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ORIGINAL ARTICLE

Evaluation of a propolis hydroalcoholic solution with glycine in the joint reduction of microorganisms and helminth eggs from leafy green vegetables

Avaliação da eficácia da solução hidroalcoólica de própolis com glicina na redução conjunta de microrganismos e ovos de helmintos em vegetais de folhas verdes

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Abstract

The main goal of this study was to evaluate the effectiveness of a propolis hydroalcoholic solution with glycine on the reduction of microorganisms and helminth eggs removal in lettuce leaves. Three experimental groups were evaluated in triplicate or replicates: microbiological (ME), parasitological (PE), and microbiological and parasitological (MPE). Before the experiments, the initial concentration of the native microbiota was assessed by counting mesophilic aerobes, molds and yeasts, using conventional microbiological techniques. Lettuce leaves were washed in tap water and subjected to different immersion treatments: propolis hydroalcoholic solution (PHS), propolis hydroalcoholic solution at pH 5.5 (PHS2), and propolis hydroalcoholic solution with glycine (PHS-glycine). In ME, propolis hydroalcoholic solution combined or not with glycine, was evaluated on the reduction of native microbiota. Residual microbiological contamination was assessed. In the PE, the effect of PHS2 and PHS-glycine was evaluated on the removal of a known number of *Ascaris suum* eggs on leaves artificially contaminated. In MPE, PHS-glycine was evaluated on the reduction of native microbiota and removal of eggs. A reduction of more than 2 log CFU/g was observed in ME. In PE, PHS-glycine removed 37.22% of eggs. In MPE, the PHS-glycine reduced 3.3 CFU/g of mesophilic aerobes, 5.0 log CFU/g of fungi, and 35.53% of eggs from vegetables. The results highlighted the solution's efficiency in reducing or eliminating different contaminants in vegetables and reinforced the need for the development of hygiene methods that do not harm human health and are reproducible at the household level.

Keywords: Antimicrobial agent; Bacteria; Fungi; Parasite; Food safety; Vegetables.

Resumo

O principal objetivo deste estudo foi avaliar a eficácia da solução hidroalcoólica de própolis com glicina na redução de microrganismos e na remoção de ovos de helmintos em folhas de alface. Três grupos experimentais foram avaliados em triplicata ou réplicas: microbiológico (ME), parasitológico (PE) e microbiológico e parasitológico (MPE). Antes dos experimentos, a concentração inicial da microbiota nativa foi avaliada quanto à contagem de aeróbios mesófilos, fungos e leveduras, usando técnicas microbiológicas convencionais. As folhas de alface foram lavadas em água da torneira e submetidas a diferentes tratamentos de imersão: em solução hidroalcoólica de própolis (SHP), em solução hidroalcoólica de própolis com pH 5,5 (SHP2) e em solução hidroalcoólica de própolis com glicina (SHP-glicina). No ME, a solução hidroalcoólica de própolis combinada ou não com glicina foi avaliada na redução da microbiota nativa. A contaminação microbiológica residual foi avaliada. No PE, o efeito de SHP2 e SHP-glicina foi avaliado na remoção de um número conhecido de ovos de *Ascaris suum* em folhas artificialmente contaminadas. No MPE, a SHP-glicina foi avaliada na redução da microbiota nativa e na remoção de ovos. Uma redução de mais de 2 log UFC/g foi observada no ME. No PE, SHP-glicina removeu 37,22% dos ovos. No MPE, a SHP-glicina reduziu 3,3 UFC/g de aeróbios mesófilos, 5,0 log UFC/g de fungos e 35,53% dos ovos nos vegetais. Os resultados destacam a eficiência da solução na redução ou eliminação de diferentes contaminantes em vegetais e reforçam a necessidade do desenvolvimento de métodos de higienização que não prejudiquem a saúde humana e sejam reproduzíveis em nível doméstico.

Palavras-chave: Agente antimicrobiano; Bactérias; Fungos; Parasitos; Segurança alimentar; Vegetais.

Highlights

- A natural solution was developed and tested for decontaminating vegetables
- The solution removed parasite eggs and did not differ from the glycine statistically
- The solution reduced or removed concomitantly microorganisms and helminth eggs

1 Introduction

Washing and sanitizing vegetables and fruits should be a recurring habit, as it decreases the risk of contamination by bacteria and parasites (Ramos et al., 2013; Hajipour et al., 2021). Chlorine compounds can be employed to decontaminate vegetables because of their effectiveness in reducing microbial load (Aworh, 2021; López-Gálvez et al., 2021).

However, the use of chlorinated solutions can pose chemical risks to human health. After chlorine comes into contact with organic matter presents in water and/or vegetables, a reaction occurs resulting in the formation of carcinogenic substances (Mendoza et al., 2022).

The ineffectiveness against parasites is another concern raised in the sanitizing processes commonly used in industries and households. This is because parasite eggs have a robust structure that makes them resistant to chemical methods and capable of enduring adverse conditions in the environment or food for several days (Amoah et al., 2017; Fidjeland et al., 2015).

These are alarming facts, considering that while bacteria require large numbers to cause pathological conditions, 1 to 10 parasite eggs may be enough to infect humans, for instance, the infection with *Ascaris* spp. (Shingare et al., 2019).

In households, where there are many food contaminating reports and minimal sanitary surveillance, the development of an ideal sanitizer, besides having a chemical composition harmless to humans, must have an antimicrobial capacity to inactivate bacteria and fungi, and mechanisms that facilitate the removal of parasite eggs from fresh food (Brasil, 2007; Matosinhos et al., 2016).

A natural antimicrobial solution can be developed through propolis extracts, a resin collected by bees that has bioactive compounds that, in synergism, present bactericidal and antifungal effects, thus enabling its use as a sanitizer or food preservative (Feás et al., 2014; Pobiega et al., 2019).

An eluent agent is required to remove parasite eggs from fresh produce, and glycine is a promising candidate. Under 1M and pH 5.5 conditions, glycine removes an expressive number of different parasite eggs in vegetables (Matosinhos et al., 2016; Pineda et al., 2021).

However, the action of a solution combining this amino acid and propolis extracts, with emphasis on vegetable decontamination, has not been properly researched.

Thus, the purpose of the present study was to evaluate the effect of propolis hydroalcoholic solution with glycine (PHS-glycine), on the reduction of microorganisms and removal of helminth eggs in lettuce leaves.

2 Material and methods

2.1 Study outline

The study was conducted in three experimental groups: microbiological experiment (ME), parasitological experiment (PE), and microbiological and parasitological experiment (MPE), where the effect of different solutions on the reduction of microorganisms and removal of helminth eggs from lettuce leaves were evaluated (Table 1).

Before the experiments, the initial concentration of native microbiota was assessed by counting mesophilic aerobes, molds and yeasts for ME and MPE, using conventional microbiological techniques (Silva et al., 2017).

After that, all lettuce leaves were washed in running tap water and subjected to different immersion treatments. Residual microbiological contamination was assessed after specific treatments (Table 1).

The evaluations conducted in ME and MPE were performed in triplicate and three replicates, and for PE, in triplicate.

Leaves without treatment, running tap water, and sodium hypochlorite were used as control groups in the ME; washing tap water and glycine in the PE; and the same control groups of ME were used for MPE.

The ME was divided into two assays: i) *in vitro*, carried out to evaluate the antimicrobial action of propolis hydroalcoholic solution (PHS) combined or not with glycine; and ii) *in situ*, carried out to assess the effect of decontamination with different solutions and with propolis hydroalcoholic solution with glycine (PHS-glycine), on the reduction of native microbiota.

In the PE, *Ascaris suum* eggs were chosen as a model, for artificial contamination of lettuces, especially for showing marked resistance to environmental stress similar to the *Ascaris lumbricoides* egg (Karkashan et al., 2015).

The effect of PHS-glycine on the removal of a known number of helminth eggs was evaluated, and whether mixing both solutions would interfere with egg recovery and visualization.

Finally, in the MPE, the effect of the PHS-glycine was evaluated over the reduction of native microbiota concomitant with helminth eggs removal.

Table 1. Treatments, characteristics, and response time to different solutions used to decontaminate vegetables.

	Treatments	Experiment	Characteristics of the antimicrobial solution and/or eluent		Time (min) of the hygienization process	
			Concentration (%)	pH	Immersion	Agitation
	Initial concentration prior to treatments	ME, PE, and MPE	NA	NA	NA	NA
1	Running tap water	ME, PE, MPE	NA	6.8	NA	NA
2	Sodium hypochlorite	ME	0.02 (v/v)	6.9	15	NA
3	PHS	ME	2 (v/v)	4.9	30	NA
4	PHS2	ME	2 (v/v)	5.5	30	NA
5	Glycine	ME	1.5 (w/v)	5.5	30	NA
6	PHS*-glycine**	ME	2 (v/v)* – 1.5 (w/v)**	5.5	30	NA
7	Washed in running tap water	PE	NA	6.8	NA	3
8	PHS2	PE	2 (v/v)	4.9	NA	3
9	Glycine	PE	1.5 (w/v)	5.5	NA	3
10	PHS*-glycine**	PE	2 (v/v)* – 1.5 (w/v)**	5.5	NA	3
11	Sodium hypochlorite	MPE	0.02 (v/v)	6.9	27	3
12	PHS*-glycine**	MPE	2 (v/v)* – 1.5 (w/v)**	5.5	27	3

ME: Microbiological experiment; PE: Parasitological experiment; MPE: Microbiological and parasitological experiment; PHS: propolis hydroalcoholic solution; PHS2: propolis hydroalcoholic solution (pH 5.5); PHS-glycine: propolis hydroalcoholic solution with glycine; NA: not applicable. *Concentration referring to propolis extract; **Concentration referring to glycine.

2.2 Obtaining the antimicrobial solutions and eluents.

The sodium hypochlorite solution at a 0.02% concentration was obtained by diluting 8 mL of the chlorinated product in 1L of distilled sterile water. To obtain the 2% PHS and PHS2, propolis extracts were used, which met the identity and quality standards according to Brazilian legislation (Brasil, 2001). Both solutions were diluted in distilled sterile water until the solution reached 200 mL. The glycine solution (1M) was created by adding distilled sterile water to 15.01 g of glycine powder until 200 mL of solution was obtained. The PHS-glycine was composed of glycine, propolis extract and distilled sterile water enough to reach 200 mL, so that the final solution had 1M of glycine and 2% of dry propolis extract.

PHS2, glycine, and PHS-glycine had their pH corrected to 5.5 for better efficiency of parasite egg removal in leafy vegetables (Pineda et al., 2021).

2.3 Acquiring and processing the lettuce samples

Leaf lettuce was used for the experiment (*Lactuca sativa var.* Crispa), of the conventional variety, collected in retail markets of Curitiba, in the state of Paraná. Besides being one of the most consumed vegetables by the Brazilian population, lettuce was selected because it is eaten raw and is frequently used in similar studies (Feás et al., 2014; Matosinhos et al., 2016).

Before treatment, a pooling was organized for each repetition, homogenizing three units of lettuce from each collection cycle in sterile vats, defoliating them and discarding the core plus two of the outermost layers. By replicating in the experiments the same cleaning method used in households, they were washed and rubbed individually for 2 min in running tap water in accordance with Amoah et al. (2007) and then were cut into dimensions of 5 x 10 cm (Feás et al., 2014) using a scalpel and a sterile template.

2.4 Microbiological Experiment (ME)

2.4.1 In vitro

Antimicrobial procedures were performed on standard strains of *Staphylococcus aureus* (ATCC 25023), *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (ATCC 35152), *Salmonella typhimurium* (ATCC 117789), and *Candida albicans* (ATCC 25023), used as yeast models. Microorganism suspensions were obtained from isolated colonies growing on agar for 24 hours and transferred to test tubes containing sterile water. Each pathogen suspension was then standardized by visually comparing its turbidity to a tube with an equivalent of 0.5 in McFarland standards (Silva et al., 2022).

The experiment was performed in a sterile 96-well plate with round bottoms and 100 µL of Mueller Hinton broth. To each well on the first row, 100 µL of the serially diluted testing solutions were added, transferring 100 µL from the first well to the well of the next row, and so on until the last row. The final aliquot taken from the last well was discarded to yield all of them the same volume.

Subsequently, 10 µL of the bacterial and fungal strains were added to every well of the plate, except for the negative control group. The solutions were evaluated from the initial concentration as described in Table 1. The positive control group consisted of chloramphenicol solution (0.01%), ketoconazole (0.1%) and ethanol (4.95% of the amount present in PHS, PHS2 and PHS-glycine).

After serial dilution of the solutions and setting control groups, the plates were incubated at 36 °C/24h, and 20 µL of 2,3,5-triphenyl tetrazolium chloride (TTC) for the bacterial strains and resazurin for *C. albicans* were subsequently added to each well. The plates were again incubated at 36 °C/2h, and after that period a visual analysis of the bacterial growth was conducted. Inhibition of microorganisms by the antimicrobial solutions was established for the wells without color changes, after the addition of TTC or resazurin (Silva et al., 2022).

2.4.2 In situ effect

The experiments were conducted on a pool (item 2.3) of 10 g lettuce leaves, randomly chosen and sorted according to the treatment in first-use plastic bags containing 200 mL of each antimicrobial or elution solutions. After the active period according to Table 1, the samples were submitted for microbiological analysis.

2.4.2.1 Microbiological analysis

Mesophilic aerobic and molds and yeasts counts were performed in triplicate according to Silva et al. (2017). Homogenization was accomplished by diluting 10 g of the samples in 90 mL of peptone water (0.1%).

The resulting solution constituted the 10^{-1} dilution, from which the serialized dilutions (10^{-2} to 10^{-5}) were obtained. For mesophilic aerobes, 0.1 mL of the corresponding dilution was inoculated by surface plating in Plate Count Agar (PCA) and incubated at 35 °C ± 1 °C for 48h ± 2h for subsequent counting of log CFU/g. For fungi, 0.1 mL of the corresponding dilution was inoculated by surface plating on agar with Dichloran Rose Bengal Chloramphenicol (DRBC) and incubated at 25 °C ± 1 °C for 5 days for subsequent counting of log CFU/g.

2.5 Parasitological experiment (PE)

2.5.1 Obtaining *A. suum* eggs for the inoculum

To obtain a purified suspension of helminth eggs, adult females collected from parasitized pigs were dissected and the uterus was removed near the last 2 cm of its bifurcation and opened. Then, the collected material was moved to a petri dish, to separate the eggs by pressing the uterus in dechlorinated water. Subsequently, the material was centrifuged (1250 x *g* for 5 min) and stored under refrigeration (4°C) in formalin

(3%). This solution resulted in the stock suspension. To quantify the eggs in suspension, the eggs contained in 10 aliquots were calculated according to Pineda et al. (2021), resulting in approximately 197 eggs on average.

2.5.2 Artificial contamination of lettuce samples

Seeding suspensions were spread on the leaves using pipettes. Another 100 µL of distilled water was used to wash the microtube, to detach the remaining eggs from the utensils' surface and disseminate them over the lettuce leaves. After this procedure, the leaves rested at room temperature (25°C) for 2h. Using tweezers, the samples were inserted into different first-use polyethylene bags (15 x 26 cm), containing tap water, PHS2, glycine solution, and PHS-glycine. Afterward, the bags were sealed and manually agitated for 3 min. The resulting liquid was submitted to parasitological analysis to determine the efficacy of removing *A. suum* eggs from lettuce leaves (Matosinhos et al., 2016; Pineda et al., 2021).

2.5.3 Assessing the efficacy of *A. suum* eggs removal from lettuce leaves

Briefly, the resulting liquid in the plastic bag was filtered through a 1 mm mesh sieve and collected in a conical goblet, kept at rest for 2 hours. The supernatant was then removed, and the sediment was centrifuged at 1250 x *g* for 5 min. After this step, the supernatant was discarded, and all the remaining sediment (1 mL) was analyzed under a microscope at 200x magnification, after arranging the slides (approximately 40 slides per sample).

Due to the presence of propolis wax after the Falcon tubes centrifugation in the PHS2 and PHS-glycine treatments, it was necessary to add 0.5 mL of ethanol (95%) followed by 1 min of vortexing to dissolve the wax, since eggs could remain in this mixture.

The recovery efficiency of parasite eggs from each treatment was expressed as a percentage, representing the ratio between the number of *A. suum* eggs removed and the initial number of eggs inoculated into lettuce (Matosinhos et al., 2016).

To confirm the absence of helminths that could eventually occur in lettuce samples under natural conditions, the leaves that did not undergo any artificial contamination and sanitization process were also submitted to parasitological analysis, using glycine as an eluent solution.

2.6 Microbiological and Parasitological Experiment (MPE)

To verify the effectiveness of PHS-glycine on the reduction of microorganisms and removal of *A. suum* eggs, lettuce leaves were previously washed in running tap water and then, artificially contaminated with *A. suum* eggs. After 2h, the leaves were placed in first-use plastic bags containing sodium hypochlorite or PHS-glycine.

Subsequently, the bags were sealed with the solutions for 27 min, followed by manual agitation for 3 min (Table 1). The resulting liquid in the plastic bag was submitted to parasitological analysis and the lettuce leaves to microbiological analysis.

The procedures for washing the lettuce leaves, preparing the inoculum, contaminating the leaves with *A. suum* eggs, and the microbiological and parasitological analyses occurred as described in segments 2.3, 2.4 and 2.5.

2.7 Statistical analysis

To verify the normality of the results, the Shapiro-Wilk test was used. Then, to verify the homogeneity of the samples, the Levene test (or F test) was used. The results of mesophilic aerobics, total fungi (ME and MPE), and percentage of *A. suum* egg removal (PE) were submitted to Analysis of Variance (ANOVA) followed by Tukey's test to identify the difference between the samples. In the MPE, the Mann-Whitney test was used to compare the results of *A. suum* egg removal between sodium hypochlorite and PHS-glycine. Statistical differences were considered at the $p < 0.05$ level and the analyses were performed using IBM SPSS Statistics software version 21.

3 Results and discussion

Food and Agriculture Organization of the United Nations recently estimated and revealed that more than 1.25 billion people have experienced food insecurity at moderate levels, and hunger as well as undernourishment scenarios which have worsened with the Coronavirus Disease-2019 (COVID-19) pandemic (Food and Agriculture Organization, 2020). Food shortage is already a reality in many parts of the world, and low-income countries are the most affected, where the population has to deal also with poor sanitary conditions, which may lead to foodborne diseases (Food and Agriculture Organization, 2014).

Considering that one of the pillars of food safety is based on food free of pathogens and chemical compounds, the development of a less harmful and effective hygiene process, especially for vegetables that are consumed raw, is of great importance for public health. Within this concept, the present study showed new and useful information on the hygiene and decontamination of leafy greens, which consists of washing vegetables in running tap water followed by sanitization with a natural solution composed of propolis with glycine.

To the best of our knowledge, this is the first study to analyze the synergistic effect of both compounds that together, exhibited an antimicrobial and eluting effect reducing or removing different groups of organisms, i.e., parasites, bacteria and fungi, that can be present on complex microbiota in the food chain of vegetables.

One of the most important initial results, derived from the *in vitro* experiments, where the developed solution (PHS-glycine) at a 2% concentration was effective against bacteria frequently found in foods, including *S. aureus*, *E. coli*, *S. typhimurium* and *L. monocytogenes* and also *C. albicans* - used only as a surrogate or a model for yeasts in this study - showed that PHS-glycine could be used as a potential sanitizer for fresh produce (Table 2).

Table 2. Minimum inhibitory concentration (MIC) of the antimicrobial solutions and/or eluents.

Solutions	Microorganisms				
	S. aureus	***E. coli***	***S. typhimurium***	***L. monocytogenes***	***C. albicans***
Sodium hypochlorite	0.02	0.005	0.02	0.02	0.02
PHS	0.25	1	2	1	1
PHS2	0.25	2	2	1	1
Glycine	NE	NE	NE	NE	NE
PHS*-glycine**	0.5* – 0.375**	1* – 0.75**	2* – 1.5**	1*– 0.75**	1* – 0.75**
Ethanol***	NE	NE	NE	NE	NE
Chloramphenicol	0.000064	0.000039	0.000039	0.000039	NA
Ketoconazole	NA	NA	NA	NA	0.05

PHS: Propolis hydroalcoholic solution at pH 4.9; PHS2: Propolis hydroalcoholic solution at pH 5.5; PHS-glycine: propolis hydroalcoholic solution with glycine; NE: no effect; NA: not applicable. *Concentration referring to propolis extract; **Concentration referring to glycine. ***Ethanol solution at 4.95%, which corresponds to the concentration level at PHS, PHS2, and PHS-glycine.

In addition, another relevant outcome of antimicrobial activity, when compared to PHS, PHS2 and PHS-glycine, showed non-interference of glycine in the bactericidal and antifungal action of propolis extract, since this would allow the use of PHS-glycine to reduce the native microbiota on lettuces leaves.

In the ME, the initial concentration of mesophilic aerobic count and molds and yeasts, that is, in lettuce leaves that have not undergone any treatment was 7.1 log CFU/g and 5.8 CFU/g, respectively (Table 3). Mesophilic aerobics and fungi (molds and yeasts) are part of the natural microbiota of vegetables *in natura*, however, high levels of such microbiota can alter its sensorial properties or even suggest the presence of pathogens or poor food preservation (Ramos et al., 2013; Mendoza et al., 2022).

Table 3. Enumeration of the initial concentration of microorganisms on lettuce leaves and respective reductions after treatment with antimicrobial / elution solutions on ME.

Treatments	Mesophilic aerobes (log CFU/g)		Molds and yeasts (log CFU/g)	
	Count	Reduction	Count	Reduction
Initial concentration prior to treatments	7.1 ± 0.16	-	5.8 ± 0.23	-
Running tap water	5.9 ± 0.05	1.2 ± 0.20[a]	4.8 ± 0.09	0.9 ± 0.14[a]
Sodium hypochlorite	4.5 ± 0.27	2.7 ± 0.38[b]	4.1 ± 0.16	1,7 ± 0.18[ab]
PHS	4.7 ± 0.10	2.4 ± 0.23[b]	3.6 ± 0.19	2,2 ± 0.30[b]
PHS2	4.8 ± 0.12	2.3 ± 0.28[b]	3.9 ± 0.32	1,8 ± 0.48[b]
Glycine	5.6 ± 0.08	1.5 ± 0.23[a]	4.3 ± 0.11	1,5 ± 0.13[ab]
PHS-glycine	4.8 ± 0.10	2.3 ± 0.15[b]	3.7 ± 0.24	2,1 ± 0.47[b]
p-value (normality)	-	0.161	-	0.579
p-value (homogeneity)	-	0.422	-	0.077
ANOVA	-	0.000	-	0.005

ME: microbiological experiment; PHS: propolis hydroalcoholic solution; PHS2: propolis hydroalcoholic solution (pH 5.5); PHS-glycine: propolis hydroalcoholic solution with glycine. Averages with equal letters in the same column show no significant differences ($p > 0.05$).

Thus, to be considered suitable for human consumption, it is recommended that, after the complete hygiene process, the vegetables present a mesophilic aerobic count below 5 log CFU/g and a total fungal count below 4 log CFU/g (Santos et al., 2010; Feás et al., 2014).

In this study, lettuce leaves were washed in running tap water using the repetitive friction of hand wash force. This method is considered more efficient when compared to the immersion cleaning method with a controlled amount of water, since it avoids cross-contamination of existing microorganisms in the water and vegetables (Banach et al., 2017). However, after washing the leaves in running tap water, there was a reduction of 1.2 logs of mesophilic aerobes and 0.9 log of fungi.

This result suggests limited efficacy of the process of washing leaves under running tap water to reduce the concentration of the native microbiota, a fact also observed by Ramos et al. (2013) and Banach et al. (2017) in the Netherlands, to shed light to the evidence that washing reduces an average of 1 to 2 log CFU of microorganisms.

Regarding the microbiological experiment (ME), the results showed that immersing the lettuce in PHS-glycine was effective in decontaminating the native microbiota. The treatment reduced more than 2 log CFU/g from both groups of microorganisms analyzed, and an even higher reduction was observed for fungi when compared to sodium hypochlorite (Table 3). Additionally, the counts of mesophilic aerobes, molds, and yeasts were reduced to the recommended levels for human consumption when treated with PHS-glycine.

Furthermore, the aerobic mesophilic results obtained by PHS-glycine were compatible with the results obtained by Feás et al. (2014) in Portugal, in which the reduction of the microorganisms in lettuce leaves after the immersion in propolis solution for 30 min was statistically similar to the sodium hypochlorite treatment.

Considering that PHS, PHS2, and PHS-glycine treatments did not differ from each other statistically we assumed that the pH change and the addition of glycine to the PHS treatment did not interfere in the propolis antimicrobial action. The results are consistent with the *in vitro* experiment (Table 2), which demonstrated similar MIC in the solutions.

The antimicrobial action of PHS-glycine demonstrated in this study against pathogens and native lettuce microbiota comes from the synergistic action of different bioactive compounds present in propolis extract, such as tannins, lignins, stilbenes, anthraquinones, anthones, ketophenol, phenylacetic acids, isoflavonoids, and flavonoids, that act on microorganisms by decreasing plasma membrane fluidity, inhibiting energy metabolism, and nucleic acid synthesis (Anjum et al., 2019).

In addition to propolis extract, several studies have evaluated the use of natural substances as food disinfectants (Lima & Souza 2021). However, to date, few studies have evaluated the use of natural substances to remove parasites in leafy vegetables, demonstrating that from a food safety perspective, parasites do not receive the same level of attention as foodborne diseases caused by bacteria or chemicals.

For this reason, the Codex Committee on Food Hygiene requested that the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) analyzed and produced a guideline regarding the current information on parasites, food risks related to public health worldwide, generating a ranking of the 24 most relevant foodborne parasites (Food and Agriculture Organization, 2014). Of this total, 13 species of parasites are related to the contamination of fresh produce, where the top three positions, belong to the helminths, overcoming the risk of infection by foodborne protozoa.

Leafy vegetables can be often involved in parasite outbreaks, due to their proximity to the soil and extensive surface area that facilitates their contamination with the pathogens such as so-called soil-transmitted helminths (STH) (Karshima, 2018). The global burden for this helminthiasis are considered high – more than a quarter of people are infected around the world, with higher prevalence in countries where there is limited access to safe drinking water, due to extreme poverty and inadequate sanitary conditions that still prevails (Jourdan et al., 2018).

Thus, the development of quick procedures for the removal of these parasites from vegetables consumed raw, and without requiring advanced technologies, should be prioritized, since the ingestion of contaminated leafy greens is one of its main paths of infection.

In PE, glycine and PHS-glycine were used to decontaminate lettuce, that is, to reduce *A. suum* eggs from leafy vegetables. The glycine solution showed a higher percentage of *A. suum* eggs removal compared to other treatments (Table 4). However, the percentage removal produced by glycine (41.96%) and PHS-glycine (37.22%) were statistically equal ($p > 0.05$) (Table 4). When considering individual trials, a higher number of eggs could be recovered when PHS-glycine was used (89 eggs), compared to the use of glycine alone (75 eggs) in the 3rd trial. Thus, the results suggested that propolis extract did not interfere with the dissociative and eluting action of glycine. Furthermore, the PE data confirm that the removal of *A. suum* eggs was caused by the elution of glycine, since tap water and PHS2 did not differ statistically ($p > 0.05$) and showed removal percentages of 2 to 3 times smaller than PHS-glycine. In none of the 12 negative control tests, *Ascaris* sp. eggs were detected in a naturally contaminated situation; however, *Trichuri*s spp., and helminth larvae were identified.

Table 4. Effect of different solutions on the removal of *A. suum* eggs from lettuce leaves on PE.

Treatments	Number of eggs recovered per test			Average of tests
	1°	2°	3°	Number of eggs (% removal)
Washed in running tap water	24	41	34	33.00 ± 8.54[a] (16.75)
Glycine	89	84	75	82.67 ± 7.09[b] (41.96)
PHS2	24	11	26	20.33 ± 8.14[a] (10.32)
PHS-glycine	70	61	89	73.33 ± 14.29[b] (37.22)
p-value (normality)				0.140
p-value (homogeneity)				0.500
ANOVA				0.000

PE: parasitological experiment; PHS2: Propolis hydroalcoholic solution (pH 5.5); PHS-glycine: Propolis hydroalcoholic solution with glycine. Averages followed by equal letters in the same column did not differ statistically ($p > 0.05$).

It is important to emphasize that the recovery efficiency is also influenced by the number of steps used in the method, since the higher the number of steps involved in procedures, the greater the chances of material loss (parasitic structures), due to intense homogenization and contact with different utensils (Collender et al., 2015).

The detection of *Trichuris* spp., and helminth larvae analyzed before the stage of artificial contamination with *A. suum* eggs, showed that washing the leaves in running tap water was not enough to remove parasites

from the vegetables. This fact highlights the relevance of research methods to find an efficient solution for reducing or eliminating different contaminants in vegetables.

The most promising result derived from the present studyt was observed in the MPE, in which washing the lettuce leaves in running tap water followed by immersion in a PHS-glycine reduced more than 3 log CFU/g of mesophilic aerobes and 5 log CFU/g of molds and yeasts compared to untreated leaves (Table 5), and concomitantly removed 35.53% of *A. suum* eggs. It is noteworthy that the percentage of *A. suum* eggs removal obtained by the PHS-glycine in this experiment was similar to PE-results (37.22%) (Table 4).

Table 5. Effect of different treatments on the reduction of the native microbiota and removal of *A. suum* on lettuce leaves on MPE.

Treatments	Microorganisms				Removal of *A. suum* eggs			
	Mesophilic aerobes (log CFU/g)		Molds and yeasts (log CFU/g)		n° of eggs removed per test			Average number of eggs removed (%)
	Count	Reduction	Count	Reduction	1°	2°	3°	
Initial concentration prior to treatments	7.6 ± 0.44	-	6.0 ± 0.96	-	197	197	197	-
Running potable water	6.2 ± 0.25	1.4[a]	3.8 ± 0.72	2.2[a]	-	-	-	-
Sodium hypochlorite	5.3 ± 0.28	2.3[ab]	1.3 ± 1.24	4.7[b]	24	26	31	27 ± 3.61 (13.71)[a]
PHS-glycine	4.3 ± 0.68	3.3[b]	1.0 ± 0.57	5.0[b]	41	90	79	70 ± 25.71 (35.53)[b]
p-value (normality)		0.236		0.147	-	-	-	0.091
p-value (homogeneity)		0.052		0.949				0.046
ANOVA and Mann-Whitney		0.045		0.002				0.050

MPE: microbiological and parasitological experiment; PHS-glycine: propolis hydroalcoholic solution with glycine. Averages with equal letters in the same column did not show significant differences ($p > 0.05$).

A similar fact was also observed in PHS-glycine treatment as a greater number of mesophilic aerobes and molds and yeasts were reduced when compared to sodium hypochlorite, although no statistical difference was observed.

The greater reduction of mesophilic aerobics observed after the PHS-glycine treatment when compared to the sodium hypochlorite treatment can be explained by the bacteria dissociation and release from the plant caused by glycine and the mechanical force applied by shaking the polyethylene bag (Ramos et al., 2013).

Although sodium hypochlorite remained twice as long in the MPE in contact with lettuce's surface and leaves, there was no greater reduction of mesophilic aerobics in ME. This may be related to the fact that sodium hypochlorite does not promote a greater reduction of microorganisms after 5 min of action on food surfaces (Ssemanda et al., 2018). In addition, sodium hypochlorite does not have an eluting effect as in the case of glycine, and, therefore, even after manually agitating the polyethylene bag, the percentage of *A. suum* eggs removal was lower than after the PHS-glycine treatment, reinforcing, one more time, that this amino acid is decisive for removing helminth eggs from fresh produce.

Furthermore, despite glycine being considered a non-essential amino acid, acquired adequately through a balanced diet, and its supplementation brings beneficial effects in certain physiological conditions, there are still no appropriate daily recommendations or standardized consumption limits (Razak et al., 2017).

It is noteworthy that the detachment and inactivation of bacteria, fungi, and parasites are influenced by factors other than washing methods, sanitizing, and elution solutions. For example, regarding the several

species of microorganisms, and their distribution on the vegetables's surface, the hydrophobic nature of microbial cell surfaces, and plant types. When compared to other root vegetables, leafy vegetables show a lower reduction and removal of bacteria and parasites after cleaning (Fallah et al., 2016), as they have an irregular porous and rough surface.

Although several physical methods do not use antimicrobial solutions for vegetable sanitization (such as ultrasound, high pressure, ultraviolet radiation, and cold plasma), these methods are expensive and limited to the food industry (Gérard et al.,2019). Moreover, these methods could damage the vegetables as they are sensitive.

Thus, in addition to the decontamination strategies, the best way to prevent contamination is with the use of Good Agricultural Practices to safeguard public health (Goodburn & Wallace, 2013).

4 Conclusions

Propolis hydroalcoholic solution with glycine (PHS-glycine) possesses antimicrobial activity against pathogens frequently found in fresh food. Furthermore, it was demonstrated the synergistic effect of the solution in the joint decontamination of the native microbiota and parasite eggs in leafy vegetables. Therefore, in households, we may have the possibility of easy and effective methods for decontaminating pathogens as well as spoilage microorganisms, by using natural solutions, such as the PHS-glycine, which can be a healthier alternative.

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