UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CÂMPUS DE JABOTICABAL

ANTIMICROBIAL PEPTIDES AS ADDITIVES TO BEEF CATTLE NUTRITION

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Animal Scientist, MSc.

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Jaboticabal, 16 de dezembro de 2021

DADOS CURRICULARES DO AUTOR

RAIZA FELISMINO SILVEIRA – Filha de Francisco Carlos da Silveira e Edilene Felismino Silveira, nascida em Fortaleza, Ceará, em 28 de março de 1993. Em agosto de 2010, iniciou o Curso de Graduação em Zootecnia na Universidade Federal do Ceará - UFC onde obteve o título de bacharel em Zootecnia em dezembro de 2014. Em fevereiro de 2015, ingressou no Mestrado no Programa de Pós-Graduação em Zootecnia, na Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp / FCAV), Jaboticabal - SP, sob orientação do Prof. Dr. Kleber Tomás de Resende e coorientação da Dra. Márcia Helena Machado da Rocha Fernandes finalizando o mesmo em julho de 2017. A partir da dissertação, o artigo *"Energy partition and nitrogen utilization by male goats fed encapsulated calcium nitrate as a replacement for soybean meal"* foi publicado na revista *Animal Feed Science and Technology.* Em agosto de 2017, iniciou seu doutorado e no ano seguinte deu início ao atual projeto sob orientação do Prof. Dr. Eduardo Festozo Vicente e coorientação do prof. Dr. Ricardo Andrade Reis. Ao longo do doutorado, publicou o artigo intitulado *"Energy expenditure of Saanen and Anglo-Nubian goats at different temperatures"* na revista *Small Ruminant Research; "Fast Determination of Short-Chain Fatty Acids and Glucose Simultaneously by Ultraviolet/Visible and Refraction Index Detectors via High-Performance Liquid Chromatography"* na revista *Food Analytical Methods;* o artigo de revisão *"Antimicrobial peptides as a feed additive alternative to animal production, food safety and public health implications: an overview*" na revista *Animal Nutrition*; o artigo *"Comparative activity of total polyphenols and antioxidant compounds from Uncaria tomentosa enhanced with citric acid"* na Revista Brasileira de Engenharia e o capítulo intitulado "Peptídeos antimicrobianos na produção animal: uma alternativa biosustentável" no livro "Tecnologias Sustentáveis e Agronegócio".

“I learned that courage was not the absence of fear, but the triumph over it. The brave man is not who does not feel afraid, but who conquers that fear.”
Nelson Mandela, *Long Walk to Freedom*

DEDICATÓRIA

À minha família, que sempre apoiou minhas escolhas e decisões.

Dedico.

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A mim. Por ter resistido, persistido e buscado forças onde achei que não mais havia para concluir essa etapa da minha vida acadêmica.

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Sumário

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Utilização de peptídeo antimicrobiano D-Ctx(Ile^{21})-Ha e suas formulações como aditivo na nutrição de bovinos de corte"**, protocolo nº 8418/19, sob a responsabilidade do Prof. Dr. Eduardo Festozo Vicente, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 16 de fevereiro de 2022.

Vigência do Projeto	30/07/2019 a 30/07/2020
Espécie / Linhagem	Bovino
Nº de animais	2
Peso / Idade	400kg, 4 anos
Sexo	Macho
Origem	Fazenda Experimental FCAV

Jaboticabal, 20 de fevereiro de 2022.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

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Peptídeos antimicrobianos como aditivos na nutrição de bovinos de corte

RESUMO

Os peptídeos antimicrobianos (PAMs), identificados nos últimos anos a partir de várias espécies de animais vertebrados e invertebrados, vem recebendo atenção como um possível substituto aos aditivos alimentares atualmente utilizados para animais. Uma dessas moléculas, extraída a partir de uma rã do cerrado brasileiro, mostrou-se uma molécula antibiótica promissora devido sua atividade biológica contra fungos e bactérias. O peptídeo Ctx(Ile^{21})-Ha e seu isômero *ent*-Ctx(Ile^{21})-Ha mostraram-se capazes de permeabilizar e destruir membranas bacterianas e por isso têm se tornado foco de estudos para a nutrição de bovinos de corte objetivando a redução das bactérias Gram-positivas, já que seu modo de ação inespecífico (tem como alvo a membranas das células) dificultam o processo de resistência bacteriana. Objetivando avaliar os efeitos da aplicação do Ctx(Ile^{21})-Ha e seu isômero *ent*-Ctx(Ile^{21})-Ha para bovinos de corte mantidos sob sistema de pastejo em comparação com os aditivos convencionais utilizados na alimentação animal, foram conduzidos quatro estudos *in vitro* com diferentes substratos combinados com os aditivos monensina e virginiamicina e doses dos peptídeos antimicrobianos. Foram avaliados pH, produção de gases, produção de metano, perfil de ácidos graxos de cadeia curta e taxa de desaparecimento de matéria seca e fibra em detergente neutro. Os PAMs reduziram a produção de gases. A produção de metano, de forma geral, foi afetada de forma significativa pela interação entre substrato e aditivo. Além disso, a taxa de desaparecimento não foi afetada de forma significativa pelo uso dos PAMs. A produção de ácidos graxos de cadeia curta apresentou diferenças significativas, com o perfil de ácidos nos tratamentos com os PAMs semelhante àqueles com monensina. Os níveis intermediários do PAM *ent*-Ctx(Ile^{21})-Ha mostraram uma redução na emissão de gases e metano, sem comprometimento da utilização de matéria seca e fibra em detergente neutro, mostrando que a redução dos gases não se deu devido à redução na utilização de nutrientes pelos microrganismos. Estudos complementares devem ser conduzidos para melhor entendimento do modo de ação e dos efeitos da adição dos PAMs à dieta de ruminantes.

PALAVRAS-CHAVE: Aditivos; Antibióticos; Monensina; Peptídeos antimicrobianos; Resistência bacteriana; Virginiamicina.

Antimicrobial peptides as additives to beef cattle nutrition

ABSTRACT

Antimicrobial peptides (AMPs), identified in recent years from several species of vertebrate and invertebrate animals, have been receiving attention as a possible substitute for feed additives currently used for animals. One of these molecules, extracted from a frog from the Brazilian Cerrado, proved to be a promising antibiotic molecule due to its biological activity against fungi and bacteria. The Ctx(Ile^{21})-Ha peptide and its *ent*-Ctx(Ile^{21})-Ha isomer proved to be capable of permeabilizing and destroying bacterial membranes and, therefore, have become the focus of studies for the nutrition of beef cattle aiming at reducing of Gram-positive bacteria, since their unspecific mode of action (targets cell membranes) hinder the process of bacterial resistance. Aiming to evaluate the effects of the application of Ctx(Ile^{21})-Ha and its *ent*-Ctx(Ile^{21})-Ha isomer for beef cattle kept under grazing system in comparison with conventional additives used in animal feed, four *in vitro* studies were conducted with different substrates combined with monensin and virginiamycin additives and doses of antimicrobial peptides. pH, gas production, methane production, short-chain fatty acid profile and rate of dry matter and neutral detergent fiber disappearance were evaluated. AMPs reduced gas production. Methane production, in general, was significantly affected by the interaction between substrate and additive. Furthermore, the disappearance rate was not significantly affected by the use of AMPs. The production of short-chain fatty acids showed significant differences, with the acid profile in treatments with AMPs similar to those with monensin. Intermediate levels of AMP *ent*-Ctx(Ile^{21})-Ha showed a reduction in the emission of gases and methane, without compromising the use of dry matter and neutral detergent fiber, showing that the reduction of gases was not due to the reduction in use of nutrients by microorganisms. Complementary studies should be conducted to better understand the mode of action and effects of adding AMPs to ruminants' diet.

KEYWORDS: Additives; Antibiotics; Antimicrobial peptides; Antimicrobial resistance; Monensin; Virginiamycin.

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CHAPTER 1 – Antimicrobial peptides as a feed additive alternative to animal production, food safety and public health implications: An overview

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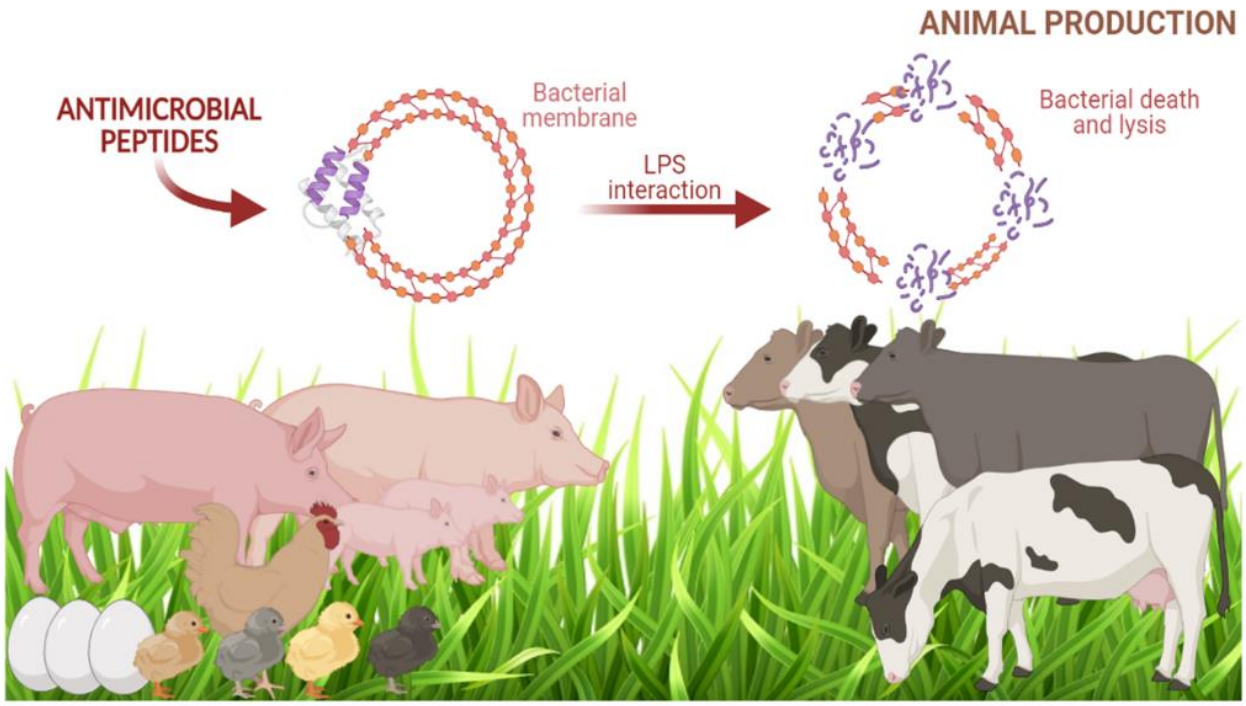
Graphical Abstract:



ABSTRACT: In the last few years, feed additives have been used in animal nutrition to improve nutrient utilization, health parameters and animal performance. However, the use of antibiotics as feed additives has allowed the occurrence of antimicrobial resistance (AMR), which can bring as a consequence, an increase in the morbidity and mortality of diseases that were previously treatable with antibiotics. In this context, antimicrobial peptides (AMP) have appeared as a promising strategy because they have multiple biological activities and represent a powerful strategy to prevent the development of resistant microorganisms. Despite the small number of studies applied in vivo, AMP appear as a potent alternative to the use of antibiotics in animal nutrition, due to an increase in feed efficiency and the prevention/treatment of some animal diseases. This review discusses the problems associated with antimicrobial resistance and the use of AMP as a strong candidate to replace conventional antibiotics, mainly in the animal industry.

Keywords: Additive; Antimicrobial peptide; Broiler; Cattle; Laying hen; Nutrition; Pig.

1 Introduction

According to the Food Outlook 2020, disclosed by The Food and Agricultural Organization (FAO, 2020), world meat demand increased 119 million metric tons. However, a reduction in pig meat production is anticipated due to the impact of African swine fever disease. In contrast, the same report shows an expansion of poultry and ovine meat production due to the increased demand. In the dairy sector, milk production is expected to experience an improvement of 1.4%, producing 860 million metric tons of milk per year.

Brazilian companies lead the world beef market, which moves more than 7 million metric tons per year between exports and imports. Since 2005, this country has internationalized the sector, purchasing large processing plants abroad. In 2018, Brazil became the major exporter, trading more than 2 million metric tons of meat, and was followed by Australia, which exported approximately 1.5 million metric tons (ABIEC, 2019). Therefore, countries need to improve the feed safety, health and process certification and quality of origin (traceability) aspects of the herd to avoid the possible serious risk of losing the positive results already achieved in international markets (Morgan et al., 2016; Conchon and Lopes, 2012).

The production of poultry meat in the world is led by the USA, China, and Brazil, which is responsible for a high economic value (FAO, 2020). In recent years, consumers have changed their perspectives when purchasing food products, focusing mainly on food safety (Heneghan, 2015). This factor is related to nutritionally

adequate usage and safe foods (Coleman-Jensen et al., 2020), with safe foods being those that do not affect consumer health (Chassy, 2010).

Various techniques have been employed in animal production to maximize food production. To achieve this, research needed to change from being solely focused on animal nutrition, replacing food nutritional value studies with an understanding of animal physiological processes and the factors that affect them (Wallace, 1994). In recent years, research has sought to manipulate and improve fermentative patterns and ruminal metabolism with additives in the diet (Meyer et al., 2009; Moya et al., 2009; Possenti et al., 2008), aiming to improve animal feed efficiency.

However, institutes such as the World Health Organization (WHO) and FAO have demonstrated concerns about the use of antibiotic additives in some situations, among them animal nutrition. Because of that, this research has been undertaken with the aim of finding some replacements for the usual additives.

2 Antimicrobial resistance (AMR)

AMR is considered to be one of the great challenges of the human health system. Every year, about 700,000 people die from uncontrolled infections (WHO, 2019). If no changes in the approaches are taken, by 2050 AMR will kill more people than cancer diseases (O'Neil, 2014). The WHO defines AMR as "the microorganism which has the capacity of stopping antimicrobial activities". AMR makes conventional treatments inefficient and infections impossible to be cured. AMR has increased in

recent years and 6 pathogens are highlighted that exhibit high multidrug resistance and virulence: *Enterobacter* spp., *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterococcus faecium* (ESKAPE). These pathogens form the ESKAPE group, a dangerous pathogen group, which takes its name from the members of the group (Mulani et al., 2019). One of the main problems related to AMR is the lack of innovation, i.e., the development of new health technologies or treatments cannot keep up with the speed that these microorganisms can readapt. The main consequences of this phenomenon are an increase in diseases' morbidity and mortality, in diseases that were previously treatable with antibiotics and even with herbal antimicrobials. In addition, AMR has also resulted in the reappearance of infectious and opportunistic diseases, such as yellow fever, Chagas disease and tuberculosis, because microorganism mutations have caused greater resistance of parasites and agents. These issues represent serious public health problems, especially in most socioeconomically vulnerable populations (Estrela, 2018).

Nowadays, 80% of animals used in food production are treated with drugs at some point in their lives (Chiesa et al., 2020). As a result, products from these animals – such as meat, milk, and eggs – can contain residues from these medicines. This has become a public health problem because these residues can affect the treatment of diseases due to AMR. In the milk industry, for example, the use of antibiotics such as chloramphenicol, β-lactams, streptomycins, sulfonamides, tetracyclines and quinolones is quite common. Their residues in milk can often cause

allergies, which increases the development of resistant bacteria in humans (Chiesa et al., 2020). In 1997, the WHO determined that “antimicrobial overuse leads to the selection of resistant forms of bacteria in the ecosystem of use” and recommended that if an antibiotic was essential to human treatments, it should not be used as a growth promoter in animals. This is because after the antibiotic administration, some residues can remain in the animal product (Menkem et al., 2019) and lead to resistance development.

In 1955, a strain of *Shigella dysenteriae* resistant to 4 different antibiotics was isolated in Japan during an outbreak of bacterial dysentery. Ten years later, half of all Japanese *Shigella* infections were caused by multidrug resistant variants (Russell and Mantovani, 2005; Shiga, 1898), which showed how fast the resistance evolves and spreads in the environment. While the transference of antibiotic resistant bacteria between human and animals is not fully understood, we do know that animals fed with antibiotics have more antibiotic resistant bacteria than the free antibiotic fed animals. Further, farm workers carry more antibiotic resistant bacteria than people who live in urban environments (Wang, 2021). Research conducted by Abbas et al. (2008) tested the drug sensitivity in *Eimeria tenella* against some anticoccidials used in broiler chicks – salinomycin, maduramicin and clopidol. They found partial resistance in chickens against all 3 anticoccidials. Other additives such as tylosin and virginiamycin are well known for cross resistance to lincosamidines, macrolides and streptogramines (Witte, 2007). In addition, in Germany evidence was found of

resistance in chickens to a streptothricin antibiotic, which had only been used in animal feeds (Witte, 2007).

As a consequence of AMR problems, international organizations, countries, academia, productive and technological sectors have been mobilizing on different levels of performance to tackle this obstacle. In 2015, the “Global Action Plan to Combat Antimicrobial Resistance” was launched because of a partnership between the FAO and the WHO, with the aim of keeping the treatment of infectious diseases effective and safe. In the fields of agriculture and animal production, the use of antibiotics to accelerate animal growth for human consumption, and their possible consequences of AMR, is a matter of great relevance due to its high economic impact. However, since 2016, the use of antimicrobial compounds as growth promoters has been banned by the European Union, considered the largest food importer in the world. Therefore, the recent call to combat AMR has led some countries to outline strategies for developing new and effectiveness antimicrobial molecules.

3 Antimicrobial peptides (AMP)

AMP are molecules with low molecular weight, belonging to a diverse and abundant group of biomolecules. AMP are produced in several types of animal and plant cells, with vast biological activity against bacteria, viruses, and fungi (Lai and Gallo, 2009). AMP are part of the natural innate immune system of animals and, in plants, form a defense system similar to the innate immunity observed in animals,

protecting them from pathogens and pests (Lehrer et al., 1993; Gabay, 1994; Boman, 1995; Hultmark 1993; Shewry and Lucas 1997; Gallo et al. 2002). AMP often share a common feature: the presence of even-numbered cysteine residues connected by disulfide bridges, which gives them high stability (Broekaert et al., 1997). In addition, their amino acid composition, amphipathicity, helicity, cationicity and size make them able to become inserted into lipid membranes, leading the target microorganism to death (Lorenzon et al., 2012; Brogden, 2005; Vicente et al., 2013; Izadphanah and Gallo, 2005). The continuous increase of multidrug resistant microorganisms' appearance has led research to shed light on these molecules, allowing the development of new therapeutic agents (Duin and Paterson, 2016; Gallo et al., 2002). Appreciation of the therapeutic potential of AMP is due to their ability to rapidly kill many microorganisms such as fungi, viruses, and bacteria, mainly those which have become resistant to multiple conventional drugs.

Despite conventional antibiotics that usually have the mechanism of action through a high affinity for a defined target in the microorganism, AMP perform multiple antimicrobial functions, which acts as a powerful strategy to prevent the development of resistance by microorganisms (Peschel and Sahl, 2006). Further, they can act on different targets in the cells such as DNA, RNA, regulatory enzymes, and other proteins (Maria-Neto et al., 2015). Specifically, their main advantage is the property to still kill multidrug-resistant bacteria. Zhang and Sack et al. (2012) demonstrated that AMP can inhibit the methicillin-resistant bacteria *Streptococcus aureus* and *Pseudomonas aeruginosa*, the latter being resistant to conventional

antimicrobials, causing severe hospital infections. Unlike direct attacks against microorganisms, AMP may offer protection by different mechanisms, such as attacking unspecific targets (i.e., plasmatic membranes), maintaining normal gut homeostasis or modulating host inflammatory responses (Wang et al., 2015; Cespedes et al., 2012; Lai and Gallo, 2009). Current studies are not enough to fully support the synergies between conventional antibiotics and AMP, although insights could be revealed by exploring different AMP together (Magana et al., 2020). In human medicine, scientists already have published studies testing the capacity of AMP to reverse drug resistance. Teng et al. (2020) tested some AMP as anticancer drugs to reverse the cells resistance to regular drugs that have been used. Interestingly, they have found, for the first time, an antimicrobial peptide that could reverse cell resistance.

3.1 AMP activity

The most studied classes of AMP are those with antibacterial activity. Most AMP can interact with bacterial membranes and there are at least 9 hypotheses of mechanisms of action: 1) electroporation; 2) carpet model, 3) membrane thinning or thickening, 4) non-lytic membrane depolarization, 5) toroidal pore, 6) oxidized lipid targeting, 7) barrel stave, 8) disordered toroidal pore, and 9) non-bilayer intermediate (Magana et al., 2020).

There are several databases where most AMP are compiled and registered. The Antimicrobial Peptide Database (APD - http://aps.unmc.edu/AP/main.php) have

3,201 AMP from 6 kingdoms (357 bacteriocins/peptide antibiotics from bacteria, 5 from archaea, 8 from protists, 20 from fungi, 352 from plants, and 2,377 from animals, including some synthetic peptides) with a large variety of activity (Wang et al., 2016c, 2009; Wang and Wang 2004). A second database is the Data Repository of AntiMicrobial Peptides (DRAMP - http://dramp.cpu-bioinfor.org/), an open-access and manually curated database harboring diverse annotations of AMP including sequences, structures, activities, physicochemical, patent, clinical and reference information with 20,434 entries, 5,619 of which are general AMP (containing natural and synthetic AMP), 14,739 AMP patents and 76 AMP in drug development (Kang et al., 2019; Liu et al., 2018; 2017; Fan et al., 2016). Finally, the DBAASP (acronym for DataBase of Antimicrobial Activity and Structure of Peptides - https://dbaasp.org/) was developed to provide information and analytical resources for the scientific community, to help in developing antimicrobial compounds with a high therapeutic index (Pirtskhalava et al., 2016). To date, there are many other databases and resources for AMP research described elsewhere (Magana et al., 2020).

Antimicrobial peptides isolated from insects are the largest group of known AMP (Wang, et al., 2016a). Among the antibacterial peptides, cecropins were extensively studied and represent an important component of insect defense systems against bacterial infection (Hoffmann, 1995). Synthetic cecropins exhibit a powerful inhibitory efficacy against *Escherichia coli, P. aeruginosa, Bacillus megatherium* and *Micrococcus luteus* (Andreu et al., 1985) and these classes of peptides act to destroy the bacterial membrane integrity (Silvestro et al., 2000). Another highlighted insect

AMP group is the defensins (Hoffmann and Hetru, 1992), which act against Gram-positive bacteria and participate in the antibacterial defense reactions in insects (Wang et al., 2016a).

Another group of animals that presents a rich arsenal of AMP to defend against noxious microorganisms are the amphibians (Simmaco et al., 2004). The magainins were isolated from an African frog's skin and its synthetic peptide form displayed antibacterial activity against numerous Gram-positive and Gram-negative bacteria such as *Escherichia coli, S. aureus* and *Klebsiella pneumonia.* The synthetic limnochariin, a peptide from amphibians' skin, showed antimicrobial activity against Gram-positive and negative bacteria (Wang et al., 2016b). Also, the synthetic hylaranins, an amphibian AMP extracted from an Oriental frog, showed antibacterial activity against *E. coli* and *S. aureus* (Lin et al., 2014). Finally, the hylins and ceratotoxin-Ha (Ctx-Ha) are cytolytic peptides isolated from the arboreal South American frog *Hypsiboas albopunctatus* that present a broad biological activity against bacteria and fungi (Castro et al, 2009; Cespedes et al, 2012; Vicente et al, 2013). From mammals, defensins and cathelicidins (CATH) are the main classes of AMP that have been identified. Defensins show a broad range of antimicrobial activity against bacteria that have demonstrated resistance to antibiotic treatments (Verma et al., 2007) and CATH exert antibacterial activity against Gram-positive and Gram-negative bacteria via electrostatic interactions with the bacterial cell membrane (Dean et al., 2011).

One of the interesting types of AMP studied are bacteriocins (Russel and Mantovani, 2005), which are ribosomal peptides released into the extracellular medium by Gram-positive and Gram-negative microorganisms and which have specific bactericidal or bacteriostatic action (Collins et al., 2010; De Vuyst; Leroy, 2007). The first bacteriocin was initially identified as an antimicrobial protein produced by *E. coli*, called colicin (Gratia, 1925).

In 1969, studies in bacteriocins produced by lactic acid bacteria aroused more interest in nisin, the first commercially applied bacteriocin. Nisin was added to the European list of food additives in 1983 and authorized for use in processed cheeses by the Food and Drug Administration (FDA) in 1988. Since then, the research field on this biomolecule has extensively increased, allowing the discovery and detailed characterization of many bacteriocins in recent decades (Rolhion et al., 2019; Hwanhlem et al., 2017; Collins et al., 2010). Recently, this compound has received great attention due to its high potential for application in the food industry – being used as natural preservatives – as well as being suggested to reduce the indiscriminate use of antibiotics in food products for humans and animals (Sabo et al., 2014). The production of bacteriocins occurs initially as a response mechanism to stimuli or environmental stress generated by microbial competition. They are usually synthesized as inactive pre-peptides with a precursor sequence in the N-terminal region (Xie and Van der Donk, 2004) and transported to the cell surface during the exponential phase and catalyzed into the active form (Aucher, et al., 2005).

Bacteriocins are also widely used in the clinical field, e.g., in the treatment of topical dermatitis (Valenta et al., 1996), stomach ulcers and colon infections and respiratory tract infections (Dicks et al., 2018). Nisin, combined with conventional antibiotics, effectively helped the membrane permeabilization of an enteric multidrug-resistant *Salmonella* strain (Singh et al., 2013). This combination of antibiotics with AMP represents a way to decrease the use of conventional antibiotics in medical applications and help to reduce resistant bacteria (Naghmouchi et al., 2011). Kamarajan et al. (2015) demonstrated that nisin ZP (a naturally occurring variant) reduced tumorigenesis in vivo. In addition, a long-term treatment with nisin ZP extended survival and induced apoptosis dose-dependently in human umbilical vein endothelial cells (HUVEC), with concomitant decrease in vascular sprout formation in vitro and reduction of intratumoral microvessel density in vivo.

Over 70,000 distinct fungi have been identified and some of these can cause serious damage to human health (Li et al., 2012). Since the AMP have pleiotropic functions, i.e., a single molecule has several characteristics that are often unrelated, they can exert strong antifungal activity and could be useful in addressing fungal infections. In addition, many AMPs are viral inhibitors (Jenssen, et al., 2006). This antiviral activity can be related to the direct interaction with the virus or is a result of an indirect effect through interactions with potential target cells. Moreover, there are some studies that show the effects of some AMP against influenza virus and human immunodeficiency virus (HIV) (Tripathi et al., 2013; Barlow et al., 2011).

4 AMP utilization in animal feed

Antibiotics have been used in the animal industry for more than 50 years (Xiao et al., 2015). Although its use brings benefits, the misuse has caused some problems, including the emergence of bacteria resistant to antibiotics and drug residues in meat products (Bacanli and Basaran, 2019).

4.1 Swine

In the swine industry, post weaning diarrhea is a serious problem for production, due to increased mortality and reduced growth performance. Approximately, 50% of piglet mortality due to diarrhea is caused by enterotoxigenic *E. coli* (ETEC) (Cutler et al., 2007). According to United States Department of Agriculture (USDA, 2018), 95.5% of swine farms in United States include subtherapeutic concentrations of antibiotics in young pigs' diets. Despite that, farmers still reported a large occurrence of diarrhea, an unsurprising fact since very often they have seen a broad spectrum of antibiotic resistance among ETEC strains (Lanz, et al., 2003; Maynard, et al., 2004).

A potential alternative to conventional antibiotics is the colicins, a class of bacteriocins produced by and effective against *E. coli*, killing bacteria by disrupting the ionic gradient (FAO, 2020). Recent studies have demonstrated that colicins are effective against ETEC strains from pigs in vitro (Cutler et al., 2007) and against a wide range of *E. coli* strains (Murinda et al., 1996; Lanz et al., 2003). The colicins are not related to any antibiotic used in human medicine and do not leave any kind of

traces in the animals (Cutler et al., 2007). Cutler et al. (2007) tested 2 doses of colicins (11.5 and 16.5 mg of colicin/kg of diet) on weaning pigs and observed a 40% higher weight gain and a 7% lower feed efficiency in the higher dose group compared to the control group (no colicin), showing that the animals have a better performance with colicin even when a small dose was used. Also, the authors did not observe any signs of post weaning diarrhea in the pigs in the higher dose group.

Other types of AMP that have been used are the synthetic AMP A3 and P5 (Yoon et al., 2012 a,b; 2014). Yoon et al. (2012a) evaluated different increasing levels of AMP A3, which were 0, 60 and 90 mg/kg of diet, as an alternative to conventional antibiotics. The study analyzed the growth performance, coefficient of total tract apparent digestibility (CTTAD) of nutrients and intestinal aspects of weanling pigs. A linear improvement on final body weight and an average daily gain, with no effect on average daily feed intake and feed efficiency were found. In addition, they observed a linear improvement on CTTAD of dry matter and crude protein, and no differences were observed between ileal total anaerobic bacteria, *Clostridium* spp. and coliform populations of pigs feed with AMP A3 compared to conventional antibiotics. Therefore, the results showed that AMP A3 is a potential molecule to replace antibiotics used as growth promoters in pigs.

In a parallel work, Yoon et al. (2012b) evaluated the increasing levels of AMP P5 on growth performance, CTTAD and intestinal aspects of weanling pigs as an alternative to conventional antibiotics. They noted a similarity between the groups fed with a conventional antibiotic and with the AMP P5 on average daily gain, also

presenting CTTAD with greater results than the negative control group. The same behavior was observed for fecal coliform concentration. As in the previous work, results also showed that the AMP P5 is a possible potential molecule for replacement to conventional antibiotics used as growth promoters to pigs. Finally, in an association analysis, Yoon et al. (2014) tested both AMP A3 and P5 with a negative control – no antibiotic – and a positive control – a conventional antibiotic. They found that both AMP have potential to improve growth performance, nutrient digestibility, intestinal morphology and to reduce pathogenic bacteria in weanling pigs and could be an alternative to the usual antibiotics used in the swine industry.

Cecropin, an AMP isolated from silkworm *Hyalophora cecropia*, have also been tested on pigs. Wu et al. (2012) evaluated the cecropin AD – a chimeric cecropin AD peptide, which has the first 11 residues from *Hyalophora cecropia* cecropin A and the last 26 residues from *Hyalophora cecropia* cecropin D (Wu et al. 2009) – and a conventional antibiotic on pigs challenged with *E. coli* to investigate whether dietary alteration with AMP application could improve performance, immune defenses and reduce intestinal inflammation. The results indicated that animals fed with a diet containing the AMP cecropin AD improved the performance and reduced the incidence of diarrhea, a similar effect to what they observed from the treatment with conventional antibiotics.

As can be seen, there are some proven strategies with AMP to replace the use of antibiotics as growth promoters. However, further studies are needed to

identify the precise in vivo mechanism of the action of these AMP to allow for safer utilization in animal nutrition.

4.2 Cattle

The rumen is an essential organ for nutrient fermentation due its capacity to produce end-products, particularly short chain fatty acids (SCFA) and microbial protein, the major energy and protein source to ruminants, respectively (Kristensen et al., 2005). Therefore, the more efficient the rumen is, the more end-products are synthetized. For this reason, in recent years, studies in rumen manipulation have widely increased. Antibiotics can increment the rumen efficiency; however, as occurred in swine industry, long-term usage may not be beneficial for ruminants and consumers (Cheema et al., 2011). Therefore, attempts have been made to replace them with better and safer alternatives.

Ruminants, as other animals, produce many AMPs that act as natural innate barriers, limiting microbial infectious diseases and, therefore, have different sizes and mechanism of actions (Brogden et al., 2003). Ruminants present 2 AMP groups, available in different tissues, macrophages, mucosal epithelial cells and polymorphonuclear leukocytes, as follows: 1) anionic peptides – a small group rich in aspartic and glutamic acids, with activity against Gram-negative and Gram-positive bacteria, and 2) cationic peptides – rich in proline and cysteine, with activity against Gram-positive and Gram-negative bacteria (Brodgen et al., 1996; 1997). These

peptides have also antiviral and antifungal activities (Jenssen et al., 2009; Lee-Huang et al., 1999; Arnold, et al., 1980).

Some ruminal Gram-positive bacteria can produce bacteriocins, an inhibitor of other bacteria species (Kalmokoff et al., 1966). A lantibiotic, bovicin HC5, was identified and isolated from *Streptococcus bovis* HC5 by Mantovani et al. (2001) with a large antibacterial activity and no bacterial adaptation demonstrated (Mantovani et al., 2001, 2002). Lee et al. (2002) tested the effects of bovicin HC5 on ruminal methane production in vitro and demonstrated that it can inhibit methane production at pH 6.7. Another study found that bovicin HC5 is even more effective in 5.5 pH, showing that the molecule can be more effective in animals fed grain rather than forage (Mantovani et al., 2002). The same study also tested the capacity of bovicin HC5 to inhibit methane production in a successive fashion. In addition, it was found that microorganisms, which received bovicin HC5, gradually lose their activity, i.e., the methanogen archaea did not adapt to the molecule. Lima et al. (2009) investigated if bovicin HC5 could inhibit the deamination of mixed ruminal bacteria and evaluated if bovicin HC5 and monensin affected the same types of ammonia producing ruminal bacteria. Interestingly, they found that bovicin HC5 and monensin have activity against the same types of bacteria.

4.3 Broiler and Layers

In the poultry industry, the greatest problem raised is based on the presence of *Salmonella* sp., which also affects human public health (Narushin et al., 2020). The

defense mechanisms of both broilers and laying hens have been studied for several years. As a result, it has been possible to identify AMP and classify them into 2 large families, defensins and CATH (Akbari et al., 2008).

Defensins are composed of between 18 and 45 amino acids, predominately comprised of cystines, cationic and hydrophobic residues. They present antimicrobial activity against bacteria, some fungi, protozoa, and even viruses (Sugiarto & Yu, 2004), immunomodulatory, anti-inflammatory, and intermediaries in regeneration of skin wounds (Xiao et al., 2020; Akbari et al., 2008). They are subdivided into α, β and θ, which are differentiated by the disulfide bridge position, size (kDa), and structure (β-sheet dimer or cyclic). Furthermore, in previous studies, it was revealed that chickens, as well as cattle, pigs and humans have β-defensins, but not α- or θ-defensins (Sugiarto & Yu, 2004). Avian β-defensins (AvD) are produced by the activation of encoded genes in response to an external or environmental factor and thus achieve homeostasis. These genes remain inactivated when chickens are not strained or are totally healthy, which is directly related to the presence of pathogenic microorganisms such as *Salmonella* sp. (Narushin et al., 2020; Akbari et al., 2008).

In laying hens, white eggs have been demonstrated to possess antimicrobial proteins and β-defensins. Also, a wide variety of cationically active AMP such as gallinacins, which can interact with pathogens cell membrane, have also been found. In addition, many of these AMP were identified as anti-biofilms that could eliminate microorganisms (Arena et al., 2020). A large list of AMP and other peptides related to anticancer, anti-inflammatory, antihypertensive and antiviral effects were also

identified in chicken yolk plasma (Arena & Scaloni, 2018). Gallinacin 11 has demonstrated an effective response against *S. typhimurium* and *Listeria monocytogenes* in chickens (Higgs et al., 2005).

Some ceratotoxins, such as the cationic AMP, Ctx(Ile^{21})-Ha are a promising peptide applied against *S. enteritidis* and *S. typhimurium*, it also showed great activity against multidrug-resistant bacteria (Roque-Borda et al., 2021a). Innovative systems such as microparticles were successfully applied to control systemic infection caused by *Salmonella* (Roque-Borda et al., 2021b). Likewise, it was reported that these microparticles would be able to reduce the mortality rate of the chicks during the first days of post-infection life and increase their weight with the passing of days (Roque-Borda et al., 2021c).

A previous study has shown that the regulation of AMP was influenced by the presence of probiotics in chickens, because their relationship was inversely proportional (Ma et al., 2020). The gene expression of AMP decreased as the probiotic concentration increased, which indicates that AMP may not be entirely necessary in the presence of probiotics. However, there are still gaps to be studied in this relationship (Schlee et al., 2008; Akbari et al., 2008; Wehkamp et al., 2004). Another important regulator could be yeast culture (YC), where the expression of genes encoding AMP is increased, such as those expressing the AvD 1, 4 and 7 peptides and CATH 1 and 3. These molecules presented beneficial results for aged laying hens, demonstrating how beneficial this combination supplemented with YC can be, and the impact it has with the age of the chickens (Zhang et al., 2020).

In recent published studies on broilers, the AMP Microcin J25 was used against *Salmonella* sp. and *E. coli.* This peptide, showing antimicrobial activity, promoted animal growth performance, which improved the fecal flora composition and intestinal structure, as well as an induction of an efficient immune response (Wang et al., 2020b). Another important agent used was potato proteins, which reduced the presence of coliform bacteria and improved broiler production performance (Ohh et al., 2009). Lactobacillins (Xu et al., 2020), which are polypeptides with antimicrobial activity produced by lactic acid bacteria (LAB), were also studied. *Lactobacillus* bacteriocin plantaricin K (PlnK) is part of this group and was expressed in vitro by genetic engineering. This peptide is particularly selective because it does not eliminate Gram-positive bacteria and exclusively eliminates pathogenic bacteria from the body. In addition to not altering the intestinal flora diversity, this peptide is a specific promoter of intestinal microbial control and has become an option for studies of selective AMP (Xu et al., 2020). A recombinant peptide, plectasin, complements the positive results already mentioned, and has exhibited an increase in duodenal lipase yield and trypsin activity, which are important for the digestion process in broilers (Ma et al., 2020).

Some studies based on broilers have also integrated the use of sublancin, from *B. subtilis* 168, which was shown to have a great biological effect against *Clostridium perfringens*, *S. aureus* and *P. aeruginosa*, also helping the proliferation and count of LAB (Özcan et al., 2019). This recombinant AMP has also been shown to generate immunological memory (e.g., humoral, and cellular) by increasing the

lymphocyte T content ($CD4^+$ to $CD8^+$ ratio), and Newcastle disease antibodies. Therefore, due to these results, a highly effective vaccine could be generated using this AMP as immunity stimulators (Liu et al., 2019).

In addition, it has also been shown that mixed-formulations of AMP and other compounds could have promising effects, such as those that use marine algae-cecropin, when *Laminaria japanica* powders were used as a feed supplement, showing a high growth performance and immune protection (Bai et al., 2019); This mixed effect could be key to the development of new antimicrobial additives as a food source for chickens.

There are other AMP such as magainin (Wang et al., 2016b), nisin (O'Connor et al., 2020), camel lactoferrin “36” and “chimera” (Daneshmand et al., 2019a,b) that are described in literature. These molecules have shown results like those already mentioned in this section. For all these reasons, AMP are essential for the survival and animal protection and become an interesting and valuable alternative for poultry production improvement.

5 Food preservatives

AMP have been applied in food preservation for more than 25 years. The most studied AMP were the bacteriocins produced by the LAB family, as they have potential application in food preservation (as shown in their application in broilers) (Kareem and Razavi, 2020). Among the most studied, plantaricins showed a high rate of microbial inhibition and activity against *S. enteritidis*, *E. coli*, *L.*

monocytogenes, *C. perfringens* and *B. cereus*, among others (Thakur, 2017). Regarding the plantaricin family, some molecules have special features, such as: plantaricin A, inducer of production of other plantaricins (E/F and J/K); plantaricin C, applied in conservation and preservation of cheese and plantaricin S, extracted from fermented green olives (Kareem and Razavi, 2020).

The use of natural preservatives in replacement of chemical agents is an important strategy to increase shelf life of minimally processed fruits and vegetables (da Costa et al., 2019, Przybylski et al., 2016). In Brazil, with the approval of nisin in 1996, the first AMP/bacteriocin was used commercially by the Health Department as a cheese preservative. In addition, pediocin peptide, isolated from *Pediococcus* spp., is mainly used as a preservative for meat products, which protects from heat treatment due to its stability over a wide pH range (2 to 8) and high temperatures (maximum at 121 ºC) (da Costa et al., 2019).

A new peptide recently isolated from broccoli, known as broccoli napin, has revealed a protective activity against pathogenic fungi, such as *Fusarium culmorum* and *Penicillium expansum*, being one of the most problematic pathogens in the agricultural sector. Moreover, the molecule has shown thermal stability, anti-tumor activity, and presented no hemolysis, protecting mainly crops and cereal-based products (Thery et al., 2020).

The α137–141 peptide was obtained from hemoglobin hydrolysis of blood residues of slaughterhouses, a residual by-product of beef production. This peptide has shown a high rate of microbial inhibition and potential antioxidant capacity. Thus,

these functions together are interesting for their application in the food industry (Przybylski et al., 2020). Other AMP, such as those isolated from the house fly (*Musca domestica pupae*) (Md-AMP), which are used to preserve pork (chilled pork), have been found to destabilize the cell membrane and bind to DNA (Dang et al., 2020). The application of some AMP in the food industry has been tested through pilot trials of innovative containers, such as polyethylene terephthalate with the mitochondrial-targeted peptide 1. This molecule was evaluated in meat and dairy foods, demonstrating satisfactory results, corroborating a great progress and efficiency of AMP (Gogliettino et al., 2020). Therefore, these recent discoveries could lead to a promising alternative for food additives.

6 Cattle disease treatments with AMPs

As mentioned, AMP have been considered a new source of biomolecules in several fields of scientific research, given their potential against many pathogenic microorganisms. In addition, the increase of microorganisms' resistance to antibiotics and the inability to discern the mechanisms of inhibition of these microorganisms have become a matter of concern, receiving immediate attention from the pharmaceutical industry to governmental and academic institutions (Lima, 2009). Many pathologies associated with animal production are related to the presence of bacteria, fungi, and viruses. However, they have already been evaluated as a method of prevention and treatment. In this section, AMP applications with successful results

in cases of diseases caused by several types of microorganisms in cattle production are described.

Trichomonas foetus and *T. vaginalis* are parasites that generate enormous economic problems due to their high morbidity rates. These microorganisms can also be transmitted by humans through sexual contact. To overcome this problem, the D-hecate peptide was evaluated, proving to be highly effective against these microorganisms at a concentration of 10 µmole/L, by promoting a serious rupture in plasma membrane (Mutwiri et al., 2000). It also exhibited a great activity in cancer cells, appearing as an alternative in this type of disease in cattle production (Leuschner & Hansel, 2004). Some food additives, such as sodium bicarbonate, helped tritrpticin, also an AMP with high biological effectiveness, against *T. vaginalis* (Infante et al., 2011).

The bovine respiratory syncytial virus is considered a serious risk disease due to economic losses. This virus was counteracted by CATH LL-37 peptide, which is exceptionally attractive for monoclonal antibodies, due to their specificity and ability to induce immunity (Caskey et al., 2019). In vitro studies were carried out with this AMP in human cells and the molecule induced direct damage to the viral envelope, modifying the viral structure and decreasing the binding and interaction with epithelial cells. In addition, studies with murines indicated that an exogenous application would help in the eradication of post-infection disease (Armiento et al., 2020; Currie et al., 2016). Therefore, it is possible to use AMP with antiviral activity when applied in cattle, with possible efficient results.

Bovine respiratory disease complex (BRDC) is one of the most common illnesses in animal production caused by *Mannheimia haemolytica, Histophilus somni* and *Pasteurella multocida.* Bovines have as a natural defense mechanism, the tracheal antimicrobial peptide (TAP), that is affected and inhibited due to the stress generated by hormonal regulation in animal production (Siracusa, 2018; Vulikh et al., 2019). Synthetic TAP was used to evaluate its direct dosage effect via the nasal route. However, results showed no effects and no variance, explained by the amount of mucosa present in the nasal passage, which could be related to peptide instability and degradation (Vulikh et al., 2019). In this way, the current challenge is to use carriers or formulations for peptide interaction with the target pathogenic bacteria, with no destabilization or degradation. Another study evidenced the antimicrobial activity of bovine NK-lysine (natural killer cell origin and lytic properties) derived peptides, such as NK1, NK2A, NK2B and NK2C exhibiting activity against BRDC, which demonstrated excellent results, modifying the structure of the cell membrane, and causing a cytosolic effusion (Dassanayake et al., 2017).

In summary, these studies indicate that the use of peptides in animal models or in vitro studies could be taken as a reference for the development of novel and safer molecules for the improvement of livestock production.

7 Public health implications and future implications

The discovery of antibiotics in 1928, by Alexander Fleming, helped to improve the life expectancy of humankind (Hu et al., 2020). Following the increasing use of

antibiotics over time, antimicrobial resistance became a severe public health problem. In recent years, antibiotics used against some diseases such as *K. pneumoniae* and *E. coli* are no longer effective in at least 50% of treated people (Peleg et al., 2010; Shaikh et al., 2015). Moreover, the last option for gonorrhea medical treatment has failed in at least 10 countries (WHO, 2019). The lack of policies to control the use of antibiotics, their misuse by humans and in livestock production have helped to achieve this threatening scenario.

Since 2015, AMR has become a worldwide priority. The WHO created the "Global Action Plan on Antimicrobial Resistance" to encourage a wise use of antibiotics and some strategies to reduce their consumption. This plan of action identified that some common medical conditions, such as tuberculosis, HIV, malaria, sexually transmitted diseases, urinary tract infections, pneumonia, blood-stream infections, and food poisoning have become resistant to a large number of conventional antibiotics (Roque-Borda et al., 2021d). Consequently, this fact has forced medical practitioners to use so-called "last-resort" drugs, which are expensive and mostly unavailable in poor countries.

Therefore, the urgent need for the development of new antimicrobials, mainly natural ones, is a powerful strategy to minimize the indiscriminate use of conventional antibiotics and could help treat and overcome some diseases, thus improving our quality of life.

8 Conclusions

Advances in animal production have demanded the increase of use of additives for productivity and to avoid mortality by pathogens. However, the increasing application of antibiotics and other growth promoters has revealed unfortunate “side effects”: the appearance of AMR, multidrug resistant bacteria, and hazardous risks for human health. To tackle this broad-spectrum problem at different levels, this review presents and discusses an interesting alternative among several researched molecules: the AMP. These compounds, which have been proven to be efficient and promising, have shown biological activity and applicability against several microorganisms, mainly in the areas of animal production, as well as being suitable for food preservation. The multiple properties of AMP make them optimistic and powerful candidates to replace conventional drugs. Moreover, the development of new natural peptide-based antimicrobials for livestock, swine and poultry can help to reduce the antimicrobial problem without affecting either animal production or human health, nor leaving any pharmacological residues that generate environmental impact issues.

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Author contributions

Raiza Felismino Silveira: conceptualization, investigation, writing – original draft. **Cesar Augusto Roque Borda**: investigation, writing – original draft. **Eduardo Festozo Vicente**: writing – review and editing, supervision, project administration, funding acquisition.

Conflict of interests

The authors declare that there is no conflict of interest.

References

ABIEC (Associação Brasileira das Indústrias Exportadoras de Carne). (2019). Beef Report 2019. Available on http://abiec.com.br/publicacoes/beef-report-2019/

Akbari, M. R., Haghighi, H. R., Chambers, J. R., Brisbin, J., Read, L. R., Sharif, S. **Expression of antimicrobial peptides in cecal tonsils of chickens treated with probiotics and infected with Salmonella enterica serovar typhimurium.** Clinical Vaccine Immunology, 15(11), 1689-1693 (2008). DOI: 10.1128/CVI.00242-08

Andreu, D., Merrifield, R.B., Steiner, H., Boman, H.G. **N-terminal analogues of cecropin A: Synthesis, antibacterial activity, and conformational properties**. Biochemistry. 24, 1683–1688 (1985). DOI: 10.1021/bi00328a017

Arena, S., Scaloni, A. **An extensive description of the peptidomic repertoire of the hen egg yolk plasma.** Journal of agricultural and food chemistry, 66(12), 3239-3255 (2018). DOI: 10.1021/acs.jafc.8b01183

Arena, S., Renzone, G., Scaloni, A. **A multi-approach peptidomic analysis of hen egg white reveals novel putative bioactive molecules and food markers of storage.** Journal of Proteomics, 103646 (2020). DOI: 10.1016/j.jprot.2020.103646

Arnold R .R., M. Brewer, J.J. Gauthier. **Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms**, Infection and Immunity. 28 (1980),893-898.

Armiento, V., Hille, K., Naltsas, D., Lin, J. S., Barron, A. E., Kapurniotu, A. **The human cathelicidin LL-37 is a nanomolar inhibitor of amyloid self-assembly of islet amyloid polypeptide (IAPP)**. Angewandte Chemie (2020). DOI: 10.1002/ange.202000148

Aucher, W., Lacombe, C., Héquet, A., Frère, J., Berjeaud, J. **Influence of Amino Acid Substitutions in the Leader Peptide on Maturation and Secretion of Mesentericin Y105** by Leuconostoc mesenteroides Journal of Bacteriology, 187 (2005). 2218-2223. DOI: 10.1128/JB.187.6.2218-2223.2005

Bacanli, M., Basaran, N. **Importance of antibiotic residues in animal food.** Food and Chemical Toxicology. 125 (2019). DOI: 10.1016/j.fct.2019.01.033

Bai, J., Wang, R., Yan, L., Feng, J. **Co-Supplementation of Dietary Seaweed Powder and Antibacterial Peptides Improves Broiler Growth Performance and Immune Function.** Brazilian Journal of Poultry Science, 21 (2019). DOI: 10.1590/1806-9061-2018-0826

Barlow, P. G., Svoboda, P., Mackellar, A., Nash, A. A., York, I. A., Pohl, J., Davidson, D. J., Donis, R. O. **Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37.** PLoS ONE. 6 (2011) e25333. DOI: 10.1371/journal.pone.0025333

Boman, H. G. **Peptides antibiotics and their role in innate immunity.** Annual Review of Immunology, 13 (1995) 61-92. DOI: 10.1146/annurev.iy.13.040195.000425

Broekaert, W. F., Cammue, B. P. A., De Bolle, M. F. C., Thevissen, K., De Samblanx, G. e Osborn, R. W. **Antimicrobial peptides from plants**. Critical Reviews in Plant Sciences, 16 (1997), 297-323. DOI: 10.1080/07352689709701952

Brogden K. A., Mark A., McCray, P. B., Tack, B. F. **Antimicrobial peptides in animals and their role in host defences**. International Journal of Antimicrobial Agents, 22 (2003) 465-478. DOI: 10.1016/S0924-8579(03)00180-8

Brogden, K. A., Ackermann, M., Huttner, K. M. **Small, anionic, and charge-neutralizing propeptide fragments of zymogens are antimicrobial**. Antimicrobial Agents Chemistry, 41 (1997) 1615- 1617.

Brogden, K. A., De Lucca, A. J., Bland J., Elliott S. **Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for Pasteurella haemolytica**. Proceedings of the National Academy of Sciences USA, 93 (1996) 412-416.

Brogden, K. A. **Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?** Nature Review (2005) 238-250. DOI: 10.1038/nrmicro1098

Caskey, M., Klein, F., Nussenzweig, M. C. **Broadly neutralizing anti-HIV-1 monoclonal antibodies in the clinic.** Nature Medicine 25 (2019), 547–553. DOI: 10.1038/s41591-019-0412-8

Castro, M. S., Ferreira, T. C. G., Cilli, E. M., Crusca Jr, E., Mendes-Giannini, M. J. S., Sebben, A., & Fontes, W. **Hylin a1, the first cytolytic peptide isolated from the arboreal South American frog *Hypsiboas albopunctatus* ("spotted treefrog").** Peptides, 30 (2009) 291-296. DOI: 10.1016/j.peptides.2008.11.003

Cespedes, G. F., Lorezón, E. N., Vicente, E. F., Mendes-Giannini, M. J. S., Fontes, W., Castro, M. S., Cilli, E. M. **Mechanism of Action and Relationship Between Structure and Biological Activity of Ctx-Ha: a new ceratotoxin-like peptide from *Hypsiboas albopunctatus***. Protein & Peptide Letters, 19 (2012). DOI: 10.2174/092986612800494011

Chassy BM. **Food safety risks and consumer health.** N Biotechnol 27 (2010)534–44. https://doi.org/10.1016/j.nbt.2010.05.018.

Cheema, U. B., Younas, M., Sultan, J. I., Iqbal, A., Tariq, M., Waheed, A. **Antimicrobial Peptides: An alternative of Antibiotics in Ruminants**. Advances in Agricultural Biotechnology 2 (2011) 15-21.

Chen, X., Zhu, F., Cao, Y., Qiao, S. **Novel expression vector for secretion of cecropin AD in Bacillus subtilis with enhanced antimicrobial activity**. Antimicrob Agents Chemother. 15 (2009) 3683-3689 DOI: 10.1128/AAC.00251-09.

Coleman-Jensen A, Rabbitt MP, Gregory CA. **USDA ERS - Measurement**. Econ Res Serv (2020). https://www.ers.usda.gov/topics/food-nutrition-assistance/food-security-in-the-us/measurement.aspx (accessed October 22, 2020).

Collins, B., Cotter, P. D., Ross, R. P. **Applications of lactic acid bacteria-produced bacteriocins**. In. F. Mozzi, R. R. Raya and G. M. Vignolo (Eds). Biotechnology of lactic acid bacteria: Novel applications (2010) 89-109.

Conchon, F. L. e Lopes, M. A. **RASTREABILIDADE E SEGURANÇA ALIMENTAR.** Boletim Técnico - n.º 91 - p. 1-25 (2012). Lavras/MG. GOVERNO DO BRASIL.

Currie, S. M., Findlay, E. G., McFarlane, A. J., Fitch, P. M., Böttcher, B., Colegrave, N., Davidson, D. J.. **Cathelicidins have direct antiviral activity against respiratory syncytial virus in vitro and protective function in vivo in mice and humans**. The Journal of Immunology, 196 (2016), 2699-2710. DOI: 10.4049/jimmunol.1502478

Cutler, S. A., Lonergan, S. M., Cornick, N., Johnson, A. K., Stahl, C. H. **Dietary inclusion of colicin e1 is effective in preventing postweaning diarrhea caused by F18-positive Escherichia coli in pigs.** Antimicrobial Agents and Chemotherapy, 51 (2007) 3830–3835. 10.1128/AAC.00360-07

Dassanayake, R. P., Falkenberg, S. M., Briggs, R. E., Tatum, F. M., & Sacco, R. E. **Antimicrobial activity of bovine NK-lysin-derived peptides on bovine respiratory pathogen Histophilus somni**. PLoS One, 12 (2017), e0183610. DOI: 10.1371/journal.pone.0183610

Daneshmand, A., Kermanshahi, H., Sekhavati, M.H. et al. **Antimicrobial peptide, cLF36, affects performance and intestinal morphology, microflora, junctional proteins, and immune cells in broilers challenged with E. coli.** Scientific Reports 9 (2019a), 14176. DOI: 10.1038/s41598-019-50511-7

Daneshmand, A., Kermanshahi, H., Sekhavati, M. H., Javadmanesh, A., Ahmadian, M., Alizadeh, M., & Aldavoodi, A. **Effects of cLFchimera, a recombinant antimicrobial peptide, on intestinal morphology, microbiota, and gene expression of immune cells and tight junctions in broiler chickens challenged with C. perfringens.** bioRxiv (2019b) 871467. DOI: 10.1101/871467

Dang, X., Zheng, X., Wang, Y., Wang, L., Ye, L., Jiang, J. **Antimicrobial peptides from the edible insect Musca domestica and their preservation effect on chilled pork.** Journal of Food Processing and Preservation, 44 (2020) e14369. DOI: 10.1111/jfpp.14369

da Costa, R. J., Voloski, F. L., Mondadori, R. G., Duval, E. H., & Fiorentini, Â. M. **Preservation of meat products with bacteriocins produced by lactic acid bacteria isolated from meat.** Journal of Food Quality, (2019). DOI: 10.1155/2019/4726510

Dean, S. N., Bishop, B. M., van Hoek, M. L. **Susceptibility of pseudomonas aeruginosa biofilm to α-helical peptides: D-enantiomer of LL-37.** Frontiers in Microbiology 2 (2011) 128. DOI: 10.3389/fmicb.2011.00128

De Vuyst, L., Leroy, F. **Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications.** Journal of Molecular Microbiology and Biotechnology 13 (2007) 194-9. DOI: 10.1159/000104752

Dicks, L., Dreyer, L., Smith, C., van Staden, A. D. **A Review: The Fate of Bacteriocins in the Human Gastro-Intestinal Tract: Do They Cross the Gut-Blood Barrier?** Frontiers in microbiology, 9 (2018) 2297. DOI: 10.3389/fmicb.2018.02297

Duin, D., Paterson, D. **Multidrug Resistant Bacteria in the Community: Trends and Lessons Learned.** Infectious Disease Clinics of North America. 30 (2016) 377–390. DOI:10.1016/j.idc.2016.02.004

Estrela, T. S. **Resistência antimicrobiana: enfoque multilateral e resposta brasileira.** In: Saúde e Política Externa: os 20 anos da Assessoria de Assuntos Internacionais de Saúde (1998-2018). (2018) 307-327

Fan L., Sun J., Zhou M., Zhou J., Lao X., Zheng H., Xu H. **DRAMP: a comprehensive data repository of antimicrobial peptides**. Scientific Reports. 14 (2016) 24482. PMID: 27075512

FAO. **Gateway to poultry production and products** |. Food Agric Organ United Nations 2020. http://www.fao.org/poultry-production-products/production/en/ (accessed October 22, 2020).

Fredericq, P. Colicins Annual Review of Microbiology 11 (1957) 7–22.

Gallo, R. L., Murakami, M., Ohtake, T. e Zaiou, M. **Biology and clinical relevance of naturally occuring antimicrobial peptides**. Colicins Annual Review of Microbiology, 110 (2002). 823-831. DOI:10.1067/mai.2002.129801

Gabay, J. E. **Ubiquitous natural antibiotics**. Science, 264 (1994) 373-374. DOI: 10.1126/science.8153623

Gogliettino, M., Balestrieri, M., Ambrosio, R. L., Anastasio, A., Smaldone, G., Proroga, Y. T., Palmieri, G. **Extending the Shelf-Life of Meat and Dairy Products via PET-Modified Packaging Activated With the Antimicrobial Peptide MTP1**. Frontiers in Microbiology, 10 (2020) 2963. DOI: 10.3389/fmicb.2019.02963

Gratia A. **Sur un remarquable example d'antagonisme entre deux souches de colibacille.** Compt. Rend. Soc. Biol, 93 (1925), 1040-1042. Referred from: Gratia, J.P. (2000) Andre Gratia: A Forerunner in Microbial and Viral Genetics. *Genetics*, 156, 471-476.

Heneghan C. **Food safety top concern for consumers: survey.** Food Dive (2015). https://www.fooddive.com/news/food-safety-top-concern-for-consumers-survey/407395/ (accessed October 22, 2020).

Hultmark, D. **Immune reactions in Drosophila and other insects: a model for innate immunity.** Trends in Genetics, 9 (1993), 178-182. DOI: 10.1016/0168-9525(93)90165-e

Hu, X., Logue, M., Robinson, N. **Antimicrobial resistance is a global problem–a UK perspective.** European Journal of Integrative Medicine 36 (2020), 101136. DOI: 10.1016/j.eujim.2020.101136

Higgs, R., Lynn, D.J., Gaines, S. et al. **The synthetic form of a novel chicken β-defensin identified in silico is predominantly active against intestinal pathogens.** Immunogenetics 57 (2005) 90–98. DOI: 10.1007/s00251-005-0777-3

Hoffmann, J. A. **Innate immunity of insects.** Current Opinion in Immunology 7 (1995), 4–10. DOI: 10.1016/0952-7915(95)80022-0

Hoffmann, J.bA., Hetru, C. **Insect defensins: Inducible antibacterial peptides**. Immunology Today. 13 (1992), 411–415. DOI: 10.1016/0167-5699(92)90092-L

Hwanhlem, N., Ivanova, T., Biscola, V., Choiset, Y., Hartlé, T. **Bacteriocin producing Enterococcus faecal isisolated from chicken gastrointestinal tract originating from Phitsanulok, Thailand: Isolation, screening, safety evaluation and probiotic properties**. Food Control 78 (2017), 187-195

Infante, V. V., Miranda-Olvera, A. D., De Leon-Rodriguez, L. M., Anaya-Velazquez, F., Rodriguez, M. C., Avila, E. E. **Effect of the antimicrobial peptide tritrpticin on the in vitro viability and growth of trichomonas vaginalis.** Current microbiology, 62 (2011), 301-306. doi.org/10.1007/s00284-010-9709-z

Izadpanah, A. e Gallo, R. L. **Antimicrobial peptides.** Journal of the American Academy of Dermatology, 52 (2005) 381-390. DOI: 10.1016/j.jaad.2004.08.026

Jenssen H., Robert E.W & Hancock. **Antimicrobial properties of lactoferrin.** Biochimie 91 (2009), 19-29. DOI: 10.1016/j.biochi.2008.05.015

Jenssen, H., Hamill, P., Hancock, R.E. **Peptide antimicrobial agents.** Clinical Microbiology Reviews 19 (2006), 491–511. DOI: 10.1128/CMR.00056-05

Kalmokoff, M. L., Bartlett, F., Teather, R. M. **Are ruminal bacteria armed with bacteriocins?** Journal of Dairy Science 79 (1996), 2297±2306.

Kamarajan, P., Hayami, T., Matte, B., Liu, Y., Danciu, T., Ramamoorthy, A., Worden, F., Kapila, S., Kapila, Y. **Nisin ZP, a Bacteriocin and Food Preservative, Inhibits Head and Neck Cancer Tumorigenesis and Prolongs Survival.** PLoS One. 10 (2015) e0131008. DOI: 10.1371/journal.pone.0131008. PMID: 26132406; PMCID: PMC4489501.

Kang, X., Dong, F., Shi, C., Zheng, H. et al. **DRAMP 2.0, an updated data repository of antimicrobial peptides.** Scientific Data, 6 (2019) 148. PMID:31409791

Kareem, A. R., Razavi, S. H. **Plantaricin bacteriocins: As safe alternative antimicrobial peptides in food preservation—A review.** Journal of Food Safety, 40 (2020) e12735. DOI: doi.org/10.1111/jfs.12735

Kristensen, N. B., Huntington, G. B., Harmon, D. L. **Chapter 17 Splanchnic carbohydrate and energy metabolism in growing ruminants**11Approved as publication No. 02-07-97 by the Kentucky Agricultural Experiment Station., (Eds): D.G. Burrin, H.J. Mersmann, Biology of Growing Animals, 3 (2005), 405-432, DOI: 10.1016/S1877-1823(09)70024-4.

Lai, Y., Gallo, R.L. **AMPed up immunity: How antimicrobial peptides have multiple roles in immune defense.** Trends Immunology 30 (2009) 131–141. DOI: 10.1016/j.it.2008.12.003

Lanz, R., P. K., Boerlin, P. **Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland**. Veterinary Microbiology 91 (2003),73–84. DOI: 10.1016/S0378-1135(02)00263-8

Lee, S. S., Hsu, J., Mantovani, H. C., Russell, J. B. **The effect of bovicin HC5, a bacteriocin from *Streptococcus bovis* HC5, on ruminal methane production in vitro**. FEMS Microbiology Letters, 217 (2002) 51–55. DOI: 10.1111/j.1574-6968.2002.tb11455.x

Lee-Huang, S., Huang, P. L., Sun, Y., Kung, H. F., Blithe, D. L., Chen, H. C. **Lysozyme and RNases as anti-HIV components in beta-core preparations of human chorionic gonadotropin.** Proceedings of the National Academy of Sciences (U S A). 96 (1999) 2678-2681.

Lehrer, R. I., Lichtenstein, A. K. e Ganz, T. **Defensins: antimicrobial and cytotoxic peptides of mammalian cells.** Annual Review of Immunology, 11 (1993) 105-128. DOI: 10.1146/annurev.iy.11.040193.000541

Leuschner, C., Hansel, W. **Membrane disrupting lytic peptides for cancer treatments**. Current pharmaceutical design, 10 (2004) 2299-2310. DOI: 10.2174/1381612043383971

Li, Y., Xiang, Q., Zhang, Q., Huang, Y., Su, Z. **Overview on the recent study of antimicrobial peptides: Origins, functions, relative mechanisms and application**. Peptides. 37 (2012) 207–215. DOI: 10.1016/j.peptides.2012.07.001

Lima, J. R., Ribon, A. O. B., Russel, J. B., Mantovani, H. C. **Bovicin HC5 inhibits wasteful amino acid degradation by mixed ruminal bactéria invitro**. FEMS Microbiology Letters 292 (2009) 78–84. DOI:10.1111/j.1574-6968.2008.01474.x

Lin, Y., Hu, N., Lyu, P., Ma, J., Wang, L., Zhou, M., Guo, S., Chen, T., Shaw, C. **Hylaranins: Prototypes of a new class of amphibian antimicrobial peptide from the skin secretion of the oriental broad-folded frog, Hylarana latouchii.** *Amino* Acids. 46 (2014) 901–909. DOI: 10.1007/s00726-013-1655-1

Liu, Y., Zhang, J., Wang, S., Guo, Y., He, T. y Zhou, R. **Una nueva "sublancina" adyuvante mejora la respuesta inmune en pollos de engorde específicos libres de patógenos inoculados con la vacuna contra la enfermedad de Newcastle.** Journal of Immunology Research. (2019). DOI: 10.1155/2019/1016567

Liu S., Bao J., Lao X., Zheng H. **Novel 3D Structure Based Model for Activity Prediction and Design of Antimicrobial Peptides.** Scientific Reports. 25 (2018) 11189. PMID:30046138

Liu S., Fan L., Sun J., Lao X., Zheng H. **Computational resources and tools for antimicrobial peptides.** Journal of Peptide Science 23 (2017) 4-12. PMID:27966278

Lorenzon, E. N., Cespedes, G. F., Vicente, E. F., Nogueira, L. G., Bauab, T. M., Castro, M. S., Cilli, E. M. **Effects of Dimerization on the Structure and Biological Activity of Antimicrobial Peptide Ctx-Ha.** Antimicrobial Agents and Chemotherapy (Print), 56 (2012), 3004-3010.

Ma, J.L., Zhao, L.H., Sun, D.D. et al. **Effects of Dietary Supplementation of Recombinant Plectasin on Growth Performance, Intestinal Health and Innate Immunity Response in Broilers. Probiotics & Antimicro.** Proteomics. 12 (2020), 214–223. DOI: 10.1007/s12602-019-9515-2

Magana, M., Pushpanathan, M., Santos, A. L., Leanse, L., Fernandez, M., Ioannidis, A. **The value of antimicrobial peptides in the age of resistance.** Lancet Infect Dis. 20 (2020) e216–30. https://doi.org/10.1016/S1473-3099(20)30327-3.

Mantovani, H.C., Hu, H., Worobo, R.W. and Russell, J.B. **Purification and characterization of a bacteriocin (bovicin HC5) from Streptococcus bovis HC5**. Microbiology (in press). (2002).

Mantovani, H. C., Kam, D. K., Ha, J. K. & Russell, J. B. **The antibacterial activity and sensitivity of *Streptococcus bovis* strains isolated from the rumen of cattle.** FEMS Microbiology Ecology. 37 (2001) 223±229. DOI: 10.1016/S0168-6496(01)00166-0

Maynard, C., S. Bekal, F. Sanschagrin, R. C. Levesque, R. Brousseau, L. Masson, S. Lariviere, and J. Harel. **Heterogeneity among virulence and antimicrobial resistance gene profiles of extraintestinal *Escherichia coli* isolates of animal and human origin.** Journal of Clinical Microbiology. 42 (2004) 5444–5452. DOI: 10.1128/JCM.42.12.5444-5452.2004

Maria-Neto, S., De Almeida, K. C., Macedo, M. L. R., Franco, O. L. **Understanding bacterial resistance to antimicrobial peptides: From the surface to deep inside.** Biochim Biophys Acta – Biomembr. 1848 (2015) 3078–88. https://doi.org/10.1016/j.bbamem.2015.02.017.

Menkem, Z. E., Ngangom, B. L., Tamunjoh, S. S. A., Boyom, F. F. **Antibiotic residues in food animals: Public health concern.** Acta Ecol Sin. 39 (2019) 411–5. https://doi.org/10.1016/j.chnaes.2018.10.004.

Meyer, N. F., Erickson, G. E., Klopfenstein, T. J., Greenquist, M. A., Luebbe, M. K., Williams, P., Engstrom, M. A. **Effect of essential oils, tylosin, and monensin on finishing steer performance, carcass characteristics, liver abscesses, ruminal fermentation, and digestibility**. Journal of Animal Science. 87 (2009), 2346-2354. DOI: 10.2527/jas.2008-1493

Morgan, A., Winck, C. A., Gianezinim M. **A influência da rastreabilidade na cadeia produtiva brasileira de carne bovina.** Espacios, 37 (2016) 20.

Moya, D., Calsamiglia, S., Ferret, A., Blanch, M., Fandiño, J. I., Castillejos, L., Yoon, I. **Effects of dietary changes and yeast culture (*Saccharomyces cerevisiae*) on rumen microbial fermentation of Holstein heifers.** Journal of Animal Science. 87 (2009) 2874-2881. DOI: 10.2527/jas.2008-1446

Mulani, M. S., Kamble, E. E., Kumkar, S. N., Tawre, M. S., Pardesi, K. R. **Emerging Strategies to Combat ESKAPE Pathogens in the Era of Antimicrobial Resistance: A Review.** Frontiers in Microbiology. 10 (2019), 539. DOI: 10.3389/fmicb.2019.00539

Murinda, S. E., R. F. Roberts, and R. A. Wilson. **Evaluation of colicins for inhibitory activity against diarrheagenic *Escherichia coli* strains, including serotype O157:H7.** Applied and Environmental Microbiology. 62 (1996), 3196–3202. PMC168116

Mutwiri, G. K., Henk, W. G., Enright, F. M., & Corbeil, L. B. **Effect of the antimicrobial peptide, D-hecate, on trichomonads.** Journal of Parasitology, 86 (2000) 1355-1359. DOI: 10.1645/0022-3395(2000)086[1355:EOTAPD]2.0.CO,2

Naghmouchi, K., Lay, C., Baah, J., Drider, D. **Antibiotic and antimicrobial peptide combinations: Synergistic inhibition of Pseudomonas fluorescens and antibiotic-resistant variants.** Research in microbiology. 163 (2011) 101-8. DOI: 10.1016/j.resmic.2011.11.002

Narushin, V. G., Laptev, G. Y., Yildirim, E. A., Ilina, L. A., Filippova, V. A., Kochish, I. I., ... & Dunyashev, T. P. **Modelling effects of phytobiotic administration on coherent responses to Salmonella infection in laying hens.** Italian Journal of Animal Science, 19 (2020) 282-287. DOI: 10.1080/1828051X.2020.1733445

O'Connor, P. M., Kuniyoshi, T. M., Oliveira, R. P., Hill, C., Ross, R. P., & Cotter, P. D. **Antimicrobials for food and feed, a bacteriocin perspective.** Current Opinion in Biotechnology, 61 (2020) 160-167. DOI: 10.1016/j.copbio.2019.12.023

Ohh, S. H., Shinde, P. L., Jin, Z., Choi, J. Y., Hahn, T. W., Lim, H. T., Chae, B. J. **Potato (Solanum tuberosum L. cv. Gogu valley) protein as an antimicrobial agent in the diets of broilers.** Poultry science, 88 (2009) 1227-1234. DOI: 10.3382/ps.2008-00491

O'Neill J. **The Review on Antimicrobial Resistance.** "Tackling a crisis for the health and wealth of nations". (2014) London, UK.

Özcan, M. A., Gülüm, L., Midilli, M., & Gören, M. **The Using of Antimicrobial Peptides in Broiler Feeding.** Turkish Journal of Agriculture-Food Science and Technology, 7 (2019) 1-6. DOI: 10.24925/turjaf.v7i1.1-6.1275

Peleg, Y., Hooper, D.C. **Hospital-acquired infections due to Gram-negative bacteria**, The New England journal of medicine 362 (2010) 1804–1813. DOI: 10.1056/nejmra0904124

Peschel, A., Sahl, H. G. **The co-evolution of host cationic antimicrobial peptides and microbial resistance**. Nature Reviews Microbiology. 4 (2006) 529–536. DOI: 10.1038/nrmicro1441

Pirtskhalava, M., Gabrielian, A., Cruz, P., Griggs, H.L., Squires, R.B., Hurt, D.E., Grigolava, M., Chubinidze, M., Gogoladze, G., Vishnepolsky, B., Alekseev, V., Rosenthal, A., & Tartakovsky, M. **DBAASP v.2: an Enhanced Database of Structure and Antimicrobial/Cytotoxic Activity of Natural and Synthetic Peptides**. Nucleic Acids Research. 44 (2016) D1104-D1112.

Possenti, R. A., Franzolin, R., Schammas, E. A., Demarchi, J. J. A. A., Frighetto, R. T. S., Lima, M. A. **Efeitos de dietas contendo *Leucaena leucocephala* e *Saccharomyces cerevisiae* sobre a fermentação ruminal e a emissão de gás metano em bovinos.** Revista Brasileira de Zootecnia. 37 (2008) 1509-1516. DOI: 10.1590/S1516-35982008000800025.

Przybylski, R., Bazinet, L., Firdaous, L., Kouach, M., Goossens, J. F., Dhulster, P., & Nedjar, N. **Harnessing slaughterhouse by-products: From wastes to high-added value natural food preservative.** Food chemistry. 304 (2020) 125448. DOI: 10.1016/j.foodchem.2019.125448

Przybylski, R., Firdaous, L., Châtaigné, G., Dhulster, P., & Nedjar, N. **Production of an antimicrobial peptide derived from slaughterhouse by-product and its potential application on meat as preservative.** Food chemistry, 211 (2016) 306-313. DOI: 10.1016/j.foodchem.2016.05.074

Rolhion N, Chassaing B, Nahori MA, et al. **A Listeria monocytogenes Bacteriocin Can Target the Commensal Prevotella copri and Modulate Intestinal Infection**. Cell Host Microbe. 26 (2019) 691-701. DOI: 10.1016/j.chom.2019.10.016

Roque-Borda, C. A., Silva, H. R. L., Junior, E. C., Serafim, J. A., Meneguin, A. B., Chorilli, M., ... & Vicente, E. F. (2021a). **Alginate-based microparticles coated with HPMCP/AS cellulose-derivatives enable the Ctx(Ile^{21})-Ha antimicrobial peptide application as a feed additive.** International Journal of Biological Macromolecules, 183, 1236-1247. https://doi.org/10.1016/j.ijbiomac.2021.05.011

Roque-Borda, C. A., Saraiva, M. D. M. S., Monte, D. F., Alves, L. B. R., de Almeida, A. M., Ferreira, T. S., ... & Vicente, E. F. (2021b). **Application of HPMCAS-coated Ctx(Ile^{21})-Ha peptide microparticles as a potential use to prevent systemic infection caused by Salmonella Enteritidis in poultry.** BioRxiv, 2021.03.19.436179. https://doi.org/10.1101/2021.03.19.436179

Roque-Borda C. A., Pereira LP, Guastalli EAL, Soares NM, Mac-Lean PAB, Salgado DD, et al. (2021c). **HPMCP-Coated Microcapsules Containing the Ctx(Ile^{21})-Ha Antimicrobial Peptide Reduce the Mortality Rate Caused by Resistant Salmonella Enteritidis in Laying Hens.** Antibiotics;10:616. https://doi.org/10.3390/antibiotics10060616

Roque-Borda CA, Silva PB da, Rodrigues MC, Azevedo RB, Filippo L Di, Duarte JL, et al. (2021d). **Challenge in the Discovery of New Drugs: Antimicrobial Peptides against WHO-List of Critical and High-Priority Bacteria.** Pharm 2021, Vol 13, Page 773 2021;13:773. https://doi.org/10.3390/pharmaceutics13060773

Russell, J.B., Mantovani, H.C. **The bacteriocins of ruminal bacteria and their potential as an alternative to antibiotics.** Journal of Molecular Microbiology and Biotechnology. 4 (2005) 347-355.

Sabo, S. S, Vitolo, M., González, J. M. D., and de Souza Oliveira, R. P. **Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria.** Food Research International 64 (2014) 527–536. DOI: 10.1016/j.foodres.2014.07.041

Schlee, M., Harder, J., Köten, B., Stange, E. F., Wehkamp, J., & Fellermann, K. **Probiotic lactobacilli and VSL# 3 induce enterocyte β-defensin 2.** Clinical & Experimental Immunology, 151 (2008) 528-535. DOI: 10.1111/j.1365-2249.2007.03587.x

Shaikh, S., Fatima, J., Shakil, S., Rizvi, S. M., Kamal, M. A. **Antibiotic resistance and extended spectrum beta-lactamases:Types, epidemiology and treatment**, Saudi journal of biological sciences 22 (2015) 90–101. DOI: 10.1016/j.sjbs.2014.08.002

Shewry, P.R. e Lucas, J.A. **Plant proteins that confer resistance to pest and pathogens**. Advances in Botanical Research. 26 (1997) 135-192. DOI: 10.1016/S0065-2296(08)60120-2

Shiga, K. **Ueber den Dysenteriebacillus *(Bacillus dysenteriae)*, *Zentralbl. Bakteriol. Parasitenkd.*** Abt. I Orig. 24 (1898) 817–824.

Silvestro, L., Weiser, J.N., Axelsen, P.H. **Antibacterial and antimembrane activities of cecropin A in Escherichia coli.** Antimicrob. Agents Chemotherapy. 44 (2000) 602–607. DOI: 10.1128/aac.44.3.602-607.2000

Simmaco, M., Mignogna, G., Barra, D. **Antimicrobial peptides from amphibian skin: What do they tell us?** Biopolymers. 47 (2004) 435–450. DOI: 10.1002/(SICI)1097-0282(1998)47:6<435::AID-BIP3>3.0.CO;2-8

Singh, R., Yadav, A. S., Tripathi, V., Singh, R. P. **Antimicrobial resistance profile of Salmonella present in poultry and poultry environment in North India**. Food Control. 33 (2013) 545-548.
DOI: http://dx.doi.org/10.1016/j.foodcont.2013.03.041

Siracusa, L. **Mecanismos de la supresión mediada por glucocorticoides de la expresión del péptido antimicrobiano traqueal en células epiteliales traqueales bovinas** (disertación doctoral) (2018).

Sugiarto, H., & Yu, P. L. **Avian antimicrobial peptides: the defense role of β-defensins**. Biochemical and biophysical research communications. 323 (2004) 721-727. DOI: 10.1016/j.bbrc.2004.08.162

Teng, Q., Luo, X., Lei, Z., Wang, J., Wurpel, J., Qin, Z., Yang, D. **The multidrug resistance-reversing activity of a novel antimicrobial peptide.** Cancers. 12 (2020) 1963. DOI: 10.3390/cancers12071963

Thakur, A. **Evaluation of antimicrobial activity of bacteriocin producing lactic acid bacteria isolated from dairy products and to assess their role in enhancing the shelf life of food** (Doctoral dissertation, UHF, NAUNI) (2017).

Thery, T., Lynch, K. M., Zannini, E., & Arendt, E. K. **Isolation, characterisation and application of a new antifungal protein from broccoli seeds–New food preservative with great potential.** Food Control. 107356 (2020). DOI: 10.1016/j.foodcont.2020.107356

Tripathi, S., Tecle, T., Verma, A., Crouch, E., White, M., Hartshorn, K.L. **The human cathelicidin LL-37 inhibits influenza A viruses through a mechanism distinct from that of surfactant protein D or defensins.** Journal of General Virology. 94 (2013) 40–49. DOI: 10.1099/vir.0.045013-0

Valenta, C., Bernkop-Schnürch, A., Rigler, H. P. **The antistaphylococcal effect of nisin in a suitable vehicle: a potential therapy for atopic dermatitis in man.** Journal of Pharmacy and Pharmacology, 48 (1996) 988-991. DOI: 10.1111/j.2042-7158.1996.tb06019.x

Verma, C., Seebah, S., Low, S.M., Zhou, L., Liu, S.P., Li, J., Beuerman, R.W. **Defensins: Antimicrobial peptides for therapeutic development.** Biotechnology. 2 (2007) 1353–1359. DOI: 10.1002/biot.200700148

Vicente, E. F., Basso, L. G. M., Cespedes, G. F., Lorenzón, E. N., Castro, M. S., Mendes-Giannini, M. J. S., & Cilli, E. M. **Dynamics and conformational studies of TOAC spin labeled analogues of Ctx(Ile^{21})-Ha peptide from *Hypsiboas albopunctatus*.** PLoS One. 8 (2013) e60818. DOI: 10.1371/journal.pone.0060818

Vulikh, K., Bassel, L. L., Sergejewich, L., Kaufman, E. I., Hewson, J., MacInnes, J. I., Caswell, J. L. **Effect of tracheal antimicrobial peptide on the development of Mannheimia haemolytica pneumonia in cattle.** PloS one. 14 (2019) e0225533. DOI: 10.1371/journal.pone.0225533

Wallace, R. J. **Ruminal microbiology, biotechnology, and ruminant nutrition: progress and problems.** Journal of Animal Science. 72 (1994) 2992-3003. DOI: 10.2527/1994.72112992x

Wang, Y., Lyu, N., Liu, F., Liu, W.J., Bi, Y., Zhang, Z., Ma, S., Cao, J., Song, X., Wang, A., Zhang, G., Hu, Y., Zhu, B., Gao, G.F. **More diversified antibiotic resistance genes in chickens and workers of the live poultry markets.** Environ Int. 30 (2021). DOI: 10.1016/j.envint.2021.106534.

Wang, G., Song, Q., Huang, S., Wang, Y., Cai, S., Yu, H., Zhang, J. **Effect of Antimicrobial Peptide Microcin J25 on Growth Performance, Immune Regulation, and Intestinal Microbiota in Broiler Chickens Challenged with Escherichia coli and Salmonella**. Animals. 10 (2020b) 345. DOI: 10.3390/ani10020345

Wang, Y., Zhang, Y., Lee, W. H., Yang, X., Zhang, Y. **Novel peptides from skins of amphibians showed broad-spectrum antimicrobial activities.** Chemical Biology & Drug Design. 87 (2016a) 419–424. DOI: 10.1111/cbdd.12672

Wang S., Zeng X, Yang Q, Qiao S. **Antimicrobial Peptides as Potential Alternatives to Antibiotics in Food Animal Industry.** International Journal of Molecular Sciences. 17 (2016b) 603. DOI: 10.3390/ijms17050603

Wang, G., Li, X. and Wang, Z. **APD3: the antimicrobial peptide database as a tool for research and education.** Nucleic Acids Research. 44 (2016c) D1087-D1093.

Wang, S., Thacker, P. A., Watford, M., Qiao, S. **Functions of Antimicrobial Peptides in Gut Homeostasis.** Curr. Protein Pept. Sci. 16 (2015) 582–591. DOI: 10.2174/1389203716666150630135847

Wang, Z. and Wang, G. **APD: the antimicrobial peptide database.** Nucleic Acids Research. 32 (2004) D590-D592.

Wehkamp, J., Harder, J., Wehkamp, K., Wehkamp-von Meissner, B., Schlee, M., Enders, C., Schröder, J. M. **NF-κB-and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium.** Infection and immunity. 72 (2004) 5750-5758. DOI: 10.1128/IAI.72.10.5750-5758.2004

Witte, W. **Impact of Antibiotic use in Animal Feeding on Resistance of Bacterial Pathogens in Humans.** In Ciba Foundation Symposium 207 - Antibiotic Resistance: Origins, Evolution, Selection and Spread (eds D.J. Chadwick and J. Goode). (2007). DOI: 10.1002/9780470515358.ch5

World Health Organization (WHO). **No Time to Wait: Securing the future from drug-resistant infections.** Report to the Secretary-General of the United Nations. (2019). Available on: https://www.who.int/antimicrobial-resistance/interagency-coordination-group/final-report/en/. Accessed in June, 2020.

Wu, S., Zhang, F., Huang, Z., Liu, H., Xie, C., Zhang, J. **Effects of the antimicrobial peptide cecropin AD on performance and intestinal health in weaned piglets challenged with Escherichia coli.** Peptides. 35 (2012) 225–30. DOI: 10.1016/j.peptides.2012.03.030

Xiao, Y., Lyu, W., Yang, H., Xu, X., Zhou, C., Lu, L., & Zhang, L. **Molecular characterization, mRNA gene expression and antimicrobial activity of two new cathelicidin genes in goose.** Poultry Science. (2020). DOI: 10.1016/j.psj.2020.03.021

Xiao, H., Xiao, H., Wu, M. M., Tan, B., Li, T., Ren, W. K. **Metabolic profiles in the response to supplementation with composite antimicrobial peptides in piglets challenged with deoxynivalenol.** Journal of Animal Science. 93 (2015) 1114-23. DOI: 10.2527/jas.2014-8229

Xie, L., van Der Donk, W. A. **Post-translational modifications during lantibiotic biosynthesis.** Current Opinion in Chemical Biology. 8 (2004) 498-507. DOI: 10.1016/j.cbpa.2004.08.005

Xu, L., Zhou, J., Qu, G., Lin, Z., Fan, Q., Wang, C., & Wang, Q. **Recombinant lactobacillin PlnK adjusts the gut microbiome distribution in broilers.** British Poultry Science (just-accepted) (2020). DOI: 10.1080/00071668.2020.1752911

Yoon, J. H., Ingale, S. L., Kim, J. S., Kim, K. H., Lee, S. H., Park, Y. K. **Effects of dietary supplementation of antimicrobial peptide-A3 on growth performance, nutrient digestibility, intestinal and fecal microflora and intestinal morphology in weanling pigs.** Animal Feed Science and Technology. 177 (2012a) 98–107. DOI: 10.1016/j.livsci.2013.10.025

Yoon, J. H., Ingale, S. L., Kim, J. S. **Effects of dietary supplementation with antimicrobial peptide-P5 on growth performance, apparent total tract digestibility, faecal and intestinal microflora and intestinal morphology of weanling pigs.** Journal of the Science of Food and Agriculture. 93 (2012b) 587-592. DOI: 10.1002/jsfa.5840

Yoon, J. H., Ingale, S. L., Kim, J.S., Kim, K. H., Lee, S. H., Park, Y. K. **Effects of dietary supplementation of synthetic antimicrobial peptide-A3 and P5 on growth performance, apparent total tract digestibility of nutrients, fecal and**

intestinal microflora and intestinal morphology in weanling pigs. Livestock Science. 159 (2014) 53–60. DOI: 10.1016/j.livsci.2013.10.025

Zhang, J. C., Chen, P., Zhang, C., Khalil, M. M., Zhang, N. Y., Qi, D. S. & Sun, L. H. **Yeast culture promotes the production of aged laying hens by improving intestinal digestive enzyme activities and the intestinal health status.** Poultry Science. (2020). DOI: 10.1016/j.psj.2019.11.017

Zhang, W., Sack, D. A. **Progress and hurdles in the development of vaccines against enterotoxigenic Escherichiacoli in humans.** Expert Revie of Vaccines. 11 (2012) 677–694. DOI: 10.1586/erv.12.37

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CHAPTER 2 - Use of antimicrobial peptide Ctx(Ile^{21})-Ha and its analogue *ent*-Ctx(Ile^{21})-Ha for an alternative feed additive in beef cattle*

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Introduction

Brazilian livestock productivity is very representative in the world economic scenario. Since 2005, Brazil is leader of the largest beef exporter country in the world, with 20% of the meat traded internationally and sales in more than 180 countries. The Brazilian cattle herd provides the development of two segments: the production of meat and milk, which have an estimated gross value of more than USD 12 billion, according to data from the Ministry of Agriculture, Livestock and Supply (MAPA). In view of these facts, the promotion of strategies to increase the sustainability of cattle farming is essential for the maintenance and growth of one of the most important sectors of our economy.

An important strategy to achieve these objectives is increasing the efficiency of nutrient used by ruminants. In the rumen, there are several species of microorganisms that degrade proteins, non-structural and structural carbohydrates, synthesizing short-chain fatty acids (SCFA), amino acids, and vitamins, which make the host virtually independent of nutrients, except for vitamins A and D. The search for a maximization of the use of essential nutrients for the animal can improve the fermentation process inside the rumen, resulting in a greater availability of these nutrients (Leopoldino et al., 2007). The bacteria present in the rumen are classified

using common substrates or fermentative characteristics. The main groups are structural and non-structural carbohydrate fermenters, such as proteolytic, lactic, pectinolytic, lipolytic, ureolytic and methanogenic (Archaea), the latter being strictly anaerobic. The genus *Methanobacterium* plays a fundamental role in this chemical balance of the rumen ecosystem by using the hydrogen gas (H_2) present in the environment, contributing to the regeneration of coenzymes, such as NAD^+ and $NADP^+$, reducing them (Daquiado et al., 2014). In this sense, some groups of microorganisms, for example, Gram-positive acetate producers, which lower the rumen pH, together with high populations of methanogenic microorganisms, have an adverse effect on the optimal rumen balance and, therefore, on the animal performance. Thus, the control or reduction of fermentative parameters, using differentiated foods with probiotics and/or additives, has been studied and discussed to positively balance the diet, leading to the highest possible efficiency of the animal nutrient absorption.

Thus, the antimicrobial peptides (AMPs) have been acquiring importance in relation to the fight against pathogenic microorganisms in the most diverse areas, in addition to the resistance that many bacteria have presenting to the usual antibiotics. Hence, the use of the antimicrobial peptide Ctx(Ile^{21})-Ha – a ceratotoxin-like peptide isolated from a Brazilian frog *Hypsiboas albopunctatus* (Ferreira Cespedes et al., 2012) – as a feed additive in ruminant nutrition emerges as an alternative candidate to optimize the availability of nutrients by decreasing Gram-positive bacteria, since this molecule is known to be more active specifically against this type of

microorganism (Vicente et al., 2013). In addition, the *ent*-Ctx(Ile^{21})-Ha peptide, a D-enantiomeric Ctx(Ile^{21})-Ha analogue peptide which can be more resistant to proteases, may also efficiently acts on methane-producing bacteria. These peptides can modulate the nutritional efficiency of beef cattle, thus reducing the methane gas emission into the atmosphere.

Materials and methods

Solid phase peptide synthesis

The peptide synthesis was carried out according (Vicente et al., 2013), using the solid phase peptide synthesis (SPPS), performed on Chemistry and Biochemistry Laboratory at São Paulo State University (Unesp), School of Sciences and Engineering, Tupã – São Paulo, Brazil. The polymer used as support was the Rink Amide resin in a substitution degree of 0.6 mmol/g, scale of 0.4 mmol/g and three times of excess from the Fmoc-amino acid equivalents used. Disposable syringes with a porous filter were used do help on products disposal and reagent excess. For coupling of the Fmoc-L and D-amino acids, the following strategies using different coupling agents were used: a) *N,N*'-diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) under stirring for two hours or b) *o*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronic hexafluorophosphate (HBTU) and the base *N*-ethyldiisopropylamine (DIEA), strategy used only in cases of recoupling – from one to two hours. To eliminate reaction subproducts, cycles of two solvent washes with DMF and dichloromethane (DCM) were used for each step. The Fmoc removal, the amino

protecting group, was carried out by adding a solution of 4-methylpiperidine 20% in dimethylformamide (DMF) for one minute and then for more 20 minutes to complete the removal reaction.

Cleavage was made by adding a solution containing triisopropylsilane, deionized water (TIS) and trifluoroacetic acid - TFA in the proportions of 2.5:2.5:95 (v, v, v, respectively), in a 20 mL volume scintillation bottle, stirring for two hours, under mild agitation with a magnetic bar. Subsequently, the peptide, resin and subproducts solution was precipitated with ice-cold ethyl ether, with three five-minute cycles at 3,500 rpm and, after that, the supernatant was stored for later analysis, if needed. The peptide was extracted with the addition of acidified solutions (0.045% TFA in ultrapure water, solution A and 0.036% TFA in acetonitrile, solution B) and subsequent centrifugation. Then, the supernatant – crude peptide – was lyophilized. After extraction, a HPLC system (High Performance Liquid Chromatography, model Prominence, Shimadzu – Tokyo, Japan) was employed to separate the impurities of cleavage reaction – peptide purification – and analyze the fractions obtained. Therefore, the Ctx(Ile^{21})-Ha peptides were identified and characterized by Mass Spectrometry. Analyzes were performed in positive electrospray mode (ESI-MS) by direct injection of the peptide samples into an Ion Trap Amazon SL mass spectrometer, Bruker (Billerica, MA, USA).

In vitro gas production, methane production and ruminal fermentation

The studies were conducted at Premix® Research Center. *In vitro* gas production kinetics were evaluated by methodologies described by Mauricio et al., 1999. Initially, a mass of 0.5 g of dry samples of a) corn silage (CS), b) *Brachiaria brizantha* cv. Marandu (BRA) and c) total diet (TD – 60% of corn silage and 40% of concentrated) were weighed in bottles with 115 mL of capacity. The substrates chemical composition can be seen on Table 1. Each substrate was weight in 18 bottles. Two trials were performed. The first one had the treatments described below.

1. Negative control (NO – bottles with substrate and ruminal liquid without any additive)
2. Positive control 1 (MON) – 5.0 mg of monensin/kg of dry matter (DM)
3. Positive control 2 (VIR) – 5.0 mg of virginiamycin/kg DM
4. Antimicrobial peptide 1 (AMP2.5) – 2.5 mg of *ent*-Ctx(Ile^{21})-Ha/kg DM
5. Antimicrobial peptide 2 (AMP5.0) – 5.0 mg of *ent*-Ctx(Ile^{21})-Ha/kg DM
6. Antimicrobial peptide 3 (AMP7.5) – 7.5 mg of *ent*-Ctx(Ile^{21})-Ha/ kg DM

The second trial had the same positive and negative controls, but the original type L-peptide Ctx(Ile^{21})-Ha was employed as described below.

1. Negative control (NO - bottles with substrate and ruminal liquid, no additives)
2. Positive control 1 (MON) – 5.0 mg of monensin/kg of dry matter (DM)
3. Positive control 2 (VIR) – 5.0 mg of virginiamycin/kg DM
4. Antimicrobial peptide 1 (AMP2.5) – 2.5 mg of Ctx(Ile^{21})-Ha/kg DM
5. Antimicrobial peptide 2 (AMP5.0) – 5.0 mg of Ctx(Ile^{21})-Ha/kg DM
6. Antimicrobial peptide 3 (AMP7.5) – 7.5 mg of Ctx(Ile^{21})-Ha/kg DM

Table 1: Chemical composition of substrates used in *in vitro* incubations.

Substrate	CS[1]	BRA[2]	TD[3]
DM[4], %	35.15	32.32	40.56
NDF[5], %	48.26	70.31	60.66

[1]Corn Silage; [2]*Brachiaria brizantha* cv. Marandu; [3]Total Diet; [4]Dry matter content; [5]Neutral detergent fiber content.

At the day of incubations, the buffer solution composed by sodium and ammonium hydroxide (Marten, G. C.; Barnes, 1979) was prepared, mixed with a solution of macro and micronutrients, and continuously infused with carbon dioxide (CO_2) for 30 minutes, in a temperature of 39ºC. After that, 50 mL was dispensed in each bottle. The ruminal fluid was collected from two Nelore cattle kept under grazing of *Brachiaria brizantha* cv. Marandu and mixed among them. A volume of 25 mL of ruminal liquid was dispensed in each bottle. Then, the bottles were sealed and maintained in a 39ºC stove with automatic agitation.

The gas pressure was measured after 4, 8, 12, 24 and 48 hours of fermentation, using a pressure transducer connected to a Datalogger (*press* DATA 800). Pressure was transformed to volume using the following equation (Eq. 1):

$$V\ (mL) = (13.156 \times P) + 0.1991 \quad (1)$$

where *V* is the gas production volume (mL) and *P* is the pressure (psi).

Each of four *in vitro* incubation analyzes were composed by 112 bottles: 6 treatments × 3 substrates × 2 times of methane collect × 3 repetitions plus 4

standards (no substrate was used). After 24 and 48 hours of fermentation, a volume of 20 µL of gas was collected and stored in vials. The vials were analyzed using gas chromatography to measure the methane concentration.

Dry matter disappearance (DMD)

After 24 and 48 hours of fermentation, the bottles were removed from the stove and immersed in ice to inhibit microbial activity. Then, the bottles were opened and the material inside was passed through a textile filter and repeatedly washed with distilled water. After that, the filters were put in a stove at 55ºC for 24 hours. After this time, the filters were weighted again and, by weight difference, the DMD was calculated.

Neutral detergent fiber disappearance (NDFD)

After DMD calculation, the filters were sealed, identified and the NDF was performed according to Detmann (2013). The filters were put in an autoclave with detergent solution in a rate of 100 ml of solution per gram of sample. The solution was heated to a temperature of 105 °C per 1 hour. Then the filters were washed with hot distilled water (approximately 90°C) to remove the detergent and after with acetone. After that the filters were conditioned in a ventilated stove at 60°C for 24 hours and then in a stove at 105°C for 2 hours. The filters were weighted again and by difference the NDFD was calculated.

End products of fermentation

After 24 and 48 hours of fermentation, bottles were removed from the stove and immersed in ice to inhibit microbial activity. The pH of the buffered rumen inoculum was measured using a pH meter (model MA522). After this procedure, 1 mL of H_2SO_4 was added to rumen inoculum and 30 mL aliquots were collected and stored at –20 ºC for subsequent determination of SCFA by high performance liquid chromatography (HPLC) according to Serafim et al. (2021). It is important to highlight that this publication was produced by our group, in order to develop a cheap and straight-forward methodology for the SCFA analysis using the HPLC system.

The SCFA HPLC retention times were presented as follows: formic acid: 4.005 minutes; lactic acid: 5.505 minutes; acetic acid: 5.814 minutes; propionic acid: 13.392 minutes.; butyric acid: 20.863 minutes. The peaks obtained in the chromatography were integrated, and their areas were used to obtain the concentration of acids (mmol L^{-1}) through calibration curves previously made for each acid.

Statistical Analysis

Mix models were used for analyzing all the variables. For the variables pH, SCFA, DMD, NDFD, methane, and gas production, linear mix models using the function *lme* of the package *nlme* of software R (Pinheiro J, Bates D, DebRoy S, Sarkar D, 2020) were fitted with the fixed effect of substrate and additive and the interaction substrate*additive and the random effect of run [$r \sim iidN\ (0, \sigma_r^2)$]. For dynamic production of gas, non-linear mix model using the function *nlme* of the

package *nlme* of software R (Pinheiro J, Bates D, DebRoy S, Sarkar D, 2020) were fitted with the fixed effect of substrate and additive and the interaction substrate*additive as fixed effect and the run [r ~ iidN (0, σ_r^2)] and bottle [b ~ iidN (0, σ_b^2)] as random effect in the parameters of the one-pool logistic model, proposed by Schofield, Pitt, and Pell (Schofield et al., 1994), presented below (Equation 2):

$$V_t = V_f \times (1 + \exp(2 - 4 \times S \times (t - L)))^{-1} \quad (2)$$

where V_f = final gas volume accumulated (mL); S = degradability rate (/h); t = time (h); L = lag-time (h). Also, the autoregressive-1 was used to correct for lack of independence in the residual and *varPower* to correct homogeneity of variance.

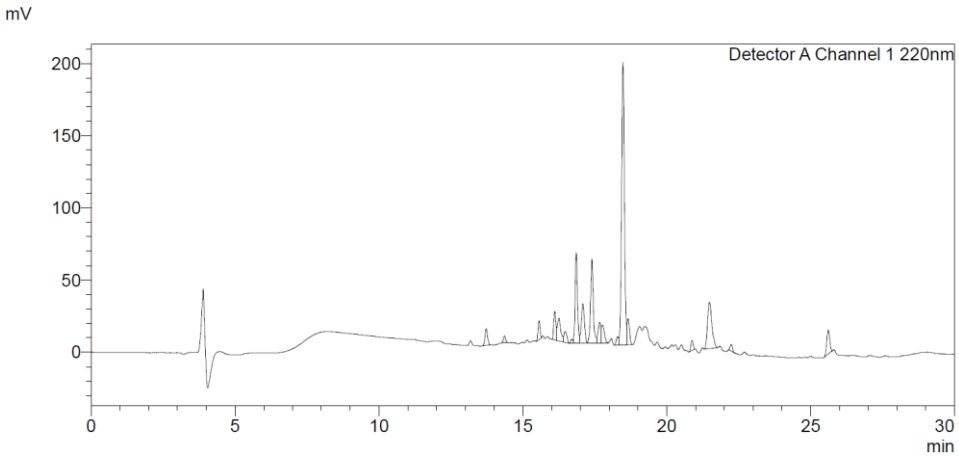
Analysis of variance were performance for both models using *Anova* function of package *stats* of R (Fox, John & Weisberg, 2011). Means were compared using pairwise comparison and Tukey's multiplicity adjustment available in the function *emmeans* of package *emmeans* (Lenth, 2019) of R (R Core Team, 2020). Orthogonal polynomial contrasts were also used to compare the effects of MON, VIR, NO. To the mean of AMP and also the linear and quadratic effect of AMP *emmeans* and *contrast* functions of the *emmeans* package of software R (Lenth, 2019) were used. The *P*-value ≤ 0.05 was considered significant.

Results

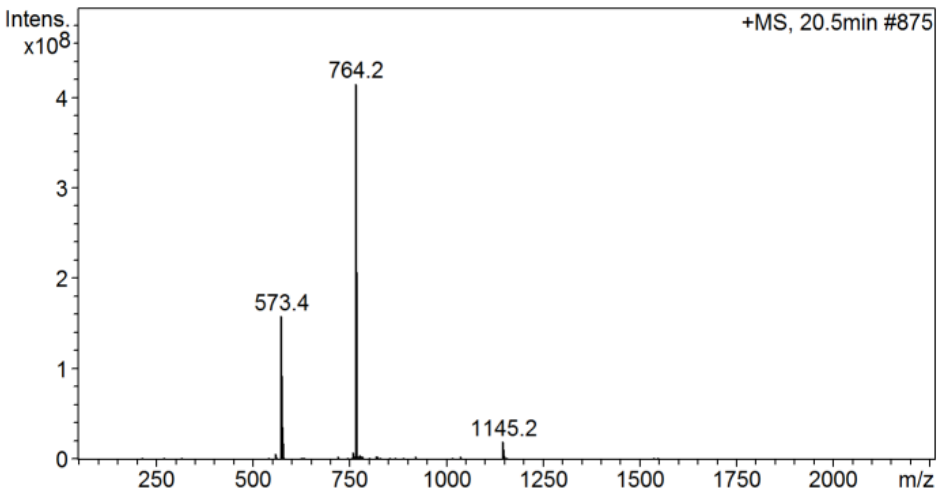
Solid phase peptide synthesis

For the peptide application in the ruminal liquid, two peptide syntheses were performed for each peptide due the high mass amount necessary to the treatments applied. The chromatographic profiles, measured at a wavelength of 220 and 280 nm, are showed on Figure 1. The first wavelength detects the peptide bonds, which absorb light at this wavelength; the second length absorbs aromatic amino acids (i.e., tryptophan), which makes the analysis more sensitive and efficient. From the crude chromatographic profile presented, there is a major peak at a column retention time of 18.5 min, when an analytical gradient with a program from 5% to 95% of solution B in 30 minutes was applied. After mass spectrometry analysis, the material of interest was confirmed through its mass/charge ratio. In order to use a greater mass of peptide, a peptide clean-up step was performed to eliminate impurities from the synthesis and all organic substances that were used in the cleavage and synthesis. This process was done using a Sep-Pak column, which contains a C18 column packed in a syringe. The crude peptide was initially solubilized in solution A and injected its entire volume into the column. Afterwards, with the peptide retained in the C18 stationary phase of the Sep-Pak column, washes were carried out with 20% of solvent B to remove impure and medium polar organic compounds. Finally, the peptide was extracted from the column with 50% of solution B, which is the proportion of non-polar solvent (acetonitrile) where it was possible to elute the peptide from the column and collect it. For the peptide Ctx(Ile^{21})-Ha we have a

theoretical molecular mass of 2,289.75 g/mol and the respective mass/charge ratios 764 (Z = +3) and 1,146 (Z = +2), a fact that confirms and ratifies the obtaining the antimicrobial peptide in this synthesis. For the peptide *ent*-Ctx(Ile^{21})-Ha we have the theoretical molecular mass of 2289.75 g/mol and the respective mass/charge of 573 g/mol (Z = +4) and 1145 (Z = +2).



(A)



(B)

Figure1: Chromatographic profiles of (A) Ctx(Ile^{21})-Ha and (B) *ent*-Ctx(Ile^{21})-Ha peptide synthesis.

pH

The results of pH are showed on the Table 2.

Table 2. Two periods pH of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD) and different feed additives.

AMP	**24 hours**						**48 hours**					
	Ctx(Ile^{21})-Ha			***ent*-Ctx(Ile^{21})-Ha**			**Ctx(Ile^{21})-Ha**			***ent*-Ctx(Ile^{21})-Ha**		
Additive/Substrate	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**
NO	6.81	6.87	6.8	6.84	6.94	6.95	6.75	6.74	6.8	6.82	6.85	6.87
MON	6.81	6.87	6.82	6.86	6.95	6.92	6.79	6.79	6.8	6.82	6.88	6.87
VIR	6.81	6.89	6.89	6.85	6.95	6.95	6.84	6.76	6.77	6.81	6.84	6.83
AMP2.5	6.83	6.9	6.84	6.86	6.93	6.94	6.86	6.78	6.8	6.82	6.88	6.88
AMP5.0	6.82	6.89	6.86	6.85	6.95	6.97	6.77	6.77	6.77	6.84	6.86	6.87
AMP7.5	6.84	6.87	6.85	6.87	6.94	7	6.78	6.78	6.78	6.83	6.89	6.87
	***p*-values**											
Substrate (S)	0.0175			0.0003			0.8869			0.0025		
Additive (A)	0.8115			0.9553			0.4491			0.0514		
Substrate*Additive (S*A)	0.1764			0.1625			0.7241			0.6091		

Gas production

The gas production was measured until 48 hours after incubation. The results are showed on Table 3. It was observed significative effects on gas production parameters for both peptides and substrates.

Considering the different substrates employed, corn silage showed a higher gas volume and degradation rate than the other substrates, for both peptides. For Ctx(Ile^{21})-Ha, the lag time was algo higher and no statistical difference was observed for this parameter for *ent*-Ctx(Ile^{21})-Ha.

Table 3. *In vitro* gas production of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD) and different peptides.

	Ctx(Ile^{21})-Ha					***ent*-Ctx(Ile^{21})-Ha**				
	CS	**BRA**	**TD**	**SEM**	***p*-value**	**CS**	**BRA**	**TD**	**SEM**	***p*-value**
FV	179	154	155	3.86	<.0001	185	149	151	2.410	<.0001
S	0.0401	0.034	0.037	0.000598	0.0265	0.0357	0.0305	0.0328	0.000437	<.0001
L	10.75	8.68	8.21	0.284	<.0001	6.3	6.3	6.3	0.358	<.0001

Among the peptides, it was also noted some significative differences. In both cases, a minor gas production was observed on the treatments with the AMP than in those without additives, or with monensin and virginiamycin. The degradation rate and lag time were affected by different additives, but no significative difference was verified.

Table 4. *In vitro* gas production of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD) and different feed additives.

Peptide	Ctx(Ile^{21})-Ha			*ent*-Ctx(Ile^{21})-Ha		
Additive	**FV, ml**	**S[1]**	**L, h**	**FV**	**S**	**L**
NO	172	0.0368	9.2	171	0.0324	6.3
MON	171	0.0363	9.2	171	0.0324	6.3
VIR	169	0.0351	9.2	170	0.0317	6.3
AMP2.5	154	0.0387	9.2	150	0.0338	6.3
AMP5.0	156	0.0380	9.2	152	0.0340	6.3
AMP7.5	153	0.0374	9.2	157	0.0337	6.3
SEM	5.46	0.000854	2.84	3.18	0.000622	0.358
***p*-value**	0.0141	0.0265	<0.0001	<.0001	0.0167	<.0001

Methane emission

According to the methodology applied, methane was measured on two periods before the bottle was opened. When analyzed isolated, as it can be seen on Table 5, the peptide Ctx(Ile^{21})-Ha has no effect on CH_4 measurements for the two measured periods. Nonetheless, the substrates and the interaction substrate and additive showed significative differences. In the first period, when feed with corn silage, it can be noted a less methane production on the treatments with monensin. On the other hand, when using other substrates, these reductions can be seen on the treatments with AMP either.

Table 5. Two periods *in vitro* methane production, mg/L, of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD), different feed additives and different levels of AMP Ctx(Ile^{21})-Ha.

Additive	Substrate					
	24 hours			48 hours		
	CS	BRA	TD	CS	BRA	TD
NO	106.8	72.9	81.6	146.8	122.5	111.6
MON	67.7	67.9	27.1	133.3	151.5	84.6
VIR	112.4	59.4	68.9	139.0	126.0	135.5
AMP2.5	116.8	56.8	47.0	103.0	146.2	157.1
AMP5.0	91.3	57.5	63.6	93.4	130.1	140.5
AMP7.5	96.0	59.4	80.4	127.6	122.5	83.4
	S	**A**	**S*A**	**S**	**A**	**S*A**
***p*-values**	0.0202	0.6313	0.1203	0.0182	0.7068	0.0092

On the second period, the effect observed previously on corn silage with monensin, can be seen on the intermediary level of the AMP. The additives did not show a significative effect, but the interaction with the substrate did. Also, in absolute values, a less methane production was detected, mainly when using corn silage and total diet.

Considering the *ent*-Ctx(Ile^{21})-Ha peptide, none of the variables had a significative effect on methane production on both periods (Table 6). However, comparing the two peptides, it can be observed a less methane emission for *ent*-Ctx(Ile^{21})-Ha.

Table 6. Two periods *in vitro* methane production, mg/L, of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD), different feed additives and different levels of AMP *ent*-Ctx(Ile^{21})-Ha.

Additive	Substrate					
	24 hours			48 hours		
	CS	BRA	TD	CS	BRA	TD
NO	94.1	69.3	72.9	119.5	122.6	106.5
MON	88.3	71.9	57.9	128.1	111.9	122.7
VIR	82.2	68.8	53.6	115.8	133.0	110.1
AMP2.5	99.0	69.1	50.7	99.1	86.8	113.7
AMP5.0	73.3	73.3	54.1	124.9	90.6	112.0
AMP7.5	79.9	70.5	56.9	141.3	106.2	105.4
	S	**A**	**S*A**	**S**	**A**	**S*A**
***p*-values**	0.2079	0.9889	0.941	0.7204	0.1317	0.4517

Dry matter disappearance

At 24 hours, the results for both peptides employed for the studies are showed on Table 7. For the AMP Ctx(Ile^{21})-Ha, no effect of additive or interaction between substrate and additive was identified. The substrate showed a significative difference, where the total diet presented a higher disappearance than others, despite the additive. On the other hand, for the AMP *ent*-Ctx(Ile^{21})-Ha analogue it was noted significative effects of substrate and additive. On the diet with corn silage, a slight improvement was identified when using monensin. Meanwhile, when analyzing *Brachiaria brizantha*, a reduction was identified on the AMP 5.0. On the treatment based on a total diet, the virginiamycin showed the highest disappearance. Until 48 hours, no significative differences were identified to additive, substrate, or their interaction for both peptides.

Table 7. Two periods *in vitro* dry matter disappearance of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD), different feed additives and different levels of AMPs.

	24 hours						48 hours					
AMP	**Ctx(Ile^{21})-Ha**			***ent*-Ctx(Ile^{21})-Ha**			**Ctx(Ile^{21})-Ha**			***ent*-Ctx(Ile^{21})-Ha**		
Additive/Substrate	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**
NO	0.413	0.364	0.558	0.546	0.405	0.504	0.576	0.526	0.587	0.489	0.508	0.506
MON	0.402	0.356	0.471	0.555	0.447	0.461	0.558	0.554	0.554	0.498	0.496	0.491
VIR	0.435	0.368	0.470	0.547	0.448	0.526	0.564	0.549	0.549	0.490	0.483	0.599
AMP2.5	0.422	0.356	0.472	0.525	0.447	0.436	0.540	0.526	0.526	0.525	0.498	0.507
AMP5.0	0.440	0.378	0.451	0.467	0.332	0.459	0.567	0.551	0.551	0.514	0.445	0.535
AMP7.5	0.434	0.374	0.458	0.500	0.426	0.403	0.548	0.531	0.531	0.52	0.476	0.502
	***p*-values**											
S	0.0222			0.0125			0.2043			0.9872		
A	0.9885			0.0098			0.7237			0.7873		
S*A	0.4338			0.1325			0.8727			0.4995		

Neutral detergent fiber disappearance

Similar to DM, the NDF disappearance was measured at two times and the results are presented on Table 8. The AMP Ctx(Ile^{21})-Ha had a significative effect of substrate on NDF parameters. Comparing the three substrates used, the *Brachiaria brizantha* showed a minor NDF disappearance. Similarly, the AMP *ent*-Ctx(Ile^{21})-Ha had the sabe effect, with minor values on *Brachiaria brizantha* utilization, but also presented an additive effect. When using corn silage, the virginiamycin caused a reduction on NDF disappearance. On the other hand, for the other two substrates, the AMP showed this reduction, at different levels. For both AMPs, the substrate presented a significative effect. Similarly, for results observed on 24 hours, the

Brachiaria brizantha treatment showed less NDF disappearance. Furthermore, a significative effect of the interaction between substrate and additive was identified for AMP Ctx(Ile^{21})-Ha.

Table 8. Two periods *in vitro* neutral detergent fiber disappearance of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD), different feed additives and different levels of AMPs.

	24 hours						48 hours					
AMP	**Ctx(Ile^{21})-Ha**			***ent*-Ctx(Ile^{21})-Ha**			**Ctx(Ile^{21})-Ha**			***ent*-Ctx(Ile^{21})-Ha**		
Additive/Substrate	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**	**CS**	**BRA**	**TD**
NO	0.817	0.692	0.819	0.882	0.767	0.853	0.887	0.780	0.810	0.904	0.862	0.920
MON	0.892	0.689	0.803	0.871	0.782	0.831	0.858	0.790	0.830	0.899	0.865	0.910
VIR	0.810	0.675	0.798	0.801	0.736	0.862	0.866	0.790	0.829	0.890	0.758	0.927
AMP2.5	0.816	0.667	0.806	0.828	0.736	0.828	0.844	0.790	0.851	0.896	0.851	0.886
AMP5.0	0.815	0.661	0.795	0.837	0.711	0.816	0.862	0.794	0.863	0.892	0.858	0.910
AMP7.5	0.813	0.687	0.805	0.857	0.738	0.809	0.861	0.784	0.853	0.895	0.852	0.901
***p*-values**												
S	<.0001			0.0054			0.0003			0.0027		
A	0.9553			0.1526			0.9743			0.8357		
S*A	0.9886			0.0439			0.1009			0.7228		

Short chain fatty acids profile

The SCFA profile for both peptides and periods are shown on Table 9. At 24 hours, were observed significative effects of substrate to acetic acid, with the total diet showing the greater values, of additive to propionic acid, with the AMP showing greater values when used with corn silage and similar values than monensin when used with total diet. Also, the butyric acid showed substrate effect with the higher

values coming from *Brachiaria brizantha* treatment. At 24 hours, a significative effect of substrate for acetic acid was observed to AMP *ent*-Ctx(Ile^{21})-Ha. No significative effects were observed to propionic and butyric acids by this time. At 48 hours, a significative effect of substrate was observed to butyric acid, similar to data from 24 hours. The higher values were observed with the corn silage substrate. To other acids, no significative difference was observed. At 48 hours a significative difference of substrate was observed to AMP *ent*-Ctx(Ile^{21})-Ha. To other acids, no significative difference was observed.

Table 9. Two periods *in vitro* Acetate (A), Propionate (P) and Butyrate (B) profile of ruminal liquid incubated with Corn silage (CS), *Brachiaria brizantha* cv. Marandu (BRA), Total diet (TD), different feed additives and different levels of AMPs.

	24 hours									**48 hours**								
	Ctx(Ile^{21})-Ha									***ent*-Ctx(Ile^{21})-Ha**								
Substrate/ Additive	**Corn Silage**			***Brachiaria brizantha***			**Total Diet**			**Corn Silage**			***Brachiaria brizantha***			**Total Diet**		
SCFA (mmol/100 mmol)	A	P	B	A	P	B	A	P	B	A	P	B	A	P	B	A	P	B
NO	56.1	16.0	11.28	55.3	21.1	6.40	58.9	19.9	9.93	62.3	20.5	10.25	68.5	20.1	5.2	66.6	21.8	3.89
MON	56.1	20.5	9.73	63.7	20.3	1.58	57.9	22.00	11.1	65.7	20.5	8.88	69.6	20.4	2.34	64.2	22.8	6.72
VIR	56.7	19.3	9.57	60.2	21.8	3.49	63.9	20.9	7.01	64.9	19.6	7.15	69.7	20.4	2.29	67.6	23.3	5.42
AMP2.5	56.4	29.8	7.18	58.8	19.6	4.66	58.6	13.7	5.74	64.5	19.5	9.67	68.3	20.2	3.64	69	20.7	8.36
AMP5.0	61.7	17.8	8.80	59.5	18.8	3.77	59.5	21.4	6.29	62.6	19.3	11.13	70.3	21.5	3.71	64.3	20.7	7.52
AMP7.5	60.6	15.1	9.75	62.9	18.6	4.29	62.9	19.6	7.82	61.9	19.8	10.67	68.6	21.4	3.61	68.1	20.4	4.11
***p*-values**																		
S	0.15	0.6972	0.0008							0.1385	0.1491	0.0065						
A	0.35	0.1151	0.5921							0.9758	0.8493	0.7372						
S*A	0.3803	0.4352	0.8413							0.7103	0.5564	0.4661						

Discussion

The AMPs have received a crescent academic attention, in order to efficiently fight against pathogenic microorganisms. For the use as an alternative feed additive, studies should be carefully conducted to understand how this substance will interact with ruminal microorganisms. According to that, this study demonstrates how the AMPs could affect the gas production, ruminal pH, methane production and the SCFA profile of beef cattle *in vitro*.

There are few studies reporting the use of AMPs in ruminants. As presented, pH was not affected by additives – neither AMPs or conventional ones – and a similar pattern was found by Ren et al. (2019) using a recombinant swine defensin PBD-mI on goats. The lack of effect of this parameter was expected, since there was not a big challenge to ruminal environment and the collect of ruminal liquid for measurements were performed on fixed times, 12 and 24 hours – in which, according to literature, few hours after feeding, the pH has its major reduction.

The initial time of gas production is related to the fast fermentable fraction of the substrate and microbial protein synthesis and the last portion is related to the fermentation of the insoluble but potentially degradable components, like NDF fraction (Groot et al., 1996). The treatments using corn silage showed a major gas production. This can be due to the stage of maturity of corn during the harvest, with a minor concentration of cell wall (Lagrange et al., 2019). On the other hand, treatments with *Brachiaria brizantha* cv. Marandu showed a minor gas production,

and this can be due to their higher NDF content and, consequently, slower degradation, which can be supported by their decreased degradation rate either.

Among additives, the antimicrobial peptides showed a minor gas production in all levels of inclusion (Table 3). Gas production has a positive correlation with greater digestibility, greater energy content of the ration used and potential reduced fill effect (Blümmel et al., 2005). According to these *in vitro* results, a greatest dry matter intake for treatments with regular feed additives could be expected because of its greater gas production. Corroborating with this data, a significative difference on DM and NDF disappearance was observed.

Bovine rumen methanogenesis is one of the factors pointed as a cause of global warming (Melchior et al., 2018). Besides that, methane represents a loss of energy by the animal, indicating a lower feed efficiency (Lee et al., 2002). Monensin is recognized by its effect on methane emission reduction on short term and by long term the studies are inconclusive, since the microorganisms can adapt themselves to monensin effects (Melchior et al., 2018). Besides that, monensin did not seem to have a primary effect on methanogenesis (Lee et al., 2002). Odongo et al. (2007) studying lactating dairy cows supplemented with monensin for six months, had a 9% decrease in methane emission and no adaptation on rumen microorganisms was observed. On the other hand, Melchior et al. (2018) did not found any effect of monensin on methane emission until fourth month with dairy cows supplemented with this additive. Also, Benchaar (2015) in a study to evaluate the effects of monensin on

dairy cows enteric CH_4 emission, showed that methane was not affected by monensin inclusion.

In the present study, both AMPs showed a reduction on methane production when compared with monensin. The addition of 2.5 mg/kg DM of *ent*-Ctx(Ile^{21})-Ha peptide reduced methane emission by approximately 30% when comparing with monensin on corn silage and *Brachiaria brizantha* substrates and almost 10% on total diet. Considering that due the mode of action of the antimicrobial peptides, as a microorganism adaptation is unusual, this reduction would be significative on a feedlot.

Similar to other research, some differences were seen on SCFA profile. Liu et al. (2017) used a mix of recombinant swine defensin and a fly antibacterial peptide in a proportion of 1:1 on juvenile goats and showed a changed on SCFA profile. In comparison, the treatment with AMP *ent*-Ctx(Ile^{21})-Ha had a higher concentration of acetic acid, similar to data described by other studies (Liu et al., 2017; Ren et al., 2019).

Conclusion

Besides the large number of studies about antimicrobial peptides, the studies with AMP applied on ruminant nutrition are very scarce. However, in this study, the intermediary level of the AMP *ent*-Ctx(Ile^{21})-Ha reduced the gas production *in vitro* and methane emission without negative effect on the nutrient utilization. Although it must be performed more trials to gather more information about these natural

antibiotic molecules, the antimicrobial peptide *ent*-Ctx(Ile^{21})-Ha demonstrated to be a promising candidate to replace the conventional and synthetic additives that have been using in animal nutrition, mainly in beef cattle.

References

Abbas, R.Z., Iqbal, Z., Sindhu, Z.D., Khan, M.N., Arshad, M., 2008. Identification of cross-resistance and multiple resistance in Eimeria tenella field isolates to commonly used anticoccidials in Pakistan. J. Appl. Poult. Res. 17, 361–368. https://doi.org/10.3382/japr.2008-00027

Benchaar, C., 2015. Diet supplementation with cinnamon oil, cinnamaldehyde, or monensin does not reduce enteric methane production of dairy cows. Animal 10, 418–425. https://doi.org/10.1017/S175173111500230X

Blümmel, M., Cone, J.W., Van Gelder, A.H., Nshalai, I., Umunna, N.N., Makkar, H.P.S., Becker, K., 2005. Prediction of forage intake using in vitro gas production methods: Comparison of multiphase fermentation kinetics measured in an automated gas test, and combined gas volume and substrate degradability measurements in a manual syringe system. Anim. Feed Sci. Technol. 123-124 Pa, 517–526. https://doi.org/10.1016/j.anifeedsci.2005.04.040

Chiesa, L.M., DeCastelli, L., Nobile, M., Martucci, F., Mosconi, G., Fontana, M., Castrica, M., Arioli, F., Panseri, S., 2020. Analysis of antibiotic residues in raw bovine milk and their impact toward food safety and on milk starter cultures in cheese-making process. Lwt 131, 109783.

https://doi.org/10.1016/j.lwt.2020.109783

Daquiado, A.R., Cho, K.M., Kim, T.Y., Kim, S.C., Chang, H.H., Lee, Y.B., 2014. Methanogenic archaea diversity in Hanwoo (Bos taurus coreanae) rumen fluid, rectal dung, and barn floor manure using a culture-independent method based on mcrA gene sequences. Anaerobe 27, 77–81. https://doi.org/10.1016/j.anaerobe.2014.01.008

Detmann, E., 2013. Métodos para analises de alimentos - INCT - Ciência Animal. J. Chem. Inf. Model. 53, 1689–1699.

Ferreira Cespedes, G., Nicolas Lorenzon, E., Festozo Vicente, E., Jose Soares Mendes-Giannini, M., Fontes, W., de Souza Castro, M., Maffud Cilli, E., 2012. Mechanism of Action and Relationship Between Structure and Biological Activity of Ctx-Ha: A New Ceratotoxin-like Peptide from Hypsiboas albopunctatus. Protein Pept. Lett. 19, 596–603. https://doi.org/10.2174/092986612800494011

Fox, John & Weisberg, S., 2011. An R Companion to Applied Regression.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for in vitro fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64, 77–89. https://doi.org/10.1016/S0377-8401(96)01012-7

Lagrange, S., Lobón, S., Villalba, J.J., 2019. Gas production kinetics and in vitro degradability of tannin-containing legumes, alfalfa and their mixtures. Anim. Feed Sci. Technol. 253, 56–64. https://doi.org/10.1016/j.anifeedsci.2019.05.008

Lee, S.S., Hsu, J.T., Mantovani, H.C., Russell, J.B., 2002. The effect of bovicin HC5,

a bacteriocin from Streptococcus bovis HC5, on ruminal methane production in vitro1. FEMS Microbiol. Lett. 217, 51–55. https://doi.org/10.1016/S0378-1097(02)01044-3

Lenth, R., 2019. Estimated Marginal Means, aka Least-Squares Means.

Leopoldino, W.M., Lana, R.P., Eifert, E.C., Arcuri, P.B., Mantovani, H.C., Martins, R.G.R., 2007. Avaliação de ionóforos pela técnica da perda do potássio celular e produção de gases in vitro. Arq. Bras. Med. Vet. e Zootec. 59, 1516–1522. https://doi.org/10.1590/S0102-09352007000600026

Liu, Q., Yao, S., Chen, Y., Gao, S., Yang, Y., Deng, J., Ren, Z., Shen, L., Cui, H., Hu, Y., Ma, X., Yu, S., 2017. Use of antimicrobial peptides as a feed additive for juvenile goats. Sci. Rep. 7, 1–11. https://doi.org/10.1038/s41598-017-12394-4

Magana, M., Pushpanathan, M., Santos, A.L., Leanse, L., Fernandez, M., Ioannidis, A., Giulianotti, M.A., Apidianakis, Y., Bradfute, S., Ferguson, A.L., Cherkasov, A., Seleem, M.N., Pinilla, C., de la Fuente-Nunez, C., Lazaridis, T., Dai, T., Houghten, R.A., Hancock, R.E.W., Tegos, G.P., 2020. The value of antimicrobial peptides in the age of resistance. Lancet Infect. Dis. 20, e216–e230. https://doi.org/10.1016/S1473-3099(20)30327-3

Maria-Neto, S., De Almeida, K.C., Macedo, M.L.R., Franco, O.L., 2015. Understanding bacterial resistance to antimicrobial peptides: From the surface to deep inside. Biochim. Biophys. Acta - Biomembr. 1848, 3078–3088. https://doi.org/10.1016/j.bbamem.2015.02.017

Marten, G. C.; Barnes, R.F., 1979. Prediction of energy digestibility of forages with in

vitro rumen fermentation and fungal enzymes systems., in: W.J. Pigden, C.C. Balch, M., Graham (Eds.), Standardization of Analytical Methodology for Feeds. International Development Research Center, Otawa, pp. 61–128.

Mauricio, R.M., Mould, F.L., Dhanoa, M.S., Owen, E., Channa, K.S., Theodorou, M.K., 1999. A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. Anim. Feed Sci. Technol. 79, 321–330. https://doi.org/10.1016/S0377-8401(99)00033-4

Melchior, E.A., Hales, K.E., Lindholm-Perry, A.K., Freetly, H.C., Wells, J.E., Hemphill, C.N., Wickersham, T.A., Sawyer, J.E., Myer, P.R., 2018. The effects of feeding monensin on rumen microbial communities and methanogenesis in bred heifers fed in a drylot. Livest. Sci. 212, 131–136. https://doi.org/10.1016/j.livsci.2018.03.019

Menkem, Z.E., Ngangom, B.L., Tamunjoh, S.S.A., Boyom, F.F., 2019. Antibiotic residues in food animals: Public health concern. Acta Ecol. Sin. 39, 411–415. https://doi.org/10.1016/j.chnaes.2018.10.004

Odongo, N.E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M.M., Hook, S.E., Gray, J.T., Kebreab, E., France, J., McBride, B.W., 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. J. Dairy Sci. 90, 1781–1788. https://doi.org/10.3168/jds.2006-708

Pinheiro J, Bates D, DebRoy S, Sarkar D, R.C.T., 2020. nlme: Linear and Nonlinear Mixed Effects Models.

R Core Team, 2020. R: A language and environment for statistical computing. R

Foundation for Statistical Computing.

Ren, Z., Yao, R., Liu, Q., Deng, Y., Shen, L., Deng, H., Zuo, Z., Wang, Y., Deng, J., Cui, H., Hu, Y., Ma, X., Fang, J., 2019. Effects of antibacterial peptides on rumen fermentation function and rumen microorganisms in goats. PLoS One 14, 1–16. https://doi.org/10.1371/journal.pone.0221815

Schofield, P., Pitt, R.E., Pell, A.N., 1994. Kinetics of fiber digestion from in vitro gas production. J. Anim. Sci. 72, 2980–2991. https://doi.org/10.2527/1994.72112980x

Serafim, J.A., Silveira, R.F., Vicente, E.F., 2021. Fast Determination of Short-Chain Fatty Acids and Glucose Simultaneously by Ultraviolet/Visible and Refraction Index Detectors via High-Performance Liquid Chromatography. Food Anal. Methods. https://doi.org/10.1007/s12161-021-01990-w

Vicente, E.F., Basso, L.G.M., Cespedes, G.F., Lorenzón, E.N., Castro, M.S., Mendes-Giannini, M.J.S., Costa-Filho, A.J., Cilli, E.M., 2013. Dynamics and Conformational Studies of TOAC Spin Labeled Analogues of Ctx(Ile21)-Ha Peptide from Hypsiboas albopunctatus. PLoS One 8. https://doi.org/10.1371/journal.pone.0060818

SUPLEMENTARY MATERIAL

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Fast Determination of Short-Chain Fatty Acids and Glucose Simultaneously by Ultraviolet/Visible and Refraction Index Detectors via High-Performance Liquid Chromatography

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Abstract

A rapid analytical method for simultaneous quantification of glucose (GLC) and seven short-chain fatty acids (SCFA) was developed for detection of ruminal fermentation parameters. The formic acid (FA), lactic acid (LA), acetic acid (AA), propionic acid (PA), butyric acid (BA), isovaleric acid (IVA), valeric acid (VA), and glucose were identified and quantified by high-performance liquid chromatography (HPLC) technique, using a reverse-phase column (C-18) and simultaneous detection in UV-Vis and Refraction Index (RID) detectors, linked in series. The method was validated following the requirements of selectivity, linearity, sensitivity, limit of detection, limit of quantification, accuracy, and precision. The developed method operates in isocratic and gradient modes, providing a better SCFA separation, which allows their determination with a high accuracy and repeatability. Therefore, this study provides a fast, reliable, accurate, straightforward, and efficient alternative method for analysis of ruminal fermentation parameters by liquid chromatography using both detectors simultaneously.

Keywords Analysis · HPLC · RID · Ruminal fermentation · SCFA · UV-Vis

Introduction

During their evolutional processes, ruminants developed the capacity of efficiently use structural carbohydrates as energy source and non-protein compounds as their protein source (Valadares Filho and Pina 2011). This ability is due to the development of anatomical structures (pre-stomachs) and the symbiosis with microorganisms, which has the potential to ferment fiber and synthesize short-chain fatty acids (SCFA), proteins, and some vitamins. The relationship between ruminants and microorganisms, regarding the consumable nutrients, is mutually beneficial.

The SCFA are organic acid compounds with one to six carbon atoms and comprise the main products from carbohydrate fermentation, composing the major energy source for ruminants (Baldwin 1998; Olson et al. 1999). These organic acids supply about 85% of energy requirement of these animals (Van Soest 2018). The most prevalent SCFA in the ruminal fluid are acetate, propionate, and butyrate; in less concentration, isobutyrate, valerate, isovalerate, and others compose the SCFA majority group (Dijkstra 1994). These acids can be used to produce adenosine 5′-triphosphate (ATP), since propionic acid acts as a glucose precursor, being part of hepatic gluconeogenesis cycle (Huntington 1990). The SCFA profile is directly correlated with the food consumption (Bergman 1990). For example, an animal which is feed with cellulose and hemicellulose will produce a higher acetate proportion, while animals supplied with soluble carbohydrate, as starch, will produce higher quantities of propionate. Thus, the acetate:propionate ratio can affect the ruminal pH and consequently the microorganism profile in the ruminal environment.

Some reported methods in literature use gas chromatography (GC) to analyze the SCFA in ruminal liquid. However, SCFA analysis by GC technique requires an extraction proceeding and consequently an additional sample preparation (Cottyn and Boucque 1968; Filipek and Dvorak 2009; Goularte et al. 2011). In contrast, liquid chromatography does not require this step, allowing a direct proceeding and only demanding a sample filtration to avoid the injection of particles that can damage the system (Mesquita et al. 2013).

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In this scenario, this study presents a new methodology for a simultaneous analysis of glucose (GLC) and seven SCFA, as follows: formic acid (FA), lactic acid (LA), acetic acid (AA), propionic acid (PA), butyric acid (BA), isovaleric acid (IVA), and valeric acid (VA) by means of high-performance liquid chromatography (HPLC) with UV-Vis and Refraction Index (RID) detectors coupled in series, using a reverse-phase chromatographic column (C-18). The validation of the method was performed by standard parameters (selectivity, linearity, sensitivity, limit of detection (LoD), limit of quantification (LoQ), precision, and accuracy) established by the Brazilian Health Regulatory Agency (ANVISA) (Armbruster and Pry 2008; ANVISA 2020).

Experimental

Safety Information

No significant hazards or risks associated with the present work were verified. All procedures were conducted in accordance with the Ethics, Bioethics and Animal Welfare Committee (protocol 8418/2019) of the School of Agricultural and Veterinarian Sciences (Unesp), Campus of Jaboticabal. Ruminal samples were obtained from two ruminally cannulated Nelore cows and feed with *Brachiaria brizantha* cv *Marandu*.

Chemicals

All acid solutions were prepared using analytical purity reagents (Sigma-Aldrich), and ultrapure water. For mobile phase, acetonitrile HPLC grade was purchased from Honeywell International Inc. and phosphoric acid (H_3PO_4) from Dinâmica.

Instrumentation

The analyses were performed in a Shimadzu HPLC system, model Prominence, equipped with an UV-Vis detector, model SPD-20, programmed to operate at a wavelength of 210 nm and a Refractive Index detector, model RID-20A. Calibration curve points were obtained by injecting 20 μL of the standard solution in different concentrations using an automatic sampler, model SIL-10AF. The system was equipped with a Shim-pack C-18 column, model VP-ODS, dimensions of 4.6 × 250 mm and particle size of 5 μm, at oven temperature of 35°C. The mobile phase was composed of phosphate buffer solution in a concentration of 20 mmol L^{-1} at pH 2.5 as polar solvent (named mobile phase A) and 100% acetonitrile as non-polar solvent (named mobile phase B).

Methodology Development

The method employed in this study was validated according to standard parameters established by the Brazilian Health Regulatory Agency (ANVISA), by Resolution n° 899, which assigns the validation criteria for analytical and bioanalytical methods. This Resolution defines parameters to be considered for validation of all analytical method where the HPLC technique is applied. Parameters required for the validation of the method are as follows: selectivity, linearity, sensitivity, LoD, LoQ, precision, and accuracy.

The method described by Kim et al. (2017), which determines SCFA in animal food using H_3PO_4 0.1% and acetonitrile as mobile phases, was modified and tested in the present work for analysis in samples containing ruminal liquid. Although, this method did not show any reproducibility on these conditions, as standard organic acids in different conditions were analyzed and exhibited a considerable shift in the chromatographic profile, which can be explained by the lack of pH control of the polar mobile phase. In aqueous solution, SCFA are mostly deprotonated. However, at a lower pH regarding their respective pK_a, they should maintain the protonated form. To overcome the peak shifting issue, the polar mobile phase A was replaced by a phosphate buffer 20 mmol L^{-1} at pH 2.5, a pH value below all the pK_a of the studied acids (FA pK_a = 3.77; LA pK_a = 3.85; AA pK_a = 4.75; PA pK_a = 4.87; BA pK_a = 4.82; IVA pK_a = 4.77, and VA pK_a = 4.82).

In parallel to SCFA quantification, the present method proposes the determination of glucose simultaneously. For this reason, analyses in UV-Vis and Refractive Index detectors are performed at the same time. For RID analysis, an isocratic method is mandatory, as any variation in mobile phase composition could interfere in the detection. However, it was not possible to separate the SCFA using a full isocratic method in a C-18 column. Therefore, the method was developed in an isocratic mode at the first 7 min of analysis, with a composition of 100% of mobile phase A. This proceeding allows to detect glucose by RID and formic, acetic, and lactic acids with UV-Vis detector, simultaneously. After this step, the RI detector was deactivated and the system started to operate in a gradient mode, eluting the remaining organic acids. At the end of each analysis, a period of 10 min was added for column conditioning and RID stabilization. The analytical method program details and parameters are described in Table 1.

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The calibration curve was constructed by diluting a stock solution of 5.0 mol L^{-1} of each standard SCFA and 1.0 mol L^{-1} of glucose, resulting in a mixture solution of 50 mmol L^{-1}

Table 1 Analysis method program parameters with the percentage variation of mobile phases A and B during the analysis time

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mode	Detector
00.00	100	0	Initial condition	RID/UV-Vis
07.00	100	0	Isocratic	RID/UV-Vis
10.00	90	10	Gradient	UV-Vis
20.00	75	25	Gradient	UV-Vis
20.01	70	30	Gradient	UV-Vis
29.00	70	30	Isocratic	UV-Vis
29.01	100	0	Stabilization	RID/UV-Vis
39.00	100	0	Stabilization	RID/UV-Vis

concentration, named working solution (WS). The curve points and linearity parameters were obtained by WS dilution to obtain the following concentrations: 1.0 mmol L^{-1}; 2.5 mmol L^{-1}; 5.0 mmol L^{-1}; 10 mmol L^{-1}; 25 mmol L^{-1}; and 50 mmol L^{-1}. All solutions were prepared independently and analyzed on the HPLC system in triplicate.

Linearity, Sensitivity, and Selectivity Parameters

Linearity is the capacity of covariance between the concentration of a given analyte in a sample and its analytical response (Armbruster and Pry 2008). That is, it is the linear interdependence between two variables and it can be measured by the correlation coefficient (r), which expresses the linear relationship between two points of the analyzed curve. If the correlation coefficient obtained is closer to values equal 1, the greater the linear relationship is resulted (Armbruster and Pry 2008; INME 2020). The method detection capacity is given by the sensitivity and can be verified by means of the slope, which is obtained with linear regression analysis. The slope is related to the substance's absorptivity: the higher its value, the more sensitive will be the method (Mesquita et al. 2013). Linearity and sensitivity of the method were evaluated by calibration curves, using peak areas versus nominal concentrations, analyzed in triplicate. Linear equation was used to determine the correlation between coefficient and sensitivity. Selectivity was determined by standard addition method (Mesquita et al. 2013). Standard aliquots of each analyte with a known concentration were added to the ruminal liquid sample (spiked ruminal sample), which was previously centrifuged at 7,000 rpm for 5 min and filtered with a syringe with a polyvinylidene fluoride (PVDF) filter of 0.45 μm. SCFA retention times were compared with those obtained in calibration curve (Fig. 1).

Limits of Detection and Quantification

The limit of detection (LoD) determines the lowest concentration of an analyte that can be detected (Armbruster and Pry 2008; ANVISA 2020). An acceptable LoD must produce an analytical signal three times greater than the noise. The limit of quantification (LoQ) is the smallest amount of analyte that can be quantified. LoQ values are accepted when they represent approximately three times the LoD value (Mesquita et al. 2013). For the analysis, LoD and LoQ were obtained by reports generated by linear regression made by LabSolution (Shimadzu) software, from the HPLC system, according to the chromatograms profiles presented in Figs. 1 and 2.

Reproducibility and Accuracy

The essays to check the method reproducibility and accuracy were performed by analyzing samples with a known concentration of 20 mmol L^{-1}, performed in five repetitions and different days. The reproducibility test was carried out by calculating the percentage of the variation coefficient obtained by Eq. 1 (Brito et al. 2003; Armbruster and Pry 2008; INME 2020):

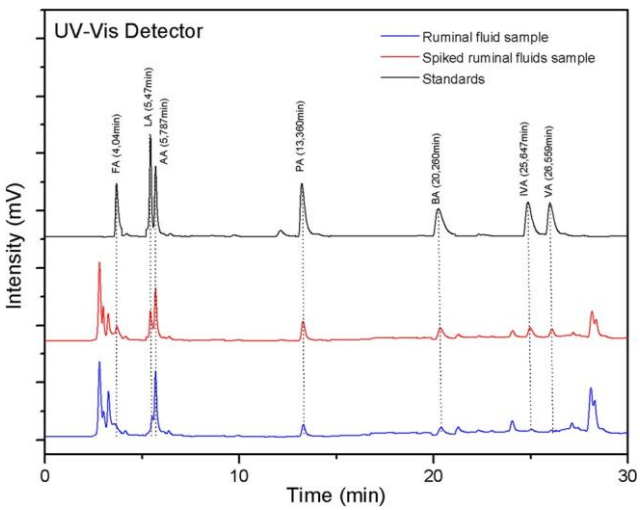

Fig. 1 SCFA UV-Vis separation profile: chromatographic profiles of ruminal fluid sample, spiked ruminal fluid sample, and SCFA standards obtained by UV-Vis detector

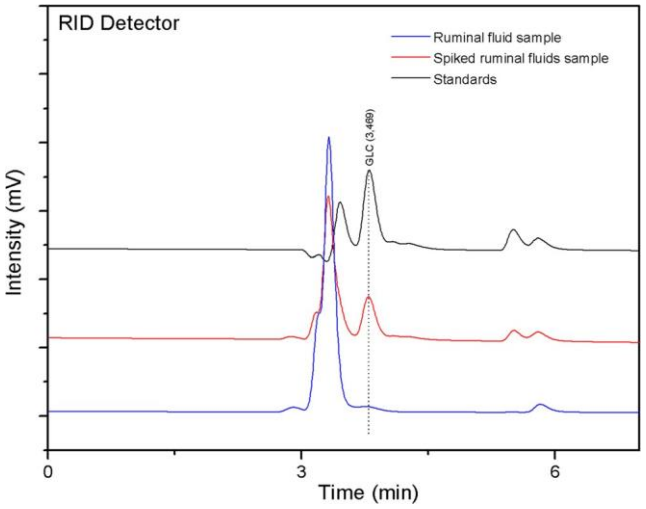

Fig. 2 SCFA RID separation profile: chromatographic profiles of ruminal fluid sample, spiked ruminal fluid sample, and SCFA standards obtained by Refraction Index detector

$$VC\ (\%) = \frac{SD}{Average} \times 10 \quad (1)$$

where SD is the standard deviation and VC is the variation coefficient, in percentage.

The accuracy of an analytical method is performed by checking the concordance between the average of the analyzed value and the theoretical value, taken as reference. In this way, it measures how close the measure of an analysis is near to the real value. The accuracy was calculated by the recovery parameter using Eq. 2 (Brito et al. 2003; Armbruster and Pry 2008; INME 2020):

$$Recovery\ (\%) = \frac{Co}{Ct} \times 100 \quad (2)$$

where Co is the obtained concentration and Ct is the theoretical concentration.

Results

Linearity, Sensibility, and Selectivity Parameters

All analytes showed a correlation coefficient remarkably close to 1, indicating that the method has a linear relationship. In addition, the developed method proved to be more sensitive for the LA sample analysis, which presented the highest angular coefficient, followed by IVA, AA, VA, FA, BA, PA, and GLC. Table 2 shows the linearity parameters of the method developed in this study and Figs. 1 and 2 show the UV-Vis and RID chromatogram profiles, respectively, acquired from the standard SCFA solution, ruminal fluid and spiked ruminal fluid samples analyzed, indicating the respective analyte peaks and their corresponding retention times. SCFA and GLC concentrations resulted from analyses of ruminal fluid samples using the present method are showed in Table 3, in triplicate.

Limits of Detection and Quantification

LoD obtained values from the analyses vary from 0.14 to 1.02 mmol L^{-1} and the LoQ values range from 0.43 to 3.10 mmol L^{-1}, which showed the lowest value for GLC and the highest for PA. These LoD and LoQ values are presented in Table 4.

Reproducibility and Accuracy

Recovery percentage results from the proposed method varied from 88.8 to 106.1%, as presented in Table 5.

Discussion

The selectivity was verified by the retention times of the analytes, where it can verify that there were no changes when comparing the chromatograms obtained from the injection of spiked ruminal fluid samples and calibration curve, prepared

Table 2 Linearity parameters: type of detector, retention time, slope, intercept, and linear correlation coefficient obtained from the reported method

Compound	Detector	Retention time (min)	Slope	Intercept	*r*
FA	UV-Vis	4.011	41,966.9	57,482.6	0.9963
LA	UV-Vis	5.471	48,357.8	4477.9	0.9999
AA	UV-Vis	5.787	45,635.6	33,795.7	0.9999
PA	UV-Vis	13.360	37,421.2	39,022.1	0.9999
BA	UV-Vis	20.260	40,688.2	14,067.2	0.9999
IVA	UV-Vis	25.647	46,881.0	7366.6	0.9998
VA	UV-Vis	26.559	42,743.3	5387.7	0.9999
GLC	RID	3.469	35,425.6	82,944.9	0.9999

Table 3 SCFA and GLC concentrations, average, and standard deviation (SD) calculated from ruminal fluid samples by the developed method

Compound	Concentration in ruminal fluid sample (mmol L^{-1})			Average	SD
	Repetition 1	Repetition 2	Repetition 3		
FA	22.49	20.09	19.71	20.76	1.70
LA	13.12	11.87	11.69	12.23	0.89
AA	58.16	64.37	63.84	62.12	4.39
PA	8.85	8.95	8.32	8.70	0.07
BA	8.97	10.20	10.08	9.75	0.87
IVA	2.46	2.90	1.96	2.44	0.31
VA	1.49	0.81	2.25	1.52	0.48
GLC	14.97	13.93	14.30	14.40	0.73

in ultrapure water. Figures 1 and 2 show that SCFA peaks exactly match with existing peaks in both ruminal fluid samples (natural and spiked), confirming the high method selectivity. Concentrations obtained by analyzing ruminal fluid samples (Table 3) indicate that the method exhibits an excellent repeatability, confirmed by the low variation (from 0.07 to 4.39) of standard deviation. In addition, calculated concentrations were still above the LoD and LoQ values. Regarding the LoD and LoQ values (Table 4), it can be clearly observed that they are similar to those acquired by de Sá et al. (2011), which presented LoD and LoQ values for glucose of 0.13 and 0.43 mmol L^{-1}, respectively; 0.24 and 0.94 mmol L^{-1} for acetic acid; 0.68 and 2.27 mmol L^{-1} for propionic acid; and finally, 0.36 and 1.19 mmol L^{-1} for butyric acid. For these reasons, the present method is in accordance with the literature and it is capable of quantifying very low concentrations (at micromolar scale) of the proposed organic analytes.

The recovery percentage values are in accordance to those described by Mesquita et al. (2013), which exhibited an average recovery ranging between 85 and 104%, when formic, acetic, propionic, butyric, isovaleric, and valeric acids' analyses were performed. In comparison, De Sá et al. (2011) obtained values between 71 and 113% when analyzing glucose, acetic, propionic, and butyric acids,

Table 4 Detection (LoD) and quantification (LoQ) limits for SCFA and glucose determination

Compound	Concentration (mmol L^{-1})	
	LoD	LoQ
FA	0.56	1.70
LA	0.24	0.72
AA	0.39	1.19
PA	1.02	3.10
BA	0.36	1.10
IVA	0.32	0.98
VA	0.35	1.06
GLC	0.14	0.43

exhibiting all results within the admissible range of 70 to 120% (Armbruster and Pry 2008; INME 2020). Values acquired for VC ranged from 0.17 to 0.60%, which are below 20%, the acceptable limit for method validation according to INMETRO guidelines, the most reliable Brazilian regulatory agency for metrology and quality (INME 2020). Moreover, VC values less than 10% are also recommended by IUPAC (Thompson et al. 2002).

There are many studies reported in literature intended to quantify SCFA using HPLC technique. However, most of these studies usually describe methods using cation-exchange columns (Guerrant et al. 1982; Stein et al. 1992; López and Gómez 1996; Castellari et al. 2001; Mesquita et al. 2013), employing a UV-Vis detection at 210 nm (Guerrant et al. 1982; Stein et al. 1992; de Sá et al. 2011) and also, as the present work, with simultaneous UV-Vis and RI detection (López and Gómez 1996; Castellari et al. 2001).

In relation to SCFA extraction, other methods performed a liquid-liquid extraction step, as described in De Baere (De Baere et al. 2013), which can be laborious and complicated. However, the presented method has the advantage to separate seven SCFA and glucose in a reverse-phase column, which can be a cheaper column option, in a simultaneous determination by UV-Vis and RI detectors. In addition, the developed method operates in isocratic and gradient modes, providing a better separation of SCFA in the examined analyses. To the best of our knowledge, this is the first time that this type of analytical method is described in the literature and it allows the employment of a reliable alternative for SCFA analysis using a HPLC system.

Conclusion

The proposed method described in this study for quantifying glucose and seven SCFA showed satisfactory results regarding the validation criteria of selectivity, linearity, sensitivity,

Table 5 Reproducibility and precision parameters: standard deviation (SD), percentage of recovery, and variation coefficient (VC) obtained from the developed method

Parameter	Short-chain fatty acid and glucose concentration (in mmol L^{-1})							
	FA	LA	AA	PA	BA	IVA	VA	GLC
Repetition 1	17.778	19.950	20.655	19.096	19.789	19.891	19.328	21.170
Repetition 2	17.754	19.888	20.600	19.244	20.063	20.014	19.535	21.231
Repetition 3	17.816	20.093	20.563	19.125	20.075	20.032	19.583	21.275
Repetition 4	17.736	19.863	20.665	19.053	19.964	19.997	19.429	21.148
Repetition 5	17.774	19.970	20.665	19.270	20.048	20.011	19.522	21.302
Average	17.772	19.953	20.630	19.158	19.988	19.989	19.479	21.225
SD	0.030	0.090	0.046	0.095	0.119	0.056	0.101	0.066
Recovery (%)	88.8	99.7	103.1	95.7	99.9	99.9	97.3	106.1
VC (%)	0.17	0.45	0.22	0.49	0.60	0.28	0.52	0.31

LoD, LoQ, precision, and accuracy. All analytes showed a correlation coefficient above 0.99, indicating that the method has a linear relationship. The recovery percentage ranged from 88.8 to 106.1%, and the variation coefficient from 0.17 to 0.60%, which is suitable for the proposed method. Calculated LoD and LoQ values acquired were in ranges of 0.14 to 1.02 mmol L^{-1} and 0.43 to 3.10 mmol L^{-1}, respectively, which are accepted and referenced values in literature. Therefore, the results demonstrate that the present method provides a reliable, fast, straightforward, and efficient alternative to analyze compounds of interest involved in ruminal fermentation, using a liquid chromatography technique with simultaneous UV-Vis and RI detection.

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Declarations

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflict of Interest J. A. S. declares that she has no conflict of interest. R. F. S. declares that she has no conflict of interest. E. F. V. declares that he has no conflict of interest.

Informed Consent Informed consent not applicable

References

Agência Nacional de Vigilância Sanitária (ANVISA); Resolução RE n° 899. (accessed 18 Jan 2020)

Armbruster DA, Pry T (2008) Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29:S49-S52

Baldwin RL (1998) Use of isolated ruminal epithelial cells in the study of rumen metabolism. J Nutr 128:293-296

Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70:567-590

Brito NM, Junior OPA, Polese L, Ribeiro ML (2003) Validação de métodos analíticos: estratégia e discussão. Pesticidas: Revista de Ecotoxicologia e Meio Ambiente 13:129-146

Castellari M, Sartini E, Spinabelli U, Riponi C, Galassi S (2001) Determination of carboxylic acids, carbohydrates, glycerol, ethanol, and 5-HMF in beer by high-performance liquid chromatography and UV-refractive index double detection. J Chromatogr Sci 39:235-238

Cottyn BG, Boucque CV (1968) Rapid Method for the Gas-Chromatographic Determination of Volatile Fatty Acids in Rumen Fluid. J Agric Food Chem 16:105-107

De Baere S, Eeckhaut V, Steppe M, De Maesschalck C, De Becker P, Van Immerseel F, Croubels S (2013) Development of a HPLC-UV method for the quantitative determination of four short-chain fatty acids and lactic acid produced by intestinal bacteria during in vitro fermentation. J Pharm Biomed Anal 80:107-115

de Sá LRV, Oliveira MAL, Matos A, Cammarota MC, Ferreira-Leitão VS (2011) Análise simultânea de carboidratos e ácidos orgânicos voláteis por CLAE para monitoramento do processo de produção de biohidrogênio por via fermentativa. In: Embrapa Solos-Resumo em anais de congresso (ALICE). In: LATIN AMERICAN WORKSHOP AND SYMPOSIUM ON ANAEROBIC DIGESTION, Ouro Preto, Brazil, pp. 5

Dijkstra J (1994) Production and absorption of volatile fatty acids in the rumen. Livest Prod Sci 39:61-69

Filipek J, Dvorak R (2009) Determination of the Volatile Fatty Acid Content in the Rumen Liqui d: Comparison of Gas Chromatography and Capillary Isotachophoresis. Acta Vet Brno 78:627-633

Goularte SR, Ítavo LCV, Santos GT, Ítavo CCBF, Oliveira LCS, Favaro SP, Dias AM, Torres Junior RAA, Bittas CMM (2011) Ácidos graxos voláteis no rúmen de vacas alimentadas com diferentes teores de concentrado na dieta. Arq Bras Med Vet Zootecnia 63:1479-1486

Guerrant GO, Lambert MA, Moss CW (1982) Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography. J Clin Microbiol 16:355-360

Huntington GB (1990) Energy metabolism in the digestive tract and liver of cattle: influence of physiological state and nutrition. Reprod Nutr Dev 30:35-47

Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO); Orientações sobre validação de métodos analíticos. (accessed 27 Jan 2020)

Kim HJ, Lee MJ, Kim HJ, Cho SK (2017) Development of HPLC-UV Method for Detection and Quantification of Eight Organic Acids in Animal Feed. J Chromatogr Sep Tech 8:1-5

López EF, Gómez EF (1996) Simultaneous determination of the major organic acids, sugars, glycerol, and ethanol by HPLC in grape musts and white wines. J Chromatogr Sci 34:254-257

Mesquita PL, Afonso RJCF, Aquino SF, Leite GS (2013) Validação de Método de cromatografia líquida para a determinação de sete ácidos graxos voláteis intermediários da digestão anaeróbia. Engenharia Sanitaria e Ambiental 18:295-302

Olson KC, Cochran RC, Jones TJ, Vanzant ES, Titgemeyer EC, Johnson DE (1999) Effects of ruminal administration of degradable intake protein and starch on utilization of low-quality warm season grass hay by beef steers. J Anim Sci 77:1016-1025

Stein J, Kulemeier J, Lembcke B, Caspary WF (1992) Simple and rapid method for determination of short-chain fatty acids in biological materials by high-performance liquid chromatography with ultraviolet detection. J Chromatogr B Biomed Sci Appl 576:53-61

Thompson M, Ellison SL, & Wood R (2002) Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report). Pure Appl Chem 74(5):835-856

Valadares Filho SC, Pina DS (2011) Fermentação Ruminal. In: Nutrição de Ruminantes, 2nd edn. FUNEP, Jaboticabal, pp 161-189

Van Soest PJ (ed) (2018) Nutritional ecology of the ruminant. Cornell University Press, Ithaca