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Performance and Intestinal Coliform Counts in Weaned Piglets Fed a Probiotic Culture (*Lactobacillus casei* subsp. *casei* CECT 4043) or an Antibiotic

PAULA FAJARDO BERNÁRDEZ,[1] CLARA FUCIÑOS GONZÁLEZ,[1] JESÚS MÉNDEZ BATÁN,[2] LORENZO PASTRANA CASTRO,[1] AND NELSON PÉREZ GUERRA[1]*

[1]*Departamento de Química Analítica y Alimentaria, Facultad de Ciencias de Orense, Universidad de Vigo, Las Lagunas s/n, 32004 Orense, Spain; and* [2]*Cooperativas Orensanas Sociedad Cooperativa Ltda. Polígono San Ciprián de Viñas, 32901, Orense, Spain*

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ABSTRACT

The production of biomass and antibacterial extracellular products by *Lactobacillus casei* subsp. *casei* CECT 4043 was followed in both batch and in realkalized fed-batch cultures. Enhanced concentrations of biomass and antibacterial extracellular products were obtained with the use of the latter fermentation technique in comparison with the batch mode. The culture obtained by fed-batch fermentation was mixed with skim milk and used to prepare a probiotic feed for weaned piglets. To test the effect of the potentially probiotic culture of *L. casei* on body weight gain, feed intake, feed conversion efficiency, and on fecal coliform counts of piglets, two groups of animals received either feed supplemented with the probiotic preparation or avilamycin for 28 days. The control group was fed nonsupplemented feed. At the end of the administration period (day 28), the groups receiving probiotic and avilamycin exhibited the highest average body weight gain values, although the mean feed intake and feed conversion efficiency values were not different among the groups ($P > 0.05$). For the entire experimental period (42 days), the control group exhibited the lowest feed intake value, the probiotic group exhibited the highest feed conversion efficiency value, and the antibiotic group exhibited the highest body weight gain ($P < 0.05$). Interestingly, no significant difference in body weight gain was observed between the probiotic and the control groups by day 42 ($P > 0.05$). Fecal coliform values decreased (although not significantly) by day 28 in the three groups. However, the mean counts returned to pretreatment levels by day 42 in all groups.

Immediately after weaning, the indigenous microflora of piglets is still not completely established, resulting in the animals being susceptible to several enteric pathogens such as *Escherichia coli* strains, *Salmonella,* clostridia, *Yersinia,* and *Campylobacter (2, 14).* Growth-promoting antibiotics have been extensively used in poultry and piglet industries for prevention or treatment of diseases produced by these pathogenic microorganisms *(24).* However, several serious side effects are associated with extensive use of antibiotics, including allergic reactions, the presence of drug residues in edible animal products, and the development of antibiotic-resistant bacteria that are pathogenic to humans or animals *(1, 33, 41).*

In recent years, probiotics and organic acids have been proposed as an alternative to the use of antibiotics in animal feeds. Probiotics are viable microorganisms that, once ingested in sufficient numbers by humans and animals, produce beneficial physiological effects by improvement of the balance of intestinal flora and the overall health of the host. Although the mechanism by which probiotics work is not clear, their action is commonly related to the elimination of pathogens by stimulating the immune system, by colonizing the gut in large numbers (competitive exclusion), and/or by the production of antimicrobial substances *(14, 43).* The suggested properties of a probiotic strain include being (i) nonpathogenic, nontoxic, and noncarcinogenic; (ii) capable of surviving in the stressful environment of the stomach (acidic pH and bile); (iii) able to adhere to host epithelial tissue; (iv) amenable to industrial scale cultivation; (v) able to produce high amounts of antimicrobial substances antagonistic to pathogen growth; and (vi) beneficial to the host animal in some way *(22).*

Promising probiotic strains include a number of lactic acid bacteria, *Bacillus* strains, and yeasts such as *Saccharomyces boulardii (14, 18).* Lactic acid bacteria are known as generally recognized as safe bacteria, which can produce antimicrobial substances (i.e., organic acids, hydrogen peroxide, and bacteriocins) with activity not only against homologous strains, but also against gastric and intestinal pathogens *(26).* In addition to these characteristics, the beneficial effect of lactic acid bacteria in the digestive tract of animals has been attributed to their ability to adhere to host epithelial tissue, which is a very important prerequisite for gut colonization *(3).*

Some commercial probiotic preparations have been assayed for their efficacies in pigs, and beneficial effects were more pronounced when high doses of probiotic were used to supplement the feed *(2).* However, some major factors restricted the use of many of these preparations in animal feed. These include low numbers of viable cells (zero in

* Author for correspondence. Tel: +34-988-387-062; Fax: +34-988-387-001; E-mail: nelsonpg@uvigo.es.

some cases), contamination, or incorrect labeling of these products *(14).* Use of a highly concentrated and viable probiotic culture, like those produced by fed-batch fermentation *(5, 20, 32),* can be an appropriate alternative to solve this problem. Thus, the direct addition of the viable culture concentrate (containing both the cells and the antibacterial substances) to piglet feed could contribute to control growth of pathogenic bacteria not only in the feed, but also in the gastrointestinal tract of the piglets *(18).*

In a previous study *(18),* we investigated the effect of feeding diets containing a potential probiotic (*Lactobacillus casei* subsp. *casei* CECT 4043) or an antibiotic (colistin sulfate) on performance and intestinal coliform contents of weaned piglets during 42 days of administration. *L. casei* CECT 4043, a strain isolated from semi-hard goat cheese *(38),* fulfills many of the probiotic criteria, as it is nonpathogenic, produces high amounts of lactic acid *(19),* is bile and acid tolerant, and is able to survive during storage with skim milk at −20°C for 3 months and in piglet feed at room temperature for 8 days *(18).* In the above-mentioned experiment, the best results were obtained in the group fed colistin sulfate. However, a significant increase in body weight gain was obtained in the group fed *L. casei* CECT 4043 as compared with the nontreated (control) group. In addition, the total coliform counts in both the probiotic and the antibiotic groups significantly decreased ($P < 0.05$) for the whole administration period *(18).*

The objective of the present study was to investigate the performance and coliform content in weaned piglets fed a growth-promoting antibiotic (avilamycin) and a culture with a more concentrated amount of *L. casei* CECT 4043 than that used in the previous study *(18),* during both the administration (1 to 28 days) and the postadministration (28 to 42 days) periods. A fed-batch fermentation technique based on successive realkalizations of the culture medium was used to obtain the viable culture concentrate in deMan Rogosa Sharpe (MRS) broth.

MATERIALS AND METHODS

Bacterial strains. *Lactobacillus casei* subsp. *casei* CECT 4043, a high lactic acid–producing strain *(19),* and *Carnobacterium piscicola* CECT 4020 (the indicator organism in the antibacterial-activity assay) were obtained from the Spanish Type Culture Collection (Valencia, Spain). Stock cultures were maintained as frozen stocks at −40°C in nutrient broth (Cultimed, Panreac Química S.A., Barcelona, Spain) containing 15% (vol/vol) glycerol and were propagated twice in MRS broth (Cultimed) before use. Working cultures maintained at 4°C on MRS agar (Cultimed) were prepared monthly from stock cultures.

Inoculum preparation and culture conditions. Working cultures were propagated twice at 30°C for 12 h in MRS broth at an initial pH of 7.0, prior to use as the inoculum. All fermentations were inoculated with 2 ml of inoculum per liter of medium.

The batch culture of *L. casei* was carried out without pH control in 250-ml Erlenmeyer flasks containing 50 ml of MRS broth, on a rotary shaker (Innova 4330, New Brunswick Scientific Co., Inc., Edison, N.J.) at 30°C and 200 rpm for 24 h. The two realkalized fed-batch cultures were carried out without pH control, at a controlled temperature of 30°C in a 6-liter bench top fermenter (New Brunswick Scientific), with an agitation speed of 200 rpm and continuous recording of the pH.

The first realkalized fed-batch fermentation was initiated as a batch process, with a working volume of 4 liters of MRS medium. The initial pH was fixed at 7.0, and the aeration flow rate was maintained at 0.5 liter/h. The batch fermentation was converted into repeated realkalized fed-batch mode by rapidly withdrawing 100 ml of the fermentation medium from the fermenter, when the culture reached the mid-exponential phase (after 12 h), as was observed in the previous batch culture of *L. casei.* These samples (100 ml) were used to perform analytical determinations. After determining the total sugar concentration in the withdrawn sample, the amount of sugar metabolized by the lactic acid–producing strain was calculated. The same volume (100 ml) of feeding substrate was fed to the residual growing culture in the fermenter to restore the initial total sugar concentration (20 g/liter) in the fermentation medium. The feeding medium consisted of a mixture of a concentrated glucose (400 g/liter) and MRS medium (20 g of glucose per liter). The first feeding substrate was mainly used to supplement the growing culture with the carbon source (glucose). The use of this highly concentrated glucose solution avoids dilution of other nutrients in the fermenter. The other feeding substrate (MRS medium) was used to supplement the fermenter not only with glucose, but also with other nutrients (nitrogen, phosphorous, vitamins, minerals, etc.) in each feeding cycle. The feeding medium was added to the fermenter using a peristaltic pump (LKB, Pharmacia, Uppsala, Sweden). Immediately after feeding, the medium was realkalized to a pH of 7.0 with 4 M NaOH. These sampling, feeding, and realkalization strategies were repeated every 12 h until the producer strain was unable to bring about a decrease in pH.

In the second realkalized fed-batch culture, both the fermentation and the feeding culture media were identical to those used in the first realkalized fed-batch culture. This second fermentation was fed and realkalized each 12 h until 108 h of cultivation. At this time, 75% of the fermenter content was removed and replenished with the same volume of sterile MRS medium (20 g of glucose per liter). Subsequently, a sample (100 ml) of fermentation medium was taken directly from the fermenter to determine the total sugar concentration in the fermentation medium. The same volume (100 ml) of feeding substrate was introduced into the fermenter to bring the culture up to the initial total sugar concentration (20 g/liter). Then, the fermenter was repeatedly fed and realkalized each 12 h until the end of the experiment.

After stopping the second realkalized fed-batch fermentation in MRS broth, the culture was mixed with 30% (wt/vol) skim milk, which has a better cryoprotective capacity than glycerol does *(8).* Subsequently, the mixture was stored frozen at −20°C until further use. This preserved probiotic culture was used to prepare the probiotic feed.

Analytical methods. The concentrations of biomass, total phosphorous, nitrogen, protein, and sugars were determined by methods described in a previous work *(20).* The lactic acid concentration was measured by high-performance liquid chromatography (Gilson, Inc., Middleton, Wis.) using an Ion column (300 by 7.8 mm) with a precolumn IonGuard. The mobile phase consisted of 6 mM H_2SO_4 at a flow rate of 0.4 ml/min at 60 to 65°C, and the refractive index of the peaks was measured by a refractometer with a refractive index detector *(20).*

Antibacterial activity of extracellular products of *L. casei.* The activity of the antibacterial extracellular products (AECPs) produced by *L. casei* was estimated by using a photometric assay *(4).* Samples from *L. casei* cultures were acidified to pH 3.5 with

TABLE 1. *Percentage composition of the experimental diet for weaned pigs*

Composition	%	Calculated nutrient content	
Barley	30.3	Metabolizable energy (MJ/kg)	14.20
Wheat	10.0	Total protein (%)	18.50
Corn	16.5	Crude fat (%)	4.00
Animal fat	1.0	Ash (%)	5.30
Full-fat soybean, extruded	5.0	Ca (%)	0.80
Soybean meal 47	15.0	Available phosphorus (%)	0.46
Potato protein	3.0	Lysine (%)	1.21
Whey powder	16.0	Methionine (%)	0.41
Dicalcium phosphate, feed grade	1.0		
Calcium carbonate	0.8		
Vitamin/mineral premix[a]	1.4		

[a] Premix contains (per kilogram): vitamin A, 1.5×10^7 IU; vitamin D, 2×10^6 IU; vitamin E, 5×10^4 IU; vitamin K, 1.7 g; thiamine, 2.1 g; riboflavin, 6 g; pantothenic acid, 15 g; pyridoxine, 3 g; cyanocobalamin, 25.0 mg; nicotinic acid, 36 g; folic acid, 0.6 g; biotin, 0.2 g; Co, 0.15 g; Cu, 11 g; Fe, 90 g; Mn, 55 g; Y, 1 g; Se, 0.25 g; Zn, 110 g.

5 M HCl, then heated at 80°C for 3 min, and centrifuged at 27,200 $\times$ *g* for 15 min at 4°C. The culture supernatants (cell-free broth) containing the AECPs were serially diluted in distilled sterile water (this step eliminated the need to correct the pH of the AECP samples). Diluted AECPs (2.5 ml) were added in sterile culture tubes. Each tube was inoculated with 2.5 ml of a culture of *C. piscicola* CECT 4020 in buffered MRS broth (pH 6.3), which was diluted to an absorbance of 0.2 with sterile buffered MRS broth (pH 6.3) just before being used. Controls consisted of three culture tubes in which the diluted samples containing the AECPs were replaced by distilled sterile water. Once mixed thoroughly, the final pH values of all the samples were very similar (6.29 $\pm$ 0.01). The tubes were then incubated at 30°C with an agitation speed of 200 rpm for 6 h, after which the optical density of each tube was recorded at 700 nm. Dose-response curves were constructed from these data. One antibacterial activity unit (AU) per milliliter was calculated as the inverse of the dilution that produces a 50% growth inhibition, which was obtained from the dose-response curves *(28).*

Treatment of piglets, preparation of experimental diets, and analysis. A total of 120 21-day-old piglets were distributed into three groups of 40 (divided into five replicates of 8 piglets): the nontreated control group, the probiotic (*L. casei*)–supplemented fed group, and the antibiotic (avilamycin)–supplemented fed group. Each group was housed separately in individual cages. The composition of the basal diet used is shown in Table 1. Each experimental group was fed ad libitum with its own diet for 28 days. After this period, all the groups were fed with the basal diet until the 42nd day of the experiment. The temperature of the room with continuous lighting was maintained at 28°C initially, and then reduced 1°C each week until it reached 24°C, at which the room temperature was maintained for the rest of the experiment.

The probiotic piglet feed was prepared weekly by supplementing the feed with 20 ml of the probiotic culture per kilogram of feed in order to administer the same amounts of viable *L. casei* cells to the animals at the beginning of each week of treatment *(18).* With this procedure, the probiotic piglet feed contained 6 g of skim milk per kg of feed. For this reason, the piglets belonging to the control group received the basal diet supplemented with 0.6% (wt/wt) skim milk. In the group receiving the antibiotic, the basal diet containing 0.6% (wt/wt) skim milk was supplemented with 0.012 g of avilamycin per kg of feed. Body weight (BW) and feed intake (FI) were measured before the treatments and on days 14, 28, and 42; thereafter the animals received the experimental diets. Body weight gain (BWG), FI, and feed conversion efficiency (FCE) were then calculated on days 14, 28, and 42.

Coliform counts in fecal samples. Coliform counts were determined in the fecal samples, which were taken directly from the rectum of each animal using a sterile cotton swab (Eurotubo, Paracuellos De Jarama, Spain). Two replicates of fecal samples of each piglet were taken simultaneously from four piglets of each replicate (40 samples per treatment). The cotton swabs were weighed before and after taking the fecal samples to determine the net weights of the samples. To be consistent, the weight of each fecal sample was multiplied by 9 to determine the amount of sterile phosphate-buffered saline to be added to each tube to yield a 10^1 dilution. The cotton swabs were vigorously shaken, and then vortexed for 2 min. Ten-fold serial dilutions in sterile phosphate-buffered saline were performed up to 10^{10} and aliquots (0.1 ml) of 10-fold serial dilutions were pour plated with eosin methylene blue agar (Levine formulation). The plates were inverted and incubated at 37 $\pm$ 1°C for 1 to 2 days. Incubated plates were observed for the optimum number of CFU: between 30 and 300 colonies per plate. The results were expressed as the number of colonies counted per gram (wet weight) of feces *(42).* Fecal samples were obtained prior to (day 0), during (day 28), and 14 days after (day 42) experimental diet administration.

All media, supplements, and reagents were obtained from Cultimed.

Statistical analysis. The data concerning growth performance (BWG, FI, and FCE) were statistically analyzed using the software package SPSS 12.0 for Windows (release 12.0.1, SPSS, Inc., Chicago, Ill.). A one-way analysis of variance (ANOVA) with the step-down multiple-stage *F* post hoc test (Ryan-Einot-Gabriel-Welsch multiple *F* test [$P = 0.05$]) was used to distinguish treatment mean differences. Normal distribution for data as well as the independence and homogeneity of variances between treatment groups were previously verified by looking at the distribution of the data (via histograms) and the Fisher *F* test (which is included in the Student's *t* test output), respectively.

Viable counts of coliforms in the feces were transformed by logarithm (log) before statistical analysis of variance. The data were statistically analyzed by the STAT software (Statsoft, Inc., Tulsa, Okla.). Preliminary analyses for these data were done by the mixed model ANOVA methodology in the ANOVA/MANOVA procedure. Measurements on the same piglet at different days were treated as repeated observations, and unstructured covariance matrix for residuals was assumed. Estimated covariances between pairs of residuals on different sampling days were not significantly different from zero. Consequently, independent residuals were assumed, and the mixed model ANOVA methodology was used. The model for coliform counts data contained the treatment group, the day of sampling, and the interaction between the group and the day of sampling as fixed class effects.

RESULTS

Production of biomass and AECPs by *Lactobacillus casei* subsp. *casei* CECT 4043. The batch production of biomass and antibacterial substances by *L. casei* was carried

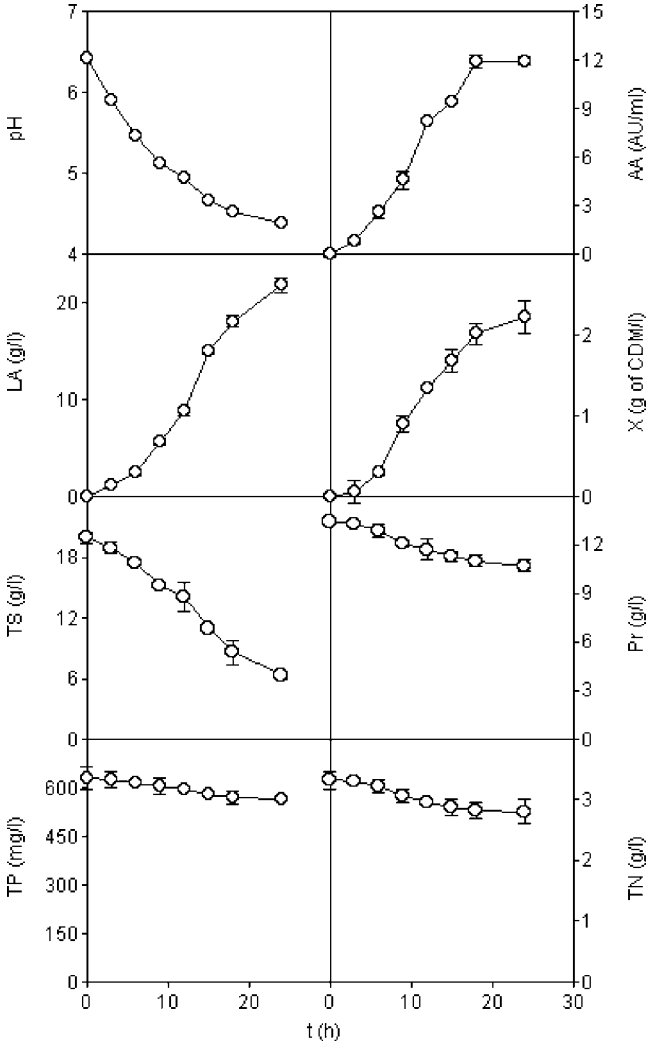

FIGURE 1. *Time course of the batch culture of* L. casei *subsp.* casei *CECT 4043 on MRS medium. X, biomass concentration; CDM, cell dry mass; AA, antibacterial activity concentration; LA, lactic acid; TS, total sugars; Pr, proteins; TP, total phosphorous; TN, total nitrogen;* t, *time. Data reported are means ± standard deviations.*

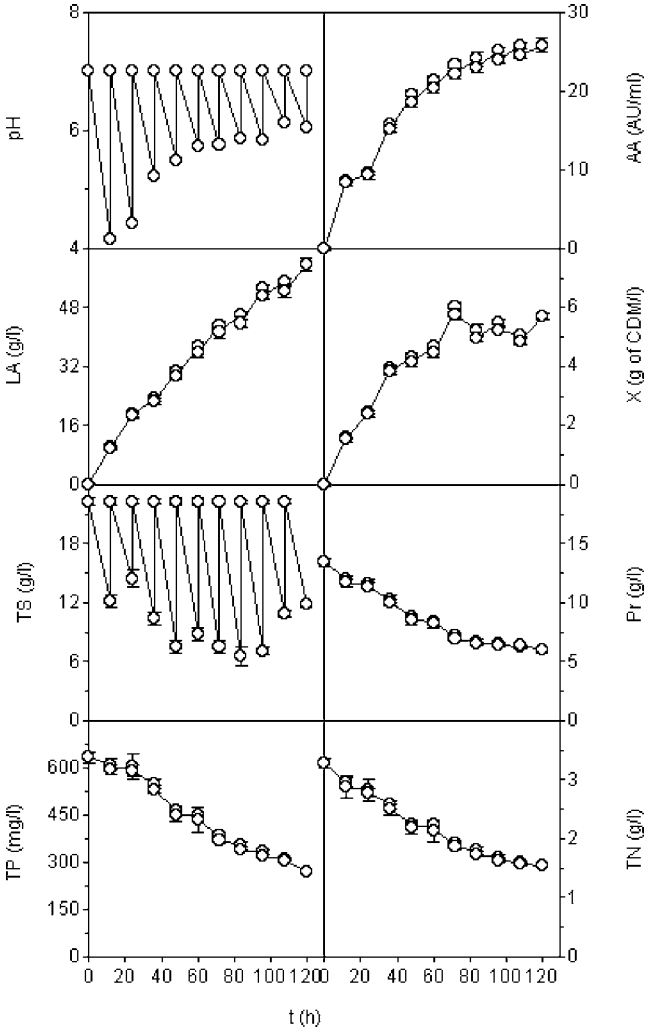

FIGURE 2. *Time course of the first realkalized fed-batch culture of* L. casei *subsp.* casei *CECT 4043 on MRS medium with feeding with MRS broth and 400-g/liter concentrated glucose (fed-batch fermentation 1). The feeding substrates were fed to the fermenter to restore the initial total sugar concentration (20 g/liter) in the fermentation medium at the beginning of each feeding cycle. X, biomass concentration; CDM, cell dry mass; AA, antibacterial activity concentration; LA, lactic acid; TS, total sugars; Pr, proteins; TP, total phosphorous; TN, total nitrogen;* t, *time. Data reported are means ± standard deviations.*

out in MRS broth. The results obtained (Fig. 1) showed that this strain is able to produce appreciable amounts of biomass (2.2 g/liter), lactic acid (21.9 g/liter), and antibacterial activity (12 AU/ml) in MRS broth. Analysis of the fermentation products showed a typical homolactic pattern, since lactic acid was the unique organic acid detected in the fermentation medium. The volumetric productivities at the end of the culture were calculated as 0.092 $g \cdot liter^{-1} \cdot h^{-1}$ (for biomass), 0.91 $g \cdot liter^{-1} \cdot h^{-1}$ (for lactic acid), and 0.5 $AU \cdot ml^{-1} \cdot h^{-1}$ (for antibacterial activity). However, the production rates of biomass and antibacterial activity began to decrease after 18 h of fermentation. For this reason, a fed-batch fermentation technique based on successive realkalizations of the culture medium was used to enhance both productions in MRS broth. In this new fermentation process, the growing culture was fed with a mixture of two sterile substrates composed of MRS broth and concentrated glucose (400 g/liter). The concentrations of biomass (5.7 g/liter), lactic acid (59.7 g/liter), and antibacterial activity (25.8 AU/ml) obtained in this culture (Fig. 2) were 2.57-, 2.73-, and 2.15-fold higher than those obtained in the previous batch fermentation (Fig. 1). *L. casei* showed again a typical homolactic fermentation pattern, as was observed in the previous batch culture. The volumetric productivities of biomass, lactic acid, and antibacterial activity in this first realkalized fed-batch culture were calculated at 0.047 $g \cdot liter^{-1} \cdot h^{-1}$, 0.50 $g \cdot liter^{-1} \cdot h^{-1}$, and 0.22 $AU \cdot ml^{-1} \cdot h^{-1}$, respectively. With regard to the nutrient consumption, it can be noted that the concentrations of protein, sugars, nitrogen, and total phosphorous decreased in parallel with the increase in biomass concentration. On the other hand, the lactic acid synthesis rate was constant (0.79 $g \cdot liter^{-1} \cdot h^{-1}$) during the first 24 h of incubation, but it decreased gradually afterward. Interestingly, the growth rate began to decrease after 72 h of fermentation, although the nutrients

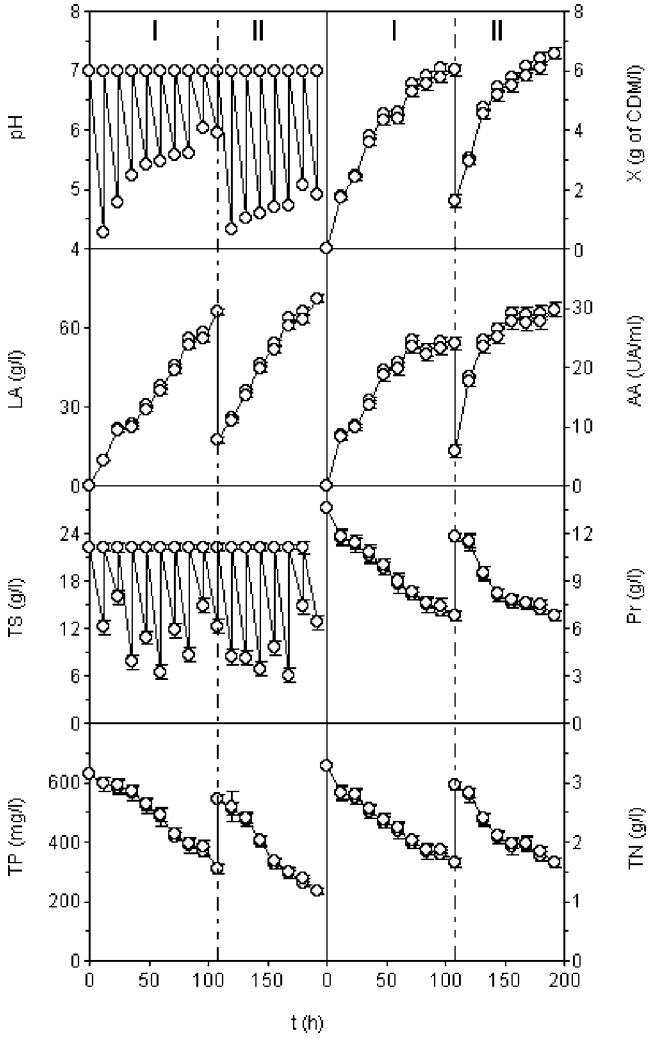

FIGURE 3. *Time course of the second realkalized fed-batch culture of* L. casei *subsp.* casei *CECT 4043 on MRS medium, with feeding with MRS broth and 400-g/liter concentrated glucose (fed-batch fermentation 2). The feeding substrates were fed to the fermenter to restore the initial total sugar concentration (20 g/liter) in the fermentation medium at the beginning of each feeding cycle. After 108 h, 75% of the fermented medium was replaced with sterile, fresh MRS medium. X, biomass concentration; CDM, cell dry mass; AA, antibacterial activity concentration; LA, lactic acid; TS, total sugars; Pr, proteins; TP, total phosphorous; TN, total nitrogen;* t, *time. Data reported are means ± standard deviations.*

(protein, sugars, nitrogen, and total phosphorous) were not completely consumed. Therefore, the decrease in growth rate could be related to the AECP accumulation in the culture medium and/or the exhaustion of some micronutrient (vitamins, ions, and/or amino acids) essential for growth of *L. casei.*

Taking into account these observations, an alternative fermentation strategy was developed for further extending the active period of the cells. This new culture was developed under identical conditions as those used in the first realkalized fed-batch fermentation, during the first 108 h of incubation. At this time, 75% of the fermented medium was replaced with sterile, fresh MRS medium, and the realkalized fed-batch cultivation was continued (Fig. 3). With this procedure, a typical homolactic fermentation pattern was observed again. Taking into account biomass production, there were two growth phases in the culture: a first growth phase of 108 h (from 0 to 108 h) was followed by a second growth phase of 84 h (from 108 to 192 h). The concentrations of biomass, lactic acid, and antibacterial activity in the second growth phase (6.6 g/liter, 71.7 g/liter, and 29.7 AU/ml, respectively) were slightly higher than those of the first growth phase (6 g of biomass per liter, 66 g of lactic acid per liter, and 24.0 AU/ml). The volumetric productivities of biomass, lactic acid, and antibacterial activity obtained in the second phase (0.061 $g \cdot liter^{-1} \cdot h^{-1}$, 0.66 $g \cdot liter^{-1} \cdot h^{-1}$, and 0.28 $AU \cdot ml^{-1} \cdot h^{-1}$) were also slightly higher than those obtained in the first phase (0.056 $g \cdot liter^{-1} \cdot h^{-1}$, 0.61 $g \cdot liter^{-1} \cdot h^{-1}$, and 0.22 $AU \cdot ml^{-1} \cdot h^{-1}$). From the detailed observation of this second fed-batch culture (Fig. 3), it can be noted that during the first 108 h of incubation, all the variables evolved in a similar way as in the previous fed-batch culture (Fig. 2).

On the other hand, the concentrations and volumetric productivities of biomass, lactic acid, and antibacterial activity obtained at the end of this second fed-batch culture were higher than those obtained in a previously reported realkalized fed-batch culture on whey *(18),* which was fed with a concentrated mussel-processing wastes medium (100 g of glucose per liter) and concentrated glucose (310 g/liter).

Growth performance of piglets fed the experimental diets. The effect of dietary probiotic or antibiotic (avilamycin) on growth performance parameters of weaned piglets was also determined (Table 2). Nine piglets died between days 28 and 42 (three in the control group, two in the group receiving the probiotic, and four in the group receiving the antibiotic) and were not replaced. The remaining animals were alive and healthy at the end of the experimental period.

The mean initial BW was 7,093 g for piglets treated with *L. casei,* 7,031 g for piglets treated with avilamycin, and 7,047 g for untreated control piglets. On day 14, average BWG in the probiotic group was significantly higher than in either the antibiotic or control group ($P < 0.05$). FI in the group fed the probiotic was higher than in the control group, but it was not significantly different from that of the group fed the antibiotic ($P > 0.05$). In addition, FCE did not differ among groups ($P > 0.05$). After 28 days of treatment, average BWG values in the groups receiving the probiotic and avilamycin were, respectively, 10.5 and 7% higher than that of the control group, although the mean FI and FCE values were not significantly different among the groups ($P > 0.05$).

At the end of the experiment (day 42), the antibiotic group exhibited the highest mean BWG value. However, no significant differences in BWG were observed between the group treated with *L. casei* and the control group ($P > 0.05$). Thus, a decline in BWG was noticed in the probiotic-treated group during the postadministration period (from 28 to 42 days) after probiotic feeding ended (Table 2). On the other hand, although the control group presented the lowest

TABLE 2. *Effect of dietary probiotic (*L. casei *CECT 4043) or antibiotic (avilamycin) administration on growth performance parameters (mean ± standard deviation) of piglets during the administration (days 1 to 28) and postadministration (days 28 to 42) periods*[a]

Criteria	Control	Probiotic	Antibiotic	*F*	df
Initial BW (g)	7,047 ± 31 A[b]	7,093 ± 36 B	7,031 ± 36 A	27.68	2, 120
Final BW (day 42) (g)	24,732 ± 275 A	25,004 ± 287 A	26,012 ± 156 B	3.81	2, 111[c]
BWG (g per piglet)					
1–14 days	2,828 ± 378 A	3,241 ± 776 B	2,828 ± 271 A	6.67	2, 120
14–28 days	6,190 ± 823 A	6,724 ± 588 B	6,821 ± 1,319 B	4.02	2, 120
1–28 days	9,018 ± 1,095 A	9,965 ± 1,284 B	9,649 ± 1,308 B	4.90	2, 120
28–42 days	8,876 ± 677 A	8,045 ± 1,518 B	9,477 ± 1,538 A	8.79	2, 111[c]
1–42 days	17,689 ± 1,507 A	17,918 ± 2,625 A	18,991 ± 754 B	4.14	2, 111[c]
FI (g per piglet)					
1–14 days	4,813 ± 745 A	5,281 ± 504 B	5,063 ± 314 AB	5.82	2, 120
14–28 days	13,536 ± 2,447 A	14,013 ± 1,481 A	13,909 ± 1,212 A	0.63	2, 120
1–28 days	18,348 ± 3,172 A	19,295 ± 1,948 A	18,972 ± 1,512 A	1.38	2, 120
28–42 days	12,520 ± 681 A	13,120 ± 1,834 A	14,549 ± 1,525 B	14.95	2, 111[c]
1–42 days	30,403 ± 3,566 A	32,250 ± 3,705 B	33,180 ± 2,771 B	5.00	2, 111[c]
FCE (g FI/g BWG)					
1–14 days	1.702 ± 0.164 A	1.692 ± 0.279 A	1.798 ± 0.098 A	2.87	2, 120
14–28 days	2.178 ± 0.192 A	2.087 ± 0.153 A	2.121 ± 0.475 A	0.71	2, 120
1–28 days	2.024 ± 0.151 A	1.9465 ± 0.115 A	2.000 ± 0.304 A	1.19	2, 120
28–42 days	1.414 ± 0.064 A	1.653 ± 0.175 B	1.563 ± 0.232 B	14.60	2, 111[c]
1–42 days	1.715 ± 0.064 A	1.809 ± 0.089 B	1.744 ± 0.084 A	10.89	2, 111[c]

[a] The piglets from the control group were not given probiotics or antibiotics. The piglets in the probiotic and antibiotic groups were given *L. casei* subsp. *casei* CECT 4043 and avilamycin, respectively. BW, body weight; BWG, body weight gain; FI, feed intake; FCE, feed conversion efficiency.

[b] Mean values within each row followed by the same letter are not significantly different by the Ryan-Einot-Gabriel-Welsch multiple *F* test ($P = 0.05$) after a significant ANOVA ($P < 0.01$).

[c] Nine piglets died between days 28 and 42 (three in the control group, two in the group receiving the probiotic, and four in the group receiving the antibiotic).

FI value, the probiotic group exhibited the highest FCE value for the whole experimental period ($P < 0.05$).

Effect of avilamycin and *L. casei* CECT 4043 dietary supplementation on the intestinal microbiota of weaned piglets. To assess any major effects of antibiotic and probiotic administration on intestinal microbiota, samples from the rectum of the pigs were taken with sterile cotton swabs and analyzed. Coliform counts were highly variable throughout the assay, with large variance of values within individual treatment groups and variations observed in counts at different time points (Fig. 4). Consequently, no significant decrease in fecal coliform counts ($P > 0.05$) was observed in the three groups when mean preadministration counts (day 0) were compared with those obtained at the end of the administration period (day 28). For example, in pigs fed *L. casei* or the antibiotic, mean coliform counts decreased (although not significantly) from 6.0×10^8 to 1.6×10^7 CFU/g and from 3.2×10^8 to 3.1×10^6 CFU/g, respectively. This represents mean reductions of 97 and 99%, respectively, when the mean preadministration counts within each treatment groups are taken as 100%. However, mean coliform counts also decreased by 96% (from 5.7×10^8 to 2.0×10^7 CFU/g) in the control group.

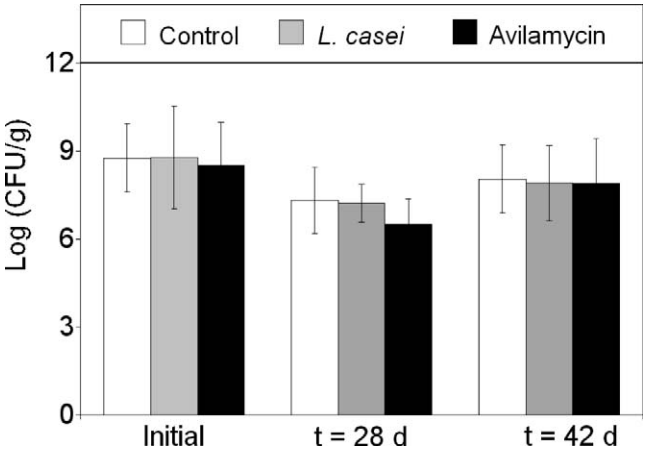

FIGURE 4. *Viable plate counts of coliforms in the feces of piglets in the nontreated control group, the probiotic (*L. casei*)–treated group, and the antibiotic (avilamycin)–treated group. Data reported are means ± standard deviations.*

On the other hand, no significant increases were observed among the three groups when postadministration (day 42) coliform counts were compared with those obtained at the end of the administration period (day 28). Thus, the counts increased from 1.6×10^7 to 8.0×10^7 CFU/g in the probiotic group, from 3.1×10^6 to 7.6×10^7 CFU/g in the antibiotic group, and from 2.0×10^7 to 1.1×10^8 CFU/g in the control group.

Finally, when all treatment groups were analyzed together (Fig. 4), no significant differences were observed

when mean preadministration counts were compared with those obtained at the end of the experiment (day 42). In the same way, no significant differences in coliform counts were observed between any of the treatment groups in the present study.

DISCUSSION

To produce the large concentrations of biomass and antibacterial substances needed for studies of their probiotic effects in weaned piglets, it is necessary to use an adequate culture medium and an efficient fermentation technique. Therefore, as an initial step to the fed-batch production of biomass and AECPs by *L. casei,* a batch culture with this strain was carried out in MRS broth. This approach allowed obtaining information on its growth kinetic in this culture medium. Interestingly, antibacterial activity was not produced in parallel with the production of lactic acid, which was the unique organic acid detected in the supernatant fluids of the culture. This observation indicates that lactic acid is far from being either the only or the major antibacterial product synthesized by *L. casei.* This fact supports the hypothesis that *L. casei* CECT 4043 is a bacteriocin-producing strain, as was reported before *(46).*

From the detailed observation of the culture, it can be noted that the rates of production of biomass and antibacterial activity began to decrease after 18 h of incubation, before sugar exhaustion. This decrease was concomitant with the observed decrease in nutrient (sugars, nitrogen, total phosphorous, and protein) consumption rates, when the culture reached a pH value below 4.5. A similar trend was observed before for *L. delbrueckii* subsp. *bulgaricus* in synthetic media *(17, 27, 36).*

Taking into account that the MRS broth is provided with high amounts of minerals, vitamins, and/or amino acids through the use of yeast and meat extracts *(45),* the exhaustion of some of these micronutrients did not seem to be a cause for the growth arrest. Thus, the accumulation of high amounts of toxic compounds (lactic acid and other growth-inhibiting factors other than lactic acid) in the medium, as well as the pH value below 4.5 reached in the culture after 18 h of incubation, could be acceptable causes for explaining the decrease in the growth rate *(19, 44).*

Regarding the accumulation of toxic compounds in the culture medium, the antimicrobial effect of lactic acid at low pH values, which produces the acidification of cytoplasm, destruction of the transmembrane proton motive force, and a loss of active transport of nutrients through the membrane, is well known. This action produces a destabilization of different functional and structural components of the cells, which interferes with viability and growth of the producing bacterium *(12, 16).* In contrast, the accumulation of bacteriocin in the medium did not seem to be a cause for the cessation of growth of *L. casei,* because the bacteriocin-producing strains have genes that encode the synthesis of immunity proteins, which protect the bacteriocin producers from their own bacteriocins *(10, 21, 23).*

Concerning the pH of the culture, it has been reported that this variable has an important influence on amino acid or peptide transport, which constitutes one of the growth rate–determining steps *(37).* For example, for *Lactococcus lactis* and *Lactococcus cremoris* strains, the optimum pH value for amino acid transport varied between 6.0 and 6.5, decreasing rapidly at higher and lower pH values *(37).* Taking into account these observations, a possible alternative to prevent the inhibitory effect of low pH on the growth of *L. casei* could be the development of a batch fermentation at a controlled constant pH of 6.0 or 6.5. However, this procedure could limit the production of bacteriocins, since small drops in pH commonly inhibit the bacteriocin synthesis *(19, 20, 47).* For these reasons, a fed-batch fermentation method based in successive realkalizations of the culture medium *(20)* was used for further enhancing biomass and AECP production by *L. casei.*

In the first fed-batch culture (Fig. 2), *L. casei* developed typically homolactic fermentation because the supernatant fluids of cultures of *L. casei* did not contain growth-inhibiting factors (e.g., acetic acid, ethanol) other than lactic acid. On the contrary, a shift from homolactic to heterolactic fermentation (with the joint production of lactic and acetic acids) was observed before in a realkalized fed-batch culture of *L. casei* in a whey-based medium *(18).* The different metabolism patterns observed in both cultures could be related to the different carbon sources present in each fermentation medium (glucose in the case of MRS broth and lactose in the case of whey medium). Thus, when lactic acid bacteria grow on rapidly metabolized sugars (like glucose), more than 90% of the metabolized sugar is converted to lactic acid, and only low quantities of other antimicrobial products (e.g., formic acid, acetic acid, and ethanol) accumulate in the medium (homolactic metabolism). Sugars metabolized at diminished rates (galactose or lactose) facilitate the shift toward the more energetically favorable heterolactic fermentation with an additional gain in ATP linked to the acetate kinase reaction *(11, 15).*

The observed decrease in biomass production rate on prolonged incubation in the first fed-batch culture of *L. casei* (Fig. 2) likely can be attributed to the accumulation of lactic acid in the culture medium, as discussed above. For this reason, a second realkalized fed-batch fermentation was carried out, in which 75% of the fermented medium was replaced with the same volume of sterile fresh MRS broth just after 108 h of fermentation. This procedure offered the possibility of supplementing the growing culture with higher amounts of micronutrients and reducing the concentrations of AECPs by 75%. From a practical point of view, this alternative proved to be an adequate method to increase the active period of growth for a period of time longer than that of the first realkalized fed-batch culture (Figs. 2 and 3). This favored the continued production of biomass and AECPs without the need to discharge the fermenter. Consequently, the nonproductive downtime of cleaning, filling, and sterilizing the fermenter between each batch was eliminated, thereby increasing the volumetric productivities of biomass and antibacterial substances.

The probiotic feed prepared with the second realkalized fed-batch culture of *L. casei* in MRS broth was used to study the effect of probiotic supplementation on performance and fecal coliform counts of weaned piglets. The

high number of viable cells obtained at the end of this second fed-batch culture (6.24×10^{10} CFU/ml) allowed the preparation of a probiotic feed with a viable cell concentration (1.25×10^9 CFU/g of feed) higher than that of the previous probiotic feed (2.52×10^8 CFU/g of feed) prepared with the *L. casei* culture obtained in whey-based media *(18).*

According to the results of the present study, the beneficial effect that the antibiotic produced on BWG during the administration period has also been extended to the postadministration period. On the other hand, the administration of *L. casei* cells during 28 days appeared to be an effective way for promoting the BWG of the treated animals. A similar effect was found when weaned piglets were fed a diet supplemented with the same probiotic strain throughout a 42-day administration *(18).* However, this positive effect disappeared in the postadministration period (from 28 to 42 days of treatment), when the piglets were fed the experimental diet without additives (Table 1). These results agree with those reported by other researchers *(2),* who observed that BWG, FCE, and carcass quality were significantly higher in pigs treated with a probiotic preparation (BioPlus 2B) during the weaning and growing stages and part of the finishing stage compared with those piglets receiving the same probiotic diet only during the weaning stage.

These differences could probably be related to an inability of the probiotic strains to colonize and persist in the gastrointestinal tract in the postadministration period. The probiotic cells could be progressively supplanted by the bacteria of the intestinal microflora once probiotic administration stopped. This leads to a decline in the numbers of probiotic bacteria in the piglet digestive tract, as was observed before for other lactobacilli strains *(14, 34, 35, 39).* In fact, some of these bacteria were capable of persisting in the porcine gastrointestinal tract for a period of between 3 and 10 days postadministration *(14, 34, 35, 39).*

Other studies showed that the administration of lactic acid bacteria reduced the intestinal coliform counts in the feces of piglets *(6, 18, 29, 30, 31, 42),* probably through competitive exclusion *(13)* and/or the production of inhibitory compounds such as organic acids, hydrogen peroxide, and bacteriocins *(18).* However, other studies have shown no effects *(9, 13).* From the results obtained in this work, it is difficult to draw clear conclusions on the impacts of probiotic administration on intestinal coliform counts, not only due to the variations observed in counts between individual animals and at different sampling time, but also by the fact that the reduction in coliform counts in both the antibiotic and the probiotic groups was also observed in the control group. On the other hand, since the growth-stimulating effects of probiotic bacteria have been observed when the piglets were exposed to stress *(1, 3, 40),* a more pronounced positive effect of the diets supplemented with *L. casei* should be expected in presence of health problems.

In conclusion, administration of the potential probiotic preparation at the dose of 20 ml/kg of feed, which is equal to 1.25×10^9 CFU of *L. casei* per g of feed, can improve the performance parameters of the piglets during the administration period. This offers the possibility of using the piglet feed as a way to administer the probiotic bacterium at levels higher than that of the recommended dose of viable probiotic (10^6 CFU of probiotic per g or ml) necessary to observe beneficial effects *(7, 25).* However, the period of administration of *L. casei* cells should be extended to the growing and the finishing stages in order to improve the performance parameters of the piglets. Finally, as probiotics are generally considered to be harmless, the findings of this study further support the fact that the use of antibiotics can be reduced without compromising the health and well-being of animals and without impacting production. This could be a way of minimizing risks for public health, such as the development of antibiotic-resistant bacteria that are pathogenic to humans or animals, as well as the presence of antibiotic residues in edible animal products.

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