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METHODS TO ASSESS BACTERIAL INTERACTION WITH CULTURED MAMMALIAN CELLS

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MINI-REVIEW

ABSTRACT

The ability to adhere to and invade eukaryotic cells are important virulence factors for many human pathogens. Mammalian cells in culture have been shown to be an useful instrument to elucidate bacterial and host factors involved in the adherence and invasion processes. As closely as possible, the cultured cells chosen for analysis should resemble cells naturally colonized and with which the microorganisms interact with in tissues. Moreover, cultured cells present some limitations that should be kept in mind while attempting to compare results obtained *in vitro* to host tissues. This review outlines the advantages and disadvantages of different methods used to study bacterial adherence and of methods used to distinguish between bacteria that are attached to the surface of the cells from those that have been internalized by them. Aside from the ability to recognize an eukaryotic cell epitope, bacterial adhesins may also be toxins and may induce cell death by both necrosis and apoptosis. This review describes some of the methods used to assess cell viability, mainly methods that detect any loss of membrane permeability and those that detect a disturbance of vital cell functions. Finaly, we have discussed how necrotic and apoptotic cells can be distinguished from each other.

Key words: Cell culture; bacterial adherence; bacterial invasion; cell viability

CHOICE OF CULTURED CELLS

Bacterial adherence endows human pathogens with the ability to withstand normal host defense cleasing mechanisms on mucosal surfaces. Adherence is, therefore, a prerequisite for colonization of epithelial surfaces. This initial host-microbial interaction is mediated by bacterial surface ligands, called adhesins (37), that bind to complementary protein or carbohydrate epitopes present on the cell surface (20, 23). Frequently, multiple ligands on the pathogen's surface serve to increase the strenght of adherence, when these ligands are engaged in concert.

Bacterial adhesins have very specific requirements for recognition of eukaryotic cell epitopes. Consequently, bacterial binding is restricted to a set of cell populations carrying their optimal receptors. This partly determines the niche a bacterium is able to occupy or the set of infections a bacterial pathogen is characterized by (12).

Cultured mammalian cells provide a simple and easily controlled model for investigating eukaryotic cell-bacterium interaction. Given the variation in expression of cell surface epitopes known to exist between species and tissues/organs, as well as among cells originating from the same tissue as a function of

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developmental and differentiation stages (13, 14), careful consideration must be made to the type of cultured cell chosen for the assay. As closely as possible, the cultured cell chosen for analysis should resemble cells naturally colonized, and with which the organism interacts with in tissues.

It may be possible to prepare and maintain cell populations from explants of normal tissue in primary culture. In fact, cells in primary cultures should be used whenever it is possible, since these cells should reflect the cells found in nondiseased tissues *in vivo*. For tissues whose cells cannot be conveniently obtained in primary culture, it is often possible to identify a transformed cell line that is derived from the tissue of interest.

A number of important limitations should be considered while attempting to compare results obtained with cultured cells to host tissue. These include the fact that the state of differentiation that a given cell type can be maintained at in culture may not reflect the state that is present in the tissue. Cultured cells frequently lose many of the traits that are characteristic of the cells *in vivo*, especially since most cell lines have been established from malignant tissue. Also, if the cell utilized has been transformed, it may be considerably altered in the repertoire of receptors that it will express, since the processes of transformation produce numerous mutations and rearrangements in the genome of the cell. Moreover, cell lines that are passed repeatedly in culture will continue to develop new mutations and rearrangements (13). The fact that two different investigators are using a cell line with the same name does not necessarily mean that they are using genetically identical cell lines. To make the transition from a differentiated, nondividing cell found *in vivo* to a rapidly dividing cell, cultured cells must be stripped of many of the properties that made them the sort of cell they were. One feature that can be lost in the process of cell passage is tissue-specific cell surface molecules that normally function as bacterial receptors, unmasking low affinity receptors. Such an occurrence may explain the surprising fact that many bacterial pathogens that are highly specific for a particular tissue when causing an infection in an intact animal are able to adhere to and invade cultured cells derived from tissue they do not infect normally. In addition, most cultured cells lose their normal shape and distribution of surface antigens. They also do not form tight junctions and are incapable of forming impermeable barriers (35). However, cell growing on porous membranes or on supporting type I collagen or EHS gels have been reported to favor cell polarization and differentiation (4, 6).

Still another problem concerning cultured cells as representative of human mucosal surfaces is that real mucosal surfaces are covered with mucus and bathed in solutions that are difficult to mimic in an *in vitro* system. The fact that there are problems with cultured cells does not mean that they have not been extremely useful. If their limitations are kept in mind, cultured cells can be a marvelous tool for discovery. Thus, cultured cells are important for generating hypothesis that can then be tested in intact animals.

METHODS TO ASSESS BACTERIAL ADHERENCE

Different approaches have been used to investigate bacterial adherence to cultured cells. In most of them, cells are exposed to bacterial suspensions for different periods and rinsed, to eliminate nonadherent microorganisms. Cultured cells are then stained with Giemsa or Gram dyes or submitted to critical point drying, to be observed under light or scanning electron microscopes. In both these approaches it is possible to determine the percentage of cells with adherent bacteria as well as the mean number of adherent bacteria per cell. In another method cells are treated with detergents to detach bound bacteria. Cell lysates are diluted and plated to furnish the concentration of adherent bacteria. Adherent bacteria can also be labeled with anti-bacterial antibodies and next treated with an anti-IgG antibody complexed to horseradish peroxidase and their presence can be quantified by an ELISA reader (1). Alternatively, one can work with microorganisms labeled with different isotopes (25, 32). Bacterial adherence is next quantified by scintillation counting. However, it is higly recomended that the initial assessment of adherence be conducted by microscopy, to ascertain that bacteria are actually binding to the cells and not to supporting matrices or dishes. For instance, while investigating *P. aeruginosa* interaction with human epithelial respiratory cells in primary culture, we have worked on the culture model described by Chevillard *et al.* (6) in which cells have been cultured on a type I collagen matrix for the expression of differentiatied functions. When the cultures were exposed to *P. aeruginosa* suspensions, we noticed that bacterial adherence to the supporting matrix was significantly higher than to the epithelial cells (33). If we have

worked on radiolabeled bacteria, all the radioactivity associated with the collagen matrix would have been attributed to bacterial adherence to the epithelial cells. Another advantage of microscopic methods to assess bacterial adherence is that they may reveal that the organisms are binding nonuniformly to cultured cells. Bacteria may be found binding to specific area of the culture that may correlate with distribution of the cellular receptors. For instance, still during our study on *P. aeruginosa* interaction with human epithelial respiratory cells, we have worked on the wound repair model described by Zahm *et al.* (42). In this model, a few epithelial cells were dettached from the collagen gel on which they have been cultured, creating a circular wound in the cell monolayers. By two hours after the culture injury, cells of the edges of the wound were seen to spread and migrate to cover the denuded area. When the wounded cultures were exposed to *P. aeruginosa* suspensions, we noticed the high affinity of bacteria for cells with the spreading phenotype from the wound borders (34). Very recently, de Bentzmann *et al.* (7) have shown that the upper membranes of these spreading cells are particularly rich in asialo GM1 residues, a major receptor for *P. aeuginosa* adhesin.

However, microscopic methods also present a few disadvantages: they are tedious and time consuming and this is particularly true for scanning electron microscopic methods.

For many bacteria, persistance in host tissues depends on the invasion of epithelial cells following attachment. Recent studies have shown that human pathogens that are not generally considered intracellular parasites can entry the epithelial cells and reside intracellularly. Research on bacterial adherence by light microscopy is often hampered by the inability to distinguish between bacteria that are attached to the surface of the cells and those that have been internalized by them. Different methods have been proposed to distinguish between attached and internalized bacteria. Some of these methods take advantage of the impermeability of eukaryotic cell membranes to large molecules, such as antibodies. While investigating the interaction of *P. aeruginosa* with Caco-2 cells, Cervante *et al.* (5) have fixed cell monolayers previously exposed to bacterial suspensions with 4% paraformaldehyde in PBS, a fixative solution that does not permeabilize cell membranes. Thereafter cell cultures were treated with anti-bacterial antibodies complexed to horseradish peroxidase, to label attached microorganisms, and counter stained with the Giemsa dye. By light microscopy, extracellular bacteria appeared stained in brown, while epithelial cells and intracellular microorganisms appeared stained in blue. Another approach taking advantage of the impermeability of cell membranes to large molecules depends on the quenching of the fluorescence of extracellular bacteria by crystal violet, as proposed by Hed (15). In this method, following the incubation of eukaryotic cells cultured on glass coverslips with fluorescent bacteria, cells are submitted to fluorescence microscopy, to determine the percentage of cells with associated microorganisms as well as the mean number of adherent bacteria per cell. In parallel, other coverslips are treated with crystal violet and re-submitted to fluorescence microscopy. Extracellular bacteria have their fluorescence quenched and appear dark and nonfluorescent while intracellular microorganisms remain bright fluorescent. However, the most current test to quantitate intracellular bacteria is the gentamicin exclusion assay in which cultured cells previously exposed to bacteria are treated with gentamicin to eliminate extracellular bacteria (24). Internalized bacteria are not usually affected by the antibiotic. Detergent-induced lysis of eukaryotic cells releases intracellular bacteria. Dilution and plating of cell lysates allows quantitation of intracellular microorganisms (38).

Once the adherence capability of a given bacteria has been established, the next step will be to characterize the molecular structure of the cellular binding epitope. Just like antibodies and lectins, bacterial adherence have a very specific structural requirements for recognition and binding of cell surface epitopes. Therefore, receptor active molecules can be characterized indirectly by adherence inhibition experiments in which bacteria are preincubated with defined soluble proteins (such as proteolytic enzymes or antibodies) or glycoconjugates (such as carbohydrates or lectins) prior to overlay on cells, to prevent attachment in a competitive fashion.

METHODS TO ASSESS THE TOXIC EFFECT OF BACTERIA ON CULTURED CELLS

Aside from the ability to recognize an eukaryotic cell epitope, bacterial adhesins may also be toxins (16). For instance, the ability to interact with a repertoire of glycoproteins and glycilipids on eukaryotic cells confers on pertussis toxin (41) and on *P. aeruginosa* exotoxin S (2) the capability on the one

hand to act as an adherence bridge for the whole bacterium and on the other hand to mediate cellular intoxication by presentation of the enzymatically active toxic subunit.

Assays to evaluate the effect of bacterial toxins on cultured cells can be divided into two major classes: those that detect an immediate or short term reponse, leading to an alteration in cell membrane permeability and those that detect a disturbance of vital cell functions.

Some of the methods used to assess cell viability depend on colorimetric assays. The major advantage of colorimetric assays over measurement of cytotoxicity by dye exclusion and microscopic evaluation is the speed with which samples can be processed. Moreover, colorimetric assays shares with radioisotope assays the advantage of precise quantitation.

MEMBRANE PERMEABILITY ASSAYS

Loss of cell viability is most often measured by assays that detect any loss of membrane integrity. These assays assume that the abnormal passage of impearmeant markers that are either in colloidal suspension or are too large or too polar for passage through the plasma membranes can be used as indicators of cell damage. Loss of membrane permeability can be assessed by i) the entry of impermeant dyes or ii) the release of cytoplasmic components.

(i) Entry of impermeant dyes. The uptake of Trypan blue is by far the commonest test used to assess cell viability, but other colloidal stains, such as erythrosin B, eosin or nigrosin, can also be used. In an attempt to allow cell observation following longer periods, after staining by Trypan blue, cells can be fixed prior to light microscopic observation. One of the features of apoptotic cells is that they retain their membrane integrity for some time after the apoptotic program has started. In consequence, loss of membrane integrity usually occurs only in final stages of the process. Therefore, cells in the early stages of apoptosis retain their ability to exclude the vital dyes and may be scored as viable. Other dead cells may be poorly stained due to an extensive loss of the cytosolic proteins stained by the dyes. Therefore, cell viability is usually overestimated (27).

The uptake of fluorescent stains that will intercalate in the cell DNA, such as ethidium bromide and propidium iodide has also been used to assess cell death (21, 39). Although both stains are structurally similar DNA intercalators, they differ in their routine usage. Ethidium bromide, that is often used to detect DNA in gels, is less water soluble and more membrane permeable. It is, therefore, less suitable than propidium iodide for cytotoxicity assays. A homodimer of ethidium bromide, ethidium homodimer-1, is reported to have a much higher DNA binding affinity as well as a lower membrane permeability than either ethidium bromide and propidium iodide. All three intercalating dyes can be excited by the mercury or xenon lamps used in fluorescent microscopes and in fluorescent microplate readers. They are also excited by argon lasers and are therefore suitable for flow cytometry (17).

(ii) Cytoplasm leakage assays. Cell membrane integrity can also be assessed by the release of cytoplasmic components into culture media. The release of the enzyme lactate dehydrogenase (LDH) is one of the most frequently used leakage assays (8, 28). LDH activity is easy to be measured by the conversion of NAD to NADH in the presence of excess lactate, detected by microplate readers. However, in at least in one comparative study of different methods to assess cell viability, the release of LDH was found to be relatively slow when compared with the rapid nuclear staining by propidium iodide, at the beggining of cell death (22).

Another leakage assay depends on cell labeling with a radioactive isotope of Chromium (^{51}Cr). The reduced form of the isotope - $^{51}Cr^{3+}$ - present in the salt $Na_2\,^{51}Cr\,O_4$ is easily taken up by living cells and is oxidized to the membrane impermeable $^{51}Cr^{2+}$ form. The release of oxidized radioactive Cr in the culture medium, detected by scintillation counters, correlates with cell death while retained radioactivity correlates with cell viability (40, 43).

VIABILITY ASSAYS

Viability assays attempt to distinguish living from dying cells by using indicators of vital cell functions. Some viability assays exploit normally functioning cytosolic enzymes that account for the production of easily detected fluorescent or colored products within the living cells.

(i) Lipophilic acetoxy methyl esters readily and passively diffuse across cell membranes. Once inside the cells, these nonfluorescent esters are transformed by endogenous esterases to fluorescent polar hydrophilic compounds to which cell membranes are

no more permeable. Dead or dying cells that still have residual esterase activity can appear fluorescent but will rapidly leak the die. Fluorescence associated with viable cells can be detected by fluorescence microscopy or by fluorescence microplate readers. A number of acetoxy methyl compounds have been used for viability tests, such as fluorescein diacetate, bis-carboxyethyl-carboxyfluorescein (BCECEF; 26) and calcein acetoxymethyl ester (30). Calcein AM has more widespread application because it has longer retention time and is less pH sensitive than other fluorescein analogs.

(ii) A second group of indicators that are taken up and metabolized by viable cells is composed by mitochondrial stains, such as Rhodamine 123 and JC-1 that are lipophilic cationic probes that interact specificaly with functional mitochondria, due to their transmembrane potential with negative interior charge maintained by proton pumps (18, 19). Both these probes can be used in association with other viable stains. Juurlink and cols. (22), have shown that mitochondrial staining of living cells and nuclear stainig of dead cells with propidium iodide are mutually exclusive phenomena: the loss of Rhodamine 123 fluorescence in living cells was shown to precede immediately the onset of staining of nucleous.

(iii) Tetrazolium salts, such as MTT and XTT are taken up by viable cells and reduced by mitochondrial succinate dehydrogenases to blue insoluble formazan that can be detected by light microscopy. The insoluble formazan is formed in the cells in propotion to the amount of dehydrogenase activity and this gives an approximation of the number of viable cells in the wells (11, 29). Formazan can also be solubilized by acidic isopropanol and detected by microplate readers. Improvements made by Denizot and Lanz (9) have made the assay a reasonable quantitative alternative to other semiautomated methods. MTT test appears to be a very useful quantitative assay for laboratories not equipped with fluorescence microplate readers. Assays based on XTT allows direct absorbance readings, therefore eliminating the solubilization step (36).

(iv) Finally, cell viability can be assessed by Alamar blue (31). This is a nonfluorescent redox indicator that is added to the culture medium. The reduction of Alamar blue by cellular metabolites present in the growth medium gives origin to a soluble fluorescent product that accumulate in the culture medium and can be detected by fluorescence microplate readers.

The major advantage of this method is that as Alamar blue is nontoxic, it allows for continuous and kinetic monitoring of cell viability for extended periods, in contrast to MTT, XTT reduction or vital dye uptake that are single end point, sample destructive methods (27). A disadvantage of the assay is that cytostatic drugs that interfere with the cellular metabolism can give misleadig results.

CONCLUSION

All the methods presented in this review do not allow the distinction between cellular necrosis and apoptosis. Cell necrosis and apoptosis are said to be easily distinguished by morphological and biochemical criteria.

The major morphological characteristic of apoptotic cells are chromatin condensation and segmentation of the nucleous while necrotic cells present a normal nuclear morphology, with flocculated chromatin and a desintegrated cytoplasm (3).

Biochemically, the major characteristic of apoptotic cells is the fragmentation of the cell DNA to multimers of 200 bp, that can be detected by different techniques (10), such as agarose gel electrophoresis of nucleosomal DNA fragments, DNA fragment assay by [^{3}H] thymidine release and TUNEL assay (in situ nick end-labeling assay).

RESUMO

Métodos de estudo da interação bacteriana com células de mamíferos em cultura

A virulência de muitos patógenos humanos parece depender de sua capacidade de aderir e de invadir células das mucosas hospedeiras. As culturas de células de mamíferos são instrumentos de grande utilidade para a elucidação dos fatores bacterianos e das células hospedeiras envolvidos nos fenômenos de aderência e de invasão celular. Na medida do possível, as células escolhidas para os ensaios devem se assemelhar às células naturalmente colonizadas e com que as bactérias interagem *in vivo*. As culturas de células também apresentam algumas limitações que devem ser levadas em consideração toda vez que pretendermos extrapolar os resultados obtidos *in vitro* para fenônemos que ocorrem *in vivo*. Neste artigo discutimos algumas vantagens e desvantagens de diferentes métodos utilizados para estudar a aderência

bacteriana e para distinguir microrganismos que permanecem aderidos à superfície das células de outras que são por elas internalizadas. Além da capacidade de reconhecer epitopos presentes nas membranas de células eucarióticas, as adesinas bacterianas também podem ter uma atividade tóxica e induzir a morte da célula hospedeira, tanto por necrose quanto por apoptose. Neste artigo apresentamos alguns dos métodos utilizados para avaliar a viabilidade celular, principalmente métodos que detectam a perda da permeabilidade das membranas e métodos que detectam distúrbios das funções celulares vitais. Finalmente, discutimos como células necróticas e apoptóticas podem ser diferenciadas.

Palavras-chave: Cultura de células; aderência bacteriana; invasão bacteriana; viabilidade celular.

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GENETICAL REARRANGEMENTS IN *AZOSPIRILLUM BRASILENSE* INDUCED BY THE PLASMID PJB4JI

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ABSTRACT

The present work was conducted with the initial purpose of looking for the characterization of *Azospirillum brasilense* strain ATCC 29.145 mutants induced by insertions of the transposon Tn-5. The strategy used to transfer such mobile element was based on the vector named pJB4JI::Mu::Tn-5 that was originally constructed to behave as suicide plasmid when within non-enterobacterial cells.

Nevertheless, when such plasmid was transferred into *A. brasilense* cells, it replicated as in enterobacteria and due to this situation it was possible to detect modifications on the indigenous plasmid content of *A. brasilense* cells. Among the changes, the absence of the *A. brasilense* resident plasmids formally named pFL1 and pFL2 was observed. Plasmid incompatibility was originally thought as an explanation for the loss of pFL1 and pFL2 plasmid bands, but using some other plasmids of the same incompatibility group as that of pJB4JI::Mu::Tn-5 it was not possible to observe the same situation.

Further analysis revealed that probably the formation of cointegrate was happening between pJB4JI::Mu::Tn-5 and the sole plasmid pFL resident of *A. brasilense* cells forming a genetical extra-chromosomal element with higher molecular weight. Thus one can state that most probably *A. brasilense* cells actually contain only one plasmid that will be referred to, from now on, as pFL1.

Key words: Genetical rearrangements, cointegrate, nitrogen fixation, plasmid incompatibility.

INTRODUCTION

The genus *Azospirillum* comprises five species: *Azospirillum brasilense, Azospirillum lipoferum* (21), *Azospirillum amazonense* (10), *Azospirillum halopreferans* (14) and *Azospirillum irakense* (7). These bacterial species have attracted generalized attention because they are nitrogen fixers (21). A number of papers involving studies of nitrogenase properties and other enzymes produced by these bacteria have been published (6, 11, 20-22, 23). In relation to carbohydrates metabolism there are also some interesting papers dealing with azospirila (9, 11, 15).

The genetical approach of this bacterial genus began in the late seventies. Lemos *et al.* (8) have proposed the possibility of the nif genes (genes for nitrogenase production) to be located in a plasmid found in the type strain of *A. brasilense*. Elmerich and Franche (4), Wood *et al.* (24) and Sing and Wenzel (19) presented different results with respect to the number of plasmids within azospirila cells. Also Plazinski *et al.* (13) have described data concerning the presence of plasmids and the localization of the nif

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genes in azospirila cells. The search for mutants of azospirila resulted in the detection of auxotrophic strains induced by chemical mutagenesis (5, 24), auxotrophic and nif⁻ mutants induced by transposon (17, 18) mutagenesis and also the use of the bacteriophage Mu (17) for such purposes. Elmerich and Franche (4) described a strategy to use the plasmid pJB4JI (2) to introduce the transposon Tn-5 within *A. brasilense* cells based on plasmid incompatibility and, have detected auxotrophic and nif⁻ mutants at a frequency of 10^{-2} mutant cells/ml. Further analysis resulted in a better understanding of the genetical architecture of *A. brasilense* cells in which a plasmid of 90 MDa (12) in the same strain used in this work is described.

We have followed the same procedures described by Elmerich and Franche (4) but found different results. This paper describes such differences.

MATERIALS AND METHODS

Bacterial Strains. *Azospirillum brasilense* type strain (ATCC 29.145) was received from P. Arruda (UNICAMP/ Brasil), *Escherichia coli* 1830 (pJB4JI), *Escherichia coli* 1843 (pJB3JI) and *Escherichia coli* J 53 (pPH1JI) were sent by J. Beringer (England) and *Escherichia coli* J 53 (pRP4) was sent by L. R. Trabulsi (EPM/ Brasil).

Culture Media. All bacterial strains were liquid grown in Luria broth. Solid TYE plates (16) containing appropriate antibiotics were used for general maintenance and selection of exconjugants.

Bacterial Conjugation Technique. According to Beringer *et al.* (2) cells at log phase of growth were harvested on 47 mm Millipore membranes and incubated for 3 - 4 h at 30°C. After that, re-suspension of the cells was carried out on phosphate buffer pH 7,0 and plating was developed according to each of the conjugal crosses. The abilities of the exconjugants to grow in the presence of certain antibiotics was a first test of the plasmids mobilization and was done on TYE plates containing either: Kanamycin (100 μg/ml) or Gentamycin (100 μg/ml) or Spectinomycin (150 μg/ml), or Tetracycline (50 μg/ml) or Carbenycilin (100 μg/ml).

Plasmid Detection Technique. Agarose gel electrophoresis was carried out according to Eckhardt (3) and the gels were photographed with Panatomic 32 ISO film when necessary. The films were developed according to the manufacturers' procedures.

RESULTS

From conjugal crosses involving as donor strain the *E. coli* 1830, harboring the transposon Tn-5, it was possible to isolate several hundreds of exconjugants able to form colonies on TYE plates containing 100 μg Km/ml or 150 μg Sp/ml. The frequency of conjugation between *E. coli* 1830 and *A. brasilense* (ATCC 29.145) is listed in Table 1.

Through agarose gel electrophoresis (3), it was possible to observe that all exconjugants from the conjugal cross cited above showed the presence of the plasmid pJB4JI (Fig. 1). What was also observed in the same set of data was that most of the exconjugants exhibiting the presence of the plasmid pJB4JI, at the same time had lost their indigenous plasmids. These plasmids named pFL1 and pFL2 were described elsewhere (8). In order to test the possibility of plasmid incompatibility between the transposon plasmid and the indigenous plasmids (pFL1 and pFL2), other conjugal crosses involving other plasmids of the same incompatibility group, as the one of the transposon vector plasmid (group P) were analyzed. These other conjugal crosses were carried out using strains of *E. coli* harboring the plasmids pJB3JI, pRP4 and pPH1JI (which originated the transposon vector plasmid pJB4JI, Beringer *et al.* (2)). The conjugation frequencies of these strains with *A. brasilense* are listed in Table 1.

From the results of the abilities of the exconjugants to grow in the presence of Km, Cb, Tc, (data not shown) it was possible to observe that these other plasmids were also stable inherited by *A. brasilense* cells. The electrophorograms of exconjugants have all exhibited the presence of the indigenous plasmids and the supposed incompatible challenge plasmids. Data shown on Fig. 2, 3 and 4.

Table 1. Frequency of conjugation involving the transfer of various plasmids of the incompatibility Group P.

Plasmid	Frequency
pJB4JI	$8{,}6 \times 10^{-5}$
pRP4	$4{,}7 \times 10^{-4}$
pJB3JI	$5{,}5 \times 10^{-5}$
pPH1JI	$6{,}2 \times 10^{-7}$

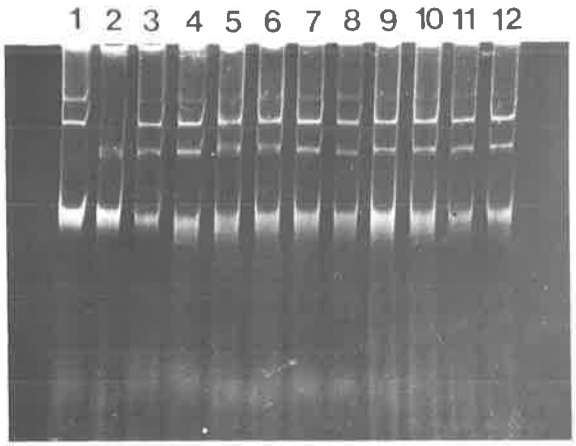

Figure 1. Electrophorogram of *A. brasilense* indigenous plasmids possible cure after the introduction of the Tn-5 vector plasmid pJB4JI.
LANES:
3, 6 and 9 *A. brasilense* (pFL1 and pFL2).
1, 4,7 and 10 *E. coli* (pJB4JI).
5 and 8 *A. brasilense* exconjugants harboring pJB4JI lacking pFL1 and pFL2.

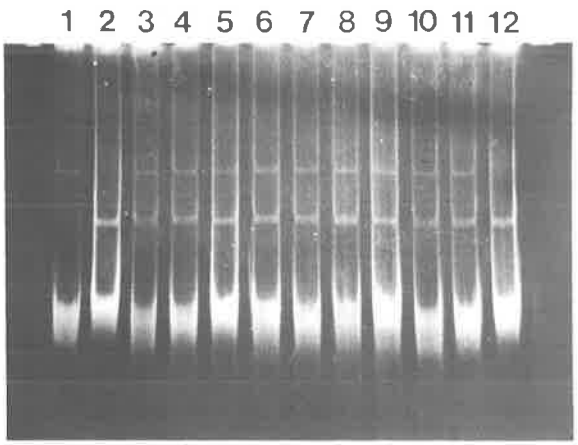

Figure 3. Electrophorogram of *A. brasilense* exconjugants harboring the plasmid pPH1JI.
LANES:
1 - *A. brasilense* (pFL1 and pFL2)
2 - *E. coli* J53 (pPH1JI)
3 - 12 *A. brasilense* exconjugants showing the plasmids pPH1JI , pFL1 and pFL2.

Figure 2. Electrophorogram of exconjugants of *A. brasilense* cells after the introduction of the plasmid pRP4.
LANES:
1 - *A. brasilense* (pFL1)
2 - *E. coli* J 53 (pRP4)
3 - 10 *A. brasilense* exconjugants harboring pRP4 and pFL1.
* - the dimer (pFL2) is not visible in this electrophorogram.

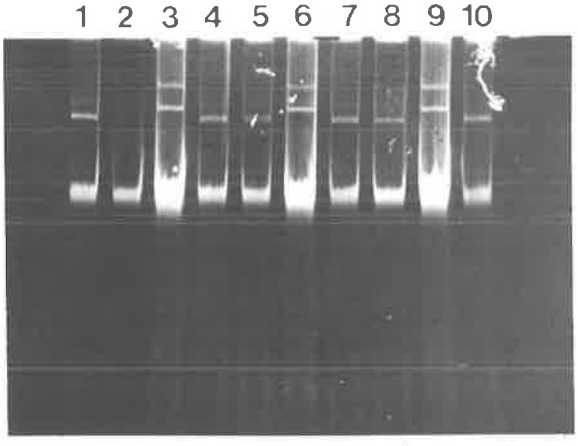

Figure 4. Electrophorogram of *A. brasilense* exconjugants harboring the transposon vector plasmid pJB4JI showing the cointegrate formation.
LANES:
1 - *E. coli* 1830 (pJB4JI)
2 - *A. brasilense* (pFL1*)
3 - 12 *A. brasilense* exconjugants exhibiting the vector plasmid pJB4JI (lower band) and cointegrate (upper band).

These results meant to us that what happened by way of the first conjugal cross using the transposon plasmid vector pJB4JI was something involving not the incompatibility grouping of plasmids but actually something that was related to the vector pJB4JI itself.

It has also been observed that after a certain time of incubation of the exconjugants harboring the transposon vector plasmid pJB4JI, the electrophorograms showed different patterns of plasmid mobility. Such change of mobility pattern might have resulted from the formation and resolution of cointegrates between the transposon vector plasmid and the indigenous plasmids found within *A. brasilense* cells. The data on the formation and

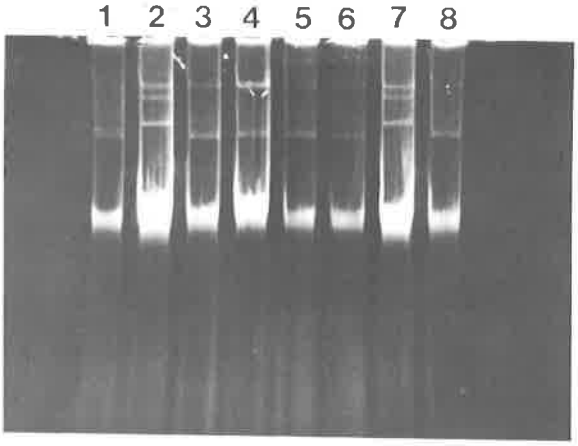

Figure 5. Electrophorogram of *A. brasilense* exconjugants harboring the transposon vector plasmid pJB4JI showing the cointegrate resolution.
LANES:
1 - 2 *E. coli* 1830 (pPH1JI)
3 - 8 *A. brasilense* exconjugants showing the formation of cointegrate (lane 7) and the cointegrate resolution (lanes 3, 4, 5, 6 and 8).

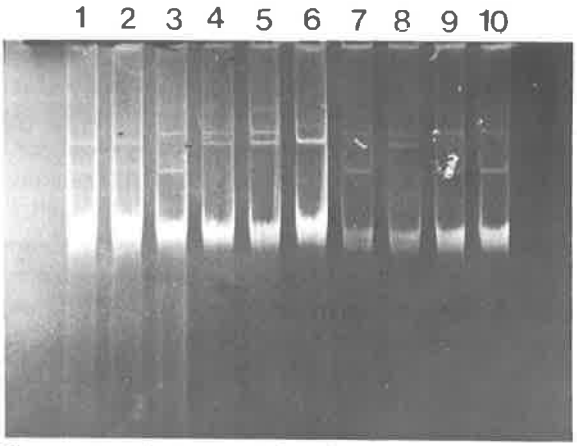

Figure 6. Eletrophorogram of *A. brasilense* exconjugants harboring the transposon plasmid pJB4JI showing the cointegrate resolution.
LANES:
1 - 2 *E. coli* 1830 (pJB4JI)
3 - 10 *A. brasilense* exconjugants showing the formation of cointegrate (lanes 3, 7, 9 and 10) and the cointegrate resolution (lanes 4, 5, 6 and 8).

resolution of cointegrates can be observed in Figures 5 and 6.

DISCUSSION

From the results obtained in the conjugal crosses done it was observed that not only the transposon vector plasmid pJB4JI but also other plasmids of the same incompatibility group are stable inherited by *A. brasilense* acting as receptor strain. Such results are in agreement with data described by Elmerich and Franche (4). In relation to the transposon Tn-5 plasmid vector pJB4JI, since it also has an insertion of a deleted bacteriophage Mu (2), it has been suspected that when this plasmid was introduced within *A. brasilense* cells it has been possible, in some cases, to cause the formation of cointegrates involving the bacteriophage Mu and the indigenous plasmids found in *A. brasilense* cells. The transposon Tn-5 is not able to induce the formation of cointegrates (1) and due to that fact it has been thought that the only way to explain cointegrate formation was to ascribe such ability to the prophage of the bacteriophage Mu.

In all the cointegrates formation cases only one kind of the cointegrates was present. This result meant to us that this could only have happened because actually the *A. brasilense* cells have only one type of indigenous plasmid instead of two as early described (8). In favor of this reasoning, in such early description it was observed that the once named pFL2 has almost the double molecular weight of pFL1, which now can be interpreted as the pFL2 being a dimer of PFL1. That is why only one type of cointegrate is formed when pJB4JI was introduced within *A. brasilense* cells. What actually happened was the formation of a cointregate involving pFL1 as the only indigenous plasmid of *A. brasilense* cells and the deleted bacteriophage Mu prophage found as insert of the transposon Tn-5 plasmid vector pJB4JI. The description of a 90 MDa plasmid within *A. brasilense* cells by Onyeocha *et al.* (12) seems to confirm this hypothesis with even more strength.

RESUMO

Rearranjos genéticos em *Azospirillum brasilense* induzidos pelo plasmídio pJB4JI

O presente trabalho foi conduzido com a proposta inicial de buscar a caracterização de mutantes da linhagem ATCC 29.145 de *Azospirillum brasilense*, induzidos por inserções do transposon Tn-5. A estratégia utilizada para transferir tal elemento móvel foi baseada no uso do vetor plasmídico denominado pJB4JI::Mu::Tn-5 que foi originalmente construído para se comportar como um plasmídio suicida quando dentro de células bacterianas não enterobacteriais.

Apesar disto, quando este plasmídio foi transferido para *A. brasilense*, ele sofreu replicações como o faz quando em células de enterobactérias e, devido a este fato, foi então possível de se detectar modificações nos plasmídios autóctones das células de *A. brasilense* . Entre estas mudanças foi observado a ausência dos plasmídios anteriormente denominados pFL1 e pFL2. Eventos de incompatibilidade plasmídica foram inicialmente pensados terem ocorrido e poderiam estar relacionados com a ausência dos pFL1 e pFL2 mas, usando outros plasmídios de mesmo grupo de incompatibilidade que o do vetor pJB4JI::Mu::Tn-5, não foi possível observar a mesma situação.

Análises posteriores revelaram que provavelmente a formação de cointegrados pode ter ocorrido envolvendo o vetor plasmídico pJB4JI::Mu::Tn-5 e o único plasmídio residente de células de *A. brasilense*, que é o pFL1, formando um elemento extra-cromossômico de muito maior peso molecular. Então, através destas análises, foi possível concluir que as células de *A. brasilense* possuem na realidade apenas um plasmídio autóctone doravante denominado pFL1.

Palavras-chave: Rearranjos genéticos, cointegrado, fixação de nitrogênio, incompatibilidade plasmídica.

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DYNAMICS OF GROWTH AND SYNTHESIS OF CAPSULAR POLYSACCHARIDES OF *ACTINOBACILLUS PLEUROPNEUMONIAE*

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ABSTRACT

The dynamics of growth and synthesis of capsular polysaccharides of a strain of *Actinobacillus pleuropneumoniae* serotype 5 under several conditions of culture was studied. The bacteria were grown in Brain Heart Infusion and Trypticase Soy Broth, both supplemented with Yeast Extract. Culturing was performed in stationary culture under CO_2 atmosphere or agitated in aerobic conditions. Bacterial mass, hexosamine concentrations, adherence to n-Hexadecane and oxygen demand were determined at several periods of incubation. The logarithmic phase ended earlier. Bacterial mass yields and hexosamine concentrations were higher in aerated than in non-aerated cultures. The maximal oxygen demand coincided with the end of the logarithmic phase of growth. Bacterial adherence to n-Hexadecane did not have any relationship with the growth phase or with the concentration of capsular polysaccharides.

Key words: *Actinobacillus pleuropneumoniae*, capsule synthesis, hydrophobicity.

INTRODUCTION

Actinobacillus (Haemophilus) pleuropneumoniae (Ap) is the primary etiological agent of swine pleuropneumonia (15). It synthesizes a polysaccharidic capsule, whose antigenic diversity allows its classification into 12 serotypes (13). Serotype 5 is the most prevalent in Brazil, being isolated mainly in the southern region (17).

It has been suggested that the protection induced in pigs by the infection with this agent (5) as well as that conferred by a bacterin produced with young cultures of *A. pleuropneumoniae* (6 hours of growth) (18) is related with the presence of capsule, a polysaccharide that contains 85% of hexosamine (5). Jacques *et al.* (9) observed that the amount of capsular material varied with the age of the culture, suggesting a relationship between capsule and immunogenicity. Very little information is available about the factors that may affect *A. pleuropneumoniae* growth, such as medium composition, pH and aeration and their relationship with capsule synthesis.

The objective of this work was to study the synthesis of capsular polysaccharides of *A. pleuropneumoniae* serotype 5 in two media commonly used for the industrial production of antigens and the effect aeration could have on it.

MATERIALS AND METHODS

Bacterial strain. A strain of *Actinobacillus pleuropneumoniae* serotype 5 (27 v), isolated from an outbreak of swine pleuropneumonia in Criciuma, Santa Catarina, Brazil, was used throughout the study.

Serotyping was carried out according to standard procedures (4).

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Culture media and growth conditions. Brain Heart Infusion (BHI, Difco, Michigan, USA) and Trypticase Soy Broth (TSB, Biobras, Montes Claros, MG, Brazil) supplemented with fresh yeast extract as a source of V factor were used.

Bacterial suspensions obtained from cultures on Brain Heart Infusion Agar (BHIA) incubated at 37°C for 18 hours in a 10% CO_2 atmosphere were used to inoculate media cultivated in shaker and in fermenter.

Cultures in Erlenmeyer flasks. Two hundred and fifty ml Erlenmeyer flasks containing 50 ml of BHI or TSB were inoculated with the suspension obtained from one plate of *A. pleuropneumoniae* grown to confluency on BHIA for 18 hs at 37°C in 10% CO_2. The flasks were incubated in an orbital shaker (New Brunswick, NJ, USA) at 37°C and 180 rpm for 30 hours or statically in an atmosphere of 10% CO_2. Samples were collected at 0, 4, 7, 8, and 24 hours of incubation.

Data are the average of at least two experiments.

Growth in fermenters. Fermenters (Multigen F-2000, New Brunswick Scientific, NJ, USA) containing 1000 ml of either BHI or TSB media were inoculated with 100 ml of an agitated culture grown in the respective medium for 7 hours at 37°C in an orbital shaker. Silicone at a final concentration of 1:5000 was used as antifoaming agent when necessary. The cultures were grown at 37°C with constant stirring at 350 rpm. Samples were collected at 0, 2, 4, 7, 9, 24 and 30 hours of culture.

Two levels of aeration were tested :

a) 1 volume of air/volume of media/minute (1 vvm);

b) No air supply.

Data expressed are the means of two or three experiments.

Assays

1- Dissolved oxygen

Dissolved oxygen was determined with a NBS DO 40 analyzer (New Brunswick, NJ, USA) through a galvanic probe (model 1016/0070). Results are expressed as percentage of saturation with air.

2- Bacterial growth

Bacterial growth was measured in a SPEC 20-D spectrophotometer (CID, São Paulo, Brazil), and expressed as Absorbance at 540 nm.

3- Capsular polysaccharide

The concentration of capsular hexosamine was determined as described by Anderson and Smith (1977) modified by Inzana (5) and Lenser *et al.* (11). Briefly, 18 mg of Cetavlon were added to the supernatant of 10 ml of culture, shaken at 4° C for 4 hours and centrifuged at 10.000 g for 30 minutes. The sediment was resuspended in 1 ml of 0,4 M NaCl and centrifuged at 10.000 g for 30 minutes. One part of ethanol 95°GL was then added to three parts of the supernatant, followed by overnight stirring at 4°C and centrifugation at 10.000 g for 30 minutes. The supernatant was discarded, the sediment resuspended in 1 ml of distilled water and dialysed against distilled water for 24 hours. The concentration of capsular hexosamine in nMol was obtained by comparison with a standard curve (22).

4. Bacterial adherence

Bacterial adherence to n-Hexadecane was carried out as described by Rosenberg *et al.* (21). The results were subjected to the "t" test. They are expressed as A_{540} of the aqueous phase.

RESULTS

Cultures in flasks. 1-Effect of CO_2 and aeration on bacterial concentration

Bacterial concentrations were higher in agitated than in stationary cultures, either in BHI or TSB. TSB gave higher bacterial yields than BHI, irrespective of agitation or CO_2 atmosphere. Yields in BHI grown under agitation were higher than those in TSB grown statically in a CO_2 atmosphere (Fig. 1).

2- Growth curve

The logarithmic phase of growth ended in agitated cultures at 4 hours of incubation in BHI and at 7 in TSB, while in stationary cultures bacterial multiplication increased continuously, in both media, during the 24 hours growth cycle, when they reached similar values to those obtained at the end of the log phase in agitated culture (Fig. 1).

Cultures in fermenters. Bacterial concentrations were higher in aerated than in non-aerated cultures, the differences among the media tested being very small.

Log phases ended between two and four hours of incubation in aerated BHI and TSB and non-aerated BHI and at 4 h in non-aerated TSB (Fig. 2).

The concentrations of capsular hexosamines decreased during growth in non-aerated cultures. In aerated BHI they increased until 9 hours of growth, decreasing subsequently to nil, whereas in TSB they increased steadily during the period of culture (Fig. 3).

Table 1. Adherence to n-Hexadecane of *A. pleuropneumoniae* cultivated in aerated and non-aerated BHI and TSB in fermenters.

Hours of growth	Air supply			
	None		1vvm	
	BHI	TSB	BHI	TSB
2	0,523*	0,678	0,629	0,629
4	0,678	0,594	0,620	0,688
7	0,629	0,699	0,648	0,629
9	0,783	0,594	0,688	0,620
24	0,688	0,611	0,495	0,594
30	0,629	0,648	0,620	0,629

*$A^{o}_{(540)}$, aqueous phase

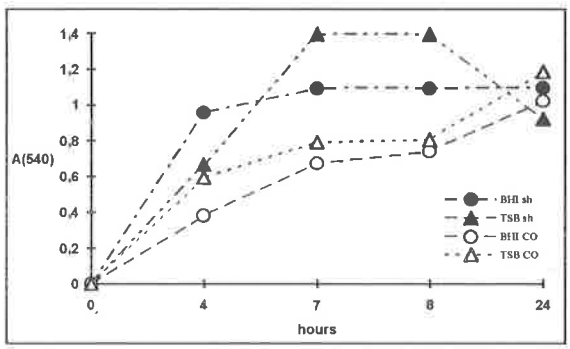

Figure 1. Growth curves of *A. pleuropneumoniae* in BHI and TSB shaken (sh) and in CO2 (CO).

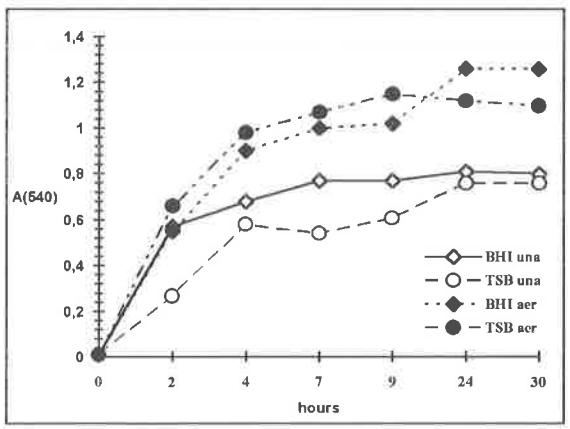

Figure 2. Growth of *A. pleuropneumoniae* cultivated in non-aerated (una) and aerated (aer) BHI and TSB in fermenters.

Bacteria grown in aerated and non-aerated BHI were more hydrophilic than those in TSB. The lower levels of adherence to n-Hexadecane, meaning higher levels of hydrophilicity, were obtained after 9 hours of growth in BHI, both in aerated and non-aerated cultures (Table 1). Differences were not significant at $P < 0,05$.

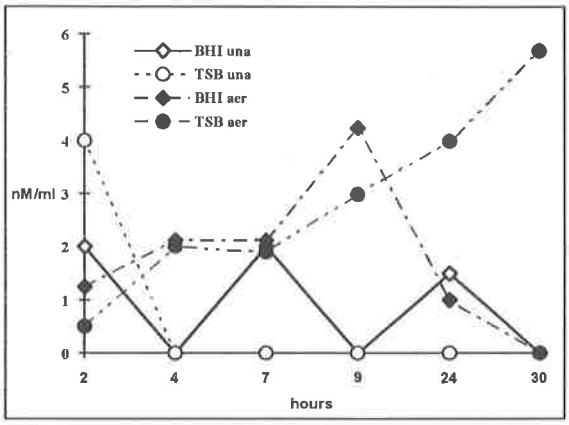

Figure 3. Capsular hexosamine concentration of *A. pleuropneumoniae* cultivated in non-aerated (una) and aerated (aer) BHI and TSB in fermenters.

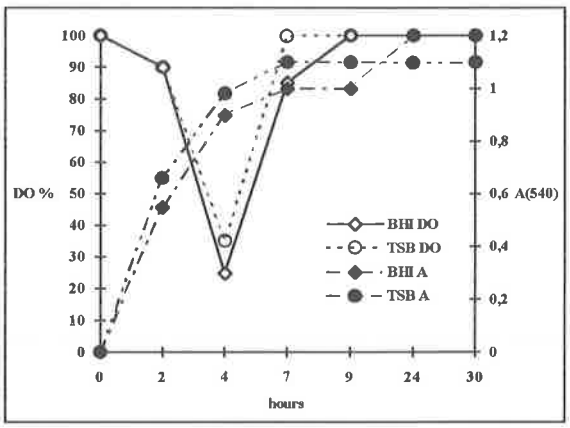

Figure 4. Oxygen demand (DO) and growth (A) of *A. pleuropneumoniae* in BHI and TSB aerated at 1 vvm.

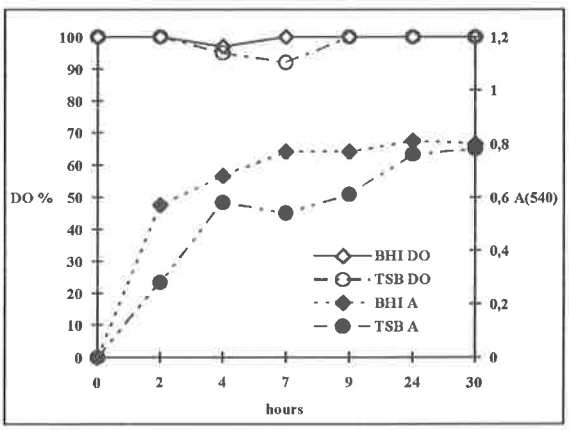

Figure 5. Oxygen demand (DO) and growth (A) of *A. pleuropneumoniae* in non aerated BHI and TSB.

The lower levels of dissolved oxygen were verified in aerated cultures of both media at 4 hours of incubation, returning to the initial values at 7 hours in TSB and at 9 in BHI (Fig. 4). In non-aerated cultures, a very small drop in dissolved oxygen was observed between the 4^{th} and 7^{th} hours of incubation (Fig. 5).

DISCUSSION

Some of the factors that influence the growth and the synthesis of capsular polysaccharides of *Actinobacillus pleuropneumoniae* serotype 5 (strain v 27) were studied in this work. Several authors (1, 2, 11) showed that an atmosphere of CO_2 is necessary for the primary isolation of this bacterium. The strain used, originally grown in CO_2, was capable of growing and producing capsular polysaccharides, even with higher yields, in aerobic conditions. This is in agreement with the results of others with other strains of this bacteria (6, 7, 16). The influence of culture medium and air supply on bacterial growth and on the synthesis of bacterial products has been already discussed (3, 10, 19). In our study, agitated cultures in TSB resulted in higher yields of bacterial mass than in BHI, but in fermenters the differences between these media were inconclusive. Bacterial mass, however, is not necessarily correlated with the synthesis of bacterial products such as pili, toxins or capsule used as antigens for the preparation of vaccines. Leite and Gil-Turnes (10) found that *Moraxella bovis* GF9 produced markedly higher yields of bacterial mass in DCF, a medium containing peptone, meat extract and salts, than in BHI, but pili were not produced in the former, whereas in the latter they were produced at the highest concentrations among the four media tested.

The media tested have at least two major differences: the nature of the peptones used, and glucose concentration. It has been shown that the nature of the peptones may influence the synthesis of some bacterial products such as the toxins of *Clostridium botulinum* type A and *Corynebacterium pyogenes* (12), among others. Poester *et al.* (19) found that not only the nature but also the brand of the peptones influenced the growth of *Brucella abortus* strain 19 cultivated in fermenters. Regarding the influence of glucose in the synthesis of bacterial exopolymers, there is opposing information. Reiman *et al.* (20) showed that low levels of glucose (0,1%) did not influence the synthesis of capsule by *Neisseria gonorrhoeae*, in disagreement with the results obtained by others. Matilla (14) verified that the synthesis of slime layer by *Staphylococcus aureus* grown on filter membranes was not affected by the addition of glucose to the medium, while Ferroni (3) observed that the concentration of exopolymers of a *Pseudomonas sp.* strain correlated positively with the concentration of glucose in TSB medium.

We detected the highest concentrations of hexosamine at 9 hours of incubation in aerated BHI medium and at 7 hours in non-aerated cultures, coinciding with the beginning of the stationary phase of growth and with the lower levels of bacterial adherence to n-Hexadecane, decreasing afterwards. Concentrations in aerated cultures were 2.1 times bigger than in non-aerated ones. However, in aerated cultures in TSB the concentrations raised steadily during the 30 hours cultivation period. The results we obtained with BHI are in accordance with those of Pijoan (18), who suggested that young cultures of *A. pleuropneumoniae* are more capsulated than older ones and with those of Jacques *et al.* (9) that detected higher concentrations of capsular polysaccharides in the early logarithmic (6h) than in the stationary phase (18h).

Inzana (5) showed that two strains of *A. pleuropneumoniae* serotype 5 produced capsular polysaccharide concentrations of 2 to 4 and 20 mg/ litre of medium, respectively, after 48 hours of incubation. These values are approximately 1.5 to 14 times larger than the greatest values obtained in our experiments. It is possible that these differences may be due to individual characteristics of the strains used or to the different growth period. Differences in yields among strains of this bacteria have already been reported (5).

Aerated and non-aerated cultures showed marked differences in their consumption of oxygen. In aerated cultures the greatest oxygen demand was produced at 4 hours of incubation, coinciding with the end of the logarithmic phase, ceasing at 7 hours in TSB and at 9 in BHI.

A direct relationship was observed among air supply, capsule synthesis and bacterial concentration. Leite and Gil Turnes (10) showed a similar effect of air on the synthesis of pili by *Moraxella bovis*. Bacterial and capsular polysaccharide concentrations were markedly higher after four hours in aerated than in non-aerated cultures. Capsular polysaccharides were not detected after 2 hours of culture in unaerated TSB, but in aerated cultures their concentration increased steadily during 30 hours of incubation. The lack of capsular polysaccharides in non-aerated cultures could be related to several factors, such as

repression of synthesis, hexosaminase production (3) or hexosamine instability due to variation of the pH of the medium.

It is accepted that capsule enhances the interaction between bacteria and water (23). The bacterial adherence to hydrocarbons test (BATH) was evaluated in our work as an alternative to the use of other more difficult and time consuming techniques to monitor capsule synthesis. Jacques *et al* (8) showed that 26 isolates of *A. pleuropneumoniae* did not adhere to n-Hexadecane , the hydrocarbon we used to test bacterial adherence, suggesting that all of them had hydrophilic cell surfaces. This may explain that in cultures in which capsular hexosamines could not be detected, as in non-aerated TSB, the levels of hydrophobicity were similar to those registered by cultures in which they were synthesized.

Our results showed differences in hydrophilicity among bacteria grown in the media tested and when air was supplied to the cultures. In aerated cultures adherence to n-Hexadecane diminished when the concentration of capsular hexosamines increased, suggesting that the variations in the synthesis or expression of capsule may determine the differences in hydrophilicity. The inconsistency of the results, however, does not allow to recommend this test to monitor capsular polysaccharide production.

Further work is needed to determine the best culture conditions to obtain maximum concentration of capsular antigens and to evaluate the efficacy of this antigen in the control of swine pleuropneumonia for other strains and serotypes of this bacterium.

ACKNOWLEDGMENTS

This research was financed by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (grant 402 123/ 89-1). S. M. Borowski had a scholarship from the Post Graduate Course in Veterinary Medicine, Universidade Federal de Pelotas, and C. Gil-Turnes a Researcher scholarship from CNPq.

RESUMO

Dinâmica de crescimento e síntese de cápsula de *Actinobacillus pleuropneumoniae*

Estudou-se a dinâmica de crescimento e síntese de polissacarídios capsulares de uma amostra de *Actinobacillus pleuropneumoniae* sorotipo 5 em diferentes condições de cultivo. A bactéria foi multiplicada em caldo infuso de cérebro e coração (BHI) e caldo de soja e tripticaseina (TSB), suplementados com extrato de levedura. Realizaram-se cultivos em frascos Erlenmeyer estáticos em atmosfera de CO_2 e agitados em aerobiose, e em fermentador com e sem aeração. Determinou-se a curva de crescimento, concentração de hexosaminas, aderência a n-Hexadecano e a demanda de oxigênio em diferentes períodos de incubação. A fase logarítmica de crescimento terminou mais rapidamente, e a produção de biomassa e de hexosaminas foram maiores nos cultivos realizados em condições de aerobiose. A maior demanda de oxigênio coincidiu com o término da fase exponencial de crescimento. A aderência ao n-Hexadecano não teve relação com a fase de cultivo nem com a concentração de polissacarídios capsulares.

Palavras-chave: *Actinobacillus pleuropneumoniae* síntese de cápsula, hidrofobicidade.

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EFFECT OF FLAVONOIDS ON *ASPERGILLUS FLAVUS* GROWTH AND AFLATOXIN PRODUCTION

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ABSTRACT

Flavonoids have been shown important antimicrobial properties and are an alternative group of fungicides for the control of contamination of stored grains. In this study, the effect on the flavonoids kaempherol, quercitin, kaempheritrin and naringinin on *Aspergillus flavus* (NRRL6513) growth and aflatoxin B_1 production was investigated. Spore suspensions were inoculated into 250ml Erlenmeyer flasks containing 50 ml of YES medium and different concentrations of flavonoids. Four repetitions were performed for each dilution of flavonoid tested, prepared from stock solutions at 2000 ppm. Fungal growth was evaluated on the basis of mycelial dry weight. Aflatoxin extraction was carried out by addition of chloroform to culture media. Extracts were analyzed by thin layer chromatography against a standard aflatoxin B_1 preparation. Concentrations of the mycotoxin were determined by photodensitometry. Maximum reduction of *Aspergillus flavus* growth was observed for naringinin at 125 ppm (60.5%), for kaenepheritrin at 300 ppm (49.4%) and for kaempherol at 100 ppm (40.0%). The lowest fungistatic effect was observed with quercitin (36.0% growth inhibition). With respect to aflatoxin B_1 production, the greatest inhibition (99.0%) was recorded for kaempheritrin at 100 ppm.

Key words: antifungal activity, aflatoxins, flavonoids, *Aspergillus flavus,* inhibition of aflatoxin production.

INTRODUCTION

Flavonoids comprise groups of secondary metabolites that occur widely throughout the plant kingdom. They have a common skeleton of 1,3 - diphenylpropane, composed of an Ar (ring B)-C_3 subunit derived from shikimate and an Ar (ring A) of poliketide origin (4). They are through to be typical secondary metabolites, without recognized harmful effects on humans and other animal species.

Besides other biological characteristics, flavonoids have showed antimicrobial properties (7), acting as a chemical barrier against invading microorganisms. Considered as natural compounds with specific antimicrobial activity, flavonoids may be an alternative to conventional fungicides, intended for the fungal control during grain storage in developing countries (8).

In this paper, the activity of four flavonoid compounds, namely kaempherol, quercetin, kaempheritrin and naringinin, on *Aspergillus flavus* growth and aflatoxin production was described.

MATERIALS AND METHODS

Fungal strain and culture conditions. *Aspergillus flavus* NRRL6513 (Northern Research Laboratory, Department of Agriculture, Ilinois, USA)

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was cultured on potato dextrose agar (Difco Laboratories, Detroit, Mich) plates for 10 days at 25°C. Spore suspension used as inoculum was prepared, washing cultures with sterile PBS. The suspension was placed in a test tube containing 50 µl of sterile Tween 80 solution and submited to spore counting in a Neubauer chamber (2).

Antifungal agents. Naringinin and quercitin were obtained from Sigma; kaempherol and kaempheritrin from *Holocalyx glaziovii* Taub (3).

Conditions of growth for aflatoxin evaluation. The semi-synthetic Yes medium, conventionally employed for testing aflatoxin production (1), was used as culture medium. Inocula (1.3×10^5 spores/ml) were transfered to 250 ml Erlenmeyer flasks that contained 50 ml of Yes medium with different concentrations of the flavonoids studied. Flavonoid stock solutions were at 2000 ppm. The concentration ranges tested were: 0, 1.5, 3, 6, 12.5, 25, 50, 100, 200 and 300 ppm for kaempheritrin; 0, 6, 12.5, 25, 50, 100, 125 and 150 ppm for kampherol; 0, 2, 5, 15, 25, 50, 75, 100 and 125 ppm for naringinin; and 0, 10, 25, 100, 200 and 300 ppm for quercetin.

Four repetitions were performed for each dilution of flavonoids tested. Cultures were incubated at 25°C for 5 days and production of aflatoxin B_1 evaluated, proceeding extraction according to Davis *et al.*, 1966 (1). Concentrations of the mycotoxin were determined by photodensitometry.

RESULTS AND DISCUSSION

Tables 1 to 4 and Figs. 1 to 4 show the correlation of fungal growth with production of aflatoxin B_1 (ng/ml), in the presence of different concentrations of the flavonoids.

Table 1 - Effect of kaempheritrin on *A. flavus* (NRRL 6513) growth and AFB_1 production.

Concentration of kaempheritrin (ppm)	dry weight of fungus (g)	% change in dry weight of fungus*	levels of AFB_1 detected (ng/ml)	% change in AFB_1 production*
0.0	0.389		9,462	
1.5	0.373	-4.0	7,803	-17.5
3.0	0.397	+2.0	15,608	+64.9
6.0	0.328	-15.5	9,852	+4.1
12.5	0.357	-8.3	7,868	-16.8
25.0	0.358	-8.0	6,154	-35.0
50.0	0.292	-24.9	2,449	-74.1
100.0	0.216	-44.3	97	-99.1
200.0	0.231	-40.5	2,445	-74.1
300.0	0.196	-49.4	190	-98.0

* Diferences in dry weight of AFB_1 production were calculed with respect to control values from untreated cultures (-) = inhibition; (+) = stmulation.

Table 2 - Effect of kaempherol on *A. flavus* (NRRL 6513) growth and AFB_1 production.

Concentration of kaempherol (ppm)	dry weight of fungus (g)	% change in dry weight of fungus*	levels of AFB_1 detected (ng/ml)	% change in AFB_1 production*
0.0	0.425		3,207	
6.0	0.3871	-9.1	1,835	-42.8
12.5	0.3673	-13.6	2,484	-22.5
25.0	0.2980	-30.0	1,427	-55.5
50.0	0.3313	-22.1	1,054	-67.1
100.0	0.2547	-40.0	1,615	-49.7
125.0	0.3229	-24.0	149	-95.5
150.0	0.319	-25.0	189	-94.1

* Diferences in dry weight of AFB_1 production were calculed with respect to control values from untreated cultures (-) = inhibition; (+) = stmulation.

Table 3 - Effect of naringinin on *A. flavus* (NRRL 6513) growth and AFB_1 production.

Concentration of naringinin (ppm)	dry weight of fungus (g)	% change in dry weight of fungus*	levels of AFB_1 detected (ng/ml)	% change in AFB_1 production*
0.0	0.500		9,438	
2.0	0.464	-7.2	11,266	+11.9
5.0	0.455	-9.0	13,202	+39.8
15.0	0.410	-18.0	5,028	-46.7
25.0	0.356	-28.7	5,587	-40.8
50.0	0.335	-29.0	2,934	-68.9
75.0	0.320	-35.9	686	-92.7
100.0	0.229	-54.1	290	-96.9
125.0	0.197	-60.5	185	-98.0

* Diferences in dry weight of AFB_1 production were calculed with respect to control values from untreated cultures (-) = inhibition; (+) = stmulation.

Table 4 - Effect of quercetin on *A. flavus* (NRRL 6513) growth and AFB_1 production.

Concentration of quercetin (ppm)	dry weight of fungus (g)	% change in dry weight of fungus*	levels of AFB_1 detected (ng/ml)	% change in AFB_1 production*
0.0	0.492		14,455	
10.0	0.405	-17.8	23,776	+64.4
25.0	0.353	-28.3	6,436	-55.5
100.0	0.370	-24.9	9,480	-34.4
200.0	0.362	-26.4	4,470	-69.1
300.0	0.315	-36.0	3,672	-74.6

* Diferences in dry weight of AFB_1 production were calculed with respect to control values from untreated cultures (-) = inhibition; (+) = stmulation.

Overall, *Aspergillus flavus* dry weight was correlated with the aflatoxin B_1 concentration, during almost all the fungal growth phase (Figs. 1 to 4). Increase of the flavonoids concentration resulted in a decrease in AFB_1 production. Naringinin at 125 ppm caused 60.5% reduction of *A.flavus* growth, whereas kaempheritrin at 300 ppm and kaempherol at 100 ppm caused 49.4% and 40% growth inhibition, respectively. Krolicki and Lames-Zarawska (5) also observed strong anti-fungal action of this flavonoid. Quercitin was less efective, with only 36% of inhibition even at 300 ppm. Weidenborner *et al.* (9) also described a reduced antifungal activity of quercitin for several species of the genus *Aspergillus*. *A. flavus* growth was stimulated by kaempheritrin at 3 ppm (Table 1).

Concerning AFB_1 production, maximum inhibition 99% was detected in the presence of kaempheritrin at 100 ppm (Fig. 1 and Table 1). Kaempherol and naringinin were slightly less efficient in reducing AFB_1 levels, with in 95,5% and 98% inhibition of AFB_1 secretion, respectively, at a 125 ppm. In contrast, the inhibitory effect of quercitin on mycotoxin production was not very pronounced (Table 4 and Fig. 4).

The differences described for kaempheritrin, kaempherol and naringinin treated cultures with respect to controls were statistically significant ($p = 0.05$) according to the F and Tukey's parametric tests. No statistically significant differences were found among untreated and quercitin treated cultures.

The results obtained in this investigation are an important step towards the development of efficient and economically viable agents for the control of AFB_1 production, setting the start of other relevant investigations. Payne (6) stated that extracts of plants that exhibit resistance to aflatoxins can be added to cultures of the secreting fungus and examined for their effect on the expression of genes involved in mycotoxin secretion. Once inhibitory compounds are identified, breeding can be initiated to incorporate genes that code for the antifungal compounds into plants with desirable agronomic lines.

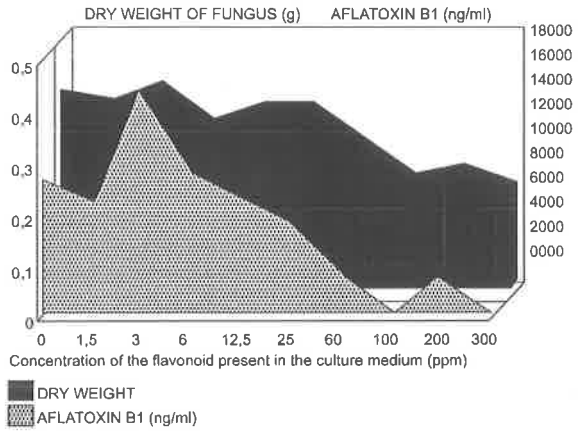

Figure 1 - Effect of different concentrations of kaempheritrin on *A. flavus* (NRRL 6513) growth and AFB_1 production

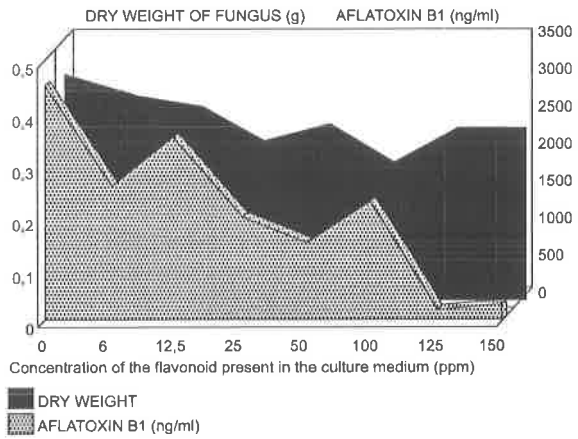

Figure 2 - Effect of different concentrations of kaempherol on *A. flavus* (NRRL 6513) growth and AFB_1 production

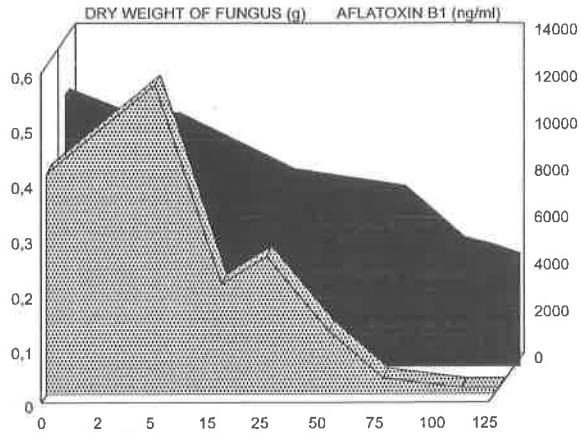

Figure 3 - Effect of different concentrations of naringinin on *A. flavus* (NRRL 6513) growth and AFB_1 production

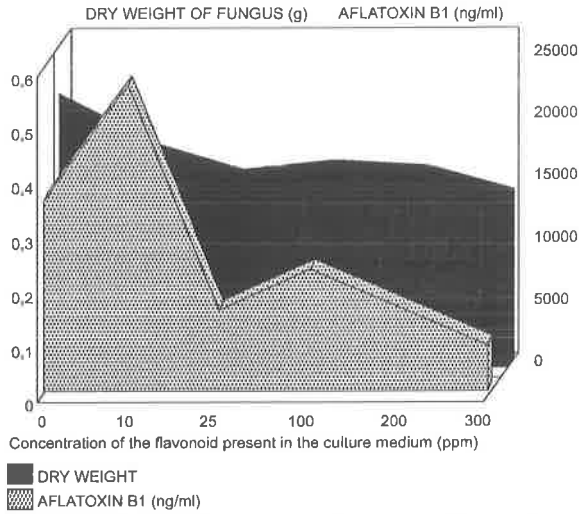

Figure 4 - Effect of different concentrations of quercitin on *A. flavus* (NRRL 6513) growth and AFB_1 production

RESUMO

Efeito de flavonóides no crescimento de *Aspergillus flavus* e na produção de aflatoxina B_1

Os flavonóides tem demonstrado efeito impediente contra microrganismos e uma alternativa no controle de fungos contaminantes de grãos armazenados. Neste trabalho, pesquisou-se a atividade de quatro flavonóides (kampferol, kampferitrina, naringinina e quercetina), no crescimento de *Aspergillus flavus* (NRRL 6513) e na produção da Aflatoxina B_1. As suspensões de esporos foram inoculadas em Erlenmeyer de 250 ml, contendo 50 ml de meio de Yes com diferentes concentrações dos flavonóides, preparados a partir de uma solução de 2.000 ppm. Para cada diluição dos flavonóides pesquisados foram feitas 4 repetições e para a determinação do crescimento fúngico procedeu-se a determinação do peso seco miceliar. A toxina foi extraída do meio de cultura, adicionando-se clorofórmio e os extratos cromatografados, em placas de camada delgada de sílica gel 60 (Merck) e quantificado pela fotodensitometria. A naringinina causou inibição de 60,5% no crescimento de *Aspergillus flavus* na concentração de 125 ppm, enquanto a kampferitrina e o kampferol resultaram na inibição de 49,4% e 40,0%, nas concentrações de 300 e 100 ppm, respectivamente. A quercetina demonstrou menor taxa de inibição (36,0%). Em relação a

produção de aflatoxina B_1, a máxima inibição ocorreu na presença de kampferitrina (99,0%) na concentração de 100 ppm.

Palavras-chave: atividade antifúngica, aflatoxinas, *Aspergillus flavus*, flavonóides, inibição da produção de aflatoxinas.

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INFLUENCE OF THE OXYGEN AND GROWTH RATE ON THE METABOLIC ACTIVITY OF *BACILLUS STEAROTHERMOPHILUS* GROWN IN GLUCOSE-LIMITED CULTURES

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ABSTRACT

Increasing the dilution rate and hence the growth rate of the culture to 0.89 h^{-1} at 50% air saturation resulted in linear increases in the rates of oxygen and glucose consumption. Analysis of these data were performed according to the equation of Pirt and it was observed that when the d.o.t. (dissolved oxygen tension) decreased from 50 to 5% air saturation the resulting effect was an increase in the maintenance rate of oxygen consumption and a decrease of the derived maximum yield value. The maintenance rate of glucose consumption and maximum yield value at 50% air saturation were further calculated, but when the d.o.t. was lowered to 5% air saturation these values could no longer be calculed since increases in dilution rate did not result in linear increases of glucose consumption.

Key words: *Bacillus stearothermophilus*, chemostat cultures, oxygen limitation, dilution rate.

INTRODUCTION

The concept of maintenance energy is essential to understanding the energy requirements for the growth of microorganisms (13). In order to quantify the specific rate of substrate consumption fulfilling functions other than growth, Pirt (14, 15), developed the equation, $q = m/Y^{max} + m$ where q = the specific rate of substrate consumption (mol g^{-1} h^{-1}); μ = the specific growth rate (h^{-1}) and m is the specific rate of substrate consumption required for maintenance purposes such as maintaining transmembrane ion gradients, turnover of cell walls and macromolecules, etc. Here Y^{max} is the theoretical maximum growth yield coefficient (g biomass per substrate consumed), when the contribution of m to total q has been discounted. Plotting q versus m (or D) yields a linear relationship with a slope equal to $1/Y^{max}$ and an intersection on the ordinate representing m. Alternatively, dividing the above equation by μ provides a reciprocal relationship between Y and μ: $1/Y = 1/Y^{max} + m/$ where Y^{max} is the true molar growth yield (g cells per mol of glucose) and $1/Y^{max}$ is the intersection on the ordinate of a plot of 1/Y versus $1/\mu$ (or 1/D), while the slope equals m. According to these equations, the maintenance coefficient does not vary with growth rate. This is probably true for energy-limited cultures, but not for growth under conditions where the carbon source is in excess and where energy is undoubtedly wasted by a variety of metabolic "slip" reactions (10). Furthermore, a linear relationship must occur between substrate consumption rate (and oxygen consumption rate) and the growth rate. Bulthuis *et al.* (3), and Antier *et al.* (1), have questioned the validity of these equations since for some organisms, experimental data measured across the entire growth range cannot be explained.

As oxygen is an important factor controlling the efficiency of substrate assimilation into biomass, as

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well as the coupling of energy metabolism (7), the effect of both growth rate and oxygen on the energetics of *Bacillus stearothermophilus* when cultured under carbon-limited conditions, at different dilution rates, in a chemostat were investigated.

METHODS

Organism and culture conditions. The organism used in this study was a near-prototrophic strain of *Bacillus stearothermophilus* that apart from the carbon and energy source, required only biotin as an organic supplement. It was obtained from the Laboratory of Microbiology, University of Amsterdam, and named *Bacillus stearothermophilus* Amsterdam. In all its characteristics it appeared to be identical with the strain *B. stearothermophilus* var. *non-diastaticus* described by Epstein and Grossowicz (4).

Organisms were routinely grown in chemostats (LH Fermentation 500 series, 1 litre growth vessel with a 700 ml working volume) in defined simple salts media (5) at 55 °C and pH 7.0. Glucose was provided as the carbon and energy source. This basal medium (excluding glucose) was prepared in 20 litre batches and sterilized by autoclaving at 121 °C for 30 min. The required amount of glucose was made up as a 50% (w/w) solution (slightly acidified with HCl), autoclaved at 121°C for 30 min and added aseptically to the bulk medium after cooling. The temperature and pH values of the cultures were controlled automatically and foaming was suppressed by the addition of a silicone-based antifoaming agent, on demand, as sensed by a foam probe. Dissolved oxygen was monitored by means of a galvanic oxygen electrode (Uniprobe Instruments) and its concentration adjusted and maintained at the desired degree of saturation by varying the stirrer speed.

Procedure. Organisms were grown firstly at a specific rate of 0.08 h^{-1} and gradually increased to 0.89 h^{-1} under fixed steady-state conditions (55 °C, pH 7.0), in a glucose-limiting medium. The d.o.t. was first set, and controlled, at about 50% saturation, then subsequently lowered to 5% air saturation. After equilibration at each d.o.t. (dissolved oxygen tension) for 1-2 days, samples of culture (10-20 ml) were withdrawn from the fermenter and analysed for glucose. From the results of these assays, the rates of glucose consumption were determined, and carbon balances constructed.

Analyses. Oxygen consumed and carbon dioxide produced by the cultures were determined by passing the effluent gas through an oxygen analyser (Taylor Servomex type OA 272) and a carbon dioxide analyser (Servomex IR gas analyser PA 404). The rates of respiration were then calculated as specified by Pennock and Tempest (12), bacterial dry weights being assessed by the procedure of Herbert *et al.* (6). Glucose was determined enzimatically, following the procedure of Bergmeyer and Bernt (2).

RESULTS AND DISCUSSION

Increasing the dilution rate and hence the growth rate of the culture to 0.89 h^{-1} at 50% saturation (oxygen in excess), resulted in linear increases in the rates of oxygen and glucose consumption, but, at 5% air saturation this linearity was only observed with the oxygen consumption rate (Fig. 1 and Fig. 3). Analysis of these data (Table 1 and 2) were performed according to the equation of Pirt (13, 14) in which $q = m/Y^{max} + m$ and $1/Y = 1/Y^{max} + \mu/m$, in order to determine the maintenance coefficient (m) for glucose and oxygen consumption and the maximum molar growth yield ($Y^{maxglucose}$ and $Y^{maxoxygen}$). In addition, a reciprocal plot 1/Y versus 1/D was constructed (Fig.2 and Fig.4) with respect to dissolved oxygen tension, and also the m and yield maximum molar growth values calculated from the two equations. When the d.o.t. was decreased from 50% to 5% air saturation the resulting effect was an increase in the maintenance rate of oxygen consumption and a decrease of the derived maximum yield value. The maintenance rate of glucose consumption and maximum yield value at 50% air saturation were further calculated, but when the d.o.t. was lowered to 5% air saturation these values could no longer be calculated since increases in dilution rate did not result in linear increases of glucose consumption (Fig. 3). During studies of the growth of *Bacillus stearothermophilus* var *non-diastaticus* was noticed that whenever the concentration of dissolved oxygen (d.o.t.) was caused to fall below 10% air saturation, a marked stimulation of catabolic rate occurred accompanied by excretion of much acid (8,9,11). In fact, assessments of the carbon balance at a d.o.t. 5% air saturation (Table 3), suggested that organic products were being excreted into the medium, causing the relationship between q and D to be non-linear. Hence, maintenance coefficients derived

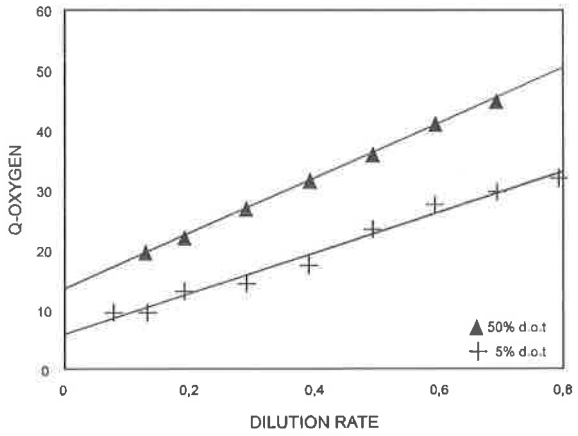

Figure 1 - Oxygem consumption rate (Q-oxygen) as function of dilution rate at a d.o.t. of 50% and 5% air saturation.

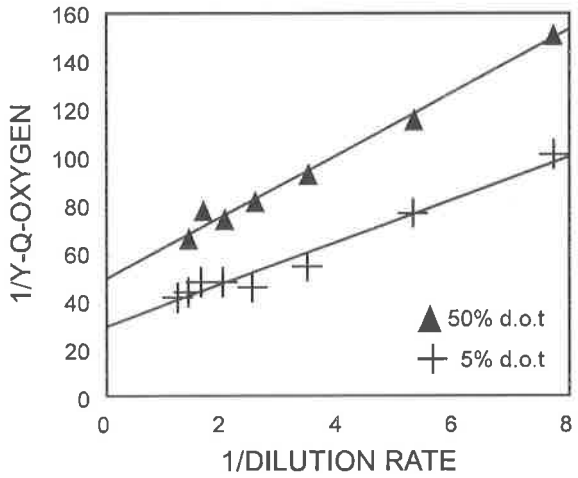

Figure 2 - Reciprocal plots of molar growth yield coefficients (Y-oxygen) as function of dilution rate at a d.o.t. of 50% and 5% air saturation.

Table 1. Linear equations inferred from Fig. 1, 2, 3, and 4.

Fig 1 (50% d.o.t.)	q_{oxygen}	=	7.85	+ 331.24D	r = 0..84
Fig. 1 (5% d.o.t.)	q_{oxygen}	=	13.57	+ 446.44D	r = 0.999
Fig. 2 (50% d.o.t.)	$1/Y_{oxygen}$	=	0.036	+ 7 7.45/D	r = 0.982
Fig. 2 (5% d.o.t.)	$1/Y_{oxygen}$	=	47.9	+ 113.34/D	r = 0.998
Fig. 3 (50 % d.o.t.)	$q_{glicose}$	=	0.35	+ 115.47D	r = 0.983
Fig. 4 (50% d.o.t.)	$1/q_{glicose}$	=	14.9	+ 00.54/D	r = 0.721

Table 2. Influence of dissolved oxygen tension on the growth parameters of a glucose-limited chemostat culture of *Bacillus stearothermophilus* grown at different dilutions rates and at a pH value of 7.0. The m and Y-values of column 1 were derived from plots of q versus D. Column 2 shows m and Y-values derived from plots of 1/D versus 1/Y.

Parameter	d.o.t. (% saturation)			
	50		5	
	1	2	1	2
m(glucose)	0.35	0.54	n.d.	n.d.
m(oxygen)	7.9	7.5	13.6	13.5
Y^{max}(glucose)	64.6	67.1	n.d.	n.d.
Y^{max}(oxygen)	32.0	32.6	21.53	21.0
Dilution rate range	0.08-0.69		0.13-0.69	

m(glucose) and m(oxygen) are the calculated rates of glucose and oxygen consumption at D=0: Y^{max}(oxygen) are the calculated maximum Yield values with respect to glucose and oxygen. The q values, relevant to any particular growth rate expressed as mmol.h^{-1} (g dry wt cells)$^{-1}$, can be obtained from the expression: $q = 1000.D/Y^{max} + m$

from linear regression of q versus D, should be examined with great care, especially when studying organisms that are capable of product-formation, as in some cases the assumption of linearity of q with D is clearly invalid, as observed here. Therefore, the discussion on maintenance energy demand still needs a greater factual input, in order to reach more solid conclusions on how to describe maintenance demands under different limitations and over a broad range of growth rates (1, 3).

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RESUMO

Influência do oxigênio e da taxa de crescimento na atividade metabólica de *Bacillus stearothermophilus* crescido em culturas limitadas de glicose

O aumento da taxa de diluição de uma cultura quimiostática de *Bacillus stearothermophilus* var *non-diastaticus*, contendo glicose como fonte de carbono para 0,89 h^{-1} a 50% de saturação com ar, resultou em aumentos lineares nas taxas de consumo

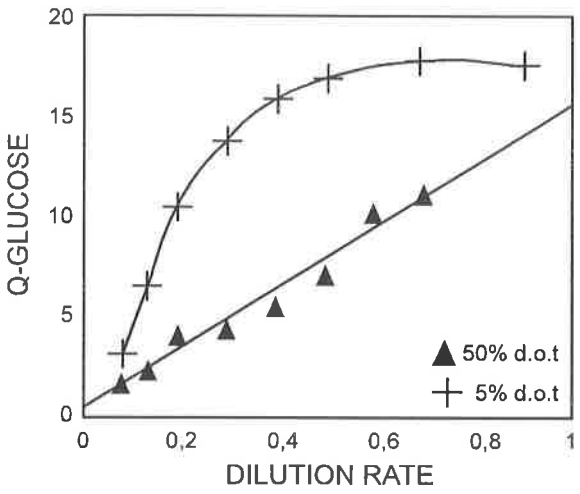

Figure 3 - Glucose consumption rate (Q-glucose) as function of dilution rate at a d.o.t. of 50% and 5% air saturation.

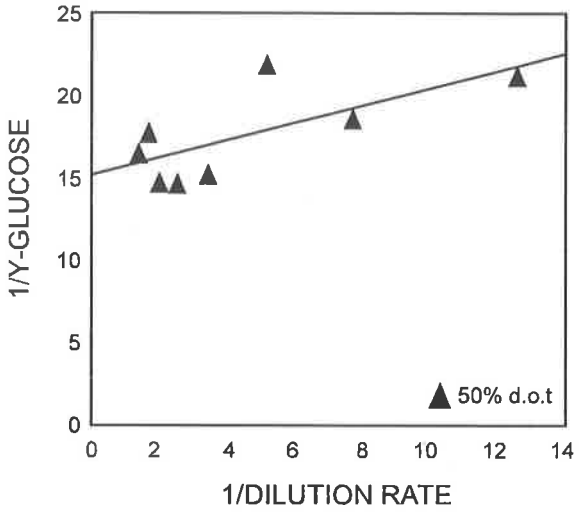

Figure 4 - Reciprocal plots of molar growth yield coefficients (Y-glucose) as function of dilution rate at a d.o.t. of 50% and 5% air saturation.

Table 3. Influence of dissolved oxygen tension on the rates of glucose and oxygen consumption, and of CO_2 production expressed by glucose-limited cultures of *Bacillus stearothermophilus* growing at a different dilution rates (Temp = 55 °C, pH = 7.0).

D (h^{-1})	d.o.t. (%saturation.)	$Y_{glucose}$ ($gmol^{-1}$)	$Q_{glucose}$	Q_{O2}	QCO2	C.Recovery (%)
			mmol.h^{-1}(g.Dwt cells)$^{-1}$			
0.08	50	47.1	1.70	9.60	8.60	117
	5	25.8	3.10	11.40	6.40	52
0.13	50	54.2	0.13	13.10	8.50	97
	5	20.0	6.50	19.80	12.60	46
0.19	50	46.3	4.10	14.40	10.20	74
	5	17.9	10.60	22.10	14.20	35
0.29	50	65.0	4.40	15.70	12.10	92
	5	21.0	3.80	27.00	15.80	34
0.39	50	69.0	5.60	17.50	14.50	92
	5	24.0	15.90	31.90	7.30	35
0.49	50	69.0	7.10	23.60	20.30	96
	5	29.0	16.90	16.30	23.30	43
0.59	50	57.3	10.30	27.80	22.60	76
	5	30.5	19.30	41.40	32.00	49
0.69	50	61.2	11.30	29.80	22.20	75
	5	38.5	17.90	45.50	37.90	62

de glicose e oxigênio. Estes dados foram analizados de acordo com a equação de Pirt e foi observado que quando o nível de oxigênio dissolvido diminuiu em função da diminuição da saturação com ar de 50% para 5%, o coeficiente da manutenção (m) para o consumo de oxigênio aumentou e o valor Y^{max} (produção máxima) decresceu. O coeficiente de manutenção para o consumo de glicose e o valor da produção máxima a 50% de saturação com ar foram também calculados, mas quando o nível de oxigênio dissolvido foi abaixado para 5% de saturação com ar, estes valores não puderam ser calculados, uma vez que o aumento da taxa de diluição da cultura não resultou em aumentos lineares no consumo de glicose.

Palavras-chave: *Bacillus stearothermophilus*, cuturas quimiostáticas, taxa de diluição.

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INHIBITION OF MICROBIAL METABOLISM BY CTAB (CETYLTRIMETHYLAMMONIUM BROMIDE) AND ITS EFFECT ON CYTOPLASM ENZYMES

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ABSTRACT

The effect of cetyltrimethylammonium bromide (CTAB) on the metabolism of various microorganisms was investigated. The rates of CO_2 release and O_2 uptake were inhibited by CTAB in a dose dependent fashion and were affected to variable degrees in the different microbial species, indicating that both respiratory and fermentative activities were inhibited. *Pseudomonas aeruginosa*, described as the most resistant of the tested microorganisms, was found to be 100 times less sensitive to CTAB with respect to metabolic activity. In contrast with intact cell responses, the activity of the enzyme lactate dehydrogenase extracted from sensitive and resistant species was equally inhibited by CTAB. Alcohol dehydrogenase was slightly more sensitive than lactate dehydrogenase. The concentration of CTAB required for complete inhibition of both enzymes was within the range of growth inhibition. This indicates that growth inhibition of microorganisms by CTAB could be due to its action on the metabolic activity of cytoplasm enzymes. Species resistance was related to differences in cell membrane permeability.

Key words: microbial inhibition; cetyltrimethylammonium bromide; enzyme inhibition; quaternary ammonium inhibition

INTRODUCTION

The antimicrobial activities of quaternary ammonium compounds have been known for many years. Studies of their action on microbial metabolism show that they are effective against both Gram positive and Gram negative bacteria and also against yeasts (8, 11, 14).

Quaternary ammonium compounds were widely accepted as large spectrum antimicrobial agents for personal hygiene and household sanitation. However, with the discovery that some species of *Pseudomonas* were extremely resistant, their usage became restricted. This resistance, shown by most Gram negative bacteria, was attributed to low cell membrane permeability to hydrophobic antibiotics and hydrophobic chemicals like crystal violet, N-phenylnaphthylamine and 8-anilino-1-naphthalenesulfonic acid (9).

Tests with various hydrophobic antibiotics showed that they were equally effective against *Pseudomonas aeruginosa* and *Escherichia coli*, with the exception of Novobiocin which was more active against the latter (10). Hence, the remarkable resistance of some species of *Pseudomonas* to quaternary ammonium compounds as compared to other species of bacteria could not be explained by the normal mechanism of cell permeation barrier to hydrophobic compounds (16).

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This work shows the effect of CTAB on the respiration and fermentation rates of various species of microorganisms, including Gram negative and Gram positive bacteria and yeasts. The effect was also tested on the enzymes lactate dehydrogenase and alcohol dehydrogenase from both intact and lysed cells, to evaluate the action of the cell membrane barrier.

MATERIALS AND METHODS

Microorganisms. The following cultures were kindly supplied by the Culture Collection of Fundação Tropical de Pesquisas e Tecnologia "André Tosello", Campinas, S.P. Brazil: *Escherichia coli* FTPT 0923, *Klebsiella terrigena* FTPT 0400, *Lactobacillus fermentum* FTPT 0694, *Leuconostoc mesenteroides* FTPT 0367, *Saccharomyces cerevisiae* FTPT 0472, *Pseudomonas aeruginosa* ATCC 10145, *Pseudomonas cepacia* ATCC 25416, *Pseudomonas mendocina* ATCC 25411, and *Pseudomonas acidovorans* ATCC 17438. *Acetobacter aceti* IZ 2023 was obtained from the Culture Collection of Escola Superior de Agricultura "Luiz de Queiróz", Piracicaba, S.P., Brazil.

Determination of Microbial Metabolic Activity. The effect of CTAB on the metabolic activity of microorganisms was measured with a Warburg apparatus (B. Brawn-Melsungen). The cultures were grown in cana 11 medium (16) for 12 hours, harvested by centrifugation (8000g, 10 min.,4°C), washed twice with 0.02M phosphate buffer, pH 7.0, and resuspended in the same buffer. Final cell concentrations were adjusted to $2x10^{-3}$ g and $2x10^{-4}$ g dry weight per ml for yeast and bacteria, respectively.

Gas exchange rates were measured in a Warburg flask containing 1.0 ml of cell suspension and 1.2 ml of 0.02 M phosphate buffer, pH 7.0. Volumes of 0.5 ml 0.2 M glucose solution and 0.3 ml CTAB at various concentrations were placed in the side arm prior to the operation. A volume of 0.2 ml 10% NaOH was added to the center well for measurement of oxygen consumption.

The reaction temperature was kept at 30°C and shaking was adjusted to 50 strokes per minute. Temperature equilibration was reached after 15 min.; the reaction was started by tilting the flask, in order to mix the content of the side arm with the cell suspension. Readings were taken at 15 min. intervals.

Determination of Enzyme Activities. Microbial cells were grown and harvested as previously described; the final bacteria and yeast cell suspensions were adjusted to $8x10^{-4}$ g/ml and $4x10^{-3}$ g/ml (dry weight), respectively, using 0.01 M phosphate buffer, pH 7.0, as medium.

Cell lysis was carried out with an ultra-sonicator (Browill Scientific, Biosonik III); 6 cycles (84w/cm) of 30 sec. each were applied at 1 min intervals on an ice bath. Intact cells were recovered by centrifugation at 8000g for 10 min. at 4°C.

The lactate dehydrogenase (LDH) activity of intact and lysed cells was measured according to the procedure of Ordal and Halvorson (11) with methylene blue as indicator. The reaction mixture in screw capped test tubes contained 5.0 ml of 0.05 M phosphate buffer pH 7.56, 0.25 ml 0.2 M D,L-sodium lactate, 1.0 ml 0.2 mg/ml methylene blue, 0.25 ml cell suspension (or lysed supernatant) and 2 ml CTAB solution at various concentrations. The reaction temperature was kept at 37°C. and enzyme activity was determined as the time required for complete disappearance of the blue color.

The alcohol dehydrogenase (ADH) activity of intact cells was also measured by reduction of methylene blue. The reaction was carried out in screw capped test tubes containing 5.0 ml 0.05 M phosphate buffer pH 7.56, 0.25 ml 99% ethanol, 1.0 ml 0.2 mg/ml methylene blue, 0.25 ml cell suspension and 2.0 ml CTAB solution at various concentrations. The mixture was adjusted to pH 9.0 with 0.1 N NaOH and incubation was carried out at 37°C. Enzyme activity was determined as the time required for complete disappearance of the blue color.

RESULTS AND DISCUSSION

Effect of CTAB on Microbial Metabolism. The addition of CTAB (cetyltrimethylammonium bromide) on cell suspensions of bacteria and yeast caused reduction of CO_2 release and O_2 uptake. The results for *Escherichia coli* and *Pseudomonas aeruginosa* are shown in Figs. 1 and 2, respectively. The rates of CO_2 release and O_2 uptake for *E. coli* were almost completely inhibited with 27.4 µM CTAB but a ten fold higher concentration of the compound (2740 µM) was required to induce the same effects in *P. aeruginosa*. The concentration of CTAB necessary to inhibit the growth of *P. aeruginosa* is also nearly 100 times higher than that for *E. coli* according to Ueno and Yokoya (16).

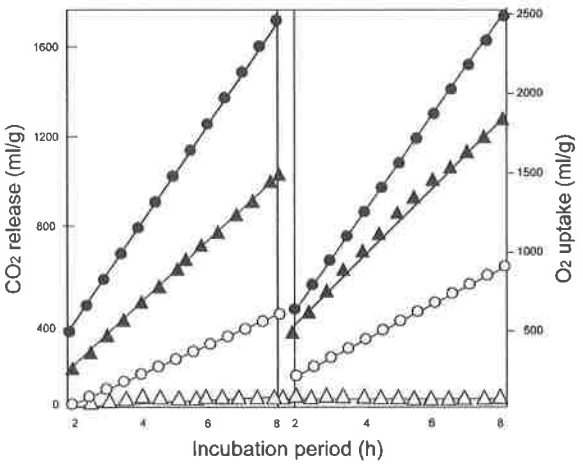

Figure 1: Effect of CTAB on gas exchange rate of *Echerichia coli*. Concentration of CTAB: •=0μM; Δ=8,2μM; O=16,5μM; Δ=27,4μM.

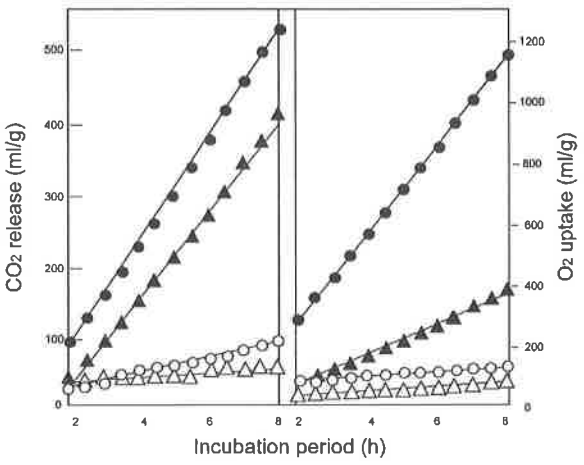

Figure 2: Effect of CTAB on gas exchange rate of *Pseudomonas aeruginosa*. Concentration of CTAB. •=0μM; =274μM; =1340μM; =2740μM.

Table 1: Changes in respiratory quotient (RQ = CO_2/O_2) with various concentrations of CTAB

CTAB (μM)	*L. mesenteroides*	Ac.aceti	*Kl. terrigena*	*E. coli*	*Sacch. cerivisiae*	*Ps. aeruginosa*
Blank (*)	-	0.44	0.66	0.66	0.55	0.16
0	2.1	1.3	0.76	0.74	4.8	0.51
2.7	2.1	-	-	-	-	-
5.5	2.8	1.0	-	-	-	-
8.2	3.8	1.0	0.58	0.63	6.1	-
16.5	-	-	0.36	0.58	7.7	-
27.4	-	-	-	0.33	1.5	-
274	-	-	-	-	-	1.2
1340	-	-	-	-	-	1.4
2740	-	-	-	-	-	0.14

(*) Reaction mixture with no glucose added

The inhibition of O_2 uptake by CTAB in *E. coli* was detected by Hugo (5) and was related to loss of viability. This was confirmed by our data on inhibition of O_2 uptake and CO_2 release by various microorganisms (Figs. 1 and 2) if compared with the survival data from Ueno and Yokoya (16) for the same microorganisms.

Quaternary ammonium compounds are known to interfere with the cell membrane and cause lysis of protoplasts (4). They also affect the transport of nutrients across the cell membrane (2,7).

Figs. 1 and 2 show that CO_2 release and O_2 uptake by *E. coli* and *Ps. aeruginosa* were linear during the experimental period (8 hs). This was also true for other microorganisms tested (data not shown), since a linear regression analysis of the data showed a correlation coefficient greater than 0.95 for all the microbial species.

The specific rates of CO_2 release and O_2 uptake of the microorganisms could be calculated from the best fitted straight lines drawn for various concentrations of CTAB. In addition, the effect of CTAB on respiratory quotient (RQ = CO_2/O_2) could be calculated from the data.

The RQ values for different microorganisms at various CTAB concentrations are shown in Table 1. As expected, the endogenous RQ values for all tested microorganisms were below 1, which indicated respiratory metabolism. With the addition of 0,3mM glucose, *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae* showed strong fermentative activity. This type of phenomenon is

Table 2: Rates of CO_2 release and O_2 uptake by different microorganisms

	CO_2 (ml/g.h.)	O_2 (ml/g.h.)
Acetobacter aceti IZ 2023	18.3	14.1
Leuconostoc mesenteroides FTPT 0367	183.1	87.3
Klebsiella terrigena FTPT 0400	173.0	229.4
Escherichia coli FTPT 0923	213.0	288.0
Saccharomyces cerevisiae FTPT 0472	165.5	34.3
Pseudomonas aeruginosa ATCC 10145	77.9	154.1

Table 3: Inhibition coefficients of CO_2 release (ICO_2) and O_2 uptake (IO_2) by CTAB on various microorganisms.

	$ICO_2.(10^{-3})$	$IO_2.(10^{-3})$
Acetobacter aceti IZ 2023	1.32	0.784
Leuconostoc mesenteroides FTPT 0367	17.29	6.98
Klebsiella terrigena FTPT 0400	10.28	7.10
Escherichia coli FTPT 0923	7.74	10.63
Saccharomyces cerevisiae FTPT 0472	6.54	0.954
Pseudomonas aeruginosa ATCC 10145	0.0498	0.0908

Table 4: Effect of CTAB on lactate dehydrogenase (LDH) activity of intact and lysed cells of various microorganisms. Activity data given in min. of methylene blue reduction time.

		CTAB concentration (M)			
	Cells	0	8.5	16.5	27.4
Escherichia coli FTPT 0923	I(*)	10	30	>480	
	L	20	30	>480	
Klebsiella terrigena FTPT 0400	I	10	40	>480	
	L	20	40	>480	
Pseudomonas acidovorans ATCC 17438	I	30	40	>480	
	L	35	50	>480	
Pseudomonas aeruginosa ATCC 10145	I	40	40	40	50
	L	30	40	>480	>480
Pseudomonas mendocina ATCC 25411	I	40	40	40	50
	L	30	45	>480	>480
Pseudomonas cepacia ATCC 25416	I	40	50	50	60
	L	30	35	>480	>480

I(*) = intact cells; L = lysed cells

Table 5: Effect of CTAB on activity of lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) of different species of bacteria. Activity data given in min. of methylene blue reduction time.

		CTAB concentration (M)				
	Enzyme	0	2.7	5.5	8.2	16.5
Pseudomonas acidovorans ATCC 17438	LDH	30	-	-	40	>480
	ADH	30	120	>480		
Leuconostoc mesenteroides ftpt 0367	LDH	10	-	-	150	>480
	ADH	20	150	>480		
Lactobacillus fermentum FPTP 0694	LDH	10	-	-	120	>480
	ADH	20	150	>480		

commonly observed with many fermentative microorganisms (3). All the other microorganisms tested had respiratory activity stimulated by sugar.

The microbial rate of CO_2 release increased to about 200 ml/g.h. with the addition of glucose, except for *Acetobacter aceti* (18.3 ml/g.h) and *P. aeruginosa* (77.9 ml/g.h.). Strong stimulation of O_2 uptake was observed with *Klebsiella terrigena*, *E. coli* and *P. aeruginosa*. *A. aceti* showed the lowest O_2 uptake stimulation by glucose (Table 2).

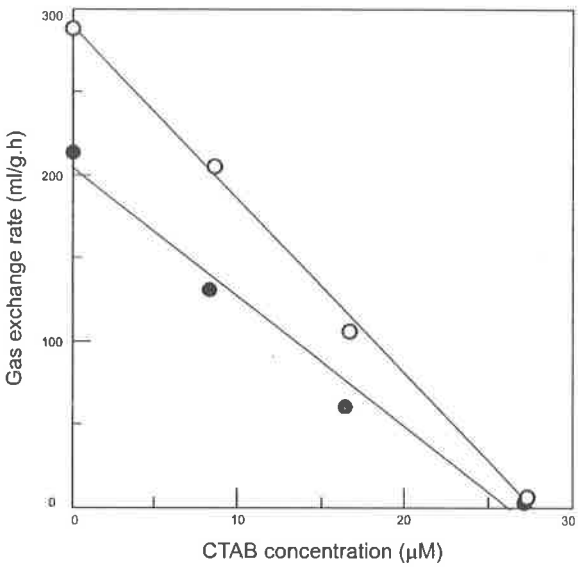

Figure 3: Reduction in CO_2 release and O_2 uptake rates with various concentrations of CTAB added to *E. coli* cells suspension. Symbols: O=CO_2 release; •=O_2 uptake.

The addition of CTAB caused some changes on RQ values, but there was no defined pattern of shift (Table 1). This indicates that both the respiratory and fermentative activities were reduced by CTAB to variable degrees.

The reduction of gas exchange rate by CTAB in the reaction mixture was linear with its concentration. The changes in CO_2 release [CO_2] and O_2 uptake [O_2] rates for *E. coli* at various concentrations of CTAB are shown in Fig. 3. The CO_2 inhibition coefficient (ICO_2=[CO_2]/[CTAB]) and O_2 inhibition coefficient (IO_2=[O_2]/[CTAB]) could be calculated from the best fitted straight lines. The ICO_2 and IO_2 calculated for the different microorganisms are shown in Table 3.

The ICO_2 and IO_2 for *A. aceti* were very low ($1.32x10^3$ and $0.784x10^3$, respectively) as compared with other microorganisms. Also, IO_2 for *S. cerevisiae* was low (0.954×10^3). These results must be due to a lower stimulatory effect of glucose on metabolism, as indicated in Table 2. All the other microorganisms showed ICO_2 and IO_2 values between $6.0x10^3$ and 10.0×10^3, with the exception of *P. aeruginosa* for which the lowest values were recorded ($0,0498 \times 10^3$ and $0,0908 \times 10^3$, respectively).

Effect of CTAB on enzyme activities. The activity of enzyme lactate dehydrogenase (LDH) was tested with intact and lysed cells of *E. coli*, *K. terrigena*, *P. acidovorans*, *P. aeruginosa*, *P. mendocina* and *P. cepacia*. The first three species were classified as CTAB sensitive and the remaining species as CTAB resistant according to the data of Ueno and Yokoya (16). Our data are shown in Table 4.

The maximum concentration of CTAB associated with enzyme activity of cell lysates was 8.5 M, and it was the same for all the tested species irrespective of their resistance to CTAB *in vivo*.

With intact cells, the activity of LDH was completely inhibited by 16.5 μM CTAB when sensitive species were tested, yet resistant species showed only a slight reduction in LDH activity even with 27.4 μM CTAB. (Table 5)

The tests on intact cells of *P. acidovorans*, *L. mesenteroides* and *L. fermentum* showed that alcohol dehydrogenase (ADH) was slightly, more sensitive to CTAB than lactate dehydrogenase (LDH). ADH was completely inhibited by 5.5 μM CTAB but LDH inhibition required 16,5 mM CTAB. (see data on Tables 4 and 5)

These results indicated that cell permeability was the major if not the sole factor responsible for the extremely high resistance to CTAB shown by some species of *Pseudomonas*.

The interference of cationic detergents on cell membranes has been pointed out as responsible for the loss of viability (13, 15). Other lethal actions of these compounds are related to their ability to coagulate cytoplasm constituent, especially proteins and nucleic acids (6).

In our experiments, the concentration of CTAB required to inhibit the cytoplasmic enzymes LDH and ADH was slightly lower than that required to halt respiratory activity of sensitive species. Furthermore, it was lower than the minimum inhibitory concentration (Mic) for the most sensitive species, namely *A. aceti* and *A. pasteurianus* (16). These results indicated that inhibition of cytoplasm enzymes could be responsible for the metabolic disorder and consequent inhibition of growth. If the cell membrane did act as permeability barrier, then its activity varied among different species. Some species of *Pseudomonas* must be extremely impermeable to quaternary ammonium compounds by a mechanism different from that associated with other hydrophobic compounds tested by Nikaido and Nakae (9).

The adaptation of resistant cells to CTAB was not observed during 8 hs. of incubation with CTAB in glucose solution. This was shown by the linear CO_2 release and O_2 uptake rates recorded throughout the

incubation period (Fig. 2). The phenomenon of adaptation has been observed with CTAB resistant species of *Pseudomonas* within 2 hs. of culture in medium containing CTAB (16). Since that medium contained organic nitrogen and minerals beside carbohydrates, this result suggests that the synthesis of cell materials was required for adaptation. Increased lipid content was detected when an antibiotic resistant type of *P. aeruginosa* was grown in medium containing sublethal amounts of quaternary ammonium compound (1). Adaptation mechanisms could include synthesis of lipids or other cell envelope components. Further studies are required to elucidate the exact mechanism of bacterial adaptation to CTAB.

RESUMO

Inibição do metabolismo microbiano por CTAB (brometo de cetiltrimetil amônio) e seu efeito em enzimas citoplasmáticas

Foi estudado o efeito do brometo de cetiltrimetil amônio (CTAB) no metabolismo de diversos microrganismos, através da medida de liberação do CO_2 e absorção de O_2. O CTAB inibiu a atividade metabólica linearmente à sua concentração e o seu grau de inibição dependeu de espécies de microrganismos testados. *Pseudomonas aeruginosa* foi 100 vezes menos afetada que as espécies de bactérias sensíveis. Em contraste com as células intactas, a atividade da enzima desidrogenase do lactato (LDH) extraída das espécies resistentes foi igualmente inibida pelo CTAB em comparação com as espécies sensíveis. A desidrogenase do álcool (ADH) foi ligeiramente mais sensível ao CTAB que a LDH. A concentração dessas enzimas foi similar àquela necessária para inibição do crescimento de bactérias sensíveis. Isso indica que a inibição do crescimento de microrganismos por CTAB pode ser devida à sua interferência nas enzimas do citoplasma. A diferença na resistência encontrada entre as diversas espécies está relacionada à diferença na permeabilidade da membrana celular.

Palavras-chave: inibição de microrganismos, brometo de cetiltrimetil amônio, inibição enzimática, inibição de quaternários de amônia

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YEASTS AND OTHERS PARAMETERS OF POLLUTION OF THE RIBEIRÃO CLARO STREAM IN RIO CLARO, SÃO PAULO

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ABSTRACT

The main water source of the Rio Claro municipal district (DAAE) was analysed for determining the level of organic contamination. Temperature, pH, light penetration, conductivity, dissolved oxygen, total dissolved phosphate, ortophosphate, ammonia, heterotrofic bacteria, total coliforms, fecal coliforms, total yeasts and fermentative yeasts were analysed during one year with samples being collected on a monthly basis. Bathing quality of the water from sites 1, 2 and 3 were classified, respectively, as Satisfactory, Very Good and Improper based on fecal coliform counts. Total yeast counts were appropriate as a complement for the appraisal of fresh water contamination by coliform counts. Seventeen yeast species belonging to genera *Candida*, *Cryptococcus*, *Kloeckera*, *Pichia* and *Rhodotorula* were found but none of them could be correlated with a level of contamination. *Candida famata*, *C. robusta-like* and *C. colliculosa* were isolated at all of the sites and between the five species found only at the more polluted site, *Rhodotorula mucilaginosa* was prevalent.

Key-words: coliforms, water contamination, yeasts.

INTRODUCTION

The presence of bacteria of the coliform group is considered as indicative of potencial health risks and is a widely used microbial method for monitoring water quality (12). The use of the coliform group as an indicator of water quality was questioned in the late 1960s, because there was little epidemiologic evidence correlating coliform levels in recreacional waters with the incidence of diseases (7). The use of *Escherichia coli* as an indicator of fecal contamination has also been questioned. This bacterium has been isolated from environments of "pristine" natural waters considered free of fecal contamination like tanks of Bromeliaceae and runoffs of tropical rainforests (8), restinga and mangrove (20).

Numerous studies have observed lower survival of *Escherichia coli* relative to many of the common nonfecal water borne pathogens like *Pseudomonas aeruginosa* (14), *Staphylococcus* and *Streptococcus* (3), mycobacteria (13), *Aeromonas* (4) and several kinds of viruses (29). Therefore, it is evident that in many situations fecal coliforms counts do not indicate the real health risks represented by the use of a water source, and some alternative methodologies have been suggested. Several studies have focused on the occurrence of molds and yeasts related to wastewater (11,16,26,27,28) and yeast counts is a monitoring method which can complement the coliforms counts, reflecting the eutrophication and levels of some non-fecal pathogens, like those derived from the body surface of bathers (15). Many studies have been done focusing on the occurrence of yeasts in polluted environments including some in Brazil (2, 16, 21, 24, 25, 27, 31). The yeasts associated to warm blooded animals and sewage include numerous fermentative

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species and there is a higher proportion of these yeasts in polluted water than in clean water (16, 24). Some of the yeast species isolated from polluted waters are opportunistic pathogens and may represent a health risk (17). However, it is important to seek a methodology to determine the yeast species or to enumerate their population in these environments in order to evaluate and to monitor the organic contamination in the water. Additionally, incubation at high temperatures has been used to select yeasts associated with man. These yeasts are introduced in the water through sewage and are mostly able to grow at 37°C (6). However, thermotolerant autochtonous yeasts typical of clean water, may interfere with interpretation and 40°C has been suggested to select human associated species from polluted waters (14, 16, 17, 19).

The objective of this paper was to study the level of fecal contamination of the Ribeirão Claro stream with groups of indicator microorganisms accepted by current legislation to confirm the validity of using yeasts for monitoring quality of freshwater.

MATERIALS AND METHODS

Three sampling sites were established on the Ribeirão Claro stream, which belong to the Piracicaba River basin. Site 1 was located on road SP-121 between kilometers 63 and 64, in a rural zone surrounded by sugar-cane crops, with possible domestic sewage. Site 2 was the water source of the Departamento Autônomo de Água e Esgoto of Rio Claro (DAAE),located in a *Eucaliptus* forest. It is an artificial impoundment coated with stones and cement of the Ribeirão Claro stream water. Site 3 was located on road SP-316 on the boundary of the Rio Claro and Santa Gertrudes municipal districts. This site is next to sugar-cane crops and receives pluvial, industrial and domestic wastes.

Samples were collected monthly during one year from each site in sterile amber bottles (1 Liter).Temperature, pH, light penetration, dissolved oxygen, ortophosphate, ammonia, heterotrofic bacteria, total coliforms, fecal coliforms, total yeasts and fermentative yeasts were analysed. Standard methodology was used for the physical, chemical and bacteriologic parameters (1). Total coliforms and fecal coliforms were determined by the Most Probable Number technique (MPN). The total yeast and fermentative yeast parameters were also quantified using the MPN method in medium for aquatic yeasts (19). The tubes were incubated at 25°C, 35°C and 40°C and results were registred after 3, 6 and 10 days of incubation. Yeasts were isolated by streaking out growth from the MPN tubes on Sabouraud Dextrose Agar and identified according to standard procedures (5, 22).

Statistical analysis was done according Sokal and Rohlf (30) considering the factors: temperature at 25°C, 35°C and 40°C and the different periods of incubation on the 3^{rd}, 6^{th} and 10^{th} days.

RESULTS AND DISCUSSION

Table 1 shows the arithmetic means of chemical and physical data. Only slight differences in pH and temperature were observed among the three sites and there were no indications suggesting that they could be influenced by external factors other than climate. Indeed, all the other parameter analysed showed that sites 1 and 2 were similar and both quite different from site 3. The physical and chemical data registered at sites 1 and 2 reflected low levels of contamination. Thus, the concentration of nitrogenated compounds was low for both sites whereas at site 3 we had a high concentration of these indicators suggesting that the aerobic bacteria responsable for nitrification were eliminated or were inactive. This conclusion was reinforced by the absence of dissolved oxygen in some samples collected at site 3. Sites 1 and 2 were also similar with respect to their phosphate concentration. Usually most of this anion is of inorganic origin and

Table 1 - Means of physical and chemical parameters of water from the Ribeirão Claro stream

Sites	Temp °C	pH	light penetration	Cond.	DO (mg/L)	TDF (µg/L)	PO_4 (µg/L)	NO_2 (µg/L)	NH_4^+ (µg/L)
1	20.3	6.48	52.1	62.5	7.5	47.5	41.4	7.8	4.0
2	21.1	6.39	59.4	57.9	6.6	42.2	29.8	7.7	3.2
3	20.8	6.41	25.6	268.6	1.8	284.3	260.2	311.5	25.2

DO = dissolved oxygen
TDF = total dissolved phosphate
Cond.= condutivity in S/cm^{-1}
PO_4 = ortophosphate

Table 2 - Geometric mean of heterotrofic bacteria counts in CFU/100 mL of water and MPN/100mL of total coliforms and fecal coliforms at three sites on the Ribeirão Claro stream.

Microorganism	site 1	site 2	site 3
Heterotrofic Bacteria	1.4×10^5	1.4×10^5	3.9×10^6
Total Coliforms	1.1×10^4	6.9×10^3	3.9×10^5
Fecal Coliforms	6.6×10^2	3.7×10^2	1.7×10^5

Site 1: SP-121 Road, between Km 63 and 64;
Site 2: DAAE Collection Station;
Site 3: SP-316 Road next to the Rio Claro and Santa Gertrudes boundary.

Table 3 - Geometric mean/100 mL of Total Yeast counts in MPN/100 mL of water.

Incubation Temp.- time	site 1	site 2	site 3
3 days	5.8×10^1	4.1×10^1	3.4×10^2
25°C - 6 days	1.3×10^2	6.8×10^1	1.5×10^3
10 days	2.6×10^2	1.5×10^2	3.6×10^3
3 days	5.1×10^1	3.6×10^1	7.8×10^2
35°C - 6 days	8.7×10^1	6.4×10^1	2.8×10^3
10 days	1.0×10^2	8.6×10^1	3.7×10^3
3 days	3.2×10^1	3.0×10^1	4.6×10^1
40°C - 6 days	3.2×10^1	3.0×10^1	7.4×10^1
10 days	3.6×10^1	3.0×10^1	1.3×10^2

Table 4 - Geometric mean/100 mL of Fermentative Yeast counts in MPN/100 mL of water.

Incubation Temp.- time	site 1	site 2	site 3
3 days	3.1×10^1	3.0×10^1	3.3×10^1
25°C - 6 days	4.3×10^1	3.8×10^1	1.3×10^2
10 days	7.6×10^1	4.5×10^1	8.2×10^2
3 days	3.3×10^1	3.1×10^1	9.2×10^1
35°C - 6 days	5.0×10^1	5.4×10^1	3.0×10^2
10 days	6.1×10^1	6.6×10^1	7.8×10^2
3 days	3.0×10^1	3.0×10^1	4.4×10^1
40°C - 6 days	3.0×10^1	3.0×10^1	6.2×10^1
10 days	3.3×10^1	3.0×10^1	6.5×10^1

the concentration determinated may be from soil runoff, since the stream runs through an agricultural region, including an urban area. At site 3, however, the concentration of TDP and PO_4 were enhanced by the presence of sewage and detergents mostly. Conductivity reflects the concentration of ions and it correlated well with other parameters, as expected.

The physical and chemical data clearly showed that site 3 was heavily disturbed mostly as a result of sewage and domestic outlet and sites 1 and 2 represent unpolluted sites. The microbial index represented by bacterial counts and yeast counts reinforced these conclusions.

Seventeen species of yeasts isolated from the three sites were identified as belonging to the following genera: *Candida*, *Cryptococcus*, *Kloeckera*, *Pichia* and *Rhodotorula*. The identification of 85 yeast isolates, isolation temperatures, and respective sites are shown in Table 5. The genera of yeasts isolated were previously observed in subtropical freshwater (2, 27) and in other aquatic environments (24, 25, 31). The prevalence of the genus *Candida* agreed with other papers, mainly in relation to environments where pollution comes from domestic sewages (25). The genus *Rhodotorula* is usually found in clean water (15), but from the samples taken in this study it was isolated only from the most polluted site, and perhaps it might have been inhibited by the MPN method which favors more fermentative species (15, 16). *Candida colliculosa*, *C. robusta* and *Candida famata* were isolated several times, regardeless of the site sampled. *Debaryomyces hansenii* and its anamorph *C. famata* is the most common fungus isolated in marine water (18) also and cannot be associated with specific water quality. *Candida pararugosa*, *C. pintolopesii*, *C. terebra*, *Cr. curvatus* and *Rh. mucilaginosa* were isolated exclusively at site 3. The later was prevalent at this site but our data is too limited to form a definitive conclusion of what this means.

According to the Brazilian Federal Legislation (9) the bathing quality of water from sites 1, 2 and 3 were classified respectively as Satisfactory, Very Good and Improper. According to the São Paulo State Legislation (10), sites 1 and 2, were graded as class 2 and site 3 as having class 4 water. This contaminated water reachs the Piracicaba river, which has a very high index of pollution due the acumulation of domestic and industrial waste. The levels of total yeasts and fermentative yeasts agreed with the pattern of bacteriological counts. The response of the yeast populations to the excess of organic input has already been reported in other studies (2, 25, 27), and was reaffirmed by our data and thus confirming that these microorganisms may be used as a complementary method for monitoring water.

The medium for aquatic yeasts of Hagler *et al.* (19) was efficient in completely inhibitting the bacterial development. Our data also showed that higher counts were obtained at 25°C which was closer to the average temperature registered at the three sites. Therefore, this might be a fact to be considered when using this

Table 5 - Yeast species isolated from Ribeirão Claro stream at 25°C, 35°C e 40°C.

Species	% of isolations	site			temperature (°C)		
	(n=85)	1	2	3	25	35	40
Candida colliculosa	11.5	+	+	+	+	+	
Candida diddensiae	1.1	+					+
Candida famata	30.0	+	+	+	+	+	+
Candida guilliermondii	2.3	+	+		+	+	
Candida maltosa	2.3	+	+			+	+
Candida parapsilosis	2.3	+		+	+		
Candida pararugosa-like	1.1			+	+		
Candida pelliculosa-like	1.1	+				+	
Candida pintolopesii-like	3.5			+	+	+	
Candida robusta-like	18.4	+	+	+	+	+	+
Candida terebra-like	2.3			+		+	
Cryptococcus curvatus	2.3			+	+		
Cryptococcus laurentii	5.7	+			+	+	
Kloeckera apis	1.1	+			+		
Kloeckera apiculata	3.5	+			+	+	
Pichia anomala	5.7		+		+	+	
Rhodotorula mucilaginosa	5.7			+	+	+	+

methodology in environments with temperatures very distinct from that. Incubation at high temperature has been used to monitor pollution (6, 19) because it can select species more associated with domestic sewage pollution but in this study the incubation at 40°C resulted in low counts for all of the sites and it is not recomended for yeast counts of sites expected to have low levels of contamination because of the high selectivity that it promotes, unless a larger volume of sample is used.

There was a significant difference in yeast counts ($p > 0.005$) among readings realized on the 3rd, 6th and 10th day of incubation. Our data suggested that for the more polluted sites it is not necessary to wait ten days for a reading, because maximum counts were obtained at six days. Longer periods of incubation are probably necessary for counts of fermentative yeasts because of the delay of some species in initiating the fermentative process. Because of this, the quantification of total yeast rather than fermentative yeast seems to be more suitable for evaluation of pollution levels. Three days of incubation is not recomended even for the more polluted sites because there were significative differences ($p > 0.005$) between the counts obtained on the 3rd and 6th day. This probably corresponded to the period necessary for the yeasts to recover their growth. Results reported in this study agree with previous work (23, 24, 27) in showing that yeast counts may be valid as an indicator of contamination in aquatic environments.

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RESUMO

Leveduras e outros parâmetros de poluição do Ribeirão Claro, (Rio Claro - São Paulo)

A água do Ribeirão Claro, principal fonte de abastecimento para o município de Rio Claro foi analisada quanto ao seu nível de contaminação orgânica a partir de três locais de coleta: a montante da captação (local 1), na estação de captação do DAAE (local 2) e a jusante deste (local 3). As amostras foram coletadas durante 12 meses consecutivos e nelas analisou-se os seguintes parâmetros: temperatura, pH, penetração de luz, condutividade, oxigênio dissolvido, fosfato total dissolvido, ortofosfato, amônia, bactérias heterotróficas, coliformes totais, coliformes fecais, leveduras totais e leveduras fermentativas. A incubação das leveduras a 25°C por três dias foi

suficiente para avaliar o nível de contaminação em ambientes pouco poluídos, devendo ser estendida para seis dias em locais mais poluídos. Quanto à balneabilidade as águas dos locais 1, 2 e 3 foram classificadas, respectivamente, como Satisfatória, Muito Boa e Imprópria. Ficou evidente que a quantificação de leveduras totais é adequada para avaliação de contaminação de água doce complementarmente às bactérias do grupo coliforme. As dezessete espécies de leveduras identificadas pertenciam aos gêneros *Candida*, *Cryptococcus*, *Kloeckera*, *Pichia* e *Rhodotorula* e nenhuma espécie em particular pôde ser associada seguramente a um determinado nível de contaminação. Os gêneros *Pichia* e *Rhodotorula* foram isolados dos locais com menor e maior nível de contaminação, respectivamente.

Palavras-chave: coliformes, poluição, leveduras.

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OCCURRENCE AND DIEL DISTRIBUTION OF YEASTS IN A PALEO-KARSTIC LAKE OF SOUTHEASTERN BRAZIL

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ABSTRACT

The seasonal and diel distribution of yeasts was studied in the paleo-karstic lake Lagoa Santa. The possible influence of environmental parameters on the total fungal counts and occurrence of yeast species was examined. The lake showed thermal and oxygen homogeneity in June, and a stratification of temperature and oxygen in the water column during the period from September to March. The yeast species diversity decreased with depth probably due to the oxydative profile of the predominant species that could limit their distribution to the highly oxygenated surface waters. The most frequent species were *Cryptococcus flavus, Rhodotorula minuta, Trichosporon cutaneum, Tr. pullulans* and *Aureobasidium pullulans*. The yeast counts and species diversity were higher during the rainy months of December and March, and lower in the dry season. *Trichosporon cutaneum* was predominant in the dry season, and it was considered to be indigenous to the lake. The predominance of oxidative polytrophic yeasts and pigmented species suggested that the mycobiota of the lake was probably carried from soils and foliar surfaces. The yeast populations of Lagoa Santa lake would be formed by the constant efflux of transitory allochtonous species to the lake by rain and the catchment basin.

Key words: Yeast diversity, diel distribution, paleo-karstic lake.

INTRODUCTION

Yeasts are normal components of the biota of most lakes and the species isolated from waters are generally oxidative types and utilize nitrate as sole nitrogen source (18, 22, 24, 25). These yeasts are known to occur in association with tree leaves and soils, and enters the aquatic environment through the runoff from the drainage basin and margins (5, 22, 27). The genus *Cryptococcus* and the yeastlike fungus *Aureobasidium pullulans* are major components in non-polluted waters (6, 31). Pink yeasts are consistently present and can make up 50% of total isolates in rivers and lakes (13, 25). Most red isolates represent the genus *Rhodotorula*, but species of *Rhodosporidium* and *Sporobolomyces* also occur (5). Some studies have shown the probable uses of yeasts as indicators of pollution complementary to the coliform bacteria (4, 6, 7, 8, 18, 20, 30, 31). The presence of allochtonous sources of metabolites leads to an increase in yeast counts, and polluted freshwaters generally have larger yeast populations than clean lake and river waters (22, 25, 31). Furthermore, a shift is noted from the prevalence of strickly aerobic yeast species in clean waters to the predominance of fermentative strains in polluted waters (6). The presence of human wastes can be related to large proportion of man-associated *Candida* species in freshwater habitats (31).

The presence and diversity of yeast species in freshwaters and estuaries may be influenced by

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hidrogen ion concentration, dissolved oxygen and total dissolved phosphates (7, 19, 23, 24, 31). The studies of Hedrick *et al.* (10), Hedrick and Soyuenc (9), and Hedrick *et al.* (11) found that the total yeast counts and the occurrence of some species are correlated with the organic nitrogen and nitrate contents of Great Lakes and their affluents. According to Hedrick *et al.* (12), the conjunction of a great number of physico-chemical factors affects the fluctuation in yeast population of lakes. Seasonal variations may also occur that are correlated to differences in water temperature or due to auto-purifying process of the water (26). Lazarus and Koburger (16) suggest that the low yeast diversity in the estuarine waters of River Suwanne, Florida was due to the depletion of nutrients and stressing environmental conditions such as high levels of salts. Quinn (22) suggests that the elevated counts of yeasts could be indicative of periodic or localized organic enrichment in aquatic habitats.

A study carried out from June, 1986 to March, 1987 in Lagoa Santa lake tries to access the seasonal and diel distribution of yeasts and to determine if any distribution was correlated with measured environmental parameters. Fungal counts and yeast diversity were higher during rainy months, suggesting the allochtonous origin of the yeast populations of the lake. Most species were oxidative type, and distributed unequally in the water column of the lake. A pattern is suggested of increase in diversity and yeast population numbers in the rainy season, differing from the occurrence of *Trichosporon cutaneum* in the dry season. We suggest *Tr. cutaneum* could be indigenous to karstic lakes of this region.

MATERIALS AND METHODS

Lagoa Santa is a paleo-karstic lake located in the Lagoa Santa Karstic Plateau of Minas Gerais (19°38'S; 43°53'W). The lake has been suffering from antropogenic activities during the last decades, including siltation and eutrophication with drastic alterations of its original biota, namely the reduction of the phytoplancton species richness (2). The lake has maximum lenght of 1,820 m and maximun width of 1,320 meters. Two sampling stations were chosen, being station I situated in the limnetic zone of the lake, with maximum depth of 7,3 m and station II in the litoral zone, 10 m far from the margin. The water samples were collected in station I, at four depths corresponding to 100%, 10%, 1% of the surface light incidence and aphotic zone, determined from the visual disappearance of the Secchi disc. Collections were made in June, September, December of 1986 and March of 1987, at 4-hours intervals during a 24 hs period. Samples were taken at station I with a van Dorn bottle of 6 liters, previously desinfected with 70% ethanol, and transferred to sterile bottles of 250 ml. In station II, water samples were collected directly in the surface of the lake, with sterile bottles of 250 ml. The samples were maintained in ice bath for the microbiological procedures.

The physicochemical parameters were obtained: temperature, measured with a Toho Dentam ET-3 termistor with precision of 1/10°C; total alcalinity, calculated by the electrometric method described by Mackereth *et al.* (17); pH, measured with a portable Micronal B278 pHmeter; dissolved oxygen, determined by the modified Winkler technique (21); and ortophosphate according to Stricklands and Parsons (28).

The samples were taken to the laboratory within 2 hours of collection. Aliquots of 0,2 ml of the samples were plated in triplicates on acidified YM agar (glucose 2%, peptone 1%, yeast extract 0,3%; malt extract 0,3%; agar 2% and chloramphenicol 15 mg%, and the pH adjusted to 4-4,5 by adittion of HCl 1N). Plates were incubated at room temperature and yeasts and molds counts were obtained after 3 to 5 days. Representatives of the yeast colony types on each plate were selected, purified and identified according to van der Walt and Yarrow (29). The yeasts were identified by the keys in Krejer-van Rij (15) and Barnett *et al.* (3). The odds measure of diversity (14) was used to determine the diversity of yeast species in each sampling station and depth, and also for the monthly collections of yeasts.

RESULTS

The physicochemical parameters measured for stations I and II are presented as the average values obtained in six collections during daytime (8:00, 12:00, 16:00 hs) and nigth (20:00, 24:00, 4:00 hs) in each month (Table 1). Temperature and dissolved oxigen values in the water column are shown in Fig. 1. Lower temperatures were obtained at night, especially in June, and higher temperatures were obtained in December and March, demonstrating the occurrence of seasonal differences. The station II have been oxygenized during all months, with lower values at night. Ortophosphate had higher values in June and September, and lower values in December and March.

Table 1 - Environmental parameters in Station I and Station II of Lagoa Santa lake

Month	Temperature (°C)		Dissolved oxigen (% of saturation)		pH		Alkalinity ($\mu eqCO_2/L$)		Orthophosphate (mg/L)	
	I[a]	II	I	II	I	II	I	II	I	II
June	21-23[b]	21-23	80-210	120-160	7,0-8,7	7,5-8,0	0,8-0,9	1,1-1,2	5-20	9-27
September	22-28	23-27	60-140	120-150	7,0-8,5	8,0	0.,9-1,2	1,1-1,2	5-20	6-27
December	21-28	24-27	20-17	100-160	6,5-10,0	8,0-8,5	1,0-1,5	1,1-1,3	5-20	3-18
March	26-31	23-30	70-160	120-160	7,0-8,8	7,0-8,5	1,1-1,3	0,7-1,3	2-17	3-18

a: Station I and Station II

b: minimum and maximum values for each environmental parameter, during six collections per day, in each month.

Table 2 - Mold and yeast counts (colony forming units (CFU)) in st I and st II of Lagoa Santa lake, during a diuturnal collection at four hour-intervals in June, September, December of 1986 and March of 1987.

Collection		JUNE					SEPTEMBER					DECEMBER					MARCH				
		I				II	I				II	I				II	I				II
		100[a]	10	1	AZ		100	10	1	AZ		100	10	1	AZ		100	10	1	AZ	
16 hs	Yeasts	18	2	8	1	18	20	8	7	1	18	38	20	6	3	121	65	84	30	5	20
	Molds	1	1	2	1	1	1	2	1	8	1	15	1	12	1	10	18	1	11	1	18
20 hs	Yeasts	12	12	4	2	12	25	36	11	1	6	96	44	82	3	196	59	8	62	2	17
	Molds	2	2	2	2	1	3	5	1	1	6	8	12	12	1	8	4	8	1	1	6
24 hs	Yeasts	12	1	7	3	18	17	17	11	6	3	52	34	60	2	68	18	43	17	1	14
	Molds	4	1	1	1	4	1	1	3	1	1	4	21	1	2	8	5	12	1	1	6
4 hs	Yeasts	13	6	6	1	13	23	18	15	3	3	64	30	30	2	49	75	50	28	15	10
	Molds	9	1	1	2	10	1	1	15	1	1	12	15	12	1	20	2	18	12	6	6
8 hs	Yeasts	20	1	6	1	26	14	3	4	1	12	40	11	12	2	130	112	28	42	12	24
	Molds	1	8	4	1	1	1	3	1	1	6	21	5	3	1	20	15	15	1	7	26
12 hs	Yeasts	5	1	12	1	21	5	8	6	1	12	25	28	8	2	51	58	60	30	22	17
	Molds	1	1	1	1	1	8	1	4	1	12	12	1	1	1	12	12	22	1	1	12

a: 100%, 10% and 1% of light incidence; AZ =aphotic zone.

The Table 2 shows the yeast and mold counts in the four depths of station I and in station II, for each month. Higher counts were generally obtained at the surface, and the lower counts were isolated in the aphotic zone. In December, a diuturnal pattern was noted, with low numbers in the surface during the day, and high counts at night. In station I, the higher counts of yeasts were usually obtained in the photic zone, during all months. The total counts were higher in March, reaching 931 CFU/ml and lower in June corresponding to 155 CFU/ml (Table 3). Total counts of molds were lower than yeast counts, varying from 51 CFU/ml in June, to 176 CFU/ml in March. The higher counts were obtained in the surface except in March, when the higher mold numbers were obtained at 10% of light incidence. In station II, the yeast counts remained low in June, September and March, the lowest counts being obtained in September (54 CFU/ml). In December the yeast counts showed to a peak, reaching 615 CFU/ml. Mold counts were usually lower than yeast numbers, with higher counts obtained in December.

Candida, Cryptococcus, Rhodotorula and *Trichosporon*, and the black yeastlike fungus *Aureobasidium* were the most frequent genera among 376 yeast strains identified as 30 species. Table 3 shows the seasonal distribution of yeasts in stations I and II. The red yeasts of the genus *Rhodotorula* were frequent in June, December and March, whereas *Cryptococcus* was the most frequent in March, represented almost exclusively by *Cr. flavus*. Yeast species diversity was higher in December (10.64) and June (5.57), and low in September (1.84) and March (2.28), in station I. In station II, the higher values were measured in September (15.0) and March (6.24), whereas the lower values were recorded in June (4.49) and December (1.58). In June, the most frequent species were *Rhodotorula minuta*, *Cryptococcus*

Table 3 - Seasonal variation of yeasts (in Colony Forming Units/ml)[a] in the station I and station II of the lagoa Santa lake in June, September and December 1986 and March 1987.

SPECIES	STATIONS	JUNE		SEPTEMBER		DECEMBER		MARCH	
		I	II	I	II	I	II	I	II
Aureobasidium pullulans						33	165	141	30
Candida famata		8	6	2		6		8	
Candida guilliermondi						30			
Candida humicola								18	
Candida karawaewi						6			
Candida peliculosa							6		
Candida pseudointermedia						99			
Candida steatolytica					6		3	36	
Candida tepae-like					6				
Cryptococcus albidus		3	6	3	6	123		12	
Cryptococcus flavus				24				462	51
Cryptococcus hungaricus		15				18	6	6	
Cryptococcus infirmominiatus		9	6	21					
Cryptococcus laurentii			6						
Cryptococcus luteolus						48		51	6
Cryptococcus macerans		24	6	15					
Debaryomyces hansenii						3	66		
Metschnikowia krissi-like			6						
Prototheca sp.		15				7	24	14	
Rodothorula acheniorum						45	15	72	15
Rodothorula auranthiaca		6				33		3	
Rodothorula glutinis		15	15						
Rodothorula lactosa			9			6		18	
Rodothorula minuta		24	39	27		84	159	84	
Rodothorula rubra		21							
Trichosporon cutaneum		15	9	141	30				
Trichosporon figueirae				6					
Trichosporon pullulans						48	105		
Trichosporon terretre				21	6	105	63		
Sporoblomyces roseus						3	3	6	
Total		155	108	260	54	694	615	931	102

a - Number of CFU is the somatory of the media of three replicates in each depth.

macerans and *Rh. glutinis*. In September, *Trichosporon cutaneum* was the most frequent species. In December, *Rh. minuta*, *A. pullulans*, *Tr. terrestre* and *Tr. pullulans* were frequently isolated. In March, *Cr. flavus* was the only yeast species present in all depths of the water column of the lake, being the most frequent species at 10% and 1% of surface light incidence. The yeast species diversity was higher at 100% (9.36) and lower at the aphotic zone (4.0), where *Cr. flavus* and *A. pullulans* summed 55% of the total yeast populations in this month. The yeasts *Cr. albidus*, *Cr. flavus*, *Rh. acheniorum*, *Rh. auranthiaca*, *Tr. cutaneum* and *Tr. terrestre* presented similar frequencies of occurrence at the three depths of the euphotic zone. These species occurred during the rainy months, except for *Tr. cutaneum* that was most frequently isolated in September. *Aureobasidium pullulans*, *Candida pseudointermedia*, *Cr. hungaricus* and *Rh. minuta* occurred only in surface waters, usually in rainy months.

DISCUSSION

Temperature and dissolved oxygen profiles suggested the occurrence of two distinct patterns which agreed with the limnological cycle described

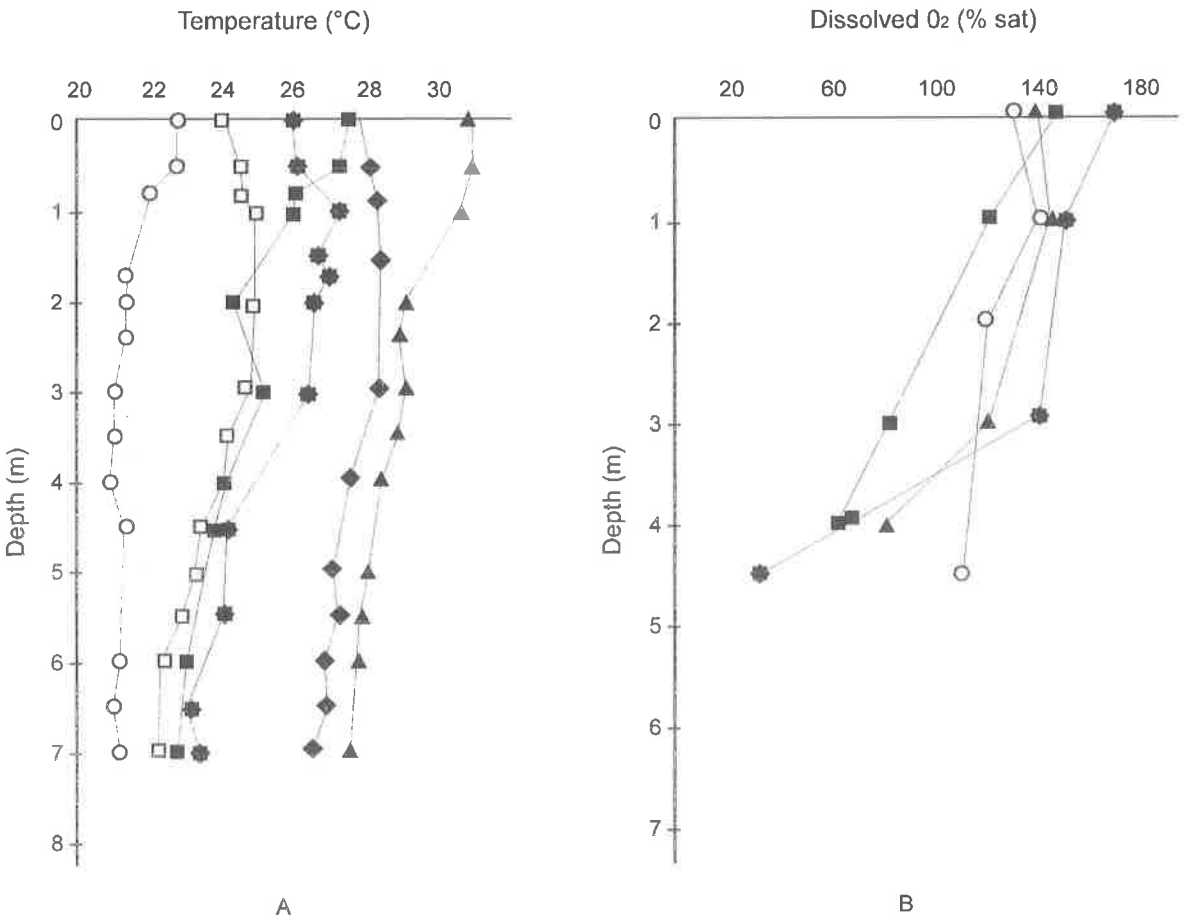

Fig. 1 - Temperature and oxygen profiles in the water colums of Lagoa Santa lake during June, September and December, 1986 and March, 1987. (A) Temperature values obtained in June (O), in September during daytime (12-20 hs. ■) and nightime (0-8 hs. □); December (✱); and March during daytime (12-20 hs.◀) and nightime (0-8 hs. ◆). (B) Percent of saturation of dissolved oxygen obtained in June (O); September (■); December (✱) and March (◀).

for the Lagoa Santa (1). In June, the lake shows a thermal homogeneity allowing the distribution of the dissolved oxygen throughout the water column. From September to March, the lake shows stratification of temperature and oxygen, exhibiting a clinograde profile. During the stratification period, temperature variation of approximately 6°C was recorded, the euphotic zone was 4,0 m deep and the aphotic zone was poorly oxygenated. The total yeast numbers were unequally distributed in the water column, decreasing with depth. This differed from the distribution of yeasts in the Great Lakes where the frequency of occurrence and species diversity increased with depth, and the numbers of yeasts in surface waters was lower than in subsurface waters (9, 10). A probable correlation could exist between yeast counts and zones of nutrient accumulation determined by the thermal stratification of the water column of the lake. Yeasts are heterotrophic organisms that tend to concentrate where nutrients are available. In Lake Ontario and Lake Superior, the total yeast population was definitely related to organic nitrogen and nitrate concentrations (9, 11). We have not observed significant correlation between yeast counts and the measured physicochemical parameters. The higher counts of yeasts in the surface of the lake would suggest their allochtonous source, as superficial waters strongly reflect the influx of materials to the lake.

The occurrence of yeast genera and species did not show any pattern of distribution in the water column.

In the deep layers, the lower frequencies of occurrence and low species diversity suggested that yeast numbers were less affected by the fluctuations of the influx of materials, and maintained low values, probably due to oxygen or nutrient limitation. The species diversity decreased with depth probably due to the oxidative profile of the yeasts isolated, that could limit their distribution in regions with low oxygen concentration, or to the allochtonous source of most species (23, 24). These species arrive to the surface waters more frequently than to deep layers, where they probably cannot maintain constant detectable populations. No pattern of diel variation was noted in yeast species distribution and occurrence.

The seasonal differences noted both in the yeast counts and species diversity are suggestive of the allochtonous influx of yeasts to the lake. In station I, total yeast counts showed strong seasonal variation, increasing in numbers in the rainy months (December and March), when the lake received an intense runoff from its catchment basin. Our results corroborated the suggestion of Quinn (22) that yeast counts are good indicators of periodic or localized enrichment in aquatic habitats. In September, during the dry season, the dominance of *Tr. cutaneum,* that summed 53% of the total isolates, lowered the yeast species diversity. In December, the rain was the probable cause of the increased diversity and population numbers, whereas in March the dominance of *Cr. flavus* (64% of the total isolates) lowered the yeast diversity. In station 2, the yeast counts were considerably low in September, being enhanced by the rain in December and March. The species diversity was low in December due to the high frequency of occurrence of *A. pullulans*, *Rh. minuta*, and *Tr. pullulans* that summed 64,4% of the yeast isolates. The yeast species composition also presented a seasonal fluctuation. The most frequent species, *A. pullulans* and *Cr. flavus* were isolated only in September, December and March. *Candida intermedia, Cr. luteolus, Debaryomyces hansenii, Rh. acheniorum, Rh. minuta, Tr. pullulans* and *Tr. terrestre* occurred in considerable numbers in the rainy months. These, and other species that occurred only in the rainy months are probably of allochtonous origin (5).

Differing from all other species, *Trichosporon cutaneum* occurred only from June to September, with higher counts in the later month (171 UFC/ml). *Trichosporon cutaneum* was isolated in another carstic lake, in the same region, with a similar seasonal pattern (24). These findings suggested that *Tr. cutaneum* could be indigenous to karstic lakes of the Lagoa Santa Plateau. Probably, it was negatively affected by the influx of allochtonous competitors or materials that would act as growth inhibitors for *Tr. cutaneum*. Also, the increase in volume of the lake during rainy months could promote an effect of dillution of the *Tr. cutaneum* stable population.

Our results suggested that the yeast mycobiota of Lagoa Santa lake is predominantly of allochtonous origin, the rain being probably the major source of yeasts to the lake, carrying species from the soils and plant materials. The predominance of oxidative polytrophic yeasts, and high frequencies of pigmented species suggested that this flora was probably carried from foliar surfaces (5, 24). The tropical lacustrine habitats present a diel pattern of physico-chemical fluctuations. The survival and colonization of the lake by the yeasts probably depend on the dynamic changes of physicochemical factors that determine the nutrient supply and availability to the populations occurring in the water. These ecological forces act together limiting the yeast diversity and population growth, but the constant efflux of materials carrying yeasts would diminish their effects on the detectable counts of yeasts in the Lagoa Santa. The majority of the yeast populations of Lagoa Santa may be seen mostly as constantly innoculated transitory species.

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RESUMO

Ocorrência e distribuição diuturna de leveduras em um lago paleocárstico do sudeste do Brasil

Foram estudadas a distribuição sazonal e diuturna de leveduras na coluna d'água da Lagoa Santa, um lago paleocárstico de Minas Gerais. A possível influência de parametros ambientais nas populações fúngicas e na ocorrência das espécies de leveduras foi examinada. O lago mostrou homogeneidade térmica e coluna d'água oxigenada durante o mês de junho, e estratificação da temperatura e oxigênio dissolvido

durante o período de setembro a março. A diversidade de espécies de leveduras diminuiu de acordo com o aumento da profundidade, provavelmente devido ao metabolismo oxidativo predominante entre as leveduras que poderia limitar sua distribuição às águas oxigenadas da superfície. As espécies mais frequentes foram *Cryptococcus flavus, Rhodotorula minuta, Trichosporon cutaneum, Tr. pullulans* e *Aureobasidum pullulans*. As contagens de leveduras e a diversidade de espécies foram maiores nos meses chuvosos de dezembro e março, e menores na estação seca. *Trichosporon cutaneum* mostrou populações elevadas na estação seca, e poderia ser considerada uma espécie indígena do lago. A predominância de espécies oxidativas politróficas e pigmentadas sugere que a micobiota da Lagoa Santa é originária de solos e superfícies foliares. As populações de leveduras da Lagoa Santa são provavelmente mantidas pelo efluxo constante de espécies transitórias nas águas da chuva e dos afluentes do lago.

Palavras-chave: Diversidade de leveduras, distribuição diuturna, lago paleocárstico.

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SURVEY OF AFLATOXINS B_1, M_1 AND AFLATOXICOL IN POULTRY AND SWINE TISSUES FROM FARMS LOCATED IN THE STATES OF RIO GRANDE DO SUL AND SANTA CATARINA, BRAZIL.

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ABSTRACT

Eighty-three samples of liver and kidney from swine and poultry for human consumption were analysed for aflatoxins B_1, M_1 and aflatoxicol residues by means of thin-layer chromatography. Aflatoxin B_1 was detected in one sample of pig liver from Rio Grande do Sul (Brazil) at a level of 27ng/g (ppb) while none of the poultry kidney and liver samples showed aflatoxins and aflatoxicol. Traces of aflatoxin M1 were found in one sample of kidney tissue. Aflatoxins B_1, M_1, and aflatoxicol recovered of artificially contaminated meat tissues were: 74% to 95.2% for aflatoxin B_1; 60 to 80% for aflatoxin M_1 and 80 to 95% for aflatoxicol.

Key words: aflatoxin B_1, aflatoxin M_1, aflatoxicol, liver, kidney, thin-layer chromatography.

INTRODUCTION

Public health defines mycotoxicosis as diseases with characteristic clinical symptoms caused by ingestion of food and feed naturally contaminated with mycotoxins. Despite the fact that more than a hundred mycotoxins are known today, the diagnosis of human mycotoxicosis is uncommon.

Feed contamination by mycotoxins has brought many problems for animals by causing either acute or chronic infection. On the other hand, the presence of mycotoxins is a potential public health hazard because exposed animals may retain residues of aflatoxins or their metabolites in their tissues (8), specially in meat, poultry and dairy products.

Nowadays the assumption that aflatoxins are a risk factor in the induction of hepatocellular carcinoma is based on the data obtained from animals, in the rate incidence of hepatic cancer in animals, and in the epidemiological studies on human exposure. Some studies show many evidencies of hepatotoxicity and hepatocarcinogenicity of the mycotoxin in various species of animals, including birds and fishes (3, 5, 6, 10 and 11). The World Health Organization recommends a systematic control of aflatoxin level in the population diet, mainly in countries located in tropical and sub-tropical areas, where the climatic conditions are favorable for the growth of the aflatoxin-producing fungi (2, 15). When animals are exposed to aflatoxins through feed, residues of the aflatoxins or their metabolites will probably be found in their livers. Quantification of the parent toxins and potentially toxic metabolites is also important for the evaluation of food safety. There is a striking lack of knowledge about the Brazilian situation on mycotoxins residues, mainly aflatoxins and its metabolites in food from animal products. To provide the much needed informatin in this area the present work aimed to investigate the presence of aflatoxins B_1, M_1 and aflatoxicol in liver and kidney from Brazilian poultry and swine destined to human consumption.

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MATERIAL AND METHODS

Forty-three samples of liver and forty-three kidneys from swine and forty samples of liver and forty samples from poultry were obtained from farms of the states of Santa Catarina and Rio Grande do Sul , Brazil . They were sent to the laboratory just after the slaughter of the animals . The type of feed the animals consumed was:

group 1 - industrial feedstuff (commercial feed);

group 2 - industrial feedstuff supplemented with grains harvested in the farm;

group 3 - only grains from the farm.

Chickens were slaughtered when 44-58 days old .There is no this kind of information about the swine.

Analysis of aflatoxins B_1, M_1 and aflatoxicol was done by thin layer crhomatography (TLC) according to the method described by Trucksess and Stoloff (13) as modified by Sabino (9). Mobile phase used: acetone-chloroform-isopropanol (10:85:5). The modifications included: thirty grams of sample extrac tion with 100ml of chloroform, clean-up by partition with 3 x 50 ml of hexane and TLC final detection. Column chromatography was not used.

RESULTS AND DISCUSSION

The limit of detection was 0.1ng/g for aflatoxin B_1 and M_1, and 1 ng/g for aflatoxicol. Recoveries were 74-95.2% for aflatoxin B_1, 60-80% for aflatoxin M_1, for aflatoxicol 80-95%. Coefficients of variation ranged from 4.41 to 14.2% for af.B_1; 8.8 to 15.25% for AfM_1 and 2.44 to 12.5% for aflatoxicol . The identity of AFB_1 and M_1 was confirmed by reaction with trifluoracetic acids (7, 12).

AFB_1 was found in only one sample of swine liver from Rio Grande do Sul at a level of 27ng/g. That animal had been fed with industrial feed (commercial feedstuff) plus grains grown in the farm. AFM_1 was only detected in one sample of kidney of poultry at a level less than 0.1ng/g. This particular animal was from Santa Catarina and had been fed with commercial feedstuff. The methodology employed (9) was found to be suitable to our work conditions, pratical and showed good repeatibility and precision as evidenced by recovery tests and relative standard deviations among replicates.

According to Horwitz (4) recovery levels as low as 60% may be accepted for trace determinations at ng/g levels. The present recoveries are within that guideline or above it. The aflatoxin B_1 and M_1 and aflatoxicol zones on TLC plates were free of interfering substances in all samples. The cleanest extracts were obtained from poultry samples. However there were some positive presuntive samples Eight swine and four poultry samples were in such a case. The presence of the false positives reinforce the need of confirmatory procedures in aflatoxins analisys in order to garantee reliability of data.

Trucksess *et al.* (14) have fed swine with aflatoxin B_1 contaminated feed and have found aflatoxicol and aflatoxins B_1 and M_1 in the tissues (kidneys, livers and muscles). They also have observed that the levels of aflatoxins B_1 and M_1 were similar in all tissues, except in the kidneys samples where AFM_1 was predominant. In addition, the amount of toxins was higher in tissues from animals that died before the slaughter than those which were sacrificed. They have found 36.5ng/g of AFB_1 in the liver of an animal that died after ingestion of a diet heavily contaminated with aflatoxin B_1. Such a result is similar to the findings of our survey (27ng/g).

There is strong epidemiological evidence that aflatoxins are carcinogenic (1). Since aflatoxins, in one hand, have been found to be widely distributed in common livestock feed and, in the other hand, it produces aflatoxicosis in farm animals, it is conceivable that tissue carry-over into meat may contribute to dietary carcinogenesis in man.

CONCLUSIONS

The present work allow us to conclude that except for a kidney poultry sample that showed traces of AFM_1, neither aflatoxins B_1, M_1 nor aflatoxicol were detected in out of 40 samples of livers and kidneys of chicken destined for human consumption. AFB_1 was present in just one sample of liver from swine out of 43 samples at the level of 27ng/g.

In order to avoid false positives results confirmatory tests should be run for reliable identification of any positive-presumptive samples.

RESUMO

Aflatoxinas B_1, M_1 e aflatoxicol em tecidos de aves e suínos provenientes dos Estados de Santa Catarina e Rio Grande do Sul, Brasil

Oitenta e três amostras de fígados e rins de aves e suínos foram analisadas para determinação de resíduos de aflatoxinas B_1, M_1 e aflatoxicol, por

cromatografia em camada-delgada. Aflatoxina B_1 foi encontrada em uma única amostra de fígado de suíno proveniente do Rio Grande do Sul (Brasil), na concentração de 27ng/g (ppb) enquanto nenhuma amostra de fígado e rim de aves apresentou aflatoxinas e aflatoxicol, porém traços de aflatoxina M_1 foram encontrados em uma amostra de fígado. Recuperações das aflatoxinas B_1, M_1 e aflatoxicol dos tecidos artificialmente contaminados foram: 74% a 95,2% para aflatoxina B_1; 60% 80% para aflatoxina M_1 e de 80% a 95% para aflatoxicol.

Palavras-chave: aflatoxina B_1, aflatoxina M_1, aflatoxicol, fígado, rim, cromatografia em camada-delgada.

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MICROBIAL CONTAMINATION OF STORED DIESEL OIL IN BRASIL

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ABSTRACT

Microbial contamination of hydrocarbon fuels, especially in refinery storage and distribution systems, is of increasing concern. Microbial activity in these systems leads to the production of a biomass at the oil/water interface. The control of this contamination can involve the use of biocidal chemicals. The aim of this paper was to select biocides of high efficiency against fungi, aerobic bacteria and sulfate-reducing bacteria isolated from a contaminated stored diesel oil system in Rio Grande do Sul. Filamentous fungi, aerobic bacteria and sulfate-reducing bacteria (SRB) were isolated from common, urban and marine diesel. Two biocides (a quaternary ammonium compound and an isothiazolone mixture) were found to show the highest efficacy against the major contaminants, fungi of the genera *Aspergillus* and *Hormoconis*, SRB and aerobic bacteria of the genera *Pseudomonas* and *Bacillus*, at low concentrations.

Key words: microbial contamination, diesel oil, biocides.

INTRODUCTION

Microbial contamination of petroleum products is a major problem in refineries and distribution systems. Changes in refinery practice worldwide, as well as the increased use of additives, have led to variations in the composition of fuel, which have been quoted to be reflected in increased microbial growth in the systems (8, 14). Many microorganisms, filamentous fungi, yeasts, aerobic bacteria and sulfate-reducing bacteria, are able to use either the hydrocarbon molecules themselves or the additives as nutrient sources in the presence of minute quantities of water. Apart from oxidizing the hydrocarbons, with the concomitant production of corrosive metabolites such as organic and inorganic acids, microbial metabolism produces more water, aiding further growth. As a consequence of this microbial activity, a large biomass can develop at the oil-water interface, resulting in operational problems due to blockage of filters and pipelines, the production of emulsions by biosurfactant activity, alteration in the quality of the fuel and corrosion of metal tanks (7, 13).

The present research aimed to determine the principal microorganisms present in the sludge formed at the bottom of diesel storage tanks in Rio Grande do Sul, Brazil, and the biocides active against these microorganisms.

MATERIALS AND METHODS

Isolation of contaminants. Microorganisms were isolated from contaminated diesel fuel from refinery storage tanks by filtration of 500ml through 0.45um pore-size filters followed by culture of the filters on nutrient and malt agar. The interfacial biomass, where present, was plated directly on to these two media for isolation of aerobic bacteria and fungi and was

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inoculated into Postgate's Medium B (11) for detection of sulfate-reducing bacteria (SRB). Three types of diesel fuel: common, urban and marine, were sampled.

After purification of the major contaminants by repeated subculture on appropriate solid media, they were identified by standard morphological methods for fungi (10) and microscopic, culture and biochemical methods for aerobic bacteria. SRB were not identified to genus level.

Biocide tests. Four antimicrobial agents, an isothiazolone mixture, glutaraldehyde, a quaternary ammonium salt (quat) and a formaldehyde-releasing agent, were tested for their activity against the principal microbial contaminants isolated. The minimum inhibitory concentrations (MICs) for aerobic bacteria, SRB and fungi were determined by dilution of the biocides in nutrient both, pH 7, for bacteria, malt extract broth, pH 5.4 for fungi and Postgate B broth, pH 7.4 for SRB, using three replicates for each organism. 2ml of medium containing biocide were inoculated with 100ul of a cell suspension containing 10^5-10^7 bacteria or 10^8 fungal propagules per ml. Growth was assessed visually (by turbidity) after incubation at 28°C for 24h or 72h for bacteria and fungi, respectively. Time-kill curves were plotted for aerobic bacteria (10^6-10^7/ ml) treated at room temperature (approx. 25°C) with the biocides in Bushnell-Haas(4) medium plus sterile diesel oil (20:1, v/v). Samples were taken at 3, 6, 9, 12 and 24h and viable cells determined using the Spiral Plater (Spiral Systems Model D) and four plates of nutrient agar for each biocide concentration.

RESULTS AND DISCUSSION

Isolation of contaminants. *Aspergillus fumigatus* and *Aspergillus flavus* were found to be major contaminants of all three types of diesel tested. In addition, *Aspergillus niger* was frequently isolated from urban diesel and *Hormoconis resinae* from marine diesel. All sediment samples from all fuel types contained viable SRB, confirming the presence of anaerobic niches within the system (5, 17). Samples of common diesel showed two different colony types of *Bacillus* as the most frequent bacterial contaminants, while urban and marine diesel showed a greater preponderance of *Pseudomonas*, a different colony type being selected from each type of fuel. The literature reports the presence of a wide variety of microorganisms in middle distillate fuels (5, 6, 14, 15). These organisms are not necessarily capable of using the hydrocarbons as carbon sources and may be growing at the expense of contaminants or of additives such as surfactants or corrosion inhibitors (7, 8). Of the microorganisms isolated in the current work, those which have previously been shown to utilize hydrocarbons as sole carbon source are the bacterial genus *Pseudomonas* (13, 16) and the fungus *Hormoconis resinae* (9). One type of SRB has also been found to metabolize hydrocarbons (1). Further work is currently under way in our laboratory to determine the hydrocarbon-utilizing capacity of the other isolates.

Biocide tests. The minimum inhibitory concentrations of the four biocides for the isolates are shown in Table 1. Biocides A (the quaternary ammonium salt) and C (the isothiazolone mixture) were the most effective against all the isolated organisms. Of the bacteria tested, the *Pseudomonas* sp. isolated from marine diesel showed the greatest overall resistance to the biocides under these growth conditions. However, this test cannot be used to predict the effects of the biocides under in-use conditions, where optimal conditions of growth in terms of nutrient, temperature, pH, etc. are not present and where the initial cell concentration is almost certainly higher at the time of biocide addition. For this reason, time-kill curves were constructed to show the efficacy of the biocides on bacterial suspensions in mineral medium with diesel as sole carbon source.

Fig. 1-4 show the time kill curves for those biocides which had a noticeable effect on the isolates and Table 2 presents the concentration of each biocide which was effective in reducing the bacterial numbers below detection level (10^3/ml) within 3h. *Bacillus* 2 was not affected to this level by any of the biocides used. The most active compounds against this isolate were the quat (Fig. 4) and the isothiazolone mixture, which reduced the numbers by 27% after 24h at a concentration of 50ppm. Isolates of the genus *Bacillus* were considerably more resistant than the pseudomonads. This could be due, in part, to the differences in the cell envelope. Brozel and Cloete (3) suggest that isothiazolones interact specifically with cysteine molecules in the outer membrane protein T of *Pseudomonas aeruginosa.* Such specific interactions, along with the presence of pores in the outer membrane, could allow increased uptake of some biocides by Gram negative cells. Both the Gram

positive isolates tested in this study belong to the genus *Bacillus*, spore-forming organisms. Although various chemicals can act as sporocides, it is generally necessary that high concentrations and/or long contact times be used, probably because of the impermeable barrier presented by the spore coat and cortex. In some cases (e.g., glutaraldehyde), the biocide may exert its lethal action directly on the spore cortex (2). Nevertheless, this biocide is considered to have low sporocidal activity (12) and this is confirmed by our results (Table 2).

Of the two aldehyde-based biocides in this study (B and D), only D (glutaraldehyde) was able to kill some Gram negative cells (*Pseudomonas* sp.2) within 3 hours at the lower concentrations used. Neither was effective against the *Bacillus* isolates. On the other hand, both A and C were bactericidal for Gram negative organisms at low concentrations and A (the quat) showed reasonable activity against the *Bacillus* spp.(Fig. 4).

Although Bloomfield and Arthur (2) consider that quaternary ammonium salts are sporostatic rather than sporocidal, our results indicate that cidal activity can be demonstrated under certain circumstances and that the quat used in these tests is the most appropriate biocide for use against these isolates. Its surfactant activity also recommends it as a useful cleansing agent for the empty storage tanks. However, it should be realized that this would not be a suitable biocide for addition to the fuel itself and, if this is the aim, then

Table 1. Minimum inhibitory concentration (MIC) of four biocides against fungi, bacteria and SRB isolated from stored diesel fuels.

Microorganisms	Biocide MIC (ppm)			
Fungus	A	B	C	D
Aspergillus fumigatus	< 3.9	250-125	< 3.9	1000-500
Aspergillus flavus	15.1-7.8	500-250	< 3.9	500-250
Aspergillus niger	15.1-7.8	1000-500	< 3.9	1000-500
Hormoconis resinae	7.8-3.9	> 1000	< 3.9	1000-500
Bacteria				
Bacillus 1	7,8-3,9	250-125	< 3.9	250-125
Pseudomonas 1	125-62.5	1000-500	< 3.9	500-250
Pseudomonas 2	< 3.9	31.5-15.1	< 3.9	31.5-15.1
Bacillus 2	< 3.9	62.5-31.5	< 3.9	500-250
SRB	7,8-3.9	500-250	< 3.9	31.5-15.1

SRB: Anaerobic sulfate-reducing bacteria.
Bacillus isolates from common diesel.
Pseudomonas 1 and 2 isolates from marine and urban diesel, respectively.

Biocide A: Didecyl,dimethyl-ammonium chloride
Biocide B: Di(2-hydroxyethoxy)methane
Biocide C: 5-chloro-2-methyl-4 isothiazo-3-one + 2-methyl-4-isothiazo-3-one
Biocide D: Glutaraldehyde

Table 2. Concentrations of biocides which reduced bacterial numbers to below detection level in 3 h.

Bacterial isolate	Biocide concentration (ppm)			
	A	B	C	D
Pseudomonas 1	10	*	1	100
Pseudomonas 2	10	50	1	10
Bacillus 1	50	*	500	*
Bacillus 2	*	*	*	*

* viable cells still detectable after 3h treatment

Biocide A: Didecyl,dimethyl-ammonium chloride
Biocide B: Di(2-hydroxyethoxy)methane
Biocide C: 5-chloro-2-methyl-4 isothiazo-3-one + 2-methyl-4-isothiazo-3-one
Biocide D: Glutaraldehyde

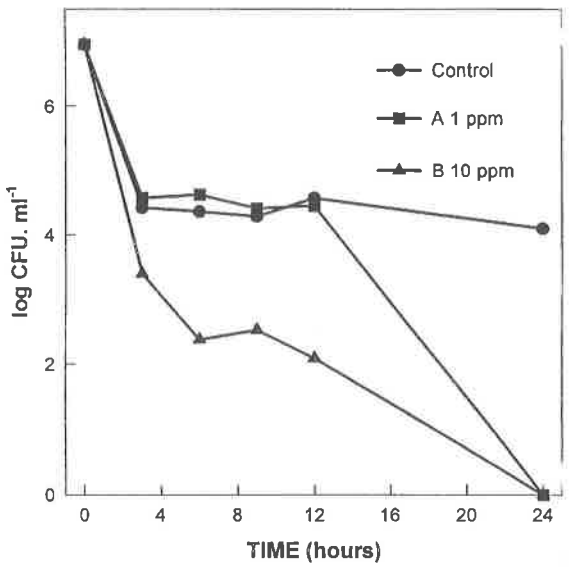

Figure 1. Time kill curve for *Pseudomonas* 2 and biocide A and B

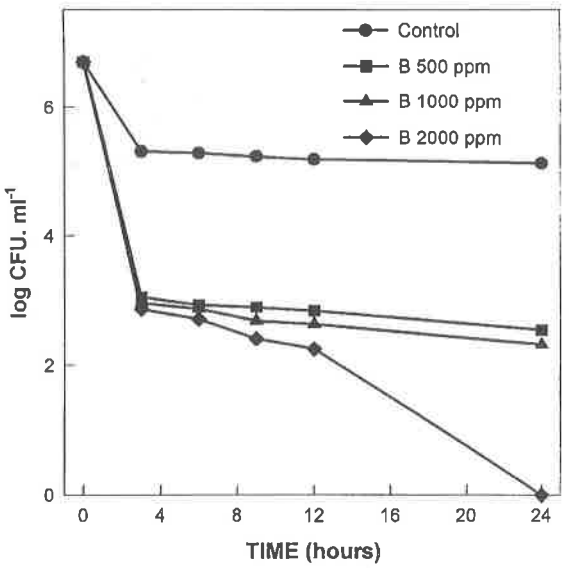

Figure 3. Time kill curve for *Bacillus* 2 and biocide B

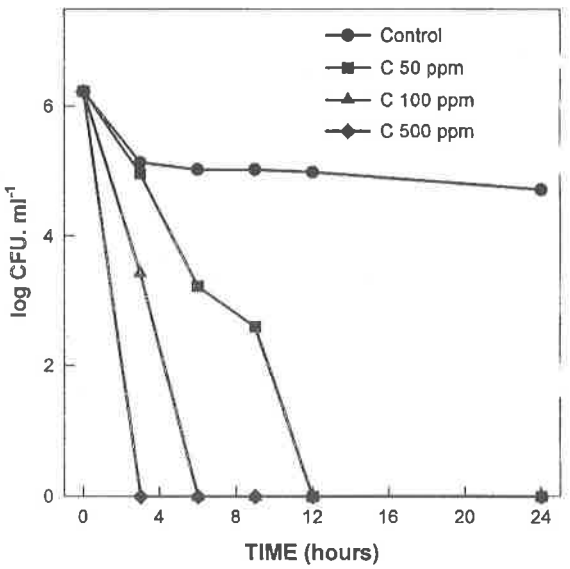

Figure 2. Time kill curve for *Bacillus* 1 and biocide C

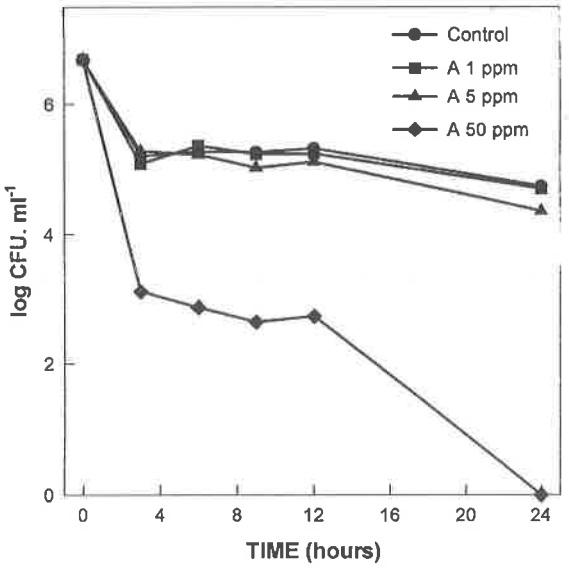

Figure 4. Time kill curve for *Bacillus* 2 and biocide A

the isothiazolone mixture, which has no adverse effects on the quality of the fuel, would be the biocide of choice.

RESUMO

Contaminação microbiana em óleo diesel armazenado no Brasil

As refinarias e sistemas de distribuição tem enfrentado problemas com a contaminação microbiana em óleo diesel armazenado. A atividade microbiana nesses sistemas leva à produção de uma biomassa na interface óleo/água, provocando problemas operacionais, como entupimento de filtros, tubulações, alteração na qualidade do combustível. O controle dessa contaminação pode envolver o uso de agentes químicos, como os biocidas. O objetivo desse trabalho foi selecionar biocidas eficientes para o controle de fungos, bactérias aeróbias e bactérias redutoras de sulfato (BSR), que foram isoladas de óleo diesel comum, urbano e naval, armazenados em

tanques de refinaria no Rio Grande do Sul. Dois biocidas (um composto quaternário de amônio e uma mistura de isothiazolona) foram selecionados por apresentarem alta eficácia, em baixas concentrações para os principais contaminantes, como fungos do gênero *Aspergillus* e *Hormoconis*, BSR e bactérias aeróbias do gênero P *seudomonas e Bacillus.*

Palavras-chave: contaminação microbiana, óleo diesel, biocidas.

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ADJUVANT EFFECT OF *STAPHYLOCOCCUS AUREUS* IN RATS INFECTED WITH *SALMONELLA TYPHIMURIUM*

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ABSTRACT

The immunomodulating action of *Staphylococcus aureus* on *Salmonella typhimurium* infected rats was studied. The experiment was carried out in two stages: Stage 1) The proliferation of *S. typhimurium* was estimated in several organs from animals pre-treated with *S. aureus*. Adult inbred rats were subcutaneously inoculated with 8×10^6 *S. aureus* and seven days later were infected i.p. with a sublethal dose (1.5×10^7) of *S. typhimurium* (Group I). Animals of a second group were exclusively challenged with the same dose of *S. typhimurium* and used as infected controls (Group II).The rats were sacrificed on days 1, 3, 6 and 9 post-infection. Peripheral blood (PB) samples were obtained for hemoculture; in addition, samples of liver (L) and spleen (S) were weighted, homogenized and cultured in MacConkey medium after appropriate dilutions. CFUs were then counted in 24 hours cultures of PB, L and S. Group I yielded values significantly lower than Group II throughout the experimental period. Stage 2) The absolute numbers and proportions of both macrophages (MØ) and lymphocytes (L) were quantified in several organs of pre-treated (Group I) and infected controls (Group II). After infection the rats were injected intravenously with 0.1 ml Iron Dextran (Fe 100 mg/ml) for selective staining of macrophages. Twenty four hours later the animals were sacrificed and the liver, spleen, intestine and mesenteric lymph nodes were removed for histological analysis; non infected controls were also included for comparison (Group III). Tissue sections stained with H.E. and by the histochemical technique of Bugelsky were used for evaluation. The numbers of Ms and Ls were higher in Group I compared with the corresponding values recorded for Groups II and III. The ranking of organs by decreasing cell numbers was as follows: mesenteric lymph nodes (follicular zone> interfollicular zone), spleen (white pulp> red pulp), intestine and liver, with a net predominance of MØs over Ls in the intestine (Peyer's patches). Our results clearly confirm the adjuvant effect of *S. aureus*.

Key words: adjuvant; *Staphylococcus aureus; Salmonella typhimurium* infection

INTRODUCTION

The modulating influence of bacteria on the immune system has been reported by several authors. Hence, bacterial extracts or substances produced by them may stimulate cellular and humoral immune responses.

It has been satisfactorily demonstrated in animal models that several infectious agents such as BCG, *Corynebacterium parvum* or *Staphylococcus aureus* activate macrophages (M s) enhancing their phagocytic activity (2, 13) and stimulate NK cells (15), T and B lymphocytes as well as the synthesis of immunoglobulins (17, 20, 21). The adjuvant effects of

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S. aureus have been expressed as tumor growth inhibition and necrosis of several animal and human tumors (4, 5, 7, 11, 18, 19), stimulation of the Mononuclear Phagocytic System (MPS) (6) and potentiation of humoral response against sheep red blood cells (SRBCs) (6). These findings thus suggest that *Staphylococcus aureus* might be able to modulate the host's immune response against microbial infections.

The present work attempts to analyze the protective effect of *Staphylococcus aureus* in rats inoculated with a virulent microorganism.

In the first stage of this study the hypothesized protective effect was investigated in rats pre-treated with *Staphylococcus aureus* and subsequently challenged with *Salmonella typhimurium*. The infection was confirmed by cultures of peripheral blood, liver and spleen. In the second stage of this study the absolute numbers and percentages of immunocompetent cells were evaluated in histological sections of several organs of these animals.

MATERIALS AND METHODS

Animals. Nine to twelve weeks old male rats with a high coefficient of inbreeding (line "m") were used throughout the experiments. This is a local line obtained from outbred rats submitted to inbreeding and maintained by Houssay and co-workers. They are registered as IIM in the VI Supplement of the International Survey in the Supply Quality and Use of Laboratory Animals, November 1964.

Staphylococcus aureus: S. aureus Cowan I strain grown in nutrient agar medium and submitted to tyndallization (70°C during 1 h for 3 days) was used. The absence of viability was demonstrated in samples cultured after tyndallization. Suspensions of the dead microorganisms used for inoculation were prepared in phosphate buffered saline (PBS); counting was performed in a hemocytometer.

Salmonella typhimurium: Salmonella typhimurium strain was maintained in nutrient agar medium, cultured in blood agar base for 24 hours at 37°C and resuspended in saline.

Rats were inoculated intraperitoneally (i.p.) with a sublethal dose of a bacterial suspension which was adjusted to a turbidity according to the Mc Farland standard N 10.

Experimental groups. Group I : This set of rats was injected s.c. with 0.2 ml of a saline suspension of *S.aureus* containing 8 x 10^6 killed microorganisms. Seven days later the rats were infected with a sub-lethal inoculum of *Salmonella typhimurium* (1.5 x 10^7 microorganisms) by the intraperitoneal route.

Group II: This set of rats was infected with the same inoculum of *Salmonella typhimurium* described above.

Group III: These rats did not receive any treatment and were used as uninfected (normal) controls during the second stage of the study.

Evaluation of *S. typhimurium* infection. Blood samples were obtained by cardiac puncture on days 1, 3, 6 and 9 post-inoculation. A suspension in PBS to a 1:100 concentration was prepared for culture in Mc Conkey medium at 37°C during 24 hours, and diluted with 10 ml of McConkey medium. All these procedures were accomplished under rigorous sterile conditions. Hemocultures in the same culture medium were performed with peripheral blood withdrawn at the beginning of the experiment and properly diluted. Tissue samples of liver and spleen removed from the animals were weighted and grounded in a mortar prior to culturing in Mc Conkey medium. Tissue weights varied between 0.10g and 0.15g.

CFUs of 24 hours cultures of peripheral blood, liver and spleen were counted and expressed as the ratio: number of *S. typhimurium* CFU/ weight or volume of the specimen under examination.

Histological examination. Group I and Group II replicate sets of animals were injected with *S. aureus* and/or *S. typhimurium* as previously described. Immediately after infection with *S. typhimurium* the animals were injected i.v. (dorsal tail vein) with 0.1 ml of Iron Dextran at a concentration of 100 mg/Fe/ ml (this substance is distributed via the blood stream to different organs and allows the selective staining of macrophages). Twenty four hours later Groups I and II rats as well as uninfected controls that received Iron Dextran (Group III) were sacrificed and the mesenteric LNs, liver, spleen and intestine were removed for histological examination.

Tissue samples were fixed in 10 % buffered formaldehyde, processed in an autotechnicom and routinely stained with H.E. to estimate the number of macrophages and lymphocytes. The Bugelsky histochemical technique used consists of Pearl's and the modified tetramethylbenzidine reaction, counterstained with 0.10 % neutral red. With this method the percentage of macrophages and lymphocytes was assessed.

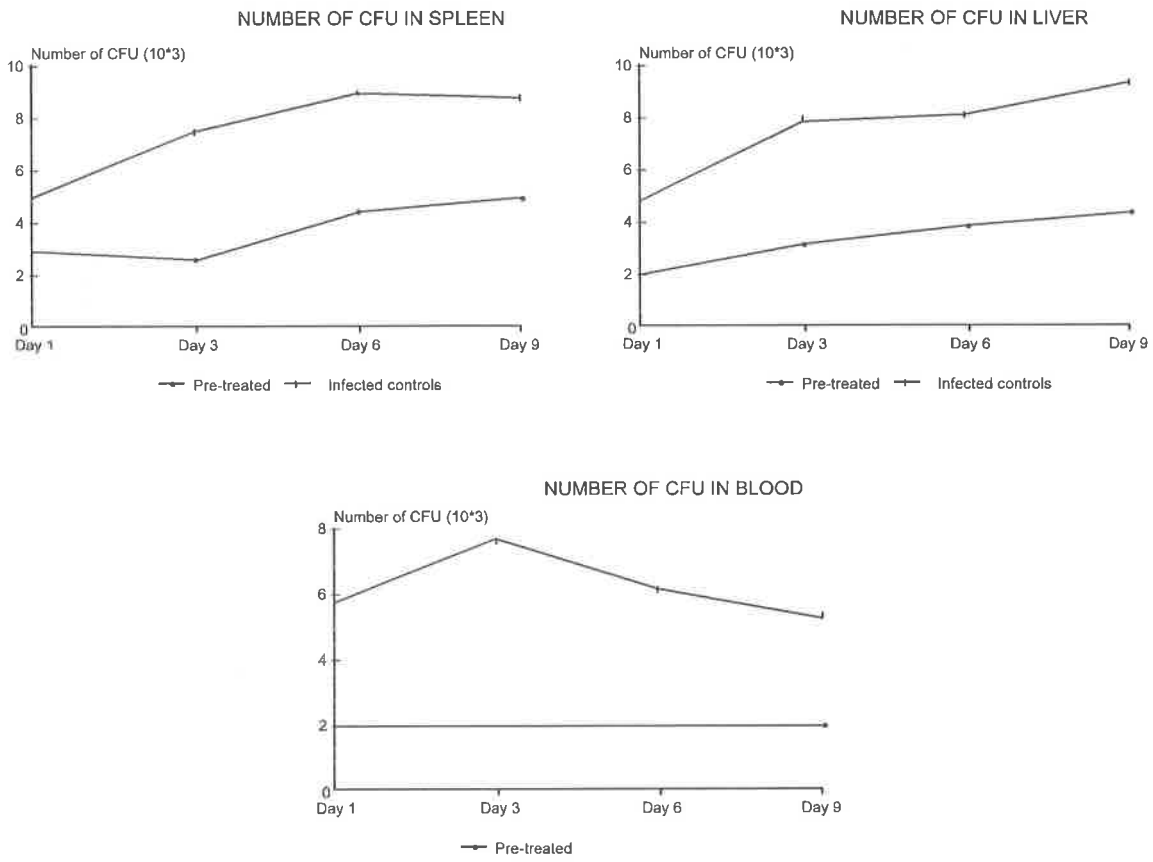


Figure 1 - Number of CFUs in cultures of liver, spleen and blood (counts of 100 fields per culture). Pre-treated with *S. aureus*, n= 19; infected controls= 18

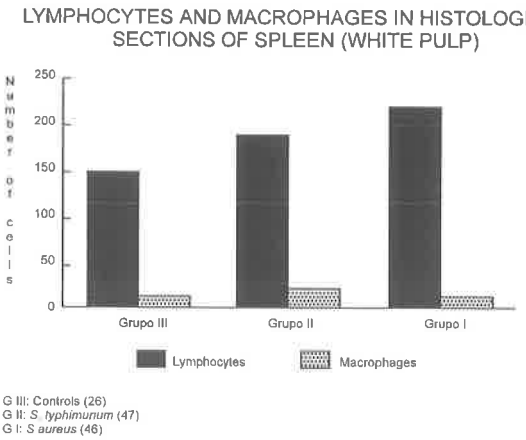
The evaluations were performed double-blind by one of the authors using an eyepiece(10 x) supplied with a grid and objective (10 x). One hundred fields were examined in serial 6m thick sections of 103 animals (50 treated and 53 controls).

Statistical analysis. Differences in the numbers of CFUs between Groups I and II on days 1, 3, 6, and 9 post-infection were submitted to statistical analysis using the unpaired Students' *t* Test. Data are presented as the arithmetic mean ± SEM. Results obtained by the Bugelsky technique were analyzed by the two-way ANOVA test.

RESULTS

A reduced number of *S. typhimurium* CFUs in peripheral blood and organ cultures of rats pre-treated with killed *S. aureus* prior to challenge with a sub-lethal dose of *S. typhimurium* was observed compared with values from cultures of non-treated infected controls (Fig. 1). The differences were recorded on days 1, 3, 6, and 9 post-infection and were statistically significant ($p < 0.01$). The resistance to *S. typhimurium* observed in pre-treated animals provided the first evidence of a protective effect of *S. aureus* in this experimental model.

The histological analysis allowed us to quantify and estimate the proportions of immunocompetent cells in *S. typhimurium* infected rats under the *S. aureus* adjuvant effect. Stained preparations of spleen, liver, mesenteric LNs and Peyer's patches revealed increased numbers of lymphocytes and macrophages in both immune and non-immune compartments of pre-treated rats as compared with those of untreated infected and non-infected controls, with a clear predominance of macrophages in Peyer's patches (Figs. 2 to 5). These differences also were statistically significant ($p < 0.001$ for lymphocytes and $p<0.01$ for macrophages) and indicate that the protective effect of



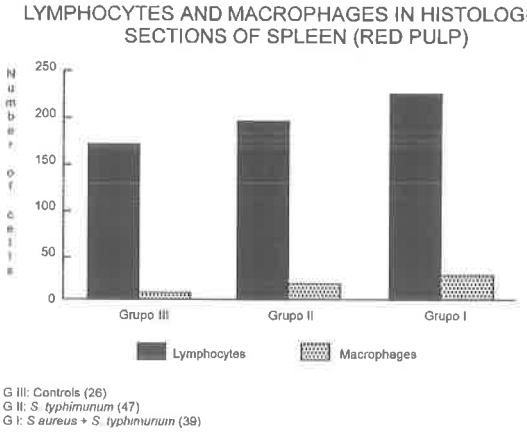


Figure 2 - Lymphocytes and macrophages in histological sections of spleen (white pulp). Uninfected controls: n=26; *S. typhimurium* infection: n=47; *S aureus* pre-treatment and *S. typhimurium* infection: n=48. Lymphocytes and macrophages in histological sections of spleen (red pulp). Uninfected controls: n=26; *S. typhimurium* infection: n=39; *S aureus* pre-treatment and *S. typhimurium* infection: n=39.

S. aureus may be linked to its stimulatory action on the host's immune system.

DISCUSSION

Our findings suggest that the protective effect of *S. aureus* may induce an increase in the number of immunocompetent cells as well as their enhanced function in pre-treated animals.

Several authors have studied the modulation of the immune response by bacteria or their released products (2, 4, 7, 8, 11, 13, 16, 18, 20, 21). It has been reported that some bacteria are able to stimulate cellular immune responses and the MPS or induce the synthesis of antibodies, cytokines and Interferon. In

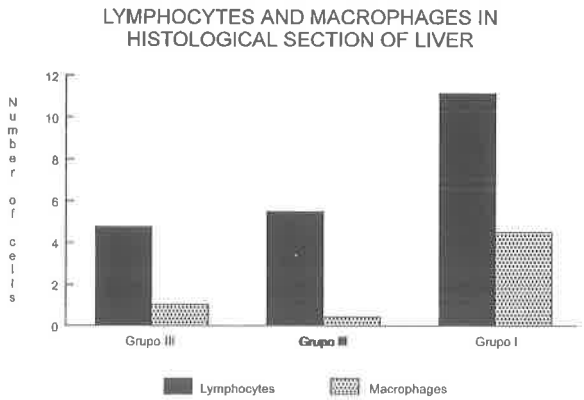


Figure 3 - Lymphocytes and macrophages in histological sections of liver. Control: n=26; *S. typhimurium* infection: n=47; *S aureus* pre-treatment and *S. typhimurium* infection: n=46.

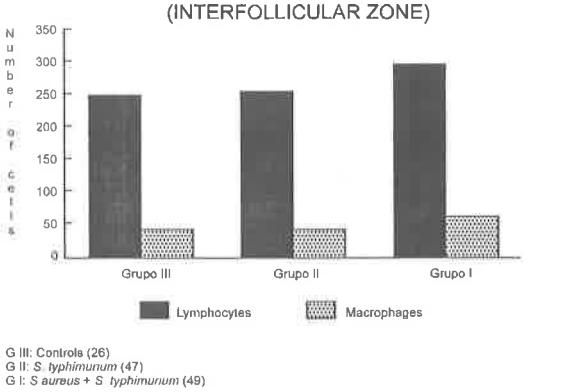
this group are included *S. aureus* Cowan I, *S. aureus* Wood 46, *Haemophilus influenza, Corynebacterium parvum, Escherichia coli, N. pharyngis, Bacillus* sp, *Streptococcus pneumoniae and Salmonella paratyphi* (8, 9, 20).

Moreover, it has been established that the extracorporeal perfusion over *S. aureus* by means of heat and formaldehyde induces the inhibition of tumor growth and necrosis in a variety of human and animal tumors (4, 5, 6, 11, 20, 23, 24).

In our study the *S. aureus* immunostimulating effect was evidenced by the inhibition of replication of the invading bacteria. It is generally accepted that facultative intracellular bacteria such as *Salmonella* are effectively killed by mechanisms of Cell Mediated Immunity (CMI) (16-24). The two important cellular effector functions that control such infections are delayed type hypersensitivity (DTH) and the ability to induce activated MØs.

The capacity of the bacteria to activate lymphocytes and MØs is primarily due to surface components, namely lipopolysacharides, peptidoglycan and protein A (10, 26).

The parameters analyzed in this paper have demonstrated that the activation of effector mechanisms of the host's immune response against an infective agent involve several phenomena and factors that determine which immunological pathway will be stimulated against the presence of a sensitizing bacterial agent. A deeper knowledge of such pathways may lead us to a better understanding of the events responsible for the immunological stimulation.



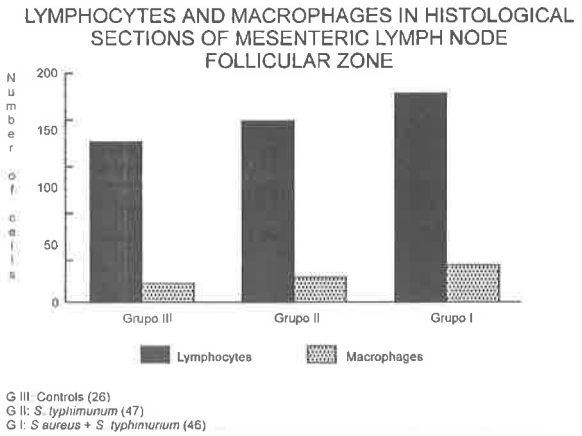
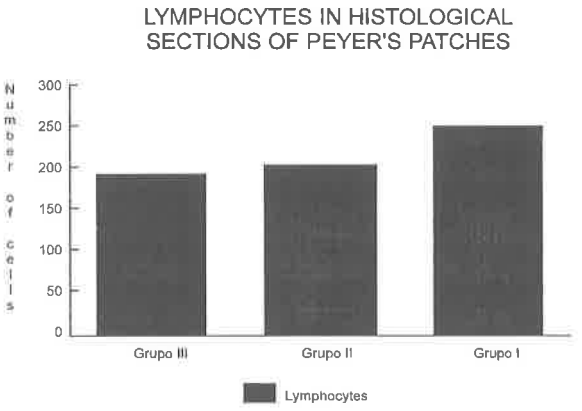


Figure 4 - Lymphocytes and macrophages in histological sections of mesenteric lymph nodes (interfollicular zone). Uninfected controls: n=26; *S. typhimurium* infection: n=47; *S aureus* pre-treatment and *S. typhimurium* infection n=49. Lymphocytes and macrophages in histological sections of mesenteric lymph nodes (follicular zone). Uninfected controls: n=26; *S. typhimurium* infection: n=47; *S aureus* pre-treatment and *S. typhimurium* infection : n=46.



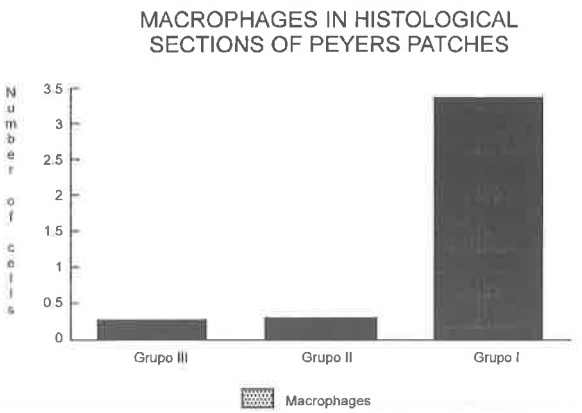


Figure 5 - Lymphocytes and macrophages in histological sections of Peyer's patches. Uninfected controls: n=26; *S. typhimurium* infection: n=54; *S aureus* pre-treatment and *S. typhimurium* infection: n=49.

RESUMO

Efeito adjuvante do *Staphylococcus aureus* em ratos infectados com *Salmonella typhimurium*

Estudou-se a ação imunomoduladora do *Staphylococcus aureus* em ratos infectados com *Salmonella typhimurium*. O trabalho foi desenvolvido em duas etapas. 1 - a multiplicação de *S. typhimurium* foi observada em vários órgãos de animais pré-tratados com *S.aureus*. Ratos adultos endocriados receberam inoculação subcutânea de 8 x10^6 *S.aureus* e, sete dias após, receberam uma dose i.p. subletal (1,5 x 10^7) de *Salmonella typhimurium* (Grupo I). Animais de um segundo grupo foram infectados somente com *Salmonella typhimurium* (mesma dose) e usados como controles infectados (Grupo II). Os ratos foram sacrificados nos dias 1, 3, 6 e 9 após a infecção. Amostras de sangue foram retiradas para hemocultura. Amostras de fígado e baço foram pesadas, homogeneizadas e, após diluições adequadas, cultivadas em agar MacConkey. Após 24h, contou-se o número de colônias no sangue, baço e fígado. O Grupo I apresentou contagens significantemente mais baixas que o Grupo II durante o período de experimentação. 2 - Os números absolutos e as proporções de macrófagos (M) e linfócitos (L) foram determinados em vários órgãos dos animais pre-tratados (Grupo I) e nos controles infectados (Grupo II). Após a infecção, os ratos receberam uma

injeção intravenosa de 0,1 ml de Ferro Dextran (Fe 100 mg/ml) para coloração diferencial dos macrófagos. 24h após os animais foram sacrificados removendo-se o fígado, baço, intestino e gânglios linfáticos mesentéricos para análise histológica. Controles não infectados foram também incluidos para comparação (Grupo III). Para avaliação, os cortes histológicos foram corados com H-E e tratados segundo a técnica histoquímica de Bugelsky. Os números de Ms e de Ls foram mais altos no Grupo I quando comparados com os valores obtidos nos Grupos II e III. Os reultados das contagens, em ordem decrescente, foram: gânglios linfáticos mesentéricos (zona folicular > zona interfolicular), baço (polpa branca > polpa vermelha), intestino e fígado, sendo que no intestino (Placas de Peyer) houve predominância de Ms e relação a Ls. Estes resultados confirmam o efeito adjuvante de *S.aureus*.

Palavras-chave: efeito adjuvante; *Staphylococcus aureus*; infecção com *Salmonella typhimurium*

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STANDARDIZATION OF A METHOD FOR THE INOCULATION OF PEANUTS (*ARACHIS HYPOGAEA L*) - TATU VERMELHO VARIETY WITH *ASPERGILLUS FLAVUS* NRRL 6513, AN AFLATOXIN B_1 PRODUCER

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ABSTRACT

This work describes the optimal analytical conditions established for inoculation of peanuts (Tatu Vermelho variety) with *Aspergillus flavus* NRRL 6513, a strong aflatoxin B_1 producer. The highest production of aflatoxin was detected after addition of 0.5 ml of a saline spore suspension (4×10^6 spores/ml) of 10-days old *Aspergillus flavus* NRRL 6513 to 1.0 g of ground and autoclaved peanuts (121°C /20 min) followed by incubation for 7 days at 26 ± 1°C.

Key words: *Aspergillus flavus*, aflatoxin B_1, peanuts, inoculation, standardization.

INTRODUCTION

Aflatoxins are metabolites of the bisfuran-isocoumarin group mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* (20). Aflatoxin B_1 poses the greatest toxicological risk because of its carcinogenic, teratogenic and mutagenic activities (46).

Consumption of food contaminated with aflatoxin B_1 is positively correlated with the incidence of carcinoma of the liver and, to a lesser extent, with carcinoma of the cervix and the gastrointestinal tract (43). Food contamination with aflatoxins is more frequent in tropical and semitropical regions such as Brazil, where the climate favors the development of toxigenic fungi, especially in peanuts, corn and beans (24, 25, 26, 32, 33, 34, 35). The high incidence of human hepatomas in the tropical regions of Kenya, Mozambique, Swaziland and Thailand (14 cases/100.000 inhabitants/year), where the ingestion of contaminated foodstuffs is a reality (13), emphasizes the need for measures and research that might contribute to the prevention and control of aflatoxin production.

The methods used for destruction of already produced aflatoxin are not fully effective (22) and also cause undesirable changes in foodstuffs, such as loss of nutrient substances and changes in aroma and flavor (36). Thus, other alternatives are being sought to prevent the production of aflatoxin in foodstuffs. One of them is the development of new types of peanuts resistant to fungal contamination and to aflatoxin production (18, 21, 44).

Another possible alternative is the use of recommended agricultural practices (18). It is known, for example, that both infestation with *Aspergillus flavus* and production of aflatoxin in peanut seeds may be affected by soil type. Mehan *et al.* (19) observed

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lower levels of contamination in soil with greater water retention and lower aeration, suggesting that the risk of contamination can be reduced depending on the plantation area.

The use of chemical substances to avoid fungal contamination and/or aflatoxin production is an intensely explored line of research. Within this context, the production of aflatoxins has been correlated in some studies with the levels of metals such as zinc, iron, aluminum, nickel, copper, manganese, among others, since the most important factor regulating aflatoxin biosynthesis is linked to trace elements (2, 10). The effect of metals on the production of aflatoxins in culture media is well described in the literature (1, 4, 5, 6, 12, 14, 15, 16, 23, 29, 30, 45).

The aim of the present study was to standardize a methodology for the inoculation of peanuts with the aflatoxin-producing strain of *Aspergillus flavus* NRRL 6513, in order to determine, in a later study, the effect of different metal concentrations on aflatoxin B_1 biosynthesis by this same strain.

MATERIALS AND METHODS

Sampling and sample preparation. An aflatoxin-free sample of peanuts kernels (*Arachis hypogaea L.* Tatu Vermelho variety), grown in "latossol roxo" soil in the Ribeirão Preto region (1991 harvest) and provided by the Agronomy Institute of Campinas was used. The sample was ground and homogenized and then sifted through a 20-mesh plastic sieve and the material was stored under refrigeration at 1°C.

Microorganism. *Aspergillus flavus* NRRL 6513 from the Northern Regional Research Laboratory, a strong producer of aflatoxin B_1, was used. To ensure fungal toxigenicity the strain was maintained on potato dextrose agar (PDA, Difco) covered with a layer of sterile vaseline and on rice medium at 26 ± 1°C. Fungal inocula were obtained by plating aseptically into a slanted PDA tube kept for about ten days at 26 ± 1°C. After incubation the spores were suspended in 5 ml 0.9% saline; spore concentration/ml was determined by counting in a Neubauer chamber. The spore suspension obtained was used for sample inoculation.

Methodology. Aflatoxin B_1 was quantified by the technique of Valente Soares (42) and confirmation was performed by the method of Przybylsky (28).

RESULTS AND DISCUSSION

Several tests were carried out until the ideal conditions for inoculation were obtained.

Quantity of sample to be used. On the basis of the work by Lillehoj *et al.* (12), who used 1.0 g ground corn, 1.0, 3.0 and 5.0 g ground peanuts were inoculated with 0.5 ml of a freshly prepared saline spore suspension after addition of two inocula containing *Aspergillus flavus* NRRL 6513 to 9 ml of a sterile 0.9% sodium chloride solution (41). After 5 days of incubation at 26 ± 1°C there was no formation of aflatoxin B_1 in the 5.0 g peanut sample, and aflatoxin formation was lower in the 3.0 g sample than in the 1.0 g sample.

Volume and type of spore suspension to be used. This is a particularly important point since the volume of spore suspension added might cause changes in the growth of *Aspergillus flavus* NRRL 6513 and in the production of aflatoxin B_1 . Several tests using 1.0 g ground peanuts as substrate and different volumes of spore suspension were performed (Table 1). The 10 days incubation time is recommended by several investigators (2, 7, 8, 37, 39, 45). The concentration of $10^5/10^6$ spores/ml was based on the data reported by Farag *et al.* (7) and Chulze *et al.* (2). It can be seen that no secretion of aflatoxin B_1 was detected in any experiment. This lack of production of aflatoxin B_1 could be possibly related to the incubation period and/or to the presence of a natural microbiota competing with *Aspergillus flavus* NRRL 6513.

When further tests were carried out using different times of incubation (3 to 14 days), with autoclaving of the samples at 121C for 20 minutes and an incubation temperature of 26 ± 1°C in all cases, again there was no formation of aflatoxin B_1.

Considering the possibility that the aqueous spore solution was not adequate, the spores were extracted with saline as described by Ujikawa and Purchio (41) and as recommended by Corrêa (personal communication, 1992). It should be pointed out that sodium chloride, the growth factor of microorganisms, at the concentration used in the present study has no effect on the production of aflatoxin B_1 (27) or on vegetative mycelial growth (40).

Further tests were performed using two types of saline suspension of *Aspergillus flavus* NRRL 6513 spores. One was obtained by the procedure described in Material and Methods and the other from two loops

Table 1. Production of aflatoxin B1 in ground peanut samples inoculated with *Aspergillus flavus* NRRL 6513.

Sample	Sterile water (ml)	Volume of aqueous spore suspension[a] (ml)	Incubation time[b] (days)	Aflatoxin B_1 (µg/ml)
No autoclaving[c]	1,5	-	5	ND
No autoclaving[d]	1,0	0,5	10	ND
	0,5	1,0	10	ND
	0,5	0,5	10	ND
	1,0	1,0	10	ND

[a] *Aspergillus flavus* NRRL 6513 at the concentration of $1x10^6$ spores/ml
[b] Incubation at 26 ± 1°C
[c] Twenty replications
[d] Duplicate
ND = not detected

Table 2. Influence of saline spore suspension on the production of aflatoxin B1 in inoculated ground and autoclaved peanut

Saline spore suspension (*Aspergillus flavus* NRRL 6513)		Incubation time (days)	Aflatoxin B_1 (µg/kg)
Obtained by	Volume (ml)		
[1]Culture on rice medium and PDA medium	0.5	3	267
		5	22240
		7	88960
		9	53400
		11	55600
	1.0	3	ND
		5	ND
		7	23722
		9	12355
		11	7413
Culture on PDA medium	0.5	3	3060
		5	5100
		7	53376
		9	62272
		11	37066

[1] Concentration: 4×10^6 spores/ml
ND = not detected
PDA = Potato dextrose agar

loaded with fungal culture in PDA medium (41). One gram of autoclaved sample was used in all assays.

The data presented in Table 2 shows that aflatoxin B_1 levels reached a maximum when 0.5mL of a 10-day old saline suspension of *Aspergillus flavus* NRRL 6513 spores was used after sample incubation for 7 days at 26 ± 1°C. It was also observed that a suspension volume of more than 0.5 ml reduced the production of aflatoxin B_1.

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RESUMO

Padronização de metodologia de inoculação de *Aspergillus flavus* NRRL 6513, produtor de aflatoxina B_1, em amendoim (*Arachis hypogaea* L.) - variedade Tatu Vermelho

Foram determinadas as condições analíticas ideais para a inoculação de uma cepa de *Aspergillus flavus* NRRL 6513, fortemente produtora de aflatoxina B_1 em amendoim, variedade Tatu Vermelho. A maior produção de aflatoxina foi verificada após a adição, em 1,0 g de amendoim moído e autoclavado (121°C/20 min.), de 0,5 ml de suspensão salina de esporos (4 x 10^6 esporos/ml) de *Aspergillus flavus* NRRL 6513, de 10 dias de idade, seguido de incubação por 7 dias a 26 ± 1°C.

Palavras-chave: *Aspergillus flavus*, aflatoxina B_1, amendoim, inoculação, padronização.

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BOVINE HERPESVIRUS ISOLATES

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SHORT COMMUNICATION

ABSTRACT

The aim of this short communication was to report Bovine Herpesvirus (BHV) isolations carried out at the Virology Laboratory of the Federal University of Santa Maria, Rio Grande do Sul state (RS), Brazil. The isolations were made from clinical cases. Bovine herpesvirus could be recovered from cases of respiratory disease (2 outbreaks), vulvovaginitis (1 outbreak), balanoposthitis (1 outbreak) and meningoencephalitis (2 outbreaks). A serological survey conducted with dairy cattle showed 18.8% prevalence of BHV infection in RS.

Key words: Bovine herpesvirus outbreaks, isolation.

Bovine herpesvirus (BHV) has been isolated worldwide from a variety of clinical manifestations, such as respiratory disease (commonly denominated infectious bovine rhinotracheitis, IBR), genital disorders (infectious pustular vulvovaginitis, IPV, and balanoposthitis), keratoconjunctivitis, abortion and meningoencephalitis (6).

Infected animals, including those with nonapparent infection, become life-long carriers. The infection may also become latent and the virus may be sporadically reactivated by natural mechanisms or by corticosteroid therapy. Transmission takes place easily because large quantities of virus are shed in respiratory, ocular and reproductive secretions of infected cattle (8). In Brazil, BHV have been isolated several times from different clinical manifestations (8). BHV infections can cause significant losses related to reproduction, milk production and feed conversion in other countries (8), and a similar, if not worse, situation may be found in Brazil. These facts associated with the worldwide distribution of BHV stresses the economical importance of this infection and the need to report outbreaks in order to emphasize the significance of the disease.

The aim of this work was to report BHV isolations from Brazilian clinical cases performed at the Virology Laboratory of the Federal University of Santa Maria, RS, Brazil.

Viral isolation was carried out using the MDBK cell line and Bovine Fetal Lung (BFL) secondary cells. The specimens submitted were generally nasal swabs, preputial and vulvar or vaginal mucosa swabs, and specimens from necropsy, such as aborted fetuses and brain. Inocula of 0.2 mL from each material were adsorbed in BFL and MDBK cells at 37°C. After 1 hour, the inocula were withdrawn and 1.0 mL of maintenance medium was added to the cell culture. The tubes were incubated and observed daily until the

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Table 1. Designation, organ of isolation, clinical signs, place and year of bovine herpesvirus (BHV) isolations at the Virology Laboratory of the Federal University of Santa Maria, Santa Maria, RS, Brazil

Designation	Specimen	Clinical signs	Place	Year
SV136/88	Brain	Meningoencephalitis	Ijuí, RS	1988
SV 35/90	Nasal swab	Respiratory	Paranhos, MS	1990
SV 56/90	Preputial swab	Balanoposthitis	Rosário do Sul, RS	1990
SV 453/93	Vulvar swab	Vulvovaginitis	Pinhal Grande, RS	1993
SV 1613/93	Brain	Meningoencephalitis	S. Martinho da Serra	1993
SV 05/94	Nasal swab	Respiratory	Lages,SC	1994*

* Animals imported from Germany

fifth day, to detect cytopathic effects (CPE). A second passage was always carried out. Samples that showed CPE were examined by the direct fluorescent antibody (FA) technique using polyclonal antibody against the whole virus, Bioveta-Gamakon, Germany(1), and by electron microscopy (EM) after embedding in epon and contrasting with uranyl acetate (2). The method described by Carbrey *et al.* (4) of serum neutralization with fixed viral dilution and variable sera was used to test serum samples.

Bovine herpesvirus could be isolated from cases of respiratory disease, vulvovaginitis, balanoposthitis and meningoencephalitis. In most of the cases, the cytopathic effect was observed in both cell lines 48-72 hours after inoculation. It was characterized by roundness, retraction and grouping of refractile cells as well as rupture and displacement of cells on the tube surface. Bovine herpesvirus was identified by FA technique and EM. BHV isolates obtained at the Virology Laboratory of the Federal University of Santa Maria from the first isolation in 1988 until June 1994 are listed in Table 1.

Isolations were obtained from BHV outbreaks, especially from respiratory and genital manifestations. The clinical sign of the respiratory disease was a profuse nasal discharge that was initially clear and later became mucopurulent. Body temperature reached 41°C. In the outbreak of animals imported from Germany, a respiratory disease could be observed along with the occurrence of abortion, vulvovaginitis, and nervous signs in calves and cows. The outbreak of bovine herpesvirus was due to the import of animals from Germany. Several animals arrived in Brazil with signs of conjunctivitis, which was uni or bilateral. Nasal discharge was clear in the early stages and became mucopurulent in the later stages. There were 35 abortions among cows with more than 6 months of gestation. Vulvovaginitis in 30 cows after parturition was also noticed. Nervous signs were present in one cow and eight calves. Eleven cows and 15 calves died. The major lesions in the cows were due to injuries during transportation from Germany to Brazil. The calves that were aborted or that died had histologic lesions similar to those described for bovine herpesvirus. In this outbreak, virus was isolated in nasal swabs from animals with clinical signs of bovine herpesvirus. The failure of virus identification from other material was probably due to the quality of the material submitted. The outbreak probably started due to reactivation of a latent infection, as described by Kahrs (8). Clinical signs were also noticed in farms that received cows imported from Germany. Before the arrival of those animals, the farmers had never observed the disease.

The isolation from Paranhos, MS, had the following history. BHV was recovered from the lungs of one necropsied animal. Approximately one hundred animals died during this outbreak. Only tissues from one animal were submitted. The animals had a history of ocular edema and diarrhea. The histological lesions observed were not related to infection.

Outbreaks of genital manifestations occurred as vulvovaginitis in Pinhal Grande, RS, 1993, and were characterized by hyperaemia, presence of papules and pustules, with a history of repeat-breeding and infertility. The disease was introduced in the dairy farm by a bull from a neighbor. The farm normally used artificial insemination but because the service was not available they decided to breed cows with a bull. The disease had a very high economic impact in the farm. The isolation of bovine herpesvirus from this outbreak of vulvovaginitis suggests that this virus is more frequently related to temporary infertility than it is generally believed, mainly due to a lack of laboratory diagnosis.

Balanoposthitis was described in Rosário do Sul county, RS, 1990. Here, bulls from an artificial insemination station presented hyperaemia of prepuce and penis mucosae, with small round eruptions covered with a mucopurulent exudate. Frequent micturition was also observed in some of the affected animals. The clinical signs started approximately 30 days after the introduction of new bulls in the station. Bovine herpesvirus was isolated from nine out of eleven preputial swabs collected. The virus could be isolated from the semen of these bulls even 13 days before the onset of clinical signs. There have been several reports of balanoposthitis similar to this case, suggesting a high prevalence of this form of the disease in the region. The epidemiological studies conducted could not explain transmission among the bulls since all were housed in individual pens, and a common factor responsible for the transmission was not identified. One possible explanation could be the respiratory route as described by Smith *et al.* (10). The isolation of bovine herpesvirus from this outbreak of acute balanoposthitis illustrates the importance of the disease in South Brazil.

The isolations of BHV from cases of meningoencephalitis occurred either as a single case (Ijuí, RS, 1988) or as an outbreak involving eight animals of two months of age (São Martinho da Serra, RS, 1993). In the first case, a one-month calf presented blindness, circling and uncoordinated gait. No significant gross lesions were observed upon necropsy. Histopathology of the brain revealed extensive mononuclear infiltration in the leptomeninges and perivascular spaces of the cortex. Vasculitis was evident in association with some of the perivascular cuffings. No inclusion bodies were detected. Other authors (9) have reported the presence of inclusion bodies in astrocytes. Neurologic signs associated with BHV have been described primarily in young animals, usually of 2 to 16 months of age (5). The findings in our region have been restricted to young calves (2 months old). Mortality in the cases described here has been 100%, as also found by others (5, 7). Several outbreaks of this form of the disease have been reported as a clinical syndrome without laboratory confirmation. Meningoencephalitis and abortion have also occurred in association with respiratory manifestations.

A serological survey conducted with 7956 serum samples collected from the dairy herd at our laboratory showed 18.8% prevalence of BHV infection in the Rio Grande do Sul state, which demonstrates its widespread dissemination. Also, abortions and respiratory and genital diseases are known to occur very commonly in the field. The apparent low frequency of BHV isolation can be more adequately interpreted if we consider that the identifications from vulvovaginitis and balanoposthitis herein described were not only the first carried out at our lab but also, to our knowledge, the first in the Rio Grande do Sul state. This probably mirrors the poor knowledge of the disease among local veterinarians and farmers, which in turn causes a scanty submission of specimens for laboratory diagnosis.

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RESUMO

Isolamento de Herpesvírus Bovino

O objetivo do presente trabalho é descrever os isolamentos de herpesvírus bovino realizados no Laboratório de Virologia da Universidade Federal de Santa Maria, RS, Brasil. Os isolamentos foram realizados de casos clínicos. Os herpesvírus bovino foram identificados pela técnica de imunofluorescência direta e pela microscopia eletrônica. Os herpesvírus foram isolados de surtos de enfermidade respiratória (2 surtos), vulvovaginite (1 surto), balanopostite (1 surto) e meningoencefalite (2 surtos). Um levantamento soro-epidemiológico realizado nas bacias leiteiras do Estado do Rio Grande do Sul indicou uma prevalência de 18,8% de animais sorologicamente positivos.

Palavras-chave: Herpesvírus bovino, surto, isolamento.

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