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Protective Effect of *Enterococcus faecium* J96, a Potential Probiotic Strain, on Chicks Infected with *Salmonella* Pullorum

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ABSTRACT

Enterococcus faecium J96 was isolated from a healthy free-range chicken and it inhibited *Salmonella* Pullorum, in vitro, due to its lactic acid and bacteriocin production. In vivo assays were carried out with 30-h-old broiler chicks. The lactic acid bacteria ($\sim 1 \times 10^9$ cells per chick) were orally administered as preventive and as therapeutic treatments. In the first case they were given to the chicks twice a day for 3 consecutive days. In the second case the lactic bacteria were administered in the same way after a 24-h challenge by *Salmonella* Pullorum (in both instances the salmonella dose was 1×10^5 cells per chick). Cecal contents, liver, and spleens were analyzed and liver and spleen fragments were also fixed in formaldehyde (pH 7.00) in order to determine salmonella translocation. The chickens that were preventively treated with *E. faecium* J96 survived the *Salmonella* Pullorum challenge. Those that were infected on the first day and then inoculated with lactic bacteria died 4 days later. Salmonellae were isolated from their livers and spleens. From these results we may conclude that *E. faecium* J96 can protect newly hatched chicks from *Salmonella* Pullorum infection but cannot act as a good therapeutic agent.

Fowl salmonellosis or Pullorum disease is a bacterial, infectious, and septicemic disease caused by *Salmonella* Pullorum, a gram-negative microorganism. The incubation time of the illness is around 3 to 6 days, but sometimes symptoms can appear 6 months later. It can cause a mortality of about 20 to 80% to a flock *(29)*; however, there could be a high mortality without any symptom at all because this bacterium is the most invasive and virulent for chicks *(11)*.

On the other hand, lactic acid bacteria are sometimes employed in probiotical supplements for poultry because, besides their lactic acid production, they could synthesize short chain fatty acids, hydrogen peroxide, bacteriocin, or bacteriocin-like substances with antimicrobial activity against spoilage and pathogenic bacteria *(5, 10, 31, 32)*. Besides, in general, they were selected because of their bile salt resistance or their capacity to adhere to intestinal epithelia *(5, 10, 12, 13)*. However, in our laboratory, we decided to choose them according to their ability to synthesize antimicrobial substances like lactic acid and bacteriocin or bacteriocin-like molecules *(1, 3)*. With this criterium, *Enterococcus faecium* J96, a strain isolated from the crop of a healthy free-range chicken, was selected for its high growth rate, bile salt resistance, and because it could in vitro inhibit *Enterococcus hirae, Listeria monocytogenes, Salmonella* Pullorum M97, and other species, such as Gallinarum and Typhimurium due to its lactic acid and bacteriocin production *(1–4)*.

Taking into account the great impact that *Salmonella* Pullorum could have on avian health and economy, the in vitro inhibitory effect of *E. faecium* J96 on this pathogenic strain and the actual trends to employ natural alternatives to fight against pathogen microorganisms, we decided to analyze its in vivo effect.

MATERIALS AND METHODS

Chicks and rearing conditions. Newly hatched broiler chicks (30 h old with 40 g average weight, ISA breed line) used in this study were obtained from a commercial hatchery of Argentina. They were reared in different isolators (12 birds in each one), supplied with *Salmonella*-free balanced food (Cargill, Argentina) and water ad libitum and under 12-h light-dark cycles. Twelve chickens were analyzed per treatment. The assays were performed in triplicate.

Bacterial cultures. *E. faecium* J96 was isolated from the crop of an adult free-range chicken *(1, 3)* and cultivated in LAPTg broth: meat peptone, 1.5 g; tryptein, 1 g; yeast extract, 1 g; glucose, 1 g; Tween 80, 0.1 ml, pH 6.5, to a final volume of 100 ml *(24)* at 37°C during 12 h in aerobic conditions.

Salmonella Pullorum M97 was provided by the Institute of Special Bacteriology ''Carlos Malbrán,'' Buenos Aires, Argentina. It was cultured in a brain-heart infusion (Merck, Darmstadt, Germany) broth at 37°C during 24 h.

Lactic acid bacterium administration. Lactic acid bacteria (200 μl with $\sim 1 \times 10^9$ cells per chick) were orally administered according to both preventive and therapeutic treatments. In the first case, they were given to newly hatched chicks (30 h old) twice a day with an interval of 12 h between each dose for 3 consecutive days (flock 4). In the second case the lactic bacteria were administered to 4-day-old chickens in the same way and after a 24-h challenge by *Salmonella* Pullorum M97 (flock 3). The number of viable bacteria of the inoculum was determined by plate count on *Streptococcus* selective agar (Merck) and the plates were incubated at 37°C during 12 h.

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Challenge with *Salmonella* Pullorum M97. At 4 days old the challenge dose, 200 μl of a 24-h culture with $\sim 1 \times 10^5$ CFU ml^{-1}, was given by gavage into the crop to untreated and treated birds with lactic acid bacteria according to a preventive scheme (flocks 2 and 4, respectively). Flock 3, as it was described before, received the same pathogen dose on 30-h-old and after were treated with *E. faecium* J96 twice a day with an interval of 12 h between each dose for 2 consecutive days. Flock 1 was bird control flock.

The number of viable *Salmonella* Pullorum M97 cells was quantified on MacConkey agar (Merck). The plates were incubated at 37°C during 12 to 24 h.

Slaughtering, sampling, and determination of different microbiological populations of birds. Four chicks from each treatment were killed by cervical dislocation 24 h and 5 days postinfection. Ceca, livers, and spleens were aseptically extracted and all of them were individually assayed for *Salmonella*. Besides, cecal contents were aseptically removed and different microorganisms were analyzed. Lactobacilli were determined on *Lactobacillus* selective agar (Merck); bifidobacteria were quantified on a *Bifidobacterium* selective medium *(20)*. Enterococcal and Streptococcal populations were quantified on *Streptococcus* selective medium (Merck); total aerobes on LAPTg agar (1.5% wt/vol) and *Enterobacteriaciae* were determined on MacConkey agar (Merck). *Salmonella* colonies were differentiated from other enterobacteria because they are translucent in this medium and because no other microbial population with this characteristic was detected on it during the assays. Lactobacilli were incubated in a microaerophilic environment for 24 to 48 h at 37°C. Bifidobacteria were grown in anaerobic conditions (90% N_2 and 10% CO_2) at 37°C for 5 days; an anaerobiosis chamber (Forma Scientific–Anaerobic System, model 1024) was employed in order to obtain this atmosphere. The other microbial populations were incubated at 37°C for 12 h.

Histological analyses. Livers and spleens were aseptically removed and fixed in formaldehyde (pH 7.00) in order to determine any injury because of the pathogen challenge or *Salmonella* translocation.

Statistical analyses. All analyses were carried out according to the honestly significant difference Tukey test and they were considered significant at the $P < 0.05$ level. Calculations were made with statistical software (Minitab Release 12.22).

RESULTS AND DISCUSSION

We decided to evaluate the effect of *E. faecium* J96 on chicks according to two oral administration schemes: as preventive and as therapeutic agent against *Salmonella* Pullorum M97 challenges. Also, we decided to analyze individually these pathogen target sites of colonization: ceca, liver, and spleen. Ceca bacterial populations were studied, livers and spleens were checked for *Salmonella* presence, and histological analyses of them were performed.

As we observed from Figure 1, the control group unexpectedly, like the other flocks tested, showed an important number of enterobacteria ($\sim 1 \times 10^8$ CFU g^{-1}), and there were no significant differences ($P > 0.05$) on the microbial populations analyzed within the same group and among the different treatments (Fig. 1). These means were calculated from 12 samples for each time. In these assays, we did not work with specific pathogen-free animals, and the values observed could explain this fact. Besides, we

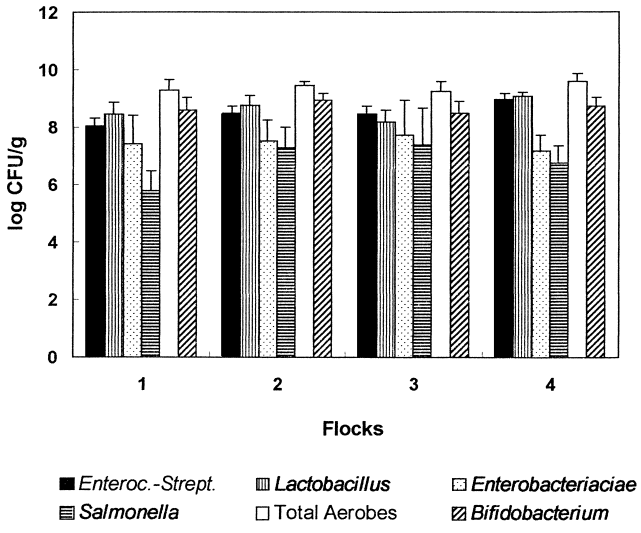

FIGURE 1. *Microbial population present on cecal contents from 5-day-old chickens individually analyzed (1, control; 2, birds without treatment and orally challenged on day 4 of life with* Salmonella *Pullorum M97; 3, birds challenged with* Salmonella *Pullorum M97 at 30 h and orally treated with* E. faecium *J96 (therapeutic model); 4, birds orally treated with* E. faecium *J96 at 30 h life during 3 consecutive days and challenged with* Salmonella *Pullorum M97 on day 4 [preventive scheme]). Bars represent the means (±SE) of three replications.*

may infer from these results that the birds with which the assays were performed could present a chronic-*Salmonella* infection but different from the one caused by *Salmonella* Pullorum M97. Perhaps they were infected since hatching, either because salmonellae were vertically transmitted from the breeders or due to a horizontal propagation in the incubator *(19, 28)*. This situation was observed before in Argentina, and it is very important because it shows the actual situation in many Argentinean farms today *(7, 26, 30)*.

When these analyses were carried out 5 days postchallenge (or 9 days old), similar results were observed (see Fig. 2). However, the chicks from flock 3 that were infected with *Salmonella* Pullorum M97 at 30 h of hatching and after that treated for 2 consecutive days with *E. faecium* J96 became ill 8 h after the first analysis (24-h postinfection data) was carried out. Mortality reached 100% at 8 days of life, and we could not perform a second determination. Obviously, the pathogen dose employed at the laboratory was so high that it caused an irreversible effect on newly hatched chicks. On the other hand, flock 2 that did not receive any protection and was challenged at 4 days old presented 50% mortality; while flock 4, where *E. faecium* J96 was administered according to a preventive model against *Salmonella* Pullorum M97 infection, was protected by lactic acid bacteria because it presented 75% survival (Fig. 3). The different mortality registered between these flocks could be explained by the fact that chick *Salmonella* susceptibility decreased naturally with age and because they could obtain a mature microflora from the environment *(10)*.

From these results, we could infer that the high mortality registered in the flock treated therapeutically with *E.*

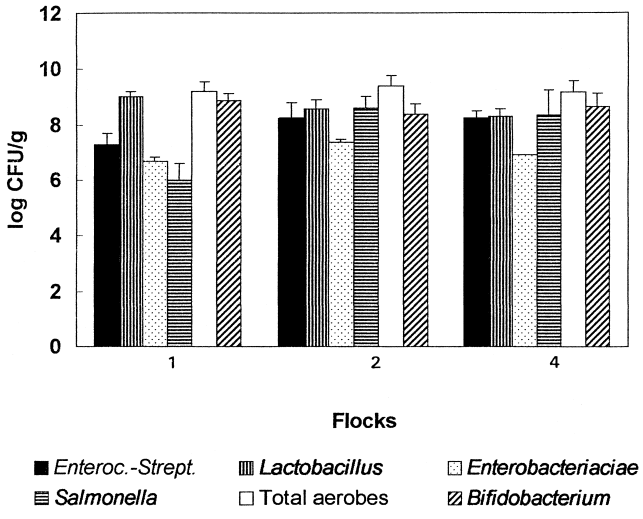

FIGURE 2. *Microbial population of cecal contents from 9-day-old chickens individually analyzed (1, control; 2, birds without treatment and orally challenged on day 4 of life with* Salmonella *Pullorum M97; 4, birds orally treated with* E. faecium *J96 at 30 h life during 3 consecutive days and challenged with* Salmonella *Pullorum M97 on day 4 [preventive scheme]). Bars represent the means (±SE) of three replications.*

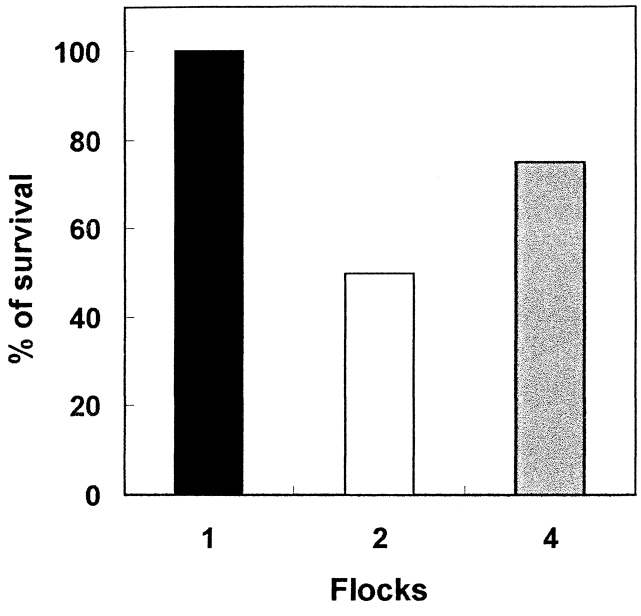

FIGURE 3. *Survival percentage of birds analyzed (1, control; 2, birds without treatment and orally challenged on day 4 of life with* Salmonella *Pullorum M97; 4, birds orally treated with* E. faecium *J96 at 30 h life during 3 consecutive days and challenged with* Salmonella *Pullorum M97 on day 4 [preventive scheme]).*

faecium J96 against *Salmonella* Pullorum M97 challenge would confirm the high invasivity of this avian pathogen that did not modify significantly the intestinal microflora (see Fig. 1), but it directly produced septicemia and death of birds. In addition, the chicken breeder line and age analyzed were important *(11, 14, 15, 25).*

However, the birds treated preventively with the lactic acid bacteria were more resistant to salmonella infection. This result shows that the protective effects were still present on flock 4 although 9 days had elapsed since the first lactic acid bacteria administration.

Both livers and spleens were histologically analyzed, but only livers are shown because lesions are more evident there. The histological analyses of livers and spleens from the control flock revealed that they were completely normal (see Fig. 4A). This result allowed us to detect and differentiate the lesions that appeared in the target organs of the treated flock with the different bacterial cultures. Flock 2, the birds of which did not receive lactic acid bacteria but *Salmonella* Pullorum M97, presented not only high mortality (50%) but also significant lesions in their livers and spleens, as can be seen in Figure 4B. Livers showed lipid infiltration, necrotic foci, and material inside the Kupffer cells (these last data not shown); in addition, the spleens were reactive. These results were interesting because, although these birds could obtain a more complete bacterial microflora from the environment and this situation reduced mortality, these new microorganisms could not protect their livers and spleens from *Salmonella* Pullorum action.

On the birds of flock 3 that presented 100% of mortality the hepatic injuries were very important. Liver cells showed a complete loss of their normal architecture (Fig. 4C). The spleens were reactive with fibrinoid exudate and necrotic foci.

Finally, flock 4 that received *E. faecium* J96 according to a preventive model against *Salmonella* Pullorum M97 challenge showed minor mortality (25%), and the histological state of spleens and livers was practically normal (Fig. 4D).

Therefore, from the results and observations performed in this work, we could infer that if *E. faecium* J96 is administered orally according to a preventive scheme with a dose of $\sim 1 \times 10^9$ CFU/chicken prior to being infected with *Salmonella* Pullorum M97, mortality could be reduced and birds protected against *Salmonella* colonization, as shown by the histological state of the target organs: liver and spleen. However, we could not say the same if the lactic acid bacteria were administered as a therapeutic agent, because once *Salmonella* Pullorum M97 colonized the intestinal tract, it would be practically impossible to reverse the situation. These results are in agreement with other reports claiming that newly hatched chicks are highly susceptible to *Salmonella* and that the sooner the competitive exclusion or probiotic culture is administered, the better is its effect *(9, 15, 17, 18, 21–23, 27).* Besides, as reported before *(1, 4), E. faecium* J96 not only inhibits in vitro *Salmonella* Pullorum but also other serovarieties, Gallinarum, Enteritidis, and Typhimurium, due to the combined action of lactic acid and bacteriocin. Therefore, *E. faecium* J96 could be a natural alternative to fight against salmonellosis today as the number of antibiotic-resistant strains is increasing *(6, 8, 16).*

Therefore, to obtain better results principally against host-specific *Salmonella* (species enterica serovar Gallinarum or Pullorum) we suggest an early treatment with *E. faecium* J96. In practical conditions, this could be achieved if the protective lactic acid bacteria culture is administered to newly hatched chicks while in the incubation chamber.

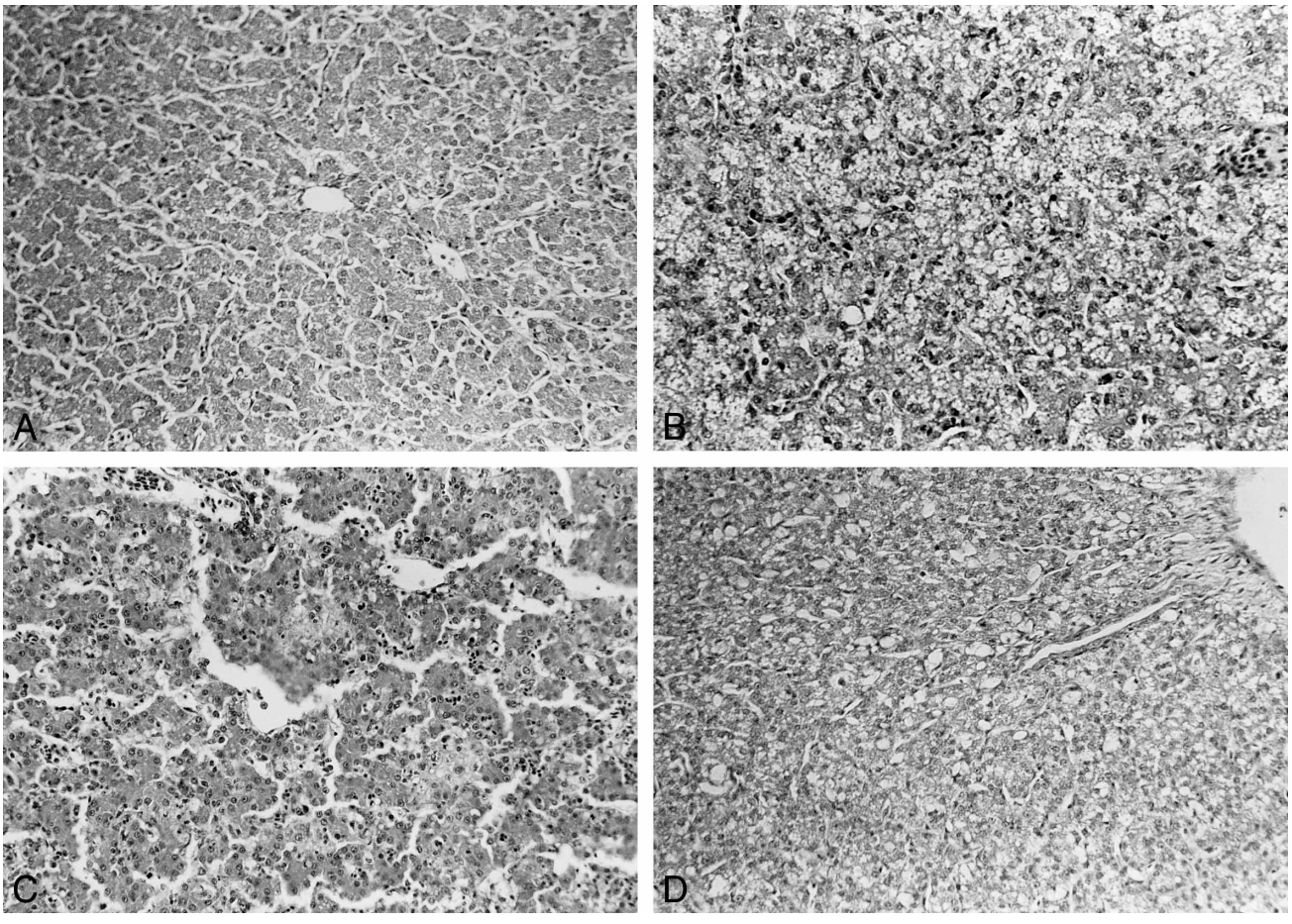

FIGURE 4. *Histological slides of livers from chickens belonging to different groups (A, control, 250×: normal cell architecture; B, flock 2, 1,000×: lipid infiltration and necrotic foci; C, flock 3, 1,000×: cells with a total loss of their normal architecture; D, flock 4, 250×: practically normal).*

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REFERENCES

1. Audisio, M. C. 1999. Estudios de bacterias lácticas con actividad antipatógena para el diseño de suplementos probióticos para aves. Tesis doctoral. Universidad Nacional de Salta, Argentina.
2. Audisio, M. C., C. Cuevas, G. Oliver, and M. C. Apella. 1997. Inhibición de *Salmonella,* especies *gallinarum* y *pullorum* por *Enterococcus faecium* J96, cepa aislada de ave. Rev. Cubana Ciencia Avícola 21:163–167.
3. Audisio, M. C., C. Cuevas, A. Pérez Chaia, G. Oliver, and M. C. Apella. 1997. Bacteriocin produced by *Enterococcus faecium* J96 active against related intestinal species. Microbiol. Alimen. Nutr. 15: 241–247.
4. Audisio, M. C., G. Oliver, and M. C. Apella. 1999. Antagonistic effect of *Enterococcus faecium* J96, strain isolated from chicken, against human and poultry pathogenic *Salmonellae* species. J. Food Prot. 62:751–755.
5. Barrow, P. A. 1992. Probiotics for chickens, p. 225–257. In R. Fuller (ed.), Probiotics: the scientific bases. Chapman & Hall, London.
6. Cherubin, C. E. 1984. Epidemiological assessments of antibiotic resistance in *Salmonella,* p. 173–200. *In* J. H. Steele and G. W. Beran (ed.), Handbook series in zoonoses, vol. 1. CRC Press, Boca Ratón, Fla.
7. Cicuta, M. M., J. C. Terrares, R. W. Roibón, F. Revidtte, M. Montenegro, M. C. Benítez, S. Boehringer, M. Morando, and C. R. de S. Kaprivka. 1996. *Salmonella gallinarum* en ponedoras de huevos para consumo. Rev. Cubana Ciencia Avícola 20:39–43.
8. Cohen, M. L., and R. V. Tauxe. 1986. Drug-resistant *Salmonella* in the United States: an epidemiologic perspective. Science 234:964–969.
9. Corrier, D. E., D. J. Nisbet, C. M. Scanlan, A. G. Hollister, D. J. Caldwell, L. A. Thomas, B. M. Hargis, T. Tomkins, and J. R. DeLoach. 1995. Treatment of commercial broiler chickens with a characterized culture of cecal bacteria to reduce *Salmonella* colonization. Poultry Sci. 74:1093–1101.
10. Edens, F. W., C. R. Parkhurst, I. A. Casas, and W. J. Dobrogosz. 1997. Principles of *ex ovo* competitive exclusion and *in ovo* administration of *Lactobacillus reuteri.* Poultry Sci. 76:179–196.
11. Froyman, R., C. L. Hofacre, B. Gautrais, and C. Day. 1998. Reduction of *Salmonella* in broilers using normal gut flora in successive grow-outs. 135th Annual Convention of the American Veterinary Medical Association, 25 to 29 July, Baltimore, Md.
12. Fuller, R. 1973. Ecological studies on the *Lactobacillus* flora associated with the crop epithelium of the fowl. J. Appl. Bacteriol. 36: 131–139.
13. Fuller, R. 1999. 2: Probiotics for farm animals, p. 15–23. *In* G. W. Tannock (ed.), Probiotics, a critical review. Biddles Ltd., Guildford and King's Lynn, UK.
14. Gast, R. K., and C. W. Beard. 1989. Age-related changes in the persistence and pathogenicity of *Salmonella typhimurium* in chicks. Poultry Sci. 68:1454–1460.

15. Gyles, N. R., H. Fallah-Moghddam, L. T. Patterson, J. K. Skeeles, C. E. Whitfill, and L. W. Johnson. 1986. Genetic aspects of antibody responses in chickens to different classes of antigens. Poultry Sci. 65:223–232.
16. Hinton, M., A. Kaura, and A. H. Linton. 1986. The ecology of drug resistance in enteric bacteria. J. Appl. Bacteriol. Symp. Suppl. 61: 77S–92S.
17. Hinton, M., G. C. Mead, and C. S. Impey. 1990. Protection of chicks against environmental challenge with *Salmonella* Enteritidis by ''competitive exclusion'' and acid-treated feed. Lett. Appl. Microbiol. 12:69–71.
18. Impey, C. S., G. C. Mead, and M. Hinton. 1987. Influence of continuous challenge via the feed on competitive exclusion of *Salmonellas* from broiler chicks. J. Appl. Bacteriol. 63:139–146.
19. Lindell, K. A., A. M. Saeed, and G. P. McCabe. 1994. Evaluation of resistance of four strains of commercial laying hens to experimental infection with *Salmonella enteritidis* phage type eight. Poultry Sci. 73:757–762.
20. Locascio de Ruiz Pesce, M. 1998. Bifidobacteria: ecología, fisiología y comportamiento inmunológico. Tesis doctoral. Universidad Nacional de Tucumán, Argentina.
21. Methner, U., P. A. Barrow, A. Berndt, and G. Steinbach. 1999. Combination of vaccination and competitive exclusion to prevent *Salmonella* colonization in chickens: experimental studies. Int. J. Food Microbiol. 49:35–42.
22. Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature 241:210–211.
23. Pivnick, H., and E. Nurmi. 1982. The Nurmi concept and its role in the control of salmonellae in poultry, p. 41–70. *In* R. Davies (ed.), Developments in food microbiology, 1st ed. Applied Science, London.
24. Raibaud, P., M. Caulet, J. Galpin, and G. Mocquot. 1961. Studies on the bacterial flora of the tract alimentary of pigs. II. Streptococci: selective enumeration and differentiation of the dominant groups. Appl. Bacteriol. 24:285–291.
25. Rolfe, R. D. 1991. Population dynamics of the intestinal tract, p. 59–75. *In* L. C. Blankenship (ed.), Colonization control of human bacterial enteropathogens in poultry. Academic Press, San Diego.
26. Sandoval, V. E., H. R. Terzolo, A. R. Moreira, G. L. Micheo, T. Eiguer, M. I. Caffer, and B. Fronchowsky. 1989. Paratifosis aviaria causada por *Salmonella* serovar. Enteritidis en Argentina. Rev. Arg. Prod. Anim. 9:295–308.
27. Schneitz, C., and M. Hakkinen. 1998. Comparison of two different types of competitive exclusion products. Lett. Appl. Microbiol. 26: 338–341.
28. Smith, H. W., and J. F. Tucker. 1980. The virulence of *Salmonella* strains for chickens: their excretion by infected chickens. J. Hyg. Camb. 4:479–488.
29. Snoeyenbos, G. H. 1991. Pullorum disease, p. 73–86. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder, Jr. (ed.), Diseases of poultry, 9th ed. Iowa State University Press, Ames.
30. Terzolo, H. R. 1999. Personal communication.
31. Vandenbergh, P. A. 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiol. Rev. 12:221–238.
32. Watkins, B. A., B. F. Miller, and D. H. Neil. 1982. In vivo inhibitory effects of *L. acidophilus* against the pathogenic *Escherichia coli* in gnotobiotics chicks. Poultry Sci. 61:1298–1308.