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THE IMPORTANCE OF BIOFILMS IN MICROBIAL DETERIORATION OF CONSTRUCTIONAL MATERIALS

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MINI-REVIEW

ABSTRACT

Constructional materials, such as concrete, stone, wood, plastic, painted surfaces and metal, are colonized by bacteria, algae and fungi, which form biofilms on the surfaces accelerating deterioration of the structure. The mechanisms of such deterioration, the major microbial genera involved and some of the factors which can affect the degree of colonization and attack are discussed. The major factor determining microbial growth on constructional materials is moisture and the importance of correct design of structures to avoid the ingress of water is pointed out.

Key words: Algae, biodeterioration, biofilms, constructional materials, cyanobacteria, fungi

INTRODUCTION

The weathering of building materials is accelerated by a superficial layer of microorganisms, called a biofilm. This contains active and dormant microorganisms and products of their metabolism, such as acids and polymers produced by the microbial cells. The polymers, known as EPS (exopolymeric substances) act as glues, trapping dust and other particulate materials and thus increasing the disfiguring effects of the biofilm. Probably the most important microorganisms on the surfaces of stone, brick and concrete are fungi, algae and cyanobacteria, which can resist dehydration and hence survive the frequently adverse conditions on these materials. A complex biofilm system can develop on such constructions (Fig. 1), the colonization process beginning with autotrophs and progressing to heterotrophic organisms (46). Since the activity of the microorganisms in the biofilm is often intense, this can lead to the rapid deterioration of these materials.

Microbial activities which are important in the deterioration of constructional materials are shown in Table 1. These activities are localized and concentrated within biofilms.

Active biofilms are formed in any location where microorganisms and moisture are present. All microorganisms are able to attach to surfaces, although the degree and rate of this process and of subsequent microbial growth depend on the type of organism, the nature of the surface and the environmental conditions (13, 69). In humid tropical environments, or in other areas of high humidity, such as bathrooms, biofilm formation can be extremely rapid (1, 42). Materials in dry conditions are the least susceptible to biofilm formation and biodeterioration. Thus the best way to

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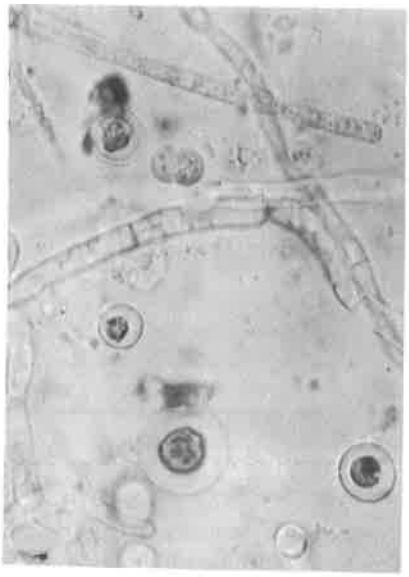

Figure 1. A typical biofilm on an external concrete painted surface, showing bacteria, cyanobacteria and fungi. Magnification x1700

reduce risks to a minimum is to maintain low humidity and to avoid large temperature fluctuations, since the latter may cause condensation (26); however, this is often impracticable. The advent of air conditioners has helped to reduce the problems within buildings, but with an increased hazard to the external structure. The air conditioner condensate falling on the outer walls of buildings commonly induces the growth of algae and fungi, which eventually leads to loss of any protective coating and crumbling of the concrete structure.

Bacterial deteriogens. Fungi and algae are not the only microorganisms involved in biofilm formation and reduced durability of structures. Bacteria can also play an important role. While fungal and algal growths are often visible to the naked eye, bacteria can be present on an apparently clean surface in sufficient numbers to exert adverse effects. Such effects can include production of inorganic acids, leading to concrete or metal corrosion (5, 14, 46), or metabolic activities which cause blistering of paint (64). Chemolithotrophic and oligotrophic bacteria may condition a surface, making it more amenable to colonization by heterotrophic microorganisms (46).

Bacteria are the only group of microorganisms in which members capable of growth in areas of low oxygen concentration are commonly found. Hence they can be very active in anoxic environments, within pipes and stagnant storage tanks, as well as beneath biofilms in otherwise well-oxygenated areas. Many bacteria are active fermenters under such conditions and produce aggressive acids, leading to corrosion of the structure or to spoilage of the product within it. Yet other bacteria (the sulfate-reducing bacteria, SRB) are the principal organisms involved in anaerobic

Table 1. Microbial activities involved in deterioration of constructional materials

Microbial activity	Damage caused	Materials affected
Surface growth	Discoloration Water retention	Concrete, ceramic tiles, stone, bricks, plaster, wood, plastic, paint, roofing tiles.
Acid production	Corrosion, erosion	Concrete, stone, metal.
Hydrolytic enzymes	Increased fragility, erosion	Wood, paint.
Chelation	Corrosion, etching	Metal, concrete, stone, glass.
H_2S production	Corrosion	Metal.
Growth of fungal filaments surfaces,	Physical damage to surface, increase in permeability	Concrete, stone, wood, plaster, painted plastics.
Inhomogeneous growth/activity	Corrosion due to concentration cells	Metal.
Metabolic activity	Blistering Embrittlement	Painted surfaces Plastics.

metal corrosion (30, 52). There is a wide literature on these organisms, whose effects are of extreme importance to the oil industry, since they not only corrode pipelines and storage tanks, but also cause souring of petroleum by their production of hydrogen sulfide gas. They are certainly the main group of organisms involved in the corrosion of underground metal structures (52) and it is well accepted that their effects are much more intense when present in the sessile state, that is, in biofilms, than when merely suspended in the surrounding medium (21, 23).

The anaerobic bacterial species, *Clostridium xylanolyticum*, causes tunneling decay of wood (56). This type of deterioration is seen as distinctive troughs under scanning electron microscopy and was originally thought to be due to fungal action (9). It is thought that anoxic microniches in the wood structure are caused by localized respiratory activity of aerobic biofilms, allowing growth of the anaerobic species, which produces a very active xylanase. Other members of the genus *Clostridium* produce cellulases, which alter the crystalline structure of the cellulose microfibrils, changing the permeability of the wood and opening the structure to fungal penetration (7, 27). Certain facultative bacteria, such as *Bacillus polymyxa*, can break down the pectin component of wood cells (39). Bacteria which attack the structure of wood cause reductions in compression strength, bending strength and modulus of elasticity (7) and such attack can occur even when the wood has been treated with preservative (17).

Some of the more important bacteria directly involved in biodeterioration of constructional materials are shown in Table 2.

Table 2. Some bacteria important in the deterioration of building materials

Material	Bacterial groups	References
Concrete & stone	Sulfur-oxidising bacteria (e.g. *Thiobacillus*), heterotrophic bacteria (e.g. *Arthrobacter*, *Pseudomonas*), nitrifying bacteria	14, 39, 66
Metal	SRB, sulfur-oxidising bacteria (e.g.*Thiobacillus*), iron bacteria (e.g. *Gallionella*), iron-reducing bacteria (e.g. *Vibrio, Shewanella*), acid-producing bacteria (e.g. *Clostridium*)	11, 14, 22, 44, 52
Painted surfaces	*Actinomyces; Alcaligenes; Bacillus; Flavobacterium; Pseudomonas*	49
Plastics & rubber	*Actinomyces, Pseudomonas, Nocardia*	72
Wood	*Acinetobacter,Alcaligenes, Bacillus, Citrobacter, Clostridium, Corynebacterium, Enterobacter, Erwinia, Pseudomonas, Rhodococcus, Serratia, Streptomyces,* a nitrogen-fixing consortium	7, 16, 39, 43, 56

Fungal deteriogens. Unlike bacteria, fungi are considered to have relatively little involvement in metal corrosion; the only well-documented cases of fungal biocorrosion are those caused by *Hormoconis resinae*. This filamentous fungus degrades many hydrocarbons, producing organic acids which corrode fuel storage tanks (67). The importance of biofilm formation by this organism on the corrosion of aircraft fuel tanks made of aluminum was demonstrated by Salvarezza and Videla (57) using scanning electron microscopy. The outline of fungal hyphae was seen, etched on the metal surface, after removal of the biofilm. This was caused by localized acidic fungal metabolites. In addition to metal, the fungus also has the ability to degrade protective coatings based on hydrocarbons, such as the synthetic rubber Buna-N (47), thus exposing the underlying material to the aggressive attack of other microorganisms, or of chemical species.

Fungi also have roles in biodeterioration of building materials such as wood, stone and plastic. This class of microorganisms is probably the most important in the degradation of wooden structures, since they not only produce enzymes which destroy the components of the wood, but can also insinuate themselves into the structure because of their filamentous form, although yeasts have also been found to be associated with wood decay (36). The damage produced by filamentous fungi can be classified into 6 types (32):

- brown rot, caused by cellulolytic fungi such as *Lentinus lepideus*
- white rot, caused by ligninolytic fungi, such as *Trametes versicolor*
- wet rot, produced in wet conditions

- dry rot, caused by a species of fungus (*Serpula lachrymans*) which can grow well in dry conditions (of little importance in Brazil)
- soft rot, which results in loss of rigidity (e.g. *Chaetomium globosum*)
- sapstain and bluestain, caused by fungi such as *Ceratocystiosis falcata* and *Gliocladium roseum*, which cause discoloration of the wood.

The type of damage depends on the fungi present and their activities, but the extent of the damage varies greatly with the type of wood. Soft woods, such as pine, are generally much more susceptible than hard woods like mahogany and heartwood is almost always more resistant than sapwood (31).

The brown rot fungi are the most important group associated with deterioration of inservice timber. These fungi degrade cellulose and hemicellulose without affecting the lignin component of the wood and cause rapid depolymerization with relatively little weight loss (28). The mechanism of colonization and degradation is still not well defined, but various cations play an important role in these processes (35) and so, as in the case of other materials such as concrete, nondestructive techniques of assay such as electron paramagnetic resonance and synchrotron X-ray fluorescence can be used with advantage (34).

There has been relatively little work on the deterioration of stone and cement by fungi, since traditionally the sulfur-oxidizing bacteria have been deemed to be most important (14). However, McCormack et al. (45) presented evidence for the acid attack of the fungus Aspergillus glaucus on concrete and Resende et al. (54) reported the production of acids by several filamentous fungi isolated from stone buildings in Brazil. A review of stone deterioration by fungi was given by May et al. (46), who also discussed the involvement of bacteria and, to some extent, algae. Fungi produce a variety of inorganic and organic acids, which can demineralize various stone substrates (29). Once again, the filamentous form of the microorganisms enables them to penetrate the weakened structure (40), especially when extra nutrients, in the form of dirt or algal and bacterial biofilms, are present (24).

The activity of various fungi on plastics, particularly polyurethanes, has been investigated by a number of workers (3, 18, 51, 68). Fungal enzymes are able to break down the polyurethanes or metabolize the plasticizers in various polymers, resulting in embrittlement and loss of strength. This is very significant in polymeric varnishes, where fungal action causes increased permeability and renders the coating useless as a protective layer (8). On the whole, however, plastics are resistant to microbial attack and the major problems of microbial growth are esthetic - discoloration of the surface - and, where internal structures such as plastic bathroom fittings are involved, health hazards. Biofilms can harbor pathogenic and toxigenic fungi such as *Aspergillus flavus*, as well as non-pathogenic but allergenic fungal spores. The latter may be, at least partially, responsible for so-called sick building syndrome (53), which causes the loss of many working days per year in commerce.

Such biofilms occur not only in bathrooms, but also on wall and ceiling materials in water-damaged rooms (2), where they can lead to debilitating respiratory problems in the inhabitants of the building. Grant et al. (26) studied the moisture requirements for fungal growth in houses and showed that the lowest water activity (Aw) allowing growth on malt agar of *Aspergillus* and *Penicillium* species commonly found in buildings was 0.76 - 0.79 at 20°-25°C. Reducing the temperature to 12°C increased this minimum to 0.87. On woodchip wallpaper, at 25°C, Aw values were higher (0.84 - 0.97), but these were reduced on paper covered with a water-based paint to 0.79 - 0.89. The authors recommended that susceptible surfaces should be kept at Aw values of below 0.8, which can be achieved by various conditions of air temperature and relative humidity.

A very wide variety of fungi have been implicated in the deterioration of building materials and some of these are indicated in Table 3.

Phototrophic microorganisms. Algae have an important role in the disfigurement of buildings. They are particularly prevalent in areas of high humidity, but can be inhibited by prolonged exposure to intense sunlight. Souza (60) was unable to isolate algae from wooden panels which had been exposed for over 2 years in Gravataí, Rio Grande do Sul, probably because of the intense insolation. The effects of algae are generally mainly noted in moist, shaded areas – rural environments or more wooded urban areas – but Wee and Lee (71) considered that the filamentous alga *Trentepohlia odorata* was the primary coloniser of high-rise buildings in Singapore, producing streaks of orange growth on many walls. As algae require little nutrient, being able to produce cell structural materials from CO_2, inorganic minerals and light, they are ready colonizers of clean surfaces such as newly painted buildings. Oil-based paints and certain paint pigments,

Table 3. Some fungi involved in deterioration of constructional materials.

Material	Fungal types	References
Concrete & stone	*Alternaria, Aspergillus, Aureobasidium, Botrytis, Cladosporium, Curvularia, Exophiala, Mucor, Paecilomyces, Penicillium, Phoma, Trichoderma, Verticillium*	41, 42, 45, 46, 54
Metal	*Aspergillus, H. resinae, Penicillium, Trichoderma*	67
Painted surfaces	*Alternaria, Aspergillus, Aureobasidium, Cephalosporium; Cladosporium, Curvularia, Exophiala, Fusarium, Geomyces, Mucor; Penicillium, Stachybotrys, Stemphyllium, Trametes, Trichoderma, Ulocladium, Verticillium*	8, 49
Plastics & rubbers	*Aspergillus, Chaetomium, Cladosporium, Cryptococcus, Fusarium, Hormoconis, Penicillium, Rhizopus, Trichoderma*	3, 47, 51
Wood	*Alternaria, Aspergillus, Aureobasidium, Candida; Ceratocystis, Ceratocystiopsis, Cladosporium, Coriolus, Cryptococcus, Gloeophyllum, Gliocladium, Hansenula, Lentinus, Penicillium, Phanerochaete, Phialophora, Phoma, Pleurotus, Postia, Rhodotorula, Serpula, Trichoderma, Verticillium*	4, 10, 16

however, are inhibitory (63) and biofilm formation on these materials is considerably delayed, as it is on new concrete, where the pH (approximately 11-12) is too high for microbial growth. After exposure to leaching by rain containing dissolved carbon dioxide, however, the material becomes conditioned and algal and fungal biofilms readily form.

The blue-green bacteria are often included in the algae, but Stanier et al. in 1978 (62) proposed that they should be classified under the rules of the Bacteriological Code. These organisms normally show a distribution similar to algae, but are more resistant to drought and extreme temperatures. Grant (25) considered that cyanobacteia are of greater ecological importance as pioneer organisms than any other class. His excellent review, although now slightly dated, should be consulted by anyone interested in terrestrial fouling by algae and blue-green bacteria. Danin and Caneva (15), working in the relatively dry environment of Jerusalem, suggested that blue-green bacteria induce weathering of stone and rock in the following steps:

1. Attachment of cyanobacterial cells in small fissures
2. Growth within the fissure
3. Water uptake and expansion of the cell mass, thus exerting pressure within the structure
4. Precipitation of carbonates and oxalates around the cells
5. Opening of the fissure due to these internal pressures
6. Entry of dust, pollen grains, etc.
7. Partial death of cyanobacterial cells and establishment of bacteria, fungi and small animals such as mites within the fissure
8. Increasing internal pressure on the superficial layer of the structure leading eventually to its detachment (spalling).

Ortega-Cal et al. (50) isolated a wide range of algae and cyanobacteria from deteriorated stone monuments in Spain and showed the penetration of the cells into the upper stone layers using scanning electron microscopy. They also applied Koch's Postulates, demonstrating that the organisms isolated in nature were able to cause deterioration of the materials in laboratory experiments when they were inoculated on to the surfaces of stone test pieces. Gómez-Alarcón et al. (24) demonstrated in the laboratory that fungi isolated from a weathered sandstone church were able to colonize sandstone cubes in the presence of algal biomass (*Monoraphidium braunii*), and that in the presence of the alga, different types of deteriogenic fungal products were formed. Such experiments are essential to prove the role of surface microorganisms in the deterioration of a structure.

Bolívar and Sánchez-Castillo (6) reported the identification of 35 cyanobacteria and 45 algae from a marble fountain in the Alhambra, Granada, Spain. They demonstrated the effect of treatment with a quaternary ammonium biocide on the populations. The most resistant types were *Chaemisiphon, Chlorosarcinopsis* and *Tetracystis*. With respect to painted surfaces, Joshi & Mukundan (37), in India, showed that cyanobacteria were the dominant photosynthetic organisms present. They sampled

Table 4. Photosynthetic microorganisms which can cause biodeterioration of building materials

Material	Algae bacteria	Photosynthetic	References
Concrete & stone	*Apatococcus, Bracteacoccus, Chlamydomonas, Chlorella, Chlorococcum, Chlorokybus, Chlorosarcina, Chlorosarcinopsis, Ecdysichlamys, Haematococcus, Friedmannia, Klebsormidium, Leptosiroid, Muriella, Neochloris, Oedogonium, Palmellococcus, Pseudodendoclonium, Scenedesmus, Stichococcus, Stigeoclonium, Tetracystis, Trebouxia, Trentepohlia, Bacilliariophyceae, Chrysophyceae, Eustigmatophyceae, Rhodophyceae, Xanthophyceae*	*Aphanocapsa, Aphanothece, Calothrix, Chaemisiphon, Chroococcus, Gloeocapsa, Hyella, Microcoleus, Myxosarcina, Nostoc, Phormidium, Plectonema, Pleurocapsa, Schizothrix, Scytonema, Synechocystis, Tolypothrix*	15, 24, 25, 50
Metal	Hydrogenase-positive *Chlorophyta*	*Nostoc, Anabaena,* purple bacteria	59, 61
Painted surfaces	*Chlorella, Chlorococcum, Eustigmatus, Trebouxia, Trentepohlia*	*Chroococcus, Lyngbya, Nostoc, Oscillatoria, Plectonema, Scytonema*	25, 37, 70, 71
Plastic	*Stichococcus*		19

surfaces painted with acrylics, cement-based coatings and oil-based enamels, and found the latter the most resistant to colonization. *Plectonema* was found in 60% of the samples, with *Lyngbya* and *Nostoc* being the next most abundant groups. The only green algae isolated from acrylic painted surfaces were *Trebouxia* and *Chlorella*. Table 4 shows some of the phototrophs which have been isolated from disfigured buildings, or have been shown to be capable of biodeterioration.

Cyanobacteria can be distinguished from algae by microscopic and microbiological culture analyses, and by the use of gene probes and other DNA analysis methods (33, 55). The differentiation of the two groups is important, since methods of control may also differ. Algae and blue-green bacteria are major deteriogens of exposed surfaces in buildings, their negative effects being esthetic (soiling) and retention of water, thus predisposing the surface to colonization by more damaging organisms such as fungi, mosses and higher plants (24, 25). Hence the treatment and control of these biofilms is important.

Control of biodeteriogenic biofilms, if their formation cannot be avoided by keeping materials clean and dry, is generally by the use of biocides (20,

46). These toxic chemicals may be used as washes (such as sodium hypochlorite), or may be incorporated into surface coatings such as paints. Schnabel (58) discusses the limitations of biocides used in the USA for the preservation of stone buildings and Morton (48) reviews the methods used for the laboratory testing of algicide activity. Surface coatings can also be used without biocides to protect susceptible materials like wood, but the coatings themselves can also be attacked by microorganisms (8). Hence treatment is not easy, especially so since microorganisms in biofilms possess an increased resistance to anti-microbial compounds (12, 65). Thus the prompt removal of the beginnings of microbial growth, or alteration of conditions to retard biofilm formation, especially reduction of humidity, is the recommended method to reduce the problems of the durability of materials associated with microbial biofilms. Engineers and architects can contribute to this by applying an awareness of the requirements of deteriogenic microorganisms to the design of constructions, ensuring that susceptible materials are not used in areas favorable for biofilm formation (i.e., humid environments), or are protected by the application of appropriate surface coatings. Where impermeable coatings are used, however, it is important to note that any ingress of moisture behind the coating will produce immense problems and such eventualities must be bourne in mind when designing and building a structure. An increased interaction between architects, engineers and microbiologists is essential to reduce the incidence of biodeterioration of buildings and constructional materials.

RESUMO

Importância de biofilmes na deterioração microbiana de materiais de construção

Materiais de construção, tais como, concreto, cantaria, madeira, plásticos, revestimentos e metal, sofrem colonização por bactérias, algas e fungos, os quais produzem biofilmes nas superfícies, aumentando a taxa da deterioração da estrutura. São apresentados os mecanismos da deterioração, os príncipais gêneros microbianos envolvidos e alguns fatores que influenciam na colonização e no ataque da estrutura. A umidade é o fator mais importante, o qual determina a taxa do crescimento microbiano em materiais de construção. Em função disso, salientou-se a necessidade do desenho correto da estrutura para evitar o ingresso da água.

Palavras-chave: Algas, biodeterioração, biofilmes, cianobactérias, fungos, materiais de construção.

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SPATIAL DISTRIBUTION AND TEMPORAL VARIABILITY OF HETEROTROPHIC BACTERIA IN THE SEDIMENTS OF PARANAGUÁ AND ANTONINA BAYS, PARANÁ, BRAZIL

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ABSTRACT

The temporal and spatial variability of heterotrophic bacteria was analyzed in sandy, sandy-silt and silty sediments of Paranaguá and Antonina Bays (SE Brazil). Samples were taken from seven stations along a salinity gradient, from the Paranaguá Bay access to Corisco Island in Antonina Bay, between November 1985 and November 1986. The results were compared with deep-water salinity, pH, temperature, dissolved oxygen and rainfall. ZoBell 2216E, at the salinities 0‰ and 32‰, was used as culture media. Principal component analysis showed that the concentration of heterotrophic bacteria was consistently lower in sandy sediments near the entrance of the Paranaguá Bay. Otherwise, the silty sediments did not differ significantly from the other sediments. Highest halophilic levels were recorded at the mouth of Maciel tidal creek. These results suggest that the amount of bacteria, in the region, is not related to sediment grain size but to currents speed which may benefit or not the organic matter deposition. Non halophile and halotolerant bacteria can be limited by high salinity. Contrary to observations in the water column, high pluviosity did not influence the heterotrophic bacteria number in the sediment from the studied area.

Key words: Heterotrophic bacteria, sediment, estuary, Paranaguá Bay, Antonina Bay.

INTRODUCTION

Marine regions near the coast, mainly estuaries, receive organic material input from rivers and land surrounding them. In tropical and sub-tropical regions the deposit is elevated as a result of mangroves with abundant fauna and flora properties which serves as a support to the food chain in the coastal region (19). However, in estuarial regions where larger quantities of heterotrophic bacteria occur (6,25,29), they develop both in the water column as well as in the sediments (29).

Several studies have shown that the number of heterotrophes is higher in superficial layers of sediment than in the water column. This fact is due mainly to larger amount of organic material, greater adhesion surface and an environment subject to few modifications. As such, being ideal for the development of bacteria (8,20,30,31,34). However it was also observed that the wind in flat regions had an influence because during turbulence a part of the organic material already settled was resuspended and, consequently, the bacteria as well (24).

Despite the number of heterotrophic bacteria is much lower than the total number of bacteria (10), they reacted fastly to environmental variations, specially at easily degradable organic material (27). This fact lead

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Hoppe (9) to place them as an important aid in organic pollution monitoring.

Regarding the Brazilian coast, few studies have been performed on heterotrophic bacteria in sediments. We could cite Pagnocca et al. (21, 22) who collected sediment from a polluted beach near the city of Pedra de Guaratiba on Sepetiba Bay (RJ). Using agar prepared with sea water as culture media, the authors noted levels of saprophytes ten times higher in the sediment than in the water column (3.8×10^4UFC.ml^{-1} in water and 4.2×10^5UFC.g. dry sed.$^{-1}$ in sediment). Kolm and Corrêa (11) analyzed the temporal and spacial variation of saprophytic bacteria in the sediments of a sandy beach in Pontal do Sul (Paraná), near the estuary entrance of the Paranaguá Bay, and observed higher levels in the high tide region.

The present work was thus developed with the objective to analyze the temporal and spatial variation of heterotrophes in the Paranaguá and Antonina Bays sediments and their relation with environmental factors.

STUDY AREA

The estuary system of Paranaguá (Lat. 25°16'34"S; Long. 48°17'42"W) is the largest lagoon complex of the Paraná State. It extends for about 50 km inland from its access, flanked by Ilha do Mel, to the west of the city of Antonina and Northward to the city of Guaraqueçaba (1) (Fig.1).

As a basis in the land drainage system the estuarine complex of Paranaguá could be divided in two main sectors: a) those formed by Antonina and Paranaguá Bays, therefore mentioned as objects of this study, and b) those composed by Laranjeiras, Pinheiros and Guaraqueçaba Bays and by the inlets of Benito and Itaqui (5, 18) (Fig. 1).

The Paranaguá Bay is located north from the "Praia de Leste" plain and continues inland from Ilha do Mel to Teixeira Island. Just after Teixeira Island the name of the bay changes to Antonina Bay (1, 5).

On the fringe of the Paranaguá and Antonina bays, on the flat islands and plains and along the river banks

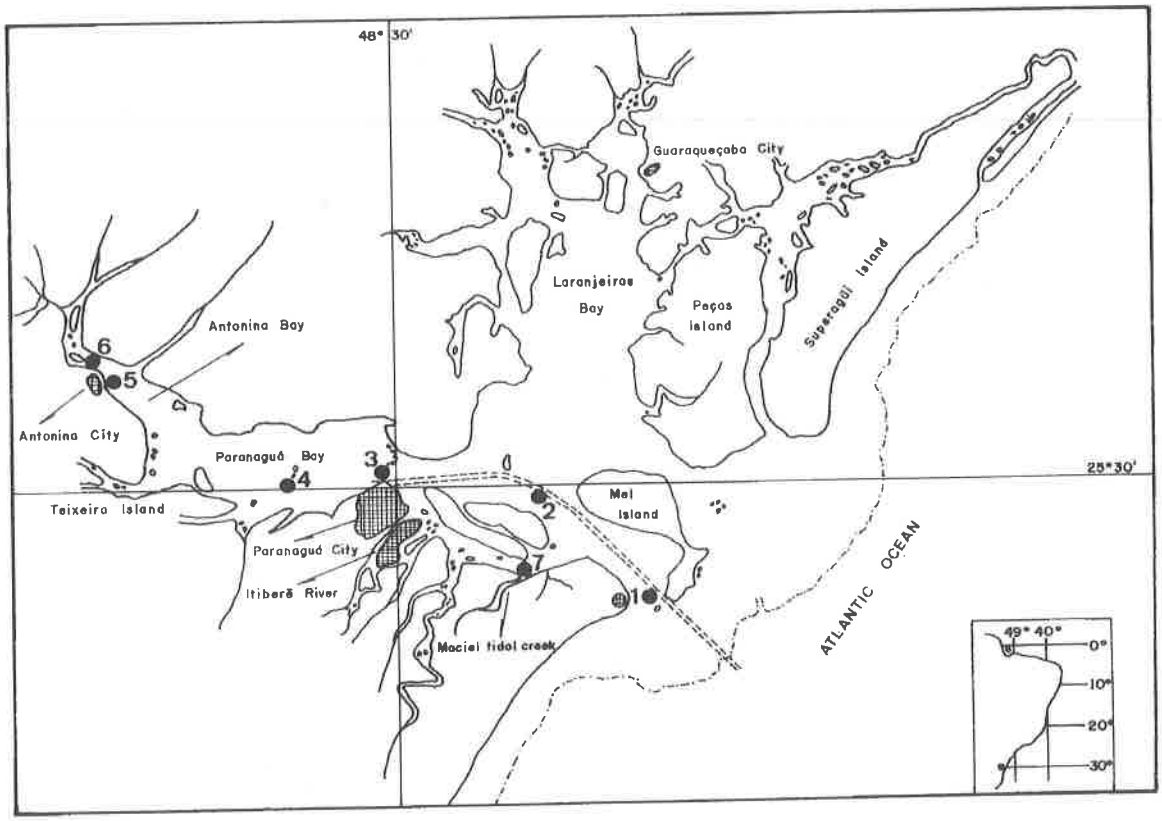

Figure 1. Paranaguá and Antonina Bays, with the seven sampling stations.

and tidal creeks mangrove formations can be found (*Rhizophora mangle, Laguncularia racemosa* and *Avicennia schaueriana*). These vegetal formations were described by Müller (18) as fringe or islet types, underdeveloped and, according to Rebello and Brandini (23), lead to organic debris development in the region. The *Spartina alterniflora* (1, 15) can be found in regions where water moves slower.

According to Lana (14) the bays could be divided in two sectors according to own hydrographic and sedimentologic characteristics. The eastern section extends from the bay access until the east of Paranaguá City. The western section extends from Paranaguá City westward. The sediments from the eastern section were composed by fine, well-selected sand, similar to that of adjacent ocean beaches and to the internal continental shelf. In the central and internal areas of low energy, those of the western sector predominate with fine sediment, poorly and most poorly sorted, formed by silt, clay and sand (1,14)

The region's average annual temperature is 21.1°C with an average amplitude of 7.9°C and an annual precipitation of 2000 mm. The weather could be defined as transitional tropical (17).

The most important cities, Paranaguá (110.889 pop.) and Antonina (17.280 pop.), are lacking adequate sewage treatment systems and as such pollute the adjacent marine areas. Beside this, Paranaguá's portuary activities require a constant dragging in the access channel. Despite the region being considered by Kolm and Absher (12) as little polluted there is a need for constant monitoring.

MATERIALS AND METHODS

Collections were performed monthly between November 1985 and November 1986. The sediment samples on the surface (> 2 cm of depth) were obtained with a Van Veen sampler at seven distinct points in the Paranaguá and Antonina Bays (Fig. 1). All samples were collected aseptically and transported to the laboratory on ice.

As no previous records of heterotrophic bacteria in the sediment of the region existed, we adopted dilutions of 1:10, 1:100 and 1:1000 in distilled water for non halophilic bacteria (Bd), and in aged sea water diluted with distilled water in a proportion of 3:1 ($S \approx 32‰$) for halophilic bacteria (Bs). Each sample consisted of one gram of moist sediment. Separation of the bacteria from the sediment was performed throught manual agitation for 3 minutes in its initial dilution. One milliliter of each sample and dilution was inoculated in culture media and the heterotrophic bacteria (HPC) were enumerated by pour plate technique. The culture media used was a ZoBell 2216E, modified by Gunkel and Gilbricht (7), prepared with destilled or sea water. The plates were incubated at 28°C for 48 hours (non halophile) and seven days (halophile). To record the results following dilutions were chosen: 1:10 for non halophilic bacteria (Bd), in station 1 and 1:100 for the others stations; 1:100 for halophilic bacteria (Bs) in stations 1, 2, 3 and 4 and 1:1000 for stations 5, 6 and 7 with an average of the parallels. The standardization of the number of heterotrophic bacteria was made by gram of dry sediment.

The dry weight and the degree of moisture of the sediment were obtained from a sediment gram submitted to an oven at 60°C for 48 hours.

Temperature (T) and hydrogen ion concentration (pH) were obtained from the field with a standard thermometer and pH-meter. The salinity (S), dissolved oxygen (OD) and pluviosity (P) were obtained from the Phytoplankton Laboratory and from the meteorological station in the Marine Studies Center of the Federal University of Paraná. All the abiotic data used refer to levels found in deep water collected a little above the sediment.

For multivaried analysis purposes the sediment types were transformed in values of 1 to 3 in ascending order of environmental energy (1=silty; 2=sandy-silt; 3=sandy).

To evaluate the main trends of data variation we logarithmized (natural logarithms), centered, reduced and treated the data of the principal components in the analysis (PCA) (2,16).

RESULTS

The data on heterotrophic bacteria from the eleven collections done in each of the seven stations along the Paranaguá and Antonina Bays are presented in Figs. 2A and 2B.

Fig. 3 illustrates the sediment type and salinity obtained in the sampled months.

Figs. 4 and 5 represent the factorial plains of the principal component analysis (PCA) resulting from the correlation sources.

The observations were plotted in factorial plains to help on axis interpretation. Only the first two principal components were analyzed. They contributed 97.99% to total variance (Table 1).

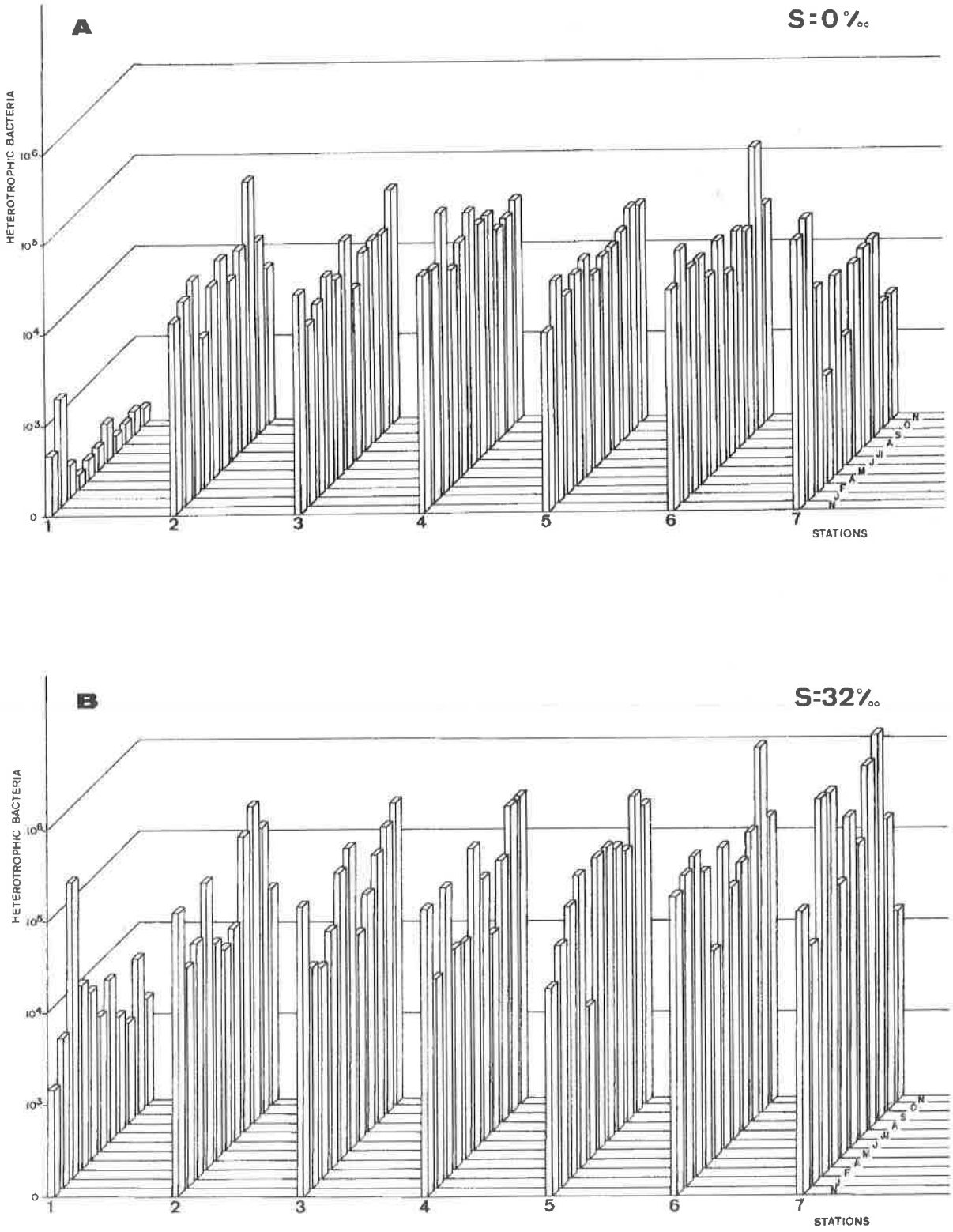

Figure 2. Absolute frequency of heterotrophic bacteria- non halophilic (A) and halophilic (B) in the months of sampling by the stations.

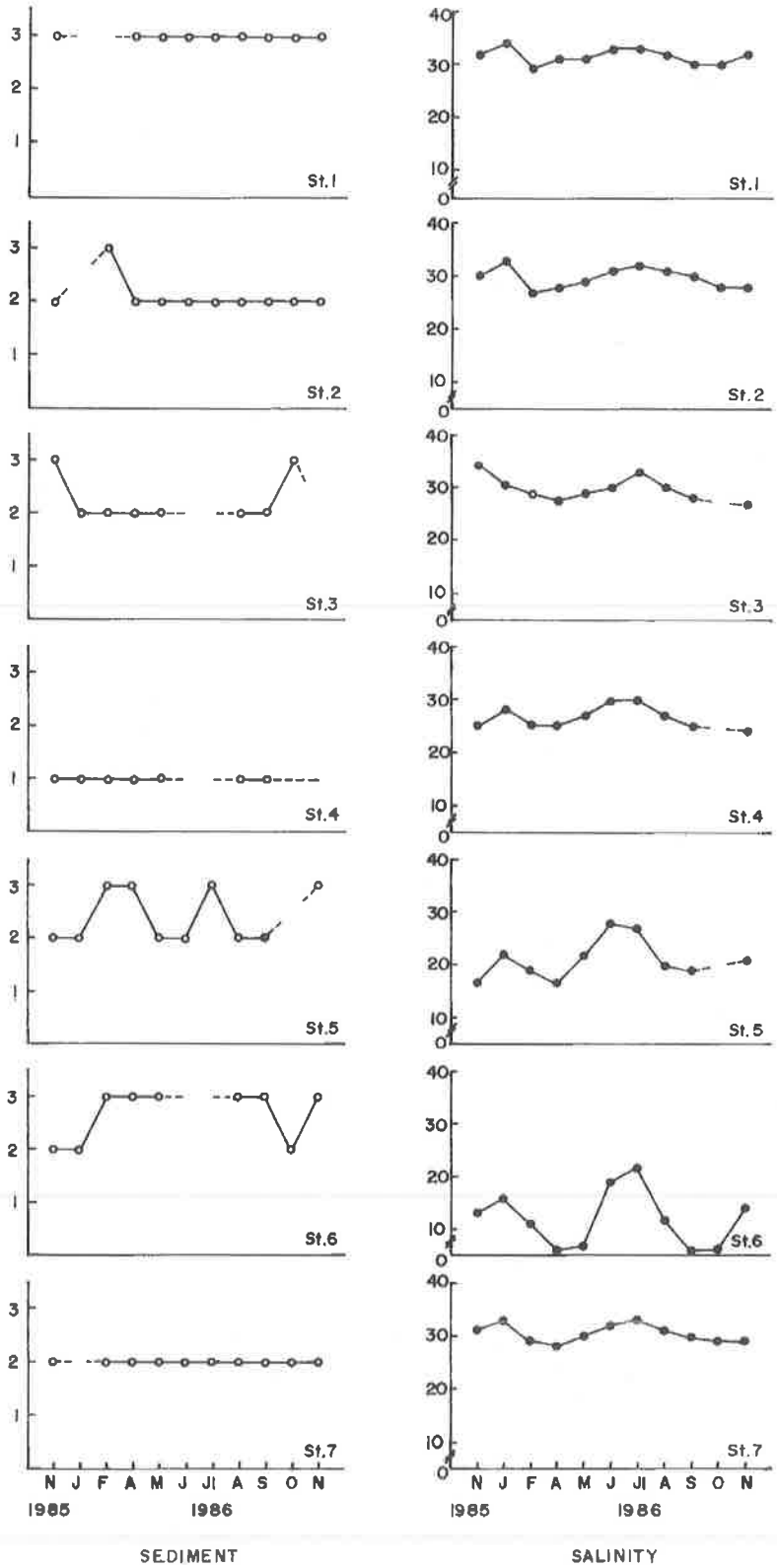

Figure 3. Types of sediment (1= silty; 2= sandy-silt; 3= sandy) and salinity of deep water.

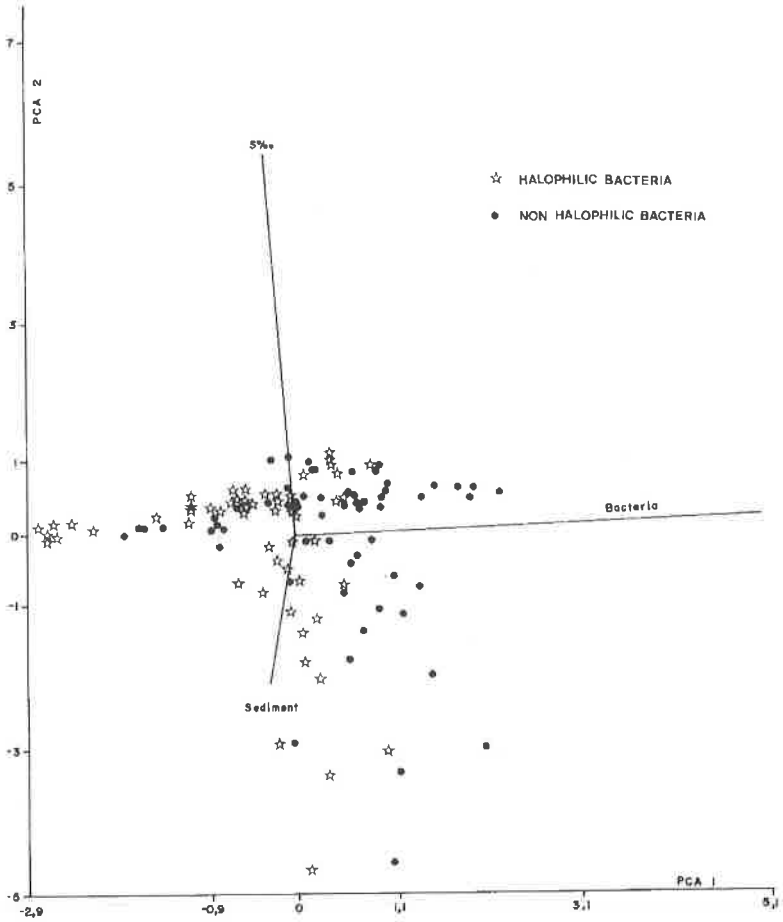

Figure 4. Position of biotic and abiotic data obtained in the space of main components according to types of bacteria.

Table 1: Variance of three first principal components of PCA, with respective individual and cumulative variances (in percentage).

Component Number	Variance Percentage	Cumulative Percentage
1	92.38424	92.38424
2	5.61119	97.99543
3	2.00457	100.00000

The first component shows approximately 92.38% variance and a positive correlation with the number of heterotrophic bacteria (non halophiles and halophiles).

Component two shows 5.61% of total variability and is positively related to salinity (S) and negatively with the type of sediment (Sed).

Throughout the studied period we consistently verified lower quantities of non halophiles than halophyiles (Fig. 2 and 4).

Lower values of heterotrophes, as much halophiles as non halophiles, were observed at the bay access (Station 1) with a minimum value of 176 UFC.g.dry sed.$^{-1}$ non halophiles, in April, 1986 (Figs. 2A and 5). The larger values occurred at the mouth of Maciel tidal creek (Station 7), with a maximum of 2.4×10^6 UFC.g.dry sed^{-1} of halophiles in September, 1986 (Figs. 2B and 5). From station 6 to station 5 there exists a salinity gradient with larger halophilic counts in the first rather than the last. Regarding all stations the differences in bacteria amount were not significant in themselves (Fig. 5).

In the same way the number of heterotrophic bacteria showed no correlation with seasons, pluviosity,

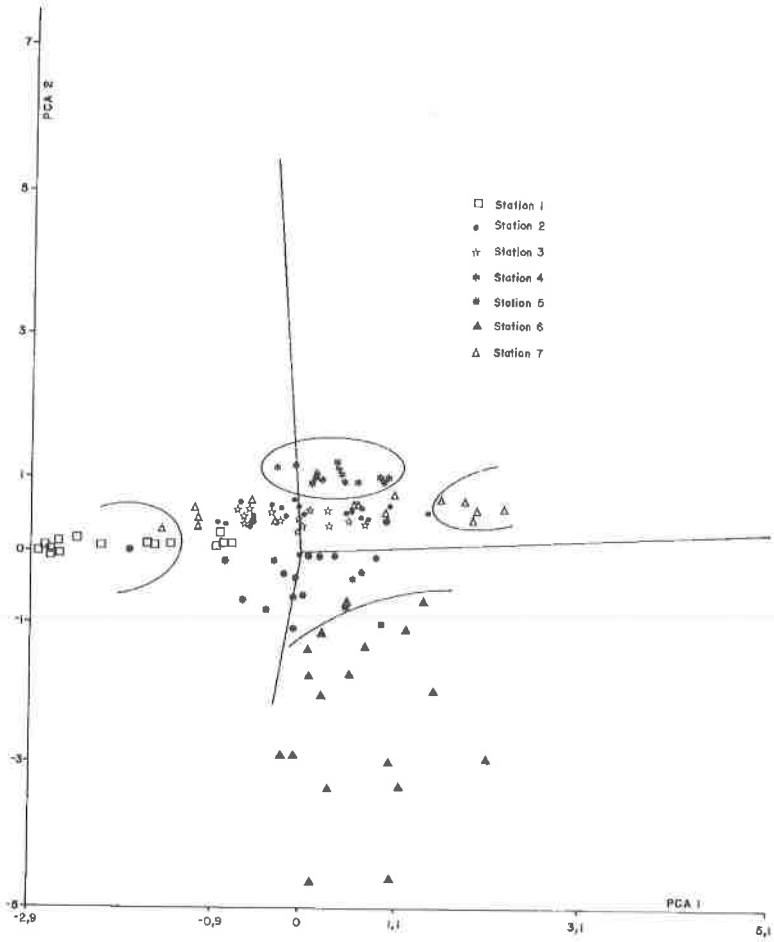

Figure 5. Position of biotic and abiotic data obtained in the space of main components according to sampling stations.

temperature, dissolved oxygen and hydrogen ion concentration.

DISCUSSION

Bacteria in natural waters and sediments have for a long time been considered important agents in the decomposition of organic matter and mineral regeneration, besides being food for superior organisms (32). However the studies of the structure of the indigenous bacteria flora that participates in the flux of matter in marine ecosystems is scarse.

The number of bacteria found in sediment superficial layers differs from those in the water column by few factors of ten (31) due to the larger accumulation of organic matter. Such observations coincide with the results obtained by Kolm and Absher (12) who found in the superficial water of Paranaguá and Antonina Bays lower quantities of saprophytic bacteria (with a maximum of 8.03×10^3 UFC.ml^{-1}) than those observed in the sediment (with a maximum of 2.38×10^6UFC.g.dry sed^{-1}).

Various authors (13,28,29,31,33) describe an inverse relationship between the size of the grains and the number of bacteria. In the Paranaguá and Antonina Bays (which will be designated under the name of Paranaguá Bay), this fact could be verified only in station 1 (Figs. 2 and 5). As described by Lana (14) the substrate found in this region is composed by fine, compact, quartz sand (70% of grains between 0.12 and 0.17 mm). It is presumed, however, that the cause of a lower bacteria level is not directly associated to the size of the grains but to the fact of being a high energy region and being considered a natural extention of the neritic adjacent environment (3,4). The strength of the waves, ebb, and the flow of the tides constantly wash

the sediment in this region, leaving it poor in organic material and thus making the development of halophilic bacteria difficult. The high salinity restricts the non halophiles and halotolerants (Figs. 4 and 5).

The sediment of station 4 is composed by very fine silt (Fig. 3) with 70% of the grains around 0.08 mm, poorly sorted, with a high water retention level, intermediate levels of limestone (9.7%) and high grades of organic material (14). Based on these informations we expected to find a greater heterotrophic amount in this region. However the values of the non halophiles as well as halophiles were similar to those from station 2 and 3 (Fig. 5). This result suggests that in the three stations could exist sufficient quantities of easily degradable organic material for an ideal bacteria development, or yet that may have occurred a measure deficiency caused by difficulties in handling the sediment.

In station 6, despite the low salinity, relatively high amounts of halophiles were found (Figs. 4 and 5). It is believed that in this region the high frequency of halotolerants in the sediment, which develop with salinities between 5 and 20‰ (26,29) and which grow in higher salinity culture media, cause it difficult to differentiate between halotolerants and halophiles.

The largest values of halophile bacteria were recorded at station 7, where a maximum of 2.38×10^6 UFC.g.dry sed^{-1} was observed in September 1986. This sediment was sampled in a depression with a maximum estimated depth of 20 m and situated at the mouth of Maciel tidal creek, as described by Kolm and Absher (12). According to Lana (14) the sediment in this area is structurally complex with 16 distinct texture classes, from fine to grains. Levels of calcium carbonate are intermediary (6.7%) and those of organic material high. Due to being a trench, it appears that deep currents are slower than those of the surface, thus favoring growth and development of bacteria. Also, the fact that the study area was located at the mouth of a sea arm and not at a river, with salinities similar to those found in external regions of the bay (Fig. 3), explains the small quantity of non halophiles.

In contrast to the observations of Kolm and Absher (12) for surface waters, high rainfall periods did not influence the development of heterotrophic bacteria in the sediments of Paranaguá Bay. There was also no clearly defined gradient for the reduction of bacteria from the inside to the entrance of the Bay.

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RESUMO

Distribuição espacial e variabilidade temporal de bactérias heterotróficas em sedimentos das Baías de Paranaguá e Antonina, Paraná, Brasil

A variação temporal e espacial da concentração de bactérias saprófitas foi observada em sedimentos arenosos, areno-lodosos e siltosos entre a barra de acesso da Baía de Paranaguá e a Ilha do Corisco na Baía de Antonina (SE Brasil). As amostras foram coletadas entre novembro de 1985 a novembro de 1986, em sete pontos amostrais distintos ao longo de um gradiente de salinidade. Os resultados foram comparados com a pluviosidade mensal e com a salinidade, pH, temperatura e oxigênio dissolvido da água de profundidade. O meio de cultura utilizado foi o ZoBell 2216E, com duas salinidades (0‰ e 32‰). A análise dos componentes principais evidenciou que, na área e no período estudado, a quantidade de bactérias heterotróficas foi consistentemente menor no sedimento do tipo arenoso da entrada da Baía de Paranaguá. Contrariamente ao esperado, o sedimento siltoso não diferiu significantemente dos demais. Os maiores valores de halófilas foram registrados na desembocadura da Gamboa do Maciel. Tais resultados sugerem que o fator limitante da quantidade de bactérias nesta região não seja o tamanho dos grãos de sedimento e sim a energia do meio ambiente e conseqüente acúmulo de matéria orgânica. Não halófilas e halotolerantes ainda podem ser limitadas pela salinidade. Ao contrário do que ocorre na coluna d'água, períodos de intensa pluviosidade não influenciaram o desenvolvimento de bactérias heterotróficas no sedimento da região estudada.

Palavras-chave: Bactérias saprófitas, sedimento, estuário, Baía de Paranaguá, Baía de Antonina.

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ASSOCIATION BETWEEN *CRYPTOCOCCUS LAURENTII* AND *EUCALYPTUS CAMALDULENSIS*

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ABSTRACT

Ninety five samples of plant debris collected from November 1993 to July 1995 under the canopies of *Eucalyptus camaldulensis* plantation in the northeastern state of Sergipe, Brazil, were examined for yeast of the genus *Cryptococcus* growth. *C. laurentii* was repeatedly isolated from samples collected under the canopies of the trees during all the period of study. The long lasting positivity suggests colonization of these microenvironments and points to a saprobiotic natural source of *C. laurentii* related to *E. camaldulensis*. Flower buds and green leaves, also examined, produced negative results. Essential oils extracted from *E. camaldulensis* showed ability to inhibit the growth of *C. laurentii* and both varieties of *C. neoformans*. These findings argue against the possibility of endophytic relation between these yeasts and *E. camaldulensis*.

Key words: *Cryptococcus laurentii*, saprobiotic source, *Eucalyptus camaldulensis*.

INTRODUCTION

Cryptococcus spp comprise encapsulated yeasts related to the heterobasidiomycetes. Most of them are plant pathogens or saprobes and only few species are known to cause infection in man and animals. Cryptococcosis is a cosmopolitan disease caused by melanine producing *Cryptococcus neoformans*. Other species, such as *C. albidus* and *C. laurentii*, are occasionally referred as agents of human infection (3, 10, 11, 20, 22, 23). It is interesting to notice that the thermotolerance to 37°C and the ability to produce melanine, occasionally observed in *C. laurentii*, turn it a potential human pathogen. *Filobasidiella neoformans* and *Filobasidium floriform* are the teleomorphs of *C. neoformans* and *C. albidus*, respectively. The teleomorph of *C. laurentii* is not known yet (14).

C. neoformans comprises the varieties *neoformans* and *gattii*. The cosmopolitan variety *neoformans* is easily isolated from environmental sources and classically related to pigeon habitats (*Columbia livia*). Probably it occurs originally in tree or other plant debris and not only in pigeon excreta (17, 18). The

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epidemiological role of pigeon habitats was overestimated because some recent data have suggested decaying wood as its original natural source (12, 15).

The natural habitat of *C. neoformans* var. *gattii* was unknown until 1990, when Ellis and Pfeiffer (5, 6) isolated a serotype B of the yeast from plant remains collected under the canopies of flowering *E. camaldulensis* trees in Australia and in San Francisco, California, USA. Pfeiffer and Ellis (24), later on, isolated this variety from a hollow of *Eucalyptus tereticornis*, which is closely related to *E. camaldulensis* and shows a similar geographical distribution. More recently, this variety was isolated from plant debris obtained under the canopies of a small plantation of *E. camaldulensis* in the surroundings of the city of Teresina, capital of Piaui, Northeast of Brazil (28).

C. neoformans clinical isolates in Brazil reveal that the variety *gattii* predominates in the Northeastern region, pointing out that this region may be an important endemic area for this variety. This was confirmed in Teresina, state of Piauí, where 31 (91,2%) out of 34 strains of *C. neoformans* isolated from non-AIDS patients in Teresina belonged to var. *gattii* (1).

E. camaldulensis is native of Australia and has been introduced in Hawaii, Southeastern California, Mexico, Brazil and regions of Africa and Southeastern Asia. Eucalyptus trees are suggested to be the only natural source for the var. *gattii* (7).

Matos et al. (21) isolated *C. laurentii* from leaves of *Eucalyptus* spp in the state of São Paulo and first suggested the association of the yeast with eucalyptus trees. Duarte et al. (4) isolated *C. laurentii, C. macerans, C. uniguttulatus, C. ater, C. neoformans* var. *neoformans, C. albidus, C. hungaricus, C. kuetzingii* and *C. heveanensis* from flowers, fruits, leaves, barks and debris of *Eucalyptus* spp trees in Santafé de Bogotá, Colombia. Natural sources of species of the genus *Cryptococcus* are insufficiently known in Brazil, especially in the Northern and Notheastern regions. Thus, we studied the association between *E. camaldulensis* trees and yeasts of the *Cryptococcus* genus in the state of Sergipe, Northeast of Brazil.

MATERIALS AND METHODS

The study was conducted in an area of 35 ha in the town of São Cristovão, Sergipe. This area had been planted with *E. camaldulensis* using seeds imported from Australia and corresponds to one of the two largest plantations of this species in the State (Figs. 1 and 2).

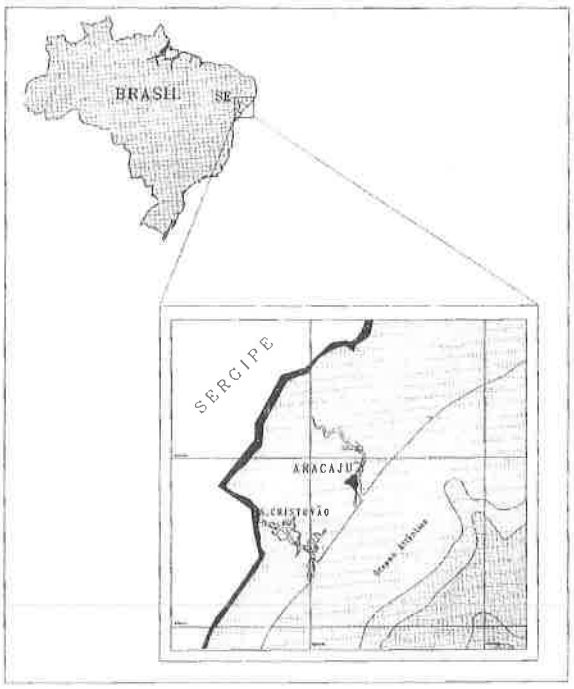

Figure 1. Area of study (São Cristovão, Sergipe, Brazil).

Figure 2. The saprobiotic association of *Cryptococcus laurentii* with *E. camaldulensis*.

From November 1993 to July 1995 five samples of plants debris (wood, bark, leaves, flowers) were collected monthly under the canopies of the trees. As flowering occurred sporadically, 15 samples of flower buds were also collected.

After homogenization of the samples, 1 g of them was suspended in 50 ml of sterile physiological saline with cloramphenicol (0,2 g/l), vigorously shaken for 3 minutes and allowed to settle for 20 minutes. The supernatant (0,1 ml) was plated in each of 10 Petri

dishes containing niger cloramphenicol agar (NSA) and incubated at 30°C. During the last three months of the investigation, 0,1 ml of supernatant was also injected subcutaneously in mice. The animals were sacrificed after 30 to 45 days and samples of the brain, lung, liver and spleen were plated in NSA and incubated at 30°C.

Isolated yeasts that did not form pseudohyphae, hyphae or ballistospores, produced urease and assimilated inositol were considered to be *Cryptococcus* spp. Species were identified on basis of assimilation tests of sucrose, maltose, lactose, dextrose, galactose, melibiose, erithritol, potassium nitrate, urea and peptone.

Internal portions of the buds were seeded in NSA plates; other buds were included in paraffin and cut sections were stained by Gomory methenamine silver stain and then analysed for the presence of fungi.

Essential oils extracted from *E. camaldulensis* and *E. citriodora* by the water vapor capture method (2) were tested for their antifungic activity against the isolated *C. laurentii* strain and *C. neoformans* var. *gattii* (11.868 HEC-INCQS) and *C. neoformans* var. *neoformans* (10.714 HEC-INCQS) standard strains. The method proposed by Grove and Randall (9) was used: 0,05 ml of pure oil or diluted in equal parts in dimethyl sulfoxide (DMSO) were put into small holes in Sabouraud agar on Petri dishes and seeded with the strain in test. After 24 to 48 hours of incubation at 30°C, zones of growth inhibition (halos), when formed, were measured.

RESULTS

C. laurentii was isolated throughout all the collection period, with 37/95 (38,9%) positive samples. Despite some sites presented higher frequency of isolation than others (Table 1), no correlation with the flowering period was observed. It is worth to point out that flowering occurred very irregularly. *C. laurentii* was isolated even from the inoculated mice, with slightly lower results than those observed with the NSA plating method. None of the positive animals presented signs of disease, the number of formed colonies was always small and the brain presented the largest average of isolated colonies of the fungus. The strains recovered from the inoculated animals were difficult to maintain in subcultures in Sabouraud agar. Culturing of flower buds resulted negative for *Cryptococcus* and the histologic sections did not show any fungal structures. The attempts to isolate fungi from green leaves and tree barks resulted also negative.

Oils extracted from leaves of *E. citriodora* were more effective in their capacity to inhibit the growth of the tested strains than oils extracted from *E. camaldulensis* more than three years old. *C. neoformans* var. *gattii* was the strain that showed the lowest sensibility to the oils. *C. laurentii* showed decreasing sensibility to the essential oils extracted from *E. citriodora, E. camaldulensis* less than one year old and *E. camaldulensis* more than three years old. The same behavior was observed with the standard strains of *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (Table 2).

DISCUSSION

Ellis and Pfeiffer (6) were the first ones to isolate *C. neoformans* var. *gattii* from the air and decomposing leaves, limbs and bark under the canopies of *E. camaldulensis* trees, suggesting a biotrophic association between the fungus and the plant, with aerial dispersion of basidiospores occurring together with the blossoming. However, attempts to isolate *C.*

Table 1. Monthly distribution of *C. laurentii* according to the collection site.

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
1993											1; 2	2
1994	1;2;5	1;2;3;4	1;2;5	1;2;5	NC	NC	1	1;2;5	1;2;3	2;3	1;2;5	2
1995	3;4;5	1;2;3	*	*	*	2	2					

1; 2; 3; 4; 5 = Collection sites with positive samples
* = Negative samples; collection sites had recently been burned
NC = Not collected
Average of isolation: 2,500 colonies of *C. laurentii* / 1 g of the sample.

Table 2. Mean diameters (in mm) of the zones of growth inhibition (halos) of *Cryptococcus* strains by *Eucalyptus* essential oils.

Oil	*E. citriodora*		*E. camaldulensis* Y		*E. camaldulensis* O	
strain	(T)	(1:2)	(T)	(1:2)	(T)	(1:2)
C. neoformans:						
var. *gattii*	> 20	9	13	11	7	7
var. *neoformans*	> 20	13	> 20	> 20	8	8
C. laurentii	> 20	15	14	11	11	11

T = Non diluted oil
1:2 = Oil diluted in equal parts with DMSO
E. camaldulensis Y = oils extracted from plants less than 1 year old
E. camaldulensis O = oils extracted from plants more than 3 years old

neoformans from flower buds, green leaves, bark and plant debris of the *E. camaldulensis* trees in Sergipe were negative. On the other hand, *C. laurentii* was isolated from litter under the canopies of the same trees, during all the study period, suggesting colonization of this environment.

The results of sensibility tests of different species of *Cryptococcus* against essential oils extracted from leaves of *E. camaldulensis* and *E. citriodora* do not support the hypothesis of an endophytic association between *C. neoformans* and eucalyptus trees. Lemos et al. (19) also demonstrated the susceptibility of *C. neoformans* to essential oils extracted from *E. maculata* as well as from other plant species.

More recently, Pfeiffer and Ellis (25) proposed that the growth of *C. neoformans* var. *gattii* associated with eucalyptus, particularly in species with great quantities of lignin and polyphenols in their composition like *E. camaldulensis*, is the main natural source of this variety. In another study, in Colombia, *C. neoformans* var. *neoformans, C. laurentii* and six other species of *Cryptococcus* were isolated from different samples (blossoms, fruits, leaves and decayed materials under the canopies) obtained from eucalyptus (4); however, *C. neoformans* var. *gattii* was not isolated.

From an ecological viewpoint, the existence of a microorganism in a certain biotope is a result of physical and chemical factors that favour its development, of the availability of suitable substrata and the interaction with other microorganisms, competitors, antagonists and predators. In this process of sequential degradation, fungi have a special participation, as a result of their heterotrophic activities on plant debris in the soil (27).

Members of Tremellales, which occur in natural habitats related to decomposing wood, produce encapsulated yeasts in their haploid state and several of them exhibit characteristics ascribed to the genus *Cryptococcus* (26). Moreover, phylogenetic tree analysis based on 17S rRNA sequences suggests proximal evolution of *Filobasidiella* and *Tremella* species (16). *C. neoformans* var. *neoformans* was demonstrated in hollows of several species of trees, and the long period of positivity suggest colonization of these microenvironments (17). In these biotopes, the relationship with the plant tissue is of a saprobiotic nature, associated with wood decomposition in the process of the hollow formation. The study suggest that *C. neoformans* probably integrates a succession of yeasts participating in the process of wood biodegration not restricted to the hollow trees.

Gonzales et al. (8) identified several species of *Cryptococcus*, among other yeasts, involved in the process of delignification of the lumber from Chilean forest. They demonstrated a succession of species involved, in which *C. laurentii* was isolated during the intermediate phase and *C. neoformans* in the final phase of the decaying process. *C. albidus* var. *diffluens* had only a small participation in the different stages of wood biodegradation.

Different species of *Cryptococcus* should participate in different stages of decomposition of eucalyptus debris and possibly other plant species, establishing a saprobiotic association. In this study, performed in young plantations (there were no trees more than five years old), it is possible that the amounts of polyphenols and lignin in the plant debris under the *E. camaldulensis* canopies were less than those in older plantations, reducing then the chance to isolate *C. neoformans*, but providing abundant growth of *C. laurentii*. It is worth considering that lignin is a polymer of very recalcitrant plant carbon that under the action

of peroxidases and phenoloxidases liberates phenolic compounds, methanol, and aromatic acids (27). Unlike other species of the genus, *C. neoformans* is characterized by its regular production of phenoloxidase, which is able to oxidize enough types of aromatic diphenols and diaminade compounds. This enzyme was recently identified as a lacase (29). Besides, it should be considered that some strains of *C. laurentii* may produce brown colonies on NSA medium (13), suggesting production of phenoloxidase, deserving further studies.

Conclusively, it is possible that the debris of *E. camaldulensis* under the canopies of the trees is progressively submitted to decomposition, in a dynamic changing process related to the occurrence and relative abundance of different species of *Cryptococcus* in these microhabitats.

RESUMO

Associação entre *Cryptococcus laurentii* e *Eucalyptus camaldulensis*

Foram coletadas de novembro de 1993 a julho de 1995 noventa e cinco amostras de restos vegetais sob as copas de *Eucalyptus camaldulensis* plantados no Estado de Sergipe, Nordeste do Brasil, objetivando o isolamento de leveduras do gênero *Cryptococcus*. *C. laurentii* foi isolado repetidamente de amostras coletadas sob as copas das árvores. A contínua positividade sugere colonização destes microambientes e indicam a ocorrência saprobiótica de *C. laurentii* relacionada a *E. camaldulensis*. Botões florais e folhas verdes, também examinados, produziram resultados negativos. Óleos essenciais extraídos de *E. camaldulensis* mostraram capacidade para inibir o crescimento de *C. laurentii* e ambas as variedades de *C. neoformans*. Estes achados tornam remota a possibilidade de relação endofítica destas leveduras com *E. camaldulensis*.

Palavras-chave: *Cryptococcus laurentii*, fonte saprobiótica, *Eucalyptus camaldulensis*

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STARVATION-INDUCED STRESS RESISTANCE DEVELOPMENT IN A GENETICALLY MODIFIED *PSEUDOMONAS FLUORESCENS* STRAIN

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ABSTRACT

The response of a genetically modified *Pseudomonas fluorescens* to nutrient starvation and starvation-induced stress cross-protection were investigated. Strain BR12 was starved for carbon, nitrogen, phosphorus and sulphur individually and for all nutrients in defined mineral media and exposed for 6 h to chemical (ethanol 20%), oxidative (H_2O_2 20μM), osmotic (NaCl 3M), cold shock (0°C) and heat shock (47°C) stresses at different incubation times. Response to starvation and stress cross-protection development were evaluated by viable bacteria counts. There was a significant increase in resistance of late log phase cultures grown in rich medium to stress, except for ethanol, in all starvation situations. Multiple nutrient starved cultures were more resistant to stress than individual nutrient starved ones. This strain inoculated in oligotrophic stream water microcosms also showed the starvation-induced stress protection mechanism but it presented a higher resistance to ethanol than cultures starved in mineral media. The acquisition of nonspecific resistance to stress can favour the persistance of Genetically Modified Microorganisms (GMMos) in apparently unfavourable conditions.

Key words: *Pseudomonas fluorescens*, GMMo, starvation response, stress resistance.

INTRODUCTION

A major stress factor faced by bacteria in most environments is nutrient limitation in conditions of fluctuating nutrient availability. This produces a series of physiological adaptations in non-differentiating organisms resulting in cells in a starvation-survival state (14,15,17). These adaptations provide a mechanism by which many bacterial species persist in the environment until the appropriate conditions become available for them to grow. Theoretically, it only takes one cell of any species per environment for survival of the species in that environment (13). The starvation for macronutrients such as carbon, nitrogen and phosphorus elicits a global cellular phenotipic response affecting cellular morphology, gene expression and other aspects of cell metabolism (21). One adaptive response is the development of cross-protection against several types of stress in starved bacteria that has been shown in *Escherichia coli* (6,8,9), *Salmonella typhimurium* (18), *Vibrio* sp. (7,17), *Pseudomonas putida* (5), and *Pseudomonas fluorescens* R2F strain

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(22). This process enables bacteria to survive in unfavourable situations that otherwise would be lethal to them.

This is an important issue to be considered in the release of genetically modified microorganisms in the environment. The changing environmental conditions may alter significantly some aspects of the expected behaviour of genetically modified microorganisms (GMMos) like persistence, gene expression, biological containment and sensibility to termination procedures. The knowledge of adaptive responses of GMMos to stress would provide a more realistic base to evaluate the risks involved in their environmental release.

The starvation response of a genetically modified *Pseudomonas fluorescens* strain as well as starvation-induced stress protection development were investigated. Although this bacterium was designed to colonize maize roots, a habitat relatively rich in nutrients, previous studies revealed that it has a high dispersion rate in the environment, resulting in its spread to bulk soil and groundwater aquifers. Consequently, its response to nutrient stress is an important aspect to predict its persistence under the oligotrophic conditions prevailing in these environments.

MATERIALS AND METHODS

Bacterial strain and growth conditions:

Pseudomonas fluorescens strain BR12 was isolated from maize rhizosphere (1, 2, 3), and genetically modified by the introduction of a Tn5 transposon containing the *cryIVB* gene of *Bacillus thuringiensis* var *morisoni* and a kanamycin resistance marker (*nptII*). It was grown overnight in Luria Broth (20) on a rotary shaker (150 rpm) at 28°C, harvested by centrifugation (5000xg) at room temperature, washed twice and ressuspended in 0,85% NaCl prior to inoculation in starvation media and in microcosms containing oligotrophic stream water.

Nutrient starvation in mineral defined media:

The individual nutrient starvation conditions were achieved by exhaustion of nutrients in defined mineral media. The general composition of defined mineral media used was: 0,4% glucose, 1,5 mM NH_4Cl, 40 mM Na_2PO_4, 20 mM Na_2SO_4 in saline (0,85%). Although, nutrients concentrations in the media composition varied for each starvation condition. The starvation for nitrogen (NH_4Cl), phosphorus (Na_2PO_4) or sulphur (Na_2SO_4) was obtained by reducing 10 times their concentration, in the medium. The carbon starvation was achieved by reducing 20 times the glucose concentration in the medium, and the multiple nutrient starvation media consisted only of saline (0,85%). Triplicate experiments were inoculated with *P. fluorescens* BR12 to a density of approximately 10^7 cells/ml, incubated at 28°C and sampled periodically during 40 days. The strain was exposed to individual nutrient such as carbon, nitrogen, phosphorus and sulphur and multiple nutrient starvation conditions.

Nutrient starvation in pristine oligotrophic water microcosms:

The strain was inoculated in a density of approximately 10^8 cells/ml, in duplicate, in microcosms containing sterile oligotrophic stream water. The water was sterilized by filtration through 0,22μm Millipore membrane filter. The microcosms were incubated at 28°C and sampled at 15, 30 and 45 days to investigate the starvation-induced stress resistance.

Nutrient starvation response:

The nutrient starvation response, in terms of the population dynamics of strain BR12, was evaluated by enumerating viable cells at 1,3,7,15,21,30 and 40 days of incubation on the starvation media. The viable bacteria were enumerated by triplicate plating of aliquots of serial dilutions of starved cultures on Plate Count agar (PCA) supplemented with kanamycin (50μg/ml).

Stress resistance development:

The development of stress resistance was tested on late logarithmic phase bacteria grown in rich medium, starved bacteria in mineral media and stream water microcosms, by exposing them to challenge protocols. These protocols consisted of the exposure of cultures and microcosm samples to 20% ethanol, hydrogen peroxide (2μM), NaCl (3M), 0°C and 47°C, in order to determine their resistance to chemical, oxidative, osmotic, cold shock and heat shock stresses, respectively (5). Aliquots of the starved cultures were added to triplicate tubes of the same media or microcosm water (to prevent an additional stress factor) and then submitted to stress conditions for a period of 6 hours. The chemical, oxidative and osmotic stress treatment tubes were incubated at 28°C. The challenge protocols were done simultaneously with the enumeration of the total viable population of starved cultures in mineral media and microcosms. The stress resistance development was evaluated by enumeration

of viable bacteria counts as described previously, after the period of stress exposure. The time dependent sensitivity was determined from the percentage of stress resistant viable cells present in total viable population of starved cultures. Geometric means of bacterial counts and arithmetic means of percentages were compared using analysis of variance (ANOVA), and differences were considered significant at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Response to stress starvation:

The viability of *P. fluorescens* BR12 cells exposed to individual and multiple nutrient starvation conditions evaluated by viable counts is shown in Fig. 1. Viable counts of cultures exposed to individual nutrient starvation conditions declined during the experiment loosing their viability between 7 and 15 days. Multiple nutrient starved cells also declined in viability until 15 days, and then they stabilized at a population size of approximately 10^3 ufc/ml and remained at this level until the end of the experiment. There was no significant ($P < 0,05$) difference between the behaviour of the cultures exposed to different starvation conditions. The *P. fluorescens* BR12 strain showed a higher sensitivity to starvation stress than other bacteria studied elsewhere (5, 6, 11, 18) and also presented a different response to the different types of starvation. *Pseudomonas putida* strain KT 2442 was found to be fully viable for one month when starved for either glucose, nitrogen or for all nutrients (5). The culturability of *Pseudomonas fluorescens* DF57 was not affected by either carbon, nitrogen or phosphorus starvation during 4 days (11). *E. coli* starved for carbon and nitrogen and *Vibrio* sp. S14 either carbon or multiple nutrient starved also presented a higher viability (6, 17). Generally, phosphorus starvation revealed a more significant role in the lost of viability in bacteria (4, 5, 6, 7, 10, 17). Sulphur limitation, in some cases (5) gave this effect as well , but this fact was not observed in our study.

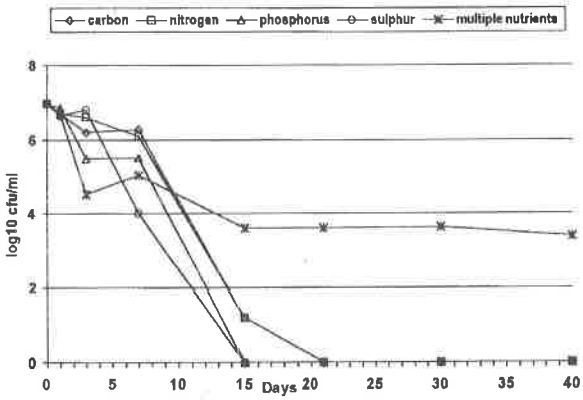


Figure 1. Response of *P. fluorescens* BR12 to individual and multiple nutrient starvation in mineral defined media.

Development of starvation-induced stress resistance:

The viable counts of resistant cells present in cultures grown in rich media and starved cultures in mineral media exposed to chemical, oxidative, osmotic, heat and cold shock stresses are shown in Figs. 2a and 2b. The time dependent sensitivity was determined

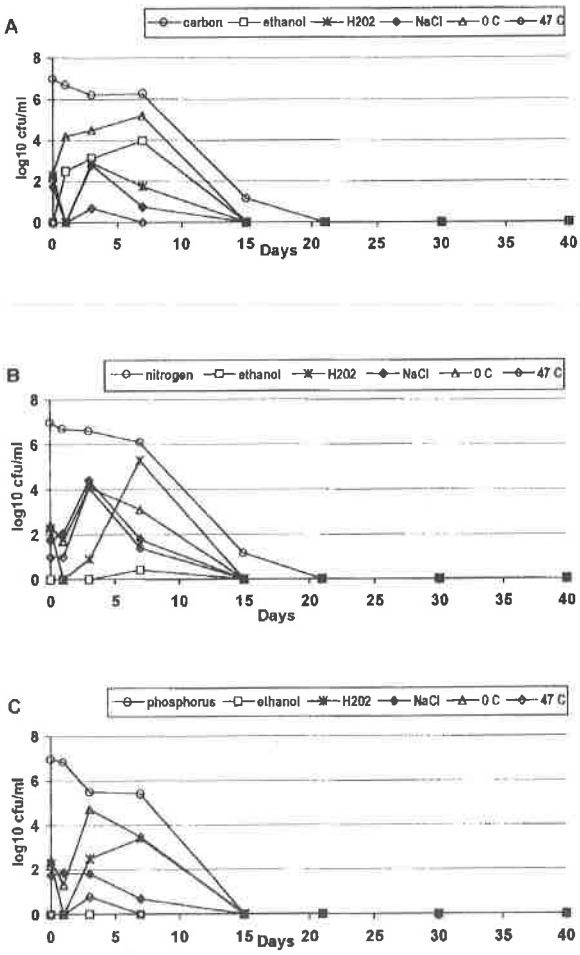
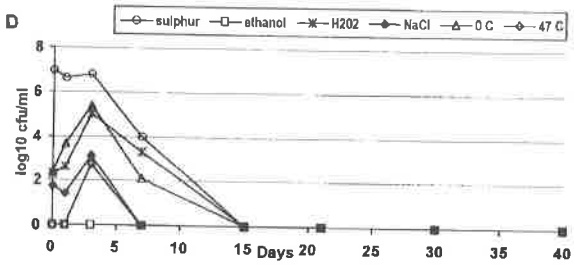


Figure 2a. Response of starved *P. fluorescens* BR12 cultures to stress: **A** – carbon, **B** – nitrogen, **C** – phosphorus.



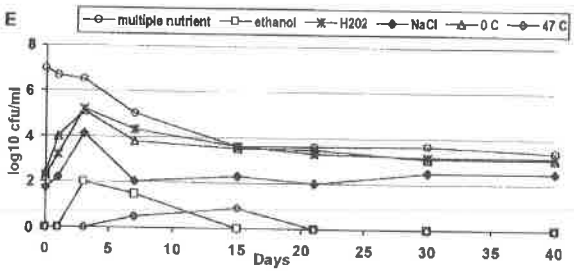


Figure 2b. Response of starved *P. fluorescens* BR12 cultures to stress: **D** – sulphur, **E** – multiple nutrients

Table 1. Resistance to chemical, oxidative, osmolarity, cold shock and heat shock stresses in individual nutrient starved and non starved *P. fluorescens* BR12 cultures expressed as % of total viable population enumerated.

	Time of Starvation (days)			
	0[a]	1	3	7[b]
Ethanol				
sulphur	0	0	0	0
phosphorus	0	0	0	0
nitrogen	0	0	0	0,0002
carbon	0	0,006	0,09	0,6
H_2O_2				
sulphur	0,002	0,009	1,6*	22*
phosphorus	0,002	0	0,1*	0,9*
nitrogen	0,002	0	0,0002*	15,5*
carbon	0,002	0	0,05*	0,003*
NaCl				
sulphur	0,0006	0,0006	0,02*	0*
phosphorus	0,0006	0,001	0,02*	0,002*
nitrogen	0,0006	0,002	0,7*	0,005*
carbon	0,0006	0	0,04*	0,0003*
0°C				
sulphur	0,003	0,1	4*	1,2*
phosphorus	0,003	0,0003	16,25*	0,98*
nitrogen	0,003	0,001	0,3*	0,1*
carbon	0,003	0,3	2*	8,5*
47°C				
sulphur	0	0	0,008*	0
phosphorus	0	0	0,002*	0
nitrogen	0	0	0,3*	0,002
carbon	0	0	0,0003*	0

[a] inoculum grown in LB broth until late log phase.
[b] after 7 days the starved cells lost their viability and the stress resistance could not be calculated.
* significant time related variations ($p < 0,05$) in stress resistance.

from the percentage of viable resistant cells present in total viable population of challenged cultures (Tables 1, 2 and 3). Except for ethanol (Tables 1 and 2), there was a stress resistance development in the cultures of *P. fluorescens* BR12 exposed to different starvation conditions, in mineral media, in relation to cells in late log phase cultivated in rich medium (LB broth). This has been observed for *E. coli* (8, 9, 12), and *Pseudomonas putida* KT 2442 (5) although these bacteria also presented starvation induced resistance to ethanol in contrast to *P. fluorescens* BR12. Similarly to *P. putida* KT2422, the strain studied did not present resistance to temperatures much higher than growth temperature, as recorded for *E. coli* K12 strains which developed heat resistance up to 57°C (8). The multiple starved cultures presented a significant higher resistance against the different types of stress tested, except for ethanol, than cultures exposed to individual nutrient starvation conditions. The latter did not present a significant variation between them in the patterns of resistance against stress conditions tested. These results differ from those recorded for *Vibrio* sp. S14 in which the starvation induced development of resistance against stress conditions such as temperature upshift, UV irradiation and cadmium chloride exposure, appears to be identical for multiple nutrients and carbon starved cells (7,17). Givskov et al (5), in their study with *P. putida* KT2442 did not recorded any difference in stress resistance induced by different types of starvation conditions. The influence of starvation on stress resistance development was not recorded until 3 days after the onset (Tables 1 and 2) which is probably the time required for exhaustion of the nutrients. The behaviour of resistance patterns evaluated by viable counts showed, in general, a dynamics characterized by a peak followed by a decline, probably due to loss of culturability of the cells as a consequence of the starvation process (16,

Table 2. Resistance to chemical, oxidative, osmolarity, cold shock and heat shock stresses in multiple nutrient starved and non starved *P. fluorescens* BR12 cultures expressed as % resistant cells of total viable population enumerated.

	Time of Starvation (days)							
	0[a]	1	3	7	15	21	30	40
Ethanol	0	0	0,003	0,03	0	0	0	0,01
H_2O_2	0,002	0,03	4,7*	18,18*	100*	55	34,9	62,5
NaCl	0,0006	0,003	0,4*	0,1*	5,5	2,5	7,9	12,1
0°C	0,003	0,2	3,7*	5,9*	87,5*	72,5	30	50
47°C	0	0	0	0,003	0,2	0,01	0	0

[a] inoculum grown in LB broth until late log phase.
* significant time related variations ($p < 0,05$) in stress resistance.

Table 3. Resistance to chemical, oxidative, osmolarity, cold shock and heat shock stresses in microcosms inoculated *P. fluorescens* BR12 cultures expressed as % of resistant cells of total viable population enumerated.

Time (days)	Stress Protocols				
	Ethanol	H_2O_2	NaCl	0°C	47°C
0[a]	0	0,002	0,0006	0,003	0
15	0,5	12	1	1	0,03
30	2	35	2,9	3,1	0
45	6,7	43	1,78	25	0

[a] inoculum grown in LB broth until late log phase.

19). The higher resistance against osmolarity and heat shock stresses for the cultures starved either for carbon, nitrogen, phosphorus or sulphur was recorded at 3 days (Table 1). The development of resistance to oxidative and cold shock stresses varied between the cultures. In the first case, the carbon starved cultures developed their highest resistances at 3 days and the nitrogen, phosphorus or sulphur starved ones developed theirs only after seven days (Table 1) and the inverse situation was recorded for cold shock stress. The multiple nutrient starved cultures presented a different pattern of development of resistance against the types of stress tested in relation to other starved cultures (Table 2). The stress resistance recorded was higher and developed more slowly reaching a peak at 15 days, except for osmolarity stress against which cultures presented an increasing resistance until the end of the experiment. The only case in which full resistance was recorded was the oxidative stress exhibited by multiple nutrient starved cultures. The starvation induced stress resistance observed in *P. fluorescens* BR12 along the experiment was much lower than that obtained for *P. putida* KT 2442 which exhibited full resistance to oxidative, heat and cold shock, ethanol and high osmolarity stresses in different times during the experiment, but higher than recorded for *P. fluorescens* R2f (22). The variation of stress resistance in relation to time was also different for these bacteria, especially for multiple nutrient starved cultures. In general, the maximal tolerance to stress is exhibited a few hours after the exposure of the culture to starvation conditions (5, 22) but for *P. fluorescens* BR12 multiple nutrient starved it takes many days (15 days).

The results indicate that the strain studied presented a higher stress resistance when individual or multiple nutrient starved in laboratory conditions. Although this pattern of resistance, obtained in culture media experiments, can be altered in microcosms studies in which bacteria are submitted to oligotrophic conditions similar to those prevailing in aquatic ecosystems. The same challenge protocols applied to *P. fluorescens* BR12 incubated in oligotrophic stream water microcosms for 15, 30 and 45 days also revealed the development of an starvation-induced stress resistance but the resistance patterns differed from those obtained in the experiment performed with defined mineral media (Table 3). A much lower pattern of resistance against osmolarity, cold shock and heat shock was recorded but, in contrast, the cells revealed a much higher resistance to ethanol than obtained in culture media studies. These results are probably subestimated because the total cultivable population of the strain in the microcosms at 15, 30 and 45 days represented only 15,8%, 7% and 0,03%, respectively of its total population enumerated by acridine orange counts. The cellular dimensions also declined from an average of 2.53 μm × 0.86 μm (biovolume of 1.47 μm^3) at the beginning of the experiment to 1.48 μm × 0.63 μm (biovolume of 0.46 μm^3) at 45 days. These results suggest that the cultures in the microcosms adopted a starvation-resistant survival state that was revealed by a progressive reduction in cultivability, viability and cellular dimensions along the experiment.

Although the starvation in environment is mainly due to the lack of carbon sources, the stress resistance observed in microcosms was higher than was recorded for carbon starved cultures. These results differ from

those obtained by van Overbeek et al (22) that observed a similar pattern of stress resistance development in *P. fluorescens* R2f, starved for carbon in soils and in culture media.

The results suggest that it is difficult to predict what kind and extent of stress resistance would be starvation-induced in bacteria in the extremely variable conditions of natural environments. This imposes a problem to the containment of genetically modified organisms released in the environment, because this mechanism of stress acquisition favours their persistence even in apparently unfavourable conditions.

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RESUMO

Resistência ao estresse induzida pela privação nutricional em *Pseudomonas fluorescens* geneticamente modificada

Uma estirpe de *Pseudomonas fluorescens* geneticamente modificada foi testada quanto à sua resposta à privação de nutrientes individuais (carbono, nitrogênio, fósforo e enxofre) e de múltiplos nutrientes e ao desenvolvimento de proteção cruzada à outros tipos de estresse decorrente da privação nutricional. A privação total foi obtida inoculando-se a estirpe (10^7 células/ml) em solução salina e a privação para os demais nutrientes em meio mineral definido, com baixas concentrações dos referidos nutrientes. Os sistemas foram incubados a 28°C por 40 dias. A resposta à privação nutricional e o desenvolvimento de resistência cruzada ao estresse foi avaliada através da enumeração de bactérias viáveis. As culturas privadas de nutrientes e em microcosmos foram submetidas por 6 h aos estresses químico (etanol 20%), oxidativo (H_2O_2 20μM), osmótico (NaCl 3M) e térmico (0°C e 47°C) para determinar a influência da privação nutricional sobre a resistência ao estresse. A privação nutricional induziu uma maior resistência a todos os tipos de estresse testados, exceto ao etanol, em relação ao inóculo cultivado em meio rico. As culturas privadas de todos os nutrientes apresentaram uma resistência significativamente maior aos estresses oxidativo, osmótico e térmico. A estirpe inoculada em microcosmos contendo água de nascente oligotrófica apresentou também uma resistência aumentada ao estresse porém, exceto pelo etanol, menor que a observada nos meios definidos. A aquisição de resistência inespecífica ao estresse pode favorecer a sobrevivência de microorganismos genéticamente modificados, no ambiente, mesmo em condições aparentemente desfavoráveis.

Palavras-chave: *Pseudomonas fluorescens*, geneticamente modificada, privação nutricional, resistência ao stress.

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ISOLATION AND CHARACTERIZATION OF *AZOTOBACTER* STRAINS FROM BRAZILIAN SOILS

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SHORT COMMUNICATION

ABSTRACT

Thirty-eight *Azotobacter* spp strains isolated from Brazilian soils were characterized for the following properties: utilization of different carbon sources, diffusible pigment production, melanin production, sodium-dependent growth, siderophore production, streptomycin resistance and antibiotic-like substances production. Four strains were identified: one as *A. armeniacus* and three as *A. paspali*.

Key words: *Azotobacter*, isolation, characterization, Brazilian soils.

Members of the genus *Azotobacter* are known to be free-living, nitrogen-fixing aerobic bacteria (20,21). These microorganisms are common soil inhabitants and are easily isolated from soils worldwide (5,8,20,21). The genus *Azotobacter* comprises six species: *A. chroococcum*, the most common species in soil; *A. vinelandii*, the best known to biochemists and geneticists; *A. beijerinckii, A. nigricans; A. armeniacus* and *A. paspali* (20). These bacterial species have attracted general attention because they can synthesize large amounts of biologically active substances: plant-growth hormones such as auxins, gibberellins and cytokinins (7), vitamins (9) and siderophores (12,14) or products with economic value: alginate (6) and polyhydroxyalkanoates, the components of biodegradable plastic (13,17).

Very little is known about the *Azotobacter* populations in Brazilian soils. Döbereiner and Pedrosa (5) reported that the occurrence of *A. chroococcum* and *A. vinelandii* is limited to soils with almost neutral pH, and thus they occur only sporadically in tropical soils as most of these are acid. However, *A. paspali* occurrence was reported in soils with a pH of about 5 and associated with the rhizosphere of *Paspalum* (5).

The objective of this work was to carry out the isolation of *Azotobacter* strains from four different Brazilian soils and to characterize them for the following properties: utilization of different carbon sources, diffusible pigment production, melanin production, sodium-dependent growth, siderophore production, streptomycin resistance and antibiotic production.

Soils. The following soil samples were collected: Dark-Red and Red-Yellow Latosols from under savanna vegetation (cerrado, Brasilia – D.F.), Red Yellow Latosol from Santa Marta Farm (Piraí,R.J.) and Red-Yellow Latosol from Mendanha Forest (Rio de Janeiro, R.J.).

Isolation procedures. Aerobic nitrogen-fixing bacteria were isolated on Winogradsky's nitrogen-free medium suplemented with 1% (wt/vol) sucrose and 15.0g of agar, pH 7.2 (20). Soil samples (10g) were

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shaken in 95 ml of sterile distilled water for 20 min. Successive decimal dilutions were made (10^{-2} to 10^{-4}) and 0.1 ml of these dilutions were spread on the surface of the nitrogen-free medium and the plates were incubacted at 30°C for 5-7 days. The large moist colonies were selected from the plates for purification and streaked onto Winogradsky's nitrogen-free agar medium with sucrose as the carbon source (20).

Cyst induction. Encystment was induced by transfering vegetative cells to the Burk's basal agar containing 0.3% n-butanol instead of glucose as the carbon source (2). These resting cells were studied using cultures at least 2 weeks old (20). Cysts were stained with acridine orange (20) and with a mixture of neutral red and light green (22). Wet mounts were prepared for fluorescence or light microscopic examinations.

Motility observation. Cultures grown for 24-48h at 30°C in Winogradsky's liquid medium supplemented with 0.1% ammonium chloride were examined for motility in wet mounts (20).

Pigment production. Production of diffusible pigments was examined on iron-deficient medium described by Thompson and Sherkman (20). The medium was inoculated by depositing 10µl of a culture suspension on the surface of the agar. The plates were incubated at 30°C for one week and were examined daily in daylight for diffusible pigment.

Utilization of carbon source. Winogradsky's nitrogen-free organic agar medium was prepared by adding 1% of organic substrate (rhamnose, caproate, caprylate, meso-inositol, mannitol and malonate). The plates were inoculated and incubated according to standard methods (20).

Resistance to antibiotics. Streptomycin (Sm) was added to Winogradsky's nitrogen-free sucrose agar medium in the following concentrations: 0.2; 2.0; 4.0; 6.0; 8.0; 25.0; 50.0 and 100 µg/ml. The inoculation was done by depositing a suspension of 10^6 cells/ml on the surface of the agar plates. The plates were incubated for 48h at 30°C and then examined for bacterial growth.

Sodium requirement for growth. The strains were grown in Winogradsky's nitrogen-free mineral broth medium supplemented with 0.1% ammonium chloride without sodium addition. The medium was inoculated and incubated at 30°C for 48h. The medium supplemented with 0.0125% NaCl was used as positive control for growth (11).

Melanin production. The production of diffusible pigment melanin in the presence of L-tyrosine and $CuSO_4 \cdot 7H_2O$ was examined by the method described by Cubo et al. (4).

Screening for antibiotic-like substances. The cross-streak test as described by Pramer and Schmidt (16) was used to establish whether or not *Azotobacter* strains were able to produce antibiotic-like substances on 0.2 strength Tryptic Soy Agar (Difco). The *Azotobacter* strains were used to make a central streak on the medium surface, then the plates were incubated at 30°C for 72h. After this period, the indicator strains were inoculated as a perpendicular streak to the azotobacter growth. The plates were reincubated at 30°C for 48h and then observed for the inhibition of the indicator strains.

Siderophore production. This test was conducted by using 0.2 strength Tryptic Soy Agar supplemented with chrome azurol S (CAS dye), a modified Schwyn and Neilands procedure (15,18).

Among one hundred aerobic nitrogen-fixing bacteria isolated from Brazilian tropical latosols, 38 strains were identified as belonging to genus *Azotobacter*: 30 strains from Dark-Red cerrado soil, 1 from Red-Yellow cerrado soil, 6 strains from Forest soil and 1 from agricultural soil. These strains formed large gram-negative ovoid cells and characteristic cysts in the presence of 0.3% n-butanol.

All 38 *Azotobacter* strains examined were motile and they did not produce diffusible pigments on the iron-deficient medium, however, two strains produced a brown-black pigment similar to melanin when grown with L-tyrosine. Siderophore production was observed for 8 strains (K-25, K-30, K-31, K-38, K-41, K-58, K-73, K-96). Sodium dependence for growth was observed only with one strain (K-31). It was possible to identify, with the tests used, 4 strains to species level, one belonging to *A. armeniacus* (strain K-25) and three to *A. paspali* (strains K-28, K-62 and K-77) (Table 1).

All *Azotobacter* strains were resistant to the minimum amount of streptomycin utilized (0.2 µg/ml), among these 2 were resistant to 0.2 µg/ml, 4 to 2µg/ml, 2 to 4 µg/ml, 2 to 6 µg/ml, 15 to 8 µg/ml, 5 to 25 µg/ml, 2 to 50 µg/ml and 6 to 100 µg/ml (the maximum amount tested). Seventeen *Azotobacter* strains showed antibacterial activity against at least one of the indicator strains examined. They were antagonistic to Gram-positive and Gram-negative bacteria. Table 1 shows the principal results obtained with the identified and non-identified strains showing Sm resistance up to 100 µg/ml and/or antagonistic activity.

Table 1. *Azotobacter* spp isolates from Brazilian soils[a].

strain	source[b]	species	melanin production	sodium-dependent growth	level of Sm resistance (μg/ml)	siderophore production	antibiotic production against[c]: G+	G–
K-20	B	nd	–	–	0.2	–	–	+ (j)
K-25	A	*A. armeniacus*	–	–	100	+	+ (d,e,f,g,h)	+ (i,j)
K-28	A	*A. paspali*	–	–	8	–	–	–
K-30	A	nd	–	–	25	+	+ (e)	+ (j)
K-31	A	nd	–	+	50	+	+ (d,e,g)	+ (j,l)
K-38	A	nd	–	–	50	+	+ (d,e,g,h)	+ (i,j,l)
K-41	A	nd	–	–	100	+	+ (d,e,g,h)	+ (j)
K-44	C	nd	–	–	8	–	+ (e,f,g,h)	–
K-52	C	nd	+	–	8	–	–	–
K-55	C	nd	–	–	100	–	–	–
K-58	C	nd	–	–	8	+	+ (d,e,f,h)	+ (j)
K-62	C	*A. paspali*	–	–	100	–	–	–
K-69	A	nd	–	–	25	–	+ (j)	–
K-73	A	nd	+	–	100	+	+ (d,e,f,g,h)	+ (i,j,l)
K-75	A	nd	–	–	8	–	–	+ (l)
K-76	A	nd	–	–	2	–	–	+ (j)
K-77	A	*A. paspali*	–	–	8	–	–	–
K-84	A	nd	–	–	8	–	–	+ (j)
K-85	A	nd	–	–	6	–	+ (g)	–
K-87	A	nd	–	–	8	–	+ (f)	–
K-88	A	nd	–	–	25	–	+ (f)	–
K-94	A	nd	–	–	100	–	–	–
K-96	A	nd	–	–	25	+	+ (f)	–

a Symbols: +, positive; –, negative; nd, not determined.

b **A**: Dark-Red Latosol from under savanna vegetation (cerrado); **B**: Red-Yellow Latossol from under savanna vegetation (cerrado), **C**: Red Yellow Latosol from Mendanha Forest.

c Test strains used: **d** (*Bacillus cereus*), **e** (*Bacillus megaterium*), **f** (*Bacillus subtilis*), **g** (*Staphylococcus aureus*), **h**(*Staphylococcus saprophyticus*) , **i** (*Escherichia coli*), **j** (*Morganella morganni*), **l** (*Pseudomonas aeruginosa*).

Azotobacter spp. are generally found in soils of slightly acid to alkaline pH, although in acid soils they can survive in microhabitats of neutral reactions (5,21). This ability was therefore important when considering the isolation of the microbial population from tropical areas, where most soils are acid. The soil is a reservoir of microorganisms with the capacity to produce antibiotics and recent evidence, both indirect and direct, has pointed to an important role for antibiotic production or antibiotic resistance in microbial interactions in soil (1,10,16,19).

Diazotrophic bacteria such as *Azotobacter* spp. have been isolated from the rhizosphere of a wide variety of plants in diverse regions of the world (5). The enrichment of streptomycin resistant bacteria in the rhizosphere of some plants has become increasingly accepted (1,10,19). Therefore, resistance to streptomycin may offer advantages to microorganisms in soil and root colonization. Nevertheless, this characteristic was not expected to be found in *Azotobacter* because all species of *Azotobacter* have been described as sensitive to 0.2 μg/ml of streptomycin (20). However, the *Azotobacter* spp isolated from Brazilian soils were resistant to this amount of streptomycin or even a higher concentration (100 μg/ml), a property that may offer advantage for these microorganisms to colonize their microhabitats in soil or in the rhizosphere, like the *Paspallum notatum* rhizosphere by *A. paspali*. An interesting aspect was that three 100 μg/ml streptomycin-resistant strains were isolated from a cerrado soil that is known to have a high population of *Streptomyces* spp that produce antibiotics, mainly streptomycin (3).

The *Azotobacter* strains isolated also produced substances like antibiotics and siderophores (iron chelators) that may account for the antagonistic activity against soil and rhizosphere microbial population. One *A. armeniacus* strain and 6 non-identified strains

inhibited Gram-positive and Gram-negative bacteria, a result contrary to *A. paspali* being the only species of this genus that is antagonistic to Gram-positive bacteria (20). The results reported here suggest that a more extensive investigation should be carried out on tropical isolates, specially regarding the biotic factors that control the microbial population in soils.

RESUMO

Isolamento e caracterização de cepas de *Azotobacter* de solos brasileiros

Trinta e oito cepas de *Azotobacter* spp. isoladas de solos brasileiros foram caracterizadas para as seguintes propriedades: utilização de diferentes fontes de carbono, produção de pigmento difusível, produção de melanina, crescimento dependente de sódio, produção de sideroforos, resistência à estreptomicina e produção de substâncias semelhantes a antibióticos. Quatro cepas foram identificadas: uma como *A. armeniacus* e três como *A. paspali*.

Palavras-chave: *Azotobacter*, isolamento, caracterização, solos brasileiros.

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SENSITIVITY OF MYCOBACTERIA TO DIFFERENT SPECIES OF *EUCALYPTUS* L'HERIT

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ABSTRACT

Sensitivity profile of 10 mycobacteria of clinical interest to eight different species of *Eucalyptus* L'Herit was studied. A Proportion Method Indirect Test was performed to detect resistant bacilli utilizing 5 and 10 mg/ml of essential oils incorporated to Lowestein-Jensen medium. *Mycobaterium tuberculosis* and other slow growing mycobacteria were more sensitive to essential oils than faster growing ones. The most effective essential oils, in decreasing order, were *Eucalyptus citriodora*, *Eucalyptus maculata* and *Eucalyptus tereticornis*.

Key words: Mycobacteria, *Eucalyptus*, sensitivity profile.

INTRODUCTION

Tuberculosis is the major cause death among infectious diseases, being responsible for almost 3 million deaths annually in the world (8,18). In Brazil, recrudescence of tuberculosis incidence is observed in spite of the intense treatment program using tuberculostatic drugs and large range vaccination with BCG. Nowadays, the number of tuberculosis cases is supposed to reach one hundred thousand cases (1). Considering São Paulo state alone, 18 to 19 thousand new cases and 1400 deaths are reported per year (5). However the control program of tuberculosis finds new challenges by the increase of multidrug-resistant tuberculosis (9) and emergence of Acquired Immunological Deficiency Syndrome – AIDS (5,12). AIDS reactivates latent tuberculous infections or eases infection by other mycobacteria resistant to tuberculostatic drugs (12,18). The chemotherapics currently used are not sufficient, due to the growing number of failures in tuberculosis treatment and lack of medicines for these mycobacteria (9). In this sense, further studies are essential in order to produce new specific drugs for these patients (10). Such agents have not been perceived as a high priority by pharmaceutical companies during the last 30 years(20) and coordinated efforts to screen antimicrobial compounds that have activity against *Mycobacterium tuberculosis* may well prove worthwhile.

Researches dedicated to the study of antimicrobial properties of higher plants, mainly of those that contain essential oils, are gaining importance (4,11,13,16). In Brazil, the use of plants to solve health problems is largely diffused as an alternative source mainly among low economic classes (15). However, no plants are available to cure tuberculosis, a wide-spread disease that affect mainly poor people. In the small number of reports related to screening for plants with antimycobacterial action, *Mycobacterium smegmatis* is the mycobacteria most frequently utilized (7,17). However, *M. smegmatis* is not pathogenic and moreover, it is a fast growing mycobacterium, while

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M. tuberculosis is always pathogenic and a slow growing species (19). The objective of the present investigation was the verification of the antimycobacterial activity of essential oils from 8 species of *Eucalyptus* L'Herit against mycobacteria of clinical interest.

MATERIALS AND METHODS

Bacterial strains. The sources of the bacterial strains used in this study are as follows: ten reference strains, obtained from the collection of the Institute Pasteur of Tuberculosis (CIP), Paris, and one strain of multidrug-resistant *M. tuberculosis* (B708/93), isolated from a clinical specimen in the laboratory of the authors. All of them were maintained on Lowestein-Jensen (LJ) medium slants at refrigeration temperature.

References strains used in this work were: *M. avium-intracellulare* CIP140310001; *M. chelonae* CIP 14040003; *M. fortuitum* CIP 1401001; *M. gordonae* CIP 140210001; *M. kansasii* CIP 140110001; *M. marinum* CIP 140120001; *M. scrofulaceum* CIP 140220001; *M. smegmatis* CIP 141330100; *M. szulgai* CIP 140240001 and *M. tuberculosis* CIP 140210001.

Essential oils from *Eucalyptus* L'Herit. Leaves of 8 different species of *Eucalyptus* L'Herit were obtained from Horto Florestal "Navarro de Andrade" of FEPASA (Ferrovia Paulista S/A), Rio Claro-SP, Brazil, and submitted to hydrodestilation process to extract essential oils, according to Brazilian Pharmacopoeia. The following species of *Eucalyptus* were analyzed: *E. botryoides* Smith, *E. camadulensis* Dehn, *E. citriodora* Hook, *E. deglubta* Smith, *E. globulus* Labil, *E. grandis* Smith, *E. maculata* Hook, *E. tereticornis* Smith.

Media and growth condition. For the performance of the tests, mycobacteria were inoculated into Lowestein-Jensen (LJ) medium containing essential oils (test tubes) and into the controls (LJ without oils). The test was performed using two different concentrations (5,0 and 10,0 milligrams per milliliter) of the essential oil. *M. marinum* and *M. chelonae* were incubated at 30°C and other mycobacteria at 37°C. The incubation period ranged from 7 to 10 days for fast growing species and 20 to 40 days for slow growing species.

Proportional Method – Indirect Test. The method recommended in the *Brazilian Manual of Tuberculosis* (2) with some modifications was utilized to determine mycobacterial resistance against different essential oils. Bacterial suspensions were adjusted to the optical density of McFarland standard nº 1 and subsequent dilutions (10^{-2}, 10^{-4} and 10^{-5}) were prepared. Two dilutions (10^{-4} and 10^{-5}) were used to inoculate the control tubes and two others (10^{-2} and 10^{-4}) to inoculate the test tubes. For each strain of mycobacteria, 2 parallel assays were performed.

Determination of sensitivity profile. To detect mycobacteria sensitivity against essential oils, the number of colony forming units grown on a drug medium (test tubes) was compared with the number on the control (LJ without oils). The ratio between resistant cells and the total viable population of the original inoculum was calculated and expressed as a percentage. A rate of resistant mutants below 1% was considered the limit of resistant mycobacteria that can be present in a bacilli population. When the rate was equal or higher than 1%, the sample was considered resistant.

RESULTS

The antimycobacterial action of essential oils derived from 8 differents species of *Eucalyptus*, as determined by the Proportional Method – Indirect Test, is shown in Tables 1, 2 and 3.

The slow growing mycobacteria were more sensitive than the fast growing. Within the slow growing mycobacteria, *M. tuberculosis* (Tables 1 and 2) was the most sensitive. Both strains of *M. tuberculosis* tested (CIP 140210001 and B708/93) were sensitive to 5 from 8 essential oils at 5,0 mg/ml drug concentration (Table 1). At higher concentration (10,0 mg/ml) they were sensitive to all oils tested, except to *E. deglupta* oil (Table 2). The second slow growing mycobacteria most sensitive to essential oils was *M. avium*. It was sensitive to 4 and 8 essential oils at 5,0 and 10, mg/ml concentrations respectively (Tables 1 and 2). For other slow growing mycobacteria, a varying degree of sensitivity, however close to *M. tuberculosis*, was verified (Tables 1 and 2). The essential oils in 5,0 mg/ml concentration were not efficient against fast growing mycobacteria, except *M. chelonae* that was sensitive to *E. citriodora* (Table 1).

E. citriodora oil presented the broadest range of action. It was active against one of the fast growing and all the slow growing mycobacteria, at the lower concentration (Table 3). In the decreasing order as to the efficiency, the *E. citriodora* oil was followed by the *E. maculata* oil, also effective against all slow growing mycobacteria, at the lower concentration and by *E. tereticornis*, efective against five slow growing ones.

Table 1. Efficiency of 8 *Eucalyptus* essential oils against mycobacteria (5 mg/ml concentration).

Mycobacterium tested	*E. botryoides* (%)*	*E. camadulensis* (%)	*E. citriodora* (%)	*E. deglupta* (%)	*E. globulus* (%)	*E. grandis* (%)	*E. maculata* (%)	*E. tereticornis* (%)
Fast growing								
M. chelonae	22,6	13,5	0,5	37,0	6,8	34,3	5,1	1,2
M. fortuitum	12,1	5,3	5,2	6,0	10,6	10,0	8,7	12,9
M. smegmatis	100	100	25,5	100	100	100	100	100
Slow growing								
M. avium	100	0	0	73,4	2,5	72,8	0	0
M. gordonae	75,2	19,2	0	15,2	82,4	19,6	0	0
M. kansasii	20,0	5,6	0,2	2,0	33,5	0	0,2	5,9
M. marinum	37,5	31,3	0	46,6	33,5	0	0	6,6
M. scrofulaceum	21,3	13,0	0	18,2	43,6	0	0	0
M. tuberculosis (reference strain)	6,0	0	0	100	35,4	0	0	0
M. tuberculosis (B-708/93)	11,6	0	0	100	31,2	0	0	0

* = Percentage of resistance.

Table 2. Efficiency of 8 *Eucayptus* essential oils against mycobacteria (10 mg/ml concentration).

Mycobacterium tested	*E. botryoides* (%)*	*E. camadulensis* (%)	*E. citriodora* (%)	*E. deglupta* (%)	*E. globulus* (%)	*E. grandis* (%)	*E. maculata* (%)	*E. tereticornis* (%)
Fast growing								
M.chelonae	0,6	0,3	0,5	26,5	0,8	0,1	0,4	0,2
M. fortuitum	6,0	4,5	0,2	4,1	6,0	0,3	0,1	0,3
M. smegmatis	100	31,3	3,0	28,3	100	3,0	13,3	4,3
Slow growing								
M. avium	0	0	0	0	0	0	0	0
M. gordonae	0	0	0	5,4	69,6	0	0	0
M. kansasii	0	0	0	0,3	0,4	0	0	0
M. marinum	0	0	0	15,0	28,6	0		
M. scrofulaceum	0	0	0	0	0	0	0	0
M. tuberculosis (reference strain)	0	0	0	33,4	0	0	0	0
M. tuberculosis (B-708/93)	0	0	0	15,6	0	0	0	0

* = Percentage of resistance.

Table 3. Number of mycobacteria species sensitive to two different concentrations of essential oils (critical proportion 1%).

Essential oils	Fast growing mycobacteria		Slow growing mycobacteria	
	5mg/ml	10mg/ml	5mg/ml	10mg/ml
E.botryoides	0	1	0	7
E.camadulensis	0	1	3	7
E.citriodora	1	2	7	7
E.deglupta	0	0	0	3
E.globulus	0	1	0	5
E.grandis	0	2	4	7
E.maculata	0	2	7	7
E.tereticornis	0	2	5	7

DISCUSSION

The method of diffusion on paper disks is an approach to determine drug sensitivity and is generally preferred by researchers. This methodology was properly standardized by Kirby and Bauer (3), being of choice in the clinical laboratories for the performance of the antibiogram tests. However, this method is not suitable for the study of active principles from plants, specially when one deals with organic compounds that are more covalent than polar. These compounds are not diffused or they diffuse poorely and irregularly on the surface of a solid medium, due to their low solubility in water. In the diffusion technique with paper disks, the diffusion of the compounds is essential. Considering that mycobacteria need a long period of incubation, the diffusion method is of limited application. Due to the high volatility and low polarity of the essential oils used in this study, the sizes of inhibition zone would result smaller than they should be.

The Method of Proportions – Indirect Test utilized in this work is adopted by all the Brazilian laboratories where antibiogram of *M. tuberculosis* is performed. This test was first described by Canetti (6) and it detects the ratio of resistant bacilli present in a sample of *M. tuberculosis* by using a fixed drug concentration, incorporated to the medium. In this work, two fixed concentration of essential oils (5,0 and 10,0 mg/ml) were incorporated to the LJ medium. The presence of egg yolk and glycerol makes possible the homogeneous and long lasting dispersion (around a month) of the essential oils in the medium. The Proportional Method Indirect Test on LJ medium proved to be a good methodology to detect mycobacteria sensitivity against essential oils. Results in Tables 1 and 2 show that, among all strains analyzed, the *M. tuberculosis* strains (reference strain CIP 140010001 and strain B 708/93) were the most sensitive. The recrudescence of tuberculosis, related or not to AIDS (12, 18) and the arise of strains with resistance to multiple drugs (9), make these data very promising. It is not a coincidence that many hospital formely specialized in the treatment of tuberculosis were located in mountain regions surrounded by different species of eucalyptus, cypress, pine-trees, etc. Benouda et al (4), testing essential oils against hospital pathogens, verified major activity of the eucalyptus against bacteria from bronchial washings. It is a popular opinion that the aromatic trees help in purification of the air.

Among the slow growing mycobacteria, *M. avium* is the most important agent of disseminated mycobacteriosis (18). It was the opportunistic mycobacterium more frequently isolated from HIV positive patients in Araraquara area (12). *M. avium* was sensitive to 4 of the 8 essential oils tested in the 5.0 mg/ml concentration (Table 1), being susceptible to all oils at 10.0 mg/ml concentration. Maruzella and Sicurella (14), studying the antimycobacterial action of 133 essential oils, verified higher susceptibility of *M. avium* in relation to other microorganisms. Thinking about modes of action of the oils against *M. avium*, the authors concluded that the essential oils may pass through mycobacterial envelope and interact with lipids of the mycobacterium wall, causing critical changes in it.

As to the fast growing mycobacteria (*M. chelonae, M. fortuitum* and *M. smegmatis*) they showed more resistance to all essential oils than the slow growing ones (Tables 1 and 2). This minor sensitivity of the fast growing mycobacteria agrees with observation of Maruzella and Sucurella (14). The slow growing mycobacteria are exposed longer to the antimicrobial agents and consequently their cell wall organization becomes more damaged. Another factor that should be considered is that mycobacterium wall is poorely permeable for water soluble drugs. The essential oils, being covalent, probably would not have difficulty to transpass the lipid rich mycobacterium wall and thus to perform their antimycobacterium activity.

Considering the spectrum of action of the essential oils tested, three *Eucalyptus* species showed a considerable broad action. In decreasing order they were: *E. citriodora, E. maculata* and *E. tereticornis* (Table 3). Kumar et al (11), studying the action of 24

essential oils against 15 bacteria, observed that all Gram negative species were sensitive to *E. tereticornis, E. camadulensis* and *E. grandis* oils. In this work (Table 3), *E. tereticornis* appeared as the third most efficient oil. These data reinforce the findings of Kumar et al (11), who showed that not all the eucalyptus essential oils have the same antibacterium activity. Probably, differences in the chemical composition of the components of the essential oils determine major or minor degrees in their antimycobacterial action.

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RESUMO

Sensibilidade de micobactérias a diferentes espécies de *Eucalyptus* l'Herit

A sensibilidade de 10 espécies de micobactérias de importância médica aos óleos essenciais de 8 diferentes espécies do gênero *Eucalyptus* foram estudados pelo Método das Proporções – Teste indireto. Os ensaios foram realizados semeando-se os bacilos em meio de Lowestein-Jensen contendo 5 e 10mg/ml de óleos essenciais. *Mycobacterium tuberculosis* e outras micobactérias de crescimento lento foram mais sensíveis aos óleos essenciais do que as micobactérias de crescimento rápido. Os óleos essenciais mais eficazes sobre as micobactérias, em ordem decrescente, foram aqueles provenientes de *Eucalyptus citriodora, Eucalyptus maculata* e *Eucalyptus tereticornis*.

Palavras-chave: Micobactéria, eucalipto, perfil de sensibilidade.

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IN VITRO DEGRADATION OF COAST-CROSS (*CYNODON DACTYLON*) BY RUMEN MICROORGANISMS ASSOCIATED WITH *SACCHAROMYCES CEREVISIAE* OR *HUMICOLA* SP

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ABSTRACT

Ruminal degradation of the dry matter and neutral detergent fiber of coast-cross (*Cynodon dactylon*) forage, when previously inoculated with *Saccharomyces cerevisiae* or *Humicola* sp., was estimated by *in vitro* incubation. The potential dry matter degradability was incresead in the presence of *S. cerevisiae*, whereas the incubation with *Humicola* sp. resulted in improvement of the neutral detergent fiber degradability. The rate of dry matter digestion was enhanced to above 15% with the inoculation with *S. cerevisiae*. The inoculation with *S. cerevisiae* or *Humicola* sp. increased the rate of digestion of the neutral detergent fiber in relation to the control treatment.

Key words: Digestion kinetics, microbial additives, rumen, tropical forages.

INTRODUCTION

Ruminants have played a major role in farming production for thousands of years, and have provided mankind with meat, milk and clothing (20). These mammals can feed on a great variety of plant forages (5). In tropical countries, the ruminant production is done mainly under extensive conditions using natural grasslands and crop residues, all of them of low nutritive quality (12). The reduced productivity of animals raised on these areas is a consequence of chemical and anatomical characteristics of the forage cell wall (NDF) (25). Plant cell walls comprise 20-80% of forage dry weight and are composed mainly of cellulose, pectin, hemicellulose and lignin (25). The amount, types and chemical interactions of these compounds vary among tissues within the forages, and these differences lead to substantial variations in plant digestibility (1).

Improved utilization of low quality roughage by ruminants could be achieved through: a) physical and chemical treatments (12); b) dietary supplementation that accommodates the nutritional requirements of the fibrolytic rumen microorganisms (6); c) use of microbial additives to manipulate cell wall degradation (3, 12, 13 21). The latter aspect has been used with great interest for the improvement of forage cell wall degradation. However, the results reported in the literature are inconsistent (7, 18, 26). The lack of experimental consistency is at least partly due to the great variation in the level of addition, ruminant species, diets and types of microbial additives (7).

Yeasts have been isolated from rumen contents, although aerobic strains are viewed only as alloctones

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or transients (14). *Saccharomyces cerevisiae* exhibits some degree of rumen viability (23). Actually this yeast has currently been used in ruminant feeding systems (7). The perception that yeast supplements are safe and wholesome, the current technology allowing the production of large quantities of yeast from inexpensive raw materials and the ample knowledge about *S. cerevisiae* physiology are the main features which make the use of this yeast attractive.

More than one mechanism of the yeast action in the rumen has been suggested including altered patterns of volatile fatty acids, enhancing pH and ammonia concentration and stimulating growth of rumen fibrolytic and lactate-utilizing bacteria (22). Thus, these mechanisms are able to improve the degradation of the forage cell wall (NDF) in the rumen.

Recently, the use of *Saccharomyces cerevisiae* and the white rot fungus *Armillaria heimii*, associated or not, were effective to improve *in vitro* and *in vivo* digestibility of forage neutral detergent fiber (12). In this study forages were inoculated with microbial additives immediately before exposure to the rumen microbial fermentation (12). The white rot fungus is essentially aerobic. Thus, they cannot grow when exposed to the rumen microbial ecosystem.

The rumen microbial ecosystem is anaerobic (20). Growth of microorganisms in an anaerobic environment may proceed by fermentation or by anaerobic respiration (11). The distinctive feature of poor quality forages and roughage is their slow rate and low extent of degradation when consumed by ruminants and exposed to the rumen fibrolytic microorganisms (1, 12). This may be explained by the thermodynamic imposition of anaerobiosis (11) and the competition between digestion and passage rates of forage cell walls in the rumen, which in turn limits the retention time of the substrate for the rumen microbial activity (20). To overcome these aspects, we hypothesized a two stage microbial treatment which has an aerobic phase outside the rumen and an anaerobic phase within the rumen. The aerobic phase was proposed to ensure the growth of selected fungi and to achieve a more potent fibrolytic activity of these microbial additives. In practice this stage is not much time consuming, because most farmers in Brazil cut the fresh forages, and only offer to the cows after they have been milked.

The aim of this study was to investigate the potential of *Saccharomyces cerevisiae* and the aerobic thermophylic cellulolytic fungus *Humicola* sp. to improve the rate and the extent of forage degradation when previously inoculated with these microbial additives.

MATERIALS AND METHODS

In vitro Procedure:

The *in vitro* degradation of dry matter (DM) and neutral detergent fiber (NDF) of coast-cross (*Cynodon dactylon*) hay was determined at 0, 6, 24, 36, 48, and 72 hours of anaerobic incubation carried out in 125 ml Erlenmeyer flasks containing 1.5 g of the sample grounded to pass through a 1 mm screen, 50 ml of buffer solution (8), 25 ml of rumen fluid and the microbial additives.

The rumen fluid was obtained from a rumen-cannulated Holstein steer fed 30% concentrate ratio (13% of crude protein expressed on a dry matter basis) and 70% coast-cross hay. Digesta were removed utilizing a manual vacuum pump adapted in an airtight container and immediately transported to the laboratory in a CO_2-filled vacuum. Rumen contents were squeezed through four layers of cheesecloth and the rumen fluid collected in a CO_2-filled flask.

The following treatments were used: a) Control (rumen fluid, buffer and forage sample); b) *S. cerevisiae* (rumen fluid, buffer and forage sample previously inoculated with 5 mg of *S. cerevisiae* marketed as compressed yeast); c) *Humicola* sp. (rumen fluid, buffer and forage sample previously inoculated with 5 mg of *Humicola* sp.); d) Negative control (rumen fluid, buffer, 5 mg of *S. cerevisiae* or *Humicola* sp). Initially the forage samples were weighed, placed into an Erlenmeyer and mixed with the 5 mg of microbial additive. To ensure the even distribution of microorganisms in the samples, 10 ml of distillated water were infused. These preparations and the negative control treatment were shaked in aerobiosis at room temperature during 2 hours. At the end of this aerobic stage, 25 ml of strained rumen fluid was included using anaerobic procedures. The Erlenmeyer flasks were capped with rubber stoppers equipped with one-way Bunsen valves, incubated at 39°C and vortexed continuously throughout the experiment. The negative control Erlemeyers were in triplicate, incubated at 0 and 24 hours.

The microbial additives were added to meet the assumption that an adult cow has 50 liter of rumen contents, and the microbial additive manufacturers recommend the intake of 10 g/day for each dairy cow. Thus 25 ml rumen fluid should be inoculated with 5 mg of these microorganisms. Fermentation were set

up in triplicate, and the results were described as the mean of each triplicate. After each incubation time the pH was measured with a digital pH-meter (Digimed/ DMPH1). At the end of the incubation the fermentation process was halted by rapid chilling to –10°C and stored at –4°C.

After each incubation period the contents of the Erlenmeyer were swirled and poured rapidly into a labeled, previously wheighed glass crucible sitting atop a filtration flask. During filtration a slight suction was applied, and approximately 25 ml of warm water was used to wash the inner surface of the Erlenmeyer and 15 ml to wash the inward of the crucible containing the forage residues. All samples and negative controls were filtered alike. The crucibles with the residues were dried over-night at 105°C and weighed after cooling in a desiccator. The samples and the residues of incubation were analyzed for dry matter (DM) and neutral detergent fiber (NDF) (2).

Mathematical Computations:

The true residue of incubation was obtained for each period by subtraction from the residue of their respective negative control. The level of degradation was calculated by determining the amount of the component (DM or NDF) that had disappeared during fermentation, and by comparing this value with the initial concentration

A spread-sheet programme (Excel 5 for Windows) was used for data handling and processing. Curve fitting was made using the program SAEG. This program uses the Marquardt's compromise limited by a 100 iterations to fit a model. The convergence criterion is achieved when there is a non-significative reduction in the residual sum of squares. The kinetics of DM and NDF degradation was described using the following model (9):

$$R(t) = B.exp(-c.t) + I,$$

where $R(t)$ is the residue after incubation on a given time t (h); B (%) is the insoluble but potentially degradable fraction, that could be calculated by the difference $[R(0) - I]$; c is the fractional rate-constant of degradation (h^{-1}); and I is the insoluble and undegradable fraction, which correspond to the asymptote reached by the model when t approaches infinity (9). To accomplish the fitting procedure, the following parameters amplitudes were taken: ($0 \leq B \leq 100$), ($0 \leq I \leq 100$) and ($0 \leq c \leq 10$). The initial estimates for the parameters B and I were taken after a visual appraisal of the degradation profiles, and the value 0.05 was assumed as the initial estimate for the first-order rate constant of degradation (c). The estimated kinetic parameters were described with each standartd deviation (Table 2). The Student's t-test was used to discriminate the differences between parameters, assuming $P < 0.10$.

RESULTS AND DISCUSSION

The inclusion of *Saccharomyces cerevisiae* increased the potential digestibility of DM for each incubation time in relation to the control treatment (Table 1, Fig. 1). At 24 and 72 hours of incubation an improvement of approximately 6-7% in the DM digestibility was observed. This was in agreement with earlier reports that digestibility increased in the order

Table 1. Dry matter and neutral detergent fiber residues after incubation[a].

Time of incubation	DM residues of incubation[b]			NDF residues of incubation[b]		
Hours	*S. cerevisiae*	*Humicola* sp	Control	*S. cerevisiae*	*Humicola* sp	Control
0	81.56 (18.44)[c]	85.32 (14.68)	84.75 (15.25)	96.57 (3.43)	95.40 (4.60)	96.72 (3.28)
6	72.96 (27.04)	78.22 (21.78)	79.10 (20.90)	81.48 (18.52)	76.27 (23.73)	89.35 (10.65)
24	50.19 (49.81)	55.23 (44.77)	53.59 (46.41)	57.25 (42.75)	56.66 (43.34)	63.18 (36.82)
36	45.56 (54.44)	51.79 (48.21)	51.25 (48.75)	54.13 (45.87)	52.40 (47.60)	56.32 (43.68)
48	44.95 (55.05)	49.99 (50.01)	49.39 (50.61)	52.06 (47.94)	47.95 (52.05)	55.23 (44.77)
72	42.07 (57.93)	44.79 (55.21)	45.32 (54.68)	49.99 (50.01)	46.39 (53.61)	53.70 (46.30)

[a] Each average comes from three observed values.
[b] Expressed in % of the initial values.
[c] Values in parenthesis correspond to the potential digestibility (100 minus the residue of incubation).

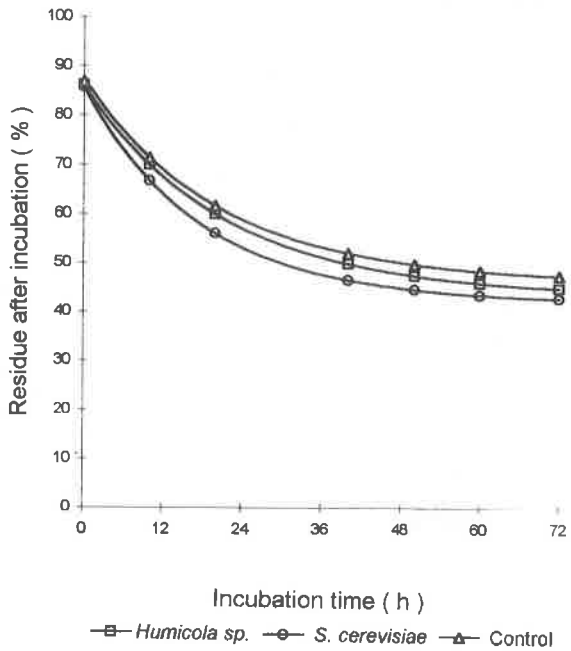

Figure 1. Potential degradability of the dry matter.

of 6 to 14% when *Saccharomyces cerevisiae* was used in vitro (4, 23). The DM undegradable residue (*I*) was almost 11% lower, indicating a positive effect on the extent of degradation (Table 2, Fig. 1). Although not significative, the digestion rate (*c*) was augmented approximately 15% with the addition of *S. cerevisiae* (Table 2). Similar results were obtained in previous experiments (4, 24).

Saccharomyces cerevisiae does not utilise starch, cellulose, nor hemicellulose because it lacks hydrolitic enzymes for these polymers (10). The specific mechanisms of action responsible for the positive effects upon the kinetic parameters remain unknown. Nevertheless, they could be explained by the yeast nutritional stimulatory effect which would lead to an increase in rumen fibrolytic microorganisms (7) or to the ruminal pH stabilization (22, 24). In this study, the addition of *S. cerevisiae* resulted in more constant values for the pH, especially on the range considered suitable (6.6-6.8) for the growth of all kinds of ruminal fibrolytic microorganisms (Fig. 3). The pH of the control treatment was lower in relation to the pH observed in the *S. cerevisiae* treatment (Fig. 3).

The incubation with *Humicola* sp. resulted in similar values for DM digestibility in relation to the control treatment. (Table 1, Fig. 1). The DM degradation rate (Table 2) and the indigestible residue (*I*) obtained after 72 hours of incubation were reduced to 3.4 and 6.7 percents (Table 2). Until 36 hours of incubation, the pH was similar to the one of the control treatment (Fig. 3). However, there was a pH reduction

Table 2. Kinetic parameters for the *in vitro* degradation.

	TREATMENTS		
Kinetic parameter	**Control DM**	***S. cerevisiae* DM**	***Humicola* sp. DM**
B	41.12[a] (2.158)[1]	43.58[a] (1.388)	42.48[a] (1.592)
c	4.96[a] (0.860)	5.72[a] (0.589)	4.79[a] (0.513)
I	46.79[a] (1.851)	42.16[b] (1.155)	43.64[a] (1.461)
r^2	96.53	98.56	98.4
	Control NDF	***S. cerevisiae* NDF**	***Humicola* sp NDF**
B	47.42[a] (1.900)	46.98[a] (0.966)	47.43[a] (1.295)
c	5.34[b] (0.622)	7.12[a] (0.470)	7.07[a] (0.624)
I	51.32[a] (1.631)	49.97[a] (0.716)	47.03[b] (0.951)
r^2	98.08	99.38	98.93

B, c, I = see test.

[1] The values in parenthesis correspond to the standart deviation; the number of degrees of freedom were calculated as follows: (18 + 18) – 2 = 34; the standard t value is 1.70.

[a, b] Means with unlike superscripts within rows differ ($P < 0.10$).

after 36 h, that could be attributed to unknown treatment effects.

Numerically the DM digestion parameters (*c* and *B*) in *S. cerevisiae* treatment were greater than the *Humicola* treatment (Table 1 and 2, Fig. 1). This could be explained by some degree of viability of *S. cerevisiae* which ocurred during the anaerobic incubation, or by its ability to use more efficiently the soluble components of the sample. If the yeast would utilize the soluble carbohydrates, a more stable ruminal pH could be expected and the lactate utilization by ruminal bacteria could be improved (22). This could explain the superior effect of the yeast treatment in this study (Fig. 1).

Digestibility of NDF was increased with addition of the micoorganisms (Table 1, Fig. 2). At 24 hours of incubation *S. cerevisiae* and *Humicola* sp. enhanced NDF digestibility by approximately 16 and 18% units (Table 1). At 72 h the increase was 8 and 15.8% (Table 1). In this study the rate of NDF degradation was lower for the control treatment and similar for *S. cerevisiae* and *Humicola* sp (Table 2). In the control treatment, the indigestible residue (*I*) of NDF was 2.7 and 9.1% higher than the one observed for *S. cerevisiae* and *Humicola* treatment (Table 2). The higher degree of NDF degradation in the microbial additive treatments could be explained by the activity of the enzymes of the cellulase complexes, xylanases, phenol-oxidases

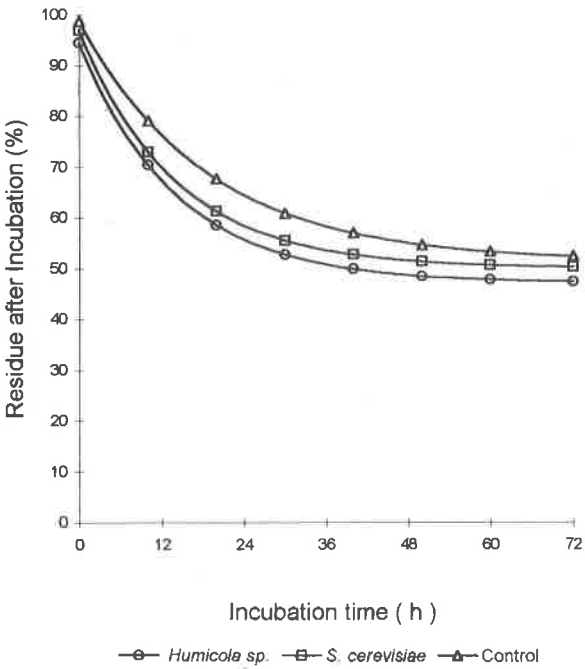

Figure 2. Potential degradability of the neutral detergent fiber.

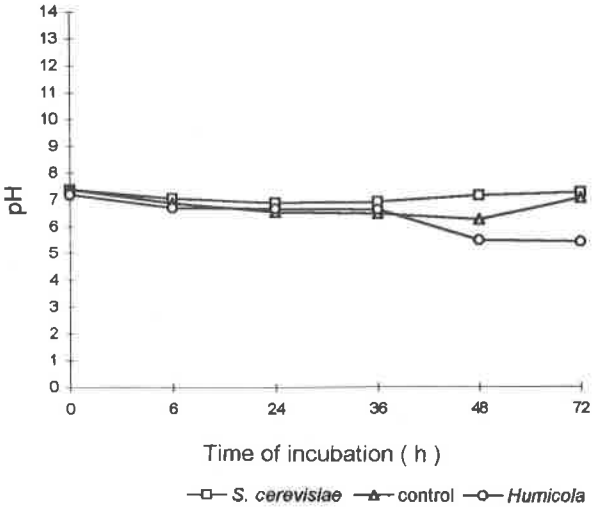

Figure 3. *In vitro* ruminal pH.

and by some degree of metabolization of lignin monomers, especially during the aerobic phase (27).

There is evidence about the synergistic effect shown by fibrolytic bacteria, associated with yeast or fungi, on the utilization of lignocellulosic materials (12, 16). In the residue of incubation, there was a reduction in the NDF percentage for all incubation times by *S. cerevisiae* and *Humicola* treatments. This could be an indication for the action of a specific enzyme in the components of the forage cell wall. This percentual alteration could be responsible for the lower NDF residue observed in the *Humicola* treatment (Fig. 2), whereas little difference was observed for DM (Fig. 1).

The positive effect observed on NDF degradation was similar to those described in earlies studies when *S. cerevisiae* was added (12, 15). The same mechanisms of action related above could be used to explain these effects upon NDF degradation (7). Despite the *Humicola* treatment, there were no reported results on the ruminal NDF degradation in the consulted literature. Kinetic studies on forage degradation have currently been made in our laboratory, but the data obtained so far with the *Humicola* sp. treatment, although promising, need more investigation to confirm its positive effect.

Humicola sp. as an aerobic fungus, probably grew in the aerobic phase. One of the reasons for this phase is to ensure fungal growth in order to produce fibrolytic enzymes. If the strict aerobic fungi are ingested by a ruminant, they would not survive in the anaerobic rumen ecosystem. In our study of ruminal forage

degradation, to compare the effect of the aerobic fungus, was necessary to use the initial aerobic incubation, because only *S. cerevisiae* can survive in the rumen ecosystem (23).

Despite the small differences between the microbial additives and the control, they should be interpreted in terms of benefits on the voluntary feed intake and on the improvement of nutrient utilization in the gastro-intestinal tract. As the costs of cattle feeding correspond to 60-80% of total costs, an improvement of 5-10% in the utilization of the nutrients is important for the reduction of the production costs. The microbial additives used in this study caused an increase of forage digestibility, as described in others reports, and thus their usage could be adopted in ruminant production systems.

CONCLUSIONS

The addition of *Saccharomyces cerevisiae* or *Humicola* sp, as microbial additives, was effective to improve the digestibility, the rate and the extent of degradation of the dry matter and the neutral detergent fiber.

The addition of *Saccharomyces cerevisiae* resulted in more stable values for the ruminal pH throughout the incubation time.

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RESUMO

Degradação *in vitro* do capim coast-cross (*Cynodon dactylon*) por microrganismosruminais associados com *Saccharomyces cerevisiae* ou *Humicola* sp

A degradação ruminal da matéria seca e da fibra em detergente neutro da forrageira coast-cross (*Cynodon dactylon*) foi estimada pela incubação in vitro inoculando-se previamente na forragem *Saccharomyces cerevisiae* ou *Humicola* sp. A degradabilidade potencial da matéria seca foi aumentada com a adição de *Saccharomyces cerevisiae* enquanto que o tratamento com *Humicola* sp resultou em maior extensão da degradação dos constituintes da parede celular vegetal. A taxa de degradação da matéria seca foi aproximadamente 15% superior quando *Saccharomyces cerevisiae* foi previamente adicionada. A inoculação com *Saccharomyces cerevisiae* ou *Humicola* sp. promoveu maior taxa de degradação dos constituintes da parede celular vegetal em relação ao tratamento controle.

Palavras-chave: Aditivos microbianos, cinética digestiva, rúmen, forragens tropicais.

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PARTIAL CHARACTERIZATION OF POLYSACCHARIDES FROM *FUSARIUM SOLANI*

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SHORT COMMUNICATION

ABSTRACT

A crude polysaccharide obtained from mycelium of *Fusarium solani* by treatment with 2% KOH/ 2h/ 100°C and fractionated by gel filtration chromatography yielded three fractions denoted L1,L2 and L3. Chemical analysis of the crude polysaccharide showed the presence of 89.5% total carbohydrate, 4% protein, 14% uronic acid, traces of phosphate and hexosamine. Mannose, galactose, glucose and an unidentified pentose, were present in a 27.5: 34: 34.5: 4 molar ratio.

Key words: *Fusarium solani*, polysaccharide, GC-MS

Fusarium species are distributed worldwide in soil, aerial and subterranial parts of plants, plant debris and other organic substrates. These fungi are common in tropical and temperate regions and they are found in desert, alpine and arctic regions, where adverse climatic conditions prevail (1). The plant pathogen, *Fusarium solani*, has a wide range of hosts and recognition plays a central role in the interactions between plants and their pathogens. Pathogens must be able to recognize the presence of their host plants in their environment and often must recognize specific surface features of their host in order to effect successful penetration and infection (2). Plant defense responses against fungal pathogens are triggered by highly specific signal compounds released during invasion of the host tissues. The release of these signals, commonly referred to as elicitors, can be either spontaneous or mediated by the exposure of the pathogen to secreted plant hydrolytic enzymes (3), making the biochemistry of the cell wall a logical area of study for control of fungal pathogens. In this work we describe the partial chemical composition of the polysaccharides from *F. solani*. *Fusarium solani*, a human isolate, strain EPM 550 was provided by Dra. Olga Fischman Gompertz, Universidade Federal de São Paulo, São Paulo, Brazil. Hyphae were grown in Sabouraud broth medium supplemented with yeast extract (0,5%) for 7 days at 25°C, with shaking at 150 rpm. The mycelium was harvested by filtration and washed several times with cold distilled water and stored at −20°C. Polysaccharides were extracted from intact hyphae of *F. solani* with hot 2% aqueous potassium hydroxide for 2h at 100°C. The extract was neutralized with acetic acid and centrifuged. The supernatant was concentrated and the polysaccharide was obtained by precipitation with ethanol (4). The precipitate was dialyzed against distilled water and lyophilized. The crude polysaccharide preparation was fractionated on a

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Sephacryl S-400 column (XK 16/100, Pharmacia Biotech), equilibrated with 0.06M NaCl. Fractions were collected in a F-100 LKB-Pharmacia collector and monitored with phenol-sulfuric acid. Three major fractions L1, L2 and L3 were obtained, dialyzed and lyophilized. The L1 fraction was further frationated on a Sephacryl S-400 column, as described above. The crude polysaccharide and L1 fraction were subjected to hydrolysis with 3N trifluoroacetic acid (TFA) for 3h at 100°C in sealed tubes (5,6). After removal of the acid under vacuum, the sugars were spotted on thin layer chromatography (TLC) plates and chromatographed in n-butanol/pyridine/ 0,1N HCl (5: 3: 2, v/v). The sugars were visualized by orcinol/ H_2SO_4. Galactose, rhamnose, glucose, mannose, glucuronic acid and N-acetylglucosamine were used as standards. For GC-MS analysis, the resulting neutral monosaccharides were reduced with sodium borohydride and acetylated with pyridine/ acetic anydride 1:1 (v/v). The sugars were characterized as their derived alditol-acetates by GC-MS on a fused silica capillary column of OV-225 (30 cm × 0.25 mm i. d.) programmed from 50°C to 220°C (50°C/ min, then hold). Total carbohydrate was assayed by the phenol-sulfuric acid method (7) with mannose as standard. Uronic acid was measured by carbazol reaction (8). Hexosamine contents were determined by p-dimethylaminobenzaldehyde reaction (9) with glucosamine as standard. Phosphate and proteins were estimated respectively by Ames *et al* (1966) and Lowry *et al* (1951). Crude polysaccharide obtained from hot KOH extraction of *Fusarium solani* mycelia was fractionated by gel filtration giving the pattern of three fractions L1, L2, and L3 shown in Fig. 1A. L1 fraction was the major fraction and yielded a single peak after further purification (Fig. 1B). Chemical analysis of the crude polysaccharide revealed the presence of 89.5% carbohydrate, 4% protein, 14% uronic acids, traces of phosphate and hexosamine. The TLC analysis of crude polysaccharide and L1 fraction demonstrated the presence of galactose, mannose, glucose and two others components not identified (Fig. 2). These results were confirmed by GC-MS analysis. Mannose, galactose, glucose and a pentose component (not identified) in a 27.5: 34: 34.5: 4 molar ratio were obtained.

Numerous data concerning the chemical composition and structure of the cell walls of different species of *Fusarium* are available (12-17), but not on the polysaccharide composition of *F. solani*. We demonstrated the presence of a polysaccharide containing mannose, galactose, glucose and a pentose

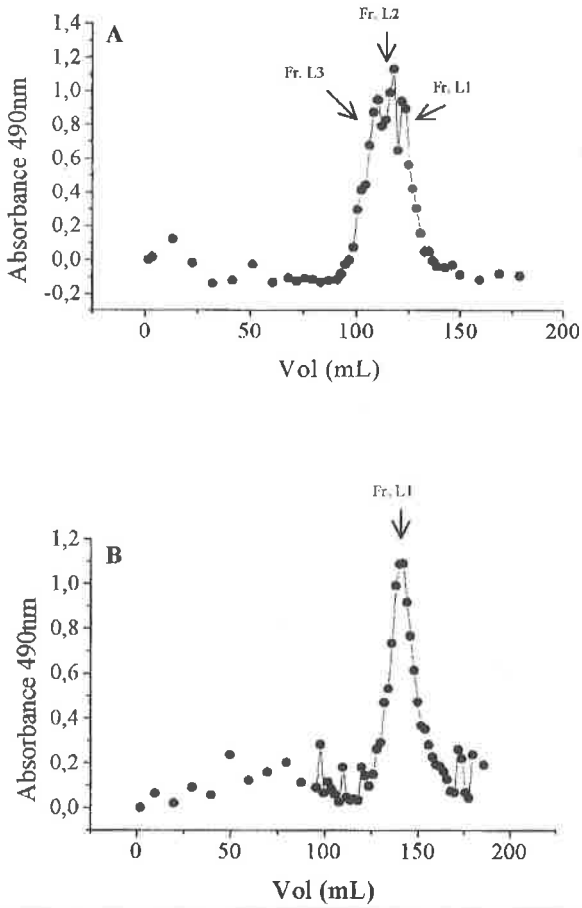

Figure 1. Chromatography of the polysaccharide of *Fusarium solani* from a Sephacryl S-400 column.(A) Crude polysaccharide (B) Fraction L1.Fractions were analyzed by the phenol-sulfuric method (•).

in a 27.5: 34: 34.5: 4 molar ratio by TLC and GC-MS. Uronic acids, protein and glucosamine are present in minor amounts. The presence of uronic acid-containing polysaccharides in the glycoproteins of *Fusarium* sp. was previously described by Ramli *et al* (1994). During the plant-fungi interaction, the first structures to enter in contact are the cell walls of the plant and its pathogenic fungus. In this way, the carbohydrates contained in these structures can be liberated, activating genes that will lead to the production of toxic metabolites. The fungal polysaccharides have been described as important elicitors of plant response, working as signaling molecules that trigger defensive responses from the host plant (19). The knowledge of the chemical structure of a possible elicitor will contribute to the advancement of studies on plant-fungi interaction.

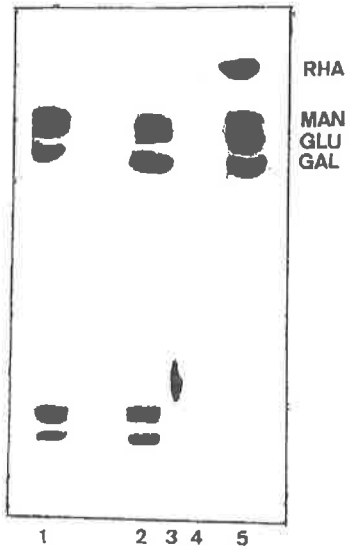

Figure 2. Thin-layer chromatography (TLC) of the monosaccharide components of *Fusarium solani* polysaccharide. Lane 1, Crude polysaccharide after total acid hydrolysis. Lane 2, Fraction L1 after total acid hydrolysis. Lane 3: N-acetylglucosamine. Lane 4, Glucuronic acid. Lane 5, Standard mix: galactose, glucose, mannose and rhamnose. Running solvent: n-butanol/pyridine/0.1N HCl (5:3:2, v/v). Detection: orcinol/H_2SO_4 reagent.

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RESUMO

Caracterização parcial de polissacarídeos de *Fusarium solani*

Um polissacarídeo bruto foi obtido do micélio de *Fusarium solani* por tratamento com KOH 2%, 2h/ 100°C e fracionado por cromatografia de gel filtração, originando 3 frações, denominadas L1, L2 e L3. O polissacarídeo bruto era constituído de 89.5% de carboidratos totais, 4% de proteína, 14% de ácidos urônicos, traços de fosfato e hexosamina. Os monossacarídeos componentes do polissacarídeo bruto, foram identificados por cromatografia em camada fina (TLC) e líquido-gás (GC-MS) como manose, galactose, glucose e uma pentose não identificada, em uma proporção molar de 27.5: 34: 34.5: 4.

Palavras-chave: *Fusarium solani*, polissacarídeo, GC-MS.

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LONG TERM FREEZE STORAGE OF *HELICOBACTER PYLORI*

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SHORT COMMUNICATION

ABSTRACT

At present there is no simple standard method available for long term storage of *Helicobacter pylori*. We evaluated the success of freeze storage of 53 strains in conventional media. Our results show that this proceedure could be applied to undertake long term research studies with this microorganism.

Key words: *Helicobacter pylori*; preservation; freeze storage

Long term storage of *Helicobacter pylori* is still a troublesome issue and at present there is no simple standard method available for the preservation of this organism. Drumm and Sherman (2) have obtained good recovery by growing strains in liquid media prior to freezing, whereas Spengler *et al* (5) also report successful results using lyophilisation. However, both procedures are cumbersome or may not be readily available. Some investigators have reported difficulties in freeze storage of *Helicobacter pylori* in conventional media (liquid broth supplemented with 15% glycerol) (6, 1,4) whilst others suggest it is satisfactory (3). In turn, several studies have evaluated different cryoprotectants (6,1,4). However, none have shown so far to provide improved strain survival. In this study we evaluated a simple method for the preservation of clinical isolates of *Helicobacter pylori* which involved freeze-storage, at –70°C and in liquid nitrogen, of liquid suspensions of organisms obtained from surface growth agar plates.

Fifty three clinical isolates of *Helicobacter pylori* were subcultured onto the surface of Columbia agar plates supplemented with heated horse blood (5%) and incubated under microaerophilic conditions (Gaspak System, Biomerieux) at 37°C for periods ranging from 3 to 7 days (culture age). Bacterial cells were harvested in tryptone soya broth (Oxoid Ltd.) to a density corresponding to a 5.0 MacFarland turbidity standard. Fifty microlitres of this suspension were then introduced into 0.5 ml aliquots of tryptone soya broth containing 15% glycerol and the final suspensions immediately frozen at –70°C and in liquid nitrogen, for periods from 2 to 10 months. Up to 5 duplicate suspensions per strain were prepared so that the influence of storage time, length of incubation prior to preparation and at –70°C versus liquid nitrogen storage could be evaluated.

Table 1 illustrates the recovery obtained from suspensions stored for periods up to 10 months in time. The difference between the yield after 2-4 and 6-10 months was not statistically significant (ANOVA $F = 2.43$; $p > 0.1$).

However, the period of incubation prior to preparation of the organisms suspensions was a major factor affecting outcome. Table 2 contains the pooled results of suspensions prepared following 3-7 days incubation on solid media and subsequently stored for 2-10 months. The decline in viability with culture age

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Table 1. Recovery of *Helicobacter pylori* samples according to storage period.

Storage period	Total stains	Total Recovered
2-4 months	48	39 (81%)
4-6 months	39	27 (71%)
6-10 months	23	15 (71%)

Table 2. Recovery of *Helicobacter pylori* samples according to incubation period.

Incubation period	Total strains	Total Recovered
3 days	33	31 (94%)
4 days	35	29 (83%)
5 days	10	7 (70%)
6 days	12	6 (50%)
7 days	17	8 (47%)

is statistically significant (ANOVA $F = 2.56, p < 0.05$). No differences were obtained between storage at –70°C and liquid nitrogen.

We have reported here successful results using conventional media for preserving *Helicobacter pylori*. These are comparable with those using different cryoprotectants (1,4) but are simpler and more economical. Thus, we believe that conventional freeze storage media proceedures could be applied to undertake long term research studies on *Helicobacter pylori*. A short incubation period prior to preparation of suspensions is important to ensure optimal recovery.

RESUMO

Estocagem de *Helicobacter pylori* em *freezer* por longo tempo

No momento, não há um método simples para estocagem por longo tempo de *Helicobacter pylori*. Neste estudo avaliamos o sucesso da estocagem em *freezer* de 53 cepas por vários meses em meios convencionais. Nossos resultados mostram que este procedimento pode ser utilizado em trabalhos de pesquisa de longo prazo com este microrganismo.

Palavras-chave: *Helycobacter pylori*, estocagem, congelamento.

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CHARACTERIZATION OF LACTOSE-FERMENTING *SALMONELLA* AGONA STRAINS ISOLATED IN A PEDIATRIC UNIT

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ABSTRACT

Eight lactose-fermenting *Salmonella* Agona strains isolated in a pediatric unit were characterized by classic and molecular methods. The strains were classified as biotype 1a, corresponding to the most frequent one in Brazil. None of the strains produced colicin. Multiple resistance to antimicrobials was observed among the strains studied. It was demonstrated that the lactose-fermenting character was encoded by a plasmid with spontaneous segregation at a frequency of 1%. This plasmid was transferable by conjugation at a frequency between 4×10^{-8} and 5×10^{-10}. The Lac^+ plasmid, which molecular weight was approximately 90 MDa, encoded both lactose fermentation and multiple resistance to antimicrobials. Replicon typing showed that this plasmid did not belong to the known types, suggesting the presence of a new replicon type. Classic methods showed that the studied strains had the same characteristics as the clone widely occurring in our area, differing only by lactose-fermenting ability. This conclusion was supported by the results of ribotyping study.

Key words: *S.* Agona, lactose-fermenting strain, plasmid, antimicrobial resistance, ribotyping

INTRODUCTION

Salmonellosis is one of the most important zoonoses, affecting annually millions of people in developed as well as in developing countries. In developing countries, in addition to the food-borne outbreaks of non-typhoid salmonellosis caused by many different serotypes, hospital salmonellosis, particularly in pediatric units, is an important public health problem.

Some *Salmonella* serotypes have the property of being predominant for a long period of time, as was the case for *Salmonella* Typhimurium throughout the world, whereas others are of only temporary importance, as was the case for *Salmonella* Agona in several countries including Brazil (8,10,33).

This serotype was introduced in the late 1970's and its isolation from food, water, animal feeds and hospital environment as well as its increase in human infections, mainly among infants, was reported by Pessôa (8). This

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serotype increased significantly and was the second most frequent in São Paulo State in the period from 1983 to 1990 (40).

Fernandes et al. (19) showed that strains of *S.* Agona belonging to biotype 1a, most of them non-colicinogenic, were the most frequent ones in São Paulo. Strains of nosocomial origin frequently showed multiple resistance to antimicrobials.

Since *S.* Agona, like other members of genus *Salmonella*, is characteristically lactose non-fermenting, the isolation of lactose-fermenting strains from hospitalized infants with diarrhea in a pediatric unit led us to undertake the present study in an attempt to characterize the strains using classical and molecular methods.

MATERIALS AND METHODS

Bacterial strains. A total of eight lactose-fermenting strains (Lac^+) of *S.* Agona (214/85, 215/85, 216/85, 379/86, 382/86, 384/86, 388/86, 391/86), isolated between 1985 and 1986 from children (eight months to 3 years of age) hospitalized with acute diarrhea were studied. For the purpose of comparison we included 26 lactose-negative strains of *S.* Agona isolated between 1978 and 1993 in distinct geographic areas in Brazil. All strains were serotyped according to Popoff and Le Minor (36) at the Laboratory of Enteric Pathogens, Instituto Adolfo Lutz. Indicator strains *E. coli* CL104 and BZB1011 (3) were used in the colicin production test. Standard strains *E. coli* ATCC 25922, ATCC 235218, *P.aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used in antimicrobial susceptibility testing. For plasmid transfer experiments *E. coli* K12 C600 F-, Nal^r was used as recipient strain. Strains *E. coli* BS-176 ST^r (L5-3C), S. Typhimurium C5, TT^r and *E. coli* HB101-AP^r (RP-4), harboring plasmids of 89,9 MDa, 60 MDa, and 34 MDa were used to determine the molecular size of the plasmid.

Biotyping. Biotyping of all strains was performed according to Duguid et al. (14) by analysing the combined reactions of fermentation in sugar peptone waters, tartrate utilization (turbidity) tests, growth on minimal medium supplemented with carbohydrates and haemagglutinating activity and fimbriae (13). The biotyping scheme was complemented with lactose fermentation test and the test for ß-D-galactosidase activity (ONPG) (16, 25). All cultures were examined for colicin production on nutrient agar (Difco) by the overlay method (3).

Antimicrobial susceptibility testing. The antimicrobial susceptibility of the strains was tested by the agar diffusion method of Bauer et al. (6) using nalidixic acid, amicacin, ampicillin, carbenicillin, cephalotin, ceftazidin, ceftraxone, colistin, chloramphenicol, cotrimoxazole, gentamicin, imipenem, kanamycin, netilmicin, tetracycline, and tobramycin. The minimal inhibition concentration (MIC) for ampicillin, kanamycin, tetracycline, chloramphenicol and gentamicin was determined according to the National Committee for Clinical Laboratory Standards (30).

Plasmid profile. Plasmid DNA was extracted and purified according to Kado and Liu (22) and electrophoresed on 0.7% horizontal agarose gels in TRIS acetate buffer; plasmids of 89.9, 60 and 34 MDa were run as molecular weight standards.

Transfer of plasmid. For plasmid transfer and evaluation of transfer frequencies, mating mixture consisted of 2 ml of donor cells and 2 ml of recipient culture in the exponential phase in 2 ml of nutrient broth; after incubation for 3 hours at 37°C, 0.1ml of the mixture was spread on MacConkey agar plates containing nalidixic acid (50ug/ml), with ampicillin (512ug/ml), gentamicin (64ug/ml), kanamycin (512ug/ml) or tetracycline (64ug/ml) added. In all conjugation assays, the initial number of donor cells was estimated to evaluate the frequencies of transfer (number of transconjugants/initial number of donor cells/ml). Transconjugants from the plates were screened for plasmid analysis and antimicrobial susceptibility testing.

Curing. All cultures of lactose-fermenting strains were subjected to treatment with acridine orange by the method described by Hirota et al. (20) for plasmid curing. A control tube (culture without acridine orange) of the plasmid curing assay was used to evaluate the rate of spontaneous loss of the plasmid.

Replicon typing. Attempts to classify the Lac^+ plasmid were made by replicon typing (26) using isotopically (^{32}P dATP) labelled specific DNA probes: repFIA, repFIB, repFIC, repFIIA, rep9, rep11, repB/O, repK, repH11, repH12, repI/M, repN, repP, repQ, repT, repU, repW, repX, and repY (12). Positive and negative controls consisted of plasmids carrying each of the probes and pBR322, respectively.

Ribotyping (21). Chromosomal DNA was extracted and purified according to Brenner et al. (7), and digested with *Bgl*I, *Bgl*II and *Eco*RI. After electrophoresis and Southern blotting (39), the membranes were hybridized with16 + 23 cDNA

prepared by the method of Popovic et al. (37). Hybridized DNA fragment sizes were estimated with DNA STAR software (DNA STAR Computer System for Molecular Biology and Genetic, London, U.K) using a *Haemophilus aegyptius* 3031 *Eco*RI DNA digest (fragments sizes: 17613, 6334, 5575, 4960, 3789, 3228, 1713, 1497 bp) as molecular marker.

RESULTS AND DISCUSSION

Lactose-fermenting *S.* Agona strains belonged to the biotype 1a and 1j, differing biotype 1a from 1j because of the production of gas. All of them were positive to the ONPG test, showing a close association with lactose fermentation. These results are in accordance with our previous study (19) and also with data reported by Duguid (14) and Barker (4,5). Homogeneous biochemical characteristics of the strains which allocate them to a unique biotype (1a) suggest that our *S.* Agona strains probably belong to the archetypal biotype with no evident mutational events, as proposed by Duguid et al. (14) for *S.* Typhimurium.

All *S.* Agona strains were negative when tested for colicin production, in agreement with Barker (3) who reported a low incidence of colicinogeny in *Salmonella*. Colicin typing seems to be more appropriate when studying a homogeneous sample of strains regarding to source, origin, and time of isolation, as is the case of nosocomial outbreaks (42).

Analysis of the antimicrobial susceptibility of lactose-positive *S.* Agona strains revealed multiple drug resistance to ampicillin, kanamycin, gentamicin, chroramphenicol, sulphatrim, carbenicillin, cephalotin, netilmicin, tobramicin and tetracycline, except for one strain which was susceptible only to tetracycline. Nevertheless, the respective lactose-negative segregant colonies of the strains were susceptible to all antimicrobials. High levels of resistance to antimicrobials were observed among these lactose-positive strains, particularly to ampicillin and kanamicin (> 5120 ug/ml), and only one strain was susceptible to tetracycline. Multiple drug resistance acquired by nosocomial isolates is likely to be a consequence of the indiscriminate use of antimicrobials, and the dissemination of strains carrying R factors represents a continuous challenge for treatment and control of hospital salmonellosis (11, 18, 19, 27, 31).

A Lac$^+$ plasmid of molecular weight of ~ 90 MDa was associated with the fermentation of lactose as we *S.* Typhimurium and *S.* Oranienburg strains which suggest that distinct plasmids could be associated with lactose fermentation in *Salmonella.*

The Lac$^+$ plasmid encoded both lactose fermentation and multiple resistance to antimicrobials. This association was first observed by the fact that the Lac+ strains were multiple resistant and the Lac- segregants were susceptible to all drugs. Moreover, analysis of 280 transconjugants clearly showed this

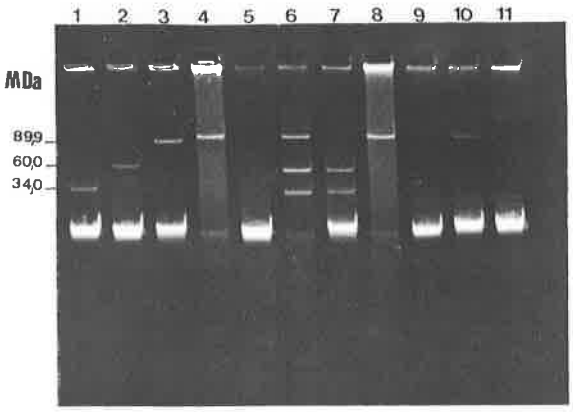

Figure 1. Plasmid profile of four lactose fermenting strains of *S.* Agona and of their spontaneous lactose negative segregants. (1) *E. coli* HB 101 (34 MDa plasmid); (2) *S.*Typhimurium C5 (60 MDa plasmid); (3) *E.coli* SM-BS176 (89.9 MDa plasmid); (4,5) Lac$^+$ *S.* Agona strain 214/85 with ~ 90 MDa plasmid and its spontaneous Lac$^-$ segregant; (6,7) Lac$^+$ *S.* Agona strain 215/85 with ~ 90 MDa plasmids and two other plasmids and its spontaneous Lac$^-$ segregant; (8,9) Lac$^+$ *S.* Agona strain 216/85 with ~ 90 MDa plasmid and its spontaneous Lac$^-$ segregant; (10,11) Lac$^+$ *S.* Agona strain 379/86 with ~ 90 MDa plasmid and its spontaneous Lac$^-$ segregant.

can see in Fig. 1, which shows this plasmid in lactose-fermenting strains and its absence in the spontaneous segregants. The role of a plasmid in lactose fermentation was reported by Le Minor et al. (24) for *S.* Typhimurium and *S.* Oranienburg isolated in the 1970's in São Paulo. Those lactose-fermenting *S.* Typhimurium strains predominated in São Paulo between 1971 and 1973, and outbreaks were observed in pediatric units, mainly affecting newborns (24, 34).

The Lac$^+$ plasmid was transferred to recipient *E. coli* (Fig. 2) at frequencies ranging from 4.0×10^{-8} to 5.0×10^{-10}, in agreement with data reported by other investigators (1,41). The molecular weights of the plasmid carried by *S.* Typhimurium and *S.* Oranienburg isolated in the 1970's were 60 MDa and 90 MDa, respectively (data not shown). Furthermore, Le Minor et al. (24) reported a non self-transferable plasmid in

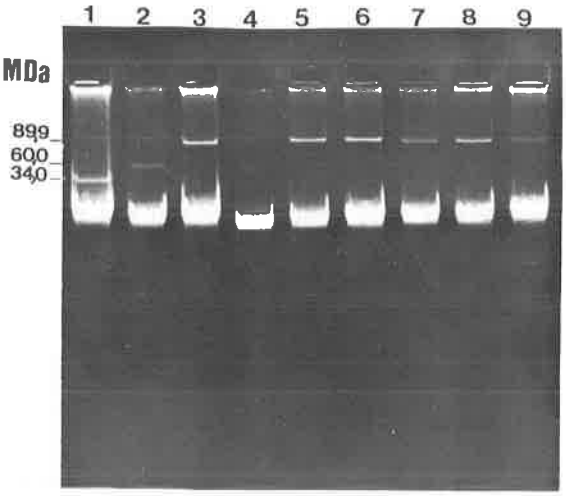

Figure 2. Lac⁺ plasmid of ~ 90 MDa isolated from recipient strain *E. coli* K_{12} C600 after conjugation assays with five lactose fermenting donor strains of *S.* Agona. (1) *E. coli* HB101 (34 MDa plasmid); (2) *S.* Typhimurium C5 (60 MDa plasmid); 3) *E.coli* SM -BS-176 (89.9 MDa plasmid); (4) *E. coli* K_{12} C600 Lac⁻ (recipient strain); (5,6,7,8,and 9) Transconjugants *E. coli* Lac⁺ colonies with ~ 90 MDa plasmid obtained from donor strains 214/85; 215/85; 216/85; 379/86, and 382/86.

association, since they expressed both the ability to ferment lactose and multiresistance to ampicillin, kanamicin, gentamicin, choranphenicol, sulfatrim, cephalotin and tetracycline (the only exception was that the transconjungants obtained from the strain 384/86 were sensitive to tetracycline). Although plasmids coding for lactose fermentation and multiple resistance to drugs had been reported in *Salmonella* (17), in *S.* Typhimurium Le Minor et al. (24) demonstrated that the ability to ferment lactose and resistance to some antimicrobials were transferred by two plasmids. On the other hand, in S. Oranienburg, the same plasmid co-transferred both characteristics (24).

In addition, it should be mentioned that we observed high frequency of spontaneous loss (10^{-2}) of the Lac⁺ plasmid and also the curing experiments with acridine orange showed negative results, as reported by some investigators for salmonellae (1,9,41).

Replicon typing, a new and more specific method than the scheme of classification based on incompatibility, has been widely used for identification and classification of plasmids associated with virulence in *Salmonella, Shigella* and *E. coli* (35, 38). Hybridization of lactose-fermenting *S.* Agona with specific DNA probes corresponding to 19 replication control genes (replicons) gave negative results, suggesting that the Lac⁺ plasmid of ~ 90 MDa, which encodes for both lactose fermentation and multiple resistance in *S.* Agona strains, probably belongs to a new replicon. Interestingly, the lactose-fermenting *S.* Typhimurium plasmid included in the assays for the purpose of comparison, also did not hybridize with the same replicons. Further research is needed to classify these Lac⁺ plasmids and to understand which characteristics they share.

Phenotypic characteristics, except lactose fermentation, did not differentiate our strains from the widely disseminated clone 1a (19). Moreover, by ribotyping these strains were genetically identical to the large majority of the lactose-negative strains. Ribotyping data revealed consistent results when chromosomal DNA was cleaved by three enzymes (*Bgl*I, *Bgl*II and *Eco*RI) (Fig. 3), classifying all lactose-positive strains into a single ribotype (ribotype A). On the other hand, among 26 non-fermenting S. Agona strains included in this study with the purpose of comparison, 20 belonged to ribotype A, and 6 strains of nosocomial origin belonged to a distinct ribotype (ribotype B).

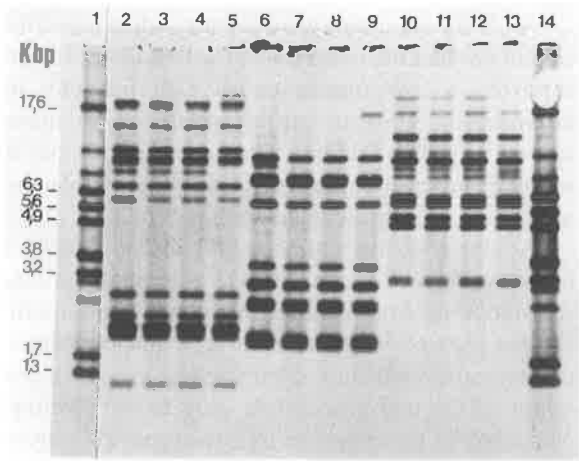

Figure 3. Ribotypes of *S.* Agona after hybridization with DIG labeled 16 + 23 S cDNA probe. Lanes 1 and 14: Molecular weight marker *H. aegyptius* 3031 *Eco*RI DNA digest. Lanes 2, 6 and 10: ribotype A, *S.* Agona Lac⁺ (382/86) after digestion with *Eco*RI, *Bgl*I and *Bgl*II, respectively. Lanes 3, 7 and 11: ribotype A, *S.* Agona Lac⁻ (31/89) after digestion with *Eco*RI, *Bgl*I and *Bgl*II, respectively. Lanes 4, 8 and 12: ribotype A, *S.* Agona Lac⁻ (792/92) after digestion with *Eco*RI, *Bgl*I and *Bgl*II, respectively. Lanes 5, 9 and 13: ribotype B, *S.* Agona Lac⁻ (613/86) after digestion with *Eco*RI, *Bgl*I and *Bgl*II, respectively.

Some investigators have shown that ribotyping can differentiate *Salmonella* serotypes according to source regardless of host or geographic origin (2, 15, 23, 28,

29, 32). In the case of *S.* Agona, ribotyping distinguished nosocomial isolates, and it is very likely that, although lactose-fermenting strains had been isolated from an outbreak in a pediatric unit, they were of unrelated non-nosocomial origin.

This study points out the difficulties in the routine diagnosis of *Salmonella* when strains harboring a plasmid coding for lactose fermentation could lead to a misdiagnosis, taking into account that the lack of lactose-fermenting ability in salmonellae is the most important characteristic for differentiating them from other enterobacteriaceae. On the other hand, dissemination of strains carrying a plasmid also encoding multiple drug resistance can constitute a serious public health problem mainly regarding the control of nosocomial outbreaks.

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RESUMO

Caracterização de cepas de *Salmonella* Agona fermentadoras da lactose isoladas em uma unidade pediátrica

Oito cepas de *Salmonella* Agona fermentadoras da lactose, isoladas numa unidade pediátrica, foram caracterizadas através de métodos clássicos e moleculares. As cepas foram classificadas em biotipo 1a, correspondente àquele mais frequente no Brasil. Todas as cepas se comportaram como não produtoras de colicinas. Acentuada multirresistência aos agentes antimicrobianos foi observada entre as amostras estudadas. Foi demonstrado que a capacidade de fermentar a lactose era codificada por um plasmídio com segregação espontânea numa frequência de 1%. Este plasmídio era transferível por conjugação numa frequência entre 4×10^{-8} e 5×10^{-10}. O plasmídio Lac$^+$, que apresentava peso molecular de aproximadamente 90 MDa, codificava tanto a fermentação da lactose como a multirresistência aos agentes antimicrobianos. Pelo método de tipagem do replicon, verificou-se que este plasmídio não pertencia aos tipos então conhecidos, sugerindo a presença de um novo tipo de replicon. A aplicação dos métodos clássicos evidenciou que as amostras estudadas apresentavam as mesmas características do clone amplamente disseminado no nosso meio e diferenciadas apenas pela fermentação da lactose. Estes resultados foram confirmados pelo estudo da ribotipagem.

Palavras-chave: *S.* Agona, cepa fermentadora da lactose, plasmídio, resistência antimicrobiana, ribotipagem.

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DISTRIBUTION OF MOLDS AND AFLATOXINS IN DAIRY CATTLE FEEDS AND RAW MILK

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ABSTRACT

Ninety six feedstuffs given to dairy cattle from four milk producing areas of the São Paulo state, Brazil (Sorocaba, Cotia, Vale do Paraíba and Itupeva) were analyzed over a one-year period (June 1992 to August 1993) for the presence of contaminating fungi, toxigenic or not, and aflatoxins. In addition, the occurrence of aflatoxins M_1 and M_2 in raw milk from the studied dairy cows was evaluated in 144 milk samples. Fungal species were isolated on potato-dextrose agar and identified by routine mycological techniques. Detection and quantification of aflatoxins were done by thin layer chromatography (TLC). The most frequent fungi recovered from feeds were *Fusarium* spp. (67.7%; main isolate: *F. moniliforme*), *Aspergillus* spp. (58.3%; main isolate: *A. flavus*) and *Penicillium* spp. (52.0%), followed by eight other fungi genera. The numbers of colony forming units (CFU/g) ranged from 3.5×10^3 to 1.1×10^6 (*Fusarium* spp.), 3.0×10^2 to 7.8×10^3 (*Aspergillus* spp.) and 1×10^2 to $3.1x \times 10^5$ (*Penicillium* spp.). Twelve (46.1%) of the 26 *A. flavus* isolates were group B aflatoxins producers and 3 of the *A. parasiticus* isolates were group B and G aflatoxins producers. The presence of aflatoxins B_1 and B_2 was observed in 14 (14.6%) of the feed samples analyzed at levels that ranged between 11.5 and 287μg/kg (AFB_1) and 19 and 40 μg/kg (AFB_2). No carryover of mycotoxins to raw milk was observed. Nonetheless, despite these negative results under the experimental conditions used, the continuous evaluation of levels of aflatoxin M_1 and M_2 in dairy products is always necessary since either favorable or unfavorable conditions for mycotoxin production may vary over different periods.

Key words: aflatoxin, *Aspergillus flavus*, feeds, raw milk.

INTRODUCTION

The need to keep certain animal species in confinement has led to a considerable increase in the incidence of physio-metabolic disturbances and infectious diseases, as well as disorders caused by parasites and nutritional impairments. The exposure of domestic animals of economic importance to feedstuffs contaminated with mycotoxins can give rise to the so-called mycotoxicoses (14). Furthermore, in dairy cattle, the biotransformed toxic principle may be eliminated in milk (15), as is the case with aflatoxins M_1 and M_2.

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+ In memoriam.

Due to their basic composition, feedstuffs constitute an excellent medium for the colonization and multiplication of fungi under the proper moisture content conditions (8). The presence of mycotoxins in these substrates poses a health risk to humans and animals. Therefore, the aim of this investigation was to identify the mycoflora and occurrence of aflatoxins in feedstuffs destined for consumption by dairy cattle. Additionally, the presence of aflatoxins M_1 and M_2 was evaluated in raw milk samples from dairy cows fed with the feedstuffs under study.

MATERIALS AND METHODS

Over a one-year period (June 1992-August 1993), 96 samples of feedstuffs from four different milk producing regions of the São Paulo state, Brazil (Sorocaba, Cotia, Vale do Paraíba and Itupeva) were analyzed. At the same time, 144 samples of raw milk were evaluated for the presence of aflatoxins M_1 and M_2. The milk samples were collected from pooled milk at the end of the day and also from individual animals fed with the studied feedstuffs.

Evaluation of moisture content

Moisture content was determined by direct heating of feed samples to 105°C till the obtention of a constant weight.

Determination of mycoflora according to Swanson *et al.* (12)

Fungi were recovered by blending a 10g portion of each feed sample in 90 ml of phosphate buffered saline (PBS). Serial dilutions to a 10^{-5} concentration were made for each material and 1 ml of each dilution was spread on two Potato Dextrose Agar (pH 5.6) plates. The plates were then incubated for 7 days at 25°C and observed daily. Fungal colonies were selected for subculturing and identified according to the method recommended for each genus (1, 6, 9).

Toxigenicity of aflatoxin-producing isolates

Aspergillus spp strains isolated were inoculated onto Coconut Agar medium and incubated for 7 days at 25°C. After, 10g of the medium were transferred to a 200 ml becker containing 30 ml of chloroform. The mixture was macerated, filtered through filter paper and the filtrate was evaporated in a water bath. The dry extract was solubilized in chloroform and chromatographed as described by Lin and Dianese (5).

Determination of aflatoxins in feed

Samples of feed were evaluated for the presence of aflatoxins according to Sabino *et al.* (10), with detection limits of 5μg/kg.

Determination of aflatoxins in milk

Samples of cow milk were evaluated for the presence of aflatoxins M_1 and M_2 as described by Sabino *et al.* (11), with detection limits of 0.5μg/l.

RESULTS AND DISCUSSION

The mycoflora detected in 96 feed samples over a one-year period is shown in Fig. 1. The fungi recovered, in decreasing order of frequency, were: *Fusarium* spp. (67.7%), *Aspergillus* spp. (58.3%), *Penicillium* spp. (52.0%), *Trichosporon* spp. (46.8%), non-sporulating fungi (38.5%), *Cladosporium* spp. (15.6%), *Scopulariopsis* spp. (9.3%), *Absidia* spp. (5.2%), *Sincephalastrum* spp. (4.1%), *Rhizopus* spp. (3.1%) and *Mucor* spp. (3.1%). Of the genus *Fusarium*, *F. moniliforme* was the predominant species (60.4%), followed by *F. proliferatum* (2.1%) and *F. leteritium* (1.0%). The *Aspergillus* isolates recovered are presented in Fig. 2 and were: *A. flavus* (23.0%), *A. glaucus* (10.4%), *A. ornatus* (9.4%), *A.chevalieri* (6.3%), *A.terreus* (4.2%), *A. parasiticus* (3.1%), *A. oryzae* (3.1%), *A. nidulans* (3.1%), *A. tamarii* (2.1%), *A. sydowi* (2.1%), *A. ruber* (2.1%), *A. ochraceus* (*A. alutaceus*) (2.1%), *A. niger* (2.1%), *A. fumigatus* (2.1%), *A. candidus* (2.1%), *A. amstelodami* (2.1%), *A.wentii* (1.0%), *A. niveo-glaucus* (1.0%), *A. montevidensis* (1.0%) and *A.carneus* (1.0%).

The three most frequent genera, namely *Aspergillus, Fusarium* and *Penicillium*, presented the largest numbers of colony forming units (CFU per gram), with respective CFU/g range values of 3.0×10^2 to 7.8×10^3, 3.5×10^3 to 1.1×10^6, 1×10^2 to 3.1×10^5 (Table 1, Fig. 3). Overall, our results fall within the tolerance limits set for feeds by the norms and standards for animal nutrition and feeding (7), which are of 10^4 to 10^5 CFU/g. With the *Fusarium* isolates, however, the highest values recorded somewhat exceeded the upper limit established as normal.

Concerning abiotic factors, the average relative humidity of the air, the environmental temperature

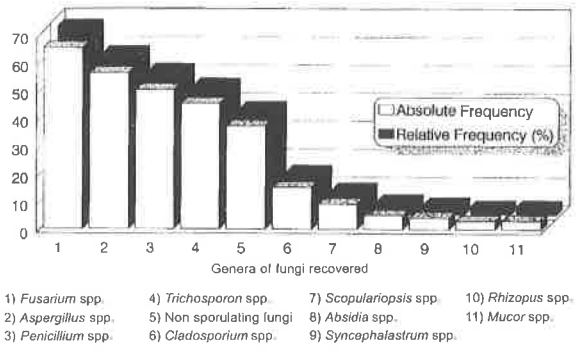

Figure 1. Absolute and relative frequency of fungi isolated from 96 samples of feeds (June 1992 to August 1993).

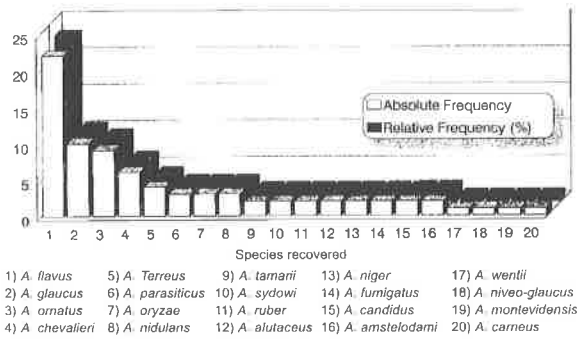

Figure 2. Frequency of *Aspergillus* spp. isolated from 96 samples of feeds (June 1992 to august 1993).

and moisture content of the feed samples studied varied between 69.2% and 78.9%, 17.5°C and 23.5°C and 9.7% and 14.6%, respectively. The maximum rainfall over the period of study was 227.6 mm. The results obtained on moisture content deserve a special mention if we consider that corn is an essential raw material in the formulation of feeds and that the national maximum value of moisture content for corn set by the Brazilian Ministry of Agriculture (14.5%) (2) was very close to the values detected in the feed samples. In fact, by and large, the numbers obtained for temperature, relative humidity and rainfall in the present investigation (Table 1) fall within those considered ideal for the isolation of fungal species.

With respect to the production of mycotoxins, 12 (46.1%) of the 26 strains of *Aspergillus flavus* were aflatoxigenic, producing group B aflatoxins, and 3 of the *Aspergillus parasiticus* strains were found to produce group B and G aflatoxins. The results of this study hence indicate that the fungal isolates recovered from feeds, although being part of the normal mycoflora, may proliferate and produce mycotoxins under favorable conditions, especially those of temperature and moisture content.

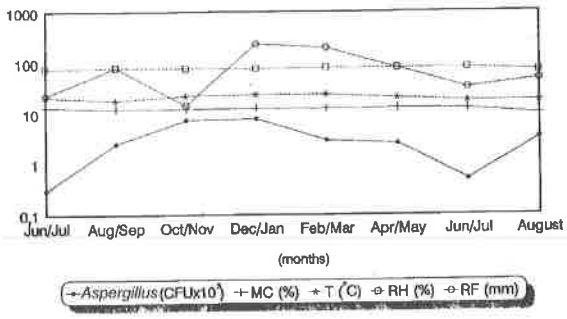

Figure 3. Distribution of Colony Forming Units (CFU) of *Aspergillus* spp. isolated from 96 samples of feeds and profile of abiotic factors (June 1992 to August 1993).

Table 1. Colony Forming Units (CFU) of the genera *Aspergillus, Penicillium* and *Fusarium* isolated from 96 samples of feeds and abiotic factors.

Period* (Months)	Moisture Content (%)	*Aspergillus*	*Penicillium* (C F U)	*Fusarium*	Rainfall (mm)	Temperature (°C)	Relative humidity (%)
June/July	14.6	3.0×10^2	3.1×10^5	1.1×10^6	22.3	20.8	75.6
August/September	12.3	2.5×10^3	7.5×10^3	1.7×10^5	79.5	18.2	78.9
October/November	12.2	7.3×10^3	3.7×10^3	3.2×10^4	14.3	21.9	76.5
December/January	12.6	7.8×10^3	4.0×10^3	2.4×10^4	227.6	23.5	74.5
February/March	12.4	2.9×10^3	1.0×10^2	3.5×10^3	192.7	23.3	78.9
April/May	12.7	2.5×10^3	5.0×10^2	1.02×10^4	76.9	20.0	77.6
June/July	12.3	5.0 v 10^2	5.3×10^3	1.1×10^5	31.5	17.5	78.3
August	9.7	3.3×10^3	1×10^3	2.7×10^4	47.0	17.5	69.2

* The isolates were obtained over a one year period (June 1992 - August 1993), covering the winter months (June to September), Spring months (October to December), summer months (January to March) and autumn months (April to June)

The occurrence of aflatoxin B_1 and B_2 was observed in 14 (14.6%) of the feed samples studied, with levels ranging from 11.5 to 287µg/kg (AFB_1) and 19 and 40 µg/kg (AFB_2) (Fig. 4). Of these samples, however, only 2 (2.1%) presented levels above the maximum 50 µg/kg recommended by the Brazilian Ministry of Agriculture for aflatoxins in feeds (3).

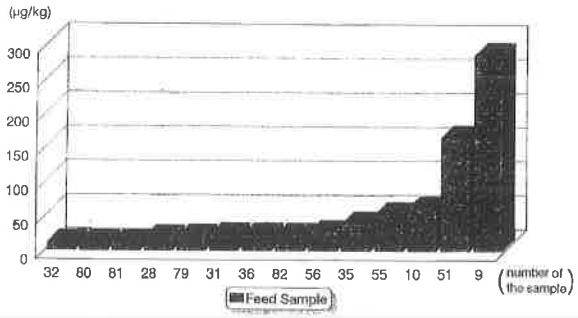

Figure 4. Levels of aflatoxin B_1 in positive samples of feeds.

The mycotoxicological analysis of the 144 samples of raw milk did not reveal the presence of aflatoxins M_1 and M_2, for which the mean conversion rate is 1.5 % according to some authors (4, 13). However, when considering these results, the detection limit of the method (0.5µg/l), the ability of the animals to convert AFB_1 to AFM_1 (which is excreted in milk), the dilution of the samples (having as a basis an average cow's milk production of 10 l/day) as well as the fact that the samples collected from pooled milk were stored in 60 l containers should be taken into account. Hence, despite the lack of detection of aflatoxins M_1 and M_2 in milk under the experimental conditions used, we believe that their potential contamination should be continually monitored, since either favorable or unfavorable conditions for their production may change over different periods.

RESUMO

Distribuição de fungos e aflatoxinas em rações destinadas ao gado leiteiro e em leite cru

A presença de fungos contaminantes, toxigênicos ou não e aflatoxinas foi estudada, no período de 1 ano, de junho de 1992 a agosto de 1993, em 96 amostras de rações destinadas ao gado leiteiro provenientes de quatro zonas leiteiras do estado de São Paulo (Sorocaba, Cotia, Vale do Paraíba e Itupeva). Paralelamente, avaliou-se a presença de aflatoxinas M_1 e M_2 em 144 amostras de leite cru de bovinos alimentados com as rações pesquisadas. O isolamento dos fungos foi efetuado utilizando Ágar Batata Dextrose e a identificação foi realizada através de técnicas micológicas usuais. A pesquisa de aflatoxinas, nas rações e no leite, foi efetuada empregando-se cromatografia em camada delgada.

As análises microbiológicas demonstraram uma população fúngica constituída de *Fusarium* spp (67,7%), seguido de *Aspergillus* spp (58,3%), *Penicillium* spp (52,0%) e outros oito gêneros fúngicos. Dentro dos gêneros *Fusarium* e *Aspergillus*, as espécies *F. moniliforme* e *A. flavus* foram as mais freqüentes. O número de unidades formadoras de colônias (UFC) variou de $3,5 \times 10^3$ a $1,1 \times 10^6$ (*Fusarium*), $3,0 \times 10^2$ a $7,8 \times 10^3$ (*Aspergillus*) e 1×10^2 a $3,1 \times 10^5$ (*Penicillium*). De um total de 26 cepas de *A. flavus*, 12 (46,1%) foram aflatoxigênicas, produzindo aflatoxinas do grupo B. Em relação ao *A. parasiticus* 3 (100%) das cepas testadas produziram aflatoxinas dos grupos B e G. As aflatoxinas B_1 e B_2 foram detectadas em 14 (14,6%) das rações estudadas com níveis entre 11,5 e 287 µg/kg (AFB_1) e 19 e 40 µg/kg (AFB_2). As análises micotoxicológicas das amostras de leite não revelaram a presença de aflatoxinas M_1 e M_2. É importante salientar que, embora não tenhamos detectado a presença de AFM_1 e AFM_2, nas condições deste trabalho experimental, acreditamos que a monitoração das micotoxinas deva ser contínua, já que as condições favoráveis ou desfavoráveis à produção da mesma mudam de um período para outro.

Palavras-chave: Aflatoxinas, *Aspergillus flavus*, ração, leite cru

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INHIBITION OF FOODBORNE PATHOGENS BY BACTERIOCIN-PRODUCING *LEUCONOSTOC* SP AND *LACTOBACILLUS SAKE* ISOLATED FROM "LINGÜIÇA FRESCAL"

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ABSTRACT

Two strains of lactic acid bacteria, isolated from "lingüiça" (a typical Brazilian meat product) stored under refrigeration, produced antagonistic substances active against selected foodborne pathogens. The proteinaceous nature of the inhibitors was demonstrated and so they were classified as bacteriocins. The inhibition due to acid production and phages was ruled out. The bacteriocin produced by *Leuconostoc* sp was active against *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*. The bacteriocin produced by *Lactobacillus sake* was active against *Listeria monocytogenes* and *Staphylococcus aureus*. Gram negative bacteria were not inhibited by the bacteriocins produced.

Key words: lactic acid bacteria, bacteriocins, foodborne pathogens, meat products

INTRODUCTION

Consumers are increasingly wary of chemical preservatives in foods, even though these substances provide the safety and diversity of our food supply. One result of these consumer trend is the increased reliance on refrigeration to assure the safety of foods free of chemical preservatives. However, it is unwise to rely too heavily on refrigeration, specially due to the ability of many pathogens, such as *Listeria monocytogenes*, to grow at near-freezing temperatures. Therefore the use of additional barriers to prevent microbial growth in refrigerated foods is recommended. One barrier may be provided through the use of lactic acid bacteria (biopreservation). Their use for food preservation is accepted by consumers as "natural" and "health-promoting" (12).

Besides many metabolic products, several authors have demonstrated the ability of lactic acid bacteria to produce antimicrobial proteins called bacteriocins, that may be active against foodborne pathogens. Bacteriocins active against *Listeria monocytogenes* were extensively reviewed by Muriana (14). Activity against *Staphylococcus aureus* was reported by Spelhaug and Harlander (20), Lewus *et al.* (9), Daeschel and Klaenhammer (2). There are also reports on the inhibition of sporulated microorganisms, such as *Bacillus cereus* (20), *Clostridium perfringens* (2, 20) and *Clostridium botulinum* (2, 15).

This study was conducted to determine the ability of two strains of bacteriocin-producing lactic acid bacteria isolated from a typical Brazilian sausage called "lingüiça" to inhibit the growth of selected bacteria. "Lingüiça" is a mixture made of minced meat (pork, beef, chicken or a mixture of the former two), curing salts and spices, filled in natural gut casings, and very popular in Brazil. Since "lingüiça" is frequently consumed undercooked, it may represent a serious

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health hazard due to the possible presence of pathogenic bacteria.

MATERIALS AND METHODS

Reference strains: The source of the cultures used in this study is shown in Table 2.

Isolation and identification of bacteriocin-producing lactic acid bacteria from pork. Two strains of lactic acid bacteria were isolated in a previous study (3) and identified according to the scheme proposed by Schillinger and Lücke (18).

Sensitivity of bacteriocin to enzymes. The method described by Lewus *et al.* (9), with few modifications, was used, as reported in De Martinis and Franco (3).

Lytic phage detection. The technique described by Lewus *et al.* (9), was used to detect presence of lytic bacteriophages.

Antimicrobial activity spectrum. The spot-on-the-lawn test, according to Lewus and Montville (8), was used in these determinations. The lactic acid bacteria were tested for activity against *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Salmonella*, non-pathogenic *Escherichia coli, Escherichia coli* O157:H7 and *Aeromonas hydrophila*.

Well-diffusion assay. The inhibitory ability of the isolates against *Listeria monocytogenes* DP-L1044 hly: Tn917-LTV3 was also tested using the well-diffusion assay (4), modified as follow. MRS broth was inoculated with each of the lactic acid bacteria isolate incubated at 25°C for 24 hours. The broth was centrifuged for 20 minutes at 3000 rpm (CELM centrifuge). The supernatant was reserved, neutralized (pH 7) with sodium hydroxide 1 N and filter sterilized (membrane GV Millipore 0.22 (m). A Petri dish was filled with 20 ml of Brain Heart Infusion Broth (Difco) plus 1% agar (Difco) seeded with 10^6 cfu/ml of *L. monocytogenes* DP-L1044. A well of 5 mm diameter was dug in the agar and it was filled with 40 µL of the culture supernatant previously prepared. The plate prepared was kept under refrigeration for 30 minutes and incubated at 30°C for 24 hours under anaerobiosis. The plates were observed for the presence of growth inhibition zones.

RESULTS AND DISCUSSION

One isolate, the strain 2a, was identified as *Lactobacillus sake* and designated *Lactobacillus sake* 2a (3). The second strain was identified as *Leuconostoc* sp and designated as *Leuconostoc* sp 3c. The morphophysiological characteristics of this isolate are presented in Table 1.

Table1. Physiological characteristics of the lactic acid bacteria strain 3c isolated in this study.

Characteristic	strain 3c	Characteristic	strain 3c
fermentation of:		gas from glucose	+
arabinose	–	NH3 from arginine	–
cellobiose	+	Growth at:	
esculin	–	8°C	+
galactose	+	15°C	+
glycerol	–	45oC	–
gluconate	–	pH 3.9	+
inulin	–	Growth in:	
lactose	–	6.5% NaCl	+
maltose	+	7.0% NaCl	+
mannitol	–	10.0%NaCl	–
melezitose	–	Slime from sucrose	+
melobiose	+	Voges-Proskauer	–
raffinose	–	Formation of:	
ramnose	–	H_2S	+
ribose	–	DL- Lactic acid	D
sucrose	+	Gram stain	+
salicin	–	Morphology	cocci
sorbitol	–	Catalase test	–
trehalose	+		
xylose	+		

The inhibitory substances produced by Leuconostoc sp 3c and *Lactobacillus sake* 2a were inactivated by α-chymotripsin and protease (*Streptomyces griseus* type XIV, Sigma) and were considered as bacteriocins, according to the definition proposed by Montville and Kaiser (11). No phages were detected against any of the indicator microorganisms studied.

Bacteriocinogenic *Leuconostoc* strains are common in meats (9, 5, 7, 22). Bacteriocin-producing *L. sake* strains had also been previously isolated from meat and meat products (6, 13, 16, 17, 19).

Leuconostoc sp 3c and *Lactobacillus sake* 2a were tested for inhibitory activity against *L. monocytogenes* DP-L1044 also using the well-diffusion assay. Only the supernatant of *L. sake* 2a generated inhibition zones, revealing that the inhibitors produced by the two strains of lactic acid bacteria isolated were not the same. Lewus and Montville (8) reported similar results for several strains tested by spot-on-the-lawn and well-diffusion assay. These authors observed false-negative results when using the well-diffusion assay and

attributed those results to the aggregation of bacteriocin molecules, inability of the inhibitor to diffuse through the agar and to dilution effects. Another possible explanation for these results is that the bacteriocin production by the *Leuconostoc* strain isolate occurs in agar, but not in broth medium. Previous studies by Barefoot *et al.* (1) demonstrated that a bacteriocin produced in agar medium would be produced in broth medium only under special conditions, such as controlled pH and presence of an inductor.

The results for the tests of the inhibitory spectrum are summarized in Table 2. The bacteriocin produced by *Leuconostoc* sp 3c presented a broader spectrum than the one produced by *Lactobacillus sake* 2a, and was active against *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus aureus*. The bacteriocin produced by *Lactobacillus sake* was active against *Listeria monocytogenes* and *Staphylococcus aureus* only. Although a slight inhibiton halo was observed when *Leuconostoc* sp 3c was tested against *E. coli* O157:H7, the inhibition zone persisted after protease treatment. When the test was carried out under aerobic conditions, the size of the halo was the same, indicating that the inhibition could not be attributed to the production of hydrogen peroxide. No lytic phages were detect against *E. coli* O157:H7 and the slight inhibition zone observed could not be attributed to any of the inhibitory substances studied. Other Gram negative bacteria were not inhibited by any of the two isolates.

Table 2. Inhibition zones (mm)* observed in the spot-on-the-lawn assay.

indicator strain (source)	*Leuconostoc* sp	*L. sake* 2a
Aeromonas hydrophila(a)	0	0
Bacillus cereus 3(a)	04	0
Escherichia coli O157:H7(b)	04[1]	0
Escherichia coli 4(a)	0	0
Listeria monocytogenes 12aOx(a)	05	12
Listeria monocytogenes 43(a)	05	05
Listeria monocytogenes 45(a)	04	08
Listeria monocytogenes DP-L1044 (c)	05	10
Salmonella agona(a)	0	0
Salmonella typhimurium (d)	0	0
Staphylococcus aureus 2(a)	03[1]	04
Staphylococcus aureus 41(a)	04	04

[1] just a slight inhibition, halo not clear
* measured from the center of the producer spot to the border of inhibition zone
(a) our strain collection, (b) EDL 933, (c) Sun *et al.*, 1990, (d) ATCC 14028

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RESUMO

Inibição de microrganismos patogênicos por *Leuconostoc* sp e *Lactobacillus sake* isolados de lingüiça frescal

Duas cepas de bactérias láticas isoladas a partir de "lingüiça frescal" (um produto cárneo tipicamente brasileiro) armazenada em temperatura de refrigeração, produziram substâncias antagonísticas ativas contra diversos microrganismos patogênicos. A natureza protéica dos inibidores foi demonstrada, sendo conseqüentemente classificados como bacteriocinas. A inibição devida à produção de ácido e devida à presença de bacteriófagos líticos foi descartada. A bacteriocina produzida por *Leuconostoc* sp foi ativa contra *Bacillus cereus, Listeria monocytogenes* e *Staphylococcus aureus*. A bacteriocina produzida por *Lactobacillus sake* foi inibitória de *Listeria monocytogenes* e *Staphylococcus aureus*. Bactérias Gram negativas não foram inibidas pelas bacteriocinas produzidas.

Palavras-chave: bactérias láticas, bacteriocinas, bactérias patogênicas, produtos cárneos

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ANALYSIS OF BRAZILIAN RABIES VIRUS ISOLATES WITH MONOCLONAL ANTIBODIES TO *LYSSAVIRUS* ANTIGENS

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ABSTRACT

Brazilian rabies virus isolates from different host species obtained from different geographical areas in the country were analysed in their reactivity profile with a panel of monoclonal antibodies (Mabs) prepared against members of the *Lyssavirus* genus of the family *Rhabdoviridae*. The panel included fourteen Mabs prepared against rabies-related viruses and two Mabs prepared to the CVS-31 rabies vaccine strain. Isolates were multiplied in mice and brain impressions examined by indirect immunofluorescence. Epitopes common to lyssaviruses were identified in all rabies viruses examined. Differential patterns of reactivity were obtained with ten of the sixteen Mabs. Two Mabs reacted only with viruses from cattle and horses with the same reactivity profile, suggesting a common origin for the viruses, most likely vampire bats.

Key words: Rabies, antigenic profile, monoclonal antibodies

INTRODUCTION

Monoclonal antibodies (Mabs) against rabies virus (RV) antigens have been used in a number of studies. Mab-based antigenic analyses of RV isolates have provided reactivity profiles that can be used to group antigenically similar viruses according to their species of origin (17,19, 24), geographic distribution (22), or both species and geographic distribution (20, 21, 25, 26), although others did not demonstrate any association at all (1). Mabs have also been used for the detection of minor antigenic differences between field and laboratory RV strains (5) and rabies-related viruses (6, 11, 12).

In Brazil, little is known about the antigenic make-up of RV field isolates (3, 4, 7, 8, 10, 15, 16, 27). The present paper reports the antigenic characterization of RV isolates obtained from some distinct regions within Brazil and from different host species, with a panel constituted of anti-lyssavirus Mabs.

MATERIALS AND METHODS

Monoclonal antibodies. A panel of fourteen Mabs prepared against rabies-related members of the *Lyssavirus* genus (Table 1) recognizing epitopes on viral nucleoproteins, was prepared elsewhere (Central Veterinary Agency, New Haw, Addlestone, Surrey, UK) by one of the authors (A.King) as described (13). The characterization of such Mabs has also been reported in detail (13). Two additional Mabs prepared against

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Table 1. Monoclonal antibodies (Mabs) used in the present study.

*Lyssavirus**:	Mabs
Lagos bat	L3, L4, L18, L23, L25, L26, L28
Mokola	M11
Duvenhage	D3, D9
Denmark bat	DB1, DB3, DB4, DB9
RV (CVS-31)	CVS1, CVS2

* Mabs prepared to antigens on the viruses listed. Mabs to rabies-related viruses are directed to nucleoprotein antigens (13). Mabs CVS1 and CVS2 have not had their protein specificity determined.

the rabies CVS-31 vaccine strain (14) were included in the panel.

Virus samples. Thirty-seven field isolates of RV obtained from rabies diagnostic laboratories from the states of Bahia, Rio de Janeiro, Rio Grande do Sul and São Paulo were analysed. Most samples (17) were obtained from dogs, although samples from cattle (12), cats (4), fox(1) and from a human case (1) were also included in the tests. The isolate 566-M was obtained from the state of São Paulo and is a neutralization escape RV variant which was only partially neutralized by antiserum prepared to CVS-31 vaccine strain (C. Zanetti, personal communication).

Virus multiplication. Viruses were multiplied by intracerebral inoculation of infected mouse brain suspensions (10% in PBS, pH 7.4) containing 5 to 500 mice intracerebral 50% lethal doses (MICLD). Two 3-4 week old, conventionally reared mice were inoculated with each virus suspension (30µl). In some experiments, suckling mice (1 to 3 day-old) were also inoculated (10µl) in order to compare the profiles of reactivity obtained. The animals were observed daily and brains collected when mice were *in extremis*. Standard vaccine strains (Challenge Virus Standard, CVS-31 and CVS-32) were also multiplied as above). Samples adapted to cell culture (CVS 247 and PV L391) were multiplied using clarified tissue culture supernatant as inoculum.

Antigenic Analysis. The determination of patterns of reactivity with the panel of Mabs was performed by indirect immunofluorescence (IIF) as described previously (23, 13) with minor modifications. Glass slide impressions of transversely cut brains were prepared from infected and control mice. Slides were fixed in acetone at –20°C for 30 minutes, covered with an appropriate dilution of each Mab (30 µl per impression) and incubated for 30 minutes at 37°C. After three washes in PBS (pH 7.4) the slides were covered with an appropriate dilution of rabbit anti-mouse IgG conjugated to FITC (Dako) in PBS (pH 7.4) containing Evans blue (final concentration 1/100 000) and incubated for further 30 minutes at 37°C. After incubation, slides were again washed as above, washed once with distilled water, covered with 50% glycerol in PBS (pH 8.5) and examined under a fluorescence microscope. Reactions were classified as strong, weak or not reactive, according to intensity of fluorescence observed.

RESULTS

The Mab profile of reactivity obtained with the RV field isolates is shown in Table 2. Mab L3, L26, DB1 and CVS2 recognized antigenic determinants in all field isolates examined. Six out of fourteen anti-lyssavirus Mabs (L18, L23, DB9, D3, D9 and M11) did not react with epitopes in any of the samples examined. Mab DB3 and DB4 recognized epitopes only on isolates from cattle, horses and on the CVS-31 RV vaccine strain. Mab CVS1 reacted weakly with only two out of twelve isolates from cattle examined. The isolate 566-M displayed a profile of reactivity similar to that of isolates of cattle origin. Mab L4, L25, L28 and CVS2 reacted with epitopes present on isolates of dogs, cats as well as on the fox and the human isolates, displaying a "scattered" profile of reactivity.

Mab DB3 and DB4 reacted only with RV of cattle and horses, allowing a subdivision of the isolates into two groups, one comprising isolates of herbivores and another including all RV of carnivores here examined.

Samples of the standard RV vaccine strain CVS with different passage histories displayed the same pattern of reactivity, regardless of whether they had been multiplied in mice or cell cultures (data not shown).

DISCUSSION

In this study, we have performed an analysis of RV field isolates obtained from certain areas within Brazil and compared their patterns of reactivity against a panel of Mabs prepared to *Lyssavirus* antigens. Common antigenic determinants were identified in all RV isolates by four of the Mabs employed. Such Mabs appear to recognize conserved epitopes on the nucleoprotein of lyssaviruses. Thus, they can be regarded as of little use for the purpose of detecting antigenic differences between isolates.

Table 2. Profiles of reactivity (*) of Brazilian field isolates of rabies virus with anti-lyssavirus monoclonal antibodies (Mabs).

Origin of isolates			Mabs									
			L					DB			CVS	
Species	**State(#)**	**N(@)**	**3**	**4**	**25**	**26**	**28**	**1**	**3**	**4**	**1**	**2**
Human	BA	1	+	W	W	+	+	+			W	+
Dog	BA	5	+		W	+	W	+			W	+
Dog	BA	1	+		W	+	W	+			W	W
Dog	BA	3	+			+	+	+			W	+
Dog	BA	2	+			+	W	+			W	+
Dog	BA	5	+			+		+			W	+
Dog	BA	1	+			+		W			W	+
Cat	BA	1*	+		W	+		+			+	+
Cat	BA	1*	+		W	+		+			W	+
Cat	BA	1	+			+		+			+	+
Cat	BA	1	+			+		W			W	W
Fox@	BA	1	+	+		+		+			+	+
Cattle	BA	1	+			+		+	+	+	W	+
Cattle	RJ	1	+			+		+	+	+	W	+
Cattle	RS	1	+	+		+		+	+	+		+
Cattle	RS	1	+			+	W	+	+	+		+
Cattle	RJ	4	+			+		+	+	+		+
Cattle	RS	1*	+			+		+	+	+		+
Cattle	BA	2*	+			+		+	+	+		+
Cattle#	SP	1	+			+		+	+	+		+
Horse	RJ	2	+			+		+	+	+		+
Mouse**			+			+		+	+	+	+	+
CVS-31			+			+		+	+	+	+	+
Total no. of field isolates examined		37										

(*) As determined by indirect immunofluorescence. Refer to text for methods.
(#) States: BA, Bahia; RJ, Rio de Janeiro; RS, Rio Grande do Sul; SP, São Paulo.
(@) number of isolates examined with the same profile.
Key for table: + = strong reaction, W = weak reaction, blank = no reaction.
* = isolates multiplied in newborn mice.
# = 566-M isolate. Mab L18, L23, DB9, D3, D9, e M11 did not react with any isolates. The CVS-31 RV vaccine strain was used as control.
@ One of the species called "fox" in Brazil, also known as "bush dog" (*Cerdocyon thous*).

Nevertheless, ten other Mabs gave rise to distinct profiles of reactivity. Scattered reactions were observed with such Mabs, but were not consistent enough to allow any conclusions to be drawn from the antigenic make-up of such isolates. However, two of them (DB3 and DB4), reacted only with samples originated from cattle and horses. Curiously, the vaccine strain CVS-31, included as a standard in the experiments, displayed a pattern of reactivity similar to that displayed by viruses of herbivorous hosts. Based on the reactivity of these two Mabs, viruses could be separated into two groups: one including samples

of cattle and horses, and a second group comprising all other RV from distinct species, including dogs, cats, fox and a human isolate.

Although we aimed to analyse viruses of bat origin, due to logistical reasons only samples originated from cattle and horses were obtained. Nevertheless, based on the more likely source of infection, RV isolates from herbivores were considered representative of viruses of bat origin. Moreover, it is unlikely that RV isolates would undergo significant antigenic variation after only one passage in cattle or horses. In fact, isolates from those host species displayed quite similar profiles of reactivity, suggesting that they have a stable antigenic profile. Also, it is quite likely that such viruses originated from a common (probably vampire bat) source.

Due to the size of the country, significant differences in the antigenic make-up of isolates from different areas were expected. However, probably due to the limited number of samples tested here, no association was found between the geographic origin of the isolates and any particular antigenic characteristic that they might possess, as deduced from the patterns of reactivity obtained with the Mab panel. Other authors have been able to associate antigenic differences to geographic distribution of isolates (18, 20, 21, 25), whereas others have not (1, 9, 27). Sacramento et al. (18), using polymerase chain reaction (PCR) analysis of fragments of viral genomes, detected differences between isolates from sites geographically close to each other within the state of São Paulo. As a more sensitive technique was employed, it is not possible to anticipate the results that would be obtained at Mab analysis of the same samples. However, Mab characterization of those isolates remains an interesting task for the close future.

As the number of samples examined here was too small, it must not be considered a representative sample for the whole country. A larger sample and the use of a wider panel, including Mabs prepared against local isolates, are expected to be capable of discriminating between isolates obtained from geographically distinct regions. Therefore, this study must be widened to include a greater number of isolates, as well as a larger number of Mabs, to provide a more comprehensive view on the antigenic composition of Brazilian RV isolates. This will be of interest, specially to determine the antigenic characteristics of samples of RV from bats, a task which has become mandatory in future studies.

RESUMO

Análise de amostras brasileiras de vírus rábico com anticorpos monoclonais contra antígenos de lissavírus

Amostras de vírus rábico obtidas de diferentes espécies animais e áreas geográficas do Brasil foram analisadas em seu perfil de reatividade frente a um painel de anticorpos monoclonais (Acms) preparados contra o gênero *Lyssavirus* da família *Rhabdoviridae*. O painel incluiu quatorze Acms produzidos contra antígenos de vírus relacionados com o vírus da raiva e dois Acms preparados contra a amostra vacinal de vírus rábico CVS-31. As amostras foram multiplicadas em camundongos e as impressões de cérebro examinadas por imunofluorescência indireta. Epítopos comuns aos lissavírus foram identificados em todas as amostras examinadas. Padrões diferenciais de reatividade foram obtidos com dez Acms. Dois Acms reagiram somente com amostras de bovinos e equinos, as quais apresentaram um padrão de reatividade sugestivo de uma origem comum, provavelmente morcegos hematófagos.

Palavras-chave: Raiva, perfil antigênico, anticorpos monoclonais.

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ABSTRACT: The abstract should be typed in a separate page and should not exceed 250 words. It should summarize the basic contents of the paper. The abstract should be meaningful without having to read the remainder of the paper. An abstract should not contain references, tables or unusual abbreviations. Abstracts are reprinted by abstracting journals and hence will be read by persons who do not have access to the entire paper. Hence the abstract must be prepared with great care. Three to five key words should also be included.

RESUMO: *Resumo* is the abstract written in Portuguese. Its preparation should follow the same recommendations for the abstract in English. The *resumo* should also contain a title in Portuguese. The rules for the title in Portuguese are the same for the title in English (see above). Three to five *palavras-chave* (key words) have also to be included. The *resumo* and the title in Portuguese should also be typed in a separate page.

INTRODUCTION: The introduction should begin on a new page and provide the reader with sufficient information so that results reported in the paper can be properly evaluated without refering to the literature. However, the introduction should not be an extensive review of the literature. The introduction should also give the rationale for and objectives of the study that is being reported.

MATERIALS AND METHODS: This section should provide enough information for other investigators to repeat the work. Repetition of details of procedures which have already been published elsewhere should be avoided. If a published method is modified, such modification(s) must be described in the paper. Sources of reagents, culture media and equipment (company, city, state, country) should be mentioned in the text. Names that are registered trade marks should be so indicated. Subheading often make this section easier to read and understand.

RESULTS: This section should, by means of text, tables and/or figures, give the results of the experiments. If a *Discussion* section is to be included, avoid extensive interpretation of results but do so in the *Discussion* section. If *Results* and *Discussion* are combined. then results should be discussed where, in the text, it is most appropriate. Tables should be numbered independently of the figures using Arabic numerals. All tables and figures must be mentioned in the text. The approximate location of tables and figures in the text should be indicated.

DISCUSSION: The discussion should provide an interpretation of the results in relation to known information.

ACKNOWLEDGMENTS: This section is optional and follows the *Discussion*. It aknowledges financial and personal assistance.

REFERENCES: Arrange the references in alphabetical order, by last name of the author. All authors must be cited. Number the references consecutively. Cite each reference in the text by its number. Journal names should be abbreviated according to the style of *Biological Abstracts* or *Chemical Abstracts*. All references given in the list should be cited in the text and all references mentioned in the text must be included in the list. List references according to the style shown in the following examples.

a. Paper in a journal
Campos, L.C.; Whittam, T.S.; Gomes, T. A.T.; Andrade, J.R.C.; Trabulsi, L.R. *Escherichia coli* serogroup O111 includes several clones of diarrheagenic strains with different virulence properties. *Infect. Immun.*, 62: 3282-3288, 1994.

b. Paper or chapter in a book
Nelson, E.B. Current limits to biological control of fungal phytopathogens. *In*: Arora, D.K.; Rai, B.; Mukerji, K.G.; Knudsen, G. (eds). *Handbook of applied mycology; soils and plants*. Marcel Dekker, New York, 1991, p.327-355.

c. Book by author(s)
Salyers, A.A.; Whitt, D.D. *Bacterial pathogenesis. A molecular approach*. ASM, Washington, 1994, 418p.

d . Patent
Hussong, R.V.; Marth, E.H.; Vakaleris, D.G. Manufacture of cottage cheese. *U.S. Pat. 3,117,870*. Jan. 14, 1964.

e. Thesis
Calzada, C.T. Campylobacter jejuni *e* Campylobacter coli - *caracterização em sorogrupos e biotipos das cepas isoladas no município de São Paulo, no período de 1983-1989*. São Paulo, 1991, 131p. (Ph.D. Thesis. Instituto de Ciências Biomédicas. USP).

f. Publication with no identifiable author or editor
Anonymous. The economy of by-products. *Álcool Alcoolquim.*, 2;33-40, 1985.

g. Communications in events (Symposia, conferences, etc)
Simão, G.S.; Silva, J.; Toledo, A.S.; Gontijo Filho, P.P. *Micobactérias não tuberculosas isoladas de pacientes com a síndrome de imunodeficiência adquirida.* XVII Congresso Brasileiro de Microbiologia, Santos, 1993, p.41.

References citing "personal communication" or "unpublished data" are discouraged, although it is recognized that sometimes they must be used. In these cases, they should be cited in the text and not in the list of references. References consisting of papers that are "accepted for publication" or "in press" are acceptable. However, references of papers that are "submitted" or "in preparation" are not acceptable.

Tables
Tables should not be included in the text. Each Table must be typed in a separate page and numbered sequentially with an Arabic number. The title of a Table should be placed in the top of it and should be brief but fully descriptive of the information in the Table. Headings and subheadings should be concise with columns and rows of data carefully centered below them.

Figures
Arabic numbers should be used for numbering the Figures. Data in Tables should not be repeated in Figures. The legend of the Figures should be placed in the bottom of them.

Photographs and line drawings
Only those photographs which are strictly necessary for the understanding of the paper should be submitted. Photoprints must be of sufficient quality to ensure good reproduction. They should be numbered on the back and identified with the nominated author's name. Legends of line drawings and photographs should not exceed the printing area. All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

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