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Lactobacillus salivarius CTC2197 Prevents *Salmonella enteritidis* Colonization in Chickens

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A rifampin-resistant *Lactobacillus salivarius* strain, CTC2197, was assessed as a probiotic in poultry, by studying its ability to prevent *Salmonella enteritidis* C-114 colonization in chickens. When the probiotic strain was dosed by oral gavage together with *S. enteritidis* C-114 directly into the proventriculus in 1-day-old Leghorn chickens, the pathogen was completely removed from the birds after 21 days. The same results were obtained when the probiotic strain was also administered through the feed and the drinking water apart from direct inoculation into the proventriculus. The inclusion of *L. salivarius* CTC2197 in the first day chicken feed revealed that a concentration of 10^5 CFU g^{-1} was enough to ensure the colonization of the gastrointestinal tract of the birds after 1 week. However, between 21 and 28 days, *L. salivarius* CTC2197 was undetectable in the gastrointestinal tract of some birds, showing that more than one dose would be necessary to ensure its presence till the end of the rearing time. Freeze-drying and freezing with glycerol or skim milk as cryoprotective agents, appeared to be suitable methods to preserve the probiotic strain. The inclusion of the *L. salivarius* CTC2197 in a commercial feed mixture seemed to be a good way to supply it on the farm, although the strain showed sensitivity to the temperatures used during the feed mixture storage and in the chicken incubator rooms. Moreover, survival had been improved after several reinoculations in chicken feed mixture.

Salmonella enteritidis, *Salmonella typhimurium*, and *Salmonella heidelberg* have been implicated in approximately 50% of the foodborne salmonellosis outbreaks in the United States (31). Many outbreaks are caused by *S. enteritidis*-contaminated shell eggs, including eggs used in such traditional recipes as eggnog and Caesar salad (19, 38). A nationwide outbreak of *S. enteritidis* caused an estimated 250,000 illnesses in 1994 when ice cream premix was transported in tanker trailers that had been transporting nonpasteurized liquid eggs containing *S. enteritidis* (12).

The extensive uses of antibiotics in animal farms with the purpose of promoting growth rate and increasing feed conversion efficiency and for the prevention of intestinal infections have led to an imbalance of the beneficial intestinal flora and the appearance of resistant bacteria. The use of probiotics in order to competitively exclude the colonization of intestinal pathogens has been proposed for poultry, specially after the European Commission banned certain antibiotics frequently included in feeding stuffs as growth promoters (8).

Since Nurmi and Rantala (22) reported that pretreatment of chicks with microflora isolated from the alimentary tract of salmonella-free adult birds could protect them from infection by *Salmonella* spp., several products have been developed. Undefined products seem to be the most effective against *Salmonella* cecal colonization (4, 13, 34), although some authors have reported their ineffectiveness (36).

The use of undefined preparations could result in the transmission of any pathogen; thus, attempts have been made to develop defined bacterial mixtures for commercial utilization (18). The experimental results about the use of defined cultures are contradictory; some authors have found them to improve broiler live weight gain and feed conversion rate and markedly reduce mortality (14, 15, 20) as well as protect the birds against *Salmonella* (3, 6, 11, 21) and coliform colonization (14, 15, 26). However a number of studies have shown that probiotics have no positive effects on broilers, neither improving body weight (16, 41, 42) nor reducing *Salmonella* carriage (1, 36, 37).

The failure of the expected benefits of some probiotics can be attributed to the inability of the strains to colonize or survive in the gastrointestinal tract or their inability to antagonize or competitively exclude the pathogenic bacteria (15). In a previous study, *Lactobacillus salivarius* CTC2197, a rifampin-resistant (Rif^r) strain isolated from the crop of chicken was selected as a potential probiotic strain because of its high degree of adhesiveness to chicken intestinal epithelial cells, antagonistic activity against some pathogenic bacteria, and competitiveness in vivo (9).

The purpose of this study was to evaluate the use of *L. salivarius* CTC2197 for reducing the colonization of *S. enteritidis* C-114 in broilers.

MATERIALS AND METHODS

Bacterial strains. *L. salivarius* CTC2197, a previously selected rifampin-resistant strain (9), was grown in MRS broth (Difco Laboratories, Detroit, Mich.) and stored frozen (−80°C) in the same medium plus glycerol (20% [vol/vol]) or in skim milk (Difco) (10% [wt/vol]) plus glucose (7.5% [wt/vol]) as well as freeze-dried in skim milk (10% [wt/vol]) plus glucose (7.5% [wt/vol]). A Christ Alpha 1-4 freeze-dryer with an LDC-1M controller (Braun, Biotech, Osterode am Harz, Germany) was used for 24 h at 0.5 Pa and −50°C to freeze-dry the culture. The freeze-dried strain was stored at 4°C, and viability was checked periodically. Briefly, serial 10-fold dilutions from the stock cultures (frozen and freeze-dried) were made in saline solution and plated in Rogosa agar (Difco Laboratories, Detroit, Mich.) incubated anaerobically at 37°C for 2 days.

S. enteritidis C-114, a noninvasive strain isolated from chicken, was obtained from the salmonella collection of IRTA-Animal Health Laboratory (Barcelona, Spain). The strain, resistant to nalidixic acid (200 mg ml^{-1}) and mercuric chloride (12 mg ml^{-1}), was stored at −70°C in bacterial storage vials (Protect, Lancashire, United Kingdom).

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TABLE 1. Composition of the acidifiers included in a commercial feed mixture based on corn and soy

Sample	Composition of the acidifiers	pH	Dosage (kg/ton)
1	Control (no acidifiers included)	6.00	
2	46% Formic acid, 5% propionic acid, 23% ammonium formate	5.87	2.52
3	61% Formic acid, 29% propionic acid	5.86	2.60
4	68% Formic acid, 20% propionic acid	5.52	2.50
5	80% Lactic acid	5.97	1.30
6	53% Phosphoric acid, 1% citric acid, 1% fumaric acid	5.92	2.00
7	32% Phosphoric acid, 0.6% citric acid, 15.6% fumaric acid	5.91	2.00

Preparation of the bacterial strains for the feeding trial. *L. salivarius* CTC2197 was grown in MRS broth for 18 h under anaerobic conditions at 37°C and then centrifuged at 4,950 × *g* for 10 min and resuspended 1:10 in phosphate-buffered saline (PBS [pH 6.0]; NaCl, 136.89 mM; KH_2PO_4, 2.50 mM; K_2HPO_4, 6.95 mM). *S. enteritidis* C-114 was subcultured in tryptic soy agar (Difco Laboratories, Detroit, Mich.) supplemented with nalidixic acid and mercuric chloride for 18 h at 37°C. The cells were suspended in General Purpose Medium Plus (GPM+; BioMérieux S.A., Marcy-l'Etoile, France) to obtain an optical density of 0.15 at 450 nm, which corresponded to 10^8 CFU ml^{-1}. The suspension was diluted 1:100 in GPM+.

Survival of the probiotic strain in different commercial mixtures at selected temperatures. The survival of the probiotic strain *L. salivarius* CTC2197 in chicken feed was studied with a freeze-dried or a liquid culture in MRS broth. The freeze-dried culture (9.89 $\log_{10}$ CFU g^{-1}) was mixed with the commercial mixture to achieve the desired *L. salivarius* CTC2197 concentration (10^5, 10^6, and 10^8 CFU g of $feed^{-1}$). The liquid culture was centrifuged at 4,950 × *g* for 10 min, the pellet was resuspended in PBS in a volume 20 times lower than the original culture, diluted in saline solution to achieve the desired concentration, and mixed 1:20 with feed. The mixtures were stored at 30°C or room temperature for several days.

Periodically, 3 g of feed was mixed 1:10 with saline solution, vortexed for 1 min, serially diluted, and plated in Rogosa agar and in Rogosa agar with rifampin (100 μg ml^{-1}) (Rogosa-rif agar). The plates were incubated anaerobically at 37°C for 2 days. A freeze-dried culture of the probiotic strain was mixed with several samples of commercial feed containing different acidifier substances (Table 1) to achieve 10^8 CFU g^{-1}. They were kept at room temperature and sampled at 0, 3, and 12 days as described above.

Ten colonies recovered from a 3-day sample of acidified feed were randomly selected, separately grown in MRS broth and confirmed to be *L. salivarius* CTC2197 by plasmid profile analysis after lysis according to Anderson and McKay (2). Fresh MRS broth was inoculated 1% with an overnight culture of these recovered colonies. The resulting overnight culture was centrifuged at 4,950 × *g* for 10 min and the pellet resuspended in a 20 times lower volume of NaCl (0.85% [wt/vol]) and used to reinoculate the acidified feed (1:20). The process was repeated three times, and in each case, periodical counts in Rogosa agar and Rogosa-rif agar were made in order to study the survival of the reinoculated strains.

In vivo trials. In all trials performed, the birds were randomly allocated into Petersime cages with six levels with four compartments at each level, each treatment occupying one level. The birds of the different treatments were dosed orally by gavage with *S. enteritidis* C-114 and/or *L. salivarius* CTC2197, as indicated below, and had free access to water and food.

At each sampling time, several chickens from each group were killed by cervical dislocation. The content of one cecum per chicken was collected, homogenized in 1:10 PBS, and serially diluted before being plated in Rogosa agar and/or Rogosa-rif agar for counts of lactobacilli and rifampin-resistant lactobacilli. The plates were incubated anaerobically at 37°C for 2 days.

The dominance of the inoculated strain was ascertained by comparing the plasmid profiles of a certain number of rifampin-resistant colonies ($\alpha = 0.05\%$ and $\beta = 0.05\%$) to the plasmid profile of the parental strain *L. salivarius* CTC2197, according to a progressive colony sampling plan based on the accumulated binomial distribution (17). The isolation of *S. enteritidis* C-114 from the other cecum was carried out by impedimetric methodology (28, 29) in a Bactometer system (BioMérieux Vitek, Inc., Hazelwood, Mo.). The impedimetric module wells were subcultured onto Modified Brilliant Green Agar (Oxoid Ltd., Basingstoke, Hampshire, England) and XLT4 (Difco). Isolated colonies were randomly selected and confirmed by growth in tryptic soy agar (Difco) supplemented with nalidixic acid (200 mg ml^{-1}) (Sigma, St. Louis, Mo.) and mercuric chloride (12 mg ml^{-1}) (Sigma).

Trial 1. One hundred twenty-eight 1-day-old Leghorn chickens were divided into four groups of 32 birds each, corresponding to the following treatments: A, control; B, inoculation with *S. enteritidis* C-114 (10^6 CFU); C, inoculation with *L. salivarius* CTC2197 (10^8 CFU); D, inoculation with *L. salivarius* CTC2197 (10^8 CFU) and *S. enteritidis* C-114 (10^6 CFU). Both strains were delivered into the proventriculus by using a syringe fitted with a semirigid cannula of 12 cm in length by 3 mm in diameter. At each sampling time (14 and 21 days after oral gavage), four chickens from each group were killed and ceca were sampled as indicated before.

Trial 2. Ninety-six 1-day-old Leghorn chickens were divided into three groups of 32 birds each, corresponding to the following treatments: B′, inoculation with *S. enteritidis* C-114 (10^6 CFU); C′, inoculation with *L. salivarius* CTC2197 (10^8 CFU); D′, inoculation with *L. salivarius* CTC2197 (10^8 CFU) and *S. enteritidis* C-114 (10^6 CFU). The bacterial strains were inoculated directly into the proventriculus, as described before, and the probiotic strain *L. salivarius* CTC2197 was also administered, during the first day, by being mixed with the feed (10^8 CFU g^{-1}) and the drinking water (10^7 CFU ml^{-1}) by using a freeze-dried culture which contained 9.89 $\log_{10}$ CFU g^{-1}. At each sampling time (14 and 21 days after inoculation), six chickens from each group were killed, and ceca were sampled as indicated before.

Trial 3. One hundred twenty 1-day-old Leghorn chickens were divided into four groups of 30 birds each. A freeze-dried culture of *L. salivarius* CTC2197 (9.89 $\log_{10}$ CFU g^{-1}) was mixed with the feed mixture administered to the chickens during the first day of the trial at different concentrations (10^5 [treatment E], 10^7 [treatment F], and 10^8 [treatment G] CFU g^{-1}) and was used to feed the birds. The birds of treatment T corresponded to the control group, which was not fed with the probiotic strain. At each sampling time (7, 14, 21, and 28 days after inoculation), six chickens from each group were killed, and ceca were sampled as indicated before and plated in Rogosa agar and Rogosa-rif agar for lactobacillus and rifampin-resistant lactobacillus counts.

Statistical analysis. Differences ($P < 0.05$ or as indicated) in lactobacillus and rifampin-resistant lactobacillus counts between the first and the last sampling times were determined by Student's *t* test (Microsoft Excel 97; Microsoft Corp., Redmond, Wash.). The same test was used to compare mean values between treatments.

RESULTS

Freezing and freeze-drying are regarded as suitable methods to preserve bacterial strains for a long period of time. The viabilities of two cultures of *L. salivarius* CTC2197 during an extended period were similar in skim milk plus glucose and in MRS plus glycerol, with an average 1.52-$\log_{10}$ decline and 1.04-$\log_{10}$ decline, respectively, after 18 months of storage at −80°C. When four freeze-dried cultures of *L. salivarius* CTC2197 were stored for 12 months at 4°C, an average 1.9-$\log_{10}$ decline of viable cells per g of culture was observed.

The survival of the probiotic strain mixed at different concentrations in chicken feed stored at different temperatures was studied. Results at 30°C are shown in Table 2. After 1 week, the culture viability at room temperature was higher than at 30°C, showing similar counts when a liquid or freeze-dried culture was used (Table 3).

When the survival of the probiotic strain in feed mixtures with different acidifiers was tested, the losses of viability of *L. salivarius* CTC2197 were very similar in all samples. The highest reduction in CFU was observed during the first 3 days, with an average 3.34-$\log_{10}$ decline. During the next 8 days, the average drop was 0.74 $\log_{10}$, with a total 4.08-$\log_{10}$ decline after 12 days. When a freeze-dried culture of *L. salivarius* CTC2197 was stored at room temperature, the results were similar, and a 4.49-$\log_{10}$ decline was observed after 12 days. However when the freeze-dried culture of *L. salivarius* CTC2197 was stored at 4°C, the counts remained steady after

TABLE 2. Survival of *L. salivarius* CTC2197 added at different concentrations in chicken feed stored at 30°C

Time (h of storage)	Form culture added as	*L. salivarius* CTC2197 ($\log_{10}$ CFU g^{-1})[a]		
0	Liquid	5.11	6.60	8.58
	Freeze-dried	5.78	6.39	8.08
6	Liquid	4.47	5.42	7.98
	Freeze-dried	3.38	5.86	6.90
24	Liquid	2.00	2.30	5.83
	Freeze-dried	4.21	3.80	4.25
Decline after 24 h of storage	Liquid	3.11	4.30	2.75
	Freeze-dried	1.57	2.59	3.83

[a] Values are the average of duplicate determinations.

12 days: 9.89 $\log_{10}$ CFU g^{-1} at the beginning and 9.73 $\log_{10}$ CFU g^{-1} at the end.

Because no differences in *L. salivarius* CTC2197 counts were detected among the seven mixtures assayed, feed no. 7 was randomly selected in order to improve the viability of the probiotic strain in feed mixture. A 2.98-$\log_{10}$ decline was detected after 3 days at room temperature. After two consecutive reinoculations, an increase of the strain survival was achieved with a 2.46-$\log_{10}$ decline after 7 days at room temperature. Subsequent reinoculations did not achieve better results (data not shown).

In vivo trials. The capability of *L. salivarius* CTC2197 to minimize *S. enteritidis* C-114 colonization in poultry was assayed in two different trials.

The results of trial 1 are shown in Table 4. The chickens challenged with *S. enteritidis* C-114 (treatment B) presented a reduction ($P < 0.001$) in counts of lactobacilli at 21 days after gavage to a level significantly lower ($P < 0.05$) than those in control chickens. Counts of rifampin-resistant lactobacilli were lower than 10^2 CFU g^{-1} among the birds that did not receive the probiotic strain and significantly different ($P < 0.05$) from the birds inoculated with it. No differences were detected, either between two treatments (A and B) at each sampling time or at each treatment during the experiment. The plasmid profile analysis confirmed that all of the rifampin-resistant strains isolated from the chickens orally dosed with *L. salivarius* CTC2197 corresponded to the inoculated strain. The groups challenged with *S. enteritidis* C-114 (treatments B and D) presented 90 and 100% colonization rates, respectively, at 14 days, and 50% of the chickens that received the probiotic strain (treatment C) were also *Salmonella* positive at this sampling time. At the end of the rearing time (21 days), 100% of the chickens inoculated with the probiotic strain were protected from salmonella colonization, whereas 70% of the chickens not treated with *L. salivarius* CTC2197 were still *Salmonella* positive.

In trial 2 (Table 4), counts of total lactobacilli, at 14 days, were significantly higher in chickens which were not inoculated with *L. salivarius* CTC2197 (treatment B′) than in birds inoculated with the probiotic strain ($P < 0.05$). At 21 days, the significant differences between the group inoculated with the pathogen (treatment B′) and the one inoculated with both strains (treatment D′) disappeared. The lactobacillus levels of treatment groups that received the probiotic strain (treatment C′) ($P < 0.05$) and both strains (treatment D′) ($P < 0.01$) increased in relation to the counts at 14 days.

As in the first trial, the levels of rifampin-resistant lactobacilli were lower than 10^2 CFU g^{-1} among the birds that did not receive *L. salivarius* CTC2197 (treatment B′) and significantly different ($P < 0.05$) from the counts in inoculated birds. In these two groups, no differences were detected, either between the two treatments at each sampling time or at each treatment during the experiment. The plasmid profile analysis showed that the rifampin-resistant lactobacillus strains isolated from the birds that received *L. salivarius* CTC2197 (treatments C′ and D′) corresponded in all cases to the probiotic strain.

The groups challenged with the pathogen presented a 100% colonization rate at 14 days. At the end of the rearing time (21 days), all of the chickens inoculated at 1 day old with *S. enteritidis* C-114 (treatment B′) were colonized, whereas no salmonella-positive birds were detected among the birds that received the probiotic strain *L. salivarius* CTC2197 (treatments C′ and D′).

In order to study if the inclusion of *L. salivarius* CTC2197 in the chicken feed during the first day of life was enough to ensure the colonization of the gastrointestinal tract, the third trial was carried out. Results obtained are shown in Table 5. Counts of lactobacilli were significantly lower 28 days after gavage in all groups ($P < 0.05$), except among chickens that received 10^8 CFU of the probiotic strain g^{-1}, where they remained steady. At 14 and 28 days, no differences among lactobacillus counts of chickens of the different groups were observed. Counts of the rifampin-resistant lactobacilli were always lower than 10^2 CFU g^{-1} among the birds that did not receive the probiotic strain. *L. salivarius* CTC2197 was recovered after 7 days from the birds fed at 1 day old with the probiotic, independently of the dose used. The levels of rifampin-resistant lactobacilli were significantly lower at the end of the study in all treated groups ($P < 0.05$), except among chickens that received 10^8 CFU of the probiotic strain g^{-1}, where they remained steady. The birds sampled at 28 days presented low levels of rifampin-resistant lactobacilli with a high standard deviation because of the presence of several chickens from which *L. salivarius* CTC2197 was not recovered. At 28 days, there were significant differences ($P < 0.05$) in the levels of rifampin-resistant lactobacilli between the control group and the groups that received 10^7 and 10^8 CFU of the probiotic strain g^{-1}.

DISCUSSION

A great number of studies exist suggesting the desirable effects of probiotic lactobacilli on the health and performance of poultry. Most of these trials only measure growth stimulation, and a few report a microbiological monitoring focused on the effect of probiotics in the pathogen population. A few reports on probiotic lactobacilli colonization and changes in

TABLE 3. Survival of *L. salivarius* CTC2197 added as a liquid or as freeze-dried culture in chicken feed and stored at room temperature

Time (days of storage)	Survival of CTC2197 ($\log_{10}$ CFU/g^{-1}) in chicken feed[a]	
	Liquid	Freeze-dried
0	7.54	6.65
2	7.76	4.62
3	6.72	4.15
6	5.57	3.71
7	5.00	4.06
Decline after 7 days	2.54	2.59

[a] Values are the average of duplicate determinations.

TABLE 4. Least-squares mean counts of lactobacilli and Rif^r lactobacilli in ceca and percentage of chickens colonized with *Salmonella*

Treatment	Count of organisms in ceca ($\log_{10}$ CFU g^{-1})[a]						% *Salmonella*-colonized birds	
	Lactobacilli			Rif^r lactobacilli				
	Day 14	Day 21	*P*	Day 14	Day 21	*P*	Day 14	Day 21
Control (A [trial 1])	8.76 ± 0.28a	8.6 ± 0.12a	NS[b]	<2.00 ± 0.00b	<2.00 ± 0.00b	NS	0	0
S. enteritidis C-114 inoculation								
B (trial 1)	9.04 ± 0.12a	7.76 ± 0.24b	<0.001	2.21 ± 0.31b	<2.00 ± 0.00b	NS	90	70
B′ (trial 2)	8.54 ± 0.29a′	8.90 ± 0.31a′	NS	<2.00 ± 0.00b′	<2.00 ± 0.00b′	NS	100	100
L. salivarius CTC2197 inoculation								
C (trial 1)	ND[c]	ND		6.75 ± 0.72a	6.10 ± 0.71a	NS	50	0
C′ (trial 2)	6.00 ± 1.31b′	7.81 ± 0.32b′	<0.05	4.18 ± 1.23a′	5.64 ± 1.56a′	NS	0	0
S. enteritidis C-114 and *L. salivarius* CTC2197 inoculation								
D (trial 1)	ND	ND		6.29 ± 0.74a	6.15 ± 0.54a	NS	100	0
D′ (trial 2)	6.62 ± 0.51b′	8.36 ± 0.82a′b′	<0.01	5.51 ± 1.11a′	6.25 ± 1.02a′	NS	100	0

[a] Values are means ± standard deviations. The minimum level of detection was 2 $\log_{10}$ CFU g^{-1}. Means of results of different treatments within the same trial were compared. Values in a column with different letters (e.g., a and a′) differ significantly ($P < 0.05$). The significance of differences between day 14 and day 21 postinoculation (in a row) for each treatment is shown.
[b] NS, not significant.
[c] ND, not determined.

the *Lactobacillus* population in the digestive tract of poultry, with a short period of time being studied, have been published (15, 25, 26).

This study was carried out in order to assess the competitiveness of a preselected probiotic strain, *L. salivarius* CTC2197 (9), during the rearing of chickens and their ability to minimize *Salmonella* colonization, as well as to analyze its survival in chicken feed.

Two different methods have been assayed to preserve the probiotic strain for a long period of time in order to have a stock of the strain available. *L. salivarius* CTC2197 showed good viability at −80°C after 18 months with skim milk or glycerol as cryoprotective agents. No differences in the effectiveness of both systems were detected, whereas Coppola et al. (5) found that skim milk had a better cryoprotective capacity than glycerol. The freeze-dried strain showed lower survival after 1 year at 4°C than the strains that underwent freezing after 18 months. These results agree with those reported by To and Etzel (39) when they compared survival of three species of lactic acid bacteria before and after freezing and freeze-drying.

Stress resistance mechanisms seem to have been developed in *L. salivarius* CTC2197 after several reinoculations in acidified chicken feed at room temperature. The best survival of the probiotic strain was achieved after a second reinoculation, when the population dropped only 2.46 $\log_{10}$ after 7 days. These results offer the possibility of using the chicken feed as a way to administer the probiotic, because of the low dose necessary to achieve the gastrointestinal colonization according to the results obtained in trial 3. When the probiotic strain was assayed in vivo, promising results were obtained. Twenty-one days after a single administration directly into the proventriculus, none of the treated chickens were colonized with salmonella in either of the trials, whereas the birds that did not receive *L. salivarius* CTC2197 maintained the colonization level (70 and 100% in the first and second trials, respectively). The great capacity of *L. salivarius* CTC2197 cells to adhere to the epithelial cells and their in vitro proven antagonism toward *S. enteritidis* can explain the ability of *L. salivarius* to exclude the pathogen in vivo. A positive correlation has been found between the adherence of bacteria and their aggregation and coaggregation abilities (23, 40). Some authors consider that coaggregation between lactobacilli and pathogens is a good host defense mechanism (27, 32). From our results, administration of *L. salivarius* CTC2197 to 1-day-old chickens achieved positive results against *S. enteritidis* C-114, comparable to those obtained when mixtures of several strains were used (7, 10, 33, 35).

The resistance of *L. salivarius* CTC2197 to rifampin, together with the plasmid profile comparison, is a selectable property with which to differentiate administered lactobacilli

TABLE 5. Least-squares mean counts of lactobacilli and Rif^r lactobacilli among chickens of trial 3

Treatment (*L. salivarius* CTC2197 inoculation)[a]	Count of organisms ($\log_{10}$ CFU g of cecum^{-1})[b]									
	Lactobacilli					Rif^r lactobacilli				
	Day 7	Day 14	Day 21	Day 28	*P*	Day 7	Day 14	Day 21	Day 28	*P*
T (control)	8.30 ± 0.59a	8.54 ± 0.29a	8.90 ± 0.31a	6.69 ± 0.45a	<0.001	<2.00 ± 0.00b	<2.00 ± 0.00b	<2.00 ± 0.00c	<2.00 ± 0.00b	NS[c]
E (10^5 CFU g^{-1})	7.55 ± 0.86ab	7.95 ± 0.82a	6.78 ± 1.18b	6.12 ± 0.72a	<0.05	5.71 ± 1.16a	6.76 ± 0.89a	5.88 ± 1.19a	3.57 ± 1.58ab	<0.05
F (10^7 CFU g^{-1})	7.47 ± 0.50b	7.43 ± 1.53a	6.29 ± 1.16b	5.67 ± 1.00a	<0.01	5.85 ± 1.24a	6.49 ± 1.48a	5.66 ± 1.69ab	3.71 ± 1.55a	<0.05
G (10^8 CFU g^{-1})	6.75 ± 1.29b	8.19 ± 0.78a	6.33 ± 0.72b	5.77 ± 1.28a	NS	5.49 ± 1.22a	7.95 ± 1.05a	3.95 ± 1.00b	3.92 ± 1.50a	NS

[a] Treatment by inoculation of *L. salivarius* CTC2197 in feed mixture.
[b] Values represent the mean $\log_{10}$ CFU (± standard deviation) per gram of cecal material. The minimum level of detection was 2 $\log_{10}$ CFU g^{-1}. Means within the same column with different superscripts differ significantly ($P < 0.05$). The significance of difference between day 7 and day 28 postinoculation (in a row) is shown.
[c] NS, not significant.

from indigenous strains. Among the birds in trial 2, the counts of rifampin-resistant lactobacilli detected were lower than expected. When three different 21-day-postinoculation birds from treatment C′ were studied, the plasmid profile analyses showed that 43.8% of the total lactobacilli corresponded to the inoculated strain, whereas only 20% were recovered in Rogosa agar with rifampin. These results indicated that *L. salivarius* CTC2197 had lost its antibiotic resistance in vivo, although this property was stable when it was studied in vitro (9). The resistance to the antibiotic is appropriate for detecting the presence of *L. salivarius* CTC2197 in the gastrointestinal tract of chicken, but not to quantify it. The same property was used by Pedersen and Tannock (24), Salvat et al. (30), and Rada and Marounek (25), but none of them reported a loss of resistance to the antibiotic during their experiments.

Three different concentrations of *L. salivarius* CTC2197 added to the chicken feed mixture were checked in order to ascertain the minimum dose capable of colonizing the gastrointestinal tract of chicken. The inoculation of the first day feed with 10^5 CFU g^{-1} was enough to ensure the presence of the probiotic strain in the digestive tracts of the birds after 1 week. Higher doses did not achieve better results. Four weeks after inoculation, there was a drop in lactobacillus levels in both the control group and in the groups receiving 10^5 and 10^7 CFU g of $feed^{-1}$, whereas the levels were stable among the chickens fed with the highest dose. The same reduction was observed among rifampin-resistant lactobacillus counts, suggesting that between 21 and 28 days, the probiotic strain had been removed from the gastrointestinal tracts of some birds, and more than one dose would be necessary to ensure the presence of *L. salivarius* CTC2197 until the end of rearing. At 28 days, the presence of several birds with counts lower than 10^2 CFU g of $cecum^{-1}$ was responsible for the low mean values and the high standard deviations reported. This also explains why no significant differences were observed at this time between rifampin-resistant lactobacillus counts of the control group and the group dosed with 10^5 CFU g^{-1}. Rada et al. (26) found good colonization results when *L. salivarius* 51R was administered as freeze-dried cells in feed at 10^6 CFU g^{-1}. Protection of chicks against *Escherichia coli* (43) and coliforms (14, 15) was also reported when probiotics were administered in feed.

The great capability of *L. salivarius* CTC2197 to reduce *S. enteritidis* C-114 colonization in vivo, together with its ability to colonize the gastrointestinal tract of chicken after a single inclusion in the feed mixture, highlights it as a suitable strain for widespread use in the avian industry in order to minimize *Salmonella* colonization.

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