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ARTICLES

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Full Length Research Paper

Comparative antimicrobial potentials of omidun obtained from yellow and white maize varieties on some diarrhoea causing microorganisms

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This study determines the comparative antimicrobial potentials of omidun "supernatant solution of fermented maize mash" from yellow and white maize varieties on some diarrhoea causing microorganisms. The pH of the two omidun varieties were determined, their antimicrobial activities was also determined using agar well diffusion. Disc diffusion assay was also done using commercially produced antibiotics. Generally, omidun from the two maize varieties had acidic pH, with mean value of 3.6 for omidun from white maize and 4.0 for omidun from yellow maize. Both maize varieties inhibited growth of the tested microorganisms but at slightly different concentrations. The mean zones of inhibition of omidun from white maize varieties (9.5 mm) was higher than zones of inhibition by yellow maize varieties (9.2 mm) on *Salmonella abaetetuba* ATCC 35460, but with no significant (p>0.05) difference. Zones of inhibitions given by omidun from the two maize varieties were the same (9 to12 mm) on *Escherichia coli* ATCC 25922 and *S. abaetetuba* ATCC 35460 (7 to 12 mm). Although, the highest concentration obtained was not as high as what was observed with commercial antibiotics. This study showed that the effect produced by omidun from the two maize varieties is not significantly different (p>0.05). Hence, the variety of maize used in this study had no significant (p>0.05) effect on the antimicrobial activity of omidun

Key words: Antimicrobial, omidun, agar well, antimicrobial assay.

INTRODUCTION

Maize or corn (*Zea mays*), is the most important cereal crop in sub-Saharan Africa and, with rice and wheat, it is one of the three most important cereal crops in the world. Maize is high yielding, easy to process, readily digested, and cheaper than other cereals. It is also a versatile crop; growing across a range of agro ecological zones. Every part of the maize plant has economic value: the grain, leaves, stalk, tassel, and cob can all be used to produce

a large variety of food and non-food products (Purseglove, 1992; Osagie and Eka, 1998). One of such food prepared from maize is "ogi", fermented cereal porridge made from maize and produced using simple processing methods. The "ogi" porridge is very smooth in texture and has a sour taste reminiscent of that of yoghurt (Banigo and Muller, 1972). The traditional preparation of "ogi" include soaking of corn kernels (maize grains) in cold water for 2 to 3 days, washing several times with clean water, wet milling to paste and sieving to remove bran, hulls and germ (Akinrele, 1970; Odunfa, 1985). The pomace retained on the sieve is discarded and used as animal feed, while the filtrate is fermented for 2 to 3 days to yield "ogi", which is a sour, starchy sediment with water on top. The top water is called "omi-eko" or "omikan" or omidun. Ogi colour depends on the colour of the maize grain used, yellow or milk white maize grain (Onyekwere et al., 1989). "Ogi" an acid fermented cereal mash made from maize or corn is traditionally produced by soaking the maize/corn grains in cold or warm water for 24 to 72 h to allow for fermentation before it is wet milled, sieved and soured. Sedimentation of the soured "ogi" for 48 to 72 h gives rise to omidun (Dada and Muller, 1983; Odunfa and Adeleye, 1985; Onyekwere et al., 1989; Teniola and Odunfa, 2001). Omidun has been traditionally found to be of medicinal importance in the South-Western part of Nigeria. It is used to soak bark of the root of some plants to treat not only fever and malaria, but is popularly used as solvent for herbal extraction, dish stain removal and as insect killers. Omidun has been used in the extraction of antimicrobial agents from some leaves such as *Bryophyllum pinnatum* and *Kalanchoe crenata*. The extracts were found inhibitory against some Gram-negative organisms (Aibinu et al., 2007). Information from indigenes also claims that omidun is popularly used in the control of diarrhoea. Series of work has been done on the microbial and therapeutic values of omidun. Omidun has been reported by Falana et al. (2011) to contain some microorganisms including *L. plantarum*. They also reported that these microorganisms have antimicrobial efficacy against some pathogenic microorganisms including *Escherichia coli* (Falana et al., 2012). However, information on comparative effects of omidun from white and yellow maize varieties is also essential. Hence, this research focused on comparative effects of omidun from yellow and white maize varieties on two tested pathogenic microorganisms.

MATERIALS AND METHODS

This study was carried out at the Department of Microbiology, Federal University of Agriculture, Abeokuta.

Collection of omidun samples and tested microorganisms

White and yellow maize varieties were obtained from a market in Abeokuta. The maize varieties were steeped separately for 72 h, wet milled, sieved and fermented for 48 h at 30±2°C by the maize natural microflora. The test organisms, *E. coli* ATCC 25922 and *S. abaetetuba* ATCC 35460, were typed cultures obtained from Lagos State University Teaching Hospital (LUTH), Idi Araba, Lagos, on Nutrient Agar slants and were taken to the laboratory for confirmation. Each was re-cultured on fresh nutrient agar plates for purity.

pH determination

The pH of omidun samples from white maize varieties and yellow maize varieties was determined using Extech model 30451 pH meter with a reference glass electrode.

Antibiotics sensitivity pattern of the test organisms

This was done using disc diffusion method of Ochei and Kolhatkar (2008). One millilitre of 18 h broth culture of each of the test organisms was transferred into sterile Petri-dishes (different organism per plate) using sterile syringe. Each plate was then overlaid with 20 ml nutrient agar, swirled carefully for even distribution of the organisms within the agar and allowed to gel. The procedure was done in triplicate for each omidun sample and the mean zone of inhibition was obtained for each triplicate. Standard commercial antibiotics disc was distributed on the seeded agar plates and incubated at 37°C for 24 h. The diameter of the zones of inhibition around the antibiotics were measured and recorded.

Agar well antimicrobial assay

This was done using agar diffusion method. One milliliter of 18 h broth culture of each of the test organisms was transferred into sterile Petri dishes (different organism per plate) using sterile syringe. Each plate was then overlaid with 20 ml nutrient agar, swirled carefully for even distribution of the organisms within the agar and allowed to gel. Wells were bored in the agar with the aid of a sterile cork borer. Different omidun samples (0.1 ml) were put into each well and sterile distilled water was used as control. The plates were incubated at 37°C for 24 h. The diameter of the zones of inhibition around the wells were measured and recorded.

Statistical analysis

The pH and diameter of zones of inhibition of the varieties of omidun samples were subjected to one-way analysis of variance (ANOVA) and Duncan Multiple Range Test to separate the means and it was determined at the 5% probability level using SPSS 16.0 for Windows (SPSS, 2007).

RESULTS

pH of omidun from white and yellow maize varieties

Omidun samples obtained from white maize varieties had pH values ranging from 2.9 to 4.2, while omidun samples obtained from yellow maize varieties had pH values ranging from 3.4 to 4.5. There was a significant difference (p<0.05) between the mean pH values of omidun from

Table 1. pH of omidun obtained from white and yellow maize varieties.

Sample code	pH of white maize	Sample code	pH of yellow maize
1	3.7 ±1.4	16	3.4 ±0.6
2	2.9±1.4	17	3.6±0.2
3	3.1±0.1	18	3.6±0.2
4	3.9±1.3	19	3.6±0.3
5	3.3±0.4	20	4.0±0.7
6	4.0±0.2	21	4.9±1.3
7	3.3±0.5	22	4.0±0.3
8	4.2±0.1	23	4.2±0.3
9	4.2±0.3	24	4.1±0.2
10	3.4±0.5	25	4.3±0.4
11	3.5±0.7	26	3.6±0.8
12	3.1±0.2	27	3.7±0.2
13	3.0±0.7	28	4.2±0.9
14	3.6±0.5	29	4.0±0.1
15	4.1±0.2	30	4.5±0.1
Mean	3.6±0.4	Mean	4.0±0.4

Mean values within a column with the same letter are not significantly different (p>0.05).

white maize varieties (3.6) and that of omidun from yellow maize varieties (4.0).

Antimicrobial sensitivity result of commercial antibiotics

Some commercial antibiotics were tested for antimicrobial activity against the two gastro enteric bacteria (Table 2). The antibiotics showed varying degree of activity to the tested microorganisms. There was a significant difference (p<0.05) in the activity of the tested microorganisms. Ciprofloxacin, Azithromycin, Cefuroxime and Ceftriazone were effective against the tested microorganisms by showing diameters of zones of inhibition greater than 14 mm. The two organisms showed resistance to Gentamicin, Tetracycline and Amoxycillin with diameters of zones of inhibition less than 14 mm. Augmentin was effective against *E. coli* ATCC 25922, but *S. abaetetuba* ATCC 35460 was resistant to it.

Comparative inhibitory effect of omidun from white and yellow maize varieties on the tested microorganisms

Similar values of zone of inhibition (ranging between 9 and 12 mm) were obtained for omidun samples from white maize varieties and yellow maize varieties on *E. coli* ATCC 25922; also 7 to 12 mm was obtained for omidun samples from white and yellow maize varieties on *S. abaetetuba* (Tables 3 and 4). The type of maize from

which omidun was prepared did not significantly (p>0.05) affect the antimicrobial activity exhibited on the gastro enteric bacteria. Though, the mean zones of inhibition of omidun from white maize on *E. coli* ATCC 25922 was 10.6 mm, while it was 10.7 mm on *S. abaetetuba* ATCC 35460, the difference was not significant (p>0.05). Also, the mean antimicrobial activity shown by omidun from yellow maize on *E. coli* ATCC 25922 and *S. abaetetuba* ATCC 35460 was 9.5 and 9.2 mm, respectively (Figure 1).

DISCUSSION

This study has revealed that omidun samples obtained from white maize and yellow maize varieties showed varying pH (Table 1). The mean pH obtained for omidun from yellow maize varieties (4.0) was significantly higher than that of omidun obtained from white maize (3.6) varieties. This is similar to what was obtained by Omemu et al. (2007) during the souring period of "ogi". Therefore, the acidic pH of omidun samples from the two maize varieties could be partly responsible for the zones of inhibition obtained in this study, this is because most bacteria are acid intolerant (Steinkraus, 1997) and cannot grow at low pH except a few such as the lactic acid bacteria (LAB) (Savadogo et al., 2006). LAB has also been reported as predominant in fermented cereal products by many authors (Omemu, 2011; Opere et al., 2012).

The issue of antimicrobial resistance of pathogens to antibiotics have necessitated the development of antimicrobial agents from other sources (Williams, 2000)

Table 2. Inhibitory effects of antibiotics on *E. coli* ATCC 25922 and *S. abaetetuba* ATCC 35460.

Antibiotics	Zone diameter (mm) against *E. coli* ATCC 25922	Zone diameter (mm) against *S. abaetetuba* ATCC 35460
Ciprofloxacin	21±0.0	20±1.4
Azithromycin	20±0.0	20±0.0
Cefuroxime	17±0.4	16±0.1
Ceftriazone	17±0.0	14±0.3
Augmentin	15±0.5	10±0.2
Amoxycillin	6±0.0	8±0.1
Tetracycline	4±0.0	6±0.4
Gentamicin	4±0.0	4±0.0

Mean values within a column with the same letter

Table 3. Inhibitory effect of omidun from white maize on test organisms.

Sample code	Zone diameter (mm) against *E. coli* ATCC 25922	Zone diameter (mm) against *S. abaetetuba* ATCC 35460
1	12±1.4	10±0.7
2	12±0.7	12±0.7
3	10±1.4	8±0.7
4	9±0.7	7±1.4
5	10±1.4	8±0.7
6	10±0.7	10±1.4
7	12±0.7	10±1.6
8	9±2.1	8±0.2
9	12±0.7	12±0.9
10	10±1.4	12±0.2
11	10±1.4	9±1.4
12	12±0.9	10±0.1
13	12±1.4	10±1.4
14	10±0.7	8±1.4
15	9±2.8	9±1.4

Mean values within a column with the same letter are not significantly different (p>0.05).

and that is one of the reasons for this research work. In the present study, similar zones of inhibitions (9 to 12 mm against *E. coli* ATCC 25922 and 7 to 12 mm against *S. abaetetuba* ATCC 35460, respectively) were obtained for the studied omidun samples from the two maize varieties (Tables 3 and 4). Hence, the type of maize from which omidun was prepared did not significantly (p>0.05) affect the antimicrobial activity exhibited on the tested gastro enteric bacteria.

The observed zones of inhibition may be due to LAB in 'ogi', which cannot be ruled in omidun. LAB had been known to produce antimicrobial substances such as organic acids, diacetyl, hydrogen peroxide and bacteriocin (Mathieu et al., 1993; Bonade et al., 2001) and has been reported to be a potential host for the production of therapeutic recombinant protein.

Although, omidun from white maize varieties showed slightly higher zones of inhibition (10.6 mm against *E. coli* ATCC 25922 and 10.7 mm against *S. abaetetuba* ATCC 35460), than omidun from yellow maize varieties (9.5 mm against *E. coli* ATCC 25922 and 9.2 mm against S. *abaetetuba* ATCC 35460). However, the varying zones of inhibition were not significant (p>0.05) and may be attributed to their varying TTA as obtained by Omemu et al. (2007) during the souring process of "ogi". It might also be attributed to the varying microbial composition of the omidun samples (Odunfa and Adeleye, 1985).

Generally, the high antimicrobial activity of omidun from white maize (Figure 1) was not as high as what was observed in most of the commercially produced antibiotics, an indication that commercial antibiotic (orthodox medicine) was more effective against the

Table 4. Inhibitory effect of omidun from yellow maize on test organisms.

Sample code	Zone diameter (mm) against *E. coli* ATCC 25922	Zone diameter (mm) against *S. abaetetuba* ATCC 35460
16	10±1.4	8±0.7
17	12±0.7	10±1.1
18	9±1.4	8±1.4
19	9±0.7	9±0.8
20	10±0.7	10±1.4
21	12±0.1	10±1.4
22	10±0.1	10±0.5
23	12±0.1	12±1.4
24	12±0.9	10±0.2
25	10±0.5	8±0.7
26	10±0.0	8±0.4
27	10±0.2	10±0.7
28	12±0.2	10±1.2
29	10±1.2	7±0.9
30	12±0.1	8±1.4

Mean values within a column with the same letter are not significantly different (p>0.05).
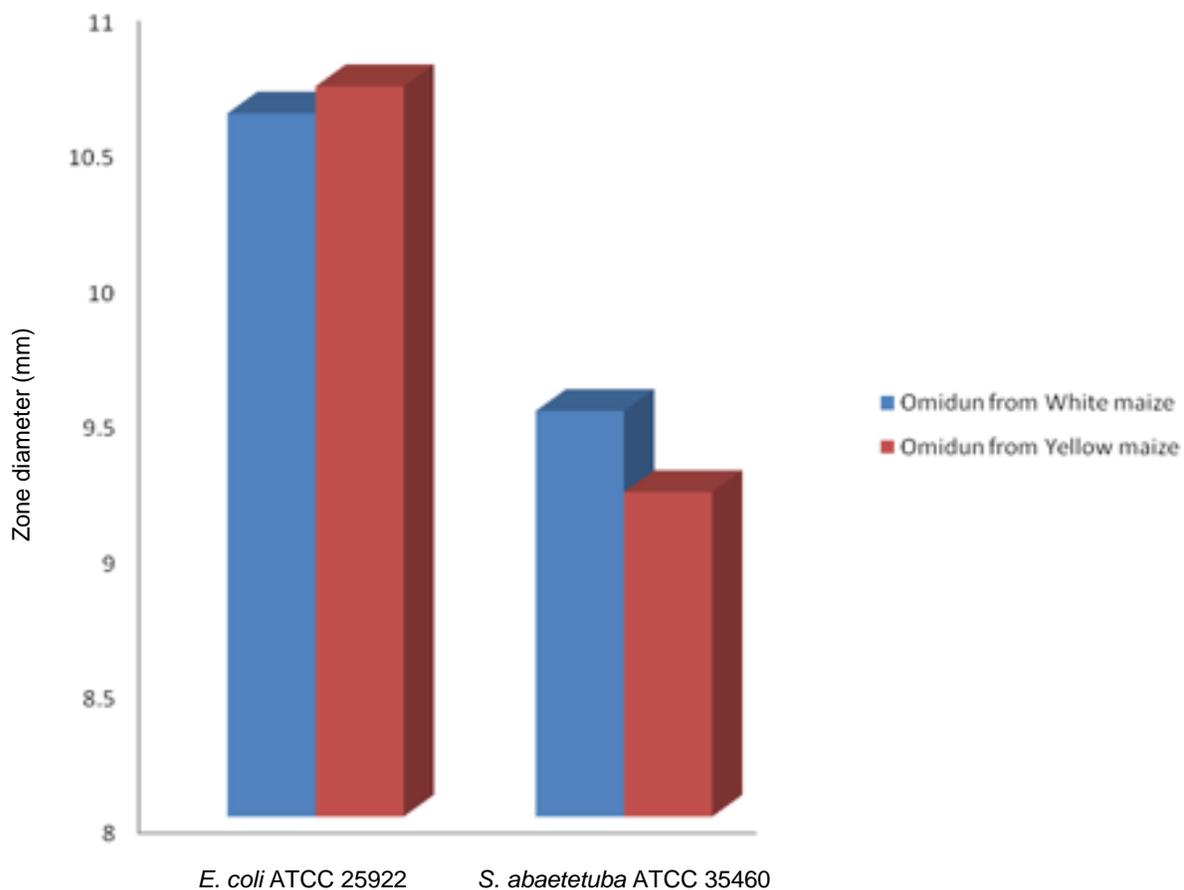
Figure 1. Mean inhibitory effect of omidun from white maize and yellow maize on *E. coli* ATCC 25922 and *S. abaetetube* ATCC 35460.

tested microorganisms than omidun samples. Antimicrobial activities of omidun samples may not be due to the action of a single active compound found in them and some of which might have been detoxified by the tested microorganisms. Moreso, disparity between activities of the two varieties of omidun and standard antimicrobial drug may be due to the mixture of bioactive compounds present in omidun samples compared to the pure compound contained in the standard antibiotics (Gatsing et al., 2010). However, many consumers may still prefer the use of LAB or their antibacterial compounds (such as omidun) to control food-borne pathogens and spoilage organisms. Many researchers such as Ogunbanwo et al. (2004) reported that, unlike synthetic chemicals, cells of LAB which might be present in omidun samples have no known adverse effects.

This work shows that both omidun obtained from white and yellow maize varieties have potential therapeutic properties, but this property was slightly higher in omidun from white maize varieties than in omidun from yellow maize varieties, although with no significant difference ($p>0.05$) and this might mean that omidun from white maize varieties possess slightly higher antimicrobial components more than omidun obtained from yellow maize variety.

Conclusion

The findings in this research work suggest the use of omidun, preferably the one obtained from white maize variety in the control of conditions caused by the tested microorganisms. However, further work is highly recommended in order to isolate, identify and quantify the active metabolite present in omidun samples from the two maize varieties (white and yellow).

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Bacteriological and physicochemical qualities of traditionally dry-salted Pebbly fish (*Alestes baremoze*) sold in different markets of West Nile Region, Uganda

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The present study aimed at estimating the microbiological and chemical characteristics of traditionally dry-salted fish product, *Alestes baremoze*. A total of 40 random dry fish samples were collected from Arua, Nebbi, Packwach and Panyimur markets. Moisture content, pH, crude protein, crude fat and sodium chloride were analysed to determine chemical quality while *Escherichia coli, fecal streptococci, Staphylococcus aureus, Salmonella, Vibrio parahaemolyticus, Bacillus cereus* and *Pseudomonas* spp. were determined to estimate the microbial quality. The moisture content of dry-salted fish collected from different markets was in the range of 37 to 41%. Mean values of sodium chloride obtained in the fish muscle were in the range of 13 to 14% and significantly differed across fish markets. Results from microbial analysis expressed as colony-forming units per gram of sample indicated that *S. aureus* was the most dominant bacteria identified in dry-salted fish sold in all markets with Nebbi market having the highest counts (9.4×10^6), Panyimur (2.2×10^6), Packwach (2.3×10^5) and Arua (9.6×10^4). *Salmonella* was absent in fish samples collected from three markets of Arua, Packwach and Panyimur apart from Nebbi market. *E. coli* counts were found to be $< 10^1$ and *fecal streptococci* counts were relatively high in fish from Panyimur market (1.1×10^3). There was presence of *B. cereus* in all the samples ranging from 8×10^1 in Arua market to <20 in Nebbi and Panyimur markets. The present study has revealed that most of the fish products sold in these markets had bacterial counts beyond the maximum tolerable limits recommended by Uganda National Bureau of Standards (UNBS). There is need to control storage temperature and also ensure proper cooking procedures in order to eliminate or reduce the microorganisms to acceptable levels.

Key words: *Alestes baremoze*, salted fish, microbial quality, fish preservation.

INTRODUCTION

Dry salting has been traditionally used as a method of fish preservation, since it lowers the water activity of fish flesh (Horner, 1997). The salt mainly contains chloride ions that are toxic to some microorganisms (Leroi et al., 2000; Goulas and Kontominas, 2005). This technique is hence used to preserve fish from spoilage owing to tissue

autolysis and microbial action (Chaijan, 2011). Bacterial spoilage is for example characterized by softening of the muscle tissue, which can however be prevented by salt, because it forms a more membranous surface that inhibits the growth of microorganisms (Horner, 1997; Rorvik, 2000). Although salting reduces the rate of autolysis, it does not completely stop enzymatic action that increases with increasing temperature.

The presence of foodborne pathogens in a fish product is a function of the harvest environment, sanitary conditions, and practices associated with equipment and personnel in the processing environment (FDA, 2001). The handling of fish products during processing involves a risk of contamination by pathogenic bacteria such as *Vibrio parahaemolyticus* and *Staphylococcus aureus* causing foodborne human intoxication (Huss et al., 1998; Shena and Sanjeev, 2007). There is substantial evidence that fish and seafood are high on the list of foods associated with outbreaks of food borne diseases around the world (Kaysner and DePaola, 2000; Huss et al., 2003). The safety of foodstuffs should be ensured through preventive approaches, such as implementation of good hygiene practices and application of procedures based on hazard analysis and critical control point (HACCP) principles.

Alestes baremoze commonly known as *Angara* in Uganda is highly marketable and valued fish in Northern Uganda, South Sudan, Sudan and in the Democratic Republic of Congo (Kasozi et al., 2014). In Sudan, *A. baremoze* is normally prepared by wet salting. After several methods of salting, fermentation and storage, the final product is called *fassiekh* (Yousif, 1989; Adam and Mohammed 2012)*. Angara* is prepared by dry salting which involves stacking the fish in salt and the formed brine is allowed to drain away while allowing it to dry under natural sunlight for two to three days. Many consumers, especially in the West Nile region appreciate the taste, special flavour and texture characteristics of this fish. Salting is not only a method to prolong shelf life, but a method to produce fish products that meet demand of consumers. Almost 90% of the total catches of *Angara* around Lake Albert are dry-salted. However, the available traditional fish processing practices expose the fish to different kinds of microbial and chemical degradation. The current wide spread practice of drying the fish directly on the ground and use of old fishing nets results in microbial contaminated fish products. There are currently no published work on the microbiological changes during production and storage of salted *Angara* yet the quality of salted and sun dried fishes are adversely affected by the occurrence of microorganisms. The need for determination of microbiological quality of dry-salted fish products is important to prevent risk of bacterial infection to the consumers. This study therefore evaluates the bacteriological and physicochemical qualities of dry-salted *Angara* sold in different markets to serve as a guide to consumers and regulatory bodies.

MATERIALS AND METHODS

Sample collection

The study was conducted in four selected markets in West Nile region of Uganda. The process of dry salting (Figure 1) is normally carried out at Panyimur landing site and it's from this site that the dry- salted fish products are obtained and transported to other markets within the region. A total of 40 dry- salted fish samples were purchased from the markets of Arua, Nebbi, Packwach where they had been on stall ready for sale for five days and from Panyimur market where they had been dried for one day (Figure 2). At least 10 samples were collected from each market. These were labeled, sealed in airtight polythene bags and later transported to the laboratory for analysis.

Physicochemical analysis

Fish samples were analysed to determine the moisture content, fat, protein, sodium chloride and pH. Moisture content was determined by oven drying of 5 g of fish fillet at 105ºC until a constant weight was obtained (AOAC, 1995). Measurement of salt content was carried out using the Volhard method according to AOAC (1985). Crude protein was determined by the Kjeldahl method using sulphuric acid for sample digestion. Crude fat was obtained by exhaustively extracting 2.0 g of each sample in a Soxhlet apparatus using petroleum ether (b.p. 40 - 60°C) as the extractant (AOAC, 2000). pH was determined after homogenizing 10 g of fish sample into 100 ml of distilled water. The pH of filtrate was then measured using pH meter (HI 8014, USA).

Enumeration and isolation of bacteria

Serial dilutions from each sample were prepared before subsequent culturing according to the microbiological techniques of AOAC (1995). The total viable count of *Angara* samples were carried out using plate count agar according to the standard methods of AOAC (1995). The microbiological parameters were conducted in duplicate, the means and standard deviations were also calculated. Plate count number was presented as colony-forming units per gram of sample (cfu/g).

Pseudomonas

Pseudomonas was determined by spread plate method where 0.5 ml of decimal dilution was spread on the surface of Pseudomonas CN Selective Agar and incubated at 37°C for 48 h. The plates containing 15 to 150 colonies were counted under florescence under UV lamp. Confirmation for the presence of *Pseudomonas* was prepared with oxidase test and fermentation of glucose on purple glucose agar.

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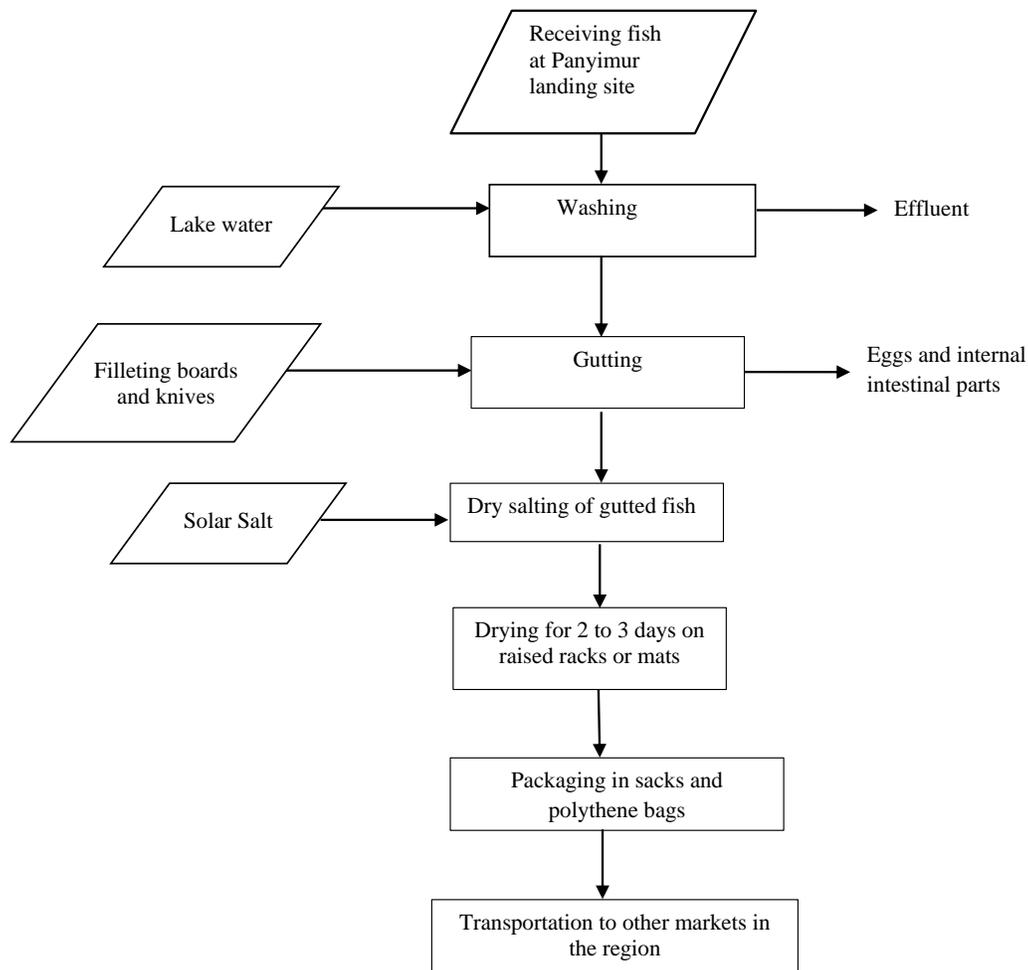
Figure 1. Traditional method for the production of salted *Alestes baremoze (Angara)* at the landing site of Panyimur.

Figure 2. *Angara* fish samples: A: Gutted *Angara* samples on ground at the landing site; B: Sun drying of split fish on raised platforms and C: *Angara* fish on stalls in different markets.

V. parahaemolyticus

V. parahaemolyticus was detected according to the General guidance for the detection of *V. parahaemolyticus* (ISO 8914:1990). Twenty five grams of each sample were weighed into sterile stomacher bag containing 225 ml alkaline peptone water and then blended for 60 s. Serial dilutions were prepared to get 10^2 and 10^3 diluents, and 1 ml aliquot of samples were transferred into 3% NaCl dilution tubes, and incubated at 35°C for 24 h. The turbid tubes were streaked on Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS)

Table 1. Physicochemical analysis of dry–salted *A. baremoze* samples collected from different markets after a storage period of five days.

Parameter	Markets				P-value
	Arua	Nebbi	Packwach	Panyimur	
Moisture content (%)	37.0±2.97	36.0±0.83	39.5±0.37	41.6±0.47	0.0014*
pH	6.3±0.01	6.9±0.045	6.6±0.02	6.8±0.01	0.0001*
Fat content (%)	12.9±0.66	16.4±0.17	14.9±0.67	16.6±0.17	0.0001*
Protein content (%)	32.0±0.81	33.0±0.69	31.7±0.61	35.1±0.88	0.0002*
Sodium chloride (%)	14.9±0.01	13.8±0.76	13.8±0.14	13.1±0.10	0.0003*

*,Significant differences across markets; Fish samples at Panyimur market had been stored for one day.

plates and incubated at 37°C for 24 h. Distinct colonies with blue green color were presumed as *V. parahaemolyticus* and yellow colonies were presumed as *Vibrio cholera*. To facilitate identification of suspect *Vibrio* isolates, the isolated colonies were further identified by API 20E system.

S. aureus

S. aureus was determined according to the method for the enumeration of coagulase-positive staphylococci (*S. aureus* and other species) using Baird-Parker agar medium (ISO 6888-1: 1999). Twenty five grams of each sample were weighed into stomacher bag containing 225 ml peptone water and then blended for 60 s. The resultant stock solution was then serially diluted and 0.5 ml diluents were spread on Baird-Parker agar plate. All inoculated plates were dried and incubated at 37°C for 48 h. Then clear zone with typical gray-black colonies was taken as presumptive evidence of *S. aureus*. Confirmation of *Staphylococcus* spp. was done using *Staphylococcus* latex test.

Salmonella spp.

Salmonella spp. was determined according to the horizontal method for the enumeration of *Salmonella* spp. (ISO 6579: 2002). Pre-enrichment was conducted with 25 g of sample diluted in 225 ml sterile buffered peptone water incubated at 37°C for 24 h. Secondary selective enrichment was performed in Rappaport-Vassiliadis peptone broth (41°C for 24 h) and Muller-Kaufmann tetrathionate broth with Novobiocin (37°C for 24 h), and streaking on Xylose Lysine Desoxycholate (XLD) agar and incubated at 37°C for 24 h. Typical *Salmonella* spp. exhibited pink colonies with black centers.

Escherichia coli

E. coli was determined by pour plate method using Rapid' *E.coli* 2 Agar (AFNOR BRD 07/1 - 07/93). Using a sterile pipette, 1 ml of each decimal dilution was inoculated to a sterile Petri dish and then 15 ml of Rapid *Ecoli* Agar was dispensed, mixed thoroughly and after setting, a thin overlay of 5 ml of Rapid *Ecoli* agar and later incubated at 44ºC for 24 h. Plates with purple colonies were counted and confirmed with Kovac's reagent and all positive colonies showed a purple layer.

Bacillus cereus

B. cereus was determined according horizontal method for the enumeration of presumptive *B. cereus* (ISO 7932:2004). Twenty five grams of each sample were homogenized in 225 ml sterile

peptone water for 60 s. Serial dilution was carried out and 0.1 ml diluents were spread on *B. cereus* Selective Agar. The inoculated plates were then incubated at 30°C for 24 h; large pink colonies with egg yolk precipitate were presumed as *B. cereus*. Confirmation was done with haemolysis test.

Fecal streptococci

Fecal streptococci was determined by spread plate method where 25 g of fish sample was taken aseptically and homogenized with 225 ml sterile peptone water for 60 s. 0.5 ml of each of decimal dilutions of the samples was spread on Typhon Soya Broth Agar and overlay with Kanamycin Esculin Azide Agar added and later incubated at 42°C for 24 h. The characteristic black colonies were counted after incubation confirmatory tests.

Statistical analysis

Data was analysed using Graph pad version 6 statistical software. Comparisons between means for physicochemical parameters were carried out using a One Way Analysis of Variance (ANOVA) and results with p values < 0.05 were considered statistically significant. Comparisons between mean values of physicochemical parameters across fish markets were done using Tukey`s multiple comparison test. Data are represented as means ± standard deviation. Results of physicochemical analysis and mean microbial counts of the dry - salted fish samples were compared with the set standards established by UNBS.

RESULTS AND DISCUSSION

Chemical analysis

Results from the chemical analysis (Table 1) revealed that moisture content significantly varied across fish markets ($F_{3, 12}$ = 4.0, p = 0.0014). The results showed that moisture content was significantly (p> 0.05) higher in fish collected from Panyimur market (41.6±0.47%) as compared to Nebbi (36.0±0.83%) and Arua (37.0±2.97%) fish markets. The relative higher moisture content in fish samples from Panyimur might be due to a shorter storage period since it is from this site that fish is distributed to other markets. Findings of this study show that values of37 to 41% of dry- salted fish collected from different markets are in accordance to 35 to 40% standard range for moisture content of dry-salted fish and fish products(UNBS, 2012). Accordingly, moisture content of

A. baremoze flesh without any processing ranged between 72 and 75% (Kasozi et al., 2014). Therefore dry salting method employed by fisher folk results in considerable loss of water due to heavy uptake of salt. The moisture content is an indicator of the susceptibility of a product to undergo microbial spoilage. It has a potential effect on the chemical reaction rate and microbial growth rate of the food product. Since moisture content is an indicator of the susceptibility of food products to undergo microbial and chemical spoilage (Horner, 1997; Chaijan, 2011; Goulas and Kontominas, 2005), traditional dry-salting of fish can result in storage stability.

The changes in the pH of dry-salted *A. baremoze* significantly varied across fish markets ($F_{3, 12} = 1.5$, $p < 0.0001$). Fish from Arua were associated with significantly lower pH as compared to other fish markets (6.3±0.01). This could be attributed to relatively higher sodium chloride (14.9±0.01%) found in samples collected from this market. Goulas and Kontominas (2005) reported that salt had a highly significant linear decreasing effect on the pH of chub mackerel after day one of storage. Similarly, Chaijan (2011) reported a rapid decrease in the pH of dry salted *Oreochromis niloticus* muscle in the first 10 min of salting. The pH decrease in fish flesh by the addition of salt is explained by the increase of the ionic strength of the solution inside of the cells (Goulas and Kontominas, 2005).

The fat content significantly varied across fish markets ($F_{3, 12} = 0.9$, $p < 0.0001$). The lowest fat content reported in fish samples from Arua market (12.9±0.66%) might be due to relatively higher sodium chloride (14.9±0.01%) since increased salt content induces lipid oxidation in muscle tissues and reported to accelerate progressively during dry salting of *Oreochromis niloticus* (Chaijan, 2011).

The protein content of processed fish significantly ($F_{3, 12} = 0.1$, $p = 0.0002$) differed across fish markets ranging from 31 to 35% (Table 1). Comparison across fish markets revealed that protein content was significantly higher (35.1±0.88%) in fish from Panyimur as compared to other fish markets. Salting of fish is usually accompanied by protein losses, as water is drawn out and meal brine is formed, with some protein dissolved into the brine (Chaijan, 2011). Since fish was only stored for one day at Panyimur, this might explain the relatively higher protein levels compared to other markets.

Mean values of sodium chloride obtained in the fish muscle were in the range of 13 to 14% and significantly ($F_{3,12} = 0.8$, $p = 0.0003$) differed across fish markets. Comparisons across fish markets revealed significantly higher ($p > 0.05$) sodium chloride levels in fish from Arua market (14.9±0.01%). Although salting effectively prevents the growth of both spoilage and pathogenic bacteria (Leroi et al., 2000); it has been reported that salt content in fish muscle enhances oxidation of the highly unsaturated lipids. Many of the fresh-fish-spoiling bacteria are quite active in salt concentrations up to 6% (Horner, 1997). Above 6 to 8%, they either die or stop growing. As the salt concentration is increased beyond 6%, bacteria of another group, consisting of a much smaller number of species, are still able to grow and spoil the fish. However, the halophiles "salt-loving" can still grow best in salt concentrations that range from 12 or 13% to saturated brine. Therefore, certain halophilic micro-organisms can multiply under the conditions of dry-salting and can also spoil the product.

Bacteriological quality

The quality of salted and sun dried fishes are adversely affected by microbial contamination. Determination of microbiological quality of processed dried fish product is very important for protecting consumer's health (Lilabati et al., 1999). The presence of potentially pathogenic bacteria in dried fishes is critical with regard to safety and quality of seafood. The acceptable microbiological limits set by UNBS for dried and salt-dried fish are indicated in Table 2 and these were compared with the results from the total plate counts of *Angara* from different markets.

Our study showed that *S. aureus* was the most dominant microorganism identified in dry-salted fish sold in all markets of West Nile region (Table 2). *S. aureus* does not appear as a part of the natural microflora of newly caught marine and cultivated fish (Herrero et al., 2003). Therefore, the presence of *S. aureus* is an indicator of poor hygiene and sanitary practices, during processing and storage. In this study, counts of *S. aureus* were above the limit of 2×10^3 cfu /10 g, recommended by the Uganda National Bureau of Standards (2012). However, lower bacterial load in fishery products might not be a serious risk, however, but food poisoning may occur if the product is handled carelessly resulting in high multiplication ($>1 \times 10^5$ cfu/g) (Varnam and Evans, 1991; Vishwanath et al., 1998).

Although *E. coli* and fecal coliform bacteria can be found in unpolluted warm tropical waters (Huss, 1993; Hazen 1988; Fujioka et al., 1988), they are particularly useful as indicators of fecal contamination and poor handling of seafood. According to UNBS (2012) absence of *E. coli* has been recommended as an upper limit for a very good quality dry salted fish. In this study, *E. coli* counts were found to be $<10^1$ cfu/g and *fecal streptococci* counts were relatively high (Table 2). Similar results have also been reported by Colakoglu et al. (2006) for *fecal streptococci* counts between $<10^1$ and 10^3 cfu/g in the fish from wholesale and between $<10^1$ and 10^5 cfu/g in retail markets. It is reported that unclean boat deck, utensils in the boat, polluted water can certainly add the fecal bacteria load (Sugumar, et al., 1995) and this might explain the high *fecal streptococci* counts of 1.1×10^3 cfu/g (Table 2) of dry- salted fish at Panyimur market situated close to a landing site where fisher folk uses the lake

Table 2. Total viable bacterial count of dry-salted Pebbly fish (*Alestes baremoze*) samples collected from different markets after a storage period of five days.

Organisms	Markets				Maximum limit[a]
	Arua	Nebbi	Packwach	*Panyimur	
Staphylococcus aureus (cfu/g)	$9.6{\times}10^4{\pm}8.1{\times}10^3$	$9.4{\times}10^6{\pm}0.1{\times}10^1$	$2.3{\times}10^5{\pm}1.0{\times}10^3$	$2.2{\times}10^6{\pm}8.1{\times}10^4$	2×10^3
Escherichia coli (cfu/g)	<10	<10	<10	<10	Absent
Feacal streptococci (cfu/g)	<20	<20	$2{\times}10^1{\pm}0.1{\times}10^1$	$1.1{\times}10^3{\pm}5.0{\times}10^1$	Absent
Salmonella / 25 g	Absent	Present	Absent	Absent	Absent
Vibrio parahaemolyticus /25 g	Absent	Absent	Absent	Absent	N/A
Bacillus cereus (cfu/g)	$8{\times}10^1{\pm}0.01{\times}10^1$	<20	$4{\times}10^1{\pm}0.01{\times}10^1$	<20	N/A
Pseudomonas (cfu/g)	<20	<20	<20	$12{\times}10^1{\pm}0.01{\times}10^1$	Absent

*Fish samples at Panyimur market had been stored for one day; [a]UNBS, 2012; N/A = Data not available.

water during the salting process.

Salmonella is highly pathogenic and this is the major reason for isolation of such bacteria from sample fishes. *Salmonella* was absent in three markets of Arua, Packwach and Panyimur apart from Nebbi market (Table 2). Incidence of *Salmonella* in the sample of fish from this market may be attributed to external contamination and poor handling at ambient temperature.

V. parahaemolyticus is an indigenous bacterium in the marine environment and can also grow at 1 to 8% salt concentrations (Huss, 1993). It occurs in a variety of fish and shellfish, including clams, shrimp, lobster, crayfish, scallops and crabs, as well as in oysters (Kaysner and DePaola, 2000) It is very heat sensitive and easily destroyed by cooking (Huss et al., 2003).

B. cereus strains are widely distributed in the environment and their spores are resistant to drying and can easily be spread with dust (Huss et al., 2003). There was presence of low density of *B. cereus* in all the samples ranging from $8{\times}10^1$ *B. cereus* (cfu/g) in Arua market to <20 cfu/g in Nebbi and Panyimur (Table 2). A small number of *Bacillus* spp. in foods is not considered significant (Beumer, 2001).

Many species of *Pseudomonas* spp. have a psychrophilic nature and are regarded as part of the natural flora of fish. The species can form aldehydes, ketones, esters and sulphides following food spoilage, causing odours described as fruity and rotten (Tryfinopoulo et al., 2002). The isolation of *Pseudomonas* spp from the collected fish samples is of high importance because this bacterium plays considerable role as a potential pathogenic bacteria for human and as an indicator of food spoilage. According to UNBS (2102), *Pseudomonas* spp. should be absent in dried and salted dried fish however this study reveals that *Pseudomonas* was detected in all samples, < 20 cfu/g in three markets of Arua, Nebbi and Packwach and $12{\times}10^1$ cfu/g for Panyimur (Table 2).

Conclusion

Bacteriological quality of most *Angara* samples analyzed

in this study did not meet the standards established by the Uganda National Bureau of Standards (UNBS) for dried and dry-salted fish. The study pointed out that *Angara* obtained from the markets was contaminated with substantial number of *S. aureus. Salmonella* and fecal *streptococci* and were also detected in fish from Panyimur and Nebbi markets, respectively. The substantial number of these microorganisms in *Angara* suggests poor personal hygiene, particularly among fish handlers and improper storage. Hence control measures such as use of good quality raw material, hygienic handling practices, potable water, good quality packaging material, hygienic processing place may be considered to improve the microbial quality of the dried fish product. Proper cooking procedures should be emphasized to eliminate or reduce the microorganisms to an acceptable level.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

In vitro antagonistic activity of native bacteria isolated from soils of the argentine Pampas against *Fusarium tucumaniae* and *Fusarium virguliforme*

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The aim of the present study was to evaluate indigenous PGPR (Plant growth promoting rhizobacteria) previously isolated from Argentina's soybean fields for their *in vitro* antagonistic effects on the control of *Fusarium tucumaniae* and *F. virguliforme*, in two separated *in vitro* assays. In assay 1, the bacteria that showed the highest significant (P < 0.05) *F. tucumaniae* mycelial growth inhibition were strains *Bacillus subtilis* 54 (70%), *B. cereus* 13 (44%), *B. cereus* 7 (44%) and *Chryseobacterium vietnamense* 110 (42%). Despite their antagonistic activity, the strains identified as *Stenotrophomonas malthophilia* and *B. cereus* were not included in any further experiments, because of their potential hazard. In assay 2, strains 54, 110 and *Pseudomonas fluorescens* 9 and 115 were tested against *F. tucumaniae* and *F. virguliforme*. In this study, native bacterial strains isolated from Argentine Pampas were tested for the first time against these pathogens. All four bacterial strains significantly inhibited mycelial growth of *F. virguliforme*. Further studies on the effects of these strains on the growth of soybean plants and on the Sudden Death Syndrome (SDS) control will uncover the mechanisms and *in vitro* antagonism potential of these bacterial isolates.

Key words: Antagonism, plant growth promoting rhizobacteria (PGPR), *Fusarium*, *Pseudomonas*, *Bacillus*, *Chryseobacterium vietnamense*.

INTRODUCTION

Soybean is the main crop in Argentina. In the last seasons, the area planted to soybean in Argentina was about 20 million hectares per year (Carmona et al., 2015). Sudden Death Syndrome (SDS) is a soybean

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disease caused by at least four *Fusarium* species, but in Argentina *F. tucumaniae* and *F. virguliforme* are the predominant. These are soil-borne pathogens commonly found in Argentina's pampas region and are important causes of crop losses (Scandiani et al., 2010). The fungus infects soybean roots and, under appropriate conditions, toxin-dependent symptoms develop in the aerial tissues after flowering and during pod fill, leading to rapid necrosis (Hartman et al., 2015). Under monoculture and no-till conditions in the argentine Pampas region the presence of SDS has intensified (Scandiani et al., 2010). Because of the difficulty in obtaining resistant soybeans varieties, the impossibility of fungicides to move towards the roots basipetally, wide host range of the pathogen and its ability to survive in the soil with resistance structures (chlamydospores), common management strategies such as genetic resistance, seed treatment with fungicides and crop rotation do not provide adequate control of SDS (Scandiani et al., 2010). In this context, biological control appears as an alternative and interesting tool.

Plant growth promoting rhizobacteria (PGPR) have been widely reported and recognized to have the potential for PGP and for their ability to antagonize the growth of fungal pathogens in crops such as maize, rice, potato, wheat and canola (Siddiqui et al., 2006). In soybean, PGPR were successfully tested against *Macrophomina phaseolina* (Simonetti et al., 2015) and *Pythium ultimum* (León et al., 2009).

Biocontrol PGPR are able to antagonize phytopathogenic fungi by different mechanisms (Siddiqui et al., 2006), including antibiosis, competition, mycoparasitism, degrading enzymes or induced resistance (Ahmad et al., 2008). PGPR could produce antibiotics or secrete lytic enzymes such as glucanases, proteases, cellulases and chitinases that degrade disease-causing fungi cells (Someya et al., 2007). Antibiotics produced by PGPR include volatile antibiotics (hydrogen cyanide, aldehydes, alcohols, ketones, and sulfides) and nonvolatile antibiotics such as polyketides (diacetyl phloroglucinol; 2,4 diacetylphloroglucinol and mupirocin), heterocyclic nitrogenous compounds (phenazine derivatives) and phenylpyrrole antibiotic (pyrrolnitrin) (Dilantha Fernando et al., 2005). PGPR could also antagonize by competition, for example by siderophore production. In addition, siderophores produced by PGPR could contribute to enhanced plant growth.

There are few reports on the antagonistic effect of bacterial isolates on soybean *Fusarium* species causing SDS (Xing and Westphal, 2007; Agaras et al., 2012). It is reported that the chance of finding bacterial strains effective for biocontrol increases if the isolates are obtained from pathogen suppressive soils and from the same environment in which they will be used (Cook and Baker, 1983). The aim of the present preliminary study was to evaluate indigenous PGPR previously isolated from soy fields in Argentina's pampas region for their *in vitro* antagonistic effects on the control of *F. tucumaniae* and *F. virguliforme*.

MATERIALS AND METHODS

Ten PGPR strains that were previously isolated and identified by Simonetti et al. (2015) for their *in vitro* antagonistic capacity against *M. phaseolina*, were tested for their *in vitro* inhibitory capacity against the fungal pathogen *F. tucumaniae* (Table 1).

The fungal strains used in this study were originally isolated from infected soybean plants showing SDS root rot and provided by Centro de Referencia de Micología (CEREMIC), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (Table 1).

Assay 1

All bacterial strains were tested for their ability to inhibit the mycelial growth of *F. tucumaniae* 149-12. Each bacterial isolate was streaked as a band on the edge of a PDA 90-mm diameter plate and incubated for 24 h at 28 ± 2°C. Then, a 6 mm diameter mycelial disc of *F. tucumaniae* 149-12 was taken from the margin of a growing colony and placed onto the centre of previously inoculated potato-dextrose-agar (PDA) plates. The Petri dishes were sealed by parafilm and incubated at room temperature in the dark. Plates containing only the fungal mycelial plug were maintained as control.

Assay 2

The bacterial strains that did not show genetic relationship with potentially hazardous bacteria in the assay 1 were tested for their ability to inhibit the growth of *F. virguliforme* 101-03 and *F. tucumaniae* 149-12 using *in vitro* dual-culture assay (Simonetti et al., 2012). Each bacterial isolate was prepared in nutrient broth (NB) and incubated for 48 h at 28 ± 2°C in order to use them in stationary phase. Fungi were maintained on PDA at 24 ± 2°C for one week. A 6 mm diameter mycelial plug was taken from the margin of a growing colony and placed centrally in a Petri dish containing PDA medium. Two drops (2 µL) of each bacterial culture previously prepared were placed in a straight line 3 cm away from the center of the plate and drops of sterile water served as control.

All these experiments were performed in triplicate. After incubation period of 11 days at 24 ± 2°C, mycelium growth inhibition was calculated as $I=[(C-T)/C]\times100$, where C is the mycelium diameter in control, and T is the mycelium diameter in bacteria-inoculated plates.

Data were analyzed using analysis of variance and differences between means were tested using Tukey test with an overall risk level of 5%.

RESULTS

In assay 1, the bacteria that showed the highest significant ($P < 0.05$) *F. tucumaniae* 149-12 mycelial growth inhibition were strains 54 (70%), 7 (44%), 13 (44%), 110 (42%), 125 (34%), 123 (32%), 116 (31%) and 48 (30%) (Figure 1A and B). Despite their antagonistic activity, the strains identified as *S. malthophilia* (48) and *B. cereus* (7, 13, 116, 123 and 125) were not included in any further experiments because of their genetic

Table 1. Identification of PGPR and fungal strains used in the antagonistic trials.

PGPR strain ID	Strain genus/species	Geographic origin	
		Location	GPS Coordinates
7	*Bacillus cereus*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
9	*Pseudomonas fluorescens*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
13	*Bacillus cereus*	Cañada de Gómez	32°49'36.76"S, 61°21'39.41"O
48	*Stenotrophomonas maltophilia*	Bouquet, Santa Fe	32°25'20.17"S, 61°54'58.79"O
54	*Bacillus subtilis*	Bouquet, Santa Fe	32°25'20.17"S, 61°54'58.79"O
110	*Chryseobacterium vietnamense*	Cañada de Gómez	32°49'36.76"S, 61°21'39.41"O
115	*Pseudomonas fluorescens*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
116	*Bacillus cereus*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
123	*Bacillus cereus*	Cañada de Gómez	32°49'36.76"S, 61°21'39.41"O
125	*Bacillus cereus*	Las Parejas	32°41'29.25"S, 61°29'21.04"O
FungalStrain ID	**Strain genus/species**	**Geographic origin**	
		Location	GPS Coordinates
149-12	*Fusarium tucumaniae*	Bouquet, Santa Fe	32°25'20.17"S, 61°54'58.79"O
101-03	*Fusarium virguliforme*	San Pedro, Buenos Aires	33°41'42.79"S, 59°42'0.60"O

PGPR bacteria strains were previously isolated and identified by Simonetti et al. (2015).

relationship with potentially hazardous bacteria (Bottone, 2010; Brooke, 2012). For this reason only strains 9, 54, 110 and 115 were used in assay 2.

In assay 2 (Table 2), all four bacterial strains significantly inhibited mycelial growth of *F. virguliforme* 101-03. Strain 110 (*C. vietnamense*) exhibited the highest inhibition on the mycelial growth of *F. virguliforme* 101-03 (31.78%) (Figure 1D). On the other hand, the only strain that significantly inhibited mycelial growth of *F. tucumaniae* 149-12 was strain 54 (*B. subtilis*) (44%) (Figure 1C) and strains 9 and 115 (*P. fluorescens*) showed no significant effect, in accordance with assay 1. However, these strains (9 and 115) significantly inhibited mycelial growth of *F. virguliforme* 101-03 (22.48 and 19.4%, respectively).

DISCUSSION

Altogether, these results suggest that strain 54 (*B. subtilis*) displays antifungal features mainly towards *F. tucumaniae* 149-12, one of the causing agents of soybean SDS. The original contribution of this study is the isolation and testing of bacteria originating from the Pampas region.

These findings are in accordance with those of Xing and Westphal (2007), who found antagonism of *B. subtilis* against 12 isolates of *F. virguliforme*. On the other hand, our results differ with those found by Agaras et al. (2012) where *Pseudomonas* strain SMMP3 antagonized the growth of several pathogenic fungi, including the *F. tucumaniae* isolate CCC 132-11.

Because good results obtained *in vitro* cannot always be dependably reproduced under field conditions, these *in vitro* results should be confirmed by *in planta* experiments. In this way, Agaras et al. (2012) carried out both greenhouse and field trials using soybean seeds inoculated with *Pseudomonas* strain SMMP3. This inoculation treatment caused a reduction of SDS ratings (incidence, severity) and a decrease in the AUDPC (area under disease progress curves) values. Nevertheless, these effects were not statistically meaningful (P>0.05). This may be due to the many factors that affect the effectiveness of the bacteria in natural conditions (Badri et al., 2009). Isolated bacterial strains should be rhizospheric competent, able to survive and colonize in the rhizospheric soil (de Souza et al., 2015).

Further studies on the effects of this strain on the growth of soybean plants and on the SDS control will uncover the mechanisms and potential of this bacterial isolate. However, it has been previously described that most cases of naturally occurring biological control result from mixtures of antagonists, rather than from high populations of a single antagonist (Myresiotis et al., 2012). Moreover, root-infecting *Fusarium* species attack soybean seedlings in the first developmental stages, thus additional tests as for example seed treatment with multiple strain inoculation might be required to improve the degree of SDS control.

Conflict of Interests

The authors have not declared any conflict of interests.
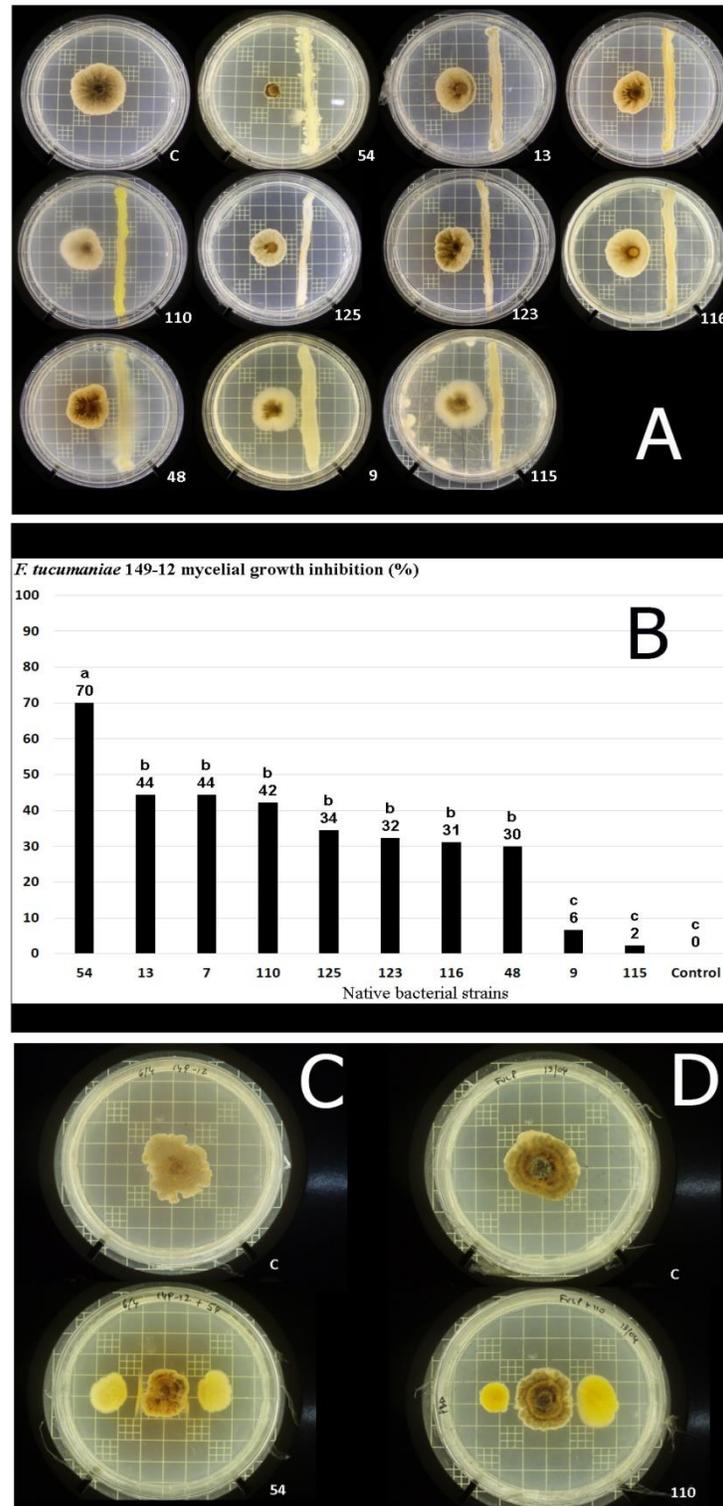
Figure 1. Photographs (A) and figure (B) of the bacteria strains tested for their ability to inhibit the mycelial growth of *F. tucumaniae* 149-12, in assay 1 (columns with different letters are significantly different P<0.05); Photographs of *B. subtilis* strain 54 and its control, tested for their ability to inhibit the mycelial growth of *F. tucumaniae* 149-12 (C) in assay 2; and *C. vietnamense* strain 110 and its control, tested for their ability to inhibit the mycelial growth of *F. virguliforme* 101-03 (D) in assay 2.

Table 2. *F. tucumaniae* and *F. virguliforme* mycelial growth inhibition caused by four selected strains of native bacteria isolated from soils of the argentine Pampas, in assay 1 and 2.

Trial	Treatment (Strain ID)	Mycelial diameter (mm)	Standard deviation	Mycelial growth inhibition (%)
	Control (*F. tucumaniae* 149-12)	15.0[c]	1.0	-
	115 (*P. fluorescens*)	14.7[c]	0.6	2.2
1	9 (*P. fluorescens*)	14.0[c]	1.0	6.7
	54 (*B. subtilis*)	4.5[a]	1.3	70.0
	110 (*C. vietnamense*)	8.7[b]	1.2	42.2
	Control (*F. tucumaniae* 149-12)	25.0[b]	2.6	-
	115 (*P. fluorescens*)	20.0[b]	1.0	20.0
	9 (*P. fluorescens*)	22.3[b]	3.2	10.7
	54 (*B. subtilis*)	14.0[a]	1.0	44.0
	110 (*C. vietnamense*)	25.3[b]	1.2	-1.3
2	Control (*F. virguliforme* 101-03)	43.0[c]	2.0	-
	115 (*P. fluorescens*)	34.7[ab]	2.1	19.4
	9 (*P. fluorescens*)	33.3[ab]	2.1	22.5
	54 (*B. subtilis*)	35.3[b]	2.5	17.8
	110 (*C. vietnamense*)	29.33[a]	1.53	31.8

Means with different letters within a column are significantly different (P<0.05).

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Full Length Research Paper

In vitro antimicrobial activity of maggot excretions/secretions of *Sarcophaga* (*Liopygia*) *argyrostoma* (Robineau-Desvoidy)

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The excessive usage of conventional antibiotics leads to the emergence of multidrug-resistant bacterial strains which threaten public health and stimulates searching for new sources of bio-therapeutic drugs. The aim of this study was to investigate the antimicrobial activity of maggot excretions/secretions from larvae of *Sarcophaga argyrostoma*, a common species of the family Sarcophagidae in Egypt. The excretions/secretions (ES) produced by third instar larvae were sterile filtered and tested against selected pathogenic strains of Gram positive (Gram+ve) bacteria, *Staphylococcus aureus* and *Bacillus subtilis;* Gram negative (Gram-ve) bacteria, *Escherichia coli* and *Pseudomonous aeruginosa*; and the filamentous fungus *Aspergillus flavus*. The ES product produced by third instar maggots proved to be more effective against Gram-ve bacteria. Larval ES, at 0.125 mg/ml concentration, were significantly potent towards *P. aeruginosa, E. coli* and *S. aureus* in a descending sequence. The minimum inhibitory concentrations of *S. argyrostoma* ES were 0.125 mg/ml for *P. aeruginosa* and *E. coli*, using the turbidimetric assay method. Twice and four times this concentration were required to inhibit growth of *S. aureus* (0.25 mg/ml) and *B. subtilis* (0.5 mg/ml), respectively. The antibacterial properties of *S. argyrostoma* ES were not affected by heating or freeze-thaw cycles when tested against *E. coli*.

Key words: *Sarcophaga argyrostoma*, antimicrobial activity, larval excretions, larval secretions, minimum inhibitory concentration.

INTRODUCTION

As necrophagous flies are living in the filthy environment filled with microorganisms, they must possess robust immune cellular and humoral components to counter infection (Wang, 2010; Hall et al., 2016). These components, dried bodies and secretions, have been used in folk medicine to treat many diseases including

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different types of infections and cancer (Ratcliffe et al., 2011). It's unclear whether the maggots produce and secrete antimicrobial molecules as a defensive mechanism or produce them to enhance the survival of their symbiotic bacteria internally and/or on vertebrate carrion. However, these facts together encourage entomologists to study these promising antimicrobial factors from larval products as health reports estimated that, 1-2% of third-world populations would experience chronic skin wounds (Brem et al., 2000).

During maggot debridement therapy (MDT), success is partly due to bactericidal properties of the fly's gut and/or exo-secretions which include the salivary gland secretions and fecal waste (Mumcuoglu et al., 2001; Kerridge et al., 2005). Maggot's ES contains several proteases and antimicrobial substances which aid in debridement, disinfecting and accelerating wound healing (Nigam et al., 2006). Maggot excretions/secretions (ES) anti-microbial components have been shown to disinfect wounds by destroying bacteria that often do not respond to commercially available antibiotics, such as methicillin-resistant *S. aureus* (MRSA) (Bexfield et al., 2004). Furthermore, maggot ES inhibited the pro-inflammatory response of human neutrophils and monocytes in the wound healing process (van der Plas et al., 2007, 2009a, b). These secretions contained factors that could break down and inhibit *S. aureus* and *P. aeruginosa* biofilms, which colonized wounds and protected harmful bacteria from both the host immune system and therapeutic antibiotics. Lately, Pöppel et al. (2016) proved that *Lucilia sericata* maggot excretion products could accelerate the wound healing by excreting a pro-coagulant chymotrypsin-like serine protease. This protease was able to reduce the clotting time and showed a potential mechanism of wound debridement by digesting the extracellular matrix proteins.

Blow flies and flesh flies were previously searched for their value for MDT and whether they are good or poor candidates (Sherman et al., 2000). The antimicrobial actions against a variety of Gram+ve and Gram-ve bacteria were studied from maggot's ES of *L. sericata* (Daeschlein et al., 2007; Huberman et al., 2007; Jaklic et al., 2008), *Lucilia cuprina* (Arora et al., 2010; El Shazely et al., 2013), *Calliphora vicina* (Barnes et al., 2010) and three *Chrysomya* species (Ratcliffe et al., 2015). Currently, there is a tendency to use modern biosurgery (that is, maggot therapy without maggots) instead of traditional biosurgery (Vilcinskas, 2011), where maggot's derivatives or active molecules could be therapeutically used in either their native or recombinant/synthetic form to face the antibiotic-resistant bacteria in hospitals and communities. Some antimicrobial factors from muscoid flies were developed as new antimicrobial and anti-tumor drugs using peptide combining patterns (Ratcliffe et al., 2011; Chernysh and Kozuharova, 2013). Pöppel et al. (2015) used insect biotechnology to characterize the transcriptomes of antimicrobial peptides (AMPs), which

are synthesized in *L. sericata* larval tissues. Previous authors identified 47 genes which encode putative AMPs and they produced 23 synthetic AMPs that showed activity against broad spectrum Gram-ve and Gram+ve bacteria. The objective of this study was to evaluate the antibacterial properties of maggot's ES of *Sarcophaga argyrostoma* on five pathogenic microbial strains as a first step in a process to find novel antibiotic-like compounds that may be used to overcome the bacterial resistance problems, and to provide insight into the maggot's antimicrobial action.

MATERIALS AND METHODS

Rearing laboratory colony

S. argyrostoma was captured from Abu Rawash, Giza provenance, Egypt. The colony was maintained for one year under a 16L:8D h cycle at 28 ± 2°C and 50% RH in the Entomology Department, Faculty of Science, Cairo University. Adults had continual access to water and granulated sugar in 45x45x45 metal cages. Females were allowed to oviposit on fresh beef meat and the larvae were reared on the same food source. Early third instar larvae were used in the experiments.

Maggot ES extraction

A modified method from Kerridge et al. (2005) was used for extraction. Briefly, approximately 200 larvae (~ 35 g) were used in each assay. Larvae were washed with ethyl alcohol for 5 min, replaced with 0.5% formaldehyde for another 5 min and finally rinsed two times with sterile phosphate saline (PBS) buffer (pH 7.2) (Dulbecco's). Larvae were incubated with 2 ml of PBS for 60 min at 27°C and 50% RH in darkness. The resultant liquid was then extracted using a pipette and centrifuged at 8,000 g for 10 min at 4°C. The supernatant was filter sterilized through a 0.22 µm membrane (Xi'an Zenlab) for antibacterial screening, collected in disinfected Eppendorf vials, and stored at -20°C. The protein concentration of the extract was determined by BCA* protein kit (Thermo Scientific) and bovine serum albumin was used as the standard.

Microbial cultures

Strains of *S. aureus* (ATCC 12600), *B. subtilis* (ATCC 6051), *E. coli* (ATCC 11775), *P. aeruginosa* (ATCC 10145) and *A. flavus* (IMI 111023) were isolated from Abo-Elrish hospital and were used to assess the biological activity of the extract. The optimal testing methods approved by the National Committee for Clinical Laboratory Standards (NCCLS) were used to evaluate the susceptibility of bacteria and filamentous fungus (NCCLS, 1993, 1997, 2002). Bacterial strains were incubated in Luria-Bertani medium (LB) agar broth at 37°C for 24 h while the fungus *A. flavus* was reactivated by incubation in potato dextrose agar at 27°C for 12–15 h.

Disc diffusion assay

The modified Kirby-Bauer method (Bauer et al., 1966) was used to evaluate the susceptibility of both bacteria and filamentous fungus to the ES extract. 100 µl of each microbial culture solution, about

Table 1. Antimicrobial activity of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by disc diffusion method.

Microorganism	Inhibition area (mm)		
	Tetracycline	Amphotericin B	ES
Gram-positive bacteria			
Staphylococcus aureus	16.00 ± 0.1	-	11.87* ± 1.4
Bacillus subtilis	14.03 ± 0.2	-	8.50* ± 1.1
Gram-negative bacteria			
Escherichia coli	24.13 ± 0.1	-	17.77* ± 0.8
Pseudomonous aeruginosa	21.10 ± 0.1	-	19.53 ± 1.06
Filamentous fungus			
Aspergillus flavus	-	28.83 ± 1.41	13.07* ± 2.1

Data expressed as mean ± S.E. Three replicates were carried out for each experiment. Tetracycline and amphotericin B were used as positive controls for bacteria and fungus, respectively; and sterile ddH$_2$O was used as a negative control. *Refers to significance ($p \leq$ 0.0001) between control and ES on each organism.

1×10^5 cells/ml, was spread onto Mueller-Hinton agar (BDH Laboratory Supplies, England) plates. 10 µl of the tested extract (2.0 or 10.0 mg/ml concentration) was added to 6.0 mm blank paper disc (Schleicher & Schuell BioScience GmbH) and discs were allowed to dry for 3 h at room temperature. Discs were then placed on agar and plates were incubated at 37°C for 24 h for bacteria and 25°C for 72 h for filamentous fungus. The radial zones of inhibition (mm) were measured. Tetracycline (10 µg/ml) and amphotericin B (40 µg/ml) were used as positive controls for bacteria and fungus, respectively; and sterile ddH$_2$O was used as a negative control. All the assays were done in triplicate.

Determining minimum inhibitory concentration (MIC) by turbidimetry

The MIC was determined according to the micro plate method (Bhuiyan et al., 2011) with modification. Briefly, ES extract was diluted in 1:2 serial dilutions using dimethyl sulfoxide (LY303366) as solvent. The initial concentration of the ES extract was 1.0 mg/ml. 100 µl of each dilution was dispensed into the wells of a 96-well, flat-bottom microtitre plate. 100 µl of each of the four bacterial culture solutions containing 1×10^5 cell/ml were dispensed into the ES wells. Tetracycline (10 µg/ml) served as control and was diluted following the above mentioned procedure using dimethyl sulfoxide while dimethyl sulfoxide served as negative control. The plates were incubated at 37°C for 24 h. The optical density (OD) of each well was read at 600 nm wavelength at zero (OD$_1$) and 24 h (OD$_2$). Each concentration was tested in triplicate and repeated three times (n=9). The bacterial growth ratio was calculated as OD$_2$/OD$_1$ using the 0.125 mg/ml concentration. The MIC was calculated as the lowest concentration of larval ES extract that inhibited bacterial growth after 24 h incubation (Wiegand et al., 2008).

Thermal stability of ES extract

A tube containing 1 ml of ES extract (0.125 mg/ml concentration) was heated in a water bath at 100°C for 5 min while another tube of ES extract was cycled from freezing to room temperature 10 times, allowing for freezing and thawing of the sample. Then, the tubes were centrifuged at 8000 *g* for 5 min and the collected supernatants were assayed against *E. coli* for antibacterial activity using the turbidimetric assay. Five replicates were used for each experiment and tetracycline was used as control.

Statistical analysis

Data were expressed as arithmetic means ± standard error (S.E.) using SPSS 16.0 statistical software. The significance of differences between the two values was assessed using a two-tailed unpaired Student's *t*-test with significance set at $p \leq 0.05$. Tukey's post hoc test was used to analyze multiple comparisons and $p \leq 0.05$ was considered as significant.

RESULTS

Disc diffusion assay failed to show any antimicrobial activity of *S. argyrostoma* ES against the five selected microbes (*S. aureus, B. subtilis, E. coli, P. aeruginosa* and *A. flavus*) at 2.0 mg/ml concentration. No zones of growth inhibition were noticed around wells containing the larval extract. However, at 10.0 mg/ml concentration, the larval extract exhibited high potency against *P. aeruginosa* almost similar to the control (Table 1). The other four organisms showed moderate potency which were significantly ($p \leq$ 0.0001, n=3) lower than the antibiotics. The highest zone of growth inhibition (mm) was recorded in *P. aeruginosa* assay and the lowest one in *B. subtilis* (Table 1).

By using turbidimetry to assay the antibacterial activity of *S. argyrostoma* ES against four bacterial strains (*S. aureus, B. subtilis, E. coli* and *P. aeruginosa*), it was clear that the extract is significantly potent ($p \leq$ 0.001, n=9) against *P. aeruginosa*, *E. coli* and *S. aureus* at 0.125 mg/ml concentration; the ES inhibited bacterial growth by 90, 76 and 61.09%, respectively, as compared to controls (Figure 1). *S. argyrostoma* ES failed to show a significant effect on *B. subtilis* ($p > 0.05$) at the same protein concentration, as its inhibition potency was less than 5% as compared to the control (Figure 1).

The MIC of *S. argyrostoma* larval ES was 0.125 mg/ml for *P. aeruginosa* and *E. coli* using the turbidimetric assay method. This concentration increased to inhibit the growth of *S. aureus* (0.25 mg/ml) and *B. subtilis* (0.5 mg/ml).
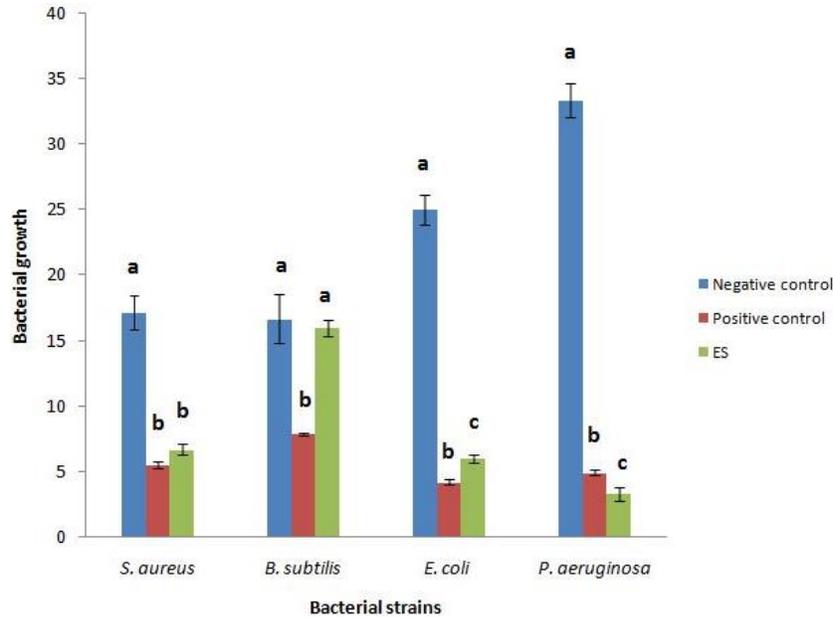
Figure 1. Bacterial growth ratio in the presence of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by the turbidimetric assay method at 0.125 mg/ml concentration. The bacterial growth ratio was expressed as OD_2/OD_1 for n=9. Data expressed as mean ± S.E. Means with different letters are significantly different from each other (p≤0.05) for the same bacterial strain. Tetracycline served as positive control and dimethyl sulfoxide served as negative control.
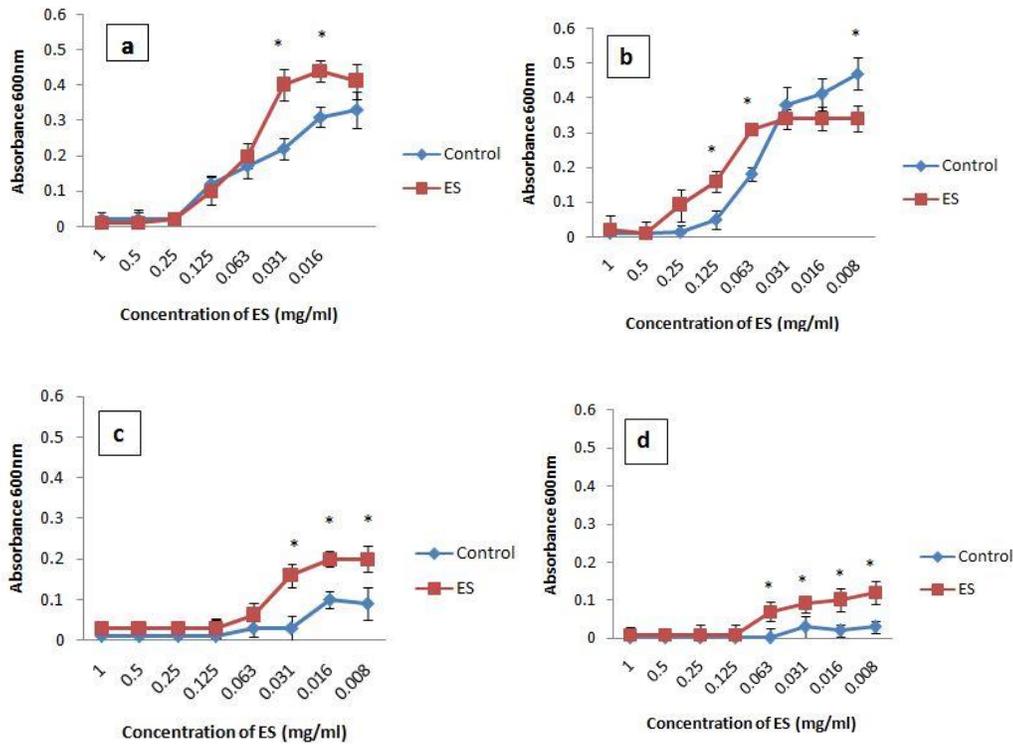
Figure 2. MICs of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by the turbidimetric assay method. Data expressed as mean ± S.E. and each point represented 3 experiments in triplicate wells (n=9). a= *S. aureus*, b= *B. subtilis*, c= *E. coli* and d= *P. aeruginosa*. *Refers to significance between control and ES *p*≤ 0.05. Tetracycline served as positive control.
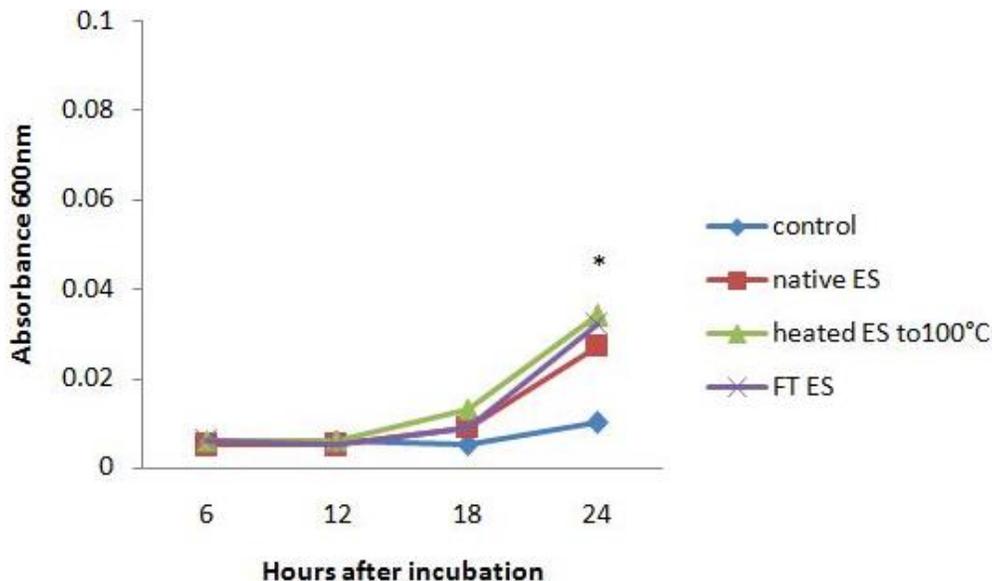
Figure 3. Heat stability of excretions/secretions (ES) of *Sarcophaga argyrostoma* larvae evaluated by the turbidimetric assay method against *E. coli*. Each point represented 5 replicates. *Refers to significance ($p ≤ 0.05$) between control and all tested ES after 24 h incubation period. Tetracycline served as positive control. FT ES = 10 cycles of freezing and thawing.

There were significant differences between potency of ES and the controls ($p ≤ 0.05$, n=9) at some concentrations in all experiments (Figure 2). The ES has a significantly lower potency than the antibiotic control at more diluted concentrations against both Gram-ve bacteria.

The ES of *S. argyrostoma* proved to be heat stable (Figure 3). It was able to withstand both boiling at 100°C for 5 min and repeated freeze-thaw cycles without significant loss of potency ($p>0.05$, n=5). 24 h incubation led to significant loss of potency ($p ≤ 0.05$) between control and both native and treated ES against *E. coli*.

DISCUSSION

The abusive usage of antibiotics leads to the emergence of multidrug resistant bacteria which form an obstacle in the battle of humans against infectious diseases. This dilemma promotes the development of new anti-infective drugs. Defensive peptides, the small molecular proteins which were extracted from different insect's species, constitute the key factors of biological antibiotics that work against several bacteria and fungi (Seufi et al., 2009). The importance of the maggot's ES is not only limited to their killing powers against several bacterial strains, but also to the factors which are contributing to the cleaning of infected wounds. Altincicek and Vilcinskas (2009) and Andersen et al. (2010) mentioned that *L. sericata* has 65 immune-inducible genes including lysozyme- and transferrin-likegenes and 3 proline-rich AMPs. Proteases induced by larval secretions may play

a crucial role in wound healing process. Valachova et al. (2014) identified the full-length cDNAs of five novel putative salivary proteases of *L. sericata*, three of them from the serine protease families which could play a significant role in debridement of wounds. Also, recent studies investigated the potentials of sterile *L. sericata* ES to prevent the formation and disrupt bacterial biofilms of *S. aureus* and *P. aeruginosa* (van der Plas et al., 2008). It was found that ES prevented and disrupted *S. aureus* biofilms immediately and enhanced the formation of *P. aeruginosa* biofilms for 10 h after incubation, and then it began to breakdown *P. aeruginosa* biofilms (van der Plas et al., 2008). A combination of ES and conventional antibiotics could ensure complete breakdown of the biofilms (van der Plas et al., 2010).

The larval ES of *S. argyrostoma* possess one or more antimicrobial factors using disc well diffusion assay. ES showed a higher antibacterial activity against Gram-ve bacteria than Gram+ve bacteria. No previous reports have been found dealing with sarcophagid larval excretion's antibacterial activity, except for the work reviewed by Natori (2010) on *Sarcophaga peregrine* immunity molecules. Sapecin, a medium-sized cationic peptide belongs to the dipteran defensins, was isolated from the culture medium of the embryonic cell line of *S. peregrine* (Matsuyama and Natori, 1988). Defensins are 4-6 kDa cyclic peptides and are the most widespread insect's AMPs (Čeřovský and Bém, 2014). *S. peregrine* sapecin possesses an N-terminal flexible loop, a central α-helix and a C-terminal anti-parallel β-sheet (Hanzawa et al., 1990). *L. sericata* defensin (lucifensin) differs from

sapecin by five amino acid residues (Čeřovský and Bém, 2014). Sarcotoxin 1A, a cecropin antimicrobial peptide from *S. peregrine*, was found to be primarily active against Gram-negative bacteria but shows moderate activity towards Gram-positive bacteria (Natori, 2010), which agrees with our results. Pöppel et al. (2015) suggested that most insects produce a broad spectrum of AMPs during innate immune responses and that the complex interaction of these AMPs mediate the efficient antimicrobial defense.

The current study showed that the larval ES has about half the lethal effect of amphotericin B on *A. flavus*. Further work, using different extraction and bioassay methods, should be done on other fungi and yeasts. A previous study succeeded in purifying an antifungal protein from the hemolymph of *S. peregrine* larvae, which worked successfully against *Candida albicans* and the protein's lethal potentials were greatly enhanced by adding sarcotoxin IA (Iijima at al., 1993). The differences between our findings and the Iijima team may be due to the differences in antimicrobial properties of ES and the insect's haemolymph. Also, Pöppel et al. (2014) separated an antifungal peptide, lucimycin, from the *L. sericata* cDNA library of genes. Lucimycin was effective against the phyla: Ascomycota, Basidiomycota and Zygomycota, but inactive against bacteria.

In the current work, the turbidimetric assay was more effective in demonstrating the potent antibacterial activity of *S. argyrostoma* larval extract by the significant inhibition of bacterial growth. Using the turbidimetric method, *L. sericata* ES showed significant activity against *S. aureus*, *Bacillus thuringiensis*, *E. coli*, *Enterobacter cloacae* and *P. aeruginosa* (Bexfield et al., 2004). On the contrary, using the standard agar diffusion to assay *L. sericata* ES showed no activity against *P. aeruginosa* and *E. coli* (Kerridge et al., 2005). Previous authors suggested that contradictory results for the same species could be due to different extraction techniques, different bioassay methods or heavier bacterial inoculates.

The MIC assay evidently demonstrated that, the larval extract of *S. argyrostoma* was more potent towards *P. aeruginosa* and *E. coli* than *S. aureus*. This is in concordance with findings of Huberman et al. (2007) and Barnes et al. (2010) on *L. sericata* hemolymph extract and ES, respectively, where significant bactericidal activity against *P. aeruginosa* than against *S. aureus* were recorded. Also, Teh et al. (2013) found that *L. cuprina* larval methanol extracts at 0.78 and 1.56 mg/ml concentrations were able to inhibit more than 50% of *P. aeruginosa* and *E. coli*, respectively, while 3.13 mg/ml was necessary to inhibit 50% bacterial growth of *Klebsiella pneumonia*.

In the heat stability test, *S. argyrostoma* ES is resistant to heating when tested against *E. coli*. The current findings are consistent with Simmons (1935) and Bexfield et al. (2004) for *L. sericata* larval extracts; Simmons concluded that the active factors in maggot's ES may not be of a viable nature. On the contrary, Kerridge et al. (2005)

recorded a complete loss of antibacterial activity of *L. sericata* boiled extracts against methicillin-resistant *S. aureus* (MRSA). Our current freeze–thaw stability test is in agreement with the findings of both Bexfield et al. (2004) and Kerridge et al. (2005) who mentioned that the antibacterial properties of *L. sericata* ES were not significantly affected by freeze–thaw cycles. Hundreds of insect's antimicrobial peptides were tested for their resistance to heating and freezing, many of them were unstable and susceptible to temperature and other factors which prohibited their development as new drugs (Kang et al., 2012; El-Bassiony et al., 2016).

Calliphorid flies received attention since they were used in MDT by ancient cultures. Continuous research efforts discovered and developed two low molecular weight antibacterial peptides in *C. vicina*, namely, the alloferons, which were found to be active as antiviral and anti-tumor factors (Chernysh and Kozuharova, 2013). Due to the biodiversity of the Sarcophagidae in Egypt and the scavenging mode of larval life, this family should produce numerous novel antimicrobial peptides or factors. At the moment, more research is needed on Sarcophagidae larval ES as suggested by this study.

In conclusion, the ES of *S. argyrostoma* has shown to be highly effective against both Gram-ve and Gram+ve bacteria. The extract's heat stability is encouraging for further investigations. Following additional isolation and characterization, this extract could potentially yield new antibacterial and/or antifungal drugs.

Conflict of interests

The authors declare that there is no conflict of interests.

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Full Length Research Paper

Fungi extract in the inhibition of the egg hatching and larval development of the *Haemonchus contortus*

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In vitro efficacy of aqueous and ethanolic extracts of *Paecilomyces lilacinus* and *Trichoderma longibrachiatum* was assessed in the egg hatching inhibition (EHI) and larval development inhibition (LDI) of *Haemonchus contortus*. For the EHI assessment, after 48 h of incubation with different concentrations of aqueous and ethanolic extracts, it was quantified blastomeres, embryonated eggs, and larvae of first stage. For the LDI assessment, a quantitative coculture adapted method was used. After seven days of feces incubation with different concentrations of aqueous extract of fungi, the infective larvae were collected and quantified, obtaining the number of developed larvae per feces gram. The aqueous extract of *P. lilacinus* at 1.96 mg mL^{-1} and the ethanolic extract of *T. longibrachiatum* at 1.90 mg mL^{-1} completely inhibited the egg hatching. In the LDI, the aqueous extract of *T. longibrachiatum* at 1.90 mg mL^{-1} showed efficacy of 92.88%. These fungi extracts showed potential to inhibition of the *H. contortus* cycle.

Key words: *Paecilomyces lilacinus*, *Trichoderma longibrachiatum*, alternative control, sheep breeding, gastrointestinal nematodes.

INTRODUCTION

Sheep are bred worldwide on family farms and for commercial agriculture, contributing to hides, meat, wool, and milk. *Haemonchus contortus* is the most common parasite in sheep reared in tropical areas. Animals with

hemonchosis may show anemia and submandibula swelling, with high mortality in young lambs and peripartal ewes. Both sexes at all age levels may be infected, decreasing weight gain and feed conversion efficiency; reduced meat, wool, and milk production; and reproductive disorders (Sczesny-Moraes et al., 2010; Taylor et al., 2010).

The frequent treatment with synthetic antihelminthics is the main method of control. However, it has promoted rapid selection of resistant helminthes populations (Duarte et al., 2012; Adiele et al., 2013; Soro et al., 2013). Resistance has been observed to the main classes of antihelminthics in different continents, impeding the animal breeding (Taylor et al., 2009). Thus, the search for alternatives is being widely done in order to decrease animal infection, the contamination of animal products, the environment and the human beings by chemical antihelminthics residues.

Researches using fungi as a biological control have shown that this alternative consists of a promising control method of gastrointestinal nematodes (Araujo et al., 2004). Nevertheless, the development of fungal formulations economically practicable and easily applicable is a challenge in the implementation of the fungal species in programs of biological control (Campos et al., 2007).

Paecilomyces lilacinus can infect eggs and adult females of Meloidogyne spp. nematode and causes death of embryos in 5 to 7 days. This fungus has given excellent results under varying conditions (Mukhtar et al., 2013). Similarly, *Trichoderma longibrachiatum* has been found to be an effective biocontrol agent for the management nemateded of root-knot and other plant nematodes. The mechanisms of *T. longibrachiatum* against nematodes are essentially unknown (Zhang et al., 2014).

Little is known about the effect of these fungi extracts in the ruminant gastrointestinal nematodes cycle. In this paper, it was assessed the efficacy of the extracts of the fungi *P. lilacenus* and *T. longibrachiatum* in the inhibition of egg hatching and of the larval development of *H. contortus*.

MATERIALS AND METHODS

Study area

The research was conducted in a rural area in city of Montes Claros in the north of Minas Gerais state, Brazil (W 43°50'33.56"; S 16°41'10.05"). The climate of the region is tropical wet with dry summer (As) according to the Köppen classification (Alvares et al., 2013), is marked by a long dry season from May to October and a rainy period in January and February.

Molecular identification

Isolated fungi were obtained from the rectum of the sheep matrix of the breed Santa Inês raised in tropical field (Freitas et al., 2012). Extraction of total DNA of microorganisms was performed according

to Rosa et al. (2009). The primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used for amplification of the rDNA region of aerobic mycelia fungi, as described by White et al. (1990). The primers used for yeasts were NL1 (5'-GCA TAT CAA AAG GAA GAG TAA GCC-3') and NL4 (5'-GGT AAG CTT GCA TGT CCG G-3'). The Polymerase Chain Reaction (PCR) was performed and amplicons produced were purified and assayed in NanoDrop ND 1000 (NanoDrop Technologies) as reported in Abrão et al. (2014). The sequencing reactions were performed in DYEnamicTM (Amersham Biosciences, USA) associated with automated sequencing system MegaBACETM 1000 (Lachance et al., 1999). The sequences of the rDNA fragments were analyzed according to Altschul et al. (1997), in the BLASTN program (Basic Local Alignment Search Tool - 2215 BLAST version 2.0) from the National Center for Biotechnology. Isolates with similarity ≥99% to a previously deposited sequence were considered to belong to the same species. After identification, the cultures were preserved as Castellani (1967).

Sample collection and aqueous and ethanolic extracts

The fungi were previously cultivated in plates containing solid Agar Sabouraud (Sabouraud Dextrose Agar, Prodimol Biotechnology, MG, Brazil) during seven days at 28°C. It was used as inoculum five discs of about 5 mm of diameter of the fungal cultures transferred to Erlenmeyers containing liquid Sabouraud, with no yeast extract. It was incubated in *termoshaker* at 30°C and 150 rpm during seven days. After this period, the cultivations were filtered twice in Whatman n° 1 paper and in Millipore membrane with 0.22 µm of porosity, using vacuum pump. The liquid obtained of each culture, called aqueous extract, was used immediately, and the fungal mass was frozen at -4°C.

To obtain the ethanolic extracts, an adapted method from Nery et al. (2010) was used. 100 mL of ethyl alcohol P.A. (99.5º GL) was added to each 50 g of fungal mass. The mixture was homogenized and transferred to a beaker tighten with plastic wrap and incubated in bain-marie at 40°C for 90 min. Later, it was filtrated through two Whatman n° 1 papers. The extracts obtained were placed in Petri plates for dehydration in an incubator with forced air circulation at 40 ± 5°C for three days and stored in freezer at -4°C.

The dry matter (DM) of the extract subsamples was determined at 105°C according to Association of Official Analytical Chemists (AOAC, 1990), to the tested concentrations calculus (Cunniff, 1995).

Egg hatching inhibition

Flotation, sedimentation, and filtration in saturated NaCl solution were conducted to obtain nematode eggs from feces (Coles et al., 1992) of two Santa Inês lambs infected with *H. contortus* and with an average fecal egg count of >1000 g^{-1}, determined using the modified McMaster technique (Gordon and Whitlock, 1939). The procedures were done in accordance with the ethical principles of the animal experimentation, approved in the 42/2008 protocol of the Ethics Committee on the Animal Experimental of the Federal University of Minas Gerais.

The treatments were conducted with five replicates: Positive control with levamisole phosphate (0.3 mg mL^{-1}); negative control with sterile purified water; and experimental treatments comprising four concentrations of each extracts (Tables 1 and 2).

Experimental mixtures comprised 100 µL of fecal suspension with an average of 80 fresh eggs and 100 µL of the extracts. The samples were homogenized and incubated in a BOD incubator at 28°C for 48 h. Subsequently, 15 µL of Lugol's solution was added to each tube, which were then stored at 4°C for counting of unembryonated and embryonated eggs, and stage one larvae (L1)

Table 1. Effects of aqueous extract and ethanolic extract of *P. lilacenus* on hatchability of *Haemonchus contortus.*

Treatment (mg mL^{-1})	Unembryonated eggs mean	Embryonated eggs mean	L1 mean	Efficacy* (%)
Aqueous extract				
1.96	142.00	8.50	0.00[a]	100.00
3.92	130.00	4.25	0.00[a]	100.00
5.89	126.25	4.25	0.00[a]	100.00
7.85	116.75	6.50	0.00[a]	100.00
Levamisole phosphate (0.3 mg mL^{-1})	97.50	39.50	0.00[a]	100.00
Sterile distilled water	12.75	18.00	86.00[b]	-
Variation coefficient (%)			47.22	
Ethanolic extract				
8.40	18.50	65.25	21.00[b]	79.05
16.80	3.00	139.25	0.75[a]	99.25
25.19	2.50	153.75	0.75[a]	99.25
33.59	4.25	76.25	0.50[a]	99.55
Levamisole phosphate (0.3 mg mL^{-1})	19.50	45.00	0.00	100.00
Sterile distilled water	0.25	0.50	100.25[c]	-
Variation coefficient (%)			27.27	

Different letters in columns indicates significant differences by Tukey's test ($P < 0.05$). *% EHI = 100 × (1 - L1 of treatment / L1 of control).

Table 2. Effects of aqueous extract and ethanolic extract of *T. longibrachiatum* on hatchability of *H. contortus.*

Treatment (mg mL^{-1})	Unembryonated eggs mean	Embryonated eggs mean	L1 mean	Efficacy *(%)
Aqueous extract				
0.59	128.50	121.25	34.5[c]	89.63
1.17	9.50	16.75	93.25[b]	71.97
1.76	6.75	10.25	98.00[b]	70.54
2.35	3.00	9.25	46.00[b]	86.17
Levamisole phosphate (0.3 mg mL^{-1})	262.00	4.50	0.00[c]	100.00
Sterile distilled water	2.00	0.75	332.75[a]	-
Variation coefficient (%)			45.70	
Ethanolic extract				
1.90	23.25	12.75	0.00[a]	100.00
3.81	9.50	3.50	0.00[a]	100.00
5.71	16.25	11.00	0.25[a]	99.49
7.62	30.5	21.25	2.25[a]	95.38
Levamisole phosphate (0.3 mg mL^{-1})l	50.75	10.25	0.00[a]	100.00
Sterile distilled water	0.75	0.00	48.75[b]	-
Variation coefficient (%)			14.15	

Different letters in columns indicates significant differences by Duncan test ($P < 0.05$). *% EHI = 100 × (1 - L1 of treatment / L1 of control).

(Coles et al., 1992).

The egg count and L1 was transformed into relative values for the initial number of eggs for replicate. The means were compared using Tukey's test ($P < 0.05$). Probit regression was employed to determine the concentrations sufficient to inhibit 50% (LC50) and 90% (LC90) of egg hatching with SAEG software (SAEG, 2007). The percent of egg hatching inhibition (EHI) and larval development was calculated using the formula modified after Coles et al. (1992):

*% EHI = 100 × (1 - L1 of treatment / L1 of control).

Inhibition of larval development

To evaluate the effectiveness of aqueous extract of *P. lilacinus* and *T. longibrachiatum* on larval development inhibition (LDI), the quantitative coproculture method (Borges, 2003; Nery et al., 2010;
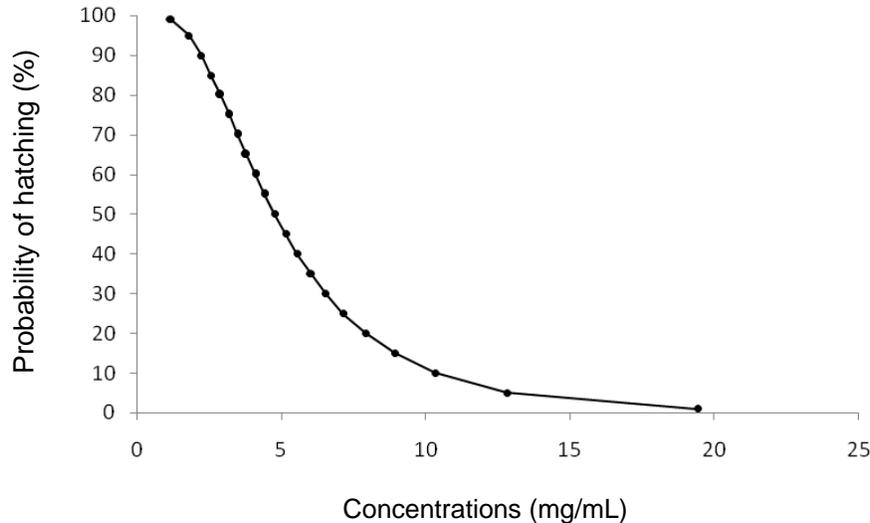
Figure 1. Probability of hatching inhibition of *H. contortus* at different concentrations of the ethanolic extract of *P. lilacenus.*

Nogueira et al., 2012) was employed using feces of lambs with *H. contortus* mono-infection.

Treatments were conducted, each with five replicates, including positive controls with 2 mL of 16 µg g^{-1} ivermectin solution (LA Ranger, Vallée, Minas Gerais, Brazil) added to 2 g of feces, and negative control of 2 mL of sterile purified water (negative control). The experimental treatments consisted of aqueous extract of *P. lilacinus* and *T. longibrachiatum* standardized at 0.28 and 1.13 mg g^{-1} and 0.20 and 0.79 mg dried weight (dw) g^{-1}, respectively.

The cultures were incubated in a BOD incubator at 28°C for seven days and assessed for presence of infective larvae (L3). The following formula, adapted from Borges (2003), was used to determine the percent reduction of larvae (L3) per gram of feces (LPGF):

% LDI = 100 × (1 - LPGF of the treated group/LPGF of the untreated group).

The data was log transformed, log (x+1), and submitted to variance analysis. The means were compared by the Duncan's test ($P < 0.05$). The LC50 and LC90 were determined by probit analysis with SAEG software (SAEG, 2007).

RESULTS AND DISCUSSION

Sequence analysis of rDNA revealed the presence of *P. lilacinus* and *T. longibrachiatum* with 99.9% of similarity. These species have been used largely in the control of plants nematodes and they are producers of lytic enzymes, which are able to destroy parasitical structures (Ferreira et al., 2008; Shuwu et al., 2015; Huang et al., 2016).

For *P. lilacinus*, it verified efficacy of 100% in the EHI in all concentrations of aqueous extract (1.96 to 7.85 mg mL^{-1}) and efficacy superior to 99% in the concentrations ≥ 16.8 mg mL^{-1} of the ethanolic extract (Table 1). The LC_{50} and LC_{90} obtained in the egg hatching inhibition, using

ethanolic extract, were 4.77 and 10.31 mg mL^{-1}, respectively (Figure 1). This way, it is suggested that these fungus metabolites can have effect in the inhibition of the embryonic development and in the egg hatching inhibition of *H. contortus* (Zhang et al., 2014).

It was not possible to estimate the LC_{50} and LC_{90} for EHI experiments with a aqueous extract of the *P. lilacinus* because the low tested concentration showed 100% of efficacy. For the aqueous extract and ethanol from *T. longibrachiatum* it was observed a dose-dependent response.

For the ethanolic extract of *T. longibrachiatum,* all evaluated concentrations presented significant reduction in the number of L1 when related to the distilled water control with efficacy superior to 95% (Table 2). The aqueous extract at 0.59 mg $m\ell^{-1}$ presented significant reduction in the average of L1 larvae hatched, and all concentrations reduced the total number of present eggs and larvae after 48 h of incubation, when compared to the distilled water control (Table 2). Thus, it is suggested that enzymes or the fungus metabolites could have caused degradation in these structures, destroying them completely and impeding the microscopic examination.

In the inhibition of larval development, the concentration of 0.79 mg g^{-1} of the aqueous extract of *T. longibrachiatum* promoted a reduction (p<0.05) in the average of the infective larvae with efficacy ≥ 92% (Table 3), showing that, even in fecal material, the metabolites presented lethal effect for the eggs and the nematode larvae. Due to the low productivity in dry matter to the aqueous extract of *P. lilacinus* used in the quantitative coproculture, the concentrations used were too low, not obtaining relevant efficacy (Table 3).

To all experiments using the aqueous and ethanolic extracts of *P. lilacinus* and *T. longibrachiatum,* it was

Table 3. Effectiveness of the aqueous extract of *P. lilacenus* and *T. longibrachiatum* on the larval development of *H. contortus* in quantitative fecal cultures.

Treatment (mg g^{-1})	LPGF*	Efficacy* (%)
P. lilacenus		
0.28	108.00[b]	22.46
0.56	93.00[b]	32.83
0.84	94.00[b]	32.04
1.13	92.00[b]	34.05
Ivermectin (16 µg g^{-1})	0.00[a]	100.00
Sterile distilled water	139.00[b]	-
Variation coefficient (%)	5.47	
T. longibrachiatum		
0.20	346.00[c]	2.40
0.39	358.00[c]	0.00
0.59	332.00[c]	6.43
0.79	26.00[b]	92.78
Ivermectin (16 µg g^{-1})	0.00[a]	100.00
Sterile distilled water	354.00[c]	-
Variation coefficient (%)	3.66	

Different letters in column indicates significant differences by Tukey test ($P < 0.05$). * % LDI = 100 × (1 - LPGF of the treated group/LPGF of the untreated group). * LPGF, number of larvae (L3) g^{-1} of feces.

verified eggs and larvae L1 with structural deformity in the barks and cuticles. The distilled water controls performed embryonic and normal larval development to the nematode. Some fungi species can destroy nematodes with toxin action (Nordbring-Hertz, 1988) and others can produce metabolites with ovicidal effects (Waller and Faedo, 1993). Nematophagous fungi can penetrate the cuticle structure of the nematode larvae because they have proteases, chitinases and collagenases involved directly in the nematode infection process (Gryndler et al., 2003). According to Braga et al. (2011), the fungus *Pochonia clamydosporia* produces a protease that destroys the eggs of fitonematodes and of *Ancylostoma* spp.

Fungi are known to have huge metabolic diversity providing a wide range of commercial compounds, including many antibiotics used in medicine. Furthermore, metabolites produced by fungi may have potential nematicides, being targets of research in biotechnology (Smedsgaard and Nielsen, 2005). A new peptide extracted of *Omphalotus olearius* fungus has demonstrated similar action to commercial nematicide Ivermectin (Mayer et al., 1997). Review by Li et al. (2007) showed 179 compounds isolated from fungi that have demonstrated activity as nematicide groups of alkaloids, peptides terpenoids, macrolide compounds, oxygen heterocycle and benzo compounds, aliphatic compounds, simple aromatic compounds.

These metabolites and enzymes with ovicidal and

larvicidal action were already identified in fungi from the genus *Aspergillus* and *Trichoderma.* The species *Aspergillus niger* produces citric acid, chymosin, lipase, protease and α-amylase (Bennett, 1998; Jiang and An, 2000; Meyer, 2008). According to De Marco et al. (2000), *Trichoderma* spp. are producers of proteases, hydrolases, chitinases, exoglucanases and endoglucanases of the type ß- glucanases (ß-1,3 and ß-1,6), and cellulases (ß-1,4- D-glucosidases), substances that perform important enzymatic activity in the biocontrol.

According to Ferreira et al. (2008), the species *P. lilacinus* is a parasite of eggs and of female adults of fitonematodes and present great relevance to the biological control. Devrajan and Seenivasan (2002) observed that *P. lilacinus* presented toxic effect to the adult fitonematodes from the genus *Melodoidyne.* This fungal species also performed efficacy *in vitro* on *Taenia saginata* eggs in conditions at the end of ten days (Braga, 2008) and on *Toxocara canis* eggs after seven days of treatment (Araújo et al. 2004).

On the commercialized bioproducts in relation to the control of pathogenic agents causers of diseases in plants, there are three bioproducts based on *A. flavus* for the fungi control, 11 based on *Paecilomyce s*sp. for the nematodes control and 55 based on *T. longibrachiatum* for the fungi and bacteria control (Sosa-Gomez, 2002). As regards the commercialized bioproducts in relation to the control of pathogenic agents that causes diseases in

plants, there are three bioproducts based on *A. flavus* for fungi control, 11 based on *Paecilomyce* ssp. for nematodes control, and 55 based on *T. longibrachiatum* for fungi and bacteria control (Sosa-Gomez, 2002).

Conclusion

The aqueous extracts of *P. lilacinus* and ethanolic of *T. longibrachiatum* at low concentrations inhibited completely egg hatching. The aqueous extract of *T. longibrachiatum* ≥ 0.79 mg mL^{-1} showed efficacy ≥ 92% to LDI of *H. contortus*. The isolated ones from these fungi were obtained naturally from the digestive tract of sheep and in future studies the probiotic effect of these species must be evaluated on the *in vive* inhibition of the cycle of gastrointestinal nematodes.

Conflict of Interests

The authors of this manuscript have no financial or personal relationship with individuals or organizations that could influence or bias the content of the paper. All procedures were performed in accordance with the principles of animal experimentation approved in the 275/2013 protocol of the Ethics Committee on the use of animals (CEUA) of the Federal University of Minas Gerais, Brazil.

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Full Length Research Paper

Microbial contamination of orthodontic appliances made of acrylic resin

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Patients and professionals involved in dentistry are constantly exposed to potentially pathogenic microorganisms, which may be present in agents such as instruments and equipment, among others. The production of orthodontic appliances, as well as other dentistry work, is carried out in a dental laboratory where there is potential for cross-infection. Nevertheless, studies which evaluate the presence of bacterial contamination of orthodontic appliances after routine procedures in the dental laboratory are not enough. Also, there is no established clinical protocol for infection control of orthodontic appliances before they are installed in the patient. This study aimed at evaluating bacterial contamination of orthodontic appliances and the effectiveness of disinfection with 2% chlorhexidine and 0.12% chlorhexidine. Two microbiological collections were done from 60 orthodontic appliances made of chemically active acrylic resin. The first collection was made before disinfection and the second was done after, in order to evaluate bacterial growth. After analysis, it was found that 85% of sampled devices introduced were contaminated and that disinfection protocol performed with 2% chlorhexidine was effective. Furthermore, the adopted disinfection protocol should have the device soaked in 2% chlorhexidine for 10 min to prevent patient contamination from contaminated orthodontic appliances.

Key words: Orthodontic appliances, chlorhexidine, disinfection.

INTRODUCTION

The control of cross infection and biosecurity are issues of great importance to dental practice and in recent years have attracted greater interest of health professionals due to the spread of infectious diseases such as AIDS and Hepatitis B. For Russo et al. (2000) and Jorge (2002), diseases of this kind have led to a general awareness of the risks of contamination and have changed the habits of professionals in dental clinics. These clinics have a high turnover of patients, as well as the multiple disease vector vehicles (equipment, instruments, hands of the

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operator and the environment itself), creating a serious risk of infection to the dentist, assistants and to patients (Sekijima; 1987; Venturelli; 2009).

In orthodontics, along with the high turnover of patients, there is direct contact with moldings and orthodontic impression models. According to Woo et al. (1992), orthodontists see blood in molding appointments an average of 3 times a week. This shows that disinfection of moldings that are made in offices for study and orthodontic appliance production should be of great concern, since the mold will always come in contact with patient's saliva and blood. Freitas et al. (2005) believe that there may be contamination both in the office and in the laboratory to which models/impressions are sent. Molds, impressions, prostheses or other orthodontic appliances that come in contact with patient's saliva - and therefore in contact with microorganisms settlers of oral environment such as *Streptococcus mutans*, *Streptococcus sanguinis*, *Staphylococcus aureus* and *Porphyromonas gingivalis* as related by Aguiar et al (2012) and Andrade et al (2011). Blood can serve as an indirect microorganism transmission route to laboratory technicians and the instruments they use. In this sense, Silva et al. (2010) and Pavarina (1999) claim that if adequate disinfection procedures are not implemented in the laboratory, micro-organisms may be transferred back from the laboratory to the patient, via prosthesis or appliances.

Recent studies as Jagger et al.(1995), Powell et al.(1990) and Silva et al.(2010) show that procedures such as immersion of alginate molds and plaster models in 1% sodium hypochlorite for 10 min are not carried out correctly in laboratories, not taking into consideration the concentration of the disinfectant substance as well as the optimum time for immersion. There is communication gap between dentists and technicians in regards to the disinfection of appliances, which further aggravates the situation.

Therefore, in this study the presence of contaminated orthodontic appliances made of acrylic resin was examined. Once contamination was confirmed, the efficacy of chlorhexidine in two different concentrations was evaluated, using a specific clinical protocol for infection control of these devices before they are installed in the patient.

MATERIALS AND METHODS

The study was an *in vitro* experimental method thus, the approval of ethics committee was not necessary. The sample consisted of 60 impressions/orthodontic appliances which were randomly divided into two groups of 30. All orthodontic devices included in the sampling were made of chemically activated acrylic resin.

Data collection for the evaluation of contamination in orthodontic appliances was carried out in two stages: the first was to collect the appliances in orthodontic laboratories, taking them to the microbiology laboratory of a dental school; and then, the contamination analysis was carried out in that laboratory. The devices were collected directly from the participating research

laboratories (the same way they would be sent to their orthodontist), and were then transported to the microbiology laboratory. After the experiment was completed, the appliances were stored back in the same container in which they would be delivered to the orthodontist.

Once in the laboratory, the devices were manipulated using sterile tweezers for sampling and individually deposited in a sterile saline solution (physiological saline solution), with sufficient volume to cover the entire surface. After 5 min, the container with saline was placed in a vibrator to mix the solution and two aliquots of 0.1 ml, then pipetted into each sample were seeded (surface seeding) into two Petri dishes (0.1 ml in each), containing blood-agar culture. The plates were incubated for 48 h and were subsequently analyzed for the presence of bacterial colony forming units (CFU). The counts for each plate were performed separately and then the mean was calculated between the plates to determine the final CFU for each analysed unit.

After this initial phase when contamination had been confirmed, evaluation of the disinfection was then carried out. The samples were randomly distributed by a draw and then underwent two disinfection protocols, one for each group. Group 1 consisted of 30 devices that were individually immersed for 10 min in a container containing 0.12% chlorhexidine, enough to cover the entire surface. Group 2 consisted of 30 devices that were individually immersed for 10 min in a container containing 2% chlorhexidine, enough to cover the entire surface.

After immersion, the appliances were washed with distilled water to remove excess chlorhexidine and individually immersed in a 3% Tween 80 solution and 0.3% L-alpha-soybean lectin based in saline, a specific solution to neutralize the action of chlorhexidine. After a lapse of 5 min, the solution containing the device was placed in a vibrator apparatus for 10 s, then two aliquots of 0.1 ml were removed from each sample and seeded into two Petri dishes (0.1 ml in each) containing medium culture Agar blood. After 48 h required for bacterial growth, the colony forming units (CFU) were individually counted on each plate and then the arithmetic mean between the plates was calculated to determine the final number of CFU for each device examined. The efficacy of the tested antimicrobial solutions was analyzed in this way.

Data were quantitatively analyzed by comparing the samples on each plate, comparing the initial collection with the experimental group. The colonies were manually counted with the aid of a magnifying glass. Thereby the number of colonies of the sample was converted into score, based on the following parameters: Score 0 for devices with no colonies/biofilm; Score 1 for devices presenting from 1 to 100 CFU; score 2 for 101 to 1000 CFU and score 3 more than 1000 CFU. These last three scores were used to analyse the efficacy of 0.12 and 2% chlorhexidine for appliance disinfection.

Descriptive statistical analysis was performed and verification of statistical difference conducted with the non-parametric Mann-Whitney test for variables that did not maintain dependence, the Wilcoxon test for paired variables and Fisher Exact test to assess the association, all with significance levels of 95%.

RESULTS

After complete data collection, it was observed that 10 different types of orthodontic appliances were part of sampling: Haas Expander, Haas Expander with digital springs, OAR with platinum grid, OAR with palatal crib and around expander, with OAR around expander, OAR with around expander and digital springs, Balters Bionator, Hawley plate, Hawley plate with anterior acrylic stopper/plug and Hawley card with digital spring. Sixty appliances were included in the first analysis (in which

Table 1. CFU assessment in cases disinfected with 0.12% chlorhexidine.

Parameter	n	Average (±SD)	Median	Q25-75	p
Initial CFU Score	30	1.97 (±0.964)	2.00	1.00-3.00	0.001
Final CFU Score	30	2.67 (±0.547)	3.00	2.00-3.00	

CFU, Colony-forming unit; SD, standard deviation; Q, quartile. Source: Original compilation.

Table 2. CFU assessment in cases disinfected with 2% chlorhexidine.

Parameter	n	Average (±SD)	Median	Q25-75	p
Initial CFU Score	30	1.03 (±0.850)	1.00	0.75-1.00	<0.001
Final CFU Score	30	0.13 (±0.571)	0.00	0.00-0.00	

CFU, Colony-forming unit; SD, standard deviation; Q, quartile. Source: Original compilation.

Table 3. Chlorhexidine concentration associated to disinfection.

Parameter	Disinfection (CFU score reduction)		No disinfection		Total		p	pfRP (IC:95%)
	n	%	n	%	n	%		
Use of 0.12% chlorhexidine	2	7.2	26	92.8	28	100	<0.001	24.7 (3.6-170.1)
Use of 2%chlorhexidine	22	95.6	1	4.4	23	100		

CFU, Colony-forming unit; SD, standard deviation; Q, quartile. Source: Original compilation.

the devices were infected before installation in the patient). For the second assessment only 15% were free of contamination from the start and has not been included at this stage. On the other hand 51 of the 60 appliances were included (equivalent to 85% of the sample). Of this total, 28 were in the first (disinfection with 0.12% chlorhexidine), and 23 in the second group (disinfection with 2% chlorhexidine).

The efficacy of chlorhexidine concentration of 0.12% and 2% in the reduced number of CFU is described in Tables 1 and 2. The difference of number of CFU increased when chlorhexidine 0.12% was used and decreased when 2% chlorhexidine was tested was statistically significant (Table 3).

DISCUSSION

The high initial contamination of the sample (85%), probably due to cross-infection, is well known in dental offices. While molding, the alginate mold usually come in direct contact with secretions from the patient, saliva and even blood, and if no decontamination protocols are carried out after the molding, the plaster is then poured over this contaminated alginate. As a result, these secretions that come from the patient come into direct contact with the plaster model, which then is sent to the

laboratory for preparation of orthodontic appliances. According to Ferreira (1995) and Silva et al. (2010), prosthetic/orthodontic laboratories believe they are not exposed to biological material and therefore disregard disinfection protocol because they do not have direct contact with the patient. In a study conducted in São Paulo with 60 laboratories, Cotrim et al. (2001) found that 63% of prosthetic laboratories believed in the possibility of contamination between office and laboratory, however, mold disinfection were not performed by 78% of those interviewed.

The appliances produced in the orthodontic laboratories may have more microorganisms at the end of the manufacturing process. This occurs because infecting microorganisms are found in the instruments used to produce appliances in the laboratories, and also inside the water used to accelerate the resin polymerization process (Barker, 2014).

According to the Ministry of Health Reports (MHR), Brazil (2000), disinfection in molding materials should be performed. For alginate, iodophors or 1% sodium hypochlorite immersion or spraying for up to 10 minutes should be used; for elastomers, immersion in 2% glutaraldehyde for 10 min and for zinc oxide / eugenol impression paste, immersion in sodium hypochlorite for 1:10 or 2% glutaraldehyde for 10 min. MHR also recommended disinfection of casts by spraying or

immersion in 1% sodium hypochlorite for 10 min. The importance of incorporating these behaviors in everyday clinical procedure is not limited to the prevention of material handling contaminated by the dentist, but also the possibility of cross infection between the work site and the technician. This may cause the dissemination of these microorganisms by dental prosthetic technician to prosthetic or orthodontic appliances of other patients, and also cross-infection back to the patient (Wang et al., 2007).

Been aware of these risks, the process of disinfection and sterilization of materials used in the manufacture of prostheses and orthodontic appliances must be reinforced. Some protocol pre- and/or post manufacture of disinfecting work should also be established as a preventive measure in controlling cross infections (Wang et al., 2007). According to the results of our research, it was found that the contamination of appliances exists and the possibility of cross infection is a reality. Thus, the disinfection of these devices is necessary before they are installed in patients as a way to curb and not further extend the possibility of a cross-infection cycle.

However, sterilization of orthodontic appliances made of acrylic by physical methods is not feasible because the boiling point of the monomer which composes the acrylic resin is 103.3°C and the heat distortion temperature is relatively low (95°C). Thus, according to Asad et al. (1993), Rodrigues et al. (1994) and Oliveira et al. (2007), the use of chemical disinfection is necessary for proper control of cross infection.

According to Jorge (2002), chlorhexidine (one of the chemical substances used for disinfection) acts on bacteria by breaking the integrity of their cytoplasmic membranes; resulting in loss of vital cellular constituents such as nucleic acid and potassium. Siqueira et al. (1998) have shown that in this way, although chlorhexidine kills vegetative forms of bacteria, it does not demonstrate effectiveness against spores except at elevated temperatures.

Despite the chemical effects of chlorhexidine, it is important to note that the concentration of the antimicrobial agent is a key factor for its action on microorganisms (Feist and Michele 1989). In this study, it was observed that the initial number of CFUs of 0.12 and 2% chlorhexidine were different, and the degree of initial contamination level of the first group (0.12% chlorhexidine) was much higher than the initial degree of contamination of the second group (2% chlorhexidine). Therefore, in this work we were unable to perform the actual comparison using different concentrations of chlorhexidine without assessing the effectiveness of a group in relation to another. However, in practice, it has been found that 0.12% chlorhexidine was not effective. From a total of 28 cases, there was a reduction of the initial CFU score in only 2 cases, the score was maintained in 13 (same level of initial contamination and final) and the score increased in 13 (final contamination level higher than the level of initial contamination). This does not corroborate with the study done by Peixoto (2007), which evaluated the efficacy of 0.12% chlorhexidine spray reducing contamination by *Streptococcus mutans* group in the acrylic surface of removable orthodontic appliances. It noted through two protocols that both of them (use of chlorhexidine once or twice a week) showed efficacy in reducing contamination of acrylic surface by *mutans Streptococcus in vivo.*

Perdiza (2009) also found that the use of 0.12% chlorhexidine digluconate spray solution used twice a week significantly reduces the level of contamination of periodontopathogenic and cariogenic microorganisms. We attribute the increase in the final result of 0.12% chlorhexidine group to the possible contamination of this product and its low concentration is not be able to eliminate or reduce its own micro-organisms.

In periodontics and orthodontics, 0.12% chlorhexidine is commonly used for intraoral disinfection because usually this concentration is enough to attack periodontopathogenic microorganisms (Lessa et al., 2007). The 2% chlorhexidine is used in more severe cases of periodontal diseases or bad oral hygiene habits. In the present study, the 2% chlorhexidine may have obtained better results due to the acrylic resin surface's accumulation of microorganisms; because there were many microscopic fissures and imperfections. The surface was nonscaling which also contributed to the *mutans Streptococcus* accumulation (Lessa et al., 2007).

The result of the second group (disinfection by 2% chlorhexidine) corroborates the work of Bambace et al. (2003) which established the efficacy of chlorhexidine aqueous solutions for the disinfection of dental office surfaces at different concentrations (0.5, 1, 2, 3 and 4%) compared to 70% ethanol gel and liquid. The study found that aqueous solutions of chlorhexidine from 1% concentration showed greater efficacy in disinfecting surfaces when compared to 0.5% chlorhexidine aqueous solution and 70% alcohol gel and liquid. Despite the results obtained in the present study, it is suggested that more research works with different concentration of chlorhexidine solution that can complement this study should be carried out.

Conclusion

It could be concluded from the findings of the present study that 85% of appliances made from acrylic resin presented infection post-manufacture in specialized laboratory cases. Also, disinfection with 2% chlorhexidine, the highest concentration examined in this study, had a statistically significant efficacy, disinfecting appliances in 91.3% of cases.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibiofilm effect of Tucumã (*Astrocaryum* sp.) endosperm against *Candida albicans*

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Candida albicans is one of the most relevant human opportunistic pathogens and highly competent to build biofilms on vital and non-vital surfaces. Facing the escalating resistance of microorganisms to current antimicrobials and fungicides, the Amazonian biodiversity may bring raw material to the development of new antimicrobial drugs. *Astrocaryum* sp. is a regional fruit, consumed *in natura* or as ice creams and other local specialties. The seeds, however, are discarded and accumulate in the environment. The present study aimed to characterize the phytochemical composition of the endosperm and to evaluate the effect of its extracts on biofilm formation and eradication by *C. albicans*. The seeds were processed to obtain extracts in hexane and ethanol. Color chromatography procedure and thin layer chromatography were used, followed by a colorimetric phytochemical prospection to identify the major secondary metabolites. Microtiter plates were inoculated with *C. albicans* and incubated for 24 h in contact with the extracts or EDTA (positive control) to test the ability of preventing biofilm formation. To evaluate the biofilm eradication effect, the target strain was inoculated in the plates and incubated for 24 h previously to the addition of the extracts or EDTA. Both hexane and ethanol extracts demonstrated significantly higher effect than EDTA on *Candida* biofilm inhibition, highlighting hexane extract that achieved the lowest percentage of adhesion (9.46±0.9%). The chemical composition indicated mainly terpenes, phenols and antioxidant compounds. These results demonstrate a pharmaceutical potential of *Astrocaryum* sp. endosperm for future developments of antifungal drugs, thus contributing to reduce the environmental impact of this biological waste of Amazonia.

Key words: Tucumã, endosperm, secondary metabolites, antifungal, antibiofilm.

INTRODUCTION

A complex biomass of different microbial prokaryotes and eukaryotes embedded in a polymeric matrix turned out to be one of the major concerns at hospital facilities, playing a relevant role at nosocomial infections (Cos et al., 2010; Kang et al., 2012). Defined as biofilm, it has been studied in the last forty years and, in that context, *Candida albicans*, opportunistic pathogen associated with the human mucous membranes, has proven to be a relevant

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and challenging organism (Pfaller et al., 2011; Mathé and Van Dijck, 2013). Although an effort to comprehend the mechanisms of attachment and inter-species interactions as well as to avoid *C. albicans* pathogenicity by using a range of fungicides and new techniques such as surface coatings and quorum sensing inhibitors (Tournu and Van Dijck, 2012), the search of new molecules is still an open field of research.

A vast number of bioactive molecules derive from plants (Bérdy, 2005) and the Amazon rainforest is known for its biodiversity, thus enhancing the chances of drug discovery from natural sources (Myers et al., 2000). In Amazonia, many native plants are used by the local population as food, artisanal medicines, cosmetic and others. Some of them may be of economic interest and yet are not well described or were not properly studied concerning their biological properties. The *Astrocaryum* sp. genus includes 24 Amazonian species (Bacelar-Lima et al., 2006). The most common species *Astrocaryum vulgare* and *Astrocaryum aculeatum*, popularly called "tucumã", are well known for their fruits, which are consumed *in natura* or in sandwiches, as filling of "tapioca" (a sort of pancake made of manioc flour), or ice cream. Rich in ß-carotene, the fruit is indicated as alternative in the combat to hypovitaminosis A (Brasil, 2002; Lorenzi et al., 2006) and pursues high content in lipids (32.3%), carbohydrates (14.5%) and proteins (3.51%) (Yuyama et al., 2008). The seed is also edible and yields 30 to 50% of white oil (Lorenzi et al., 2006).

The main way of obtaining the fruits is not by cultivation, but by collecting them from natural occurring trees. Traditionally, whole families work together on harvesting and peeling the fruits, separating the flesh from the seeds and selling them at the local markets. Despite of the potential industrial uses in cosmetics (Lorenzi et al., 2006), biofuels (Lira, 2012) or fish feeding (Brandão, 2011), the *Astrocaryum* sp. hard shells (epicarp) and seeds (endosperm) are mostly left in great amounts in the environment after the manual removal of the fruit flesh.

Recently, hydro-alcoholic extracts obtained from the pulp and peel of *A. aculeatum* were described to pursue an inhibitory effect against Gram positive bacteria and *Candida albicans* (Jobim et al. 2014). In the present work, the aim was to evaluate *in vitro* the ability of *Astrocaryum* sp. endosperm extracts to inhibit or eradicate *Candida albicans* biofilm as a contribution to enhance the economic value of this residues, thus indicating their potential application in the pharmacology.

MATERIALS AND METHODS

Plant material

The *Astrocaryum* sp. seeds were obtained at a local Market in the urban central zone in Manaus, state of Amazonas, Brazil. Observation of families working on site peeling and removing the fruit flesh of *Astrocaryum* sp., revealed that the final destination of

Figure 1. Open *Astrocaryum* sp. seed, showing the white and thick endosperm.

the seeds was in the trash. So the authors obtained circa 30 kg of seeds by donation.

The obtained seeds of *Astrocaryum* sp. were immediately transferred to the Laboratory of Biology of Natural Products at the Federal Institute of Amazonas (Manaus, Brazil). They were visually inspected and separated from dirt and other materials. After washing them under tap water, they were dried in a circulating air oven at 40°C for 48 h. The hard epicarp was cracked in a microwave oven after 20 s (Figure 1). The endosperm was removed and processed to obtain the extracts.

Phytochemical analysis

The dry endosperm samples were pulverized to enhance the contact with the solvents, hexane (HE) and ethanol (EE). They were left for extraction by maceration for a period of nine days with sequential changes of the solvent every three days. The obtained extracts (HE and EE) were filtered and concentrated in a rotary evaporator (model 801, FISATOM, Perdizes – São Paulo, Brazil) and stored at -24°C in a freezer.

Each extract was submitted to column chromatography procedure (CCP) using Silica Gel 60 (0.063 to 0.200 mm, Merck, Germany) and the eluents hexane, dichloromethane and ethanol. Subsequently, the obtained fractions were analyzed through thin layer chromatography (TLC) to identify the major compounds. The fractions were developed by TLC plates (Silica Gel 60 F254, Macherey-Nagel, Germany). The presence of secondary metabolites was indicated by anisaldehyde and cerium sulphate; antioxidant, phenols or steroids by 2,2-diphenyl-1-picrylhydrazyl (DPPH); alkaloids by Dragendorff; flavonoids, catechins and tannins by ferric chloride III; terpenes, steroids and aromatic compounds by sulfur vanillin and flavonoids by aluminum chloride. All TLC plates were submitted to UV light at 254-365 nm. A partition using hexane:methanol (1:1) was used to treat the ethanolic extract. The different parts were used in a phytochemical prospection, with a sequence of colorimetric methods (Santos et al., 2014) to determine the most relevant groups of secondary metabolites obtained within the extracts. Briefly, the methods should indicate the presence of 1. steroids and triterpenes, 2. phenols and tannins, 3. alkaloids, 4. coumarins, 5. anthocyanins, anthocyanidins, chalcones, aurones and flavonoids, and 6. flavonols, flavanons, flavanonols and xhantones.

Anti-candida assay

A standard strain of *C.albicans* (ATCC10231) was kindly provided by Oswaldo Cruz Foundation (FIOCRUZ – Rio de Janeiro, Brazil). After cultivation on Sabouraud Dextrose agar (SDA), an overnight culture in brain heart infusion broth (BHI) was used in the tests. The inoculum concentration was determined using a Neubauer chamber and standardized to a concentration of 10^6 cells/ml. Each extract was diluted in a 10% dimetilsulphoxyde (DMSO) solution resulting in a 20 mg/ml standard HE or EE solution to be used as test compounds.

The assay was prepared to evaluate the ability of inhibition and eradication of *C. albicans* biofilm. To evaluate the biofilm inhibition (BI), each 96-microtiter plate was inoculated as follows: 100 µl tripticase soy broth enriched with 1% dextrose (TSB-D) was added to each well followed by 100 µl of each extract (HE or EE), and last of all, 100 µl of microbial suspension (10^6 cells/ml) was inoculated. Three experimental controls were used: TSB-D + inoculum as negative control, where the target strain should grow without interference; ethylenediaminetetracetic acid (EDTA 17%) – as positive control of biofilm inhibition/eradication, and TSB-D solely as a sterility control. All plates were incubated at 35±2°C for 24 h. Each extract and control was tested in triplicate. To access the ability of the extracts and EDTA to eradicate the biofilm (BE), they were added to the wells 24 h later than *C. albicans*, so that the yeast could build a biofilm.

After the incubation period (24 h BI; 48 h BE), each microplate had the results recorded by a microplate reader. The supernatant was carefully washed out (3x with sterile saline 0.9%) and the plates were left at 60°C for 1 h in a Pasteur oven. After this, 120 µl of a crystal violet solution (0.06%) was added to each well and kept at room temperature for 5 min. The plates were washed gently and 40 µl of Dimetilsulfoxyde (DMSO) was added to each well in order to perform the last screening by the microplate reader. The effect of DMSO against *C. albicans* was also evaluated following the same procedure for both BI and BE.

Data analysis

For each microtiter plate, three readings were performed on a microplate reader (TP Reader Plus ELx808, BioTek Instruments Inc., Winooski, USA) at 630 nm. Plates to test BI were read immediately after inoculation (T_0), after 24 h incubation (T_{24}), and after staining with crystal violet solution (T_{24b}). Plates to test BE were read after inoculation (T_0), after 24 h incubation (T_{24}), and after staining with crystal violet solution (T_{48b}). For each well a difference was calculated ($T_{24b} – T_0$ or $T_{48b}-T_0$). To relate the results of the potential inhibitors to the negative (untreated) control, the latter was considered as 100% biofilm formation and a percentage value for each extract and EDTA was calculated (Trentin et al. 2011). Any value lower than 100% was considered inhibition or eradication of biofilm. A comparison between means gave the significance between each extract and EDTA using a Student's *t*-test (GraphPad® Software) considering p ≤ 0.001.

RESULTS

Phytochemical composition

The hexane extract (HE) yielded 41.49% against 31.8% of the ethanol extract (EE). Classic CC and TLC methods indicated the presence of terpenes, flavonoids and phenols in the HE. By the EE terpenes and antioxidant compounds were detected mainly. The partition of EE resulted in 3 phases:

1. Soluble in methanol
2. Soluble in hexane and
3. A hydrophilic orange precipitate.

When submitted to the phytochemical prospection, the phases 2 and 3 reacted positively for flavonols, flavanons, flavanonols, xanthones, phenols and tannins. No alkaloids or coumarins were detected by any of the extracts tested.

Antibiofilm effect and data analysis

As summarized in Table 1, the scores below 100% indicate that the formation of *C. albicans* biofilm (BI) was inhibited by all concentrations tested either by the *Astrocaryum* sp. extracts or EDTA. Comparing the mean values by the Students *t*-test, however, the HE was significantly (p ≤ 0.001) more effective than EDTA at four different concentrations. The strongest inhibitory effect over the cells attachment was achieved by HE at the concentration of 1.5 µg/ml. Concerning the eradication of the adhered cells (BE) all tested compounds were less effective than by the BI-assay at the same concentrations. Also EDTA showed values over 100% at five different concentrations. A dose-dependent effect was demonstrated by the %BI values obtained either by the extracts or EDTA. A gradual increase in biofilm formation was observed along with the concentration reduction. As expected, DMSO did not show any interference with *C. albicans* attachment or biofilm removal during the entire experiment.

DISCUSSION

The endosperm of Arecaceae representatives presents a high content of lipid compounds. Previous works dedicated to the nutritional composition of *Astrocaryum* mesocarp (fruit flesh) found mainly hydrophobic components. Yuyama et al. (2008) demonstrated the lipid content of 32.29 and 61.60%, respectively in the *in natura* and dehydrated mesocarp of *A. aculeatum*. The presence of β-carotene and other antioxidant compounds was previously described for *A. aculeatum* fruits. No literature described until now, the presence of secondary metabolites in the endosperm of *Astrocaryum* sp. The present study gives information about the phytochemical groups of compounds found in the endosperm of *Astrocaryum* sp., and the ability of the seed extracts to interfere with *Candida albicans* adhesion under *in vitro* conditions.

A similar protocol used for bacterial biofilms (Kwasny and Opperman, 2010; Trentin et al. 2011) was applied here to induce *C. albicans* biofilm formation. Jin et al.

Table 1. *Candida albicans* biofilm inhibition (%BI) and eradication (%BE) in response to *Astrocaryum* sp. endosperm extracts.

Concentration	%BI			%BE		
(µg/ml)	HE	EE	EDTA	HE	EE	EDTA
3.000	11.41±1.6*	14.24±6.5	23.94±0.1	46.28±22.1	46.12±17.8	43.57±0.0
1.500	9.46±0.9*	13.35±5.4	26.41±0.5	71.11±20.7*	67.64±11.7*	162.98±0.3
0.750	12.92±5.8	18.66±3.8	25.42±0.2	120.62±12.5	125.73±26.6	139.73±0.5
0.375	20.54±3.5	24.50±5.1	28.98±0.0	164.41±12.1	121.37±28.1	135.21±0.6
0.188	28.59±4.9	28.59±1.6*	36.60±0.1	240.18±67.2	220.84±25.5	183.52±0.0
0.094	32.48±3.8	29.31±5.0	37.69±0.2	183.82±16.9	173.06±45.8	197.52±0.2
0.047	39.80±5.3	39.53±7.0	53.51±0.0	110.46±11.1	136.27±39.0	80.14±0.1
0.023	33.14±1.3*	39.00±6.2	47.08±0.1	53.42±16.3	38.37±4.1	46.50±0.0
0.012	38.58±0.9*	39.07±1.7	43.13±0.1	59.07±43.1	27.92±2.7	19.64±0.3
0.006	39.53±4.7	48.57±1.6*	39.96±0.0	29.12±1.5	28.67±6.1	26.41±0.7
0.003	43.19±3.2	52.26±1.0	50.94±0.2	34.84±5.3	29.80±7.6	23.25±1.2
0.001	53.71±7.3	52.29±9.1	48.57±0.3	69.53±35.7	31.83±8.3	31.15±0.4

Percentage values are given as mean ± standard deviation and were calculated in relation to the untreated controls: biofilm inhibition = 1.011_{OD} and biofilm eradication = 0.443_{OD}. HE: extract obtained in hexane; EE: extract obtained in ethanol. EDTA: ethylene-diamin-tetracetic-acid as control of inhibition/eradication of biofilm; *Statiscally significant compared to EDTA values ($p \leq 0.001$).

(2004) described that the adhesion of *C. albicans* cultures treated with glucose was higher than those cultivated with galactose. In this study, a TSB-broth enriched with dextrose (1%) was used with an average of 1.011_{OD} biofilm formation on polystyrene microtiter plates after 24 hours incubation.

The *Astrocaryum* sp. endosperm extracts as well as EDTA have demonstrated the ability to avoid *C. albicans* adhesion at some extension. The inhibition of biofilm (%BI) was significantly stronger by HE than by EDTA at four different concentrations. At the concentration of 1.5 µg/ml, the HE induced the lowest percentage of *C. albicans* adhesion (9.46%) while the lowest value by EDTA (23.94%) was registered by doubling the concentration. The EE showed a significant inhibitory value compared to EDTA by only two concentrations, scoring the lowest percentage of biofilm formation (28.59%) at 0.188 µg/ml. The tested extracts and controls showed a similar increase in the cell adhesion along with the reduction of concentration.

EDTA is a well-known di-valent cation chelator with the ability of reducing biofilm formation and contributes to remove mature biofilms (Dunne, 2002; Zehnder, 2006). However, it was demonstrated by Ramage et al. (2007) that EDTA inhibits the hyphal formation by *C. albicans*, thus reducing its adhesion ability to polystyrene microplates and interfering only minimally on removing mature biofilms. The present study corroborates those findings indicating a higher effectivity of EDTA on biofilm inhibition than on its removal.

Overall values of %BE were higher than %BI by the *Astrocaryum* sp. extracts and EDTA at the same concentrations. This could be due to the complexity achieved by *Candida albicans* biofilms in which a variety of genes not expressed by planktonic cells lead to resistance enhancement in the attached ones (Mathé and Van Dijck, 2013). Increased values of persistent biofilm (%BE above 100%) were detected at the intermediary dilutions by EDTA (from 1.5 to 0.094 µg/ml) as well as by the extracts (from 0.75 to 0.047 µg/ml). The reason of promoting cell detachment at the highest concentration and again at low concentrations cannot be fully understood and should be further investigated. Both *Astrocaryum* extracts and EDTA showed better results on preventing biofilm formation than on eradication, but HE and EE were significantly ($p \leq 0.001$) more effective than EDTA.

The phytochemical tests indicated the group of terpenes as the major constituents in HE. Terpenes were described as Candida biofilm inhibitors by enhancing the cell wall permeability of planktonic cells and altering the cell surface in such a way that it could avoid their attachment (Braga and Dal Sasso, 2005; Dalleau et al., 2008). Polyphenols, mainly flavonoids, detected in the hydro-alcoholic extracts of *A.aculeatum* fruits and peel were effective against *Enterococcus faecalis*, *Bacillus cereus*, *Listeria monocytogenes* and *C. albicans*. The authors found rutin and the combination of rutin and gallic acid isolated from *A. aculeatum* ethanolic extract to be possibly associated to redox mechanisms that led to inhibition of growth and biofilm formation. In this study, the chemical composition indicated the major presence of terpenes and phenols with antioxidant effect (HE reacted positively to DPPH) that could suggest that these compounds could be the responsible for the better results achieved by HE on biofilm inhibition, thus corroborating

with previous studies.

CONCLUSIONS

This is the first study on the chemical composition and antibiofilm activity of extracts obtained from *Astrocaryum* sp. endosperm. Biofilm formation by *C. albicans* was significantly inhibited by hexane extracts compared to EDTA in lower concentrations. The phytochemical prospection revealed a major composition of terpenes and phenols, thus indicating the need of isolation and characterization of the bioactive compounds. These results indicate the potential use of these usually discarded seeds in the future developments of drugs, especially related to *Candida albicans* biofilm prevention.

Conflict of Interests

The authors have not declared any conflict of interests.

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