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The Effect of Serial Culture and Storage on The Protective Potential of a Competitive Exclusion Preparation

Efeito do Cultivo Seriado e Estocagem Sobre o Potencial de Ação do Método de Exclusão Competitiva

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ABSTRACT

The competitive exclusion method (EC) was used to protect day-old birds against colonisation of the intestinal tract by Salmonella *enterica serovar* Enteritidis *(SE). The culture prepared in nutrient broth incubated at 37ºC/24 h inhibited SE growth in the caecal contents. The beneficial effect was also observed after serial passage of the feces culture for up to 14 times. The material obtained after 12 serial sub-cultures after storage for 28 days at 4-6ºC also kept its action on SE.*

RESUMO

Cultura de fezes (Método de Exclusão Competitiva - EC) utilizada para prevenir a colonização cecal de aves por *Salmonella enterica* serovar *Enteritidis* (SE) foi submetida a cultivos seriados para evitar a presença de patógenos e, após o tratamento mais adequado, foi armazenada em temperatura de refrigeração antes do seu uso por até 63 dias. Os resultados mostraram que o cultivo repetido por 14 vezes não prejudica a ação protetora da cultura (CE), a qual continua inibindo a colonização cecal por SE. O produto submetido a 12 cultivos e armazenado durante 28 dias em temperatura de refrigeração também continua eficaz.

INTRODUCTION

Salmonella *remains a major cause of human food-poisoning throughout the world and it is likely that poultry are the most important reservoir. Since the beginning of the pandemic caused by* Salmonella enterica *serovar Enteritidis in the 1980's, increased efforts have been made to control the pathogen at source in poultry flocks (Rodrigue* et al*., 1990; Barrow, 2000).*

Because of the costs associated with improved hygiene and housing, biological methods have been increasingly explored. Antibiotics have been used but may select for resistance in the pathogen or components of the gut microflora (Smith &Tucker, 1975; Barrow, 1998). They can be of variable effectiveness and may even prolong faecal shedding by disrupting the normal flora.

This inherent inhibitory activity of the intestinal microflora has been exploited by the administration to newly-hatched chicks of preparations of intestinal contents or faeces, cultured in vitro*. This has been called competitive exclusion (CE) or the "Nurmi concept" after its discoverer (Nurmi & Rantala, 1973) and it has been applied in many countries (Mead, 2000). However, one of the problems associated with its use is the potential for the introduction of other intestinal pathogens (virus, protozoa and bacteria) into uninfected flocks with the intestinal cultures. Attempts have been made to avoid this problem by using mixtures of cultures of defined bacterial strains. These, however, are less protective than undefined CE preparations (Day, 1992). An alternative is to passage the culture such that non-bacterial pathogens are eliminated. A concern over this approach is whether the protectiveness of the preparation is maintained on repeated passage (Snoeyenbos* et al*., 1978; Mead & Ympey, 1984; Mead &Ympey, 1987). The results of this sort of analysis have been mixed with some authors claiming success (Pivnik* et al*., 1982; Nisbet* et al*., 1993) whilst others suggest that the numbers of some bacterial types in the preparations become reduced after laboratory passage which contributes to reduced effectiveness (Weinack* et al*., 1979).*

The present paper aims to contribute to this debate by analysing a gut flora preparation which has been passaged several times in vitro*, in addition to being stored, and tested frequently for its inhibitory activity against* S. Enteritidis *in chickens.*

MATERIALS AND METHODS

Bacteria

A spontaneous mutant of S. Enteritidis *phage type 4, resistant to both nalidixic acid and spectinomycin, was used. Cultures were prepared in nutrient broth (Oxoid CM67), incubated in a shaking water bath (100 strokes/ min) at 37ºC overnight.*

Competitive exclusion mixture

Fresh faeces were obtained from adult birds reared in the Faculty of Agronomic and Veterinary Science (FCAV-Unesp) and were inoculated in nutrient broth (1:10 weight for volume) that was incubated statically and aerobically at 37ºC for 24 hours. The culture was tested for the absence of Salmonella*. This culture was sub-cultured into fresh nutrient broth (1:10), incubated under the same conditions and this was repeated 14 times. The culture was also stored at 4ºC for up to 63 days.*

Birds

Newly hatched chickens were obtained from a commercial broiler hatchery. Groups of six birds were housed together in a box separate to other groups. Feed with no additives , and drinking water were provided ad libitum*. A heat source was provided. Swabs from the cloacae were incubated to confirm absence from* Salmonella*.*

Infection protocol

Birds were inoculated orally with 0.1 mL of cultures of the competitive exclusion mixture. They were challenge with Salmonella *by placing into the box, a seeder bird of the same age and source which had received no flora preparation but which had been infected with 0.1mL of a 1:1000 dilution of the* Salmonella *culture (approximately 1.2 x 10^6 viable cells per mL).*

Experimental procedure

For each passage number tested, eight groups of six chickens were studied. Four of these were inoculated with the competitive exclusion mixture and one day later all groups were infected with the challenge Salmonella *strain. Three birds from each group were killed 3 and 6 days later respectively and the number of* Salmonella *in their caecal contents were enumerated.*

An additional experiment was set up in which a competitive exclusion culture, which had been passaged 12 times, was stored at 4-6°C for up to 63 days. This was tested at various times after storage in 8 groups of 6 chickens in the same way as indicated above.

Log_{10} viable bacterial counts were analysed by ESTAT (1992) and the protective effect of the exclusion mixtures was estimated.

Bacterial enumeration

Decimal dilutions of caecal contents were made in PBS, pH 7.4 and 0.1 mL aliquots were cultured on Brilliant Green agar containing sodium nalidixate (100 ug/mL) and espectinomycin (100 ug/mL). Plates were incubated at 42°C for 24 hours.

RESULTS AND DISCUSSION

Serial passage of the faecal culture for up to 14 times had no discernible effect on its inhibitory activity with no isolations of the challenge strain at all from the treated groups, in contrast to the control groups (Table 1). All the differences between the treated and control groups were highly significant ($p<0.05$). The material obtained after 12 serial sub-cultures was tested for its resilience after storage up to 63 days. At 28 days storage no effect was observed, the material being fully inhibitory. However, after this time the material deteriorated in quality with increasing numbers of birds having high counts of the challenge organism in the caeca (Table 2).

Milner & Shaffer (1952) observed that day-old birds were very susceptible to Salmonella *enterica serovar Typhimurium while by day 14, they were much more resistant. This acquired resistance is related to the maturity of the immune system and the gradual acquisition of native gut microflora (Fowler & Mead, 1990). Nurmi & Rantala (1973) demonstrated the beneficial effects of the gut microflora against colonisation by S. Infantis when they accelerated the process of microflora colonisation by administration to young birds of faecal cultures obtained from adult birds. This was named competitive exclusion, which has been used extensively for control of avian salmonellosis.*

Some commercial products are available (Mead, 2000). However, their efficacy is dependant on several factors, including microbial composition, which is optimal with material obtained from native flocks (Barnes, 1979). Cultures are required to be incubated anaerobically. Rambousek et al*. (1995) demonstrated that a product prepared under aerobic conditions could be effective in preventing intestinal colonisation of chickens by* Salmonella*. Later, Oliveira* et al*. (2000) obtained similar results, challenging newly hatched chickens by contact, simulating the main route of infection of S.* Enteritidis *in the field. The present study was carried out to assess the inhibitory effect of faecal cultures prepared under aerobic conditions after serial incubation. Serial culture of the faecal broth culture has been suggested as a technique to purify the material (Snoeyenbos* et al*., 1978; Mead & Ympey, 1984; Mead &Ympey, 1987) although the process may also eliminate beneficial micro-organisms (Mead, 2000). The results indicate that serial passage aerobically up to 14 times did not seriously affect its efficacy. This is a somewhat surprising result given the extreme oxygen sensitivity of some of the obligate anaerobes present, which are essential to the inhibitory effect. However, the redox conditions in such cultures borders on anaerobic, oxygen being absorbed from the culture by the facultative anaerobes.*

Competitive exclusion material must ideally be obtained from donors free of pathogens. The use of healthy donor birds from a monitored local flock may be a useful application of this since the inhibitory activity of the microflora from such birds would be expected to be greater than that obtained from intensively reared birds (Barnes, 1979). Because many poultry industries have integrated operations and have good laboratory facilities, the storage of the exclusion culture at 4-6°C over several weeks was investigated. The results presented here showed that the product kept well over 28 days and continued to be effective against S. Enteritidis.

***Table 1**- Number (log_{10}) of viable cells of S.* Enteritidis *NalSpec^r (SE NalSpec^r) present in the caecal contents of the birds challenged 24 hours after the treatment with faecal culture (CE) submitted to 5, 7, 12, or 14 serial cultures.*

Treatment	Log_{10} viable number of SE NalSpec^r per gram of caecal contents	
	3 days post contact infection	6 days post contact infection
CE5	N*(N-N)	N (N-N)
None	5,50 (N - 9.00)	5,39 (N - 9.00)
CE7	N (N -N)	N (N -N)
None	6.36 (N - 9.17)	4.95 (N - 9.00)
CE10	N (N -N)	N (N -N)
None	5.35 (N - 9.39)	6.86 (6.23 - 8.25)
CE12	N (N -N)	N (N -N)
None	5.35 (N - 7.76)	6.86 (6.23 - 7.55)
CE14	N (N - 6.78)	N (N -N)
None	7.84 (N - 9.39)	4.07 (N - 8.25)

*CE5: five serial dilutions of the CE. N = log_{10}<2.0; *Log_{10} median count per gram from 24 birds (range in parentheses).*

***Table 2**- Number (log_{10}) of viable cells of S.* Enteritidis *NalSpec^r (SE NalSpec^r) present in the caecal contents of the birds challenged 24 hours after the treatment with faecal culture (CE) submitted at 12 serial cultures and stored for 28, 35, 42 and 63 days at 4-6°C.*

Treatment	Log_{10} viable number of SE NalSpec^r per gram of caecal contents	
	3 days post contact infection	6 days post contact infection
CE/28d	N* (N-N)	N (N-N)
None	4.79 (N - 9.00)	5.72 (N - 7.60)
CE/35d	N (N -6.63)	3.09 (N - 8.77)
None	6.21 (N - 9.00)	6.26 (N - 7.60)
CE/42d	N (N -N)	N (N - 6.27)
None	5.22 (N - 9.00)	4.79 (N - 7.60)
CE/63d	2.66 (N - 7.36)	3.66 (N - 8.90)
None	3.96 (N - 8.38)	3.83 (N - 7.41)

*CE/28d: CE submitted to 12 serial dilutions and storage at 4-6°C/28 days. N + log_{10} <2.0. *Log_{10} median count per gram from 24 birds (range in parentheses).*

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