African Journal of Microbiology Research

Volume 9 Number 35, 2 September, 2015
ISSN 1996-0808

Academic Journals

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African Journal of Microbiology Research

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academicJournals

Vol. 9(35), pp. 1964-1973, 2 September, 2015
DOI: 10.5897/AJMR2015.7510
Article Number: 37D544255539
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Fermented milk enriched with passion fruit peel flour (*passiflora edulis*): Physicochemical and sensory aspects and lactic acid bacteria viability

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Received 4 April, 2015; Accepted 8 June, 2015

This study aimed to evaluate the physicochemical parameters, total and thermotolerant coliforms, lactic acid bacteria viability, instrumental color, and sensory analysis of fermented milks added to passion fruit peel flour (PFPF), throughout 29 days of storage, except composition. Four fermented milk treatments were prepared as follows: 1, fermented milk without addition of PFPF; 2, fermented milk added with 1% PFPF; 3, fermented milk added with 2% PFPF; 4, fermented milk added with 3% PFPF. According to the results obtained, acidity and pH values were inversely proportional, and microbiological analyses of coliforms showed no contamination, lactic bacteria were viable up to the 15th day of storage, treatment 3 showed the highest water holding capacity and syneresis decreased by raising the levels of PFPF. Fermented milk with the lowest level of addition of PFPF showed better scores and was the most preferred among panelists.

Key words: Whey, milk, pH, acidity, viable lactic acid bacteria.

INTRODUCTION

Passion fruit (*Passiflora edulis*) belongs to the family *Passifloraceae* (Sebrae, 2005) and is a fruit of tropical and subtropical climates; the fruit consists of approximately 52% peel, 34% pulp and 14% seeds and cultivation is aimed at the juice and pulp industry (Zeraik et al., 2010). Environmental care and waste reduction are increasing concerns of the food industry; therefore, viable alternatives to the use of food waste in the development of new products for human consumption must be proposed (Garmus et al., 2009).

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Table 1. Formulations of fermented milk drinks enriched with passion fruit peel flour (PFPF).

Ingredients	Treatments with PFPF (%)			
	0	1	2	3
Whole milk (%)	60	60	60	60
Milk whey (%)	40	40	40	40
Sucrose (%)	10	10	10	10
PFPF (%)	-	1	2	3
Starter culture (mg/L)	400	400	400	400

Passion fruit by-products (peel and seeds) may have technological and biological characteristics of interest to the food industry (Martinez et al., 2012). According to Ambrosio-Ugri and Ramos (2012), after drying, passion fruit peel is ground to obtain passion fruit flour or passion fruit fibre. The passion fruit peel is composed of flavedo, which corresponds to the outer layer of yellow-green color, rich in insoluble fibre and albedo, corresponding to the white inner layer rich in soluble fibre, in particular pectin, with small amounts of mucilage (Janebro et al., 2008).

Dietary fibres helps the bowel function and are considered prebiotic; soluble fibres retard intestinal passage, gastric emptying and glucose uptake, helping to reduce blood cholesterol, insoluble fibres accelerate intestinal transit, increasing the fecal volume, slowing down glucose hydrolysis, contributing to the reduction of some colon diseases (Pereira, 2002) and can serve as a substrate for beneficial microorganisms such as probiotics (Gallina, 2009).

According to the World Health Organization (FAO, 2002), probiotics are defined as live microorganisms that when administered in adequate amounts, confer a health benefit to the host. Awaisheh et al. (2005) reports that bacteria belonging to the genus *Lactobacillus*, which colonize the small intestine of humans and combat pathogens such as *Salmonella* spp., and those of the genus *Bifidobacterium*, which colonize the large intestine of human and inhibits the growth of *Escherichia coli* and *Candida* spp., are major microbial species with probiotic properties. Lee et al. (1999) claims that products containing *L. acidophilus* and *B. bifidum* have the capacity of improving the peristaltic movements of the intestine, increasing absorption of nutrients, controlling or preventing intestinal infections by blocking the receptors of pathogens, inactivating the effects of enterotoxin and favoring the development of microorganisms resistant to pathogens, especially against *Escherichia coli*.

Gallina et al. (2012) have reported that the main technological challenge for the processing industry is the viability and stability of probiotic cultures and that probiotic foods should contain specific strains of probiotic microorganisms and maintain adequate levels of viable cells during product storage without interfering with flavor and texture.

Since the functional foods take an important place in the daily meal of the consumers, new studies must be carried out to: test ingredients, explore more options of food matrix that have not yet been industrially utilized, reengineer products and processes (Coman et al., 2012).

The aim of this work was to evaluate physical, chemical and microbiological parameters, sensory analysis and morphological structure of each fermented milk enriched of passion fruit peel flour (PFPF).

MATERIALS AND METHODS

Development of Fermented milks

Refrigerated milk and milk whey were obtained from enzymatic coagulation of Mozzarella cheese in a dairy industry located in the city Rio Verde (GO, Brasil).

The processing of fermented milks was conducted at the Laboratory of Products of Animal Origin - Instituto Federal Goiano, Rio Verde Campus, GO, Brazil (IF Goiano). Milk and whey were filtered to eliminate physical contamination. Four treatments consisting of 4 L were prepared, with proportion of 60% milk and 40% milk whey added with 10% sucrose; subsequently, the mixture was submitted to heat treatment at 90°C for 3 min. After pasteurization, fermented milk was cooled to 42°C for the addition of the Bio Rich® lyophilized culture (*Lactobacilus bulgaricus, Lactobacilus. acidophilus, Streptococcus termophilus* and *Bifidobacterium)* and incubated at 42°C up to pH 4.5.

After coagulation, fermented milks were removed from the oven and cooled to reach 20°C, then clot breaking was performed using glass rod in circular movements for 1 min. After clot breaking, passion fruit peel flour was added in the following proportions: 1, fermented milk without addition with passion fruit peel flour (0%); 2, fermented milk added with 1% passion fruit peel flour; 3, fermented milk added with 2% passion fruit peel flour and 4, fermented milk added with 3% passion fruit peel flour. The formulations for each drink are presented in Table 1. After the addition of PFPF, fermented milks were packaged in aseptic polypropylene packages, identified and stored at 5°C for sensory evaluation, scanning electron microscopy (SEM), pH, acidity, syneresis, water holding capacity, viable lactic acid bacteria, and instrumental color during the 29 days of storage.

Physicochemical analyses

All analyses were performed in triplicate, except for the sensory analysis that was performed only once on the eighth day of storage.

Milk

Milk samples were collected for chemical composition evaluation at the Laboratory of Milk Quality, Research Center, School of Veterinary, Food and Animal Science - Federal University of Goiás, using MilkoScan 4000 equipment (Foss Electric A/S. Hillerod, Denmark) to obtain fat, protein, lactose and non-fat solid (NFS) results, expressed in percentage (%).

Milk whey

Analyses of pH, acidity, fat and protein were performed in Ecomilk equipment (Cap Lab) milk analysis.

Fermented milks

Fat

Fat was analyzed using the Gerber method as IAL (2005). About 10 ml of sulfuric acid were transferred to Gerber butyrometer and 11 ml of sample and 1 ml of isoamyl alcohol were added, sealed and centrifuged at 1200 ± 100 rpm for 15 min.

Protein determination

For crude protein analysis, total nitrogen was determined by the micro-Kjeldahl method according to official method No.960.52 of AOAC International (1997). Total nitrogen was converted into crude protein using factor 5.95 (Alencar; Alvarenga, 1991). The equipment used was digester block (Tecnal, TE-0070) and nitrogen distiller (Tecnal, TE-0363).

Titratable acidity determination

For titration of samples, 10 ml of diluted fermented milk were added to 10 ml of distilled water with five drops of 1% phenolphthalein solution, followed by titration with 0.1 N sodium hydroxide solution up to the appearance of a persistent pink color for approximately 30 seconds (Brazil, 2006). Acidity was determined according to the following equation: lactic acid (%) = (V x f x 0.9) /m (IAL,2005).

pH

For pH assessment, bench Bel Engineer digital potentiometer was used. The electrode was inserted into the sample after homogenization without touching the bottom or the sides of the package, so the reading was carried out (Brazil, 2006).

Microbiological analyses

Analyses of total coliforms, *Escherichia coli* and estimation of viable lactic acid bacteria were performed at the Laboratory of Food Microbiology (IF Goiano) at storage times of 1, 8, 15, 22 and 29 days.

About 25 g of fermented milk were weighed and added to 225 ml of sterile peptone water and after homogenization, the solution was diluted to concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The enrichment step for total coliform count with difference for *Escherichia coli* used 1 ml aliquots of concentrations of 10^{-1}, 10^{-2} and 10^{-3}, which were transferred into test tubes containing 10 ml of lauryl sulfate broth (LST) and incubated at 35°C for 24 h. Then, the presence of coliforms was confirmed using Brilliant Green Bile Broth (BG) incubated at 35°C for 24 h and in *E. coli* broth (EC) incubated for 24 h in water bath at 45°C.

For viable cell count, the method used was “pour plate” in-depth plating, using MRS agar (Kasvi). Serial dilutions were made from 10^{-1} to 10^{-6} and plating performed in triplicate and incubated at 35°C for 48 h (Macedo, 1997). The results were presented as Log^{-1} CFU/ml.

Syneresis determination

To determine syneresis, 30 g of yogurt were filtered in funnel and distributed on filter paper and after 5 h of draining, the supernatant was removed and weighed, and the syneresis rate was expressed in percentage (%), which was obtained by the ratio between supernatant weight and the total sample weight multiplied by 100 (Riener et al., 2010).

Water holding capacity

The water holding capacity (WHC) was determined in triplicate according to the modified method of Parnell-Clunies et al. (1986), being expressed as percentage (%), according to the following Equation:

WHC (%) = 100 x (initial sample weight - supernatant weight) / sample weight

Color parameters

Instrumental color parameters (L*, a* and b*) of fermented milk samples were analyzed in triplicate in colorimeter (HunterLab, 1998) at the Post-Harvest Laboratory of Plant Products (IF Goiano).

Sensory evaluation

This study has been submitted and approved by the ethics research committee of Instituto Federal Goiano, with number 20/2013.

Analyses were performed at the Laboratory of Sensory Analysis (IF Goiano). Fifty-one untrained panelists aged 18-56 years, 64.3% females and 35.7% males, participated at the sensory analysis. Fermented milk samples were coded with three-digit numbers and presented under white light in 50 ml white cups (± 20 g fermented milk) to each of the panelists.

The sensory analysis used acceptance and ordination tests: for acceptance, evaluation was based on scores awarded by panelists through a nine-point hedonic scale, where value one (1) represented "dislike extremely" and nine (9) "liked extremely", assessing flavour, aroma, texture and colour. The ordination test was analyzed, in which panelists put on an increasing order the samples they liked the most and those they liked less (IAL, 2005).

Scanning electron microscopy

Fermented milk samples were lyophilized in lyophilizer equipment (Enterprise II / Terroni®). Then, samples were defatted and analyzed in scanning electron microscope (JSM - 6610 / Jeol®) for the acquisition of images.

Statistical analyses

The experimental design adopted in the analysis was a completely randomized design (CRD) and syneresis, water holding capacity and were presented by means of regression while pH, acidity, viable lactic acid bacteria values, color parameters results were analyzed by comparison among treatment means using the Tukey’s test. Analyses were performed using SISVAR and Sigma Plot 11.0 software.

Table 2. Average results of fat and protein of milk drinks enriched by increasing levels of passion fruit peel flour.

Parameter	PFPF (%)			
	0	1	2	3
Fat (%)	1.43 ±0.15a	1.40 ±0.4a	1.40 ±0.17a	1.23 ±0.15a
Protein (%)	2.19 ±0.03c	2.44 ±0.11bc	2.59 ±0.02b	3.24 ±0.17a

*Small letters in the line do not differ from each other according to Tukey's test at 5% significance level.

RESULTS AND DISCUSSION

The composition of the milk used in the manufacture of fermented milks (fat 3.6% ± 0.04; acidity 0.16 ± 0.02, density 1.030 g/100 ml; cryoscopy -0.530ºH; NFS 8.4% ±0.03; protein 3.12% ± 0.15 and pH 6.70) shows that milk was in accordance with Normative Instruction N°. 62/2011, which establishes the following minimum physicochemical parameters: fat, 3.0 g/100 g; acidity from 0.14 to 0.18 g of lactic acid/100 ml; relative density from 1.028 to 1.034 g/ ml; Cryoscopic index from -0.512 °C at 0.531 °C; NFS at least 8.4%; protein 2.9%; whey showed fat 0.31% ± 0.16; acidity 0.10 ± 0.07; density 1.026 g / 100 ml; cryoscopy of -0.500 ºH; NFS 0% ± 6.25; protein 1.10% ± 0.08 and pH 6.54.

The inclusion of increasing levels of PFPF in the preparation of fermented milks did not affect the fat content (Table 2); however, there was an increase of the protein content of fermented milks with the addition of PFPF, which was due to its average protein content of 15.4%, considered high.

The fat and protein values of the present study are lower than those observed by Gallina et al. (2011) in fermented milks with and without addition of probiotics and prebiotics (fat 2.8% and protein from 4.03 to 4.28%) by Toledo et al. (2013) in yogurts added of pulp and passion fruit flour (*Passiflora edulis*) (fat from 2.42 to 2.88% and protein from 2.98 to 4.20%) and Gerhardt et al. (2013) in fermented milks added of ricotta whey and collagen hydrolysate (fat from 2.90 to 3.10% and protein from 2.99% to 4.44%).

The titratable acidity and pH results of fermented milks throughout the storage period were inversely proportional. Fermented milk with 0% PFPF showed lower acidity while pH was the highest on the fifteenth day of storage. In this period, fermented milk with 3% PFPF showed high acidity and the lowest pH; at the end of the twenty-ninth day of storage, titratable acidity decreased and pH increased, and fermented milk with 0% PFPF showed the lowest acidity and fermented milk with 3% PFPF showed the highest pH (Table 3).

The titratable acidity results of this study were higher than those reported by Gallina et al. (2012), who worked with fermented milk produced from symbiotic fermented milk added of guava pulp and found variations from 0.41 to 0.42%, but the pH values were lower to those reported by the author who observed values from 4.40 to 4.42. Results similar to those of this study were observed by Gerhardt et al. (2013) in fermented milks using whey ricotta and collagen hydrolysate, which ranged from 0.72 to 0.91%; pH results corroborate those found by Toledo et al. (2013) in yogurts added with passion fruit pulp and flour (*Passiflora edulis*), ranging from 4.49 to 3.63.

Similar values were observed by Costa et al. (2013) in a study with fermented milk made with different stabilizers / thickeners (titratable acidity from 0.55 to 0.61% and pH from 3.95 to 4.07), and those reported by Gonçalves and Leão (2013) in yogurts added of mixed flours from apple, passion fruit and grape waste (titratable acidity from 0.84 to 0.89% and pH from 4.20 to 4.60).

The 29th day of storage the samples of fermented milk showed a decrease in acidity. This can be explained by the fact that the 29th day of storage the samples fermented milk showed incidence fungus samples, which caused decrease in acidity. Coelho et al. (2009), when evaluating shelf life in yogurt for 60 days there were decreased acidity for the high count of yeasts and molds. Franco and Langraf. (2003) reported that some species of yeasts and molds using lactic acid, leading consequently to an increase in the pH.

Regarding the count of coliforms, none of the samples showed typical colony formation, which results are similar to those reported by Tebaldi et al. (2007) in 20 samples of fermented milks commercialized in southern Minas Gerais and by Araújo et al. (2012) in passion fruit-flavored sundae-type yogurt, where microbiological results for analysis of coliform bacteria showed no turbidity with acidification (turning) and gas production, indicating absence of this microorganism in the samples analyzed. Paula et al. (2012) observed presence of <10 CFU ml^{-1} of coliforms at 30°C and 45°C in fermented milk.

The viable cell count results of Figure 2 shows that 3% PFPF concentration up to the fifteenth day of storage showed the highest number of CFU/ml, followed by concentrations of 2 and 1%, which is in accordance with the current legislation that establishes minimum number of viable bacteria per milliliter (10^6 CFU) during the validity period (Penna, 2002). On the fifteenth day of storage, it was observed that the 0% PFPF showed

Table 3. Acidity (%) and pH fermented milk with increasing levels of flour passion fruit peel during storage.

Treatment (%)	Storage period (Days)									
	1		8		15		22		29	
	Acidity	pH	Acidity	pH	Acidity	pH	Acidity	pH	Acidity	pH
0	0.63±0.01Ab	4.36±0.09Aa	0.68±0.2Bb	4.23±0.01Aab	1.12±0.16Aa	4.20±0.01Aab	1.32±0.04Aa	4.10±0.01Bb	0.56±0.06Ab	4.11±0.01ABb
1	0.74±0.03Abc	4.31±0.05ABa	0.87±0.07ABbc	4.20±0.05Aa	1.05±0.01Aab	4.14±0.05ABb	1.27±0.03Aa	4.00±0.01Bb	0.70±0.06Ac	4.16±0.02ABb
2	0.73±0.02Ab	4.26±0.09 Ba	1.09±0.3Aa	4.25±0.01 Aa	1.16±0.07Aa	4.06±0.05ABb	1.36±0.04Aa	4.02±0.01Bb	0.70±0.06Ac	4.02±0.01ABb
3	0.72±0.02Ab	4.18±0.02 Bb	1.19±0.1Aa	4.12±0.05 Ab	1.19±0.03Aa	3.98±0.05Bb	1.34±0.03Aa	3.97±0.01Bb	0.80±0.09Ab	4.21±0.2 ABb

* Capital letter on same column do not differ from each other, same lowercase letters on the same lines do not differ according to Tukey's test at 5% significance level. 0% = no addition of PFPF (0%); Treatment 1%= 1% of PFPF; Treatment 2% = 2% of PFPF; Treatment 3% = 3% of PFPF.

Table 4. Viable lactic acid bacteria (CFU/mL) in fermented milks with increasing levels of passion fruit peel flour during storage.

Treatment (%)	Storage period (Days)				
	1	8	15	22	29
0	8.62x10^6±0.07Aa	9.05 x10^6±0.11Aa	5.05 x10^6±0.10Bb	4.67 x10^6±0.8Ab	0±0Ac
1	7.56 x10^6±0.30Aa	9.30 x10^6±0.12Aa	8.46 x10^6±0.01Aa	1.00 x10^6±0.1Bb	0±0Ab
2	7.26 x10^6±0.20Aa	9.27 x10^6±0.15Aa	8.50 x10^6±0.5Aa	1.00 x10^6±0.1Bb	0±0Ab
3	9.27 x10^6±0.12Aa	9.96 x10^6±0.03Aa	8.41 x10^6±0.6Aa	0±0Bb	0±0Ab

*Capital letter on same column do not differ from each other, same lowercase letters on the same lines do not differ according to Tukey's test at 5% significance level. 0% = no addition of PFPF (0%); Treatment 1%= 1% of PFPF; Treatment 2% = 2% of PFPF; Treatment 3% = 3% of PFPF.

values lower than 10^6 CFU/ml. On the twenty second day of storage 3%, 2% and 1% PFPF drastically decreases while the 0% PFPF concentration remained constant, showing a decrease in CFU/ml only on the twenty-ninth day of storage (Table 4).

The lactic acid bacteria results corroborate those found by Gallina et al. (2011) assessing the viability of lactic acid and probiotic bacteria during shelf-life, which remained within adequate levels (10^6 CFU/ml) up to 15 days. Coman et al. (2012), while working with fermented milk added with different percentages of wheat flour and oat bran in concentrations (control, 2, 4 and 6%) with 2 types of sepas (L. rhamnosus IMC 501®, L paracasei IMC 502®, SYNBIO®) for each treatment; up to 28 days of storage all treatments showed lacticas viable whith levels above 10^6 CFU/ml. Higher values were found by Matta et al. (2012) in symbiotic rice-based drink after 22 days of storage which showed from 10^9 to10^{10} CFU/ml and those reported by Ribeiro et al. (2014) with fermented milk made with *Camellia sinensis* that after 30 days of storage showed 10^7 CFU/ml.

The increase in PFPF levels led to a decrease in syneresis values over a period of twenty nine days of storage, and fermented milk with the highest concentration (3% PFPF) resulted in the lowest syneresis value, while fermented milk with 0% PFPF showed the highest syneresis value,

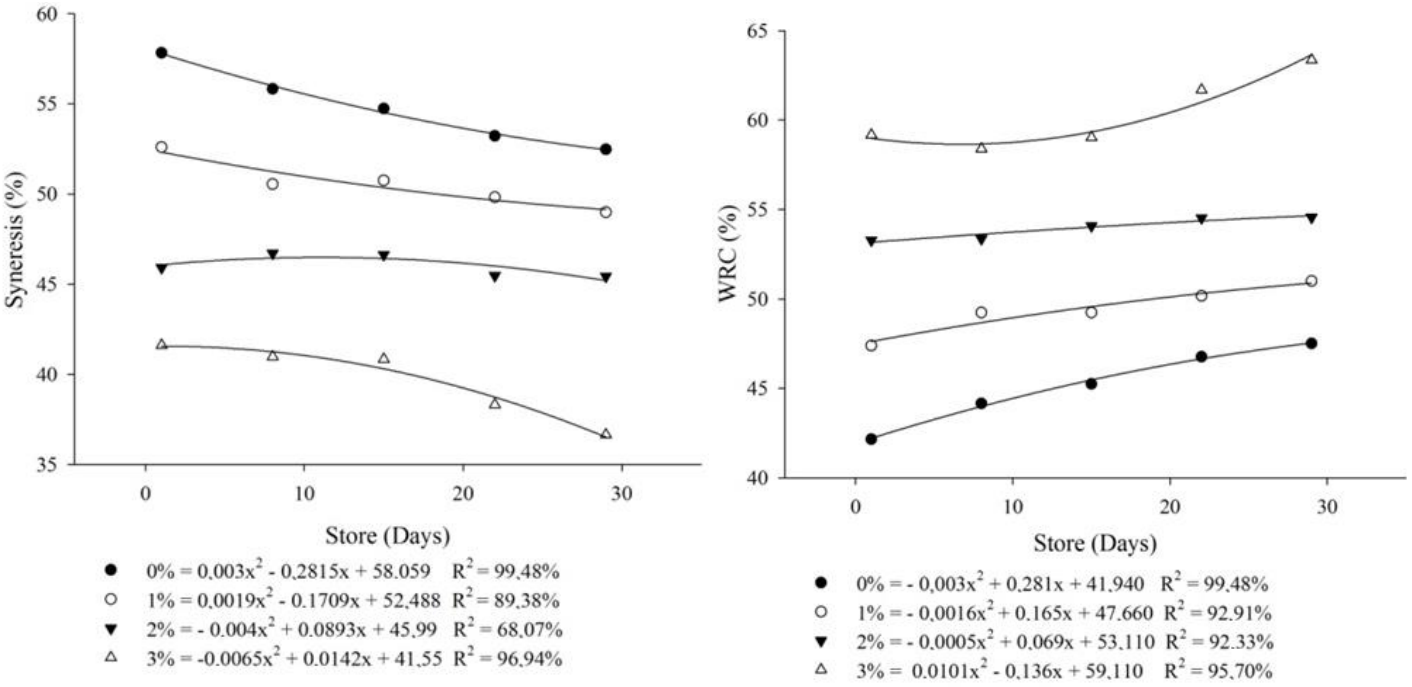

Figure 1. Syneresis and water holding capacity of dairy drinks with increasing levels of passion fruit peel flour during storage.

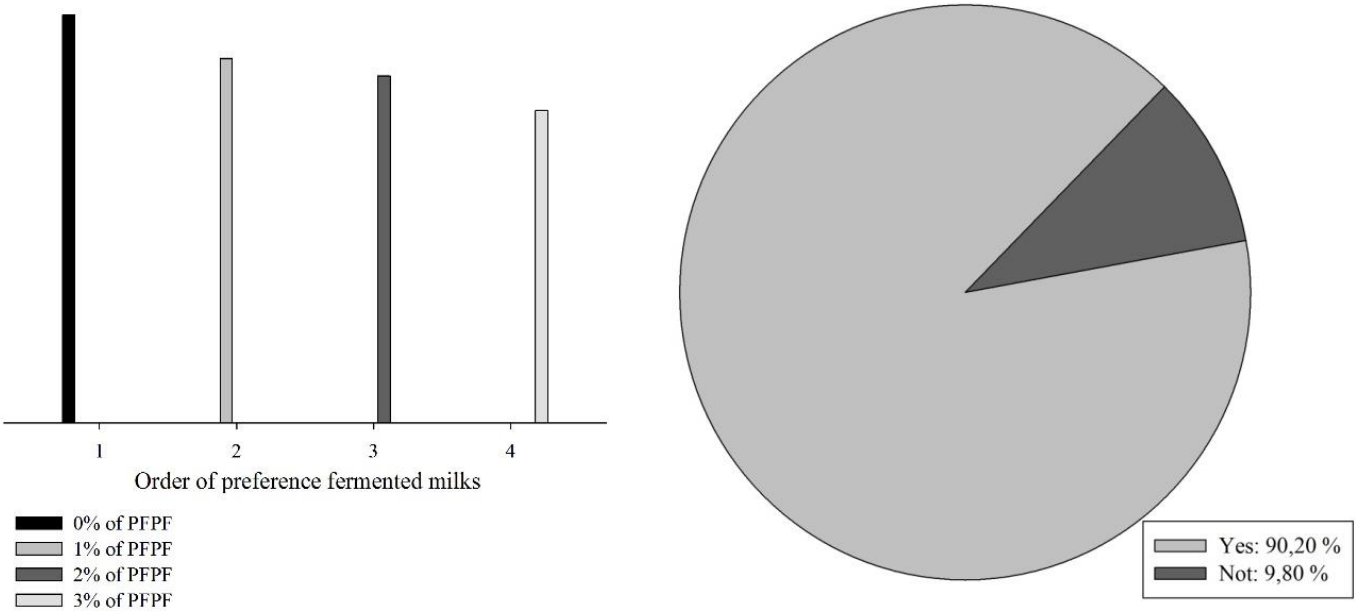

Figure 2. Hedonic scale of preference and purchase intent of fermented milks with increasing levels of passion fruit peel flour.

indicating that there was a better whey release. WHC showed increased values in fermented milk with 3% PFPF and decreased values for 0% PFPF, these results show that syneresis and WHC have an inverse relationship (Figure 1).

Similar results were observed by Toledo et al. (2013) in a study with yogurt added of passion fruit pulp and flour (*Passiflora edulis*), who found that samples with lower PFPF content showed higher whey release and consequently higher syneresis values. The same behavior was observed by Antunes et al. (2007), who studied nonfat probiotic yogurt in combination with starter

Table 5. Mean L * coordinate values of milk drinks with increasing levels of passion fruit peel flour during storage.

Treatment (%)	Storage period (days)				
	1	8	15	22	29
0	89.86±0.02Abc	89.04±0.06Ad	89.42±0.06Ac	90.87±0.04Aa	90.50±0.02Aab
1	72.22±0.50 Bc	72.21±0.19Bc	73.11±0.11Bab	73.45±0.16Ba	72.46±0.17Bbc
2	65.34±0.77Cb	65.80±0.72Cab	66.13±0.37Ca	66.19±0.16Ca	65.36±0.80Cb
3	62.67±1.13 Db	62.67±0.26Db	64.95±0.37Da	62.51±0.14Db	62.38±0.13Db

*Capital letter on same column do not differ from each other, same lowercase letters on the same lines do not differ according to Tukey's test at 5% significance level. 0% = no addition of PFPF (0%); Treatment 1%= 1% of PFPF; Treatment 2% = 2% of PFPF; Treatment 3% = 3% of PFPF.

Table 6. Mean a *coordinate values of milk drinks with increasing levels of passion fruit peel flour during storage.

Treatment (%)	Storage period (Days)				
	1	8	15	22	29
0	1.99±0.04Dc	1.87±0.04Dab	1.99±0.02Da	1.71±0.02Cb	2.06±0.01Da
1	4.40±0.16Cb	4.01±0.04Cb	4.31±0.14Cb	4.33±0.10Cb	4.51±0.14Cb
2	6.39±0.14Ba	5.72±0.19Bb	5.89±0.14Ba	6.26±0.08Ba	6.11±0.25Bb
3	7.01±0.21Aa	6.69±0.13Aa	6.44±0.13Aa	6.93±0.03Aa	7.03±0.04Aa

*Capital letter on same column do not differ from each other, same lowercase letters on the same lines do not differ according to Tukey's test at 5% significance level. 0% = no addition of PFPF (0%); Treatment 1%= 1% of PFPF; Treatment 2% = 2% of PFPF; Treatment 3% = 3% of PFPF.

Table 7. Average b * coordinate values of milk drinks with increasing levels of passion fruit peel flour during storage.

Treatment (%)	Storage period (Days)				
	1	8	15	22	29
0	12.45±0.08Db	11.60±0.06Dc	13.25±0.06Ca	13.34±0.01Da	13.32±0.02Ca
1	19.64±0.29Cbc	19.27±0.12Cc	19.26±0.04Bc	20.04±0.07Cab	20.79±0.16Ba
2	22.93±0.18Bbc	22.26±0.12Bcd	21.81±0.19Ad	23.05±0.06Bb	24.27±0.29Aa
3	24.08±0.43Aa	24.24±0.08Aa	22.49±1.29Ab	24.24±0.04Aa	24.72±0.08Aa

*Capital letter on same column do not differ from each other, same lowercase letters on the same lines do not differ according to Tukey's test at 5% significance level. 0% = no addition of PFPF (0%); Treatment 1%= 1% of PFPF; Treatment 2% = 2% of PFPF; Treatment 3% = 3% of PFPF.

culture and milk whey protein concentrate and found lower syneresis levels and increased whey retention capacity in formulations added of protein concentrate, and by Gerhardt et al. (2013), who prepared 11 treatments of fermented milks varying formulations with ricotta whey and collagen hydrolysate and observed that samples containing less collagen (0.65 and 0.5%) showed higher syneresis values. The results indicate that the addition of solids to fermented milks had an effect on syneresis.

The L* parameter (Table 5) indicates brightness and can determine values between zero (0) and one hundred (100), called black and white, respectively. The inclusion of increasing levels of PFPF in the preparation of fermented milks influence the brightness parameter (L*), and with increasing proportion of flour in treatments, there was a decrease in L*, corroborating results by Toledo et al. (2013) in the characterization of yogurts added of passion fruit pulp and flour (*Passiflora edulis*).

Parameter a* (Table 6) showed significant difference (P<0.05) among treatments, and increasing flour levels led to an increase in a* values. Treatment with 0% was negative (-a*) towards green while treatments with 1, 2 and 3% flour were positive (+ a*) towards red.

Parameter b* (Table 7) showed the addition of increasing levels of PFPF in fermented milks increased the b* chromaticity coordinate values. According to Caldeira et al. (2010), b* values greater than zero go

Table 8. Average acceptance of milk drinks with increasing levels of passion fruit peel flour after 8 days of storage.

Treatment (%)	Aroma	Flavour	Acidity	Viscosity	Appearance	Color
0	7.42±1.31a	7.85±1.41a	7.31±1.28a	5.04±1.50c	7.35±1.30 a	7.22±1.49a
1	5.60±1.93b	5.10±2.22b	5.54±2.04b	5.80±2.40 a	6.08±1.96b	6.22±1.61b
2	4.96±2.02cb	3.86±2.17c	4.58±1.78cb	4.52±2.29b	5.22±2.27cb	5.76±1.78cb
3	4.36±1.99c	3.20±2.21c	4.22±2.16c	4.38±2.51b	4.96±2.15c	5.28±1.99c

*Means with same letters in the column do not differ by the Tukey's test at 5% significance level.

towards yellow and b* values less than zero go towards blue. Yellow coloration is related to the use of milk whey.

The test results are presented in Table 8; four types of fermented milks presented significant differences ($P<0.05$) in the aroma, flavour, acidity, viscosity, appearance and colour, presented notes between ranging from 4.4 to 7.4 (disliked slightly to liked moderately), from 3.20 to 7.85 (disliked moderately to liked moderately), from 4.22 to 7.31 (disliked slightly to liked moderately), from 4.38 to 5.04 (disliked slightly to indifferent), from 4.96 to 7.35 (disliked slightly to liked moderately), from 5.28 to 7.22 (indifferent to liked moderately).

However, fermented milk without addition of PFPF obtained higher score in relation to parameters of fermented milks added of PFPF.

Fermented milks added with PFPF showed sand-like texture; description corroborated by Espirito Santo et al. (2013) when studying probiotic yogurt enriched with passion fruit fibre. According to Gonçalves and Leão (2013), yogurt added with mixed flour containing passion fruit peel and apple bagasse showed acceptability between 5 (not liked nor disliked) and 6 (liked slightly).

Figure 2 shows the preference of consumers regarding the addition of PFPF to fermented milks, in which panelists evaluated from the most preferred to the least preferred fermented milk, and it was observed that among the 50 panelists, the control drink (without PFPF) was the most preferred, followed by fermented milks added of 1% PFPF and 2% PFPF and the least preferred fermented milk was that added of 3% PFPF.

According to Figure 2, it was observed that fermented milks added with passion fruit peel fibre were rejected, and according to Espírito Santo et al. (2013), this rejection is explained by the fact that panelists were unfamiliar with the consumption of yogurt added of fibre, which is corroborated by Ribeiro et al. (2010), when reporting that the Brazilian yogurt market is dominated by yogurt with fruit flavor (about 95% of the market), and colorful and sweet yogurts are preferred by consumers.

However, some sensory properties of fermented milks such as aroma, flavor, acidity, viscosity, appearance and color may have been underestimated by panelists because they are not used to consume fermented milks added of fibre. When the 51 panelists were asked if they would buy the fermented milk they liked the most, 90.2% responded yes and only 9.8% responded they would not buy.

Fermented milks added with passion fruit peel flour were intended for fast freezing in Ultrafreezer (Terroni®) at -80°C and then freeze-dried in lyophilizer equipment (Enterprise II/Terroni®). After lyophilization, samples were degreased by soxhlet method, stored in plastic bags and placed in desiccator with silica gel and then transported to the Laboratory of Electron Microscopy (LabMic) at the Federal University of Goiás. Samples were mounted on stubs and covered with gold plating. At the end of this procedure, stubs were examined in scanning electron microscopy (JSM - 6610 / Jeol®). Figure 3 shows the scanning electron microscopy images of fermented milk drinks added with passion fruit peel flour at 500x magnification

The images at Figure 3 show the microstructure of a protein matrix and with the addition of flour passion fruit peel showed incidence of surface holes, which are called pits (Martins et al., 2009) and are more present the fermented milk added with 3% PFPF (Figure D), with higher porosity compared to control treatment. It was observed that fibres do not show a smooth and homogeneous surface, but rather quite irregular surface covered by recesses and protrusions.

Conclusion

The results allowed concluding that the use of passion fruit peel for the production of flour is an alternative for the reuse of this product, as it is rich in nutrients. Fermented milks produced achieved physical and chemical parameters established by brazilian law, with no contamination by total and thermotolerant microorganisms throughout the 29 days of storage.

The viability of fermented milks showed efficiency up to the fifteenth day of storage; and in relation to acceptance, the results demonstrated that fermented milks added of PFPF reached satisfatory sensory acceptance.

Conflict of interest

Authors declare there is no conflict of interest for this research.

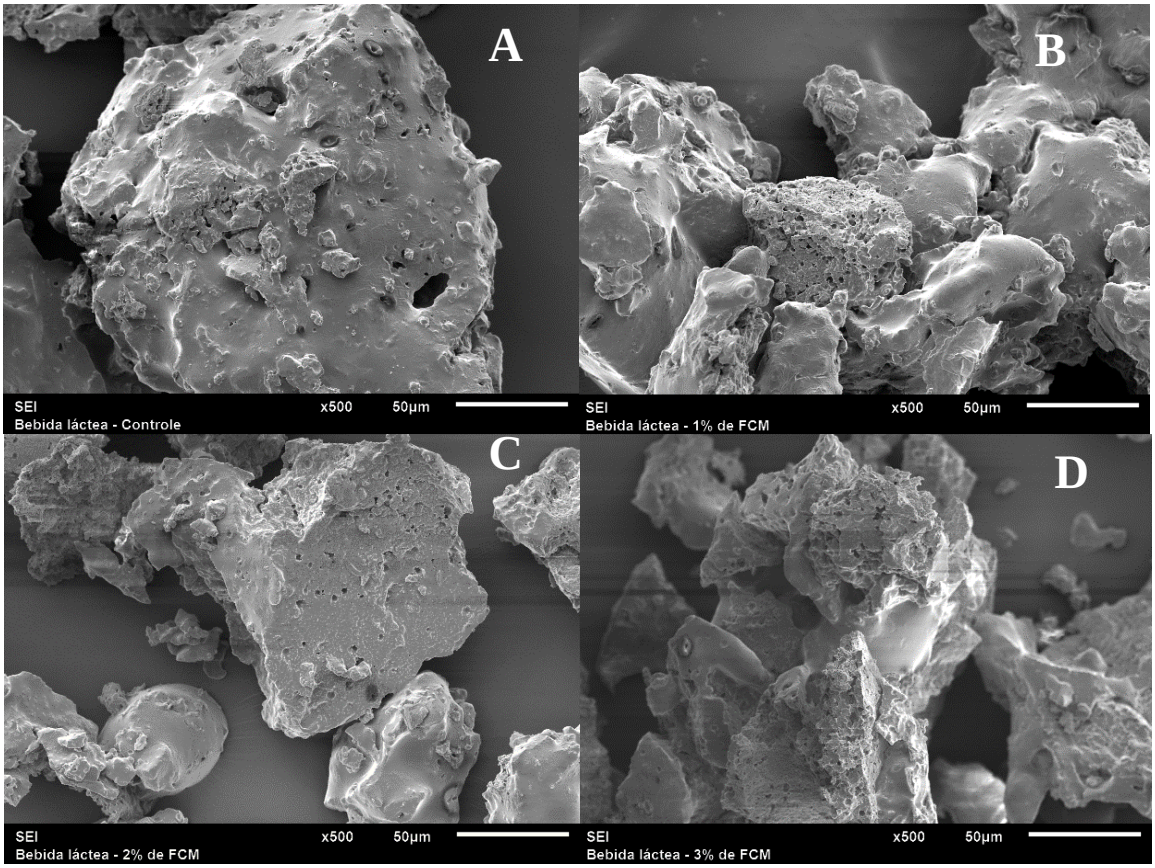

Figure 3. Scanning electron microscopy. (A) fermented milk drink with no addition of PFPF, (B) fermented milk drink with 1% PFPF, (C) fermented milk drink with 2% PFPF, (D) fermented milk drink with 3% PFPF.

REFERENCES

Alencar MLCB, Alvarenga MG (1991). Farelo de arroz (I): composição química e seu potencial como alimento. Arq. Biol. Tecnol. 34: 95-108.

Antunes AE, Marasca ETG, Moreno I, Dourado FM, Rodrigues LG, Lerayer ALS (2007). Desenvolvimento de buttermilk probiótico. Cienc Tecnol Aliment 27:83-90.

Ambrosio - Ugri MCB, Ramos ACH (2012). Elaboração de barra de cereais com substituição parcial de aveia por farinha da casca de maracujá. Tecnol. 21:69-76.

Araújo TF, Ferreira EG, Souza JRM, Bastos LR, Ferreira CLLF (2012). Desenvolvimento de iogurte tipo sundae sabor maracujá feito a partir de leite de cabra. Inst Latic Cândido Tostes 67:001.

AOAC - Association of Official Analytical Chemist (1997). Official methods of analysis of AOAC International. Gaitherburg: AOAC International. 16ed.

Awaisheh SS, Haddadin MSY, Robinson RK (2005). Incorporation of selected nutraceuticals and probiotic bacteria into fermented milk. Int. Dairy J. 15:1184-1190.

Brazil (2006). Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa nº 68, de 12 de Dezembro de 2006. Métodos analíticos oficiais físico-químicos para 0% de leite e produtos lácteos (revoga Instrução Normativa nº 22, de 14 de Abril de 2003). Diário Oficial da União, Brasília, 14 de Dezembro 2006, Seção 1, Página 8.

Caldeira LA, Magnavita APA, Santos TRD, Fernandes SAA, Ferrão SPB (2010). Desenvolvimento de bebida láctea sabor morango utilizando diferentes níveis de iogurte e soro lácteo obtidos com leite de búfala. Cienc Rural, 40:1-6.

Coelho FJO, Quevedo PS, Menin A, Timm CD (2009). Avaliação do Prazo de Validade do Iogurte. Cien. Anim. Bras. 10(4):1155-1160.

Costa AVS, Nicolau ES, Torres MCL, Fernandes PR, Rosa, Sarah IR, Nascimento R (2013). Desenvolvimento e caracterização físico-química, microbiológica e sensorial de bebida láctea fermentada elaborada com diferentes estabilizantes/espessantes. Semina Cienc. Agrar. 34:209-226.

Coman MM, Cecchini C, Verdenelli MC, Silvi S, Orpianesi C, Cresci A (2012). Functional foods as carriers for SYNBIO®, a probiotic bactéria combination. Int. J. Food Microbiol. 157: 346-352.

Espirito-Santo AP, Lagazzo A, Sousa ALOO, Perego P, Convertia A, Oliveira MN (2013). Rheology, spontaneous whey separation, microstructure and sensorial characteristics of probiotic yoghurts enriched with passion fruit fiber. Food Res. Int. 50: 224-231.

FAO - Food and Agriculture Organization of the United Nations (2002). Working group report on drafting guidelines for the evaluation of probiotics in food. Ontario, Canada.

Franco BDGM, Landgraf M (2013). Microbiol alimen. São Paulo: Atheneu, 2003. 182 p.

Gallina DA (2009). Leites fluídos disponíveis no mercado brasileiro: algumas considerações. Ind Latic 14:38-44.

Gallina DA, Silva AAT, Trento FKHS, Carusi J (2011). Caracterização de leites fermentados com e sem adição de probióticos e prebióticos e avaliação da viabilidade de bactérias láticas e probióticas durante a vida-de-prateleira. Cient Cienc Biol Saúde 13:239-244.

Gallina DA, Antunes AEC, Ferreira NCA, Mendonça JB, Norbona RA (2012). Caracterização de bebida obtida a partir de leite fermentado simbiótico adicionado de polpa de goiaba e avaliação de viabilidade das bifidobactérias. Inst Latic Cândido Tostes 67:45-54.

Garmus TT, Bezerra JRMV, Rigo M, Córdova KRV (2009). Elaboração de biscoitos com adição de farinha de casca de batata (*Solanum tuberosum* L.). Bras de Tecnol Agroindustrial 03:56-65.

Gerhardt A, Monteiro BW, Gennari A, Lehn DN, Souza CFV (2013). Physicochemical and sensory characteristics of fermented dairy drink using ricotta cheese whey and hydrolyzed collagen. Inst Latic Cândido Tostes, 68: 41-50.

Gonçalves CR, Leão MF (2013). Produção de iogurte com adição das farinhas mistas a partir dos resíduos de maça, maracujá e uva. Enciclop Biosf 9:3618-3631.

Hunterlab (1998). User's manual with universal software versions 3.5. Reston.

IAL - Instituto Adolfo Lutz (2005). Normas analíticas do Instituto Adolfo Lutz: métodos químicos e físicos para análise de alimentos. São Paulo: IMESP. 4ed.

Janebro DI, Queiroz MSR, Ramos AI, Sabaa-Srur AUO, Cunha MAL, Diniz MFFM (2008). Efeito da farinha da casca do maracujá-amarelo (Passiflora edulisf. flavicarpa Deg.) nos níveis glicêmicos e lipídicos de pacientes diabéticos tipo 2. Bras Farmacogn 18:9.

Lee YK, Nomoto K, Salminen S, Gorbach SL (1999). Handbook of probiotics. New York: John Wiley & Sons Inc.

Macedo REF (1997). Desenvolvimento de bebida láctea fermentada a base de extrato hidrossolúvel de soja e soro de leite de búfala por cultura mista de *Lactobacillus casei* Shirota e *Bifidobacterium adolescentis*. 112f. Dissertação (Mestrado em Tecnologia Química) - Universidade Federal do Paraná, Curitiba.

Martinez R, Torres P, Meneses MA, Figueroa JG, Perez-Alvarez JA, Viuda-Martos M (2012). Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dietary fibre concentrate. Food Chem. 135: 1520-1526.

Martins MA, Mattoso LHC, Pessoa, JDC (2009). Thermogravimetric evaluation of açaí fruit (*Euterpe oleracea* Mart.) agro industry waste. Bras Frutic, 31: 4.

Matta CMB, Jurkiewicz CH, Kunigk L, Roson BM (2012). Influência de prebióticos e de farinha integral de aveia na sobrevivência de bactérias probióticas em bebida simbiótica à base de arroz. Alim. Nutr. 23:55-63.

Parnell-Clunies EM, Kakuda Y, Mullen K, Arnott DR, Deman JM (1986). Physical properties of yogurt: a comparison of vat versus continuous heating systems of milk. J. Dairy Sci. 69: 2593-2603.

Paula JCJ, Almeida FA, Pinto MS, Teodoro VAM, Costa RGB (2012). Use of coalho cheese whey in the pasteurized dairy drink elaboration. Inst Latic Cândido Tostes, 67:13-20.

Penna ALB (2002). Probióticos: uma alternativa para o setor lácteo. In: Congresso brasileiro de ciência e tecnologia de alimentos, 18, Porto Alegre. Anais. SBCTA pp. 4045-4050.

Pereira J (2002). Tecnologia e qualidade de cereais: arroz, trigo, milho e aveia. Lavras: UFLA/FAEPE.

Ribeiro MM, Minim VPR, Minim LA, Arruda AC, Ceresino EB, Carneiro HCF, Cipriano, Paula Aguiar (2010). Estudo de mercado de iogurte da cidade de Belo Horizonte/MG. Ceres 57:151-156.

Ribeiro OAS, Boari CA, Fonseca CM, Figueiredo SP, Neumann D, Abreu LR (2014). Bebida láctea fermentada formulada com *Camellia sinensis*. Bol Centro Pesqui Process Aliment, 32: 289-304.

Riener J, Noci F, Cronin DA, Morgan DJ, Lyng JG (2010). A comparison of selected quality characteristics of yoghurts prepared from thermosonicated and conventionally heated milks. Food Chem. 119:1108-1113.

SEBRAE - Serviço de Apoio às Micro e Pequenas Empresas do Paraná (2005). Oportunidade Sebrae/PR:A Cultura do maracujá. Sebrae 1:5-14.

Tebaldi VMR, Resende JGOS, Ramalho GCA, Oliveira TLC, Abreu LR, Piccoli RH (2007). Avaliação microbiológica de bebidas lácteas fermentadas adquiridas no comércio varejista do sul de Minas Gerais. Cienc Agrotec 31:1085-1088.

Toledo NMV, Ramos PBM, Canniatti-Brazaca SG (2013). Caracterização de iogurtes adicionados de polpa e farinha de maracujá (Passiflora edulis). In: Simpósio sobre Inovação na Indústria de Lácteos, Campinas-SP. ITAL - Simpósio sobre Inovação na Indústria de Lácteos.

Zeraik ML, Pereira CAM, Zuin VG, Yariware JH (2010). Passion fruit: a functional food. Bras Farmacogn 20:459-471.

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Vol. 9(35), pp. 1974-1977, 2 September, 2015
DOI: 10.5897/AJMR2015.7629
Article Number: 103450855541
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Seroprevalence of *Ehrlichia canis* in dogs from Monterrey, Mexico

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Received 17 June, 2015; Accepted 17 August, 2015

Infection by *Ehrlichia canis* in dogs causes the worldwide tick-borne disease called canine monocytic ehrlichiosis (CME), and the presence of *E. canis* has been serologically demonstrated in all continents, with prevalence ranging from 0.2 to 80%. In southern Mexico, a prevalence of 44% was found, whereas in the northwest part of the country it varies from 21 to 49%. In the present study, a commercial kit for the detection of antibodies against *E.* canis was used in 391 dogs from the city of Monterrey, which is located at northeast of Mexico. A total of 54 samples were positive, giving a prevalence of 13%. According to sex, prevalence was 14% for males and 13% for females. Positive animals varied in age from 21 to 132 months old and only 10 of them presented ticks. As in the southern and northwest parts of Mexico, CME is present in northeast region, although with a lower prevalence.

Key words: *Ehrlichia canis*, dogs, serology, Mexico.

INTRODUCTION

The dog can be infected by different species of *Ehrlichia*, and *Ehrlichia canis* is the most important species; it is transmitted by *Rhipicephalus sanguineus* (Pusterla et al., 1998), although the American dog tick, *Dermacentor variabilis*, has also been shown to be a vector transmitter of this disease (Johnson et al., 1998).

E. canis is the primary causal agent of Canine Monocytic Ehrlichiosis (CME), a worldwide tick-borne disease (Kamani et al., 2013; Stich et al., 2008); it is an obligate intracellular gram-negative bacterium that multiply in eukaryotic cells, like monocytes and macrophages, developing leucopenia and thrombocytopenia (Stich et al. 2008).

CME can be divided in an acute phase, beginning from 8 to 20 days after infection, involving anemia, anorexia, ataxia, conjunctivitis, depression, fever, leucopenia, ocular discharge, thrombocytopenia and vomiting that end with a partial recovery of the dog, followed by an months-to-years subclinical phase. The chronic phase can be mild or severe with recurrent clinical and hematologic signs like pancytopenia, hemorrhage, monocytosis, lynphocytosis and weight loss (Stich et al., 2008). It is thought that *E. canis* is the only agent responsible for the development of CME. It has been

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suggested that this bacteria could be the causal agent in the human granulicytic ehrlichiosis and it has even been successfully isolated from human patients with symptoms similar to those caused by other infections by *E. chafeensis* and *E. ewingii* (Perez et al., 2006; Nicholson et al., 2010).

Serologic evidence of previous studies around the world indicated that *E. canis* is present among dogs throughout all continents, where serologic studies have found a prevalence ranging from 30 to 80% in some countries of Africa (Azzag et al., 2015; Inokuma et al., 2006; Eoghain and Raoult, 2004; Ndip et al., 2005; Davoust et al., 2006), whereas in some Asian countries it was of 0.2 to 30% (Inokuma et al., 1999; Rajamanickam et al., 1985; Stich et al., 2008).

In Europe, a prevalence ranging from 2 to 50% have been found (Solano-Gallego et al., 2006; Cocco et al., 2003; Pusterla et al., 1998; Sainz et al., 1995). A study realized in the USA detected most often *Ehrlichia* antibodies in dogs in the Southeast, with 1.3% of samples testing positive, whereas other regions showed lower numbers ranging from 0.3 to 0.6%. (Bowman et al., 2009). Different results were found in Oklahoma, where the prevalence of *E. canis* was 10.8% by serology and 3.1% by the polymerase chain reaction (PCR) method (Murphy et al., 1998). Among dogs belonging to the U.S.A. military forces, seropositivity to *E. canis* ranged from 8% in cold zones (above 45° latitude) to 24% in temperate places (between 40 and 45° latitude); a 13% prevalence was found in tropical zones (below 40° latitude) (Keefe et al., 1982).

Several studies on *E. canis* prevalence have been realized in Brazil. Melo et al. (2011) reported a prevalence of 74.4% in urban and of 67.5% in rural dogs (overall frequency of 70.9%), whereas Witter et al. (2013) informed a seroprevalence of 70.1%; in this last study the frequency of *E. canis* infection was of 23.3% by PCR. On the other hand, also in Brazil a prevalence of *E. canis* of 41.5% by IFA and of 9.4% was found in cats (Braga et al., 2014).

In Grenade, 43.8% of dogs tested were positive for *E. canis* (Yabsley et al., 2008). In Mexico, studies performed in the southern area found 44% of seropositive dogs to *E. canis* with ELISA testing (Rodriguez-Vivaz et al., 2005), 36% prevalence by PCR and 45% in dogs located at animal shelters (Pat-Nah et al., 2015), whereas at the northwest region a prevalence of 49% was found (Tinoco-Gracia et al., 2007). In another study (Haro-Álvarez et al., 2007), a 21.6% prevalence, with 40% of the dog population in contact with E. canis, have been reported.

Although much have been said about the presence of this disease in Mexico, currently there are no reports of it in the northeast region; therefore, the goal of the present study was to estimate the seroprevalence of ehrlichiosis in dogs from the city of Monterrey, located in this part of Mexico.

MATERIALS AND METHODS

Blood samples were obtained from 391 dogs of different breeds in the city of Monterrey, using as inclusion factor only animals with fixed address, age over 6 months. It was decided to sample only one animal per house in case of having more than one dog. The examination of the dogs started with physical evaluation followed by blood sampling. All dogs showed no symptoms of any disease.

This study was carried out during 2014 in the city of Monterrey, Nuevo Leon, located in the northeast of Mexico, with a territorial extension of 451.30 square kilometers. Location coordinates are 25°40´17´´ N, 100° 18´31´´ W. Altitude is 530 m above sea level. The climate of the region has an average of 21°C, but because of annual thermal oscillation of 18°C, with important contrast among seasons. In summertime, temperatures above 30°C are common with an average in July and August of 34°C. In Winter, cold air arrive constantly to the region, often accompanied of humidity from the coast, making the temperature descend drastically, and every year at least two to three days are recorded with 0°C or less. The average annual precipitation is of 600 ml spread mainly in summer, with September as the rainiest month. The city was divided in quadrants in accordance with its cartographic plan. From this map, the 15 most urbanized quadrants were chosen, since the others belonged to non-well developed neighborhoods and little human population. Sampling was performed according to the dog population density and owner cooperation, and only one animal per city block and only one animal per house. To determine the sample size, calculations were made in basis of the population's representative sample (infinite), with precision level of 5%, confidence level of 95% and a power of statistical test of 80% in order to ensure reliability of the results and that they could be translated to the population under study. Sample size was determined using Epidat 3.1. For the *in vitro* diagnosis for detection of antibodies against *E. canis* in the samples, a commercial kit canine SNAP*4Dx (IDEXX labs, Inc. USA) was used. Before starting the procedure, samples must be at room temperature. The sera, either fresh or refrigerated, were utilized after no more than a week from the sampling. Sensitivity and specificity of the kit for the disease are reported with a minimum of 98.8% and 100%, respectively, and detects antibodies generated against peptides from the proteins p30 and p30-1 of *Ehrlichia*. (O'Connor et al., 2004, 2006).

RESULTS AND DISCUSSION

For the present work, 391 blood samples were taken from dogs located in the city of Monterrey, Mexico; antibodies against *E. canis* were found in 54 samples, resulting in a prevalence of 13.8%. Regarding to sex, animal's samples comprised 173 males and 218 females of which 25 males and 29 females were positive, giving a prevalence of 14.5 and 13.3% respectively (Table 1).

Positive animals varied in age from 21 to 132 months old; and according to size, 19 were small, 27 medium and eight large. Only 10 positive animals presented ticks (*Rhipicephalus sanguineus*). The distribution of positive animals by breed is presented in Table 2; the biggest percentage of positive dogs was for mixed-breed.

Comparing the frequencies found in the present work to other studies on the subject can be difficult due to the wide range of prevalence reported according to the continent in which such studies were performed (from 0.2 to 80% in Africa, Asia and Europe), as can be seen in the

Table 1. Distribution of positive animals for *Ehrlichia canis* by sex.

Sex	Number of dogs sampled	Number of positive dogs	%
Female	218	29	13.3
Male	173	25	14.5
Total	391	54	13.8

Table 2. Number of positive animals to *Ehrlichia canis* according to breed.

Breed	Number of positive animals
Basset Hound	1
Boxer	3
Bull Terrier	3
Chihuahua	2
Cocker Spaniel	2
Collie	1
Mixed breed	16
Doberman	1
French poodle	5
Great Dane	2
Maltese	4
Labrador	4
Schnauzer	2
German shepherd	1
Shar Pei	2
Shih tzu	4
Westhighland	1

Introduction section. However, when we compare our work with studies done in the U.S.A., we find that a very similar prevalence (10.8%) was found in Oklahoma by serology, although in this same paper the prevalence was 3.1% by PCR (Murphy et al., 1998). This low prevalence of *E. canis* by PCR in the U.S.A. is confirmed by other work that informed 1.3% in the Southwest and 0.3 to 0.6% in other areas of that country (Bowman et al., 2009). On the other hand, a very large prevalence of *E. canis* in dogs has been informed in both Grenade (Yabsley et al., 2008) and south Mexico (Rodriguez-Vivaz et al., 2005); in the first, the prevalence was of 43.8% and in the second of 44%. Other studies in Mexico concluded that the prevalence of *E. canis* is high, ranging from 40 to 49% in both the northwest and south part of the country (Haro-Álvarez et al., 2007; Pat-Nah et al., 2015; Tinoco-Gracia et al., 2007). These results are in disagreement with the ones presented in our work. Therefore, a wide range of results regarding the prevalence of *E. canis* in dogs exist in the literature. One possible explanation to this disagreement could be the diagnostic method. Work in this subject indicate that the IFA method may be better than ELISA (Jimenez-Coello et al., 2009); using the IFA method these authors found a 8.7% prevalence, which is closer to the results informed in the present work; the previously mentioned work also indicates that the sampling method can also have an influence in the results. We think that both the sampling method and the technique used in the present study give an accurate view of the actual prevalence of *E. canis* infection in dogs located in the northeast region of Mexico; the prevalence we found is close to the ones reported in the U.S.A. and in the work done by Jimenez-Coello et al. (2009) in Mexico, but much lower to the prevalence informed in both the south and northwest areas of Mexico, as well as in other parts of the world as mentioned above.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We wish to thank the Programa Integral para el Fortalecimiento Institucional (PIFI) program of the Universidad Autónoma de Nuevo León for financial support to this work.

REFERENCES

Azzag N, Petit E, Gandoin C, Bouillin B, Ghalmi F, Haddad N, Boulois H-J (2015). Prevalence of selected vector-borne pathogens in stray and client-owned dogs from Algiers. Comp. Immunol. Microbiol. Infect. Dis. 38:1-7.

Bowman D, Susan EL, Lorentzen L, Shields J, Sullivan MP, Carlin EP (2009). Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: Results of a national clinic-based serologic survey. Vet. Parasitol. 160:138-148.

Braga IA, dos Santos LG, de Souza Ramos DG, Melo AL, da Cruz Mestre GL, de Aguiar DM (2014). Detection of Ehrlichia canis in domestic cats in the central-westwrn region of Brazil. Braz. J. Microbiol. 45:641-645.

Cocco R, Sanna G, Cillara MG, Tola S, Ximenes L, Pinnaparpaglia ML, Masala G (2003). Ehrlichiosis and Rickettsiosis in a canine population of Northern Sardinia. Ann. N.Y. Acad. Sci. 990:126-130.

Davoust B, Boni M, Gomez J, Lafay L, Casali F, Leroy E, Parzy D (2006). Surveys on seroprevalence of canine monocytic Ehrlichiosis among dogs living in the Ivory Coast and Gabon and evaluation of a quick commercial test kit dot-ELISA. Ann. N.Y. Acad. Sci. 1078:464-469.

Eoghain GN, Raoult D (2004). Antibodies reactive with *Bartonella henselae* and *Ehrlichia canis* in dogs from the communal lands of

Zimbabwe. J. S. Afr. Vet. Assoc. 75:116-120.
Haro-Alvarez P, Lopez-valencia G, Tinoco-Gracia L, Renteria-Evangelista T, Medina-Basulto G (2007). Seroprevalence and traceback of animals suspected of carrying Ehrlichia canis, in dogs attended in veterinary clinics in Mexicali, Baja California, Mexico. JAVA 6:850-854.
Inokuma H, Ohno K, Yamamoto S (1999). Serosurvey of *Ehrlichia canis* and *Hepatozoon canis* infection in dogs in Yamaguchi Prefecture, Japan. J. Vet. Med. Sci. 61:1153-1155.
Inokuma H, Oyamada M, Davoust BM, Boni M, Dedeure J, Bucheton B, Hammad A, Watanabe M, Itamoto K, Okuda M, Brouqui P (2006). Epidemiological survey of *Ehrlichia canis* and related species infection in dogs in Eastern Sudan. Ann. N.Y. Acad. Sci. 1078: 461-463.
Jimenez-Coello M (2009). Vector Borne Zoonot. Dis. 9:209-211.
Johnson EM, Ewing SA, Barker RW, Fox JC, Kocan KM (1998). Experimental transmission of *Ehrlichia canis* by *Dermacentor variabilis*. Vet. Parasitol. 74: 277-288.
Kamani J, Lee CC, Haruna AM, Ping-Jun, Chung P-J, Weka PR, Chung Y-T (2013). First detection and molecular characterization of *Ehrlichia canis* from dogs in Nigeria. Res. Vet. Sci. 94:27-32.
Keefe TJ, Holland CJ, Salyer PE, Ristic M (1982). Distribution of Ehrlichia canis among military working dogs in the world and selected civilian dogs in the United States. J. Am. Vet. Med. Assoc. 181:236-238.
Melo AL, Martins TF, Horta MC, Moraes-Filho J, Pacheco RC, Labruna MB, Aguiar DM (2011). Seroprevalence and risk factors to Ehrlichia spp. and Rickettsia spp in dogs from the Pantanal Region of mato Grosso State, Brazil. Ticks Tick Borne Dis. 2:213-218.
Murphy GL, Ewing SA, Whitworth LC, Fox JC, Kocan AA (1998). A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. Vet. Parasitol. 79:325-339.
Ndip LM, Ndip RN, Esemu SN, Dickmu VL, Fokam EB, Walker DH, McBride JW (2005). Ehrlichial infection in Cameroonian canines by *Eherlichia canis* and *Ehrlichia ewingii*. Vet. Microbiol. 111: 59-66.
Nicholson WL, Allen KE, McQuiston JH, Breitschwerdt EB, Susan EL. (2010).The increasing recognition of rickettsial pathogens in dogs and people. Trends Parasitol. 26:205-212.
O'Connor TP, Esty KJ, Jancy L, Hanscom JL, Shields P, Philipp MT (2004). Dogs vaccinated with common Lyme disease vaccines do not respond to IR_6, the conserved immunodominant region of the VlsE surface protein of *Borrelia burgdorferi.* Clin. Diagn. Lab. Immunol. 11:458-462.
O'Connor TP, Hanscom JL, Hegarty BC, Groat RG, Breitschwerdt EB (2006). Comparison of an indirect immunofluorescence assay, western blot analysis, and a commercially available ELISA for detection of *Ehrlichia canis* antibodies in canine sera. Am. J. Vet. Res. 67:206-210.
Pat-Nah H, Rodriguez-Vivas RI, Bolio-Gonzalez ME, Villegas-Perez SL, Reyes-Novelo E (2015). Molecular diagnosis of Ehrlichia canis in dogs and ticks Rhipicephalus sanguineus (Acari:Ixodidea) in Yucatan, Mexico. J. Med. Entomol. 52:101-104.
Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y (2006). Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. Ann. N.Y. Acad. Sci. 1078:110–117.
Pusterla N, Pusterla JB, Deplazes P, Wolfensberger C, Muller W, Horauf A, Reusch C, Lutz H (1998). Seroprevalence of *Ehrlichia canis* and granulocytic *Ehrlichia* infection in dogs in Switzerland. J. Clin. Microbiol. 36:3460-3462.
Rajamanickam C, Wiesenhutter E, Zin FM, Hammid J (1985). The incidence of canine haematozoa in Peninsular Malaysia. Vet. Parasitol. 17: 151-157.
Rodriguez-Vivaz, RI, Albornoz REF, Bolio GME (2005). *Ehrlichia canis* in Yucatan, Mexico: seroprevalence, prevalence of infection and associated factors. Vet. Parasitol. 127: 75-79.
Sainz A, Tesouro MA, Rodriguez F, Mayoral I, Mazzucchelli F (1995). Seroprevalence of *Ehrlichia canis* infections in pólice dogs in Spain. Prev. Vet. Med. 23:179-182.
Solano-Gallego L, Trotta M, Razia L, Furlanello T, Caldin M (2006). Molecular survey of *Ehrlichia canis* and *Anaplasma phagocytophilum* from blood of dogs in Italy. Ann. N.Y. Acad. Sci. 1078:515-518.
Stich RW, Shaefer JJ, Bremer WG, Needham GR, Jittapalapong S (2008). Host surveys, ixidid thick biology and transmission scenarios as related to the thick-borne pathogen, *Ehrlichia canis*. Vet. Parasitol. 158:256-273.
Tinoco-Gracia L, Quiroz-Romero H, Quintero-Martinez T, Renteria-Evangelista TB, Barreras-Serrano A, Hori-Oshima S, Lopez-Valencia G, Tamayo-Sosa AR, Quezada-Iniguez VA, Moro-Manuel VJ (2007). Seroprevalence of Ehrlichia canis in dogs from a mexico-U.S. border desert region: Pilot study. JAVA 6:758-760.
Witter R, Nunes Vecchi S, dos Anjos Pacheco T, Tomé Melo AL, Borsa A, Sinkoc AL, Mendonca AJ, Moura Aguiar D (2013). Prevalencia da erliquiose monocitica canina e anaplasose trombocitica em caes suspeitos de hemoparasitose em Cuiaba, Mato Grosso. Semina: Ciencias Agrarias 34:3811-3822.
Yabsley MJ, McKibben J, Macpherson CN, Cattan PF, Cherry NA, Hegarty BC, Breitschwerdt EB, O`Connor T, Ramaswamy R, Paterson T, Perea ML, Ball G, Friesen S, Goedde J, Henderson B, Sylvester W (2008). Prevalence of *Ehrlichia canis*, *Anaplasma platys*, *Babesia canis vogeli*, *Hepatozoon canis*, *Bartonella vinsoni berkhoffii*, and *Rickettsia spp*. in dogs from Grenada. Vet. Parasitol. 151:279-285.

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Vol. 9(35), pp. 1978-1983, 2 September, 2015
DOI: 10.5897/AJMR2015.7588
Article Number: 646437D55547
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Detection of *Mycobacterium bovis* in bovine carcasses by multiplex-PCR

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Received 20 May, 2015; Accepted 20 July, 2015

The causative agent of bovine tuberculosis (BTB) is *Mycobacterium bovis*, a bacterium belonging to the *M. tuberculosis* complex (MTC). The definitive diagnosis is achieved through isolation and identification of *M. bovis* from clinical samples, using a combination of traditional culture and biochemical methods, which is considered the "gold standard". This procedure is cumbersome and time-consuming. We evaluated a *multiplex*-PCR (m-PCR) assay for the direct detection of *M. bovis* DNA from tissue with BTB-suspected lesions. A dairy herd consisting of 270 adult cattle where 34 animals were positive to the tuberculin skin test has been reported. At 30 days after the tuberculin test, all 34 reactive animals were slaughtered and subjected to a necropsy procedure. A pool of tissue samples representative of each animal (lung and mediastinal, scapular and retropharyngeal lymph nodes) were collected and subjected to bacteriological culture and m-PCR. *Mycobacterium* spp. was isolated in 50% (17/34) of the collected samples. When using m-PCR directly from tissue fragments, it was possible to detect *M. bovis* in 67.6% (23/34) of the collected samples including 15 samples isolated by bacteriological culture. High performance liquid chromatography (HPLC) was used to differentiate the 17 isolated strains of *Mycobacterium* spp., from the *Mycobacterium tuberculosis* complex (MTC) or other *Mycobacterium* sp. not belonging to the MTC. The use of m-PCR assays directly from tissue samples may be a valid supplementary tool for the *post mortem* diagnosis of BTB, since this is a a faster and more specific technique than bacterial culturing, reducing the diagnosis time for diagnosis of the disease from three months to two days.

Key words: Bovine tuberculosis, high performance liquid chromatography (HPLC), *Mycobacterium tuberculosis* complex, multiplex polymerase chain reaction (PCR), tissues with macroscopic lesions.

INTRODUCTION

Bovine tuberculosis (BTB) is a major infectious disease among cattle in many countries. Although cattle are the main host and reservoir of this chronic infection, other mammals, including humans, are also susceptible to *Mycobacterium bovis* (Medeiros et al., 2010). Zoonotic TB can also be considered a socio-economic disease, as it causes direct economic losses in the agribusiness and hampers commercial exchange of animals and products (Zumárraga et al., 1999). Many countries around the world perform the control or eradication of BTB by their official control of infectious diseases, based on test-and-slaughter policy. Brazilian policies regarding the control and eradication of BTB include the National Plan for Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT), established in 2001 and reviewed in 2004, which is based on the slaughtering of all reactive animals to the tuberculin test (Brazil, 2006). According to Pollock et al. (2005) new tools, such as additional diagnostic tests, are needed to make a quick diagnosis of the disease and develop vaccines in order to prevent bovine tuberculosis.

There is a lack of official data regarding the current prevalence of BTB in Brazil. Based on official reports, there was a national average prevalence of 1.3% of cows infected from 1989 to 1998 (Brazil, 2006). Since the implementation of the PNCEBT in Brazil, the prevalence of the disease was reported to range from 0.7 to 3.3% (Furlanetto et al., 2012). According to the epidemiology of the disease, there is a higher incidence of BTB in dairy herds when compared to beef herds, due to the difference between the breeding systems of these animals.

BTB is usually diagnosed "*in vivo*", based on delayed hypersensitivity reactions (intradermal tuberculin tests), which may lack high sensitivity and specificity. However, a definitive diagnosis is still established by the isolation and identification of the etiological agent (*M. bovis*) from lymph nodes or lungs, obtained during necropsy or at slaughter, using a combination of traditional culture and biochemical methods, which is considered the "gold standard method". These methods are laborious, unreliable and time-consuming; it may take more than 90 days to grow the microorganism, and an additional 2 weeks for biochemical identification (OIE, 2009). Several alternative approaches have been attempted for the rapid and specific diagnosis of BTB, but molecular methods, especially the polymerase chain reaction (PCR) assay, are the most promising (Carvalho et al., 2015).

BTB lesions in cattle are most often found in organs rich in reticuloendothelial tissue, particularly the lungs and associated lymph nodes (Corner et al., 1990). Other studies conducted on naturally infected cattle experimentally infected with *M. bovis*, demonstrated that lesions are most commonly present in the lower respiratory tract, however the upper respiratory tract and its associated tissues also displays disease in many cases (Neill et al., 1994; Rodgers et al., 2007). Although tubercles are not pathognomonic of BTB, identifying *M. bovis* or its DNA confirms the disease.

PCR has been successfully applied by our group and other researchers in the detection of members from the *M. tuberculosis* complex (MTC), and DNA amplification of specific sequences is especially useful for this (Cardoso et al., 2009; 2015). However, the success of the PCR assay depends on the availability of intact and impurity-free DNA. Thus the presence of contaminants can interfere with the PCR technique, becoming an obstacle for its implementation (Cardoso et al., 2009). Vitale et al. (1998) showed that the QIAamp Blood and Tissue Kit (Qiagen®) was able to circumvent these problems, supplying DNA templates suitable to be amplified by PCR in most biological samples. We adopted this procedure to evaluate the efficiency of an m-PCR targeting for the *RvD1Rv2031c* and *IS6110* sequences, specific for *M. bovis* and MTC, respectively, to identify *M. bovis* DNA from tissues of slaughtered, skin-test positive, animals. The results were compared with those obtained from the skin test and conventional culture for *M. bovis*.

MATERIALS AND METHODS

Study design

This study was conducted on a dairy herd comprised of 270 adult crossbred Holstein and Gir cows, located in Macaé city, Rio de Janeiro State, in Southeastearn of Brazil. Prior to the study, 34 adult cows had positive reactions to a single intradermal tuberculin test (SITT) and were kept in quarantine for 90 days, waiting for confirmatory tests to be conducted, in order to avoid bacillus transmission. After 90 days, a comparative intradermal tuberculin test (CITT) was performed in these same 34 cows (Group A - reagents), plus 16 randomly selected cows that were negative to the first SITT test (Group B - control), totaling 50 animals. After 30 days of the PPD injection, all CITT-reactive cattle (Group A) were slaughtered and subjected to a necropsy procedure (OIE, 2009). Mediastinal, scapular and retropharyngeal lymph nodes, as well as lung samples of lungs, independently of the macroscopic tuberculous lesions, were collected and analyzed by bacteriological culturing and PCR.

Intradermal tuberculin test

Intradermal tuberculin tests (both SITT and CITT) for BTB diagnosis were performed on all 50 cows, in accordance with the regulations of the Ministry of Agriculture, Livestock and Supply (Brasil, 2006). For the SITT, 0.1 mL of bovine PPD (bovPPD–*M. bovis* strain AN5,

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1 mg protein/mL; Instituto Biológico, São Paulo - SP, Brazil) was inoculated in the cervical area of each cow. After 72 h, the innoculation site was measured with a caliper, and the cow was considered reactive if a swelling >4.0 mm occurred at the injection site. The CITT consisted of the same procedure, plus an inoculation of 0.1 mL avium PPD (*M. avium* strain D4, 0.5 mg protein/mL; Instituto Biológico) in the cervical area, approximately 20 cm from the bovPPD inoculation. Cattle were considered reactive if the difference between the thicknesses of both innoculation site were>4.0 mm.

Isolate culturing and identification

All CITT-reactive cows were killed 30 days after the PPD injection, and a thorough necropsy was conducted. Mediastinal, scapular and retropharyngeal lymph nodes, as well as lung samples, independent of macroscopic tuberculous lesions, were collected. A total of four tissue fragments were collected per animal. A pooled samples from each animal was packed in the same package and taken to the laboratory frozen. Prior to bacteriological analysis, the tissue samples were decontaminated by three different treatments: hexadecylpyridinium (HPC) to 0.75%, sulfuric acid (H2SO4) at 6% and NaOH at 4% according to standard methods (OIE, 2009) and inoculated on two slopes of solid, egg-based Lowenstein-Jensen (LJ) media with 0.5% pyruvate, and two slopes of Stonebrink media, which were incubated at 37°C and observed once weekly for 12 weeks.

DNA templates were extracted from colonies by suspension in 200 µl of distilled water for 10 min at 100°C. The isolated microorganisms were confirmed by m-PCR (Figueiredo et al., 2009) and identified by HPLC.

The HPLC was performed according to Furlanetto et al. (2014). A suspension of acid-fast bacteria grown in LJ medium was collected by a swab and saponified with 2 ml KOH 25% in methanol:H_2O (v:v) autoclaved for 1 h at 121°C, 15 psi, to cleave the mycolic acids bound to the cell wall. Mycolic acids were then separated by acidification with HCl:H_2O (v:v) and extraction into chloroform. After conversion to ultraviolet (UV)-absorbing *p*-bromophenacyl esters (Pircen®) and clarification with HCl:H_2O:metanol (1:1:2, v:v:v), the mycolic acids were analyzed on a reverse-phase C18 100 x 4.6 mm column (Kromasil®) using high performance liquid chromatography. A methanol and dichloromethane (methylene chloride) gradient generated by microprocessor-controlled pumps was used to separate the mycolic acid esters, which were detected with a UV detector at 260 nm. Reproducible chromatographic patterns containing combinations of different diagnostic peaks were obtained by using reference strains (*M. abscessus* ATCC 19977, *M. africanum* ATCC 25420, *M. agri* ATCC 27406, *M. aichiense* ATCC 27280, *M. asiaticum* ATCC 25276, *M. aurum* ATCC 23366, *M. avium* ATCC 25291, *M. bovis* ATCC 19210, *M. bovis* BCG INCQS 00062, *M. chelonae* ATCC 35752, *M. flavescens* ATCC 14474, *M. fortuitum* ATCC 6841, *M. gastri* ATCC 15754, *M. godornae* ATCC 141470, *M. intracellulare* ATCC 13950, *M. malmoense* ATCC 29571, *M. mucogenicum* ATCC 49650, *M. scrofulaceum* ATCC 19981, *M. simiae* ATCC 25275, *M. terrae* ATCC 15755, *M. tuberculosis* ATCC 25177, *M. vaccae* ATCC15483, *M. triviale* ATCC23292). The chromatographic pattern for each strain was examined for differences in the heights for pairs of peaks. HPLC patterns were grouped according to species, and the values calculated for each ratio were combined, sorted in numerical order, and examined regarding their ability to discriminate species, using the range of the relative standard deviation (RSD) of the absolute retention times (ART) and the relative retention times (RRT).

DNA preparation from tissues of CITT-reactive cows

DNA was extracted from the pooled samples (lymph nodes and lung), in order to obtain a representative aliquot of each animal, based on a a modification of a QIAamp Blood and Tissue Kit (Qiagen®) already described by Furlanetto et al. (2012). A small piece of tissue (approximately 1 g) was macerated and diluted and a aliquot of the 1 mL was taken. The pellet was suspended in 180 µl of lysis buffer (20 mg/mL lysozyme in 20 mM Tris-HCl, pH 8.0; 2 mM EDTA and 1.2% Triton) and incubated for 1 h at 37°C. After this step, DNA extraction followed the manufacturer's recommendations. DNA was quantified in a Nanodrop ND1000 (Thermo Scientific, USA).

m-PCR assay

The m-PCR was performed according to Figueiredo et al. (2009). The reaction mix (50 µL) contained 5 µl of 10× PCR buffer (Invitrogen®), 200 µM dNTP (GE Healthcare®), 2.5 U of recombinant *Taq* polymerase (Invitrogen®), 2.0 mM $MgCl_2$, 50 ng of each DNA template, and 0.2 µM of each primer JB21 (5´-TCGTCCGCTGATGCAAGTGC-3´) and JB22 (5´-CGTGAACGTAGTCGCCTGC-3´), targeting the *RvD1-Rv2031c* sequence, specific for *M. bovis* and INS1 (5'-CGTGAGGGCATCGAGGTGGC-3') and INS2 (5'-GCGTAGGCGTCGGTGACAAA-3'), targeting the *IS6110* region, specific for MTC. Amplification was carried out in a GeneAmp PCR System 9600 (Applied Biosystems®) with the following cycling parameters: 94ºC for 5 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 68ºC and 1 min at 72ºC, with a final extension at 72ºC for 7 min. PCR products were checked by electrophoresis on 1.5% agarose gels stained with ethidium bromide (10 µg/mL).

Clinical samples were considered positive when double bands of 500 (*M. bovis*) and 245 bp (MTC) DNA were observed. DNA templates from reference strains *M. avium* (ATCC 13950), *M. fortuitum* (ATCC 6841), *M. terrae* (ATCC 15755), *M. vacae* (ATCC 15483), *M. xenopi* (ATCC 33501), *M. flavescens* (ATCC 14474) and *M. scrofulaceum* (ATCC 19981) were used as negative controls to control cross-contamination and confirm the specificity of the m-PCR assays.

RESULTS AND DISCUSSION

From the 34 cows considered CITT-reactive, only nine animals presented macroscopic lesions compatible with granuloma in the lungs, were considered suggestive lesions. However, tissues from all 34 animals were collected, pooled and submitted to the culture and m-PCR assays. Seventeen (50%) of the samples were culture positive for *Mycobacterium* sp, where the presence of *M. bovis* was confirmed in 15/17 (88.2%) isolates, by m-PCR assays (Figueiredo et al., 2009) and HPLC analyses (Figure 1B). The others two isolated mycobacterium (m-PCR negative assay) were identified as *M. fortuitum* by HPLC analysis (Figure 1D). The totality of the remaining samples, 17, failed to grow in culture.

Decontamination with 0.75% HPC yielded *M. bovis* recovery from 10 samples, whereas 4% sodium hydroxide or 6% sulphuric acid yielded only recovered, *M. bovis* from six and five samples, respectively. The proportion of positive samples was higher for HPC than for each of the other two methods. When using both 0.75% HPC and 6% sulphuric acid methods for decontamination, it was possible to identify 13 of 15 (86, 6%) infected cows.

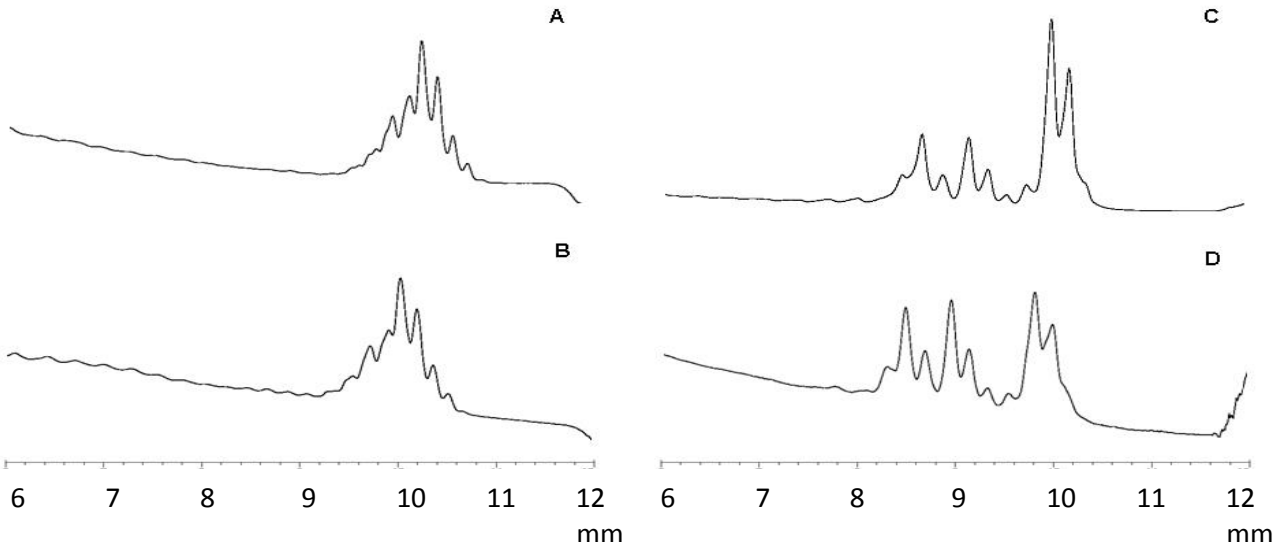

Figure 1. Representative reverse-phase HPLC chromatograms of mycolic acid methylesters from reference strains and isolated microorganisms. (A) Chromatogram from *M. bovis* ATCC 19210 (representative of MTC); (B) representative chromatogram of 15 *Mycobacterium bovis* (CITT-reactive and m-PCR positive), (C) chromatogram from *M. fortuitum* ATCC 6841; (D) representative chromatogram from two isolated *Mycobacterium fortuitum* (CITT-reactive and m-PCR negative).

It was possible to identify all isolates (17) by HPLC, while the m-PCR technique identified only *M. bovis* (15). HPLC was more efficient than m-PCR adopted here because the mycolic acids from the cell wall generate characteristic chromatograms of each species or group. On the other hand, this is a technique that requires more expensive equipment and expertise for deployment as a BTB routine testing (Figure 1).

Multiplex PCR tests of tissue samples from CITT-reactive cows were able to amplify the target DNA in 23/34 (67.6%) of the assayed samples (Figure 2). *M. bovis* by m-PCR assays were identified in 10 samples where no culture growth was observed, which means that 59% of negative-culturing samples came from infected cows.

PCR assays have been successfully applied to detect MTC and *M. bovis* from clinical cattle samples (Cardoso et al., 2009; Figueiredo et al., 2009). In the present study, the PCR test was sensitive enough to detect *M. bovis* in a large proportion (59%) of the samples that failed to grow in culture. This was also emphasized by Liébana et al. (1995) and Zanini et al. (2001). For Miller et al. (2002) and Araujo et al. (2005), the efficiency of the culture method used as a first criterion for *M. bovis* identification is low because of the small number and live bacilli presence in some tissues, because of a short delay in delivering the tissues to the laboratory or because of the sensitivity of the mycobacteria to sodium hydroxide used in the Petroff method.

For the remaining 11 CITT-reactive cows, where both culturing and m-PCR assays failed to identify *M.bovis*, it is possible that there was an inhibitory effect during the PCR assay (Al-Soud and Radstrom, 2001; Cardoso et al., 2009). Some authors (Zanini et al., 2001; Cardoso et al., 2009) also observed less than 100% sensitivity. PCR assays are not able to detect samples that contain a small numbers of pathogens, mainly in paucibacillary tissue samples. The 11 samples from CITT-reactive cows, not confirmed by culturing and m-PCR tests, probably presenting paucibacillary lesions (low amount of *M. bovis* bacillus), fit the characteristics of a recent intra-herd infection. It is generally accepted that the CITT is related to *M. bovis* infections and not necessarily to disease (Neill et al., 1994).

Conclusions

Our results indicate that m-PCR is able to detect *M. bovis* DNA directly in tissue samples and represents a valid additional tool for the post mortem diagnosis of BTB. Multiplex PCR assay is faster and more specific than culture-based diagnosis in *M. bovis* detection and can reduce the diagnosis time from 90 days to approximately two days. Moreover, the m-PCR test is useful when the bacilli are non-viable and cannot be detected by culture methods, being a valuable aid during the sanitary inspection of slaughterhouses for the condemnation of carcasses that show suspected lesions of the bovine tuberculosis.

Conflict of interests

The authors did not declare any conflict of interest.

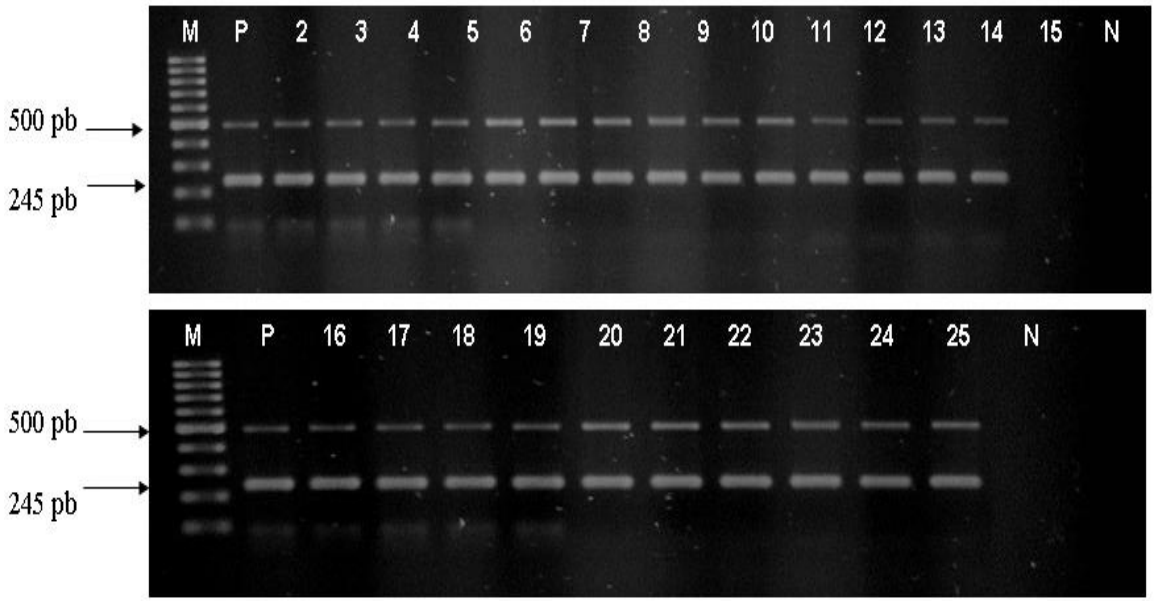

Figure 2. Direct detection of *M. bovis* DNA in tissue samples from CITT-reactive animals. DNA templates obtained from tissue samples were tested by m-PCR. Amplicons were resolved on 1.5% agarose gels stained with ethidium bromide; lane M: 100 bp DNA ladder (Promega®); lane P, positive control *M. bovis* IP; lane N, negative control (without mycobacterial DNA); lane 2-25, 500 bp (*M. bovis*) and 245 bp (MTC) amplified fragments from tissue samples from CITT-reactive cattle; lane 15, negative control with DNA pool of *M. avium* (ATCC 13950), *M. fortuitum* (ATCC 6841), *M. terrae* (ATCC 15755), *M. vacae* (ATCC 15483), *M. xenopi* (ATCC 33501), *M. flavescens* (ATCC 14474) and *M. scrofulaceum* (ATCC 19981). Three independent PCR tests were performed for each cow, using the same template DNA.

ACKOWLEDGEMENTS

The authors would like to acknowledge the financial support from FAPERJ, FAPEMAT, CAPES and CNPq.

REFERENCES

Al-Soud WA, Radstrom P (2001). Purification and characterization of PCR-inhibitory components in blood cells. J. Clin. Microbiol. 39(2): 485-493.

Araujo CP, Leite CQ, Prince KA, Jorge Kdos S, Osorio AL (2005). *Mycobacterium bovis* identification by a molecular method from post-mortem inspected cattle obtained in abattoirs of Mato Grosso do Sul, Brazil. Mem. Inst. Oswaldo Cruz. 100(7):749-752.

Brazil (2006). Ministry of Agriculture, Livestock and Supply. National Plan for Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT),. Brasília: MAPA/SDA/DAS, 188p. (Available from http://www.agricultura.gov.br/arq_editor/file/Animal/programa%20nacional%20sanidade%20brucelose/Manual%20do%20PNCEBT%20-%20Original.pdf).

Cardoso MA, Cardoso RF, Hirata RD, Hirata MH, Leite CQ, Santos AC, Siqueira VL, Okano W, Rocha NS, Lonardoni MV (2009). Direct detection of *Mycobacterium bovis* in bovine lymph nodes by PCR. Zoonoses Public Health 56(8): 465-470.

Carvalho RCT, Furlanetto LV, Maruyama FH, Araujo CP, Barros SL, Ramos CA, Dutra V, Araujo FR, Paschoalin VMF, Nakazato L, Figueiredo EES (2015). Evaluation of the efficiency of nested q-PCR in the detection of *Mycobacterium tuberculosis* complex directly from tuberculosis-suspected lesions in post-mortem macroscopic inspections of bovine carcasses slaughtered in the state of Mato Grosso, Brazil. Meat. Sci. 106:11-15.

Corner L, Melville L, McCubbin K, Small KJ, McCormick BS, Wood PR, Rothel JS (1990). Efficiency of inspection procedures for the detection of tuberculous lesions in cattle. Aust. Vet. J. 67(11): 389-392.

Figueiredo EES, Silva FGS, Campos WN, Furlanetto LV, Medeiros L, Lilenbaum W, Fonseca LS, Silva JT, Paschoalin VMF (2009). Identification of *Mycobacterium bovis* Isolates by a multiplex PCR. Braz. J. Microbiol. 40(2): 231-233.

Furlanetto LV, Conte-Jr CA, Figueiredo EES, Duarte RS, Lilenbaum W, Silva JT, Paschoalin VM F (2014). HPLC Protocol for Identification of *Mycobacterium* spp. from Clinical Samples of Human and Veterinary. J. Microbiol. Res. 4(6):193-200.

Furlanetto LV, Figueiredo EES, Conte-Jrúnior CA, Silva FGS, Duarte RS, Silva JT, Lilenbaum W, Paschoalin VMF (2012). Prevalence of bovine tuberculosis in herds and animals slaughtered in 2009 in the State of Mato Grosso, Brazil. Arq. Bras. Med. Vet. Zootec. 64(2): 274-280.

Liébana E, Aranaz A, Mateos A, Vilafranca M, Gomez-Mampaso E, Tercero JC, Alemany J, Suarez G, Domingo M, Dominguez L (1995). Simple and rapid detection of *Mycobacterium tuberculosis* complex organisms in bovine tissue samples by PCR. J. Clin. Microbiol. 33(1):33-36.

Medeiros LS, Marassi CD, Figueiredo EE, Lilenbaum W (2010). Potential application of new diagnostic methods for controlling bovine tuberculosis in Brazil. Braz. J. Microbiol. 41(3): 531-541.

Miller JM, Jenny AL, Payeur JB (2002). Polymerase chain reaction detection of *Mycobacterium tuberculosis* complex and *Mycobacterium avium* organisms in formalin-fixed tissues from culture-negative ruminants. Vet. Microbiol. 87(1): 15-23.

Neill SD, Cassidy J, Hanna J, Mackie DP, Pollock JM, Clements A, Walton E, Bryson DG (1994). Detection of *Mycobacterium bovis* infection in skin test-negative cattle with an assay for bovine interferon-gamma. Vet. Rec. 135(6): 134-135.

OIE (2009). World Organisation for Animal Health - Bovine Tuberculosis.

(Available from http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.04.07_BOVINE_TB.pdf

Pollock JM, Welsh MD, McNair J (2005). Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. Vet. Immunol. Immunopathol. 108(1-2):37-43.

Rodgers JD, Connery NL, McNair J, Welsh MD, Skuce RA, Bryson DG, McMurray DN, Pollock JM (2007). Experimental exposure of cattle to a precise aerosolised challenge of *Mycobacterium bovis*: a novel model to study bovine tuberculosis. Tuberculosis (Edinb). 87(5):405-414.

Vitale F, Capra G, Maxia L, Reale S, Vesco G, Caracappa S (1998). Detection of *Mycobacterium tuberculosis* Complex in Cattle by PCR Using Milk, Lymph Node Aspirates, and Nasal Swabs. J. Clin. Microbiol. 36(4): 1050-1055.

Zanini MS, Moreira EC, Lopes MT, Oliveira RS, Leao SC, Fioravanti RL, Roxo E, Zumarraga M, Romano MI, Cataldi A, Salas CE (2001). *Mycobacterium bovis*: polymerase chain reaction identification in bovine lymphonode biopsies and genotyping in isolates from Southeast Brazil by spolygotyping and restriction fragment length polymorphism. Mem. Inst. Oswaldo Cruz. 96(6):809-813.

Zumarraga MJ, Martin C, Samper S, Alito A, Latini O, Bigi F, Roxo E, Cicuta ME, Errico F, Ramos MC, Cataldi A, Van Soolingen D, Romano MI (1999). Usefulness of spoligotyping in molecular epidemiology of *Mycobacterium bovis*-related infections in South America. J. Clin. Microbiol. 37(2):296-303.

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Vol. 9(35), pp. 1984-1988, 2 September, 2015
DOI: 10.5897/AJMR2015.7652
Article Number: 2E2DBD955552
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antioxidant and *in-vitro* anthelminthic potentials of methanol extracts of barks and leaves of *Voacanga africana* and *Rauwolfia vomitoria*

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Received 30 June, 2015; Accepted 17 August, 2015

***Voacanga africana* (Stapf) and *Rauwolfia vomitoria* (Afzel) (Apocynaceae) are traditional plants widely used in folkloric medicine. Methanol extracts of *V. africana* bark (VAB) and leaves (VAL), and *R. vomitoria* bark (RVB) and leaves (RVL) were evaluated for antioxidant and anthelmintic potentials. The antioxidant properties of the extracts were determined by the DPPH free radical scavenging method using ascorbic acid as reference antioxidant. The IC_{50} values were then determined. Four concentrations (20, 30, 40 and 50 mg/mL) of extracts were evaluated for *in-vitro* anthelmintic activity by determining the effects of the extracts on the paralytic and death time of *Pheretima posthuma* using albendazole (ABZ) (10 mg/mL) as reference standard. Results reveal that, all the extracts exhibited some level of antioxidant activity with IC_{50} values of 187, 43, 610 and 967 µg/mL for VAL, RVB, VAB and RVL, respectively. VAB and RVB demonstrated significant anthelmintic activity. RVB at a concentration of 50 mg/mL had a paralytic time of 11.17 ± 0.088 min ($p < 0.001$) with reference to ABZ. It also demonstrated a concentration dependent reduction in death time of the worms at all concentrations tested. VAB demonstrated a concentration dependent effect on the worms with decreasing paralytic and death times upon an increase in extract concentration. It also showed significant paralytic and death times ($p < 0.001$) at concentrations of 30, 40 and 50 mg/mL with reference to albendazole.**

Key words: Paralytic time, free radical, death time, anthelmintic activity, *Pheretima posthuma, Voacanga africana, Rauwolfia vomitoria.*

INTRODUCTION

Medicinal plants have been used for decades in the management of various ailments in ethno-medicine.

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Research has it that about 80% of the world's population especially in developing countries use plant materials as their source of primary health care (Farnsworth et al., 1985). Despite all the advancement in medical science, mankind still depends on medicinal plants as remedies to a number of ailments.

Anthelmintic resistance is now a serious problem particularly among farm animals and complete de-worming of farm animals is currently very difficult (Prichard, 1994). High levels of drug resistance in human helminth infections such as soil-transmitted helminths (STH), (*Ascaris lumbricoides*, hookworms (*Necator americanus* and *Ancylostoma duodenale*) and *Trichuris trichiura)* have resulted from periodic mass administration of anthelmintic drugs to school age children and other at-risk groups (Vercruysse et al., 2011). Thus in the face of drug resistance it is imperative that new molecules be sought to curb the menace.

Many medicinal plants have been used in the management of helminth infections and these include: *Carica papaya* (Levecke et al., 2014), *Annona senegalensis*, (Alawa et al., 2003) and essential oil of *Ocimum gratissimum* (Linn.) and eugenol, (Pessoa et al., 2002).

The seeds of *Voacanga africana,* (Stapf), are known to contain up to 10% indole alkaloids including voacamine and voacangine as well as many related compounds. Similar alkaloids are also found in the bark but in limited quantities (Bisset, 1985). Studies conducted have revealed that *V. africana* is a plant with a reservoir of alkaloids from which numerous alkaloid-based chemical compounds can be synthesized. Ibogaine, an alkaloid from *V. africana* has demonstrated numerous CNS effects (Kombian et al., 1997). *V. africana* has also been extensively studied for its alkaloids as well as its CNS and gastro-protective effects.

Rauwolfia vomitoria, (Afzel), (Apocynaceae) is a plant with numerous therapeutic uses (Irvine, 1961). Also known as serpent wood, (Kutalek and Prinz, 2007), the plant is traditionally used as treatment for snake bites, fever and nervous disorders (Kutalek and Prinz, 2007). The root, according to Prajapati et al. (2003) is a good anthelmintic and an antidote to snake venom. The root extracts are also known to possess good antioxidant effects (Okolie et al., 2011). Methanol extracts of the bark has demonstrated anti-ulcer activity in different models (Tan et al., 2000).

It is in this view that the leaves and barks of *V. africana* and *R. vomitoria* were evaluated to determine their effects on helminths and also explore their antioxidant potentials.

MATERIALS AND METHODS

Collection and preparation of plant material

Leaves and bark of *R. vomitoria* and *V. africana* were obtained from the forecourt of the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST. They were authenticated by Dr. G. H. Sam, a Botanist in charge of the physique garden and herbarium of the Department of Herbal Medicine, KNUST, where voucher specimens (KNUST/HM/2015L029 and KNUST/HM/2015L030 respectively) have been kept. The samples were thoroughly washed under running water to get rid of debris. The barks were sun dried for 5 days, whiles the leaves were dried at room temperature (25 to 28ºC) for 5 days. The dried leaves and barks of *V. africana* and *R. vomitoria* were milled into powder using a laboratory mill machine (Type 8, Christy & Norris, UK). The powdered plant materials (50 g each) were each extracted by cold maceration for 72 husing methanol (70%v/v) and concentrated under reduced pressure using rotary vapour (Buchi, Germany). They were finally evaporated to dryness at 40°C in a hot-air oven and the weights of the extracts obtained recorded. The extracts were stored in a desiccator until needed. All chemicals used in the study were obtained from BDH, England, unless otherwise stated.

Experimental organism

Adult Indian earthworms (*Pheretima posthuma;* class Annelida; subclass Megascolecidae) which have anatomical and physiological resemblance to human intestinal roundworms (Vidyarthi, 1967), were collected from the soil close to the Wiwi River in the Botanical Garden of KNUST. The earthworms were washed with 0.9% saline solution to remove all debris.

In-vitro anthelminthic bio-assay

An *in-vitro* anthelmintic bio-assay was performed according to the method described by Bhawar et al. (2009). *P. posthuma* samples, 4.0 to 5.0 cm in length and 0.10 to 0.20 cm in width were used. Extract solutions of concentrations of 20, 30, 40 and 50 mg/mL were prepared using a mixture of DMSO and distilled water in the ratio 2:8. Albendazole at a concentration of 10 mg/mL was used as the reference standard. A solution of 0.9% saline was used as the negative control.

Experimental procedure

The earthworms were placed in Petri dishes (five worms per Petri dish) into which the various extract solutions and reference standard were added. Observations were made for the times taken for the various extracts to cause paralysis and death of the individual worms. Paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Death was noted when the worms lost motility followed by a fading away of their body colour.

Determination of antioxidant activity

The antioxidant activity of the extracts were determined according to the method described in a previous study by Agyare et al. (2015) using the free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) (Sigma- Aldrich, Damstadt, Germany). Solutions of the extracts and standard antioxidant (ascorbic acid) (Sigma-Aldrich, Damstadt, Germany) of concentrations 1.0, 3.0, 10.0, 30.0, 100.0, 300.0 and 1000.0 µg/mL were prepared in methanol. DPPH solution of concentration 5.0×10^{-6} M was prepared in methanol in a dark room. Three millilitres of this solution was added to 1.0 mL of the methanol test extracts and standard antioxidant. The tubes were kept in the dark for 30 min after which absorbance (A_1) of excess DPPH in the extracts and standard solutions were measured at a wavelength of 517 nm using a UV spectrophotometer. The absorbance (A_o) reading for a blank solution containing equivalent volumes of methanol and DPPH was used as control. The

Table 1. Paralysis time of RVB and RVL extracts against *P. posthuma.*

Extract conc. (mg/mL)	Time (min)		
	RVB	RVL	0.9% saline
20	19.38 ± 0.409	31.08 ± 0.260	Na
30	16.50 ± 0.500	26.64 ± 0.049	Na
40	15.68 ± 0.266	24.02 ± 0.044	Na
50	11.17 ± 0.088***	21.68 ± 0.095	Na
Paralysis time ABZ 10 mg/mL	15.48 ± 0.180		

RVB, *R. vomitoria* bark;RVL, *R. vomitoria* leaves; ABZ, Albendazole; Na, No activity; conc., concentration; ****p* < *0.001.*

Table 2. Paralysis time of VAB and VAL extracts against *P. posthuma.*

Extract conc. (mg/mL)	Time (min)		
	VAB	VAL	0.9% saline
20	16.62 ± 0.347	34.01 ± 0.720	Na
30	12.51 ± 0.289***	31.46 ± 0.395	Na
40	9.43 ± 0.536***	27.62 ± 0.968	Na
50	7.03 ± 0.491***	22.55 ± 0.569	Na
Paralysis time of ABZ (10 mg/mL)	15.48 ± 0.180		

VAB, *V. africana* bark; VAL, *V. africana* leaves; ABZ, Albendazole; Na, No activity; conc., concentration; ****p* < *0.001.*

Table 3. Death time of VAB and leaves extracts against *P. posthuma.*

Extract conc. (mg/mL)	Time (min)		
	VAB	VAL	0.9% saline
20	24.59 ± 0.356	141.71 ± 0.918	Na
30	19.07 ± 0.261	138.72 ± 1.254	Na
40	16.43 ± 0.780***	125.65 ± 0.872	Na
50	14.77 ± 0.117***	113.99 ± 1.014	Na
Death time ABZ (10 mg/mL)	21.03 ± 0.258		

VAB, *V. africana* bark; VAL, *V. africana* leaves; ABZ, Albendazole; Na, No activity; conc., concentration; ****p* < *0.001.*

percentage of free radical scavenged was calculated from the equation [% scavenging = $((A_o-A_I)/ A_o \times 100)$]. The IC_{50} was determined as the concentraion of samples which scavenged 50% of free DPPH radicals. The experiment was performed in replicates.

Statistical analysis

All results were plotted and analysed with GraphPad Prism 5.0 for windows (GraphPad software, San Diego, CA, USA) and analysed by two-way ANOVA followed by Bonferroni post-test analysis which recognises **p < 0.05, **p < 0.01, ***p < 0.001* as statistically significant.

RESULTS AND DISCUSSION

Anthelmintic activity

The extracts of *R. vomitoria* and *V. africana* demonstrated a concentration dependent paralytic and death times on *P. posthuma* (Tables 1 to 4).

Antioxidant activity

VAL and RVB demonstrated relatively high antioxidant activity with reference to their IC_{50} as compared to ascorbic acid (Table 5 and Figure 1)

Studies conducted on the leaves and barks of *V. africana* and *R. vomitoria* revealed some pharmacological activity of the two plants. The methanol leaves and bark extracts of both plants demonstrated both anthelmintic and antioxidant activity. VAB demonstrated very potent anthelmintic activity with significant (*p <0.001*) paralytic and death times with reference to albendazole. VAB demonstrated a concentration dependent activity with

Table 4. Death time of RVB and leaves extracts against *P. posthuma.*

Extract conc. (mg/mL)	Time (min)		
	RVB	RVL	0.9% saline
20	35.10 ± 0.608	185.68 ± 0.344	Na
30	27.02 ± 0.908	175.41 ± 0.455	Na
40	24.84 ± 0.182	167.10 ± 1.539	Na
50	21.67 ± 0.733	143.35 ± 1.413	Na
Death time ABZ 10 mg/mL	21.03 ± 0.258		

RVB, *R. vomitoria* bark;RVL, *R. vomitoria* leaves; ABZ, Albendazole; Na, No activity; conc., concentration.

Table 5. IC_{50} of extracts and standard antioxidant.

Sample	IC_{50} µg/mL
VAL	187
VAB	610
RVL	967
RVB	43
ASCORBIC ACID	22

IC_{50}, concentration of agent that scavenged 50% of DPPH.

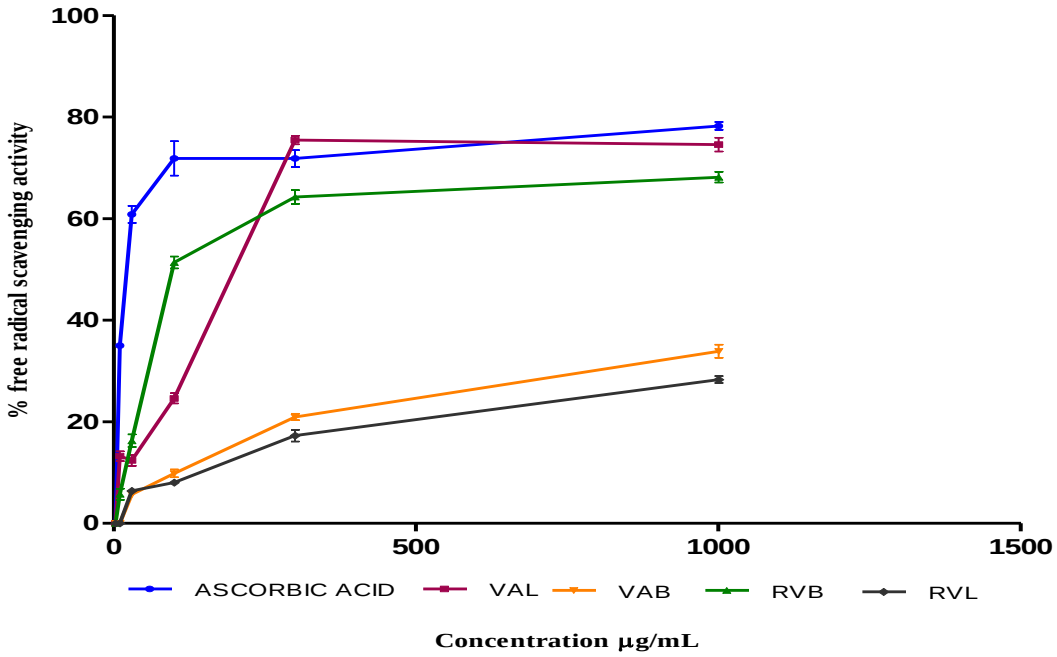

Figure 1. Antioxidant activity of methanol extracts of barks and leaves of *R. vomitoria*, *V. africana,* and ascorbic acid.

significant paralytic and death times ($p < 0.001$) at concentrations of 40 and 50 mg/mL (Tables 1 to 4). RVB also demonstrated anthelmintic activity at all concentrations with a significant ($p < 0.001$) paralytic time at 50

mg/mL. The anthelmintic activity of plants have been attributed to the presence of some phytochemicals in the plants particularly tannins which are polyphenolic compounds (Olusegun-Joseph et al., 2012). Research has shown that some synthetic phenolic anthelmintics such as niclosamide, interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation or by binding to the free protein of the gastrointestinal tract of the worms. This eventually leads to death (Olusegun-Joseph et al., 2012). Tannins are presumed to exert the same effects on the worms. *V. africana* is known to possess tannins as some of its phyto-constituents (Ayoola et al., 2008). The anthelmintic effects demonstrated by the extracts could possibly be attributed to the presence of tannins in the extracts. Studies conducted on both plants have also revealed a wide array of alkaloidal content. The alkaloids can also cause paralysis of the worms by acting on its central nervous system (Mute, 2009), which could have also accounted for the anthelmintic effects of the extracts. The study revealed that the anthelmintic activities of the leaves of both plants are weaker (giving long paralysis and death times) than the barks that showed significant anthelmintic activities at concentrations of 30, 40 and 50 mg/mL ($p < 0.001$).

The study also revealed that the extracts possess good antioxidant activities. The IC_{50} values clearly depict the extent of antioxidant activity of the various extracts (Table 5). It was evident that the bark extracts of *R. vomitoria* demonstrated the highest free radical scavenging activity (IC_{50} = 43 µg/mL) with the lowest being RVL (IC_{50} = 967 µg/mL). The situation was however the opposite with *V. africana*, in which the leaves rather demonstrated much scavenging activity (IC_{50} = 187 µg/mL) than the bark (IC_{50} = 610 µg/mL). The antioxidant properties could be due to the presence of flavanoids and tannins which are known to exert antioxidant activity (Agyare et al., 2015; Marja et al., 1999). These two plants are already known to contain phytochemicals which include tannins and flavonoids (Okolie et al., 2011; Korochi et al., 2009) and might therefore account for the antioxidant activity. The two plants; *V. africana* and *R. vomitoria* could therefore, be potential sources of antioxidant compounds.

Conclusion

The methanol extracts of *R. vomitoria* and *V. africana* demonstrated anthelmintic activity with the bark extracts demonstrating significant anthelmintic activity. The extracts also demonstrated antioxidant activity at concentrations tested.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Agyare C, Baiden E, Apenteng JA, Boakye YB, Adu-Amoah L (2015). Anti-infective and Anti-inflammatory propertis of *Portulaca oleraceae* (L). Don. J. Med. Plant Res. 2 (1):1-6

Alawa CBI, Adamu AM, Gefu JO, Alanusi OJ, Abdu PA, Chiezey NP, Alawa JN, Bowman DD (2003). In vitro screening of two Nigerian medicinal plants (*Vernonia amygdalina* and *Annona senegalensis*) for anthelmintic activity. Vet. Parasitol. 113(1):73-81

Ayoola GA, Folawewo AD, Adesegun SA, Abioro OO, Adepoju-Bello AA, Coker HAB (2008). Phytochemical and antioxidant screening of some plants of apocynaceae from South West Nigeria. Afr. J. Plant Sci. 2(9):124-128

Bhawar GB, Bhalke RD, Lodha KR, Karmase BC, Londhe CD (2009). Phytochemical investigation and in vitro anthelmintic activity of *Bauhinia racemosa* (L) (Leguminaceae). Pharmacology 1: 300-303

Bisset N (1985). Phytochemistry and Pharmacology of Voacanga Species. Agricultural University Wageningen Papers 85 (3): 81–114.

Farnsworth NR, Akerele O, Bingel AS (1985). Medicinal Plants in Therapy. Bull. World Health Organ. 63(6):965-981.

Irvine F (1961). *Rauwolfia vomitoria*: woody plants of Ghana. Oxford University Press. 281

Kombian SB, Saleh TM, Fiagbe NIY, Chen X, Akabutu JJ, Buolamwini JK, Pittman QJ (1997). Ibogaine and a total alkaloid extract of *Voacanga africana* modulate neuronal excitability and synaptic transmission in the Rat Parachial Nucleus *In vitro.* Brain Res. Bull. 44 (5):60-610

Korochi AR, Juliani HR, Kulakowski D, Arthur H, Asante-Dartey J, Simon JE (2009). *Voacanga africana*: Chemistry, Quality and Pharmacological Activity. African Natural Plants Products: New discoveries and challenges in chemistry and quality. Am. Chem. Soc. 20:363-368

Kutalek R, Prinz A (2007). African Medicinal Plants in Yaniv Z and U. Bachrach (eds) Handbook of medicinal plants. New Delhi: CBS Publishers.

Levecke B, Buttle DJ, Behnke JM, Duce IR, Vercruysse J (2014). Cysteine proteinases from papaya (Carica papaya) in the treatment of experimental *Trichuris suis* infection in pigs: two randomized controlled trials. Parasit. Vectors 7:255

Marja P, Kahkonen AI, Hopia HJ, Vuorela JR, Kalevi PT, Kujala S, Marina H (1999). Antioxidant activity of plant extracts containing phenolic compounds. J. Agric. Food. Chem. 47:3954-3962.

Mute V (2009). Anthelmintic effect of *Tamarind indica* linn leaves juice extract on *Pheretima posthuma*. Int. J. Pharm. Res Dev. 7:1-6

Okolie N, Israel E, Falodun A (2011). *In-vitro* evaluation of antioxidant potentials of *Rauwulfia vomitoria* root extracts and its inhibitory effects on Lipid peroxidation as indication for aphrodisiac properties. Pharm. Chem. J. 45(8):476

Olusegun-Joseph TS, Ofodile LN, Oguntoku T (2013). *In -vitro* evaluation of anthelmintic activity of crude extracts of leaves of *Dalbergiella welwitschii*. Int. J. Pharm. Pharm Sci. 5(1):32 -33

Pessoa LM, Morais SM, Bevilagua CM, Luciano JHS (2002). Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn and eugenol against *Haemonchus contortus.* Vet. Parasitol. 109 (1-2): 59-63

Prajapati ND, Purohit SS, Sharma AK (2003). A Handbook of Medicinal Plants: A Complete Source Book, Agrobias, Jodhpur, India.

Prichard R (1994). Anthelmintic resistance. Vet. Parasitol. 54(1-3):259-68

Tan PV, Penlap VB, Nyasse B, Nguemo JDB (2000). Anti-ulcer actions of the bark methanol extracts of *Voacanga africana* in different experimental ulcer models in rats. J. Ethnoparmacol. 73 (3): 423 - 428.

Vercruysse J, Albonico M, Behnke J M, Kotze AC, Prichard RK, McCarthy JS, Montresor A, Levecke B (2011). Is anthelmintic resistance a concern for the control of human soil-transmitted helminths? Int. J. Parasitol. Drugs Drug Resist. 1(1): 14-27.

Vidyarthi RD (1967). A Textbook of Zoology. S. Chand and Co. New Delhi. pp. 329-370.

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