the intracellular environment through molecular mimicry or "Trojan Horse" due to the low rate of cell recognition of Fe^{3+} conveyors, resulting in incorporation of Ga^{3+} and alteration in

metabolism, cellular respiration and synthesis of DNA in the target cell (Rzhepishevska *et al*., 2011).

Our motivation was to contribute to the studies that have been conducted around the world regarding the obstacles imposed by the development of drug multiresistant bacteria. In this way, by virtue of the positive expression of data in the literature about the antimicrobial potential of Ga^{3+} and the need for new alternatives to solve the issue of microbial resistance, this work has promoted investigations in order to assist in the development of new generations of antimicrobials by trials of minimum inhibitory concentrations (MIC) of $Ga(NO_3)_3$ against *E. coli* and *S. aureus* pathogens.

Objectives and hypothesis

The main objective of this work was to determine the Minimal Inhibition Concentration (MIC) by microdilution tests and verify if $Ga(NO_3)$ is efficient against planktonic strains.

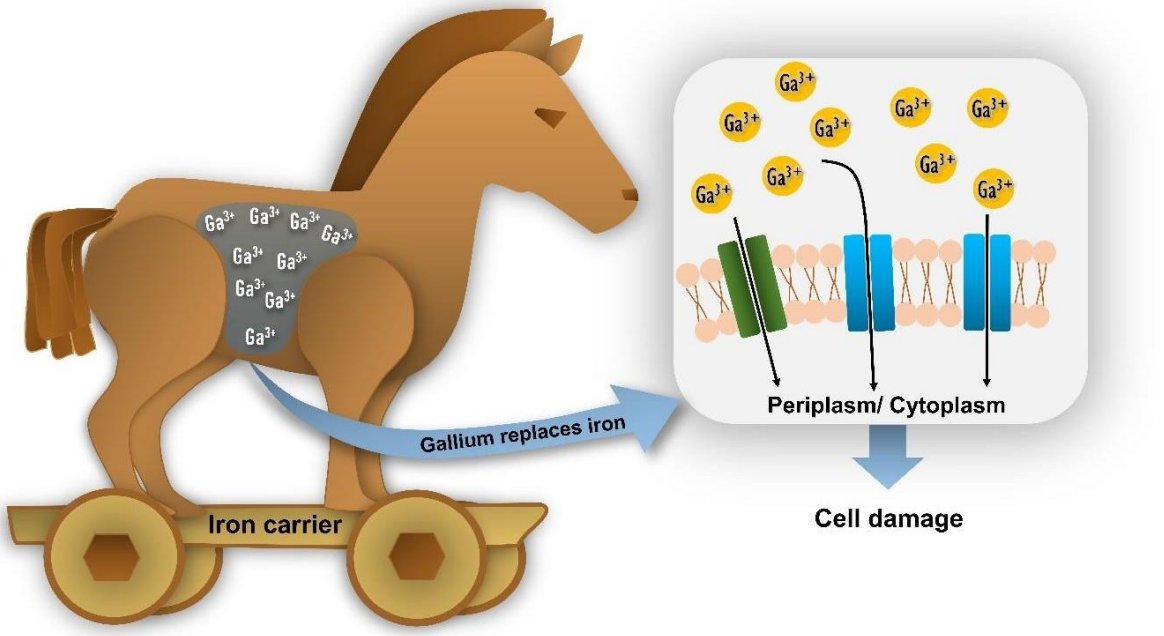
For this, specific tasks were assigned as secondary objectives:

- Investigate, through literature, the MIC required for several species of microorganisms, especially for the strains of *E. coli* and *S. aureus*;
- Perform microdilution and define the MIC for the strains tested;

CHAPTER 1

Gallium: a decisive "Trojan Horse" against microorganisms

Graphical Abstract



Abstract

Controlling multidrug-resistant microorganisms (MRM) has a long history with the extensive and inappropriate use of antibiotics. At the cost of these drugs being scarce, new possibilities have to be explored to inhibit the growth of microorganisms. Thus, metallic compounds have shown to be promising as a viable alternative to contain pathogens resistant to conventional antimicrobials. Ga^{3+} can be highlighted, which is an antibacterial agent capable of disrupting the essential activities of microorganisms, such as metabolism, cellular respiration and DNA synthesis. It was observed that this occurs due to the similar properties between Ga^{3+} and iron (Fe^{3+}), which is a fundamental ion for the correct functioning of bacterial activities. The mimetic effect performed by Ga^{3+} prevents iron transporters from distinguishing both ions and

results in the substitution of Fe^{3+} for Ga^{3+} and in adverse metabolic disturbances in rapidly growing cells. This review focuses on analyzing the development of research involving Ga^{3+}, elucidating the intracellular incorporation of the "Trojan Horse", summarizing the mechanism of interaction between gallium and iron and comparing the most recent and broad-spectrum studies using gallium-based compounds with antimicrobial scope.

Keywords: Gallium, "Trojan Horse", microorganisms.

Introduction

Considered a primary issue for developing alternatives in the pharmacological scope, bacterial resistance has become an ongoing problem that hinders conventional treatments of infections caused by microorganisms (Chitambar, 2016). The importance of using alternative means with antimicrobial effects is emphasized, as is the case of metals and metallic compounds, that have been used due to their ability to reach different groups of molecules, unlike antibiotics, which target specific locations. Therefore, metals and metallic compounds would have less tendency to generate resistance from bacteria (Rzhepishevska *et al*., 2011).

Identified in 1871 by Dmitri Mendeleev, gallium is a semi-metal and has to states of oxidations, +1 or +3; the latter is considered a relevant property in relation to its involvement with antibacterial and antitumor effects (Mendeleev, 1871; Chitambar, 2010; Bonchi *et al*., 2014). This therapeutic effect is justified considering its ability to intercept the pathogenic metabolic processes dependent on Fe^{3+}, resulting in damage to the cellular metabolism (Kaneko *et al*., 2007; Mjos *et al*., 2016).

For decades, Ga^{3+} has been identified as a promising antimicrobial agent due to its characteristics similar to those of Fe^{3+} (Dudley *et al*., 1950; Rasey *et al*., 1982). Along these lines, gallium is known for its strategy similar to the "Trojan Horse", as it is able to replace Fe^{3+}

in intracellular transport (Mjos *et al*., 2016). This happens as a result of the main iron transporters (transferrin, heme and specific receptors), which do not differentiate both ions. Consequently, the incorporation of Ga^{3+} occurs into the cytoplasm, interrupting Fe^{3+} dependent biological functions (Bonifacio *et al*., 2017; Dong *et al*., 2019; Graves *et al*., 2019).

Many of the critical bacteriological processes are linked to Fe^{3+} such as metabolism, cellular respiration, DNA synthesis (Kaneko *et al*., 2007). In order to perform such operations, a change from oxidized iron (Fe^{3+}) to reduced iron (Fe^{2+}) must occur. However, as there is an intervention of Ga^{3+}, which cannot be reduced under physiological conditions, bacterial cells suffer from the deleterious effects due to the interruption of cellular functioning (Valappil *et al*., 2008; Antunes *et al*., 2012; Valappil and Higham, 2014).

Therefore, this review aims to highlight the most recent and relevant publications on the antimicrobial activity of gallium to promote new insights for studies involving the fight against infections caused by multidrug-resistant bacteria and to explore new ways of using gallium compounds for therapeutic purposes.

Gallium: interactions and chemical properties

Gallium is a metallic by-product from the extraction of aluminum and zinc ores, and it is a chemical element belonging to group IIIA of the periodic table (Chitambar, 2017). In addition, several chemical properties of gallium make it similar to iron. Among them are the atomic, ionic and covalent radius, crystalline structure (Fig. 1), ionization potential, electronegativity, electronic affinity and tendency to ionic bonding (Table 1) (Hegge *et al*., 1977).

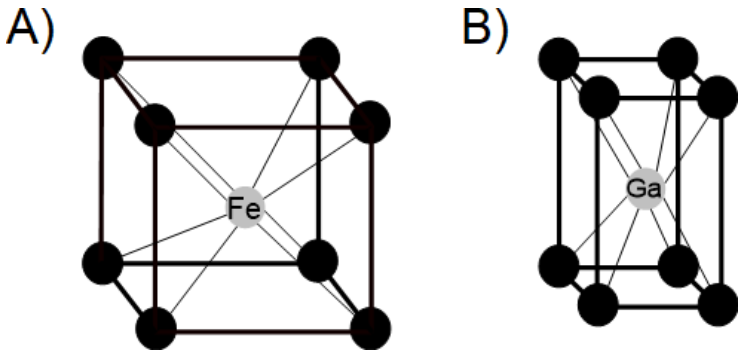


Figure 1 Structural similarity between Fe^{3+} and Ga^{3+}. A: Body-centered Cubic Crystal Structure. B: Body-centered orthorhombic crystal structure. Drawn with ChemSketch program (ACD/ChemSketch).

Identified as the main iron-transporting proteins, transferrin, lactoferrin and ferritin act in the formation of complexes with Ga^{3+}, which are as stable as the complexes with Fe^{3+}. The first study that considered the interaction between gallium and iron transporters was carried out by Hartman and Hayes (1969), in which demonstrated gallium binding to blood plasma proteins. They also assumed that these proteins were alpha globulins and beta and, transferrin as the dynamic component of the system..

In this context, Gunasekera *et al.* (1972) affirmed that about 70% of the associations of gallium in blood were formed with serum proteins, such as albumin, transferrin and haptoglobin, responsible for maintaining and regulating human metabolism. Other studies have also illustrated the transport of gallium compounds in blood, such as Clausen *et al.* (1974), who stated that Gallium Citrate (^{67}Ga) performs a solid interaction with transferrin. This result is corroborated by Sephton and Harris (1975) study in which the responses suggested only the action of transferrin in the absorption process of ^{67}Ga.

Table 1 Chemical properties compared between Fe^{3+} and Ga^{3+}

Chemical Properties	Iron	Gallium
Atomic number	26	31
Atomic mass	55.84	69.72
Atomic radius	2.04	1.87
Covalent Radius	1.24	1.23
Ionic Radius (+3) Å	0.55	0.62
3rd Ionization Potential (eV)	30.65	30.72
Reduction potential	+0.77	-0.68
Electronegativity	1.83	1.81
Electronic affinity (eV)	0.15	0.43
Crystalline structure	Body-centered cubic	Body-centered orthorhombic

In 1977, Hegge *et al.* showed that gallium behaves similarly to iron in relation to ferritin, transferrin or hemoglobin. Hoffer *et al*. (1977) demonstrated a strong link between ^{67}Ga and lactoferrin in research involving the intravenous injection of ^{67}Ga in lactating women and also less affinity with transferrin, which transported it to the mother's breast. However, only in 1982, in studies on the cytotoxicity of gallium nitrate, Rasey *et al.* recognized the increase in the inhibitory effects of $Ga(NO_3)_3$ by performing the intracellular transport of Ga^{3+} due to its ability to imitate Fe^{3+} in cellular metabolism using transferrin supplementation.

In contrast, iron can exist in a divalent (Fe^{2+}) or trivalent (Fe^{3+}) state, unlike gallium, which is found only as Ga^{3+} in bacterial systems (Bernstein, 1998; Chitambar, 2017). Under physiological conditions, Ga^{3+} intercepts Fe^{3+} dependent bacterial metabolic processes, enters the cell and causes disorders in eukaryotic and prokaryotic organisms. This occurs due to the dissimilarity

of the reduction potential of the compared ions, since gallium has +3 oxidation state and cannot be reduced under such biological conditions (Table 1). Therefore, as a potential antimicrobial agent, Ga^{3+} replaces Fe^{3+}, not only interrupting the reduction process, but also changing the protein's conformation (Logan *et al*., 1981; Harris and Pecoraro 1983).

Effects of iron on bacterial metabolism under the biochemical context

Iron is a key element for several metabolic pathways of most organisms, such as signaling functions and cellular information (Ramos 2004; Wandersman and Delepelaire, 2004; Miethke and Marahiel, 2007). However, iron is a component that is not freely bioavailable under aerobic conditions due to its insolubility in aqueous media (Braun and Killmann, 1999).

Under aerobic conditions and at neutral pH, iron is essentially in the oxidized state, insoluble form of Fe^{3+}, which is added to form an oxy-hydroxide polymer at a solubility concentration of $1.4x10^{-9}$ M. In contrast, in the absence of oxygen and pH 7, free iron in solution is reduced to Fe^{2+} and its concentration under these conditions is given as 10^{-18} M, which is unsatisfactory for the requirement of the bacteria (Ratledge and Dover, 2000).

Another adversity for bacteria is the harmful effect caused by the excess of iron to these organisms. When the intracellular Fe^{2+} activates the Fenton reaction (1), under the presence of hydrogen peroxide (H_2O_2), it promotes the formation of reactive forms of oxygen (OH^-), which are harmful to various macromolecules, such as proteins, lipids and nucleic acids (Ramos 2004; Wandersman and Delepelaire, 2004).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+}OH \cdot + OH^- \quad (1)$$

However, such insufficient wealth added to the complexity of obtaining iron from hosts results in the development of refined strategies for the acquisition of iron by bacteria (Ramos, 2004; Weinberg, 2009). During evolution, some species of pathogens solved this nutritional

limitation by direct and indirect methods: the first involves the iron gain by various sources through cognate receptors, located on the cell surface, through the influence of siderophores; the second is related to iron from host protein receptors, such as transferrin, lactoferrin before being carried into the cell (Wandersman and Delepelaire, 2004; Miethke and Marahiel, 2007; Krewulak and Vogel, 2008; Weinberg, 2009).

In this scenario, both gram-negative and gram-positive bacteria perform iron assimilation. Gram-negative bacteria have an outer membrane or lipopolysaccharide layer with a protective function against bacteriophages and hydrophobic materials (Cescau *et al*., 2007; Krewulak and Vogel, 2008). Moreover, porins are found on the inner membrane surface - quarterly β-barrel transmembrane proteins - which allow passive diffusion of lower molecular weight solutes (<600 Da) (Nikaido, 2003).

Iron transport begins with the Fe^{3+} uptake from the extracellular medium to the periplasmic space, and then the main complexes of Fe-siderophore ions (transferrin, lactoferrin, hemoglobin) are conducted from the periplasmic medium to the cytoplasm. However, as these units exceed the molecular weight limit, there is a need for the participation of specific receptors of the extracellular membrane in order to be displaced to the intracellular media (Touati, 2000; Krewulak and Vogel, 2008).

In gram-negative bacteria, there are several iron absorption pathways, which are operated through binding proteins (siderophores, transferrin, lactoferrin, heme or heme associated with host hemoproteins) (Fig. 2), that require specific receptors for the outer membrane (Killmann *et al*., 1993; Ratledge and Dover, 2000). Afterwards, the outer membrane receptors transport Fe^{3+} or Fe^{2+} to the periplasmic medium by interacting this cluster with the Ton complex (TonB, ExB or ExbD). Once in the periplasmic space, the siderophores that carry Fe^{3+} bind with unique periplasmic proteins and deliver them to the inner membrane ATP-

binding cassette (ABC) transporter at cost of ATP (Andrews *et al*., 2003; Schalk and Guillon, 2013).

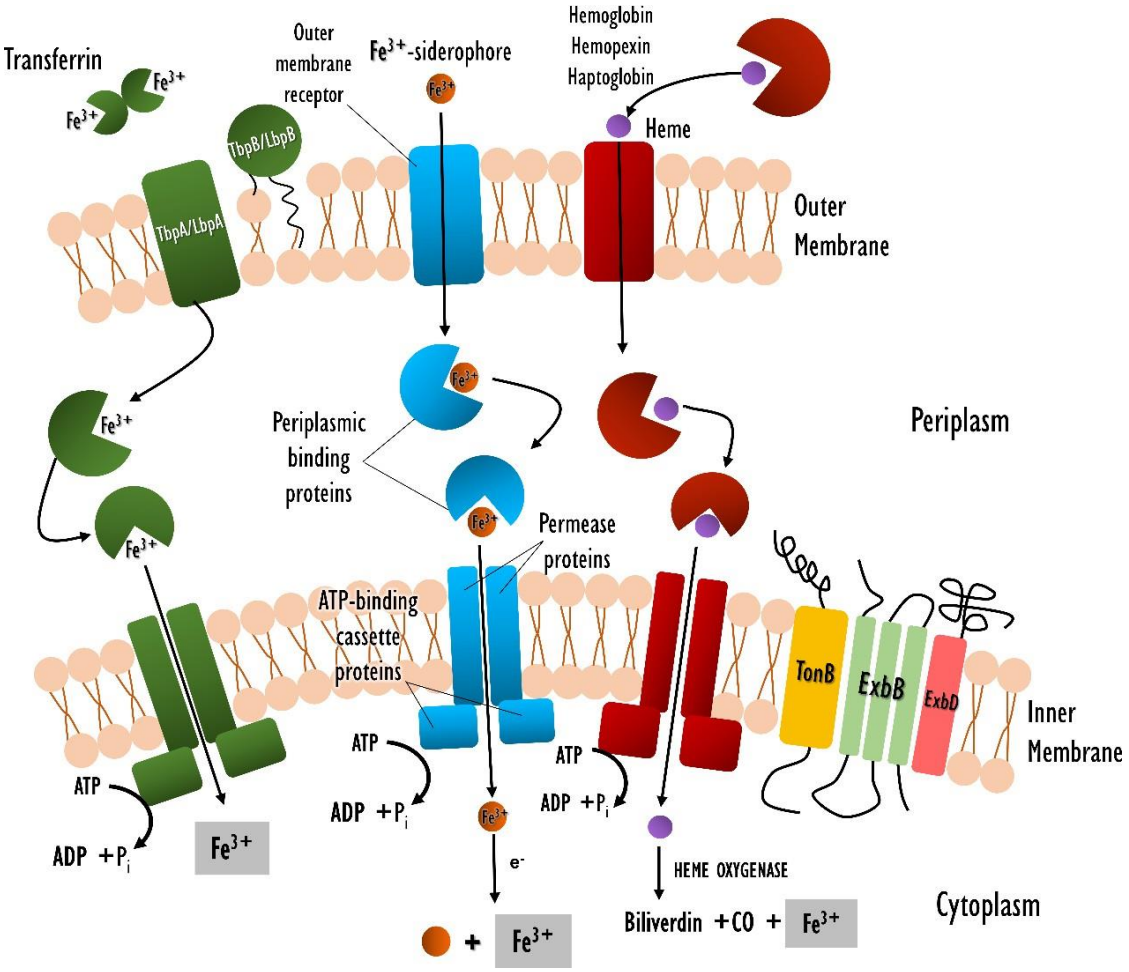


Figure 2 Representation of Fe^{3+} metabolism in gram-negative bacteria.

In addition, it should be noted that due to the admitted atomic weight of the Fe^{2+} ions, they are moved freely to the intracellular medium through the porins, and then moved by transport systems (Feo System - main in pathogenic and non-pathogenic microorganisms) to the cytoplasm (Kammler *et al*., 1993; Große *et al*., 2006; Cao *et al*., 2007; Lau *et al*., 2015). Depending on the amount of iron available, Fe^{2+} binds to the FUR (Ferric uptake regulator) by suppressing or stimulating the expression of transcription genes, which regulate iron uptake (Andrews *et al*., 2003).

In the absence of an outer membrane, gram-positive bacteria have only one barrier that connects with the cytoplasm (Fig. 3) (Lau *et al*., 2015). Thus, in these organisms, there is a presence of siderophore-binding proteins that are connected to the cell membrane through bonds with the groups of fatty acids. These proteins bound to Fe^{3+} ions forming a complex, which is directed to the permease proteins and delivered to the ABC carrier protein. Immediately, the ATP molecule loses a phosphorus ion and releases Fe^{3+} to Fe^{2+} through reductases (Krewulak and Vogel, 2008).

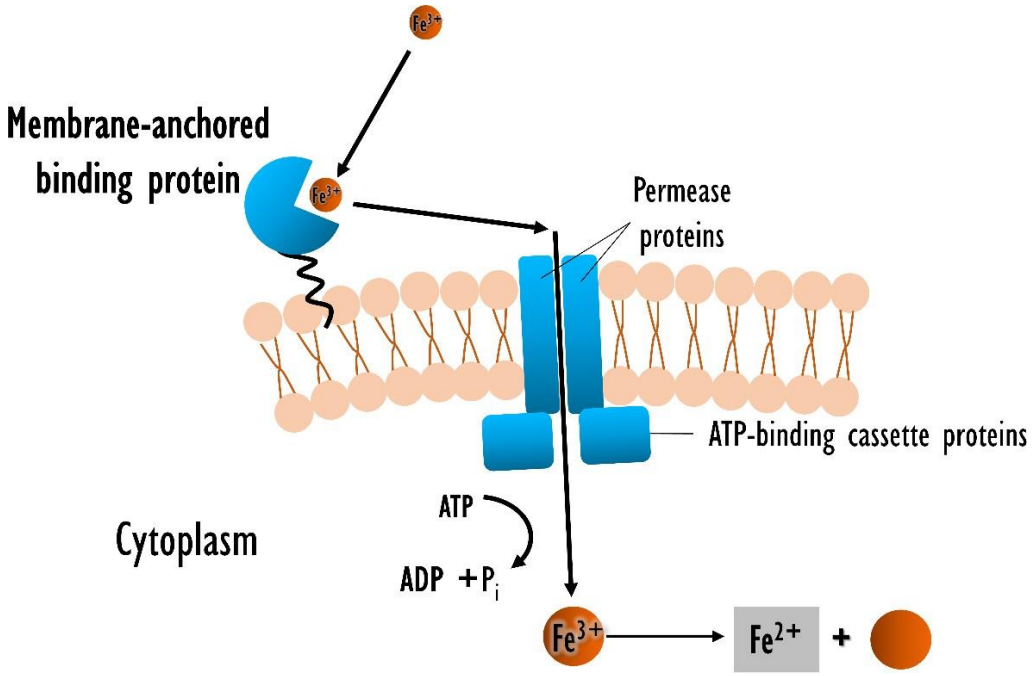


Figure 3 Representation of Fe^{3+} metabolism in gram-positive bacteria.

Finally, the iron inserted in the cytoplasmic space can be incorporated by several metalloproteins in order to carry out metabolic processes of pathogenic organisms, or it can be stored in bacterioferritin or in DNA-binding proteins from starved cells that are similar to ferritin (Miethke and Marahiel, 2007; Krewulak and Vogel, 2008; Lau *et al*., 2015).

Antimicrobial action of gallium-based compounds

Investigations on the activity of Ga^{3+} are found, mostly, in planktonic pathogenic bacteria, which microorganisms are dispersed in liquid media and, eventually, can adhere to a surface and form biofilms (Madigan *et al.*, 2016). However, despite the wide variety of microorganisms species and culture conditions tested against Ga^{3+} compounds, the most relevant publications were discussed according to the growth of the pathogen and the place of occurrence of biological processes in table 2 on account of the difficulty of direct comparison between the data.

Table 2 – Comparison of the antibacterial activity of gallium-based compounds

Microrganisms	Ga-based Compound	Medium	Inhibitory concentration	Reference
A. baumannii	$Ga(NO_3)_3$	TSB + TSA	MIC=0.0156 to 0.50 µM	(Choi *et al*., 2019a)
		MH	MIC= 2.00 µM	(Xu *et al*., 2017)
	GaPP	TSB + TSA	MIC= 0.00079 to 0.0126 µM	(Choi *et al*., 2019a)
	GaMP		MIC= 0.00039 to 0.0125 µM	
	GaPP	BM2+CAMH	MIC= 0.0008 µM	(Choi *et al*., 2019b)
	GaN	MS+HS	IC_{90}= 32 µM	(Runci *et al*., 2017)
		MH+DMHB+RPMI-HS	MIC= 2 µM	(Hijazi *et al*., 2018)
	GaM		MIC= 1 µM	
	GaPPIX		MIC= 16 µM	
		LB + MH	MIC= 0.0316 µM	(Arivett *et al*., 2015)
	Ga-MPIX	MH	IC_{90}= 16µM	(Chang *et al*., 2016)
			IC_{90}= 128 µM	
Candida albicans	Ga(dfo)	TSB	IC_{50}= 71 µM	Huayhuaz *et al*., 2017)
E. coli	$Ga(NO_3)_3$	Nutrient broth	MIC= 20 µM	(Zhu *et al*., 2015)
		MH	MIC= 1.00 µM	(Xu *et al*., 2017)
		LB	MIC= 31250 µM	(Gugala *et al*., 2019)
		DMB	MIC= 391.02 µM	(Graves *et al*., 2019)

Table 2 – Comparison of the antibacterial activity of gallium-based compounds

(To be continued)

Microorganisms	Ga-based Compound	Medium	Inhibitory concentration	Reference
E. coli	$Ga(NO_3)_3$	M9	MIC= 312 µM	(Neill *et al*., 2020)
	Ga(dfo)	TSB	IC_{50}= 42 µM	(Huayhuaz *et al*., 2017)
	Ga-D2	MH+MHII	MIC= 0.23 µM	(Pandey *et al.*, 2019)
E. faecalis	$Ga(NO_3)_3$	MH	MIC= 1.00 µM	(Xu *et al*., 2017)
Klebsiella pneumoniae		MH	MIC= 2.00 µM	
		BM2+CAMH	MIC= 0.0007 µM	(Choi *et al*., 2019b)
	GaMP		MIC= 0.0003 µM	
	Ga-D2	MH+MHII	MIC= 12.5 µM	(Pandey *et al.*, 2019)
	GaCi	LB+MH	MIC= 0.00048 µM	(Thompson *et al*., 2015)
Mycobacterium avium	GaM	7H9	IC_{90}= 94 – 132 µM	(Fecteau *et al.*, 2014)
	GaN		IC_{90}= 140 – 190 µM	
	GaTP		MIC= 0.0029 µM	(Choi *et al*., 2018)
M. abscessus			MIC= 0.0058 µM	
	$Ga(NO_3)_3$		MIC= 25 µM	(Abdalla *et al.*, 2015)
	GaPPI		MIC= 0.1 µM	
M. tuberculosis	GaTP		MIC= 0.0058 µM	(Choi *et al*., 2017)
P. aeruginosa	$Ga(NO_3)_3$	DCAA	MIC= 12.5 µM	(Frangipani *et al*., 2014)
			MIC= 8 µM	(Bonchi *et al.*, 2015)
		MH	MIC= 211 µM	(Kurtjak *et al*., 2016)
			MIC= 2.00 µM	(Xu *et al*., 2017)
		LB	IC_{90}= 6.25 µM	(Hijazi *et al*., 2017)
			MIC= 12 and 24 µM	(Zemke *et al*., 2020)
			MIC= 15630 µM	(Gugala *et al.*, 2019)
		TSB	IC_{90}= 4 – 6 µM	(Goss *et al*., 2018)
		TSB + TSA	MIC= 0.0039 – 0.0062 µM;	(Choi *et al*., 2019a)

Table 2 – Comparison of the antibacterial activity of gallium-based compounds

(To be continued)

Microorganisms	Ga-based Compound	Medium	Inhibitory concentration	Reference
P. aeruginosa	$Ga(NO_3)_3$	BM2+CAMH	MIC= 0.0019 to 0.0039 µM;	(Choi *et al*., 2019b)
		MSM	MIC_{50} = 50 and 25 µM	(Tovar-García *et al*., 2019)
		M9	IC_{90}= 7.8 and 15,6 µM	(Guo *et al*., 2019)
	$Ga(NO_3)_3$ + PVD	LB + CAA	MIC > 2 µM	(Ross-Gillespie *et al*., 2014)
	GaPPIX	LB	IC_{90}= 0.38 µM	(Hijazi *et al*., 2017)
	Ga@C-dots		MIC= 4.16 µM	(Kumar *et al*., 2017)
	Ga@HAp	MH	MIC= 0,582 µM	(Kurtjak *et al*., 2016)
	Ga-D2	MH+MHII	MIC= 3.8 µM	(Pandey *et al.*, 2019)
	GaPP	TSB + TSA	MIC= 0.0063 – 0.0506 µM	(Choi *et al*., 2019a)
	GaMP		MIC= 0.0062 to 0.0502 µM	
		BM2+CAMH	MIC= 0.0014	(Choi *et al*., 2019b)
	GaM	Nutrient broth	IC_{50}= 0.1404 µM	(Piatek *et al*., 2020)
	Ga(dfo)	TSB	IC_{50}= 103 µM	(Huayhuaz *et al*., 2017)
Rhodococcus equi	GaM	Minimum medium without iron	MIC= 8 µM	Coleman *et al*., 2016)
S. aureus	$Ga(NO_3)_3$	MH	MIC ≥ 128 µM	(Garcia *et al.*, 2016)
			MIC= 2.00 µM	(Xu *et al*., 2017)
		LB	MIC= 15630 µM	(Gugala *et al.*, 2019)
	GaMP	BM2+CAMH	MIC= 0.0007 µM	(Choi *et al*., 2019b)
			MIC= 0.0002 µM	
	GaPP		MIC= 0.0003 µM	
	Def-GaPP	LB	MIC= 0.0081 µM	(Richter *et al*., 2017)
	GaN	MH+DMHB+RPMI-HS	MIC= 0.06 µM	(Hijazi *et al*., 2018)

Table 2 – Comparison of the antibacterial activity of gallium-based compounds

(To be continued)

Microorganisms	Ga-based Compound	Medium	Inhibitory concentration	Reference
S. aureus	GaM	MH+DMHB+RPMI-HS	MIC= 0.12 µM	(Hijazi *et al*., 2018)
	GaPPIX	TSB	MIC= 0.03 µM	(Morales-de-Echegaray *et al*., 2018)
			MIC= 0.0088 µM	(Morales-de-Echegaray *et al*., 2020)
		Nutrient broth	MIC < 0.00031 µM	(Ooi *et al*., 2018)
	Ga(dfo)	TSB	IC_{50}= 565 µM	(Huayhuaz *et al*., 2017)
	Ga-D2	MH+MHII	MIC= 0.94 µM	(Pandey *et al*., 2019)
V. splendidus	$Ga(NO_3)_3$	Marine agar 2216E	MIC > 160 µM	(Song *et al*., 2019)

TSB: trypticasein soy broth; TSA: tryptone soy broth; Ga(NO3)3: gallium nitrate; MIC: minimum inhibitory concentration; BM2: minimum means; CAMH: Müeller-Hinton (Cation-Adjusted); HS: human serum; DMHB: MH broth deprived of iron; RPMI-HS: tissue culture medium supplemented with 10% complemente free human serum; GaPPIX: gallium complex with protoporphyrin IX; LB: Luria-Bertani; MH: Müeller-Hinton; GaMPIX: gallium-mesoprotoporphyrin; Ga(dfo): gallium-deferiprone; DMB: Davis minimal broth; M9: M9 minimal salts medium; Ga-D2: gallium(III) complex of ciprofloxacin-functionalized desferrichrome; MHII: Müeller-Hinton II; GaMP: gallium mesoporphyrin; GaCi: gallium citrate; GaM: gallium maltolate; GaN: gallium nitride; GaPPI: gallium complex with protoporphyrin; GaTP: gallium meso-tetraphenylporphyrin; 7H9: Middlebrook 7H9 broth; DCAA: medium of deferred casamino amino acids; MSM: minimum succinate medium; PVD: pyoverdine; CAA: medium of casamino acids; Ga@C-dots: gallium doped in carbon (C)-dots; Ga@HAp: gallium and hydroxyapatite nanoparticles; Def-GaPP: deferiprone and gallium-protorporphyrin

Several authors have analyzed the antibacterial effects of $Ga(NO_3)_3$ alone or this compared to the Ga^{3+} complexes on the cultivation of microorganisms. Through table 2, it was possible to verify that, in order to inhibit the growth of *P. aeruginosa* cultures, higher concentrations of $Ga(NO_3)_3$ than Ga^{3+} complexes are needed (Bonchi *et al*. 2014; Ross-Gillespie *et al*. 2014; Goss *et al*. 2018; Guo *et al*. 2019; Tovar-García *et al*. 2019; Piatek *et al*. 2020).

According to this scenario, Frangipani *et al*. (2014) proposed an assay to potentiate the effect of gallium on the culture of *Pseudomonas aeruginosa* through siderophores and synthetic

chelators. It was found that the Ga^{3+} complex with piochelin and siderophores was more efficient than $Ga(NO_3)_3$ alone in the culture inhibition process, requiring 3.13 µM of the solution (Frangipani *et al*. 2014).

When using the gallium compound with hydroxyapatite, Kurtjak *et al.* (2016) were able to inhibit the cultivation more effectively by applying 0.2 µM, in contrast to 211 µM $Ga(NO_3)_3$ (Kurtjak *et al*. 2016). Other research obtained values similar to Ga@HAp after using the combination of gallium-protoporphyrin IX: MIC = 0.38 µM and gallium doped in C-dots, MIC = 0.34 µM for one of the strains of *P. aeruginosa* tested (Hijazi *et al*. 2017; Kumar *et al*. 2017).

In the same context, Choi *et al*. (2019) conducted two studies at different times with distinct pathogens that corroborate to previous data. One of them explored the inhibition capacity of gallium protoporphyrin (GaPP), gallium mesoporphyrin (GaMP) and nanoparticles encapsulating these compounds in *P. aeruginosa* and *Acinetobacter baumannii* cultures. In iron-depleted media, all the strains were affected by GaPP and GaMP when applied concentrations ranging from 0.0003 to 0.0502 µM. In the secondary study, similar data was obtained for the respective strains, however, in intra and extracellular growth conditions, the expressive inhibition was observed in *P. aeruginosa* grown in macrophages, which needed 5 µM of Ga^{3+} complexes and nanoparticles. In addition, minor concentrations of Ga^{3+} compounds were required to methicillin-resistant *S. aureus*, according to table 2 (Choi *et al*. 2019b).

When analyzing the minimum inhibitory concentrations for *S. aureus* cultures, Garcia *et al*. (2016) found evidence of a minimum of 128 µM of $Ga(NO_3)_3$ needed to inhibit the growth of the strain. In contrast, Richter *et al* (2017) used the deferiprone and protoporphyrin-Ga^{3+} complex, which was more effective to inhibit the strain cultures than the MICs for individual compounds, 0.0081 µM, 0.0162 µM and above 0.0648 µM, respectively. Among other publications that apply protoporphyrin IX combined to Ga^{3+}, the authors also recognized the

requirement of lower concentrations for Ga^{3+} complexes, ranging from 0.02–0.06 µM, compared to the amounts of $Ga(NO_3)_3$ (Hijazi *et al*. 2018; Morales-de-Echegaray *et al*. 2020).

Most of the assays analyzed involving strains of *Escherichia coli* verify the minimum concentration of $Ga(NO_3)_3$ required to inhibit the growth of the microorganism (Zhu *et al*. 2015; Xu *et al.* 2017; Gugala *et al*. 2019; Graves *et al*. 2019). Zhu *et al.* (2015) cultivated strains of *E. coli* in nutrient broth supplemented with $Ga(NO_3)_3$, of which 20 µM of the compound was needed to reduce bacterial growth. Strains cultivated in different culture media showed differences in the MIC values, 1.00 µM and 391.02 µM respectively (Xu *et al*. 2017; Graves *et al*. 2019). Gugala *et al.* (2019) also used $Ga(NO_3)_3$, but added it to Luria-Bertani broth and obtained a much higher MIC, 31250 µM. Notwithstanding this data, Pandey *et al.* (2019) introduced a study in *E. coli* strains regarding the antimicrobial potential of the Ga^{3+} complex of ciprofloxacin-functionalized desferrichrome (D2), which exhibits much lower MIC, 0.23 µM.

It was observed in cultures of *Mycobacterium abscessus* in Middlebrook 7H9 broth that the minimum inhibitory concentration was lower when supplemented with gallium tetrafenylporphyrin (GaTP) than $Ga(NO_3)_3$ concentrations, 0.005 – 0.5 µM , 25 – 50 µM respectively (Abdalla *et al.* 2015; Choi *et al.* 2018). However, Abdalla *et al.* (2015) also tested the Ga^{3+} combined to protoporphyrin and the result corroborates to other assays who obtained lower values when using gallium complexes (MIC= 0.1, 0.5 µM).

For *Acinetobacter baumannii* cultures, the researchers carried out supplements with a complex based on gallium, such as implementing gallium and protoporphyrin IX and gallium nitride (GaN). It was observed that even adding different gallium compounds, like GaN, they are able to inhibit *A. baumannii* culture by applying small amounts of Ga^{3+} (Table 2) (Chang *et al.* 2016; Runci *et al*. 2017). In contrast, other strains, such as *Corynebacterium*

pseudotuberculosis and *Rhodococcus equi* were grown with supplies of gallium maltolate (GaM). In the first case, concentrations of GaM were not sufficient to inhibit the growth of the strains. However, for *R. equi* only 8 µM was needed to exhibit evidence of proliferative reduction (Norman *et al*. 2014; Coleman *et al*. 2016).

Moreover, Lindgren *et al.* 2016 proposed a hypothesis that gentamicin, combined with gallium, would potentiate the inhibitory effect on strains of *Francisella tularensis*. As a result, the addition of this complex to infected bone marrow macrophages showed that 100 µM of gallium alone and 0.0194 µM of gallium combined to gentamicin inhibit the intracellular growth of the strain, also in responding positively to the treatment derived from this complex.

Thompson *et al.* (2015) studied the topical formulation of gallium citrate (GaCi) during the treatment of wounds infected with *Klebsilla pneumoniae* strains. The authors concluded that the minimum inhibitory concentration varies between 0.00048 – 0.0077 µM of GaCi, which represent concentrations that corroborate with the other authors' assays, who used Ga^{3+} combined with other elements or in the form of complexes. Finally, other pathogens are being tested for growth reduction through the addition of $Ga(NO_3)_3$, such as *Vibrio splendidus*, and it was observed that the growth of the culture is completely inhibited through the use of MIC> 160 µM (Song *et al*. 2019).

In summary, the results presented in Table 2 show the antimicrobial potential of Ga^{3+} ions by affecting the growth of most relevant bacterial pathogens in recent years, either by its use as $Ga(NO_3)_3$ or in Ga^{3+} complexes. In the meanwhile, the latter have been highlighted as an inhibitory compound more efficient than $Ga(NO_3)_3$. For this, more extensive and comparative studies are needed to validate this information.

Conclusion

Considering the need for iron ions (Fe^{3+} e Fe^{2+}) for processing critical activities of bacterial pathogens and the demand for efficient incorporation systems of these ions into the intracellular medium, they become attractive factors for implementing and studying new antimicrobial alternatives. Thus, due to the complexity of developing these agents with applicable drugs, the most used shortcuts to reaching the bacterial cytoplasm is the mimicry of Ga^{3+} of the Fe^{3+} ion.

It is known that the Ga^{3+} and Fe^{3+} ions have similar properties, such as nuclear radius, coordination chemistry, ionization potential, electronegativity, among others, which result in the indiscrimination of receptors located on the cell membrane. Consequently, Ga^{3+} is offered an opportunity to be incorporated into the intracellular medium and promote harmful effects to the system due to its inability to change to reduced ion under physiological conditions.

Research involving Ga^{3+} is still worthy of proper consideration, specially when implementing gallium-based compounds as antimicrobial agents. New complexes, that require smaller concentrations than $Ga(NO_3)_3$ to reduce microbial growth, should be taken into account, either by the form of incorporation into human systems, whether by subcutaneous application or oral ingestion, their individual use or associated with other components, in the same way as their development as a therapeutic component, mainly against multi-resistant bacteria. However, considering the progress from its discovery to the present day, components from Ga^{3+} are considered promising in the generation of antimicrobials to come.

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CHAPTER 2

Evolution of gallium applications in medicine and microbiology: a timeline

Abstract

Characterized as a semi-metal, gallium is a chemical element not found freely in the environment, but extracted as a by-product from other minerals. Despite of this, there are several gallium compounds with various applications, such as in the production of semiconductors, light emitting diodes (LEDs); commercially as a potential cost reducer; pharmacology as cancer-related hypercalcemia, non-Hodgkin' lymphoma, breast and bladder cancer mainly and antimicrobial treatments. The latter will be emphasized in this work due to the contemporary emergence of the development of compounds with antimicrobial potential as a result of the spread of multidrug-resistant bacteria. So, this article discusses the main works, from the discovery of gallium to those that culminated in the current research in microbiology of the last two decades. The antimicrobial activity of gallium can be confirmed through the experimental data and be a promising mean to other investigations, especially due to its iron mimicry ability and the capacity to disrupt microorganisms' metabolism.

Key-words: gallium; microbiology; medicine

Introduction

Gallium is a chemical element with atomic number 31, belonging to period 4 and group 13 of the periodic table, which is also called IIIA, being often characterized as a semi-metal. It has a silver color both when solid and liquid. Its melting temperature is 29.7646 °C, which means it is possible to change its physical state from solid and liquid with certain ease at temperatures near room temperature. However, its boiling temperature is much higher, reaching 2229°C (Elvers, 2011; Haynes *et al*., 2017). Gallium has no natural occurrence in its isolated form as pure metal. Therefore, the semi-metal and its compounds are usually obtained as by-products of the extraction of minerals with major composition of other chemical elements. This occurs with bauxite, which is an aluminum raw material. It can present small concentrations of

Ga, as well as in sphalerite, a zinc ore, or even in the extraction of coal. Since gallium has these characteristics for its obtainment, literature points out that economic and geopolitical factors can influence gallium's availability to the market (Frenzel *et al*., 2016).

Its most commonly found isotopes in nature are ^{69}Ga and ^{71}Ga, with respective abundance of 60.108% and 39.892%, respectively. More than twenty other gallium isotopes are known, although they are radioactive, such as ^{67}Ga, which has a half-life of 3261 days and tends to undergo radioactive decay by electron capture (EC), forming ^{67}Zn. Another example is ^{72}Ga, with a half-life of 14.10 hours and that undergoes β^- decay, producing ^{72}Ge (Haynes *et al*., 2017).

Gallium's chemical reactivity majorly involves the formation of trivalent ions (Ga^{3+}) in compounds such as gallium arsenide (GaAs), gallium nitrate ($Ga(NO_3)_3$), gallium nitride (GaN) or gallium(III) oxide (Ga_2O_3), which also have several widespread applications in current days. It's worth mentioning that there are existing compounds that contain gallium with other oxidation numbers, such as gallium sulfide (GaS) or gallium telluride (GaTe) (Elvers, 2011; Shenoy *et al*., 2016).

Of the various existing applications for gallium compounds, the production of semiconductors has great relevance and importance. Although electronics has been developed primarily around the silicon element (Si), compounds such as GaAs can be applied for this purpose based on their wide forbidden band energy gap semiconductor characteristic. GaN also demonstrates the capacity to be used as semiconductor, but its commercial viability has not yet reached desired levels, and its cost-effectiveness is constantly compared to silicon or silicon carbide (SiC) (Baliga, 2013). Gallium nitride has also been applied in the production of light emitting diodes (LEDs), since it is found among materials capable of emitting light with different wavelengths in the visible light range, as well as in laser diodes (LDs). More recent studies demonstrated that GaN's optical properties have potential for commercialization, reaching an even broader range of wavelengths and potential reduction in production costs (DenBaars *et al*., 2013).

In the medical context, gallium was studied primarily in hypercalcemia: a condition found in people with cancer, especially those affected by metastatic stage breast cancer (Warrell Jr. *et al*., 1986). The reason for the intensification of calcium bone resorption is still controversial, but tumor associated hypercalcemia is related to increasing the dissolution of hydroxyapatite crystal with the release of excessive amounts of calcium in circulation

(Bockman, 1980). Another drugs have been reported in the literature as bone resorption inhibitors, such as biophosphonates, salmon calcitonin, calcimimetic drugs, antibodies as prednisone, IV fluids and diuretics, as well as new alternative media, such as gallium nitrate, which promotes inhibition of bone resorption in addition to increasing calcium concentration in bones (Mundy *et al* 1983; Warrell Jr. *et al* 1984; Bockman *et al* 1985). In consequence other antineoplastic activities in clinical tests involve gallium nitrate and patients with non-Hodgkin's lymphoma and bladder cancer (Straus 2003, Chitambar, 2017). One of the mechanisms includes the disruption of iron homeostasis, which is vital for tumor cell development. This occurs due to the binding of gallium with transferrin, and its incorporation into the intracellular medium, resulting in inhibition of ribonucleotide reductase: an enzyme that contains iron and is related to deoxyribonucleotide synthesis (Chitambar *et al.,* 1983, Seligman *et al.,* 1993).

In its most common cationic form, Ga^{3+}, some physicochemical properties similar to other metallic ions can be noted, such as Al^{3+} or Fe^{3+}, which allow their applications in microbiology as an antibacterial agent. Among these physicochemical characteristics, the ionic charge of +3 and the ionic radius can be highlighted. For the case of ions with coordination number equal to 6, Ga^{3+} has an ionic radius of 0.62 Å, while Al^{3+} has 0.56 Å and Fe^{3+} has 0.55 Å, which are relatively similar values (Haynes *et al.,* 2017; Kaneko *et al.,* 2007). This similarity allows $Ga^{3+}$to be applied in interrupting or inhibiting certain microbial activities, such as in *Pseudomonas aeruginosa*, replacing the Fe^{3+} transported by other molecules (Lemire *et al*., 2013). In such manner, these microorganisms are not able to reduce gallium as they reduce iron in ordinary metabolism, which causes complications in some biological processes in which Fe acts as a cofactor, such as the enzymatic activity of ribonucleotide reductase or the electron transport chain. The observation of this phenomenon boosted research of gallium compounds in microbiology, especially due to the fact that it has already been approved by the Food and Drug Administration (FDA) for treatment of hypercalcemia of malignancy, pushing the semi-metal further as a potential candidate as an antibacterial agent that could respond to the demand for novel bactericidal drugs (Kaneko *et al*., 2007).

Although briefly reporting on the various gallium and gallium compounds' applications, the present work aims to highlight the historical development of the research involving gallium in oncology, pharmacology and other fields, culminating in its recent antimicrobial applications in microbiology. Our timeline begins with its discovery and isolation, discusses the different relevant applications for its diffusion in distinct areas of research, points out the main studies

that have converged for its application in microbiology and performs a brief review of the main findings involving gallium in microbiology in the last two decades.

The discovery of gallium and its convergence to microbiology

Predictions of the existence of elements not yet discovered were elaborated by Dmitri Mendeleev, in 1871 (Mendeleev, 1871). Among the four presupposed elements, *eka-aluminium* could be found, which proved to be a good predictor of the properties of the gallium chemical element. However, only in 1875 was P.E.F. Lecoq of Boisbaudran able to extract a few milligrams of gallium chloride from ore samples, which were submitted to spectroscopic analysis and a new spectrum was found, belonging to a new element: gallium, whose name comes from Latin term, *Gallia*, referring to the Gallic region of southern France (de Boisbaudran, 1875).

In the mid-1940s, the first studies were carried out involving the experimental administration of gallium and radio-gallium (^{72}Ga) in animals, given its physiological properties favorable to common and neoplastic bone metabolism. Thus, after chemical and image analyzes, it was verified that gallium is able to enter bones and accumulate itself in regions with great osteogenic activity (Dudley and Maddox, 1949). In the 1950s, an intense deposition of ^{72}Ga was verified in areas with osteogenic activity and in bone tumors, but only a subtle increase in its concentration in soft tissue tumors (Dudley *et al*., 1950). These works were accompanied by other tests to determine the role of gallium citrate (^{67}Ga) in different routes, subcutaneous or intravenous, as to determine the ideal dose and the time of decay of gallium in bones after its administration. Thus, it was noted that the routes of administration were not significant. However, the concentration of ^{67}Ga in bones was proportional to the administered dose (Dudley and Marrer, 1952).

In 1968, the studies were directed to ^{67}Ga as a scanning agent capable of detecting malignant nodes in the neck region and the premise that the location of the isotope in the tumor area could be related to the particularities of gallium and its binding to proteins (Edwards and Hayes, 1969). In a report developed by Winchell *et al*., (1970), the location of gallium was tested in malignant tumors through a technique similar to that used by Edwards and Hayes (1969): intravenous infusion of radioactive gallium with no carrier in the form of ^{67}Ga (Winchell *et al*., 1970). In this case, it was possible to visualize the site of the isotope in different areas, such as the liver, spleen and skeleton (Lavender *et al*., 1971).

Other surveys support the hypothesis that high gallium concentrations in bloodstream, under low administered quantities, may be related to the binding of gallium to serum proteins. Through studies with gel filtration, it was possible to verify that such components were indeed proteins and that gallium deposition in bones was related to the exceeded binding capacity to serum (Hartman and Hayes, 1969). Among the analyses of the 1970s, the tests of Edwards and Hayes (1970) in non-bone tumors of rats and mice can be highlighted, which showed that gallium concentrates mostly in viable cells rather than in necrotic tumor cells (Edwards and Hayes, 1970; Hayes *et al*., 1970). In addition, at that time, the first studies on the interaction of Ga^{3+} and proteins were also performed.

In such manner, the work of Woodworth *et al*. (1970) acted as a precursor of research involving the interaction of conalbumin, a glycoprotein of the egg albumen, with Ga^{3+}, Fe^{3+} and siderophilin or transferrin with Fe^{3+}, through proton magnetic resonance imaging. These assays showed that, by virtue of the enlargement or changes in phenolate resonance, Fe^{3+} showed a decrease in signal intensity in the aromatic region while Ga^{3+}, in bonding with the protein, intensified the ionization of the tyrosyl residues (Woodworth *et al*., 1970). Lavender *et al*. (1971) indicated larger concentrations of gallium in malignant neoplasms and metastases, but also amounts of Ga^{3+} in inflammatory lesions, which may be associated with the absorption of proteins, such as fibrinogen and globulins present in injuries (Lavender *et al*., 1971). In this period, Hart and Adamson (1971) tested the toxicity and antitumor activity of salts from the IIIA group. As a result, all evaluated metals exhibited antitumor activity, but when executing the inoculation by a different route, only $Ga^{3+}$and In(III) were able to prevent tumor growth. Thus, of four types of solid rodent tumors tested, in three $Ga(NO_3)_3$ was effective in inhibiting growth, denoting its therapeutic potential (Hart *et al*., 1971; Hart and Adamson, 1971).

Subsequently, surveys began to indicate complexes being formed between proteins and gallium. To this extent, through the cross electro-immuno-diffusion technique, Gunasekera *et al*. (1972) identified these proteins as transferrin and haptoglobin. In addition, although it was already known that the trivalent ions (iron, indium and chromium) are exclusively bonded to transferrin, Ga^{3+} was able to distinguish itself from these elements due to its ability to bond to non-specific proteins (Gunasekera *et al*., 1972). Just as Woodworth *et al*. (1970), Aisen *et al*. (1973) used the isoelectric focusing technique to isolate specific conalbumin complexes and metal ions. This method enabled the formation of metallic complexes or mixed isotopes, which were analyzed according to their contribution to protein spectra. Thus, it was verified that, when the internal location of the conalbumin was occupied by Ga^{3+}, the external-bonded Fe^{3+} signal

was similar to the transferrin signal (Aisen *et al*., 1973). This provided the foundation to the development of more solid work on gallium mimicry and its relationship with iron conveyors.

Later on, Clausen *et al*. (1974) used the immunoelectrophoretic technique combined with autoradiography to investigate the effect of ^{67}Ga incubated in human serum. As a result, it was demonstrated that the ^{67}Ga radioisotope was strongly associated with the transferrin and had a weak bond with the β-lipoprotein (Clausen *et al*., 1974). Other works, such as Sephton and Harris (1975), corroborate with this gallium-protein connection. In this investigation, the ^{67}Ga absorption in tumoral cells of mice was analyzed, which was increased by adding certain serum types in culture. This result is related to transferrin, a macromolecular component in the serum that, according to the authors, acted as a tumor tracker carrier, which was ^{67}Ga in this case.

The possibility of transferrin acting as a carrier for the gallium ion was also strengthened by evidence in the study of Larson *et al*. (1979). This work sought to verify the absorption of ^{67}Ga in EMT-6 breast sarcoma in mice. In this manner, it was noted that, in the absence of transferrin, ^{67}Ga was found at a lower concentration when compared to the medium supplemented with transferrin. Thus, these clues pointed out that this increased EMT-6 absorption was mediated by a transferrin-specific cell receiver (Larson *et al*., 1979).

In this sense, only in the 1980s was the idea of mimicry between Ga^{3+} and Fe^{3+} emerged through Rasey *et al*. (1982). In this research, the cytotoxicity of $Ga(NO_3)_3$ titrated in EMT-6 tumoral cells of mice was studied. It was found that, under the exposition of low doses of $Ga(NO_3)_3$, there was inhibition of sarcoma cells growth and this result could be increased by virtue of the addition of transferrin to the medium. It was noted that the presence of transferrin raised both the toxicity of the cells and the absorption of gallium, which directed the researchers to mention the mimetic effect of Ga^{3+} to Fe^{3+} ions at a metabolic level with its intracellular transport through transferrin (Rasey *et al*., 1982).

After the revelation of the mimicry between Ga^{3+} and Fe^{3+} ions, other works with different perspectives on this regard were conducted. Thus, in 1983, Harris and Pecoraro conducted their own investigation in relation to the bonding of gallium to transferrin through macroscopic constants determined by UV spectroscopy. As a product, it was verified that there is a bond between gallium and transferrin in a weaker way than the thermodynamic association with the ferric ion.

Despite the postulates involving intracellular gallium transport, the exact mechanism of the deposition of ^{67}Ga in tumors was still uncertain in the 1980s. In view of this, Weiner *et al*. (1985) reported that, in patients with inflammatory diseases, there is strong evidence that lactoferrin, a regulator with bactericidal characteristics, would be activated and directed to the inflammatory site and, during that displacement, could transport high concentrations of ^{67}Ga to the abscess, which would subsequently be transferred to ferritin through receptors. In addition, the authors had the objective of examining this process of ^{67}Ga final translocation to ferritin by lactoferrin.

In these circumstances, tests have emerged that explored the specificity of siderophore transport systems from synthetic siderophore analogues. Müller *et al.* (1985) studied this process in *Rhodotorula pilimanae*, which has ineffective binders for iron transport. Thus, they used Ga^{3+} to investigate whether the exchange of binders involves the redox catalysis. Subsequently, it was noted that Ga^{3+}, by replacing Fe^{3+}, presented similar iron absorption rates, since Ga3+ could not be reduced to the +2 oxidation state in living organisms. Therefore, Ga^{3+} complexes were able to elucidate the dependence on Fe^{3+} mechanism in microorganisms

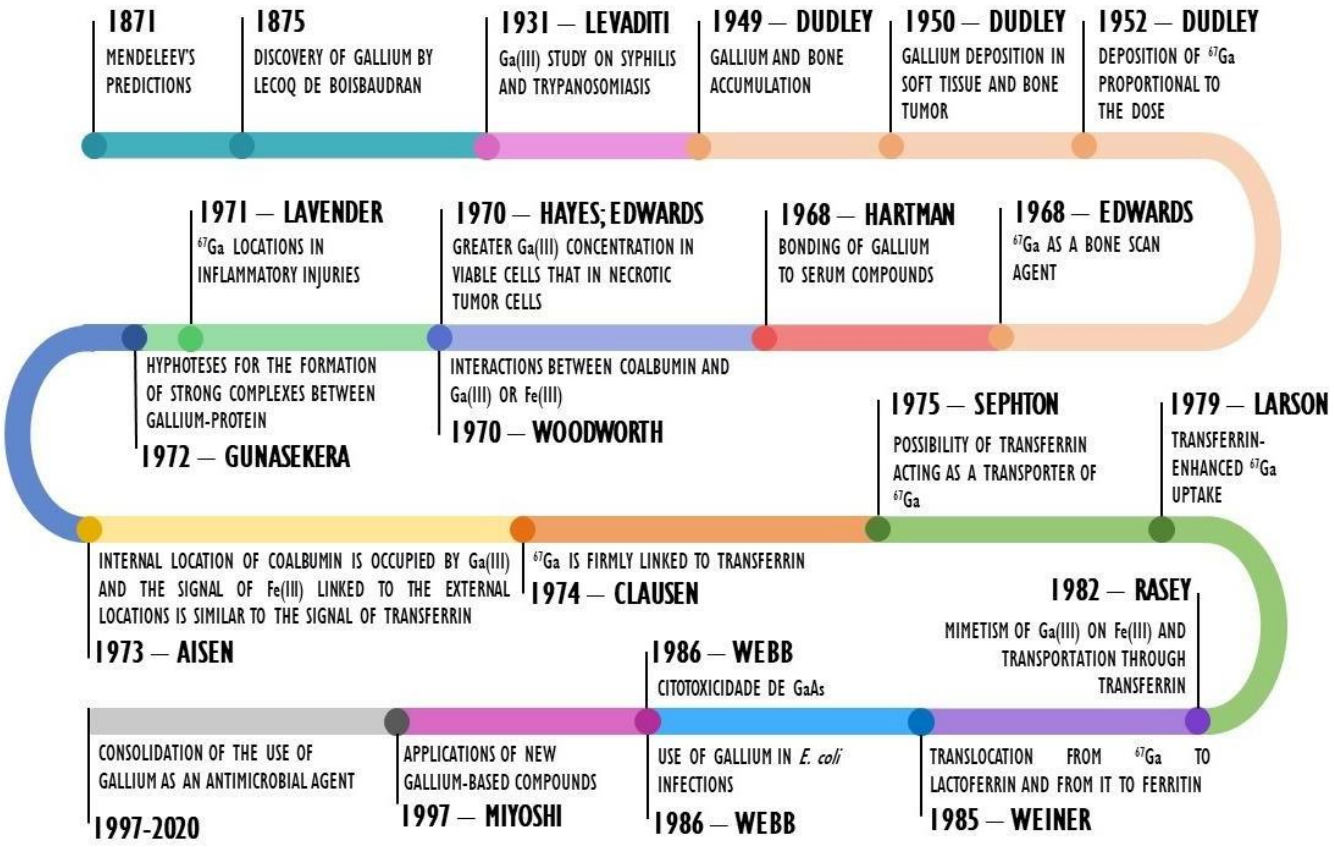
Other research that shows the versatility of gallium were carried out by Bach *et al*. (1986), which studied three patients with the acquired immunodeficiency syndrome (AIDS) with concomitant infection by *Mycobacterium avium-intracellulare* (MAI). It is known that MAI infections are difficult to control and diagnose and, due to its complexity, may result in deaths in AIDS patients. Therefore, in view of gallium imaging potential, it was used to diagnose nodal infections by MAI during feverish state identification. Therefore, authors suggest the use of gallium as a diagnostic agent in order to assist in the development of more effective strategies for discovering the origin of this pathology.

In addition to diagnostic characteristics, Ga^{3+} also stood out in 1986 as a reducing agent for Fe^{3+} in *Escherichia coli*. Elaborated by Hubbard *et al*. (1986), the investigation denoted deficiencies of bioavailable Fe^{3+} in *E. coli* cultures and the consequences of the addition of Ga^{3+} to the medium. In view of this, the Fe^{3+} limitation on the microorganism metabolism, added to the absorption of Ga^{3+}, further reduced the ferric concentration in the system. This result showed the Ga^{3+} as a culprit in the damage to Fe^{3+} metabolism of the studied microorganism.

Other gallium-based compounds were tested for their cytotoxicity by several authors such as Webb *et al*. (1986), who verified the pulmonary damage in mice after being exposed to gallium arsenide (GaAs), or Al-Aoukaty *et al*. (1992), which studied the interaction between

gallium citrate and *P. fluorenscens*, and Miyoshi *et al*. (1997), which observed the effects of the relation between low concentrations of gallium-porphyrin analogue ATX-70 and toxicity in human leukemia cells. In these cases, Ga^{3+} toxicity was attested because of its ability to replace Fe^{3+} and cause losses to the vital cellular system (Al-Aoukaty *et al*., 1992; Miyoshi *et al*., 1997; Webb *et al*., 1987, 1986). Through the 19 century, the convergence of all findings involving gallium culminated in the year of 2000 in expanding studies on the bacterial inhibition mechanism by metallic elements, especially those based in gallium (Bonifacio *et al*., 2017; Eby, 2005; J. L. Graves Jr *et al*., 2019; Kaneko *et al*., 2007; Valappil *et al*., 2012). As a result, according to the Fig. 1, this review also intends to highlight recent surveys covering the antimicrobial gallium activity, as well as present the properties of gallium and gallium compounds and their effects on microbial metabolism.

Figure 1 – Timeline: Evolution of the studies involving gallium compounds in biotechnology process.



Recent research involving gallium in microbiology

In the year of 2000, Bernstein *et al*. (2000) verified the behavior of gallium maltolate (GaM) in living organisms, comparing it with gallium nitrate. The synthesis of this compound was done using maltol and gallium nitrate nonahydrate as precursors. The tests were performed on dogs and subsequently in healthy individuals, and the authors observed that doses of up to 500 mg of GaM did not cause serious side effects in healthy humans, in addition to being excreted in much lower quantity in the urine when administered orally, compared to intravenous gallium nitrate. The authors discuss the possibility that the latter leads to $Ga(OH)_4^-$ formation, which would be more easily transported to kidneys for excretion, while oral gallium maltolate would lead to the bonding of the Ga^{3+} to transferrins, similar to Fe^{3+}.

In the same year, studied the effect of gallium in the metabolism of different microorganisms in human macrophages. Tests were performed with *Mycobacterium tuberculosis*, *M. avium* and *M. intracellulare*, verifying that $Ga(NO_3)_3$ was able to inhibit the growth of the three microorganisms both in broth conditions and in human macrophages. The same hypothesis was tested for the gallium-transferrin complex, obtaining similar inhibition results. In addition, the authors noted that the required concentration of gallium for inhibition, which was found to be in the safe standards determined by the FDA, is dependent on the amount of iron available, indicating a competition between the two metals in microorganisms (Olakanmi *et al*., 2000).

In this context, Harrington *et al*. (2006) investigated the effect of gallium nitrate *in vitro* in inhibiting the growth of *Rhodococcus equi*. In cultures, the compound was able to inhibit the growth of microorganisms, but the presence of excess iron contradicted this effect, as had been observed in previous surveys. For *in vivo* studies, the authors adopted gallium maltolate as an inhibitor of *R. equi* in mice, considering the prior reports of its better bioavailability in relation to the use of $Ga(NO_3)_3$. The researchers noted an inversely proportional effect between the bacterial concentration in the living tissues and the concentration of GaM in the group of tested mice, although they also mentioned that the study would have more statistical significance if a greater number of organisms had been surveyeed.

A similar investigation was done a year later by Martens *et al*. (2007), in which foals received intragastric doses of gallium maltolate in order to suppress the growth of *R. equi* both in macrophages and other tissues. The authors considered the gallium concentration of 700 ng/mL of the serum as sufficient for the treatment since, in other works, it was able to greatly

reduce the concentrations of the microorganism in the intracellular medium, in addition to lowering it in mice tissues, reaching a reduction of up to 90%. The results showed that there was an appropriate bioavailability of gallium in the foals tested with a dose of 20 $mg \cdot kg^{-1}$, being possible to apply the GaM as treatment for *R. equi* infections, although it had been reported that they still lacked studies with the application of multiple doses.

The competition between gallium and iron was alsotested *in vitro*, which verified that there was inhibition of the growth of *P. aeruginosa* when using gallium nitrate. In addition, the ratio of 1:5 between gallium and iron was necessary for the growth of *P. aeruginosa* to return to the control test's levels. For the case of biofilm formation, in which inhibition was also observed, this proportion increased to 1:10, demanding even more iron for biofilms to reach control growth levels. The capacity of the gallium nitrate to eliminate already consolidated biofilms was identified and in *in vivo* tests executed in mice confirmed the previous observations, with inhibition of biofilms (0.5µM) and the loss of the antibacterial effect in the presence of excess iron (Kaneko *et al*., 2007).

Halwani *et al*. (2008) synthesized liposomal gentamicin formulation containing gallium (Lipo-Ga-GEN), in an attempt to improve the gentamicin antibiotic with the antibacterial effects of Ga^{3+} to also reduce the formation of biofilms by *P. aeruginosa* in lung cells. Liposomes were employed as antibiotic and gallium carriers, and the authors found that the Lipo-Ga-GEN combination had better effects when compared to the introduction of gentamicin or Ga in their isolated form, observing a higher effect on bacterial suppression and in eliminating biofilms. In addition, the researchers noticed an improvement in the toxicity levels of gallium when carried by liposomes, with more cell viability compared to the metal in its isolated form).

The combination between the desferrioxamine (DFO) siderophore and gallium, calling it DFO-Ga was investigated in the inhibition of biofilms of *P. aeruginosa*. In results similar to the previous ones, the authors identified a more powerful antimicrobial effect when there was a combination of DFO-Ga with gentamicin, although DFO-Ga had already showed inhibition on its own in other assays. This effect was also noticed against biofilms that had already been developed. In *in vivo* tests performed in rabbits, keratitis was induced in cornea tissues. Subsequently, topical treatment was carried out with gentamicin and its combination with DFO-Ga, which showed the most promising results in fighting infection, even reducing the dimensions of the scar tissue (Banin *et al*., 2008).

The antimicrobial effect of gallium continued to be researched in subsequent years. Valappil *et al*. (2008) produced phosphate-based glasses (PBGs) from NaH_2PO_4, P_2O_5 and $CaCO_3$, and doped them with gallium using Ga_2O_3. The materials were investigated in regards to their degradation, release of Ga^{3+} ions and inhibition of microorganisms such as *Staphylococcus aureus*, *Clostridium difficile*, *P. aeruginosa* and *E. coli*. The performed tests demonstrated that the material has more effective antibacterial property against *P. aeruginosa*, *E. coli* and *S. aureus*. These effects were observed to a lesser extent in *C. difficile* and methicillin-resistant *S. aureus* (MRSA). The authors emphasized that, from the different doping concentrations that were carried out, the presence of Ga_2O_3 in 1 mol % was the most promising one. The PBG studies carried on with the controlled release of Ga with antimicrobial effect against planktonic *P. aeruginosa* and its biofilms. The researchers increased the concentration of calcium in the material, testing 14, 15 and 16 mol%, with the objective of reducing the degradation of the material and reaching a more controlled release of Ga^{3+}. The highlight among their results was the material with 14 mol% doping, which presented the most intense antibacterial effect and was able to provide Ga^{3+} ions to the medium for longer time.

The studies of DeLeon *et al*. (2009) were focused on inhibiting *P. aeruginosa*, *S. aureus* and *Acinetobacter baumannii* in lesions caused by burns in mice. The chosen gallium compound was GaM injected subcutaneously, for which toxicity was not identified. It avoided the death of animals when injected in doses of 25 $mg \cdot kg^{-1}$ and 100 $mg \cdot kg^{-1}$ in response to *P. aeruginosa* infections. Although *S. aureus* and *A. baumannii* did not cause mortality, they were also among the microorganisms inhibited by GaM treatment, which was most efficient when applied in the first 24 h after the lesion occurred, in addition to preventing the infection from spreading.

Baldoni *et al*. (2010) researched the inhibitory effect of GaM for planktonic phases and biofilms of *S. aureus* and *Staphylococcus epidermidis*, for which cultures of methicillin resistant and susceptible microorganisms were employed. The authors identified that the minimum inhibitory concentration (MIC) of Ga was higher for *S. aureus* (0.842 to 4.54 μM) in relation to *S. epidermidis* (0.21 to 0.45 μM), but the compound was able to act against all planktonic cultures in which it was applied. Calorimetric tests were performed, detecting the amount of heat generated by different cultures in the presence or absence of GaM, verifying that the addition of gallium maltolate at sub-inhibitory concentrations was able to reduce the amount of heat generated .

One year later, Rzhepishevska *et al*. (2011) investigated the antibacterial properties of gallium citrate (Ga-Cit) and gallium desferrioxamine B (Ga-DFOB) in cultures of *P. aeruginosa, S. epidermidis, S. aureus, E. coli, Klebsiella pneumoniae, Vibrio cholerae* and *Enterococcus faecalis*. The authors observed that *P. aeruginosa* was the most sensitive microorganism to gallium, for which Ga-Cit had a smaller concentration for inhibition of 90% of the culture (IC_{90}) than Ga-DFOB did. It was still possible to identify that Ga-Cit was also more effective against *E. coli, K. pneumoniae* and *V. cholerae*, while Ga-DFOB had better inhibition against *S. epidermidis*. The researchers also conducted an investigation in the metabolism of *P. aeruginosa* in the presence of Ga-Cit, evidencing that there were changes in the concentration of different amino acids and other metabolites such as glutamate

Antunes *et al*. (2012) studied the inhibition of *A. baumannii* by using gallium nitrate. In *in vitro* tests, as observed in other previous studies, gallium was able to inhibit the growth of the microorganisms, with IC_{90} varying between 2 and 80 μM of $Ga(NO_3)_3$. In the model for blood infections of the bacteria in humans, inhibition was also noted. Another phenomenon that corroborated previous works was the fact that the presence of excessive Fe^{3+} reduced the effectiveness of Ga^{3+} as an antibacterial agent. In the *in vivo* part of their investigation, the authors found that the survival rate of *Galleria mellonella* increased when treated with doses of 1.2 $mmol \cdot kg^{-1}$ of gallium nitrate for *A. baumannii* infections.

At this point in time, several other gallium compounds were already commonly found in research, such as those based in gallium-thiosemicarbazones, which were effective against *P. aeruginosa* and *Candida albicans*. The already known gallium bactericidal effects were combined with the ability of iron chelation of thiosemicarbazones, reaching better results overall (Lessa *et al*. 2013). The incorporation of Ga^{3+} into other chemical compounds proceeded with other works, which combined Ga^{3+} ions with hydroxyapatite, calling it HAp(Ga). It is reported that this incorporation is possible on the irregular surface of the material, which presents ions such as HPO_4^{2-} and PO_4^{3-}, while in the inner part a conversion to gallium and calcium phosphates was observed. Kurtjak *et al*. (2016) identified that gallium liberation occurred more uniformly when the HAp(Ga) was produced with the introduction of Ga^{3+} during the conversion of octacalcium phosphate (OCP) into hydroxyapatite crystals. For the inhibition of *P. aeruginosa* growth, it was noted that all materials that were produced had MICs of 837.73 μM or smaller, but the HAp(Ga) synthesized by ion exchange presented greater toxicity in mouse fibroblasts (Kurtjak *et al*. 2016a).

Goss *et al*. (2018) used $Ga(NO_3)_3$ to investigate its antimicrobial effect against *P. aeruginosa*. , they also identified that iron is an essential nutrient for these microorganisms, including situations of cystic fibrosis (CF). When added in excess, iron was again able to reduce the inhibitory properties of Ga^{3+}, as verified in several other research. The authors also demonstrated that the activity of bacterial ribonucleotide reductase was inhibited at about 40% with the concentration of 20 μM of $Ga(NO_3)_3$, although the increasing of this concentration had not produced better inhibitory effects. On the other hand, the inhibition values for the bacterial catalase enzyme reached approximately 70% when Ga^{3+} was added in a concentration of 60 μM.

In addition, it was possible to observe that gallium was able to act in conjunction with antibiotics such as colistin and piperacillin/tazobactam, while also not negatively interfering with human macrophages and their ability to eliminate *P. aeruginosa*. In their *in vivo* tests, $Ga(NO_3)_3$ increased survival rates in mice and reduced the amount of bacteria in pulmonary infections. For humans with cystic fibrosis, intravenous treatment did not cause negative side effects on a preliminary clinical trial, but an improvement in the pulmonary activity of the patients instead (Goss *et al*., 2018b).

The antibacterial activity of compounds such as gallium nitrate, gallium maltolate and gallium-protoporphyrin IX (GaPPIX) were further tested by Hijazi *et al*. (2018), in which ESKAPE species of microorganisms were used. These were *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* species, adding up to 24 different strains. In their assays, three different media were used for tests. The authors highlight the fact that GaPPIX's bactericidal properties were not negatively affected by the increase in iron concentration in the medium, whereas the addition of hemin managed to suppress GaPPIX effects, strongly suggesting its action as an analog of heme.

In simulated biological fluid tests, the addition of gallium compounds with the concentration of 28 μM produced bacteriostatic effect for GaM and gallium nitrate. GaPPIX reduced the concentration of viable cells in some microorganisms. Iron-rich media tests showed that all species were resistant to gallium's effects, with the exception of *S. aureus* and *A. baumannii* for GaPPIX. However, the GaPPIX antibacterial effects could be reduced in the presence of albumin (Hijazi *et al*., 2018b). A similar study was conducted a year later, in which Choi *et al*. (2019) verified the simultaneous use of $Ga(NO_3)_3$ with gallium protoporphyrin (GaPP) or gallium mesoporphyrin (GaMP), observing that gallium nitrate and GaPP were

effective against several common microorganisms, such as *K. pneumoniae, P. aeruginosa,* and MRSA. The same synergetic effect between gallium nitrate and gallium porphyrin were noted in further *in vitro* research with *M. avium, M. tuberculosis* and *Mycobacterium abscessus,* in which the latter showed most promising results. The authors also identified inhibition of enzymes such as catalase and aconitase, which suggests the efficacy of gallium-compounds based on antimicrobial treatments.

Conclusion

Characterized as a semimetal extracted from minerals, gallium has characteristics that make it viable for materials science, pharmacology and medicine. Mostly, the chemical reactivity of gallium involves the formation of trivalent ions (Ga^{3+}) in compounds such as Gallium Arsenide (GaAs) and Gallium Nitride (GaN), in the production of semiconductors and production of diodes that emit light (LEDs) or in laser diodes (LDs), with the potential of reducing production costs; Gallium nitrate ($Ga(NO_3)_3$) act as an antineoplastic and antimicrobial agent, through the intervention of iron homeostasis of the target cell, in the latter its development is impeded due to the inhibition of the synthesis of deoxyribonucleotides. This is due to the physicochemical characteristics of gallium, such as the ionic charge +3, the ionic radius, for example, which reveals a similarity with the Fe^{3+} ion. Thus, 3+ gallium enters the cell through iron transporters such as transferrin and is directed to the intracellular medium, interrupting metabolism, cellular respiration, DNA synthesis from tumor cells or even microorganisms.

Since the first predictions of its existence in 1871, gallium has presented itself as a versatile chemical element in different areas of research that progressed to its involvement as an antineoplastic agent, a detection agent in inflammatory and tumoral lesions, until it was considered an element capable of forming strong complexes with proteins and able to enter the intracellular medium and act in the first applications in research in 1931 as an antimicrobial agent. From that period onwards, studies involving gallium as a growth inhibitor of multiresistant bacterial strains have intensified in recent decades as a consequence of the misuse of classic antibiotics. It was possible to verify that, in different microorganisms, gallium and its different compounds have been presented as potential therapeutic means in times of combating multiresistant pathogens.

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CHAPTER 3

Determination of the Minimum Inhibitory concentration (MIC) of Gallium Nitrate against bacteria isolated from nosocomial infections.

Abstract

Under the "Era" of new agents with the potential to contain the advance of multi-resistant bacteria to traditional drugs, the semimetal Gallium (Ga^{3+}) has stood out due to its physicochemical characteristics. This inorganic antimicrobial was investigated and the effect of gallium nitrate was tested on common bacteria isolated from healthcare-associated infections and the minimum inhibitory concentration (MIC) was determined. In the present work, the results showed that the MIC_{50} of Gallium Nitrate ($Ga(NO_3)_3$) against *E. coli* and *S. aureus* were 11.36 µM and 1.42 µM, respectively. This research has shown promising results for $Ga(NO_3)_3$ inhibition of bacteria growth commonly found in nosocomial infections due to the concentration data, which are lower than those found in the literature.

Key-words: Gallium nitrate; antimicrobial; MIC.

1. Introduction

For decades, medical practices such as invasive procedures have led to the development of HAI (health care-associated infections), also known also as nosocomial infections (NI): conditions acquired after the admission in the hospital environment that can affect various body systems, with the prevalence of infections in the respiratory, cardiovascular, urinary and gastrointestinal tracts (Bereket *et al*., 2012; Magill *et al*., 2014 and 2018). Therefore, especially the traditional prophylactic methods and the uncomplied use of antibiotics have taken to the development of microorganisms resistant to antibiotic or nosocomial pathogens (Chikere *et al*., 2008). These latter result in hospitalized patients with infections other than increases mortality (up to one-third), morbidity rates, long hospital stays and extensive use of antibiotics (Bereket *et al*., 2012, WHO, 2020).

It was found that healthcare-associated infections are caused mostly by Gram-negative bacteria (34% to 64%), followed by gram-positive bacteria (16% to 40%) and fungi (9% to

19%) of the cases and up to one-third of the patients with infections were originated by multidrug-resistant bacteria (Markwart *et al*., 2020). Based on data there are several bacterial strains that are responsible for hospital infections, but the most commonly strains isolated from HAI are *Escherichia coli* and *Staphylococcus aureus*, (Bereket *et al*., 2012; Hanlon, 2005; McGowan, 2006, WHO, 2020).

In most nosocomial infections, these microorganisms leave the planktonic growth to multiply in biofilms and become more challenging to treat due to their greater resistance to traditional medicines and to adverse conditions (Struelens *et al*, 2004; Bogino *et al*., 2013; Li, Tan, 2012). This alarming issue results in the rise of new means of controlling multiresistant bacterial infections, like non-essential metals. These alternative means are less likely to develop multi-drug resistance as a result of acting in various ways, like the interference in the regulation of reactive oxygen species (ROS) and antioxidant depletion, protein dysfunction and loss of enzyme activity, nutrient assimilation and membrane function, genotoxicity, intervention of Fe^{3+} uptake, while antibiotics have specific targets such as DNA, RNA, cell wall or protein synthesis (Kohanski *et al*., 2010, Rzhepishivska, 2011, Lemire *et al*., 2013).

There are several chemical elements with potential to be antimicrobial agents, either through interactions based on interaction chemistry, in which groups of the donors of biomolecules interact with transition metals and assign them to specific activities, forming coordination complexes, either through molecular mimicry, under which some chemical elements are used from the low recognition of proteins to enter cells through coordination geometry, with the aim of identifying classes of toxic metals and non-essential ions (Lemire *et al*., 2013; Finney and O'Halloran, 2003; Waldron *et al*., 2009;). Another way is through the "theory based on hard-soft acids", as in the association of soft acids with soft or borderline bases found in proteins, making their antimicrobial potential proportional to their binding to the group of molecules found in proteins (Pearson, 1963; Workentine *et al*., 2008).

As a result of its toxicity to bacteria, antimicrobial metals compounds such as gallium (Ga^{3+}) – a transition metal element from group 13 of the periodic classification with relevant chemical properties that are similar to Fe^{3+} – make it a pioneering antimicrobial by interfering on iron regulation in microorganisms. Primordial bacterial activities such as metabolism, cellular respiration and DNA synthesis are Fe^{3+} dependents, so the Ga^{3+} strategy relies on its mimicry to "trick" the Fe^{3+} receptors and enter the cell (Chitambar, 2017, Antunes *et al*., 2012). In the cell, Ga^{3+} cannot be functional, resulting in disorders of the microorganisms' metabolism.

Only in 1931 the first experimental study on the therapeutic properties of Ga^{3+} on syphilis and trypanosomiasis resulted in its title of "antimicrobial agent" (Levaditi *et al*., 1931). Nowadays, gallium has been positively reported in relation to the growth inhibitory activity of different strains, such *as Acinetobacter baumanni, Escherichia coli, Klebsiella pneumoniae, Mycobacterium, Pseudomonas aeruginosa, Rhodococcus equi,* Staphylococci (Choi *et al*., 2019a, 2018; Coleman *et al*., 2016; Garcia *et al*., 2016; Xu *et al*., 2017; Zhu *et al*., 2015).

In this work we aimed to determine the minimum inhibitory concentration (MIC) of $Ga(NO_3)_3$ in solution against the most common bacterial strains reported in hospital infections: *Escherichia coli* and *Staphylococcus aureus.*

2. Material and methods

2.1. Chemicals, microbial strains and culture conditions

Two bacterial strains were selected: a gram-negative, *Escherichia coli* (ATCC 11229) and a gram-positive, *Staphylococcus aureus* (ATCC 6538). Initially, the strains were cultivated under anerobic conditions, at 37°C overnight, in Tryptic Soy Agar (TSA). In the microdilution test, *E. coli* and *S. aureus* were grown on the 96-well microplate, aerobically, at 37°C in Mueller-Hinton Broth (MHB).

The analysed gallium compound was $Ga(NO_3)_3 \cdot x$H2O (crystalline, 99.9% trace metals basis) was purchase from Sigma-Aldrich and dissolved in Sodium Citrate buffer $(CH_2COONa)_2 \cdot 2H_2O$) solution (0,1M). This combination resulted in the acidification of the medium. Then, the pH of the solution was measured and ajusted with sodium hydroxide (NaOH) solution (1M) for a pH of 7-8. All mentioned above were maintained at temperature of 5 – 8°C.

2.2. Susceptibility testing

In order to determine the Minimal Inhibitory Concentration (MIC) of $Ga(NO_3)_3$, 200 µL of MH broth and $Ga(NO_3)_3$ concentrations (from 0.17 to 90 µM) were transferred to 96-well microplates and inoculated with 10^7 CFU mL^{-1}, according to McFarland standards, of *E. coli* and *S. aureus* previously grown only within the same broth used. According to Xu *et al.* 2017, $Ga(NO_3)_3$ solution has a few flocs in higher concentrations, so it was added 10 µL of 0.2% EDTA solution to each well in order to eliminate any interference in the absorbance reading.

Afterwards, the microplate was incubated for 24 h at 35ºC and read at 540 nm with a microplate reader (Synergy HTX multimode reader). So the MIC value was interpretated as the lower concentration of $Ga(NO_3)_3$ in which absorbance changed no more than 0.05 when comparing to the blank value. Therefore, the tests were conducted in quadruplicate to each strain (Tsukatania *et al*., 2012).

2.3. Statistical analyses

Statistical analyses employed one-way analysis of variance (ANOVA) followed by Turkey's test (significance level of $p<0.05$), performed using OriginPro 2021b 9.85v.

3. Results

3.1. Determination of the MIC of $Ga(NO_3)_3$ against bacteria strains

Initially, when $Ga(NO_3)_3$ was diluted in water, the pH of the solution was reduced to the range of 2.3 – 2.5 and there was a change in the turbidity that interfered in the spectrometry of the experiment. In aqueous solution, there are H^+ and OH^- ions, so when $Ga(NO_3)_3$ is added, gallium ions react with sequestrate OH^- ions which results in precipitate formation, identified as gallium hydroxide ($Ga(OH)_3$) (Ristic *et al.* 2005). The precipitate changes the turbidity and the pH of the solution. Thus, to contain these variations, the dilution of $Ga(NO_3)_3$ was performed in sodium citrate buffer solution (1M) in order to solve the turbidity problem and aqueous 0.1 M NaOH was added dropwise to adjust and maintain the pH at fixed values of pH= 7 – 8.

The MICs of $Ga(NO_3)_3$ for the two different strains, *E. coli* and *S. aureus*, were investigated in MH broth. Overall, as seen in Fig. 1, gram-positive strain showed a lower absorbance when compared to the absorbance values of gram-negative strain. The MIC_{50} average values for *E. coli* and *S. aureus* were 11.25 µM and 1.40 µM, respectively.

Low concentrations of $Ga(NO_3)_3$ reduced the cultures by half (p <0.05) when compared to the control of the pathogens. It was also possible to verify that $Ga(NO_3)_3$ interfered more with the *E. coli* culture than the *S. aureus* one.

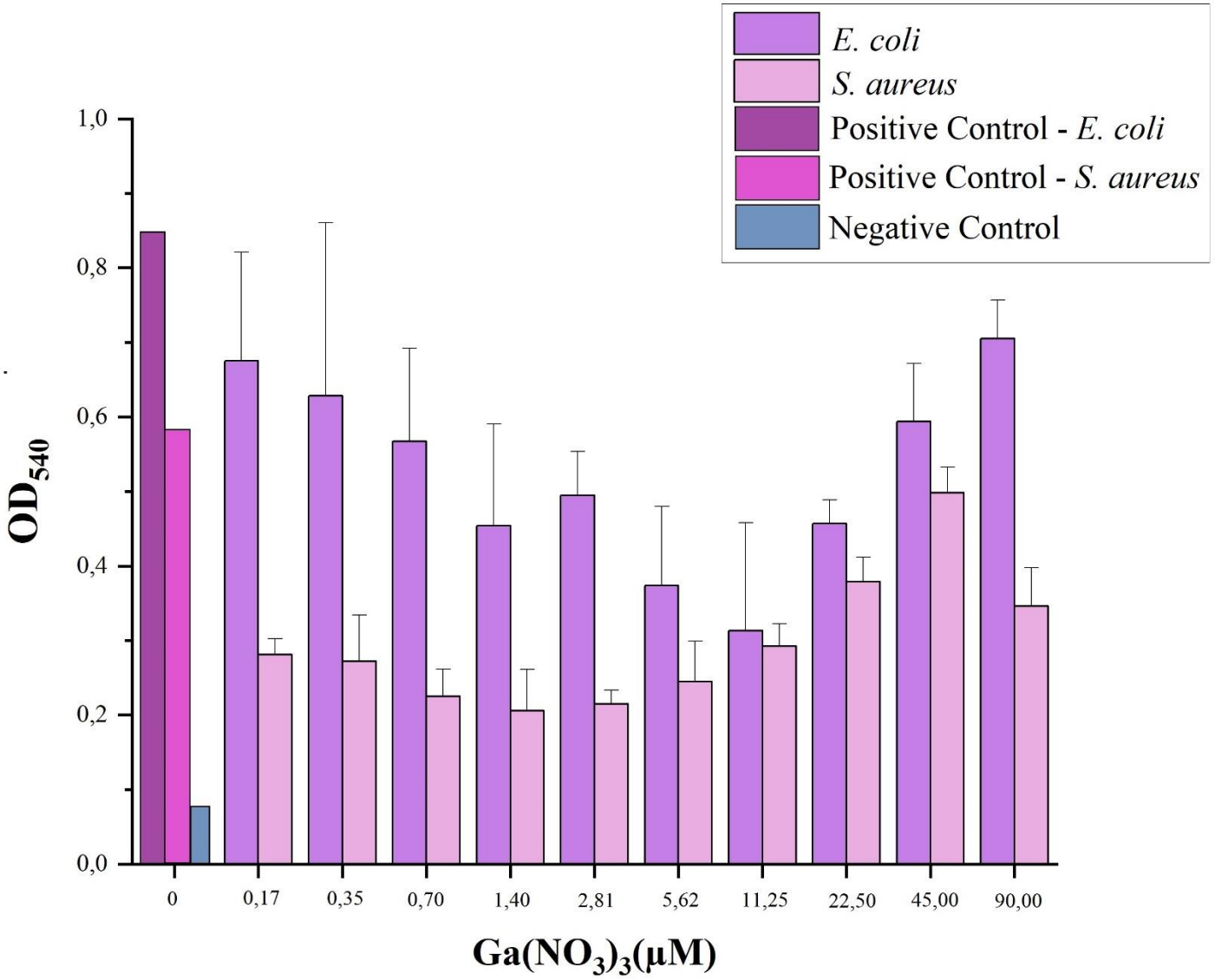

Fig. 1. Antimicrobial susceptibility of $Ga(NO_3)_3$ against *S. aureus* and *E. coli*.

4. Discussion

In this work, after the dilution of $Ga(NO_3)_3$ in aqueous medium, a complex started to appear and interfere with the turbidity and causing acidification of the medium (Tsukatania *et al*., 2012). According to Zhao *et al.* 2008, the precipitate was $Ga(OH)_3$. When $Ga(NO_3)_3$ was diluted in sodium citrate solution, there was no change in turbidity and the required pH was adjusted with NaOH. This corroborates with Rzepishevska *et al*, (2011) research, who reported the use of gallium citrate (Ga-Cit) in order to prevent precipitation of $Ga(OH)_3$ and investigate the minimum inhibitory concentration against gram-negative and positive planktonic strains with minor precipitation.

Furthermore, as highlighted in the present study, $Ga(NO_3)_3$ proved to be an efficient antimicrobial agent against nosocomial pathogens, in which the MIC required to inhibit 50% of *E. coli* and *S. aureus* strains in solution were 11.25 µM and 1.40 µM, respectively. That means the lower concentration of the antimicrobial tested, expressed in micromolar, reduced the absorbance by 50% when compared to that found in the positive control to the strain.

It was also possible to notice that gram-positive *S. aureus* was more resistant to treatment with $Ga(NO_3)_3$, which can be attributed to the difficulty in permeabilizing the compound, due to the peptidoglycan layer present in gram-positive strains. As predicted, the gram-negative strain, which have only one barrier preventing access to the cytoplasm, needed lower concentrations than the gram-positive.

This data corroborates to those that established in the literature. Recent research by Gugala *et al*. (2019) on the effectiveness of antimicrobials in isolated species of *E. coli*, *P. aeruginosa* and *S. aureus* verified the from the determination of the minimum inhibitory concentration of $Ga(NO_3)_3$ against these strains, being 31.25 µM, 15.63 µM and 15.63 µM, respectively. Graves *et al*. (2019) used the experimental evolution to develop *E. coli* strains resistant to $Ga(NO_3)_3$ and this assay allowed them to determine the MIC of the strain through the use of 391.02 µM of antimicrobial compound.

In 2020, Neill *et al.* conducted a study of the antibacterial activity of $Ga(NO_3)_3$ against the representative pathogen *E. coli* under physiologically oxygen-reduced (anaerobic) growth conditions. Therefore, to reduce the growth of the strain by 50%, 312µM were needed under aerobic conditions and 2500µM under anaerobic conditions; 90% of growth was inhibited using 625µM and ≥104 µM in both physiological situations.

As expected, the inhibitory concentrations were similar to those found in the literature for gram-negative and positive strains. However, despite the gram-negative requiring a higher concentration of gallium and the gram-positive one needing a lower concentration, our study was able to reduce the cultivation of both strains by 50% with a lower amount when compared to literature data. Thereby, it was shown that gallium nitrate has an effective role as an antimicrobial agent against microorganisms found in representative strains of nosocomial infections even in lower concentrations. In addition, besides representing a microbial growth inhibitory tool, $Ga(NO_3)_3$ also has the potential to be used in the deposition of biomaterials in order to prevent healthcare-associated infections. In order to advance in studies involving medical devices and pathogenic microorganisms, further studies are needed, especially

regarding the antimicrobial effect of $Ga(NO_3)_3$ on other strains commonly found in hospital environments and test its involvement in hospital tools.

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CHAPTER 4

Conclusion

The primary objective of this work was to promote microdilution assays of Gallium Nitrate $(GaNO_3)_3$ in a solution containing *E. coli* and *S. aureus* inoculums and them verify its effectiveness. The motivation behind this research involved i – the emerging issue related to combating, preventing and treating infections caused by drug-resistant bacteria; ii – reduction of alternatives for containing these diseases; iii – lack of consolidated research in the literature on the amount of gallium compounds needed to inhibit microbial growth; iv – the potential of Ga^{3+} that can still be exploited.

To achieve these objectives, it was decided to study in depth the literature since the period when Gallium (Ga^{3+}) was discovered to its application in the most cited studies in the area of Medicine and Microbiology and how was its evolution until the present days. Thus, based on a solid literature review, it was possible to carry out chapter 3, which reports the research itself and the MIC_{50} data.

In general, the review of references presented here was important to establish a solid microdilution protocol due to adversities found throughout the project, such as turbidity and acidification of the solution of $Ga(NO_3)_3$ diluted in aqueous medium. After this issue was solved with the dilution of $Ga(NO_3)_3$ in sodium citrate buffer solution and pH adjustment with NaOH, a 50% reduction of *E. coli* and *S. aureus* cultures was observed. Finally, these data showed the possibility of lower concentrations for the growth reduction of strains tested in relation to data present in literature.