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Production and antimicrobial activity of sophorolipid against *Clostridium perfringens* and *Campylobacter jejuni* and their additive interaction with lactic acid

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ABSTRACT

The poultry industry experiences enormous economic losses due to microbial contamination, leading to searches for natural antimicrobials. Sophorolipid is a glycolipid produced by *Starmerella bombicola*, that acts as an emulsifier and antimicrobial agent. The aim of this research was to produce, characterize and apply sophorolipid from *S. bombicola* against the chicken pathogens *Clostridium perfringens* and *Campylobacter jejuni*, which are responsible for diseases that cause great damage to the poultry industry. The application of sophorolipid against these microorganisms has not yet been reported and to aggregate value to the product, sophorolipid was associated with lactic acid, a nontoxic molecule already utilized in the food industry. Sophorolipid production reached 69.83 $g.L^{-1}$ with a productivity of 0.24 $g.L^{-1}.h^{-1}$ and a yield of 46.41% at 288 h in a bioreactor. The structural characterization analyses confirmed the predominance of the lactonic form. Sophorolipid demonstrated antimicrobial activity against the tested bacteria and the combined treatment of sophorolipid and lactic acid represented an additive interaction. Therefore, this combination could be an alternative to use as a new natural sanitizer in the poultry industry, reducing the microbial contamination of these main pathogens.

1. Introduction

The poultry industry in Brazil has a great social and economic impact, being ranked second in the worldwide production of chicken meat and first in export (ABPA, 2018). In contrast to this scenario of economic growth, maintaining the safety of the food supply system is one of the major challenges faced by the poultry industry. Technological advances have brought conditions that are difficult to animal health, such as super populated production systems, which provide ideal conditions for the multiplication of pathogens, resulting in disease outbreaks and economic losses to poultry producers (Aranda et al., 2019; Wilde et al., 2019).

Necrotic enteritis is an important chicken disease caused by *Clostridium perfringens*, an anaerobic endospore forming pathogenic bacterium, that accounts for annual worldwide losses of up to $6 billion dollars (Wade et al., 2015). This disease results in poor productivity by diminishing the weight of chickens, carcass condemnation and a high mortality rate of up to 50% (Van Immerseel et al., 2004).

Another great concern in the poultry industry is the pathogen *Campylobacter jejuni*, which is considered the most common cause of human gastrointestinal infections worldwide, causing 37,600 deaths per year globally (World Health Organization (WHO), 2015). This species may colonize chicken gastrointestinal tracts in an asymptomatic form, leading to the spread of this microorganism during production and processing, which results in contamination of live animals and processed carcasses (Skarp et al., 2016).

Antibiotics have been used to promote animal growth and as a prophylactic treatment of poultry pathogens (Lin et al., 2019). However, the impacts of this practice have been associated with the selection and dissemination of antimicrobial resistant bacteria (Aidara-Kane et al.,

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2018), resulting in restrictions, regulations and challenges regarding antibiotic use; therefore, the poultry industry is in search of alternative strategies.

Sophorolipid is a glycolipid produced by the yeast *Starmerella bombicola* in the lactonized and acidic forms with different degrees of acetylation on the sophorose moiety. The hydroxy fatty acid portion can vary in saturation degree, hydroxylation position (terminal or subterminal) and chain length, mostly with 16 and 18 carbons (Asmer et al., 1988; Van Bogaert et al., 2011).

This molecule has gained considerable importance because of its antimicrobial activities against a broad range of Gram positive and Gram negative bacteria. There are reports about its action against foodborne pathogens, such as *Salmonella spp.*, *Listeria spp.* (Zhang et al., 2016a) and *Escherichia coli O157:H7* (Zhang et al., 2017), and bacteria responsible for dental caries (Solaiman et al., 2017), acne formation (Ashby et al., 2011) and associated with common hospital infections (Pontes et al., 2016). Additionally, the antibiofilm activity of sophorolipid has been described (Banat et al., 2014; Díaz De Rienzo et al., 2015; Haque et al., 2016).

The antimicrobial activity of sophorolipid is characterized by plasma membrane disruption, causing the lysis and possible leakage of cytoplasm contents of pathogens (Kulakovskaya et al., 2014; Silveira et al., 2018; Zhang et al., 2016b). Additionally, the lactonic forms of this biosurfactant have better surface tension lowering properties and antimicrobial activities than acidic structures (Van Bogaert et al., 2007).

The application of organic acids on meat surfaces is a common procedure and the use of lactic acid could reduce the initial microbial load of meat products by immediate bactericidal and bacteriostatic effects, resulting in an extended shelf life (Prasai et al., 1992). This acid is also regularly utilized for the control of poultry pathogens at concentrations up to 5% (Mani-López et al., 2012).

Considering the antimicrobial properties of sophorolipid and the need to use alternative and safe molecules to control these poultry pathogens, this work evaluated for the first time, the action of sophorolipid from *S. bombicola* against *C. perfringens* vegetative cells and *C. jejuni* and its association with lactic acid for potential application in the poultry industry.

2. Material and methods

2.1. Sophorolipid production

Starmerella (Candida) bombicola (ATCC® 22214™) was maintained cryopreserved in stock solution containing 25% $v.v^{-1}$ glycerol at - 80 °C. The inoculum was prepared by transferring 0.5 mL of the stock solution to 25 mL of medium containing the following in $g.L^{-1}$: glucose (100), yeast extract (10) and urea (0.1), and incubated with 150 rpm for 24 h at 30 °C. The inoculum was standardized at 10% $v.v^{-1}$. The fermentation was conducted in a 5.0 L fed batch bioreactor (FerMac 320 Electrolab Biotech Ltd., United Kingdom) containing 3.5 L of working volume. The medium comprised the following in $g.L^{-1}$: glucose (77.5), yeast extract (2.5) and oleic acid (75) (Minucelli et al., 2017). Cultures were grown at 30 °C, at 450 rpm, with the pH controlled in 3.5 and an aeration rate of 1.0 vvm for 288 h. Oleic acid (20 $g.L^{-1}$) was added every 48 h until 192 h, and glucose (45 $g.L^{-1}$) was added at 144 h and 192 h. Every 24 h and at the end of fermentation, the sophorolipid, biomass, residual sugar and oil were measured. Sophorolipid was extracted three times with ethyl acetate, forming two phases; the organic phase was roto evaporated, and then extracted with a methanol:water solution (4:1 $v.v^{-1}$). The residual oleic acid content was removed with hexane, and both phases were quantified by gravimetry (Minucelli et al., 2017). The aqueous phase was centrifuged, and the biomass was determined gravimetrically. Residual glucose was quantified by the Somogyi Nelson method (Nelson, 1944; Somogyi, 1945).

The analysis of variance (ANOVA) was performed with the dates to estimate the significance differences ($p < 0.05$) between means by using the statistical software R version 3.4.1.

2.2. Sophorolipid characterization

Sophorolipid was detected by thin layer chromatography (TLC) by spotting on silica gel 60 F_{254}, and a solvent system comprising chloroform:methanol:water (65:15:2, $v.v.v^{-1}$) with 1% acetic acid was used. The plates were treated with vanillin sulfuric acid at 110 °C for 20 min and observed under UV light (Pekin et al., 2005). In addition, 1′, 4″-Sophorolactone 6′,6″-diacetate (Sigma Aldrich, USA) was used as the sophorolipid standard.

High performance liquid chromatography (HPLC) Shimadzu Corp. LC-6AD with the diode array detector SPD-M20A (λ 207 nm) and the Shim-pack column Shimadzu CLC-ODS (M)® C_{18} (4.6 × 250 mm; 4.6 μm; 12 nm) were also used for the detection of the sophorolipid. An acetonitrile water gradient was used that started with acetonitrile:H_2O (30:70, $v.v^{-1}$) for 5 min, increased to acetonitrile:H_2O (80:20, $v.v^{-1}$) in 25 min and was maintained there for the next 25 min (Wadekar et al., 2012).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were used to confirm the sophorolipid structure, utilizing a Bruker Avance III spectrometer operating at 400.13 MHz for ^{1}H and equipped with a direct detection probe (5 mm). All NMR experiments were performed in $CDCl_3$ as solvent using standard pulse sequences. Chemical shifts (σ) for ^{1}H and ^{13}C spectra were expressed in parts per million relative to tetramethylsilane (TMS) (Minucelli et al., 2017).

The surface tension was measured at 25 °C with a Fisher tensiometer (Krüss K6) in an aqueous solution of sophorolipid in the concentration range of 0–1 $g.L^{-1}$ (Du Nouy, 1925). Critical micelle concentration (CMC) was determined by relating the concentration of sophorolipid with the corresponding surface tension.

2.3. Antimicrobial study

2.3.1. Minimum inhibitory concentration (MIC)

The antimicrobial activities of sophorolipid and lactic acid were tested against *Clostridium perfringens* (ATCC® 3624™) vegetative cells and *Campylobacter jejuni* (ATCC® 33560™) by the microdilution method using 96 well plates (Clinical and Laboratory Standards Institute (CLSI), 2012). Strains were preserved in brain heart infusion broth (BHI) containing 25% $v.v^{-1}$ glycerol at - 80 °C. *C. perfringens* was first grown in BHI in anaerobiosis conditions created with the anaerobic gas generator (Mitsubishi™ AnaeroPack) and *C. jejuni* in thioglycolate (TC) agar in microaerophilia created with the Microaerobac system (Probac) for 48 h at 42 °C for both bacteria.

Bacterial suspensions were adjusted to 10^{8} CFU mL^{-1} (0.9% NaCl) using a 0.5 McFarland scale. These suspensions were diluted in the respective media and plated at a density of 5.0×10^{5} CFU.$well^{-1}$. Sophorolipid at 25% ($w.v^{-1}$) in ethanol was diluted in media to reach concentrations from 0.0078% to 1% for *C. jejuni* and from 0.0015% to 0.2% for *C. perfringens*. Lactic acid ranged from 0.04% to 5%. Positive (untreated bacteria) and negative (only medium) controls were performed. A vehicle control with ethanol was also conducted since this was used to dissolve the sophorolipid. Bacteria were incubated for 48 h at 42 °C, and the MIC was recorded as the lowest concentration of sophorolipid or lactic acid that completely inhibited microbial growth visually.

2.3.2. Combined effect of sophorolipid and lactic acid

The interaction activity of sophorolipid (SL) with lactic acid (LA) was evaluated by checkerboard assay, which consisted of broth dilution in a double-antimicrobial gradient as described by Traub and Kleber (1975). Sophorolipid dilutions (0.0015%–0.025% for *C. perfringens* and 0.5%–0.03% for *C. jejuni*) were prepared in the horizontal rows, and the lactic acid dilutions (0.019%–2.5%) were prepared in the vertical columns of the 96 well plates. The fractional inhibitory concentration (FIC) and

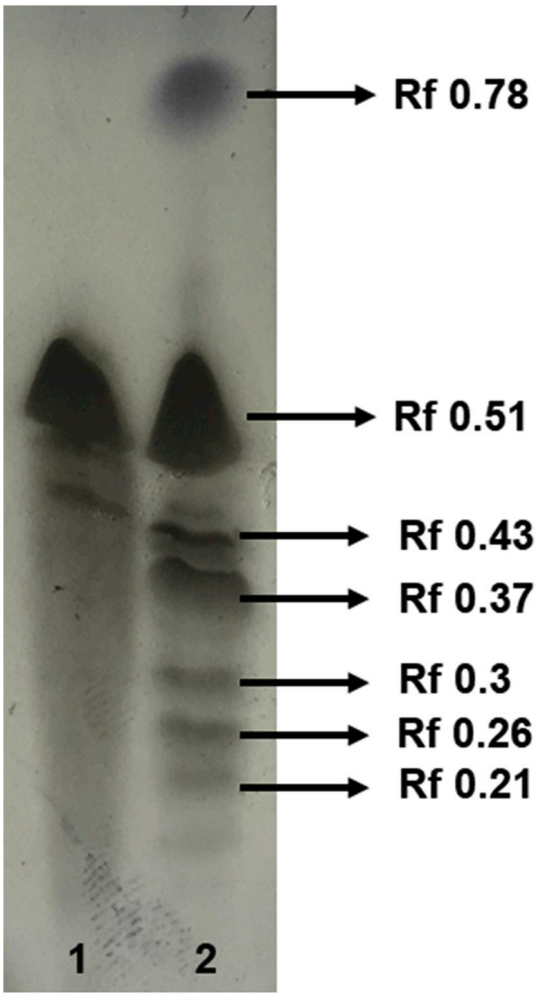

Fig. 1. Thin layer chromatograph (TLC) analysis of sophorolipid by *Starmerella bombicola* with developing solvent system chloroform: methanol: water (65:15:2, v/v/v) with 1% acetic acid. (1) Standard 1′,4″-Sophorolactone 6′,6″-diacetate and (2) sophorolipid.

fractional inhibitory concentration index (FICI) were calculated by employing the MIC of the compounds alone and combined. The FICI classification was the following: ≤0.5, synergistic; >0.5 to 1, additive; >1 to <4; indifferent and ≥4, antagonistic. The FICI (∑FICI) was determined using the following equation (Chin et al., 1997):

$$\sum FICI = FIC_{SL} + FIC_{LA}$$

∑FICI= (MIC of SL in combination) / (MIC of SL) + (MIC of LA in combination) / (MIC of LA)

3. Results and discussion

3.1. Sophorolipid production and characterization

Sophorolipid production reached 69.83 $g.L^{-1}$ with productivity a of 0.24 $g.L^{-1}.h^{-1}$ and a yield of 46.41%. Fermentative parameters influence sophorolipid production and the structures formed, affecting the proportions of acidic and lactonic forms (Ribeiro et al., 2015; Van Bogaert et al., 2011). Casas and García-Ochoa (1999), Davila et al. (1992) and Hu and Ju (2001) reported that the lactonic structures are highly produced during the stationary phase after 100–250 h of fermentation. Therefore, the fermentation time is a substantial parameter to direct the production of specific sophorolipid structures, which define particular applications, such as antimicrobial activity (Van Bogaert et al., 2007; Zhang et al., 2016a).

TLC showed the presence of acidic structures with Rf values of 0.21 and 0.26, whereas the spots with higher Rf values of 0.3, 0.37, 0.43, and 0.51 were attributed to lactonic forms, as expected considering their lower polarity (Fig. 1) (Asmer et al., 1988; Mousavi et al., 2014; Pekin et al., 2005). The HPLC analysis in comparison with the standard showed that the main structure detected in the chromatographic profile was lactonic C18:1 diacetylated (retention time of 43.39 min) (Fig. 2).

These results were confirmed by the combined analysis of one- and two-dimensional ^{1}H NMR experiments (Fig. 3). The one-dimensional ^{1}H NMR spectrum showed signals assigned to the two anomeric protons from the sophorose moiety resonating at 4.56 and 4.46 ppm. The HSQC spectrum showed correlations for these proton signals with their anomeric carbons at 104.0 and 102.3 ppm, respectively. Another seven glucose CH protons were observed between 3.40 and 3.80 ppm as

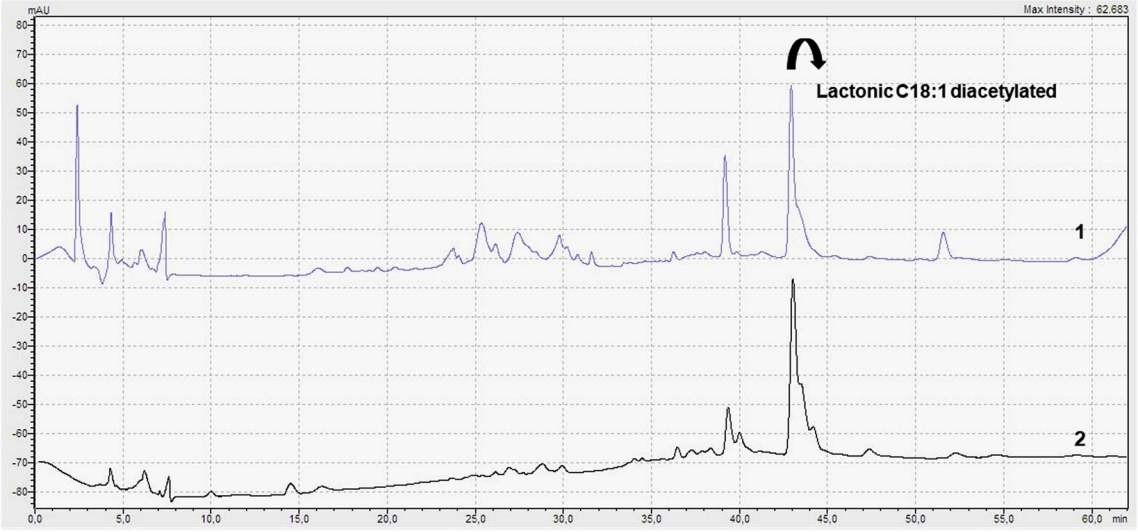

Fig. 2. High performance liquid chromatograph (HPLC) analysis of sophorolipid in comparison with standard. (1) Standard 1′,4″-Sophorolactone 6′,6″-diacetate and (2) sophorolipid.

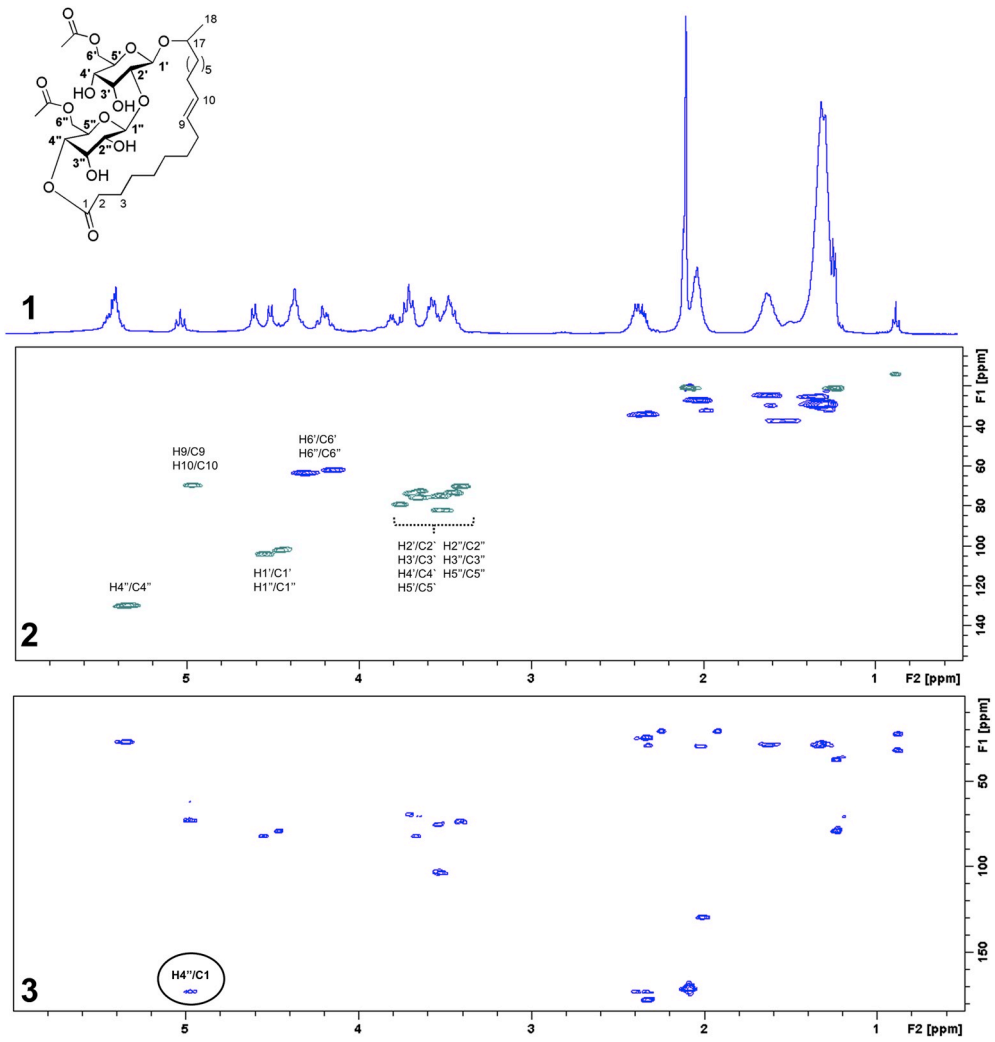

Fig. 3. NMR experiments of the sophorolipid from *Starmerella bombicola*. (1) ^{1}H, (2) HMQC and (3) HMBC spectra.

positive phased HSQC signals, while the H-4″ proton resonates at higher frequency (4.97 ppm) due to its proximity to the lactone carboxyl. Moreover, the corresponding HMBC signal showed a multiple bond correlation for H-4″ and the carbonyl carbon at 173.4 ppm, evidencing the continuous connectivity throughout H-4″ and C-1. The carbon signals of the two cetyl carbonyls were observed at slightly higher fields at 171.7 and 170.6 ppm.

Both CH_2 groups of the glucose units were easily identified by negative phased HSQC signals at 4.32 and 4.15 ppm. In particular, methylene bound to the lactone carbonyl was identified at 2.35 ppm (H-2). The HSQC signal at δ_H 5.35/δ_C 129.9 confirmed the presence of the vinyl group (–CH═CH–).

The sophorolipid structural analyses using TLC, HPLC and NMR in combination with literature reports (Daverey and Pakshirajan, 2010; Kaur et al., 2019; Minucelli et al., 2017; Price et al., 2012) determine that the produced sophorolipid was mainly lactonic in structure.

Sophorolipid surface tension reduction of water is demonstrated in Fig. 4. A gradual decrease from 70 mN m^{-1} to 34 mN m^{-1} was observed at 25 °C with an increase in sophorolipid concentration at a CMC of 65 mg.L^{-1}, which then remained constant. These results are in accordance with the literature (Develter and Lauryssen, 2010; Jadhav et al., 2019; Minucelli et al., 2017; Otto et al., 1999).

Sophorolipid antimicrobial activity is also related to antiadhesive properties due to their amphiphilic nature. This glycolipid can reduce the interfacial and superficial tension of compounds and surfaces, thus promoting alterations in the adhesion of microorganisms (Pontes et al., 2016; Valotteau et al., 2017). Lactonic sophorolipid exhibits lower CMC than acidic forms, being more hydrophobic and less soluble in water molecules (Kulakovskaya et al., 2014), which could also explain the better antimicrobial action reported involving this type of sophorolipid.

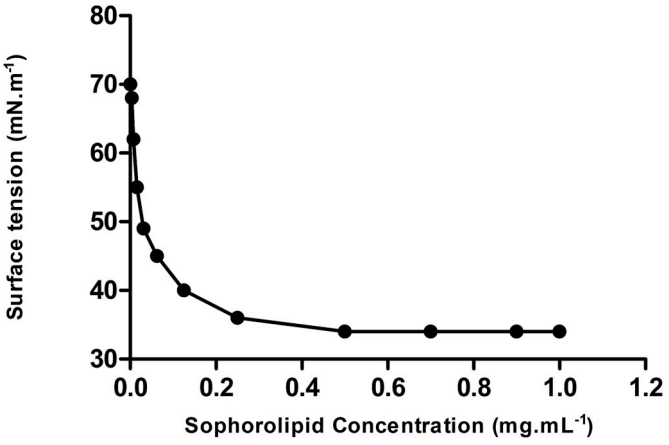

Fig. 4. Relationship between the surface tension and the concentration of sophorolipid.

Table 1
Minimum inhibitory concentration and fractional inhibitory concentration of sophorolipid and lactic acid against *Clostridium perfringens* and *Campylobacter jejuni*.

Bacteria	Sophorolipid (%)			Lactic acid (%)			FICI	Interaction
	MIC	MIC_C	FIC	MIC	MIC_C	FIC		
Clostridium perfringens	0.003	0.0015	0.5	0.07	0.03	0.43	0.93	Additive
Campylobacter jejuni	1	0.5	0.5	0.07	0.03	0.43	0.93	Additive

MIC - minimum inhibitory concentration.
MIC_C – minimum inhibitory concentration in combination.
FIC – Fractional inhibitory concentration.
FICI – Fractional inhibitory concentration index: ≤0.5, synergistic; >0.5 to 1, additive; >1 to <4; indifferent and ≥4, antagonistic.

3.2. Antimicrobial activity

The results of the antibacterial tests with sophorolipid and lactic acid are demonstrated in Table 1. Although there are many works describing the antimicrobial action of sophorolipid in a broad range of bacteria, the susceptibility of the tested pathogens with great importance in the poultry industry has never been assessed. This novel discovery brings new possibilities for the implementation of sophorolipid as a natural sanitizer during the processing steps of the poultry industry, enhancing the safety of food products. Sophorolipid could be incorporated in the washing/rinsing cabinet, known as inside/outside bird wash (IOBW) or in the immersion chiller tank systems, where the carcasses remain for long periods of 45–110 min (Blevins et al., 2018).

The mechanisms of action of sophorolipid antimicrobial activity are related to their surfactant effect, which involves synergistic interactions between the sophorose and the fatty acid moieties, resulting in the destabilization and alteration of the membrane permeability of pathogens, characterized by plasma membrane disruption and extravasation of the cellular content (Dengle-Pulate et al., 2013; Fracchia et al., 2015; Oliveira et al., 2015; Valotteau et al., 2017). In this work, *C. perfringens* was more susceptible to sophorolipid action than *C. jejuni*. This could be explained by the differences in cell wall structures since *C. perfringens* is a Gram positive bacterium and *C. jejuni* is a Gram negative bacterium.

The cell wall of Gram positive bacteria is made of one membrane layer surrounded by a thick peptidoglycan layer. While in Gram negative bacteria, the cell wall is composed of a thin peptidoglycan layer and two membranes, making it more difficult for sophorolipid to interact with the cell envelope of Gram negative organisms than that of Gram positive organisms (Dengle-Pulate et al., 2014; Zhang et al., 2016a). These differences in the cell envelope also confer different charges to the bacteria. Gram negative species have less hydrophobic and more negative characteristics, making them less affected by the surface changes promoted by sophorolipid, possibly explaining the lower antibacterial potential obtained for *C. jejuni*.

The combined treatment of sophorolipid and lactic acid was capable of keeping the antibacterial efficiency at only ½ MIC of each compound, representing an additive interaction. Lactic acid is a natural antimicrobial largely used in the decontamination of meat products (Anang et al., 2007; Chaine et al., 2013; Cil et al., 2019; Liu et al., 2016). This action is related to intracellular acidification and disruption of the transmembrane proton motive force, causing depletion of cellular energy (Alakomi et al., 2000; Mani-López et al., 2012; Ricke, 2003).

Latic acid has a low molecular mass (90.08 Da) and water soluble nature; thus, this molecule can easily penetrate the outer membrane of bacteria and modify its integrity, increasing the permeability of membranes and enabling other compounds (macromolecules and hydrophobic components) to penetrate (Alakomi et al., 2000; Nikaido, 1996; Stanojević-Nikolić et al., 2016). The presence of lactic acid may also maximize the effect of other antimicrobial agents, as shown in this study in combination with sophorolipid.

Although the use of organic acids in the poultry industry is very common and effective, their use is limited since they may affect sensory aspects of the product (color changes and negative flavor) (Mani-López et al., 2012; Nagel et al., 2013). Organic acids have been associated with other antimicrobials agents, enabling a lower concentration of the acid to a sensory acceptable level (Hulankova et al., 2013; Nagel et al., 2013). Thus, the sophorolipid association with lactic acid could be advantageous because of the lower concentration achieved in combination, possibly resulting in fewer impacts on the sensory characteristics of the product with the same antimicrobial efficacy.

4. Conclusions

Sophorolipid produced by *S. bombicola* in a fed batch bioreactor achieved 69.83 $g.L^{-1}$, a productivity of 0.24 $g.L^{-1}.h^{-1}$ and a yield of 46.41% at 288 h. Structural analyses demonstrated the predominance of the lactonic form, and HPLC detected lactonic C18:1 diacetylated in comparison with the standard. Sophorolipid presented antibacterial activity against the poultry pathogens *C. perfringens* and *C. jejuni*. The combination of this biosurfactant with lactic acid resulted in an additive interaction, keeping the same antibacterial efficacy with half concentration of each compound. This study suggests that sophorolipid and its combination with lactic acid have the potential to be a new natural sanitizer for applications in the poultry industry.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

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