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COMPARISON OF EXPERIMENTAL COMPETITIVE-EXCLUSION CULTURES FOR CONTROLLING *SALMONELLA* COLONIZATION IN BROILER CHICKS

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ABSTRACT

The efficacy of three different types of experimental competitive exclusion (CE-A, CE-B and CE-C) cultures against *Salmonella* Kedougou (SK) NCTC 12173, resistant to nalidixic-acid resistant (Nal^R), in one-day-old broiler chicks, in four treatments with three replicates in each treatment, was evaluated. The mean logarithmic counts of *Salmonella* program of feces were 0.4 L in the group treated with CE-A, derived from the whole cecal contents of an adult bird with a mixture of aerobic and anaerobic bacterial cultures; 1.22 in the group treated with CE-B, containing aerobic bacterial culture; 1.00 in the group treated with CE-C, containing anaerobic bacterial culture; and 6.64 in the control group. The percentage of colonized birds varied from 10% to 26.66% in the treated groups and was 63.33% in the control group. A good protection (76.66% to 90%) was obtained in all treatments whereas lower protection was verified with experimental products containing only aerobic or anaerobic cultures. These results showed that a mixture of aerobic and anaerobic cultures can be effective for reducing SK colonization in broiler chicks.

Key words: Competitive exclusion, *Salmonella*, broiler chicks.

INTRODUCTION

Salmonella infections are mainly assymptomatic in poultry, but are associated with widespread human illness from this source. Therefore, there is continuing interest in finding ways of preventing flock infection and hence contamination of poultry products with *Salmonella* (24).

The work of Nurmi and Rantala (22) highlighted the link between susceptibility to *Salmonella* infection and the delayed development of the microbiota in the gastro-intestinal (GI) tract of young chickens. It also provided a simple, practical solution to the problem through the early establishment of an adult-type microflora that markedly increased the resistance of the bird to *Salmonella* colonization. The protective effect depends upon the administration of viable bacteria that must include certain obligate anaerobes.

Treated flocks can be expected to have fewer *Salmonella*-positive birds than untreated controls and lower levels of cecal colonization in those birds that become infected (20).

The complexity of the protective microbiota appears to be important. Attempts to use simpler defined mixtures of treatment bacteria, including conventional probiotic preparations, have been less successful (32).

Several commercial competitive exclusion (CE) products have become available (14,20) and these are based on anaerobic cultures of cecal material from suitable adult donor birds that have been extensively screened to ensure the absence of avian and human pathogens. The products are of undefined composition, but most have been partly characterised and are known to include the principal organisms that occur naturally in the ceca of adult chickens. Products aimed at protecting chickens against *Salmonella* colonization may also be used for turkeys, since there is reciprocal protection between the two (1,29).

As an intervention measure, CE treatment is of particular value in controlling horizontal transmission of *Salmonella* among chicks. For optimum efficacy, however, chicks need to be free from *Salmonella* prior to treatment and reared under conditions of good biosecurity, especially during the first two days following

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treatment when the administered organisms are becoming established. Advantages of the treatment include the ease of application (usually by spray-inoculation in the hatchery) and the protection provided against any salmonellas capable of invading the GI tract (20). The response to treatment is relatively rapid and there appears to be full compatibility with other intervention measures such as vaccination and treatment of feed with organic acids. CE treatment is particularly appropriate for the broiler, where any period of *Salmonella* shedding leads to external contamination of the bird and subsequent spread of the organisms among processed carcasses.

This study was initiated to determine the efficacy and protective effect of experimental CE cultures to reduce *Salmonella* spp. in broiler chicks.

MATERIALS AND METHODS

Experimental animals

Trials were carried out on newly hatched male chicks (Ross) obtained directly from an industrial hatchery. Chicks were housed in boxes, 1.70 m^2. Chicks were reared on new wood shaves litter in floor-pens. In each trial, 30 randomly selected chicks were placed in each pen. Temperatures were at least 29ºC under brooder lamps in each box. Non chlorinated water was used, feed was commercially prepared non-medicated broiler starter crumbles.

Challenge organism

Salmonella Kedougou (SK) strain NCTC 12173 nalidixic-acid resistant (Nal^R) at the final concentration 20 µg/mL, originally isolated from chickens, being non-pathogenic for humans, non-invasive in chicks but able to colonise the ceca, was used for the infective treatment (21).

Preparation of CE cultures

Three cecal cultures were prepared from cecal content of adult *Salmonella*-free donor hen. The cecum was aseptically removed from the adult chicken and it was placed in a sterile petri dish. Then, the dull edge of a sterile scalpel blade was used to gently remove adherent cecal contents. Next the epithelium was scraped, and a sterile syringe was used to collect the rinsate from the petri dish. The cultures were prepared by inoculating 1.0 g of fresh cecal content and epithelium into three bottle contents 1 L of BHI (Brain heart infusion broth) supplemented with 0.5% cysteine-HCl, according with Bailey *et al.* (3) and Blankenship *et al.* (6). In order to obtain CE-B, the bottle was incubated at 37ºC for 24 h using a shaker (100 rpm) under aerobic conditions. The anaerobic bacterial cultures (CE-C) were incubated in an anaerobiosis jar containing an anaerobic system (Anaerobac system – Probac do Brasil Ltda). These cultures were tested for the presence of *Salmonella* spp. using XLT4 agar.

The bacterial cultures were centrifuged at 12,000 x g for 30 min at 4ºC. After that the pellet was suspended in PBS 0.1 M (pH 7.4) supplemented with glycerol 30%. According to the pellet volume, the bacterial cultures were diluted 1:10. The bacterial suspension was frozen at -20ºC for 24 h, and afterward was kept at -80ºC until the experimental study.

Three experimental frozen cultures were obtained: CE-A derived from the whole cecal contents with a mixture of aerobic and anaerobic bacterial cultures; CE-B containing aerobic bacterial cultures, and CE-C containing anaerobic bacterial cultures.

Experimental protocol

Day-old chicks were divided into five groups of 30 birds. Three CE experimental cultures were suspended 1:100 in PBS 0.1 M (pH 7.4) and giving to the chicks into the crop, three replicated groups of chicks per treatment. 24 h later, all chicks except those in the negative control group were challenged with SK into the crop with 0.1 mL of a cell suspension containing 3.6 x 10^6 CFU/mL (21).

On the fifth day after challenge the chicks were killed by cervical dislocation and the cecal contents were examined for *Salmonella* colonization from all treatments. After opening the ceca were aseptically removed and mixed with 9 mL of 1% peptone water into sterile plastic bags, and tenfold dilution was prepared for surface plating on XLT4 agar containing 20 µg/mL nalidixic-acid. The volume of inoculum required was 0.1 mL for each sample of cecal suspension (21). The plates were incubated for 24 h at 37ºC, and the number of colony- forming unit (CFU) of *Salmonella* Kedougou Nal^R (20 µg/mL) per gram of cecal content was calculated (21).

Statistical analysis

Viable counts were converted into logarithmic form and analysed using non-parametric tests. Data were assessed using Kruskall-Wallis test, Mann-Whitney test and Spearman correlation (30), as appropriate.

The efficacy of treatment cultures in protecting chicks against *Salmonella* infection was expressed using: a) number of chicks with different levels of *Salmonella* per g of cecal content; b) percentage of birds with infection; c) Infection Factor (IF) and Protection Factor (PF). IF is the logarithmic number colony forming units of *Salmonella* organism per gram (IF= log10 CFU/g^{-1}) of cecal contents and PF is the value obtained by dividing the control group IF by that IF from the treated group (21).

RESULTS

The results of the trials are shown in Tables 1 and 2. The three experimental treatments were shown to be effective in controlling *Salmonella* colonization. Treatment 1 (CE-A) caused

a significant reduction of SK colonization when compared to the other two treated groups (Table 1). Treatments 2 (CE-B) and 3 (CE-C) resulted in significant reduction in colonization when compared to the control group – T4 (Table 1).

Results obtained from treated (CE-A, CE-B and CE-C) and untreated groups are presented in Table 2, where IF and PF were calculated according to Mead *et al.* (21). The IF value of 6.64 indicated strong colonization of cecum by SK in the control group, while in pre-treated chicks the IF values were between 0.41 and 1.22. PF values varied from 5.44 to 16.19. The results of these trials were far below the limit of acceptance, and even below the value 4.0 that Mead *et al.* (21) suggested as a minimum for a treatment material to be effective against *Salmonella* in field conditions.

Non-treated controls, challenged with the same SK strain, IF of 6.64, demonstrating a strong colonization (Table 2). All groups showed values higher than four, but the best results were obtained in chicks treated with experimental frozen cultures containing a bacterial mixture produced under aerobic and anaerobic conditions (CE-A). In this case the PF was 16.19, which represents a considerable increase of protection it compared to the other groups. Chicks treated with experimental frozen cultures containing aerobic (CE-B) or anaerobic (CE-C) bacteria, showed PF of 5.44 and 6.64, respectively.

Results for the different treated groups showed significant differences among them ($p<0.05$). A positive correlation between treatment and reduction of SK recovery was observed. Protection was demonstrated in all treated groups and varied from 76.66% to 90%.

Table 1. Influence of treatment with experimental CE cultures on recovery of *Salmonella* Kedougou from ceca.

Treatment	Log10 *Salmonella* UFC/g faeces			Mean Log10 *Salmonella* UFC/g Faeces	Positive ceca
Replication	1	2	3		(%)
T1 CE-A	2.34 (1/10)*	1.19 (1/10)	2.74 (1/10)	2.09[a]	10.00
T2 CE-B	3.15 (2/10)	2.36 (3/10)	2.63 (2/10)	2.71[b]	23.33
T3 CE-C	2.56 (2/10)	2.16 (2/10)	2.44 (2/10)	2.39[c]	20.00
T4 positive control	2.57 (6/10)	2.06 (7/10	2.35 (6/10)	2.33	63.33

a,b,c = no common superscript differ significantly ($p<0.05$).
* (positive chicks/number of chicks challenged).

DISCUSSION

Different methods may be used to increase resistance against *Salmonella* infections (24). CE treatment is used to prevent *Salmonella* colonization. Oral administration of defined mixtures of bacterial isolates from faecal and cecal contents of adult chickens protect young chicks against infection with *Salmonella*. Defined bacterial mixtures give protection comparable to that of faecal or cecal cultures of unknown bacterial composition (31).

Our results showed that aerobic bacterial mixture protected chicks against SK colonization (IF= 1.22, PF=5.44), different from results obtained by Snoeyenbos *et al.*, (29). Nevertheless, the experimental aerobic culture used in this trial had *Lactobacillus sp*, in its composition (data not published), a bacteria with proven capability to prevent growth of *Salmonella* in the cecum of chickens (19). The mechanisms of the protective effect are unknown. However, this protective effect could be due to antibacterial factors produced by *Lactobacillus sp* (25,36), or to enhancement of the intestinal immunity (23).

Table 2. Data for control and treated chicks related to Infection Factor (IF) and Protection Factor (PF).

Treatments	Number of chicks infected							
	0	1	2	3	4	5*	Infection Factor (IF)	Protection Factor (PF)
T1 CE-A	27	0	0	1	2	0	(27x0)+(1x3)+(2x4)=11/27 = 0.41	6.64 / 0.41= 16.19 90%
T2 CE-B	23	0	0	1	5	1	(23x0)+(1x3)+(5x4)+(1x5)=28/23 = 1.22	6.64/ 1.22 = 5.44 76.66%
T3 CE-C	24	0	0	1	4	1	(24x0)+(1x3)+(4x4)+(1x5) = 24/24 = 1	6.64 / 1= 6.64 80%
T4 Positive control	11	0	0	4	14	1	(11x0)+(4x3)+(14x4)+ (1x5)=73/11 = 6.64	6.64/6.64 = 1 36.66%
T5 Negative control	30	0	0	0	0	0	(30x0)= 0/30= 0	6.64/0 = 0 100%

* These represent the *Salmonella* CFU/Log 10.

The efficacy of CE was demonstrated in the group treated with anaerobic culture, with 80% of protection and a reduction of *Salmonella* colonization, which is similar to results obtained by Snoeyenbos *et al.* (29), Barnes *et al.* (2) and Schneitz *et al.* (26). Some mechanisms could explain this protective effect. Following CE treatment, the factors involved in protecting recipient birds against *Salmonella* are likely to be the same as those affecting the normal, adult microbiota. These mechanisms have recently been reviewed by Schneitz and Hakkinen (27). They include: a) creation of a restrictive physiological environment, involving microbial production of volatile fatty acids and a low oxidation-reduction potential; b) competition among different microbes for receptor sites; c) production of antibiotic-like substances, such as bacteriocins, by some microorganisms and d) microbial competition for essential nutrients. It seems unlikely that any single mechanism is wholly responsible for the protective effect of CE treatment.

The CE treatment with an experimental culture containing undefined aerobic and anaerobic cultures resulted in IF= 0.41 and PF=16.19, similar to those obtained by Stavric (33), with a protective effect of 90% and a reduced SK colonization (10%). It seems that successful protection against *Salmonella* colonization requires complex bacterial mixtures (34). However, protection is similar in defined and undefined complex mixtures (15,17,27). According to Mead *et al.* (21) this treatment was more effective because it has a higher value of PF, similar to those obtained by Stavric *et al.* (31).

Present CE products are essentially undefined in composition, but contain a complex mixture of viable bacteria, some of which may play a part in the development of colonization resistance. The mechanisms of the protective effect are unknown, and may never be determined because of the complexity of the intestinal environment and the interactions between host and microbes that can occur (4).

The intestinal environment has indigenous microbes that modulate the immune response by increasing or decreasing the amounts of mediators secreted by immunocompetent cells (4,10,11,37). The microbiota also provides a substantial amount of antigenic material for the mucosal immune system and can influence the oral immune response (13,35).

Related to the host, genetic control of disease resistance is highly complex and can involve any of the body's defense systems and their interaction (9). It is well established that the responses of chickens to disease agents may vary. Differences in susceptibility among poultry breeds are attributable to varying genetic resistance and it has been shown that resistance of chicks to *S.* Typhimurium is linked to a dominant autosomal gene (7). Thus, different breeds of poultry respond differently to a standard *Salmonella* challenge. With some strains, mortality is high (97% or more) while in others it is no more than 3%. These observations are consistent and reproducible, suggesting that resistance is genetically determined and the situation for disease in the chick (18) is the same as that for resistance to cecal colonization by *Salmonella* (5). Among different breeds of chicken, variations have been reported in resistance to infection by *S.* Pullorum (8,16), *S.* Gallinarum (8,28) and *S.* Enteritidis (8,12).

Since there are many factors in the chick that could affect the microbiota and their interactions, mechanisms associated with host genetic resistance, microbial interaction on the intestinal environment, intestinal immunity, and others, can play an important role in the protective effect of CE products. The obtention of protection against *Salmonella* colonization is a complex process. While differences exist between animal species in the operation of the immune system, genetic resistance and others, they are functionally very similar. Information on the topic from any source can be used for further understanding the CE mechanism to protective effect.

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RESUMO

Comparação de culturas experimentais de exclusão competitiva para o controle da colonização de *Salmonella* em pintos de corte

Esse estudo avaliou a eficácia de três diferentes tipos de culturas de exclusão competitiva (EC-A, EC-B e EC-C) contra *Salmonella* Kedougou (SK), amostra NCTC 12173, resistente ao ácido Nalidíxico (Nal^R), em pintos de um dia de idade, utilizando-se 4 tratamentos, em três repetições. A média logarítmica de *Salmonella* por gramas de fezes foi de 0,41 para o grupo tratado com a EC-A, contendo uma mistura de culturas bacterianas aeróbias e anaeróbias, derivada de conteúdo cecal de uma ave adulta; 1,22 no grupo tratado com a EC-B, contendo culturas bacterianas aeróbias; 1,00 no grupo tratado com a EC-C, contendo culturas bacterianas anaeróbias e 6,64 no grupo controle positivo. A porcentagem de aves colonizadas variou de 10 a 23,33% nos grupos tratados e foi de 63,33% no grupo controle. Uma boa proteção (76,66 a 90%) foi obtida em todos os tratamentos, sendo que a menor proteção foi verificada com os produtos experimentais contendo somente culturas de bactérias aeróbias ou anaeróbias. Os resultados sugerem que o uso de misturas de culturas aeróbias e anaeróbias pode ser efetivo para o uso na exclusão competitiva contra SK em frangos de corte.

Palavras-chave: Exclusão competitiva, *Salmonella*, frangos de corte.

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