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Aerobic and Facultative Bacterial Community of Fruits and Seeds of *Bertholletia excelsa*, "Castanha-do-Para", Collected in the Amazon Forest

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The contamination of Brazilian nuts, *Bertholettia excelsa*, by pathogenic microorganisms, is the main concern of health authorities in countries that consume the product, which is an excellent nutrient in the human and animal diet. This research work aimed to identify the common bacterial biota from fruits and seeds collected in the Brazilian amazon forest, in the states of Amapa, Amazonas and Acre, and review the role of these microorganisms in distinct ecosystems. The aerobic and facultative bacteria species found in the Brazilian nut, in this study, are members of the following families: Enterobacteriaceae, Enterococcaceae, Streptococcaceae, Moraxellaceae, Aerococcaceae, Aeromonadaceae, *Micrococcaceae,* Pasteurellaceae, Pseudomonadaceae, Sphingomonadaceae, Burkholderiaceae and Dermacoccaceae. Some of the isolated microorganisms are pathogenic to man and animals, and others are innocuous, of biotechnological interest. These data would contribute to the understanding and control of microbiological contamination of the Brazilian nut, besides to ensure the high level of competitiveness of this product in the international market, which also favor the sustainable development of the Brazilian nut rational exploitation by the Amazonian forest inhabitants.

Key words: Brazilian nut; bacteria; contamination; human health.

The Brazilian nut is an edible seed produced by the colossal tree, *Bertholletia excelsa* (Humb. & Bonpl., Lecythidaceae), that can reach 50 meters of height, found in the drained areas of the Amazon forest in Brazil, Peru, Bolivia, Colombia, Venezuela, Guyana, Surinam and French Guyana[36, 34].

The fruit measures 10-12 cm, weighs 0.5 to 2.5 kg and encloses about 10 to 25 seeds. During the rainy season, in the Amazon tropical forest, the Brazilian nut fruits fall down and spread out on the soil. Therefore, before their collection, there is plenty of time to the fruits' shell to be microbiologically enriched and, certainly the contaminated water in the soil enters the fruit, and also the nuts. Besides the natural microbial contamination in the soil, man also contributes to this task, by breaking the fruits in the forest soil and washing the nuts in the nearby rivers. Depending on the location, the collectors wash the nuts, but the transportation from the forest to the warehouses is dramatic. Some places are distant

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from warehouses, therefore rivers and dust roads are crossed by collectors carrying the nuts in cotton bags or baskets, favoring microbial proliferation in the products[34].

Previous work by Ribeiro *et al*[34] reported the detection of microorganisms in Brazilian nuts, in their fruits and in shelled and unshelled seeds. These microorganisms are commonly found in the forest soil and natural running water.

In this research work, fruits directly collected from the trees, and picked up from the forest soil, as also seeds obtained from warehouses, were analyzed in order to identify isolated aerobic and facultative bacteria.

MATERIALS AND METHODS

Collection of fruits and seeds

The Brazilian nut samples were collected in the Amapa, Amazon and Acre states in Brazil (Figure 1). In the Amapa state/Laranjal do Jari county, in the coordinates: S 00°33'50.2"/W 052°18'24.4", fruits were harvested directly from the tree and picked up from the soil, as also the seeds were collected in the warehouses. Seeds were cleaned in the warehouses by steam vapor treatment, usually for 20 minutes, and after, one layer of seeds were spread out on clean surfaces to dry. The warehouses' infrastructure was distinct in each area, but usually high humidity and temperature were common, similar to the data described elsewhere. In the Amazon state, Itacoatiara county, the fruits were harvested directly from the tree, in the coordinates: S 03°00'49.5"/W 058°49'56.4". In other locality, also in the Amazon state, Manicore, the fruits were picked up from the soil, and seeds were obtained from the warehouses in the coordinates: S 5°47'37.1"/W 61°25'29.5". In the Acre state, Acrelandia, the fruits were harvested directly from the tree, picked up from the soil and collected from warehouses, in the coordinates: S 10°01'57.4"/W 066°48'26.2". The areas selected for the collection of Brazilian nut products, were located in a perimeter of 1 km of the above registered coordinates. All samples were directly collected in sterile bags.

The rainy season in Laranjal do Jari county lasts from December to May, while in Itacoatiara, Manicore and Acrelandia counties lasts from November to April[32, 44]. About 30 days before the rainy season, the fruits were harvested in sterile bags directly from the trees by climbing them. Also, around 20 days after the rainy season, the fruits were collected from the forest soil in sterile bags. The sampling areas were characterized by annual high humidity, between 80.3 to 84.8 mean relative humidity; the mean of annual temperature oscillated between 25.3 °C to 27.5 °C, as also the total monthly precipitation averaged between 293.3 mm to 211.5 mm. These data refer to the years 2011 and 2012, when the samples were collected[18]. The seeds collected in the warehouses were also enclosed in sterile bags, before being processed for commercial purposes. All collected samples were kept separately in sterile closed bags, maintained at steady temperature (25-28 °C) in thermal box, transported to the Laboratory of Bioprospection (University of Brasilia, Federal District, Brazil) and processed before reaching 48 hours after collection.

Isolation and identification of aerobic and facultative bacteria

The entire fruit and seeds, without any treatment, were separately and directly transferred from sterile bags, under aseptic conditions, to flasks (one fruit per flask and 25 seeds per flask) containing 500 mL of Brain Heart Infusion broth/BHI (Sigma-Aldrich, St. Louis, US) in a laminar flow, in triplicate, and incubated at 37 °C for 24 hours. Small aliquots of cultured fruits and seeds in BHI broth, were spread out on Nutritive Agar (Sigma-Aldrich, St. Louis, US) utilizing a 10 uL calibrated platinum wire, incubated at 37 °C for 24 hours. The isolated colonies were characterized morphologically (colour, appearance and size), and processed for Gram staining. Additional preliminary biochemical assays likewise, growth in Blood Agar (Sigma-Aldrich, St. Louis, US) for Gram negative bacilli, Azide Agar (Sigma-Aldrich, St. Louis, US) for Gram positive cocci, Agar MacConkey (Sigma-Aldrich, St. Louis, US) for Gram negative bacilli, complemented by the catalase test and growth in Urea Agar and TSI Agar (Sigma-Aldrich, St. Louis, US), were carried out, and the final identification of isolated colonies was run automatically in the MicroScan autoSCAN "Walkaway" system (W/A)-(Dade/Baxter, West Sacramento, US), utilizing

commercial kits (bioMerieux, Marcy-l'Étoile, France). All the isolation and identification procedures were repeated three times.

RESULTS AND DISCUSSION

The isolated and identified bacteria species are listed in Table 1, according to the locations (Amapa, Amazon and Acre states) where the fruits and seeds were collected, and also discriminated the identified microorganisms in fruits harvested directly from the tree, picked up from the soil, and seeds obtained from warehouses. The identified microorganisms were classified according to their morphological, tinctorial and biochemical pattern in species belonging to the families Enterobacteriaceae, Enterococcaceae, Streptococcaceae, Moraxellaceae, Aerococcaceae, Aeromonadaceae, *Micrococcaceae*, Pasteurellaceae, Pseudomonadaceae, Sphingomonadaceae, Burkholderiaceae and Dermacoccaceae.

Despite a common climate conditions, but different geographical locations, the microbiota profile of fruits and seeds were distinct, even though at the genus level, a relative uniformity was observed. The genus *Citrobacter* was found in fruits in all areas of collection. Among the species isolated, the *Citrobacter amalonaticus*

Fig. 1. Geographic location of the areas in the Amazon region where the fruits and seeds of *Bertholettia excelsa* were collected. The areas of collection are depicted in the map according to their geographic coordinates: Acrelandia (S 10°01'57.4"/W 066°48'26.2"), Manicoré (S 5°47'37.1"/W 61°25'29.5"), Itacoatiara (S 03°00'49.5"/W 058°49'56.4"), and Laranjal do Jari (S 00°33'50.2"/W 052°18'24.4").

composes the common intestinal microbiota of human beings, and it is not well established its role in human disease, besides together with other enterobacteria members, it has been isolated from cockroaches in Malaysia[21], while *Citrobacter freundii* is reported to be associated to mortality in 22% of human patients with predominant infection in the intraabdominal region, as reported in a hospital in Taipei[6], as also in gallbladder disease [7], becoming a worldwide emerging nosocomial pathogen with escalating incidence of multidrug resistance, even though it colonizes natural environment, particularly in health care settings, which make it difficult to eradicate. Another species, the *Citrobacter farmeri* should be considered as a possible cause of fatal meningitis in cancer patients[38]. Therefore, the source of Brazilian nut contamination by these microorganisms is diverse, but most probably from man and animals' fecal samples, and mainly carried by insects, birds and rodents.

Another common genus, the *Enterobacter*, was found in all areas, as also the *Enterococcus*. The *Enterobacter aerogenes* and*Citrobacter freundii*, among other enterobacteria, are commonly found in nosocomial environment, implicated in urinary tract infections[23] as also, the *Enterobacter cloacae* and *Enterobacter aerogenes* were isolated from processes in newborn sepsis[22]. The *Enterobacter asburiae* has been utilized for biotechnological purposes, even though many diseases could be caused by this agent[16, 25]. The *Enterococcus casseliflavus* has been isolated from food fermented products and also from wounds, so the production of virulent factors, antibiotic resistance, biofilm and gelatinase expression besides DNAse and lipase activity, has been evaluated, as also the susceptibility to the majority of antibiotics[8, 6]. Despite the *Enterococci* group forms part of the normal microbiota of the human and animal gastrointestinal tract, the *Enterococcus faecalis* has been isolated from nosocomial infection, involved in diseases in the genitourinary apparatus, bacteremia, wound infections, endocarditis, as also skin and soft tissue infections[9, 28]. Probably, birds and rodents feces are the source of the *Enterococci* group, found in fruits in the trees as also in the forest soil.

The *Enterococcus durans* has been

Table 1. Identified species of bacteria of fruits harvested directly from the tree and picked up from the soiland seeds (Bertholletia excelsa) obtained from warehouses in the Acre, Amazon and Amapa states,Brazil.

Bacterial organisms	Fruits harvested from the tree	Fruits collected from the soil	Seeds obtained from warehouses
Enterobacteriaceae (Gram -)			
Citrobacter amalonaticus		+[1]	
Citrobacter freundii	+[2,3]	+[1]	+[1,2]
Citrobacter farmeri	+[2]		+[2]
Enterobacter aerogenes	+[1]	+[1,2]	+[2,3]
Enterobacter cloacae	+[1]	+[1]	+[1,3]
Enterobacter asburiae		+[2]	
Enterococcus casseliflavus	+[1]	+[1,3]	+[1,3]
Enterococcus faecalis		+[1]	
Enterococcus durans	+[3]		
Enterococcus gallinarum	+[2]		+[2]
Enterococcus faecium		+[3]	+[3]
Enterococcus raffinosus	+[3]		
Klebsiella pneumoniae	+[3]	+[3]	+[1,3]
Klebsiella oxytoca			+[3]
Kluyvera ascorbata		+[1]	
Kluyvera intermedia	+[1]	+[1]	
Proteus mirabilis		+[1]	+[1]
Pseudomonas aeruginosa	+[1]	+[1,2]	
Morganella morganii	+[2,3]		+[2]
Providencia rettgeri	+[2,3]		+[2]
Pseudomonas mendocina		+[2]	+[2]
Serratia marcescens	+[2]		+[2]
Moraxellaceae (Gram -)			
Acinetobacter Iwoffii			+[2]
Aerococcaceae			
Aerococcus viridans			+[2]
Aeromonadaceae (Gram -)			
Aeromonas salmonicida			+[2]
Pasteurellaceae (Gram -)			
Pasteurella canis			+[2]
Pasteurella pneumotropica			+[2]
Sphingomonadaceae (Gram -)			
Sphingomonas paucimobilis			+[2]
Burkholderiaceae (Gram -)			
Burkholderia cepacia	+[3]		
Micrococcaceae (Gram +)			
Kocuria varians	+[2]	+[3]	+[2]
Kocuria rosea		+3	
Dermacoccaceae (Gram +)			
Dermacoccus nishinomiyaensis	+[3]		
Streptococcaceae (Gram +)			
Lactococcus lactis ssp lactis		+[1]	

[1]Acre state, [2]Amazon state and [3]Amapa state

exclusively isolated from fruits directly harvested from the tree in the Amapa state, as we previously reported, probably associated to the nut maturation[34]. The *Enterococcus gallinarum*, a commensal lactic acid bacteria, was isolated from fruits directly harvested from the tree, and seeds obtained from warehouses, in the Amazon state. In spite of the apathogenic role of the *Enterococci* group, likewise *E. gallinarum*, it has been reported the multidrug resistance of these organisms in nosocomial environment[47]. The *Enterococcus faecium* and *Enterococcus raffinosus* were isolated from samples collected in the Amapa state. The first, *E. faecium*, was found in fruits picked up in the soil, and seeds obtained from warehouses, while *E. raffinosus* was isolated from fruits harvested directly from the tree. As a common characteristic of the *Enterococci* group, of facultative pathogen, an increased multidrug resistance has been reported[8].

The *Klebsiella* genus was present in the Acre and Amapa state, but it is not dismissed the possibility that it would be present in the collected area of the Amazon state. The *Klebsiella pneumoniae* was classically, in the past, associated with the etiology of pneumonia, mainly among alcoholics and diabetic patients. Besides, *Klebsiella pneumoniae* has been isolated from urinary and biliary tract infections, osteomyelitis and bacteremia[24]. Recently, new variants of this pathogen have emerged, critically raising concerns due to its natural augmented virulence and multiresistance to commonly prescribed antibiotics[37]. Some strains of the endophytic *Klebsiella oxytoca* fix atmospheric nitrogen[6], therefore it is a common and beneficial soil organism.

The *Kocuria* genus was detected in the Amazon and Amapa state, but apparently, absent in the Acre state. *Kocuria* species are apathogenic Gram positive organisms, despite commensals, immunocompromised subjects could develop disease related conditions[27]. The *Pseudomonas* genus was found in the Acre and Amazon state, but not in the Amapa state, and the *Morganella* genus was isolated in the Amapa and Amazon state. The *Pseudomonas aeruginosa* is frequently isolated from wound infections and airways of patients with cystic fibrosis, posing a challenge for antimicrobial therapy[10, 26], likewise *Morganella morganii*, which is an opportunistic microorganism involved in different human pathological conditions[43]. Another species, the *Pseudomonas mendocina*, commonly isolated from the environment, has been utilized for biotechnological purposes[13].

The *Providencia* genus was grown from samples collected in the Amapa and Amazon state, but not from collected samples in the Acre state. The *Providencia rettgeri* has been isolated from vertebrates and insects, causing diseases in man, mainly in nosocomial environment[11], therefore it would not be uncommon to find it in fruits collected directly from the tree.

The genera *Dermacoccus* and *Burkholderia* were exclusively found in the Amapa state. The members of the *Kocuria*, *Lactococcus* and *Dermacoccus* genera secrete enzymes[14, 34, 35] that most probably play a role in the seed maturation. The *Burkholderia cepacia* is a Gram negative bacillus, originally linked to plant diseases, therefore commonly found in the environment, but it causes high mortality in humans, associated with respiratory diseases [17].

The genera *Kluyvera*, *Lactococcus* and *Proteus* were just found in the Acre state, while the genera *Pasteurella*, *Serratia* and *Sphingomonas* were solely isolated from areas in the Amazon state. The *Kluyvera ascorbata* is not usually found associated to clinical entities, but it is frequently reported to be isolated from urinary tract infections in children, and rarely in elderly[30], even though some authors claim that in both immunocompetent and immunocompromised hosts[40], this enterobacteria could be found, involved in pathogenic conditions[19, 29, 45].There are no reports indicating that *Kluyvera intermedia* causes diseases in man and animals. Possibly, this species constitutes the common intestinal microbiota of vertebrates, similarly in the rainbow trout, *Oncorhynchus mykiss*[31]. The *Lactococcus lactis* has been utilized for a long time in food fermentation and recently, it has been envisioned its employment to vector antigens and plasmids in vaccine development due to its innocuity, safety and adaptability to reproduce in distinct host niches[2, 4]. The *Proteus mirabilis* is a morphologically versatile bacteria, commonly found in the environment, etiologically linked to human pyelonephritis, urolithiasis, prostatitis and

catheter-associated urinary tract infections[1, 20]. The genera *Serratia, Pasteurella* and *Sphingomonas* were solely isolated from samples collected in the Amazon state. The *Serratia marcescens* is an environmental microorganism opportunistically causing disease in vertebrates including man, and also known as an entomopathogenic agent[41]. As an opportunistic and zoonotic organism, the *Pasteurella pneumotropica* is usually isolated from laboratory mice and rats, so rodents would be the source of contaminated brazilian nut fruits, while *P. canis* is usually reported infecting humans after dog bites[15, 39]. The *Sphingomonas paucimobilis* is a low virulent Gram negative bacillus commonly found in soil and water, including nosocomial environment, infecting immunocompromised patients and health children[3].

The *Acinetobacter Iwoffii* was found exclusively in fruits collected directly from the tree, in the Amazon state. It has been suggested the role of *Acinetobacter Iwoffii* in the etiology of gastric cancer as 25% of these casualties have not been linked to *Helicobacter pylori*, and experimental studies with mouse models have shown the same histological changes in gastric mucosae of animals inoculated with *Acinetobacter Iwoffii*, as it occurs in gastric cancer, etiologically linked to *Helicobacter pylori*[33, 46].

The *Aerococcus viridans* was isolated from seeds stored in warehouses in the Amazon state, so it could not be discarded the possibility of local contamination. This organism is a Gram positive airborne cocci, widely distributed in nosocomial environments, but infrequently found in human infections, nevertheless bacteremia, endocarditis and urinary tract infections have been etiologically associated with *Aerococcus viridans.* Probably, the fastidious characteristic of this organism could underestimate its participation in human infections[12].

The *Aeromonas salmonicida*, solely found in seeds stored in warehouses in the Amazon state, is a fish pathogen, of significant economic impact in fisheries worldwide[42]. Probably, the seeds were contaminated from local fish products.

CONCLUSIONS

Certainly, the traditional culture methods, based on growth capability, and the microbial inoculum amount, does not represent the real microbiota profile, but it represents real possibilities of contamination to human beings and domestic animals, as potential inoculum for infection and disease development.

In order to determine the most real profile of the microbiota, in the Brazilian nut, it would be necessary to carry out metagenomic analysis, which meanwhile, is not the main objective of this study, but to evaluate the sanitary conditions of the Brazilian nut as a food product, having in mind that the seeds go through a decontamination process before commercialization.

Ongoing studies are devised to analyze the drug resistance profile of the isolated microorganisms in order to build a probable route of these microorganisms circulation. The dynamics of a microbiota ecosystem would help us to better understand the role of human interference when utilizing indiscriminately antimicrobial drugs.

It is not known the interactions determined by microbial metabolites of the commonly isolated bacteria and fungi species found in the Brazilian nut, if these microbial relationship would trigger or inhibit the production of toxins, specially by aflatoxigenic fungi. Up to date, there are no reports in the literature concerning the bacterial biota of the Brazilian nut, except our own results.

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