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APPLICATION OF PROBIOSIS TO POULTRY

S. H. Khan and F. A. Ansari

Department of Microbiology, University of Karachi, Karachi 75270, Pakistan

ABSTRACT

Probiotics for chickens are designed either to replace beneficial organisms that are not present in the alimentary tract or to provide the chicken with the effects of beneficial bacteria. *Salmonella typhi* colonizes the intestinal tract of poultry and causes food-borne illness in humans. Reduction of *S. typhi* colonization in the intestinal tract of poultry reduces potential carcass contamination during slaughter. The purpose of this study was to isolate, identify and determine the effect of avian-specific *Lactobacillus acidophilus* as probiotic, on the colonization, weight gain and disease resistance in chicken. Two groups, each containing five chicks, were designated as "probiotic" and "control" group. At placement, probiotic group chicks were orally administered with *L. acidophilus* for 2 weeks. As a result, an increased weight gain of this group was observed comparable to the control group. Competitive exclusion of intestinal microflora and resistance to *Samonella typhi* in *L. acidophilus* fed probiotic group was also observed. Antibiotic resistance pattern of the crop isolated probiotic and other Lactobacillus strain was determined. Production of antimicrobial substance by *L. acidophilus* was tested against Gram-positive and Gram-negative bacteria. Antibacterial activity was found against *S. aureus* and *M. luteus*. These findings indicate that probiosis can be applied as an alternative to antibiotic use and, for improving the production efficiency in the poultry industry.

Key-words: Probiosis, Poultry, Lactobacilli, *Salmonella typhi*

INTRODUCTION

The microbial flora of the alimentary tract can have a considerable effect on the health and performance of poultry. Disturbance of the flora could thus lead to detrimental effects by allowing colonization of pathogens or by the growth depressing bacteria. Probiotics are viable bacterial cultures or components of bacterial cells that have beneficial effects on the health of the host (Fuller, 1989; Salminen *et al.*, 1998). Probiotics have been used as alternative tools for helping newly hatched chicks colonize normal microflora as conventionally hatched chicks do. Due to their several negative effects, antibiotics have gradually been replaced by probiotics in controlling intestinal pathogenic bacteria (Fuller, 1992). 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 (Martin *et al*., 2000).

The concept of using *Lactobacillus* species for disease treatment and prevention as well as health restoration and maintenance is not new. There exists a huge literature on the use of various *Lactobacillus* strains as probiotic agents. In general terms, a group of requirements have been identified as important properties for lactobacilli to be effective probiotic organisms (Reid, 1999). These include the ability to (i) adhere to cells; (ii) exclude or reduce pathogenic adherence; (iii) persist and multiply; (iv) produce acids, H_2O_2, and bacteriocins antagonistic to pathogen growth; (v) be save and therefore noninvasive, non carcinogenic, and non pathogenic; and (vi) co aggregate and form a normal, balanced flora.

Lactobacillus acidophilus (type strain ATCC 4356), Gram positive, non-sporing, microaerophilic, previously known as *Bacillus acidophilus* is one of the most thoroughly studied probiotics (Salminen *et al*., 1998). Most studies on the efficacy of probiotics in poultry production have been done with *L. acidophilus* and have been reviewed extensively (Barrow, 1992; Starvic and Kornegay, 1995; Jin *et al*., 1997). The principle is that ingestion of large numbers of the lactobacilli may result in replacement of undesirable intestinal organisms by harmless or beneficial organisms. Yeo and Kim (1997) used *L. casei* in broiler diets and found out that average daily gain during 0 to 3 week of age was significantly improved. Tortueuro (1973) reported that weight gain and feed conversion rate were greater in groups treated with *L. acidophilus* in drinking water than in control. Miles et al. (1981) conducted an interesting experiment at three geographical locations to study the effect of a living *Lactobacillus acidophilus* culture on egg quality. Feeding the *Lactobacillus* resulted in significantly increased egg production at one location (Arizona), a numerical improvement at the second (Florida), and no difference at the third location (South Dakota). It is difficult to understand the rationale of using non-avian strains (Barrow, 1992). Morishita *et al.* (1971) found that whereas avian strains of *L. acidophilus* colonized well, a human *L. acidophilus* strain was rapidly eliminated from the alimentary tract.

In the present report, we describe the results of oral administration of crop isolate *L. acidophilus* on Misri chicks. 2 groups (Probiotic and control) of chicks of either sex (5 per goup) at 4 weeks of age were used. Probiotic group was fed with *L. acidophilus* (10^9/day). Feeding was done by hand and continued for 14 days. Chicks of both

groups were weighed before and after 2 weeks of oral probiotic administration. Antibiotic resistance pattern and production of antimicrobial substance by *L. acidophilus* was tested against Gram positive and Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strain and growth conditions:

Lactobacillus acidophilus was isolated from milk as well as from crop of healthy farm chicken. Isolation and identification was accomplished using simple methods usually carried out for the bacteriological examination of food, milk and water samples (Cullimore, 1999). *Lactobacillus acidophilus* isolated from crop was adopted as strain of choice due its host origin. It was stored in De Man-Rogosa-Sharpe (MRS) agar (BioM laboratories, Cerritos, USA) at 4^{o} C and subcultured twice in MRS broth (BioM) for 24 h at 37^{o} C prior to experimental use.
Salmonella typhi suspension, enumeration and inoculation were performed by the same procedure described above. However culture media used were Tryptone Soy Agar (TSA) and Tryptone Soy Broth (TSB) instead of MRS agar and MRS broth respectively.

Experimental design:

Twenty eight-day-old chicks were divided in two groups. One group (Probiotic) was fed with *L. acidophilus* suspension at a concentration of 10^{9} CFU/ml (total number, 5), and the other was fed only sterilized PBS. Feeding was carried out once a day for 14 days. The normal diet was given to the chicks of both groups. 42 days old chicks of both groups were weighed in order to count their weight increase during that period. 45 days old chicks (3 days after stopping oral administration of probiotic suspension), both the groups of chicks were challenged with high doses (10^{8} CFU/chick) of *S. typhi*. Chicks were observed after 24 hours for observable and comparable signs & symptoms of *S. typhi* infection, between control and probiotic group.
On days 0, 7 and 14 of oral probiotic administration and 14 days after oral administration had been stopped, CFU of the faecal coliforms and *Lactobacilli* of both groups was determined using MacConkeys agar (Merck) and MRS agar (BioM).

Antimicrobial Agent Production:

Production of antimicrobial agent by *L. acidophilus* was tested against *Pseudomonas aeruginosa*, *S. typhi*, *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus leuteus* and *L. acidophilus* itself, by well diffusion assay. *Lactobacillus acidophilus* culture supernatants free of cells, used in the experiments, were derived from fresh overnight (o/n) *L. acidophilus* in MRS broth centrifuged twice at 2500 RPM for 20 min., supernatant was decanted and stored. Fresh o/n *L. acidophilus* culture grown in MRS broth prior to centrifugation was also used. *Lactobacillus acidophilus* culture supernatants and fresh culture were used at their native pH (3.0 – 4.0), and were adjusted to pH 7.0 with NaOH. Sterile MRS broth control at its original pH was used. Well diffusion assay was carried out using 50 µl aliquots of *L. acidophilus* supernatants and o/n *L. acidophilus* cultures at their native pH and pH adjusted against the bacteria mentioned earlier. Plates were incubated at 37^{o} C for 24 to 48 h under normal aerobic conditions, and the diameters of inhibition zones around the wells were measured.

Antibiotic Susceptibility Test:

Antimicrobial susceptibility test disks were plated on agar plates inoculated with the bacterium. After 16 – 18 hours, the plates were examined and the diameters of the clear zones around the disc indicating no bacterial growth were measured. The diameters of the zones for the individual antibiotic were translated into susceptible, intermediate, or resistant categories by referring to the standardized values (Koneman et al., 2001). Thirteen antibiotics including Ampiclox (25 µg), Amoxil (25 µg), Chloramphenicol (10 µg), Erythromycin (15 µg), Gentamicin (10 µg), Orbenin (5 µg), Ofloxacin (10 µg), Penicillin G (10 units), Piperacillin (100 µg), Streptomycin (25 µg), Tetracycline (10 µg), Tobramycin (10 µg), and Trimethoprim (5mcg) were used.

RESULTS

Effect on weight gain:

All the chicks of both the groups were weighed before (at 28 days of age) and after (at 42 days of age) 14 days oral administration of probiotic suspension. Average weight of all the chicks was determined. Initial weight of all

the chicks was nearly the same (Approx. 250 g). The results showed that after 14-day probiotic administration, a considerable weight gain was observed in the probiotic group i.e. 78.6 g or 31 % more weight gain than chicks of the control group (Table 1).

Table 1. Effect of *Lactobacillus acidophilus* on body weight gain of chicken.

	Body weight[1] (g)		
Treatment Group	**Initial weight**	**Final weight**	**Gain**
Control	250	309	59
Probiotic	250	387.6	137.6

[1]n = 5 (Average weight)

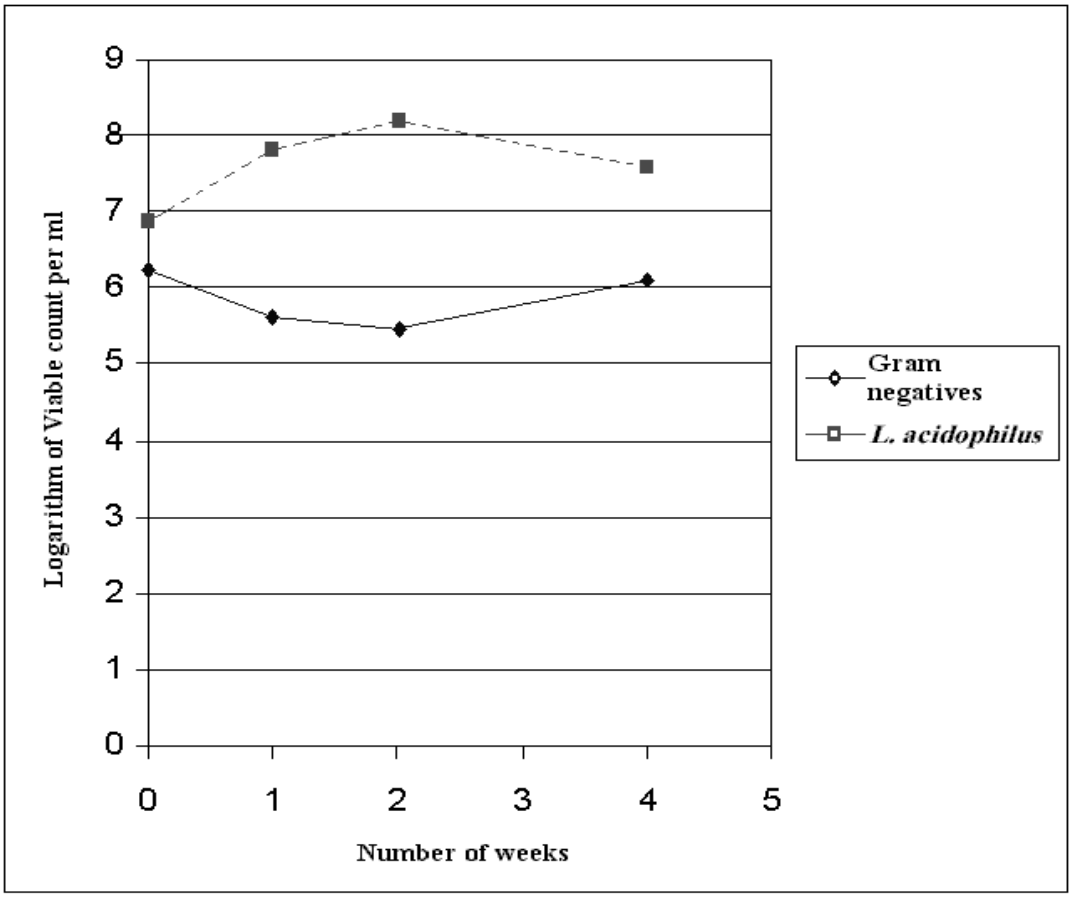

Fig. 1. Effect of subsequent doses of L. acidophilus on Gram negatives and Lactobacilli shedding in Faeces.

Microbial Examination of Faeces:

On MacConkey's Agar, majority or nearly all of the colonies were *lac*$^+$, Gram negative and scattered rods. For convenience, these were considered as *E. coli.*

On MRS agar, the colonies were very minute in size, Gram positive. Majority resembled Lactobacilli upon microbial examination; however, some Gram-positive cocci were also found. Fig. **1** clearly shows a competitive exclusion of the Gram negatives including *E. coli* due to the subsequent *L. acidophilus* dosing.

From Fig. **1,** we can also observe that the no. of *Lactobacilli* increased during the dosing.

However, a decline and incline in the no. of *Lactobacilli* and *E. coli* respectively, 2 weeks after the dosing was stopped, shows that the microbial count of faeces revert back to the integer similar to the one which was recorded before the dosing was started. And yet, the *lactobacilli* count was fairly increased as compared to the original count.

Competitive Exclusion (CE) of *Salmonella*:

Three chicks from each group were challenged with 3×10^8 CFU/chick of *S. typhi*. After 4-5 days, chicks of the control group showed mild infection and other signs & symptoms i.e. low consumption of feed, weakness, fixed joints etc. However, the mortality rate was 0%. Whereas, all 3 probiotic group chicks remained healthy and negative for any signs of infection, thus manifesting the CE of *Salmonella*.

Antibiotic Susceptibility:

Four of the 9 strains obtained from Milk & Fowl crop identified as *L. acidophilus* were tested to determine their disc sensitivity or resistance to 13 commonly used antibiotics. All 4 of the *L. acidophilus* strains were sensitive to Chloramphenicol, Erythromycin, and Trimethoprim. In contrast, the *L. acidophilus* strains were highly resistant to Ampiclox, Amoxil, Gentamicin, Orbenin, Piperacillin, Streptomycin, Tetracycline and Tobramycin.

The *L. acidophilus* strains also exhibited variable resistance to Penicillin G and Ofloxacin. The resistance was variable in that *L. acidophilus* isolated from crop was highly resistant and those isolated from milk showed significant zones of inhibition around the antibiotic containing discs, however these 3 strains were also designated as resistant according to the standards (Data not shown).

Antimicrobial Agent Production:

The *L. acidophilus* used in this study was tested for the production of an antimicrobial agent against *P. aeruginosa, S. typhi, E. coli, S. aureus, M. luteus* and *L. acidophilus*. A detectable zone and a weak zone of inhibition on the lawns of *S. aureus* and *M. luteus* respectively, were observed. These zones were smaller in case of cell supernatant. No zone of inhibition was observed against the control well and in the case of live suspension or cell supernatant at neutral pH.

DISCUSSION

The present study aimed to investigate the potential probiotic properties of *L. acidophilus* intended for use in poultry. To the best of our knowledge and based on the available literature reviewed earlier in the introduction, there are no truly effective probiotics available at this time for use in the poultry industry. In this study, the efforts made over the past years have been summarized to determine if *Lactobacilli* particularly *L. acidophilus* could serve this purpose.

The information that is available on colonization by some potentially useful organisms was kept in mind and therefore the identified *L. acidophilus* from the crop was chosen in this study to carry out probiotic functions. Is this study, we are not concerned with the mechanism of resistance or sensitivity to these antibiotics. In part, this antibiotic resistance pattern is not all that surprising. The purpose of the antibiotic susceptibility test was just to determine their antibiotic resistance pattern to familiarize with the antibiotics that could be used with the probiotic in any further studies.

Lactobacillus acidophilus was tested for the production of antimicrobial agent against some Gram negative and Gram-positive bacteria of clinical importance. Broth culture and cell supernatant; both at neutral and original pH were used. Whereas *L. acidophilus* itself was not effected. This antibacterial activity could be due to any of the substances described earlier. High acidity as a result of low pH can also be a reason, as no antimicrobial activity was detected at neutral pH. The pH of a 24 hour fresh broth culture of *L. acidophilus* was noted which ranged from 3-4 and the viability of the organisms present in probiotic suspension declare the resistance of the strain against the highly acidic environment and other antibacterial substances that it produces.

However, the resistance of the Gram-negative bacteria to the antibacterial activity speculates the production of a bacteriocin or antibiotic confined to Gram-positive bacteria. No exact reason for this in vitro antimicrobial activity can be stated at this level of study. There is a need of many experimental trials at analytical and molecular levels to proclaim a valid statement.

As far as Competitive Exclusion (CE) is concerned, an exclusion of Gram negatives and increase of *lactobacilli* in the probiotic group faeces was observed as a result sequential dosing of *L. acidophilus*. This exclusion was constant within the 14 days of dosing and recidivated, rather terminated after the subsequent doses were stopped.

The decline in number of the Gram negatives can either be due to the intestinal colonization of lactobacilli leading to competitive exclusion or, due to the production of an antibacterial substance that may be active against the Gram

negative bacteria in vivo environment. Likewise, much further study has to be carried out including anatomization, characterization of the organisms inhibited during the CE by lactobacilli, and the organisms that are involved in the colonization of crop and gut of the chick.

The most gratifying result & observation of this present study are the significant and noteworthy weight gain of the Probiotic group chicks, which were orally administered by the *L. acidophilus* strain. An average weight gain of 137.6 grams as compared to the control group (Average weight gain 59 grams) in 14 days, testify the potential characteristics of the probiotic strain used in this study. Such beneficial role and the rationalization behind it have been reviewed beforehand in detail.

Many remains to be learned about the action of the *L. acidophilus* culture used in this study. Also, a need for conducting field trials in this concern with increased varieties and quantity of fowl is imperative.

From this point of view, it is deplorable that still only a few groups of scientists are working practically in this area and have come out with prominent results.

To the best of our knowledge and based on the available literature, there are no truly effective probiotics available at this time for the poultry industry. In conclusion, the findings in our study indicate that a *Lactobacillus* particularly *L. acidophilus* based probiotic technology could become 1) a cost-effective means to improve production efficiency in the poultry industry, and 2) a biological alternative to use of antibiotics as growth promoter for poultry and other food animals.

It remains to be seen if *L. acidophilus* probiosis alone or in combination with decreased levels of feed stuff antibiotics will be used to alleviate this public health problem and to satisfy the need for cost effective means to increase productivity in the food animal industries. *L. acidophilus* mode of action as a probiotic remains to be determined. Although much remains to be learned about the mode of action underlying its beneficial effects on the host, there can be little doubt that *L. acidophilus* is finding its "honorable place" in Metchnikoff's belief that "there are many useful microbes, amongst which the lactic bacilli have an honorable place."

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