ISSN 0001-3714

Revista de Microbiologia

SBM
Sociedade Brasileira de Microbiologia

São Paulo — Brasil

Volume 24 Número 2 Abr. - Jun. 1993

FICHA CATALOGRÁFICA

Preparada pela Biblioteca do
Instituto de Ciências Biomédicas da Universidade de São Paulo

Revista de Microbiologia/Sociedade Brasileira de Microbiologia.
— Vol. 24, nº 2(abr/jun 1993)
— São Paulo: SBM, [1970] -
v.:il ; 27 cm

Trimestral
1970 - 1993, 1-24
ISBN 0001-3714

1. Microbiologia I. Sociedade Brasileira de Microbiologia

NLM-QW4

Revista de Microbiologia

Publicação da Sociedade Brasileira de Microbiologia
São Paulo — Brasil

Filiado à IUMS — INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES

Os artigos publicados na Revista de Microbiologia são indexados em: Current Contents (USA); CNRS - Centre National de la Recherche Scientifique (França); Chemical Abstracts Service(USA); Cambridge Scientific Abstract (USA); Commonwealth Mycological Institute (England): Hamdard National Foundation (Paquistão); IMLA - Index Medicus Latino Americano (Brasil); Institut Nautchnoi Informatsii (URSS); Periodica (México); Sumarios Correntes Brasileiros; UMI - University Microfilms International (USA).

Auxílio Financeiro: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); FINEP.

SOCIEDADE BRASILEIRA DE MICROBIOLOGIA
AV. Prof. Lineu Prestes, 1374
05508 - São Paulo - S.P.
Brasil
Fone (011) 813-9647
TELEX 11 35085
FAX 88 79875

Sociedade Brasileira de Microbiologia

Filiada a IUMS – INTERNATIONAL UNION OF MICROBIOLOGICAL SOCIETIES

Objetivos

A Sociedade Brasileira de Microbiologia, fundada em 28 de setembro de 1956, é sociedade civil, sem fins lucrativos, dedicada a agremiar microbiologistas brasileiros; a promover o desenvolvimento da microbiologia através do estímulo à pesquisa científica e suas aplicações, melhorar as qualificações profissionais dos microbiologistas; e manter intercâmbio, a cada dois anos, na última semana do mês de julho, o Congresso Brasileiro de Microbiologia e edita a Revista de Microbiologia, com distribuição trimestral. Para informações sobre a SBM, escrever para qualquer membro da Diretoria.

Endereço

Av. Prof. Lineu Prestes, 1.374
05508 – São Paulo – SP – Brasil
Fone: (011) 813-9647 – Telex: 011 35085 –

REVISTA DE MICROBIOLOGIA
Depto. de Microbiologia – ICB II – USP
Av. Prof. Lineu Prestes, 1374 – Cid. Universitária
CEP 05508-900 – São Paulo - SP - BRASIL
Site: www.revmicro.cjb.net

REVISTA DE MICROBIOLOGIA
PUBLICAÇÃO DA SOCIEDADE BRASILEIRA DE MICROBIOLOGIA
VOLUME 24 ABRIL - JUNHO 1993 NÚMERO 2
REV. MICROBIOL. (S. PAULO), 24(2)

CONTEÚDO - CONTENTS PAG

Rev. Microbiol., São Paulo, **24**(2): 73-77, 1993

TEACHING OF MEDICAL MICROBILOGY: COMMENTS ON BASIC VERSUS APPLIED ASPECTES RELATED TO PROFESSION

Augusto Cezar Montelli*

SUMMARY

Comments about education and medicine teaching were made. The learning and the curriculum in according with the priorities of the country's health had special emphasis.

Students and teachers of Botucatu considered that the improvement of the 2nd year medical microbiological course was good with predominantly basic contents. The students prefer a balanced content or predominantly applied to medicine.

Three phases of the teaching of microbiological contents are proposed:

1st - in the basic cycly (2nd year); 2nd - into the course of Clinical Laboratory (3rd or 4th year); 3rd - into the clinical cycle, following a program of integration.

Key-words: Medical microbiology, teaching, objectives

May be it would not be so difficult to give an opinion about this question if it would be based on personal and/or institutional experience. Therefore, to make a complete and a deep analysis of the matter certainly will constitute a complex task and a huge challenge if it would be considered the modern scientific progress in microbiological and molecular biological areas and also the real needs of society.

Taking the characteristics of the readers of this journal into account it is not necessary to enumerate or make comments on the latest aims that have been reached in this scientific area. Thus, it must be emphasised that the origin of Education is strictly linked with the History of Humanity. Simões Barbosa says that: "Education does not exist to preserve values and traditions but to renew them. It means that Education is not only the citizen right or a consumer goods to be used individually". It transcends these concepts when it is seen as "an instrument of transformation that is capable to provoke social changes". Differently from the educational ideals, the school of nowadays is very distant of being an institution where the necessary social transformations should start. On the contrary, its real function in society has been that one which consists in reaching an education system that could perpetuate certain ways of existing besides being resistant to changes.

The scarce mobilization and participation of community in managing its own collective decisions make possible that the orientation or passive receptive tendency become predominant and be expressed in the development of the conduct that is also well marked by an attitude of constant expectation in the other's behavior as: giving an opinion, criticizing or even managing their acts.

If this orientation should be taken into consideration in Education area, we would face a kind of student who would be incapable to take advantages of the several options of learning that could be offered by the school. The whole responsability for the educational results is practically in the hands of the teachers and the qualities of the students although have been despised, in this case, are frequently decisives to the good process of teaching-learning.

Just the opposite of this tendency, the participative orientation in teaching involves the capacity of devel-

*. Departamento de Clínica Médica - Disciplina de Laboratório Clínico - Faculdade de Medicina de Botucatu - UNESP

opment in students and also in the teachers generating attitudes and antecipated actions which could change reality.

The main objective in the student is to expand the capacity of/or to select, to apply and criticise information; in teachers the development of promoting and orientating the participation and criticism.

These concepts were approached by Viniega-Velasquez who still adds: "The supperation of the complex problems of a country in any field of activity, has as its point of starting the large knowledge of reality which constitutes an essential basis to begin the strategies of an effective and viable solution. Thus, the major role of superior Education does not consist in answering the pressing demands of specific sectors of society acting in an automatic, immediate and little reflexive way. The irreplaceable role of the University is to dopt community which is its support with professionals who are capable of promoting antecipated actions before the several problems of the country. These actions become viable when they are steeped in knowledge of the outlined situation".

Considering that medical knowledge in general is continously expanding in such a rhythm that unables any teacher of keeping updated have been endeavoured all efforts in order to be adjusted to this reality which has been rapidly modified although it has already been verified a progressive ampliation into the displacement between what is known as new from what has been taught. Maybe this situation is still persisting due to the insidious thought that the most important aspect is to involve the multiple contents of knowledge in learning than the manner that they are assimillated.

According to Viniega-Velasquez "to insist on the purpose of incorporating the contents rapidly modified from knowledge as the center of learning it will take us in a short period of time to a serious problem: we shall be uncapable of facing the troubles that society can generate".

During the "World Conference on Medical Education" held in Edinburgh, Scotland, from August 7 to 12, 1988, was divulged the "Declaration of Edinburgh" which had provoked a significant impact on th International Medical Education. Since then, several appreciations and suggestions of actions into the medical school itself have been made in order to arrive at a desirable progress. Among them it can be mentioned:

"The target of Medical Education is to form doctors who can be able to promote health for the whole population in a sense that would not be only by rending curative services to people who can bear the costs or to those who can have an easy access to these services".

Despite the huge progress obtained by Biomedical Sciences in this century, this aim has not been achieved in several places yet.

Nevertheless, this problem can not be considered as a new one even though the efforts that were previously made to establish a large socially conscience in Academic Medical Schools have not got a remarkable succes.

These considerations show that this progress can be aimed through the actions inside the Medical School itself as follows:

* To organize the curriculum and also the evaluation systems in such a way that the extent of professional capacity and social values and not only the act of fixing and memorizing the information could be realized

* To be assured that the curriculum contents will reflect the priorities of national health and the availability of the resources as well.

Thus, it can be detected than even in this such relevant conference on International Medical Education the final proposals pointed out to essential aspects of learning and also to the curriculum.

In relation to this was emphasized the inclusion of priorities respecting the country's health.

The relevance of social demands in our sphere reflects in posgraduation policy itself. Recently this preocupation was demonstrated by Eunice R. Durham when she was director of CAPES (Coordination for the improvement of superior level personal) who also referred to the present system of master and doctorate as being both clearly turned to the constitution of an academic competence which has been guided to form teachers and researchers into a system that basically supplies the university. She concluded saying that the expansion and the valoration of the courses turned to professional development and to the applied research are, nowadays, one of the most urgent needs of society and also a priority respectinf posgraduation policy.

The experience of renewing medical teaching held in the Faculty of Medicine at Universidade Federal de Minas Gerais, has as its main objective to offer to the students not only a biological comprehension but also the social aspects of the health-diseases processes.

Salgado and Paula Castro who insiminated and lived in this process made the following statement: "The amateurish in Medical Education which did not do much harm some years ago, is still so disastrous when it faces the new social and technical realities, that is imperious and undelayable to have correctly

adjusted the curriculum of Medicine to the present and mainly to the future growing needs of the Brazilian population".

Therefore, the option that seems to be of great validity is resumed in the objective diagnostic of the teaching conditions in Medical School and also in the needs of society which involves it in such a manner that the process of the curriculum development can be started. In other words, "the implementation of the curriculum must be constantly evaluated and progressively improved".

Taking all these arguments into account and now - the teaching of Microbiology into a medical context - we would ask: how could be better defined its objective? a basic formation or its application to profession? In 1975, in "The variation on dissonance", Prof. Suassuna had already written in the introuction to his work "Teaching of Microbiology in the Faculties of Medicine" that to get a definition of the specific aims of the Discipline, the general targets of the school should be watched closely. He had also put in evidence that the fundamental difficulty lives in the conception of what should be a "good doctor". After more than 16 years, surely this difficulty has become much larger than before.

So, in order to expand the arguments to be brought into this discussion about the proposed theme, we looked for the opinion of the students and teachers at the University of Botucatu.

In the Faculty of Medicine of Botucatu the curriculum contents of Microbiology has two different aspects:

1. Microbiology course. It is given, offered during the first semester of the second year medical by the Discipline of Microbiology at the Institute of Biosciences. I have been participating in this course for 28 years as a volunteer teacher.

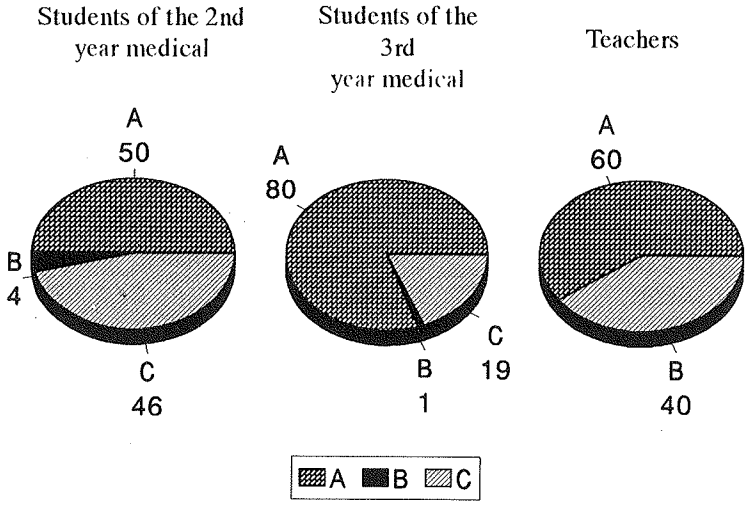
2. Cycle of Clinical Microbiology of the Cinical Laboratory Course at the Department of Medical Clinic. It is given during the second semester of the third year medical. This course has been under my responsability for 17 years.

Thus, in 1991, at the end of each one of these courses, we had an evaluation made by students through a questionaire especially elaborated. Item as "The content of Microbiology to be taught"was included. Sixty three per cent of the students of the second year and 79% of the third year had expressed their opinion and after had been added up became an importamt point of the group to be analysed.

In the same way was also collected the opinion of the teachers of Microbiology Discipline who participated in the course of the second year. The results allowed us to construct the graphics of the figures 1 and 2. After the answers had been analysed we managed to reach the following results:

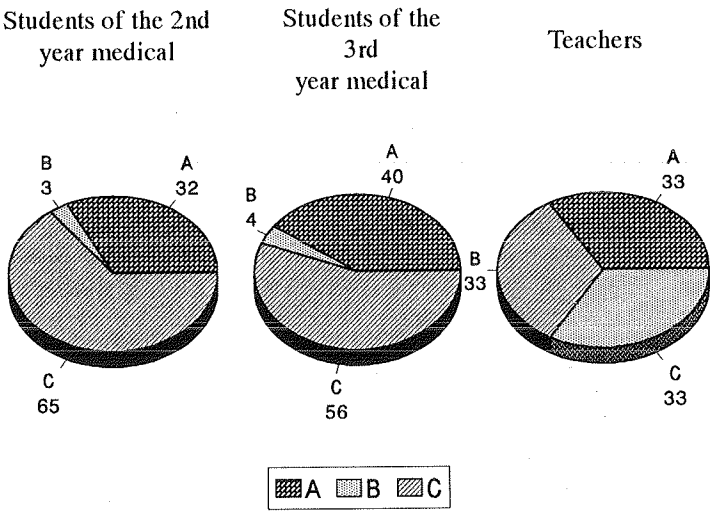
1. At first, the improvement that was obtained by the students of the 2nd year, in a scale from good to bad, was considered good by both students (66%) and teachers (80%).

FIGURE 1 - In 1991, during the course of microbiology at the 2nd year medical you acquired knowledge



A- Predominantly basic
B- Predominantly applied to medicine
C- Basic and applied (in a balanced way)

FIGURE 2 - In your opinion the course of microbiology at the 2nd year should have as its fundamental aim



A- A formation predominantly basic to the discipline
B- A formation predominantly applied toMedicine
C- Both types of formation (in a balanced way)

2. In relation to knowledge that was offered by the 2nd year medical course the students of the second year (50%), 3rd year (80%) and the teachers (60%) agreed, almost in its majority, that they were predominantly basic.

3. Respecting the fundamental objective that the courses would have aimed the answer that mostly appeared was "it should be offered to the stdents as a basic and applied formation in a balanced way". The results were obtained between the students of the 2nd year (56%) and the 3rd year (65%).

4. Among the teachers the same question had generated a homogeneous distribution of the three alternatives of answering which were made: formation - predominantly basic (33%) or applied (33%).

5. Respecting the contribution to medical formation that was offered by the course of Microbiology the students of the 2nd year answered in its majority that it was vast (36%) or medium (63%).

Even without having a deep analysis of what is the meaning of basic or its application to the teaching of Microbiology it can be detected by the available data that the students and teachers who were consulted considered that the 2nd year medical course has demonstrated that its contents are predominantly basic. On the other hand, between the students of the 2nd and 3rd years predominated the wish of having a balanced content, or, predominantly applied to profession.

Regarding this aspect the teachers were homogenuosly divided although it must be emphasized that their opinions were just related to the curriculum of the 2nd year medical course.

For that reason, we think that the contents considered basic in Microbiology must have their place assured in the curriculum, mainly at the beginning of the course (1st and 2nd years.) and also it must be kept at a level more or less deep, depending on the human resources in the area.

At the 3rd year medical course when the students have started dominating the knowledge and the skill in propedeutics programmes of Microbiology it must be as much as possible predominantly applied and taught in an integrated manner (For example: at an integrated course of Pathology and Clinic of the organs and systems).

In this sense, it can interact with disciplines as Pathology, Pediatry, Infectious Diseases, Medical Clinic and Preventive Medicine.

Thus, the teaching of Microbiological contents can be projected to three different and progressive phases: the first one into the basic cycle, the second with the course of Clinical Laboratory or Clinical Pathology (in the 3rd and or the 4th year medical course) and the 3rd into the clinical cycle following a reasonable program or integration which could be introduced through the interdisciplinary seminars and also by the discussions of clinical cases in hospitals,

ambulatories or in Centers of Health.

It is known that in our country the formation of the general doctor or at least, of the clinical doctor has been discussed for years, as the fundamental objective of several schools. It is also well known that almost the totality of this aspect has been only a speech without any evidence of reality. What really happens is that the medical schools have been produced, in general, professionals of unconcerned formation and sometimes an anguished candidate to specialization. Therefore, it must be left to the microbiologists of the Faculties of Medicine the responsability of giving the best formation in this area of knowledge in order to be assured to the future professional a large competence respecting the approach of the infected patient and consequently in the practice of the antibioticotherapy which can be reasonably conducted

According to the participative orientation in medical education, we will finish this explanation emphasizing that even though the microbiological contents are assured as it was previously proposed, the efficacy of learning only will be granted when the priority occurs into "The methods of knowledge".

It means that the emphasis must appear in the territory of the methods of production, assimilation and in the criticism of knowledge.

RESUMO

Ensino de Microbilogia Médica: Formação Básica ou Aplicada à Profissão

São feitas considerações sobre Educação e Ensino Médico, enfatizando-se aspectos de aprendizagem e de currículo adaptado às proridades de saúde do país.

Estudantes e docentes de Botucatu consideraram que o aproveitamento do curso de microbiologia do 2º ano médico foi bom e com conteúdo predominantemente básico. Houve preferência dos estudantes por conteúdo equilibrado ou predominantemente aplicado à profissão.

Propomos ensino de microbiologia em 3 fases distintas: a primeira, no ciclo básico (2ºano); a segunda, junto ao curso de Laboratório Clínico (3º ou 4º ano) e a terceira, dentro do ciclo clínico , obedecendo a programa de integração com outras disciplinas.

Palavras-chave: Microbiologia médica, ensino, objetivos

REFERENCES

1. ABEM - Declaração de Edinburgo. *B. Assoc. Bras. Educ. Med. Rio de Janeiro*, **XX(3)**, Julho-Setembro, 1988.
2. MONTELLI, A.C. et al. - Diagnóstico da situação e perespectivas do ensino na Faculdade de Medicina de Botucatu. *Rev. Bras. Educ. Med.*, **4**:53-62, 1980.
3. MONTELLI, A.C. & MAGALDI, C. - Curso de graduação da Faculdade de Medicina de Botucatu - características e aspectos evolutivos (1963-1988). *Rev. Bras. Educ. Med.*, **13**:15-24, 1989.
4. PAULA CASTRO, L. & SALGADO, J.A. - Educação Médica: um permanente desafio. *B. Assoc. Bras. Educ. Med.*, Rio de Janeiro, **XX(1)**, Janeiro-Fevereiro 1988.
5. SIMÕES BARBOSA, F. - Educar para a Democracia. *B. Assoc. Bras. Educ. Med.*, Rio de Janeiro, **XX(4)**, Outubro-Dezembro, 1988.
6. SUASSUNA, I. - O ensino de microbiologia nas Faculdades de Medicina. *Rev. Microbiol. (São Paulo)*, **6**:63-72, 1975.
7. VINIEGA-VELASQUEZ, L. - Uma nueva estrategia para la educación médica de posgrado. Parte I. *Rev. Invest. Clin.*, **42**: 150-156, 1990.
8. VINIEGA-VELASQUEZ, L. - Uma nueva estrategia para la educacion médica de posgrado. Parte II. *Rev. Invest. Clin.*, **42**:240-244, 1990.

Received for publication in 21/12/92

Rev. Microbiol., São Paulo, **24**(2): 78-83, 1993

ENTEROTOXIN PRODUCTION IN STRAINS OF *CAMPYLOBACTER JEJUNI*, ISOLATED FROM SWINE DIARRHEA

Angela Cleusa de Fatima Banzatto de Carvalho[1]
Eduardo Nascimento Mós[2]
Ruben Pablo Shocken-Iturrino[3]

SUMMARY

One hundred and twenty-six strains of *Campylobacter jejuni* isolated from swine with diarrhea were examined for the production of enteroxin, by culture filtrate inoculation into ileal loops in several animal species.

Four (3,1%) of the strains tested produced fluid in ligated ileal loop of albino rabbit, 19 (15%) in rat and 14 (11,1%) in swine. By the test of intragastric inoculation in suckling mouse, none of the strains revealed capacity to produce enterotoxin, although two strains were considered suspicious. Twelve (54,5%) of the strains that induced ileal fluid in rat and swine produced cytotoxic effect in monkey kidney cells (Vero), affecting up to 60% of the monolayer. However, no alteration was observed in hamster kidney cells(BHK).

Key word: *Campylobacter jejuni*, enterotoxin, swine diarrhea, pathogenicity.

INTRODUCTION

The thermotolerant species of the genus *Campylobacter* (*C. jejuni, C. coli*) are microorganisms habitually present in intestinal flora of different species of domestic and wild animals (19,32,35) and of man (30). Their presence, however, is not always accompanied by obvious signs of disease and these bacteria may act as pathogenic or commensal organisms. These species can be isolated from the feces of both apparently healthy animals (34) and of animals with diarrhea (2,4,9,37).

The diarrhea caused by *Campylobacter* sp may be of the secretory type, suggesting the involvement of an enterotoxin (27). However, the mechanisms of virulence attributed to *C. jejuni* and to *C. coli* are still a matter of debate, although invasiveness and enterotoxin and cytotoxin production are believed to be the three major potentially pathogenic properties of these organisms (1,6,16)

The first attempts to demonstrate the presence of enterotoxin using classic methods such as intragastric inoculation into newborn mouse pups (15, 20) and into a ligated intestinal loop of the rabbit (36) were negative (8,10,13,20). However, BUTZLER & SKIRROW (3) reported possible evidence that some of the strains studied were enterotoxigenic. RUIZ-PALÁCIOS et al. (27) later demonstrated the production of heat-labile enterotoxin (LT) in strains isolated from diarrehic feces using the technique of inoculation into a ligated intestinal loop of rats and in cell culture. New findings were confirmed by other investigators using different techniques (6,8,12,18,21,23).

The objective of the present investigation was to

1 - FCAVJ-UNESP - Departamento de Medicina Veterinária Preventiva
2 - ICB-USP - Departamento de Microbiologia
3 - FCAVJ-UNESP - Departamento de Microbiologia

study the ability of *C. jejuni* strains isolated from pigs with diarrhea to produce enterotoxin using the technique of inoculation into a ligated intestinal loop of rabbits, rats and swine, and of production of cytotoxic effects in cultures of monkey kidney cells (Vero) and of hamster kidney cells (BHK).

MATERIAL AND METHODS

The study was conducted on 126 *C. jejuni* strains isolated from the diarrehic feces of swine with ages varying between 10 to 60 days raised in different municipalities of the Ribeirão Preto region, State of São Paulo, from 1988 to 1990. Colonies similar to those described by SMIBERT (33) were considered as being of the genera *Campylobacter* and the identification of the species was done following the method of SKIRROW & BENJAMIN (31). For short-time cultures, the strains were kept in thioglycolate broth containing 0.16% agar and 2.0 ml liquid vaseline at 37ºC, whereas for long-term storage the strains were kept in brucella broth containing 15% neutral glycerol at -70ºC.

Only *Campylobacter jejuni* samples were studied and not *C. coli* in virtue of the number of isolates of these second bacteria which number was not representative when compared with the first specie.

For enterotoxin preparation, the *C. jejuni* strains were cultured in brucella broth, pH 6.7, in glass bottles with screw caps. The material was incubated on a water bath with shaking (150 rpm) (28), at 42ºC for 72 h in jars with a reduced oxygen atmosphere. After incubation, the culture was centrifugated at 20.000 rpm for 10 minutes at 4ºC (18) and the supernatant was filtered through a Millipore filter, divided into small volumes for immediate use or stored at 4ºC for later testing within a maximum of two days.

The culture filtrates were tested on a ligated intestinal loop of adult albino Norfolk 2000 rabbits weighing 1500-2000 g by the method of SAHA et al. (28) that consists in the inoculation of 1.0 ml of supernatant of the culture in the ileal loops of 5 cm, being 8 to 10 loops by animal. The positive control was a known enterotoxinogenic *Escherichia coli* (K99 STa^+, gently given by F.Ávila) strain cultured on a brain and heart infusion (BHI) broth, and the negative control was sterile brucella broth. The animals were sacrificed 18 hours later and the loops examined to determine the presence of dilatation and of fluid accumulation, and the fluid volume/loop length ratio was calculated.

A similar technique was used to inoculate adult male Wistar rats weighing 150-200 g and aged 7 to 8 months, except that the number of loops used was 6 to 8 and the volume of culture filtrate inoculated was 0.5 ml (28). The positive and the negative controls used were the same as mentioned above.

Lancrace pigs aged 6 to 8 weeks were used for intestinal loop inoculation by the technique of GYLES (11), that consist in inoculating 15 to 20 ileal loops by animal, of 10 cm each, with 10 ml of the supernatant of the culture. The animals were sacrificed 18 hours after injection, the loops were examined and the fluid volume/loop length ratio was calculated. Positive and negative controls were also used.

The test of intragastric inoculation of suckling 2-3 day old albino mice was also performed. The animals were separated from their dams and divided at random into groups of 4 animals according to the technique of DEAN et al. (5). The pups were maintained at 28ºC for 4 hours and sacrificed with ether, their abdomen was opened, and the intestine was removed from the carcass and weighed. An intestinal weigh/carcass weight ratio (IW/CW) of 0.07 to 0.09 was considered to indicate suspected enterotoxin production.

Baby Hamster kidney (BHK) and monkey kidney (Vero) cell were cultured in milk bottles according to the technique described by RIZZO et al. (26) in this method the milk bottles with a completely monocelular layer are left without the maintenance medium and then 1 ml of the fluid inducing cultures filtrates diluted 1/2 and 1/5 was added and left in contact for 40 minutes at 37ºC, after this period the excess was removed and serum-free maintenance medium was added and incubated at 37ºC for 24-48 hours. Readings were taken at 6 hour intervals and morphological cell changes described by JOHNSON & LIOR (12) as rounded cells and total or partial destruction of the layer were recorded. A cell culture inoculated with sterile brucella broth was used as control.

Data were analyzed statistically by the Tukey test at the 5% level of probability according to NETER & WABBERMAN (24) and the efficiency of the techniques used was determined by the McNemar test (29).

RESULTS

Of the 126 *C. jejuni* strains studied in the different animal species by inoculation of the culture filtrate into a ligated intestinal loop, 4 (3.1%) pro-

duced fluid in adult albino rabbits at volumes ranging from 1.6 to 3.0 ml/cm intestine, 19 (15%) in adult rats, and 14 (11.1%) in swine. The variation in the fluid accumulated in the species are presented in TABLE 1. The Tukey test showed that the difference among the volumes obtained for each animal species was non significant. The McNemar test revealed that the sensitivity of the test was identical when rats and pigs were used.

Intragastric inoculation of newborn mice for the detection of enterotoxin revealed 2 suspected strains (1.5%).

Cytotoxic effects on Vero cells were observed in 12 (36.3%) intestinal fluid-inducing strains (rat-pig), with 10 to 60% of destruction of the cell monolayer at dilutions of 1/2 and 1/5. No change was observed in BHK cell cultures.

TABLE 2 shows the effects of *C. jejuni* culture filtrates according to the different methods used.

TABLE 1 - Mean amounts of accumulated intestinal fluid (ml/cm/intestine) induced by 126 cultures* of *C. jejuni in* the ligated intestinal loop of adult rabbits, rats and swine

Strain N	Rabbits**	Rats**	Swine**
10	-	0.23±0.0508	-
12	2.51±0.0230	0.20±0.0063	-
14	2.73±0.0134	0.20±0.0015	-
16	3.00±0.0417	-	-
33	2.00±0.0842	0.22±0.0144	-
114	-	-	2.46±0.0329
115	-	0.35±0.0288	-
127	-	0.35±0.0230	1.02±0.0098
131	-	0.25±0.0242	3.51±0.0490
140	-	0.22±0.0230	3.35±0.0866
141	-	0.22±0.0259	-
142	-	0.34±0.0305	2.01±0.098
145	-	0.31±0.0202	3.35±0.0866
146	-	0.23±0.0150	1.67±0.0392
149	-	0.24±0.0305	4.03±0.0173
150	-	0.21±0.0063	2.87±0.0721
157	-	0.26±0.0098	2.6±0.0415
161	-	0.22±0.0144	1.8±0.0173
164	-	0.25±0.0098	2.77±0.0144
171	-	0.20±0.0063	-
172	-	0.19±0.0132	-
174	-	-	2.62±0.0577
178	-	-	2.32±0.0196

* 122, 107 and 112 cultures did mot induce intestinal fluid production in rabbits, rats and swine, respectively.
** Mean ± S. E. M. of 3 determinations per strain.
- Strains that not produced fluid.

TABLE 2 - Effects of *C. jejuni* culture filtrates in the experiments carried out by the different methods used.

Strain N	Ligated intestinal loop of			Newborn mice	Cytotoxic effect on	
	rabbits	rats	pigs		BHK cells	Vero cells
10	-	+	-	-	-	-
12	+	+	-	-	-	-
14	+	+	-	-	-	-
16	+	-	-	-	-	-
33	+	+	-	-	-	-
114	-	-	+	-	-	-
115	-	+	-	-	-	+
127	-	+	+	-	-	-
131	-	+	+	-	-	+
140	-	+	+	-	-	+
141	-	+	-	-	-	-
142	-	+	+	+\-	-	+
145	-	+	+	-	-	+
146	-	+	+	-	-	+
149	-	+	+	-	-	+
150	-	+	+	-	-	+
157	-	+	+	-	-	-
161	-	+	+	+\-	-	+
164	-	+	+	-	-	-
171	-	+	-	-	-	+
172	-	+	-	-	-	-
174	-	-	+	-	-	+
178	-	-	+	-	-	+

(+) positive
(-) negative
(+ / -) suspect

DISCUSSION

The frequency of fluid-inducing strains (3.1%) identified in the ligated intestinal loop of the rabbit, although low, agreed with that observed by McCARDELL et al. (23) in a study of *C. jejuni* strains of human origin. There is agreement that the use of the technique of culture filtrate inoculation into the ligated intestinal loop of the rabbit for the determination of fluid production induced by *Campylobacter* should be better studied, since it is possible that the rabbit is not the most indicated animal for this type of test.

The variation in fluid production observed in the rat for the 19 (15.0%) strains was within the mean values detected by RUIZ-PALÁCIOS et al. (27) an similar to those obtained by FERNANDEZ et al. (6) and SAHA et al. (28), but lower than those reported by KLYPSTEIN & ENGERT (15). The results observed by most investigators and in the present study using the same conditions demonstrate that different *C. jejuni* strains may or may not produce fluid accumulation in the ligated intestinal loop of the rat, and when they do, the volumes are variable.

The obvious alterations in the 14 (11.1%) inoculations into the intestinal loops of swine ranged from an increase in fluid content up to congestion and hemorrhage in the intestinal wall, with the presence of hemorrhagic and viscous fluid being observed in some cases. These alterations have also been reported by JOHNSON & LIOR (12), KLIPSTEIN & ENGERT (15), McCARDELL et al. (22), and PINOCHET et al. (25) as the result of the action of the enterotoxin present in strains inducing fluid production. The fluid volumes observed ranged from 1.02 to 4.03 ml/cm intestine and could not be compared with those reported by MANNINEM et al. (20) and PINOCHET et al. (25) since these investigators did not measure them in their studies. Despite the promising results obtained, we believe that this technique needs improvement for the study of *Campylobacter* in terms of inoculum dose, ideal period of observation after inoculation and reliability of the method.

When comparing fluid-producing strains in the ligated intestinal loops of rats and swine, not all strains that were positive for rats were found to be also positive for swine and vice-versa, demonstrating that enterotoxin is not always detectable and that detection may depend on the technique employed, on factors related to the animal itself and on the susceptibility to, or quantity of toxin produced. Statistical analysis showed that the difference in the volumes obtained for each species was not significant at the level of 5% with the Tukey test. The non parametric analysis test of McNEMAR (29), to check the sensitivity of the tests showed that it was identical when rats or pigs were used.

When the production of enterotoxin was studied by the test of DEAN et al. (5), (intragastric inoculation into newborn mice), the majority of the strains tested was unable to produce sufficient amounts of enterotoxin to be considered positive, although 2 strains were considered to be suspected, because the ratio weight of intestine/weight of carcass was very close to reach the limit to be considered as positive. Negative results have also been obtained by JOHNSON & LIOR (13), MANNINEM et al. (20) and RUIZ-PALACIOS et al. (27). However, the fact that satisfactory results were not obtained by this technique does not invalidate the use of the method, since the ability to produce enterotoxin may be plasmid related or may be due to the partial loss of toxic activity in stored culture filtrates. An additional possibility is that the technique is not sensitive enough to reveal small amounts of these substances, although it has been reported to be the safest method for the detection of Sta enterotoxin in *E. coli* strains. (7).

Although the results obtained from cytotoxicity in BHK cell cultures were negative, the possibility that this type of cell will be used should not be ruled out. For Vero cells, the fluid-producing strains had a cytotoxic effect on as much as 60% of the cell carpet according to the dilution used, in agreement with the results obtained by most of the investigators who work with this cell type.

RESUMO

Produção de enterotoxina por *Campylobacter jejuni* isolada de suínos com diarréia.

Cento e vinte e seis amostras de *Campylobacter jejuni* isoladas de suínos com diarréia foram examinadas para produção de enterotoxina, através da inoculação do filtrado de cultura, em alça ileal de várias espécies de animais.

Quatro (3.1%) das amostras testadas produziram fluidos intestinais em alças ligadas de coelhos albinos, 19 (15%) em ratos albinos e 14 (11,1%) em suínos. Pelo teste a inoculação intra-gástrica em camundongos rescém-nascidos nenhuma das amostras revelou capacidade de produção da toxina embora duas tenham sido consideradas suspeitas. Doze (54,5%) das amostras indutoras da produção de fluido intestinal

em ratos adultos e em suínos produziram efeito citotóxico em células renais de macaco (Vero) afetando mais de 60% do total das células . No entanto, não foram observadas em células renais de hamsters (BHK).

Palavras-chave: *Campylobacter jejuni*, enterotoxina, diarréia suína, patogenicidade.

REFERENCES

1. BLASER, M.J. et al. - *Campylobacter* enteritis clinical and epidemiological features. *Ann. Inter. Med.* **91**:179-85, 1979
2. BRADBURY, W.C.; MUNROE, D.L.G. - Ocurrence of plasmid and antibiotic resistance among *Campylobacter jejuni* and *Campylobacter coli* isolated from healthy and diarrheic animals. *J. Clin. Microbiol.* **22**:339-46, 1985.
3. BUTZLER, J.P. ; SKIRROW, M.B. - *Campylobacter enteritis Clin. Gastroenterol.* **8:**737-65, 1979.
4. CHO, H.H. et al. Biotype, serotype and drug susceptibility of thermophilic *Campylobacter* isolated from diarrheal animal feces. *Korean J. Vet. Pub. Health* **11:**25-31, 1987
5. DEAN, A.G. et al. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. *J. Infec. Dis.* **125:**407-11, 1972
6. FERNANDEZ, H. et al. - Culture supernatants of *Campylobacter jejuni* induce a secretory response in jejunal segments of adults rats. *Infect. Immun.* **40:**429-31, 1983.
7. GIANELLA, R.A. Suckling mouse model for detection heatstable *Escherichia coli* enterotoxin characteristics of the model. *Infect. Immun.* **14**:95-9, 1976.
8. GOOSENS, H.; DE BOECK, M.; BUTZLER, J.P. A new selective medium for the isolation of *Campylobacter jejuni* from human faeces. *Eur. J. Clin. Microbiol.* **2:**389-94, 1983.
9. GORGEM, M. et al. - Ocurrence of *Campylobacter* spp in swine.
   I - Cultural investigations on faeces intestinal contents and gall bladder and infection experiments. Berlinger und Munchener Tierarztliche Wochenshrift , (1983), 96(3):86-89. *Apud Vet. Bull.*, v.**53**, n8, p. 723, 1983. (n. de ref. 5150).
10. GUERRANT, R. et al. Campylobacteriosis in man: pathogenic mechanisms and review of 91 bloodstreams infections. *Am. J. Med.*, v.**65**, p.584-92,1978.
11. GYLES, C.L. Heat-labile and heat-stable forms of the enterotoxin from *E. coli* strains enteropathogenig for pigs. *Ann. N.Y. Acad. Sci.* **176:**312-22, 1971.
12. JOHNSON,W.M.; LIOR, H. - Toxins produced by *Campylobacter jejuni* and *Campylobacter coli*. *Lancet* **1:**229-30, 1984.
13 .....Cytotoxic and cytotonic factors produced by *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter laridis*. *J. Clin. Microbiol.* **24:**275-81, 1986.
14. .....A new heat labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp *Micro. Pathol.* **4**:115-26, 1988.
15. KLIPSTEIN, F.A. et al., ENGERT, R.F. Properties of crude *Campylobacter jejuni* heat-labile enterotoxin . *Infect. Immun.* **45:**314-19, 1984.
16. KLIPSTEIN, F.A. et al. - Pathogenic properties of *Campylobacter jejuni*: assay and correlation with clinical manifestations. *Infect. Immun.* **50:**43-9, 1985.
17. LINDBLOM, G.B. et al. - Enterotoxigenicity and frequency of *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter laridis* in human and animal stool isolates from differentcountries. FEMS *(Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* **66:**163-8, 1990.
18. LINDBLOM, G.B. et al. Enterotoxin production and serogroup of *Campylobacter jejuni* and *Campylobacter coli* from patients with diarrhea and from health laying hens. *J. Clin.Microbiol.* **27:**1272-6, 1989.
19. LUECHTEFELD, N.W. Animal reservoirs of *Campylobacter jejuni*, In Newell,B.G. ed. *Campylobacter epidemiology pathogenesis and biochemistry.* Lancaster MTP Press, p.249-251, 1982.
20. MANNINEM, KI et al. Pathogenicity of *Campylobacter jejuni* isolated from animals and humans. *Infect. Immun.* **38**:46-52, 1982.
21. MATHAN,V.I. et al. Enterotoxigenic *Campylobacter jejuni* among children in South India. *Lancet*, v. **27**:981, 1984.
22. McCARDELL, B.A. et al. Production of cholera like toxin by *Campylobacter jejuni/coli*. Lancet. 1:448-9, 1984a.
23. McCARDELL, B.A. et al. *Campylobacter jejuni* and *Campylobacter coli* production of a cytotonic immunologically similar to cholera toxin *J. Food Prot.* **47:**943-49, 1984b.
24. NETER,J. & WASSERMAN, W. *Applied linear statistical model: regression, analysis of variance experimental designs.* Homewood, Illinois: Richard D. Irwin Inc. 1974, 842p.
25. PINOCHET, L.V. et al. Estudio preliminar de la enteropatogenicidade de *Campylobacter coli* en cerditos lactantes. *Avances en Ciencias Veterinárias* **3:**106-8, 1988.
26. RIZZO, E.; TUCHIYA, H.N.; MARTINEZ, C.H. *Técnicas básicas de cultura celular.* Instituto Butantã, São Paulo, p. 133, 1983.
27. RUIZ-PALACIOS, G.M. et al. Cholera like enterotoxin produced by *Campylobacter jejuni* . *Lancet* **2**:250-52, 1983.
28. SAHA, S.K.; SINGH, N.P.; SANYAL, S.C. Enterotoxigenicity of chicken isolates of *Campylobacter jejuni* in ligated ileal loops of rats . *J. Med. Microbiol.* **26:**87-91, 1988.
29. SIEGEL, S. *Estatística não paramétrica para as ciências do comportamento.* ed. McGraw-Hill do Brasil, Ltda., 1981, p.69-74.
30. SKIRROW, M.B. *Campylobacter enteritis* in a "new"disease *Br. Med.J.*, **2**:9-11, 1977.
31. SKIRROW, M.B. ; BENJAMIN, J. "1001" *Campylobacters*: cultural characteristics of intestinal *Campylobacters* from man and animals. *J. Hyg.* **85**:427-42, 1980.
32. SMIBERT, R.M. The genus *Campylobacter* . *Ann. Rev. Microbiol.* **32**:673-709, 1978.
33. SMIBERT, R.M. Aerobic/microaerophilic, motile, helical/vibroid gram-negative bacteria genus *Campylobacter* . Apud *Bergeys manual of systematic bacteriology,* Baltimore, Williams and Wilkins, v.1, sect. 2, p. 111-18, 1984.
34. STICHT-GROW, V. *Campylobacter* in healthy slaughter pigs: a possible source of infection for man. *Vet. Rec. 110:*104-06, 1982.
35. SVEDHEM, A. ; KAIJSER, B. Isolation of *Campylobacter jejuni* from domestic animal and pets: probable origin of human infection. *J. Infec.* **3**:37-40, 1981.
36. TAYLOR, J. ; SANGSTER, N; LANSER, J.A. Factor influencing the response of ligated rabbit-gut segments to injected *Escherichia coli* . *J. Path. Bacteriol.* **76**:491-9, 1958.
37. TURKSON, P.K.; LINDOVISI, K.J.; KAPPERUD, G. Isolation of *Campylobacter* spp and *Yersinia enterocolitica* from domestic animals and human patients in Kenya. *APMIS (Acta Pathol. Microbiol. Immunol. Scand.)* **96:**141-46, 1988.

Rev. Microbiol., São Paulo, 24(2): 84-87, 1993

ISOLATION OF *CAMPYLOBACTER JEJUNI* FROM DOGS WITH DIARRHEA

Elsa Masae Mamizuka[1]
Denise Saretta Schwartz[2]
Maria de Fátima Borges Pavan[1]
Mitika Kuribayashi Hagiwara[2]

SUMMARY

Thermophilic *Campylobacter jejuni* has been considered as a possible etiologic agent of diarrhea in dogs, but data presented in most reports are conflicting. To assess the role of *C. jejuni* as an enteropathogen, fecal samples from healthy and from diarrheic dogs were analysed and attempts to isolate the microorganism were made. Seventeen strains of *C. jejuni* biotype 1 were isolated from 100 fecal samples; eleven out of fifty-nine diarrheic feces and six out of forty-one non-diarrheic feces were positive for that microorganisms. The isolation rate was higher in dogs up to six months of age. There was no difference between the isolation rate from diarrheic and non-diarrheic dogs. It has not been possible to imput *Campylobacter* as the main pathogen in dogs with diarrhea, since 70% of positive cultures for this bacterium showed another enteropathogenic agent concomitantly. Independently of being or not the etiological agent of diarrheal disease in dogs, we must emphasize the role of this animal as a possible source of infection to human beings.

Key words: *Campylobacter* sp; thermophilic *Campylobater* ; dogs; diarrhea.

INTRODUCTION

Besides other microorganisms such as *Salmonella* sp; *Vibrio* sp and enteropathogenic *E. coli*, the worldwide distributed thermophilic species of *Campylobacter* (*C. jejuni*/ *C. coli*) are recognized nowadays as causes of diarrhea in human and domestic animals (3, 5, 6, 11, 13). Human infections occur by the ingestion of contaminated water or food such as fresh milk, pork or avian meat and also, by direct contact with birds, swine, dogs and cats (2, 8). Like humans, domestic pets are susceptible to *C. jejuni* and *C. coli* infections that lead to either assymptomatic carriage or acute gastroenteritis. Although those microorganisms had been considered as causes of diarrhea in dogs (9), the available data are conflicting and do not allow a definite conclusion about their pathogenicity. According to Hosie et al. (10) and Bruce et al. (3), the prevalence of *Campylobacter* infections is almost the same in healthy and sick dogs. Some evidences suggest that *Campylobacter* might be an opportunistic pathogen (11, 12, 15, 17), found in association with other known infectious agent such as parvovirus, coronavirus, paramyxovirus, entheropathogenic bacteria like *Salmonella* sp or intestinal parasites.

The aim of this study is to evaluate the prevalence of *C. jejuni* and *C. coli* in owned healthy dogs and dogs with enteritis in order to assess their role as

1 Department of Clinical and Toxocological Analysis. Faculty of Pharmaceutical Sciences, University of Sao Paulo, SP, Brasil.
2 Faculty of Veterinary Medicine and Zootechny - Botucatu
3 Faculty of Veterinary Medicine, University of Sao Paulo

a pathogen.

MATERIAL AND METHODS

Fecal specimens were obtained from dogs from 1 month to 3 years age with or without diarrhea examined at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine and Zootechny at the University of São Paulo. Fresh samples were introduced into Cary & Blair medium, in a proportion of three parts of medium to one part of feces, and sent to the Laboratory of Clinical Microbiology from the Department of Clinical and Toxicological Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo and processed within twelve hours.

Fifty nine samples were obtained from dogs with diarrhea and forty one from healthy dogs.

The isolation of *C. jejuni/coli* was performed made according to the methodology described elsewhere (14). Briefly, stool specimens were inoculated into a selective plate medium (blood agar base plus vancomycin, polymyxin B, trimethoprin, cefalotin and amphotericin B, enriched with hemine, sodium pyruvate, sodium metabissulfite and ferrous sulphate). Plates were incubated at 42ºC for 48 hours in a candle jar (microaerophilic atmosphere) and examined at 24 and 48 hours. Grey, small mucoid non hemolytic flat colonies were Gram stained. If the Gram stained smears showed vibrio forms, further isolation and biochemical tests were carried out. Organisms were considered *C. jejuni/coli* when they were oxidase and catalase positive, grew at 37º and 42ºC but not at 25ºC, and were susceptible to nalidixic acid (13). For the differentiation of species and biotypes, sodium hipurate hydrolysis and capacity of producing H_2S in Kligers medium (16) were used. Pathogenic enterobacteria were searched for using the methodology described by Ewing (4). The test of two proportions was used for statistical analysis, according to Goldstein (7) with alfa = 0, 05.

RESULTS

Eleven samples belonging to the group of dogs with diarrhea (18, 6%) and six samples coming from non diarrheic dogs (14, 6%) were positive for *C. jejuni* biotype 1 as identified by biochemical profile and susceptibility to nalidixic acid (table 1). There was no statistical difference between the prevalence of infection in both groups. As observed in table 2, the frequency of *C. jejuni* was higher among dogs up to six months of age in both groups studied (28, 6% and 27, 7%, in diarrheic and non diarrheic dogs, respectively). Among diarrheic dogs (table 3) other causes of diarrhea were found in association to *Campylobacter*: *Ancylostoma caninum* and parvovirus infection in three samples each one, paramyxovirus (distemper virus) (one sample) and Coccidia (one sample). In the remaining three cases no other causes of diarrhea could be identified. No enteropathogenic bacteria were isolated from diarrheic or healthy dogs.

TABLE 1 - Isolation of *Campylobacter jejuni* from feces of dogs with diarrhea and healthy dogs. São Paulo, 1992.

Campylobacter jejuni Isolation	Nº (%) of dogs	
	healthy	diarrheic
Positive	06 (14,6)	11 (18,6)
Negative	35 (85,4)	48 (81,4)
Total	41 (100)	59 (100)

TABLE 2 - Isolation of *Campylobacter jejuni* from healthy dogs and dogs with diarrhea, according to age. São Paulo, 1992.

AGE (months)	Nº (%) OF DOGS			
	DIARRHEIC		NON-DIARRHEIC	
	Positive	Total	Positive	Total
0 - 6	10 (28,6)	35	05 (27,7)	18
> 6	01 (4,2)	24	01 (4,3)	23

TABLE 3 - Concurrent infection found in diarrheic dogs bearing *Campylobacter jejuni*. São Paulo, 1992.

Dog nº	Age	Pathogen
1	3m	parvovirus
2	2m	parvovirus
3	3m	*Isospora sp*
4	3m	Paramyxovirus
5	3m	None
6	4m	*Ancylostoma sp*
7	2m	None
8	1m	*Ancylostoma sp*
9	1m	*Ancylostoma sp*
10	3m	Parvovirus
11	3y	None

m= months, y = years

DISCUSSION

As mentioned by other authors (1, 3, 11) only *C. jejuni* biotype 1 was isolated from feces of dogs, but the magnitude of isolation was lower than compared to those reported by Bruce et al. (3) and Jaramillo (11). This difference could be explained by the fact that almost all dogs studied were owned individually, thus having less opportunity to acquire the infection by means of direct contact. *C. jejuni* was not considered a primary cause of diarrhea in dogs, since the isolation rate was almost the same in both, healthy and sick dog groups. Moreover, about 70% of the diarrheic dogs from which *C. jejuni* was isolated had presented concurrent viral or parasitic infections.

The role of *Campylobacter* as a pathogenic microorganism may be emphasized in dog nº 3 (Table 3). The dog had presented a recurrent colitis lasting three weeks. Besides oocysts of Coccidia *(Isospora* sp), a simultaneous infection by *C. jejuni* was evidenced. After seven days of treatment with eritromycin the dog was recovered and new attempt to isolate the microorganism was unsuccessful although oocysts of *Isospora* sp were still present in the sample.

Another aspect that needs to be pointed out is concerned to the role of companion animals as a source of infection to their owners. Keeping this possibility in mind, *C. jejuni* should be included for the differential diagnosis in cases of acute or chronic diarrhea in dogs and an appropriate therapy should be indicated when isolation attempts are successful.

RESUMO

Isolamento de *Campylobacter* de fezes de cães com diarréia.

Embora as espécies termofílicas de *Campylobacter (C. jejuni/C.coli)* possam ser consideradas como possíveis agentes etiológicos de diarréia em cães, os dados existentes na literatura são conflitantes. Assim, objetivou-se, neste trabalho, verificar o papel desempenhado por esse agente como causa de diarréia em cães, pesquisando-o no material fecal de animal normais e de cães com diarréia. Das 100 amostras analisadas, foram isoladas 17 cepas de *C. jejuni* biotipo 1, na proporção de 18,6% (11/59) de isolamento no grupo com diarréia e 14, 6% (6/41) no grupo de animais normais, tendo sido os animais da faixa etária de 0 a 6 meses os mais frequentemente acometidos. Não foi possível caracterizar-se *C. jejuni* como patógeno primário entre os cães, uma vez que em cerca de 70% dos casos positivos no grupo de animais diarréicos essa bactéria foi encontrada concomitantemente com outro agente enteropatogênico. Porém, independentemente da sua condição de agente etiológico de diarréias em cães, deve-se enfatizar o papel desses animais como possível fonte de infecção humana.

Palavras-chave: *Campylobacter* sp, *Campylobacter* termofílico, cães, diarréia.

REFERENCES

1. BLASER, M.J.; POWERS, B.W.; CRAVENS, J. & WANG, W.L. - *Campylobacter* enteritis associated with canine infection. *Lancet,* **2**:979-81, 1978.
2. BLASER, M.J.; WEISS, S.H.; BARRTT, T.J. *Campylobacter* enteritis associated with a healthy cat. *J.Amer. Med. Associat.*, **247:**816, 1982.
3. BRUCE, D.; ZOCHOWSKI, W. FLEMING, G.A. *Campylobacter* infection in cats and dogs. *Vet. Rec.* **107:**:201-2, 1980.
4. EWING, W.H. - *Edward's and Ewing's identification of Enterobacteriaceae* 4.ed. Elsevier Science Publishing Co, New York, 1986.
5. FOX, J.G.; KRAKOWKA, S; TAYLOR, N.S. Acute onset of *Campylobacter* associated gastroenteritis in adult beagles. *J.Am.Vet.Med.Assoc.,* **187:**1268-70, 1985.
6. FOX, J.G.; MOORE, R.; ACKERMAN, J.I. *Campylobacter*

jejuni-associated diarrhea in dogs. *J.Am.Vet.Med. Associat.*; **183:**1430-33, 1983.

7. GOLDSTEIN, A. - *Biostatistics: an introductory text.* 2. ed. New York, McMilan, 1965.
8. GRANT, I.H.; RICHARDSON, N.J.; BOKKENHEUSER, V.D. Broiler chicken as potential source of *Campylobacter* infections in humans. *J.Clin.Microbiol.*. **11:**508-10, 1980.
9. HASTINGS, D.H. - Canine and feline enteritis associated with progressively motile bacteria in fresh fecal samples. *Vet. Med. Small Anim.Clin.*, **74:**1113-16, 1979.
10. HOSIE, B.D.; NICHOLSON, T.B.; HENDERSON, D.H. - *Campylobacter* infection in normal and diarrhoeic dogs. *Vet. Rec.*, **105;** 80, 1979.
11. JARAMILLO, H.F. - *Espécies termofílicas de Campylobacter: aspectos bacteriológicos, epidemiológicos e patogênicos.* Tese. Escola Paulista de Medicina., São Paulo, 1983.
12. NAJAR, G.P.S. - *Campylobacter* enteritis in dogs and cats (correspondence) *Canad. Vet. J.*, **21:**13-19, 1980.
13. PAVAN, M.F.B.; MAMIZUKA, E.M.; MARTINEZ, M.B. *Campylobacter* termofilico em fezes de crianças com diarréia: estudo com controles. *Rev.Microbiol.* **18**:243-48, 1987.
14. PAVAN, M.F.B.; MAMIZUKA, E.M.; MARTINEZ, M.B. - Diagnóstico rápido (presuntivo) de diarréia aguda causada por *Campylobacter* sp termofílico *Rev. Farm. Bioq. Univ. S.Paulo*, **23:**152-58, 1987.
15. SKIRROW, M.B. *Campylobacter* enteritis: a new disease *Brit.Med.J.*, **2:**9-11, 1977.
16. SKIRROW, M.B.; BENJAMIN, J. - Differentiation of enteropathogenic *Campylobacter*. *J. Clin. Path.*, **33**:1122, 1980.
17. SMIBERT, R.M. - *Campylobacter* In: *Bergey's Manual of Systematic Bacteriology*. Krieg & Holt S.G. eds., 1984, p.111-18.

Received for publication in 21/08/92

Rev. Microbiol., São Paulo, 24(2): 88-93, 1993

PROTICINE TYPING OF *PROTEUS MIRABILIS* STRAINS

Ana Terezinha Tavechio*
Tania Mara Ibelli Vaz*
Sonia Shizue OkitaBuschinelli*
Sueli Aparecidsa Fernandes*
Chifumi Takeushi Calzada*
Kinue Irino*

SUMMARY

A total of 250 *Proteus mirabilis* strains (200 isolated from urine, 31 from cerebrospinal fluid and 19 from blood) were examined for production of bacteriocine spontaneously and induced by mitomycin C. The proticine types were determined by Senior's typing scheme. By using the two methods of proticine detection, 90.40% of the strains examined were identified as producers and typing was done on 83.63% of these strains. Types 1, 2 and 3 were the most frequent among the 10 types described in the scheme followed. Two additional types (designated 14 and 15) were identified among the strains studied. The bacteriocine typing using mitomycin C induction should be used in epidemiological studies of nosocomial infections caused by *P.mirabilis* as well as other species of this genus.

Keywords: *Proteus mirabilis,* bacteriocine, proticine typing

INTRODUCTION

Bacteria of the genus *Proteus* have been associated with cases of urinary tract infections, septicemia, meningitis and osteomyelitis in children as well as in adults (4, 5, 21). These infections still represent serious risks to hospitalized patients because of the natural and easily acquired resistance of *Proteus* spp to antimicrobial agents (19). *P. mirabilis* has been the most frequently associated species, mainly for urinary tract infections (10,12,18).

Infections by *Proteus* spp may have an exogenous or endogenous origin and the characterization of strains through specific markers is essential for the study of nosocomial infections. Among several reported methods concerning strains differentiation, bacteriocine typing is one of the simplest available.

The first proticine typing scheme was developed by Cradock-Watson (6) in 1965 and consisted in determining the sensitivity patterns of 18 indicator strains for spontaneously produced proticines from *Proteus* . By this scheme, 139 (60.70%) out of 229 *P. mirabilis* and 1 out of 10 *P. vulgaris* strains were found to produce bacteriocines which were classified in 26 distinct types.

In 1975, Al-Jumaili (2) established a proticine

*Secção de Bacteriologia - Instituto Adolfo Lutz - Av. Dr. Arnaldo, 351 - 9º andar - São Paulo - CEP 01246.902

typing system based on the sensitivity of *P. mirabilis* and *P. vulgaris* strains to 12 proticine preparations which consisted in supernatants from cultures exposed to mitomycin C. None of the 1805 strains examined by this system was found to be untypable and 48 different sensitivity patterns were found.

In the following year, Senior (17) developed a combined typing system based on proticine production and sensitivity using, respectively, 14 indicator strains and 14 proticine-producing strains that were selected among Cradock-Watson's indicator strains and 250 strains examined against the former ones. A total of 90 distinct types were identified by this scheme.

The purpose of this study was to type *Proteus mirabilis* strains isolated from urine, blood and cerebrospinal fluid cultures, using the spontaneous proticine-producing method and proticine production after mitomycin C induction.

MATERIALS AND METHODS

Media - All from Difco, dissolved in distilled water, adjusted to pH 7.4 and sterilized by autoclaving:

1) Conservation medium - 10g/l peptone, 5g/l NaCl, 4g/l meat extract, 12 g/l agar;

2) Tryptone water - 10g/l tryptone, 5 g/l NaCl;

3) Proteose - peptone nº3 (PP3) - 20g/l PP3, 5g/l NaCl

4) Nutrient broth - 5g/l peptone, 1g/l meat extract, 2g/l yeast extract, 5g/l NaCl.

5) McConkey agar - 50 g/l of the powder were rehydrated. This medium was used throughout these experiments as it prevented spreading and was found to be suitable for bacteriocine production (06,19).

Bacterial strains - A total of 250 strains of *Proteus mirabilis* were studied: 200 isolated from urine during the period of 1985-1987, 19 isolated from blood (1973-1987) and 31 isolated from cerebrospinal fluid (1973-1987) in the Bacteriology section of the Instituto Adolfo Lutz , São Paulo, Brazil. The presumptive identification of the strains were done in IAL medium (15) and the final biochemical identification was performed by the methods described by Edwards and Ewing (7) and the Bergey's Manual of Systematic Bacteriology(13).

The standard set of 24 proticine-indicator strains isolated by Cradock-Watson (6) were received from the Central Public Health Laboratory, Colindale, London.

All bacterial strains were stored on Conservation medium, at room temperature. Their reactivation was done on Nutrient broth (18 h, 37ºC) and then, just one well isolated colony of each strain was picked-up from McConkey agar and used in all tests.

Spontaneous proticine production - To verify the spontaneous production of proticine (6,17), one colony from a young culture on MacConkey agar of each strain was inoculated in 1% Tryptone water and incubated at 37ºC for 18 hours. After this incubation period, each strain was streaked across the surface of McConkey agar in glass Petri dishes and incubated at 37ºC for 18 hours. The growth (central streak) was removed and the plates were sterilized by chloroform vapor (drenched in filter paper and put inside Petri dish lid) for 10 minutes, which was eliminated by keeping the plates open for 30 minutes in the incubator. Cultures (3h at 37ºC) of 24 indicator strains in 1% Tryptone-water were streaked across these plates (8 strains by plate) at right angles to the lines of first streak and incubated at 37ºC for 18-24 hours.

Induced proticine production - All non-spontaneous proticine-producer strains were treated with mitomycin C (Sigma) by Al-Jumaili's method (2). These strains were cultured in 1 ml of PP3 for 18h at 25ºC. After addition of 9 ml of PP3 and an incubation period of 1 h at 25ºC with shaking, mitomycin C was added in the final concentration of 1 µg/ml and the cultures were further incubated for 18-24 hours in the same conditions. The cultures were then centrifuged at 3000 rpm for 20 minutes and the supernatants were sterilized by chloroform in a 1% final concentration for 5 minutes, centrifuged again and kept at 4ºC.

To verify the proticine presence after mitomycin C induction, the 24 indicator strains were cultured in 1% Tryptone-water for 2 h at 37ºC and flooded over the surface of MacConkey agar plate. After incubation at 37ºC for 30 minutes, 15µl of each supernatant were droped on the agar surface (18 supernatants/plate), dried at room temperature and incubated for 18h at 37ºC.

Some supernatants produced zone of lysis resembling the ones caused by phage action, i.e., plaque formation inside or at the edge of the zone. To confirm the phage action on these supernatants a methodology

TABLE 1 - Cradock-Watson (CW) scheme of Proticine typing of *Proteus* strains by their spectrum of inhibitory activity against 24 standard indicator strains.

Proticine type of producer strain	CW Standard producer strain nº	Inhibition of CW indicator strain nº																							
		175	664	794	816	337	784	537	823	1	11	52	712	106	122	261	276	4	380	384	792	335	565	630	818
1	535/A	+	+	+	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
2	362	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
3	977	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
4	404/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
5	867	-	-	-	-	-	-	-	-	-	-	+	-	±	+	+	+	-	-	-	-	-	-	-	-
6	237	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	677	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	+
8	927	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	516	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	349	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-
*14	?	±	-	+	+	+	-	±	-	±	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-
*15	?	+	-	-	-	-	+	+	-	-	+	-	+	+	+	+	+	+	-	+	+	-	-	+	±

* = New proctine type; + = Inhibition of indicator; ± = Partial or variable inhibition; - = no inhibition of indicator

described by Vieu (20) was applied with some modification as follows: the material from the zone of lysis was inoculated in Nutrient Broth and incubated at 37°C under shaking till the broth became cloudless. After centrifugation, the supernatants were treated with toluene, submitted to 10-fold serial dilution and streaked on McConkey agar plate which had been flooded with a culture of the respective indicator strain. The bacteriophage presence was confirmed by plaques of lysis along the streaks.

Classification of strains by proticinogenic types - To classify in proticinogenic types, the inhibition spectrum of the strains under test for the indicator strains were compared with Cradock-Watson scheme adapted by Senior (17) as described on table 01.

RESULTS

A total of 137 (54.80%) strains included in this study produced proticine spontaneously (Table 2). Among the remaining 113 strains, 89 (78.76%) were identified as proticine producers after mitomycin C induction (Table 3) . The supernatants that demonstrated phage action were not scored for proticine production. With the two methods, a total of 226 strains (90.40%) were identified as proticinogenic.

Among the 226 proticinogenic strains, 189 (83.63%) were classified in distinct types. Of 125 strains distributed among 08 of the 10 proticine types described by Senior (17), 64 strains had distinct proticine patterns (designated here as 14 and 15) and 37 did not fit in any distinct pattern.

TABLE 2 - Frequency of spontaneous proticine production according to the source of P. mirabilis strains.

Source of strains	Number of strains	Number of strains with proticine production	
		Negative	Positive
Urine	200	90 (45.00%)	110 (55.00%)
CSF*	31	15 (48.39%)	16 (51.61%)
Blood	19	8 (42.10%)	11 (57.89%)
Total	250	113 (45.20%)	137 (54.80%)

* Cerebrospinal fluid

TABLE 3 - Frequency of induced proticine production according to the source of P. mirabilis strains.

Proticine types (Cradock-Wat Watson)	number of strains	number of strains isolated from:		
		Urine	CSF*	Blood
1	60 (31.74%)	55 (35.71%)	3 (14.29%)	2 (14.28%)
2	17 (8.99%)	14 (9.10%)	2 (9.53%)	1 (7.14%)
3	25 (13.22%)	16 (10.39%)	5 (23.80%)	4 (28.58%)
4	14 (7.41%)	12 (7.80%)	2 (9.53%)	-
5	-	-	-	-
6	3 (1.59%)	2 (1.30%)	1 (4.76%)	-
7	1 (0.53%)	1 (0.65%)	-	-
8	-	-	-	-
9	1 (0.53%)	-	-	1 (7.14%)
10	4 (2.12%)	4 (2.59%)	-	-
14**	50 (26.46%)	40 (25.97%)	5 (23.80%)	5 (35.72%)
15**	14 (7.41%)	10 (6.49%)	3 (14.29%)	1 (7.14%)
None	37 (16.37%)	29 (15.84%)	5 (19.23%)	3 (17.64%)

* Cerebrospinal fluid

TABLE 4 - Number and frequency of the proticine types according to the source of P. mirabilis strains

Proticine types (Cradock-Watson)	number of strains	number of strains isolated from:		
		Urine	CSF*	Blood
1	60 (31.74%)	55 (35.71%)	3 (14.29%)	2 (14.28%)
2	17 (8.99%)	14 (9.10%)	2 (9.53%)	1 (7.14%)
3	25 (13.22%)	16 (10.39%)	5 (23.80%)	4 (28.58%)
4	14 (7.41%)	12 (7.80%)	2 (9.53%)	-
5	-	-	-	-
6	3 (1.59%)	2 (1.30%)	1 (4.76%)	-
7	1 (0.53%)	1 (0.65%)	-	-
8	-	-	-	-
9	1 (0.53%)	-	-	1 (7.14%)
10	4 (2.12%)	4 (2.59%)	-	-
14**	50 (26.46%)	40 (25.97%)	5 (23.80%)	5 (35.72%)
15**	14 (7.41%)	10 (6.49%)	3 (14.29%)	1 (7.14%)
None	37 (16.37%)	29 (15.84%)	5 (19.23%)	3 (17.64%)

* - Cerebrospinal fluid
** - New proticine types

As shown in Table 4, the most frequent proticine type among urine culture isolates was the type 1 (35.71%) followed by type 3 (10.39%). The type 3 (23.80%) and type 1 (14.29%) were the most common among the cerebrospinal fluid isolates. Among the blood culture isolates the type 3 (28.58%) and 1 (14.28%) were the most common.

Among 39 spontaneous-proticine-producer strains with "atypical" sensitivity patterns according to Senior's scheme, 34 (87.17%) were classified after mitomycin C induction.

DISCUSSION

Although nosocomial infections due to bacteria of the genus Proteus represent an important therapeutic problem due to the facility that these microorganisms acquire multiple resistance to antimicrobial agents, little attention has been done to the epidemiological study of these infections (1). The identification of the source and dissemination ways of these infections have great impact on the control measures that can be applied in a hospital environment (4,5).

Different typing methods have been used in the differentiation of P. mirabilis strains (2, 3, 6, 9, 11, 14, 16). However, some of them, such as biotyping (8,16) and phage typing (8) have been less used because their limited epidemiologic value, low specificity or small number of types determined. Bacteriocine typing has also been less used due to the low percentage of strains that produce proticine spontaneously.

In the present study, the use of the spontaneous and the mitomycin C induction methods allowed the detection of proticine production in 90.40% of the strains examined. The fact that this percentage is much higher than the one obtained with only the spontaneous method (54.80%) and that the induction method allowed typing of more strains indicates that mitomycin C induction seems to be a necessary step on the bacteriocine typing process for P. mirabillis.

Although the use of mitomycin C for the detection of proticine has been questioned because possible bacteriophage induction (17), it was possible to differentiate the proticine action from the phage one.

Among the 10 proticine types described by Senior (17), 8 were detected in the present study. Two different types were identified among the strains studied and they were designated types 14 and 15 (Table 1), because Senior had already mentioned the existence of 3 additional types in his scheme. However, he did not describe the pattern given by these types.

The proticine types 1, 2 and 3 seem to be more frequently associated with urinary infections as well as to septicemia and meningitis in our environment. The proticine type 14 also seems to be frequently associated to strains isolated from these infections.

P. mirabilis producers of proticine type 3 are, according to Senior (18), the most frequently type associated to severe and persistent infections of upper urinary tract. However, strains belonging to proticine type 1 (35.71%) were the most frequent among the isolates from urine examined in this study. Proticine

type 3 was the second most frequent among these isolates. It is possible that this difference is due to the fact that almost all strains were isolated from patients not hospitalized. On the other hand, the proticine type 3 was the most frequent among strains isolated from blood (22.73%) and cerebrospinal fluid (30.77%).

The bacteriocine typing system described here is relatively simple and should be used in studies of nosocomial infections by *P. mirabilis,* as well as for other species of the genus *Proteus.*

RESUMO

Proticinotipagem de cepas de *Proteus mirabilis*

Um total de 250 cepas de *Proteus mirabilis* (200 isoladas de urina, 31 de líquido cefalorraquidiano e 19 de sangue) foram examinadas para produção de bacteriocina, espontaneamente ou induzida por mitomicina C. Os proticinotipos foram determinados pelo esquema de Senior. Com a utilização conjunta dos 2 métodos de detecção de proticinas, 90,40% das cepas examinadas foram identificadas como produtoras e 83,63% destas foram tipáveis. Os tipos 1,2 e 3 foram os mais frequentes entre os 10 descritos no esquema de Senior. Dois tipos adicionais (designados 14 e 15) foram identificados entre as cepas estudadas. A bacteriocinotipagem pela produção espontânea e indução com mitomicina C poderá ser usada em estudos epidemiológicos de infecções nosocomiais causadas por *P. mirabilis*, assim como por outras espécies do gênero.

Palavras-chave: *Proteus mirabilis*, bacteriocina, proticinotipagem.

REFERENCES

1. ADLER, J.L.; BURKE, J.P.; MARTIN, D.F. and FINLAND, M. - *Proteus* infections in a general hospital. II. Some clinical and epidemiological characteristics with an analysis of 71 cases of *Proteus* bacteremia. *Ann.Inter.Med.*, **75:**531-536, 1971.
2. AL-JUMAILI, I.J. - Bacteriocine typing of *Proteus. J. Cli. Path.*, **28**:784-787, 1975 a.
3. AL-JUMAILI, I.J. - An evaluation of two methods of bacteriocine typing of organisms of the genus *Proteus. J. Clin. Path.* **28**:788-792, 1975 b.
4. BURKE, J.P.;INGALL, D.;KLEIN; J.O.; GEZON, H.M. and FINLAND, M. - *Proteus mirabilis* infections in a hospital nursery traced in a human carrier. *N. Engl. J. of Med,* **284:**115-121, 1971.
5. CHOW, A.W.; TAYLOR, P.R.; YOSHIKAWA, T.T. and GUZE, L.B. - A nosocomial outbreak of infections due to multiply resistant *Proteus mirabilis* . Role of intestinal colonization as a major reservoir. *J. Infec. Dis.* **139**:621-627, 1979.
6. CRADOCK-WATSON, J.E. - The production of bacteriocine by *Proteus* species. *Zbl.Bakt. Hyg. I Abt. Orig. A.* **196**:385-388, 1965.
7. EDWARDS, P.R. and EWING, W.H. - *Identification of Enterobacteriaceae.* 4th. ed. Atlanta - Georgia, 1986.
8. FRANCE, D.R. and MARKHAM,N.P. - Epidemiological aspects of *Proteus* infections with particular reference to phage typing. *J. Clin.Path.***21:**97-102, 1968.
9. KUSEC, J.W. and HERMAN, L.G. - Typing of *Proteus mirabilis* by bactericin production and sensitivity as a possible epidemiological marker. *J. Clin. Microb.* **12:** 112-120, 1980
10. LECOMTE, F.; GRISE, G.; MOREL,A.; LEMELAND, J.F. - Infection des voies urinaires en pratique medicale de ville. Etude des donnees cliniques, de la repartition des especes microbiennes responsables et de leur sensibilite a seize antibiotiques. *Path. Biol.* **34:**483-489,1986
11. LOUVOIS, J. - Serotyping and the Dienes reaction on *Proteus mirabilis* from hospital infections. *J. Clin. Path.*, **22:**263-268, 1969.
12. MUSHER, D.M.;GRIFFITH, D.P.;YAWN, D.and ROSSEN, R.D. - Role of urease in pyelonephritis resulting from urinary tract infection with *Proteus. J. Infect. Dis.*,**131:**177-180, 1975.
13. PENNER,J.L. - *Genus Proteus in Bergey's Manual of Systematic* Bacteriology, Vol. 1, Krieg, N.R. and Holt, J.G. (ed) Williams & Wilkins, Baltimore/London, 1984 pp 491-494.
14. PENNER, J.L. and HENNESSY, J.N. - Separate O-Grouping schemes for serotyping clinical isolates of *Proteus vulgaris* and *Proteus mirabilis. J.Clin.Microbiol.* , **12:**304-309, 1980.
15. PESSOA, G.V.A. et SILVA, E.A.M. - Milieu pour la identification presomptive rapide das enterobacteries, des aeromonas et des vibrions. *Ann. Microbiol.*, **125A:**341-347, 1974.
16. POPOVICI, M.; NACESCU, N. & ISRAIL, A. - Relations phage bacteries a le interieur des especes *Proteus vulgaris, Proteus mirabilis Arch. Roum. Path. Exp. Microbiol.* **27:**135-142, 1968.
17. SENIOR, B.W. - Typing of *Proteus* strains by proticine production and sensitivity. *J. Med. Microbiol.*, 10: 7-17, 1977.
18. SENIOR, B.W. - The purification, structure and synthesis of proticine 3. *J. Med. Microbiol.* **16:**323-331, 1983.
19. TRACY, O. and THOMPSON, E.J. - An evaluation of three methods of typing organisms of the genus *Proteus. J. Clin. Path.*, **25:**69-72, 1972.
20. VIEU, J.F. - *Cours de Microbiologie Systematique (Bacteriologie - Isolament des Bacteriophages et Techniques de estudes)* - Inst. Pasteur.
21. WILLIAMS, E.W.; HAWKEY, P.M.; PENNER, J.L.; SENIOR, B.W. and BARTON, L.J. - Serious nosocomial infection caused by *Morganella morganii* and *Proteus mirabilis* in a cardiac surgery unity. *J. Clin. Microbiol.* **18**:5-9, 1983.

Received for publication in 05/08/92

Rev. Microbiol., São Paulo, **24**(2): 94-97, 1993

CAPSULAR TYPES AND TOXIN PRODUCTION BY STRAINS OF *PASTEURELLA MULTOCIDA* ISOLATED FROM PIGS IN SOUTHERN BRAZIL

José R. F. Brito[1]
Itamar A. Piffer
Ingon Wentz
Maria A. V. P. Brito

SUMMARY

A total of 116 *Pasteurella multocida* isolates from 24 pig herds in Southern Brazil were classified according to their capsular type. All the isolates were tested for toxin production by using the guinea pig skin test and the Vero cell cytotoxic assay. Most isolates (101) were recovered from piglets with atrophic rhinitis. Of these, 31 belonged to capsular type A, 67 belonged to capsular type D and the remaining three isolates were not classified into the A, B, D or E *P. multocida* capsular types. Thirteen isolates of which nine were type A, two were type D and two were non-tipable, were recovered from pneumonic lungs of weaned piglets. Two other non-typable isolates were recovered from the subcutaneous tissue of a septicaemic piglet and from the joint of an arthritic pig. Forty-three (62.3%) of the 69 type D and two (5%) of the 40 type A *P. multocida* isolates were found to be toxigenic by the guinea pig skin test. None of the non-typable isolates were toxigenic. Except for the two type A toxigenic isolates there was an agreement between the guinea pig skin test and the Vero cell assay.

Key words: *Pasteurella multocida*, toxin production, capsular types, respiratory infection, swine.

INTRODUCTION

The family *Pasteurellaceae* forms a heterogeneous group of bacteria adapted to parasitic life in different hosts. *Pasteurella multocida* is one of the best known members of this family. Swine throughout the world are affected by pasteurellosis (4) and toxin-producing strains of *P. multocida* have been incriminated as having a central role in the aetiology of severe atrophic rhinitis (6, 13). Pasteurella organisms from pigs with atrophic rhinitis are usually of capsular type D whereas those recovered from pneumonic lungs are usually of type A (10). Differentiation of toxigenic from non-toxigenic isolates of *P. multocida* is essential for control, diagnosis and epidemiological studies of atrophic rhinitis (7, 12).

Swine atrophic rhinitis has been recognized in Southern Brazil for more than a decade (1, 9). This report describes the identification of the capsular types and the detection of toxin production by a number of *P. multocida* strains, which have been isolated in our laboratory since 1982, mainly from respiratory tract infections of weaned piglets.

1. CNPSA/EMBRAPA, Caixa Postal 21, 89700-000 Concórdia, SC.
Corresponding author; Present address: CNPGL/EMBRAPA, Rodovia MG 133, Km 42; 36155-000 Coronel Pacheco, MG

MATERIALS AND METHODS

Pasteurella multocida strains. A total of 116 field isolates were examined. They were obtained from nasal swabs (77 isolates), tonsils (24 isolates), lungs (13 isolates) and other tissues (2 isolates) of pigs from 24 herds in Southern Brazil.

All isolates were recovered initially on sheep blood agar and were identified as *P. multocida* by the following criteria established by Carter (2): Gram negative cocobacilli, non haemolytic, hydrogen sulfide production in TSI agar, non-motile, indole production, presence of cytochrome oxidase, no growth on MacConkey agar, nitrate reduction, glucose fermentation, non fermentation of lactose and variable reactions from sucrose and mannitol. All cultures were stored in defibrinated blood at -70°C until needed (2).

Identification of capsular type. The strains were classified according to Carter (2) into capsular types A or D by performing the staphylococcal hyaluronidase test and the acriflavine test with all the isolates. Those which were hyaluronidase positive and acriflavine negative were identified as type A. Type D isolates were hyaluronidase negative and acriflavine positive. Isolates which were neither type A nor D by the above tests were identified by the indirect haemagglutination test (IHA) by using specific antisera (2). Anti-pasteurella capsular sera A, B and E were given by Dr. L. Renault (Laboratoire Vétérinaire, Societé Sanders, Paris, France); anti-pasteurella capsular serum D was prepared by us according to Carter (2). All antisera were prepared in rabbits. Reference strains (A, B, D and E types) were used as control in the tests. Types B and E *P. multocida* were provided by Dr. G.R. Carter (Virginia Tech, Blacksburg, Virginia, USA). Types A and D strains belonged to our culture collection.

Preparation of crude toxin. *P. multocida* isolates stored at -70°C were recovered on sheep blood agar. Four individual colonies were passaged into fresh 3 ml brain heart infusion broth (BHI, Difco) and incubated at 37°C for 48 hours. The cultures were centifuged at 1,500 g for 10 minutes, the supernatants were sterilized by filtration (0.22 μm Millipore membranes) and subsequently tested in the cell culture assay and the guinea pig skin test.

Cell culture. Vero cell monolayers were grown in 96-well tissue culture plates with flat bottoms, using a mixture of equal parts of Ham F10 and 199 media (Flow Laboratories) containing 10% foetal calf serum, 20 IU penicillin, 20 μg streptomycin and 20μg amphotericin B per ml. The plate wells were seeded with 0.2 ml cell suspension/well, containing $2x10^5$ cells. Confluent monolayers were obtained in 24 hours, at 37°C, in a humidified atmosphere of 5% C02.

Assay for *P. multocida* toxin. This assay was performed according to Pennings & Storm (8). Shortly, the cell culture medium in the plates was replaced by the same medium without serum immediately before the assay. Serial fourfold dilutions of each sample were made separatly in the same cell culture medium, starting from 1:10 up to 1:10240. The medium was gently aspirated from the wells and 0.2 ml of each dilution was added. The cell cultures were further incubated for five days. Cell morphology was observed daily with an inverted microscope under 10x10 magnification. A known toxin-producing strain and a non-toxigenic strain of *P. multocida* were included in each plate as positive and negative controls respectively. Wells with non-inoculated bacterial culture supernatant were used for cell growth evaluation. All tests were run in duplicates.

Guinea pig skin test. This test was performed according to de Jong et al. (6). Briefly, adult guinea pigs weighing about 500 g were inoculated intradermally on the back with 0.2 ml of the *P. multocida* culture supernatants referred above. Each guinea pig was injected with four preparations and observed for five days. Controls were the same as those used in the Vero cell assays.

RESULTS

Table 1 shows the distribution of the capsular types of *P. multocida* according to the site of isolation in the piglets studied.

TABLE 1 - Capsular types of *P. multocida isolated* from piglets in Southern Brazil (1982-1991).

Site of Isolation	Nº of Strains with capsular types		
	A	D	Not Defined
Nasal cavity	21	54	2
Tonsil	10	13	1
Lung	9	2	2
Other tissues	0	0	2
Total	40	69	7

The majority of the 116 *P. multocida* isolates were recovered from piglets with atrophic rhinitis, being 77 from nasal secretions and 24 from tonsils. Thirteen isolates were recovered from pneumonic tissues of weaned piglets. One isolate was recovered from subcutaneous tissue of a septicaemic piglet and another one from the joint of an arthritic piglet. Sixty-nine (59.5%) isolates were classified as capsular type D, most of which were recovered from the nasal cavity (54 isolates; 78.3%) and from the tonsils (13 isolates; 18.8%). Only two isolates of capsular type D were isolated from the lungs. Capsular type A *P.multocida* were recovered from the nasal cavity (21 isolates), lungs (9 isolates) and tonsilar tissue (1 isolate).

The association between capsular type and the production of *P. multocida* toxin is shown on Table 2. Most of the toxin producing isolates were recovered either from the nasal secretions or the tonsils . Of the 69 type D *P. multocida*, 43 were toxin-producing strains as detected by the guinea pig skin test and the Vero cell assay. The two type D isolates recovered from the lungs were toxigenic. The remaining 26 type D strains were negative in both assays. Most type A strains (38/40) were non-toxigenic. One type A strain was positive only in the guinea pig skin test and the remaining strain was positive only in the Vero cell assay. All non typable isolates were found to be non-toxigenic.

DISCUSSION

The results in this study confirm the role of type D *P. multocida* in respiratory diseases, more especially the importance of these organisms in atrophic rhinitis, as demonstrated by the high number of strains classified as toxigenic type D. Similar findings have been reported elsewhere (3, 6, 11). Our results differ from those of Kobisch et al. (7) who found a higher proportion of toxin-producing strains in the capsular type A. We have found a correlation between results from the guinea pig skin test and the Vero cell assay for toxin production with the 69 isolates classified as type D. These results agree with those of Pennings & Storm (8) and Rutter & Luther (12) but not with those of Kobisch et al. (7) who found the cell assay less sensitive than the guinea pig skin test or ELISA. The two type D *P. multocida* recovered from lung tissues in this study were also toxigenic, as reported by Rutter et al. (14).

Our results also agree with other reports in which it has been shown that most lung isolates of *P. multocida* are of type A (7, 10). Only one of the type A *P. multocida* recovered from the lung was found to be toxigenic, but only in the guinea pig skin test, whereas another type A isolate, from the tonsilar tissue, was found to be toxigenic, only in the Vero cell assay. These may suggest some heterogeneity of the *P. multocida* toxin as previously described for other bacterial toxins (15). It may also suggest additional heterogeneity within the *P. multocida* species as indicated by Harel et al. (5) .

The findings that both type A and D *P. multocida* are present in piglets with respiratory disease and that the majority of type D isolates are toxigenic gives evidence that considerable effort has yet to be made to control respiratory diseases, more specifically atrophic rhinitis. Attention should also be paid for the complete diagnosis which means including toxigenic characterization and capsular typing of isolated strains, as opposed to routine diagnosis carried out in most laboratories in Brazil, which are based only on biochemical identification of the isolates. The Vero cell assay is suited for the detection of toxigenic *P. multocida*

TABLE 2 - Association between *P. multocida* toxin and capsular types.

Capsular type	Number of isolates	Agreement between tests	Positive results in Guinea pig	Vero cell	Negative results in both tests
D	69	69	43	43	26
A	40	38	1	1	38
Not defined	7	7	0	0	7

because it is relatively inexpensive, allows the testing of large number os samples and avoids the use of animals.

ACKNOWLEDGMENTS

The authors are grateful to Maria B. B. Fávero, Marni L. F. Ramenzoni, I. Muller, R. Vizotto and Márcia Pelisser for technical assistance. Three of the authors (J.R.F.B.; I.A.P. and M.A.V.P.B.) are recipients of CNPq research fellowships.

RESUMO

Grupos capsulares e produção de toxina de *Pasteurella multocida* isoladas de porcos no sul do Brasil

Um total de 116 amostras de *P. multocida* isoladas de 24 rebanhos suínos do Sul do Brasil foram classificadas por seu grupo capsular e testadas quanto à produção de toxinas utilizando-se os testes de dermonecrose em cobaias e o ensaio citotóxico em células Vero. A maioria das amostras (101) foram isoladas de leitões com rinite atrófica. Destas, 31 foram classificadas como tipo capsular A, 67 como tipo capsular D e três não foram tipadas como A, B, D ou E de P. multocida. Treze amostras, sendo nove do tipo A, duas do tipo D e duas não tipáveis, foram isoladas de pulmões pneumônicos de leitões desmamados. Duas outras amostras não tipáveis foram isoladas do tecido subcutâneo de um animal septicêmico e da articulação de um leitão com artrite. Quarenta e três das 69 amostras de *P. multocida* do tipo D e duas dentre as 40 amostras do tipo A eram toxigênicas. Houve concordância entre os testes de toxicidade em cobaias e em células Vero, exceto para as duas amostras toxigênicas do tipo A. Nenhuma das amostras nao tipáveis era toxigênica.

Palavras chave: *Pasteurella multocida*, toxina dermonecrótica, tipos capsulares, trato respiratório, suínos.

REFERENCES

01. Brito, J.R.F.; Brito, M.A.V.P.; Piffer, I.A. & Freitas, A.R. Rinite atrófica dos su~nos. III. Prevalência da doença e da infecçao por *Bordetella bronchiseptica* em suínos de "pedigree" do Estado de Santa Catarina. *Arq. Esc. Vet. UFMG.* **34:** 65-75, 1982.
02. Carter, G.R. - Serotyping of *Pasteurella multocida*. *Methods Microbiol.,* **16:** 247-258, 1984.
03. Eamens, G.J.; Kirkland, P.D.; Turner, M.J.; Gardner, I.A.; White, M.P. & Hornitzky, C.L. - Identification of toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs by in vitro characterisation. *Aust. Vet. J.* **65:** 120-123, 1988.
04. Farrington, D.O. - Pneumonic pasteurellosis. - Leman, A.D.; Straw, B.; Glock, R.D.; Penny, R.H.C.; Scholl, E. eds. In: Diseases of swine. 6th ed. Iowa, Iowa State University Press, p. 434-444, 1986.
05. Harel, J.; Côté, S. & Jacques, M. - Restriction endonucle ase analysis of porcine Pasteurella multocida isolates from Que bec. *Can. J. Vet. Res.,* **54:** 422-426, 1990.
06. de Jong, M.F.; Oei, H.L. & Tetenburg, G.J. - AR pathogenicity-tests for *Pasteurella multocida* isolates. In: *INTERNATIONAL PIG VETERINARY SOCIETY CONGRESS,* 6., 1980. Copenhagen. Proceedings. Copenhagen, IPVS, 1980. p. 211.
07. Kobisch, M.; Blanchard, B.; Morvan, P.; Labbé, A.; Toquin, D.; Menec, M. & Morvan, H. - L'exotoxine de Pasteurella mul tocida d'origine porcine. Étude comparative de trois techniques de detéction. *Rec. Med. Vet.,* **167:** 407-411, 1991.
08. Pennings, A.M.M.A. & Storm, P.K. - A test in Vero cell mono layers for toxin production by strains of Pasteurella multocida isolated from pigs suspected of having atrophic rhinitis. *Vet. Microbiol.,* **9:** 503-508, 1984.
09. Piffer, I.A.; Avila, L.A.F. & Brito, J.R.F. - Rinite atrófica dos suínos: isolamento e identificaçao de Bordetella bronchisep-tica. *Arq. Esc. Vet. UFMG,* **30:** 291-296, 1978.
10. Pijoan, C.; Morrison, R.B. & Hilley, H.D. - Serotyping of *Pasteurella multocida* isolated from swine lungs collected at slaughter. *J. Clin. Microbiol.,* **17:** 1074-1076.
11. Rimler, R.B. & Brogden, K.A. - *Pasteurella multocida* isolated from rabbits and swine: serologic types and toxin production. *Am. J. Vet. Res.,* **47:** 730-737, 1986.
12. Rutter, J.M. & Luther, P.D. - Cell culture assay for toxigenic *Pasteurella multocida* from atrophic rhinitis of pigs. *Vet. Rec.* **114:** 393-396, 1984.
13. Rutter, J.M. & Mackenzie, A. - Pathogenesis of atrophic rhinitis in pigs: a new perspective. **Vet. Rec.. 114:** 89-90, 1984.
14. Rutter, J.M.; Taylor, R.J.; Crighton, W.G.; Robertson, I.B. & Benson, J.A. - Epidemiological study of *Pasteurella multocida* and *Bordetella bronchiseptica* in atrophic rhinitis. *Vet. R e c .*, **115:** 61S-619, 1984.
15. Soderholm, J.; Smyth, C.J. & Wadstrom, T.A. - A simple and reproducible method for crossed immunoelectrofocusing: comparative studies on microheterogeneity of diphteria toxin from different preparations. *Scand. J. Immunol.,* **4:** 107-113, 1975. Suppl.

Received for publication in 24/09/92

Rev. Microbiol., São Paulo, 24(2): 98-103, 1993

VACCINATION AGAINST AVIAN COLIBACILLOSIS. PROTECTION AGAINST HOMOLOGOUS AND HETEROLOGOUS *ESCHERICHIA COLI* SEROGROUPS

Clarice Rachman Ajzental[1]
Edir Nepomuceno da Silva[2]
Raphael Lucio Andreatti Filho[3]

SUMMARY

Groups of one day old chicks were subcutaneosly vaccinated with 0.2 cc per chick of oil based bacterins prepared with ***Escherichia coli*** belonging to each one of the following six serogroups: 02:K88:F41, 021:K99:H52:F41, 078:K88:F41, 088:K88:F41, 0119:K88:F41 and 0119:K^-:F^-. The chicks were challenged at twenty eight days of age by air sac inoculation with cultures from each ***E. coli*** serogroup. The survival chicks were killed seven days post challenge, necropsied and the severity of air sac lesions scored from zero to four. Serum samples collected from Trial I survival chicks and control birds were tested by a rapid seroagglutination test using ***E. coli*** antigens derived from homologous and heterologous serogroups to the vaccination. The groups challenged with the homologous serogroup to the vaccination showed protection, measured by mortality index, ranging from 37,5% to 100%. The best protection (100%) using homologous vaccine was obtained with the 0119:K88 infected chicks while vaccination with 02:K88 was the least effective (37,5%). All the control, not vaccinated chicks, died after challenge. The groups challenged with a heterologous serogroup used in the vaccination showed protection ranging from zero to 100%. One hundred per cent of cross protection was seen in the groups vaccinated with 021:K99 and challenged with 088:K88, vaccinated with 078:K88 and challenged with 021:K99. The vaccination with the serogroup 021:K99 gave the best average of cross protection (42,1%) and the vaccination with 088:K88 the worst (26,7%) . None cross protection was seen in the groups vaccinated with 02:K88 and challenged with 021:K99 and 078:K88; vaccinated with 021:K99 and challenged with 02:K88; vaccinated with 088:K88 and challenged withh 02:K88 and 0119:K88; vaccinated with 0119:F^- and challenged with 078:K88 and 0119:K88. There were correlation of protection with score of air sac lesions and seroagglutination tests with ***E. coli*** antigens. The results showed that there are antigens responsible for crossprotection among ***E. coli*** serogroups.

Key words: ***Escherichia coli***, chickens, vaccine, vaccination, avian collibacilosis.

INTRODUCTION

Escherichia coli is part of the intestinal microbiota of mammals and birds. In chickens, this bacteria has been associated with several extra intestinal infections, and the infections of the respira-

1 Bolsista IC FAPESP - Proc. 87/3171-4. FMVZ/USP.
2 Departamento de Tecnologia de Alimentos - Faculdade de Engenharia de Alimentos - UNICAMP - Caixa Postal 6121 - 13081 Campinas, SP.
3 Departamento de Clínica Veterenária - Faculdade de Medicina Veterinária e Zootecnia - UNESP - Caixa Postal 502 - 18610 Botucatu, SP.

tory tract are of the most economic importance, causing mortality and carcass condemnations during processing (3,15).

The clinical picture is manifested by air saculitis, pericarditis, perihepatitis and peritonitis, being *E. coli* the main causative agent, either per se or associated with other microorganisms, particularly respiratory viruses (1, 3, 7, 15). Actually, the minimization of the colibacillosis problems on poultry farms is the adoption of better management practices and medication. The vaccination, if suitable, can be an additional help on the control of avian colibacillosis.

E. coli 01, 02 and 078 are the main serogroups isolated from disease conditions in chickens and turkeys (6,7).

Chickens vaccinated with *E. coli* bacterin showed protection after being challenged with homologous strains but not with heterologous ones (2). This can be a difficulty to the vaccination practice, due to the great number of different serogroups causing infection in chickens. *E. coli* strains belonging to different serogroups carry common protective antigens, like the adesins responsible for calves and piglets diarrhea. There is some indication of the presence of antigens on the outer membrane of different *E. coli* serotypes pathogenic for chickens, capable of giving cross protection to challenge with different serogroups (7).

Our purpose was to verify if the inactivated oil based bacterins, prepared with different *E. coli* serogroups, pathogenic for chickens, could give any cross protection to the experimental challenge with the heterologous strains.

MATERIALS AND METHODS

***E. coli* strains, vaccine and antigen preparations.** Six *E. coli* serogroups were used: 02:K88, 021:K99:H52, 078:K88, 088:K88,0119:K88,all with pili type I, F41, and the unpiliated serogroup 0119:K$^-$. All of them are pathogenic for day-old chicks, and were isolated from air sac of broilers showing air saccullitis (19). They were serogrouped in the *Escherichia coli* Reference center, Pennsylvania State University, USA.

All strains were cultured in CFA agar contained Roux bottles for both, the antigen production used in the agglutination tests and for oil based bacterin preparation as previously recommended (20).

The number of colony forming units (CFU) of *E. coli* was determined by plating 0.1 ml of cultures and decimal dilutions using phosphate buffer saline (PBS) pH 7,2 on McConkey agar.

Chickens, vaccination and challenge. Day old males Hissex Brown, free from *Mycoplasma gallisepticum* were used. They were wing banded and kept in heated wire cage receiving commercial feed and water *ad libitum*.

Groups of day old chicks were subcutaneously vaccinated in the neck with 0.2 cc per chick of oil based bacterin prepared with *E. coli* belonged to each one of the serogroups. Chicks were challenged at 28 days by thoracic air sac inoculation with 0.5 cc of brain heart infusion (BHI) cultures incubated at 37ºC for 8 hours from each *E. coli* serogroup.

The survival chicks were killed seven days post challenge, necropsied and the severity of air sac lesions scored from zero to four as following: zero, meant no macroscopic lesions on the air sac; 1, indicated mild air sac opacity; 2, denote an extensive opacity of the inoculated air sac, but, yet, with some areas without lesions; 3, showed an intense opacity with exsudate in the inoculated air sac extending to part of the abdominal air sacs; and 4, intense opacity with bilateral exsudate in the abdominal and thoraxic air sacs on dead birds.

Serum samples collected from Trial I survival chicks and control birds were tested by rapid seroaglutination test with *E. coli* antigens prepared with homologous and heterologous serogroups to the vaccination. The control group was not challenged in this Trial.

Experimental Trials

Trial I. Seven groups with 10 chicks each were used. Six were vaccinated with bacterin prepared with *E. coli* belonging to each one of the serogroups and, one group was the control not vaccinated. The challenge, using homologous strains to the vaccination, was done with 10^7 CFU of all *E. coli* serogroups, but serogroup 078 was with 10^8 CFU.

Trial II Six groups with 30 chicks each were vaccinated with bacterin prepared with *E. coli* belonging to each one of the serogroups. After vaccination, each group was divided in six subgroups for challenge. It was done using heterologous strains to the vaccination. Control not vaccinated subgroups were used. The challenge was done with 10^7 CFU of all *E. coli* serogroups, but serogroup 02 and 021 were with 10^8 CFU.

RESULTS AND DISCUSSION

Table 1 shows survival index of groups of chicks challenged with homologous and heterologous *E. coli* strains. The vaccinated groups challenged with ho-

mologous strains to the vaccination showed protection ranging from 37,5% to 100%. The best protection (100%) using homologous vaccine was obtained with the 0119:K88 infected chicks while vaccination with 02:K88 was the less effective (37,5%). All the control, not vaccinated chicks, died after challenge. The groups challenged with a heterologous serogroup used in the vaccination showed protection ranging from zero to 100%. The groups vaccinated with 021:K99 and challenged with 088:K88, vaccinated with 078:K88 and challenged with 021:K99, gave the highest cross protection with 100%. Vaccination with serogroup 021:K99 gave the best cross protection (42,1%) :while with the 02:K88, the worst average. (20,8%) No cross protection was seen in the groups vaccinated with 02:K88 and challenged 021:K99 and 078:K88; vaccinated with 088:K88 and challenged with 02:K88 and 0119:K88; vaccinated with 0119:F$^-$ and challenged with 078:K88 and 0119:K88. These results can be affected, in part, by the variability in virulence of the ***E. coli*** strains (19), once a very virulent one can break the cross protection and not be detected by this methodology and by the small numbers of chicks in the experimental groups. Published results showed a high correlation of homologous protection in chickens vaccinated and challenged with strains from serogroups 02 and 078, but, low protection when the chickens were vaccinated with serogroups 01 and challenged with heterologous strains from the serogroups 02 and 078 (7, 8, 16).

The results on Table 2 show the average rates of macroscopic lesions in the air sacs of vaccinated chicks and challenged with homologous and heterologous ***E. coli*** serogroups.. These results also confirmed the highest protection gotten by challenge with homologous serogroups and the presence of cross protection factors among homologous and heterologous ***E. coli*** serogroups. This methodology has been previously used by other authors and their results agree with ours (7,8,11,12,14,17,18).

The surviving groups of chicks challenged with homologous strains to the vaccine had the lowest average rates of macroscopic air sac score lesions. It ranged from 0.7 to 2.5 for the serogroups 0119:K88 and 02:K88, respectively. All the control, not vaccinated chicks, dead after challenge, had the highest score lesion(4.0).

The cross protection measured by air sac score lesions was low. The best results of cross protection were seen in 021:K99 and 078:K88 vaccinated groups challenged with 088:K88 strain with score index of 2.5 for both. In some groups it was not possible to

TABLE 1 - Survival birds among groups of chickens vaccinated and challenged with homologous and heterologous *Escherichia coli* serogroups.

Vaccine Sero groups	Challenge Serogroups						% of Cross Protection
	02:K88	021:K99	078:K88	088:K88	0119:K88	0119:F-	
02:K88	3/8[1]	0/5	0/5	3/5	1/5	1/4	20,8%
021:K99	0/4	8/9	1/4	4/4	2/4	1/3	42,1%
078:K88	1/5	3/3	4/8	2/4	1/5	1/4	38,1%
088:K88	0/3	1/3	1/3	7/9	0/3	2/3	26,7%
0119:K8 8	1/5	2/4	1/5	2/5	10/10	2/5	33,3%
0119:F$^-$	2/4	4/5	0/4	1/4	0/4	8/10	33,3%
Control	0/5	0/5	0/5	0/5	0/5	0/5	

1. Number of survival birds / total challenged.

TABLE 2 - Score of air sac lesions from chickens vaccinates and challenged with homologous and heterologous *Escherichia coli* serogroups.

Vaccine Sero groups	Challenge Serogroups					
	02:K88	021:K99	078:K88	088:K88	0119:K88	0119:F-
02:K88	2/5[1]	4.0	4.0	3.3	3.8	3.8
021:K99	4.0	1.1	4.0	2.5	3.1	3.5
078:K88	3.8	3.2	1.6	2.5	3.5	3.4
088:K88	4.0	3.2	3.4	1.5	4.0	3.1
0119:K8 8	3.9	3.9	4.0	2.6	0.7	4.0
0119:F$^-$	3.9	2.7	4.0	3.4	4.0	1.1
Control	4.0	4.0	4.0	4.0	4.0	4.0

1. Mean of air sac lesions score from zero to four.

determine cross protection scoring air sac lesions because all chicks died after challenge or there was only one survival chick. We adopted score 4.0 for dead chick for the purpose of these trials as it has been used by other authors.

The rapid seroaglutination test (SAT) results, from serum samples collected from Trial I survival chicks, using ***E. coli*** antigens prepared with homologous and heterologous serogroups, are shown in Table 3. Two serum samples were missed. The homologous antigens gave 100% positive strong reactions on SAT for all vaccinated groups. The heterologous antigens gave variable results with weak reactions: group vaccinated with 021:K99 gave 57.1% (4/7) positive reaction on SAT using antigen prepared with 088:K88; chicks vaccinated with 021:K99 were 42.9% (3/7) positive to the antigen 02:K88 and, the vaccinated group with 0119:F$^-$ were 37,5% (3/8) positive at SAT with antigen 078:K88. The lowest level of positive reactions on SAT was found among the groups vaccinated with 021:K99 and tested with antigens 078:K88 (2/7) and 0119:K88 (1/7), vaccinated with088:K88 and tested with antigen 0119:F$^-$ (2/7), vaccinated with 0119:K88 and tested with antigens 078:K88 and 0119:F$^-$ (1/10 for each one), vaccinated with 0119:F$^-$ and tested with antigens 021:K99, 078:K88 and 0119:K88. No other combination showed seroconversion. The SAT once more, confirmed the results of higher protection afforded by vaccination and challenge with homologous serogroup and, they also revealed, the presence of antigenic cross relation among different ***E. coli*** serogroups. However, the used methodology with a not purified agglutination antigen, does not elucidate what antigens and antibodies are envolved, and new serological tests should be used in attempt to identify those ones. Similar results showing higher antibody response to homologous antigen has been reported before (17). It has been demonstrated that the antibodies produced by ***E. coli*** vaccination have protector effect to the birds and their offspring during the first days of life (12,17).

Vaccines prepared with piliated (K88) and unpiliated strains (F$^-$) belonging to serogroup 0119 F$^-$ were used to demonstrate the participation of somatic and pili antigens on immunity determination. The results of mortality and air sac lesions after challenge (Tables 1 and 2) showed that the somatic and K88 antigens were not fundamental in the immunity determination. Although, an extensive work has been done showing that the pili is a pathogenicity factor in ***E. coli***

TABLE 3 - Seroagglutination test from chicken sera using *Escherichia coli* antigens derived from homologous and heterologous serogroups to the vaccination.

Vaccine Sero groups	Serogroups of antigens used in the test					
	02:K88	021:K99	078:K88	088:K88	0119:K88	0119:F-
02:K88	2/2[1]	0/2	0/2	0/2	0/2	0/2
021:K99	3/7	7/7	2/7	4/7	1/7	0/7
078:K88	0/4	0/4	4/4	0/4	0/4	0/4
088:K88	0/7	0/7	2/7	7/7	0/7	0/7
0119:K8 8	0/10	0/10	1/10	0/10	10/10	1/10
0119:F$^-$	0/8	1/8	3/8	0/8	2/8	8/8
Control	0/9	0/9	0/9	0/9	0/9	0/9

1. Serum samples with positive reaction / total tested.

strains (1,2,4,5,9,10,19), our results with K88 antigen did not agree with the previous ones.

Our results indicated that the use of a vaccine prepared with only one *E. coli* strain is not the best approach to prevent chicken colibacillosis under field conditions. Although, it can give certain degree of cross protection to heterologous challenge strains. There are some indications that the vaccine could be prepared with different serogroups (7,8,11).

In fact, it is necessary to identify the *E. coli* antigens related to protection and to find an adequate method and media for their identification and expression. Besides that, the inactivation procedure to maintain the antigenic properties is of particular importance. It has been demonstrated that the ultrasonication following irradiation produces a better antigen with a broader spectrum of protection (13).

ACKNOWLEDGMENTS

The authors thanks all the Laboratory staff from FMVZ/USP that helped in this research, as well as FAPESP for the financial support.

RESUMO

VACINAÇÃO CONTRA A COLIBACILOSE AVIÁRIA. PROTEÇÃO CONTRA SOROGRUPOS HOMOLOGOS E HETERÓLOGOS DE *ESCHERICHIA COLI*

Grupos de pintos de um dia de idade foram vacinados, via subcutânea, com 0,2 cc/ave de bacterinas oleosas preparadas, individualmente, com cada um dos seis sorogrupos de *Escherichia coli*: 02:K88:F41, 021:K99:H52:F41, 078:K88:F41, 088:K88:F41, 0119:K88:F41 e 0119:K$^-$:F$^-$. Aos 28 dias de idade foram desafiados, por injeção no saco aéreo com inóculos dos diferentes sorogrupos. No sétimo dia após o desafio, as aves sobreviventes foram sacrificadas, necropsiadas e as lesões nos sacos aéreos classificadas por meio de esquema, variando de zero a quatro de acordo com sua severidade. Amostras de soros coletadas das aves sobreviventes do experimento I, após o desafio, e do grupo controle, foram submetidas a prova de soroaglutinação rápida com antígenos homólogos e heterólogos de *E. coli* utilizados na vacinação. Os grupos de aves vacinados e desafiados com as cepas homólogas à utilizada na vacina apresentaram proteção, medida pelo índice de mortalidade, que variou

de 37,5% a 100,0%, com a média de 74,0% de proteção. O sorogrupo 0119:K88 foi o que conferiu melhor proteção (100%) e o 02:K88, o pior (37,5%). Todos os controles não vacinados e desafiados morreram. Os grupos vacinados e desafiados com cepas heterólogas apreentaram proteção de zero a 100%, com média de 26,0%. Maiores índices individuais de proteção cruzada ocorreram nos grupos vacinados com 021:K99 e desafiado ccom 088:K88, vacinado com 078:K88 e desafiado com 021:K99 , com 100% de proteção cruzada. A vacinação com o sorogrupo 021:K99 foi a que conferiu a maior média de proteção cruzada (42,1%) e o 088:K88, a pior (26,7%). Nenhuma proteção cruzada foi observada nos grupos vacinados com 02:K88 e desafiado com 021:K99 e 078:K88; vacinados com 021:K99 e desafio com 02:K88; vacinados com 088:K88 e desafio com 02:K88 e 0119:K88; vacinados com 0119:F$^-$ e desafio com 078:K88 e 0119:K88.

Maior proteção conferida para o desafio com sorogrupos homólogos e a existência de proteção cruzada entre diferentes sorogrupos também foram confirmadas nas observações das lesões nos sacos aéreos e através da prova de soroaglutinação rápida.Certamente, existem antígenos protetores comuns entre diferentes sorogrupos de ***E. coli.***

Palavras-chave: *Escherichia coli,* galinhas , aves, vacinas, vacinação, colibacilose.

REFERENCES

1. Andreatti F^o, R. L. Destino de amostras patogênica e apatogênica de ***Escherichia coli*** em aves experimentalmente inoculadas e estudo de lesões. Dissertação de mestrado. Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo. São Paulo, SP. 133p., 1989.
2. Arp, L.H. Consequences of active or passive immunization of turkeys against ***Escherichia coli*** 078. *Avian Dis.*, 24:808-815, 1980.
3. Arp, L.H. Pathology of spleen and liver in turkeys inoculated with ***Escherichia coli***. *Avian Path.* 11:263-279, 1982.
4. Brée, A.; Dho, M.& Lafont, J.P. Comparative infectivity for axenic and Specific-Pathogen-Free chickens of 02 ***E. coli*** strains with or without virulence factors. *Avian Dis.*, 33:134-139,1989.
5. Dominick, M.A.; Schmerr, M.J.F.; &Jensen, A.E.. Expressions of type 1 pili by ***E. coli*** strains of high and low virulence in the intestinal tract of gnotobiotic turkeys. *Amer. J. Vet. Res.* 46:270-275, 1985.
6. Gross,W.B. Colibacillosis. In: Calnek,B.W. et al., eds. Diseases of Poultry . 9 ed Ames, Iowa State University Press, 1984, p. 138-144, 1991
7. Gyimah,J.E.; Panigrahy, B.; Hall, C.F.; Williams, J.D. Immunogenicity of an oil emulsified ***E. coli*** bacterin against heterologouys challenge. *Avian Dis.*, 29:540-545, 1985.
8. Gyimah, J.E.& Panigrahy, B. Immunogenicity of an ***Escherichia coli*** (serotype 01) pili vaccine in chickens. *Avian Dis.* 29:1078-1083, 1985.
9. Gyimah, J.E.; Panigrahy, B. & Williams, J.D. Immunogenicity of an ***Escherichia coli*** multivalent pilis vaccine in chickens *Avian Dis.*, 30:687-689, 1986.
10. Gyimah, J.E. & Panigrahy, B. Adhesin-receptor interactions mediating the attachment of pathogenic ***Escherichia coli*** to chicken tracheal epithelium. *Avian Dis.*, 32:74-78,1988.
11. Jayappa, H.G. & Garcia, R. Efficacy of a new ***Escherichhia coli*** bacterin in controlling colibacillosis in chickens and turkeys. In: Congresso Latinoamericano de Avicultura, 9. Acapulco, Mexico. p.874-882, 1985.
12. Kumar, S.; Boclair,W & Rosenberger, J.K. Field Studies on an ***Escherichia coli*** vaccine for use in poultry. In Congresso Latinoamericano de Avicultura, 9. Acapulco, Mexico. p. 158-169, 1985.
13. Melaned, D.; Leitner, G. & Heller, E.D. A vaccine against avian colibacillosis based on ultrasonicc inactivation of ***Escherichia coli***. *Avian Dis.* 35:17-22,1991.
14. Myers, R.K. & Arp, L.H. Pulmonary clearance and lesions of lung and air sac on passively immunized and inimmunized turkeys following exposure to aerosolised ***Escherichia coli.*** *Avian Dis.* 31:622-628, 1987
15. Nagaraja, K.V.; Emery, D.A.; Jordan, K.A.; Sivanandan, V.; Newman, J.A. & Pomeroy, B.S. Effect of ammonia on the quantitative clearance of ***Escherichia coli*** from lungs, air sacs and livers of turkeys aerosol vaccinated against ***Escherichia coli*** . *Amer. J. Vet. Res.* 45:392-395, 1984.
16. Panigrahy, B.;Gyimah, J.E, Hall, C.F.; Williams, J.D. Immunogenic potency of an oil emulsified ***Escherichia coli*** bacterin. *Avian. Dis.* 28:475-481, 1984.
17. Rosenberger, J.K.; Fries, P.A. & Cloud, S.S.; In vitro and in vivo characterization of avian ***Escherichia coli***. III Immunization. Avian Dis. 29:1108-1117,1985
18. Sandhu, T.S. & Layton, H.W. Laboratory and field trials with formalin inativated ***Escherichia coli*** (078) - ***Pasteurella anatipestifer*** bacterin in white pekin ducks. *Avian Dis* 29:128-135,1985
19. Silva, E.N. Características de patogenicidade em amostras de ***Escherichia coli*** de origem aviária. Tese de livre docência. Faculdade de Medicina Vetrinária e Zootecnia da Universidade de São Paulo. São Paulo,SP. 186p, 1986.
20. Stone, H.D.; Brugh, M.; Hopkins, S.R.; Yoder, H.W. & Beard, C.W. Preparation of inactivated oil-emulsion vaccines with avian viral or mycoplasma antígens. *Avian Dis* **22:**666-674,1978.

RECEIVED FOR PUBLICATION IN 7/29/92

Rev. Microbiol., São Paulo, **24** (2): 104-110, 1993

"CRITICAL PERIOD" FOR IRREVERSIBLE BLOCK OF VACCINIA VIRUS REPLICATION

Marco Antônio da Silva Campos
Erna Geessien Kroon

SUMMARY

Hela cells were infected with vaccinia virus and treated with cycloheximide 30 to 60 minutes after infection. The cells extracts were prepared and the proteins analysed in a 12.5% SDS-PAGE, and an early viral protein profile was observed, which is similar to that of infected cells treated with cytosine arabinoside, an inhibitor of DNA replication. In order to get some more evidence that an early viral protein could be synthesized in these conditions, enzymatic activity of thymidine kinase was assayed *in vitro* (thymidine kinase is an early viral protein), and was yielded 30% activity compared with infected non treated cells extracts. The synthesis of viral DNA was measured through the incorporation of [^{3}H]-thymidine to DNA, in infected HeLa cells, to better localize the block in the virus growth cycle, and a strong inhibition of viral DNA synthesis was observed. Results suggest that the irreversible block in the virus multiplication, happened after the early protein synthesis and before DNA replication.

Key-words: protein synthesis inhibition, vaccinia virus, cycloheximide

INTRODUCTION

The inhibition of the cell protein synthesis, in cells infected with vaccinia virus, increases with the higher multiplicity of viral infection and 95% of these inhibitions shown to occur 1 to 4 hours after the infection, with 5o PFU/cell of vaccinia virus. Viral particles irradiated with UV light were defectives in their ability to initiate viral protein synthesis, but in spite of this, inhibited the host protein synthesis (23). Experiments in systems without cells, *in vitro,* demonstrated the existence of a factor in the vaccinia virus core, which inhibited the protein synthesis of the cell (1, 7, 8, 20).

The viral multiplication cycle can be analysed by the use of a diversity of inhibitors of macromolecular synthesis. The inhibition of the viral DNA synthesis by cytosine arabinosid (ara C), blocked the expression of the late genes (6, 26) and the cycloheximide (CHX) inhibited the protein synthesis in general, at post-transcriptional level, in a reversible way (19). The transcription of the early viral genes ocurred even under the influence of these inhibitors, because it is independent of *de novo* synthesis of viral enzymes (4, 29). However, experiments of hibridization showed that, in the presence of the protein synthesis inhibitors, there appears only viral mRNA produced normally in the first two hours of infection (15). So, the "turn-off" of the early viral mRNA synthesis and the "turn-on" of the late viral mRNA synthesis are prevented by

Laboratório de Vírus. Depto. de Microbiologia. ICB, UFMG, Cx. Postal 2486, CEP 30270-901. Belo Horizonte, MG. BRASIL

the inhibitors of the protein synthesis. The presence of the CHX during the period of 30 to 60 minutes after the HeLa cells infection by vaccina virus, in a high multiplicity of infection, produced, based on MOSS and FILLER (24), an irreversible block in the viral DNA replication and in the viral protein synthesis, even after the inihitors have been removed. The same authors postulated that the failure in the viral mRNA translation is related to the inhibition of host protein synthesis, induced by vaccina virus. NOLL (25) proposed the hypothesis that some virus, not specifically or only the vaccine virus, interferes with the host protein synthesis, inactivating specific initiators factors. Based on this model, the viral protein synthesis would be irreversibly blocked, if the host factor were inactivated before the synthesis of new viral factors. In this way, could be explained the failure of vaccinia viral mRNA to be translated after the withdrawal of the inhibitor, since the presence of the drug the virus-induced inhibition of host protein synthesis is established, but viral protein were not produced, although this didn't occur in low multiplicity of infection, because in these conditions the host protein synthesis was not totally inhibited within the period of CHX treatment. MOSS and FILLER (24) concluded that there was interference in the synthesis of the initiation factors of protein, specific to virus, required for translation.

Our study describes the synthesis of early vaccinia virus proteins in cells infected with high multiplicity of virus and treated with CHX in the two first hours of infection, in which event only the block of viral late protein synthesis became irreversible.

MATERIAL AND METHODS

Culture of cells. HeLa S3 (ATCC CCL 2.2) were maintained in exponential grow in MEM (10), added with 5% of new born calf serum and 2 x 10^5 U.I. of penicillin and 25 mg/litre of streptomycin.

The primary culture of the chicken embryos fibroblasts (CEF) was realized according to DULBECCO and VOGT (9).

Virus - The vaccinia virus WR, received from JOKLIN, W., Durham, USA, was multiplicated in CEF, and purified according to JOKLIK, W.(12). For titration, CEF were inoculated with the serial virus suspension dilutions, which were incubated at 37ºC (5% CO2) during 48 hours. The medium was withdrawen from the plates and these were stained with violet of gentian 0,3% (p/v), and the areas of lise of the monolayers were counted.

Infection of HeLa S_3 cells - The cells (3 x 10^6) were infected with the indicated multiplicity, in Petri plates (diameter of 30 mm), with 45 min. of adsorption, and treated or not by inhibitor, according to the experiment. The viral suspension was then substituted by MEM, with or without inhibitor, as indicated. The cells were them washed with PBS at 4ºC, collected, centrifuged at 500 g and the sediment was frozen at -20ºC.

Treatment of the cells with inhibitors - With CHX: three conditions were utilized. In the first one, which we called treatment in the "critical period", the CHX was added 15 minutes before the infection and kept for two hours after the end of viral adsorption, and in these conditions there isn't multiplication of the virus, and the effect of the CHX is irreversible, even after the inhibitor withdrawal, according to MOSS and FILLER (24). To obtain a reversible effect of the CHX, two other conditions were used, according to the already mentioned work (24), in which the CHX was present 2 hours later, when the inibitor was withdrawen. In these three conditions, the dose of CHX was 300 ug/ml (24). The viral infection was of 50 PFU/cell or double infection, with 30 PFU/cell of active virus followed by another "infection" with the equivalent to 50 PFU/cell of virus, pre-treated 5 minutes with UV light (23).

With ara C: the inhibitor was put (50 ug/ml) 15 min. before the infection and the drug remained until the material was collected.

With CHX and ara C: were utilized the two inhibitors in the same sample, the ara C was kept from 15 min. before the infection until the collect and the CHX was kept in the "critical period".

Analyses of the polipeptides by incorporation of [^{35}S]-methionine - The nutrient medium of the infected cells was discarded and substituted by methionine-free MEM. At indicated times, 10 uCi/ml of L-[^{35}S]-methionine (with specific activity of 800 uCi/mM) were added to the cells(27). After one hour of incubation at 37ºC (5% CO_2) this medium was discarded and the cells were washed three times with PBS. The cells were collected and washed twice with PBS and centrifuged at 500 g at 4ºC. The sediment was resuspended in 60 ul of distilled sterile water and frozen at -20ºC during 30 min., thawed and subjected to sonic vibrations at 3 A during ten seconds on ice bath.

PAGE - The cells were infected and the samples were adjusted to contain 100 ug of protein according to LOWRY, O. *et al.* (21). The electrophoretic separation was made in PAGE 12.5% (18). As

molecular weight markers there was used a mix of non-radiative proteins (SDS - 7, Sigma), of 14.2 to 66 kDa. After staining, drying and exposing the gel to a film for X-ray, the film was superput to the stained dried gel and notes were made of the relative molecular mass, in the margin of the film.

DNA viral synthesis in HeLa S3 cells - The synthesis of DNA of the vaccinia virus was measured through the incorporation of [^{3}H]-thymidine to the viral DNA (6). HeLa cells were infected with 50 PFU/cell of vaccinia virus WR, and 2 or 4 hours after the end of the adsorption, the [^{3}H]-thymidine was added to the medium (10 uCi/ml, specific activity 50 Ci mM) during 10 min. The cells were collected in 0.5 ml of sample buffer (0.01 M TRIS pH 7.3, 0.003 M MgCl2, 0.01 M KCl, 0.5% (p/v) Nonidet P4O) and the mechanic rupture was made with sonic vibration during 5 min. at 3 A. The sample (5ul) was mixed with 1 ml of distilled water at 4ºC and at 1 ml of TCA of the precipitation was filtered on Whatmann GF/C menbrane, which was dried for one hour at 60ºC, and the scintillation's liquid (POPOP in toluol) was added and it was counted in scintillator.

Determination of the enzymatic activity of the thymidine kinase (TK) - HeLa cells were infected with 50 PFU/cell of the virus, collected 8 hours after the infection and frozen. The enzymatic activity was determined in cells extracts with 10 ug of protein/sample, in 15, 40 and 65 min. (11). The blank value of the incorporation of [^{3}H]-thymidine was determined by extracts of infected cells with vH5TK1 (recombinant vaccinia virus non productor of the TK enzyme, received from GRUN, J., FRG) which was subtracted from the value of another samples.

Inactivation by ultraviolet light - Purified virus (100 ul) was diluted in 4900 ul of solution 1mM TRIS pH 8.8. An UV germicid lamp of General Electric GI5T8 was used with a distance of 9 cm on open Petri plate with the virus (with agitation by sterile magnetic bar), and the irradiation was made during 5 min. (23). Titrations of the irradiated samples were realized in dillutions of 10^{-1}, 10^{-2}, 10^{-3}.

RESULTS

To the study of the synthesis of the viral and cellular proteins in the infected cells, in conditions of treatment in the "critical period" with CHX, many PAGE were realized, after incorporation of [^{35}S]-methionine.

The experiment of the FIG 1A was made with extracts from cells infected with 50 PFU/cell of the vaccinia virus WR. Under these conditions there was a much accentuated cytopathic and cytotoxic effect, to which were added the actuation of the inhibitor drugs ara C and/or CHX, and still the procedure of the washing of the cells after tratment with CHX, which caused with frequency the loss of the cells of the Petri plate. However, with double infection, using the first infection the active virus (30 PFU/cell) and immediately after, the virus inactivated with UV light (50 PFU/cell), a complete host protein synthesis inhibition was observed, without accentuated cytotoxic effect and loss of cells (FIG 1B).

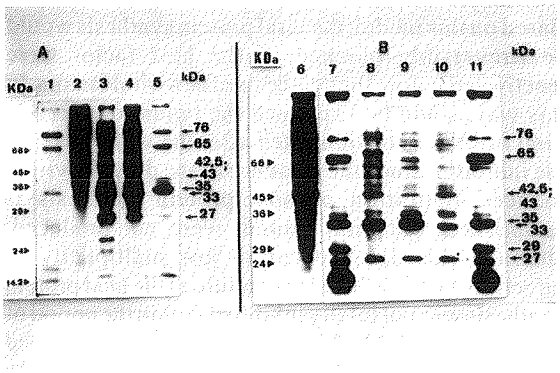


FIGURE 1 - Protein synthesis inhibition of HeLa cells by infection with vaccinia virus WR in autoradiograph of PAGE at 12.5%.

A - cells were infected with 50 PFU/cell in the time 0 and the adsorption was conducted. Six hours after the end of the adsorption the medium was changed to methionine-free medium and [^{35}S]-methionine (10 μCi/ml) was added during one hour. Cells extracts were prepared according to material and methods. In the test were used 100 μg of protein/sample in a PAGE (12.5%) and the autoradiograph was made. **B** - in the time 0, 30 PFU/cell of virus was added to the cells and immediately after the equivalent to 50 PFU/cell of UV inactivated virus. The incorporation was made from the seventh to the eighth hour and was conducted as in A. Lanes 1 and 11 - infected cells; 2 and 6 - non-infected cells; 3 and 9 - cells infected and treated with CHX (300 μg/ml) from 15 min. before until 2 hours after the adsorption; 4 and 8 - cells infected and treated with ara C (50 μg/ml) from 15 min. before the adsorption up to the collect; 5 - cells infected and treated with CHX (300 μg/ml) from 2 hours until 4 hours after the end of adsorption; 7- cells infected and treated with CHX (300 μg/ml) from 2 hours before until immediately before the adsorption; 10- cells infected and treated with ara C (50 μg/ml) from 15 min. before the adsorption up to the collect and treated with CHX (300 μg/ml) from 15 min. before until 2 hours after the adsorption.

From the experiments of the FIG 1 (A and B) we can observe that, lanes 2 and 6 have a dense profile, when we apply 100 ug of protein at PAGE, the predominant band being with 43 kDa. The lanes 1, 5,

7 and 11 presented the bands of 27, 29, 35, 65 and 76 kDa. The lanes 3, 4, 8, 9 and 10 presented the bands of 27, 33 and 42.5 kDa.

Synthesis of the thymidine kinase - This test showed that, when HeLa cells are infected and treated with CHX in the "critical period", after the removal of the inhibitor, there is a significant production of the TK viral enzyme (ca. of 30% of the produced by the virus, when non-treated previously with CHX) .

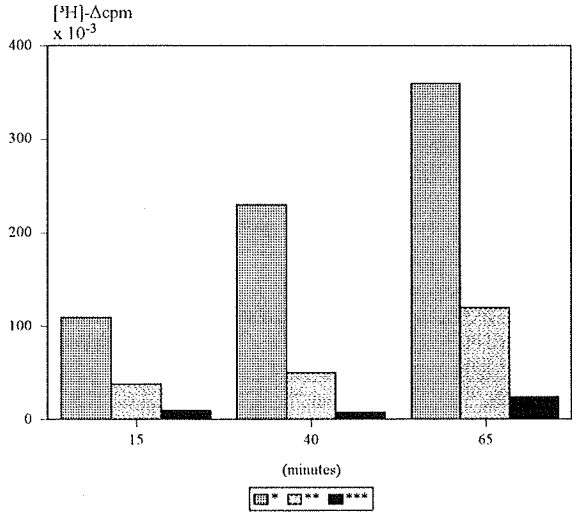


FIGURE 2 - Enzymatic activity of thymidine kinase.
HeLa cells were infected with 50 PFU/cell of vaccinia virus and collected 8 hours after the end of the adsorption. In the test were used extracts of cells with 10 ug proteins/samples, with [^{3}H]-thymidine, in times of 15, 40 and 65 min. (samples in triplicats were used).

* ▩- value of cells infected by vaccinia virus WR minus value of infected cells by $vH5_{TK1}$ (vaccinia virus recombinant non productor of TK)
** ▩ - value of cells infected by vaccinia virus WR (treated with CHX in the "critical period") minus value of infected cells by $vH5_{TK1}$
*** ■ - value of cells infected by vaccinia virus WR treated by CHX continually after the infection minus value of infected cells by $vH5_{TK1}$

DNA synthesis - In order to verificate the viral DNA synthesis in these conditions, an incorporation of [^{3}H]-thymidine was made (FIG 3).

Experiments of incorporation in cells, infected and treated with araC were realized, as control, because this drug inhibit the synthesis of DNA (26). In the cells infected and treated with CHX in the "critical period" (column 5) there was observed a lower incorporation than with the cells infected and treated with ara C (column 3). So we can conclude that the synthesis of DNA becomes strongly inhibited, under these

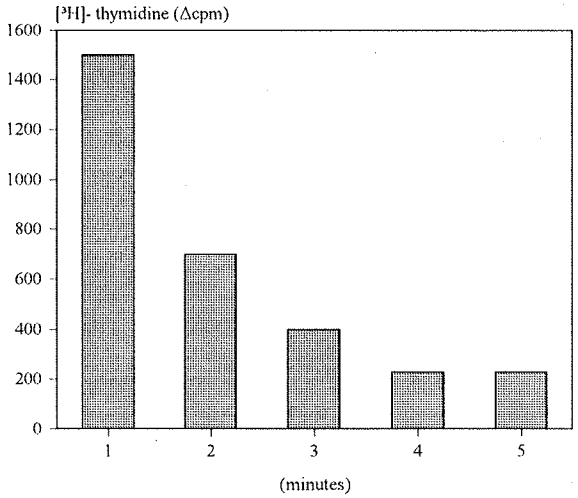


FIGURE 3 - DNA synthesis in HeLa cells infected by vaccinia virus WR. Cells were infected with 50 PFU/cell in the time 0. After 2 hours from the end of adsorption the [^{3}H]-thymidine was added to the medium during 10 min., and the incorporation was read in scintillator. 1 - infected cells, non treated with drug; 2 - non infected cells; 3 - cells infected and treated with ara C (50 ug/ml) from 15 min. before the adsorption up to the collect; 4 and 5 - cells infected and treated CHX (300 ug/ml) from 15 min. before the adsorption until 2 hours after the same, when the sample 5 received the [^{3}H]-thymidine during 10 min. and was collected, while the sample 5 received the [3H]-thymidine during 10 min., 4 hours after the end of the adsorption (Samples in triplicates were used).

conditions. Non-infected cells (column 2) presented an incorporation of [3H] less than the half of the value of the sample of infected and non treated by drug cells (column 1). Cells treated with CHX from viral adsorption to collect (column 4 - control of the efficiency of the drug) presented an incorporation identical to the one in column 5.

DISCUSSION

Inhibition of the synthesis of proteins in HeLa cells infected with vaccinia virus - The 43 kDa protein is a cellular protein. The 76, 65, 35 and 29 kDa proteins are viral, because they weren't present in the host cell, and late viral, because they were synthesized after the DNA replication, which is around 2 hours after the begining of the infection (13). The 33 kDa protein is viral because it was absent in the host cell, and is an early viral protein, because it was synthesized in the presence of ara C, inhibitor of the DNA synthesis (26). The 27 kDa protein is viral because it was absent

in the host cell, and is an early and late viral (early-and-late) because it was synthesized before and after the viral DNA replication. The 42.5 kDa protein is an early viral protein (5, 7). Analysing these results, beyond the profile of the non infected cells (samples 2 and 6), we can divide the profiles in two groups: one in which late and early-and-late proteins were present (samples 1, 5, 7 and 11) and another presenting early and early-and-late proteins (samples 3, 4, 8, 9 and 10). Therefore, we can verify that, when infected cells were treated with CHX in the "critical period" (samples 3 and 9), proteins with an electrophoretic profile, characteristic of early viral proteins were synthesized, when the inhibitor is withdrawen.

The contested irreversibility in relation to early proteins. According to MOSS and FILLER (24), the presence of CHX in the first 2 hours of infection of HeLa cells with high multiplicity of vaccinia virus, produces an irreversible block in the viral DNA replication and in the viral protein synthesis, even after the withdrawal of the inhibitor. However, in the present work in PAGE, in experiments with extracts of infected cells with vaccinia virus in this conditions, there was observed the presence of proteins with relative molecular mass similar to the viral proteins found in cells infected and treated with ara C (early viral proteins) (FIG 1, A and B). This suggests that some proteins of the samples treated with CHX in the quoted conditions, could be early viral proteins. The CHX inhibits the synthesis of protein at peptidyl transferase level (19), threfore after the transcription, not impeding the formation of the mRNA, which became accumulated(4, 29). So, it was concluded that the treatment with CHX in the 'critical period", of the cells infected with vaccinia virus, made a synchronization of the early phase of the viral multiplication possible. After the elimination of CHX, all the mRNA are present in sufficient quantity to produce proteins detectable by the employed methods. In another experiment (4) CHX was applied during the whole time of infection, and the protein profile in PAGE was without bands (control of drug efficiency) (data not shown).

As the TK is an early protein of the vaccinia virus (22, 31), its enzymatic activity was detected, in virus infected cells and treated with CHX in the "critical period", to show the production of functional early protein. Although the results had showed that the function of TK in these conditions of infection and treatment of the cells, was around one third of the one in the cells infected without treatment, it was at least 4 times higher than the value of the enzyme activity obtained in infected cells treated with CHX from the begining of the infection to the harvest. Therefore, the synthesis of the early vaccinia virus proteins occurs, even in the conditions described by MOSS and FILLER (24) as irreversible. These differences certainly are due to the differents methods of analysis, because MOSS and FILLER (24) reached their conclusion from the absence of the synthesis of viral and cellular proteins, throgh the incorporation of aminoacids, with a mix of the same ones containing [14C], twenty years ago, a test less sensible than the one effectuated in this work, with the direct visualization of viral bands in PAGE, when the proteins are marked with [35S]-methionine (FIG 1). The profile of the proteins in PAGE, in these samples, didn't demonstrate any protein with characteristic molecular weight of the late class.

The experimental conditions utilized in this study are very important; for example, proteins of the vaccinia virus were obtained by CARRASCO and BRAVO (5), in two dimensional PAGE, with extracts from cells infected and treated with CHX in the first 3 hours of the infection. The cells were infected with a multiplicity of 20 PFU/cell, not filling therefore the requisites of a condition of treatment with CHX in the "critical period", which is of 50 PFU/cell or by double infection and, so, there didn't occur inhibition of the DNA synthesis nor of the viral late proteins.

From proteins of HeLa cells, the one which has more expression in two dimensional PAGE is the one with a relative mass of 43kDa, which is the actin (5). This protein wasn't observed, or any others from cellular origin, in extracts of HeLa cells infected with high multiplicity of vaccinia virus, in PAGE (FIG 1, A and B), which confirms MOSS (23).

In the vaccinia virus early multiplication phase, there is a protein with a relative molecular mass of 42,5 kDa (5, 7). The sensibility of PAGE isn't sufficient to differentiate between proteins with molecular mass of 42,5 and of 43 kDa. So, in the cells infected by vaccinia virus and treated with ara C (lanes 4 and 8 from the FIG 1) the protein with molecular mass approached this value, was considered as having 42,5 kDa (early viral protein), because it was cited thus in the literature. The same occurs with the equivalent protein in the cells infected by vaccinia virus and treated with CHX in the "critical period"(lanes 3, 9 and 10 from FIG 1). Many authors affirm that there is an inhibitor agent of the synthesis of the cellular protein in the vaccinia virus core (1, 7, 8, 20), and it is therefore not necessary to make *de novo* synthesis of viral proteins to the occurrence of the phenomenon. So, in the samples

with the profile of early viral proteins and the absence of viral late proteins, we suppose that this selective inhibition had occurred, and the protein in question (with molecular mass approached of 42,5 kDa) in this case is the early viral protein of the vaccinia virus.

Synthesis of viral DNA. The viral DNA synthesis was analysed (FIG 3) in HeLa cells, infected by vaccinia virus WR and treated with CHX in the "critical period", and we observed that the incorporation of [^{3}H]-thymidine by cells was lower than the incorporation in cells treated with ara C. This result indicate a synthesis of viral DNA extremelly inhibited, it was therefore concluded, that there is an irreversible effect in relation to the viral DNA synthesis, in these conditions. The incorporation of [^{3}H]-thymidine in column 2 (non-infected cells) was a cellular DNA synthesis. Because there is a strong inibition of cellular DNA synthesis in infections of cells with highmultiplicity of vaccinia virus (13, 17), the value of this incorporation is only valid in the comparison between column 1 with column 2 (to compare viral DNA incorporation with cellular DNA incorporation).

Mechanism of protein synthesis inhibition - As the DNA synthesis was strongly inhibited (FIG 3), and the late viral protein synthesis was blocked (FIG 1, A and B), while the early viral protein synthesis occurred (FIG 1, A and B), in conditions of cells infected and treated with CHX at "critical period", if there is a cellular factor that the virus needs to multiplicate itself in infected cells(24), this factor is necessary to the viral DNA replication and not for the viral early protein synthesis . Or, because of the syncronization and accumulation of early viral mRNA, caused by cycloheximide, early viral protein, synthesized after the inibitor's withdrawal, could have caused the irreversible effect in the viral DNA synthesis, because there was a modification of temporal regulation of the vaccinia replication cycle. Without the occurrence of the viral DNA replication, which according to a regulation cyclic model (16), provides molds of free DNA to intermediary and late genic expression, regulating the whole process, the viral multiplication is interrupted. The VLTF (vaccinia late transcription factor) (30), according to the same model (16), is synthesized just in the intermediate phase of the infection, one more reason for which there isn't translation (because there isn't transcription) of late genes, when we utilize the treatment with CHX in the "critical period"of cells infected with vaccinia virus. Although the VITF-A and the VITF-B (vaccinia intermediate transcription factors) were synthesized in early phase of infection, the intermediate genes aren't expressed in infected cells, without that the DNA viral synthesis having ocurred (28). Because the VETF (vaccinia early transcription factor) (2, 3) still according to this model (16), is synthesized in the late phase of previous infection, this factor is still present, together with the viral RNA polimerase (14), even with the presence of proteic inhibitors, and the transcription (4, 29) and translation of early genes (FIG 1, A and B) are then realized in infected cells.

As a result of viral double infection, we obtained a complete inibiton of the cellular protein synthesis (FIG 1 B), which confirms once again that the viral structural proteins have an important role in this inhibition process.

RESUMO

"Período crítico"para o bloqueio irreversível da replicação do vírus vaccínia.

Células HeLa foram infectadas com virus vaccínia e tratadas com cicloheximida de 30 a 60 minutos após a infecção. Extratos de células foram preparados e as proteínas analisadas em um SDS-PAGE 12,5%, e um perfil de proteínas precoces de virus vaccínia foi observado, o qual era similar ao de células infectadas e tratadas por citosina arabinosídeo, um inibidor da replicação de DNA. A fim de obter mais evidências de que proteínas precoces do vírus vaccínia poderiam ser sintetizadas nestas condições, foi feito um ensaio da atividade enzimática da timidina quinase *in vitro* (a timidina quinase é uma proteína precoce do virus vaccínia) e foi obtida 30% de atividade enzimática quando comparado com extratos de células infectadas e não tratadas pela droga. Para melhor localizar o bloqueio no ciclo de replicação do vírus vaccínia, a síntese de DNA viral foi medida através da incorporação de [^{3}H]-timidina em células HeLa infectadas, e uma forte inibição da síntese de DNA viral foi observada. Os resultados sugerem que o "período crítico"para o bloqueio irreversível na multiplicação viral é após a síntese de proteínas precoces e antes da replicação de DNA.

Palavras-chave: inibição da síntese proteica, virus vaccínia, cicloheximida.

ACKNOWLEDGEMENTS

We thank Dr. Romain Rolland Golgher (UFMG, MG) for data discussion and for suggestions. Marco Antonio da Siva Campos had scholarship of CAPES and Dra. Erna Geessien Kroon has fellowship of CNPq. This work was supported by CNPq.

REFERENCES

1. BEN-HAMIDA, F.; BEAUD, G.. In vitro inhibition of protein synthesis by purified cores from vaccinia virus. Proc. Natl. Acad. Sci. USA, 75:175-179, 1978.
2. BROYLES, S.S.; YUEN, L.; SHUMAN, S.; MOSS, B.. Purification of a factor required for transcription of vaccinia virus early genes. J. Biol. Chem., 263:10754-10760, 1988.
3. BROYLES, S.S.; MOSS, B.. DNA-dependent ATPase activity associated with vaccinia virus early transcription factor. *J. Biol. Chem.*, **263:**10761-10765, 1988.
4. CAMPOS, M. A. S. .Inibição da síntese de proteínas em células infectadas com vírus vaccínia: expressão de genes celulares e virais. Belo Horizonte. Instituto de Ciências Biológicas. Universidade Federal de Minas Gerais. 93 p. tese de mestrado em Microbiologia, 1992.
5. CARRASCO, L.; BRAVO, R.. Specific proteins synthesized during the viral lytic cycle in vaccínia-virus infected HeLa cells: analysis by high-resolution, two dimensional gel electrophoresis. *J. Virol.*, **58:**569-577, 1986.
6. CHEN, X.; ROSEL, J.; DUNKER, R.; GOLDSCHMIDT, R.; MAURER-SCHULTZE, B.; JUNGWIRTH, C.. Reversible inhibition of poxvirus replication by cicloheximide during the early phase of infection. *Virology,* **124:**308-317, 1983.
7. COOPER, J. A.; MOSS, B.. Transcription of vaccinia virus mRNA coupled to translation *in vitro. Virology,* **88:**149-165, 1978.
8. DAMASO, C. R. A.; MOUSSATCHÉ, N.. Vaccinia core transcription prevents "in vitro"depolymerization of polyribossomes in rabbit reticulocyte lysate. *Brazilian J. Med. Biol. Res.*, **20:**763-766, 1987.
9. DULBECCO, H.; VOGT, M.. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.*, **99:**167-182, 1954.
10. EAGLE, H.. Aminoacid metabolism in mammalian cell cultures. *Science,* **130:**432-437, 1959.
11. HRUBY, D. E. BALL, A. . Cell-free synthesis of enzimatically active vaccinia virus thymidine kinase. *Virology,* **113:**594-601, 1981.
12. JOKLIK, W. K. The purification of four strains of poxviruses. *Virology,* **18:**9-18, 1962.
13. JOKLIK, W. K.; BECKER, Y.. The replication and uncoating of vaccinia DNA. *J. Mol. Biol.,* **10:**452-474, 1964
14. KATES, J. R.; BEESON, J.. Ribonucleic acid synthesis in vaccinia virus. Synthesis of polyriboadenilyc acid. *J. Mol. Biol.*. **50:**19-33, 1970.
15. KATES, J. R.; McAUSLAN, B. R.. Messenger RNA synthesis by a "coated" viral genome. *Proc. Natl. Acad. Sci. USA,* **57:**314-320, 1967.
16. KECK, J.G.; BALDICK Jr., C. J.; MOSS, B.. Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. *Cell,* **61:**801-809, 1990.
17. KIT, S; DUBBS, D. R.. Biochemistry of vaccinia-infected mouse fibroblasts (strain L-M). Effects on nucleic acid and protein synthesis. *Virology,* **18:**274-285, 1962.
18. LAEMMLI, U.K. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature,* London, **227**:680-685, 1970.
19. LEWIN, B.. Genes IV. 4. ed. Oxford University Press and Cell Press, 1990.
20. LOBO, D.S.; REBELLO, M.A.; MOUSSATCHÉ, N.. Core transcription restores "in vitro" inhibition of protein synthesis induced by vaccinia virus. *Biochim. Biophys. Acta.* **868:**183-189, 1986.
21. LOWRY, O.H.; ROSEBROUGH, N. J.; FARR, A.L.; RANDELL, R.J.. Protein measurement with Folin phenol reagent. *J. Biol. Chem.*. **193:**265-275, 1951.
22. McAUSLAN, B.R.. The induction and repression of thymidine kinase in the poxvirus infected cell. *Virology,* **21:**383-389, 1963.
23. MOSS, B.. Inhibition of HeLa cell protein synthesis by the vaccinia virion. *J. Virol.,* **2:**1028-1037, 1968.
24. MOSS, B.; FILLER, R.. Irreversible effects of cycloheximide during the early period of vaccinia virus replication. *J. Virol.,* **5:**99-108, 1970.
25. NOLL, H.. Chain initiation and control of protein synthesis. *Science,* **151:**1241-1245, 1966.
26. ODA, K.; JOKLIK, W. K.. Hibridization and sedimentation studies on 'early 'and 'late' vaccinia messenger RNA. *J. Mol. Biol.,* **27:**395-419, 1967.
27. OSTERHOFF, J.; JÄGER, M.; JUNGWIRTH, C.; BODO, G.. Inhibition of poxvirus-specific functions induced in c h i c k embryo fibroblasts by treatment with homologous interferon. *Eur. J. Biochem.,* **69:**535-543, 1976.
28. VOS, J.C.; SASKER, M.; STUNNENBERG, H.G.. Promoter melting by a stage specific vaccinia virus transcription factor is independent of the presence of RNA polymerase. *Cell,* **65:**105-113, 1991.
29. WOODSON, B.. Vaccinia mRNA synthesis under conditions which prevent uncoating. *Bioch. Biophys. Res. Comm.,* Duluth, **27:**169-175, 1967.
30. WRIGHT, C.F.; MOSS, B.. Identification of factors specific for transcription of the late class of vaccinia virus genes. *J. Virol.,* **63:**4224-4233, 1989.
31. ZÖLLER, B.; KREMPIEN, U.; JUNGWIRTH, C.. Synthesis of early vaccinia virus-specific enzymes under conditions of immediate early gene expression. *Eur. J. Biochem.,* **143:**589-591, 1984.

Received for publication in 19/05/92

Rev. Microbiol., São Paulo, **24**(2): 111-117, 1993

BEHAVIOUR OF *STAPHYLOCOCCUS AUREUS* AND OF *ESCHERICHIA COLI* AND INJURY FORMATION DURING PRODUCTION AND STORAGE PHASES OF PARMESAN CHEESE

Celso Guimarães Barbosa[1]
Paschoal Guimarães Robbs[2]
Valdir Favarin[3]

SUMMARY

The behaviour of *S. aureus* and *E. coli* during production and storage phases of Parmesan cheese was studied. The product was prepared following the usual technology, being the milk used inoculated with *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, after pasteurization, to provide concentrations of approximately 10^6 and 10^7 cells/ml, respectively. Cheese pieces were kept at 15 ± 2ºC for 130 days and later at 21 ± 2ºC until day 200. In order to assess injury formation, counts were made on all of the samples of *S. aureus* and *E. coli*, both by traditional methodology and those employing the recovery period of stressed cells (15). pH and acidity were constantly determined. The ripening process of Parmesan cheese prompted the reduction of four log cycles on *S. aureus* counts. In the case of *E. coli* the multiplication occurred at the beginning of ripening (about one log cycle), which stabilized later on, with a count decrease to four and five log cycles in relation to the beginning of ripening. Thermal injury occurred on both organisms during heating phase of curd at 50ºC, especially on *E. coli*. Sublethal acid injury on *E. coli* began to occur faster and in smaller concentrations of lactic acid (0,50%) than it did for *S. aureus* (0,70%), during ripening.

Key words: *S. aureus, E. coli,* bacterial injury, Parmesan cheese.

INTRODUCTION

In the Brazilian market, several types of cheese are found, of which the Minas cheese, which can be soft or cured, is outstanding; there is the "Prato" (Edam-like) cheese, noted for its semi-cooked curd, and the Parmesan cheese, which has a low humidity content. Among these, only Parmesan cheese, owing to its long ripening period (over four months), is most of times made from crude milk. In cheese production, when milk pasteurization is not conducted properly and when certain hygienic/sanitary and processing cares are not taken, several microorganisms, such as

1 Department of Mathematics, UFRJ, Antiga Estrada Rio-São Paulo,km 47, 23851-970 Seropédica, Rio de Janeiro, Brazil
2 Department of Food Technology, UFRJ, Fellow of CNPq
3 Department of Food Technology, UFRJ

Staphylococcus aureus and *Escherichia coli* may represent danger to public health. The multiplication of *S. aureus* during and after the manufacture of various cheese, has caused the involvement of these products in several intoxication outbreaks (22). Also *E. coli*, especially the enterotoxigenical and enteroinvading strains, have been reported as causing such outbreaks (13).

Some researchers have studied the behaviour of *S. aureus* in cheese consumed in Brazil, particularly in "Minas" cheese (1, 5) and Parmesan cheese (19). On other types of cheese not traditional in Brazil, some studies have been carried out (8, 19). As to the behaviour of *E.coli* during cheese manufacturing periods, this has been less researched.

During the manufacture of Parmesan cheese, in which curd heating is employed, stress or thermal injury of microbe cells may occur, depending on processing conditions. Now by the time of ripening, a period in which an acidity increase is observed, there is the possibility of occurrence of acid injury. The Department of Food Technology of Federal Rural University of Rio de Janeiro (UFRRJ) runs a large program on the microbiology of dairy products, which also comprises the microbiological stability aspect. This paper is an attempt to verify the behaviour, as well as the development of sublethal injuries in *S.aureus* and *E. coli* during the production and marketing phases of Parmesan cheese.

MATERIALS AND METHODS

Preparation of *S. aureus* and *E. coli*. *S. aureus* strain ATCC 25923 and *E. coli* ATCC 25922 were used. Stock cultures kept at 5ºC on biazed nutrient agar (Difco), were chopped into tubes containing lactosed broth which, after incubation at 35 ± 1ºC for 12 hours. Cells were then centrifuged and washed three times in saline solution at 0.85% at 6000 rpm, on an EXCELSA 2 centrifuge manufactured by FANEM.. After last washing, cells were resuspended in 50 ml peptonated water at 0.1%, optical density being read on spectrophotometer model SPECTRONIC 20 from Bausch & Lomb, at 570 nm. Along with this, number of viable microorganisms from suspension was determined by counting on plates, on Baird-Parker agar media (BP-Merck agar), for *S. aureus*, and violet red bile agar (VRBA-Merck) for E. coli. On relating the absorbance measure of suspension and the number of viable microorganisms from it, a factor was obtained which was used to inoculate the pre-determined cell count into the samples.

Cheese preparation. Pieces were produced in a local, small-size dairy industry. After quick pasteurization of milk (72ºC for 15 seconds), and cool ing at 32ºC, the following were simultaneously inoculated: lactic culture (on a 15% ratio) of *Lactobacillus* and of *Streptococcus thermophilus*; suspensions of viable cells in concentration of approximately 10^6 and 10^7 cells/ml, respectively; clot in an amount needed to obtain coagulation in 25-35 minutes, and calcium chloride on a ratio of 25 g per 100 liter milk. On following steps, the traditional manufacturing methodology was used, salting being not made. Cheese produced (two pieces weighting 7.5 kg each) were stored first in incubator room at 15 ± 2ºC for 130 days, and later in a B.O.D. oven f rom FANEM at 21 ± 2ºC up to end of experiment (200 days) .

Sampling. Physico-chemical and microbiological analyses were conducted from manufacture through ripening. Samples were obtained on following phases: (a) inoculation of bacterial suspension at 33ºC (zero time); (b) clotting, at 34ºC (at 22 minutes); (c) curd heating, at 40ºC (at 33 minutes); (d) whey removal, at 50ºC (44 minutes); (e) ripening at times of 1, 2, 3, 4, 11, 18, 25, 32, 46, 60, 74, 88, 102, 116, 130, 144, 158, 172, 186, and 200 days.

Samples obtained on cheese processing were collected in sterilized glass vials, kept in isothermal boxes with ice, and analyzed within one hour at the most. In order to obtain cheese aliquots on later steps, a sterilized tubular extractor (a cork driller) was used, with which cheese cylinders were drawn from inside the pieces, on sites from which the surface layer had pre- previously been removed. Afterwards the holes were filled with sterilized vaspar (vaseline and paraffin, 1:1).

Physico-Chemical and Microbiological Determinations

pH and acidity determination. pH and acidity analyses were conducted in triplicate, following the techniques described in the Handbook on Physico-Chemical methods from the Animal Reference National Laboratory (LANARA), Ministry of Agriculture (3). pH was measured on a pHmeter model CG 711 from SCHOTT MAINZ; on liquid samples, readings were made directly, while on cheese samples 5 g were previously homogenized in 50 ml distilled water prior to mensuration. For acidity analysis, cheese samples were macerated in a vial and subjected to previous extraction with ethylic alcohol p.a. at 95% (neutralized) for 24 hours. After filtration, it was titred with a NaOH solution 0.1N, acidity being expressed in lactic acid

(% w/w).

Microbiological analysis. Traditional methodologies were used, as recommended by LANARA (4) as well as those enabling the recovery of injured cell, recommended by the American Public Health Association (APHA) (12, 15).

For the analysis of *S. aureus* after the traditional methodology, 25 g aliquot were employed which were homogenized for two minutes in 225 ml peptonated water at 0.1%, in a sterilized blender cup. From such suspensions (1:10) decimal dilutions were made in peptonated water at 0.1%, which were inoculated (0.1 ml from each dilution with eight replications) following the surface plate method, on Petri plates containing Baird-Parker agar. These were incubated at 37 ± 1ºC and read after 24 and 48 hours. On the technique using recovery of injured cells of *S. aureus*, 25g cheese aliquots were homogenized as previously described, however, in 225 ml casein-peptone soymeal-peptone broth (CASOY-Merck broth), and incubated at 35 ± 1ºC for two hours. Afterwards, decimal dilutions, inoculations and incubations were made following description of the traditional methodology.

On the analysis of *E. coli* by the traditional methodology, samples and decimal dilutions were prepared as seen previously on the traditional analysis of *S. aureus*. Afterwards, 0.01 ml aliquots from each dilution (eight replications) were inoculated, following the surface plate method, on Petri plates containing violet red bile agar. Incubation was made in an oven at 35 ± 1ºC for 24 hours. In the technique using the recovery of injured *E. coli* cells, decimal dilutions of samples mentioned in previous item were inoculated by the surface plate method, on plates containing a thin layer (approx. 5 ml) of casein-peptone soymeal-peptone agar (CASOY-Merck agar). After incubation at 35 ± 1ºC for two hours, plates were overlaid with approximately 15 ml violet red bile agar and incubated in an oven at 35 ± 1ºC for 24 hours, when characteristic colony counts were made.

Statistical analysis. Essays were designated taking into consideration an entirely randomized design, with two factors (employed methodology and assessment period) and four replication per treatment. For the assessment of results from microbiological counts, values thereof were analyzed by Fisher-Snedecor's F test and the Tukey's test, both at a 5% significance level (17). For percent calculations of non-detected cells, the mathematical equation described by RAY (14) was used.

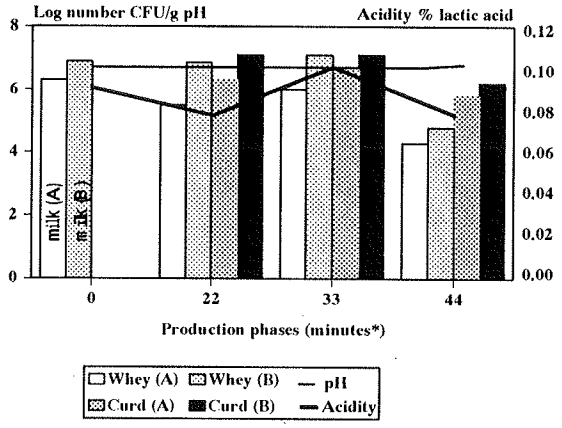


FIGURE 1 - Behaviour of *S. aureus* population on inoculated Parmesan cheese (≅ 10^6 *S. aureus* cells/ml milk and ≅ 10^7 *E. coli* cells/ml milk), as assessed by the traditional methodology (A) and the methodology employing stressed-cell recovery (B); of pH and acidity during the production phases. (*) 0 min: inoculation of bacterial suspension (33ºC)
22 min: milk clotting (34ºC)
33 min: curd heating (40ºC)
44 min: whey removal (50ºC)

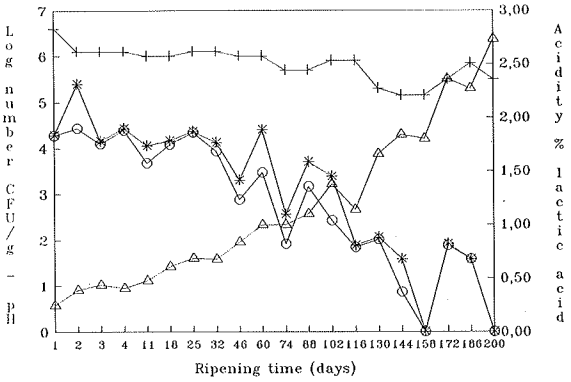


FIGURE 2 - Behaviour of *S. aureus* population on inoculated Parmesan chee cheese (≅ 10^6 *S. aureus* cells/ml milk and ≅ 10^7 *E. coli* cells/ml milk), as assessed by the traditional methodology (- o -) and the method of employing stressed-cell recovery (-*-); of pH (-+-) and acidity (-Δ-) during the storage phases.

RESULTS AND DISCUSSIONS

On *S. aureus* behaviour. Results concerning pH and acidity determinations, as well as *S. aureus* counts following the two methodologies during manufacture and storage phases are seen in Figures 1 and 2.

It is noted from Figure 1 that there was, after clotting, a greater hold of *S.aureus* to the curd than to whey. Indeed, MINOR & MARTH (10), on reviewing *S. aureus* in dairy products, reported work with Colby cheese produced with crude milk inoculated with *S. aureus*, refer to counts 13 to 32 times higher of the bacterium in the curd than in whey.

From Figure 1, it is also noted that there was practically no variation on count from milk inoculated with *S. aureus* ($2,0 \times 10^6$cfu/ml) for the curd after whey removal (at 50ºC), which was $1,2 \times 10^6$cfu/g. Seemingly, there was a balance between the increase phases (curd multiplication or retention) and count decrease phases (temperature effect). Multiplication during initial phases of Cheddar cheese manufacture was already observed by TATINI et al. (18), who at first found a fast, and later, a slow increase in *S. aureus* count during pressing, final counts being six to 12 times as much as first counts. They also concluded that an approximate 10-fold increase was the result from cell's physical retention. Still with regard to *S. aureus* behaviour on initial phases of Parmesan cheese preparation, (Figure 1), at first a marked drop in counts was noted (from $1,2 \times 10^6$ to $1,9 \times 10^4$cfu/g), of nearly two log cycles, from curd after whey removal to shaped product (first day), (Figure 2), thus indicating possible decreases owing to temperature (50ºC) effect. After first day of preparation, *S. aureus* counts kept practically constant up to day 32 when it started to drop progressively (Figure 2). Within the stability period, acidity was lower than 0.70% lactic acid. Decrease began to be effectively observed when acidity reached 0.68% and pH 6.1. From this point on, decrease was marked and steady although some oscillation occurred, probably owing to sampling factors. At the end of experiment, decreases were already higher than four log cycles in relation to first day. Acidity increase resulting from production of lactic acid by lactic bacteria is essential to the control of *S. aureus* population in cheese. A very interesting study on this microorganism's behaviour during the marketing period of "Minas" and "mussarela" cheese was carried out by ASSIS (1). On the former cheese type, sensible decrease in counts was observed when acidity reached the 0.50% level and, on the "mussarela" cheese type, 0.80% lactic acid. Until such concentrations were reached, however, an increase in counts was observed for both types of product. DELAZARI et al. (5) also found development of *S. aureus* in "Minas" cheese, which was more marked on first days after processing. Increases in *S. aureus* counts on phases following processing were also detected on "Minas" cheese and on "mussarela" cheese by ASSIS (1). On "Burgos" cheese, NUNEZ et al. (11) also verified similar behaviour, there being an increase in *S. aureus* count on first day after manufacture which kept steady up to day 3 of storage at 15ºC, followed by a gradual decrease on days 5 and 7. Therefore, it is seen that acid concentration is critical, and, if certain value is not reached, there is an increase in risk potential resulting from *S.aureus* development. That was also found by ZEHREN & ZEHREN (22) on Cheddar cheese and by TATINI et al. (18), on Cheddar and Colby cheese. Thus the lactic acid culture used is fundamental. However, the measure isolated from the pH in cheese has a relative importance, since in high pH values (above 5.0) inhibition may occur, depending on the lactic acid concentration. This results from the buffering power of casein. WALKER et al., cited by MINOR MARTH (10), shows this fact when they managed to recover staphylococci at the end of 120 days (approx. 1% of first day count), and verified that during ripening the pH level ranged from 5.10 to 5.75 and did not found any evidence of correlation between pH and staphylococci counts. Also the inhibiting effect of certain starter strains, especially *Streptococcus lactis*, upon *S. aureus*, should be taken into consideration (8, 11).

On E. coli behaviour. The results regarding to pH and acidity determinations, as well as *E. coli* counts following the two methodologies, during fabrication and storage phases, are expressed in Figures 3 and 4. It is observed from Figure 3, that *E. coli* counts in curd and whey kept practically constant up to 30 minutes (heating phase, at 40ºC).

Similarly to what occurred with S. aureus, counts in curd were higher than those in whey, however, on smaller proportions. At the time of whey elimination at 44 minutes, when the temperature reached 50ºC, decreses from two to three log cycles (per traditional count) occurred both on whey and curd, thus higher than those observed for S. aureus. On studying *E. coli*'s behaviour during the manufacture period of Cottage cheese VECCHIONACCE et al (20), verified that bacterial counts did not vary very much during inoculation, acidification, clotting, and cutting phases of curd at a 38ºC temperature. Using a low inoculum level ($2,5 \times 10^4$ *E. coli*/ml), they found that curd heating

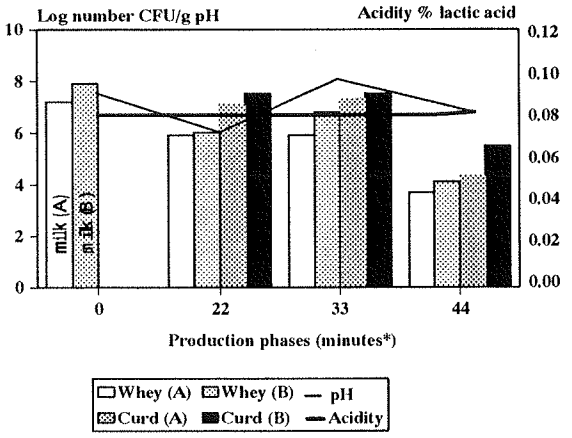


FIGURE 3 - Behaviour of *E. coli* population on inoculated Parmesan cheese (≅ 10^6 *S. aureus* cells/ml milk and ≅ 10^7 *E. coli* cells/ml milk), as assessed by the traditional methodology (A) and the methodology employing stressed-cell recovery (B); of pH and acidity during the production phases.

(*) 0 min: inoculation of bacterial suspension (33ºC)
22 min: milk clotting (34ºC)
33 min: curd heating (40ºC)
44 min: whey removal (50ºC)

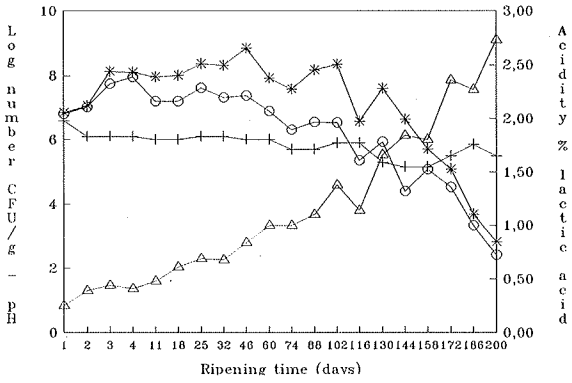


FIGURE 4 - Behaviour of *E. coli* population on inoculated Parmesan cheese (≅ 10^6 *S. aureus* cells/ml milk and ≅ 10^7 *E. coli* cells/ml milk, as assessed by the traditional methodology (--o--)and the methodology employing stressed-cell recovery (- * -); of pH (- + -) and acidity (-Δ-) during the storage phases.

from 43 to 49ºC, thus close to the 50ºC temperature used in this piece of research, caused the reduction of approximately one log cycle, and that at 54ºC there occurred entire destruction of the microorganism.

It is observed from Figure 4, that immediately after molding (first day), an increase was observed in the Parmesan cheese of approximately two log cycles (per traditional count) in relation to curd's count after whey removal at 50ºC, and which continued, in a slower fashion, up to day 4, when it remained practically stabilized up to day 46. At this phase, the acidity was 0.84% lactic acid and the pH was around 6.0. From then on, a gradual decrease in counts was observed, which reached five log cycles on the 200th day of ripening. Some papers on *E. coli's* behaviour during preparation and ripening phases of cheese show results similar to those obtained in this paper . THOMPSON & MARTH (1 9), verified that during ripening of Parmesan cheese, coliforms were gradually decreased up to full elimination at seven months at the most. On the other hand, KORNACKI & MARTH's paper (9), on a cheese similar to Colby cheese, showed that an inoculum with approximately 10^3 *E. coli* cells/ml milk yielded a cheese with counts higher than 10^8 *E. coli*/g immediately after fabrication.

On injury formation. Concerning injury formation during the processing phase (from zero to 44 minutes) of Parmesan cheese, from Figure 1 it is observed that significantly different *S. aureus* counts occurred between the two methodologies employed, except for the whey sample obtained at 44 minutes. Higher counts were obtained when injured cell recovery was used, through a two hour preincubation at 35ºC in liquid medium. During the ripening phase (Figure 2 - samples of 1 to 200 days), this also occurred, although the statistical tests showed significant differences only on count averages of samples obtained on days 2, 11, 60, 88 and 102 of ripening. Therefore, the use of the injured cell recovery methodology, generally made it possible to obtain higher *S. aureus* counts than by the traditional methodology, although differences were significant only on samples in which per cent cells not detected by the traditional methodology were above 40.0%.

Thermal injury of *S. aureus* cells possibly occurred on the phase in which curd reached around 50ºC and acid injury began to be noted from the period between 32 and 46 days of ripening, when acidity levels increased considerably. From then on a significant difference between counts averages of both methodologies was observed.

The development of acid injury on *S. aureus* cells was studied by EL-BANNA & HURST (7). Using a pH between 4.0 and 4.5 they verified that the microorganism's cells, in the presence of lactic acid,

died a little faster at 37ºC than at 46ºC. They did not manage to detect the bacterium after two days at pH 4.0 using the temperature of 37ºC, but it was possible to detect it after seven days at pH 4.5 Using pH 5.0, there was a cellular increase at both temperatures. The authors stress that such results were already expected, since antimicrobial activity of organic acids is due mainly to non-dissociated molecules, which occur in larger proportion with lower pH values. Thus, 35% from total acetic acid occur in a non-dissociated form at pH 5, whereas 85% occur at pH 4. Similarly, 6 and 39% from total lactic appear in the non-dissociated form at pH 5 and 4, respectively (2). Therefore, small pH increases may mean large increases in the ratio of non-dissociated molecules. ZAYAITZ & LEDFORD (21) also on studying acid injury effect upon *S. aureus,* verified that a difference of at least 25% between counts in non-selective medium (trypticase soy agar-TSA) and in selective medium (TSA + 7% NaCl) evidence acid injury on *S. aureus* cells. In the case of Parmesan cheese, formation of acid injury of said microorganism probably augmented after the acidity reached values around 0.70% This was also found by ASSIS (1) at levels between 0.50% and 0.80%, for "Minas" and mussarela cheese. Indeed, at increased acidity conditions, greater differences between counts begin to be observed from 46 days of ripening, which well characterizes stressed cell formation.

Regarding *E. coli*, during the first phases of Parmesan cheese processing (Figure 3), samples generally showed count averages significantly different following the two methodologies, higher counts occurring when pre-incubation was used. Also during ripening, (Figure 4), was this observed, except after 158 days when count levels were low, which impaired statistical interpretation. Thus, in a general way, the methodology using injured-cell recovery made it possible for larger bacterial counts to be made than after the traditional methodology counts being significantly higher when recovery percentages were above 29.2%.

Thermal injury formation on *E. coli* at the processing of this cheese happened when the temperature reached levels of 50ºC . Indeed, DRAUGHON & NELSON (6) on comparing methods of direct plating for recovery of thermally injured *E. coli* cells, concluded that there was only significant differences between these counts and those obtained per selective media when cells were heated at 59ºC . This was not noted at 62ºC and 69ºC, possibly owing to the strong lethal power of such temperatures, which did not make it possible for sub-lethal injury formation to occur. Same was observed by SINGH et al. (16), who, on studying the reactivation of thermally injured *E. coli* cells in milk, verified that exposure to the temperature of 60ºC for 10 minutes, did not make it possible for the recovery in solid medium to be done. This, however, occurred in whole or unskimmed milk, in a period of 10 days.

Acidity was also accounted for decrease in *E. coli* counts from day 46. At this time the acidity level was already high, and gradually increased, which undoubtedly caused larger differences between the counts after the two methodologies (Figure 4), and larger percentages of cells recovered by pre-incubation. Thus the occurrence of *E. coli* injury during the production of Parmesan cheese may be attributable, besides other factors, to the thermal heating which occurred at first steps (cooking) and to acidity development which begins to be observed from the third day and is more strongly expressed after a more advanced period in ripening (around 48 days), when a cellular death process starts, with consequent decrease in count per both methodologies.

In conclusion we can say that: a) thermal injury occurs in *S. aureus* and especially in *E. coli* at the whey removal phase at 50ºC; b) counts of both microorganism are higher in curd than in whey. c) *S. aureus* counts remain stabilized at the onset of ripening and later undergo large decreases; d) *E. coli* counts increase at the onset of ripening, stabilizes, and then decreases; e) methodologies employing recovery phase of stressed cells generally make it possible to obtain higher counts than per traditional methodologies, both for *E coli* and for *S. aureus*; f) in *S. aureus* sublethal acid injury is clearly observed around first month of ripening, a phase in which acidity (above 0.7%) already stressed the microorganism; g) in *E. coli,* sublethal acid injury begins to be observed immediately after first days of ripening process, at acidity levels lower (0.50%) than is needed to cause stress on *S. aureus.*

RESUMO

Comportamento de *Staphylococcus aureus* e de *Escherichia coli* durante as fases de produção e de estocagem do queijo parmesão.

Foi estudado o comportamento de *S. aureus* e de *E. coli* durante as fases de produção e de estocagem do queijo parmesão. O produto foi preparado pela tecnologia usual, tendo sido o leite, após a pasteurização, inoculado com *S. aureus* ATCC 25923 e com *E. coli* ATCC 25922 para fornecer concentrações de aproximadamente 10^6 e 10^7 células/ml, respectivamente. As peças de queijo foram mantidas a 15 ± 2ºC por 130 dias e posteriormente a 21 ± 2ºC até o 200º dia. Para se avaliar a formação de injúrias, em todas as amostras foram feitas contagens de *S. aureus*

e de *E. coli* pelas metodologias tradicionais e pelas que empregam período de recuperação de células estressadas (15). O pH e a acidez foram sempre determinados. O processo de maturação do queijo parmesão provocou reduções de quatro ciclos log nas contagens de *S. aureus*. No caso de *E. coli*, ocorreu multiplicação no inicio da maturação (cerca de um ciclo log) havendo posteriormente estabilização e decréscimo das contagens, que chegaram a reduzir de quatro a cinco ciclos log em relação ao início da maturação. A injúria térmica ocorreu nos dois microrganismos na fase de aquecimento da massa a 50ºC, especialmente em *E. coli*. A injúria ácida subletal, em *E.coli*, começou a ocorrer mais rapidamente e em menores concentrações de ácido lático (0,50%) do que em *S. aureus* (0,70%), durante a maturação.

Palavras-chaves: *S. aureus*, *E. coli*, injúria bacteriana, queijo parmesão.

ACKNOWLEDGMENTS

We thank Drs. ANITA TIBANA and ALBA LUCIA S. NOLETO, of the Department of Medical Microbiology of Federal University of Rio de Janeiro, for the provision of microorganisms cultures, and the employees and faculty of the Food Technology Department of UFRRJ for their valuable help.

REFERENCES

1. ASSIS, E. M. *Comportamento de Staphylococcus aureus e formação de injúria durante o periodo de comercialização dos queijos Minas e mussarela*. Lavras, ESAL, 1990. 95p. Dissertação (Mestrado em Ciências dos Alimentos), Escola Superior de Agricultura de Lavras, 1990.
2. BAIRD-PARKER, A. C. Organic acids. In: *INTERNATIONAL COMISSION ON MICROBIOLOGIGAL SPECIFICATION FOR FOODS. Microbial Ecology of foods*; factors affecting life and death microrganisms. New York, Academic Press, v. 2, p. 126-135, 1980.
3. BRASIL. Ministério da Agricultura. Secretaria Nacional de Defesa Agropecuária. Laboratório Nacional de Referência Animal. *Métodos analíticos oficiais para controle de produtos de origem animal e seus ingredientes*. I. Métodos físico-químicos. Brasilia, 1981.
4. -------- Ministério da Agricultura. Secretaria Nacional de Defesa Agropecuária. Laboratório Nacional de Referência Animal. *Métodos analíticos oficiais para controle de produtos de origem animal e seus ingredientes*. II. Métodos microbiológicos. Brasília, 1981.
5. DELAZARI, I.; LEITÃO, M. F. F.; GERALDINI, A. M.; EIROA, M. N. U. & VALLE, J. L. E. Desenvolvimento de *Staphylococcus aureus* e produção de enterotoxina em queijo tipo Minas. *Coletânea do ITAL*. **9:** 163-174, 1978.
6. DRAUGHON, F. A. & NELSON P. J. Comparison of modified direct-plating for recovery of injured *Escherichia coli*. *J. Food. Sci.* **46:** 1188-1191, 1981.
7. EL-BANNA, A. A. & HURST, A. Survival in foods of *Staphylococcus aureus* grown under optimal and stressed conditions and the effect of some food preservatives. *Can. J. Microbiol.* **29:** 297-302, 1983.
8. GAYA, P.; MEDINA, M.; BAUTISTA, L. & NUNEZ, M. Influence of lactic starter inoculation, curd heating and ripening temperature on *Staphylococcus aureus* behaviour in Manchego cheese. *Internat. J. Food. Microbiol.* **6:** 249-257, 1988.
9. KORNACKI, J. L. & MARTH, E. H. Fate of nonpathogenic and enteropathogenic *Escherichia coli* during the manufacture of Colby like cheese. *J. Food Protec.* **45:** 310-316, mar. 1982.
10. MINOR, T. E.& MARTH, E. H. *Staphylococcus aureus* and staphylococcal food intoxications. A review. III. Stapnylococci in dairy foods. *J. Milk Food Technol.* **35**: 77-82, mar. 1972.
11. NUNEZ, M.; CHAVARRI, F. J.; GARCIA, B. E. & GAYTAN, L. E. The effect of lactic starter inoculation and storage temperature on the behaviour of *Staphyloccocus aureus* and *Enterobacter cloacae* in Burgos cheese. *Food Microbiol.* **3 :** 235-242, 1986.
12. ORDAL, Z. J.; IANDOLA, J. J.; RAY, B. & SINSKEY, A. G. Detection and enumeration of injured microorganisms. In: SPECK, M. L. *Compendium of Methods for the Microbiological Examination of Foods. Washington,* APHA, cap. 7, p. 163-169, 1976.
13. RASH, K. E. & KOSIKOWSKI, F.V. Behaviour of enteropathogenic *Escherichia coli* in Camembert cheese made from ultrafiltered milk. *J. Food Sci.* **47**: 728-732, 736, 1982.
14. RAY, B. Methods to detect stressed microrganisms. *J. Food Protec.* **42:** 346-355, apr. 1979.
15. RAY, B. & ADAMS JR., D. M. Repair and detection of injured microorganisms. In: SPECK, M. L. *Compendium of Methods for the Microbiological Examination of Foods.* Washington, APHA, cap. 7, p. 112-123, 1984.
16. SINGH, R. S.; BATISH, V. K., GHANDER, H. & RANGANATHAN, B. Reactivation of heat injured *Escherichia coli* cells in milk. *Milchwissenchaft.* **40:** 398-401, 1985.
17. SNEDECOR, G. W.& COCHRAN, W. G. *Statistical methods.* Ames, Iowa Press, 6a. ed., 593 p., 1976.
18. TATINI, S. R., JEZESKI, J. J., MORRIS, H. A., OLSON JR., J. C. & CASMAN, E. P. Production of staphylococcal enterotoxin A in Cheddar and Colby cheeses. *J. Dairy Sci.* **54:** 815-825, 1971.
19. THOMPSON, T. L.& MARTH, E. H Changes in Parmesan cheese during ripening: Microflora - coliforms, enterococci, anaerobes, propionibacteria and staphylococci. *Milchwissenschaft.* **41**: 201-205, 1986.
20. VECCHIONACCE, R. A.; BASSETE, R.& METHA, R. S. Survival of *Escherichia coli*, strain W, during the manufacture of Cottage cheese. *J. Dairy Sci.* **61:**1704-1708, 1978.
21. ZAYAITZ, A. E. K. & LEDFORD, R. A. Characteristics of acid- injury and recovery of *Staphylococcus aureus* in a model system . *J. Food Protec* **48:** 616-620, july 1985.
22. ZEHREN, V. F. & ZEHREN, V. F. Relation of acid development during cheesemaking to development of staphylococcal enterotoxin. *A. J. Dairy Sci.* **51:** 635-639, may 1968.

Received for publication in 7/10/92

Rev. Microbiol., São Paulo, 24(2): 118-124, 1993

BEHAVIOUR OF *STAPHYLOCOCCUS AUREUS* AND OF *ESCHERICHIA COLI* AND INJURY FORMATION DURING PRODUCTION AND STORAGE PHASES OF "PRATO" CHEESE

Celso Guimarães Barbosa[1]
Paschoal Guimarães Robbs[2]
Sonia Maria da Costa Raimundo[3]

SUMMARY

The behaviour of *S. aureus* and of *E. coli* during production and storage of "Prato" (Edam-like) cheese were studied. The product was prepared according to the usual procedure. After pasteurization, the milk was inoculated with *S.aureus* FRI A-100 and *E. coli* ATCC 25922 in concentrations corresponding to 10^6 and 10^7 cells/ml, respectively. The cheeses samples were kept at 12 ± 2ºC for 5 days followed by storage at 15 ± 2ºC up to the end of experiment (98 days). In order to assess cell injury, the *S.aureus* and *E. coli* counts were made according to the traditional methodologies and those employing a period for recovery of stressed-cells (19). Both pH and acidity were constantly determined. *S.aureus* counts increased during the cheese manufacture, whereas *E. coli* remained practically constant. However *E. coli* counts presented marked decrease since the onset of the ripening on curing period, with acidity levels, corresponding to 0.60% and 0.70%. On the other hand, *S.aureus* counts started to decrease after 21 days and corresponding acidity varying from 0.75% to 0.80%. At the end of three months ripening, the results indicated decrease in *S. aureus* and *E. coli* counts, corresponding to eight and seven to to eight log cycles, respectively. Sub-lethal acid injury was more noticeable on *E. coli* than on *S. aureus*..

Key words: *S. aureus, E. coli,* bacterial injury, "Prato" cheese.

INTRODUCTION

"Prato" cheese has a commercial expression similar to that "Minas" cheese in Brazil. However, due to a more standardized technique in its manufacture, the former is better characterized commercially. It's a cheese having a semi-cooked curd, pressed, with a minimum ripening period of thirty days (14).

In cheese manufacture, raw milk, improperly pasteurized milk and inadequate hygienic-sanitary measures can result in microbial contamination, including *Staphylococcus aureus* and *Escherichia coli*, thus representing danger to the public health. The multiplication of *S. aureus* prior to and during the manufacture of various types of cheese, has caused various intoxication outbreaks (5, 25). Also *E. coli* , specially has enterotoxigenic or enteroinvading strains,

1. Mathematics Department, UFRJ, BR 465, km 7, Seropédica, Rio de Janeiro, Brazil
2. Food Technology Department, UFRJ, Fellow of CNPq
3. Food Technology Department, UFRJ

are reported to cause many serious outbreaks (17).

During "Prato" cheese ripening, a period characterized by acidity increase, stress or acid cell injury may be observed. ASSIS (1), studying cell injury during the marketing period of "Minas" and "muzzarela" type cheeses, observed the possibility of *S. aureus* sub-lethal injury due to acidity.

This paper is part of the program in dairy microbiology, with the objective of studying the behaviour of *S. aureus* and *E. coli*, and their sub-lethal injury development during the manufacture and commercialization of "Prato" cheese.

MATERIALS AND METHODS

Cultures - *S. aureus* strain ATCC25923 and *E. coli* ATCC 25922 were used. Stock cultures kept at 5ºC on biased nutrient agar (Difco), were chopped into tubes containing lactose broth which, after incubation at 35 ± 1ºC for 12 hours, were transferred to Erlenmeyer vials containing same medium. These were kept under blending at 180 rpm at 35 ± 1ºC for 12 hours and cooled at 5°C for two hours. Cells were then centrifugated and washed three times in saline solution at 0.85% at 6000 rpm, on an EXCELSA 2 centrifuge (FANEM). After last washing, cells were re-suspended in 50 ml peptonated water at 0.1%, optical density being read on spectrophotometer SPECTRONIC 20 (BAUSCH-LOMB), at 570nm. Along with this, number of viable microorganisms from suspensions was determined by counting on plates, on Baird-Parker agar (BP-Merck agar), for *S. aureus*, and violet red bile agar (VRBA-Merck) for *E. coli*. On relating the absorbance measure of suspension and the number of viable microorganisms from it, a factor was obtained which was used to inoculate the pre-determined cell count into the samples.

Cheese manufacture - The cheese were manufactured in a small dairy plant. After pasteurization (72ºC for 15 s) and cooling (32ºC), the milk was inoculated with 1.5% lactic cultures (*Streptococcus lactis* and *Streptococcus cremoris*) and viable suspensions of *S. aureus* and of *E. coli*, in such volumes as to obtain concentrations correspondent to 10^6 and 10^7 cells/ml of milk respectively; rennet (0.0025%), calcium chloride (25 g/100 l milk), and annato (0.008%). The traditional procedure for "Prato" cheese manufacture was followed with salting (2%) before full whey removal. The cheese obtained (nine two-kilo pieces) were initially stored in ripening room at 12 ± 2ºC for five days and then transfered to 2 B.O.D. (FANEM) at 15 ± 2ºC till the end of experiment (98 days).

Analysis - Physico-chemical and microbiological analysis carried out during cheese manufacture and throughout the ripening period. Samples were taken after milk inoculation with the bacterial suspension (zero time) ;after setting (40 minutes); after cooking the curd (60 minutes); at the beginning of the prepressing (80 minutes); and at 1, 2, 3, 4, 5, 9, 13, 17, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, and 98 days of curing.

The samples obtained during cheese manufacture were collected in sterilized glass vials, kept in an ice-water bath, and analyzed within a period of one hour. For the obtention of cheese aliquots on following phases, cross-sections cuts were made on the pieces after the elimination of the surface layer. The exposed surfaces were lined with sterilized "vaspar" (vaseline and paraffin, 1:1).

Physico-chemical - pH and acidity determination. Both pH and acidity were determined in triplicate, according to the methodology of the Handbook on Physico-Chemical Methods from the Animal Reference National Laboratory (LANARA), Ministry of Agriculture (3). pH was measured on a pHmeter CG711 (SCHOTT MAINZ) ;on liquid samples, readings were made directly, while on cheese samples 5g were previously homogenized in 50ml distilled water prior to mensuration. For acidity analysis, cheese samples were macerated in a vial and subjected to previous extraction with ethylic alcohol p.a. at 95% (neutralized) for 24 hours. After filtration, it was trited with a NaOH solution 0.1N, acidity being expressed in lactic acid (%w/w).

Microbiological analysis - The routine microbiological analysis were carried out according to the procedures of the LANARA (4). The recovery of injured cells was assessed according to American Public Health Association (APHA) (15, 19).

For the analysis of *S. aureus* after the traditional methodology, 25g aliquots were employed which were homogeneized for two minutes in 225ml peptonated water at 0.1%, in a sterilized blender cup. From such suspensions (1:10) decimal dilutions were made in peptonated water at 0.1% which were inoculated (0.1 ml from each dilution with eight replications) following the surface plate method, on Petri plates containing Baird-Parker agar. These were incubated at 37 ± 1ºC and read after 24 and 48 hours.

On the technique using recovery of injured cells of *S. aureus*, 25 g cheese aliquots were homogeneized as previously described, however, in 225 ml casein-peptone soy meal-peptone broth (CASOY-Merck broth), and incubated at 35 ± 1ºC for two hours. Afterwards, decimal dilutions, inoculations and incubations were made following descriptions of the traditional methodology.

On the analysis of *E. coli* by the traditional methodology, samples and decimal dilutions were prepared as seen previously on the traditional analysis of *S. aureus*. Afterwards, 0.01 ml aliquots from each dilution (eight replications) were inoculated, following the surface plate method, on Petri plates containing violet red bile agar. Incubation was made in an oven at 35 ± 1ºC for 24 hours. In the technique using the recovery of injured *E. coli* cells, decimal dilutions of samples mentioned in previous item were inoculated by the surface plate method, on plates containing a thin layer (approx. 5 ml) of casein-peptone soymeal-peptone agar (CASOY-Merck agar). After incubation at 35 ± 1ºC for two hours, plates were overlaid with approximately 15 ml violet red bile agar and incubated in an oven at 35 ± 1ºC for 24 hours, when characteristic colony counts were made.

Experimental design and the statistical analyses - Essays were designated taking into consideration an entirely randomized design, with two factors (employed methodology and assessment periods) and four replications per treatment. For the assessment of results from microbiological counts, values thereof were analyzed by Fisher-Snedecor's F test and the Tukey's test, both at 5% significance level (22). For percent calculations of non-detected cells, the mathematical equation described by RAY (18) was used.

RESULTS AND DISCUSSIONS

Behaviour of *S. aureus* - The results of pH, acidity and of *S. aureus* counts using both methodologies during the manufacture and curing are shown in Figures 1 and 2. It indicated in Figure 1 that *S. aureus* counts during processing increased and that counts in the curd were larger than in whey. MINOR & MARTH (12), reported that Colby cheese, produced from crude milk inoculated with *S. aureus* showed counts 13 to 32 times higher than the whey.

It was also observed that in Figure 1 there occurred an increase from 4.0 x 10^5 cfu/g (inoculated milk) to 3.3x10^6 cfu/g during setting (40 minutes) and from 8.8x10^6 cfu/g (60 minutes) to 3.3x10^7 cfu/g in the curd (80 minutes), indicating a gradual population increase in the curd, prompted by either bacterial multiplication or the physical retention of cells during syneresis. The bacterial multiplication during the initial phases of Cheddar cheese manufacture was already observed by TATINI et al. (23), as being fast, followed by a slow increase in *S. aureus* counts during pressing, to extent of 6 to 12 times the initial count. They also concluded that an increase of approximately 10 times, was a result of the physical retention of cells. MINOR & MARTH (12), working with Cheddar cheese, report 15 to 45- fold increases in staphylococci counts in cooked curd in relation to milk. It was observed that for "Prato" cheese (Figure 1), such increases corresponded to approximately two log cycles, thus being much larger than those observed for Cheddar cheese. Figures 1 and 2 also indicated an increase in *S. aureus* count from 3.3 x 10^6 cfu/g in the curd at the moment of whey removal, up to 9.6x10^7 in the cheese with one day. The rapid multiplication of *S. aureus* in "Minas" cheese whey, which is not cooked (34 - 36ºC) was observed by SANTOS & GENIGEORGIS (20).

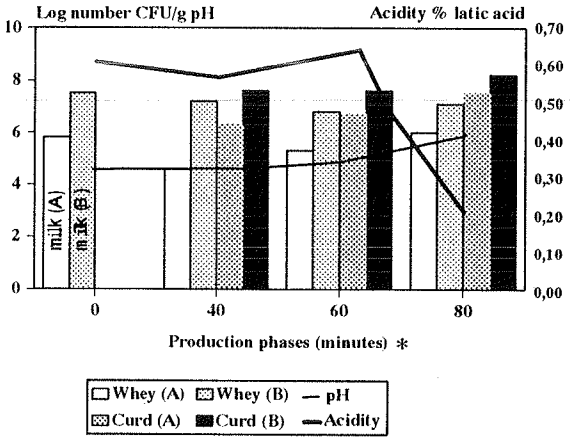


FIGURE 1 - Behaviour of S. aureus population in inoculated "Prato"cheese (≅ 10^6 S. aureus cells/ml milk and ≅ 10^7 E. coli cells/ml milk), as assessed by the traditional methodology (A) and by the methodology employing stressed-cell recovery (B); of pH and acidity during production phases.

(*) 0 min: inoculation of bacterial suspension (33ºC)
40 min: milk clotting (34ºC)
60 min: curd heating (41ºC)
80 min: begining of prepressing (38ºC)

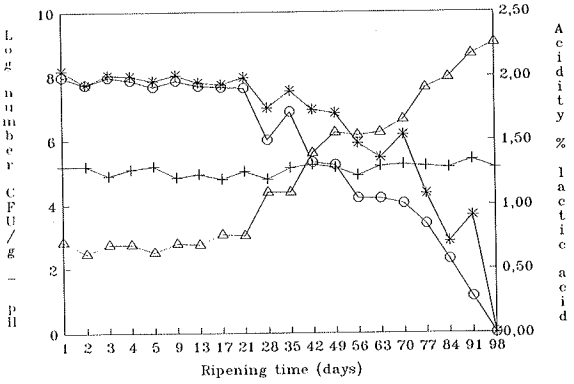


FIGURE 2 - Behaviour of S. aureus population in inoculated "Prato"cheese (≅ 10^6 S. aureus cells/ml milk and ≅ 10^7 E. coli cells/ml milk), as assessed by the traditional methodology (- o -) and by the methodology employing stressed-cell recovery (- * -); of pH (- + -) and acidity (- Δ -) during ripening.

These authors found increases of approximately four log cycles, with *S. aureus* domination in the microbiota present in the first four hours, when the initial inoculation was 10^5 cells/ml. OTERO et al. (16) working with Burgos cheese verified that, with an *S. aureus* inoculum corresponding to 10^3 or 10^5 cells/ml, the multiplication increased one log cycle in the first day after manufacture.

The *S. aureus* counts kept practically constant from the first to 21 days ripening, when the acidity reached 0.76% lactic acid and the pH 5.1. After this period of curing a progressive reduction was observed till the end of the experiment.

The acidity resulting from the production of lactic acid by lactic bacteria is essential to control *S. aureus* in cheese. ASSIS (1), working with "Minas" and "Muzzarela" cheeses, observed a significant decrease in *S. aureus* counts when the cheese acidity reached 0.50 and 0.80%, respectively in "Minas" and "Muzzarela". She also observed *S. aureus* growth between these acidities were reached. DELAZARI et al. (6) also found growth of *S. aureus* in "Minas" cheese, mostly during the first days of curing. *S. aureus* growth during the first days of curing was also observed by SILVA (21) and ASSIS (1) in "Minas" cheese, and also in "Muzzarela" by ASSIS. (1). A similar behaviour was also observed in Burgos type cheese by NUNEZ et al. (13); *S. aureus* growth occurred during the first day following manufacture , kept stable up to the third day at 15°C; followed by a gradual decrease till the seventh day. Therefore, acid concentration is critical and if a certain value is not reached, the potential risk resulting from the presence of *S. aureus* is increased. This was also found by ZEHREN & ZEHREN (25) on Cheddar cheese and by TATINI et alli (23) on Cheddar and Colby cheeses.

Behaviour of *E. coli* - The results of pH, acidity, and of *E. coli* counts using both methodologies during manufacture and curing, are shown in Figures 3 and 4. It was indicated in Figure 3 that *E. coli* counts during manufacture kept practically constant and that the curd showed higher counts than the whey. It was also observed that the *E. coli* counts in the first day wee closer to those of the inoculated milk. The *E. coli* counts decreased gradually from the first day of manufacture to total absence after 98 days of curing as shown in Figure 4; thus resulting in a decrease higher than seven log cycles. BARBOSA (2) working with Parmesan type cheese observed a small increase during the first days of manufacture, followed by a slower decrease, that corresponded to five log cycles till the end of ripening and that the velocity of acidity production undoubtedly slower had influence on this result.

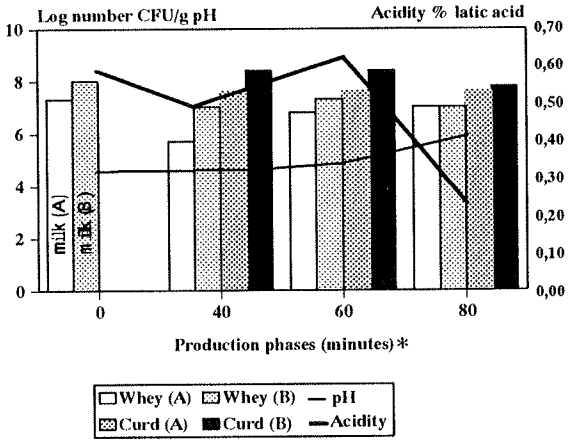


FIGURE 3 - Behaviour of E. coli population in inoculated "Prato"cheese (≅ 10^6 S. aureus cells/ml milk and ≅ 10^7 E. coli cells/ml milk), as assessed by the traditional methodology (A) and by the methodology employing stressed-cell recovery (B); of pH and acidity during production phases.

(*) 0 min: inoculation of bacterial suspension (33°C)
40 min: milk clotting (34°C)
60 min: curd heating (41°C)
80 min: begining of prepressing (38°C)

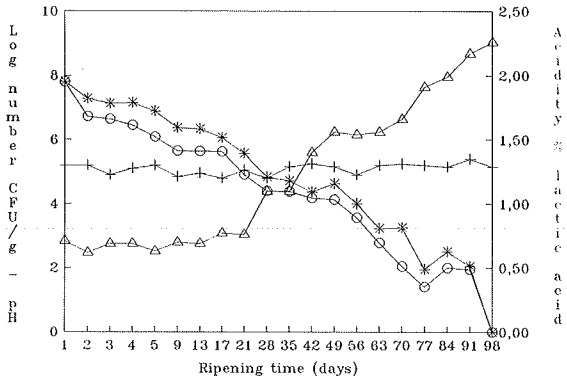


FIGURE 4 - Behaviour of E. coli population in inoculated "Prato" cheese (≅ 10^6 S. aureus cells/ml milk and ≅ 10^7 E. coli cells/ml milk), as assessed by the traditional methodology (- o -) and by the methodology employing stressed-cell recovery (- * -); of pH (- + -) and acidity (- Δ -) during ripening.

KORNACKI & MARTH (11) working with a cheese similar to Colby, showed that an inoculum corresponding to approximately 10^3 *E. coli* cells/ml of milk gave origin to a product having more than 10^8 *E. coli*/g after manufacture and a gradual decrease during the ripening period. The survival of enteropathogenic *E. coli* in Cammembert cheese and in salted cheese similar to Colby in a work carried out by FRANK et al. (9, 10), indicated higher inhibition of *E. coli* in Cammembert cheese, and absence after 6 weeks of curing.

The interaction between *E. coli* and homofermentative lactic cultures were studied by FRANK & MARTH (8). They verified that all *E. coli* strains inoculated in sterilized milk were more inhibited in their growth when 2% lactic culture followed by storage at 21ºC.

Process-injury of microorganisms - The process-injury during the 80 minutes, shown in Figure 1, indicated a significative difference in *S. aureus* counts between the two methodologies applied. Larger counts were obtained when recovery of the injured cells was used, pre-incubation the samples for two hours at 35ºC in liquid medium. During the 98 days ripening period, as shown in Figure 2, this difference was also observed. Therefore it was concluded that the injured-cell recovery method resulted in higher *S. aureus* counts (23.1%) than did the traditional one.

The development of acid injury on S. aureus cells was studied by EL-BANNA & HURST (7), at pH between 4.0 and 4.5 in the presence of lactic acid. ZAYAITZ & LEDFORD (24), also studying the effect of acid-injury of *S. aureus* observed a difference of, at least, 25% between counts on non-selective medium (tripticase soy agar - TSA), and selective medium (TSA + NaCl), evidencing injury of *S. aureus* probably increased after acidity reached values of 0.70%. This was also observed by BARBOSA (2) in working with yogurt and by ASSIS (1), in working with "Minas" and "Muzzarela" cheeses, when the acidity corresponded to 0.50 and 0.80%, respectively. Greater cell recovery is observed with 28 days of ripening, when the acidity is high, characterizing the presence of stressed cells.

During the manufacture and ripening of "Prato" cheese as shown in Figures 3 and 4, the *E. coli* counts were significantly higher when pre-incubation was used; except in the first day and after 21 days of ripening when the count levels were low. Thus, with the methodology employing the recovery of injured cells it is possible to obtain larger counts than with the traditional methodology, from the second day till the end of the ripening period when the recovery percentages were above 64.2%. The concentration of lactic acid (0.71%) in "Prato" cheese during manufacture (first day) was already sufficient to bring about a stressing action, which can be noted from the second day onwards, besides the gradual and steady drop in counts till the end of ripening. Thus, the occurrence of injury of *E. coli* during "Prato" cheese manufacture may be ascribed, among others factors, to acidity development, that starts immediately after manufacture and becomes more intense with ripening (21 days). The cell death process, however, occurs from the onset of ripening, being characterized by decrease in counts independently of the enumerating method applied.

This study indicated that the population of *S. aureus* and *E. coli* is larger in the curd than in the whey, the population of *S. aureus* increases during manufacture, whereas of the *E. coli* remains practically constant. The population of *S. aureus* remains constant during the first days of ripening and then decreases till the end of this process. The population of *E. coli* begins to decrease during the first days of ripening. The pre-incubation methodology increased the enumeration of *E. coli* and of *S. aureus* over the traditional method, indicating the presence of injured cells. The *S. aureus* sub-lethal acid injury becomes clear during the third and fourth weeks of ripening, where the acidity (above 0.80%) stresses the microorganisms, while the same effect on *E. coli* starts immediately on the first days of ripening, with lower acidity (0.60%).

RESUMO

Comportamento de *Staphylococcus aureus* e de *Escherichia coli* durante as fases de produção e de estocagem do queijo prato.

Estudou-se o comportamento de *S. aureus* e de *E. coli* durante a produção e cura do queijo prato. O produto foi obtido segundo a tecnologia usual, a partir do leite pasteurizado, inoculado com *S. aureus* FRI A-100 e *E. coli* ATCC 25922 em número correspondente a, aproximadamente, 10^6 e 10^7 células/ml, respectivamente. Os queijos foram mantidos a 12 ± 2ºC por cinco dias e posteriormente a 15 ± 2ºC até o final do experimento (98 dias). Para se avaliar a possibilidade de injúrias, a enumeração de *S. aureus* e de *E. coli* foram feitas de acordo com a metodologia tradicional, além da que emprega a recuperação de células estressadas, segundo RAY & ADAMS JR. (19). As populações de *S. aureus* aumentaram nas fases iniciais de obtenção do queijo, enquanto as de *E. coli* permaneceram praticamente constantes. As populações de *E. coli* sofreram grandes reduções a partir do início do período de maturação, quando a acidez já correspondia a 0.60%-0.70%, enquanto as de *S. aureus* já começaram a reduzir a partir do 21º dia, com uma acidez da ordem de 0,75% a 0,80%. No final de três meses de maturação, ocorreu reduções correspondentes a oito ciclos log nas populações de *S. aureus* e de sete a oito ciclos nas de *E. coli*. A injúria ácida subletal manifestou-se mais cedo quando se tratava de *E. coli*.

Palavras-chave: *S.aureus, E.coli,* injúria bacteriana, queijo prato.

ACKNOWLEDGMENTS

Thanks to doctors ANITA TIBANA and ALBA LÚCIA S. NOLETO, of the Medical Microbiology Department of the Federal University of Rio de Janeiro, for providing the cultures and to the Food Technology Department personnel of the Federal Rural University of Rio de Janeiro, for they valuable help and contribution.

REFERENCES

1. ASSIS, E.M. Comportamento de *Staphylococcus aureus* e formação de injúria durante o período de comercialização dos queijos Minas e Muzzarela. Lavras, ESAL, 1990. 95p. Dissertação (Mestrado em Ciências dos Alimentos) Escola Superior de Agricultura de Lavras, 1990.
2. BARBOSA, C.G. *Comportamento de Staphylococcus aureus e de Escherichia coli e formação de injúria durante as fases de produção e de estocagem dos queijos parmesão e prato e do iogurte.* Itaguaí, UFFRJ, 1990. 92p. Dissertação (Mestrado em Ciência e Tecnologia de Alimentos), Universidade Federal Rural do Rio de Janeiro, 1990.
3. BRASIL. Ministério da Agricultura. Secretaria Nacional de Defesa Agropecuária. Laboratório Nacional de Referência Animal. *Métodos analíticos oficiais para controle de produtos de origem animal e seus ingredientes.* I - Métodos físico-químicos. Brasília, 1981.
4. ------- Ministério da Agricultura. Secretaria Nacional de Defesa Agropecuária. Laboratório Nacional de Referência Animal. *Métodos analíticos oficiais para controle de produtos de origem animal e seus ingredientes.* II - Métodos microbiológicos. Brasília, 1981.
5. BRYAN, F.L. Epidemiology of milk-borne diseases. *J. Food. Prot.* **46:**637-649, 1983.
6. DELAZARI, I; LEITÃO, M.F.F.; GERALDINI, A.M.; EIROA, M.N.U. & VALLE, J.L.E. Desenvolvimento de *Staphylococcus aureus* e produção de enterotoxina em queijo tipo "Minas". *Coletânea do Ital.* **9**:163-174, 1978.
7. EL-BANNA, A.A. & HURST, A. Survival in foods of *Staphylococcus aureus* grown under optimal and stressed conditions and the effect of some food preservatives. *Can. J. Microbiol.* **29 :** 297 - 302, 1983.
8. FRANK, J.F. & MARTH, E.H. Inhibition of enteropathogenic *Escherichia coli* by homofermentative lactic acid bacteria in skimmilk. I - Comparison of strains of *Escherichia coli*. *J.Food Prot.* **40:**749-753, 1977.
9. FRANK, J.F.; MARTH, E.H. & OLSON , N.F. Survival of enteropathogenic and nonpathogenic *Escherichia coli* during manufacture and ripening of Cammembert cheese. **J. Food Prot. 40:**835-842, 1977.
10. Behaviour of enteropathogenic *Escherichia coli* during manufacture and ripening of brick cheese. *J. Food Prot.* **41:**111-115, 1978.
11. KORNACKI, J.L. & MARTH, E.H. - Fate of nonpathogenic and enteropathogenic *Escherichia coli* during the manufacture of Colby like cheese. *J. Food Prot.* **45:**310-316, 1982.
12. MINOR, T.E. & MARTH, E.H. - *Staphylococcus aureus* and staphylococcal food intoxications. A review. III. Staphylococci in dairy foods. *J. Milk Food Technol.* **35:**77-82, 1972.
13. NUNEZ, M.; CHAVARRI, F.J.; GARCIA, B.E. & GAYTÁN, L.E. The effect of lactic starter inoculation and storage temperature on the behaviour of *Staphylococcus aureus* and *Enterobacter cloacae* in Burgos cheese. **Food Microbiolog. 34:**235-242, 1986.
14. OLIVEIRA, J.S. de Fabricação dos principais queijos. In ------ *Queijos - Fundamentos tecnológicos.* São Paulo. Ícone, 1986, cap.6, p. 105-137.
15. ORDAL, Z.J. ; IANDOLA, J.J. ; RAY, B. & SINSKEY , A.G. - Detection and numeration of injured microorganisms. In: SPECK, M.L. *Compendium of Methods for the Microbiological Examinations of Foods.* Washington, APHA , cap. 7, p. 163-169, 1976.
16. OTERO, A.; GARCIA, M.C.; GARCIA, M.L.; PRIETO, M. & MORENO B. Behaviour of *Staphylococcus aureus* strains, producers of enterotoxin C1 or C2, during the manufacture and storage of Burgos cheese. *J. Appl. Bacteriolog.* **64:**117 - 122, 1988.
17. RASH, K.E. & KOSIKOWSKI, F.V. Behaviour of enteropathogenic *Escherichia coli* in Cammembert cheese made from ultrafiltered milk. *J. Food Sci.* **47:**728-732, 736, 1982.

18. RAY, B. & ADAMS JR., D.M. - Repair and detection of injured microorganisms. In SPECK, M.L. *Compendium of Methods* **for** *the Microbiological Examination of Foods.* Washington, APHA, cap. 7, p. 112-123, 1984.
20. SANTOS, E.C. dos & GENIGEORGIS, C. Survival and growth of *Staphylococcus aureus* in commercially manufactured Brazilian Minas cheese. *J. Food Prot.* **44:**177-184, 1981.
21. SILVA, C.A.M. *Avaliação da qualidade microbiológica do queijo Minas frescal.* Rio de Janeiro, UFRJ, 1981. 65p. Dissertação (Mestrado em Ciências Biológicas - Microbiologia) Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, 1981.
22. SNEDECOR, G.W. & COCHRAN, W.G. - *Statistical methods.* Ames, Iowa Press , 1976. 6ª ed. 593p.
23. TATINI, S.R.; JEZESKI, J.J.; MORRIS, H.A.; OLSON JR.; J.C. & CASMAN, E.P. - Production of staphilococal enterotoxin A in Cheddar and Colby cheeses. *J. Dairy Sci.* **54:** 815-825, 1971.
24. ZAYAITZ, A.E.K. & LEDFORD, R.A. - Characteristics of acid - injury and recovery of *Staphylococcus aureus* in a model system. *J.Food Prot.* **48:**616-620, 1985.
25. ZEHREN, V.F. & ZEHHREN, V.F. - Relation of acid development during cheesemaking to development of staphylococcal enterotoxin *A. J. Dairy Sci.* **51:**635-639, 1968.

Received for publication in 6/10/92

Rev. Microbiol., São Paulo, **24**(2): 125-131, 1993

QUANTIFICATION AND BEHAVIORAL CHARACTERIZATION OF *BACILLUS CEREUS* IN FORMULATED INFANT FOODS. I - GENERATION TIME*

Sonia Maria da Silva[1]
Leon Rabinovitch[1,2]
Paschoal Guimaraes Robbs[3]

SUMMARY

The ability of *Bacillus cereus* to grow in three different types of reconstituted formulated food, and the growth rates of the bacterium in each food, were investigated. The foods tested were: caramel-flavored porridge, chocolate-flavored porridge and bean soup with noodles, all of which are commonly served in public institutions.

Generation times (gt) were measured at 25°C , and 45°C, both for *B. cereus* that were naturally present in the products and for *B. cereus* that had been inoculated in the form of *B. cereus* NCTC 2599 spores into sterilized and unsterilized food. The gt values were calculated from growth curves obtained by counting colony formation units (CFU) on selective indicator-agar medium (MYP).

Intrinsic microflora were not observed to have an deleterious effect on the growth of *B. cereus* NCTC 2599 in any of the experiments at the indicated temperatures.

In experiments using both caramel and chocolate-flavored porridge , naturally occurring *B. cereus* displayed similar characteristic to *B. cereus* that were inoculated, and where incubation temperatures were found to have a significant effect on growth rates: mean generation times of 54.20, 31.30 and 56.00 minutes were recorded at temperatures of 25°C, 35°C and 45°C, respectively.

Our results demonstrate that *B. cereus* is able to multiply rapidly in all three foods, especially in bean soup with noodles, at temperatures around 35° C, in the presence or absence of indigenous microorganisms.

We conclude that care should be taken to observe basic hygiene practices during the preparation (rehydration) and consumption of these products, especially with regard to storage times and temperatures.

Key words: *B. cereus*, spores, food microbiology, generation time.

INTRODUCTION

Bacillus cereus has been well characterized as a significant causative agent of food-related illness in humans (9,12, 14, 18,20). The literature also states that several distinct clinical forms of gastroenteritis can result from the ingestion of foods contaminated with this bacterium (7, 8, 13). Two different syndromes have been observed. The diarrheic syndrome occurs 8 to 12 hours after ingestion of proteinaceous or vegetable foods, of puddings and of milk desserts; it is

1. Faculdade de Farmácia da Universidade Federal Fluminense, Rua Dr. Mário Vianna 523,Niterói, RJ 24240, Brasil.
2. Instituto Oswaldo Cruz/FIOCRUZ Av. Brasil 4365, Rio de Janeiro, RJ 21045. Cx. Postal: 926
3. Departamento de Tecnologia de Alimentos, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ 23851
* This paper constitutes part of the Master thesis of one of the authors

characterized by abdominal pain, heavy diarrhea, rectal cramps, and occasionally nausea and vomiting (7, 8, 24), with the symptoms usually lasting 12 to 24 hours (6). The emetic syndrome, on the other hand, is associated exclusively with foods containing flour or starch - in particular, boiled rice and is characterized by a rapid onset of clinical symptomatology within 1 - 5 hours, with nausea, vomiting and queasiness, followed in certain cases by diarrhea lasting 6 - 24 hours (7, 8, 16, 17).

Delazari et al (2) investigated 805 different samples of dehydrated food, including flours, soups, whole and skimmed milk, and enriched soya-based mixtures, and found that 44.4% of the contaminated samples contained between 10^4CFU/g and 10^5CFU/g of *B. cereus*. In the enriched mixtures 98.5% of the food samples displayed bacterial counts of between 10^2CFU/g and 10^3CFU/g . Subsequently, Blakey and Priest (1) demonstrated that this bacterium also occurs naturally in beans and lentils: when these products were incubated for 48 hours in sterile distilled water, *B. cereus* populations were observed to reach concentrations of over 10^6CFU/g at 37°C, and over 10^5CFU/g at 22°C. Meanwhile, Garcia et al (4) detected 2.6×10^7CFU/g of *B. cereus* in a dehydrated food made rice, soya and banana rehydrated with milk and incubated for 12 hours at 23°C ± 2°C. At 7°C, however, these authors detected no signs of bacterial growth.

McKnight et al (15) assessed the frequency of *B. cereus* in pasta made with and without eggs, and found contamination rates, respectively, of 58.3% and 87.5%, with a mean of 70.9%. *B. cereus* contamination levels were variable, with 52.1% of the samples displaying populations of between 10 CFU/g and 10^2CFU/g, and 20.1% between 10^2CFU/g and 10^3CFU/g.

B. cereus can be commonly detected in unprocessed agricultural products that are subsequently transformed into formulated foods; the latter frequently contain ingredients such as starchy materials, powdered milk, cereals and pulses. For this reason, the suppliers of unprocessed agricultural products must be constantly monitored in order to ensure that adequate standards are maintained for dehydrated foods served in public institutions; indeed, strict rules defining standards and control methods relating to *B. cereus* already exist. As for the retail trade, the Brazilian Ministry of Health (resolution no. 13/78) stipulates maximum allowable limits for *B. cereus* in a variety of foods, especially those containing starch.

For the most part, *B. cereus* is present in such foods in the form of spores, which are resistant to heat during cooking and which subsequently germinate as the foods cool. Since these foods act as an excellent culture medium, storage times and temperatures play a vital role in determining the risks associated with their consumption. Of particular concern are foods served in nurseries or schools and foods given to pregnant or nursing mothers. Failure to observe correct procedures with regard to storage times and temperatures can easily result in dangerous concentrations of bacteria.

In the present investigation, it was establish the potential risks associated with *B. cereus* contamination level in products usually served in public institutions. Behavioral studies of naturally occurring and inoculated *B. cereus* were also done and were carried out at various storage temperatures, using reconstituted formulated foods.

MATERIALS AND METHODS

Food-samples. Three types of sample, all of them dehydrated foods produced for the FAE (Foundation for the Assistance of Schoolchildren), were used: caramel-flavored porridge, chocolate-flavored porridge and bean soup with noodles.

Microorganism. *B. cereus* NCTC 2599, in the form of spores preserved in sterile distilled water and treated at 10^6CFU/ml, were used. The suspension was stored in the refrigerator (3°C ± 1°C).

Methodology for the study of *B. cereus* development in foods. On the basic of the instructions printed on the food-labels, it was decided that aqueous suspensions containing 157 g (equivalent to four soup - spoons) of dehydrated food per 1000 ml should be used throughout. The quantity by weight of dehydrated food was established by taking the average weight of one hundred samples each containing four label-recommended soup spoons of food. Each type of food was submitted to three different experiments, with each experiment using six 100 ml servings of the rehydrated product aseptically distributed in six 250 ml flasks with screw lids. In the first experiment, the product was prepared for consumption without further intervention; in the second, *B. cereus* spores were inoculated into the prepared food at a concentration of 10^2 spores/g; and in the third, the prepared food was sterilized prior to the inoculation of bacterial spores (again, 10^2 spores/g).

In all three experiments, two flasks of each product were at least incubated at 25°C, two at 35°C, and two at 45°C. At time zero, one of each two flasks was placed in the refrigerator while the other was immedi-

ately incubated at the set temperature. After 12 hours, all remainder flasks in the refrigerator were removed and also incubated at the set temperature. Samples of 1 ml of all flasks were taken after 0,3, 6, 9, 12, 15, 18, 21 and 24 hours, and (with the exception of the 0-hour and 3-hour samples) 10-fold serial dilutions in phosphate buffer at pH 7.0 were prepared (23). Aliquots of 0.1 ml taken from the serially diluted samples were plated out on phenol red/mannitol/ egg-yolk/polymyxin/agar (MYP), as described by Mossel at al (20). The plates were incubated at 35°C, and counts were taken at 12 and 48 hours.

Characterization of *B. cereus* - Vegetative cell dimensions - *B. cereus* are large - celled (> 1 μm width), therefore measurements of vegetative cells were carried out under an optical microscope using suspensions in sterile saline solution taken from cultures grown for 16 - 18 hours at 35°C in nutrient agar. The length and width of ten vegetative cells were measured for each slide culture. Cell dimensions were computed, as the average of the various measurements taken. The presence of para-sporal-body, normaly found in *B. thuringiensis* but not in *B. cereus*, was also searched by this procedure.

Morphology and position of spores. Suspensions (in sterile saline solution) of the cultures isolated as strains of *B. cereus* were examined periodically under optical microscopy (phase-contrast 2.000 x magnification). The bacteria were grown in soil-extract agar (AES) (11) at 35°C for 5 days (time of maximum sporulation). Para-sporal-body were also searched by this way.

Mortality tests. Cultures were grown in nutrient broth containing glucose 0,5% at 35°C, for 16 - 18 hours, prior to insertion of a single drop between a slide and a coverslip for observation using phase-contrast microscopy.

Biochemical and physiological tests. The following attributes were tested: carbohydrate fermentation (glucose, arabinose, xylose, trehalose and mannitol); anaerobic growth; acetyl-methyl-carbinol production; methyl red reaction; tyrosine decomposition; hydrolysis of gelatin; indol production; nitrate reduction; deamination of phenylalanine; growth in 7% sodium chloride; breakdown of casein; utilization of sodium citrate; hydrolysis of starch; hemolysin production. All cultures were incubated at 35°C (5, 11, 21).

Determination of generation times (gt). Counts of the *B. cereus* colonies plated on MYP agar were carried out every 3 hours at all incubation temperature. On the basis of these counts, it was possible to derive a graph showing bacterial population increases in the three foods under study. The specific rate of growth (μ) was calculated for each experiment from the onset of the exponential growth phase onwards. With the latter defined graphically as occurring when there was an exact intersection of points x_2 with t_2 and x_1 with t_1 the value of x was defined as the log of the number of CFUs in 1ml of the food suspension, and t as the time in hours. Finally, the known equation $\mu = \frac{\Delta \ln x}{\Delta t}$ was used, with the value of μ providing the means to calculate individual generation time using the formula $gt = \frac{\ln 2}{\mu}$ (min).

Maintenance of isolated *B. cereus* samples. Spore samples were maintained in AES medium (11) in the refrigerator.

RESULTS AND DISCUSSION

Growth of *B. cereus* in bean soup with noodles. Two different situations were investigated: a) when the bacterium was naturally present, and b) when it was inoculated into the food following sterilization. As can be seen in Table 1, in the three experiments carried out without prior sterilization of the food, the gt was always shorter at 45°C than at 25°C.

TABLE 1. Generation times (min) during the exponential growth phase of *B. cereus* NCTC 2599 following inoculation of this bacterium into rehydrated soup, with and without sterilization.

Food	Experiment	Incubation Temperature		
	Nº.	25ºC	35ºC	45ºC
Bean soup with noodles	19a	59.37	44.40	26.36
	21a	62.54	54.31	36.53
	23a	57.33	35.59	41.32
	18b	60.23	37.53	26.13
	20b	43.56	26.20	22.19
	22b	62.20	47.45	36.30
Mean		57.54	41.31	31.47

a - without sterilization
b - with sterilization

The gt values ranged from 43.56 min to 62.54 min at 25°C, from 26.20 min to 54.31 min at 35°C, and from 22.19 to 41.32 min at 45°C. Average gt values were 57.54 min at 25°C, 41.31 min at 35°C and 31.47 min at 45°C. To establish a minimum significant difference for these averages, they were submitted to the Tukey test at the 5% level (26). The minimum significant difference (m.s.d.) was 13.34 min, indicating that there was no significant difference in the gt values obtained at 35°C and 45°C. However, at 25°C the gt was significantly greater than at 35°C and 45°C. Thus, for this food product, temperatures between 35°C and 45°C - equivalent, in practice, to a temperature range between lukewarm and barely touchable (45 - 47°C) were found to pose a considerable hazard, since they allow intense multiplication of *B. cereus* (19).

Growth of naturally ocurring or artificially added *B. cereus* in chocolate and caramel-flavored porridges. Comparing the gt values obtained at the three different experimental temperatures (Table 2), it can be seen that lowest gt values occurred at 35°C, with a range of 23.21 min to 43.04 min. At 25°C, the gt values varied between 46.38 and 60.00 min. At 45°C, meanwhile, the gt values ranged between 38.20 min and 68.07 min, except for one experiment (no. 3) in which the gt value was considerably higher (125 min) than in the rest. Morita & Woodburn (19) also encountered a disproportionaly high gt value (152) when they added vitamins of the B-complex to rice incubated at 23°C, following inoculation of *B. cereus* strains B-4AC and 158/73.

On the basis of the gt values shown in this table, the following averages were calculated for the three different temperatures: 25°C = 54.20 min; 35°C = 31.30 min; and 45°C = 56.04 min.

Statistical analysis of these averages, using the Tukey 5% test (26) showed that there was no significant difference between the results obtained at 25°C and at 45°C. However, the difference at 35°C was significant, with considerably lower gt values than at other temperatures. Thus, with this group of products (porridges), the behavior of *B. cereus* is different to that exhibited with bean soup. In the former, 35°C is the temperature that best favors bacterial growth; in the latter, however, growth is favored as much at 45°C as at 35°C.

TABLE 2 - Generation times (min) during the exponential growth phase of naturally occurring **B. cereus** and inoculated NCTC 2599 in chocolate and caramel-flavored porridges (see Material and Methods).

Food	Experiment Nº.	Incubation Temperature		
		25ºC	35ºC	45ºC
Chocolate flavored porridge	3n	58.14	28.10	125.00
	6n	52.00	25.26	48.56
Caramel flavored porridge	9n	50.20	37.00	38.20
Chocolate flavored porridge	2i	56.55	35.48	48.28
	5i	46.38	43.04	40.00
	7i	57.33	33.56	68.07
Caramel flavored porridge	11i	60.00	23.21	63.51
	12i	54.31	32.50	43.36
	15i	50.20	27.16	41.59
	17i	53.32	24.14	44.20
Mean		54.20	31.30	56.04

i = inoculated
n = naturally occurring

Growth of *B. cereus* in sterilized and unsterilized chocolate and caramel-flavored porridge. The gt values recorded in the exponential growth phase of *B. cereus* strain NCTC 2599, following inoculation of this bacterium into porridge with and without prior sterilization, are shown in Table 3. The lowest gt values was observed at 35°C, in a range of 23.21 min to 43.04 min; at 25°C, the range was 37.33 to 68.07 min; and at 45°C, 28.10 min to 68.07 min.

The average gt values for each temperature were: 25°C = 54.33 min; 35°C = 29.01 min; and 45°C = 49.01 min.

Statistical analysis of these average values showed that there was no significant difference between incubation at 25°C and 45°C. At 35°C, however, the difference w significant. In addition, naturally occurring microorganisms had no effect on the growth of *B. cereus*, since at any given temperature no difference arose as a result of sterilization or the lack of it.

TABLE 3 - Generation times (min) during the exponential growth phase of **B. cereus** NCTC 2599 following inoculation into recontituted porridges, with and without sterilization.

Food	Experiment Nº.	Incubation Temperature 25ºC	35ºC	45ºC
Chocolate	2a	56.55	25.48	48.28
flavored	5a	46.38	43.04	40.00
porridge	7a	57.33	33.56	68.07
Caramel	11a	60.00	23.21	63.51
flavored	12a	54.31	32.50	43.36
porridge	15a	50.20	27.16	41.59
Chocolate	1b	37.33	28.10	30.58
flavored	4b	50.49	38.27	42.21
porridge	8b	40.28	30.31	33.02
Caramel	10b	58.00	27.20	28.10
flavoured	13b	68.07	26.20	64.55
porridge	14b	59.20	25.27	62.04
Mean		54.33	29.01	49.01

a - without sterilization
b - with sterilization

The microbiological quality of all formulated foods - including infant products - varies depending on the temperature at which the food is kept following preparation (2,3,21). The number of *B. cereus* present in the product can pose risks to health if populations exceed 10^6CFU/ml and if the product is kept at inadequate temperatures, i.e. below 55°C, according to Goepfert (10). In our experiments, generation times were generally found to vary between 22.19 min and 68.07 min, depending on the temperature and on the type of product. McKnight et al (15), meanwhile, recorded average gt values of 45.9 min at 30°C for *B. cereus* in cooked noodles. Clearly, then, it is easily possible for a population increase of one logarithmic cycle to occur within 1-1.5 hours, which means that it is essential to maintain adequate temperatures when keeping these kinds of food warm.

With regard to the initial levels of *B. cereus* in the school tested in our investigation (dried soup and porridge), it was apparent that these foods had been manufactured under properly controlled conditions and that *B. cereus* levels were therefore generally low. This is clear from the low incidence of bacteria detected in the initial phases of our experiments (< 10^2 UFC/ml). Nonetheless, when soup and porridge are distributed for consumption, problems can occur if they have been prepared, and then kept warm or stored, under conditions of inadequate hygiene. Poorly washed pans, ladles and other instruments can act as sources of *B. cereus* and thereby increase initial bacterial levels. Risks to health may be further compounded by inadequate control over storage times and temperatures, leading to an outbreak of illness (or an isolated case). The present investigation demonstrated that B. cereus can multiply rapidly in the three products tested, especially at temperatures ranging between 35°C and 45°C, whether or not the product initially contains micro organisms. It should be emphasized that the same observation can be applied to many other foods consumed in a typical Brazilian diet.

However, the crucial point with regard to the foods tested in our study is that they are produced for children, and therefore special care should be taken both in the production of these dried foods and in their subsequent preparation and storage as rehydrated food.

Thus, given that population increases of one log cycle can occur within an hour and a half (gt $\bar{x}$ = 30 min), if the initial population of *B. cereus* in soup (after rehydration) is 10^2CFU/ml, it would only take about 6 hours at 35°C - 45°C for the population to reach 10^6CFU/ml. This times-period would decrease to 4.5 hours or 3.0 hours if the initial bacterial concentration was 10^3CFU/ml or 10^4CFU/ml, respectively. It would therefore appear that the Brazilian present standard for infant foods (10^2CFU/g of dehydrated food)is adequate and safe, since only in the event of a serious lapse in hygiene during preparation and distribution could *B. cereus* concentrations reach dangerous levels.

RESUMO

Crescimento de *Bacillus cereus* em alimentos infantis.

Foi avaliada a possibilidade do desenvolvimento, bem como a velocidade de crescimento do *Bacillus cereus* em três alimentos formulados reconstituídos, a saber: mingaus sabores caramelo e chocolate, e sopa de feijão com macarrão. Tais produtos são distribuidos em programas institucionais.

Foram determinados os tempos de geração (tg) nas temperaturas 25°C, 35°C e 45°C, tanto de *B. cereus* naturalmente presentes nos produtos, quanto de esporos de *B. cereus* NCTC 2599 inoculados nestes alimentos previamente esterilizados ou não. As determinações dos tg, foram feitas a partir das curvas de crescimento obtidas por contagens de unidades formadoras de colônias (UFC) suspeitas, crescidas em meio seletivo-indicador (MYP).

Não foi observada, em nenhum experimento, a influência da microbiota natural dos produtos sobre o crescimento do *B. cereus* amostra tipo NCTC 2599, nas temperaturas estudadas. Nos mingaus sabores caramelo e chocolate, o *B. cereus* nativo e o inoculado tiveram comportamento bastante próximos, quando a temperatura de incubação teve uma grande influência sobre a multiplicação, sendo verificado a 25°C um tg médio de 54,20 min, a 35°C de 31,30 min e a 45°C de 56,00 min.

Os resultados demonstram que o *B. cereus* pode se multiplicar rapidamente nos três alimentos especialmente na sopa de feijão com macarrão, na faixa de 35°C - 45°C, na presença ou ausência da microbiota natural. Assim, devem ser tomados os cuidados higiênicos-sanitários básicos durante as fases de preparo (reidratação) e consumo dos produtos, especialmente os relativos à temperatura de manutenção e tempo de estocagem.

Palavras-chaves: *B. cereus*, esporos, microbiologia de alimentos, tempo de geração .

ACKNOWLEDGMENTS

We would like to express our thanks to the technical staff of the Laboratory of Bacterial Physiology in the Department of Bacteriology of the Oswaldo Cruz Institute, to Marise Sacramento de Magalhães for providing various drugs, to Prof. Valdir Favarin of the Department of Food Technology at the Federal Rural University of Rio de Janeiro (UFRRJ) for supplying food samples, and to Prof. Celso Guimaraes of the Department of Mathematics at UFRRJ for statistical analysis. Thanks also to Rosangela da Costa for typing the manuscript.

REFERENCES

1. BLAKEY, L.J. & PRIEST, F.G. - The occurence of *Bacillus cereus* in some dried foods including pulses and cereals. *J. Appl. Bacteriol.*, **48:** 297 - 302, 1980.
2. DELAZARI, I.,LEITÃO, M.F.F., GERALDINI, A.M. & EIROA, M.N.U. - *Bacillus cereus* em Alimentos Desidratados. *Bol. ITAL.*, Campinas, **60:** 31 - 40, 1978.
3. DELAZARI, I.,LEITÃO, M.F.F., GERALDINI, A.M. & EIROA, M.N.U. - Microbiologia de Produtos Institucionais. *Bol. ITAL.*, Campinas, **17:** 315 - 325, 1980.
4. GARCIA, V., LASTRETO, C. & MONTOYA, A. - Evaluación de la presencia de *Bacillus cereus* en un alimento infantil deshidratado. *Rev. Lat.amer. Microbiol.*, **23:** 141 - 144, 1981.
5. GIBSON, T. & GORDON, R.E. - In: Bergey's Manual of Determinative Bacteriology, 8 ed., Baltimore, Williams & Wilkins, p. 529 - 550, 1974.
6. GILBERT, R.J., & PARRY, J.M. - Serotypes of *Bacillus cereus* from outbreaks of food poisoning and routine foods. *J. Hyg. Camb.*, **78:** 69 - 74, 1977.
7. GILBERT, R.J. - *Bacillus cereus* gastroenteritis. In: Riemann, H. & Bryan,F. L. (eds). Food-borne Infections and Intoxications, p. 459 - 518, 2nd ed., Academic Press., 1979.
8. GILBERT, R.J., TURNBULL, P.C.B., PARRY, J.M. & KRAMER, J.M. - *Bacillus cereus* and other *Bacillus* species: their part in food poisoning and other clinical infections, In: Berkeley, R.C.W. & Goodfellow, M., (eds). The Aerobic Endospore - Forming Bacteria. Classification and Identification, p. 297 314. Soc. Gen. Microbiology, Academic Press, Inc., London, 1991.
9. GILBERT, R.J. & KRAMER, J.M. - *Bacillus cereus* Food Poisoning. In: Cliver, D.O. & Cochrane, B.A. (eds). Progress in Food Safety, p. 85 - 93, Madison-Wisconsin, Food Research Institute, 1986.
10. GOEPFERT, J.M., - *Bacillus cereus*. In: Speck, M.L., (ed). Compendium of Methods for the Microbiological Examination of Foods, p. 417 - 423, Washington, D.C., American Public Health Association. 1976.
11. GORDON, R.E., HAYNES, W.C. & PANG, C.H. - N. - The genus *Bacillus*. Washington, D.C., United States Department of Agriculture. Handbook No. 427, 1973.
12. HOLMES, J.R., PLUNKETT., PATE, P. ROPER, W. & ALEXANDER, W.J. - Emetic food poisoning caused by *Bacillus cereus*. *Arch. Intern. Med.*, **141:** 766 767, 1 981 .
13. JOHNSON, K.M. - *Bacillus cereus* foodborne illness: an update. *J. Food. Prot.*, **47**: 145- 153, 1984.
14. KRAMER, J.M., TURNBULL, P.C.B., MUNSHI, G. & GILBERT, R.J. - Identification and caracterization of *Bacillus cereus* and other *Bacillus* species associated with foods and food poisoning. In: Isolation and Identification Methods for Food Poisoning Organisms edited by Corry, J.E.L. & Skinner, F. A ., Academic Press, London and New York, p. 261 - 286, 1982.
15. MC KNIGHT, I.C.S., LEITÃO, M.F.F. & LEITÃO, R.F.F. - *Bacillus cereus* em macarrões industrializados. II - Ocorrência em produtos comerciais e sua multiplicação no alimento preparado para consumo. *Rev. Microbiol.*, **21:** 268-275, 1.990 .
16. MELLING, J., CAPEL, B.J., TURNBULL, P.C.B. & GILBERT, R.J. - Identification of a novel enterotoxigenic activity associated with *Bacillus cereus*. *J. Clin. Pathol.*, **29:** 938 - 940, 1976.
17. MELLING, J. & CAPEL, B.J. - Characteristics of *Bacillus cereus* emetic toxin. FEMS - *Microbiol. Lett.*, **4:** 133 - 135, 1978.
18. MIDURA, T., GERBER, M., WOOD, R. & LEONARD, A.R. - Outbreak of food poisoning caused by *Bacillus cereus*. *Public Health Reports*, **85:** 45 - 48, 1 970 .
19. MORITA, T.N. & WOODBURN, M.J. - Stimulation of *Bacillus cereus* growth by protein in cooked rice combinations. *J. Food Scien.*, **42:** 1232 - 1235, 1 977 .
20. MOSSEL, D.A.A., KOOPMAN, M.J. & JONGERIUS, E. - Enumeration of *Bacillus cereus* in foods. *Appl. Microbiol.*, **15:** 650 - 653, 1967.

21. ROBBS, N.K., ROBBS, P.G. & FAVARIN, V. - Microbiologia de Alimentos Formulados para o Programa Nacional de Merenda Escolar. Arq. Universidade Federal Rural do Rio de Janeiro, Itaguai, jul/dez, 193 - 203, 1983.
22. SPIRA, W.M. & GOEPFERT, J.M. - *Bacillus cereus* - induced fluid accumulation in rabbit ileal loops. *Appl. Microbiol.*, **24:** 341 - 384, 1972.
23. Standard Methods for the Examination of Dairy Products, p. 62, 13th American Public Health Association, Washington, D.C., 1972.
24. TURNBULL, P.C.B. - Studies on the production of enterotoxins by *Bacillus cereus*. *J. Clin. Pathol.*, 29: 941 - 948, 1976.
25. TURNBULL, P.C.B. - *Bacillus cereus* toxins. *Pharmac. Ther.*, **13:** 453 - 505, 1981.
26. Vieira, S. & Hoffmann, R. - Estatística Experimental, 178p. Ed. Atlas S. A., São Paulo, 1989.

Received for publication in 06/10/92

Rev. Microbiol., São Paulo, **24**(2): 132-139, 1993

CELL WALLS PROTEINS AND THEIR INVOLVEMENT IN THE FLOCCULATION OF *PICHIA STIPITIS*

Nei Pereira Jr. [1]
John D. Bu'Lock [2]

SUMMARY

The concern of this work is related to flocculation in strains of *Pichia stipitis* (D - xylose fermenting yeast). Investigations in this area enabled us to realize that, similar to other cell aggregation systems, proteins play an important role in the phenomenon. The evidence that proteins are strongly involved in flocculation was corroborated by irreversible deflocculation caused by protease and by the reversible deflocculation caused by denaturating agents such as urea and sodium dodecyl sulphate (SDS).

The results are discussed in the light of the basis of protein chemistry. Through quantitative analysis, the protein content in the cell wall of flocculating and non-flocculating strains was found to be higher in the former than in the latter. The elemental analysis also revealed a higher nitrogen content and a less phosphorus content in the cell wall of flocculating strains than the corresponding cell walls of non-flocculating ones.

Key words: *Pichia stipitis,* flocculation, cell wall protein, cell aggregation.

INTRODUCTION

Flocculation is a phenomenon when single cells, suspended in a liquid, aggregate to form flocks, followed by rapid sedimentation or flotation. The latter process may be caused by inclusion of gas bubbles if the flock presents an open or fluffy structure.

In yeasts, flocculation has been reported in cells of *Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Hansenula wingei, Schizosaccharomyces pombe, Citromyces matritensis, Pichia amethionina, Saccharomyces kluyveri, Schizosaccharomyces japonicus, Kluyveromyces marxianus* and *Kluyveromyces bulgaricus* [3,5,10,13,17,24]

The industrial interest in flocculation stems from two principal advantages that it promotes. First, it facilitates cell separation allowing the suppression of the costly centrifuges (widely used on a industrial scale), and its substitution by simple settlers, providing an easy and economical way to separate cells. Secondly, the flocculating nature of certain yeast cells has provided a means of maintaining a high cell concentration within the continuous fermenter [11], without the need for inert support materials. Yeast flocculation is therefore the simplest natural mode of cell immobilization, and it can be also named as auto-immobilization.

Flocculation is of utmost importance, especially

1. Departament de Engenharia Bioquímica - Escola de Química - CT/U.F.R.J.Ilha do Fundão - Cidade Universitária Rio deJaneiroCEP: 21949 - Brasil
2. Department of Chemistry - Faculty of Science The University Manchester, Manchester M 13 9PL, England

during new technology process design for energy production. It constitutes, many times, the 'great technological secret'. It is mainly associated with brewing, although it has been considered in the selection of yeast strains for other alcohol fermentations [4,11,19]. In addition to its industrial relevance, flocculation also provides an important tool for the genetic control of cell surface recognition phenomena.

This important microbial characteristic has been intriguing researchers since the end of the last century, although even at the present time the knowledge of flock formation has not been elucidated completely. There is a great deal of work in the study of flocculation of brewer's yeast *Saccharomyces cerevisiae*. There is little to show that the factors involved in yeast flocculation are particularly exclusive, thus, the information on this model aggregation system, and on others, will be used here as a basis for comprehending the phenomenon with the yeast used in the present work. From the available literature, one can draw that, basically, flocculation is a result of the interplay between the yeast genetics (internal factor) and the environment as a wholle[22] (external factor). It seems that the inheritable character finds expression in the cell wall, through the syntesis of an aggregating substance. The partial characterization of some cell wall polypeptides, present only in flocculating yeasts, indicates their strong role in cell aggregation[9,23,24]. Additionally, the fact that pH, temperature and ionic environment[3,10,19] can influence flocculation corroborates the participation of some proteinaceous component in cell aggregation. Flocculation is a superficial phenomenon, since it is also manifested by isolated cell wall prepared from flocculating yeasts[17]. Therefore, the cell wall region is regarded as the crucial area responsible for cell flocculation. The work of Miki et al[15] is recommended for a better understanding about cell-interaction in flock formation in yeast.

This work, which falls into two categories, aims to examine the flocculation in cells of *Pichia stipitis*, one of the yeasts considered for alcohol fermentation of D-xylose[7,19,21,25,27]. The first is related to the examination of some external agents which may prevent or induce the phenomenon. The second category is related to the assessment of the cell walls of flocculating and non-flocculating strains, in order to ascertain whether or not there are differences in their cell wall composition. Investigations in this subject area are of interest, particularly in microbial systems which, to date, have not been examined in terms of their flocculation characteristics.

MATERIALS & METHODS

Microorganisms: The D-xylose fermenting yeast strains of *Pichia stipitis* (5773, 5774, 5775 and 5776) were obtained from the Central Bureau voor Schimmelcultures (CBS) - The Netherlands. The maintenance medium had the same composition as the grown medium (provided below), being solidified with agar (15 g/L).

Growth medium composition: Cells were propagated in a medium with the following composition: D-xylose, 20 g; urea, 1.25 g; KH_2PO_4, 1.10 g; yeast extract, 1.5 g in 1000 mL of distilled water. A solution of mineral salts and citric acid was also used in the medium preparation. This solution was prepared 25-fold more concentrated than the actual concentration of its components in the medium. Its concentrated composition (in g/L) was as follows: $MgSO_4$.7aq, 12.50; $CaCl_2$.2aq, 1.25; citric acid, 12.50; $FeSO_4$.7aq, 0.90; $MnSO_4$.4aq, 0.25; $ZnSO_4$.7aq, 0.30; $CuSO_4$.5aq, 0.025; $CoCl_4$.6aq, 0.050; $NaMoO_4$.2aq, 0.035; H_3BO_3, 0.050; KI, 0.009; $Al_2(SO_4)_3$, 0.0125. The initial pH was adjusted to 6.0 with 1M of HCL or NaOH, and D-xylose was sterilized separately from the other components to prevent damage to the nutritive value of the medium by caramelization reactions.

Inoculum development: All inocula were prepared by transfering a loopful of cells from the fresh slope medium to 500 mL-conical flasks containing 200 mL of medium. The inoculated flasks were incubated at 25ºC on a rotatory shaker, at 180 rpm for 48 h. A second transfer was made by inoculating 10 mL of the former cultures in the same conditions as described above.

Flocculation measurements: The extent of flocculation was evaluated indirectly by the percentage of cells remaining in suspension(R%). This parameter was determined after yeast cells of known total cell concentration (C_T) were suspended by vigorous shaking, and immediately after, placed in a 50 mL-measuring cylinder. After a pause of 5 minutes in order to allow the sedimentation of the yeast flocks, 10 mL was removed from the supernatant to determine the free-cell concentration (C_F). Both C_T and C_F were determined by dry weight on pre weighed membrane filters (Milipore; 0.22 um pore size, 47 mm diameter), after vacuum filtration of the samples. The cells were washed twice with an equal volume of distilled water, and allowed to dry in an oven at 80 ºC to constant weight.

The R values were calculated by the following formula: $R\% = 100\ C_F/C_T$. Obviously, high values denote a higher dispersion, and low values a higher flocculation. A milimetrically graduated cylinder (h=33.0 cm x d=1.9 cm) was also used to measure the sedimentation rate (intensity of flocculation) of the flocks formed by strain CBS 5775. The method consisted of: after inverting the cylinder a few times and leaving the cell suspension to stand undisturbed the height of sediment was registered as it settled at intervals of 1 minute. The tests were performed at room temperature.

Microscopic observation: The morphological observation of the yeast cells under study was made with a microscope Leitz-Laborlux 12, with a magnification of x 400.

Cell breakage: The cells were harvested by centrifugation at 3000 g (Sorvall RC-5B) for 10 min at 2 ºC and washed twice with 20 mM of Tris-Cl buffer pH 8.7, containing 1 mM of PMSF, which was added to avoid endogenous degradation and therefore preventing proteolytic artefacts during the cell breakage and cell wall washes. Additionally all operations were conducted close to 0 ºC for the same reason. The cells were then broken by mechanical shaking with glass beads (0.45 - 0.50 mm) in a Braun homogenizer. Centrifuged cells and buffer were mixed in an approximately ratio of 1:1 (vol:vol) and cold glass beads were added up to the liquid meniscus, filling a 10 mL-container. The container was shaken at about 4000 cycles/min. To minimize heating effects, three series of 30 sec shakes were used. Before and between each agitation period the containers were cooled with CO_2 snow for about 10 sec. Then, the beads were sieved from the broken-cell suspensions and they were washed with 100 mL of cold buffer with PMSF. At that stage an assessment was made by microscopic observation, using a magnification of x 400 with phase-contrast illumination. Slides were prepared to observe before and after cell breakage. The broken cells of strains CBS 5774 and CBS 5775 would still aggregate, forming flocks and had to be separated by suspending them in a solution of urea (4 M). Over 94% of cell breakages in all cases could be roughly estimated.

Cell wall separation: After cell breakage the cell walls were separated by centrifugation at 3000 g/ 10 min and at 2 ºC. Then, they were washed five times in cold buffer containing PMSF, followed by washes with NaCl at decreased concentrations (5%; 2% and 1%, successively, two times each) to extraction - constituent proteins from the cell wall, as recommended by Vega & Domingues[26].

Finally, they were washed ten times again in cold buffer with PMSF. In between each wash the cell walls were centrifuged in the same conditions as described above. Thereafter the cell wall preparations were submitted to a high vacuum and lyophilized ($p=10^{-2}$ m bar; T=-60ºC; t = 20 h) in a freeze-dryer. The dry matter was determined by weighing 20 mg of lyophilized cell wall in pre-weighed containers, which was allowed to dry at 80 ºC up to constant weight.

Analyses of cell walls:

Elemental composition: The percentual composition of the elements: C, N, H, S, in the freeze-dried cell wall preparations, was determined on an Elemental Analyser. The elements: Na, K and P were analysed, after acid digestion of the samples (5 mg) in a volumetric mixture of HNO_3, H_2SO_4 and $HClO_4$ (4:1:1, respectively), in an Inductively Plasma Optical Emission Spectrometer at 10,000 ºK. The intensity of emission of an element could be correlated with its concentration in the sample, through a calibration curve prepared previously.

Protein determination: The cell wall protein content of *Pichia stipitis* strains was quantified by the BIO-RAD Assay[2]. To measure the cell wall protein content, they had first to be extracted from the cell wall. This was done by weighing 10 mg of freeze-dried cell wall and adding 2 mL of NaOH (1M) at 100 •ºC for 10 min.

Thereafter, the whole sample was quantitatively transfered to a volumetric flask and the volume made up to 100 mL, from which a 500 µL- aliquot was taken and added to 200 mL of Dye Reagent Concentrate (BIO-RAD Laboratories - Munchen - FRG), plus 300 mL of distilled water in a 1.5 mL- spectrophotometer cuvette. The absorbance was measured at a wave length of 595 nm against reagent blank in a double-beam spectrophotometer. Bovine serum albumin (Sigma, UK) was used as standard and was treated in the same manner. The calibration curve allowed linear readings in the range of 2 - 25 µg/,µL.

RESULTS AND DISCUSSION

Figure 1 shows the flocculating character of strains 5774 & 5775 and the dispersed character of strains 5773 & 5776. The cells of strain 5774 are oval in form and its flocks are compact, which settle down quickly in the spent medium, however the supernatant

produced is cloudy, containing some free cells in suspension. This strain initiates the fermentation presenting a dispersed behavior, achieving its optimum of flocculation after 25 hs of fermentation, and displaying, after this time, a mean R value of 22.3% (Fig. 2). This was an indication that its cells might be synthesizing some component to bring about aggregation, prior to its maximum expression of flocculation. Strain 5775 possesses lengthened cells and also exhibited aggregation characteristics, yet its flocs are large and presented an open and a fluffy structure. In the standardizing of the test for quantifying flocculation, it was necessary to fix a time for sedimentation, which for obvious reasons, needed to be the shortest time possible. Consequently, flocculation with strain CBS 5775 could not be depicted in Fig. 2. The velocity of sedimentation of its flocs varied according to the concentration of biomass, requiring increasingly extended sedimentation times in order to produce clear supernatants (Fig. 3). It can be seen that as the cell concentration decreases, or in other words, as the porosity (ε) increases the sedimentation rate also rises. It is evident that, in the flocculation of this strain, sedimentation is hindered, due to the open nature of the flocks, which accentuates the 'particle-particle effect 'or 'population effect'. This occurs as a result of the collision between flocs, provoking a decrease in their velocity of sedimentation. However, when biotechnological applications are intended, a high cell concentration is a prerequisite to obtaining high yields and productivity values. The hindered sedimentation shown by strain CBS 5775 may constitute a problem in cell separation. An effective flocculating microorganism should have the ability to form more compact flocks which sediment rapidly to the bottom of the fermenter or settling device, as displayed by strain CBS 5774.

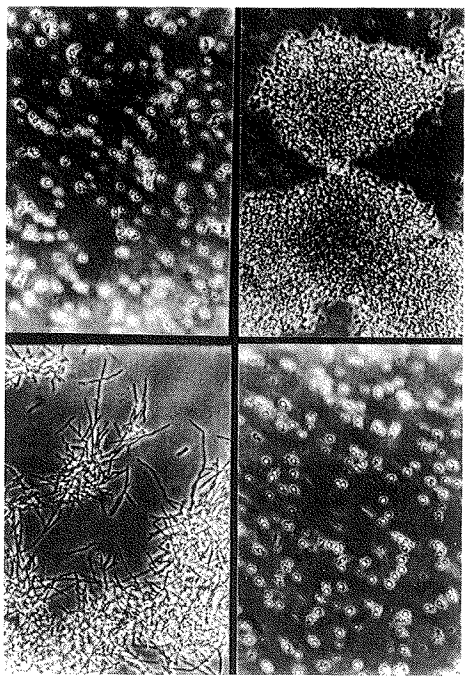


FIGURE 1: Microscopic observation of *Pichia stipitis* strains top left: CBS 5773; top right: CBS 5774 bottom left: CBS 5775; bottom right: CBS 5776

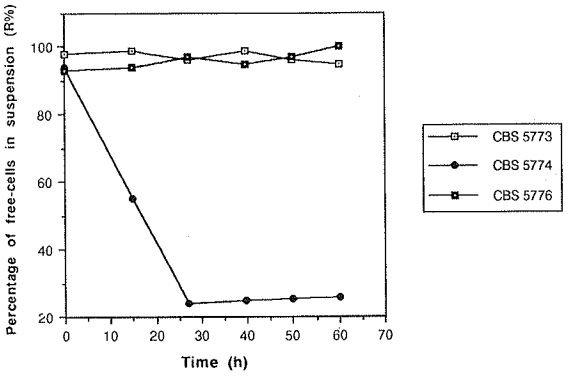


FIGURE 2: Flocculation assay during ethanol fermentation of D-xylose. Strains of *Pichia stipitis*. Sedimentation time (T_s) = 5 min.

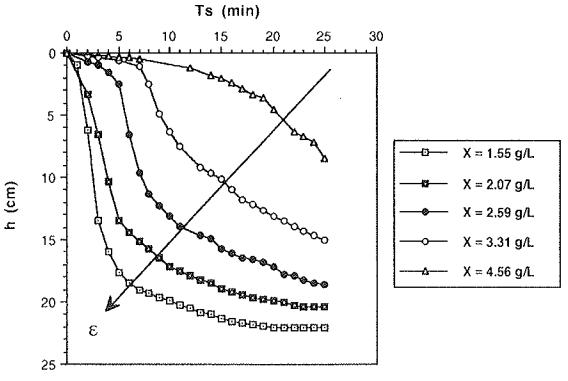


FIGURE 3: The hindered sedimentation of the flocs of *Pichia stipitis* CBS 5775 h = height of sedimentation; TS = sedimentation time X = biomass concentration. Cells supended in spent medium; pH = 5.0

As mentioned previously, there is an agreement that cell wall proteins are involved in the phenomenon of flocculation. Therefore, a broad-spectrum protease from *Streptomyces griseus* type XXV-Sigma (pronase E), containing no detectable DNase, RNase nor Nickase activities, was used to study whether or not flocculating cells of *Pichia stipitis* CBS 5774 would disperse by the action of the enzyme. A temporal test was then carried out, suspending the cells in 0.2 M (Na_2HPO_4/NaH_2PO_4) buffer. After the addition of the enzyme, the time course was followed and its action measured through the parameter R% (Fig. 4). A control (flocculating cells + buffer) was set up since the buffer used was composed of sodium salts, which are reported to be dispersing agents of aggregates of *Saccharomyces cerevisiae*[6], however, it did not show to be effective in disaggregating flocks of *Pichia stipitis*. On the other hand, the enzyme showed to promote deflocculation. In less than 1 hour the flocks were almost completely dispersed. Besides, the enzyme-treated flocks continued to be completely dispersed (R=97.3%) after two washes and resuspension in buffer, denoting the irreversible deflocculation caused by protease. The results indicated the involvement of some structural-protein component on flocculation.

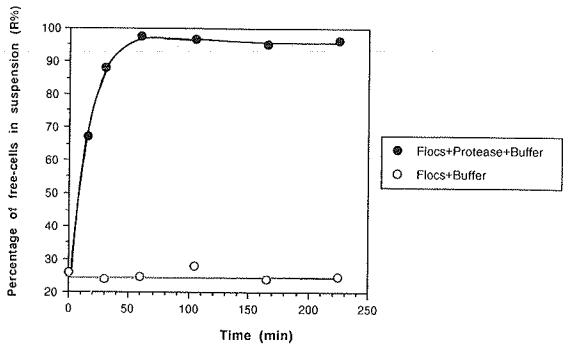


FIGURE 4: Time course of enzymatic deflocculation of *Pichia stipitis* CBS 5774. C_T=3.95 g/L; Protease conc.=0.71 mg/mL; T=35 °C; pH=7.5; N=120 rpm.

The action of potentially denaturating agents such as urea and sodium dodecyl sulphate (SDS) was also investigated. First, an instantaneous test was carried out, increasing urea concentration from 0 - 5 M. The results are displayed in Fig. 5. Deflocculation is increasingly observed as the urea concentration rises. The cells which were put in contact with the highest urea concentrations (4 and 5 M) were then washed twice in distilled water and resuspended in spent medium. Flocculation was partially regained. R% values of 45.2 and 56.8% were obtained from cells tested for 4 and 5 M of urea, respectively.

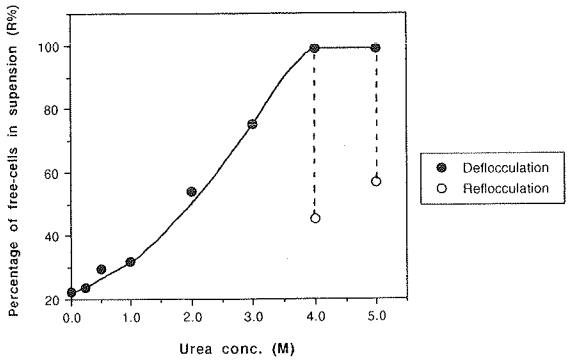


FIGURE 5: The disaggregating action of urea in flocks of *Pichia stipits* CBS 5774 and the partial reflocculation after removal of this denaturating agent. C_T=5.30 g/L; pH=5.5; Ts= 5 min.

The findings can be discussed in the light of the basis of protein chemistry since they are related to the phenomenon of flocculation. It is well known that the denaturation of proteins causes an extreme conformational alteration in their structures. Denaturation leads to an unfolding of the protein to a more or less random coil conformation. In the denaturated state the amide groups of the peptide chain form hydrogen bonds with surrounding water molecules rather than with each other[14], The biochemical characteristics are lost and physical properties such as sedimentation constant, viscosity, aqueous solubility and light absorption are also altered. The process of protein denaturation can also increase susceptibility to proteolytic degradation [28]. It is likely that the refolding of the cell wall proteins (after the removal of urea), in order to attain its best structural conformation to bring about flocculation, was not completed. This could be due to a partial degradation the cell wall proteins may have undergone. In this way, total refolding would not have been possible, and flocculation was not completely restored.

The action of another potentially denaturating agent, sodium dodecyl sulphate (SDS) was also examined in a temporal test with flocks of strain CBS 5774 (Fig. 6). This experiment was carried out in spent

medium, containing 10 g/L of SDS. Its effect of totally disaggregating the flocs was attained within 6 hours. It is well known that SDS is an anionic detergent which binds strongly to proteins, causing their denaturation and giving them a constant negative charge per unit of mass. Therefore, it is expected that the cells in such a condition would repel each other, being dispersed as a consequence. The cells were then allowed to be in contact with this detergent overnight on a rotatory shaker (120 rpm) at 25 ºC when once more, the percentage of free cells in suspension was determined. Thereafter, they were centrifuged and washed twice with distilled water, being resuspended in free-cell spent medium without SDS. Flocculation was totally regained. The possible explanation for the reversible deflocculation can be ascribed to an additional property of SDS, which also seems to provide the 'protective coat' around the protein, preserving its integrity [28]. In that way, the protein is not so susceptible to structural degradation, as was indicated when urea was investigated. The protein refolding seems to happen immediatelly after the removal of SDS.

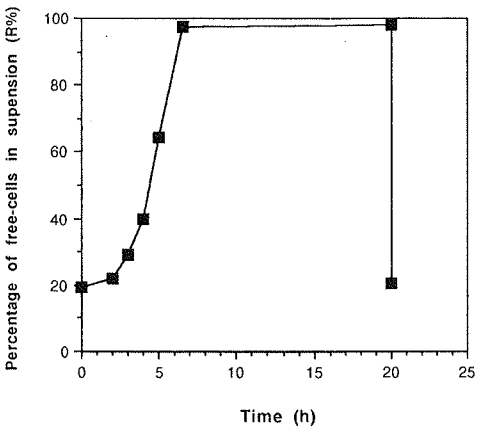


FIGURE 6: Reversible deflocculation caused by SDS 1% (w/v) in cells of Pichia stipitis CBS 5774, suspended in spent medium. CT = 3.0 g/L; pH = 5.5, T = 25ºC; N = 120 rpm

The previous results prompted an investigation of the cell wall of flocculating and non-flocculating strains of *Pichia stipitis*, aiming to assess whether the acquisition of flocculation was associated with changes in their cell wall composition. Initially determinations of the protein content in the cell wall were performed. The results (corresponding to a mean of 5 analyses for each strain) show a considerable increase in the total protein content of flocculating strains when compared with non-flocculating ones (Table 1).

TABLE 1 - Percentage of protein in the cell waal of *Pichia stipitis* strains: BIO-RAD assay[2].

Strain (CBS)	Protein / cell wall dry weight (% W / W)
5773 (F-)	21.74 (+ / - 0.37)
5774 (F+)	31.31 (+ / - 0.74)
5775 (F+)	32.62 (+ / - 0.56)
5776 (F-)	22.19 (+ / - 0.42)

Not very recently, Lyons and Hough[12] asserted the protein content present in flocculating and non-flocculating strains of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* did not correlate with the ability to flocculate. Marfey et al[13] treated intact cells of flocculating and non-flocculating strains of *Saccharomyces carlsbergensis* with trypsin, followed by B-mercaptoethanol. The treatment solubilized 15% of material from both types of cells based on dry-weight. In the solubilized fractions, it was found that the flocculating strain contained less protein than the corresponding fraction isolated from the non-flocculating cells. Stewart[22] also reported lower protein contents for two flocculating strains of *Saccharomyces cerevisiae*, compared to two other non-flocculating strains. By contrast, in a more recent article, Teixeira et al [24] found a higher protein content in the cell wall of a flocculating strain of *Kluyveromyces marxianus* when compared to a non-flocculating one, which is more in agreement with our results for the flocculating and non-flocculating strains of *Pichia stipitis*. Nevertheless, it is not only the higher protein content in the cell wall of flocculating strains *per se* which brings about cell-aggregation. It is more certainly like that a specific polypeptide takes part in the phenomenon.

The previous results are also in accordance with those obtained from the elemental analysis (Table 2), in which is shown that the nitrogen content of the cell wall of flocculating strains was found to be much higher than that of non-flocculating ones.

TABLE 2 - Elemental analysis of the cells walls of *Pichia stipitis* strains: (% of the cell wall dry weight)

Element	CBS 5573 (F-)	CBS 5774 (F+)	CBS 5775 (F+)	CBS 5776 (F-)
C	42.24	48.86	49.01	44.92
H	8.06	8.26	8.30	7.86
N	3.34	5.70	5.87	3.59
S	0.52	0.45	0.56	0.56
P	1.50	0.89	0.99	1.52
Na	0.35	0.45	0.11	0.34
K	0	0	0	0

It has also been claimed that flocculating cells of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* contain more phosphorus than non- - flocculating cells [12,16] Yet our results showed exactly otherwise, with non - flocculating strains of *Pichia stipitis* containing more phosphorus than the corresponding flocculating ones.

The results demonstrated that the cell walls of flocculating strains of *Pichia stipitis* contain quantitatively more protein than the corresponding cell walls of non- flocculating strains. It remained to be known if there were qualitative differences in the cell wall proteins of such strains. These results were already obtained and will be presented in another paper (in preparation). We can anticipate that, through the use of the polyacrylamide gel electrophoresis technique, a polypeptide with an apparent molecular weight of 65 KDa was detected in the cell wall proteins derived from the flocculating strains of *Pichia stipitis*, while it is missing in the protein extracts from the non-flocculating ones.

RESUMO

Proteínas de parede celular e seu envolvimento na floculação de *Pichia stipitis*

O interesse deste trabalho está relacionado à floculação em amostras de *Pichia stipitis* (levedura fermentadora de D-xilose). Investigações nesta área mostraram que, similarmente a outros sistemas de agregação celular, proteínas desempenham um importante papel no fenômeno. Evidências de que proteínas estão fortemente envolvidas em floculação foram confirmadas pela defloculação irreversível causada por protease e pela defloculação reversível causada por agentes desnaturantes como uréia e dodecil sulfato de sódio. Os resultados são discutidos a luz da química básica de proteínas. Através de análise quantitativa, o conteúdo proteico na parede celular de amostras floculantes mostrou-se maior do que em amostras não-floculantes. A análise elementar também revelou um maior conteúdo em nitrogênio e um menor conteúdo em fósforo na parede celular das amostras floculantes do que as correspondentes não-floculantes.

Palavras-chave: *Pichia stipitis*, floculação, proteínas da parede celular, agregação celular.

Acknowledgment: This study was supported by grants from CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico.

REFERENCES

1. ATKINSON, B. & DAOUD, I.S. Microbial flocs and flocculation in fermentation process engineering. *Adv. Biochem. Engin.*, **4:** 41-124, 1976.
2. BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254, 1976.
3. BUENO NETTO, C.L. Fermentation alcoolique par levures floculees: étude des facteurs limitants et potentialités technologiques. Ph. D. Thesis. INSA de Toulouse. France, 1984.
4. BU'LOCK, J.D.; COMBERBACH, D.M. & GHOMMIDH, C. A study of continuous ethanol production using a highly flocculent yeast in the gas lift tower fermenter. *Chem. Eng. Journal.* **29**:B9-B24, 1984.
5. CALLEJA, G.B. Cell aggregation.In The Yeasts. vol 2, 165-201. eds. A.H. Rose and J.S. Harrison. Academic Press, 1987.
6. CASTELLON-VOGEL, M.A. & MENAWAT, A.S. A method to disperse aggregates of a flocculent yeast for photometric analysis. *Biotechnol. Prog.* **6:** 135-141, 1990.
7. DU PREEZ, J.C.; BOSCH, M. & PRIOR, B.A. The fermentation of hexose and pentose sugars by *Candida shehatae* and *Pichia stipitis*. *Appl. Microbiol. Biotechnol.* **2** : 228-233, 1986.
8. ESSER, K.; HINRICHS, J. AND KUES, U. Genetic control of flocculation of yeast with respect to application in biotecnology. In Flocculation in biotechnology and separation systems. 383-398. ed. Y.A. Attia. Elsevier Science Publishers. Amsterdam. 1987.
9. HOLMBERG, S. Isolation and characterization of a polypeptide absent from non- flocculent mutants of *S. cerevisiae*. *Carlsberg Res. Commn.* **43**: 401-413. 1978.
10. HUSSAIN, T; SALHI, O.; LEMATRE, J.; CHARPENTIER, C. & BONALY, R. Comparative studies of flocculation and defloculaton of *Saccharomyces cerevisiae* and *Kluyveromyces bulgaricus* . *Appl. Microbiol. Biotechnol.* **23:** 269-273, 1986.
11. KURIYAMA, K.; SEIKO, Y.; MURAKAMI, T.; KOBAYASHI, H. & SONODA, Y. Continuous ethanol fermentation with cell recycling using flocculent yeast. *J. Ferment. Technol.* **63:**159-165, 1985.

12. LYONS, T.P. & HOUGH, J.S. Further evidence for the cross-bridging hypothesis for flocculation of brewer's yeast. *J. Inst. Brew.* **77:** 300-305, 1971.
13. MARFEY, P.; SORENSEN, S.B. & OTTESEN, M. Studies on yeast flocculation. Comparison of enzymatic digests of flocculent and nonflocculent cells of Saccharomyces carlsbergensis. *Carsberg Res. Commun.* **42:** 353-367, 1977.
14. METZLER, D.E. Membranes and cell coats. In Biochemistry. 252-300. Academic Press. 1977.
15. MIKI, B.L.A.; POON, N.H.; JAMES, A.P. AND SELIGY, V.L. Possible mechanism for flocculation interactions governed by gene Flo 1 in *Saccharomyces cerevisiae. J.Bacteriol.* **150 :** 878-889, 1982.
16. MILL, P.J. Phosphomannans and other components of flocculent and non- flocculent walls of *Saccharomyces cerevisiae. J. Gen. Microbiol.* **44:** 329-341, 1 966.
17. NISHIHARA, H.; TORAYA, T. AND FUKUI, S. Flocculation of cell walls of brewer's yeast and effects of metal ions, protein-denaturants and enzyme treatments. *Arch. Microbiol.*, **131:** 112-115, 1982.
18. NUÑES, M.J. AND LEMA, J.M. Cell immobilization: Application to alcohol production. *Enz. Microb. Technol.* **9**:642-649, 1987.
19. PEREIRA JR., N. Investigations of D-xylose fermenting yeasts. Ph. D. Thesis. The University of Manchester. UK. 1991.
20. ROBERTSON, A.J.; GERLACH, J.H.; RANK, G.H. AND FOWKE, L.C. Yeast cell wall, membrane, and soluble marker polypeptides identified by comparative two- dimensional electrophoresis. *Can. J. Biochem.* **58:** 565-572, 1980.
21. SLININGER, P.J.; BOTHAST, R.J.; LADISCH, M.R. AND OKOS, M.R. Optimum pH and temperature conditions for xylose fermentation by *Pichia stipis. Biotechnol. Bioeng.* **35:**727-731, 1990.
22. STEWART, G.G. Yeast flocculation - Practical implications and experimental findings. The Brewers Digest (March), 42-62, 1975.
23. STEWART, G.G. The genetic manipulation of industrial yeast strains. *Can. J. Microbiol.* **27:** 973-990, 1981.
24. TEIXEIRA, J.; GONÇALVES, M.H.; GAMA, F.M.; MORADAS-FERREIRA, P. & MOTA, M. Partial characterization of cell wall from a flocculent strain of *Kluyveromyces marxianus. Biotechnol. Lett.* **1 1:** 579-582 .1 989.
25. TRAN, A.V. & CHAMBERS, R.P. Ethanol fermentation of red oak acid prehydrolyzate by the yeast *Pichia stipitis. E n z . Microbiol. Technol.* **8:** 439- 444, 1986.
26. VEGA, R. AND DOMINGUEZ, A. Cell wall composition of the yeast and mycelial forms of *Yarrowia lipolytica. Arch. Microbiol.* **144:** 124-130. 1986.
27. WAYMAN, M.; SEAGRAVE, C. AND PAREKH, S.R. Ethanol fermentation by *Pichia stipitis* of combined pen tose and hexose sugars from lignocellulosics prehydrolysed by SO_2 and enzimatically saccharified. *Proc. Biochem.* **22:** 55-59, 1987.
28. WILSON, K. AND GOULDING, K.H. A Biologist's guide to principles and techniques of practical biochemistry. eds. A.J. Willis & M.A. Sleigh. Edward Arnold. London, 1989.

Received for publication on 9/06/92

Rev. Microbiol., São Paulo, **24**(2): 140-143, 1993

EFFECT OF VINASSE ON GERMINATION OF *METARHIZIUM ANISOPLIAE* (METSCH.) SOROKIN "IN VITRO" AND IN THE SOIL

Norma Suely Sobral da Silveira[1]
Galba Maria de Campos-Takaki[1]
Maria Menezes[2]

SUMMARY

The effect of vinasse on ***Metarhizium anisopliae*** spores germination "in vitro" and their survival after addition of the residue in the soil was studied. The germination was evaluated at 8, 16 and 24 hours intervals in different treatments (Vinasse pH 3.5, 6.5 and Potato/Dextrose medium control). *M. anisopliae* survival in soil with vinasse was observed 15 and 45 days after the soil inoculation. The results showed that vinasse inhibited the germination of *M. anisopliae* in 55.76 and 42.69%, respectively, in the pH 3.5 and 6.5. The application of vinasse to the soil artificially inoculated with *M. anisopliae* reduced significatively the number of colonies recovered and altered qualitatively the environmental microbiota in the studied area.

Key Words: Vinasse, *Metarhizium anisopliae*, germination.

INTRODUCTION

Soil has been considered an important source of entomopathogenic fungi (7,15). Particular condition of this environment plays a fundamental role in the capacity of pathogens introduced for insect control to persist under field conditions and cause epizooties.

Ling & Donaldson (8) carrying out studies on abiotic and biotic factors, which affected the stability of the *Beauveria bassiana* (Bals.) Vuill., found that organic matter from cultural remains, as well as applications of fertilizers, improved the soil microbiota, altering the survival of fungal spores. These authors suggest that the success in the soilborne insect control with fungi depend on adoption of agricultural practices which encourage the persistence of these soilborne pathogens.

The utilization of vinasse, residual liquid of alcohol distillation, as fertilizer in cultivated areas has increased in the last few years and it has shown promising results (14). Some consequences verified in the soil, due to its use: pH elevation; increase in nutrient availability; in cation and water retention capacity; improvement in physical structure; and intensification of microbial activity (2, 4, 9, 11, 12, 16).

The *Metarhizium anisopliae* is the most important organism used for insect control nowadays. However, until now investigations have not been done about the factors that can interfere with its permanence in agroecosystems. The purpose of this paper is to evaluate the effect of vinasse on spores germination of *M. anisopliae* "in vitro"and in field conditions.

MATERIAL AND METHODS

- Microorganisms - *Metarhizium anisopliae* (PL43) URM3187, isolated from *Mahanarva posticata* (Stal.) and characterized in Entomological Laboratory of Escola Superior de Agricultura "Luiz de

1 Laboratório de Imunopatologia Prof. Keizo Asami, Depto. de Antibióticos - Universidade Federal de Pernambuco, 50.739-901/Recife - PE.
2 Área de Fitossanidade, Depto. de Agronomia - Universidade Federal Rural de Pernambuco, Dois Irmãos, 52171-900 Recife - PE.

Queiroz"- USP

- Substances - The vinasse utilized "in vitro" tests was derived from ethanol manufacture, with the following composition: organic matter, 8.5%; nitrogen (NO_3), 0.03%; phosphorus (P_2O_3), 0.7 g/l; potassium (K_2O), 0.83 g/l; initial pH 3.5. In order to evaluate the effect of vinasse on *M. anisopliae* survival in soil, in field conditions, vinasse with the following characteristics was used: organic matter, 2.39 g/l; total nitrogen (N), 0.42 g/l; phosphorus (P_2O_3), 0.24 g/l; potassium (K_2O), 0.8 g/l; pH 4.9.

- Effects of vinasse on spores germination - The spores were removed from the *M. anisopliae* colonies grown on Potato Dextrose agar medium for 15 days, transferred to flasks containing 30 ml of sterile distilled water resulting in a suspension containing 1.8 x 10^6 spores per mililiters. One ml of the spore suspension was transferred to test tubes with 9 ml of the following media: a) vinasse, pH 3.5; b) vinasse, pH 6.5; and c) Potato/Dextrose (control). The tubes were incubated at 28ºC, for 8, 16 and 24 hours intervals. Samples were removed, stained with lactophenol cotton blue and examined at optical microscopy. The percentual of germination inhibition was established by modified method (3).

- M. anisopliae survival in field conditions - The evaluation of effect of vinasses was carried out inoculating the soil with 4 x 10^7 spores of *M. anisopliae* in rice substrate. The fungus spores and vinasse were pulverized on the surface of 1 m^2 of soil in areas cultivated with sugar cane. The cultivated areas had not been previously treated with *M. anisopliae*. The amount of vinasses used in the experiment was 20 liter/m2 of soil, as recommended (6) for higher sugar cane productivity.

Determination of *M. anisopliae* survival was done in 15 and 45 days after the fungus inoculated in soil. Five samples of 5g of soil were removed from soil surface for each evaluation period. Each sample was added to 100 ml of distilled water and kept in a shaker for 5 minutes. After this treatment the samples were reunited, homogenized and, from this suspension, a 10^{-3} dilution was prepared. In the reisolation for the selective medium described was used (10).

The evaluation was done 7 days after the incubation, by counting the amount of *M. anisopliae* colonies in each sample. Other fungus present in the plate dish were transferred to test tubes with potato dextrose agar medium for qualitative analysis.

RESULTS AND DISCUSSION

The effects of vinasse on *M. anisopliae* spores

TABLE 1 - Percent of germination (GER %) of *Metarhizium anisopliae* spores maintened in vinasse, at different incubation periods.

pH	GER (%)	Hours	GER (%)	pH/Hours (GER %)				DMS (5%)
				pH	8	16	24	
3.5	40.73 b	8	23.41 c	3.5	1.40 bC	48.87 bB	71.93 bA	19.20
6.5	51.44 b	16	68.63 b	6.5	11.67 bB	61.80 bA	80.87 abA	19.20
Control	81.25 a	24	81.39 a	Control	57.17 aB	95.22 aA	91.37 aA	19.20
DMS (5%) C.V.=15.94	11.09		11.09		19.20	19.20	19.20	

germination is shown in Table 1. The most significant interference of this residue was observed in pH 3.5, however, the inhibition effect produced by vinasse was decreased after 16 hours of incubation as is shown in figure 1.

The inhibition of spores germination of *M. anisopliae* spores in vinasse may be due to the high concentration of potassium in the residue, its reducible action, as well as the high level of acidity. These characteristics have been previously (11) when they pointed out that vinasses is a potential agent of aquatic environment pollution. According to the results obtained, it is probable that spores germination of *M. anisopliae* have been delayed in vinasse due to high osmotic pressure in the medium, since, under these conditions, the absorption of water becomes difficult, hindering spores hydration. Besides, the delay may have been caused by changes in the permeability of the spores cellular walls, which affects the cellular metabolism. Changes of this kind hinder the uptake of exogenous nutrients available in the medium and necessary to the germination process. Gloria & Orlando (6) suggest that there are limitations to vinasse utilization on some kind of soil due to the fact that the high potassium concentration of this residue works as saline agent, causing a decrease in the vegetable production in some treated areas. Santos et al. (12) observed that the increasing application of this residue inhibits the emergence of maize seedlings.

On the other hand, in Table 2 the effects of vinasse in field conditions, and the average number of colonies recovered from soil artificial by inoculated with *M. anisopliae* spores are shown.

Those results indicated a significative influence of this residue on the survival of *M. anisopliae* when comparing with non-treated areas.

The increase in fungal diversity species isolated in treated area with vinasse has been noticed (4). In the present study, qualitative changes in the soil microbiota were noted and previously described (13). It was observed substantial increase in population of *Rhizopus nodosus* in the area treated with vinasse. The increase in the population of this organism of quicker growth than *M. anisopliae* furthered by vinasse composition, associated with the release of toxic metabolic in the soil, probably made possible the predominance of this fungus in soil environment.

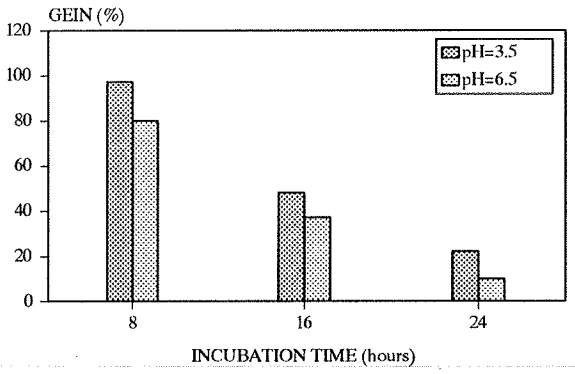


Figure 1 - Percent of germination inhibition (GEIN %) of *Metarhizium anisopliae* spores in vinasse, at different periods of incution and pH values.

TABLE 2 - Colonies of *Metarhizium anisopliae* recovered from soil either artificially treated or not with vinasse. Data transformed at Vx + 5.

Vinasse	colonies (n.)	Incuba tion Time (days)	colonies (n.)	Vinasse/Incubation Time[colonies(n.)]			DMS (5%)
				Vinasse	15	45	
No treated	3.70 a	15	3.57 a	No treated	4.85 aA	2.54 aB	1.25
Treated	2.26 b	45	2.39 b	Treated	2.29 aB	2.24 aA	1.25
DMS (5%)	0.88		0.88		1.25	1.25	
C.V.=27.22%							

RESUMO

Efeito de vinhaça na germinação de *Metarhizius anisopliae* (Metsch.) Sorokin "in vitro"e no solo

Neste trabalho foi estudado o efeito da vinhaça na germinação de esporos de *Metarhizium anisopliae* "in vitro"e sobrevivência deste fungo após adição do resíduo no solo. O efeito da vinhaça na germinação de *M. anisopliae* foi avaliado a intervalos de 8, 16 e 24 horas, em diferentes tratamentos (Vinhaça pH 3.5 e pH 6.5; e no meio de Batata/Dextrose como Controle). A sobrevivência de *M. anisopliae* em solos contendo vinhça foi observada após 15 e 45 dias de inoculação. Os resultados indicaram que a vinhaça inibiu a germinação de *M. anosopliae* em 55.76 e 42.69 nos pH 3.5 e 6.5, respectivamente. O solo contendo vinhaça e inoculado artificialmente com esporos de *M. anisopliae* apresenteram uma redução significativa do número de colonias reisoladas deste fungo, bem como, ocorreu alteração qualitativa da microbiota na área estudada.

Palavra-chave: Vinhaça, *Metharhizium anisopliae*, germinação.

ACKNOWLEDGMENTS

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq.

REFERENCES

1. COCHRANE, V.W. - *Physiology of Fungi. New York,* John Wiley & Sons, 1958.
2. COSTA, S.M.G. - *Efeito da adição de vinhaça na dinâmica da microflora do solo de cerrado de Corumbataí, SP.* Rio Claro, Universidade Estadual Paulista "Julio de Mesquita Filho". 1983. (Msc. Thesis.)
3. EDINGTON, L.V.; KHEW, K.L. & BARRON, G.L. - Fungitoxic spectrum of benzimidazole compunds. *Phytopathology*. **61**:42-44, 1971.
4. FERNANDES, M.J. & MAIA, L.C. - *População fúngica em solos da Zona da Mata de Pernambuco, antes e após adição de vinhaça.* In: CONGRESSO NACIONAL DE BOTÂNICA, 38., São Paulo, 1987. Resumos...São Paulo, Universidade de São Paulo, 1987. p. 296
5. GLÓRIA, N.A. - Utilização agrícola da vinhaça. *Brasil Açuc.*, **86**:397-403, 1975.
6. GLÓRIA, N. & ORLANDO FILHO, J.A. - Aplicação de vinhaça: um resumo, sobre o que foi pesquisado (Parte 2). *Alcool & Açuc.*, **4**:24-35, 1984.
7. IGNOFFO, C.M.; PUTTLER, B.; MARSTON, M.L.; HOSTETTER, D.L. & DICKERSON, W.A. - Season incidence of the entomopathogenic fungi *Spicaria rileyi* associated with noctuid pest of soybeans. *J. Invert. Pathl.*, **25**:135-137, 1975.
8. LING, A.J. & DONALDSON, M.D. - Biotic and abiotic factors affecting stability of *Beuveria bassiana* conidia in soil. *J. Invert. Pathol.*, **38**:191-200, 198
9. NEVES, M.C.P.; LIMA, I.T. & DOBEREINER, J. - Efeito da vinhaça sobre a microflora do solo. *Rev. Bras. Ciên. Solo,* **7**:131-136, 1983.
10. PEREIRA, J.C.R.; DHINGRA, O.D. & CHAVES, G.M. - A selective medium for population estimations of *Metharhizium* in soil. *Trans. Br. Mycol. Soc.*, **72**:495, 1979.
11. RODELA, A.A. & FERRARI, S.E. - A composição da vinhaça e efeitos de sua aplicação como fertilizante na cana-de-açúcar. *Brasil Açuc.*, **90**:6-13, 1977.
12. SANTOS, G.A.; ROSIELLO, R.O.P.; FERNANDES, M.S. & O'GRADY, P.C. - Efeitos da vinhaça sobre o pH do solo, a germinação e o acúmulo de potássio no milho. *Pesq. Agropec. Bras.* **16**:489-493, 1981.
13. SILVEIRA, N.S.S.S. - *Microflora associada à rizosfera da cana-de-açúcar (Saccharum spp), sua interação com Metarhizium anisopliae (Metsch) Sorokin e o efeito da vinhaça na sobrevivência deste fungo "in vitro"e no solo.* Recife. Universidade Federal de Pernambuco, 1990. (MSc. Thesis).
14. SOBRAL, A.F.; LIRA, L.J. & GUIMARÃES, V. O efeito da suplementação mineral da vinhaça na fertilização da cana-de-açúcar. *Brasil Açuc.*, **106**:11-15, 1988.
15. SPRENKEL, R.K. & BROOKS, W.M. Winter survival of the entomogenous fungus *Nomurae rileyi* in North Carolina. *J. Invert. Pathol.*, **29**:262-266, 1971.
16. TAUK, S.M.; Shoehein-Crusius, i.h. & PETENATE, A. Modelo ecológico dos nutrientes, microorganismos e atividade enzimática de solo sob vegetação de cerrado tratado com vinhaça. Corumbataí, SP, *Rev. Microbiol.*, **21**:99-109, 1990.

Received for publication in 19/08/92

Rev. Microbiol., São Paulo, 24(2): 144-149, 1993

STUDIES ON SPORE IMMOBILIZATION CONDITIONS FOR DEVELOPMENT OF *PENICILLIUM CHRYSOGENUM* BIOPARTICLES.

Maria Lucia G.C. Araujo
Carlos Osamu Hokka*

ABSTRACT

Cultural conditions in shaking flasks for obtention of *Penicillium chrysogenum* IFO 8644 bioparticles of suitable size for its utilization in tower type fermentor were set up. Immobilization was realized by spore entrapment in selected diatomaceous earth particles ranging from 125 to 292 μm. The effect of agitation speed, phosphate concentration and spore concentration on the bioparticle diameter was examined in a 72 hours culture at 26°C utilizing medium with sucrose and corn steep liquor as main components. Experiments were performed according to central composite design technique and the statistical analysis of the results enabled to correlate bead size with the studied variables through an empirical equation describing a second order model. It was observed that the main variable affecting bioparticle diameter is the spore concentration whose increase reduces the pellet size.

Furthermore, experiment in which only the spore concentration was varied confirmed the general pattern predicted by the empirical expression established through the factorial design experiments.

Key-words: mold bioparticle, immobilized cell, pellet size

INTRODUCTION

The immobilization of filamentous fungi in inert carriers for antibiotic production in tower type bioreactors is being propose as a promising alternative to overcome oxygen transfer limitation caused by highly viscous broths produced in conventional fermentors (2,4,6,7,16) . The feasibility of its operation for long periods in continuous (4,6,11)or repeated fed-batch systems (6,10,14,15) has been demonstrated in penicillin production process.

Gbewonyo and Wang (5) developed a technique to immobilize mycelial cells on diatomaceous earth particles (Celite) obtaining bioparticles ranging from 200 to 600 μm . Further the same authors (6) observed higher penicillin productivity utilizing confined cells on Celite in a bubble column as compared with free mycelial culture. This fact was attributed to the higher mass volumetric mass transfer coefficient ($k_{\ell}a$) when immobilized cells are utilized. Still, Gbewonyo et al. (7) relate the successful scale up of a 200 liter tower fermentor utilizing the same technique, emphasizing the relatively low cost of Celite and the simple procedure for cell immobilization on it, making the process a promising one for industrial application. Keshavarz et al. (9) evaluated some parameters affecting spore uptake and growth to develop method for large scale immobilization of spores onto Celite. They found that immobilized biomass was almost independent of spore loading and the free cells obtained were much less than the confined one. Oh et al . (15) utilizing the method developed by Gbewonyo

Departamento de Engenharia Química Universidade Federal de São Carlos. Caixa Postal 676 - CEP 13560 São Carlos - SP
* Corresponding author

and Wang (5) investigated the effect of phosphate concentration on bioparticle size in a fluidized-bed bioreactor and an optimal feed rate of phosphate was determined.

The control of particle size for proper substrate and oxygen internal transfer are emphasized by various authors whether in Celite confined systems (10,15) or in natural pellets of fungi (1,12) and as pointed out by Gbewonyo and Wang (5) the internal mass transport resistances can be a major handicap of the pellet growth form.

Based on the above mentioned, the objective of the present work was to correlate bioparticle diameter, obtained in shaking flasks with spore concentration, phosphate concentration and agitation speed through experiments planned according to the factorial design technique (central composite design.)

MATERIALS AND METHODS

Microorganism and Media: *Penicillium chrysogenum* IFO 8644 kindly provided by ICBiotech, Osaka, Japan was utilized throughout this work. The medium composition in g/l for strain maintenance and spore formation was: 7.5 glycerol, 7.5 glucose, 2.5 corn steep liquor, 5.0 peptone, 0.05 $MgS0_4.7H_20$, 0.06 KH_2P0_4, 4.0 NaCl and 20.0 agar; pH = 6.0. In the experiments for bioparticle formation the composition in g/l was: 30.0 sucrose, 20.0 corn steep liquor, 2.0 $(NH_4)_2S0_4$, 0.25 $MgS0_4.7H_20$, 5.0 $CaC0_3$, 5.0 soy bean oil. Metals were provided by adding 20 ml/l of salts solution composed of (in g/l): 3.0 $FeSO_4 . 7H_2O$, 2.5 $CuSO_4 . 5H_2O$, 10.0 $ZnSO_4 . 7H_2O$. The pH was ad justed to 6.5 . Phosphate concentration was varied according to the experimental design conditions and ranged from 0.4 to 2.0 g/l as KH_2P0_4 .

Spore confinment: The procedure was based on the technique developed by Gbewonyo and Wang (5) and modified to our conditions. Celite 560 (John Manville Corp., Denver Co., USA) kindly supplied by Rhodia S.A. - São Paulo SP, Brazil, was washed in water to remove fines and particles between 125 and 292 μm were selected by wet sieving. The Celite beads were dryed at 250°C for 4 hours and 2g were added to each 250 ml erlenmeyer flasks. The flasks were autoclaved at 121°C for 30 minutes and dried at 60°C overnight. After cooling, 10 ml of spore suspension with known concentration were added to the flasks, gently mixed and kept static for 2 hours. The liquid was drained and the particles were washed twice with 10 ml sterile water. The above mentioned spore suspension was prepared by adding 100 ml of sterile water to a 10 days culture developed on the agar medium at 26°C in 1 liter Roux bottles. Spore number was determined by using a hemacytometer (Neubauer Chamber) and the concentration (ranging from $0.2X10^8$ to $4.0X10^8$) adjusted by proper dilution with sterile distilled water.

Bioparticle preparation: Previously sterilized medium (40 ml) with proper phosphate concentration, was added to each flask and kept for 4 hours at 26°C. Agitation speed was kept low (ca. 100 rpm) to avoid loss of the spores from Celite, as suggested by Jones et al. (8). After this period the agitation speed was increased to the desired values according to the experimental design condition (ranging from 170 to 330 rpm). A rotary shaker (Equipamentos Científicos Superohm Ltda., Piracicaba, SP, Brazil) describing 19 mm circle was utilized. After 72 hours, when the sugar was almost totally consumed, the diameter of 60 to 80 particles were measured under a microscope (Amplival from Carl Zeiss, Jena - Germany) with a microscale in the ocular.

Statistical analysis: Response surfaces, linear and non-linear regression as well as analysis of variance were realized with the aid of SAS Statistical Analysis System Inc.-SAS User's Guide: Statistics, Cary, NC, 1982) in the Computer Center of UFSCar, S.Carlos, SP, Brazil.

RESULTS AND DISCUSSION

Initially, the effects of the variables: X_1', spore concentration, X_2', phosphate concentration, and X_3', agitation speed on the dependent variable Y, bioparticle diameter were investigated through 2^3 factorial design with three replicated center points. Spore concentrations in the suspension for adsorption were 1.0, 2.1 and $3.2X10^8$ spores/ml and agitation speed, 204, 250 and 296 rpm. These values were chosen based on the literature (4,5) where it is mentioned a concentration of 10^8-10^9 spores/ml and an agitation speed of 200 to 220 rpm. Phosphate concentration (0.4, 1.0 and 1.6 g/l) was examined as it has been reported (1) its remarkable effect on the bead size.

Table 1 presents the results obtained, where the independent variables are in both original, Xi', and coded units, Xi. In Figure 1, a microphotograph of a typical bioparticle obtained is shown. As it can be observed, small spheric pellets with radially projecting hyphae were formed resulting in fluffy bioparticles. This morphology as mentioned by Kim et al. (10), facilitates the intraparticle mass transfer.

TABLE 1 - Results from 2^3 factorial central composite design experiments utilized to obtain a linear model

Spore conc.		Phosphate conc.		Agitation speed		Biopart. diameter
$10^{-8}ml^{-1}$ (X_1')	coded (X_1)	g/l (X_2')	coded (X_2)	rpm (X_3')	coded (X_3)	µm (Y)
1.0	-1	0.4	-1	204	-1	282
3.2	1	0.4	-1	204	-1	197
1.0	-1	1.6	1	204	-1	241
3.2	1	1.6	1	204	-1	222
1.0	-1	0.4	-1	296	1	397
3.2	1	0.4	-1	296	1	252
1.0	1	1.6	1	296	1	337
3.2	1	1.6	1	296	1	166
2.1	0	1.0	0	250	0	207
2.1	0	1.0	0	250	0	181
2.1	0	1.0	0	250	0	200

A linear model was adjusted through regression by the least square method. The analysis of variance showed that this model explain roughly the studied region at 10% significance level.

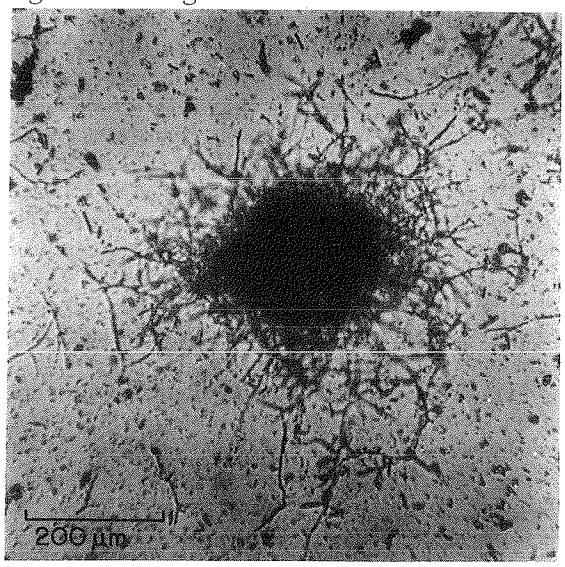


FIGURE 1 - Microphotograph of a typical bioparticle obtained after 72 hours cultivation of spores immobilized in celite.

TABLE 2 - Results from additional conditions of the 2^3 factorial central composite design to enlarge the bioparticle diameter range and utilized to obtain a second degree model.

Spore conc. diameter		Phosphate conc.		Agitation speed		Biopart.
$10^{-8}ml^{-1}$ (X_1')	coded (X_1)	g/l (X_2')	coded (X_2)	rpm (X_3')	coded (X_3)	µm (Y)
0.2	-1.732	1.0	0	250	0	762
4.0	1.732	1.0	0	250	0	133
2.1	0	0.0	-1.732	250	0	311
2.1	0	2.0	1.732	250	0	204
2.1	0	1.0	0	170	-1.732	218
2.1	0	1.0	0	330	1.732	353
2.1	0	1.0	0	250	0	194
2.1	0	1.0	0	250	0	190

The mathematical expression with the variables in the coded form was:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 \qquad (1)$$

where the coefficients values with their deviation are:

$$a_0 = 248.2\ (\pm\ 15.6);\ a_1 = -52.5\ (\pm\ 18.3)$$

$$a_2 = 20.2\ (\pm\ 18.3);\ a_3 = 26.2\ (\pm\ 18.3)$$

and $F_{(regression)}=3.83$; $F(0.9, 3, 7) = 3.07$

In order to enlarge the bioparticle diameter range and to improve the definition of the response tendency, the region of study was expanded, as a second degree aproximation provides a better representation over a larger region including the studied one (3). This factorial group of points was augmented with a star design consisting of six axial points and two additional center points. The experiments were carried out in the same manner as the first group, and from the results (Table 2) it is evident the important effect of spore concentration on the bioparticle diameter. The data from the first and second groups (complete central composite design) enabled the obtention of a mathematical expression through non-linear regression, describing a second order model as follows:

$$Y = b_0 + b_1X_1 + ... + b_{11}X_1^2 + --- + b_{12}X_1X_2 + --- + b_{23}X_2X_3 \quad (2)$$

where the coefficients values are:

b_0= 194.4 (± 41.0); b_1= -107.8 (± 24.5);
b_2 = -24.8 (± 24.5); b_3=31.7 (± 24.5);
b_{11}= 71.3 (± 23.8); b_{22}= 8.0 (± 23.8);
b_{33}= 17.3 (± 23.8); b_{12}= 5.0 (± 32.4);
b_{13}= -26.5 (± 32.4); b_{23}= -16.2 (±32.4).

The analysis of variance indicates that the model explains fairly well this region with a 10% significance level as:

$$F_{(regression)} = 3.5713;\ F\ (0.9,9,9) = 2.44$$

However, the analysis of the coefficients of equation (2) from the model fitting suggested that the interaction effects of the variables as well as the second order terms in X_2 and X_3 are not relevants. Then, the original expression was reduced and through non-linear regression the following expression was obtained:

$$Y = \alpha_0 + \alpha_1X_1 + \alpha_2X_2 + \alpha_3X_3 + \alpha_{11}X_1^2 \quad (3)$$

where:

α_0 = 215.8 (±23.5); α_1 = -107.8 (± 21.2);
α_2 = 24.8 (± 21.2); α_3 = 31.7 (± 21.2);
α_{11} = 67.6 (± 20.1).

TABLE 3 - Analysis of variance for the full regression

Source	Sum of Squares	D.F.	Mean square	Ratio
Model	1597733.5	5	319546.7	50.6
Error	88351.5	14	6310.8	
Total	1686085.0	19		
Total (corr.)	345442.4	18		

R^2 =0.744; F = 10.18

It can be observed from the analysis of variance, shown in Table 3, that the regression is significant at 1% level as:

$$F_{(regression)} = 10.18;\ F\ (0.99,4,14) = 5.04$$

The coefficient values of the above expression show the remarkable effect of the spore concentration on the bioparticle diameter as compared with the other variables, especially the phosphate concentration. This must be due to the fact that corn steep liquor presents in its composition (13) some amount of phosphorilated compounds.

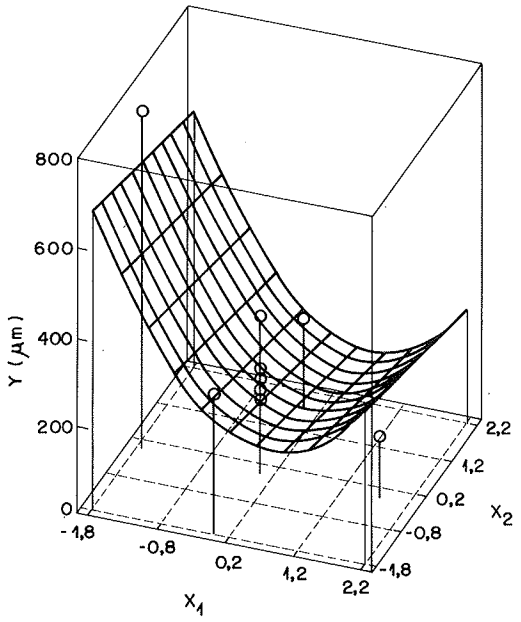


FIGURE 2 - Surface representing the effect of spore concentration, X_1, and phosphate concentration X_2, in coded units on the bioparticle size, Y. The dots indicate the experimental data.

In order to allow visualization of equation (3), Figure 2 shows a three dimensional plot of the surface representing bead size, Y, against spore concentration X_1, and phosphate concentration X_2, while in Figure 3, Y is plotted against X_1 and X_3, agitation speed. In the figures, the experimental points plotted correspond to those where X_3 and X_2 are zero, respectivelly, and the important effect of spore concentration can be observed.

Based on the above results, experiments were performed to confirm this effect, keeping constant the phosphate concentration, 0.5 g/l, and agitation speed, 250 rpm, and varying the spore concentration. The phosphate concentration was established based on values normally utilized in this process, as its influence is the less significant when compared with the

other variables. Regarding agitation speed, as it exerts a fairly higher influence utilized in order to nullify its effect in equation (3). The other conditions were the same, and concentration in the suspension ranged from 2.5 x 10^6 to 4.3 x 10^3 spores/ml and the bead presented average diameters between 119 to 996 μm.

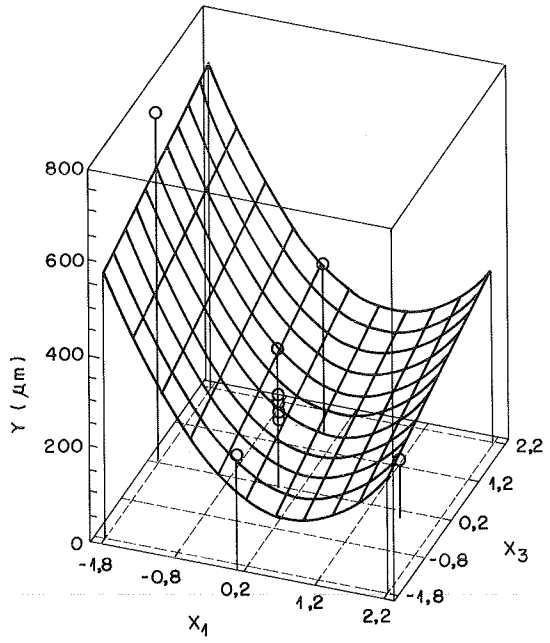


FIGURE 3 - Surface representing the effect of spore concentration, X_1, and agitation speed, X_3, in coded units on the bioparticle size, Y. The dots indicate the experimental data.

The results are shown in Figure 4 and as it can be observed the size decreases sharply as spore concentration is increased up to ca. 10^8/ml, tending to level down thereafter.

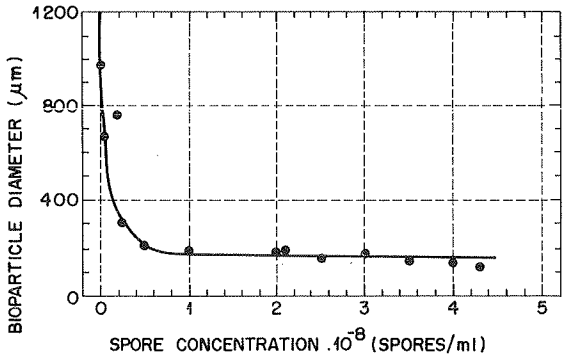


FIGURE 4 - Effect of spore concentration on bioparticle diameter with 0.5 g/l phosphaste concentration and 250 rpm agitation speed.

This behaviour is fairly well depicted by the empirical correlation except for the region of high concentration where it was not observed an increase in bead size. We can conclude that, for the range of technological interest the results obtained from experimental design technique are very useful to obtain bioparticles of convenient size in order to minimize internal oxygen limitation and allow utilization in tower type bioreactors. Besides, phosphate concentration and agitation speed, in the usual ranges employed, are much less effective than spore concentration. Still, interactions effects between the variables seem not to occur. Further studies in this laboratory are being directed towards the investigation of the influence of bead size on the penicillin production rate as well as the behaviour of tower type fermentor with pellets obtained through this technique.

RESUMO

Condições de imobilização de esporos de *Penicillium chrysogenum* para obtenção de biopartículas.

Foram estabelecidas condições de cultivo em frascos agitados, para a obtenção de biopartículas de *Penicillium chrysogenum* IFO 8644 de tamanho adequado para utilização em biorreator tipo torre. A imobilização foi realizada por confinamento de esporos em partículas de diatomito selecionado com diâmetro variando de 125 a 292μm. Foram examinados os efeitos da velocidade de agitação, concentração de fosfato e concentração de esporos no diâmetro da biopartícula formada em cultura de 72 horas a 26°C, utilizando meio com sacarose e água de maceração de milho como principais componentes. Os experimentos foram conduzidos de acordo com a técnica do planejamento fatorial em dois níveis com ponto central. A análise estatística dos dados permitiu correlacionar tamanho de biopartícula com as variáveis estudadas, através de uma equação empírica descrevendo um modelo de segunda ordem. Verificou-se que a variável mais importante foi a concentração de esporos, cujo aumento reduziu o tamanho das partículas. Ainda, experimentos nos quais apenas a concentração de esporos foi variada, confirmaram o comportamento geral previsto pela equação empírica estabelecida através de planejamento experimental.

Palavras - chave: biopartícula de fungo, célula imobilizada, tamanho de biopartícula.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. T.Yoshida for his advise during the stay of one of the authors (C.O.H.) at I.C.Biotech, Japan under JSPS scholarship, when this work has started. Financial support from PADCT/ FINEP (proc. 43 . 89. 0195 . 00) and CNPq, Brazil, as well as the scholarship to one of the authors (M.L.G.C.A.) from CNPq are also acknowledged.

REFERENCES

1. Aiba, S. and Kobayashi, K.: Comments on oxygen transfer within a mold pellet . *Biotechnol . Bioeng .*, **17**:583-588, 1971 .
2. Berk, D., Behie, L.A., Jones, A., Lesser, B.H. and Gaucher, G.M. : The production of the antibiotic patulin in a three phase fluidized bed reactor II.Longevity of the biocatalyst. *Can. J. Chem. Eng.*, **62**:120-124, 1984.
3. Box, G.E.P., Hunter, W.G. and Hunter, J.S.: *Statistics for Experimenters*, p.518-519 . John Wiley & Sons, New York, 1978.
4. Deo,Y.M. and Gaucher, G.M.: Semicontinuous and continuous production of penicillin G by *Penicillium chrysogenum* cells immobilized in k- carragenan beads. *Biotechnol. Bioeng.*, **26** 285-295, 1984.
5. Gbewonyo, K. and Wang, D.I.C.: Confining mycelial growth to porous microbeads: a novel technique to alter the morphology of non-Newtonian mycelial cultures . *Biotechnol . Bioeng .*, **25**: 967-983, 1983.
6. Gbewonyo, K. and Wang, D.I.C.: Enhancing gas-liquid mass transfer rates in non-Newtonian fermentations by confining mycelial growth to microbeads in a bubble column. *Biotechnol. Bioeng.*, **25**: 2873-2887, 1983.
7. Gbewonyo, K., Meier, J. and Wang, D.I.C.: Immobilization of mycelial cells on Celite. *Methods Enzymol.*, **135**:318-333, 1987.
8. Jones,A., Wood, D.N., Razniewka, T., Gaucher, G.M., and Behie, L.A.: Continuous production of penicillin G by *Penicillium chrysogenum* cells immobilized on Celite biocatalyst support particles. *Can. J. Chem. Eng.*, **64**:547-552, 1986.
9. Keshavarz, T., Walker, E., Eglin, R., Lilley, G., Holt,G., Bull, A.T. and Lilly, M.D.: Immobilization of *Penicillium chrysogenum*: spore growth on Celite. *Appl. Microbiol. Biotechnol.*,**30**:487-491,1989.
10. Kim,J.H.,Oh,D.K.,Park,Y.H. and Wallis, D.A.: Production of penicillin in a fluidized bed bioreactor using a carrier-supported mycelial growth. *Biotechnol. Bioeng.*, **28**: 1838-1844, 1986.
11. Kobayashi,T., Tachi, K., Nagamune,T., and Endo, I. : Continuous production of penicillin in a fluidized bed bioreactor. *J. Chem. Eng. Japan*, **23**:505-507, 1990.
12. Konig, B. and Schügerl, K.: Strategies for penicillin fermentation in tower-loop reactors. *Biotechnol. Bioeng.*, **24**:259-280, 1982.
13. Miller,T.L. and Churchill,B.W. : Substrates for large-scale fermentations, p.122-136. In Demain,A.L. and Solomon,N.A. (ed), *Manual of Industrial Microbiology and Biotechnology*. American Society for Microbiology, Washington D.C.1986.
14. Morikawa,Y., Karube,I. and Suzuki,S.: Penicillin G production by immobilized whole cells of *Penicillium chrysogenum*. *Biotechnol. Bioeng.*, **21**: 261-271, 1979.
15. Oh,D.K., Hyun,C.K., Kim, J.H. and Park,Y.H.: Production of penicillin in a fluidized-bed bioractor:control of cell growth and penicillin production by phosphate limitation. *Biotechnol. Bioeng.*,**32**:569-573 ,1989.
16. Park,Y.H., Kim, E.Y., Seo,W.T., Jung, K.H. and Yoo,Y.J.: Production of cephalosporin C in a fluidized bed bioreactor. *J. Ferm. Bioeng.*, **67**:409- 414, 1989.

Received for publication in 13/11/92

GUIDELINES TO AUTHORS

Revista de Microbiologia (Published by the Brazilian Society for Microbiology) publishes original research papers, research notes and ocasionally reviews covering all aspects of Microbiology. A paper is a comprehensive contribution to the subject; a note or short communication is a concise account of new and significant findings and a review is an authoritative, critical account of recent and current research in a specific field to which they author has made notable contributions.

All submissions should be written in Portuguese or preferencially in English. The decision to accept manuscript is made by the Editorial Board.

Submission of a paper to this Journal is understood to imply that it has not previously been published and that it is not being considered for publication elsewhere.

ORGANIZATION AND FORMAT OF THE MANUSCRIPT. Type every portion of the manuscript double-space with a wide margin at the left on UNE A-4 format sheets and the pages should be numbered sequentially. Papers must be restricted to a maximun of 15 printed pages including figures and tables (this corresponds to aproximately 25 typewritten pages)

The front page should include title, name(s), of the author(s), institution affiliation(s) and complete address(es). Three to five keyword should also be included.

Paper should be divited into: Abstracts in English and in Portuguese (not excceding 250 words). Introduction, Materials and Methods, Results, Discussion, Acknowledgments and References. Results and Discussion can be combined.

Abbreviations and symbols should follow the recommendations of the IUPAC-IUB Comission and the Metric System is to be used thoughout.

Cite each listed references by numbers in the text. References should be numbered and arranged in alphabetical order as indicated in the following examples.

Ingram, L.O. & Conway, T. - Expression of different levels of the ethalogenic enzymes from *Zymomonas mobilis* in recombinant strains of *Escherichia coli. Appl. Environ. Microbiol.*, 54:397-404, 1988

Fleming, H.P. - Fermented Vegetables. - In: Rose, A.H., ed. - Economic Microbiology. London, Academic Press, p. 228-258, 1982.

Krieg, N.R. & Holt, J.C., eds. - Bergey's manual systematic bacteriology. Baltimore, Willians & Wilkins, v. 1. 1984.

Only those photographs which are strictly necessary for the understanding of the paper should be submitted. Fotoprints must be of sufficient quality to ensure good reproduction. They should be numbered on the back and identified with the first author's name written in pencil. Legends for linedrawings and photographs should not exceed the printing area (13x20 cm). All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

Tables should be compiled on separate sheets with a descritive title and numbered indepently of the figures using Arabic numerals.

Please indicate with a soft pencil the approximate location of tables and figures in the left margin on the page.

NOTES. Notes should be restricted to 6 typewritten pages and intended to present experimental observations and descriptions of techniques or methodological changes of interest. They should be written according to the guidelines given for papers, but without the heading divisions, and their abstracts should not exceed 50 words. Figures and tables should be restricted to maximum of 2 figures or 2 tables, or 1 table and 1 figure.

REVIEWS. Review articles should deal with microbiological subjects of broad interest. Specialists will be called upon to write them. In addition to an abstract, they may contain a list of contents.

PROOFS. On acceptance of the paper, one galley proof will be sent to the nominated author to check for typesetting accuracy. The corrected proofs shoulds be duly returned within 10 days. If delays were observed, the proofs will be corrected by the editorial staff and published. Broader changes implying recomposition of the text will be at author's expense. Fifteen offprints of each paper are supplied free of charge. Additional reprints will be billed at cost price if requested upon returning the corrected galley proofs.

Papers must be submitted, in triplicate, to the Executive Director.

Revista de Microbiologia
Av. Prof. Lineu Prestes, 1374
Cidade Universitária - USP
05508 - São Paulo - SP - Brasil