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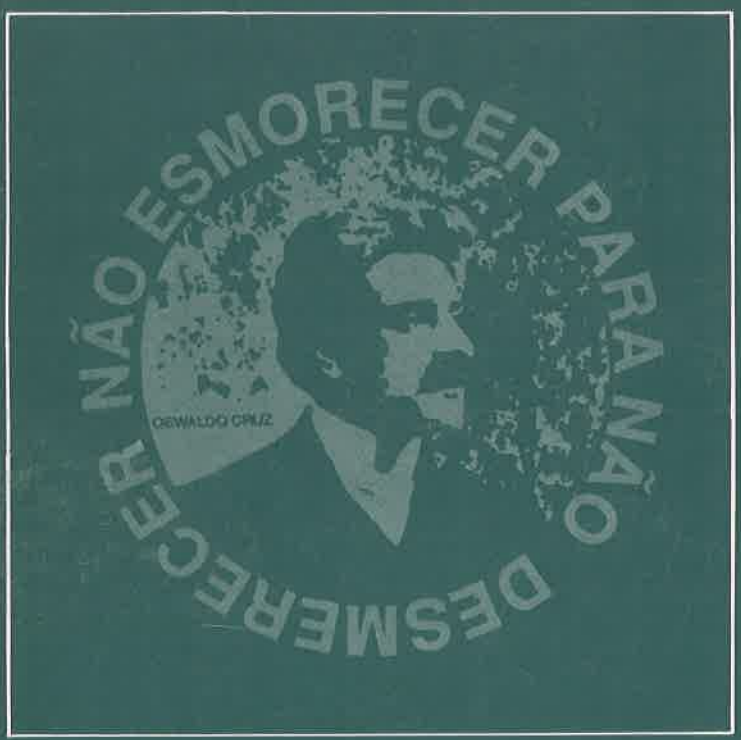

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The Brazilian Society for Microbiology

Filiated to IUMS - International Union of Microbiological Societies

A Sociedade Brasileira de Microbiologia, fundada em 28 de setembro de 1956, é sociedade civil, sem fins lucrativos, dedicada a agremiar microbiologistas brasileiros, promover o desenvolvimento da microbiologia através do estímulo à pesquisa científica e suas aplicações, melhorar as qualificações profissionais dos microbiologistas e realizar o Congresso Brasileiro de Microbiologia para manter o intercambio entre estudantes e profissionais da área. Edita a Revista de Microbiologia, com distribuição trimestral.

The Brazilian Society for Microbiology was founded on the 28th of September, 1956, and represents a nonprofitable society dedicated to the association of Brazilian microbiologists, to promote development in the field of Microbiology by encouraging scientific research work and its applications thus improving the professional qualifications of microbiologists, and to maintain a scientific exchange during the Brazilian Conference for Microbiology. The Society publishes the quarterly Revista de Microbiologia (Journal of the Brazilian Society for Microbiology).

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EDITORES SECCIONAIS / *Section Editors*

Editorial / *Editorial*

Ao completar 25 anos de publicação ininterrupta, a Revista de Microbiologia, editada pela Sociedade Brasileira de Microbiologia, está passando por diversas modificações.

Visando a sua descentralização, a Revista de Microbiologia conta agora com 9 Editores Seccionais (nomes e endereços estão na página anterior). Os autores que pretendem publicar seus trabalhos devem encaminhá-los ao Editor Seccional mais relacionado com o tema dos mesmos. Cabe a cada Editor Seccional a recepção dos trabalhos e o gerenciamento de todo o processo de avaliação, feita por revisores por ele(a) designados. Uma vez aceitos para publicação, cabe à Diretoria da Revista o gerenciamento dos processos de revisão do inglês e de impressão desses trabalhos.

O Corpo Editorial foi modificado visando atender mais adequadamente as necessidades da Revista. Cabe ao Corpo Editorial a definição das políticas de publicação, bem como o julgamento final em caso de dúvidas.

A partir do volume 26, a Revista de Microbiologia passa a ser também denominada "Journal of the Brazilian Society for Microbiology".

O item "instruções aos autores" foi revisto e ampliado, de forma a fornecer informações mais completas e permitir melhor uniformização na preparação dos trabalhos que são encaminhados para publicação, principalmente no que diz respeito às referências bibliográficas.

Estas modificações são importantes para que os trabalhos desenvolvidos pelos pesquisadores brasileiros sejam reconhecidos internacionalmente e para que a Revista de Microbiologia e a Sociedade Brasileira de Microbiologia possam ser melhor divulgadas. A Diretoria acredita que essas alterações deverão contribuir muito para a modernização da Revista de Microbiologia e pede o apoio e a colaboração de toda a comunidade científica brasileira e internacional.

Having completed 25 years of ininterrupted publication, the Revista de Microbiologia, *edited by the Brazilian Society for Microbiology, is undergoing several modifications.*

Aiming at its decentralization, the Revista de Microbiologia *now comprises a body of Section Editors (their names and addresses are listed on the previous page). Authors should submit their manuscript to the Section Editor of the subject area closest to their own work. Section Editors are responsible for the receipt of manuscripts and handling of their assessment by referees that they designate. Once accepted for publication, manuscripts are sent to to the Board of Directors of the* Revista de Microbiologia, *which proceeds to the evaluation of the English language and printing of the text.*

The Editorial Board was modified to provide a service of improved quality and also more directed to the needs of the Revista de Microbiologia. *It is a function of the Editorial Board to define publishing policies as well as to reach the final decision on a manuscript in case of doubt.*

Starting from Volume 26, the Revista de Microbiologia *will also be named* "Journal of the Brazilian Society for Microbiology".

The item "guidelines to authors" was reviewed and extended so as to provide more comprehensive information on the preparation of manuscripts submitted to publication.

The modifications introduced are important for the international recognition of Brazilian researchers and also for a more efficient circulation of the Revista de Microbiologia *and acknowledgment of the Brazilian Society for Microbiology. The Board of Directors believes that these changes will contribute much to the modernization of the* Revista de Microbiologia *and expects support and collaboration of both the Brazilian and the International scientific community.*

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ISOLATION AND CHARACTERIZATION OF LACTOCOCCAL BACTERIOPHAGES FROM CHEESE WHEY [1]

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Walter Vieira Guimarães
Elza Fernandes de Araújo
Arnaldo Chaer Borges

ABSTRACT

Samples of whey from cheese industries of the State of Minas Gerais, Brazil, were used for isolation and characterization of lactococcal bacteriophages. Ten bacteriophages were observed under electron microscope and their DNA cleaved with several restriction enzymes and analyzed in agarose gel. Seven bacteriophages were specific for *Lactococcus lactis* subsp. *cremoris* and three for *L. lactis* subsp. *lactis*. Electron micrographs showed that all of them had a similar morphology, with isometric heads and short non-contractile tails. Based on their DNA restriction patterns and their genome's size they could be classified into four distinct groups. All three bacteriophages specific for *L. lactis* subsp. *lactis*, were in the same group.

Key words: lactococcal bacteriophages, cheese whey.

INTRODUCTION

The lactic acid bacteria *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* are used in cheese industries to bring about the fermentation of lactose with gradual acid production essential for the manufacture of cheese (11). The growth of these starter cultures can be inhibited by antibiotics, bacteriocins and bacteriophages (2). Lack of acidification during cheese manufacturing has been mainly related to the bacteriophages that cause loss of culture viability (16). Considerable effort has been directed to the isolation and use of bacteriophage-resistant starter cultures. The resistance to bacteriophages can be induced by mutation or by expression of bacteriophage-resistant plasmids in the cells (8, 15). However, when a new starter strain is released a new bacteriophage will soon infect it, making the selection of suitable cultures difficult (10).

The sources of bacteriophages in cheese industries are not well established, but it has been suggested that they may come from raw milk, soil, animal intestines, reinfection of equipment employed in the process and also from prophages present in lysogenic cultures (10, 14, 16).

Studies of these bacteriophages based on morphology, serology and host range indicate that the vast majority of them belong to Group B (1). They have small isometric heads and non-contractile tails, with or without appendices (3, 7). Analyses of these bacteriophages at the genetic and molecular

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levels have been undertaken (3). A comparison of DNA restriction patterns of six bacteriophages from *L. lactis* subsp. *cremoris* showed a high degree of similarity between five of them and also with bacteriophage DNA patterns of *L. lactis* subsp. *lactis* (5).

The aim of this study was to isolate and characterize lactococcal bacteriophages from cheese industries located in the State of Minas Gerais, Brazil.

MATERIALS AND METHODS

Lactococcus lactis subsp. *lactis* was obtained from Visbyvac Laboratory and *Lactococcus lactis* subsp. *cremoris* from Christian Hansen Laboratories (from now on these bacteria will be referred to as *L. lactis* and *L. cremoris*). Cultures were routinely propagated at 30°C in M17 broth and stored at -20°C in M17 broth containing 15% glycerol (6). They were used as hosts to isolate bacteriophages present in cheese whey samples collected in cheese industries from Minas Gerais, Brazil.

Whey samples were centrifuged for 10 min. at 3,000 x g and the supernatants filtered in Millipore membranes (0.45 µm). Volumes of 0.1 ml of filtered suspensions were transferred to tubes containing 0.05 ml of 1.0 M $CaCl_2$ sterile solution and 0.1 ml of log phase cell suspensions of *L. lactis* or *L. cremoris*. After 30 min. at room temperature, 3.0 ml of M17 semi-solid medium, previously melted and cooled to 50°C, were added to each tube. These suspensions were poured onto plates containing the M17 basal agar and incubated for 18 hours at 30°C. Several single isolated plaques were picked, mixed with log phase cultures and plated on M17 agar. After incubation for 18 hours at 30°C, 5.0 ml of M17 broth were added to each plate and all plates kept for 12 hours at 4°C. The bacteriophage suspensions were collected for titer determination and storage at low temperature (6).

For electron microscopy examination bacteriophages were concentrated by PEG-6000 precipitation and purified in CsCl gradients (12). Bacteriophages were absorbed to the coated grids and stained with 3% uranyl acetate (9).

DNA was extracted from the bacteriophages and digested with the restriction enzymes EcoRI; PstI; BamHI; HindIII; SalI or RsaI. Restriction digests were loaded onto horizontal 0.75% agarose gel containing 0.5 µg/ml ethidium bromide and electrophoresed at 35 mA for 1 to 6 hours in TBE buffer (12). Gels were photographed under UV light with an Ashai-Pentax Camera with orange filter.

The size of DNA fragments were determined (13) using the program developed at the Center of Biotechnology, Federal University of Rio Grande do Sul, employing fragments of lambda CI857 DNA cleaved with HindIII as standards.

TABLE 1 - Bacteriophages isolated from cheese whey samples

Bacteriophages	Date of Collection	HOST
Lc1	October/87	*L. lactis* subsp. *cremoris*
Lc2	January/88	*L. lactis* subsp. *cremoris*
Lc3	January/88	*L. lactis* subsp. *cremoris*
Lc4	January/88	*L. lactis* subsp. *cremoris*
Lc5	May/89	*L. lactis* subsp. *cremoris*
Lc6	May/89	*L. lactis* subsp. *cremoris*
Lc7	May/89	*L. lactis* subsp. *cremoris*
Ll1	May/89	*L. lactis* subsp. *lactis*
Ll2	May/89	*L. lactis* subsp. *lactis*
Ll3	May/89	*L. lactis* subsp. *lactis*

RESULTS

Bacteriophages isolated from whey collected in cheese industries during the years of 1987, 1988 and 1989 and their host specificity are listed in TABLE 1. All the bacteriophages from 1987 and from 1988 samples were specific for *L. cremoris*; however, of the six bacteriophages isolated from samples collected during 1989, three were specific for *L. cremoris* and three for *L. lactis*.

Electron micrograph of the bacteriophage preparations revealed that they were morphologically similar, with isometric heads and apparently non contractile tails. FIGURE 1 shows Lc4 bacteriophage particles. The isometric heads measured approximately 43 nm in diameter and the tails 142 nm in length. Some bacteriophages had striated tails and distal appendices.

Bacteriophages Lc2, Lc3 and Lc4 isolated from 1988 whey samples had similar DNA restriction patterns when cleaved with EcoRI, PstI, HindIII, SalI and RsaI. The DNAs of bacteriophages Lc1, Lc2, Lc3 and Lc4 treated with EcoRI and PstI are shown in FIGURE 2. The Lc1 bacteriophage, isolated from a 1987 sample, had a different DNA restriction pattern

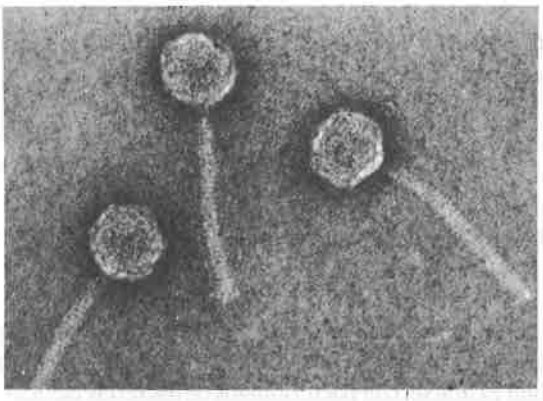

FIGURE 1 - Electron micrograph of Lc4 bacteriophage (260.000x).

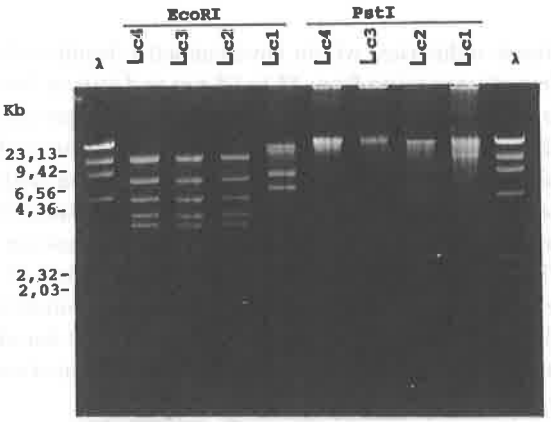

FIGURE 2 - DNAs of Lc1, Lc2, Lc3 and Lc4 bacteriophages treated with EcoRI and Pst1. Lambda DNA cleaved with HindIII.

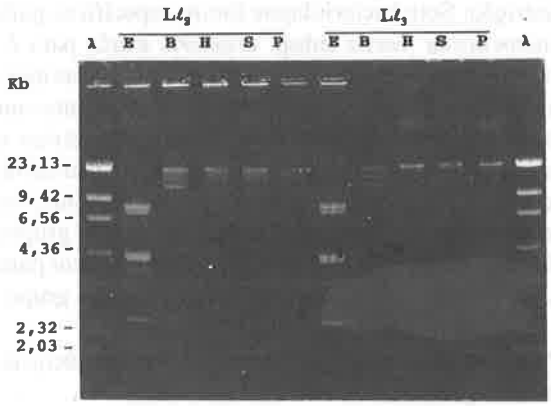

FIGURE 3 - DNAs of Ll2 and Ll3 bacteriophages treated with EcoRI(E); BamHI(B);HindIII(H); SalI(S) and Pst1(P). Lambda DNA cleaved with HindIII.

when treated with the same enzymes. The DNA of the Lc5 and Lc6 bacteriophages from 1989 samples had a similar pattern when treated with EcoRI which was, however, distinct from that of the Lc2, Lc3, Lc4 and Lc7 isolates. Similar DNA patterns were observed between bacteriophage Lc7 from a 1989 sample and bacteriophages Lc2, Lc3 and Lc4 from 1988 samples (Figure not shown).

The DNAs from Ll1, Ll2 and Ll3 *L. lactis* bacteriophages were cleaved only with EcoRI and BamHI enzymes and their patterns were similar. FIGURE 3 shows the Ll2 and Ll3 DNA patterns. It was observed that the DNA restriction patterns differ between *L. cremoris* and *L. lactis* bacteriophages.

Size determination of the ten bacteriophage genomes based on the DNA restriction fragments after cleavage with EcoRI indicated four distinct groups. The *L. cremoris* bacteriophages were divided into three groups, with genome sizes ranging from 20.39 to 31.60 kb (TABLE 2). All three *L. lactis* bacteriophages had a 31.44 kb genome and made up a single group.

DISCUSSION

The bacteriophages isolated from cheese whey samples had small isometric heads 43 nm in diameter and short tails 142 nm long. This morphology is similar to that normally found in bacteriophages from

TABLE 2 - Classification of the lactococcal bacteriophages by their genome size.

GENOME GROUP	BACTERIOPHAGES	BACTERIAL H O S T	SIZE (kb)
1	Lc1	*L. lactis* subsp. *cremoris*	25.18
2	Lc2, Lc3, Lc4, Lc7	*L. lactis* subsp. *cremoris*	31.60
3	Lc5, Lc6	*L. lactis* subsp. *cremoris*	20.39
4	Ll1, Ll2, Ll3	*L. lactis* subsp. *lactis*	31.44

cheese industries, which have isometric heads with diameters varying from 45 to 65 nm and tails of 100 to 250 nm in length. Bacteriophages with prolate heads of sizes varying between 55-65 x 40-48 nm and tail lengths of 80 to 110 nm as well as those with isometric heads ranging from 80 to 110 nm in diameter and tails 500 nm long can be found in cheese industries. Their frequencies, however, are lower when *L. cremoris* and *L. lactis* are utilized as starter cultures (3, 8). These types of bacteriophages were not found among the samples collected in cheese industries from the State of Minas Gerais.

According to the size of their genome, four distinct bacteriophage groups were identified. Three groups were specific for *L. cremoris* and one for *L. lactis*.

The *L. lactis* bacteriophages were obtained only from samples collected in 1989 whereas those specific for *L. cremoris* were found in the 1987-1989 samples. It is possible that bacteriophages grouped into a distinct group according to their DNA restriction pattern and genome's size derive from a common ancestor.

The procedure of culture substitution normally used in cheese industries is important to avoid loss of culture viability, since the *L. lactis* and *L. cremoris* bacteriophages can keep their infectivity for a long time (4). In cheese industries of New Zealand it was also observed that bacteriophage populations varied according to the starter strain used (10).

In this study all the isolated bacteriophages had the same morphological characteristics with, however, a distinct host range. The differences in restriction patterns between the *L. lactis* and *L. cremoris* bacteriophage DNAs indicate different base sequences in their genomes. These distinct sequences can alter the adsorption of bacteriophage proteins and consequently affect host specificity.

The characterization of bacteriophages frequently found in cheese industries of the State of Minas Gerais constitutes the first step towards the local development of stable bacteriophage-resistant *L. lactis* and *L. cremoris* cultures.

ACKNOWLEDGMENTS

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RESUMO

Isolamento e caracterização de bacteriófagos de *Lactococcus lactis* e *Lactococcus cremoris*

Amostras de soro, coletadas em indústrias de queijo de Minas Gerais, Brasil, foram usadas para isolar e caracterizar bacteriófagos de bactérias lácticas. Dez bacteriófagos foram observados em microscópio eletrônico e seus DNAs foram analisados em gel de agarose após a clivagem com várias enzimas de restrição. Sete bacteriófagos foram específicos para *Lactococcus lactis* subsp. *cremoris* e três para *L. lactis* subsp. *lactis*. As micrografias eletrônicas mostraram que todos os bacteriófagos apresentavam morfologia similar, contendo cabeça isométrica e cauda curta não contrátil. Baseando-se no padrão de restrição do DNA e no tamanho do genoma, os bacteriófagos foram classificados em quatro grupos distintos. Todos os três bacteriófagos específicos para *L. lactis* subsp. *lactis* se situaram no mesmo grupo.

Palavras-chave: bacteriófagos de bactérias lácticas, soro de queijo

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COMPETITION BETWEEN A GENETICALLY MODIFIED *PSEUDOMONAS FLUORESCENS* AND ITS PARENT IN SUBTROPICAL SOIL MICROCOSMS

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Leda Cristina Mendonça-Hagler[1*]
Allen Norton Hagler[1]
Jan Dirk van Elsas[2]

ABSTRACT

The ecological fitness of a *Pseudomonas fluorescens* strain (Br12) modified with a transposon Tn5::*cry*IVB chromosomal insertion in relation to its parent strain (Br5) and to the indigenous microbiota was assessed in two subtropical soils of fine and course texture planted with maize. Both strains had similar growth rates when grown separately in rich LB broth at 28°C, but the modified strain was at a slight growth disadvantage during mixed growth. The introduced populations showed a gradual decline in both kinds of unplanted soils. The decline rates were affected by soil type and temperature. In unplanted clay soil, strain Br12 was less competitive than its cointroduced parent, its proportion in the total surviving inoculant populations dropping from the initial 50 to 23% after 30 days. A similar proportion of Br12 strain (27%) was observed in the bulk and rhizosphere soil of clay soil planted with maize, however a slightly higher one (32%) was found in the rhizoplane. The percentage of the modified strain in unplanted sandy soil relative to total inoculant densities dropped slightly (from 50 to 37%) independently of temperature; in planted sand microcosms, its relative amount dropped to 37% - 30% in bulk soil, rhizosphere soil and in the rhizoplane. In the introduced cell mixtures, both strains made up a progressively lower percentage of the total heterotrophic bacteria and of the total fluorescent pseudomonads in the soil microcosms, with slight disadvantages for the modified strain as compared to its parent. However, in clay soil at 25°C, both strains kept up stable proportions of the total fluorescent pseudomonads, from day 5 to the end of the experiments (day 25). The effect of the genetic modification on ecological fitness was apparently minor compared with the effects of the soil environment acting on the introduced strains in the presence of the indigenous microbial soil communities.

Key words: competition, genetic engineering, *Pseudomonas fluorescens*, survival, soil microcosm.

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INTRODUCTION

The use of bacteria, either unmodified or genetically modified to express beneficial functions, for several applications including, for example, crop protection in agriculture (2, 4, 6-7) is a powerful approach. Although the potential for successful applications is high, several problems, which are primarily related to biosafety issues, should be solved before field applications can be conceived. Genetically modified microorganisms (GEMMOs) considered for environmental release are often modifications of indigenous microorganisms, since such strains are expected to survive and perform well following release. To be effective in the environment, such introduced GEMMOs must compete with the indigenous microorganisms during the period needed to complete the intended task. Generally, GEMMOs might either have a lower ecological fitness than wild-type strains because of the extra metabolic load due to the presence of the inserted genes (22, 25), or have an ecological advantage when selective pressure for the inserted gene is present. This makes ecological studies on GEMMO performance under natural conditions urgent.

Several studies on the competitive abilities of genetically engineered strains in natural environments have indicated that the GEMMO derivatives often did not reveal enhanced fitness as compared to their parent strains (9, 12, 20, 23). A modified plant pathogen, *Pseudomonas solanacearum*, with different levels of gene expression, also showed no enhanced survival in relation to that of its parent (27). The behaviour of both GEMMOs and wild strains was similar to that observed in numerous studies in soil (3, 10, 21-22), that is, a decline of the introduced populations was noted. A few other studies, however, have reported a selective advantage for GEMMOs introduced into the environment (11, 15).

Various strains of the group of fluorescent pseudomonads have been selected to serve as carrier organisms for the delivery of beneficial genes, e.g. the *Bacillus thuringiensis cry*IVB gene, to the rhizosphere of crop plants, and their ecological behaviour in soils, from both temperate and subtropical climate zones, has been addressed (1, 24). Introduced fluorescent pseudomonads in both microcosms and field studies often declined at rates that depended on various factors such as soil type, temperature (18, 21, 24-26). Studies on soil composition comparing soils from temperate, tropical and subtropical zones reported many significant differences that include, in general, the following features: tropical and subtropical soils are deeper, show lower cation exchange capacity (Ca^{++}, Mg^{+}, K^{+}, Na^{+}) and higher anion exchange capacity ($PO_4^{=}$, $SO_4^{=}$, Cl^{-}, NO_3^{-}). These soils are poor in silicates and rich in aluminium and iron content. In natural conditions, they are more aggregated and acid, presenting higher phosphorus fixing rates and lower ammonia and potassium fixing rates. Microbial activities are stronger, with high organic matter decomposition and consequent humus production. Another significant difference is the intensity of soil erosion resulting from summer storms (14). For these reasons it is extremelly important to determine the GEMMOs behaviour in different types of tropical and subtropical soils. In early studies, the behaviour of *cry*IVB-loaded transposon Tn5 containing GEMMOs in the subtropical soils was grossly akin to that of the parent strain in single inoculation studies, with certain soil conditions resulting in diminished survival of the GEMMO (1). These experiments, however, did not assay the possibility of subtle differences in fitness which are visualized when GEMMO and parent populations evolve in soil in direct competition for colonization of soil and root microsites (24). In the present paper, we show the competitiveness of a modified *Pseudomonas fluorescens* versus its parent in two subtropical soils of different texture, at different temperatures and with or without growing maize plants.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas fluorescens Br17 was isolated from the rhizosphere of maize in a Brazilian clay field soil. This strain was selected among the dominant strains in this habitat, as judged from rhizosphere viable plate counts (Araujo *et al.* in preparation). A spontaneous rifampicin (Rp) resistant mutant, denominated strain Br5, was selected on LB agar (16) with 50 µg/ml of Rp. Construction of a modified derivative carrying the *Bacillus thuringiensis* var *morrisoni cry*IVB gene in transposon Tn5, designated strain Br12, was previously described (1). Briefly, transposon Tn5 carrying *cry*IVB was inserted into the chromosome of strain Br5 by a standard filter

mating procedure (17) using *Escherichia coli* strain S17.1 with a suicide plasmid containing Tn5::*cry*IVB, as a donor. The modified transposon Tn5 used was previously described (23). The cultures were maintained at -20°C in LB broth (tryptone 1%; yeast extract 0.5%; NaCl 0.5%; ph 7.2) with 20% glycerol.

Determination of growth rates in competition

Parent strain Br5 and modified strain Br12 were grown in LB broth supplemented with the appropriate antibiotics, kanamycin (Km) or Rp (50µg/ml of both) at 28°C in a gyratory shaker (180 rpm). Nonselective LB was then used to study the growth rates of both strains in pure culture and in competition (using mixed inoculation with a 1:1 starting ratio). The growth of each strain was determined by measurements of optical density at 600 nm, and by plating serial tenfold dilutions onto selective LB agar at 2-hour intervals (Br12 was enumerated on LB containing Rp + Km and the total cfu on LB containing Rp). The numbers of Br5 were calculated by subtracting counts on LB with Rp + Km from those on LB with Rp.

Soils

A sandy soil (obtained from Embrapa - Empresa Brasileira de Pesquisa Agro-pecuaria, Rio de Janeiro) and a clay soil (latosol, red yellow) from the Pinheral farm, Rio de Janeiro, Brazil, were used in this study. Characteristics of these soils have been described (1). The water holding capacities (WHC), determined by a standard procedure (13), were 28% for the sandy soil and 49.5% for clay soil (w/w). About 1 month before sampling, the clay soil, which originally presented pH 4.9, was modified in the field by the addition of manure (about 15 kg/m$^{2)}$ and $CaCO_3$ (about 20 kg/m^2) setting the pH at about 6.9. Soil samples were collected from the upper soil layer (0-30 cm), sieved (4 mm mesh), air dried to a moisture content of about 10% and brought to 50% of the WHC with either a cell suspension or distilled water. The survival and competition experiments were done in soil microcosms, with two replicates for each system.

Competition studies in soil microcosms

Soil microcosms were inoculated with strains Br5 and Br12 grown in 500 ml of LB broth at 28°C for 24h. Bacterial cells were harvested by centrifugation (6000 x g, 15 min), and washed twice in sterile saline. Pellets were resuspended in sterile distilled water, and the bacterial densities were adjusted to provide approximately 10^8 cells g^{-1} soil at adequate moisture content. Strains Br5 and Br12 were mixed at an initial proportion of about 1:1 and the resulting suspensions were mixed into the soils for 10 min using sterile spatulas. Portions of 70 or 500 g of each soil were rewetted to a final moisture level of 50% of WHC and were packed to a bulk density of 1.5 (wet weight basis) in plastic beakers which composed the soil microcosms.

Seeds of maize (*Zea mays*) were surface-sterilized using acid hypochlorite solution, rinsed with sterile water (three times), and dried for 3 hours in a sterile air stream. This procedure was sufficient to remove all culturable bacteria, as evidenced by plating seed homogenates on LB agar plates. The sterilized seeds were germinated on sterile wet filter paper during 2 or 3 days. They were then placed in the top layer (1 cm) of each microcosm (two seeds per container) and covered with soil.

Soil microcosms were incubated at 25°C (greenhouse) or at environmental temperature (which ranged from 28 to 42°C, with an average of about 38°C). Sterile distilled water was added during the experimental period to compensate for moisture loss due to evaporation and plant growth. Control pots without inoculants were also incubated and remoisted with sterile distilled water.

Microcosm sampling

Recovery and enumeration of bacteria from unplanted microcosms were done at 3 h (zero value), and at 5, 10, 15, 20 and 30 days after inoculation. Microorganisms were extracted by suspending 10 g soil samples in 95 ml of 0.1% sodiumpyrophosphate (NaPP) solution supplemented with 0.1% Tween 80 (28) and blending (low speed) at room temperature for 3 min. Serial tenfold dilutions were then prepared in 0.25% strength Ringer's solution (5). To determine cfu numbers of both Br5 and Br12, appropriate dilutions were plated on S1 medium (8) containing 50 µg/ml of Rp for enumerating the total Br5 + Br12 populations, or 50 µg/ml of both Rp and Km for counting Br12. Plates were incubated at 28°C for 24 to 48 h. The numbers of cfu of Br5 were calculated as

described before. The inserted markers in Br12 were shown to remain stable under the conditions used, as evidenced by colony hybridization.

Total fluorescent pseudomonads were counted on S1 and King's B agar (BBL) without antibiotics. Total heterotrophic bacteria were enumerated on 0.1 strength trypticase soy agar (BBL) by the pour plate technique, which was used to avoid the spread of fast-growing *Bacillus* colonies on the plates.

For planted soil microcosms, samples were taken after 5, 10, 15, 20 and 25 days of incubation, in order to determine bacterial numbers in non-rhizosphere and rhizosphere soil and in the rhizoplane. Soil was removed from the containers and the plants were carefully dug out. Roots plus tightly adhering soil were separated from the remaining non-rhizosphere soil. The roots were then cut into pieces, placed in 250 ml sterile flasks containing 95 ml of 0.1% NaPP solution and shaken (45 min/200 rpm), resulting in the rhizosphere soil suspensions. Non-rhizosphere (bulk) soil (10 g) was shaken similarly, producing the bulk soil suspension. After shaking, roots were picked from the rhizosphere soil suspensions, rinsed with sterile distilled water, placed in 250 ml sterile flasks containing 95 ml of 0.1% NaPP solution and 10 g of glass beads (3 mm diam) and shaken (45 min/200 rpm). This resulted in the rhizoplane bacterial suspensions. Roots and soil were removed from each sample and their dry weights determined. Viable counts were done on serial dilutions of all samples (bulk and rhizosphere soil and rhizoplane) as described above.

Molecular analysis using the *cry*IV B gene probe

To confirm the presence of the genetically modified strain in the total culturable populations obtained from soils, colony hybridizations with a labelled *cry*IVB probe were done. Molecular techniques used were according to Sambrook *et al.* (16). The *cry* IVB gene probe was produced from *Escherichia coli* strain S17.1 (pSUP101::Tn5::*cry* IVB), grown in LB broth with appropriate antibiotics and incubated at 37°C (24h). Cells were harvested by centrifugation at 6000 x g at 4°C for 15 min. Plasmid DNA was then extracted and purified (16). The 3.7-kb *cry* IVB fragment used as a probe was obtained by *Eco*RI and *Hin*dIII digestion of the plasmid obtained, followed by low-melting point agarose gel purification. The probe was radiolabeled by nick translation using a BRL labeling kit (no. 8160 SB) and ^{32}P-dATP as recommended by the manufacturer.

Plates used for enumerating Br5 and Br12 containing well-separated colonies were used for colony lifts using QIBRANE membrane filters. Colonies were lysed and DNA denatured as described (16), and filters were dried. Hybridizations with the radiolabelled *cry* IVB gene probe were done in accordance with Sambrook *et al.* (16). The numbers of probe-positive signals were compared with Br5 and Br12 cfu counts in order to assess marker stability.

Statistical analysis

All experimental data were submitted to analysis of variance (ANOVA) or regression analysis, using GENSTAT-5 (Rothamsted Experimental Station), and differences were considered to be significant when $P<0.05$.

RESULTS

The growth characteristics of strains Br5 and Br12 were considered since growth is an important parameter determining competitiveness of introduced strains. Both strains Br5 and Br12 cultured in LB broth at 28°C exhibited generation times of 45 min in the exponential growth phase. During batch growth for about 15 generations in direct competition in LB broth, both strains increased their cell numbers over 24 h, from about 10^6 to 10^{10}-10^{11} cfu/ml. Strain Br12 grew somewhat slower than Br5, since it gradually made up a smaller part of the total cfu counts, from 50% initially to 26% after 24 h (not shown).

Experiments in unplanted soil microcosms showed that the total introduced mixed populations declined progressively at different rates, as affected by soil type and temperature (FIGURE 1A). In clay soil at both 25°C and the 38°C mean environmental temperature, the total introduced populations survived well over 30 days of experimental period, and there was no effect of temperature. However, survival of the total populations in the sandy soil was noticeably impaired, with a significantly higher death rate recorded at 38°C than at 25°C; the introduced mixed populations became undetectable in the sandy soil after 20 and 30 days of incubation at environmental temperature and at 25°C, respectively.

A different trend was observed on survival of the

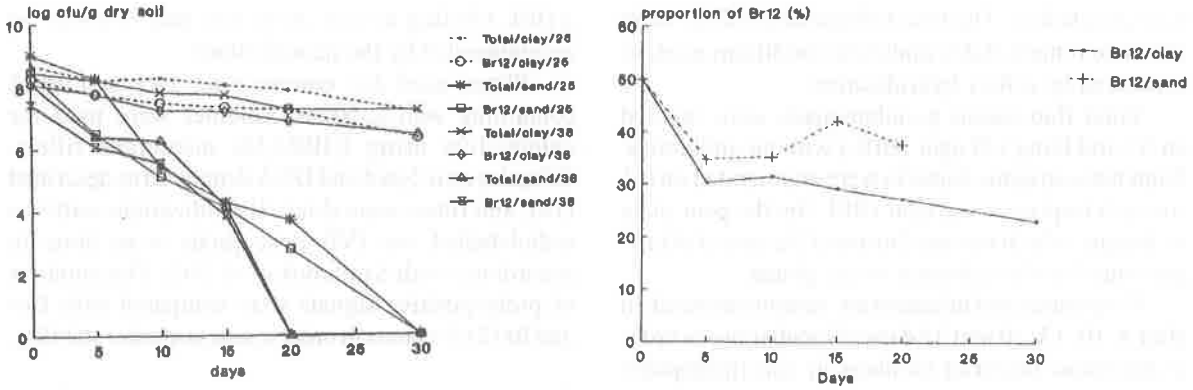

FIGURE 1 - Competition between the modified strain Br12 and its parent in unplanted clay and sand microcosmos. Total inoculant and specific Br12 viable counts at two different temperatures (25°C and environmental temperature)

GEMMO Br12 in competition with its parent strain Br5 in unplanted systems. For instance, in clay soil at 25°C, the proportion of strain Br12 in the total inoculant populations dropped steadily from the initial 50% to 23% in 30 days. In the sandy soil this proportion dropped slightly (to 38%) at both temperatures, showing no clear decreasing trend related to temperature differences (FIGURE 1B).

The introduced mixed populations survived very well in bulk and rhizosphere soil of the planted clay microcosms at 25°C and at environmental temperature, with no significant decline in cfu counts either. There was no difference in the dynamics of the total or the GEMMO inoculant populations at these temperatures, so only the results obtained at 25°C are shown (FIGURE 2A). The total inoculant counts in the rhizosphere were similar to those in bulk soil at both temperatures. In the rhizoplane, the GEMMO Br12 culturable numbers fluctuated around 10^3 - 10^4 cfu per g dry root, with a slight trend to decrease (FIGURE 2A). At the end of the experimental period, the proportions of the modified strain Br12 in bulk and rhizosphere soil and on the rhizoplane showed a fall from the initial 50% to 27-32% of the total surviving inoculant populations, whereas somewhat fluctuating values revealed similar trends observed in unplanted clay soil (FIGURE 2B).

In the planted sandy soil microcosms the introduced mixed populations showed fast declines in both bulk and rhizosphere soil samples at both temperatures, from initially 10^8 to about 10^5 (25°C) or 10^4 (environmental temperature) cfu per g dry soil

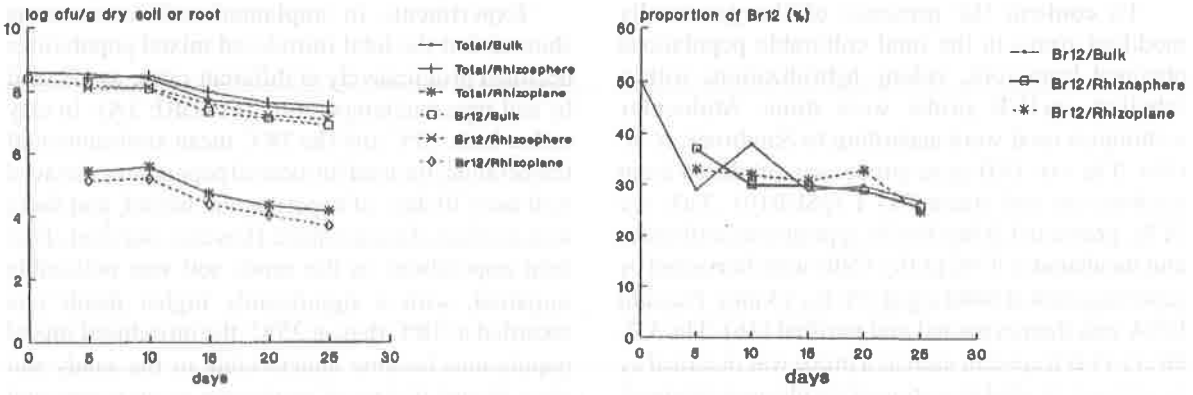

FIGURE 2 - Competition between the modified strain Br12 and its parent in clay microcosms planted with maize, at 25°C. Total inoculant and specific Br12 viable counts in bulk rhizosphere and rhizoplane of maize.

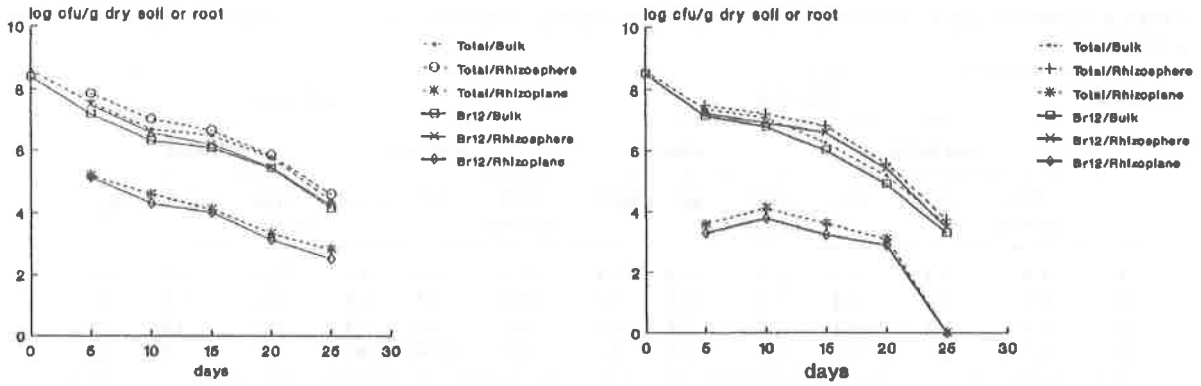

FIGURE 3 - Competition between the modified strain Br12 and its parent in sand microcosm planted with maize. A. Total inoculant and specific Br12 viable counts in bulk, rhizosphere and rhizoplane of maize, . B. Idem, at environmental temperature

after 25 days (FIGURE 3A, B). Rhizoplanic inoculant counts also dropped, from about 10^5 on day 5 to 10^3 (day 25) cfu per g dry root at 25°C, or from about 10^4 cfu per g dry root on day 5 to undetectable levels on day 25 at environmental temperature. The proportions of Br12 in the total surviving inoculant populations dropped by day 5 from the initial 50% to levels of around 30-37% in bulk, rhizosphere and rhizoplane samples.

Colony hybridizations performed on enumeration plates obtained from both planted and unplanted soils throughout the experimental periods showed, by comparison of the number of *cry*IVB probe positive hybridization signals to the counts on both selective media used, that the cfu counts obtained accurately reflected the numbers of culturable cells of Br12 recoverable from soil. This indicated that the cassette consisting of the Tn5-encoded Km resistance marker and the *cry*IVB gene was stable, and that the Km resistance was adequately expressed when recovering cells from soil on selective agar plates. In addition, comparisons of counts in initial samples, with high inoculum densities, on selective (Rp containing) and unselective LB agar revealed that there was no significant loss of the Rp resistance marker in both strains Br5 and Br12.

The introduced mixed inoculant populations as well as the modified strain present in the mix made up a progressively smaller part of the total heterotrophic bacterial populations, which remained relatively stable over the experimental period (TABLE 1). At 25°C the proportions dropped from 3-16% (5 days) to 0.6-1.2% (20 days) in planted and unplanted clay soil, with no apparent effect of the rhizosphere, and from 9-17% (5 days) to 0.01-0.04% (unplanted sand) or from 12-28 to 0.5-1.0% (planted sand). With one exception, the proportions of the mixed inoculant in rhizosphere and rhizoplane samples of the 2 soils in relation to the total fluorescent *Pseudomonas* counts also showed diminishing values, from the initially high proportions measured after 5 days to much lower levels after 25 days (TABLE 2). Interestingly, throughout the experimental period (15-25 days), both Br5 and Br12 made up a relatively stable proportion (around 20% for Br5 and around 6-9% for Br12) of the total fluorescent *Pseudomonas* counts on the maize rhizoplane in clay soil, indicating an ecological stability of this introduced organism which was similar to that of the indigenous fluorescent pseudomonads under these conditions.

DISCUSSION

The present paper assessed the competitive ability of a modified *P. fluorescens* strain as compared to its parent after introduction into two subtropical soils of different texture. To ascertain the competitive power of a GEMMO is an important pre-release task, since such strains might either be able to disturb the soil ecosystem by outcompeting indigenous wild strains or might have suffered a fitness decrease due to the very modification, limiting its usefulness for application.

The GEMMO Br12 apparently had a subtle growth disadvantage when growing in competition

TABLE 1. Proportion of genetically modified Br12 and its parent Br5 with respect to total heterotrophic bacteria in two soils of different textures, at 25°C .

Days	Clay Soil						Sandy Soil					
	unplanted			planted			unplanted			planted		
	THB cfu x 10^9	Br5	Br12	THB cfu x 10^9	Br5	Br12	THB cfu x 10^6	Br5	Br12	THB cfu x 10^7	Br5	Br12
5	1.9	7.3	3.2	2.1	15.8	7.9	33.9	17.4	9.3	13.2	28.2	12.6
10	2.0	3.1	1.4	2.2	11.5	5.2	15.5	1.5	0.8	6.6	6.8	4.1
15	2.3	2.2	0.9	2.5	2.1	0.8	3.6	0.4	0.4	5.6	4.8	2.6
20	2.5	1.2	0.6	2.7	1.2	0.6	3.0	0.04	0.01	5.1	1.0	0.5

THB, Total heterotrophic bacteria; Br5, *P.fluorescens* parental strain; Br12, genetically modified strain;
* cfu/g dry soil; all other values are percentages of THB counts.

TABLE 2. Proportion of genetically modified *P. fluorescens* Br12 and its parent Br5 in total fluorescent pseudomonads in the maize rhizosphere and rhizoplane at 25°C.

Days	Clay Soil						Sandy Soil					
	Rhizosphere			Rhizoplane			Rhizosphere			Rhizoplane		
	TFP* ufc x 10^8	Br5	Br12	TFP* ufc x 10^5	Br5	Br12	TFP* ufc x 10^6	Br5	Br12	TFP* ufc x 10^4	Br5	Br12
5	7.6	34.7	14.1	25.7	9.8	4.2	85.1	31.6	16.6	10.7	16.8	9.1
10	5.4	54.0	31.0	79.4	19.0	14.4	47.9	26.3	10.4	8.9	35.5	20.1
15	2.6	34.0	15.5	26.9	19.5	9.1	17.0	22.4	12.6	3.5	27.3	17.8
20	2.2	19.0	7.8	4.0	18.2	8.4	4.1	12.0	4.2	1.6	14.4	7.5
25	1.4	22.0	9.3	0.8	22.4	6.0	0.71	5.4	2.0	0.8	7.9	5.6

TFP, total fluorescent pseudomonads; Br5, *P. fluorescens* parental strain; Br12, genetically modified strain.
* cfu/g dry soil or root; all other values are percentage of TFP populations

with its parent in LB broth at 28°C. On the other hand, in separate inoculation experiments, the growth rates appeared similar between the two strains. Decreased fitness in liquid culture of a Tn5::*cry* IVB loaded *P. fluorescens* strain as compared to its parent has been described before (23). The higher energy demand due to the constitutive expression of several genes in the insert may have caused the subtle changes in fitness of the modified strain as compared to the parent, which became apparent only upon growth in direct competition.

Previous studies with *Pseudomonas* strains showed that soil texture is a factor affecting the survival of introduced populations and the proportions between modified strains and their parents (20-22, 24). Our results are in line with these results in that the mixed *P. fluorescens* strains Br5 and Br12 survived better in the (subtropical) clay soil than in the sandy soil. The apparent insensitivity of the inoculants to temperature effects in clay soil and their sensitivity in sandy soil may be explained by assuming that the clay exerted a protective effect on the inoculants which was absent in sandy soil. Temperature increases in a loamy sand soil have been shown to affect inoculant survival; this effect may be attributed to direct abiotic and more indirect biotic factors (26). Moreover, protection of inoculants in soil from deleterious effects due to high temperature by clay particles also is a known factor in microbial ecology.

The apparently decreased fitness of the *cry* IVB containing GEMMO Br12, most apparent in the subtropical clay soil, may have been related to the diminished fitness in batch growth in competition if inoculant growth is assumed to play a substantial role in colonization and survival in soil. Poor possibilities for growth in the sandy soil, possibly leading to the

rapid death observed, may have resulted in the seemingly more stable proportions of the GEMMO found. Surprisingly, the maize roots caused only minor changes in the GEMMO/total proportions in the sandy soil. Therefore, growth of the inoculants and colonization of the rhizosphere and rhizoplane were probably severely limited due to competition by the indigenous microflora. Other results obtained with *cry*IVB-modified fluorescent pseudomonads substantiate the small loss of competitiveness in soil reported herein. Thus, a different *Pseudomonas fluorescens* strain, R2f, with an insert similar to the one used in this work, has been shown to be less competitive in bulk soil and in the rhizosphere and rhizoplane of wheat than its parent when introduced into a Dutch loamy sand soil (23). Further, recent derivatives of *P. fluorescens* R2f modified with *cry* IVB-*npt*II or with *npt*II, introduced with the parent into loamy sand microcosms were also found to be somewhat less competitive than the parent (20).

The data shown here, even though pointing to a generally small ecological disadvantage for the GEMMO Br12 in competition with the parent Br5 in soil, also suggested that Br12 was able to maintain population densities grossly akin to those of the parent strain in the experimental period. Our results showed that often the shifts in the proportions of the GEMMO occurred in the 5 days following introduction, possibly due to the somewhat more advantageous growth characteristics of strain Br5 as compared with Br12. The initial colonization phase, in which the ability of inoculants Br12 and Br5 to occupy favorable soil microsites faster than indigenous bacteria plays a pivotal role, is probably critical for inoculant population densities in later phases. Since the initial differences in competitiveness between Br12 and Br5 were generally small in the systems tested, the overall effects were similarly small.

Our data on total fluorescent pseudomonads and heterotrophic bacterial counts showed relatively stable levels of these populations during the experimental periods. This suggested that conditions in soil and rhizosphere were favorable for the maintenance of these microbial groups. However, with the exception of the rhizoplane counts in the clay soil, the proportions of the introduced bacteria in planted and unplanted clay and sand microcosms in both the total heterotrophs and in the total fluorescent pseudomonads decreased steadily. This is in line with the postulated general ecological weakness of many incoming bacteria not protected by carrier materials, and may be related to the localization of such inoculants primarily in open spaces in soil, where both abiotic and predatory stress factors are fully operative (19). Apparently, the rhizoplane of maize growing in the clay soil was well colonized by both Br5 and the GEMMO Br12, and both remained stably present, as evidenced both from direct counts (FIGURE 2) and from the proportions of these strains in the total fluorescent pseudomonads. Even though strain Br12 colonized well, it slightly lagged behind its parent Br5 in colonization power. The developing roots may have provided new substrate utilizable and colonizable by the inoculants initially present in relatively protected microsites in the clay soil. Such protective microsites were apparently missing in the sandy soil, since here the inoculant numbers in the rhizoplane followed a pattern of decline, both in terms of absolute numbers (FIGURE 3B) and as proportions of the total fluorescent pseudomonads (TABLE 2). Previous studies on maize root colonization by strain Br12 showed proportions of 46 to 1.3% of total fluorescent pseudomonads persisting in different parts of the root zone (1), also suggesting that Br12 may compete well with indigenous microorganisms on the maize rhizoplane.

This study demonstrates that, in subtropical soils, soil type and temperature significantly influence the survival of introduced bacteria and the competitiveness of a modified strain compared to its parent. The competitiveness of the modified strain generally diminished when environmental conditions improved bacterial survival, like for instance those encountered in the clay soil. The stable colonization of maize roots by the GEMMO Br12 in the subtropical clay soil in the presence of the parent strain suggests that the delivery of a beneficial gene such as *cry* IVB to the rhizoplane of maize is a viable approach, even in the context of its somewhat diminished competitive ability. In addition, plant development and microbial populations in the rhizosphere and rhizoplane were apparently not affected by the presence of the GEMMO, suggesting ecosystem stability with regards to these criteria. However, prior to field studies, further studies with larger microcosms of greater complexity and involving target insect larvae are needed to better predict potential benefits and to assess unforeseen risks of the application of GEMMOs like Br12.

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RESUMO

Competição entre *Pseudomonas fluorescens* modificado geneticamente e a estirpe selvagem em microcosmos de solos subtropicais

A capacidade de adaptação ecológica da estirpe *Pseudomonas fluorescens* (Br12), modificada pela inserção cromossomal do transposon Tn5::*cry*IVB, em relação à estirpe selvagem correspondente (Br5) e à microbiota indígena, foi avaliada em dois solos subtropicais com diferentes texturas (fina e grossa), plantados com milho. Ambas estirpes apresentaram crescimento similar quando cultivadas separadamente em meio líquido LB à 28°C. Quando as estirpes foram cultivadas, misturadas nas mesmas condições, a estirpe Br12 apresentou crescimento desvantajoso em relação à estirpe Br5. Ambas populações introduzidas mostraram um declínio gradual nos dois tipos de solos não plantados. As taxas de declínio foram afetadas pelo tipo de solo e temperatura. Nos solos argilosos não plantados a estirpe Br12 apresentou competitividade menor do que sua parental Br5, em experimentos com introdução simultânea, ocorreu uma queda na proporção inicial de 50% para 25% após 30 dias. Uma proporção similar para a estirpe Br12 (27%) foi observada nos solos argilosos de não rizosfera e de rizosfera de milho, contudo um ligeiro aumento (32%) foi observado em rizoplano. Em solos arenosos não plantados, independente da temperatura, ocorreu um pequena queda, de 50 para 37%, nas proporções da estirpe modificada em relação à densidade total do inoculante. Nos solos arenosos plantados as proporções variaram de 37 a 30% nos solos de não rizosfera, rizosfera e rizoplano. Ambas as populações das estirpes introduzidas, em inóculos mistos, resultaram em percentuais gradativamente menores das populações de bactérias heterotróficas totais e de pseudomonadaceas fluorescentes totais, com pequena desvantagem para a estirpe modificada, quando comparada com sua parental selvagem. Entretanto, em solos argilosos a 25°C, ambas as estirpes, Br12 e Br5, mantiveram proporções estáveis durante todo o período experimental em relação às densidades de pseudomonadaceas fluorescentes totais. O efeito da modificação genética sobre a capacidade de adaptação ecológica foi aparentemente menor do que a influência dos efeitos ambientais sobre as populações das estirpes introduzidas em comunidades microbianas indígenas de solo.

Palavras-chave: competição, geneticamente modificado, *Pseudomonas fluorescens*, sobrevivência, microcosmos de solo.

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EVIDENCE FOR TWO ARSENATE RESISTANCE MECHANISMS IN *THIOBACILLUS FERROOXIDANS*

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ABSTRACT

Thiobacillus ferrooxidans is able to grow in the presence of high free arsenic levels which are generated during the recovery of gold from arsenopyrite-pyrite ores by leaching. The mechanisms of such resistance are not known for these bacteria. A strain isolated from Morro Velho Mines (Nova Lima, MG, Brazil) was grown in inorganic 9K medium containing sodium arsenate concentrations of 0, 2, 4, 6 or 8g.L^{-1}, in semi-continuous cultures kept in a 10 L fermentor. Cell growth was little altered by arsenate levels lower than 4g.L^{-1}. At this concentration, growth was considerably slower due to a lag phase of about 30h. When cells were transferred to medium containing arsenate concentrations of between 4g.L^{-1} and 8g.L^{-1} the growth profile was improved. This phenomenon was not observed in 500ml flask cultures (agitation rate: 150rpm). The comparison of electrophoretic profiles (PAGE-SDS) of cell-free extracts from cultures of bacteria grown without arsenate or in the presence of 8g.L^{-1} arsenate revealed, in the latter case, four major bands with apparent molecular weights of about 16,500; 22,500; 24,000 and 33,500 Da, respectively. These results suggest that there are two mechanisms of arsenates resistance in *T. ferrooxidans,* one dealing with low and the other with high levels of available arsenate.

Key words: arsenate resistance, *Thiobacillus ferrooxidans*

INTRODUCTION

Thiobacillus ferrooxidans is an acidophilic, diazotrophic, autotrophic bacterium that is able to obtain energy through the oxidation of ferrous iron to ferric iron or sulfur compounds to sulfuric acid. Because of these properties, *T. ferrooxidans* is the most important bacterium in most leaching operations.

Arsenic is found in many sulfide ores, such as copper and gold ores. Biohydrometallurgic processing of such ores results in arsenic dissolution (5) which is toxic for bacterial and animal cells. Arsenic resistance of microorganisms used in the leaching of metals from these sulfide ores would be an attractive and industrially significant characteristic.

Some attempts were made to construct

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recombinant plasmids between *T. ferrooxidans* and an *Escherichia coli* strain which contained arsenic resistance genes (9). Recently, naturally arsenic-resistant *T. ferrooxidans* strains were isolated from brazilian gold mines (Morro Velho Mines, Nova Lima, MG, Brazil).

Arsenic resistance mechanisms are now relatively well understood in *Staphylococcus aureus* and *E. coli* (1,2,3,6,7,10,13), but there is no information about this phenomenon in *T. ferrooxidans*.

Our data allow some hypothesis to be made concerning the mechanisms of adaptation of these bacteria to increased arsenate concentrations.

MATERIALS AND METHODS

Bacteria

A strain of *T. ferrooxidans* was isolated from the Morro Velho Mines, Nova Lima, MG Brazil (Pitangui strain) and was maintained by weekly serial cultures in inorganic 9K medium containing ($g.L^{-1}$): $(NH_4)_2SO_4$, 3.00; KCl, 0.10; K_2PO_4, 0.50; $MgSO_4$, 0.50; $FeSO_4.7H_2O$, 2.25 (11).

Medium

Thiobacillus was grown for maintenance and during experiments in the inorganic 9K medium (11) ajusted to pH 1.5 with sulfuric acid. Arsenic, when added, was used as sodium arsenate at the following concentrations: 2, 4, 6 or $8g.L^{-1}$.

Culture conditions

For flask cultures, the bacteria were grown in 500mL flasks containing 50mL of medium and incubated at 30°C on a rotary shaker (150rpm). Each flask was inoculated with 5ml of a culture suspension with an absorbance of 0.1.

Semi-continuous growth was done in a bench top fermentor (Model MF 114 New Brunswick Scientific Company). During the operation, aeration, agitation and temperature were kept at 1vvm (culture medium volume/air volume/minute), 400rpm and 30°C, respectively. As first inoculum, a 2L-Kitasato flask containing 500mL of cultured bacteria after 24 hs of incubation was transferred to the fermentor vessel containing 10L of the same medium. At the end of the growth period (when about 70% of the iron in the medium was oxidized), all but one liter of the fermentor vessel contents were removed by air pressure; 9L of sterile medium were then added and the incubation continued. At the end of the next culture period, all but one liter of broth were removed and new medium added as described above.

At different times depending on the experiment (see FIGURES), samples or flasks were withdrawn from the fermentation vessel or from the shaker for analysis.

Analysis

The concentration of ferrous iron in the medium was determined by volumetric titration with potassium dichromate. Ferrous iron oxidation rate (FIOR) was calculated as follow:

$$\text{FIOR } (mg.L^{-1}.h^{-1}) = \frac{Fe^{2+}\text{ initial} - Fe^{2+}\text{ final}}{\text{culture time}}$$

After each culture, a bacterial pellet was obtained by centrifugation and suspended in a Tris-EDTA-$MgCl_2$ buffer (10mM, 1mM, 1mM), pH 7.5. This suspension was disrupted by sonication and a cell-free extract obtained by centrifugation at 45,000 g for 60min (2). Protein content was determined by the Bradford method, as modified by Peterson (8). Polyacrylamide gel electrophoresis-SDS (PAGE-SDS) of the cell-free extract was performed using a PhastGel system (Pharmacia) and a 12.5% acrylamide gel. Macromolecules in the gel were stained with silver.

RESULTS AND DISCUSSION

FIGURE 1 shows that the presence of $2g.L^{-1}$ arsenate level did not alter the rate of ferrous iron oxidation by *T. ferrooxidans* in 9K medium. When the arsenate concentration was raised to $4g.L^{-1}$, a lag phase of about 30h was observed and the oxidation was completed only after approximately 80h. The oxidation of ferrous iron was remarkably faster (about twofold) in fermentor cultures containing $6g.L^{-1}$ or $8g.L^{-1}$ arsenate. These results could perhaps be explained by two arsenate resistance mechanisms. The first, apparently constitutive, would apply to arsenate concentrations ranging from 0 to $4g.L^{-1}$,

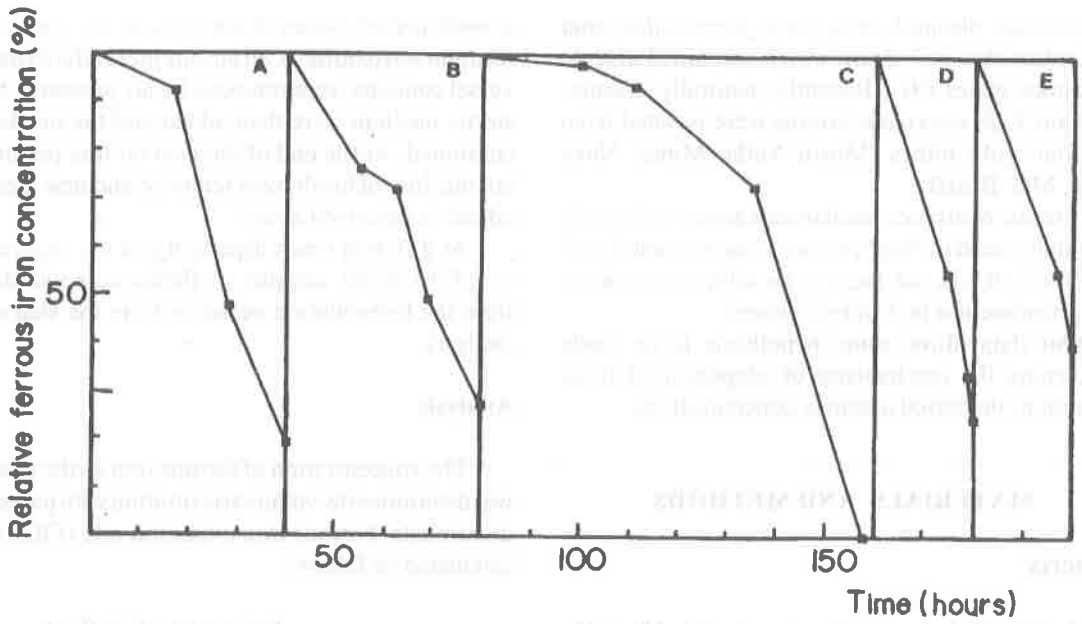

FIGURE 1 - Relative ferrous iron concentration (%) during the semi-continuous growth of *Thiobacillus ferrooxidans* in 9K medium containing 0 (A), 2 (B), 4 (C), 6 (D) or 8 $g.L^{-1}$ (E) sodium arsenate. The cultures were carried out in a 10L fermentor. One liter of each previous culture was used to inoculate 9L of new medium with increased arsenate concentration. Culture A was inoculated with 500mL taken from a Kitasato flask containing culture in 9K medium. Aeration, agitation and temperature were adjusted at 1vvm, 400rpm and 30°C, respectively.

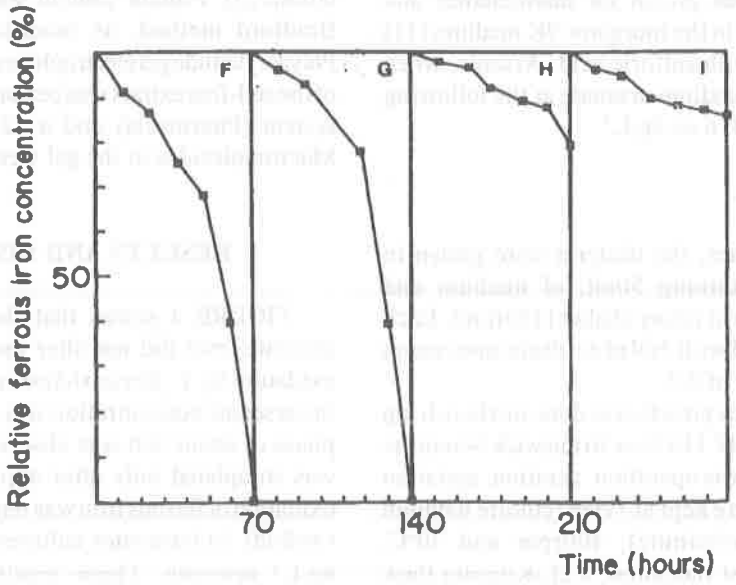

FIGURE 2 - Relative ferrous iron concentration (%) for the flask culture of *Thiobacillus ferrooxidans* in 9K medium containing 0 (F), 2 (G), 4 (H) or 6 $g.L^{-1}$ (I) sodium arsenate. Incubation was carried out at 30°C on a rotary shaker (150rpm).

when a possible threshold for the induction of a more efficient arsenate resistance mechanism seems to have been reached . It is interesting to note that the appearance of this new arsenate resistance mechanism was accompanied by more effective ferrous iron oxidation. However, the phenomenon observed in FIGURE 1 could also be explained by the selection of a mutant population during growth in the presence of 4g.L^{-1} of arsenate. FIGURE 2 shows that the biphasic phenomenon described above could not be repeated when a similar experiment was carried out in flask cultures, a fact that may have resulted from a less efficient aeration under such culture conditions. However, relatively efficient arsenate resistance was observed at an arsenate concentration of 2g.L^{-1}, reinforcing the hypothesis that the first mechanism of resistance is a constitutive feature. The biphasic behaviour of *T. ferrooxidans* cells during growth in the presence of increased arsenate concentrations in semi-continuous cultures as compared to flask cultures can be seen in FIGURE 3, where the overall ferrous iron oxidation rate (mg.L^{-1}.h^{-1}.) is shown for each arsenate concentration. FIGURE 4 shows the electrophoretic profiles of cell-free extracts from bacteria grown under various conditions. Similar SDS-PAGE profiles are observed for cell-free extract from bacteria cultured without arsenate or in the presence of 4g.L^{-1} arsenate, for both fermentor (A and C) and flask (F and H) cultures. On the other hand, four major bands can be noted when the bacteria are grown in 9K medium containing 6 or 8g.L^{-1} arsenate (D and E). The apparent molecular weight of these bands are of 16,500; 22,500; 24,000 and 33,500 Da, respectively.

Plasmid-determined arsenate and arsenite resistance has been known to exist in *Staphylococcus aureus* and *Escherichia coli* since 1968 and 1970 and was shown to be inducible (6,1) Arsenate is known to be an analog for the phosphate transport systems, which are apparently two (12). One of the arsenate resistance mechanisms is probably associated with the elimination of the less selective of these two systems (12).

Another adaptive device utilized by bacteria to produce resistance to toxic agents are efflux systems. The *ars* operon encodes the pump genes responsible for the efflux of arsenite in *E. coli* and *S. aureus* (2). The *ars* operon has four genes, designated *ars*R, *ars*A, *ars*B and *ars*C (7). The operon is regulated by the ArsR protein, which is a *trans*-acting repressor (13). ArsA and ArsB proteins together form the basic anion pump. The ArsA is the catalytic subunit, with

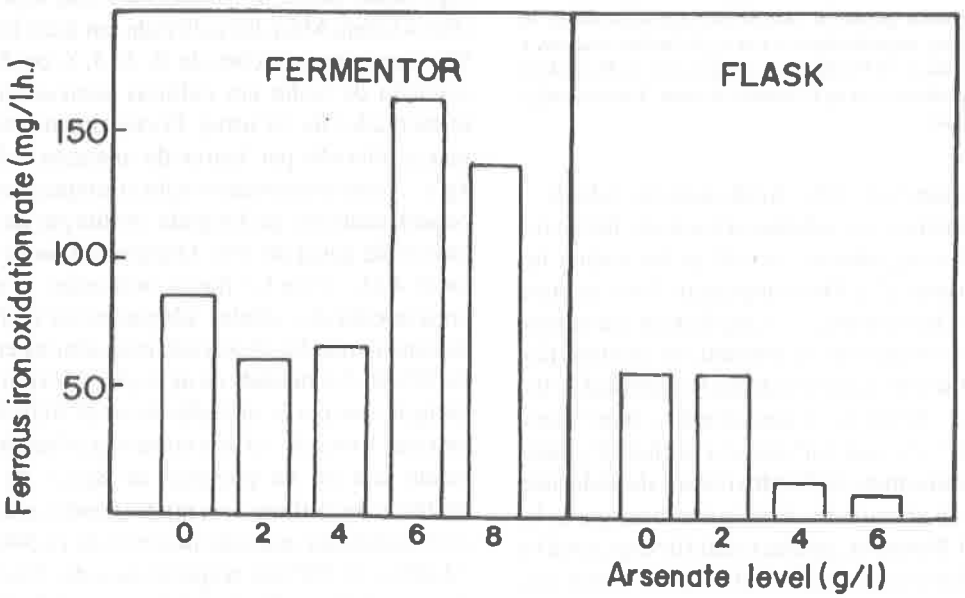

FIGURE 3 - Ferrous iron oxidation rates (mg.L^{-1}.h^{-1}.) during semi-continuous and flask culture growth of *Thiobacillus ferrooxidans* in 9K medium containing increasing sodium arsenate levels. For culture conditions and codification, see FIGURES 1 and 2.

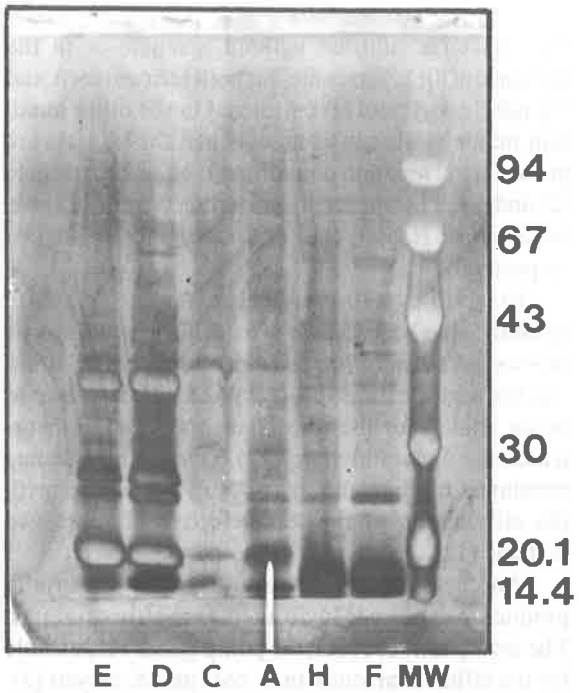

FIGURE 4 - Electrophoretic profiles of cell-free extracts from of *Thiobacillus ferrooxidans* grown in 9K medium and with various culture conditions. Polyacrylamide gel electrophoresis-SDS (PAGE-SDS) was performed using a PhastGel system (Pharmacia) and a 12.5% acrylamide gel. Macromolecules in the gel were stained with silver. MW = standard molecular weight (Phosphorylase b, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsine inhibitor, 20,100; Ó-lactoalbumin, 18,400 Da). **A, C, D** and **E** = semi-continuous growth of *Thiobacillus ferrooxidans* in 9K medium containing, respectively, 0, 4, 6 or 8 $g.L^{-1}$ sodium arsenate. **F** and **H** = flask culture of *Thiobacillus ferrooxidans* in 9K medium containing, respectively, 0 or 4$g.L^{-1}$ sodium arsenate. Volume applied with 0.7µg protein.

an ATPase activity. The ArsB protein, which is located in the inner membrane of bacteria, forms the anion-conducting subunit, as well as the anchor for the ArsA protein (3). The cytoplasmic ArsC protein is a 16,000 Da monomer (3) and has an enzymatic function: the reduction of arsenate to arsenite (2). Arsenite is the only form which can be extruded by the anion-pump. Arsenite is considerably more toxic than arsenate, reacting with protein sulfhydril groups to inactivate enzymes (4). For this reason, the reduction of arsenate to arsenite by ArsC protein seems to be paradoxical. However, this fact could be explained by the loss of phosphate, which would occur if a relatively non-specific arsenate efflux system was used instead of the arsenite efflux mechanism described above.

The two arsenate resistance mechanisms described above (decrease of arsenate influx and increase of arsenate efflux) could be a possible explanation for the two levels of arsenate resistance observed in our work. Further studies are being carried out in our laboratory to investigate this possibility.

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RESUMO

Evidência sugerindo dois mecanismos de resistência ao arsenato em *Thiobacillus ferrooxidans*

Thiobacillus ferrooxidans é capaz de crescer na presença dos altos níveis de arsênio livre que são gerados durante a recuperação de ouro a partir de arsenopirita por lixiviação. Os mecanismos dessa resistência não são conhecidos nessa bactéria. Uma cepa desta bactéria, isolada na Mina Morro Velho (Nova Lima, MG), foi cultivada em meio inorgânico 9K com concentrações de 0, 2, 4, 6 ou 8 $g.L^{-1}$ de arsenato de sódio em culturas semi-contínuas em fermentador de 10 litros. O crescimento celular foi pouco alterado por teores de arsenato inferiores a 4$g.L^{-1}$. Para essa concentração, o tempo de cultura foi consideralmente prolongado em função de uma fase latente de cerca de 30h. Quando níveis de arsenato entre 4$g.L^{-1}$ e 8$g.L^{-1}$ foram utilizados, o perfile de crescimento das células adaptadas foi melhor. Este fenômeno não foi observado nas culturas em frascos de 500mL (velocidade de agitação: 150rpm). A comparação dos perfis eletroforéticos (PAGE-SDS) dos extratos livres de células obtidos das bactérias cultivadas sem ou na presença de 8$g.L^{-1}$ de arsenato destacou, no último caso, quatro bandas maiores com peso molecular aparente de cerca de 16.500, 22.500, 24.000 e 33.500 Da, respectivamente. Esses resultados sugerem que há dois mecanismos de resistência em *T. ferrooxidans* para baixo e alto níveis de arsenato, respectivamente.

Palavras-chave: resistência ao arsenato, *Thiobacillus ferrooxidans*

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MOTILE *AEROMONAS* SPP. IN RETAIL VEGETABLES FROM SÃO PAULO, BRAZIL

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ABSTRACT

A total of 90 retail vegetable samples, including lettuce (30), water-cress (30) and escaroles (30), were examined for detection and enumeration of motile *Aeromonas* spp. The isolation procedures employed were direct plating onto starch-ampicillin agar (enumeration) and/or selective enrichment in trypticase-soy broth containing ampicillin (establishment of positive or negative samples), both involving a 24 h incubation period at 28°C. *Aeromonas* species were detected in 43 samples (47.8%) and their numbers varied from less than 10^2 to up to 2.0×10^6 CFU/g. Water-cress samples presented the highest numbers of *Aeromonas* spp. Sixteen (37.2%) of the 43 positive samples, 11 of which were water cress, gave counts of 10^4 CFU/g or higher. The number of water-cress positive samples (70.0%) was significantly higher ($p<0,05$) than that of lettuce (43.3%) and of escarole (30.0%). No significant differences were found between both methods of detection of *Aeromonas* spp. when lettuce and water-cress were analysed. For escarole samples, however, positivity was significantly greater after selective enrichment. Of the 143 strains isolated and identified as *Aeromonas* spp., 138 were *A. caviae* (96.5%) , 4 were *A. hydrophila* (2.8%) and 1 (0.7%) was considered atypical due to its different biochemical profile. Results show that the vegetables examined might constitute a health hazard to consumers due to the frequency of detection of motile *Aeromonas* spp. and to the numbers present in positive samples.

Key words: *Aeromonas* spp., *A. hydrophila*, *A. caviae*, vegetables, food.

INTRODUCTION

The importance of motile *Aeromonas* species, previously known as *Aeromonas* of the *A. hydrophila* group (7), has been increasing in the last decade concerning their role as gastroenteritis agents. Buchanan (6) suggested that 13% of the reported cases of gastroenteritis might be ascribed to these bacteria. Several studies have tried to associate diarrhea with the presence of *Aeromonas* spp. in stools (3,9,12,13,27,28). However, experimental infection studies in primates (32) and human volunteers (26) were unsuccessful.

Nishikawa and Kishi (28) observed an increased frequency of *Aeromonas* spp. in stools during summer which was not matched by a marked increase in the

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isolation of the microorganisms from river and tap water. On the other hand, the authors recovered high numbers of motile *Aeromonas* from many different food samples, which lead them to conclude that infections caused by these bacteria are probably more food-borne than water-borne. Additionally, a big outbreak (1) and some other cases of food-borne infections caused by *Aeromonas* spp. have been described (19,35). Foods involved in some of these episodes revealed contamination levels of about 10^4 and 10^6 CFU/g (35).

An increasing number of studies are reporting the isolation of motile *Aeromonas* from different kinds of food in several countries, including the United States (4,8,29,31), England (11), Australia (16,23), New Zealand (15), Denmark (18), Sweden (20), Japan (28) and Brazil (22,34).

Beuchat (5) emphasizes the importance of *Aeromonas*' psychrotrophic nature, mainly in retail foods that can be eaten raw, like vegetables, and which are kept refrigerated, even during transport and sale.

Majeed *et al.* (24) suggest that gastroenteritis by *Aeromonas* spp. may possibly be a consequence of pre-formed toxin ingestion from contaminated food. Majeed and Mac Rae (25) detected the production of virulence factors like enterotoxin and haemolysin by *A. hydrophila* strains also at 4°C and Krovacek *et al.* (21) observed cytotoxin, enterotoxin and haemolysin production at the same temperature.

In the present study we investigated the occurrence and numbers of motile *Aeromonas* spp. in lettuce, water-cress and escarole samples and comparing the isolation procedures used, namely, direct plating onto starch-ampicillin agar (31) and after enrichment in trypticase-soy broth containing ampicillin (29), as well as identifying the isolated strains at the species level.

MATERIALS AND METHODS

Samples

A total of ninety vegetable samples, including 30 of lettuce, 30 of water-cress and 30 of escarole, were collected at 20 different retail markets and grocery shops in São Paulo, Brazil. The vegetables were transported to the laboratory immediately after purchase and the analysis started within less than two hours of collection.

Isolation procedures

Sampling was achieved by aseptically transferring 25 g to a sterile blender, which were then homogenized in 225 mL of 0.1% peptone saline solution by blending for 2-3 minutes. Tenfold serial dilutions up to 10^{-6} were subsequently made using the same diluent.

For *Aeromonas* spp. enumeration, 0.1 mL of each dilution was surface-spread onto starch-ampicillin agar (SAA) (31) and plates incubated at 28°C for 24 hours.

For *Aeromonas* spp. detection, 10 mL of the initial 10^{-1} dilution were transferred to tubes containing 10 mL of trypticase-soy broth (twice concentrated) with 2 mg/100mL of ampicillin (TSB-A). After gentle homogenization, tubes were incubated at 28°C for 24 hours. Following incubation, a loopful of enrichment broth culture was streaked onto the surface of SAA and plates incubated at 28°C for 24 hours.

After incubation, plates were examined for large, yellow to honey colonies surrounded by a yellow ring and surrounded by a clear zone; plates were flooded with Lugol iodine solution according to Palumbo *et al.* (31).

Presumptive counting proceeded in plates exhibiting 30 up to 300 colonies. Confirmation or correction of the presumptive counting was done after the identification of the isolated strains.

Up to 5 colonies from the counting plate and from the one resulting from enrichment were picked and placed on trypticase-soy agar (TSA) slants and triple-sugar-iron agar (TSI). Tubes were incubated at 28°C for 24 hours.

Identification of colonies

Presumptive colonies showing acid reaction both in the butt and on the slant of TSI agar, with little or no gas, were tested for oxidase and catalase in 24 hours cultures on TSA. Oxidase and catalase positive strains were scored as possible *Aeromonas* spp.

Using a 24 hours culture on TSA, suspected colonies were confirmed by Gram staining, motility, nitrate reduction, indol and acetoin production, glucose O/F, growth at 37°C and in 0%, 3% and 6% NaCl, utilization of sucrose, mannitol, inositol, salicin, arabinose, arginine, ornithine and lysine, gas production from glucose, esculin hydrolysis (2,7,29,33) and monotrichous flagellation (14).

Strains characterized as motile *Aeromonas* spp.,

were submitted to the ''CAMP-test'', according to Figura and Guglielmetti (10).

RESULTS AND DISCUSSION

Motile *Aeromonas* spp. were detected in 43 (47.8%) of 90 vegetable samples examined, either by one or by both isolation methods employed. The three kinds of vegetables analysed were positive for these microorganisms. Water-cress samples showed significantly higher positivity rates (70.0%) when compared to lettuce (43.3%) and escaroles (30.0%), according to the two proportion test with approximation through normal distribution at the 5% significance level (TABLE 1).

A great variation was observed between *Aeromonas* spp. numbers in the different vegetable samples. These numbers ranged from less than 10^2 to $2.0x10^6$ CFU/g. Sixteen (37.2%) of 43 positive samples showed counts of 10^4 CFU/g or higher, 11 of which consisted of water-cress (TABLE 2).

Callister and Agger (8) detected *Aeromonas* spp. in 38.1% of 21 vegetable samples using direct plating onto starch-ampicillin agar. The authors reported numbers of these microorganisms varying from 10^2 to $2.3x10^4$ CFU/g.

In the present investigation, positivity for *Aeromonas* spp. was higher (47.8%) in comparison with that observed by Callister and Agger (8). This difference between results might be explained by the simultaneous use of direct plating and enrichment

TABLE 1 - Absolute and relative positive results for *Aeromonas* spp. from vegetable samples as obtained by direct plating onto starch ampicillin agar and/or selective enrichment in trypticase soy broth containing ampicillin, São Paulo, 1993.

Samples	Lettuce		Water-cress		Escarole		Total	
	nr	%	nr	%	nr	%	nr	%
DP (+)	8	26.7	16	53.3	4	13.3	28	31.1
SE (+)	9	30.0	17	56.7	9	30.0	35	38.9
DP (+) & SE (-)	4	13.3	4	13.3	0	0	8	8.9
SE (+) & DP (-)	5	16.7	5	16.7	5	16.7	15	16.7
DP (+) & SE (+)	4	13.3	12	40.0	4	13.3	20	22.2
SE (+) & DP (+) in relation to total of positive samples (+)	-	30.8	-	57.1	-	44.4	-	46.5
nr. analysed	30	100.0	30	100.0	30	100.0	90	100.0
Positive (+)	13	43.3	21	70.0	9	30.0	43	47.8

DP = direct plating
SE = selective enrichment
(+) = positive samples
(-) = negative samples

TABLE 2 - Distribution and enumeration of *Aeromonas* spp. in vegetable samples, São Paulo, 1993.

Enumeration CFU/g*	Lettuce		Water-cress		Escarole		Total	
	nr	%	nr	%	nr	%	nr	%
$<10^2$	22	73.4	14	46.7	26	86.7	62	68.9
10^2 Á 10^3	1	3.3	-	-	2	6.7	3	3.3
10^3 Á 10^4	3	10.0	5	16.7	1	3.3	9	10.0
10^4 Á 10^5	3	10.0	9	30.0	1	3.3	13	14.5
10^5 Á 10^6	1	3.3	1	3.3	-	-	2	2.2
$\geq 10^6$	-	-	1	3.3	-	-	1	1.1
TOTAL	30	100.0	30	100.0	30	100.0	90	100.0

* CFU/g - colony forming units per gram

procedure in the present study. The higher numbers of microorganisms found in this study (up to $2.0x10^6$ CFU/g) exceed those reported by Callister and Agger (8). Water-cress samples contributed the most to the highest numbers of *Aeromonas* spp. recorded.

Nishikawa and Kishi (28), examining 43 vegetables and their products, including cabbage, carrot, cucumber, eggplant, lettuce, green peas, onion, pasta, potato, radish-sprouts, rice, spinach, tofu, tomato and vegetable salad, isolated *Aeromonas* spp. from 51.2% of the samples.

Fricker and Tompsett (11) detected *Aeromonas* spp. in 21.6% of 97 pre-made salad samples and Knochel and Jeppesen (18) in only 10.2% of 177 mayonnaise salad samples which, however, contained higher numbers of the microorganisms, exceeding 10^5 CFU/g.

Hudson and de Lacy (15) recovered *Aeromonas* spp. from 9.1% of 44 green salad and coleslaw samples. A coleslaw salad without mayonnaise was the only positive sample of that kind. The authors concluded that mayonnaise and other salad dressings may lower the pH thus preventing grow of *Aeromonas* spp. or even promoting death of these bacteria. According to them, this fact could possibly explain the low *Aeromonas* spp. detection rates observed for the salad samples analysed by them as well as others mentioned above.

Leitão and Silveira (22) detected *Aeromonas* spp. in 66.7% of 15 lettuce samples analysed and their numbers varied from $2.3x10^3$ to $2.4x10^5$ CFU.

Rodrigues (34) analysed 100 food samples of animal and vegetable origin. *Aeromonas* spp. were detected in 18% of the vegetable ones.

In this study, positivity values for both isolation methods were significantly different for escarole samples but not for lettuce or water-cress samples, at a 5% significance level. In the former case, the enrichment procedure showed a better performance, probably due to the fact that no escarole sample resulted positive through direct plating and negative after selective enrichment.

TABLE 1 shows that, for some samples, *Aeromonas* spp. was detected only after enrichment. This fact can be easily explained by the presence of low numbers of these bacteria or, alternatively, the presence of injured microorganisms. At the same time, other samples revealed the presence of *Aeromonas* spp. only when isolated by direct plating. A reasonable explanation for this is the possibility of not tranferring *Aeromonas* spp. from the enrichment broth to the agar or the competition between *Aeromonas* spp. and bacteria also resistant to ampicillin in the enrichment broth.

In terms of competition between *Aeromonas* spp. and other bacteria, Palumbo and Buchanan (30) reported that *A. hydrophila* reached higher numbers in ground pork without its natural microflora. Kirov *et al.* (17) observed that an *A. sobria* strain isolated from chicken did not grow well in competition with a *Pseudomonas fragi* strain under refrigeration temperature.

Okrend *et al.* (29) observed that the use of enrichment procedures improved the recovery of *Aeromonas* spp. mainly when these bacteria were found in low numbers or injured by freezing.

Majeed *et al.* (23) detected *Aeromonas* spp. in 26% and in 59% of lamb meat and offal samples examined, by direct plating onto starch-ampicillin agar and after enrichment in 0.1% alkaline peptone water, respectively.

Of the 187 isolated strains suspected of being *Aeromonas* spp. in the present investigation, 143 (76.5%) were confirmed as so. Palumbo *et al.* (31), Callister and Agger (8), Majeed *et al.* (23) and Leitão and Silveira (22), using the same agar, confirmed 80% ,74% , 100% and 79% of the isolated strains, respectively.

The predominant species isolated in this study was *A. caviae*. Of the 143 strains confirmed as *Aeromonas* spp., 138 (96.5%) were *A. caviae*, 4 (2.8%) were *A. hydrophila* and 1 (0.7%) was considered as atypical due to its different biochemical profile. TABLE 3 shows the distribution of the different species isolated in relation to the vegetable analysed.

Callister and Agger (8) identified 29 strains as *A. hydrophila* and 16 as *A. caviae* from a total of 45 strains isolated from vegetables. Nishikawa and Kishi (28) identified 17 strains as *A. caviae* and 10 as *A. hydrophila* from a total of 27 strains isolated from foods of vegetable origin.

The results of this investigation show that the vegetables examined, particularly water-cress, may represent a health risk to consumers considering the detection and numbers of motile *Aeromonas* spp. present, as they are usually eaten raw. Although the precise infecting dose for these bacteria has not been established yet, clinical and epidemiological evidence lead to the idea that foods containing high numbers of these microorganisms may result in infection,

TABLE 3 - Positive samples for *Aeromonas* spp. and for different species of the genus isolated from several types of vegetables (São Paulo, 1993)

Vegetable	Number and % of *Aeromonas* positive samples							
	Aeromonas spp.		*A. hydrophila*		*A. caviae*		*A. caviae* + atypical *Aeromonas*	
	nr	%*	nr	%**	nr	%**	nr	%**
Lettuce	13	30.2	2	15.4	11	84.6	-	-
Water-cress	21	48.9	1	4.8	19	90.4	1	4.8
Escarole	9	20.9	-	-	9	100.0	-	-
Total	43	100.0	3	7.0	39	90.7	1	2.3

* % to total positive samples.

** % positive for each stated species in relation to total *Aeromonas* spp positive samples of the typeof vegetable analysed.

especially among susceptible people like the elderly, individuals with underlying malignancies and young children. Therefore, it may be advisable to take adequate hygienic measures to avoid direct ingestion of vegetables contaminated with *Aeromonas* spp., whose numbers may increase in these foods while they are kept under refrigeration (8). It may also be advisable to avoid recontamination of prepared food through contamination of kitchen utensils and working surfaces by raw vegetables containing high numbers of *Aeromonas* spp.

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RESUMO

Aeromonas spp. móveis em hortaliças comercializadas em São Paulo, Brasil

Em um total de 90 amostras de hortaliças, incluindo 30 de alface, 30 de agrião e 30 de escarola, foi verificada a ocorrência de bactérias móveis do gênero *Aeromonas*, empregando-se os métodos de semeadura direta em ágar amido-ampicilina (contagem) e após enriquecimento em caldo tripticase-soja adicionado de ampicilina (teste de presença/ausência). As incubações foram feitas a 28°C, durante 24 horas. Foi detectada a presença dessas bactérias em 43 (47,8%) das amostras analisadas, com contagens variando de $<10^2$ a $2,0x10^6$ UFC/g. As amostras de agrião foram as que se revelaram, na contagem, com maiores números de *Aeromonas* spp. Das 43 amostras positivas para *Aeromonas* spp., 16 (37,2%) revelaram-se com números iguais ou superiores a 10^4 UFC/g, sendo que 11 eram de agrião. Dentre as amostras de hortaliças analisadas, as de agrião revelaram-se com positividade para *Aeromonas* móveis (70,0%) significativamente maior em relação às de alface (43,3%) e de escarola (30,0%) a nível de 5% . Não foram observadas diferenças significativas entre as positividades obtidas através do método de semeadura direta em placas e do teste de presença/ausência para as amostras de alface e de agrião. Para as de escarola, a positividade foi significativamente mais alta no teste de presença/ausência. Do total de 143 cepas confirmadas como sendo do gênero *Aeromonas*, 138 (96,5%) eram de *A. caviae*, 4 (2,8%) de *A. hydrophila* e 1 (0,7%) que, pelas características bioquímicas, foi considerada como *Aeromonas* atípica. Dos resultados obtidos, pode-se depreender que, dado os níveis de contaminação observados, essas hortaliças podem representar risco aos consumidores.

Palavras-chave: *Aeromonas* spp., *A. hydrophila*, *A. caviae*, hortaliças, alimentos.

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RECOVERY OF ACID INJURED SALMONELLAE FROM ARTIFICIALLY CONTAMINATED MAYONNAISE

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ABSTRACT

Laboratory procedures for the recovery of acid-injured salmonellae (10^3 CFU/g) from artificially contaminated mayonnaise samples (pH ranging from 4.0 to 4.5) stored at 4°C and at room temperature (30°C) were evaluated. Aliquots were withdrawn at every 24 hours and submitted to the following procedures: direct pour plating onto tryptic soy agar (TSA), repair period in TSA at room temperature for 4h, followed by overlaying with SS agar and incubation at 35°C for 24h; direct plating onto TSA, repair period at room temperature for 4h followed by overlaying with Hektoen enteric agar and incubation at 35°C for 24h; pre-enrichment in buffered peptone water at 35°C for 24h and selective enrichment in Kauffmann's tetrathionate, selenite-cystine and Rappaport-Vassiliadis broths and incubation at 35°C for 24h, at 35°C for 24h and at 43°C for 24h, respectively, followed by plating onto SS agar and Hektoen enteric agar and incubation at 35°C for 24h. In samples held at room temperature no surviving salmonella cells were detected after 24h, regardless of the method of analysis. In samples held at 4°C, surviving cells were observed after up to 72h at pH 4,0 and 4,2 and up to 96h in samples at pH 4,5 only when pre-enrichment and selective enrichment steps were used. No significant difference was observed between the performances of the three enrichment broths.

Key words: salmonellae, acid injury, laboratory methods, mayonnaise

INTRODUCTION

Microbial cells may suffer injury when exposed to an acid environment. Such injury may be irreversible, when cells are considered dead, or reversible, when cells are sublethally damaged and remain recoverable. Injured microorganisms may be revived or allowed to repair the damage if incubated in an appropriate environment (6).

Establishment of adequate laboratory procedures for detection of sub-lethally damaged salmonella cells in food remains a challenge. Many studies have been carried out on this subject and all used a methodology based on repair of injury in appropriate culture conditions (1,2,6). Direct plating of food samples onto salmonella selective agar is not recommended for isolation of colonies because selective agents in these media may be extremely inhibitory to stressed cells (4,7). Prior to plating, repair of cell damage is necessary. For repair, a pre-enrichment step in a non-selective broth is used, followed by a selective enrichment procedure and plating onto appropriate selective agars. Stranz and Zottola (8), proposed an alternative plating technique

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for recovery and enumeration of stressed salmonella cells. The technique is based on the inclusion of a 4h repair period at room temperature in non-selective agar and subsequent overlaying on selective agar . The main advantage of this alternative procedure is that it reduces analytical time, since the repair period replaces the pre-enrichment and selective enrichment steps and isolated colonies become available in 24h.

This study was conducted to evaluate these laboratory procedures for isolation of damaged salmonellae in an acid egg-based food product. Artificially contaminated mayonnaise samples, with pH 4.0, 4.2 and 4.5, stored under refrigeration (4°C) and at room temperature (30°C), were used as the experimental model.

MATERIALS AND METHODS

Mayonnaise samples: Commercial mayonnaise samples were obtained from a local source. The reported composition of the product was: eggs, vegetable oil, vinegar, salt, sugar, lemon juice and antioxidant. The pH was about 4.0. Samples with pH 4.2 and 4.5 were obtained by adding 0.1N NaOH.

Test microorganism: *Salmonella typhimurium* ITAL-SLML 001, provided by Instituto de Tecnologia de Alimentos, Campinas, Brazil, was used. The strain was stored in tryptic soy agar under refrigeration.

Culture media: The media used included: tryptic soy agar (TSA), SS agar (SSA), Hektoen enteric agar (HEA), triple sugar iron agar (TSIA), lysine iron agar (LIA), tryptic soy broth (TSB), selenite-cystine broth (SCB) and Kauffmann's tetrathionate broth (TTB). All these media were from Difco Laboratories Ltd. Buffered peptone water (BPW) was prepared according to Edel and Kampelmacher (5). Rappaport-Vassiliadis broth was formulated according to Vassiliadis (9).

Experimental contamination: Cultures of *Salmonella typhimurium* were grown at 35°C for 24 h in TSB, with gentle shaking. Decimal dilutions of the cultures were made and dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated ontoTSA for size determination of the inoculum (CFU/ml) used in the experimental contamination. 3 ml of a 10^{-5} dilution were added to 297g of mayonnaise and homogenized, resulting in a final concentration of ca 10^3 CFU/g of sample. Half of the contaminated sample was kept in a refrigerator (4°C) and the other half was incubated at 30°C.

Methods for isolation of salmonellae: 25 g of each contaminated sample were removed immediately after inoculation and at every 24h. After homogenization with 225 ml of BPW, the following procedures were used:

procedure 1: 1 ml of the homogenate was pour-plated ontoTSA (10-12 ml) and incubated at 35°C for 24 h;

procedure 2: 1 ml of the homogenate was transferred to a Petri dish and mixed with 5 ml of TSA. The plates were held at room temperature for 4 h (repair period). Afterwards, the plates were overlaid with 10 ml of HEA and incubated at 35°C for 24 h;

procedure 3: same as procedure 2 but using SSA for the overlay;

procedures 4 and 5: the remaining homogenate in BPW was incubated at 35°C for 24 h. Portions of 1 ml were transferred to 10 ml of TTB (procedure 4) and to 10 ml of SCB (procedure 5). Both broths were incubated at 35°C for 24 h and streaked on HEA and SSA and incubated at 35°C for 24 h;

procedure 6: After incubation of BPW at 35°C for 24 h, 0.1 ml was transferred to 10 ml of RVB and incubated at 43°C for 24 h. The broth was streaked on SSA and HEA and incubated at 35°C for 24 h.

In procedures 1, 2 and 3, colonies were enumerated and three of them submitted to biochemical identification in TSI and LIA. In procedures 4, 5 and 6, growth of typical colonies of salmonellae on SSA and HEA, subsequently confirmed in TSIA and LIA, was recorded as present (+) or absent (-).

The experimental contamination of mayonnaise was done three times, using three different samples. Control experiments were done using uninoculated mayonnaise samples submitted to the above mentioned laboratory procedures.

RESULTS AND DISCUSSION

As shown in TABLE 1, the isolation procedures that included plating onto non-selective agar with a 4h repair period at room temperature before addition of selective agar were inadequate for the recovery of salmonellae from mayonnaise: all contaminated samples resulted salmonella negative, regardless of the pH and temperature of storage. TABLE 2 indicates that when conventional laboratory procedures using pre-enrichment, selective enrichment and plating on selective agar were used, salmonellae were recovered

TABLE 1: Recovery of salmonellae from mayonnaise samples artificially contaminated with 3.5 log CFU/g and stored at 4°C or 30°C, using direct plating with and without a repair period

laboratory procedure	pH of sample	temperature of storage	counts* (log CFU/g) after 0 h	24 h	48 h
direct pour-plating onto TSA (proc.1)**	4.0	4°C or 30°C	2.2	<1	<1
	4.2	4°C or 30°C	2.8	<1	<1
	4.5	4°C or 30°C	3.0	<1	<1
with 4h repair period on TSA overlaying with HEA (proc.2)**	4.0	4°C or 30°C	2.1	<1	<1
	4.2	4°C or 30°C	2.7	<1	<1
	4.5	4°C or 30°C	3.0	<1	<1
with 4h repair period on TSA overlaying with SSA (proc.3)**	4.0	4°C or 30°C	2.1	<1	<1
	4.2	4°C or 30°C	2.1	<1	<1
	4.5	4°C or 30°C	2.1	<1	<1

* = average of six experiments (three at 4°C and three at 30°C)
** = see text for description of procedures

TABLE 2: Recovery of salmonellae from mayonnaise samples artificially contaminated with 3.5 logCFU/g and stored at 4°C or 30°C, using conventional methods (pre-enrichment, selective enrichment and plating)

laboratory procedure	pH of sample	temperature of storage	presence of salmonellae* after 0h	24h	48h	72h	96h
proc. 4, 5 and 6**	4.0	4°C	+	+	+	-	-
		30°C	+	-	-	-	-
	4.2	4°C	+	+	+	-	-
		30°C	+	-	-	-	-
	4.5	4°C	+	+	+	+	+
		30°C	+	-	-	-	-

+ salmonellae present
- salmonellae absent
* most frequent result in three experiments
** see text for description of procedures

but only from samples maintained under refrigeration.

Failure to detect survivors in samples held at room temperature, regardless of the isolation procedure, indicates that salmonella cells are irreversibly damaged as a consequence of the acid environment. Results of the direct plating procedure onto non-selective agar (procedure 1) show that the acid environment caused an immediate reduction in the counts of viable cells, which was dependent on the pH of the sample. AS can be seen in TABLE 1, counts of viable salmonellae at time 0h were 1.3, 0.7 and 0.5 log CFU/g lower than the initial inoculum in samples at pH 4.0, 4.2 and 4.5, respectively. Refrigeration of mayonnaise seems to protect salmonella cells from acid injury to some extent: as survivors were recovered after up to 48h from samples at pH 4.0 and 4.2 and up to 96h from samples at pH 4.5. It must be emphasized that recovery of stressed cells was possible only when conventional laboratory procedures were used and that the performance of the three enrichment procedures was the same. Similar effects were reported by Blakenship (3), who observed that the injury of *S. bareilly* by acetic acid increased with an increase in both acid concentration and temperature. The effects of various compounds on the recovery of *S. bareilly* indicated that it was dependent on protein and RNA synthesis.

The ineffectiveness of the procedure that includes a 4h repair period at room temperature can be explained by the amount of sample analyzed: while in the repair period method (procedures 1, 2 and 3) 0.1g of sample is plated, in conventional methods (procedures 4, 5 and 6) 25g of sample are submitted to enrichment. It must be pointed out as well that enrichment in liquid media is more effective than in solid media, since in the former case damaged cells can both recover from injury and multiply (2).

Another point to be considered is that injured microbial cells have an extended lag phase related to their metabolic cellular damage. It is during the extended lag phase that injured cells repair the injury and synthesize the proteins and nucleic acids needed for growth (2). Consequently, the 4h period may be insufficient for adequate recovery from acid stress.

These results indicate that the method which includes a resuscitation period of 4h at room temperature for the recovery of stressed salmonellae from acid foods is very limited. It can only examine relatively small samples and is suitable only for samples with a high level of surviving salmonella cells. To monitor this pathogen in this type of food, the conventional pre-enrichment plus selective enrichment procedures prior to plating onto selective agars must be used.

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RESUMO

Recuperação de salmonelas injuriadas pelo pH ácido de maionese artificialmente contaminada

Este estudo refere-se à avaliação de métodos laboratoriais para o isolamento de salmonelas (10^3 UFC/g) de amostras de maionese artificialmente contaminadas (pH entre 4.0 e 4.5), mantidas a 4°C e em temperatura ambiente. De cada amostra contaminada, alíquotas foram retiradas a cada 24h e submetidas aos seguintes procedimentos: plaqueamento direto em superfície em agar soja tríptica (TSA) seguido de periodo de reparo de 4h em temperatura ambiente e posterior adição de agar SS e incubação a 35°C por 24h; plaqueamento direto em superfície em TSA seguido de periodo de reparo de 4h em temperatura ambiente e posterior adição de agar Hektoen enteric e incubação a 35°C por 24h; pré-enriquecimento em água peptonada tamponada a 35°C por 24h, enriquecimento seletivo em caldo tetrationato de Kauffmann, em caldo selenito-cistina e em caldo Rappaport-Vassiliadis, com incubação a 35°C por 24h, a 35°C por 24h e a 43°C por 24h, respectivamente, seguido de plaqueamento em agar SS e agar Hektoen enteric com incubação a 35°C por 24h. Nas amostras mantidas em temperatura ambiente, nenhuma salmonela sobrevivente foi detectada 24h após o início dos experimentos, independentemente do método de análise. Nas amostras mantidas refrigeradas, salmonelas sobreviventes foram observadas até 72h após o início dos experimentos nas amostras com pH 4.0 e 4.2 e até 96h naquelas com pH 4.5. Essa observação somente foi possível quando as metodologias que empregam etapas de pré-enriquecimento e enriquecimento seletivo foram utilizadas. Não foi notada nenhuma diferença significativa no comportamento dos três caldos de enriquecimento utilizados.

Palavras-chave: salmonelas, injúria ácida, métodos analíticos, maionese.

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THE EFFECT OF CARBOHYDRATE ADMINISTRATION ON EXPERIMENTAL INFECTION WITH *SALMONELLA* SEROTYPES IN CHICKENS

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ABSTRACT

Carbohydrates and cultures of faecal microflora were administered to newly hatched chicks to prevent infection with *Salmonella typhimurium, Salmonella enteritidis, Salmonella agona and Salmonella infantis*. Birds were killed 72 hours after challenge and the number of viable *Salmonella* organisms in their caecal contents estimated. Carbohydrates did not promote efficient control of infection with the *Salmonella* serotypes tested whereas cultures of faecal microflora completely prevented infection with all serotypes.

Key words - *Salmonella* control, carbohydrates, competitive exclusion

INTRODUCTION

In their first days of life, chickens are more susceptible to infection by paratyphic salmonellas. Contamination at this time may result in clinical disease and also in a greater number of birds excreting the organisms in faeces and for longer periods of time than do chickens infected at a later age (20; 28). The gradual emergence of resistance is thought to be due to the establishment of a natural microflora in the alimentary tract (1; 24). Oral administration of material derived from native gut microflora of adult chickens to newly hatched chicks has been shown to prevent the infection by enteric salmonellae; this is known as the Nurmi Concept or Competitive Exclusion (CE) (23). Nevertheless, under field conditions, CE treatment has been considered efficient only when the presence of *Salmonella* is minimal (13; 27). Prevention of *Salmonella* infection may help to avoid the disease in birds and also the occurrence of foodborne disease from avian sources. Besides Competitive Exclusion, other attempts to increase the resistance of young chickens to *Salmonella* organisms have been tried. Instead of receiving faeces from adult hens, young birds are given carbohydrates which may act as substrates for non-pathogenic bacteria thus stimulating their growth, or as inhibitors of adhesion between pathogenic bacteria and the intestinal epithelium. Of the sugars tested, lactose and D-mannose have been shown to possess some protective action (8; 25).

Control of salmonellosis in poultry under field conditions has not been an easy task. Preventive methods such as hygiene, disinfection and control measures of food and foodstuff quality are impaired by modern mass production systems and by the lack of investments in human resources. For this reason, farmers are advised to include certain drugs in the dietary formulation without appropriate criteria for

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use. Considering that carbohydrates are not harmful to chickens or human beings, the present work was undertaken to assess the effect of adding carbohydrates to the drinking water and diet of newly hatched chicks as a preventive measure against *Salmonella* infection of the intestinal tract. Treatment by ingestion of a culture of faeces from laying hens of a commercial flock was also included in the analysis.

MATERIALS AND METHODS

Chickens

One-day-old hybrid broiler chicks were purchased from a commercial hatchery. Samples of cloacal swabs taken from birds on delivery showed them to be free of *Salmonella* infection. The birds were randomly assigned to groups of 10 animals each, reared in wooden boxes with paper bedding, kept under an electric heat source and given water and a balanced unmedicated commercial ration ad libitum (Starter diet).

Bacterial strains

Strains *S. typhimurium* F98, *S. infantis* and *S enteritidis* used in the trials were obtained from Dr. Paul Barrow, Compton Laboratory, UK. The *S. agona* strain was provided by Instituto Adolfo Lutz, São Paulo, Brazil. Mutants resistant to nalidixic acid and spectinomycin (Nal/Spec) were produced by the method of Smith and Tucker (1980).

Unless otherwise indicated, all *Salmonella* strains were cultured overnight in 10 ml of nutrient broth, at 37°C and with agitation. They contained approximately 10^9 cfu/ml.

The four *Salmonella* serotypes were chosen for challenge of chicks because of their association with human and avian salmonellosis. *S. infantis* is also a good colonizer of the intestinal tract and very difficult to control. *S. agona* is very common in Brazil, having been isolated from humans (Non-published data from Instituto Adolfo Lutz of São Paulo, Brazil) and from the poultry industry (2).

Experimental design

The trials were designed to test the inhibition of *Salmonella* by lactose, D-mannose, dextrose and saccharose which were added to the drinking water at 2.5% and 5% concentrations as well as to the diet. Additionally, 5% fat-free dried milk as a source of lactose was incorporated into the diet for assessment. As a standard positive control for inhibition, 0.1 ml of a culture of faeces from commercial laying hens was administered by gavage into the crop of newly hatched chicks. The culture of faecal material was prepared in nutrient broth and incubated overnight at 37°C. Previous results have indicated that static aerobic incubation produces an inhibition comparable to that obtained by anaerobic culture (Berchieri Jr. and Barrow, unpublished results). The faecal culture was found to be free of *Salmonella* sp before use. Forty-eight hours later chickens were challenged by gavage with 10^5 viable organisms of the appropriate *Salmonella* strain in 0.1 ml volume. Chicks were killed after a further three days and the number of viable cells of the challenge *Salmonella* strain per gram of caecal contents estimated. Appropriate control groups were included in all experiments.

Bacterial enumeration

Viable counts of bacteria were determined by the method of Miles *et al.* (19) using brilliant green agar (Difco) containing nalidixic acid (100µg/ml) and spectinomycin (100µg/ml).

RESULTS

The effect of adding different carbohydrates and fat-free dried milk to water and feed of chicks on the number of distinct viable *Salmonella* serotypes in their caecal contents is shown in TABLE 1. High caecal counts were observed in untreated birds. Administration of a culture of adult chicken faeces prevented the establishment of infection. The addition of D-mannose, dextrose and saccharose to the drinking water had no beneficial effect. A reduction in the viable *Salmonella* counts was obtained by using lactose in the feed or water. This effect was more pronounced when lactose was diluted in the water, being greater for *S. enteritidis* and much less noticeable for *S. infantis*. High *Salmonella* counts were always recorded for some birds of each group even when the median values were low. The addition of fat-free dried milk to the diet caused a reduction in *S. typhimurium, S. enteritidis* and *S. agona* numbers but had no effect on *S. infantis*. Treatment with the

TABLE 1 - Number of *Salmonella* organisms Nal/Spec in the caecal contents of chickens

Treatments	log_{10} viable number of *Salmonella* organism Nal/Spec per gram of caecal contents			
	STM F98	*S.enteritidis*	*S.agona*	*S.infantis*
Lactose 2.5% (drinking water)	2.7* (N -8.6)	N (N - N)	7.1 (5.2-8.8)	8.1 (N -9.0)
Lactose 2.5% (diet)	6.3 6.3	2.9 (N -6.7)	6.6 (N -9.0)	8.3 (6.5-9.1)
D-mannose 2.5% (drinking water)	8.9 (8.9-9.0)	8.3 (7.4-7.8)	8.6 (8.0-8.9)	8.5 (6.0-9.4)
Dextrose 2.5% (drinking water)	8.2 (N -9.2)	8.9 (7.6-9.8)	8.3 (7.2-9.0)	9.1 (9.1-9.1)
Saccharose 2.5% (drinking water)	9.0 (7.8-9.3)	8.0 (6.5-9.0)	8.3 (6.6-9.0)	8.6 (6.4-9.4)
Fat-free dried milk 5.0% (diet)	N (N - N)	5.3 (N -8.8)	3.7 (N -7.6)	9.0 (8.9-9.1)
Faecal culture	N (N - N)	N (N - N)	N (N - N)	N (N - N)
Control	8.6 (7.4-9.8)	8.3 (6.5-9.2)	7.7 (6.2-9.0)	9.4 (7.5-9.5)

STM F98 = *S. typhimurium* strain F98
* The median count per gram is shown with the range in parenthesis.
N = <2.0

TABLE 2 - Number of Nal/Spec *Salmonella* organisms per gram of caecal contents from chickens.

Treatments	log_{10} viable number of Nal/Spec *Salmonella* strains			
	STM F98	*S.enteritidis*	*S.agona*	*S.infantis*
	Inoculum of 10^4 cells of challenge organisms			
Lactose 5% (drinking water)	2.3* (N** -5.4)	2.5 (N -6.3)	4.5 (N -8.5)	6.8 (4.4-8.6)
Control	7.8 (5.2-8.5)	5.6 (N -8.2)	8.0 (6.6-8.8)	8.0 (6.4-8.7)
	Inoculum of 10^3 cells of challenge organisms			
Lactose 5% (drinking water)	N (N - N)	N (N - N)	7.9 (7.1-8.6)	4.1 (N -7.2)
Lactose 5% (diet)	____***	____	5.7 (N -8.9)	4.4 (N -8.6)
Control	6.4 (N -8.4)	7.4 (N -8.4)	7.2 (6.0-8.2)	5.4 (N -7.5)

STM F98 = *S. typhimurium* strain F98
* The median count per gram is shown with the range in parenthesis.
** N= <2.0
*** Not done

culture of faecal microflora induced a good reduction of all serotypes tested.

A combination of 2.5% lactose in the drinking water with the oral administration of a competitive exclusion culture induced very good inhibition, similar to that caused by CE alone (results not shown).

The effects of adding 5% lactose to the feed or water on intestinal colonization by the four *Salmonella* serotypes are presented in TABLE 2. In this case, the inoculum used contained approximately 10^4 or 10^3 *Salmonella* organisms per dose. A decrease of caecal counts below control values was observed for *S. typhimurium* and *S.enteritidis*, but not for *S. agona* and *S. infantis*.

DISCUSSION

Most *Salmonella* organisms have the ability to infect chicks and persist in their alimentary tract. Industrial procedures for rearing chickens favour the presence of these bacteria in poultry farms and their control becomes very difficult to achieve through standard measures of hygiene and sanitation (21). Acquired immunity may be generated by vaccination but the effect is not immediate (3) whereas pre-colonization by protective organisms or a complex intestinal microflora may induce the rapid onset of resistance (15). Oral administration of certain carbohydrates added to feed or drinking water has also been shown to effectively reduce *Salmonella* caecal counts; it is thought that the sugars enhance colonization by desirable inhibitory bacteria in the gut or that they prevent the adherence of some pathogenic bacteria to the intestinal epithelium (8; 25).

Dextrose and saccharose failed to inhibit the intestinal colonization by *Salmonella*. These sugars are digested in the alimentary tract and are absorbed before they reach the caecum (10). The utilization of D-mannose to block adherence of *Salmonella* to the caecal epithelium has been suggested by results from "in vitro" studies (17; 26). It was shown "in vivo" (25) that D-mannose was capable of reducing the percentage of broilers contaminated with *S.*

typhimurium. However, in the present study, the establishment of all *Salmonella* serotypes in the caeca of birds treated with D-mannose was not different from that of the control groups. It has been demonstrated (16) that there is no advantage in giving D-mannose to broilers to control *Salmonella* infection, even when this sugar is offered to the birds for the entire rearing production period (16). Lactose is not digested by the bird, so it can reach the caeca and be assimilated by the native gut microflora (14). The concomitant production of lactic acid by the native microflora is thought to inhibit the establishment of *Salmonella* organisms (11; 14; 25). Various workers have demonstrated that lactose reduces caecal colonization by *S. typhimurium* (18; 25), *S. enteritidis* and *Salmonella senftenberg* (4; 5). The beneficial effect of lactose was also observed in this experiment with serotypes *S. typhimurium* and *S. enteritidis* but not with *S. agona* and *S. infantis*. The fat-free dried milk treatment caused some inhibition of *S. typhimurium*, *S. enteritidis* and *S. agona*, as also observed by Deloach *et al.* (9). The results presented herein do not encourage the use of lactose as they show that lactose cannot ensure that chickens will reach slaughter-age free of all *Salmonella* serotypes.

Other workers have suggested that lactose may help prevent the infection with a small inoculum of *Salmonella* organisms (7; 22; 30; 33). Lactose was administered in the drinking water of birds at a 5.0% concentration for all tested serotypes; in the case of *S. agona* and *S. infantis* the same concentration of the sugar was incorporated into the diet. Birds were challenged using an inoculum of 10^3 or 10^4 org per dose. Once again, the present findings showed that lactose may not be effective for all *Salmonella* serotypes as has been reported for *S. typhimurium* by previous works (7; 22; 30; 31).

It has been suggested that lactose administration helps the development of a beneficial intestinal microflora (6;7;12;14). In the present study, no additional effect was found when treating chicks with a faecal culture together with lactose but different conditions may have been required to show this. The flora itself was completely inhibitory thus an additive effect would not have been detectable.

According to the data obtained, the administration of carbohydrates to young chickens to prevent *Salmonella* infection may not be as beneficial as has been reported. Even lactose, which to some extent inhibited intestinal colonization by *S. typhimurium* and *S. enteritidis*, was unable to induce a good reduction in the colonization of all four *Salmonella* serotypes used for challenge. Besides, lactose was found to cause an intense diarrhea, as also observed by Tellez *et al.* (29).

To resume, lactose and other carbohydrates appear to be less effective as control agents of salmonellosis than has been previously asserted. Their differential effect on distinct *Salmonella* serotypes was interesting and may be a fruitful area for future work. Such studies may not only lead to a better understanding of the mechanism of inhibition caused by carbohydrate administration on the establishment of bacterial populations but also improve our knowledge on intestinal colonization by *Salmonella* sp and other enteric bacteria.

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RESUMO

Efeito da administração de carboidratos na infecção experimental de galinhas com sorotipos de *Salmonella*

Carboidratos e cultura fecal foram administrados a pintos de um dia de idade para prevenir a infecção por *Salmonella typhimurium, Salmonella enteritidis, Salmonella agona, e Salmonella infantis*. As aves foram sacrificadas 72 horas após o desafio, e o número de organismos viáveis de *Salmonella* nos conteúdos cecais foi estimado. Os carboidratos não se mostraram adequados para o controle dos sorotipos de *Salmonella* utilizados neste estudo, enquanto a cultura fecal mostrou uma proteção completa da infecção contra todos os sorotipos testados.

Palavras-chave: salmonelose aviária, controle, carboidratos, exclusão competitiva

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SEROTYPES AND VIRULENCE OF *SALMONELLA* SP. ISOLATED FROM FRESH WATER

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ABSTRACT

Twenty six *Salmonella* isolates of different serotypes were found in samples of fresh water. All 26 strains were checked for the presence of plasmids. Plasmids were found in eleven isolates. Four of the plasmid-containing strains and two of the plasmid-free strains were tested for invasiveness in mice. At least in the case of *S. typhimurium*, it seems reasonable to link virulence with plasmid harbouring.

Key words: fresh water; *Salmonella*; virulence.

INTRODUCTION

Water can represent a habitat for enteropathogenic bacteria which enter host organisms by ingestion of such water, thereby exerting their pathogenicity. One such bacterial genus is *Salmonella* sp.

Salmonella sp initially colonize and invade the ileal mucosa, proliferating within the lamina propria. In systemic illness, the mesenteric lymph nodes are contaminated and infection proceeds through the lymphatic system, reaching the liver and spleen. In the latter case, bacteria can invade the bloodstream and lead to the patient's death (4,5,14,20).

It is generally believed that chromossomal genes are responsible for *Salmonella*'s virulence yet various authors have associated it with the presence of plasmids. A plasmid of approximately 60MDa has been linked with the virulence of *S. typhimurium* (10,11,12,13,16). In the case of *S. enteritidis, S. dublin* and *S. choleraesuis* plasmids of 37MDa, 56MDa and 30MDa seem to play such role, respectively (2,13,19).

Salmonella sp. has been isolated from various types of water (1,6,9,17,18). However, virulence of the isolates was not investigated in the majority of these studies. Therefore, the capacity of the isolates to act as enteropathogens has not been demonstrated. In order to clarify the question and evaluate the possibility of a link between contaminated water and human disease, the presence or absence of *Salmonella* sp. in several water samples from Araraquara, S.P., Brazil, was investigated in the present work. It was also analysed whether the serotypes isolated from water were the same as those associated with human gastroenteritis. Additionally, plasmid profiles were characterized in all *Salmonella* sp. isolated and some were checked for invasiveness in mice.

MATERIALS AND METHODS

Water samples

Two hundred and eight fresh water samples (126 without any treatment and 82 with hypochloride treatment) were analysed. Samples were collected in

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Araraquara, State of São Paulo, Brazil, and included: 62 from lakes, 19 from reservoirs; 14 from rivers, 16 from springs; 6 from artesian wells; 9 from non-artesian wells; 73 from treated swimming pools and 9 from tap water.

Salmonella sp. isolation and characterization

Four liters of water were concentrated onto 0.45 µm pore size membrane filters which were then fragmented. For enrichement of the microorganisms, half of each filter was placed in selenite broth and incubated at 42°C and half in tetrathionate broth and incubated at 37°C. Isolations were performed on SS Agar and Brilliant Green Agar. Identification was carried out as described by Ewing (7).

Plasmid DNA analysis

Plasmid DNA was prepared by alkaline lysis, as described by Birnboim and Doly (3). For the molecular weight calculation of the plasmids detected, *E. coli* standards carrying the following plasmids were used: pTP124 (120MDa); pJPNII (66MDa); pRP4 (35MDa); pSa (23MDa); pRK290 (13,2MDa); pBR325 (2,1MDa).

Invasion test

Six *Salmonella* sp. were chosen for further testing, 4 containing plasmids (*S. miami, S. typhimurium, S. heidelberg, S. anatum*) and 2 plasmid-free (*S. miami, S. agona*).

The invasion test was done according to the methodology reported by Helmuth *et al.* (13) using swiss albino mice inoculated via their intragastric tracts. On the 2nd, 4th, 7th and 20th days of infection, three animals were slaughtered and their livers and spleens removed. The organs were ground up in a 0.85% saline solution. The resulting homogenate was serially diluted (from 10^{-1} to 10^{-8}) and 0.1 ml of the dilutions plated on SS agar plates. After 18 - 24 hours of incubation, the number of colony-forming units (CFUs) in the original organs was calculated, taking the mean for every three animals.

RESULTS

Twenty-six strains of *Salmonella* sp were isolated from 208 water samples (6 from lakes, 14 from

TABLE 1 - Serotypes of *Salmonella* sp. isolated from 208 water samples.

Serotype	Nr. of isolations	Source
Salmonella oranienburg	5	3 Reservoir, 2 Lake
Salmonella arizonae	3	2 Reservoir, 1 Lake
Salmonella miami	3	2 River, 1 Reservoir
Salmonella heidelberg	2	River
Salmonella typhimurium	1	Reservoir
Salmonella give	1	Lake
Salmonella abaetetuba	1	River
Salmonella agona	1	Reservoir
Salmonella jos	1	Lake
Salmonella glostrup	1	Reservoir
Salmonella anatum	1	Reservoir
Salmonella morehead	1	Reservoir
Salmonella 4,12:i:-	3	1 Lake, 2 Reservoir
Salmonella 4,12:r:-	1	River
Salmonella 4,5,12:i-	1	Reservoir
Total	26	

TABLE 2 - Molecular weight (MW) of the plasmids found in 11 of the 26 *Salmonella* sp. isolated from water.

Salmonella strain	Plasmid (MW)
S. heidelberg (67R22)	3MDa
S. heidelberg (57R22)	3MDa
Salmonella 4,12:r:- (57R12)	3MDa
S. typhimurium (69R28)	66MDa
S. miami (80R)	29MDa
S. miami (93R17)	29MDa
S. anatum (29R15)	69MDa
Salmonella 4,12:i:-(25Ec18)	34MDa
Salmonella 4,12:i:- (28R19)	34MDa
Salmonella 4,5,12:i:- (69R20)	34MDa
Salmonella 4,12:i:- (24R19)	34MDa

TABLE 3 - Invasiveness in mice of six *Salmonella* sp. isolated from water

Sample	Plasmid Profile	Invasibility Highest bacterial count	
		Liver	Spleen
S. miami	29MDa	-	-
S. miami	-	$6,7x10^{2(a)}$	$1,7x10^{2}$
S. typhimurium	66MDa	$7,2x10^{7}$	$6,8x10^{8}$
S. heidelberg	3MDa	$8,0x10^{1}$	$2,7x10^{1}$
S. agona	-	-	-
S. anatum	66MDa	$4,0x10^{2}$	$4,0x10^{1}$

(a) = colony-forming units

reservoirs and 6 from rivers). *Salmonella* sp. was not found in water samples of springs, wells, artesian and non-artesian wells, and treated water (swimming pools or drinking water).

Twenty-one of the 26 strains isolated were identified as belonging to 12 well-known serotypes whereas 5 remained unproperly characterized (TABLE 1).

Plasmid profiles were determined for each of the 26 *Salmonella* sp. isolated. It was observed that eleven of the strains carried plasmids of various molecular weights (TABLE 2).

TABLE 3 details the results of the invasiveness compared with plasmid profile.

DISCUSSION

The majority of serotypes identified did not correspond to those found in the feces of Brazilian patients with gastroenteritis (8,15,21). This finding is in accordance with those of Benassi *et al.* (1) and Gonzalez *et al.* (9). However, 2 strains were classified as *S. typhimurium* and *S. agona* which are often isolated from diarrheal illness.

In a previous and extensive study on *Salmonella* sp. occurrence in water from reservoirs with poor protection, Martins *et al.* (17) isolated a high percentage of *S. agona* and *S. infantis*, which corresponded to serotypes commonly found in sewage. However, most of the isolates were not previously associated with diarrhea.

This demonstrates that water can carry *Salmonella* sp. of human or animal origin.

Six Salmonella sp. were chosen for further testing, 4 plasmid-containing strains (*S. miami, S. typhimurium, S. heidelberg, S. anatum*) and 2 plasmid-free (*S. miami, S. agona*). The first intention was to carry out this study with isogenic strains, one plasmid-containing and the other cured, or, as an alternative, two separate isolates of the same serotype with and without plasmids. The latter approach was adopted by Helmuth *et al.* (13) in an attempt to link virulence to plasmids of various molecular weights. However, in the present work, only in the case of *S. miami* plasmid-free and plasmid-containing strains were available. Various attempts were made to cure the plasmids present in *S. typhimurium* but these proved unsuccessful, indicating an extremely stable plasmid (data not shown).

The *S. miami* strain carrying a 29MDa plasmid was found to invade neither livers nor spleens of the test animals while the plasmid-free strain exhibited an invasive capacity, albeit at a very low level. Unfortunately, it is impossible to establish a relationship between virulence and presence of these plasmids on the basis of these results.

The *S. heidelberg* strain (carrying a 3MDa plasmid) and the *S. anatum* strain (carrying a 66MDa plasmid) studied were also found to be weakly invasive, being isolated in small numbers in both livers and spleens.

The plasmid-free *S. agona* strain used in this study was not found to be invasive. This lack of invasive capacity in a serotype thought to be pathogenic due to its frequent occurrence in clinical cases is quite interesting. However, it must be remembered that this result may be due to the type of test-animal used or to competition with the intestinal microbiota, or reduction of the infecting bacterial population by the acidity of the animal's stomach.

The *S. typhimurium* strain harbouring a plasmid of 66MDa was found to be highly invasive, being detected in high numbers in liver and spleen of the slaughtered animals. We cannot assume that this 66MDa plasmid is playing any role in the invasiveness due the lack of a plasmid-free strain. However, the size similarity of this plasmid with the virulence plasmid previously described for this serotype (10,11,12,13,16) and its invasive capacity can be taken at least as an indirect support for the proposed link between the 66MDa plasmid and the virulence of the *S. typhimurium* strain isolated from water.

Comments on the virulence of *Salmonella* sp. isolated from water are rare in the literature. In the study carried out by Dondero *et al.* (6), all the *Salmonella* sp strains isolated from water showed no virulence when inoculated into mice. *S. typhimurium* and *S. agona*, serotypes highly associated with human disease, were among the strains isolated from water, suggesting that water can be a vehicle for low-virulence strains or that prolonged exposure to an aquatic environment can reduce bacterial virulence.

The present study demonstrates that the waters in Araraquara region probably do not represent a high risk of *Salmonella* disease, since the majority of the isolated strains showed no virulence in mice, the only exception being *S. typhimurium* which was strongly invasive. In conclusion, water can carry not only human pathogens participating in the

epidemological chain of gastroenteritis but also strains which do not represent a hazard to human health.

ACKNOWLEDGMENTS

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RESUMO

Sorotipos e virulência de *Salmonella* sp. isolada de água doce

A partir de amostras de água doce isolaram-se 26 cepas de *Salmonella* sp. de sorotipos diversos. A presença de plasmídios foi verificada nessas amostras sendo que onze delas apresentavam plasmídios de diferentes pesos moleculares. Quatro amostras contendo plasmídios e duas sem plasmídios foram analisadas quanto à capacidade de invasão em camundongos. Com respeito a *S. typhimurium*, é possível sugerir uma correlação entre virulência e a presença de plasmídio.

Palavras-chave: água, *Salmonella*, virulência

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PURIFICATION AND CHARACTERIZATION OF EXTRACELULLAR AMYLOGLUCOSIDASE FROM *CANDIDA* SP. AND ITS USE FOR THE PRODUCTION OF GLUCOMALTOSE SYRUP

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ABSTRACT

An amylolytic enzyme from *Candida* sp. ATCC 90238 was purified and its enzymatic characteristics studied. The molecular weight of the purified enzyme was calculated as 120.000. Paper chromatograms of soluble starch hydrolysates produced by the enzyme indicated that it is an amyloglucosidase. Maltotriose and soluble starch were efficiently hydrolyzed to glucose whereas maltose was the least efficiently degraded substrate. The enzyme showed different characteristics when compared to amyloglucosidases derived from *Aspergillus niger* and *Rhizopus* sp., which completely hydrolyzed maltose. The *Candida* sp. enzyme is adequate for the production of glucomaltose syrup.

Key words: *Candida* sp., amyloglucosidase, glucomaltose, pullulanase, α-amilase

INTRODUCTION

The production of amylolytic enzymes by microorganisms has been widely investigated. Very few yeasts, however, have been reported to produce amylolytic enzymes and they include *Endomycopsis fibuligera* (14), *Candida tropicalis* (8), *Lipomyces starkeyi* (4), *Schwanniomyces castelli* (1), *Saccharomyces diastaticus* (9,10) and *Schwanniomyces alluvius* (11, 15).

Extracellular amylolytic enzymes from *E. fibuligera* and *S. alluvius* were purified and it was found that they consisted of α-amylase and amyloglucosidase (11, 15).

A hyperamylolytic enzyme-producing yeast was previously isolated in this laboratory and some characteristics of the crude enzyme reported (5). The strain was later deposited in the American Type Culture Collection (ATCC) and classified as *Candida* sp. ATCC 90238. The aim of the present investigation was to purify the yeast enzyme, characterize its activity and utilize it for the production of glucomaltose syrup.

MATERIALS AND METHODS

Microorganisms: Extracellular amylolytic enzyme-producing *Candida* sp. ATCC 90238 and pullulanase-producing *Klebsiella* sp. (7) were isolated from soil as previously described (5,7).

Reagents: Bacterial (*Bacillus licheniformis*) - amylase was kindly donated by Novo Lab., Brazil;

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Maizena (starch) was purchased from Refinação de Milho Ltd., Brazil.

Production of the enzyme: The slant culture of *Candida* sp. was inoculated into 500 ml Erlenmeyer flasks containing 100 ml of culture medium supplemented with 1% yeast extract, 2% peptone and 3% soluble starch. Incubation was carried out at 30°C for 3 days. After incubation, the cultures were centrifuged and the supernatants used as a crude enzyme preparation. Pullulanase was also used as crude enzyme preparation, obtained as previously described (7).

Purification of the enzyme: Ammonium sulfate was added to the crude enzyme solution to give 80% saturation. The resulting precipitate was removed by centrifugation, dissolved in 50 ml of deionized water and dialyzed against 0.05 M acetate buffer, pH 5.6. The dialyzed solution was applied to a DEAE-Sephadex A-50 column (4x50 cm) previously equilibrated in 0.05 M acetate buffer, pH 5.6, and the column eluted with a saline concentration gradient (0.1 - 1.0 M). The enzyme fraction obtained from this column was dialyzed against the same buffer and further purified using a CM-cellulose column. The enzyme fraction from the latter column was dialyzed against deionized water and concentrated by ultrafiltration using an Amicon Model TCF 2 equipment.

Amyloglucosidase assay: The reaction mixture of 0.5 ml of 1% soluble starch in 0.1 M acetate buffer, pH 5.6, and 0.5 ml of enzyme was incubated at 50°C for 30 min. The reducing sugars formed were determined by the method of Somogyi and Nelson (12). One unit of amyloglucosidase activity was defined as the amount of enzyme that liberates mol of reducing sugars (calculated as glucose) in 1 min, under the stated conditions.

Paper chromatography: Descending paper chromatography was run at 25°C for 16 h using Whatman n°.1 filter paper and a solvent system of n-butanol-pyridine-water (6:4:4 v/v.). Sugar analysis was performed by HPLC with a differential refractometer detector (IR) and a YMC-Pack Polyamine-II column. The mobile phase was acetonitrile/water (75:25 v/v) with a flow rate of 1 ml/min. Samples were passed through a membrane filter before injection.

Determination of molecular weight: The molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out in a vertical slab gel apparatus according to the method of Laemmli (2). Protein bands were stained with Coomassie Blue R-250. The molecular weight marker proteins were ferritin (220.000), phosphorylase b (94.000), bovine serum albumin (67.000), ovalbumin (43.000), carbonic anhydrase (30.000) and soybean trypsin inhibitor (20.100).

Determination of protein: Protein concentration was determined according to the method of Lowry *et al.* (3).

RESULTS AND DISCUSSION

Enzyme purification:

The crude enzyme solution (750 ml) with extracellular enzyme activity was purified as described in materials and methods and results are summarized in TABLE 1. A 9-fold purified amyloglucosidase was obtained. SDS-gel electrophoresis of the purified enzyme solution revealed a single protein band with a molecular weight of 120.000.

Characterization of enzyme activity:

The optimum pH for the amyloglucosidase activity of the purified enzyme was between 5.5 and 6.0 the pH stability was between 4.3 and 6.0. The optimum temperature for activity was 60°C. The paper chromatograms showed that glucose was the only detectable product yielded by the purified enzyme

TABLE 1. Purification of Extracellular Amyloglucosidase from *Candida* sp. ATCC 90238.

Purification step	Total enzyme activity (Units)	Specific activity (Units/mg protein)	Yields (%)
Crude enzyme*	450	0.05	100
Ammonium sulfate fractionation	312	0.5	60
DEAE-Sephadex A-50 column chromatography	138	2.9	31
CM-Cellulose column chromatography	31	2.0	7

*750 ml of the crude enzyme extract were applied for purification

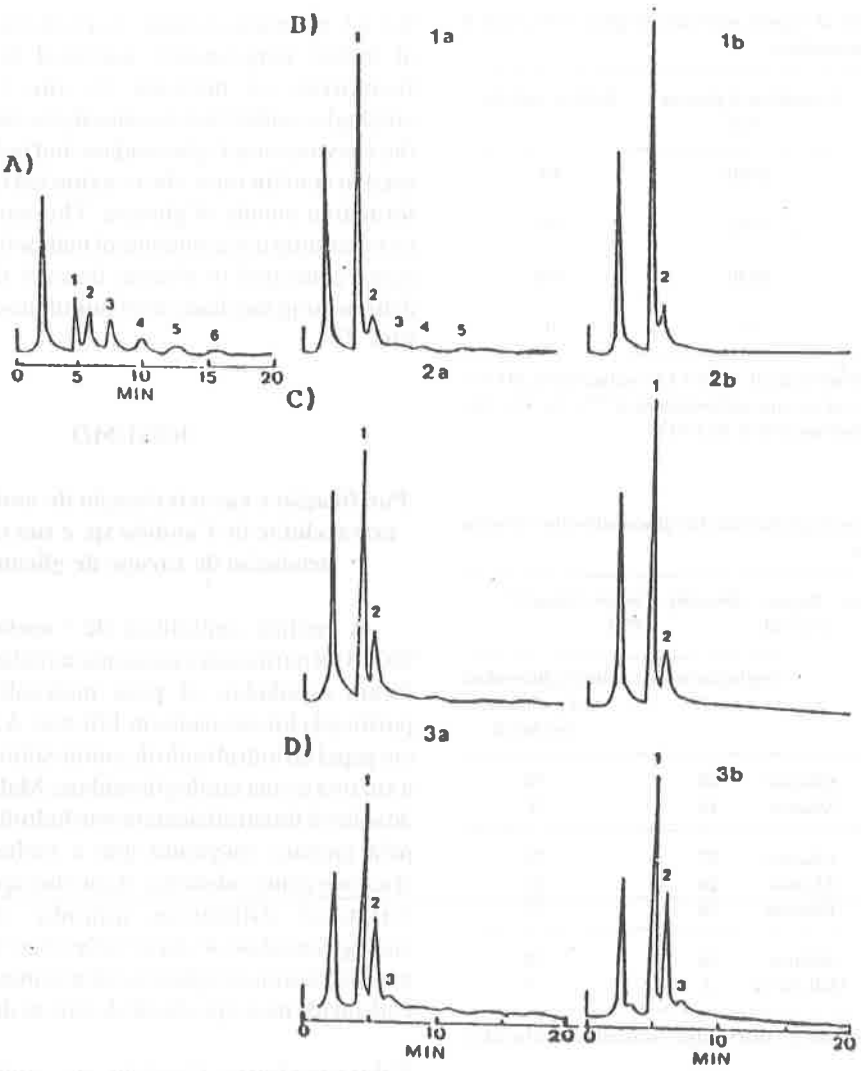

FIGURE 1. HPLC of products of the conversion of liquefied starch to sugars by *Candida* sp. amyloglucosidase.
A), Liquefied starch; B), Incubation at 50°C; C), Incubation at 55°C; D), Incubation at 60°C; 1a, 2a and 3a correspond to reactions with amyloglucosidase only; 1b, 2b and 3b correspond to reactions with amyloglucosidase and pullulanase combined. 1, Glucose; 2, Maltose; 3, Maltotriose; 4, Maltotetraose; 5, Maltopentaose; 6, Maltohexaose.

when using soluble starch as substrate. These results indicated that the enzyme is an amyloglucosidase (α-1,4-glucan glucanohydrolase, E.C. 3.2.1.3). On the other hand, when the enzyme acted on starch liquefied by bacterial α-amylase, glucose and maltose became detectable by paper chromatography and by HPLC (FIG. 1). A further test for the hydrolysis of maltose and maltotriose by the enzyme was thus performed. It was found that maltotriose was completely hydrolyzed to maltose and glucose, whereas maltose was partially hydrolyzed to glucose. The hydrolysis of different substrates to glucose by the enzyme was next studied by incubating 2% solutions of each substrate (starch, maltose, maltotriose and pullulan) with the purified enzyme (100l) at 55°C for 1 h and them measuring the glucose formed by HPLC. Results are shown in TABLE 2. It can be concluded that maltotriose was the most efficient and maltose the least efficient substrate for enzyme action. After 24 h of reaction, maltose had not undergone hydrolysis. It has already been demonstrated that amyloglucosidases from *Aspergillus niger,*

TABLE 2. Hydrolysis of various substrates to glucose by purified *Candida* sp. amyloglucosidase.

Substrates* (g/ml)	Formation of glucose (%)	Relative activity
Soluble starch	3400	85
Maltose	1550	38
Maltotriose	4040	100
Pullulan	0	0

*1 ml of the respective substrates at 2% in 0.1 M acetate buffer, pH 5.6, were mixed with 100 l of enzyme and incubated at 55°C for 1 h. The glucose formed was then measured by HPLC.

TABLE 3. Conversion of liquefied starch to gluconaltose by *Candida* sp. amyloglucosidase.

Reaction Temperature (°C)	Sugars formed	Quantity of sugars formed,[1] (%)	
		Amyloglucosidase only	Amyloglucosidase and pullulanase combined
50	Glucose	69	76
	Maltose	10	9
55	Glucose	57	71
	Maltose	24	21
	Glucose	50	57
60	Maltose	30	39
	Maltotriose	3	3

[1] The conversion of liquefied starch to sugars was calculated based on liquefied starch.
Mixtures of substrate and enzyme were incubated for 72 h.

Aspergillus awamori and *Rhizopus* sp. preferably hydrolyze long chain oligosaccharides to glucose rather than short chains, but that they eventually completely hydrolyze all of them to glucose (6, 13). For this reason, a further experiment was performed by incubating a mixture (40ml) of liquefied starch (15%) and the amyloglucosidade from *Candida* sp. (2.6 units of the enzyme) at 50°C, 55°C and 60°C for 72 h, with shaking. The sugars were then analyzed by HPLC. The results are illustrated in TABLE 3 and FIG. 1. It was observed that the *Candida* sp. enzyme was unable to completely hydrolyze maltose to glucose even after a prolonged reaction time. Therefore, this enzyme behaves differently to other fungal amyloglucosidase. Carrying out the reaction at higher temperatures appeared to increase the formation of maltose by the *Candida* sp. amyloglucosidase but decreased glucose yields. When the enzymes amyloglucosidase and pullulanase were used in combination, the reaction led to an increased formation mainly of glucose. This can be explained by remaining trace amounts of maltooligosaccharides being converted to glucose through the action of a debranching mechanism of pullulanase, as shown in FIG. 1.

RESUMO

Purificação e caracterização de amiloglicosidase extracelular de *Candida* sp. e sua utilização na produção de xarope de glicomaltose

A enzima amilolítica de *Candida* sp. ATCC 90238 foi purificada e suas características enzimáticas foram estudadas. O peso molecular da enzima purificada foi estimado em 120.000. A cromatografia em papel do hidrolisado de amido solúvel indicou que a enzima é uma amiloglicosidase. Maltotriose e amido solúvel foram eficientemente hidrolisados a glicose pela enzima, enquanto que a maltose foi menos eficiente como substrato. A enzima apresenta características diferentes quando comparada à amiloglicosidase *de Aspergillus niger* e *Rhizopus* sp. que hidrolisam completamente a maltose. Esta enzima é adequada para a produção de xarope de glicomaltose.

Palavras-chave: *Candida* sp., amiloglicosidase, glicomaltose, pululanase, α-amilase

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EFFECT OF THE CARBON SOURCE ON α-AMYLASE PRODUCTION BY *BACILLUS SUBTILIS* BA-04

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ABSTRACT

The effect of the initial concentration of different carbon sources on α-amylase production by a *Bacillus subtilis* strain was studied. The microorganism produced α-amylase in media containing glucose, soluble starch, dextrin and lactose. Longer fermentation times for reaching the maximum specific enzyme production rate were observed with higher initial carbohydrate concentrations. The highest enzyme production was observed in media containing glucose at 0.75g/L and 30.0g/L or dextrin at 10.0g/L. The overall specific enzyme activity showed that the amount of enzyme produced per cell mass unit in culture media containing dextrin was higher than that produced in media containing soluble starch, glucose or lactose. Results on overall specific enzyme activity also showed that at low carbohydrate concentrations the levels of enzyme production were lowest using glucose as carbon source and fell below the most reduced production, detected in media containing lactose.

Key words: α-amylase, *Bacillus subtilis*

INTRODUCTION

The effect of the carbon source on α-amylase (EC 3.2.1.1) production by *Bacillus* has been studied in order to define the medium composition for industrial enzyme production and to study its inducibility. In general, α-amylase substrates such as glycogen, starch, maltose, maltotriose and α-1,4 oligosaccharides are considered carbon sources appropriate to induce α-amylase production by *Bacillus* strains (2, 3, 8). Some results of α-amylase production in media containing these carbohydrates suggest that the high enzyme production is not elicited by the polysaccharides originally added but to some degradation products formed and consumed during fermentation (9). The production of α-amylase by *Bacillus* strains is greatly influenced not only by the nature but also by the concentration of the carbon source (4, 3, 6). YOO *et al.* (10) showed that although maximum enzyme production by a *Bacillus amyloliquefaciens* strain was greater in a medium containing maltose than in one containing glucose, the overall specific enzyme activity was almost the same in both cases. According to the authors, the results show that both glucose and maltose repressed enzyme synthesis, but glucose was more effective.

The aim of this work was to study the effect of the carbon source on the production of α-amylase by *Bacillus subtilis* Ba-04, correlating enzyme synthesis with microbial growth in each culture medium.

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MATERIALS AND METHODS

Microorganism. *Bacillus subtilis* Ba-04 from the Instituto de Tecnologia de Alimentos collection was used throughout this study. A stock culture was maintained on nutrient agar slants immersed in mineral oil at 5°C. Fermentations were carried out with microorganism from the stock culture maintained on nutrient agar slants for 3 days at 30°C.

Enzyme production. The medium composition (g/L) was: 2.5 $(NH_4)_2$ SO_4; 0.5 $MgSO_4$. $7H_2O$; 0,5 KCl; 1.0 K_2HPO_4; 10.0 bacto peptone (Difco); 5.0 yeast extract (Difco) and a carbon source. Sterile $CaCl_2$ at 0.418g/L was added to the medium as a concentrated solution. The pH of the medium was adjusted to 7.0 with KOH 5N before sterilization at 121°C for 15 minutes. The inoculum was obtained by transferring a loopful of the microorganism to 100ml of culture medium in a 500ml Erlenmeyer flask which was then incubated for 18 hours at 37°C with shaking (200rpm). The enzyme was produced in 1000ml Erlenmeyer flasks containing 400ml of the culture medium inoculated with 20ml of the inoculum diluted with sterile water to 24mg of dry cell mass. Flasks were incubated for 72 hours at 37°C with shaking (200rpm). Samples for enzyme assay and cell mass determinations were taken from the same flask. The results in the tables and graphs are the average of two experiments.

Dextrinizing activity. This was determined in the clear solution obtained by centrifuging the fermented broth at 13,000g for 10 minutes at 5°C. The method used was that described by MEDDA and CHANDRA (5) but modified using 50°C and pH 6.0 (0.1 M citric acid - sodium phosphate buffer). One unit of enzyme activity (DU) was defined as the amount of enzyme which brings about the hydrolysis of 1mg of starch per minute in the presence of 5.0mg of substrate. Specific enzyme production rate, μ_p, was defined as $1/X.\Delta E/\Delta t$, where X was the maximum cell concentration in a given time interval and $\Delta E/\Delta t$ was the mean enzyme production rate in the same time interval. The overall specific enzyme activity was defined as E_{max}/X_{max}, where E_{max} was the maximum enzyme activity within 72 hours of fermentation and X_{max} was the maximum cell mass concentration within the same time interval.

Cell mass concentration. Samples of 10ml of the culture broth were centrifuged at 13,000g for 10 minutes. The precipitate was washed once with distilled water and resuspended in water. After an appropriate dilution with distilled water, the optical density of the cell suspension was read at 660nm and converted to dry weight through a calibration curve.

Cell disruption. Ten milliliters of an 18h culture in which the initial glucose concentration was 10.0g/l were centrifuged for 5 minutes at 13,000g. The precipitate was washed twice with distilled water and resuspended in 2.0ml of 0.1M citric acid - sodium phosphate buffer at pH 6.0. The cell suspension was incubated with 3.0ml of the same buffer containing 20mg of lysozyme (Sigma product containing 50.000 units/mg protein) for 90 minutes at 37°C. After centrifugation the enzyme's activity was determined in the supernatant liquid.

RESULTS

FIGURE 1 shows a typical growth curve and α-amylase production by *Bacillus subtilis* Ba-04. A similar behavior was observed with all carbon sources. Regardless of the carbohydrate used, the enzyme was not detected before 10 hours of fermentation. In general, the α-amylase concentration in the culture broth increased between 24 and 72 hours. Some inactivation occurred in the medium containing

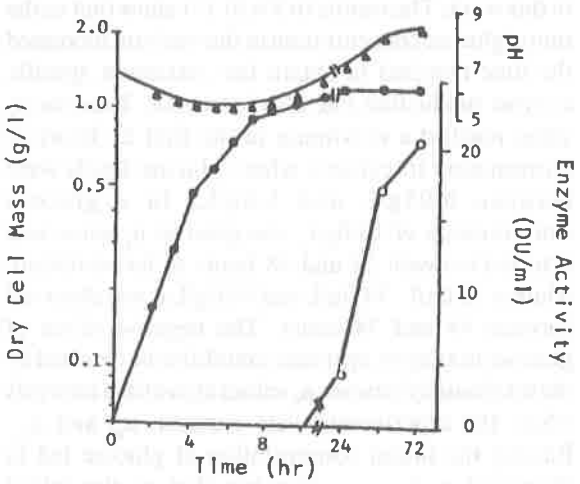

FIGURE 1 - Cell growth and α-amy lase production by *B. subtillis* Ba-04 in medium containing glucose at 10.0g/l. -▲- pH; -●- dry cell mass; -○- enzyme activity.

TABLE 1. Effect of the initial concentration of the carbon source on microbial growth and α-amylase production.

initial	glucose									soluble starch								
concen-tration	μ_p (DU/mg.hr)			X (mg/ml)			E (DU/ml)			μ_p (DU/mg.hr)			X (mg/ml)			E (DU/ml)		
(g/l)	μ_{P1}	μ_{P2}	μ_{P3}	X_1	X_2	X_3	E_1	E_2	E_3	μ_{P1}	μ_{P2}	μ_{P3}	X_1	X_2	X_3	E_1	E_2	E_3
0.05	0.50	0.21	0.09	0.54	0.41	0.40	6.5	9.2	10.4	...	...	...	...	...	...	...	...	...
0.10	0.50	0.12	0.17	1.10	0.70	0.64	13.2	16.2	20.6	...	...	...	...	...	...	...	...	...
0.20	0.51	0.23	0.26	0.84	0.81	0.86	10.3	15.1	20.5	...	...	...	...	...	...	...	...	...
0.50	0.54	0.38	0.02	1.12	1.01	1.01	14.5	24.6	25.0	0.43	0.30	0.0	0.65	0.47	0.42	6.8	11.5	11.5
0.75	0.53	0.42	0.03	1.34	1.12	0.86	17.0	30.8	30.9	...	...	...	...	...	...	...	...	...
1.0	0.53	0.39	0.05	0.55	0.79	0.64	7.0	14.4	15.5	0.54	0.42	0.02	0.56	0.42	0.38	7.2	12.9	13.1
1.5	...	...	...	...	...	...	...	...	...	0.52	0.34	0.05	0.59	0.35	0.28	7.3	12.1	12.5
2.0	...	...	...	...	...	...	...	...	...	0.53	0.40	0.06	0.62	0.38	0.36	7.8	13.7	14.2
4.0	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
5.0	...	...	...	...	...	...	...	...	...	0.41	0.49	0.17	0.69	0.49	0.41	6.8	14.9	16.9
10.0	0.12	0.53	0.13	1.11	1.11	1.11	3.2	17.2	20.6	0.41	0.59	0.27	0.65	0.59	0.41	6.4	15.7	19.5
20.0	0.14	0.32	0.47	1.21	0.70	0.70	4.1	13.5	21.4	0.25	0.75	0.30	0.71	0.80	0.73	4.2	18.6	24.4
30.0	0.12	0.49	0.76	0.91	0.91	0.72	2.6	13.3	29.9	0.13	0.25	0.62	0.71	0.63	0.45	2.3	6.6	16.0
40.0	0.14	0.43	0.64	0.70	0.76	0.84	2.3	10.0	23.1	...	...	...	...	...	...	...	...	...
50.0	0.03	0.10	0.00	0.98	1.14	1.07	0.7	3.3	2.3	...	...	...	...	...	...	...	...	...

μ_{P1}, μ_{P2}, μ_{P3} = μ_p at the time intervals (0-24)hr, (24-48)hr and (48-72)hr respectively.
X_1, X_2, X_3 = dry cell mass concentration at 24hr, 48hr and 72hr respectively.
E_1, E_2, E_3 = enzyme concentration at 24hr, 48hr and 72hr respectively.

glucose at 50.0g/L (TABLE 1) where enzyme concentration was 3.3DU/ml after 48 hours of fermentation and 2.3DU/ml after 72 hours.

Since some *Bacillus* strains are reported to be very sensitive to glucose (3,4), concentrations of this sugar varying from 0.05g/L up to 50.0g/L were used in this work. The results in TABLE 1 show that as the initial glucose concentration in the medium increased the time required to obtain the maximum specific enzyme production rate also increased. Thus the μ_p value reached a maximum in the first 24 hours of fermentation in cultures where glucose levels were between 0.05g/L and 1.0g/L. In a glucose concentration of 10.0g/l, the greatest μ_p value was detected between 24 and 48 hours of fermentation, while at 20.0g/L, 30.0g/L and 40.0g/L it was observed between 48 and 74 hours. The negative effect of glucose on enzyme synthesis could also be detected by the relationship between μ_p values at two time intervals where the experiments were pursued, μ_{P1} and μ_{P2}. Raising the initial concentration of glucose led to decreased $\mu_{P1}/\mu_{P2\ ratios}$, showing that as the initial concentration of glucose increased a longer fermentation time was necessary to obtain the bulk of enzyme synthesis. Treatment of the bacterial cell at the end of the exponential growth phase with lysozyme showed that the concentration of the intracellular α-amylase was very low and could not be responsible for the high enzyme concentration in the fermentation broth after cell lysis. These results suggested that an intense enzyme synthesis takes place after the beginning of the stationary growth phase. Results from media with glucose at 0.1g/L and 0.75g/L also illustrate this behavior. In both cases cell concentration decreased within 72 hours but the decrease was greater at 0.1g/L, where the higher μ_{P1}/μ_{P2} ratio was obtained. The opposite was observed at the initial glucose concentration of 40.0g/L, where cell concentration after 48 hours of fermentation was 8.5% higher than that observed after 24 hours and μ_{P2} was three times higher than μ_{P1}.

There was cell lysis after 24 hours of fermentation in all soluble starch media, except for the 20.0g/L concentration. The greatest specific enzyme production rates with soluble starch were detected during the first 24 hours at concentrations 0.5g/L to 2.0g/L, between 24 and 48 hours at concentrations of 5.0g/L, 10.0g/L and 20.0g/L and between 48 and 74 hours at 30.0g/L (TABLE 1). As the initial amount of soluble starch increased both enzyme synthesis and

TABLE 2. Effect of the initial concentration of the carbon source on microbial growth and -amylase production

initial	dextrin									lactose								
concen-tration	μ_P (DU/mg.hr)			X (mg/ml)			E (DU/ml)			μ_P (DU/mg.hr)			X (mg/ml)			E (DU/ml)		
(g/l)	μ_{P1}	μ_{P2}	μ_{P3}	X_1	X_2	X_3	E_1	E_2	E_3	μ_{P1}	μ_{P2}	μ_{P3}	X_1	X_2	X_3	E_1	E_2	E_3
1.5	0.64	0.26	0.04	0.58	0.58	0.43	8.9	12.6	13.2	...	...	...	...	...	...	...	...	...
2.0	0.67	0.33	0.06	0.55	0.41	0.34	8.8	13.2	13.8	...	...	...	...	...	...	...	...	...
4.0	0.56	0.69	0.21	0.60	0.39	0.34	8.1	18.0	20.0	...	...	...	...	...	...	...	...	...
5.0	...	...	...	...	...	...	...	...	...	0.19	0.19	0.62	0.49	0.49	0.29	2.2	4.5	11.8
10.0	0.19	0.88	0.71	0.55	0.64	0.37	2.5	16.0	27.0	0.19	0.18	0.70	0.56	0.61	0.62	2.5	5.2	15.6
20.0	0.09	0.85	1.15	0.23	0.33	0.39	0.5	7.2	18.0	0.26	0.25	0.31	0.31	0.49	0.60	1.9	4.8	9.3
30.0	0.10	0.69	0.85	0.21	0.32	0.32	0.5	5.8	12.3	0.14	0.14	0.24	0.47	0.55	0.66	1.6	3.4	7.2

μ_{P1}, μ_{P2}, μ_{P3} = μ_P at the time intervals (0-24)hr, (24-48)hr and (48-72)hr respectively.
X_1, X_2, X_3 = dry cell mass concentration at 24hr, 48hr and 72hr respectively.
E_1, E_2, E_3 = enzyme concentration at 24hr, 48hr and 72hr respectively.

the rations μ_{P1}/μ_{P2} and μ_{P1}/μ_{P3} decreased, similarly to the behavior in media containing glucose. Cell lysis also seemed to be associated with enzyme synthesis when using soluble starch as carbon source. Thus, the highest cell lysis detected between 24 and 48 hours occurred at the 1.5g/L initial soluble starch concentration, when the highest ratio μ_{P1}/μ_{P2} was observed. At 20.0g/l the cell concentration in the medium increased between 24 and 48 hours and the μ_{P1}/μ_{P2} value was the lowest observed.

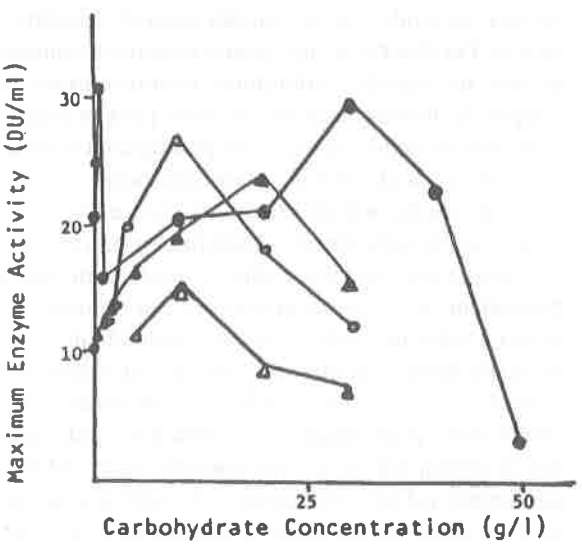

FIGURE 2 - Effect of the carbon source on the α- amylase production. -●- glucose, -▲- soluble starch, -○- dextrin, -△- lactose.

Increasing the initial concentrations of dextrin also resulted in a decrease of μ_{P1}/μ_{P2} and μ_{P1}/μ_{P3} values (TABLE 2). At the initial dextrin concentrations of 1.5g/Land 2.0g/L the highest μ_P value was detected within the first 24 hours of fermentation. At 10.0g/L and 4.0g/L the highest μ_P value was between 24 and 48 hours of fermentation and at 20.0g/L and 30.0g/L between 48 and 72 hours. μ_P also seemed to be dependent on the physiological state of the cell as its maximum was detected at the time interval where cell lysis had occurred.

Cell lysis was not observed within the first 48 hours of fermentation for the four concentrations of lactose studied. The results in TABLE 2 show long periods of slow growth when enzyme synthesis was very low. The highest μ_P values were obtained after between 48 and 72 hours of fermentation for all the lactose concentrations studied, suggesting that the greatest enzyme production would occur after cell growth took place.

The maximum α-amylase production was detected in media containing glucose at 0.75g/L and 30.0g/L or dextrin at 10.0g/L (FIG. 2). Lactose was the least suitable carbon source for enzyme production. During 72 hours of fermentation the enzyme concentrations in media containing glucose at 30.0g/L and dextrin at 10.0g/L were 29.9DU/ml and 27.0DU/ml, respectively. Lactose at 10.0g/L as carbon source resulted in a maximum enzyme concentration of 15.6 DU/ml.

The overall specific enzyme activity, E_{max}/X_{max}, was highest in media with dextrin, followed by

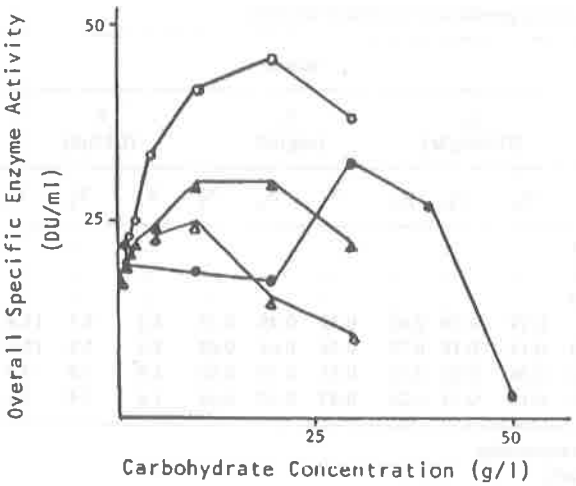

FIGURE 3 - Effect of the carbon source on the overal specific enzyme activity, -● glucose, -▲- soluble starch, -O- dextrin, -△- lactose.

soluble starch (FIG.3). An exception was observed at a carbohydrate concentration of 30.0g/l when E_{max}/X_{max} was higher in medium containing glucose than in medium with soluble starch. When lactose was employed at low concentrations, the amount of enzyme produced per cell mass unit was higher than that in media with glucose.

DISCUSSION

Glucose at 50.0g/Lappears to favour the production of some substances like proteases (10) propionate, lactate and 2,3 butanediol (1) which inactivate -amylase. As the glucose, soluble starch and dextrin initial concentrations increased, the fermentation time had to be extended in order to reach the maximum μ_p value. The difference between these μ_p values reveals that increasing the initial concentrations of carbohydrate led to a decrease in enzyme produced per cell mass unit at the beginning of fermentation, indicating that the sugars or their first degradation products cause inhibition of enzyme synthesis. Data from the literature show that both maltose and glucose repress α-amylase synthesis by different *Bacillus licheniformis* and *Bacillus amyloliquefaciens* strains (3,4,7).

Although the pattern of enzyme synthesis was the same in media with glucose, soluble starch and dextrin, the amount of enzyme synthesized per cell mass unit was higher in the latter case. This behavior might also reflect some stimulation of enzyme synthesis after the inhibitor had been consumed or had its effect overcome. The experiments with soluble starch and dextrin suggest that the carbohydrates as added to the culture media were not the true inducers of enzyme synthesis by *Bacillus subtilis* Ba-04, since the greatest sugar concentrations were associated with the lowest μ_{P1}/μ_{P2} values.

The low enzyme production per cell mass unit in culture media with glucose was counterbalanced by a high cell mass concentration, resulting in a high enzyme concentration in the culture medium. Similarly, the low enzyme production in media with high levels of dextrin appeared to be due to a low cell growth. The less appropriate carbon source for α-amylase synthesis was shown to be lactose, which did not cause either a great cell mass production or a marked enzyme production per cell mass unit.

RESUMO

Efeito da fonte de carbono sobre a produção de α-amilase por *Bacillus subtilis* Ba-04

Foi investigado o efeito da concentração inicial de diferentes fontes de carbonos sobre a produção de-amilase por uma linhagem de *Bacillus subtilis*. O microrganismo produziu α-amilase em meios de cultura contendo glicose, amido solúvel, dextrina e lactose. Foi observado que quanto maiores as concentrações iniciais de carboidrato maiores foram os tempos de fermentação necessários para ocorrer a máxima velocidade específica de produção da enzima. As maiores produções de α-amilase ocorreram em meios de cultura contendo glicose a 0,75g/L e 30,0g/L ou dextrina a 10,0g/L. Resultados obtidos para a atividade enzimática específica global mostraram que a quantidade de enzima produzida por unidade de massa celular no meio de cultura contendo dextrina foi maior do que em meio de cultura contendo amido solúvel, glicose ou lactose. A baixas concentrações de carboidrato, a produção de enzima por unidade de massa celular em meio contendo glicose foi inferior até mesmo à observada em meio de cultura contendo lactose, onde houve a menor produção de α-amilase.

Palavras-chave: α-amilase, *Bacillus subtilis*

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REMOVAL OF IRON FROM TALC BY ACIDS PRODUCED BY FUNGI

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ABSTRACT

A fungal strain was selected out of fifteen other strains as having the best potential to remove iron from talc. Iron was removed by products of fungal metabolism which acted as leaching agents in the culture medium filtrate.

Key words: fungi; bioleaching; industrial minerals; talc; *Aspergillus*

INTRODUCTION

The presence of iron can affect the quality of industrial minerals and various methods for iron removal have been studied(6). The efficiency of physical methods is not always good whereas chemical methods are suitable but more expensive. Oxalic acid is a good leaching agent for the removal of iron. It is a strong chelating agent for iron produced by some strains of fungi(3) with moderate acid strength.

The use of oxalic acid generated by fermentation for the leaching of iron can be a feasible process. Moreover, some other organic acids (e.g. citric acid) and metabolic products which are also secreted into the medium exert a positive effect on the leaching process(5). So, the use of organic acids produced by microorganisms combined with conventional methods such as flotation can be a suitable leaching technique.

Bacteria and fungi are able to attack ores and solubilize metals(10). In the case of oxidized ores, fungi act more efficiently than bacteria(5). Metal solubilization is connected to the production of organic acids, mainly oxalic and citric, and other metabolites. *Aspergillus niger* is one of the best oxalic and citric acid producers. Based on these considerations, only fungal strains were isolated and only strains of *A. niger* were tested.

The use of microbiologically-produced acids for the removal of metals from different minerals has been studied(1,2,5) yet the removal of iron from talc by these acids has not been reported to date. Talc is used by various industries (cosmetics, paper, paints, ceramic, and others), each one having different quality standards(4). The removal of iron from talc can improve its quality, allowing its use in applications with more stringent requirements. Brazil is the world's third largest talc producer but Brazilian talc receives poor treatment and only 4% of the total production has good enough quality to be used in noble applications(8). The present study was undertaken to select a fungal strain able to promote iron leaching from talc.

MATERIALS AND METHODS

A flotation-treated talc sample from Paraná, Brasil(7) was used in this study. The iron content of the concentrate was 0.29%. Eight strains of fungi were isolated from suspensions of two talc samples (run of mine ore) in a 0.9% NaCl solution. The supernatant was inoculated onto malt-extract agar plates and, after growth, the fungi were subcultured. These eight isolates were named according to the

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TABLE 1 - Acidity produced by the strains tested and iron leaching from talc by the culture filtrate.

		Fermentation				
	Strain	Acidity (meqAcid/l)	Final pH	Dry matter (g/l)	Final glucose (g/l)	Leached Iron (mg/l)
	KPM 1	158	1.80	3.2	0.02	33.8
I	KPM 2	16	2.96	2.9	0.11	2.6
S						
O	KPM 3	70	1.96	2.6	0.09	16
L						
A	KPM 4	26	2.04	5.0	3.00	1.8
T						
E	ITA 1	18	5.25	1.8	14.26	0.7
D						
	CETEM 1	93	2.15	3.7	0.03	13.5
	A.niger 5002	38	2.04	5.0	0.15	4.9
C	*A.niger*	111	1.86	3.3	0.02	21.7
O	190					
L	*A.niger*	30	1.95	5.9	0.04	1.1
L	1220					
E	*A.niger*	23	2.53	2.1	0.10	4.3
C	1549					
T	*A.niger*	29	1.95	6.6	0.12	2.1
1	1628					
O	*A.niger*	152	1.90	3.0	0.07	24.4
N	2014					
	A.niger EMB	124	1.84	3.1	0.06	16.6

sample they were isolated from. These isolates and seven other strains of *Aspergillus niger* from the collection of the Escola de Quimica - Universidade Federal do Rio de Janeiro were tested. Some of them did not form conidia and thus their inocula for acid production were prepared from mycelial fragments (strains KPM 2, KPM 5, KPM 6, ITA 1, *A. niger* 1549). Inoculation of the other strains was done with a suspension of conidia. The strains were maintained on Sabouraud-dextrose-agar at 4°C after incubation at 32°C for fourteen days.

Initial attempts to solubilize iron were done by growing the fungi in the presence of iron oxide which, however, remained occluded inside the mycelial pellets. Therefore, the overall process was conducted in two stages: the fermentation step for the production of acid followed by the leaching of iron by the fermentation filtrate.

The acid production medium contained 20 g of commercial glucose, 3 g of NH_4NO_3, 1 g KH_2PO_4 and 0.2 g of $MgSO_4.7H_2O$ per liter of deionized water. The initial pH of the medium was 7.0(6). Volumes of 150 ml of medium inside 500 ml conical flasks were inoculated with 10^5 conidia/ml or mycelial fragments from a Sabouraud-dextrose-agar culture, and incubated for seven days in a rotary shaker at 160 rpm and (32+/-1°C). After fermentation, the content of each flask was filtered and the filtrate then used for iron leaching. Dry matter contents, glucose concentrations, acidity and pH were measured at the end of the fermentation process.

Leaching was conducted inside 250 ml shaken flasks (168 rpm) at room temperature (22+/-2°C). Twenty grams of talc were treated with 80 ml of fermented medium and leaching proceeded for 4 h. In the control test, sterile unfermented culture medium at pH 3,0 was used as the leaching solution. The leached iron was measured in the liquid after filtration.

Mycelium dry mass was measured by filtering through pre-weighted paper followed by washing with distilled water, drying for 24 h at 80°C, cooling and weighting of mycelia. Glucose concentration was determined by the Somogyi method(9). The acidity was determined volumetrically using 0,1N NaOH and phenolphtalein as indicator. The pH was measured with a pHmeter and the leached iron by an atomic absortion spectrophotometer, Varian Techtron, model AA6.

RESULTS AND DISCUSSION

The results are shown in TABLE 1 and represent the average of duplicate determinations. All the strains tested exhibited growth except KPM 5 and KPM 6. Glucose was totally consumed in almost all fermentations and the pH of every medium decreased. A correlation was found between acid production and iron removal. The best acid-producing strains were also the best promoters of the removal of the metal. Organic acids produced from glucose by fungal metabolism were probably the iron leaching agents. The control test showed that there was no participation of the unfermented culture medium on iron leaching. The iron-solubilizing capacity of each strain was different, probably due to differences in concentration and distinct types of metabolic products from each. Best leaching results were obtained using the fermented medium of strains KPM 1, *A. niger* 2014

and *A. niger* 190. Strain KPM 1 showed growth and sporulation similar to that of the species *A. niger*.

The leaching of sulphide ores by bacteria is a direct consequence of the microbial metabolism and in such cases it is considered necessary to grow the microorganism in the presence of the ore(10). In this study, it was confirmed that there is an indirect action of the fungi on the iron content of the talc and that there is no need to grow the fungi in the presence of the mineral. The amount of iron removed from talc was not optimal, but experiments are in progress to select the best conditions of fermentation and leaching for iron removal with strain KPM 1.

ACKNOWLEDGMENTS

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RESUMO

Remoção de ferro de talco por ácidos produzidos por fungos

Uma linhagem fúngica foi selecionada dentre quinze outras, como sendo aquela de maior potencial de remoção de ferro de talco. A remoção do ferro pelo filtrado do meio de cultura demonstrou que algum produto do metabolismo do fungo foi o responsável pela lixiviação.

Palavras-chave: fungos, biolixiviação, minerais industriais, talco, *Aspergillus*

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PRODUCTION OF FRUITY AROMA BY *NEUROSPORA* SPECIES ISOLATED FROM BEIJU

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ABSTRACT

Eight strains of *Neurospora* sp. were isolated from beiju in various regions of the State of Maranhão, Brazil. Growth media from cultures of these *Neurospora* sp. exhibited a pleasant fruity aroma, which was absent in cultures of *Neurospora* strains of the NRRL collection and other strains of the microorganism isolated from soil in an area of São Paulo. The fruity aroma was chemically characterized as ethyl hexanoate by Dynamic Headspace/Gas Chromatography. The *Neurospora* strains from the State of Maranhão also produced 3-methyl-1-butanol, 1-octen-3-ol, ethyl acetate and ethanol.

Key words: fruity aroma; *Neurospora sp.*; ethyl hexanoate

INTRODUCTION

Several microbial species produce volatile fruity aromas during growth in culture medium (5). These microorganisms are potential sources of natural fruit essences which are desirable in certain foods, cosmetics and pharmaceutical products. Park *et al.*, (4) reported that a strain of *Neurospora* sp. producing a pleasant odor was isolated from beiju, a naturally fermented cassava mass used in a region of the state of Maranhão, Northeast of Brazil, to produce a traditional indigenous alcoholic beverage. This strain of *Neurospora* was deposited in the American Type Culture Collection at their request and classified first as *Neurospora* sp. ATCC 46892 (1) and subsequently as *Neurospora sitophila* ATCC 46892 (2). The nature of the fruity odor from this *Neurospora* strain was identified as ethyl hexanoate by Yoshizawa *et al.* (9); Yamauchi *et al.* (6, 7) reported the presence of alcohol acyltransferase activity in the cell-free extract of this *Neurospora* sp. and also that it was synthesizing ethyl hexanoate from ethyl alcohol and n-hexanoyl coenzyme A. The extract produced little or no acetate esters. Such result suggested the absence of alcohol acetyltransferase. Subsequently, Yamauchi *et al.* (8) examined 12 other strains of *Neurospora* from IFO culture collections for the production of ethyl hexanoate and found that none produced it except for the *Neurospora* strain from beiju.

The aim of this study was to analyze another collection of beiju samples in order to isolate *Neurospora* strains and examine them for the production of ethyl hexanoate and other volatile substances.

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MATERIALS AND METHODS

Isolation of *Neurospora* sp. from beiju

Samples of beiju were collected from small breweries in various regions of the State of Maranhão. Approximately 10g of beiju were crushed and 1g of powder suspended in 100 mL of sterile water. An inoculum of 0.1 mL of the suspension was plated onto potato dextrose agar. After two days of incubation at 30C, colonies resembling *Neurospora* sp. were transferred to a slant of the same culture medium. Besides those obtained from beiju, the other strains of *Neurospora* used in this study were *Neurospora tetrasperma* NRRL 2164, *N. crassa* NRRL 2223, *N. sitophila* NRRL 2884 and *N. intermedia* NRRL 5506 kindly donated by the Northern Regional Research Laboratory, USDA, Peoria, USA, as well as twenty strains isolated from soil in the State of São Paulo, Brazil

Production of fruity aroma

All *Neurospora* strains were inoculated into 250 ml Erlenmeyer flasks containing 50 mL of culture medium supplemented with 5% malt extract and kept at 30°C for 6 days, under agitation (250 rpm). After incubation, 0.5 ml samples of medium were transferred to 40 ml serum bottles, which were next sealed air-tight with Teflon septa and aluminum caps and stored at - 20C.

Analysis of volatile compounds from culture medium

Analysis of volatile compound was performed as described by Yang and Min (10). Serum bottles containing 0.5 mL of culture medium were placed at room temperature for 10 min and transferred to a sample heater of Dynamic Headspace/Gas Chromatography (Teckmar Co., Cincinnati, OH, USA). The sample was purged with nitrogen gas for 0.5 minute and volatile compounds were transferred and adsorbed onto a tenax trap (12 x 1/8 inch, Teckmar Co.). The adsorbed volatile compounds were thermally desorbed from the trap by heating at 160°C for 4 min; volatiles concentrated at the capillary interface and were then automatically injected into a capillary column (DB-Wax, 30m x 0.32 mm ID, 0.25 m film thickness, J.W. Scientific Inc., Folson, CA., USA). Gas Chromatography (Model 5890, Hewlett Packard Co., Palo Alto, CA, USA) and Integrator (3390 A Hewlett Packard) were used for detection of volatile compounds. The volatile compounds were identified by coincidence of each relative retention time with that of authentic standards.

RESULTS AND DISCUSSION

As shown in TABLE 1, growth media from cultures of beiju-derived *Neurospora sitophila* ATCC 46892 and seven additional *Neurospora* sp isolates exhibited a pleasant fruity aroma, whereas those from *Neurospora* strains of NRRL culture collections and other strains isolated from soil in an area of São Paulo did not. Identification of volatile compounds from culture media was attempted by Dynamic Headspace/ Gas Chromatography and the results are presented in FIG. 1. TABLE 2 shows the amount of each volatile compound. It was found that *N. sitophila* ATCC 46892 produced ethyl hexanoate, 3-methyl-1-butanol, 1-octen-3-ol, ethyl acetate and ethanol. Ethyl hexanoate has a strong fruity aroma and, as shown in TABLE 1, all media from cultures of beiju-derived *Neurospora* sp. presented a fruity odor due to the presence of ethyl hexanoate. Strain ATCC 46892 yielded the greatest amounts of ethyl hexanoate (59 ppm in TABLE 2) as compared to the other strains of *Neurospora* sp from beiju. It is also interesting to note that strain ATCC 46892 produced 40 ppm of 1-octen-3-ol, yet Yamauchi *et al.* (8) did not detect production of such component by the same strain when using a

TABLE 1. Production of fruity aroma by various strains of *Neurospora*.

Strains	Origin	Odor
Neurospora sitophila ATCC 46892	Beiju	Fruity
Neurospora sp. Nº 1	Beiju	Fruity
Neurospora sp. Nº 2	Beiju	Fruity
Neurospora sp. Nº 3	Beiju	Fruity
Neurospora sp. Nº 4	Beiju	Fruity
Neurospora sp. Nº 5	Beiju	Fruity
Neurospora sp. Nº 6	Beiju	Fruity
Neurospora sp. Nº 7	Beiju	Fruity
Neurospora tetrasperma NRRL 2164	NRRL	No odor
Neurospora crassa NRRL 2223	NRRL	No odor
Neurospora sitophila NRRL 2884	NRRL	No odor
Neurospora intermedia NRRL 5506	NRRL	No odor

1. Twenty strains of *Neurospora* sp. isolated from the State of São Paulo did not produce a fruity odor.
2. ATCC - American Type Culture Collection
3. NRRL - Northern Research Regional Laboratory.

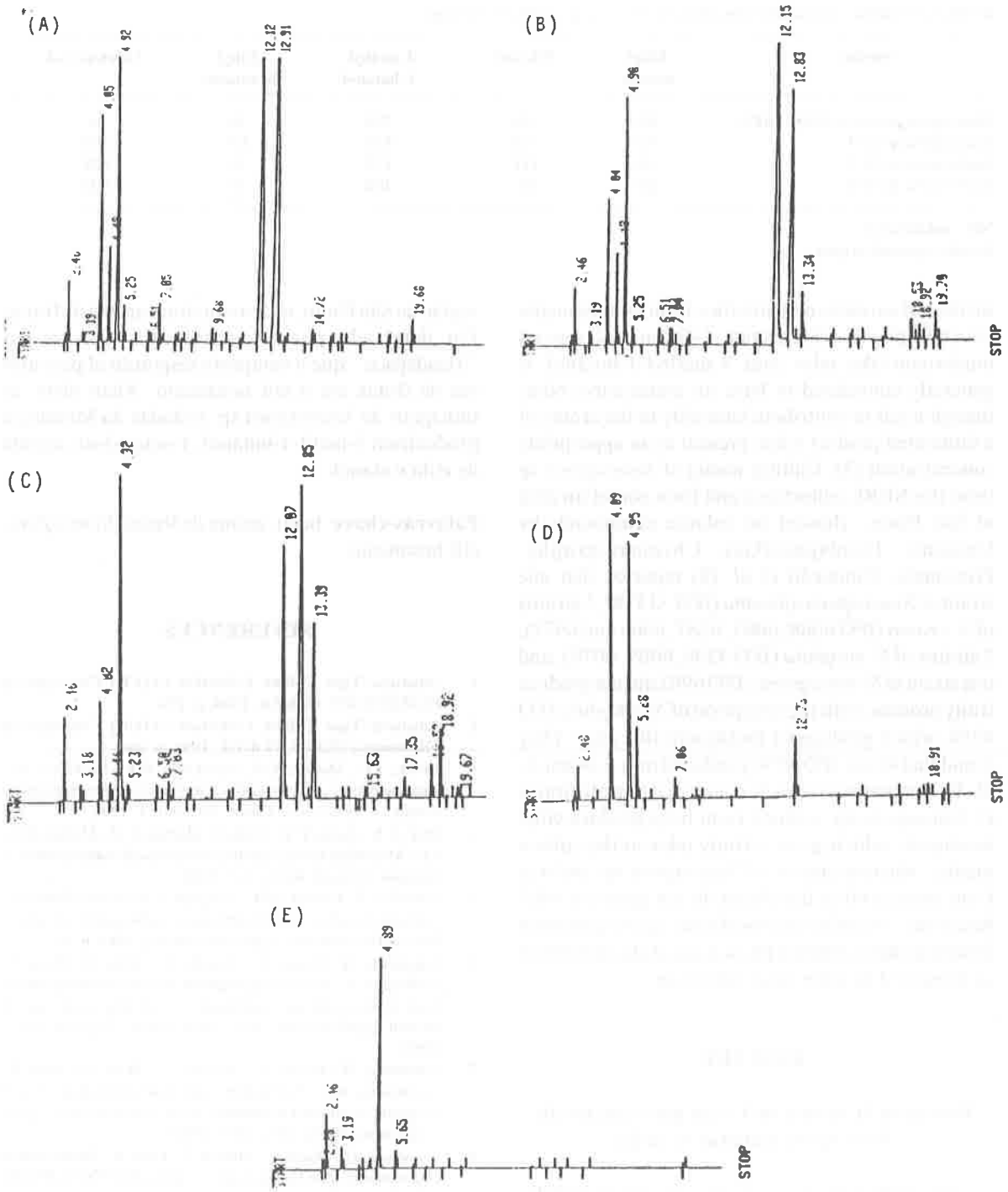

FIG.1. Dynamic Headspace/Gas Chromatography of Volatiles of *Neurospora* species.
1, Ethyl acetate; 2, Ethanol; 3, 3-Methyl-1-butanol; 4, Ethyl hexanoate; 5, 1-Octen-3-ol.
A, *Neurospora sitophila* ATCC 46892; B, *Neurospora* sp. Nº 6; C, *Neurospora* sp.
Nº 5; D, *Neurospora* sp. Nº 1; E, *Neurospora sitophila* NRRL 2884

TABLE 2. Volatile compounds produced by *Neurospora* sp. isolated from beiju.

Strains	Ethyl acetate	Ethanol	3-methyl-1-butanol	Ethyl hexanoate	1-Octen-3-ol
Neurospora sitophila ATCC 46892	4.8	128	318	59	40
Neurospora sp. Nº 1	9.0	111	ND	5.7	ND
Neurospora sp. Nº 5	0.9	111	117	16	125
Neurospora sp. Nº 6	2.8	99	208	26	ND

ND = not detected
Results expressed in ppm

method of extraction of volatiles from culture media which employed ethyl acetate. 1-Octen-3-ol causes a mushroom-like odor and 3-methyl-1-butanol is generally considered to have an unattractive odor, though tends to contribute favorably to the aroma of a fermented product when present at an appropriate concentration (3). Culture media of *Neurospora* sp from the NRRL collections and from soil of an area of São Paulo showed no volatile compounds by Dynamic Headspace/Gas Chromatography. Previously, Yamauchi *et al.* (8) reported that one strain of *Neurospora africana* (IFO 31378), 5 strains of *N. crassa* (IFO 6068, 6067, 6187, 6966 and 6977), 3 strains of *N. sitophila* (IFO 4596, 6069, 6070), and one strain of *N. tetrasperma* IFO 6982 did not produce fruity aromas, with the exception of *N. sitophila* IFO 4596, which produced a mushroom-like odor. They found that strain IFO 4596 produced only 1-octen-3-ol. To summarize, it can be concluded that all strains of *Neurospora* sp. isolated from beiju produce ethyl hexanoate, which gives a fruity odor to the culture media, whereas strains of *Neurospora* sp. isolated from sources other than beiju do not generate ethyl hexanoate. Therefore, strains of *Neurospora* sp isolated from beiju have different physiological characteristics as compared to other *Neurospora* sp.

RESUMO

Produção de aroma de frutas por espécies de *Neurospora* isoladas de beiju

Oito linhagens de *Neurospora* sp. foram isoladas de beiju, em várias regiões do Estado do Maranhão, Brasil. As linhagens apresentaram um agradável aroma de frutas no meio de cultura, enquanto que, as linhagens de *Neurospora* da coleção de cultura NRRL e outras linhagens de *Neurospora* isoladas de solo na região de São Paulo, não produziram aroma de frutas. Foi detectado por Cromatografia Gasosa por "Headspace" que o composto responsável pelo aroma de frutas era o etil hexanoato. Além disto, as linhagens de *Neurospora* sp. isoladas do Maranhão produziram 3-metil-1-butanol, 1-octen-3-ol, acetato de etila e etanol.

Palavras-chave: beiju, aroma de frutas, *Neurospora*, etil hexanoato

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LACTOBACILLUS ACIDOPHILUS AS DIETARY ADJUNCT: "IN VITRO" SUSCEPTIBILITY TO GASTRIC JUICE, BILE SALTS, LYSOZYME AND CHEMOTHERAPEUTIC AGENTS

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ABSTRACT

Lactobacillus acidophilus NCFM-S, sensitive to bacteriocin, as well as *L. acidophilus* NCFM-R_1 and *L. acidophilus* NCFM-R_2, both bacteriocin-resistant, were evaluated "in vitro" for their ability to grow in the presence of artificial gastric juice, bile salts, lysozyme and of eight chemotherapeutic agents. The aim of the study was to investigate how the degree of resistance to bacteriocin, an inhibitory substance secreted by *L. acidophilus* NCFM against related species, would relate to each strain's ability to survive conditions present in the human gastrointestinal tract. All the strains were sensitive to artificial gastric juice after 24 hours of incubation at 37°C. Oxgall at 0.3% also appeared to be inhibitory in all three cases, however strain NCFM-R_2 was the least affected. After 30 min. contact with a 100 µg/ml solution of lysozyme, strain NCFM-R_2 showed the lowest percentage inhibition. Evaluation of the action of ampicillin, kanamycin, neomycin, penicillin-G, streptomycin, chloramphenicol, tetracycline and sulfanilamide on *L. acidophilus* growth showed that strain NCFM-R_2 was again slightly more resistant to inhibition as compared to the other two. These results indicate that the NCFM-R_2 strain of *L. acidophilus* should be more appropriate for use as dietary adjunct than strains NCFM-R_1 and NCFM-S, since its resistance to bacteriocin was found to be coupled to a reduced susceptibility to the stressing conditions evaluated herein. These features thus make strain NCFM-R_2 better able to grow within the human gastrointestinal tract in the presence of other lactobacilli.

Key words: *Lactobacillus acidophilus*, gastrointestinal tract, dietary adjuncts, probiotics.

INTRODUCTION

Ever since Metchnikoff's theories (27) about the beneficial effects of lactobacilli on the intestinal microflora were put forward, there has been great interest in studying the benefits of consuming milk fermented by these microorganisms (26). Some researches have demonstrated that it is possible to increase the counts of lactobacilli in the human gastrointestinal tract in a few days by ingestion of cultured low-fat acidophilus milk. However, *Lactobacillus acidophilus* in this type of preparation has to be ingested continuously in order to obtain the expected effect on the intestinal microflora (16, 26).

The consumption of lactic products containing viable cells of lactobacilli is recommended due to the

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therapeutic effects that these microorganisms can have on the host, such as production of antibiotics and other inhibitory substances (34), competitive antagonism (12), deconjugation of bile salts (19) and anticarcinogenic action (28). Nutritionally, lactobacilli are associated with the synthesis of ß-vitamins (31); enzyme production resulting in partial hydrolysis of milk proteins, fat and lactose and enhancement of product digestibility (34); an anticholesterolemic effect (14); and increase in lactose utilization by the non persistent lactase(24).

There are many reports on the antagonistic action of *Lactobacillus acidophilus* towards some pathogenic microorganisms such as *Staphylococcus aureus* (1, 4, 39), *Pseudomonas putrefaciens* (35), *Escherichia coli* (15, 20) and *Salmonella tiphymurium* (15). The exact mechanism whereby dietary cultures of *L. acidophilus* may inhibit intestinal pathogens is not completely understood(13, 25). However, it is known that *L. acidophilus* produces bacteriocins in addition of others types of inhibitory compounds (2, 8). By definition, bacteriocins are active only against closely related species of bacteria and thus may not be of much benefit in controlling intestinal pathogens. However, they can be very important to the establishment of selected strains of *L. acidophilus* in the intestinal tract in the presence of other lactobacilli (13).

In order to exert both its therapeutic and nutritional effects in the gastrointestinal tract, *L. acidophilus* has to be viable and able to adhere to intestinal cells (3). The two first barriers met by these microorganisms ingested with food are the low pH and hydrochloric acid present in the stomach (3). If they survive gastric digestion they become strong candidates for the interaction with the gastrointestinal microflora (23). After passing through the stomach barrier, the microorganisms reach the duodenum, where the secretion of bile salt takes place. Thus, resistance to bile salts is an important factor to guarantee the establishment and growth of microorganisms used as dietary adjuncts within the intestinal tract (5, 9, 19, 22). Additionally, certain enzymes of the gastrointestinal system, such as lysozyme, are also deleterious to microorganisms (18, 33). Lysozyme has been detected in tears, nasal mucus, saliva, blood serum, plasma, milk, and many other tissues and secretions of human and animal origin (32, 38). Finally, antibiotic therapy drastically affects the balance of the intestinal microflora (18). According to Speck, (36), the use of lactobacilli after antibiotic treatment is recommended to restore the intestinal microflora and to inhibit the development of other organisms responsible for infections and other disturbances. In summary, for a microorganism to be useful as a dietary adjunct it must be able to bypass the natural barriers mentioned above.

In this study we analyzed the resistance of three strains of *L. acidophilus* to some conditions of the human intestinal tract "in vitro". Of these, we included gastric juice, bile salts, lysozyme and chemotherapics. Bacteriocin-resistant and bacteriocin-sensitive *L. acidophilus* strains were used in the evaluation. We found that *L. acidophilus* NCFM-R_2, a bacteriocin-resistant strain, was better able to bypass the *in vitro* conditions which mimic natural barriers than the other strains evaluated.

MATERIALS AND METHODS

Origin and maintenance of cultures

The following lactobacilli were used: *L. acidophilus* -S, -S standing for the sensitivity of the strain to the bacteriocin produced by *L. acidophilus* NCFM when grown in MRS broth (De Man, Rogosa and Sharpe-Difco, São Paulo, Brazil) at pH 6.0 (8); *L. acidophilus* NCFM-R_1 and *L. acidophilus* NCFM-R_2, both resistant to this bacteriocin. Strain NCFM-S was obtained from the Dairy Foods Laboratory of the Oklahoma State University, Stillwater, USA. The NCFM-R_1 and NCFM-R_2 strains were isolated from strain NCFM-S according to Ferreira and Gilliland (8).

Cultures were maintained under refrigeration in non-fat powdered milk reconstituted to 10% and sterilized at 121°C/15 min. All the cultures were subcultured three times in MRS broth for 18 hours(37°C) prior to use.

Testing the resistance to gastric juice

One percent aliquots of the *L. acidophilus* strains activated as stated above were inoculated into Erlenmeyer flasks containing 20 ml of artificial gastric juice (sodium chloride, 2g; pepsin, 3.2g; concentrated hydrochloric acid, 7ml; distilled water, 1000ml; final pH 1.2 to 3.0) prepared as described (11). The gastric juice solution was sterilized by filtering through a Millipore filter (0.45 µm pore

size). The pH of the gastric juice samples was maintained within the range of 2.0 to 2.3. Inoculated gastric juice samples were kept at 37°C and the *L. acidophilus* counts performed immediately and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 and 24 hours after incubation. Inoculated MRS broth maintained under the same conditions was used as control. The experiment was repeated three times for each strain.

Testing the resistance to bile salts

Each of the three strains were inoculated (1.0%) into screw cap tubes containing 5ml of sterilized MRS broth supplemented with 0.3% Oxgall. A control tube with MRS broth devoid of Oxgall was prepared for each strain. Tubes were kept at 37°C in a water bath and the growth after 8 hours of incubation followed turbidimetrically at 620nm in a Spectronic 20 (Bausch & Lomb) (17). The experiment was repeated three times.

Testing the resistance to chemotherapics

Fresh cultures of *L. acidophilus* were inoculated onto MRS agar in order to obtain spread growth (0.1 ml of inoculum in approximately 15 ml agar). After inoculation, paper discs (6.0mm diameter, Laborclin, Brazil) containing selected chemotherapics (listed in TABLE 1) were placed over the agar and subsequently incubated at 37°C for 48 hours. Two discs of testing material were prepared for each strain. The diameter of inhibition zones was measured at the end of the incubation period.

Testing the resistance to lysozyme

TABLE 1 - Chemotherapeutic agents used on *L. acidophilus* cultures.

CHEMOTHERAP.AGENTS[a]	CONCENTRATION (µg/DISC)
Ampicillin (AM)	10
Kanamycin (KA)	30
Neomycin (NE)	30
Penicillin G (PE)	10*
Streptomycin (SR)	10
Chloramphenicol (CM)	30
Tetracycline (TE)	30
Sulfanilamide (SF)	50

* Concentration expressed in units/disc.
[a] Paper discs obtained from Laborclin, Brazil.

The active strains of *L. acidophilus* were centrifuged (2000g; 15 min.; room temperature) and the supernatant discarded. Cells were resuspended in TES buffer (10mM tris, 10mM EDTA, 5mM NaCl, pH 7.0) and centrifuged as above. The cell pellets obtained were finally resuspended in TES buffer containing 100 µg/ml lysozyme (E.C.3.2.1.17; Sigma Chemicals), to the absorbance of 0.9 units (at 420 nm) measured in a Spectronic 20 (Bausch & Lomb), as described (21). For each strain, a control sample containing TES buffer without lysozyme was run. The tubes were incubated in a water bath at 37°C and the turbidity determined at 420nm immediately after contact with lysozyme (time 0) and after 30 minutes of incubation. Lysis was expressed as the variation of percent turbidity after incubation of the sample containing lysozyme in relation to the control sample. The whole procedure was repeated three times.

Statistical analysis

The data obtained from the lysozyme and chemoterapics experiments were analyzed by the SAEG system (System of Statistical Analysis and Genetics) developed by the Federal University of Viçosa, Minas Gerais, Brazil. Qualitative treatment data were compared by the Newman-Keuls test (28). The level of significance for the comparisons was 5% ($p<0.05$). Gastric juice and bile salt data were assessed by analysis of the average and standard deviation.

RESULTS AND DISCUSSION

Resistance to gastric juice

The ability of the *L. acidophilus* strains to resist incubation at 37°C in artificial gastric juice is shown in TABLE 2. The number of microorganisms at time 0 was similar for all the strains. After 3 hours, the reduction on viable cells was of 4 and 3 log cycles for strains NCFM-R_1 and NCFM-R_2 respectively, whereas that for strain NCFM-S was of 2 log cycles (from 3.7×10^5 to 1.7×10^3 CFU/ml). Pettersson *et al.* studied the behaviour of *L. acidophilus* NCDO 1748 in the presence of human gastric juice for the same time period and observed a reduction from 7.1×10^8 CFU/ml to 2.6×10^6 CFU/ml (2 log cycles). The authors classified the strain as resistant to gastric juice. So, by

analogy, we could consider NCFM-S a resistant strain. However, according to Davenport (5), the time that food is kept in the stomach depends on its composition. When the food is poor in fat, its gastric drainage ends in four hours. Therefore, since the reduction observed after 4 hours in this study was of 3 log cycles for strain NCFM-S and of 4 log cycles for the other two strains, all the strains were considered susceptible to the action of artificial gastric juice. Nonetheless, our analysis does not take into account the protection that food as a whole should exert on the bacterial cells, reducing the action of gastric juice upon them. Furthermore, when the passage of food containing the microorganism through the stomach is faster, a higher number of survivors is to be expected.

Resistance to bile salts

Results on resistance to bile salts are shown in FIGURE 1. The growth of strains NCFM-S and NCFM-R_2 was similar. These microorganisms grew alike in medium with and without bile salts. *L. acidophilus* NCFM-R_1 performed less well than the other two strains in both media; it was more inhibited by bile salts than NCFM-S and NCFM-R_2. Gilliland *et al.* (17) demonstrated the resistance of seven strains of lactobacilli isolated from calves to 0.3% Oxgall. In that study, strains that did not reach 0.3 units of absorbance (620nm) after six hours of incubation at 37°C were considered sensitive to bile salts. So the strains studied herein should be considered sensitive to bile salts, as they only reached 0.3 of

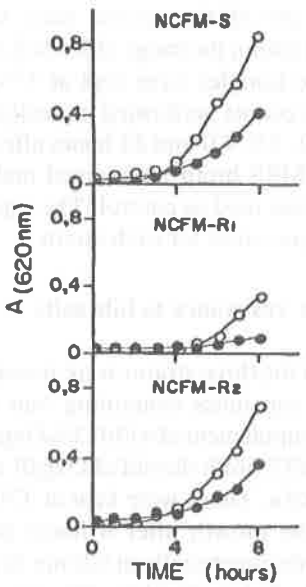

FIGURE 1 - Growth of *L. acidophilus* NCFM-S, *L. acidophilus* NCFM-R_1 and *L. acidophilus* NCFM-R_2 in MRS broth with (●) and without (○) 0.3% Oxgall added.

absorbance after eighth hours of incubation in MRS containing 0.3% Oxgall. However, it must be considered that these experiments do not allow for the recirculation of bile salts in the human intestinal tract 6) that removes deconjugated salts, which are more inhibitory than the conjugated ones (9,10). Thus strains that were considered sensitive according to our experiment may be resistant in "in vivo" conditions.

TABLE 2 - Survival (log # CFU/ml) of *L. acidophilus* NCFM-S, NCFM-R_1 and NCFM-R_2 in artificial gastric juice at 37°C.

Strain	Time(hours)							
	0	0.5	1.0	1.5	2.0	3.0	4.0	24.0
NCFM-S	5.57 ±0.37a	5.49 ±0.69	3.95 ±0.55	3.94 ±0.87	3.79 ±1.02	3.22 ±1.20	2.11 ±1.39	0.84 ±0.32
NCFM-R_1	5.24 ±0.10	3.80 ±1.41	3.79 ±0.87	2.44 ±1.39	2.77 ±0.59	1.74 ±0.72	1.38 ±0.69	0.87 ±0.28
NCFM-R_2	5.33 ±1.17	4.52 ±1.00	3.73 ±1.02	3.57 ±0.99	3.47 ±0.86	2.37 ±0.54	1.33 ±0.06	1.11 ±0.10

[a] Results are expressed as means ± SD of three experiments.

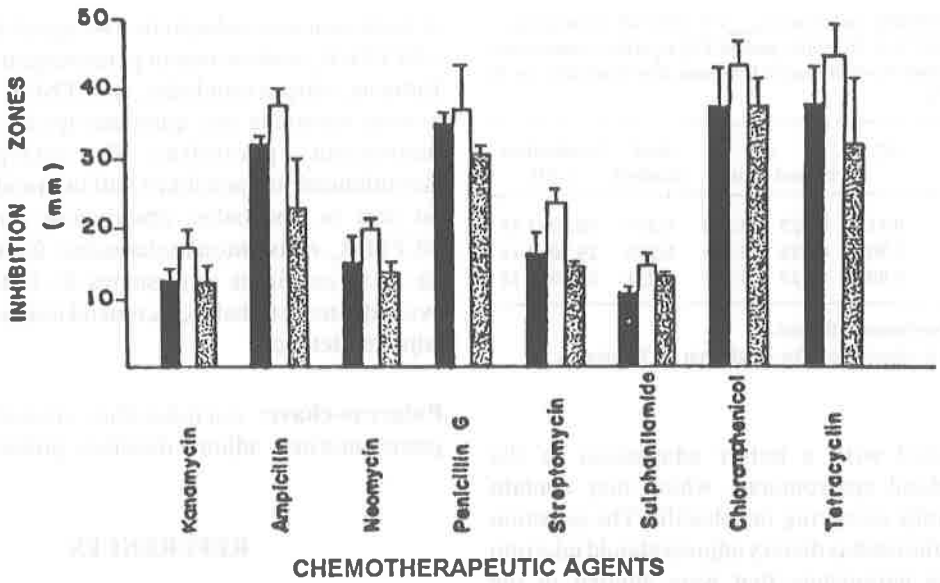

FIGURE 2 - Effect of different chemotherapeutic agents on *L. acidophilus* NCFM-S (■), *L. acidophilus* NCFM-R_1 (□) and *L. acidophilus* NCFM-R_2 (▨).

Resistance to chemotherapeutic agents

The antibiotics involved in this evaluation can be placed into three groups according to their mechanism of action: 1) streptomycin, neomycin and kanamycin, which act on protein synthesis and determine the synthesis of modified proteins, resulting in abnormal membranes with altered permeability; 2) tetracycline and chloramphenicol which inhibit protein synthesis - the first inhibiting the fixation of messenger-RNA to the ribosome by competing with it and the latter inhibiting the fixation of transport-RNA to ribosome; 3) ampicillin and penicillin, which inhibit the formation of adequate bacterial cell walls (37). The last compound sulfanilamide, not an antibiotic, competes with paraminobenzoic acid, essential for the synthesis of nucleic acid components.

Results are shown in FIGURE 2. It can be observed that all strains were affected by the chemotheraphics in the following order of increasing susceptibility: antibiotics acting on protein synthesis (chloramphenicol and tetracycline); antibiotics acting on the cell wall (penicillin and ampicillin); antibiotics altering the permeability of membranes (streptomycin, neomycin and kanamycin) and sulfanilamide. According to the statistical analysis, for each strain, the behaviours towards individual chemotherapics did not differ significantly between them.

When different strains were compared, *L. acidophilus* NCFM-R_2 presented an equal or higher resistance than *L. acidophilus* NCFM-S to the chemotherapics studied. *L. acidophilus* NCFM-R_1 was more sensitive in almost all occasions. The tendency of NCFM-R_2 to be more resistant to these drugs is an important characteristic which justifies its use as a dietary adjunct. Resistance to chemotherapeutic agents favours the maintenance of a high number of *L. acidophilus* cells in the intestinal tract and this contributes to the balance of intestinal microflora in patients being treated with the compounds(7).

Resistance to lysozyme

The incubation of lactobacilli with a 100 μg/ml lysozyme solution for 30 minutes resulted in approximately 30%, 29% and 26% growth inhibition for strains NCFM-S, NCFM-R_1 and NCFM-R_2, respectively (TABLE 3). All strains presented some sensitivity to lysozyme though differences in their responses were not statistically significant.

The behaviour of *L. acidophilus* NCFM-R_2 observed in this experiment added to the previous ones suggests that this microorganism is more indicated as dietary adjunct than the other two. The ability of this strain to resist to bacteriocin appears to

TABLE 3 - Turbidity variation (A_{420nm}) of TES buffer containing *L. acidophilus* NCFM-S, NCFM-R_1 and NCFM-R_2 in the presence (test) and absence (control) of 100 µg/ml lysozyme after incubation for 30 minutes at 37°C.

strains	A_1[a]	A[b]		Atest - Acontrol	% inhibition ± SD
		control	test		
NCFM-S	0.912	0.025	0.302	0.277	30.29±3.19
NCFM-R_1	0.902	0.032	0.296	0.265	29.30±2.19
NCFM-R_2	0.898	0.027	0.265	0.238	26.49±3.18

[a] A_1=Initial absorbance of the test.
[b] A=Decrease in absorbance after incubation for 30 minutes.

be associated with a better adaptation to the gastrointestinal environment, which may contain other naturally occurring lactobacilli. The selection of strains to be used as dietary adjunct should take into account the parameters that were studied in the present investigation.

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RESUMO

Lactobacillus acidophilus como adjunto dietético: resistência "in vitro" ao suco gástrico, sais biliares, lisozima e quimioterápicos

Lactobacillus acidophilus NCFM-S, sensível à bacteriocina, *L. acidophilus* NCFM-R_1 e *L. acidophilus* NCFM-R_2, resistentes à bacteriocina, foram avaliadas "in vitro" quanto à resistência a suco gástrico artificial, sais biliares, lisozima e quimioterápicos. O objetivo do trabalho foi saber se a resistência à bacteriocina, uma substância inibitória secretada por *L. acidophilus* NCFM contra espécies homólogas, poderia estar relacionada com a habilidade das estirpes de sobreviver no trato gastrointestinal humano. As três culturas apresentaram sensibilidade após 24 horas em presença de suco gástrico artificial a 37°C. Oxgall a 0,3% pareceu ser inibitório para as três culturas, apesar de haver menor inibição para a estirpe NCFM-R_2. Após 30 minutos de contato com uma solução de 100 µg/ml de lisozima, a NCFM-R_2 mostrou menor percentagem de inibição. Entre as estirpes estudadas, a NCFM-R_2 mostrou-se a mais resistente aos quimioterápicos ampicilina, neomicina, penicilina G, estreptomicina, cloranfenicol, tetraciclina e sulfanilamida. De acordo com os resultados, observou-se que a estirpe NCFM-R_2, resistente à bacteriocina, foi menos afetada pelas condições estressantes do trato intestinal avaliadas neste trabalho, recomendando seu uso como adjunto dietético.

Palavras-chave: *Lactobacillus acidophilus*, trato gastrointestinal, adjunto dietético, probiótico.

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MICROSCOPIC AGGLUTINATION FOR THE IDENTIFICATION OF *CANDIDA ALBICANS* SEROGROUPS "A" AND "B"*

Celeste Fava Netto[1]
Claudete Rodrigues Paula[1]

SHORT COMMUNICATION

ABSTRACT

A microscopic reading of the agglutination reaction is proposed to substitute the naked eye agglutination evaluation used in the identification of *Candida albicans* serogroups "A" and "B". The microscopic method may obviate some doubtful results obtained with the conventional naked eye technique.

Key words: *Candida albicans*; serogroups, microscopic agglutination readings.

The agglutination reaction for the identification of *Candida albicans* serogroups "A" and "B" is usually evaluated by readings taken with the naked eye (1, 2, 3). We are proposing an alternative technique in which a microscopic reading is used.

Reagents are delivered onto a thoroughly clean microscope slide, according to the following procedure:

1 - addition of 10 µL of specific anti-*Candida albicans* serogroup "A" serum;

2 - addition of 5 µL of a suspension of the unknown strain of *Candida albicans* (turbidity equivalent to tube 10 of McFarland scale), prepared in physiologic saline containing merthiolate 1:5000;

3 - homogenization with a stick to obtain a spot of 1 cm x 1cm;

4 - microscopic reading, using a 10x ocular and a 20x objective, and an adequate illumination. The agglutinated cells are clearly seen.

5 - inclusion of a control slide containing a drop of the suspension of the unknown strain in physiologic saline.

Results should be interpreted as follows:

agglutination = strain serogroup "A" positive

non agglutination = strain serogroup "B" positive

This technique also allows the use of a partially absorbed antiserum. Microscopic readings should be done within 3 minutes, without agitation of the slide.

RESUMO

Aglutinação microscópica para identificação de *Candida albicans* sorogrupos "A" e "B"

Neste trabalho está sendo proposta a leitura microscópica para visualização da reação de aglutinação em substituição à leitura realizada a olho nu na identificação de *Candida albicans* sorogrupos "A" e "B". A leitura microscópica pode dirimir qualquer dúvida encontrada na leitura a olho nu.

Palavras-chave: *Candida albicans*, sorogrupos, aglutinação microscópica

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FIGURES

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