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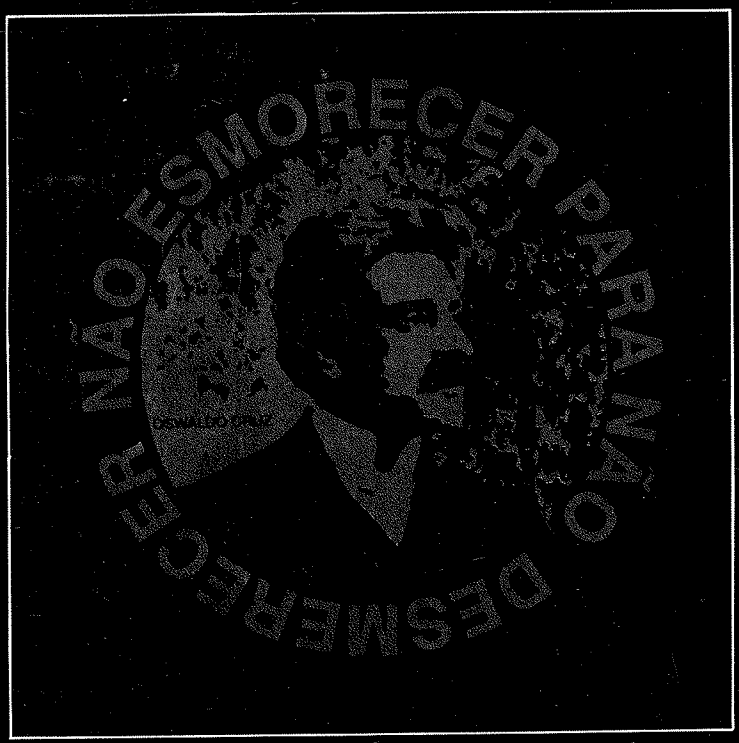

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Continuem prestigiando a Revista e a Sociedade Brasileira de Microbiologia, pois nós passamos mas as Instituições permanecem.

Flávio Alterthum

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Rev. Microbiol., São Paulo, 23(4): 211-216, 1992

EFFECT OF COLOSTRUM AND HUMAN MILK ON THE ADHERENCE TO HeLa CELLS OF COMMON AND RARE ENTEROPATHOGENIC *ESCHERICHIA COLI* SEROTYPES FOUND IN BRAZIL

Carmen M. Saraiva Giampaglia[1]
Maria de Lourdes Monteiro da Silva[2]

SUMMARY

The effect of colostrum and human milk upon the localized adherence of enteropathogenic *Escherichia coli* (EPEC) to HeLa cells was studied. The tests were performed with 8 EPEC strains belonging to serotypes 055:H$^-$, H6, and H7; 086:H34; 0111:H$^-$ and H2; 0119:H6 and 0142:H6. A pool of 5 samples of colostrum was used and the milk was collected from 3 women at 4, 9, and 14 months after delivery (milk 1, 2, and 3, respectively). High percentages of adherence inhibition were observed when colostrum or milk were added during the infection period of the HeLa cells with EPEC. Incubation of HeLa cells or bacterial cultures with colostrum or milk before the adherence tests also allowed remarkable adherence inhibition mainly with colostrum or milk 1. The serotypes more affected were 055:H$^-$ 0111:H$^-$, 0111:H2 and 0119:H6, the last three more frequently isolated in acute diarrhea in Brazil.

Key Words: Bacterial adhesion, colostrum immunology, infantile diarrhea, *Escherichia coli.*

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) are the main cause of acute diarrhea in children up to 1 year old in developing countries (17). Morbidity rates due to acute diarrheic disease were found to be highest in the 6 to 11-month age group, while the mortality rates were greatest in infants under 1 year of age and children 1 year old (26). EPEC strains belong to specific serotypes and 0111:H$^-$, 0111:H2 and 0119:H6 are the aetiological agents most frequently found in Brazil (27).

The mechanism of virulence of EPEC strains was shown to be the adherence of the bacteria to the intestinal mucosa (4, 21, 28). An attaching and effacing activity of EPEC strains in epithelial cells in pig and rabbit intestine was first described by Moon et al (19). These studies were corroborated by Knutton et al (15) who demonstrated that EPEC adherence is a two-stage mechanism: an initial attachment of bacteria to the intestinal mucosa, promoted by plasmid-encoded adhesins, and subsequent effacing of microvilli and intimate EPEC attachment with destruction of the intestinal brush border.

Adherence of EPEC strains to tissue-culture cells was first demonstrated by Cravioto et al (6) using HEp-2 cells. Scaletsky et al (25) studied the adherence of *E. coli* strains to HeLa cells describing two distinct patterns: localized adherence (LA)

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characteristic of EPEC strains and diffuse adherence (DA) presented by other classes of *E. coli*. An enteroaggregative adherence pattern was later described by Nataro et al (20). Localized adherence was described in 93% of enteropathogenic serotypes whereas its occurrence in non-enteropathogenic serotypes was 14% (24). LA was found to be mediated by a plasmid of approximately 60 megadaltons (2). Two outer-membrane proteins of 29-32 kDa (23) and 94kDa (3) and an inducible bundle-forming pili composed of a reapeting subunit of 19,500 daltons (12) were related to EPEC adherence.

Breastfeeding has been described as an important protection factor from intestinal and respiratory infections (14). The amount of specific secretory immunoglobulin A (sIgA) in milk has been shown to relate to proctection against certain pathogens such as *Vibrio cholerae* (13) and enterotoxin-producing *E. coli* (5, 8, 12). Nonimmunoglobulin fraction of human milk has also been related to protection against infections due to *Streptococcus pneumoniae* and *Haemophilus influenzae* (1). We have recently described the inhibition of one EPEC serotype (0111ab:H2) adherence to HeLa cells by colostrum and human milk (11).

This communication reports the effect of colostrum and human milk upon the adherence to HeLa cells of common and rare EPEC serotypes found in Brazil, under different test conditions.

MATERIAL AND METHODS

Bacterial strains – Eight EPEC LA$^+$ strains belonging to different serotypes were used (Table 1). All strains were isolated from feces of patients with acute diarrhea in São Paulo, Brazil. O and H antigen determinations were performed as decribed by Edwards and Ewing (10).

Colostrum and human milK – Five 5ml samples of human colostrum were collected no more than 72h postpartum and used as a pool. Human milk was collected from 3 women at 4, 9 and 14 months after delivery (milk 1, 2, and 3, respectively). Both colostrum and milk were mantained in aliquots at -20°C until used. Milk was delipidated after a 30min 1,500g centrifugation at 4°C.

Adhesion assays – HeLa cells were grown as previously described (16) using Lab-Tek Chamber Slides (Nunc Inc., Naperville, Illinois) with 8 chambers per slide for tissue culture. For quatification purposes exponential-phase bacterial cultures grown in Tryptic Soy Broth (TSB, Difco Laboratories) were used at a concentration of $2x10^8$ bacteria ml^{-1} in all experiments. Adherence assays were carried out under three different conditions:

Condition A: An amount of 100µl of exponential-phase bacterial cultures were added to each chamber containing 150µl of colostrum or each sample of milk plus the same volume of Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 1% D-Mannose (DMEM-FBS-DM). The chamber slides were incubated for 30 min at 37°C (infection period). Unattached bacteria were then removed by washing six times with PBS. A subsequent incubation of 3h was carried out with 300µl of the same medium without colostrum or milk. The cells were then washed with PBS, fixed with methanol and stained with May-Grünwald and Giemsa as previously described (25).

Condition B: HeLa cells were preincubated with 150µl of colostrum or each sample of milk with the same volume of DMEM-FBS-DM for 1h at 37°C. After this period the colostrum or milk was replaced by 300µl DMEM-FBS-DM and 100µl of the exponential-phase bacterial culture. The system was then incubated for 30min at 37°C, the non-attached bacteria were removed by washing with PBS followed by a subsequent incubation time of 3h with DMEM-FBS-DM and so on, as described for Condition A.

Condition C: Bacterial cultures were preincubated with colostrum or milk 1:1 (v:v) for 1h at 37°C. After this period the adhesion test was carried out inoculating 200µl of the suspension above in chambers containing 200µl of DMEM-FBS-DM. The chamber slides were incubated for 30min at 37°C and the test was performed as described for Condition A.

TABLE 1 – Localized adherence frequencies of enteropathogenic *Escherichia coli* strains.

Strain	Serotype	Adherence frequencies(%)[a]
49-81HSJ	055 : H$^-$	49.0 (± 18.0)
111-1-85HMJ	055 : H6	90.5 (± 2.5)
340-2CII	055 : H7	95.5 (±3.5)
96-82HSP	086 : H34	87.0 (±6.0)
0041-1-85HMJ	0111 : H$^-$	92.0 (±1.0)
34-81HC	0111 : H2	93.5 (±4.5)
53-81HSJ	0111 : H6	51.0 (±3.0)
51-81HSJ	0142 : H6	92.0 (±1.0)

[a] Results represent mean ± SD of three determinations.

At least one hundred HeLa cells of each preparation were observed under light microscopy. In all experiments the effect of colostrum or milk upon EPEC adherence to HeLa cells was determined by calculating the percentage of cells with six or more attached bacteria in relation to the control, carried out under same conditions without colostrum or milk.

RESULTS

Colostrum and the three samples of milk all inhibited the adherence of EPEC strains whether the number of HeLa cells with attached bacteria or the clusters' sizes per cell are considered (Fig. 1).

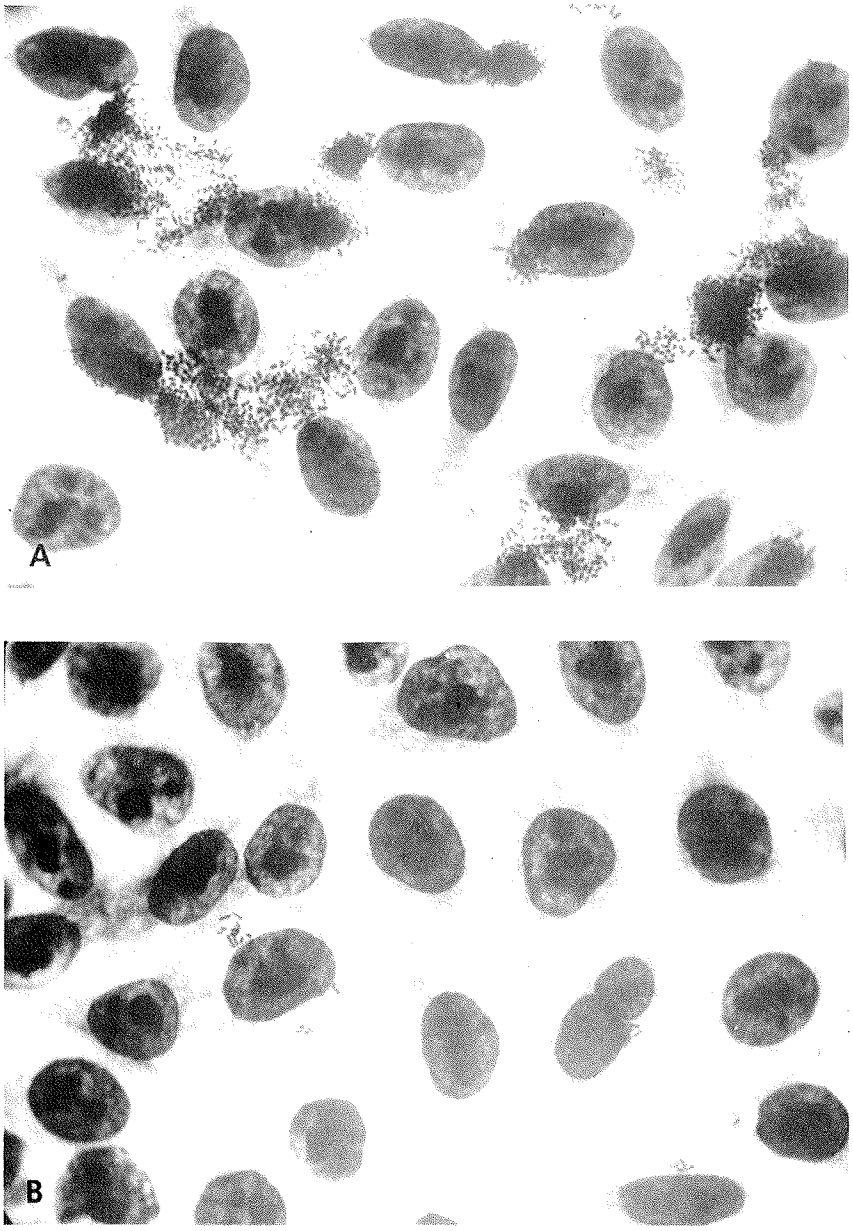

FIGURE 1 – *E. coli* 0111:H2 attached to HeLa cells. A, Control assay. B, Effect of milk 2 during the 30 min infection period (inhibition of adherence: 86%) (1000X).

TABLE 2 – Adherence inhibition of EPEC strains by colostrum and human milk during the 30 min infection period (A) and after 1h preincubation with HeLa cells (B) or bacterial culture (C).

	Inhibition of Adherence to Hela Cells (%)[a]											
	Colostrum			Milk 1			Milk 2			Milk 3		
Serotype	A	B	C	A	B	C	A	B	C	A	B	C
055 : H-	92	83	NT	88	93	78	91	67	63	91	0	0
055 : H6	96	41	81	66	8[b]	12[b]	24	0	0	69	1[b]	0
055 : H7	94	20	55	24	9	0	71	0	0	94	0	0
086 : H34	55	89	49	64	8[b]	5[b]	28	0	9[b]	72	0	0
011 1 : H-	97	89	71	92	88	55	96	0	29	88	0	0
0111 : H2	96	90	96	73	35	13	86	0	12	79	0	0
0119 : H6	44	37	60	89	77	84	75	0	NT	81	0	0
0142 : H6	87	39	68	NT	NT	NT	61	0	0	79	6[b]	0

[a] Calculated in relation to bacteria adherence frequency in control experiments.
[b] No statistically significant difference between adherence frequencies of control assays in χ^2 test ($P > 0.05$).
NT, not tested.

Colostrum inhibited the adhesion of the bacteria in frequencies usually higher than 40% (Table 2). The three samples of milk also showed high percentages of inhibition, mainly when present during the infection period of the test. Milks 2 and 3 were less effective than milk 1. Increasing frequency of adherence was observed in some cases.

As regards the assay condition, a high inhibition of adherence was always observed when the colostrum or milk was present in the system during the infection period. Pre-incubation of HeLa cells or the bacterial cultures with colostrum or milk were efficient mainly when colostrum or milk 1 were used.

All EPEC strains showed inhibition of adherence when treated with colostrum or milk, the percentages varying as to the sample of colostrum or milk used and the assay condition. In general, strains of serotypes 055:H-, 0111:H-, 0111:H2 and 0119:H6 were more affected.

DISCUSSION

The main finding of this study was the significant inhibition effect of colostrum and human milk on the adherence of EPEC to HeLa cells. The inhibition was observed in all strains, whether or not they belonged to the more frequent EPEC serotypes often isolated from acute diarrhea in Brazil.

Inhibition of adherence was more effective when colostrum or milk was present in the system to which the bacteria were added. Two factors may be involved in such inhibition: SIgA and non-immunoglobulin or oligossacharide milk components (14). Specific induction of SIgA antibodies occurs largely through the common mucosal immune system, whereby antigens presented to the mucosa-associated lymphoid tissues, such as intestinal Peyer's patches, stimulate B lymphocytes committed to IgA systhesis. These cells enter the circulation via lymphatics and finally home to several remote secretory tissues (22). In a recent study Cravioto et al. (7) showed that both sIgA and oligossacharides present in breast milk are capable of inhibiting the attachment *in vitro* of EPEC strains.

Our results showed that adherence of both common EPEC serotypes isolated in Brazil, such as 0111:H-, 0111:H2 and 0119:H6 as well as rare serotypes (055:H-, H6 and H7, 086:H34 and 0142:H6) was inhibited by both colostrum and milk. The nature and antigenicity of adhesins of different EPEC serotypes has not so far been studied. Our results suggest that these adhesins may be antigenically related since one mother's milk inhibits the adherence of strains belonging to serotypes to which she may not have been exposed.

Several bacterial and viral antigens have been used to evoke sIgA antibodies experimentally in humans, but only a few oral vaccines have been developed for clinical application (22). Dluholucký et al (9) attempted in 1980 to produce antimicrobial activity in colostrum giving killed *E. coli* 0111 orally to expectant mothers. These authors observed inhibition of the growth of *E. coli* by 7 of 47 samples of colostrum used. A bacteriostatic and bactericidal effect of a pool of the three samples of milk upon *E. coli* 0111 was not observed in our experiments during the period equivalent to the adhesion assay (data not shown).

In this work we showed that colostrum and milk inhibit the adherence of EPEC strains in an *in vivo* system, suggesting that an analogous process may occur *in vivo*, protecting their offspring against acute diarrhea. Social improvements which would permit mother to breastfeed their children would contribute to the lowering of infant mortality by diarrhea in third world countries such as Brazil.

RESUMO

Efeito do colostro e leite materno sobre a adesão a células HeLa de *Escherichia coli* enteropatogênicas isoladas no Brasil.

Neste trabalho foi estudado o efeito do colostro e leite materno sobre a adesão localizada de *Escherichia coli* enteropatogênica (EPEC) a células HeLa. Para tanto, foram utilizadas 8 amostras de EPEC pertencentes aos sorotipos 055:H$^-$, H6 e H7; 086: H34; 0111:H$^-$ e H2; 0119:H6 e 0142:H6. Cinco amostras de colostro foram utilizadas em forma de "pool" e o leite foi coletado de 3 mulheres com 4, 9 e 14 meses de amamentação (leites1, 2 e 3, respectivamente). Foram observados altos índices de inibição de aderência quando o colostro ou o leite eram adicionados ao sistema durante o período de infecção das células HeLa com as amostras de EPEC. Incubação prévia das células HeLa ou das culturas bacterianas com colostro ou leite também provocaram altos índices de inibição, principalmente na presença de colostro e leite 1. Os sorotipos mais afetados foram 055:H$^-$, 0111:H$^-$, 0111:H2 e 0119:H6, sendo os últimos três os mais freqüentemente isolados em casos de diarréia aguda no Brasil.

Palavras-chave: Aderência bacteriana, colostro, imunologia, diarréia infantil, *Escherichia coli*.

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CLONING OF THE MERCURIC ION-RESISTANCE OPERON OF pBH100 INTO *ESCHERICHIA COLI* 5K USING pAT153 AS VECTOR

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Spártaco Astolfi-Filho[2]
Edmar Chartone-Souza[1]

SUMMARY

Transconjugants were initially obtained by crossing the wild strain *Escherichia coli* BH100 bearing plasmid pBH100 (95 Kb) which codes for resistance to tetracyclin, kanamycin, chloramphenycol, streptomycin and inorganic mercury, with *E. coli* 5K. In order to clone the "Hg operon", recombinant plasmid were constructed from fragments of plasmids pBH100 and of the cloning vehicle pAT153 digested with the enzyme *Bam*H I. After transformation of *E. coli* 5K, genetic and electrophoretic analysis permitted the detection of recombinant plasmids that were denoted pATHg1, pATHg2 and pATHg3. pATHg3 simultaneously showed less stability (37%) and lower size (12 Kb). Restriction analysis of the plasmid pATHg1 digested with *Bam*H I and *Hind* III showed bands of 24, 4 and 1 Kb and two bands of 21 and 8 Kb, respectively.

Key Words: Mercury resistance, *Escherichia coli*, cloning, pAT153.

INTRODUCTION

Mercury ions are toxic to bacteria mainly because they strongly bind to sulfhydryl groups, thus inhibiting biosynthesis and enzyme activity (5). Bacterial resistance to mercury and organomercury is determined by plasmids that may simultaneously code for resistance to other heavy metals and antibiotics (16, 17, 18). Several transposons associated with mercury resistance have been described (18).

The biochemical mechanism of plasmid encoded resistance to inorganic mercuric depends on the enzyme mercuric reductase which reduces Hg^{2+} to the elemental form of mercury (Hg^0). In the degradation of organomercurial compounds there is the additional involvement of the enzyme organomercurial-lyase which cleaves carbon-mercury bounds. According to Summers (1986)[18] other mechanisms of mercury biotransformation have been observed in bacteria. Gene cloning and expression for inorganic mercury resistance have permitted a better understanding of this mechanism including the mapping and sequencing of several of the genes involved (6, 12, 18).

Genetic analysis and DNA sequencing have permitted the identification of six genes in the *mer* region of plasmid R100 (4, 10, 15), i.e., *mer*R, which codes for a regulating protein responsible for the induction of the system; *mer*T, which is responsible for mercury transport into the cell; *mer*P, which acts on mercury absorption; *mer*C, whose function has not yet been fully elucidated and whose product may be involved in transport or

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regulation; *mer*A which codes for the enzyme mercuric reductase, and finally gene *mer*D, whose product and function are unknown.

Plasmids which code for broad-spectrum resistance contain an additional gene, *mer*B, which codes for organomercurial-lyase (6).

Considering the relevance of the genetic study of bacterial resistance to mercury with respect to public health and the possibility of using bacterial strains and/or the enzymes responsible for the mechanism of this resistance to clean up the environment polluted by industrial waste contaminated with mercury, we undertook the present study with the objective of cloning a mercury resistance operon from a R plasmid (pBH100) of the wild strain *E. coli* BH100.

MATERIAL AND METHODS

E. coli BH100 is a wild strain bearing two R plasmids of respective sizes of approximately 95 and 15 Kb. The first (pBH100) is a conjugative plasmid and codes for resistance to inorganic mercury, tetracyclin, kanamycin, chloramphenicol and streptomycin, and the second (pAp) is a non-conjugative but can be mobilized by the first and codes for resistance to ampicillin. Plasmid pBH100 was transferred by conjugation to *E. coli* 5K (8), with chromosomal resistance to streptomycin.

The DNA of the plasmid pBH100 from the transconjugant *E. coli* 5K and of plasmid pAT153 (20) was isolated by the method of Birnboin and Doly (1979)[1] and treated with the restriction enzyme *Bam*H I (Biolabs). Extraction and determination of the size of plasmidial DNA were also carried out by the method of Meyers et al. (1976)[9]. To obtain recombinant plasmids "in vitro", the DNA fragments were incubated in the presence of DNA-ligase T_4 (8). The transformation of *E. coli* 5K cells sensitive to mercury was performed by the method of Dagert and Elrich (1979)[3].

Restriction analysis of pATHg1 was performed with *Bam*H I and *Hind* III (Biolabs); pAT153 was digested with *Bam*H I and λ phage DNA (standard) with *Hind* III and *Eco*R I (Biolabs): see Maniatis et al. (1982)[8].

Electrophoresis was carried out on 0.8% agarose gel (Sigma type II) using TEB buffer. The level of resistance to $HgCl_2$ was determined by the dilution method in nutrient agar (Difco) containing 0.5% NaCl (Merck), pH 7.0.

The stability of the resistance markers ampicillin and mercury dichloride (recombinant plasmids) was determined by analysis of 100 colonies after several growths in nutrient broth (Difco) without selective pressure.

RESULTS

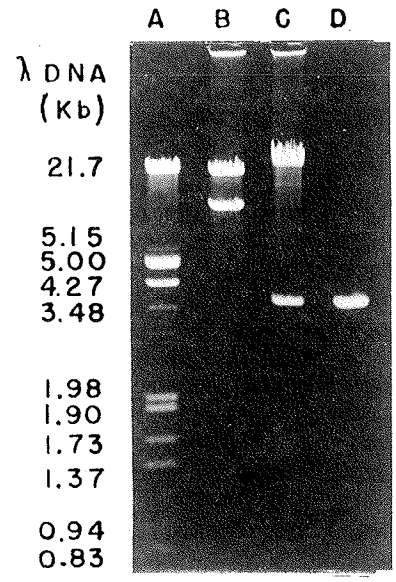

FIGURE 1 - Restriction analysis of plasmid pATHg1 digested with *Bam*H I and *Hind* III. A, λ phage DNA (standard) digested with *Eco*R I and *Hind* III - fragments of 21.7, 5.15, 5.0, 4.27, 3.48, 1.98, 1.90, 1.73, 1.37, 0.94, and 0.83 Kb; B, plasmid pATHg1 digested with *Hind* III - fragments of 21 and 8 Kb; C, plasmid pATHg1 digested with *Bam*HI - fragments of 24, 4 and 1 Kb; D, plasmid pAT153 digested with *Bam*H I - fragment of 4 Kb.

To clone the "Hg operon" of the conjugative plasmid pBH100 hybrid plasmids were constructed from vector pAT153 and from plasmid pBH100 treated with endonuclease *Bam*H I. After the use of a ligase, this preparation was utilized to transform competent *E. coli* 5K cells and the recombinant clones were selected on the basis of relevant characteristics. Transformant clones bearing the plasmids pATHg1 (29 Kb), pATHg2 (29 Kb) and pATHg3 (12 Kb) were obtained in this manner (results no presented). In order to determine approximate sizes of those plasmids the following standard plasmid were used; RP4 (54 Kb), Sa (36 Kb) and pBR322 (4 Kb).

Eletrophoresis was performed on 0.8% agarose gel. The level of $HgCl_2$ resistance in the trans-

formant clones bearing plasmids pATHg1, pATHg2, pATHg3 was 8 μg/ml. The stability of the recombinant plasmids in these transformants was 100% for the clones bearing plasmids pATHg1 epATHg2and 37% for the clone bearing pATHg3.

Restriction analysis of plasmid pATHg1 digested with *Bam*H I showed three bands of 24, 4 and 1 Kb; when the plasmid was treated with *Hind* III, two bands of 21 and 8 Kb were observed (Figure 1).

DISCUSSION

Genes for resistance to $HgCl_2$ of plasmid pBH100 originally located in the wild strain *E. coli* BH100 were cloned and are being expressed in *E. coli* 5K. These results agree with those obtained by investigators who used the restriction enzyme *Bam*H I, among others, and concluded that there were no restriction sites for this enzyme within the genes (operon) for mercury resistance in pDU1003 derived from pR130 (15) or Tn 21 and Tn 501 (11). However, the *mer* operon is known to have restriction sites for the enzyme *Eco*R I (4, 7, 11). In agreement with these data is also the attempt at cloning and expression of the Hg operon after treatment of pBH100 with *Eco*R I, which did not produce positive results (13).

It can be seen that plasmid pATHg3 simultaneously presented the least stability (37%) and lowest size (12 Kb) when compared with plasmids pATHg1 and pATH2 (29 Kb). Thus, we may assume that the additional 17 Kb DNA segment present in plasmids pATHg1 and pATHg2, but not in pATHg3, may partly contain the full DNA sequence related to plasmid stability a property that deserves further study (2).

The level of resistance to $HgCl_2$ coded by plasmids pATHg1, pATHg2 and pATHg3 was 8 μg/ml, less than the level coded by pBH100, which is approximately 10 μg/ml. Nascimento et al.(1992)[14], using the mini-plasmid technique (19) whose presence of a special vector is not required to clone, found the level of 10 μg/ml for pHg, a recombinant clone derived from pBH100.

The study of stability and the attempt at improving the expression of genes for mercury resistance by greater reductase production, and as a consequence of greater mercury volatilization, may be of great academic importance and lead to the development of a process for cleaning up the environment polluted with this element.

RESUMO

Clonagem do operon de resistência ao íon mercúrio do plasmídio pBH100 em *Escherichia coli* 5K, usando o vetor pAT153.

Inicialmente, o plasmídio pBH100 (95 Kb) que codifica resistência para tetraciclina, canamicina, cloranfenicol, estreptomicina e mercúrio inorgânico, foi transferido, por conjugação, da linhagem selvagem *Escherichia coli* BH100 para a receptora *E. coli* 5K. Para clonar o "operon Hg" plasmídios recombinantes foram construídos "in vitro" a partir da ligação (ligase) de fragmentos de pBH100, contendo os marcadores de resistência ao mercúrio, com o veículo de clonagem pAT153, ambos digeridos com a endonuclease *Bam*H I. Após transformação de *Escherichia coli* 5K análises genética e eletroforética permitiram a detecção dos plasmídios recombinantes pATHg1, pATHg2, pATHg3. Análise de restrição do plasmídio pATHg1, digerido com *Bam*HI e *Hind* III, evidenciou três bandas de 24, 4 e 1 Kb e duas bandas de 21 e 8 Kb, respectivamente. O pATHg3 apresentou menor tamanho (12 Kb) e menor estabilidade (37%).

Palavras-chave: resistência ao mercúrio, *Escherichia coli*, clonagem, pAT153.

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ISOLATION OF MUTACINS IN CELL FREE EXTRACTS AND ANALYSES OF SOME PHISICAL AND BIOLOGICAL PROPERTIES

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SUMMARY

It's suggested that bacteriocins produced by *S. mutans*, also named mutacins, may influence the colonization and selection of bacteria in oral cavity. To study their characteristics, mutacins were obtained from supernatant of broth-grown cultures of two strains of *S. mutans*. After precipitation by ammonium sulphate followed by dialyses, the bacteriocins solutions were submmited to several agents to determine their stability. These preparations showed to be sensible against proteolytic and lipolytic enzymes, but resistant against lisozyme and saliva. Their titre increased after detergent treatment. They were stable under 100°C, and in pH between 2.2 to 7.0, with a maximum activity between 3.0 to 5.0. The activity of these lipoproteins in low pH sugests a possible role in the ecosystem of the dental plaque.

Key Words: bacteriocins, mutacins, *Streptococcus mutans.*

INTRODUCTION

The role of *S. mutans* in the etiology of dental caries in man is already established (18). In cariogenic plaque *S. mutans* shows a marked increase in number, while other species, such as *S. sanguis*, decrease. Besides other factors as lowering the pH (9) and decrease in oxidation-reduction potencial (15, 23), bacteriocins produced by *S. mutans* may also play a role in the alterations occurring in streptococcal microflora. There is evidence that these inhibitors may influence the microbial composition of plaque in vitro (4), and *in vivo* (10, 12, 17, 22), and they are also considered as possible agents in preventing dental caries(11, 14). In order to evaluate their phisical and biological properties, there is a need to obtain these substances in a cell free state. However, the bacteriocins of *S. mutans* are not immediately obtained from culture fluids, with few exceptions (2, 8), and a protein extraction method is often necessary (5, 6, 13).

The aim of the present study was to isolate, in cell free solutions, two bacteriocins of *S. mutans* with different inhibitory spectra, described in a previous study (19). We also analyse some of the biological and phisical properties of these crude bacteriocin solutions.

MATERIAL AND METHODS

Organisms and Media - The bacteriocin pro-

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ducing *S. mutans* strains, 11-1 and 47-1, were isolated from saliva of children, and their identification and inhibitory spectrum described19. *S. mutans* 11-1 and 47-1 could inhibit the growth of *S. sanguis* I (ATCC 10.556), *S. sanguis* II (ATCC 10.557), *Rothia dentocariosa* (ATCC 14.190), and *S. mutans* CM7 - serotype "e", but not *S. sobrinus*. Strain 47-1 could also inhibit *A. viscosus* (WVU627) and *S. mutans* 1A5 - serotype "c". *S. saguis* I (ATCC 10.556) was selected as the indicator strain. The strains were stored at -20°C in defribrinated sheep blood. Subcultures were started by adding a drop of this solution to fresh Tryptic Soy Broth (TSB - Difco).

Crude bacteriocin preparation - The methodology used was a modification of Fukushima's description (5) (1982). The same procedures were done for each strain of *S. mutans* tested.

Fifty milliliters of overnight culture of producer strain were inoculated in 4 l of TSB with 2 percent of yeast extract, according to our previous study (19), followed by incubation under aerobic condition for 36h. Cells and cell debris were removed by centrifugation at 6,000g for 50 min. Solid ammonium sulphate (390g/l) was slowly added to the supernatant solution, which was left stirring at 4°C for 16 h. The precipitate was collected by centrifugation at 6,000g for 50 min, and dissolved in 20 ml of 0.05M tris-hydrocloride buffer at pH 8.6, and dialysed for 3 days against 3 l of the same buffer, changed daily. The crude bacteriocin solutions obtained were stored at-20°C for up to three months, until use.

Bacteriocin assay - The titres were measured by the spotting drop method (7). A drop of 0.1ml of overnight culture of the indicator strain, wich 0.15 A of absorbance, at wave lenght of 500nm, was mixed with 7.5 ml of melted soft TS agar and poured on the surface of a TS agar plate.

Drops of 25µl of serial dilutions of the bacteriocin solution in 0.05M tris-hydrocloride buffer at pH 8.6, were placed on the seeded plate with a micropipette. After overnight incubation at 37°C under microaerophilic condition (candle jar), the highest dilution to give a translucent area of inhibited growth was determined. This procedure was performed before storage at -20°C, and after defrosting the solutions, before any analyses was made.

Bacteriocin concentration - The activity of the crude bacteriocin solutions were detected only undiluted, so the solutions were concentrated 20 ml of each bacteriocin solution were dialysed against 400ml of 20 percent polyethilene glycol (PM 20,000) for 16 h. After this procedure the bacteriocin assay was repeated. The volume of the bacteriocins solutions decreased ten times, but titre only doubled. These concentrated bacteriocin solutions were used only in the saliva treatment experiment.

Saliva treatment - Paraffin stimulated saliva was obtained from three individuals, aged 20-30 years, at least 2 h after eating. In order to remove microorganisms, the saliva were filtered using a membrane 0.22µm pore associated to a pre filter (Millipore) or centrifuged at 10,000 g for 10 min. The concentrated bacteriocin solutions were mixed with equal volume of saliva from each individual, and maintained at 37°C for 1h, when the bacteriocin titre was determinated as described above. The inhibitory activity of the saliva was also tested as control.

Effect of various enzymes - The enzymes obtained from Serva (Germany) were tested in proper buffers as follows: trypsin (31 U/mg) and Alfachymotrypsin (30 µ/mg) in tris-hydrocloride buffer at pH 8.0; lipase (17U/mg) in tris-hydrocloride buffer at pH 8.0, containing 0.01M $CaCl^2$; phospholipase D (2.6 U/mg) in tris-hydrocloride buffer at pH 7.2, containing 0.01M $CaCl^2$; pronase E (7.4DMC-U/mg), papain (10.4 U/mg) and lysosyme (25,000 U/mg) in tris-hydrocloride buffer at pH 7.4. Bacteriocins 11-1 and 47-1 were tested by incubating the bacteriocin with each enzyme at a concentration of 500µg/ml for 60 min at 37°C. As control, pure enzymes and bacteriocins were tested separately

Effect of heat treatment - Temperature stability was assessed by heating the bacteriocins solutions at 60°C, 80°C and 100°C for 15 and 30 min and at 121°C for 15 min. Samples from the tubes were removed at intervals and cooled in an ice bath before bacteriocin titres were determinated.

Effect of pH on bacteriocin activity - The pH stability of 11-1 and 47-1 bacteriocins was examined by dialysins 2.0 ml of each bacteriocin solution for 16 h against 100 ml of each of the following buffer: 0.05M phosphate buffer at pH 7.0; citrate buffer at pH 3.0-6.0; and glycine-HC buffer at pH 2.2. The solutions were mixed, even when precipitation ocurred, using a mixer (Phoenix), and the titre determinated immediately. Controls were made using the bacteriocins solutions dialysated against tris-hydrocloride buffer at pH 8.6, and the buffer solutions separately.

Effect of detergent treatment - Detergents were mixed with an equal volume of bacteriocin solution. The mixtures were kept at room temperature for 1 h and the activity was measured. Sodium

dodecyl sulphate (0.01%), tween 80 (0.1%), and sodium deoxycolate (0.1%) were used. The inhibitory activity of the detergents were also tested against the indicator strain as control.

RESULTS

The crude solutions obtained from both strains showed inhibitory only when tested undiluted. The inhibitory titer remained the same after storage for three months at -20°C. The concentration with polyethilene glycol decresead the volume ten times, but the activity only double. So, we decided to make the further analyses using the crude solution without concentration, except for the saliva experiment.

Saliva from three individuals, either filtered or centrifuged, did not affect bacteriocin activity (table 1).

TABLE 1 - Bacteriocin activity after treatment with saliva, different enzymes and temperatures.

Treatment		bacteriocins 11-1	11-1
saliva			
filtered	1	R	R
	2	R	R
	3	R	R
centrifuged	1	R	R
	2	R	R
	3	R	R
Enzymes			
Papain		S*	S
Alfa-chymotrypsin		S	S
Lysozyme		R	R
Pronase E		S	S
Trypsin		S	S
Phospholipase D		R	R
Lipase		S	S
Temperature/Time			
60°C	15 min	R	R
	30 min	R	R
80°C	15 min	R	R
	30 min	R	R
100°C	15 min	R	R
	30 min	R	R
121°C	15 min	S	R

S or R: bacteriocin activity sensible or resistant

We observed that bacteriocin activity of the two crude extracts was sensible to papain, trypsin, pronase, alfa-chimotrypsin, and lipase but resistant to phospholipase and lysozyme (table 1).

The bacteriocins were not affected by heating at 100°C for 30 min. The bacteriocin 47-1, but not 11-1, was destroyed after heating at 121°C for 15 min (table 1).

Addition of tween 80, sodium dodecyl sulphate and sodium deoxycolate resulted in increased activity (table 2).

Both bacteriocins were stable and active at pH 2.2 - 7.0; the activities increased at pH between 3.0 - 5.0, demonstrated in table 3.

TABLE 2 - Bacteriocin activity after treatment with detergents. Results of three experiments.

Detergents	bacteriocins 11-1		47-1		Contr*
	1:2**	1:5	1:2	1:5	
sodium dodecyl sulphate	+++	++-	++-	++-	---
tween 80	+++	---	++-	+--	---
sodium deoxycolate	+++	---	---	+--	---

+: inhibition of indicator strain.
-: no inhibition.
* control with buffered solutions only.
* dilutions.

TABLE 3 - Titre of bacteriocins 11-1 and 47-1, in cell free solutions, after dyalises for 16h against different pH buffered solutions solutions. Results of experiments in duplicate.

Treatment	bacteriocins 11-1			11-1			Contr*
(pH)	1:0*	1:2	1:5	1:0	1:2	1:5	
8.6 (Control)	++	--	--	++	--	--	-
7.0	++	--	--	++	--	--	-
6.0	++	--	--	++	++	--	-
5.0	++	++	--	++	++	--	-
4.0	++	++	--	++	++	++	-
3.0	++	++	--	++	++	+-	-
2.2	++	--	--	++	++	--	-

+: inhibition of indicator strain.
-: no inhibition.
* control with buffered solutions only.
* dilutions.

DISCUSSION

Bacteriocins of strains 11-1 and 47-1 were not inhibited by saliva. These results are similar to those obtained by Delisle (3) (1976) with a partially-purified bacteriocin from the supernatant of culture fluid of *S. mutans* strain BHT, and by Fukushima et al (6) (1985) with a purifild bacteriocin from strain RM-10, but different from those by Kelstrup & Gibbons (16) (1969). On the other hand bacteriocins showed sensibility to proteolytic enzymes, which might destroy them *in vivo*. The proteinaceous nature of the inhibitors plus their biological activity formally qualify them for inclusion in the broad family of bacteriocins (20). The two bacteriocins were also inactivated by lipase, different from most studies (5, 13), but probably similar to the lipoprotein isolated by Weerkamp et al (21) (1976).

Both solutions showed activity after heating at 100°C, and the one produced by strain 47-1 was active even after being submmited to121°C. These results showed a different between the two substances tested, and are similar to those obtained by Ikeda et al (13) (1982), but different from Fukushima et al (6) (1985).

The increased activity demonstrated after detergent treatment indicates that the bacteriocins studied might also have a fibrilar structure, similar to the bacteriocin RM-10, described by Fukushima et al (6) (1985), which separate under the action of detergents.

In the present study we observed increased activity of both bacteriocins under low pH. These results are in accordance to our previous data (19) in solid media, where an increased activity was observed under pH 5.5 compared to pH 7.0. Caufield et al (1) (1985) also showed enhance in inhibition zones between pH 8.0 and 7.0. Ikeda et al (13) (1982) purified bacteriocin C3603, which was stable between pH 1.0 to 12.0. On the other hand, the bacteriocin RM-10, isolated by Fukushima et al (6) (1985), showed stability at pH 7.0 and 6.0, but descreased at lower values. The increased bacteriocin titre described here might be due to altered conformation of the molecules in different pH. Although this characteristic has not been described for the mutacins, it was already shown for the staphylococcin (20). The increased activity in low pH might be of ecological importance in the dental plaque, where these acid values are commonly reached.

RESUMO

Isolamento de mutacinas em extratos livres de células e análise de algumas propriedades físicas e biológicas.

É sugerido que as mutacinas, bacteriocinas produzidas por *S. mutans*, exercem influência na colonização inicial e seleção de bactérias na cavidade oral. Para o seu estudo, as mutacinas foram obtidas em solução livre de células, a partir do sobrenadante da cultura de duas cepas de *S. mutans*. Após precipitação com sulfato de amônia, seguida de diálise, as soluções foram submetidas, *in vitro*, a vários agentes, para determinar a estabilidade da sua ação inibitória. Estas preparações foram sensíveis a enzimas proteolíticas e lipolíticas, mas resistentes a ação da saliva e da lisozima.

Houve um aumento do título inibitório após tratamento com detergentes. Mostraram estabilidade a 100°C e a pH entre 2,2 e 7,0, com máximo de atividade em pH entre 3,0 e 5,0. A atividade destas lipoproteínas em pH baixo indica uma possível ação no ecossistema da placa dental.

Palavras-chave: bacteriocinas, mutacinas, *streptococcus mutans*.

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ANTAGONISTIC EFFECT AGAINST *CLOSTRIDIUM PERFRINGENS* OF A DIFFUSIBLE COMPOUND PRODUCED BY A *PEPTOSTREPTOCOCCUS SP* FROM HUMAN INTESTINAL FLORA IN MICE

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SUMMARY

A *Peptostreptococcus* sp strain was isolated from human intestinal flora which produces a diffusible substance with an antagonistic effect against *Clostridium perfringens* in gnotobiotic mice. This diffusible compound was active against the target strain either in the presence or in the absence of a complex intestinal microflora of human origin when tested in gnotobiotic mice.

Key Words: *Peptostreptococcus, Clostridium perfringens,* Antagonism.

INTRODUCTION

The normal flora of the gastrointestinal tract represents an important barrier against gut infections by pathogens. The protective effect could be obtained by growth inhibition of a pathogenic agent (5,7) or by modulation of its toxin production (1). Little is known about the factors involved in these barrier effects. The possible mechanisms of these bacterial interactions include: changes in oxidation-reduction potential or in pH; production of bacteriocins, fatty acids, and H_2O_2 + peroxidase; competition for substrates or for adhesion sites; and local immunity. Soluble fecal inhibitor produced by a strain of *Bacillus licheniformis* has been successfully detected in the gastrointestinal tract of gnotobiotic models. But the antibiotic-like substance was no longer detected when indigenous bacteria were associated to the *Bacillus* strain (3). Bacteriocin-producing bacterial strains are commonly found in the mammalian intestinal tract. However, it is known that the antagonism which can be evidenced "in vitro" between bacteriocinogenic and sensitive strains does not become expressed "in vivo", in the digestive tract of gnotobiotic mice. Moreover, it was shown that certain sensitive strain exerted a barrier effect against strain which produced a bacteriocin "in vivo" (4).

This work describes the isolation of a *Peptostreptococcus* sp from the dominant human intestinal flora which produces a diffusible antagonistic substance against *Clostridium perfringens* in gnotobiotic mouse intestines.

MATERIAL AND METHODS

Animals and diet - Adult gnotobiotic C3H

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mice were kept in Trexler-type isolators fitted with a rapid transfer system (La Calhéne, Vélizy-Villacoublay, France). The animals were fed *ad libitum* a commercial diet for rodents (U.A.R., Epinay-sur-Orge, France) sterilized by gamma irradiation (40 kGy).

Bacterial strains - Target strains for demonstrating antagonistic compound production were strains CpA of a *Clostridium perfringens,* which was a variant belonging to serotype A and unable to form thermoresistant spore, and strains of *C. perfringens* (also of the serotype A, as determined by Dr. M. Popoff, Service des Entérobactéries, Institut Pasteur, Paris) isolated from patients with ulceration of the intestinal mucosa (pouchitis). Spores of a strictly thermophilic *Bacillus subtilis* were used as transit marker (7). The *Peptostreptococcus* strain was isolated from the predominant bacterial flora of a patient.

Association of axenic mice with bacterial strains - All *C. perfringens* were grown in soft medium W (6). The *Peptostreptococcus* sp and the dominant anaerobic bacteria from human fecal flora were grown on brain heart infusion agar (Difco Laboratories, Detroit, Michigan) in an anaerobic chamber (La Calhéne, Vélizy-Villacoublay, France). A suspension containing 10^8 spores/ml of the transit marker was extemporaneously mixed with the CpA inoculum containing 10^8 vegetative cells/ml. Each animal was inoculated through the orogastric route with 1 ml of bacterial inocula. The order of inoculations is given in the text.

Bacterial counts - Feces were collected individually from the anus of animals, diluted 100-fold in liquid medium LCY (6), and homogenized with an Ultraturrax (OSI, Paris, France). *C. perfringens* counts were made by mixing a 1-ml amount of adequate serial 10-fold dilution with 14 ml of W medium (5) supplemented with 0.013% neomycin sulfate. The resulting mixture was poured into 8 x 400 mm tube (Touzard & Matignon, Vitry-sur-Seine, France) and incubated at 37°C overnight. *Peptostreptococcus* sp count was made in an anaerobic chamber by plating on brain heart infusion agar. Incubation was done at 37°C and colonies were counted one day later. Spores of transit marker were counted by plating on agar medium MS containing 0.8% meat extract (Merck A G, Darmstadt, Germany), 0.2% yeast extract (Difco), 0.1% glucose, 40 mg of manganese sulfate per liter, and 1% Bacto-agar (Difco), pH 6.3. Incubation was done aerobically at 55°C for 24 h.

Detection of inhibitory diffusible substance by in vitro *test* - Feces were collected individually from the anus of gnotobiotic mice harboring the antagonistic strain and put down in a Petri dish containing 14 mL of solid W medium, 0.1 mL of a 20 mg/mL neomycin sulfate solution, and 1.0 mL of a *C. perfringens* culture before medium solidification. The Petri dish was kept in an anaerobic jar Gas-Pack System Biomerieux, Lyon, France) for 24 hrs at 4°C and then incubated for 18 hrs at 37°C. The presence of an antagonistic substance against *C. perfringens* was demonstrated by an inhibitory halo around the feces.

Isolation of the Peptostreptococcus sp - A patient with ulcerative colitis had undergone pouch-anal anastomosis. Fresh fecal sample was collected from this patient and introduced in an anaerobic chamber. A 1-g sample of feces was homogenized in 9 mL of LCY and 10-fold dilutions were made. Aliquots of 0.1 mL were plated onto brain heart infusion agar and incubated at 37°C for seven days. Ninety six colonies were picked from the dominant strict anaerobic flora and the bacteria were identified to the genus level by their appearance on the medium and by supplementary tests. The *Peptostreptococcus* was selected among the 96 bacterial strains as described in the results. *C. perfringens* levels were determined on W solid medium supplemented with 0.013% neomycin.

RESULTS AND DISCUSSION

Several types of enteritis or enterotoxemia in animals and humans are caused by *Clostridium perfringens.* Recently this pathogen was involved also in antibiotic associated infection of the gastrointestinal tract (2). The multiplication of *C. perfringens* in high populational level in the gut does not occur due to the intestinal microbiological barrier. The ecological barrier effect is generally exerted by the association of a few different strains, and rarely by a single one (5, 7).

Figure 1 shows the drastic antagonistic effect against *C. perfringens* A (CpA) of the 96 bacterial strains of dominant anaerobic bacteria from an ileo-anal pouch flora. The strict anaerobic bacteria group in this association was constituted of 25 *Peptostreptococcus,* 60 *Clostridium,* 2 *Bifidobacterium,* and 1 *Veillonella.* After a second challenge with CpA and the transit marker, the latter was eliminated rapidly and the inoculum of the former was apparently killed immediatly in the gastrointestinal tract of the animals. This suggests a very potent bactericidal effect against CpA exerted by the 88 strict anaerobic bacteria associated with the

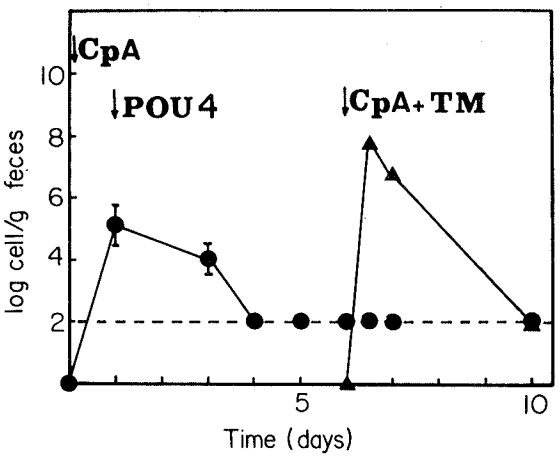

FIGURE 1 – Antagonistic effect of bacterial strains from human intestinal fecal flora against *Cloristridium perfringens* A (●) in mice. Transit maker (▲). Arrows show the inoculation times of *C. perfringens* (CpA), the 96 strains (POU4), and *C. perfringens* with the transit marker (CpA + TM). The 96 strains were constituted of the following genus: 25 *Peptostreptococcus*, 60 *Clostridium*, 2 *Bifidobacterium*, and 1 *Veillonella*.

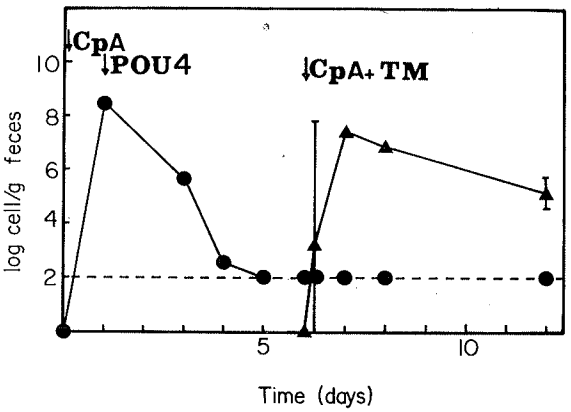

FIGURE 2 – Antagonistic effect of 15 of bacterial strains from human intestinal fecal flora against *Clostridium perfringens* A (●) in mice. Transit marker (▲). Arrows show the inoculation times of *C. perfringens* (CpA), the 15 strains (POU4), and *C. perfringens* with the transit marker (CpA + TM). The 15 strains were constituted of the following genus: 4 *Peptostreptococcus*, 9 *Clostridium*, 1 *Bifidobacterium*, and 1 *Veillonella*.

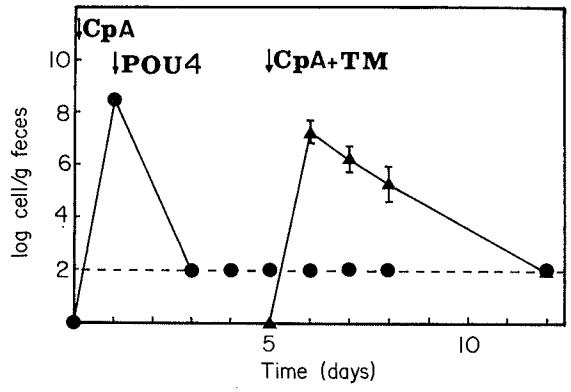

FIGURE 3 – Antagonistic effect of 4 *Peptostreptococcus* sp from human intestinal fecal flora against *Clostridium perfringens* A (●) in mice. Transit marker (▲). Arrows show the inoculation times of *C. perfrigens* (CpA), the 4 *Peptostreptococcus* (POU4), and *C. perfringens* with the transit marker (CpA + TM).

mice. Microscopical examination and supplementary tests on the 88 strains had shown that these were apparently constituted of only 15 morphogically different microorganisms and 73 of their repetitions. The group of 15 of bacterial strains was formed of 4 *Peptostreptococcus*, 9 *Clostridium*, 1 *Bifidobacterium*, and 1 *Veillonella*.

Figure 2 shows the antagonistic effect of these 15 strains against CpA in mice previously monoassociated with the target strain. On the second challenge, the transit marker was eliminated slower than in figure 1, but the flashing bactericidal effect of the human microflora was obtained again. On microscopical examination of the feces of the gnotobiotic mice associated with these 15 bacterial strains we had noted that *Peptostreptococcus* genus was the unique morphological form present in the dominant flora. This fact suggested that this genus was responsible for the bactericidal effect against CpA.

Figure 3 shows the antagonistic effect of the four *Peptostreptococcus* strains against CpA. Elimination profiles similar to those obtained in figure 2 were observed on the second challenge with CpA and the transit marker. Using an *in vitro* antagonistic test, an inhibitory halo of the CpA culture was obtained for three of four *Peptostreptococcus*. Differences between these three strains being few, we have concluded that they were the same microorganism.

Figure 4 shows the barrier effect of this *Peptostreptococcus* against CpA in gnotobiotic mice. The elimination profile of the transit marker was slower than in the previous figures but the killing effect on CpA was the same. Population levels of the *Peptostreptococcus* were of 9.68 log cell/g of wet feces.

Figure 5 shows the inhibitory halo against various *C. perfringens* cultures when feces from *Peptostreptococcus*-monoassociated mice were used. There was no halo when feces from germfree mice were used in the inhibitory test. This

suggests the production of an antagonistic diffusible substance. The three serotype A of *C. perfringens* were isolated from patients with intestinal mucosa inflammation (pouchitis).

This work is the first report of an antagonistic relationship between an inhabitant of the normal human intestinal flora and a pathogenic target strain obtained *in vivo* either in the presence as in the absence of a normal complex flora in gnotobiotic mice. Curiously, the drastic barrier against the target strain was not observed in the patient from which the *Peptostreptococcus* was originated. *C. perfringens* population in the feces of this patient was about 7.08 log cell/g. This phenomenon could be probably explained by the different population levels of this strain between the gnotobiotic mice (9.68 log cell/g) and the patient (7.34 log cell/g).

Studies are in course on the chemical identity and the bacteriological spectrum of the diffusible compound produced by the *Peptostreptococcus.*

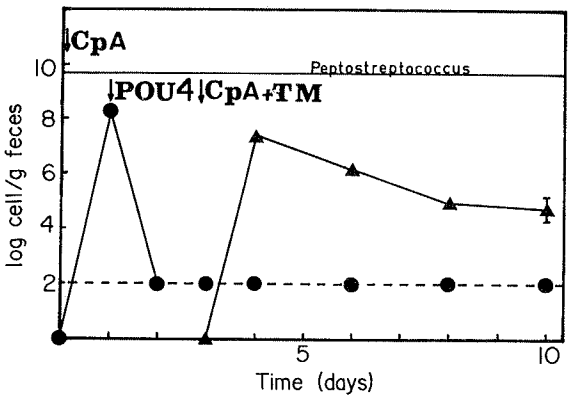

FIGURE 4 – Antagonistic effect of a *Peptostreptococcus* sp from human intestinal fecal flora against *Clostridium perfringens* A (●) in mice. Transit maker (▲). Arrows show the inoculation times of *C. perfringens* (CpA), the *Peptostreptococcus* (POU4), and *C. perfringens* with the transit marker (CpA + TM).

RESUMO

Efeito antagonista contra *Clostridium perfringens* de uma substância produzido por *Peptostreptocuccus* sp. em camundongo gnotobiótico.

Um *Peptostreptocuccus* sp foi isolado de uma flora intestinal fecal humana. Esse microorganis-

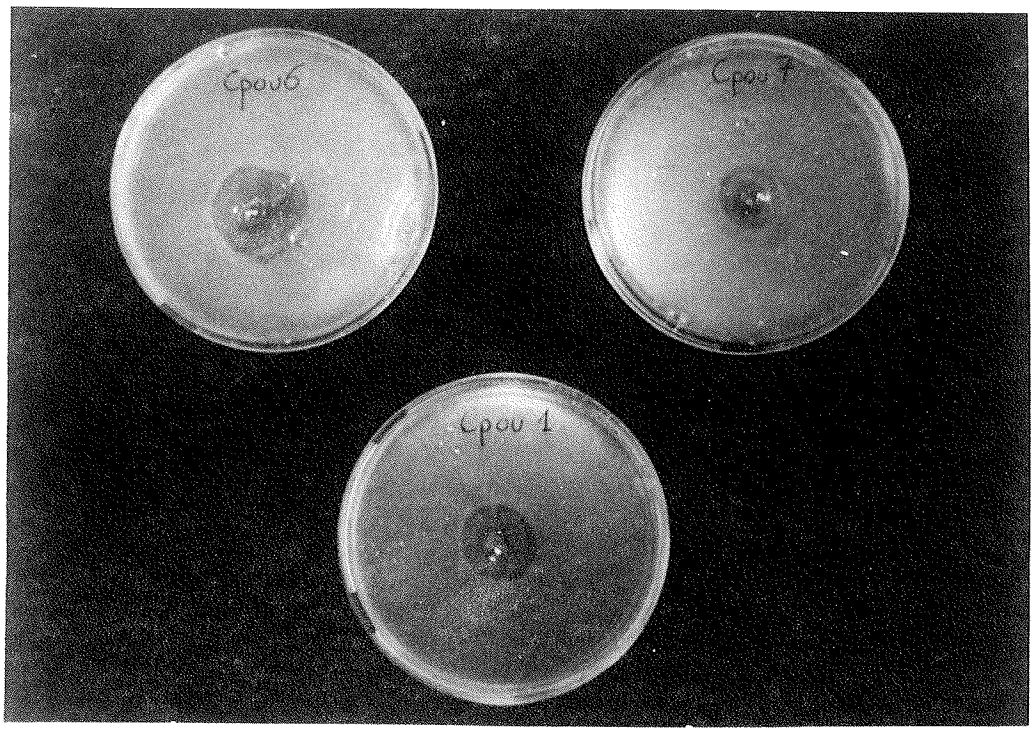

FIGURE 5 – Inhibitory effect against various serotype A of *Clostridium perfringens* (Cpou6, Cpou7) exerted by feces from mice monoassociated with a *Peptostreptococcus sp* isolated from human intestinal fecal flora.

mo produz uma substância difusível apresentando um efeito antagonista contra *Clostridium perfringens* em camundongo gnotobiótico. Esse composto diluível foi ativo contra a cepa alvo tanto na presença como na ausência de uma microflora intestinal complexa de origem humana quando foi testado em camundongo gnotobiótico.

Palavras-chave: *Peptostreptococcus, Clostridium perfrigens*, Antagonismo.

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ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA): OPTIMAL CONDITIONS FOR DETECTION OF ANTIBODIES PRODUCED AGAINST *CORINEBACTERIUM PSEUDOTUBERCULOSIS*

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Charlotte H. Langenegger[2]
Roberto Meyer[3]

SUMMARY

Enzyme-linked immunosorbent assay (ELISA) was used to measure the antibody level to exotoxin, "linfadenina" and to solube antigen extracted with SDS and sonic disruption of *Corynebacterium pseudotuberculosis* using sera from healthy and naturally infected goats.

Our results indicate the exotoxin and "linfadenina" as the best antigens to detect specific antibodies against *Corynebacterium pseudotuberculosis.*

Key Words: caseous lymphadenitis, goats, Elisa, serological test.

INTRODUCTION

Caseous lymphadenitis (CLA) of goats and sheep is a chronic disease caused by *Corynebacterium pseudotuberculosis* and characterized by suppurative, necrotizing inflammation of one or more lymph nodes.

CLA is a common disease in goats in many countries; in Northeastern of Brazil the prevalence in goat flock is appoximately 27.7% (14). CLA causes a significant financial loss estimated in US$ 2 million per year in the sheep meat industry (13) due to condemnation of mutton carcaces at abattoirs (2, 11).

Several researchers have studied different serological tests such as passive haemagglutination (5), anti-hemolysis inhibition test (15), sinergystic hemolysis inhibition test (3), immunofluorescence (1), DIG-ELISA (7), and enzyme-linked immunosorbent assay - ELISA (6, 8, 10, 12, 13) to detect animals with CLA. Among these assays, the ELISA has been studied using different antigenic preparations. Shen & col. (12) used as antigen fraction of the cellular wall of *Corynebacterium pseudotuberculosis* obtained after the breaking of bacterial cells. Johnson & col. (7) used the exotoxin to develop the DIG-ELISA. Sutherland & col. (13) compared the use of exotoxin and cell wall fractions and Maki & col. (10) compared these two last antigens with whole bacterial cells.

The purpose of the present study was to compare four different antigen preparations for the detection of specific antibodies against *Corynebacterium pseudotuberculosis* in the sera of natu-

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rally infected and healthy goats, using the indirect ELISA.

MATERIAL AND METHODS

Goats - Fifty goats maintained in confinement were chosen from seven different flocks located in Rio de Janeiro, Brazil. Twenty-five goats were naturally infected and *C. pseudotuberculosis* was confirmed as the etiological agent after isolation from subcutaneous abscesses. The other twenty-five came from flocks with no abscesses and were used as control group.

Antigen preparation - *Corynebacterium pseudotuberculosis,* strain 1002, was grown on triptose-agar medium enriched with yeast extract and lactalbumine (DIFCO), and incubated at 37°C during 48 hours. After culture, the *Corynebacterium pseudotuberculosis* suspension was centrifuged at 3000g at 4°C for 30 min. The settled cells were washed three times with 0.85% saline solution (SS) and submitted to the different treatments mentioned below.

Extraction with SDS - After washing, the bacterial mass was suspended in 1% PBS (phosphate buffer solution pH 7.2, 0.1M) - SDS (duodecyl sulfate sodium) solution on the proportion 1:20 and incubated overnight at room temperature and agitation. The suspension was centrifuged at 3000g for 30min. and the supernatant was dialyzed against saline solution. Part of this supernatant was submitted to ultra-filtration, using Amicon filter with membranes YM-100 and YM-30 to obtain a fraction of molecular weight between 30-100 kD. The protein concentration was measured by the Lowry method and that fraction was used at 10µg/ml.

Sonic disrupton - The bacterial pellet suspended in distilled water was submitted to fifteen pulses of two minutes each in ice bath using Branson Sonic apparatus at 100Hz of frequency. This material was centrifuged at 3000g for 30 min., and the supernatant was centrifuged at 10000g for 30 min. The pellet, resuspended in saline solution, was submitted to ultrafiltration with the membranes mentioned above and the fraction 30-100kD was obtained. This preparation was used at a protein concentration of 10µg/ml and it was also measured using Lowry method.

Exotoxin - The supernatant of a brain-heart infusion (DIFCO) culture of *C. pseudotuberculosis,* incubated at 37°C during 72 hours, was centrifuged at 1500g during 30 min., filtrated in millipore 0.45µm filter and diluted 1:2 with 0.06M carbonate buffer pH 9.6.

Linfadenina - This antigen preparation was provided by "Unidade de Apoio ao Programa Nacional de Pesquisa em Saúde Animal - Empresa Brasileira de Pesquisa Agropecuária (UAPNPSA-EMBRAPA, RJ)" and its obtention was described by Langenegger & col. (9).

ELISA - The ELISA was performed in duplicated using 96 wells U-shaped polystyrene plates coated overnight at 4°C with the antigens mentioned above, diluted in 0.06M, pH 9.6 carbonate buffer. The plates were washed three times with phosphate buffer solution (PBS) containing 0.05% tween 20 and 5% PBS-skim milk was placed in each well. After washing the plates again three times with PBS-tween, sera diluted 1:200 in 1% PBS-skim milk was added to each well and incubated at 37°C during 2 hours. The plates were washed again and peroxidase-conjugated rabbit anti-goat lgG (SIGMA Co., St. Louis, MO.) diluted in 0.05% PBS-tween 20 was placed in each well and incubated at 37°C during 1 hour. A solution containing H_2O_2 and OPD (O-phenildiamine) was used to develop the reaction and the color resulting was measured by Labsystem Uniskan II ELISA reader (492nm).

Statistical analysis

The data were analyzed according to student's t-test using a computerized statistic packaged (Statworks).

RESULTS AND DISCUSSION

Figure 1 is a histogram of an ELISA assay that shows the mean of antibody level against four different *Corynebacterium pseudotuberculosis* antigen preparations expressed in optical density values on sera from healthy and naturally infected goats. These results clearly show the efficacy of the proposed technique to detect specific antibodies against *Corynebacterium pseudotuberculosis* especially when exotoxin and linfadenina were used as antigen.

Maki & col. (10) obtained good results using exotoxin as antigen. High titers were found, especially when samples from sheep three months postinoculation were tested. On the other hand, Sultherland & col. (13) detected more sheep with caseous lymphadenitis lesions by ELISA using cell wall as antigen rather than using exotoxin.

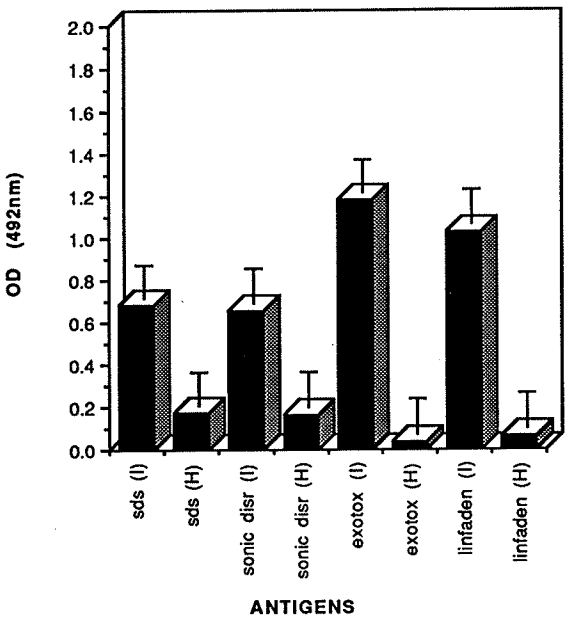

FIGURE 1 - Optical density values from sera of health (H) and naturally infected (I) goats submitted to ELISA using four different antigens. Data are expressed as mean ± S.E.

The 30-100 kD fraction from SDS and sonic disruption antigens was used in our assays based upon Meyer & col. studies (data not shown). They observed a good performance by ELISA using this fraction instead of <10 kD, 10-30 KD, >100 kD fractions.

When the fraction 30-100 kD extracted by SDS and sonic disruption was used to analyze the naturally infected group by ELISA, some lower titers were observed. These results may be due to the strong link between the SDS and the cell wall proteins making the removal of SDS by dialysis difficult and affecting the antigen adherence to the plates when used as a coat. This lack of adherence could have been a hindrance to antibody-antigen binding. Another possibility could be the resistance of *C. pseudotuberculosis* to break open by sonic disruption due to the thick lipid surface. This resistance to cell disruption may result in a poor concentration of relevant low-epitope density antigens in this preparation.

According to the Figure 1, when sera from healthy goats were submitted to ELISA using antigen extracted by SDS and sonic disruption, the OD values were higher when exotoxin and linfadenina were used. This finding may be due to epitopes on the cell wall of the bacteria that can croossreact with antibodies raised against other organisms in the sera.

However, when exotoxin and linfadenina OD values were analyzed from healthy and naturally infected groups, the specificity of this test to detect *Corynebacterium pseudotuberculosis* infection was confirned.

RESUMO

Determinação das condições ótimas para detecção de anticorpos contra *Corynebacterium pseudotuberculosis* através de método imunoenzimático (ELISA).

O ensaio imunoenzimático (ELISA) foi utilizado para mensurar os níveis de anticorpos contra a exotoxina, "linfadenina" e os antígenos solúveis extraídos por SDS e por ultra-som do *Corynebacterium pseudotuberculosis,* nos soros de caprinos normais e naturalmente infectados.

Nossos resultados indicam a exotoxina e a "linfadenina" como os melhores antígenos, dentre os estudados, para detectar anticorpos específicos contra o *Corynebacterium pseudotuberculosis.*

Palavras-chave: Elisa, teste sorológico, cabras linfoadenite caseosa.

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IN VITRO ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS AGAINST CLINICAL ISOLATES OF DERMATOPHYTES

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SUMMARY

Six essential oils were tested *in vitro* against 16 strains of dermatophytes *Epidermophyton floccosum, Microsporum canis, Trichophyton mentagrophytes* and *T. rubrum)* isolated from different pacients with dermatophytosis. The oils were obtained from medicinal plants (*Annona classifora, Cinnamomum zeylanicum, Cymbopogon citratus, Ocimum gratissimum, Protium heptaphyllum, Xylopia frutescens),* utilized by native people of Northeastern region of Brazil for the treatment of skin infectious diseases. The results showed that the essential oils (with exception of the sample obtained from *P. heptaphyllum)* possess excellent activity causing inhibition upon 81% of the studied strains of dermatophytes. Only 2 strains of *T. rubrum* and one of *M. canis* were resistant.

Key Words: antifungal activity, dermatophytes, essential oils, medicinal plants.

INTRODUCTION

One of the main scopes of our work has been the evaluation of antimicrobial properties of plant extracts used in folk medicine (2, 5) in order to verify whether the popular use has a therapeutic basis. When possible, purified fractions or potencial active compounds obtained from the plant extracts are also tested (1, 6, 7, 13).

In the Northeastern region of Brazil, medicinal plants are frequently used by the inhabitants for the treatment of skin infectious diseases. Several species utilized for this purpose are fragrant plants that contain essential oils (constituted mainly by volatile terpenes as pinene, camphene, cineol, citral, limonene and aromatic compounds as eugenol, cinnamaldehyde, thymol) (4), which are known to have antimicrobial properties (3, 9, 11, 14, 15, 16, 17).

This paper concerns the investigation of the antifungal activity of essencial oils obtained from 6 medicinal plants that were selected on the basis of a bibliografic review and on interviews with rural people. The oils were tested against 16 isolates of dermatophytes.

MATERIAL AND METHODS

Plant material and oil samples - The plants

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were collected in João Pessoa, PB, in the botanical reserve of Departamento de Ecologia e Sistemática da Universidade Federal da Paraíba. Essential oils were obtained from different parts of the plants (roots, stems, barks, leaves, flowers or fruits) which were dried at room temperature. Just prior to analysis they were ground and submitted to steam distillation for 1-2 h. The aqueous solution containing essential oils was extracted with CH_2CL_2 and after evaporation of the solvent chemical analysis was carried out by gas liquid chromatography.

Essential oil constituents were identified by the retention times and coinjection with authentic compounds and by comparison of their mass spectra with those reported for reference compounds.

The plants studied and some chemical constituents of the oils are presented in table 1.

Microbiological assays – All test organisms were clinical isolates obtained from different patients with dermatophytosis. The following microrganisms were used: *Trichophyton rubrum* (45T, 54T, 69T, 75T, 78T, 84T, 101T, 118T), *T. mentagrophytes* (6T, 12T, 16T, 29T), *Microsporum canis* (72T, 73T, 85T) and *Epidermophyton floccosum* (27T). Cultures were maintained on Sabouraud dextrose agar (DIFCO) plates at 4°C.

Two to three weeks before the assay, subcultures of the different dermatophyte strains were prepared by inoculation onto Sabouraud dextrose agar slants and incubation at 25°C. The growth on agar was then scraped and washed from the surface with saline. The suspension was homogenized by shaking with glass beads and by filtering through sterile cotton to remove mycelial fragments. Test inocula were prepared by adjusting the suspension with saline to give 90% light transmission at 530 nm. This procedure results in cell densities of approximately 10^6 cell/ml (12).

The disk plate agar diffusion method (8, 12) was used to test the samples. 1ml of the inoculum suspension was carefully mixed with 20ml of melted Sabouraud dextrose agar and the agar poured in Petri plates. After solidification, paper disks (CECON-SP) impregnated with 0.02ml of essential oil were distributed on the agar surface.

The plates were incubated at 28°C and the activities measured as the diameter (mm) of the inhibition zone surrounding the disk, after 10-14 days. At least two replicates were made for each sample. Two sets of control were run simultaneously, the organism control (seeded plate with no sample added) and antibiotic control (disk impregnated with 0.02ml ketoconazole at concentration of 100μg/ml).

TABLE 1 - Some chemical constituents of the essencial oils studied.

Essencial oils from	Constituents
Annoma crassiflora MART ANNONACEAE (araticum)	δ-3-carene, L-limonene, β-myrcene, L-phellandrene, α-pinene, trans-ocimene
Cinnamomum zeylanicum BLUME LAURACEAE (canela)	α-pinene, β-pinene, limonene, camphene, 1, 8-cineol, cinnamaldehyde
Cymbopogon citratus STAPF GRAMINAE (capim santo)	E-citral, Z-citral, 2-β-pinene
Ocimum gratissimum L. Lamiaceae (alfavaca)	1, 8-cineol, trans-caryophyllene, β-pinene, myrceneno, eugenol, trans-anetol
Pratium heptaphyllum MARCH BURSERACEAE (almiscar)	β-terpinolene, L-phellandrene, α-pinene
Xilopia frutescens AUBL ANNONACEAE (embiriba vermelha)	γ-elemene, β-cubebene, L-limonene, cycloisoloneifoline

RESULTS AND DISCUSSION

The results of the microbiological assays, presented in table 2, showed that the essential oils obtained from *A. classifora, C. zeylanicum, C. citratus, O. gratissimum* and *X. frutescens* possess excellent activities causing inhibition upon 81% of the studied strains of dermatophytes.

Only strains 69T, *(T. rubrum)* and 72T *(N. canis)* were resistant to all samples. Strain 118T *(T. rubrum)* was sensitive only to *C. zeylanicum.* The oil obtained from *P. heptaphyllum* was completely inactive.

The diameter of the inhibition zones produced by the oils are comparable to (or in some cases greater than) those obtained for the antifungal ketoconazole (100 μg/ml).

Although zone size depends on the sensitivity of the organism to the antibiotic and large zone sizes frequently means good sensitivity, it is not possible to compare the antimicrobial potency of the samples among themselves or in relation to the classical antifungal since zone is also a measure of diffusibility (8).

Yousef and Tawil (1980) evaluated the bacteriostatic and fungistatic activities of essential oils and compared the inhibition zone sizes produced by the oils with the minimal inhibition concentrations. The data showed that not always larger inhibitory zones correspond to lower MIC (17).

TABLE 2 - Activity of essencial oils against dermathophytes measured as the mean diameter (N≥2) of the inhibition zones in mm.

Essencial oils from	Dermatophyte Strains															
	T. rubrum								*T. mentagrophytes*				*M. canis*			*Epiderm. floc.*
	45T	54T	69T	75T	78T	84T	101T	118T	6T	12T	16T	29T	72T	73T	85T	27T
A. crassiflora	16	16	0	18	16	24	24	8	10	12	14	14	0	15	14	21
C. citratus	20	20	0	20	22	22	22	0	17	18	19	28	0	20	28	20
C. zeylanicum	28	28	0	26	24	30	28	22	26	30	28	30	0	27	30	23
O. gratissimum	24	30	0	25	30	15	20	0	26	22	20	28	0	26	28	24
P. heptaphyllum	0	0	0	8	8	0	0	0	0	8	0	0	0	0	0	0
X. frutescens	22	20	0	16	25	16	21	0	15	15	20	22	0	20	22	20
Dermatophyte control	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ketoconazole control	25	20	22	22	21	20	20	20	20	23	20	20	21	22	20	20

+ = normal growth

From the results we can conclude that, from the 6 essential oils tested, 5 strongly inhibited the growth of the majority of the dermatophyte strains studied, this being consistent with the results obtained by several researchers which have studied the antimicrobial activity of other essential oils. These studies, made with essential oils obtained from different aromatic plants collected in different parts of the world and having different chemical constituents, showed that a great number of them were active against many species of microorganisms (3, 9, 11, 14, 15, 16, 17).

Little is known however on the mechanism of antimicrobial activity of essential oils. Compounds isolated from them (and from plants in general) widely differ in their chemical structures from those of classical antibiotics and no correlation can be made with the already known modes of action. Despite the several investigations on the antimicrobial activity of essential oils, they were not followed by studies of the molecular mode of action. Knobloch et al (1986) investigated the influence of terpenoids on the reaction mechanisms of primary energy metabolism, in particular NADH and succinate dehydrogenase activities, membrana-bound respiratory electron flow and oxidative phosphorilation. All reactions studied were inhibited by all terpenoids (at 5 mM conc) independently of having different chemical functions (hidrocarbon, aldehyde, ketone, phenol ou alcohol). They concluded that terpenoids inhibit the reactions because their lipophilic properties enable them to dissolve in the membrane and because their functional groups interfere with the enzyme protein structures (10).

Our data showed that the popular use of the studied medicinal plants, against local skin infectious diseases can have a therapeutic basis since the plants contain antifungal components.

RESUMO

Atividade antifüngica in vitro de óleos essenciais frente a isolados clínicos de dermatófitos

Óleos essenciais de *Annona crassiflora* (araticum) *Cymbopogon citratus* (capim santo), *Cinnamomum zeylanicum* (canela), *Ocimum gratissimum* (alfavaca), *Protium heptaphyllum* (almiscar), *Xylopia frutescens* (embiriba vermelha) foram ensaiados contra várias cepas de *Microsporum canis, Trichophyton rubrum, Trichophyton mentagrophytes* e *Epidermophyton floccosum* isolados de pacientes com dermatofitoses. Essas plantas são popularmente utilizadas no Nordeste brasileiro para tratamento de infecções da pele e seus anexos, causadas por bactérias, vírus e fungos. Cinco dos óleos essenciais ensaiados mostraram excelente atividade antifúngica inibindo fortemente o crescimento de 81% das cepas estudadas. O óleo de *P. heptaphyllum* foi completamente inativo.

Palavras-chave: atividade antifúngica, dermatófitos, óleos essenciais, plantas medicinais.

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COAGGLUTINATION ASSAY FOR DETECTION OF ROTAVIRUSES

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SUMMARY

Rotaviruses present in human and porcine faecal specimens were detected using a coagglutination test. A suspension of *Staphylococcus aureus,* protein A-positive, coated with group A rotavirus diluted antiserum agglutinated specifically faecal extracts known to be positive for rotavirus by the EIERA test. A total of 89 faecal samples absorbed with *S. aureus* were tested by the coagglutination test and compared with an enzyme immunoassay (EIERA). The mixture of staphyloccocal cells and diluted serum showed a stability up to 6 months when stored at 4°C. Statistical analysis of the results showed a close correlation between the two tests (C=0.91). It was concluded that the coagglutination test was a simple, rapid, cheap and sensitive assay for the detection of rotavirus in faecal specimens for routine work.

Key Words: rotavirus, coagglutination assay.

INTRODUCTION

Rotaviruses are now recognized as one of the most important causes of acute diarrhea in infants and animals ¬(6, 7, 13, 16). The genus Rotavirus belong to family *Reoviridae* (11). They are classified serologically into six distinct groups or serogroups (A to F) (1) based on the presence of antigens on the inner capsid detectable by serological tests such as ELISA, immunofluorescence and immunoelectron microscopy (6, 12, 18). Rotaviruses within serogroup A are also classified into subgroups according to non-neutralizing epitopes present on the inner capsid proteins which are detectable by an ELISA assay (6). Furthermore this group of rotaviruses can be classified into serotypes which are defined by ELISA and PCR tests (4, 9, 15). Up to now, it has been suggested that between 9 and 11 serotypes of rotavirus exist (4, 15) and their specifities are related to the outer capsid proteins (15). Over the last ten years many serological assays for the quick detection of rotaviruses strains in stools have developed, mainly with regard to serogroup A, whose strains are among those most frequently isolated from human and animals stools.

In the present study we show that the coagglutination test is a simple, fast and cheap procedure for the direct identification of rotaviruses from specimens of stools, using as control an ELISA assay specific for group A of rotaviruses.

MATERIAL AND METHODS

Virus strain and immune sera - The simian SA11 serogroup A rotavirus strain was propagated in MA-104 cell culture to obtain antigens for the

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tests, as previosly described (13). Antiserum was obtained in rabbits by 3 intramuscular inoculations with a mixture of SA11 purified antigen and complete Freund's adjuvant (v/v), in 7 days intervals. After 21 days the animals were bleed out. In contraimmunoelectrophoresis (2) the antiserum shows a positive reaction untill 1:64 dilution, against SA11 antigen.

Preparation of Staphylococcal antigens - The Cowan-1 strain of *S. aureus* which produces large amounts of protein A was used for both preparation of the antigens for the agglutination test and to absorb the faecal specimens. This microorganism was grown in Trypticase Soy Broth (TSB), pH 7.0 at 37°C in a fermentor and after 18h (150 rpm) the culture was centrifuged at 1500 g and washed three times with phosphate buffered saline (PBS) pH 7.4. The cells were then resuspended in PBS containing 0.5% formalin and incubated overnight at 4°C. Afterwards, the bacterial suspension was washed twice with PBS and the pellet (1:10 suspension) was heat-treated at 80°C for 10 min and washed twice with PBS. The cells were resuspended to 50% with PBS, containing 0.5% sodium azide and stored at 4°C (5, 14).

Antibody coating of Staphylococci - To determine the optimum amount of antiserum for coating staphylococcal cells, 1 part of a 50% staphylococcal cell suspension was coated with 2 parts of an increasing reciprocal dilutions (10, 20, 30, 50, and 100) of antiserum. The sensitized cell suspension was left at room temperature for 20 min and gently shaked intermittently, followed by storage at 4°C for 20 min. The antibody-coated staphylococci were washed three times with PBS and resuspended to a final concentration of 2.5% (v/v) in PBS containing 0.05% sodium azide and 0.1% bovine serum albumine. Staphylococci coated with rabbit preimune serum and uncoated staphylococcal cells, both at 2.5% suspension, were used as controls in the coagglutination test.

Faecal specimens - Faecal specimens were obtained from children and piglets with diarrhea. Suspensions of the faecal specimens in PBS were clarified by centrifugation at 900 g for 10 min at 4°C and stored at this temperature. These suspensions were also used in the EIERA assay. Equal volumes of specimens and a 50% (v/v) suspension of staphylococci in PBS were mixed and incubated for 15 min at room temperature and afterwards at 4°C for another 15 min. Thereafter, the suspension was centrifuged at 900 g for 30 min at 4°C and the supernatants (faecal extracts) were saved and examined for rotavirus by the coagglutination test (5).

Coagglutination assay - Three separated drops (0.25µl) of the faecal extracts were placed on a glass slide, after the reagents have been previously left at room temperature for 10 min. A drop of the 2.5% suspension of staphylococci coated with antiserum against rotavirus was added to one of the drops and a drop of each of the two control reagents to the other 2 drops of faecal extracts. The mixtures were homogenized by continous circular motion of the glass slide during 2 min and specimens developing agglutination only with the suspension of staphylococci coated with hiperimune serum were taken as positive for rotavirus.

Elisa test - EIERA diagnostics kits from Fundação Oswaldo Cruz, R. J., Brazil (12), were used accordingly the author's instructions.

Statistical analysis - The results obtained by EIERA and coagglutination assays were analysed for sensitivity, specificity and concordance values, according Jenicek, M. and Cleraux, R. (10).

RESULTS

Table 1 shows results obtained in the determination of the optimal amounts of antiserum for coating the staphylococcal cells, using differents dilutions of SA11 antigen obtained from cell cultures. With regard to the antiserum that we have prepared, the dilution 1:20 was considered optimum in terms of specificity and sensitivity in the

TABLE 1 - Determination of optimal-dilution of antiserum for coating staphylococcal cells (2.5%) vs SA11 antigen by check board titration.

Antigen dilution (SA11)	Antiserum dilution						
	10	20	30	50	100	sta[a]	sta[b]
undiluted	4[c]	4	3	2	0	0	0
1:10	3	4	4	4	0	0	0
1:20	2	4	2	4	3	0	0
1:40	1	4	3	4	2	0	0
1:80	3	4	4	4	2	0	0
1:160	3	4	0	0	0	0	0
1:320	3	3	0	0	0	0	0

a Staphylococcal cells 2.5% with pre-immune serum
b Staphylococcal 2.5% uncoated
c The agglutination was classified from strong (4) to no agglutination (0)

TABLE 2 - Coagglutination test for rotavirus detection in positive faecal specimens with staphylococci cells coated with different dilutions of antiserum.

Faecal extract dilution	Antiserum dilution						
	10	20	30	50	100	sta[a]	sta[b]
1:2	4[c]	4	3	2	0	0	0
1:4	4	4	3	2	0	0	0
1:8	2	2	2	0	0	0	0
1:16	2	1	0	0	0	0	0
1:32	0	0	0	0	0	0	0
1:64	0	0	0	0	0	0	0

a Staphylococcal cells 2.5% coated with pre-imune serum
b Staphylococcal cells 2.5%, uncoated
c The agglutination was classified from strong (4) to nc agglutination (0)

TABLE 3 - Results of coagglutination test of rotaviruses from human and porcine faecal specimens, as compared with the EIE assay.

Associaton	Faecal Specimens		Total
	Human	Porcine	
EIE+COA+	13	19	32
EIE+COA-	1	3	4
EIE-COA+	0	4	4
EIE-COA-	21	28	49
Total	35	54	89

Sensivity value = 0.88
Specifity value = 0.92
Concordance value = 0.91

coagglutination test. When the same dilutions were analysed with faecal specimens rotavirus positive in EIERA test, (diluted from 2 to 64 in PBS) the dilution 1:20 also proved to be sensitive (Table 2). Based on these results we used these coated staphylococcal cells to test 89 faecal specimens from children and pigs (Table 3).

From 36 strains positive in the EIERA assay, 32 gave the same result in the coagglutination test. Statistical analysis from the results (Table 3) showed values of 0.88 e 0.92 with regard to sensitivity and specifity, respectively with a concordance of 0.91.

Another objetive of this work was to evaluate the time which coated staphylococcal cells maintain their stability for detecting rotaviruses in faecal specimens. As shown in Table 4, the staphylococcal suspension cells, when stored at 4°C, showed reproducible results for up to 6 months. However when the same test was made 12 months later the reagent stored as described was no longer stable, giving a false positive reaction.

TABLE 4 - Results of coagglutination test of rotaviruses from the same human and porcine strains in different times, with the same staphylococcal suspension cells.

Strains	Time (months)			EIERA
	0	6	12	
Human	3/4[a]	3/4	4/4	3/4
Porcine	6/14[b]	9/14	12/14	10/14

a Number of human strains tested
b Number of porcine strains tested

DISCUSSION

In this study, staphylococci coated with antibodies against rotavirus were used for rapid detection of this viral agent in human and porcine faecal specimens. A rabbit hyperimmune serum against group A rotavirus was used for coating the staphylococci. The results showed that the reactivity of the reagent is variable according to the amount of antibody against rotavirus, when using either the SA11 antigen or the faecal specimens. Thus, it is necessary to determine the optimal dilution of each antiserum for use in the test. The use of diluted serum (1:20) showed to be viable to sensibilization of staphylococcal cells, given a good results with SA11 antigen as too as the faecal extracts (Tables 1 and 2). If the faecal specimens were examined directly by coagglutination test, most of them reacted with the two control reagents utilized (data not shown). These non-specific reactions, were eliminated by mixing the faecal specimens with a suspension of uncoated staphylococci and the use of a control represented by staphylococci coated with pre-imune serum.

The coagglutination assay showed of 0.88 and 0.92 values for sensitivity and specifity respectively, and when compared with an ELISA assay used for group A rotavirus detection gave a concordance of 0.91. Based on the results, we suggest that the coagglutination test could be used for rou-

tine diagnostic purposes. The stability of the staphylococci suspensions, coated with pre and hiperimmune sera last for up to 12 months at 4°C, showing that these preparations gave very reliable results up to 6 months, without any false positive reaction. Furthermore, when compared with other assays for rotavirus detection such as the ELISA assay, the coagglutination test used as described in this investigation is simple, easy to perform and cheap, making it suitable for laboratories with limited facilities.

RESUMO

Teste de coaglutinação para detecção de rotavirus.

O teste de coaglutinação foi utilizado para a detecção de rotavírus em fezes de origem humana e de suínos. Suspensão de *Staphylococcus aureus*, produtor de proteína A, foi sensibilizada com uma diluição seriada de antissoro anti-rotavírus do grupo A mostrando que quando foi utilizada a diluição a 1:20, o teste foi capaz de detectar tanto antígeno SA11 como também o extrato fecal, ambos diluídos. Um total de 89 amostras de fezes absorvidas com *S. aureus* foram testadas por coaglutinação e por um ensaio imunoenzimático. A análise estatística dos resultados obtidos mostrou uma concordância de 0,91 entre os dois testes o que levou-nos a concluir que a coaglutinação é um método simples, rápido, sensível e pouco dispendioso para a detecção de rotavírus diretamente do material fecal. Além da utilização do soro diluído para a sensibilização de *S. aureus* ficou demonstrado também que, esta mistura, quando estocada a 4°C pode ser utilizada por até 6 meses após o seu preparo, sem implicar em resultados falso-positivos.

Palavras-chave: rotavirus, coaglutinação.

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A RAPID AND LOW COST ADAPTATION OF STAPHYLOCOCCAL TNase DETECTION IN MILK

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SUMMARY

Modification of the method for recovery of thermonuclease (TNase) from foods in which staphylococci had grown was investigated. Replacement of bovine serum albumin (BSA) in Tris buffer with proteose peptone nº3 gave better protection of the TNase when held for several hours before assay. Partial concentration of brain heart infusion broth and milk in which enterotoxigenic staphylococci had been grown by dialysis against sucrose was less sensitive than the use of trichloro-acetic acid precipitation with centrifugation, but was adequate for detecting the TNase. These modifications can be useful in developing countries where BSA and high-speed centrifugation are not available.

Key Words: thermonuclease, staphylococci, dialysis.

INTRODUCTION

Detection of staphylococcal enterotoxins in food is the best way to incriminate the food in staphylococcal food poisoning; however, the cost involved in their assay resulted in the search for alternatives for screening the foods for their possible implication in food poisoning. Thermonuclease (TNase) has been used for this purpose because it is produced by essentially all enterotoxigenic staphylococci and is heat stable (6, 14, 18). It is detectable in food even when the staphylococci have been destroyed (17) and remains active for long storage periods (3, 8). TNase in contaminated food is present in nanogram quantities, thus it is necessary to extract it and concentrate the extract by acid precipitation followed by centrifugation and steaming for 15 minutes (5, 18). However, this process results in the loss of enzyme activity unless a protective agent such as bovine serum albumin (BSA) is added before the steaming (8). The use of Tris buffer or brain heart infusion (BHI) broth did not prove to be protective (8). TNase is a protein with a molecular weight of 16,807 daltons; it requires calcium ions for its activity and has an optimum pH of 8.6 (4).

In this project we investigated low cost components for replacement of BSA to protect the TNase activity. The pH and concentration of Ca^{2+} ions in Tris buffer were analyzed in the resuspension of the TNase extracted from milk. Also, the possibility of enzyme concentration by dialysis

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against sucrose and/or polyethylene glycol 6000 (PG-6000) was tested, in order to dispense centrifugation to simplify TNase analysis in foods under the conditions existing in Brazil.

MATERIALS AND METHODS

Microorganisms - *Staphylococcus aureus* strains FRI-196E, producer of TNase and enterotoxins A (SEA) and D (SED), and FRI-100, producer of SEA were used in the experiments.

Standard TNase - The standard TNase used to evaluate the effect of heating on the enzyme activity was purified from *S. aureus* strain Foggi with an activity of 50,000 units/mg protein (Micrococcal nuclease, Sigma Chemical Co., St. Louis, MO, USA).

Detection of TNase - TNase was detected in Toluidiose blue-DNA agar (TDA)(10), using the diffusion medium at pH 10 with 4 hours incubation at 50°C (9). Wells in the agar in which the samples to be tested were placed were 7 mm in diameter.

Extraction of TNase - TNase was extracted by the method of Tatini et al. (18) and by a modification of this method. In brief, the modified method was as follows: adjustment to pH 4.5, centrifugation at 7,800xg for 15 min, addition of trichloro-acetic acid (TCA) (0.15 M), centrifugation at 7,800xg for 15 min, precipitate dissolved in 0.5 M Tris buffer containing 1% proteose peptone nº3 (PP-3).

Evaluation of the thermal inactivation of nuclease activity - The standard nuclease at concentrations of 0.0 to 2,500 ng/ml in 0.05M Tris buffer with 0.1% bovine serum albumin (BSA) was treated in a boiling water bath for 15 min; the enzyme activity was analyzed in TDA agar at pH 9.0 and 10.0.

Protective effect of different substances on TNase - The protective effect of proteose peptone nº2 and PP-3, tryptone, tryptose, yeast extract, meat extract, and brain heart infusion (BHI) broth at concentrations of 0.5 to 5% on 500 ng/ml of micrococcal nuclease was analyzed. The activity of nuclease in the presence of these solutions was compared with the enzyme in water, 0.05 M Tris buffer at pH 8.5, and the buffer supplemented with 0.1% and 0.5% BSA. Each mixture was analyzed in TDA agar.

Effect of pH, Ca^{2+}, and Tris buffer concentration on TNase - Enzyme activity of 500 ng/ml of the standard TNase was analyzed in the presence of 0.05 and 0.5 M Tris buffer, pH 8.5 and 10.0, the addition of 1.1 and 11.0 ug/ml of $CaCl_2$ in the buffer containing 1% PP-3. The enzyme activity in the suspensions were observed for seven days in the TDA agar.

Preparation of inoculated samples - BHI broth was inoculated with *S. aureus* strain FRI-196E culture, with 0.3 absorbance at 600 nm, in the proportion of 0.1 ml culture in 100 ml sample; preheated pasteurized milk was inoculated with either strain FRI-196E or FRI-100. Incubation was at 37°C for 18 hours. The BHI broth culture was centrifuged at 7,800xg for 15 min at 4°C and merthiolate was added at a final concentration of 1:20,000; each 15 or 20 ml of this supernatant was assayed by different extraction procedures. The artificially contaminated milk was divided into two portions, one was heated for 20 min before extraction and the other was unheated. A separate 500 ml sample of milk was inoculated and incubated for 15 hours; a 2.5 ml sample was taken for CFU determination and direct TNase determination and a 25 ml sample was taken for the membrane dialysis concentration procedure at 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15 hours.

Precipitation of TNase with TCA - BHI culture supernatant fluids (15 ml) prepared as above, were treated with 0.0 to 40.% of 3.0 M TCA. The precipitates obtained by centrifugation at 7,800xg for 10 min at 4°C were resuspended in 1.5 ml of Tris buffer, pH 10.0, with or without 1% PP-3. The suspension was diluted 1:10 to 1:400, and evaluated in TDA agar.

Effect of pH, Ca^{2+}, and Tris buffer on extracted TNase - The same procedure described above for standard TNase was repeated with extraction of TNase from BHI broth culture; 5% TCA, 3.0 M, was added to 20 ml of culture supernatant, the precipitate removed by centrifugation, and resuspended in 1.5 ml of Tris buffer + 1% PP-3. Tris buffer was used at concentrations of 0.05 or 0.5 M, pH 8.5 or 10.0, and CaCl2 was either not present or was used at 1.1 ug/ml.

Extraction of TNase from milk after growth of staphylococci - Inoculated milk samples prepared as described above were analyzed for TNase, using the procedures giving the best results with the purified TNase in BHI medium. The contaminated milk samples, without heating, were adjusted to pH 4.5 and centrifuged with refrigeration in two portions. One portion was filtered through gauze covered with cotton; the other portion was adjusted to pH 4.5 and centrifuged at 7,800xg for 10 min. Both the heated and unheated milk samples were treated with 3.0 M TCA (5%), centrifuged at 7,800xg for 15 min; the precipitates were resuspended in 1.5 ml of 0.05 or 0.5 M Tris buffer at pH 10, with 1% PP-3, containing no $CaCl_2$ or 1.1 ug/ml. The TNase activity in the extracts was analyzed in TDA agar.

Concentration of TNase with PG-6000 and commercial sugar - The inoculated milk and BHI

broth were prepared as described above with concentration by applying PG-6000 or commercial sucrose directly to the dialysis tub (nº 133-81, 1363 from Inlab) containing the samples. The samples were concentrated to volumes of 10 to 30% of the initial volumes; the TNase activity in the concentrates was analyzed in TDA agar. An uninoculated milk sample was used as a control.

Hydrolysis of DNA by sucrose and PG-6000 was analyzed in the TDA agar with 0, 10, 20, 30, 40, 50, 55 and 60% concentrations of these substances in water, 0,05 and 0.5 M Tris buffer, pH 8.5, the same PP-3, BHI broth and milk. The capacity of the dialysis tubing in preventing the passage of sucrose into the samples was analyzed by detection of the sucrose inside the membrane by the Benedict test. The dialysis tubing containing 100 ml of H_2O was placed in a beaker containing a high concentration of sucrose and was allowed to remain until the volume was reduced to about 20 to 30 ml; the concentrated fluid was analyzed with the Benedict reagent.

Concentration efficiencies - Concentration efficiencies of sucrose and PG-6000 on the TNase in inoculated milk, both heated and unheated, were compared to the TNase in BHI broth. The casein in unheated milk was separated by centrifugation at 7,800xg for 15 min after adjusting the pH to 4.5. The supernatant fluids to which merthiolate (1:20,000) was added, were adjusted to pH 7.0 and concentrated. The heated milk was centrifuged without pH adjustment and treated with merthiolate, (1:20,000). The supernatant fluid was divided into two portions, one adjusted to pH 7.0 before dialysis, and the other without pH adjustment.

Relationship of TNase production to S. aureus *growth* - Milk (500 ml) was inoculated with 10^3 CFU/ml of S. aureus strain FRI-100 and incubated at 37°C for 20 hours. Samples of 2,5 ml for staphylococcal count and 25ml for dialysis experiments were taken at 0, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 15 hr. Staphylococcal counts were made on plate count agar. The 25 ml samples were heated for 20 min at 100°C. The pH was adjusted to 4.0-4.5 to precipitate the casein which was removed by filtration. The supernatant fluid was adjusted to pH 7.0 and dialyzed against sucrose. Tests for TNase were made after 2 and 4 hr of dialysis.

RESULTS

According to Fig. 1, the heat treatment for 15

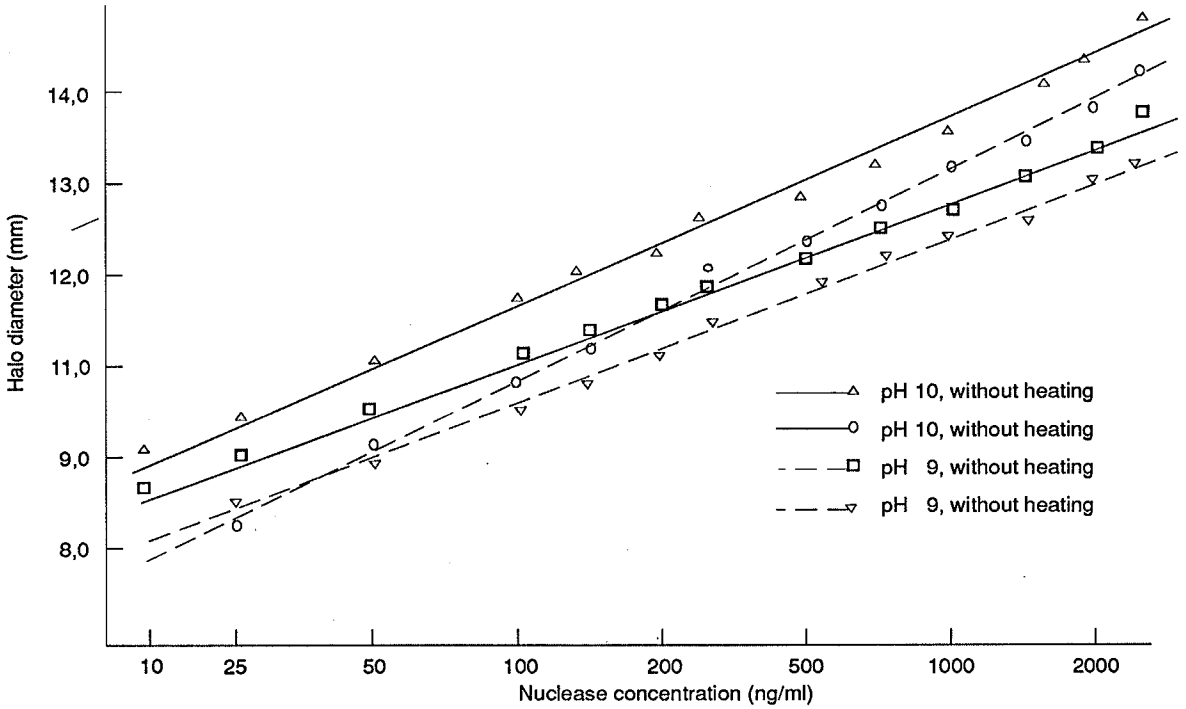

FIGURE 1 - Effect of TDA agar, pH and heating of standard nuclease for 15 min on the enzyme activity. Incubation at 37°C for 4 hours.

min of standard TNase suspended in 0.05 M Tris buffer, pH 8.5, treated with 0.1% BSA, resulted in some loss of enzyme activity.

The protective effect of the different substances on 500 ng/ml of standard staphylococcal TNase increased as the amount of the substances was increased, with PP-3 and meat extract giving the best results of these two substances, PP-3 was slightly superior to meat extract (Table 1). A rapid loss of activity was observed when the nuclease was dissolved in water or Tris buffer without protective components. All of the components studied

TABLE 1 - Protective effect of proteose peptone nº 3, and bovine soroalbumin on staphylococcal TNase[a].

		TNase activity (mm)[b]				
		Concentration (%)				
Protective agent	Time[c]	2.5	1.0	0.5	0.1	0.0
H_2O	0					12.1
	24					11.0
	48					9.0
BSA	0			15.0	14.5	
	24			13.0	11.5	
	48			11.2	11.0	
PP-3	0	16.0	15.0	14.5	12.7	
	24	16.0	14.5	14.0	13.0	
	48	15.5	14.0	13.0	11.5	
Meat extract	0	15.0	14.8	14.0	13.0	
	24	15.5	14.5	14.0	12.8	
	48	14.8	14.0	13.5	11.5	

a - Suspendend in 0.05 M Tris buffer, pH 8.5.
b - Diameter of activity in TDA agar, pH 10, 50°C, 4 hours.
c - Time of holding before determination of activity.

gave better protection than 0.1% BSA, with the exception of tryptone when the nuclease solutions were held for 24 hours at 4°C.

The standard TNase remainded stable for seven days when placed in Tris buffer, either 0.05 or 0.5 M, to which 1% PP-3 was added. The addition of $CaCl_2$, either 1.1 or 11.1 ug/ml, had little or no effect on the stability. Slightly higher values were obtained when the pH of the buffer was adjusted to 10 (results not shown).

The best recovery of TNase from BHI broth inoculated with *S. aureus* strain FRI-196E was with 0.15 and 0.3 M TCA (Table 2). The addition of 1% PP-3 protected against the loss of TNase activity for four days. There was little difference in the recovery of TNase, whether 0.05 of 0.5 Tris buffer containing 1% PP-3 was used to dissolve the TCA precipitate (only results with 0.05 M Tris are shown). The recovery and stability was slightly less at pH 8.5 than at pH 10 (results not shown).

TABLE 2 - Recovery of TNase from BHI broth inoculated with *S. aureus* strain FRI-196E by TCA precipitation[a].

		TNase activity (mm)[b]			
TCA	Buffer[c]	Undil	1:10	1:100	1:400
0.00 M	Tris	18.0	15.5	12.5	11.0
	Tris + 1% PP-3	19.0	16.5	14.5	12.5
0.075 M	Tris	18.5	16.5	13.5	11.5
	Tris + 1% PP-3	20.5	18.5	15.5	14.5
0.15 M	Tris	19.5	16.5	13.5	11.5
	Tris + 1% PP-3	21.0	18.5	16.0	14.2
0.30 M	Tris	19.2	14.0	13.5	10.0
	Tris + 1% PP-3	21.2	19.0	16.8	13.8
0.60 M	Tris	19.2	13.5	13.0	10.5
	Tris + 1% PP-3	21.2	18.5	15.2	13.8

a - Method of Tatini et al., 1976.
b - Diameter of activity in DNA agar, pH 10, 50°C, 4 hours; held 24 hours before testing.
c - TCA precipitate dissolved in 1.5 ml of 0.05 M Tris buffer, pH 10.

The best recovery of TNase from milk was with 0.15 M TCa, with increased recovery from the heated milk. Better results were obtained when Tris buffer at pH 10.0 was used to redissolve the precipitate (Table 3). The larger the amount of TCA used, the lower the pH of the resuspension and less stable was the TNase (results not shown). The best recovery of TNase was from milk that had been heat treated before precipitation with 0.3 M TCA. There was essentially no difference in the recovery whether 0.05 or 0.5 M Tris buffer at pH

TABLE 3 - Recovery of TNase from milk inoculated with *S. aureus* strain FRI-196E by TCA precipitation[a].

Heat treatment	Casein pptn.	Tris buffer	TNase activity (mm)		
100°C/20 min	pH	Concn.[b]	0[c]	24	72
Uninoculated	–	–	8.5	8.5	9.0
Untreated	–	–	9.0	9.0	9.0
None	4.5	–	9.0	9.0	9.0
		0.05 M	14.0	13.0	13.0
	–	–	15.0	15.0	14.0
Heated		0.05 M	17.0	16.5	16.0
	4.5	–	14.5	15.0	15.0
		0.05 M	18.0	17.5	16.0

a - Method of Tatini et al., 1976.
b - TCA precipitate dissolved in 1.5 ml of 0.05 M Tris buffer at pH 10.0.
c - Time of holding before testing.

10 containing 1% PP-3 was used to redissolve the precipitate. There was some decrease in stability after 72 hours.

An attempt was made to replace TCA precipi-

tation and centrifugation with concentration by dialysis. Two substances, sucrose, and PG-6000, were used in the experiments. Only partial concentration was achieved, 70 or 80%, which could be accomplished in 3 hours using dialysis tubing with sucrose as the concentrator. Longer treatment resulted in increased loss of TNase and increasing amounts of sucrose in the concentrate. Concentration of TNase present in BHI culture supernatant fluid after growth of *S. aureus* strain FRI-196E was possible with both PG-6000 and sucrose, however, slightly greater interference in the TNase test was noted with PG-6000. The use of dialysis tubing for concentrating the TNase in milk after growth of *S. aureus* strain FRI-196E in place of TCA precipitation resulted in slightly lower TDA values (Table 4). Better recovery was accomplished when the milk was heated before concentration.

TNase detectable in the culture supernatant fluid after 5 hr of incubation when the count was approximately 10^7 CFU/ml (Table 5). However, if the test was continued for an additional 20 hr at room temperature, TNase was detectable after 4 hr of incubation when the count was 10^6 CFU/ml. The analysis of the 4 hr incubation samples could be enhanced by using 50 ml of milk culture instead of 25 ml, with readings of 7.5 and 8.0 mm after 4 and 8 hr dialysis, respectively. Allowing the test to continue for another 20 hr at room temperature resulted in readings of 10.0, 11.5, 11.7 mm after dialysis of 2, 4, and 8 hr, respectively. The blank reading for milk was 7.0 mm. Essentially no interference from the sucrose was detectable until after 10 hr of dialysis.

TABLE 4 - Recovery of TNase from milk inoculated with *S. aureus* strain FRI-196E by dialysis[a].

Sample	pH[b]	Heat treatment[c]	Concentration[d]	TNase activity (mm)[e] 0	24	72
Milk, uninoc.	–	–	ND	8.5	8.5	–
Milk, inoc.	NA	NB	ND	9.0	9.0	–
	7.0	NB	ND	–	9.5	10.0
	NA	B	ND	15.0	–	14.5
	7.0	B	ND	14.5	15.5	14.0
	7.0	NB	S	10.0	10.0	11.0
	NA	B	S	15.0	16.5	15.0
	7.0	B	S	15.0	16.5	15.5
	7.0	NB	PG	9.0	10.0	10.0
	NA	B	PG	16.0	15.5	15.5
	7.0	B	PG	16.0	16.0	16.0
Sucrose	–	–	–	11.0	12.0	12.0
PG-6000	–	–	–	13.0	13.5	13.0

a - Samples placed in dialysis tubing.
b - NA, not adjusted, about pH 5.0; adjusted to 4.5, then to 7.0 before dialysis.
c - NB, not boiled; B, boiled.
d - ND, not dialysed; S, sucrose; PG, PG-6000.
e - Diameter of activity in TDA agar, pH 10, 50°C, 4 hours.

TABLE 5 - Relation of TNase production to growth of *S. aureus* strain FRI-100 in milk.

Incubation hours	CFU/ml	TNase activity (mm)[a] O[b]	2	4
0	10^3	7.0 (7.2)c	7.0 (7.2)	7.0 (7.2)
2	3.2×10^4	7.0 (7.2)	7.0 (7.2)	7.0 (7.2)
3	2.3×10^5	7.0 (7.2)	7.0 (7.2)	7.0 (7.5)
4	1.5×10^6	7.0 (7.2)	7.0 (8.0)	7.0 (8.2)
5	7.0×10^6	7.2 (7.8)	7.8 (10.0)	8.0 (10.0)
6	1.4×10^7	7.7 (9.5)	8.3 (12.5)	9.0 (12.8)
7	6.0×10^7	8.8 (11.0)	10.0 (14.0)	10.5 (14.0)
8	2.3×10^8	10.0 (12.5)	11.5 (14.0)	12.0 (14.0)
9	2.4×10^8	10.4 (13.0)	12.0 (14.5)	12.5 (14.5)
10	2.8×10^8	10.5 (13.0)	12.2 (15.0)	12.5 (15.0)
15	1.9×10^8	11.5 (13.0)	12.2 (15.0)	13.7 (15.0)
20	–	11.5 (–)	13.0 (–)	13.5 (–)

a - Diameter of activity in TDA agar, pH 10, 50°C, 4 hours.
b - Hours of dialysis against sucrose.
c - TNase reading after 4 hours at 50°C (reading after an additional 20 hours at room temperature).

DISCUSSION

Frequently foods involved in what appears to be staphylococcal food poisoning have been heated, resulting in destruction of any staphylococci that may have been present. Analyzing the food for staphylococcal enterotoxin is difficult in the developing countries because of the cost of the reagents necessary for doing this. Thus, to avoid unnecessary testing, a screening test was devised based on the presence of TNase, a characteristic product of *S. aureus* strains. The detection of this product is an indication that staphylococci had grown to sufficient numbers to produce enterotoxins, thus, justifying examination of the food for enterotoxin.

The methods developed for analyzing food for TNase are adequate, but require procedures such as centrifugation that may not always be available in the developing countries. Also, maintaining the stability of the TNase during extraction and storage requires the addition of protective substances such as BSA which is expensive. An alternate product, PP-3, has been used (7) and in this study was found to give superior protection to that of BSA (Table 1). This material is relatively inexpensive and available to scientists in developing countries.

Precipitation of TNase with TCA followed by high-speed centrifugation (23,500xg) is an effi-

cient method for concentrating TNase; however, it has been shown that low-speed centrifugation is inadequate to recover all of the TNase (7). Inclusion of concentration step resulted in sufficient concentration to detect small amounts of TNase. The material used that was most effective was sucrose which is relatively inexpensive. The concentration could be done in a short time before appreciable loss of TNase occurred or interfering quantities of sucrose diffused into the sample.

Comparison of the results of concentration with those of precipitation, with centrifugation at 7,800xg, showed concentration by dialysis against sucrose to be less sensitive, but adequate in most instances. It is recommended that centrifugation be used whenever possible to make detection as sensitive as possible. If TCA precipitation is employed, 0.15 M TCA appears to give the best results as higher concentrations render the precipitate too acid. The precipitate can be redissolved in either 0.05 or 0.5 M Tris buffer, however, 0.5 at pH 10 appears to give the most consistent results (Table 2). The addition of $CaCl_2$ to the Tris buffer had little effect on the stability of the TNase, however, it may be beneficial to add it to the Tris buffer used in the extraction of solid foods that were suspected of containing TNase.

The sensitivity of testing milk was increased by heating in a boiling water bath for 20 min plus precipitation of the casein at pH 4.5 (Table 3). It is possible that the diffusion of TNase after the removal of the heated casein was better than with it present.

The methods presented here do indicate that TNase can be detected when the staphylococcal count is 10^6 (Table 5) which is below the count that was found necessary before production of enterotoxin in foods could be demonstrated (13).

There are reports showing that TNase was not produced in adequate amounts prior to the production of enterotoxin (2, 11) in foods such as pasta and custards. This could be a reflection on the extraction procedure as Park et al. (15) improved the extraction by the addition of non-fat dry milk to foods before acid precipitation at pH 3.8, enhancing recovery of TNase from most foods (15). However, one needs to use caution when such foods are involved, as the failure to detect TNase does not necessarily indicate the absence of enterotoxin. This would be particularly true if the food was involved in what appeared to be staphylococcal food poisoning.

Although other organisms such as *Bacillus* and *Streptococcus* (1, 12, 16) can produce TNase at detectable levels, it is unlikely that these organisms would be present where at the staphylococci had grown sufficiently to result in food poisoning. The TNase produced by the other organisms appeared to be less heat stable and were not extractable at pH 3.8, whereas staphylococcal TNase could be extracted at pH 3.8.

RESUMO

Detecção de termonuclease de estafilocócica em leite através de método rápido e barato.

Um método visando detecção direta de termonuclease (TNase) em leite foi desenvolvido, com a finalidade de triagem de alimentos contaminados com estafilococos enterotoxigênicos. A substituição de soroalbumina bovina (BSA) do tampão tris por proteose peptona nº3 concedeu melhor proteção à TNase mantida por várias horas, antes de proceder o ensaio. O método de diálise com sacarose foi adequada para detectar TNase em caldo BHI e leite contaminado com estafilococos enterotoxigênicos, embora seja inferior ao método de precipitação com tricloroacético seguida de centrifugação. Estas modificações poderão ser úteis em condições onde existem dificuldades referentes à disponibilidade de BSA e centrifugação a alta rotação para detecção direta de TNase estafilocócica em alimentos.

Palavras-chave: termonuclease, estafilococos, diálise.

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YERSINIA SPP: A STUDY OF THE BIOLOGICAL CHARACTERISTICS OF STRAINS ISOLATED FROM MINAS FRESCAL CHEESE (WHITE CHEESE)

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SUMMARY

Fifty samples of 10 different brands of white cheese purchased from supermarkets in Rio de Janeiro were analysed. Fecal contamination was established according to the MPN technique. Total coliforms counts ranged from 2,.7x10^3/g to 2.4x10^6/g and fecal coliforms from 2.0x10^1/g to 2,1x10^6/g. Among the samples examined, 80% showed poor hygienic conditions according to the standards of the National Sanitary Vigilance Committee (DNVS).

Samples were also investigated for the presence of *Yersinia* spp using three different methodologies. Enrichment at 26°C/48 hs following alkaline treatment and plating onto Desoxychcolate Citrate agar resulted in the isolation of 11 strains of *Y. frederiksenii*-0: 16a, 16b - Xo. Direct plating onto Mac Conkey agar allowed the isolation of one strain of *Y. intermedia*-NAG-Xo whereas the cold-enrichment technique at 4°C for 7-21 days gave negative results for all the samples tested. *Yersinia* spp isolates were assayed for the production of heat-stable enterotoxin, autoagglutination, calcium dependency, binding to crystal violet, pyrazinamidase activity and extracellular enzymatic activity as "in vitro" biologic markers. Only one strain of *Y. frederiksenii* showed protease activity.

Isolates were also tested for "in vitro" susceptibility to antibiotics. They showed uniform resistance to Ampicillin, Amoxicillin, Carbenicillin, Cephalexin, Cephalothin, Colisthin and Sulphazotrim. All *Yersinia* were shown to be susceptible to Nalidixic Acid, Amikacin, Chloramphenicol, Gentamicin, Norfloxacin, Polymyxin B and Tobramicin. The Nitrocefin method demonstrated the production of Beta-lactamase for all strains studied.

Key Words: White cheese, *Yersinia* spp., Biological characteristics, Susceptibility.

INTRODUCTION

The raw material indicated for the fabrication of white cheese is pasteurized milk, after undergoing physical and chemical processes in order to become a proper product, with specific characteristics.

This kind of food provides favourable conditions for contamination and maintenance of microorganisms, due to high nutritional value, humidity rate and physical structure, as well as with the precarious conditions of production, transportation and storage.

Some species of the genus *Yersinia* constitute a serious public health problem, particularly be-

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cause of their ability to multiply at refrigeration temperatures (24). Therefore this cheese - which must be kept at low temperature until the moment it shall be consumed - may be a proliferation medium and a transmission vehicle for Yersinioses; episodes have been related in which it was said to be foodborne (2, 24).

Although the occurrence of *Yersinia* spp in food, including raw or pasteurized milk and several by-products, like cheese, has been investigated in some countries (11, 15), only reports on isolates from raw or pasteurized milk are available in Brazil (25).

The present lack of data concerning the occurrence of this microorganism in milk by-products, more specifically in white cheese, prompted us to investigate the presence of *Yersinia* spp in it, aiming at determining its biological characteristics, as well as its antimicrobial susceptibility profile.

MATERIALS AND METHODS

Origin and processing of the white cheese samples analysed - Sample collection and dilution techniques followed the recommendations of the Standard Methods for Examination of Dairy Products (3).

Fifty white cheese samples (100 g each) of different brands were purchased at supermarkets in Rio de Janeiro from March to August, 1988, respecting their shelflife validity. The samples were transported immediately, in an ice bath, to the laboratory and processed within two hours.

Colimetric rates were determined by means of the most probable number technique, as a parameter for the microbiological quality of the cheese samples collected (4).

Yersinia *spp strains isolation and identification methods* - First, the cheese samples were processed in accordance to the centrifugation technique used for the isolation of *Yersinia* spp from raw milk (25) and water (10). Different methodologies used for the isolation of *Yersinia* spp were compared. Thus, the samples were centrifuged and the sedimented material analysed through three different methodologies, namely: direct plating; cold enrichment at 4°C in phosphate buffer saline (PBS), pH 7,4 with 1% sorbitol, 0.15% bile salts and 0.5% peptone (26) and 48 hours pre-enrichment in PBS at 26°C, undergoing blending and posterior alkaline treatment (24). Material processed according to these three methodologies was spread onto Mac Conkey agar (Merck), Desoxycholate Citrate agar (Biobrás) and *Yersinia* Selective agar (Merck), and then incubated at 25°C for 24-48 hours. Colonies resembling Yersiniae taken from each plate were inoculated into triple sugar iron agar (TSI-Biobrás) and Lysine Iron agar (LIA-Biobrás), and incubated at 25°C for 24 hours. Isolates displaying characteristic reactions were inoculated into modified Rugai-EPM and MIO (Biobrás), and incubated at 25°C for 24 hours (10). The strains identified as *Yersinia* were confirmed by the API-20 E (Analytab Products Inc) system.

Those isolates biochemically identified as *Yersinia* spp were sent for biochemical confirmation, serotyping and phage typing to the Brazilian National Center for *Yersinia* located in Araraquara, São Paulo, Brasil.

Evaluation of the biological characteristics of the Yersinia *spp isolates* - Production of heat-stable enterotoxin (ST): ST production was investigated according to the methodology described by Nunes & Ricciardi (17).

Autoagglutination test: Autoagglutination was assayed in accordance to the method of Aulisio & cols. (1)

Calcium dependence test: The calcium dependence test was done through the method Berche & Carter (6).

Violet crystal binding test: Violet crystal binding capacity was done in accordance to the methodology proposed by Bhaduri & cols. (7)

Pirazinamidase activity test: Pirazinamidase activity was determined through the method proposed by Kandolo & Wauters (14).

Production of exoenzymes: Lecithinase, DNAse, Lipolytic and Proteolytic activities.

The methodology proposed by Janda & Bottone (13) was used in the specific medium for the different activities, and the plates incubated for 48 hours at 25°C and 37°C.

Antibacterial susceptibility testing - Antibacterial susceptibility testing was performed at 26°C on Mueller Hinton agar plates by the standard disk method (5).

Together with the strains of *Yersinia* spp, *Staphylococcus aureus* (ATCC 25923) was used as standard strain in the susceptibility tests.

The antibacterial agents (Cecon) used were: Amikacin (30 ug), Amoxacillin (10 ug), Ampicillin (10 ug), Carbenicillin (100 ug), Cephoxitin (30 ug), Cephalexin (30 ug), Cephalothin (30 ug), Chloramphenicol (30 ug), Colisthin (10 ug), Gentamicin (10 ug), Kanamycin (30 ug), Nalidixic

acid (30 ug), Nitrofurantoin (300 ug), Norfloxacin (10 ug), Polymyxin B (300 U), Sulphazotrim (25 ug), Tetracycline (30 ug) and Tobramycin (10 ug).

Assay for Beta-lactamase was done through the fast Nitrocefin method (22).

RESULTS AND DISCUSSION

In the 50 cheese samples analysed the colimetric rates varied from 10^3 to 10^6 MPN/g, and from 10^1 to 10^6 MPN/g, for total and fecal coliforms, respectively. The large number of microorganisms indicating fecal contamination found in 80% of the white cheese samples studied showed poor hygienic-sanitary conditions, according to the standards established by DNVS - National Sanitary Vigilance Committee (9) - representing a serious risk for the population.

No correlation was observed in the samples between colimetric rates and *Yersinia* spp positivity rates: brand "H", positive for *Yersinia* spp, showed small number of fecal coliforms (2.0×10^1 MPN/g of cheese), which may suggest the presence of reasonable concentrations of bactericidal and bacteriostatic agents (Table 1).

As an outcome of the different methodologies used in the search for *Yersinia* spp, pre-enrichment followed by alkali treatment and spreading onto Desoxychcolate Citrate agar showed a better performance (91.7%) when compared to the techniques of direct plating (8.3%) with spreading onto Mac Conkey agar and cold enrichment, that did not show efficiency for the isolation of *Yersinia* spp. These findings confirm other reports (8, 24, 26), according to which the alkali treatment reduces or even eliminates many of the competing organisms, thus facilitating *Yersinia* spp isolation. It could be observed that the Desoxychcolate Citrate agar medium was efficient for the isolation of *Yersinia* spp strains from white cheese followed by Mac Conkey agar, in accordance to several authors (10, 25).

TABLE 1 - Relation between the number of *Yersinia* spp and the number of total and fecal coliforms assayed in 50 samples of white cheese.

Cheese Sample		Number of coliforms (MPN)		Number of strains of *Yersinia* spp isolated
Brand	Nº	CT[a]	CF[a]	
A	5	$1,0 \times 10^6$	$3,6 \times 10^5$	–
B	5	$2,4 \times 10^6$	$9,7 \times 10^5$	5
C	5	$1,0 \times 10^6$	$1,0 \times 10^5$	–
D	5	$1,8 \times 10^5$	$2,3 \times 10^2$	6
E	5	$1,9 \times 10^6$	$9,6 \times 10^5$	–
F	5	$1,6 \times 10^6$	$1,1 \times 10^6$	–
G	5	$1,4 \times 10^6$	$7,1 \times 10^5$	–
H	5	$2,7 \times 10^3$	$< 2,0 \times 10^1$	1
I	5	$1,1 \times 10^5$	$9,6 \times 10^4$	–
J	5	$2,4 \times 10^6$	$2,1 \times 10^6$	–

CT = Total coliforms
CF = Fecal coliforms
a = Mean values obtained by arithmetic average

Four (8%) of the 50 cheese samples examined were positive for *Yersinia* spp, the highest positivity rates corresponding to brands "B" (two strains), "D" (1 sample) and "H" (1 strain). Isolates were identified as *Yersinia frederiksenii* 0:16a, 16b (11 samples) and *Yersinia intermedia* NAG (1 sample), belonging all to phagetype Xo (Table 2), and representing a high isolation rate (24%) as compared to the findings of Schiemann (21) - who could get no isolation at all from cheddar and italian cheese - and Pianetti et al. (19) - who got a low rate (0.62%) in sixty milk by - products samples (cheese, whipped cream, etc.) from which only one strain of *Y. frederiksenii* 0:4, 33-Xo was isolated (from cottage cheese). This isolation rate can be related to the fact that we have worked using three different techniques, while other authors used only one.

The *Y. intermedia* NAG strain isolate from white cheese, the pathogenic potential of which has not yet been clearly defined, has been considered in many papers to be responsable for clinical cases in humans (20, 24). The same happens to *Y. frederiksenii*, which showed the highest isolation rate, being isolated from animals and humans, healthy or not (18, 19), although those two strains are usually found in environmental sources and food (10, 11, 25).

This investigation failed to isolate *Yersinia enterocolitica*, contrarily to some authors (11, 21), who report such isolation from mild cheese and "queijo coalho" (a stiff fatty cheese made in northeast Brazil).

If we take into account the raw material (milk) used in the fabrication of cheese, there are works which relate a high incidence of *Yersinia* spp in raw and pasteurized milk (11, 24, 25), showing some correspondence with the works mentioned in this paper, as to the cheese strains isolated. Nevertheless, the *Y. frederiksenii* strain isolated in this investigation differed from those isolated by Tibana & cols. (25), suggesting that

TABLE 2 - Positivity observed in white cheese samples in relation to the presence of *Yersinia* spp.

Cheese Sample	Number of sample examined	Sample positive for *Yersinia* spp	Number of strains of *Yersinia* spp	*Yersinia* spp Biotype	*Yersinia* spp Serotype	*Yersinia* spp Phagotype
A	5	0	0	–	–	–
B	5	2	5	*Y. frederiksenii*	0: 16a, 16b	Xo
C	5	0	0	–	–	-
D	5	1	6	*Y. frederiksenii*	0: 16a, 16b	Xo
E	5	0	0	–	–	–
F	5	0	0	–	–	–
G	5	0	0	–	–	–
H	5	1	1	*Y. intermedia*	NAG*	Xo
I	5	0	0	–	–	–
J	5	0	0	–	–	–
Total	50	4	12			

*NAG = Not agglutinable.

their presence may be due to contamination during the processing of the product, while the presence of *Y. intermedia* may be due to its resistance to cheese preparation technique, since it has been previously detected in raw milk (25).

From an epidemiological point of view, besides isolating and classifying *Yersinia* spp strains it is important to determine their biological characteristics. In our study, it was not possible to detect enterotoxin ST, positive autoagglutination or calcium dependent strains. These results confirm previous reports which consider *Yersinia* spp isolated from food to be inadequate for the production of enterotoxin ST (25), although accounts were given of its production in environmental strains (10). Regarding autoagglutination and calcium ion dependence, studies suggest that these tests showed be used mainly in isolates originated from humans, involved in clinical cases.

In relation to the violet crystal binding, *Yersinia* spp tested did not have the ability to bind to the dye when incubated at 25°C and 37°C, remaining white (CV). The surface cell components (essencial polypeptides of the outer membrane proteins - OMP) are supposed to facilitate binding to the violet crystal in colonies bearing virulence plasmidium (40 - 42 MDA) when cultivated at 37°C instead of 25°C (7), showing direct corrrelation with autoagglutination and calcium dependence tests.

Another test appllied, the pyrazinamidase activity test, showed once again the characteristic of non-pathogenicity for the strains tested. All of them being positive (Pyz +); this data is in accordance to the works of Kandolo & Wauters (14), where virulence plasmidium free *Y. enterocolitica* and other strains considered "environmental" were Pyz +, also showing correlation with the previously mentioned tests.

Finally, the production of exoenzymes in the twelve strains isolated was verified, and protease activity was observed in only one *Y. frederiksenii* strain 0:16a, 16b - Xo. Lecithinase, lipase and DNAse activities have not been observed in the tested isolates. In epidemiological studies, the enzymatic profile permits the differentiation of the genus *Yersinia* spp in the potentially pathogenic and non-pathogenic strains. No correlation seems to exist between this activity and other virulence tests (8).

Thus, in the evaluation of the biological characteristics of *Yersinia* spp samples, unmistakeable efficiency is shown by new kinds of test, like the pyrazinamidase activity and violet crystal binding, as more practical and less expensive laboratorial resources to be used in the verification of *Yersinia* spp pathogenic potential, which can be applied at food analysis routine.

In what concerns the action of antimicrobials, the twelve *Yersinia* spp samples (11 *Y. frederiksenii* 0:16a, 16b - Xo and 1 *Y. intermedia* NAG-Xo) were sensitive to Norfloxacin (NOR 10), and equally resistant to Amoxacillin (AMO 10), Ampicillin (AP 10), Carbenicillin (CP 100), Cephalexin

(CF 30), Cephalothin (CF 30), Cephalothin (CF 30) and Sulphazotrim (SFT 25).

Susceptibility to other antimicrobials varied, depending on the strains studied: 10 (91%) - of the 11 *Y. frederiksenii* strains were sensitive to Nalidixic acid (AN 30), Amikacin (AMI 30), Chloramphenicol (Cl 30), Gentamicin (Gn 10), Kanamycin (KN 30), and Tobramycin (TOB 10); 9 strains (82%) were sensitive to Polymyxin B (PB 300 U); 6 strains (55%) to Cephoxithin (CT 30); 3 strains (27.3%) to Nitrofurantoin (NT 300) and Tetracycline (TT 30); and only 1 strain (9.1%) was sensitive to Colisthin (COL 10).

As for the *Y. intermedia* isolate, its behaviour towards antimicrobials may be defined as sensitive to Nalidixic Acid (AN 30), to Amikacin (AMI 30), to Cephoxithin (CT 30), to Chloramphenicol (CO 30), to Gentamicin (GN 10) to Kanamycin (KN 30) to Polymyxin B (PB 300 U), to Tetracycline (TT 30), and to Tobramicin (TOB 10), and resistant to Colisthin (COL 10) and Nitrofurantoin (NT 300).

As to the detection of the Beta-lactamase resistant strains, these findings confirm the reports made by some authors who suppose this resistance is related to the production of Beta-lactamase, according to the *Yersinia* spp strains (12,16). It should be emphasized that all the strains studied produced Beta-lactamase, what had only been previously reported to happen in *Y. frederiksenii* and *Y. intermedia* isolated from humans and animals, healthy or not, in Rio de Janeiro (18), and in *Yersinia* spp isolated from food, in the same city (25).

The present study shows that food originated *Yersinia* spp, although considered "avirulent", may be an important risk factor to the consumer due to its multiresistant behaviour towards many antimicrobials.

RESUMO

Yersinia spp: Estudo das características biológicas das amostras isoladas de queijo tipo Minas Frescal.

Foram analisadas 50 amostras de dez diferentes marcas comerciais de queijo tipo "Minas Frescal", comercializados em supermercados do Rio de Janeiro. Foram pesquisados os microrganismos indicadores de contaminação fecal pelo método do número mais provável. Os coliformes totais variaram de 2,7 x 10^3/g a 2,4 x 10^6/g e os coliformes fecais de 2,0 x 10^1/g a 2,1 x 10^6/g. Das amostras examinadas, 80% mostraram precárias condições higiênico-sanitárias, de acordo com os limites estabelecidos pelos padrões do Ministério da Saúde (DNVS).

Paralelamente, foram pesquisadas *Yersinia* spp por três diferentes metodologias. O método do pré-enriquecimento a 26°C/48 horas e posterior tratamento alcalino seguido de semeadura em agar desoxicolato citrato permitiu a identificação de 11 amostras de Y. frederiksenii - 0:16a, 16b - Xo. Pelo método do plaqueamento direto, apenas uma amostra de *Y. intermedia*-NAG-Xo no meio agar Mac Conkey; enquanto o enriquecimento a 4°C por 7 a 21 dias não permitiu o isolamento de nenhuma amostra. Essas amostras de *Yersinia* spp foram submetidas aos testes de produção de enterotoxina termoestável, autoaglutinação, cálcio-dependência, capacidade de ligação ao cristal violeta, atividade de pirazinamidase, atividade enzimática extracelular, com objetivo de avaliar o seu comportamento biológico. Todas se comportaram negativamente, com exceção de uma amostra de *Y. frederiksenii* que foi protease positiva.

Finalmente, o perfil de sensibilidade das *Yersinia* spp isoladas, mostrou uma multiresistência em relação à vários antimicrobianos (Ampicilina, Amoxacilina, Carbenicilina, Cefalexina, Cefalotina, Colistina e Sulfazotrim). Apresentaram uma melhor sensibilidade ao Ácido Nalidíxico, Amicacina, Cloranfenicol, Gentamicina, Norfloxacina, Polimixina B e Tobramicina. Todas as amostras de *Yersinia* spp isoladas no presente estudo tiveram a capacidade de produzir ß-lactamase, através do método de Nitrocefin.

Palavras-chaves: Queijo tipo Minas Frescal, características biológicas, sensibilidade a antibióticos.

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COMPARISON OF TWO PLATING MEDIA FOR THE ISOLATION OF *LISTERIA* SP FROM SOME BRAZILIAN DAIRY AND MEAT PRODUCTS

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SUMMARY

The present study deals with the comparison of two selective agars for *Listeria* sp isolation. The United States Department of Agriculture - Food Safety and Inspection Service (USDA-FSIS) methodology was used. Besides the use of lithium chloride phenylethanol moxalactam agar - LPM, recommended by USDA-FSIS, modified Vogel-Johnson agar - MVJ was used to improve the isolation rate of *Listeria*. A total of 140 samples of meat and milk products (20/product) obtained in Campinas, SP, Brazil, were examined. The results indicated the superiority of MVJ agar in promoting *L. monocytogenes* isolation and pointed out the use of more than one isolation medium to minimize false negative results.

Key Words: *Listeria*, isolation media, dairy products, meat products.

INTRODUCTION

In the last few years, some new culture media were developed for the isolation of *Listeria* from foods, as can be seen in RALOVICH (13) and in BRACKETT & BEUCHAT (2). The efficiency of these media has been compared by some researchers like BUCHANAN et alii (4); CASSIDAY et alii (5); HEISICK et alii (7); LOESSNER et alii (9); PINI & GILBERT (12); RALOVICH (13) and TRUSCOTT & MCNAB (14).

Among all proposed methods, the method known as USDA-FSIS (11) shows good results for isolation of *Listeria* from milk and meat products. The recommended isolation medium is the lithium chloride phenylethanol moxalactam agar (LPM), which must be examined under obliquely reflected light.

BUCHANAN et alii (3) developed a selective medium, modified Vogel-Johnson agar (MVJ), that eliminates the need of using reflected light. This medium is very useful for laboratories without an appropriate source of transmitted light.

The aim of the present study was to evaluate the performance of MVJ and LPM agars for detection of *Listeria* spp in some Brazilian retail level foods.

The products examined were ground beef, sausage, frankfurters, "minas frescal" cheese (a Brazilian soft cheese eaten fresh) and milk (raw plain, pasteurized plain and pasteurized 3.2% fat). Twenty samples of each type of product were investigated between January and May, 1989. Raw plain milk was obtained from a milk processing plant, from Campinas, São Paulo State, Brazil, immediately after homogenizing. The other samples

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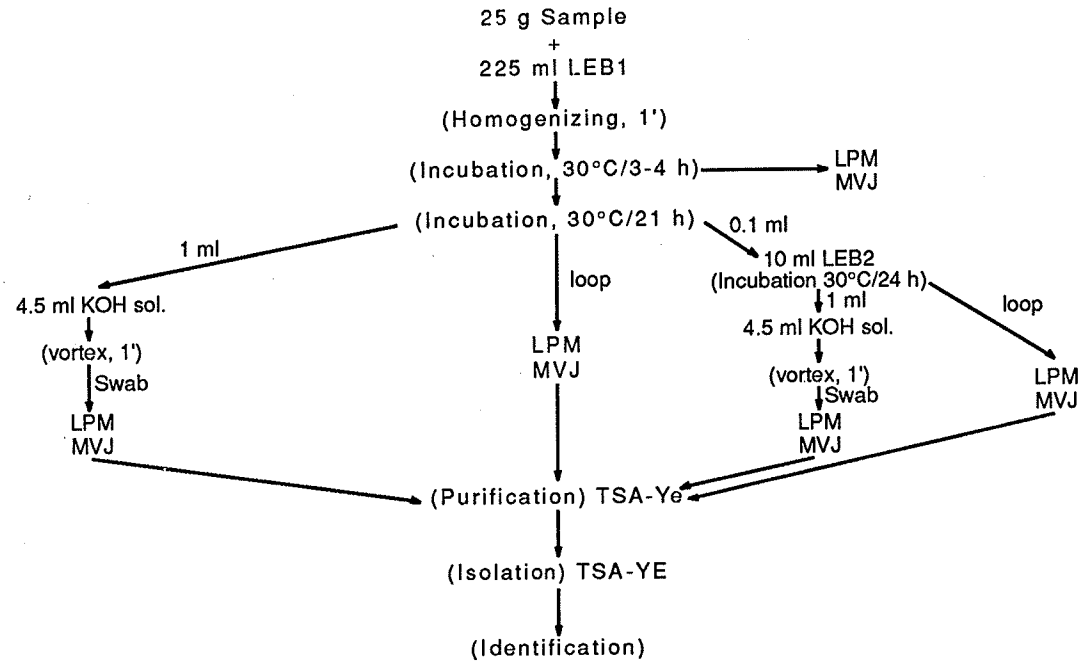

FIGURE 1 - Flow chart of the methodology used. LEB 1 and LEB 2 = primary and secondary enrichment broth; LPM = Lithium chloride phenylethanol moxalactam agar; MVJ = modified Vogel-Johnson agar; TSA-YE = tryptic soy agar supplemented with yeast extract added.

were collected in different supermarkets of the same city. The samples were transported to the laboratory in an insulated container and were examined within 2 hours.

Isolation media were prepared in the laboratory as described by LEE & MCCLAIN (8) and BUCHANAN et alii (3). The primary and secondary enrichment broth (LEB1 and LEB2), tryptic soy agar supplemented with yeast extract and other media used were prepared according to MCCLAIN & LEE (11).

As shown in figure 1, we used both LPM and MVJ agars in the USDA-FSIS methodology (11). LPM agar plates were incubated at 30°C/24-48 h and MVJ agar at 35°C/24-48 h.

After incubation, three colonies that were presumptively positive for *Listeria* sp from each LPM and MVJ agar plates were picked and streaked on tryptic soy agar with yeast extract (TSA-YE) for purification. After incubation for 24 h at 35°C, colonies with "typical" bluish color were transferred to a TSA-YE tube for further investigation. The following tests were done: tumbling motility using a phase contrast microscope; Gram stain; typical motility and indol production in SIM motility medium; catalase; oxidase; acid and H_2S) production from triple sugar iron agar. Cultures that gave typical reactions in these tests were considered as belonging to genus *Listeria*. For species identification the following tests were used: nitrite reduction; methyl red and Voges Proskauer; acid production from xylose, rhamnose, mannitol and dextrose; hemolisin production (horse blood agar and CAMP test). The mouse pathogenicity test as recommended by Lovett (10) was also done.

RESULTS AND DISCUSSION

As can be seen in Table 1, 59 (42%) samples were positive for *Listeria* spp in MVJ and LPM agars simultaneously; 5 samples (4%) were positive only on MVJ agar and 3 (2%) only on LPM agar. However, when only *L. monocytogenes* is considered (Table 2) 9 samples (6%) were positive simultaneously on both media, 32 samples (23%) were positive only on MVJ agar and 4 (3%) only on LPM agar. No difference in media selectivity was observed in the different types of food examined.

Extensive research has been done to compare the efficacy of media used in the *L. monocytogenes* recovery from various foods (1, 4, 6, 9, 14).

TABLE 1 - Recovery of *Listeria* sp from foods using two different selective agars.

Samples (20 each)	Number of positive samples (%)			
	only on MVJ*	only on LPM**	on both	Total
Ground beef	0 (-)	0 (-)	20 (100)	20 (100)
Frankfurters	1 (5)	0 (-)	16 (80)	17 (85)
Raw sausage	4 (20)	0 (-)	16 (80)	20 (100)
Raw plain milk	0 (-)	1 (5)	1 (5)	2 (10)
Pasteurized plain milk	0 (-)	0 (-)	0 (-)	0 (-)
Past. 3.2% fat milk	0 (-)	0 (-)	0 (-)	0 (-)
"Minas frescal" cheese	0 (-)	2 (10)	6 (30)	8 (40)
Total (140)	5 (4)	3 (2)	59 (42)	67 (48)

* MVJ = modified Vogel-Johnson agar
** LPM = lithium chloride phenylethanol moxalactam agar

TABLE 2 - Recovery of *Listeria monocytogenes* using two different selective agars.

Samples (20 each)	Number of positive samples (%)			
	only on MVJ*	only on LPM**	on both	Total
Ground beef	9 (45)	3 (15)	1 (5)	13 (65)
Frankfurters	9 (45)	0 (-)	5 (25)	14 (70)
Raw sausage	13 (65)	1 (5)	2 (10)	16 (80)
Raw plain milk	0 (-)	0 (-)	0 (-)	0 (-)
Pasteurized plain milk	0 (-)	0 (-)	0 (-)	0 (-)
Past. 3.2% fat milk	0 (-)	0 (-)	0 (-)	0 (-)
"Minas frescal" cheese	1 (5)	0 (-)	1 (5)	2 (10)
Total (140)	32 (23)	4 (3)	9 (6)	45 (32)

* MVJ = modified Vogel-Johnson agar
** LPM = lithium chloride phenylethanol moxalactam agar

The list of selective media for *Listeria* is extensive, but no one medium is clearly superior.

Results outlined in Table 2 suggest that MVJ agar is more effective than LPM agar because it detected *L. monocytogenes* in 91.1% of the positive samples while the recovery rate (%) for LPM agar was 28.9%.

Although LPM agar had provided a lower number of positive samples, its use was important since some samples (3 ground beef and 1 sausage) were positive to *L. monocytogenes* only on this agar (Table 2).

Our results are quite different from those of BUCHANAN et alii (4), who reported that LPM and MVJ agars were approximately equivalent with regard to recoveries. However, it must be considered that in our study, a different methodology was applied.

LOESSNER et alii (9) related that MVJ agar was found to be inhibitory to *listéria*. However, our results suggest that this can vary.

In view of the public health implications associated with *L. monocytogenes*, the results of this study suggest that two different plating media should be used to improve the isolation of this pathogen from foods. Anyway, the search for a more sensitive medium must continue.

RESUMO

Comparação de dois meios seletivos no isolamento de *Listeria* sp, a partir de alguns produtos lácteos e cárneos

Este trabalho tem por objetivo avaliar o desempenho de dois meios seletivos para o isolamento de *Listeria* e *L. monocytogenes*. Utilizou-se a metodologia recomendada pelo United States Department of Agriculture - Food Safety and Inspection Service (USDA-FSIS) que preconiza para o isolamento o ágar cloreto de lítio feniletanol moxalactam - LPM e empregou-se, concomitantemente, o ágar Vogel-Johnson modificado - MVJ. Um total de 140 amos-

tras de produtos láteos e cárneos (20 por produto), coletadas em Campinas, SP, Brasil, foram analisadas. Os resultados demonstram a superioridade do ágar MVJ no isolamento de *L. monocytogenes* e indicam a necessidade do uso de mais de um meio de isolamento a fim de minimizar a ocorrência de resultados falso negativos.

Palavras-chave: *Listeria*, meios de isolamento, produtos lácteos, produtos cárneos.

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PHOSPHORRUS UPTAKE BY MYCORRHIZAL AND NONMYCORRHIZAL *PINUS* ROOTS

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SUMMARY

Ectomycorrhizae of *Pinus caribaea* var. *hondurensis* Morelet with the fungus *Pisolithus tinctorius* (Persoon) Coker et Couch grown under two different P sources were studied, in an effort to understand the mechanism of P absorption by these symbionts. Mycorrhizal plants yielded significantly more root and top fresh and dry matter, higher Vmax and lower Km and Cmin than the nonmycorrhizal ones. The source of P did not influence significantly these parameters. The results suggest that the increased P uptake by mycorrhizal plants was due to an increase in the number of absorption sites per unit of weight of root and mainly to an increase in the affinity of these sites to P. Moreover mycorrhizal plants, opposite to nonmycorrhizal ones, seem to be able to uptake P from the soil solution when this ion is present in rather low concentrations.

Key Words: P uptake, mycorrhiza, *Pinus*, *Pisolithus*.

INTRODUCTION

The concentration of inorganic phosphate (Pi) in the soil solution of most soils around the world is usually very low and inadequate for plant growth (5). This is usually overcame by aplication of fertilizers to these soils. However, since P sources are nonrenewable, researchers are looking for more efficient plants or symbiotic associations as far as the acquisition and/or utilization of P is concerned.

Several authors have reported that micorrhizal plants are able to accumulate more P than nonmycorrhizal ones, especially when the availability of this ion is a limiting factor (10, 11). The increase in P uptake has been attributed to an increase in the surface area of the root system and, as a consequence, to an increase in the soil volume exploited (11); to an increase in the activity of extracellular acid phosphatases able to release the phosphate from organic compounds in the soil (3, 16); and to an increase in the efficiency of the uptake system localized in the fungus plasmalemma (4, 11).

The kinetics of P uptake has been studied both in endo-and ectomycorrhizae (6, 9, 12, 13, 14), with excised roots as well as with intact root systems (4). Some authors have reported a raise in the Vmax values, suggesting that the increase in P uptake would be due to an increase in the number of absorbing sites per unit area of the root (14). Others have failed to show such effects on Vmax, alternatively suggesting that there would be in increase an the affinity of the absorption sites for P(9).

The subject remains essentially controversial and more studies are recquired, with as much different mycorrhizal systems as possible, to understand this symbiotic association as far as P absorption is concerned. Therefore, the purpose of this research was to study P uptake in a mycorrhizal

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system of *Pinus caribaea* and *Pisolithus tinctorius* which has extensively been used in Brazil.

MATERIAL AND METHODS

The fungus *Pisolithus tinctorius*, isolate 185 (Pt 185) was initially grown in Petri plates containing 20 ml of a modified Merlin-Norkrans medium (15) at 25 ±1°C for 21 days. Then, in order to allow the fungus to adapt to the subsequent experimental conditions, two 6 mm discs were placed in Erlenmeyer flasks containing 125 ml of Clark's nutrient solution, pH 5.5, 1/5 of the ionic strength (8), with 1 mM P, 0.5% glucose, 50 µg thiamine, 10 µg biotin and 100 µg folic acid per liter of solution. After incubation at 25±1°C for 21 days the Erlenmeyer flask content was rinsed, homogenized with 10 ml of sterile demineralized water and used as mycelium inoculum.

Seeds of *Pinus caribaea* var. *hondurensis* surface sterilized with 30% H_2O_2 during 30 minutes and washed with demineralized water were sown in polyethylene pots with 1.6 liters of a sterilized mixture of Januaria AQ-16 soil and washed sand (3:1), v/v). Seven days after germination, plants were watered with 50 ml of the same Clark's nutrient solution above mentioned but free from P, and then every 30 days. In between, they were watered daily with demineralized water to soil field capacity. On the 30th day plants were randomly divided into 4 groups and applied the following treatments: 1) 30 µmol of NaH_2PO_4; 2) same as "1") and inoculation with Pt 185; 3) 30 µmol of $Ca_3(PO_4)_2$; 4) same as "3") and inoculation with Pt 185, all with four replicates, in order to verify whether a low solubility P salt would enhance colonization. After 6 month of growth in greenhouse, the plants were transferred to a controlled environmental room which had a day length of 16 hours with 230 $\mu E.m^{-2}.S^{-1}$, at 25±2°C. One week later, the soil-sand mixture was carefull removed under water flow, the root system rinsed with demineralized water and plants transferred to glass vessels with 500 ml of the Clark's nutrient solution, previously mentioned. Next day, this nutrient solution was changed to a 0.2 mM $CaSO_4$ solution and left for another period of 24 hr. One hour before the P uptake experiment, the solution was renewed and 10 µmoles of P as NaH_2PO_4 added. Finally, the solution was changed by another with same composition but with 5 µCi of ^{32}P ($NaH_2{}^{32}PO_4$, free from carrier) and 0.5 ml aliquots were taken during a period of time of 8 hours. The radioactivity of the samples were measured in a Beckman liquid scintillation spectrometer with open window. Kinetic constants were determined according to Claassen & Barber (7), using a graphico-mathematical method of estimation as proposed by RUIZ (18). The minimum concentration (Cmin) at which net P influx approach zero was estimated graphically from the curves. The porcentage of mycorrhizal infection was estimated according to AMBLER & YOUNG (2), after fixing in acetic acid:glycerol:water (1:2:1; v/v) (1) and staining with trypan blue in 0.05% lactophenol (17).

RESULTS AND DISCUSSION

Mycorrhizal plants yielded greater amounts of root and top fresh and dry matter than the nonmycorrhizal ones (Table 1). The P source, despite the lower solubility of $Ca_3(PO_4)_2$, did not influence the yield of fresh and dry matter by mycorrhizal and nonmycorrhizal plants.

TABLE 1 - Fresh and dry matter yield and percentage of infection of mycorrhizal and nonmycorrhizal *Pinus* grown under two P sources.

P source	Root condition	Fresh wt		Dry wt		Infection
		Root	Top	Root	Top	%
NaH_2PO_4	nonmycorrhizal	8.15b	5.53b	1.82b	1.85b	ND
	mycorrhizal	12.30a	9.68a	3.65a	3.38a	87
$Ca_3(PO_4)_2$	nonmycorrhizal	7.23b	7.23b	1.70b	2.35b	ND
	mycorrhizal	12.55a	9.25a	3.90a	3.09a	85

* Means followed by the same letter within a column are not statistically different at P=0.05 by Scott-knott mean test; ND = not detected.

Mycelia of Pt 185 were obsorved only on the roots of the inoculated plants and the mean percentage of infection was 86%. No single root system of these plants exhibited less than 80% infection.

Mycorrhizal plants depleted P of the absorption solution at higher rates than did the nonmycorrhizal ones (Figure 1). Mycorrhizal plants absorved about 50% of the available P during a period of one hour while nonmycorrhizal absorved only about 30% after 8 hours. Results like that were expected since mycorrhizal plants developed a greater root system and therefore had a greater root surface area of absorption. The top was also greater and therefore the plant had a greater demand for essential nutrients.

The higher P uptake by mycorrhizal plants was also evident in per unit of weight basis (Table 2). Independently of the P source, mycorrhizal roots exhibited greater values of Vmax than the nonmycorrhizal ones. Similar results have been found in mycorrhizal excised roots of *Fagus sylvatic*a (12, 13) and *Pinus radiat*a (6), taken from the field. This enhanced P uptake by mycorrhizal roots, however, is still a matter of controversy. Most of the time, this has been attributed to an increase in absorptive area provided by the mycelia outside the root (5, 10, 11). In fact, it has been calculated that ectomycorrhizal fungi may increase

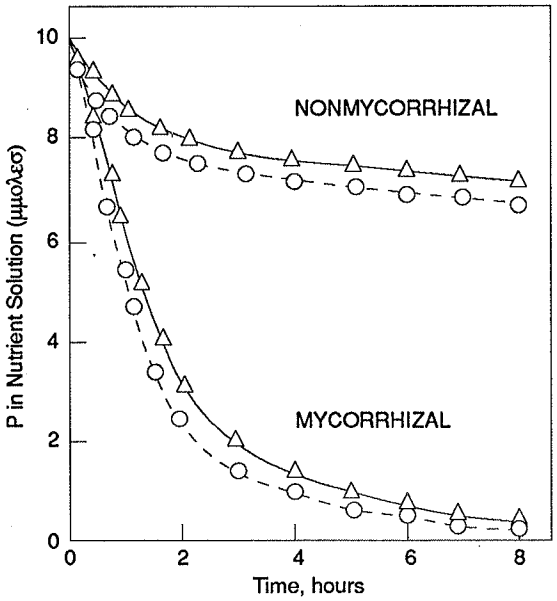

FIGURE 1 - Depletion of P from absorption solution by mycorrhizal and nonmycorrhizal roots grown in the presence of NaH_2PO_4 (—△—) and $Ca_3(PO_4)_2$ (- - ○ - -).

TABLE 2 - Kinetic constants of phosphorus uptake by mycorrhized and nonmycorrhized *Pinus* plants grown *under* two P sources.

P source	Root condition	Vmax	Km	Cmin
		μmol/hr.g Fw	μM	μM
NaH_2PO_4	nonmycorrhizal	0.23b	16.44a	11.98a
	mycorrhizal	0.30a	3.89b	0.32b
$Ca_3(PO_4)_2$	nonmycorrhizal	0.21b	17.00a	12.65a
	mycorrhizal	0.27a	4.26b	0.42b

* Means followed by the same letter within a column are not statistically different at P = 0.05 by Scott-knott mean test.

the effective absorbing surface area as much as a thousand times (11). Despite the morphological differences between ecto- and endomycorrhizae, an increase in Vmax values was also found in soybean infected with *Glomus mosseae* indicating an effective increase in the number of uptake sites per unit surface area of root system (14).

In our study with intact ectomycorrhizal root systems, besides an increase on Vmax, it was also observed a decrease in Km and in Cmin. So, mycorrhizal *Pinus* plants not only have a greater absorbing surface area but they also have a greater affinity for P and increased ability to uptake this ion at at very low concentration. Similarly, Cress et alii (9) also found a decrease in Km in mycorrhizal tomato roots and argued that probably the most important effect of mycorrhization would be an increase in the affinity of the absorbing sites for P.

Thus, *Pinus* plants mycorrhized with *Pisolithus Linctorius*, besides having a greater number of absorbing sites, are more efficient in the P uptake and will grow better in most soils throughout the world than the nonmycorrhizal ones.

RESUMO

Absorção de P por raízes micorrizadas e não micorrizadas de *Pinus*

Ectomicorrizas de *Pinus caribae* var. *hondurensis* com o fungo *Pisolithus tinctorius*, tratadas com duas diferentes fontes de P, foram estudadas quanto ao mecanismo de absorção deste íon. As plantas micorrizadas produziram significativamente mais matéria fresca e seca de raiz e parte aérea apresentaram maiores valores de Vmax e menores valores de Km e de Cmin do que as não

micorrizadas. A fonte de P não influenciou significativamente estes parâmetros. Os resultados sugerem que o aumento na absorção de P foi devido a um aumento no número de sítios de absorção por unidade de peso radicular e a um aumento na afinidade destes sítios por P. Além disso as plantas micorrizadas se mostraram capazes de absorver P da solução do solo mesmo quando a concentração deste íon era extremamente baixa.

Palavras-chave: Absorção de P. micorrizadas, *Pinus, Pisolithus.*

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PLANT GROWTH PROMOTING RHIZOBACTERIA AND THEIR EFFECT ON RAPESSEED (*BRASSICA NAPUS L.*) AND POTATO (*SOLANUM TUBEROSUM L.*) SEEDLINGS

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SUMMARY

The increasing awareness of the detrimental effects of pesticides on the environment has renewed interest in the use of bacteria as inoculants to increase plant growth and to control plant pathogens.

In this research native bacterial strains were isolated and selected from roots of potato (*Solanum tuberosum L.*) and rapeseed (*Brassica napus L.*). The main objective was to obtain and to assay plant growth promoting rhizobacteria for these two crops. Potato true seeds and rapeseed were inoculated with the isolated bacterial strains in pots under controlled environmental conditions. Twelve bacterial strains that promoted growth were selected and studied in relation to: 1) the *in vitro* inhibition of a number of important plant pathogens, 2) their perfomance as biological inoculants under field conditions.

Results indicated that it is possible to isolate and select native strains of plant growth promoting rhizobacteria with inhibitory activity toward important plant pathogenic microorganisms such as *E. carotovora, P. solanacearum, S. scabies* and *R. solani.* The selected PGPR might be used as biological inoculants on rapeseed and potato tubers and effectively increase plant growth under field conditions.

Key Words: Plant pathogen, rhizobacteria, inhibition, *Solanum tuberosum, Brassica napus.*

INTRODUCTION

Recent studies have shown that the application of bacterial inoculants to seeds may increase yield in some important crops. These studies indicate that selected bacteria inoculated on plant seeds act as growth promoters. These reports also demonstrate that the main source of these bacteria is the soil (12, 24).

Field experiments recently conducted with potato tubers and seeds of other crops showed that inoculation with selected bacteria can increase plant productivity by 30% when compared with non-inoculated treatments (15). Today some new commercial products are being offered to farmers which are made up with specially selected soil microorganisms. These biological products are oriented toward the control of plant pathogens and enhancement of plant growth (20, 22).

Plant growth promoting rhizobacteria (PGPR) act through a competitive mechanism with undesired soil and rhizosphere microflora (2). This competitive ability allows the establishment of PGPR in the root microenvironment and induces

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the inhibition of plant pathogens, allowing a better plant growth (14, 22). However, a limiting factor for constructing these bacterial inoculants is the access to specific and efficient native strains adapted to the particular agroclimatic systems in which they will be used(6).

The main objectives of this study were to isolate and select native strains potentially useful for plant growth of rapeseed (*Brassica napus*) and potato (*Solanum tuberosum*), and to study the *in vitro* inhibition of selected strains toward several plant pathogens. Inoculation tests in pots were included to determine the performance of superior PGPR strains for dry matter yield.

MATERIAL AND METHODS

Isolation of green fluorescent bacteria from plant material - Plant material for bacterial isolation was obtained from the rhizosphere of randomly selected growing plants at the Santa Rosa Experimental Station of the Austral University, Province of Valdivia. Other plant samples were gathered from field crops growing around the city of Paillaco, Province of Valdivia. Bacteria were selected from rhizosphere, rhizoplane and inner tissue of potato and rapeseed samples.

Isolation of bacterial cultures from rapeseed plants was conducted taking drops from 2 g of homogenized soil in sterile water and plated on King's Medium B (KB) (21). Also, cut roots 3 cm long were immersed in sterile distiled water, vigorously shaken and placed on top of KB agar plates. From larger roots, samples were taken directly from vascular tissue and streaked immediately on KB plates. For the isolation of bacteria from potato tubers the following samples were used: a) normal potato skin, b) inner potato tissue, and c) inner potato tissue of potato tubers disinfected 10 mins previously with 5% sodium hypochlorite. From all these potato and rapeseed samples, isolations were directed toward the selection of green pseudomonads, fluorescent under UV light on KB agar. Plates were incubated at 28°C during 24 a 48 h, storing pure cultures at 4°C.

Selection of plant growth promoting rhizobacteria (PGPR's) - Selection of PGPR's was conducted in several phases that included: climatic growth chamber tests with the isolates and assays to determine antagonistic capabilities toward plant pathogens.

Climatic growth chamber assays - All fluorescent and a few non-fluorescent isolates were tested to select and to establish their potential as PGPR. Thirty six other bacterial isolates included in the culture collection of the Plant Biotechnology Laboratory of the Austral University of Chile, were also tested. This collection was gathered from several plant samples forwarded to the laboratory and is made up of fluorescent and saprophytic isolates. All the bacterial isolates were used in pot assays conducted in climatic chambers.

The effect of the isolates on the promotion of plant growth was studied in pots filled 10 cm deep with a organic-rich soil, adding 5 g/Kg of soil of calcium in order to reach the pH near 6.0. Temperature inside teh chambers was 12-15°C with a photoperiod of 14h light and 10h darkness. Rapeseed seedlings (cv. Norin 16) and true potato seed (TPS) were used in the tests. TPS dormancy was interrupted treating seeds with giberellic acid 1,500 ppm (19). Seed germination was determined before planting (17).

Twenty five TP and rape seeds, surface sterilized with 5% w/v sodium hypochlorite, were used per pot. After washing and the excess water drained, the seeds were immersed, for each isolate, for 30 m in a 24h old bacterial suspension of 1 x 109 cfu/ml (O. D. 1.0= 600-um) (3). Three pots were used for each isolate tested. Non-inouclated controls were also used and considered of 5 pots immersed in sterille water.

At the period of four weeks after plant emergence, the fresh and dry matter weight and height of the seedlings were determined. Inoculated treatments that showed higher yield were characterized as due to Plant Growth Promoting Rhizobacteria (PGPR). Statistical differences were determined between treated plants and non-inoculated controls by means of ANOVA and t tests.

Determination of bacterial antagonism under in vitro *conditions* - The inhibition spectrum of selected strains characterized as PGPR's was determined on the following plant pathogens: *P. fluorescens* biotype II (*P. marginalis*), *P. syringae*, *P. solanacearum* (Race 3), *E. carotovora* subspecie *carotovora* and *atroseptica*, *Bacillus* spp, (pectolytic), *S. scabies*, *R. solani* and *Botrytis cinerea* (Table 1). Each PGPR isolate was grown on KB and incubated for 24 h at 28°C. To each plate 1 ml of chloroform was added and kept closed for 1h by exposing the colonies to the fumes. When all the fumes were exhausted, a second layer of soft agar (40°C) was added on top of the agar which contained the plant pathogenic bacteria to test for inhibition. The plates were incubated at 28°C for 24 h.

The plant pathogenic fungi were tested for inhibition as follows: four PGPR's were grown separately on edges of PDA plates during 48h at 28°C, then, in the centre of the plate, was placed a disc of agar containing mycelia of each fungus isolate. In all cases, clear zones of inhibition or growth retardance were considered to be an inhibition effect.

The effect of cellular filtrates of 6 PGPR on 3 isolates of Race 3 of *P. solanacearum* was determined as follow: PGPR isolates I19, P22, P27, P25, P13B and P21A, were grown on casaminoacids broth (CAA) (21) at 180 rpm during 24 h at 24°C. Later, 2ml of sterile filtered broth (Millipore filters 0,22 um) were obtained for each isolate and stored inside sterile test tubes. A *P. solanacearum* suspension (O.D. 1.0 = 600 nm) was prepared and 0.15ml was placed in test tubes containing 4ml of CAA peptone glucose medium (21). To the test tubes, increasing volumes of filtrates from the PGPR's were added. The concentrations were: 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 ml per test tube. Finally the tubes were incubated at 28°C and growth of *P. solanacearum* was detected with a spectrophotometer at 600nm. Tubes with medium free of bacteria and active growing cells of *P. solanacearum* were used as controls.

Bacterial inoculants prepared with selected PGPR's - The same isolates used in the previous experiments were field tested on inoculated seeds. Each isolate was grown on KB medium, and inoculated into casaminoacid broth and cultured at 180 rpm during 24h at 28°C. The broth was centrifuged at 8000 rpm for 15m and re-suspended in a lower volume to reach an O.D. of 1.8 at 600nm. Rapeseed cv. Rapanui and potato tubers cv. Desiree were coated with equal volumes of a bacterial suspension and arabic gum. The seeds were finally covered with $CaCO_3$. Seeds coated with casaminoacids alone were used as non-inoculated treatments.

Bags of 2Kg each containing a organic-rich soil were used as cell carriers. Pure cultures of PGPR isolates were inoculated alone and in equal proportions as combined mixtures. From a 24 h CAA broth culture each isolate was re-inoculated in a fresh CAA broth and incubated at 28°C for 24 h at 180 rpm. To each soil bag was added 1.5 litres of bacterial suspension.

In September 1989 a field experiment was conducted at the "Carillanca" Experimental Station (INIA-Temuco). Spring rapeseed was sown on top of carrier inoculated with selected with selected isolates 128, P22 and P27. These isolates proved superior as plant growth promoters to controls in pots experiments. Treatments with proportional mixtures of isolates were also used. Two treatments of carrier inoculated with casaminoacids alone and seed alone were used as controls. A randomized block was designed to analyse data from the field experiment. Seeds were continuously delivered to the 5 m long furrows, with two levels of fertilization: normal (150 mgN/kg, 160 mgP/kg, 100 mgK/kg) and a 75% lower than normal. Two months after emergence, plants from each treatment were evaluated, recording their height and fresh and dry matter weights.

Another experiment was conducted in November 1989 at the Austral University of Chile "Santa Rosa" Experimental Station. By using the same methods described above, potato tubers and rapeseed were used to test 12 PGPR isolates in the field. A complete randomized block was used for each crop at normal fertilization (16, 20). Ten tubers per 3m row repeated three times were used for the potato experiment. All plants were tested 30 days after emergence, a second test was done in rapeseed after flowering, and for potato at harvest. In all case measurements of height and weight were recorded and data statistically analysed using ANOVA and t test.

RESULTS

Isolation of green fluorescent bacteria – Sixty nine isolates were obtained from the rhizosphere and plant material of rapeseed and potato. Table 2 shows that 28 isolates are related to rapeseed and 41 to potato. In both cases, most of the isolates were obtained from soil. All these isolates plus 36 others included in the strain collection of the Biotechnology Laboratory were tested for selection of PGPR's.

Selection of Plant Growth Promoting Rhizobacteria (PGPR) - The effect of 67 bacterial isolates on potato and rapeseed seedlings was not homogeneous. Recorded values such as plant height, plant number, dry matter per pot were variable. Inoculated potato seedlings showed highly significant differences when compared to the non-inoculated treatment. On the other hand, 26.9% of the rapeseed treatments showed the same effect. These results are presented in Figures 1 and 2 for potato and rapeseed respectively.

Sixty seven percent of the isolates tested

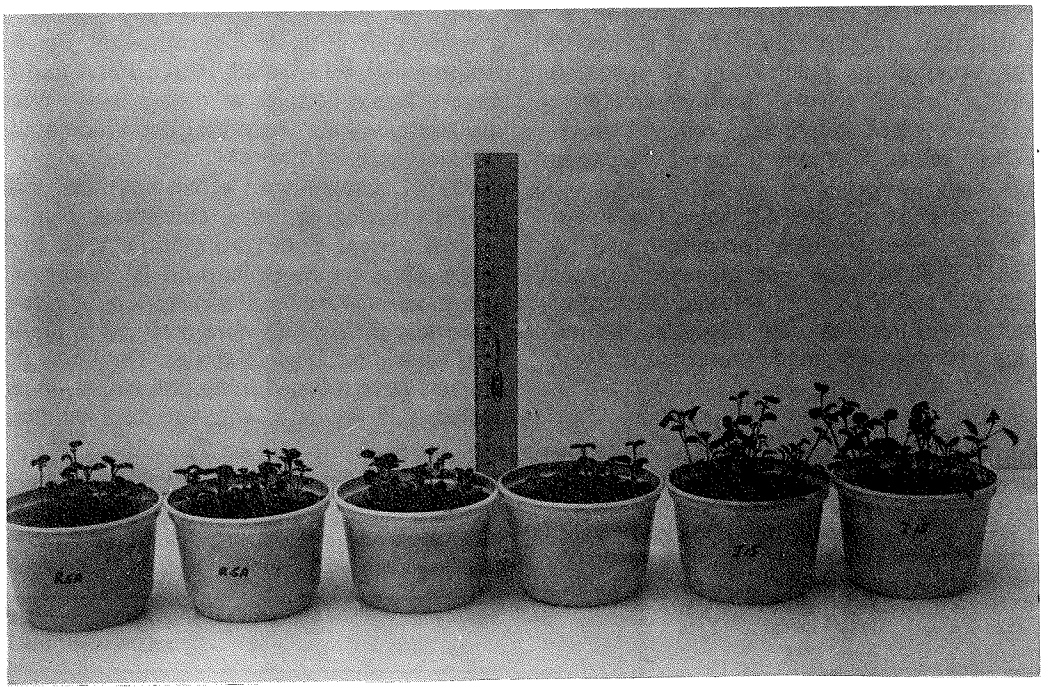

FIGURE 1 - Effect of the bacterial inoculation on potato plantlets in the selection process of plant growth promoting bacteria. The central pots are non inoculated controls, the pots to the left are inoculated with the strain R5A and the pots of the right strain I15.

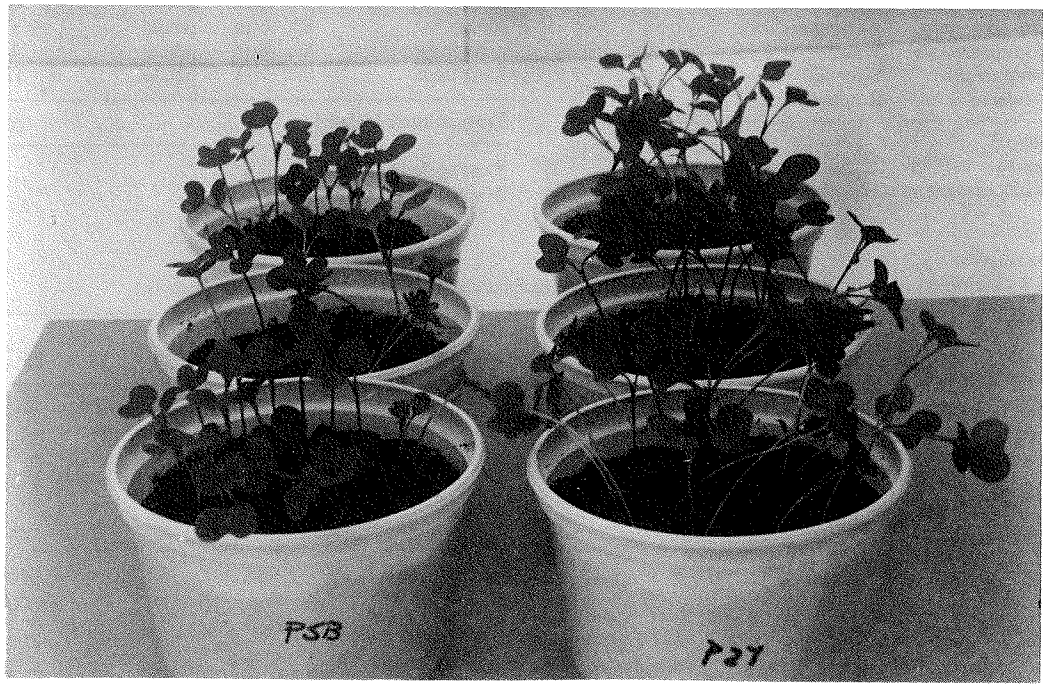

FIGURE 2 - Effect of bacterial inoculation on rapeseed plantlets in the selection process of plant growth promoting bacteria. The pots to the left were inoculated with the strain P5B with similar growth to the control. The pots of the right were inoculated with strain P27 presented a higher plant growth.

had no statistical effect on the yield of the tested seedlings growing in pots. Only in rapeseed 4,5% of the isolates showed detrimental effects such us lower plant height and reduced emergence. This effect was not detected in potato seedlings.

From the above results 12 isolates were selected, 6 for rapeseed and 6 for potato, and labelled as PGPR isolates. For rapeseed isolates P27, p22, 128 and R5A were selected because of their better performance than controls. Isolates R11C and 119 were selected because they showed a marked effect on seed germination. For potato, isolates 17B, P13B, P25, P28C, P21A and P28B, were selected because of their positive effect on dry matter values of seedlings and increased rates of emergence.

The antagonistic effect of the selected PGPR's under *in vitro* conditions was tested against several plant pathogenic agents. In Table 3 the effect on the isolates on 19 strains of *E. c.* subsp *carotovora* and *atroseptica* is presented. Important differences were detected in the inhibitory effects on *Erwinia* strains. Only two PGPR's isolates have a broad inhibition action, especially isolate P25 which is able to cause inhibition of

TABLE 1 - Some chemical constituents of the essencial oils studied.

Strains	Isolated from	Nº os strains tested
P. fluorescens1	Potato tuber	6
P. fluorescens	Potato tuber	14
P. marginalis	Potato tuber	1
P. marginalis	Sugar beet	1
P. syringae	Wheat	1
P. solanacearum2	Potato tuber	3
Bacillus sp.	Potato tuber	1
Streptomyces scabies	Potato tuber	1
E. c. subsp3 carotovora	Potato tuber	11
E. c. subsp atroseptica	Potato tuber	9
E. c. subsp atroseptica	Asparagus	1
E. c. subsp carotovora	Weath	1
Rhizoctonia solani	Sclerotia	1
Botrytis cinerea	Raspberry	1

1 = biotipe II
2 = Race 3
3 = Erwinia carotovora subspecie

TABLE 2 - Strains of green fluorescent bacteria isolated from materials associated rapeseed (*Brassica napus* L.) and potato (*Solanum tuberosum* L.).

Sample	Locality	Associated plant material	Nº strains collected
soil1	Paillaco	rapeseed	15
soil and roots	Paillaco	rapeseed	6
root	Paillaco	rapeseed	2
lateral root	Paillaco	rapeseed	5
soil2	Sta. Rosa	potato	21
roots	Sta. Rosa	potato	7
non-washed roots	Sta. Rosa	potato	1
inner tissue	Sta. Rosa	potato	12
Total cultures isolated			69

1 = rapeseed as previous crop
2 = potato as previous crop

TABLE 3 - Inhibition spectrum of 12 plant growth promoting rhizobacteria (PGPR) on 19 strains of *Erwinia*.

PGPB (1)	Strains of Erwinia carotovora																	
	6	5	7	9	10	11	12	13	14	15	18	19	20	22	23	24	25	26
D8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
I19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R5A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R11C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P22	–	–	+	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–
P27	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P25	+	+	+	+	+	–	–	+	+	+	+	–	+	+	+	+	+	+
P21A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P28B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P28C	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
P13B	–	–	+	+	+	–	+	+	–	–	–	–	–	+	–	–	–	+
17B	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–

(1) 19 strains in both subespecies of Erwinia
+ = Inhibition
- = No inhibition

16 strains of *Erwinia*. Table 4 illustrates the inhibitory effect of the 12 PGPR isolates against other plant pathogenic agents. Plant pathogenic fungi are not most sensitive and are affected by all isolates. Isolate P25 has a broad inhibition action followed by isolates P22, P27 and P13B respectively. *S. scabies* is the most sensitive of the pathogens tested and is inhibited by all PGPR's. Among the *P. fluorescens* biotype II (*P. marginalis*) strains the response was erratic, and isolate D10 was the most effective. Table 5 presents the effect of the 12 PGPR's isolates on *P. solanacearum*, the bacterial wilt agent. The only isolate that did not cause inhibition was the non-fluorescent isolate 17B.

The inhibitory effect on the growth of 3 strains of *P. solanacearum* was studied with cell-free filtrate at pH 7.0. The filtrate was obtained from six selected PGPR's. Lower concentrations of a fresh culture of PGPR may inhibit strain C at high bacterial concentration. At 24h, is possible to detect minor absorbance when the concentration of PGPR increases. This effect is more notable after 48h of incubation. These results confirmed the inhibitory effects observed in Petri dishes.

Effect of PGPR's on potato and rapeseeds plants growing under field conditions - Seeds inoculated with different PGPR's sown at the Carillanca Experimental Station, showed visual differences at the early stages of plant growth when the plots were examined two months after planting. Statistically significant differences were found in dry matter content at both fertilization levels.

Significant differences (LSD 1%) were found between single bacterial treatments and the controls or mixed treatments at normal fertilization state. However, at 75% below normal fertilization, differences were found only between mixed and soil carrier treatments. The non-inoculated treatment was only better than the unibacterial treatments when a 75% fertilization was used.

In rapeseed and potato assays in the Santa Rosa Station (Valdivia), no statistical differences were found when the treatments were analysed as a whole. In rapeseed, the values obtained for some of the treatments were greater than the control

TABLE 4 - Inhibition effects on plant pathogens by 12 plant growth promoting rizobacteria strains (1).

	Plant growth promoting rhizobacteria (PGPR)											
Plant Pathogens (1)	I28	I19	P22	P27	R5A	R11C	P13B	P25	P28B	P28C	P21A	17B
Bacillus spp	–	–	–	–	–	–	+	+	+	+	+	–
*P. fluorescens** 3	–	–	+	–	–	–	–	–	–	–	–	–
P. fluorescens 10	–	+	–	+	–	–	–	–	–	–	–	–
P. fluorescens I22	–	+	+	–	+	+	+	–	–	+	–	–
P. fluorescens I23	–	–	–	+	–	–	+	+	–	–	–	–
P. fluorescens I24	–	+	–	–	–	–	+	+	+	–	–	+
P. fluorescens I39	–	–	–	–	–	–	+	–	–	–	–	–
P. fluorescens D9	–	–	+	+	–	+	–	+	–	–	+	–
P. fluorescens D10	+	+	+	+	+	+	+	+	–	–	+	–
P. fluorescens D11	–	–	–	–	+	+	–	–	–	–	–	–
P. fluorescens D17	–	–	+	+	+	+	–	+	+	–	–	+
P. fluorescens D32	–	–	–	–	–	–	–	+	–	–	–	–
P. fluorescens D34	–	–	–	–	–	–	–	+	+	–	+	–
P. fluorescens D35	–	–	+	–	+	–	+	–	–	–	–	–
P. fluorescens D36	–	–	–	+	–	–	–	+	–	–	–	–
P. fluorescens D37	–	–	–	–	–	–	–	+	–	–	–	–
P. fluorescens D38	–	–	–	–	–	–	+	–	–	–	–	–
P. syringae	–	–	+	–	–	–	–	+	–	–	–	+
S. scabies	+	+	+	+	+	+	+	+	+	+	+	+
R. solani R1	–	–	+	+	+	+	–	+	+	+	+	–
R. solani R4	+	–	+	+	+	–	+	+	+	+	+	–
R. solani R9	+	+	+	+	–	+	+	+	+	+	+	–
R. solani R10	–	+	+	+	+	+	+	+	+	+	+	–
Bitrytis spp	+	–	+	+	+	–	+	+	+	+	+	–

(1) Phytobacteriology Institute Collection
+ = Inhibition
- = No inhibition
* = *Pseudomonas fluorescens* biotipe II

(strains P27 and R11C). In potato, the matter of all treatments was more homogeneous and superior to the control in net values. However, no significant differences were found between PGPR-inoculated and control treatments.

DISCUSSION

From different samples of soil and plant material obtained from potato and rapeseed plants it was possible to isolate bacterial strains with potential to be used as inoculants to promote plant growth. The results indicated that these bacteria are normal inhabitants of soil and confirm reports from other countries, indicating that these bacteria are distributed world-wide, a very important and interesting characteristic of the PGPR (16, 22).

30% of the isolates studied in the selection assays showed to be PGPR's and only 4.5% induced some kind of detrimental effect on plant growth of rapeseed treated seeds. This effect was not detected in treated potato seeds. These results strongly suggest that PGPR from soil and plant samples need specific tests and procedures in order to isolate them and finally to use them as specific inoculants.

The experience gathered from this research suggests a great variability among strains of the same taxonomic group, especially among the green fluorescent *Pseudomonas*. This reality is demonstrated by the variable responses of the plants after bacterial inoculation. Therefore, the selection of a PGPR is a complex process in which only a very low percentage of the microbial population studied can be used as a potential agricultural inoculant.

The search for isolates from the *Pseudomonas* was not arbitrary. This bacterial genus has been extensively studied and among its advantages are: a) they are strong and versatile bacteria from a nutritional point of view, and this characteristic makes them very active and competitive in different environments (1, 24); b) they produce important antibacterial and antifungal compounds, such as antibiotics or bacteriocins (4); and c) some strains produce iron chelating compounds, this enables them to produce an indirect control over other undesidered microorganisms (11).

Two thirds of the strains isolated and later studied did not produce effects on any of the plants tested, neither were any statiscally significant differences found from the control treatments. These results confirm other reports where only 10 to 20% of the total bacterial isolates from the rhizosphere present potential as possible PGPR's (2, 8, 16).

The absence of phytopathogenic strains from the isolations suggests agreement with other reports (18, 24), that the rhizobacterias are primarily saprophytic. This characteristic makes them and ideal group to be studied as potential agents for biocontrol purposes. However, any isolate that is potentially usable as PGPR must be thoroughly studied and its possible pathogenic ability must be tested on several plant species.

TABLE 5 - Inhibition of three strains of *Pseudomonas solanacearum* Race 3(1) induced by 12 plant growth promoting bacteria (PGPB).

	Strains of *P. solanacearum* (1)		
PGPB	C	B	D
I28	+	+	+
P22	+	+	+
17B	-	-	-
P27	+	+	+
R11C	+	+	+
R5A	+	+	+
P21A	+	+	+
I29	+	+	+
P25	+	+	+
P13B	+	+	+
P28B	+	+	+
P28C	+	+	+

(1) Phytobacteriology Institute Collection
+ = Inhibition
- = No inhibition

TABLE 6 - Effect of plant growth promoting rhizobacteria isolates I28, P22 and P27 on rapeseed plants cultivated at the Estación Experimental Carillanca (INIA-Temuco), grown at two fertilization levels.

Treatments	Dry matter (g/pot) and level of significance	
	Fertilization rate	
	Normal	75% Normal
I28	119.87 **	68.20 ns
P22	92.88 **	54.03 ns
P27	87.39 **	68.24 ns
Mixture (1)	77.42 ns	75.19 **
Control (2)	77.38 ns	80.22 **
Control (3)	78.03	73. 69

LSD* = significative to 5%
ns = non significative
(1) = same concentration of strains I28, P22 and P27
(2) = inoculated with soil and CAA culture medium, without bacteria
(3) = non inoculated, normal sowing

Due to the great microbial diversity found in rhizosphere samples the broadening of the spectrum of study to other genera beside *P. fluorescens* can be justified only if the possibilities to find other microorganisms potentially useful as biological control agents are real (1, 2, 7).

From our results it is possible to conclude that among the PGPR's studied only two or these may have a broad inhibition spectrum under *in vitro* conditions toward the plant pathogens used. This indicates that most strains which cause broad inhibition are found in low numbers, and to detect this particular isolate requires complex procedures. Other authors (1, 22) found similar results, by studying larger number of rhizobacteria and finding only 1 to 4% among them to be potentially superior PGPR's.

Among the plant pathogens the fungi were more sensitive to inhibition than bacteria. The bacteria pathogens, especially the green fluorescent pseudomonads were not greatly affected. Probably this latter group has similar competition mechanisms of the PGPR's tested. These results agree with other reports (20) where the inhibition reported was greater in fungi, especially in isolates of *R. solani, Pythium* spp., *Gaeumannomyces* spp, and *Phytophthora*. This affirmation is more clear when the sensitivity of pathogenic *Pseudomonas marginalis* is compared with the response observed on isolates of the genus *Erwinia* (9, 13, 25).

The pathogenic-opportunistic *P. fluorescens* biotype II isolates tested in this study were not strongly affected by the PGPR's. This show the need and importance of obtaining other different isolates which have more efficient and diverse mechanism of biological action, such as production of antibiotics, bacteriocins, siderophoses or other similar substances. This action should be extended toward the plant pathogenic group *P. fluorescens* since around 10% of the fluorescent pseudomonads can cause damage to several crops including potato, rapeseed and wheat (5).

The PGPR's caused inhibition to about six of the plant pathogens tested, but inhibition was not even. This possibly indicates that the inclusion of a mixture of strains in seed coatings will improve the biological control action by broadening the spectrum of inhibition.

The potential of the PGPR's as biological control agents is evidenced by their ability to *in vitro* inhibit important plant pathogens as *P. solanacearum* and both subspecies of *E. carotovora*. These species cause bacterial wilt and soft rot respectively and are responsible for important agricultural losses in our country and in the rest of the world as well. To date no chemical control are available (7, 13, 14). This potential is shown also by the great sensibility of *R. solani* and *S. scabies* strains to the pathogens responsible for black scurf and common scab to our PGPR. Both cause yield damage in our country and other places of the world, primarily to potato.

Field assays conducted with the PGPR's showed promising results since statistically significant difference were obtained in rapeseed plants, grown from inoculated seeds. This difference was initially observed during the first stages of plant development but only when a rich soil was used as cell carrier. However, when bacteria are directly coated on seeds this difference was not detected. This effect could be due to a higher death rate of the inocula on the seed surface prior the rot emergence, when the PGPR's have to compete with native strains and in the precence of seed toxins.

The soil-inoculated plants presented larger differences in relation to control treatments at the flowering stage probably due to a higher amount of fertilizer and a better plant growth. Kloepper et al (12) pointed out significant differences between several carriers used and rapeseed varieties. These authors and others (10) also indicated, that the enhance in plant growth is maximized only under favourable agricultural conditions.

The results allow the conclusion that it is feasible to establish a future program to use PGPR's to increase plant growth in rapeseed. The inoculum production of selected PGPR for field conditions can be achieved by using the procedures and technology available today for seed inoculation. Similar results were presented by other reports for other crops such as sugar beet and radish (15, 23), confirming the potential of PGPR as an alternative to increase plant growth.

In potato, in spite of having higher growth values than the control, it is not possible at this point to confirm that seed tuber inoculation may increase yield. This is also demonstrated by the statistical analysis, which showed no differences. However, in pot assays, PGPR - inoculated treatments showed significant growth improvements over the control.

We believe that it will be necessary to conduct new assays using a variety of seed carriers to evaluate the beneficial effect of these bacteria especially under field conditions. Indeed, the enhance of plant growth observed in the pots, with true potato seeds is an important fact. The use of bacterial coatings may have important implica-

tions in countries that use their own potato seeds. This also could allow not only more vigorous plants to grow in nurseries but to have seedlings bioprotected from damage of plant pathogens under controlled conditions.

RESUMO

Bactérias produtoras do crescimento vegetal e seus efeitos sobre a batata (*Solanum tuberosum*) e colza (*Brassica napus*).

O uso dos inoculantes bacterianos como agentes de controle biológico ou promotores do crescimento vegetal é uma área de pesquisa que tem sido desenvolvida como alternativa aos pesticidas químicos.

Neste estudo isolaram-se cepas de bactérias nativas com o objetivo de obter cepas promotoras específicas do crescimento vegetal da batata (*Solanum tuberosum* L.) e da colza (*Brassica napus* L.). Inocularam-se sementes botânicas de colza e batata utilizando cepas isoladas e dispostas em macetas em condições controladas. Selecionaram-se doze cepas de bactérias que foram estudadas em relação a:

1) Capacidade de inhibição "in vitro" de alguns agentes patógenos, e 2) sua capacidade de estimular o crescimento em experimentos no campo.

Os resultados permitem concluir que é possível obter cepas promotoras do crescimento vegetal, as quais possuem a capacidade de inibir agentes patógenos tais como *E. carotovora, P. solanacearum, S. scabies* e *R. solani.* As bactérias promotoras do crescimento vegetal podem ser inoculantes biológicos potenciais em culturas de colza e batata, que promovem efetivamente um maior crescimento vegetal além de proteger as sementes do ataques dos agentes fitopatógenos.

Palavras-chave: fitopatógenos, rizobactéria, *Solanum tuberosum, Brassica napus.*

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ON THE INTERACTION OF SOME MESOIONIC COMPOUNDS WITH *SACCHAROMYCES CEREVISIAE* BY BIOLOGICAL MICROCALORIMETRY

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SUMMARY

The sensitivity of *Saccharomyces cerevisiae* towards mesoionic compounds of types 1,3,4-thiadiazolium-2-aminide and 1,3,4-triazolium-2-thiolate has been evaluated by biological microcalorimetry and the results have been compared with those obtained by the classical method of agar diffusion. Both methods have yielded a similar biological response though the former is shown to be a rapid and precise method which can also indicate differing modes of action of the studied compounds.

Key Words: *Saccharomyces cerevisiae*, biological microcalorimetry, agar plate diffusion, mesoionic compounds.

INTRODUCTION

The heat effects produced by all microbial metabolic processes taking place in a culture can be measured by microcalorimetric techniques, methods which have been developed over the last 15 years (3, 8, 13).

Cultures of metabolizing yeast cells, under growing and non-growing conditions, produce heat output profiles (thermograms) which are often shown as power-time curves (dQ/dt versus time).

These profiles are altered when substances interfering with the metabolism of the yeast are added to the culture. The simplest case of microbial metabolism to describe in terms of heat production is metabolism without growth. This can be achieved with microbial cells suspended in buffered glucose. Under these conditions, the heat changes detected by flow microcalorimetry are believed to result solely from the respiration of the cells and a curve, similar to that produced from an enzyme-substrate reaction (zero-order process) is obtained, as shown in figure 2. These curves (dQ/dt vs. time) show no return of the thermogram to the steady baseline level due to continuing respiration of yeast cells.

Studies of dose/response data evaluated by biological microcalorimetry have been done by Beezer and co-workers since 1977 (2 to 9, 12, 13).

In these studies, the microcalorimetric assays rely upon inhibition of the observed respiration of *Saccharomyces cerevisiae* in buffered glucose

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when an antifungal drug is added to the system. The experiments must be conducted under standardized conditions that include the standardization of inocula through storage in liquid nitrogen, anaerobic incubation conditions and the use of a strict timetable to assure addition of the drug at the same point in cell metabolism each time. (1, 2, 3).

It was shown that the power-time curves for *Saccharomyces cerevisiae* respiring in buffered glucose in the presence of antibiotics can be altered in two different ways revealing differences in their modes of action. For some substances the curves show a signal returning to the baseline implying that all cells added have ceased respiring. The substances apparently lead to cell death. For other antibiotics the curves show reduction of power to another plateau, showing that they simply reduced the metabolic activity (2, 3, 9). Figure 2 shows typical thermograms obtained for *S. cerevisiae* in the absence and presence of antibiotics with different modes of action.

This paper concerns the study of the bioactivity of nine new mesoinic compounds of types 1,3,4-thiadiazolium-2-aminide and 1,3,4-triazolium-2-thiolate by biological microcalorimetry and the comparison of the results with those obtained by the agar diffusion technique.

In view of some disadvantages of the classical agar diffusion assay, biological microcalorimetry is proposed as an alternative method for screening compounds for useful antimicrobial properties.

MATERIAL AND METHODS

Compounds - The structures of the mesoionic compounds can be found in Figure 1. Their preparation, identification and purity have been described elsewhere (11).

Inocula - *Saccharomyces cerevisiae* NCYC 239 was utilized in all experiments.

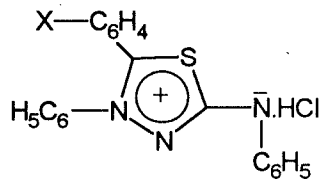
The yeast was mantained on yeast-beef agar (Oxoid) slopes. Yeast suspensions were prepared in a enriched medium (modification of Antiobiotic Medium nº3 Oxoid) (1, 13) and after incubation for 24h at 25°C were subcultured several times. A growth curve was constructed by optical density measurements, in order to determine optimum time for harvesting. When the cells were in the late exponential-early stationary phase of growth, they were removed from the medium by centrifuging and resuspended in sterile saline.

Ampoules were charged with 1.8ml of yeast suspension and were first exposed to liquid nitrogen vapor and then plunged into liquid nitrogen.

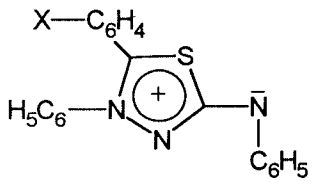
Composition of phthalate buffer-glucose - ($gl^{-1}o$): D-glucose, 1.80; potassium hydrogen phthalate, 14.91; KOH, 0.36. The pH was adjusted to 4.5 with KOH solution.

Calorimeter - The calorimeter used was the LKB Flow microcalorimeter.

Microcalorimetry assays - The compounds were screened against *Saccharomyces cerevisiae* (NCYC 239) under non-growing conditions, utiliz-



C



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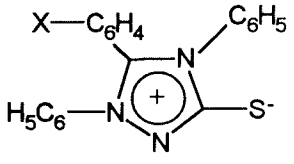

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Compounds	X
C_1, D_1, E_1	H
C_2, D_2, E_2	4-0CH_3
C_3, D_3, E_3	4-NO_2

FIGURE 1 - Structures of the assayed mesoionic compounds: C, conjugate acids; D and E free bases.

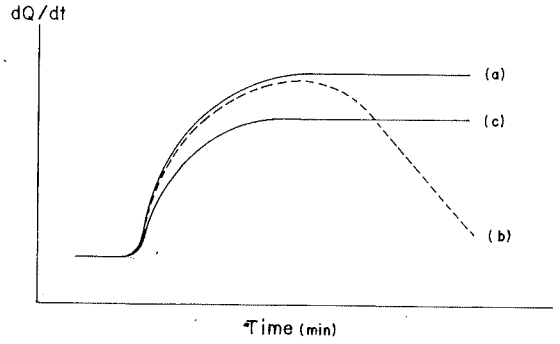

FIGURE 2 - Representative power-time curves showing the effect of antifungal agents on *S. cerevisiae* cells respiring in buffered glucose: (a) control; (b) addition of an antifungal agent that kills the cells; (c) addition of an antifungal agent that simply reduces the metabolic activity.

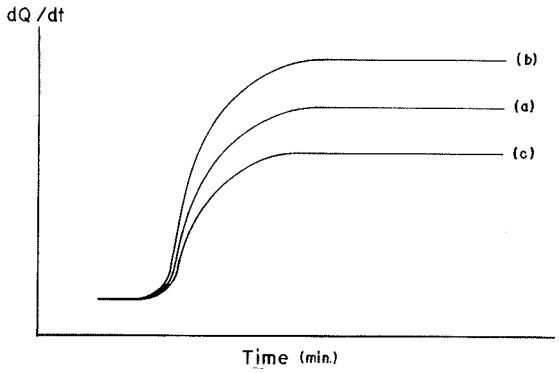

FIGURE 3 - Representative power-time curves showing the effect of mesoionic compounds on *S. cerevisiae* cells respiring in buffered glucose: (a) control (cells in buffer containing DMSO); (b) cells in the presence either of C-1, C-2, D-1, D-3, E-1 or D-2 at low concentration; (c) cells in the presence either of C-3, E-2, E-3 or D-2 at higher concentration. Note that figure 3 shows only the general trends, not the real deflections observed for each compound.

ing liquid nitrogen stored inocula in phthalate buffer (pH 4.5) containing glucose as reaction medium.

Glucose buffer (50 ml) is passed through the microcalorimeter cell to establish a steady instrumental baseline deflection. During the establishment of the baseline, the liquid nitrogen stored ampoules are thawed at 40°C for 3 min. The yeast suspension (0.6 ml) is shaken for 1 min and then inoculated into glucose buffer (47 ml) 2 min after complexion of thawing. 2 min later the solvent (DMSO, 3 ml) is added to the reaction medium in order to establish the control curve. 30s after this addition the inlet tube to the calorimeter is inserted. The flowing incubation medium reaches the calorimeter 3 min after the inlet tube insertion; at this instant the first signal for the incubation vessel contents is detected. The outflow from the calorimeter is returned to the incubator ca. 4 min after inoculation, thus making a closed system. After each incubation the calorimeter tubing is flushed by deionized water.

Once obtained the control curve each compound is dissolved in DMSO (3 ml) and added to 47 ml of medium plus cells for presentation to the microcalorimeter and the experiment is repeated.

Each experiment requires approximately 30 minutes.

Agar Diffusion - The compounds were tested against *S. cerevisiae* by the cup-plate agar diffusion techniques (10), in concentrations of 1 and 0.5 mg ml^{-1}.

RESULTS AND DISCUSSION

Nine newly synthetized compounds have been tested.

Addition of different concentrations of mesoionic compounds to respiring cells has led to different power-time curves.

Figure 3 shows the representative power-time curves observed for *S. cerevisiae* in the absence and presence of mesoionic compounds. The control curve was obtained for the cells in glucose buffer plus the same volume of solvent used to dissolve the samples (3 ml).

The individual power-time curves are not shown but the observed deflections from the control varied with the nature and concentration of the drug assayed.

The biological response, i.e., the percentage inhibition of activity is measured by the percentage deflection observed from the control when the mesoionic compound is added. These deflections are measured fifteen minutes from the detection of the first microcalorimetric response.

The percentage inhibition of activity of yeast cells in the presence of mesoionic compounds are presented in table 1. They have been calculated from each individual power-time curve and can be expressed by

$$\% \text{ inhibition} = \frac{P\mu c - Ptc}{P\mu c} \times 100$$

where Puc = Power (untreated cells)
Ptc = Power (treated cells)

The general trends of the power-time curves shown in figure 3 suggest differing modes of action for these compounds. Apparently, none of the tested

TABLE 1 - Percentage inhibition of activity of *S. cerevisiae* yeast cells in the presence of mesoionic compounds as evaluated by biological microcalorimetry.

Compound (mg/ml)	Dose	Response (%)
C, 1	1.3	- 7.8
C, 2	1.3	- 3.9
C, 3	1.3	16.88
D, 1	1.3	-3.90
	0.6	- 6.41
	0.3	- 7.79
	0.16	- 11.69
D,2	1.3	5.70
	0.6	- 1.29
	0.3	- 6.49
	0.16	- 6.33
D, 3	1.3	-6.49
	0.6	- 6.49
	0.3	- 6.49
	0.16	0.00
E, 1	1.3	- 5.19
E,2	0.3	1.30
E,3	0.3	2.60

compounds can lead to cell death, since none of the recorded power-time curves showed a signal decreasing to the base line. For compounds C-3, D-2, E-2 and E-3 the curves obtained showed reduction of power to another plateau value. The response suggests that these mesoionic compounds act as fungistatic antibiotics (3). For compounds C-1, C-2, D-1, D-2, E-1 (in all concn tested) and ooo, at low concentration, the curves showed increase of power to another plateau value, suggesting either that some damage to the cell membranes has allowed the uptake of glucose to be higher or that the added drugs are raising the metabolic activity (3). Further work is in progress to elucidate this point.

Unlike previous results obtained for two different series of compounds (9, 12) where the dose-response data have shown the usefulness in establishing a linear Free Energy Relationship (LFER), there is no evidence at all in this work of such quantitative relationship for the study compounds.

As the purpose of the microcalorimetric assays was the screening of the new mesoionic compounds for antifungal activity we decided to compare the results with those obtained by the agar diffusion assay, since this technique is the most widely used either in the search for new antibiotics or for clinical sensitivity testes. The agar diffusion method measures the inhibition of growth and in spite of certain difficulties it is well suited for preliminary antimicrobial screening.

The antifungal activity of the mesoionic compounds against *S. cerevisiae* evaluated by the agar plate diffusion assay has shown that only compound C-3 has a measured response at the tested concentrations. This is in agreement with the results obtained by biological microcalorimetry since C-3 has the highest percentage of inhibition (ca. 17%), i.e. the highest antifungal potency. Compounds D-2, E-2 and E-3 that presented weak inhibitory activity by biological microcalorimetry did not show inhibition zones in the agar plate assays. This is probably due to the poor ability of the compounds to diffuse in agar medium, since diffusion rate is usually the determining parameter.

This improved sensitivity gives an advantage for microcalorimetry over the classical microbiological methods. Other advantages are: the time taken for the assay that is less than 1 hour in comparison to 24-48h for agar diffusion assay; improved reproducibility (ca. 2% for microcalorimetry and 5-10% for agar diffusion); the technique and the analysis of results are very simple and there is no possibility of subjective errors. A disadvantage is the high instrument cost.

In conclusion, biological microcalorimetry can provide a rapid, simple and precise method of screening newly synthesised compounds for antimicrobial activity since the data reveal the existence of a complementary behaviour between microcalorimetry and classical microbiological methods. Unlike classical diffusion methods, biological microcalorimetry can distinguish modes of action of the study compounds towards *S. cerevisiae* yeast cells.

RESUMO

Interação de compostos mesoiônicos com *Saccharomyces cerevisiae* medida pela microcalorimetria biológica.

Avaliou-se a sensibilidade de *Saccharomyces*

cerevisiae frente a compostos mesoiônicos dos tipos 1,3,4-tiadiazólio-2-aminida e 1,3,4-triazólio-2-tiolato através de microcalorimetria biológica de fluxo comparando-se os resultados com os obtidos através do método de difusão em ágar. Apesar dos resultados fornecidos pelos dois métodos terem sido compatíveis, há vantagem no uso do método microcalorimétrico por ser mais rápido e preciso, podendo, além disso, distinguir entre diferentes modos de ação.

Palavras-chave: *Saccharomyces cerevisiae*; microcalorimetria biológica; difusão em ágar, compostos mesoiônicos.

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POST-HARVEST CHEMICAL TREATMENT OF UNSHELLED MOIST PEANUTS WITH SODIUM OTHO-PHENYLPHENATE TC CONTROL AFLATOXIGENIC FUNGI. I. FIELD TESTS

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I. V. Zambello
E. L. Fonseca

SUMMARY

The effect of sodium ortho-phenylphenate (SOPP) was analysed to control aflatoxigenic fungi in unshelled moist peanuts, under field conditions. Its efficiency was tested by spraying peanuts, under field conditions. Its efficiency was tested by spraying peanuts in the windrow immediately prior to combine harvest with 0.1% SOPP solution in the rainy and in the dry season crops of 1986 and with a 0.5% solution in both crops of 1987.

In 1986 crops were observed heavy fungi development. In 1986 rainy season crop, the percentage of contaminated samples, during whole storage period was 11.25% in the treated lot, with an average level of contamination (B_1 + G_1 aflatoxins) of 23 µg/kg, against 100% contamination in the control lot, with an average of 3427 µg/kg, maybe related to the high initial moisture content of this lot. In 1986 dry season, the percentage of contaminated samples, was 10% in the treated lot, with an average of 27 µg/kg, against 3% of control lot, with an average of 49 µg/kg.

In the rainy season of 1987, 100% of the samples were contaminated, with an average level of 2780µg/kg for the treated lot and 6524µg/kg for the control lot. In the dry season of 1987, 8.5% fo the samples from the treated lot were contaminated with an average of 96 µg/kg against 38.5% of the control lot, with an average of 70 µg/kg.

The spray operation in the field was deficient because total coverage of pods with SOPP solution was not attained indicating the need of optimizing product application method.

Key Words: Aflatoxin, peanuts, chemical control, sodium ortho-phenylpenate, post-harvest, fungi.

INTRODUCTION

Among all known mycotoxins, aflatoxins are the most worrying due to its toxicity, carcinogenic properties and occurrence as natural contaminant in wide range of food crops.

In Brazil, peanut is the most susceptible product to contamination due to the underground na-

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ture of the crop, climate conditions and agricultural practices during harvest, drying and storage.

Contamination may be minimized by use of adequate techniques from harvest to storage, however very often the climate conditions during this period are adverse and do not favor rapid and efficient drying of peanuts. Thus, the use of chemical agents to control fungal growth and aflatoxins production can be an option for obtaining better quality product and this was the recommendation made during the conference "Mycotoxins in Human and Animal Health" in 1976, in the University of Maryland (8).

Chemical substances can reduce the *Aspergillus flavus* Link growth and aflatoxin production in peanuts plants windrowed in field (2, 3, 5).

The efficiency of several fungicides was tested, *in vitro*, by Fonseca et alii (4), in the control of *A. flavus*, spraying SOPP, Ferbam, Thiran and Captafol on unshelled peanuts, immediately after digging during four consecutive years. Although the selected fungicides were efficient in *in vitro* fungal control, it was not possible to conclude about their efficiency in the field, as in dry seasons harvests, both treated and control lots were not contaminated. In rainy seasons harvests all lots were contaminated. The authors concluded that in dry season, use of fungicide would not be necessary, once climate conditions is enough to control fungal development if good post-harvest practices are applied. When a rainy condition occurs at harvest time, it seems that fungicides are washed out allowing fungal growth.

The present work aimed to test efficiency of unshelled peanuts spraying with SOPP solution, immediately before combine harvest and not immediately after digging, as previously tested by Fonseca et alii (4).

MATERIAL AND METHODS

This experiment was conducted in the region of Marília, SP, Brazil, during the peanut rainy and dry crops of 1986 and 1987.

In each crop, 240 bags of unshelled peanuts were divided in two lots of 120. One lot was treated with SOPP solution and the other, without treatment, was considered as control.

Digging, windrowing and combine harvesting were made according to the usual practices of the region. After digging and windrowing, daily samples were taken to control moisture content to the levels of 18-14%. When peanuts is harvested and stored with this moisture level, there will be a great probability of fungal growth and consequent aflatoxins production.

1986 CROPS - When peanuts reached the desired levels of moisture, an area corresponding to 120 bags was sprayed with 0,1% SOPP solution utilizing a tractor, in the concentration indicated by the manufacturer to control *A. flavus, in vitro*.

Immediately after spraying, peanuts were combine harvested and transported to the "Posto de Sementes" of Marília, where they were submitted to a pre-cleaning in a ventilation machine. Fifteen samples from each lot were drawn to check moisture and aflatoxin to determine the initial condition of the lots which following were stored in 120 bags stacks. In the initial period of storage (Period A), the moisture of the lots was determined weekly and when it reached 11%, 40 samples in the rainy season and 30 samples in the dry season were taken from each lot to determine aflatoxins. After three weeks, moisture reached 11% and during the two following months (Period B), 5 samples from each lot were taken ervery two weeks to analyze aflatoxins end at the end of the periods, the stacks were broken down and 20 samples were taken from each lot for aflatoxins analysis.

1987 CROPS - The procedure was the same as 1986 crops, but SOPP concentration was raised to 0,5% due to the intense fungal growth observed in the previous crops.

Analytical Methodology

Moisture: In the field, a portable equipment of the resistance type (ELOTEST) was utilized. In the laboratory, the Oven Method (1) was used.

Aflatoxins: The modified methods of Pons Jr. et alii (6) conjugated with Velasco & Morris (10) were utilized. The Modifications were: a) the ratio peanut kernels: water in the slurry was 1:15; a total of 50 g of the slurry was transferred to 250 ml erlenmeyer flask and 100 ml acetone was added for extration; b) the clean up procedure was made with lead acetate solution without boiling (7); c) the chloroform amount for partition was 2 x 25 ml (9).

RESULTS AND DISCUSSION

The moisture content of peanuts in both treated and control lots of 1986 and 1987 crops immediately before storage are presented in Table 1,

TABLE 1 – Mean values of moisture content (%) of unshelled peanuts immediately before storage.

Crop Season	Treatments	
	SOPP	CONTROL
Rainy / 86	14.3	22.0
Dry / 86	16.8	16.6
Rainy / 87	17.1	18.4
Dry / 87	18.5	19.0

and the results of aflatoxins analyses, in the Tables 2 and 3.

1986 CROPS Rainy season crop- Both treated and control lots showed and intense fungal growth in internal and external parts of stacks. In the treated lot only nine samples were contaminated with aflatoxins during the whole storage period (periods A + B) which represented 11.25% of analyzed samples, with a mean of 23 µg/kg (aflatoxins $B_1 + G_1$).

In the treated lot, 8 samples were initially contaminated with a mean value of 82 µg/kg (always $B_1 + G_1$), due to the heavy rains during the harvest, raising moisture content to 22%. No rain occurred when the treated lot was harvested and its initial moisture was 14.3%. During the whole storage period (periods A + B), 100% of the control lot were contaminated with a mean value of 3427 µg/kg of aflatoxins.

Dry season crop - In both treated and control lots, great development of genus *Rhizopus* and *Aspergillus* was observed. In the treated lot, 7 samples were contaminated, representing 10% of all analyzed samples during the whole storage period (periods A + B) with a mean value of 27 µg/kg of aflatoxins. In the control lots, only two samples (3% of all analyzed samples) were contaminated with a mean value of 49 µg/kg during the whole storage period, which indicates that environmental condition was not favorable for aflatoxins production.

1987 CROP - Rainy season crop - No visible fungal development was observed in the treated peanut, however, 100% of samples analyzed during whole storage period (periods A + B) were contaminated with a mean value of 2780 µg/kg of aflatoxins. At the beginning only two samples (13.3%) were contaminated with a mean value of 9 µg/kg. The control lot, such as in 1986, was initially contaminated. All samples had aflatoxins with the mean value of 489 µg/kg due to the intense rains that occurred during the harvest time.

Dry season crop - It was observed, intense fungal growth in the treated lot and six samples analyzed, during all storage period (periods A + B) were contaminated with a mean of 95 µg/kg of aflatoxins. The control lot which, at the beginning, was not contaminated with aflatoxins, although presented an intense fungal growth and 27 samples were contaminated (38.5%) with a mean level of 70 µg/kg during all storage period.

TABLE 2 - Mean values of aflatoxins content (µg/Kg, $B_1 + G_1$) of contaminated peanut samples of rainy and dry seasons of 1986.

Treatments	Before storage			During storage Period A			During storage Period B		
	Analyzed samples	Contam. samples	Mean value	Analyzed samples	Contam. samples	Mean value	Analyzed samples	Contam. samples	Mean value
SOPP (0.1%) Rainy / 86	15	0	ND	40	1	2	40	8	44
CONTROL Rainy / 86	15	8	82	40	40	2932	40	40	3922
SOPP (0.1%) dry / 86	15	1	7	30	2	41	40	5	14
CONTROL dry / 86	15	0	ND	30	0	ND	40	2	49

SOPP - sodium ortho-phenylphenate solution.
Period A - when moisture reached 11%.
Period B - after moisture reached 11%, till the end of experiment.

TABLE 3 - Mean values of aflatoxins content (µg/Kg, B_1+G_1) of contaminated samples in rainy and dry season crops of 1987.

Treatments	Before storage			During storage Period A			During storage Period B		
	Analyzed samples	Contam. samples	Mean values	Analyzed samples	Contam. samples	Mean value	Analyzed samples	Contam. samples	Mean value
SOPP (0.5%) Rainy / 87	15	2	9	30	30	1991	40	40	3570
CONTROL Rainy / 87	15	15	489	30	30	6961	40	40	6087
SOPP (0.5%) dry / 87	15	0	ND	30	4	66	40	2	126
CONTROL dry / 87	15	0	ND	30	7	47	40	20	92

SOPP - sodium ortho-phenylphenate solution.
Period A - when moisture reached 11%.
Period B - after moisture reached 11%, till the end of experiment.

In the four crops of this experiment, it was observed that field spraying of peanuts was not efficient to control aflatoxin contamination. An efficient coverage of the pods with SOPP solutions could not be obtained due to the difficulties inherent to spraying in the windrow such as irregular surface of the field and position of the inverted pods. Also it was not possible to obtain treated and control lots in the same day and with the same characteristics, specially in the rainy season crops due to tho intense rains. This adversely affected the analysis of the behavior of the treatment in both years, and did not permit to draw better conclusions towards the efficiency of the treatment.

It is concluded that the spraying operation needs to be optimized in order correctly evaluate the SOPP efficiency in controlling aflatoxigenic fungi, what will be tried in the coming experiments.

RESUMO

Controle de fungos produtores de aflatoxinas no amendoim em casca pelo ortofenilfenato de sódio. I. Condições de Campo.

O objetivo deste trabalho foi testar a eficiência do ortofenilfenato de sódio (OFS) no controle de fungos produtores de aflatoxinas no amendoim em casca, em condições de campo.

O experimento foi conduzido na região de Marília e a eficiência do produto foi testada através da pulverização, no campo, do amendoim em casca ainda enleirado, imediatamente antes de ser despencado e ensacado, com solução de OFS nas concentrações de 0,1% nas safras das águas e seca de 1986 e de 0,5% nas safras das águas e seca de 1987.

Na safra das águas de 1986, a percentagem de amostras contaminadas, durante todo o período de armazenamento, foi de 11,25% para o lote tratado, com nível médio de contaminação (aflatoxinas B_1 + G_1) de 23 µg/kg e de 100% para o lote testemunha com nível médio de 3427 µg/kg. Isto deve ter acontecido em virtude do elevado teor inicial de umidade deste lote. Na safra de seca de 1986 a percentagem de amostras contaminadas, no mesmo período, foi de 10%, no lote tratado, com nível médio de contaminação de 27µg/kg e de 3%, no lote testemunha, com nível médio de 49 µg/kg.

Na safra da águas de 1987, 100% das amostras, analisadas durante todo o período de armazenamento, estavam contaminadas. No lote tratado, as amostras apresentaram um nível médio de contaminação de 2780 µg/kg) e no lote testemunha este valor foi de 6524 µg/kg no lote testemunha. Na safra da seca de 1987, 8,5% das amostras analisadas estavam contaminadas com nível médio de 96µg/kg no lote tratado e no lote testemunha 38,5% das amostras retiradas estavam contaminadas com nível médio de 70 µg/kg.

Verificou-se, nas quatro safras, que a aplicação do produto, no campo, foi deficiente, pois não se conseguiu a cobertura completa das va-

gens com a solução de OFS. Os resultados indicam que há necessidade de se otimizar a aplicação do produto para que se possa avaliar a sua eficiência no controle de fungos potencialmente aflatoxigênicos.

Palavras-chave: Aflatoxina, amendoim, controle químico, ortofenilfenato de sódio, pós-colheita, fungo.

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