The Ecological Role of Probiotics in *in vitro* Culture for the Improvement of Health in the Poultry Industry

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Abstract: *The general objective of this work was to isolate from yoghourts cultured strains of lactobacilli with potential for use as probiotics in poultry farming. Three yoghourts were cultured to see the presence of lactobacilli in the Rogosa agar base culture medium. It was found that only one yoghourt (number 1) showed the growth of lactobacilli. This yoghourt was immediately selected for further cultivation. Afterwards, the Lactobacillus strains were isolated and fortified under CO_2 and then inoculated into a solution of peptone water, which constituted inoculums to be administered to an experimental group of poultry. Another group served as controls. Pathogenic strains of Escherichia coli were also administered to both groups (experimental and control, each comprising five hens). The results showed significant weight gain from the experimental group (positive effect on immunity) and freedom from disease after an incubation period (inhibitory effect of the lactobacillus strains on the Escherichia coli strains), whereas the control group showed less weight gain than the experimental group and development of colibacillosis after an incubation period. The positive effects of the Lactobacillus strains observed on the poultry of the experimental group proved the ecological role of these microorganisms in improving the health of the poultry by inhibiting the effects of pathogenic Escherichia coli strains. This suggests reassuring prospects for the reduction of antibiotic use in both human and animal care.*

Keywords*: Probiotics, Antibiotics, Poultry, Lactobacilli, Pathogenic strains, Escherichia coli.*

Introduction

The use of antibiotics currently poses several problems on the market: on the one hand, those related to efficacy, harmful side effects and bacterial resistance, and on the other hand, those related to the decline in the function of the immune system and the diarrhea associated with the use of antibiotics, as well as the metabolites found in the environment [25]. Faced with these multiple problems, the use of antibiotics becomes limited in certain diseases, particularly in gastrointestinal diseases.

The intestinal microbiota is the set of bacteria that inhabit the digestive tract. It is sustained by food residues, secretions and tissue desquamation. In return, the microbiota plays an active role in good health. Among the major functions of the microbiota are the fermentation of available substrates in the colon, the role as a barrier to colonisation by pathogenic microorganisms,

the development and maturation of the intestinal immune system, and interactions with epithelial cells that have essential roles in maintaining host health [2, 3, 5, 8, 16, 21, 23, 26, 27, 40]. In poultry, the gastrointestinal tract has some anatomical features that differ from other animal species [15, 17, 30]. Colibacillosis is probably the most frequent and important bacterial infection in avian pathology due to *Escherichia coli* with an incubation period of one to three weeks. They can lead to mortality, reduced performance and seizure at slaughter [31] (Table 1).

Table 1. Composition of the digestive microbiota of the poultry [7]

Majority groups	**Number of viable bacteria (log_{10} CFU/g content)**						
	Ruffle	**Gizzard**	**Intestine 1(2)**	**Intestine 3**	**Intestine 5**	**Intestine 7**	**Caeca**
Lactobacilli	8.7	7.3	8.0	8.2	8.2	8.6	8.7
Streptococci	4.0	3.7	4.0	4.0	3.7	4.2	6.7
Escherichia coli	1.7	nd	2.0	1.7	1.7	2.7	5.6
Yeast	2.7	nd	1.7	nd	1.7	nd	2.0
Clostridium welchi	nd	nd	nd	nd	nd	nd	1.7
Bacteroides	nd	nd	nd	nd	nd	nd	8.7

CFU: colony forming unit; nd: organism not detected, i.e., quantity with a log_{10} of less than 1.7/g; (1) adult broilers from a farm (6 individuals), consuming a diet consisting of cereals and fish meal (10-15%), without antibiotics; (2) the intestine was divided into 7 parts: different portions were studied (the 1st, 3rd, 5th and 7th parts).

The Table 1 shows the composition of the digestive microbiote in chicken. The digestive tract of the poultry contains an extremely rich and diversified microbial population, composed of many different micro-organisms.

The treatment of choice for enteric disease involves the use of antibiotics, but does not prevent recurrence of the disease after stopping the medication. The problem of bacterial resistance has been compounded by the use of antibiotics to prevent infections before they occur. Inappropriate prescribing of antibiotics for infections against which they are ineffective (especially viral) results in the destruction of antibiotic-sensitive bacteria and contributes to the proliferation of resistant strains. In addition, the administration of antibiotics to poultry and livestock has contributed to the emergency of resistant strains, particularly among salmonella populations [29].

One of the most recent alternatives proposed is the use of probiotics. According to several researchers, probiotics are well placed to take over from antibiotic additives because of their interesting nutritional and antimicrobial abilities. In 2001, the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) gave an official definition of probiotics as "*live micro-organisms that, when ingested in sufficient quantities, exert positive health effects beyond the traditional nutritional effects*" [35]. Microorganisms must have various survival properties to meet the definition of probiotics [18]. They must exhibit positive activity and persist during their passage through the digestive tract.

In fact, "to be effective on the intestinal microbiota", probiotics must arrive alive in the colon and "in sufficient numbers". They must therefore not be degraded after passing through the stomach and "must be able to resist gastric acidity and pancreatic juice". Among the microorganisms used in probiotics, we often find lactic acid bacteria, natural hosts of the human intestinal microbiota. The most studied probiotics belong to two genera: *bifidobacteria* and

lactobacilli [6, 13, 22, 24, 28]. In animal nutrition, many bacterial and fungal genera are used as probiotics [34].

The Rogosa agar base culture medium is used for the growth and isolation of lactobacilli. It is used for the enumeration of *Lactobacillus* in dairy products (yoghourt), meat, food products and biological samples of animal origin [20]. When the culture is read, the lactobacilli appear as large white colonies [19].

Most probiotic bacteria are lactic acid producing bacteria (lactic acid bacteria). The latter inhibits the growth of coliforms in the gastrointestinal tract of piglets and this effect has been attributed to the reduction of the pH of the environment (lactic acid has a strong acidifying effect) [32].

The aim of this work was to demonstrate the ecological role of probiotics in in vitro culture through their beneficial effects on health, with a view to reducing the use of antibiotics in healthcare.

Materials and methods

All the fieldwork took place at the Veterinary Laboratory of Kinshasa, in the Bacteriology and Animal Health Departments. Two flaps were used for the poultry experiment, one for the experimental group (Group A) and one for the control group (Group B).

Group A species were labelled as *P*1*GE, P*2*GE, P*3*GE, P*4*GE & P*5*GE* and those in Group B as *P*1*GT, P*2*GT, P*3*GT, P*4*GT & P*5*GT*, where *P* is hen; 1, 2, 3, 4, 5 are hen numbers in their respective groups; *G* is group; *E* is experimental and *T* is temperature.

It should be noted that prior to start the handling, the hens had developed coccidiosis but after diagnosis of the disease by the veterinary surgeons, they were treated with Amprolin-300 ws 100 g (3 g in 1.5 L of drinking water/day for 5-7 days) and antibiotics. They were all cured.

Throughout the experiment, hens of the same breed (Isabrown), age (2 months) and sex (males) were fed with poultry feed A_2 with an intake of 100 g/hen/day, i.e., 1000 g/day for the whole flock.

The methodology consisted of isolating lactobacilli from yoghourt on the one hand and preparing samples of pathogens (*E. coli*) on the other hand in order to carry out inoculations in poultry and observe the effects. The yoghourt samples were cultured on Rogosa agar base, poured into petri dishes and incubated at 37.5 °C for 72 hours. Each sample was read after incubation. In order to fortify the growing lactobacilli, the seeded dishes were placed in an anaerobic jar, further incubated at room temperature of 37 °C for 72 hours in a CO_2 enriched atmosphere.

The different grown species of lactobacilli were considered as a whole. Chickens in both groups were weighed before the *Lactobacillus* and *E. coli* pathogen inoculation sessions using a manual scale. In the experimental species, three inoculation sessions of the lactobacilli fortified in peptone water took place within a week, every 72 hours with 2 ml of inoculum per head at each session and administered orally in such a way as to travel through the digestive tract until they reached the target, i.e., the colon of the poultry, where they exerted the beneficial effect on health. Inoculation and collection was done using a sterile single-use syringe.

After administering the lactobacilli strains to the experimental species, a series of three inoculation sessions of *E. coli* strains were carried out in both groups, within a week, every 72 hours with 2 ml of inoculum per species at each session. Here, we were not able to determine the approximate concentration of cells in the 2 ml of inoculum but it was a more question of seeking the effect of probiotic on the poultry of the experimental group according to the definition of the WHO: "*the probiotics ones are living micros-organisms which, when they are introduced in sufficient quantity, exert the positive effects on health, beyond the traditional nutritional effects.*" (http://www.passeportsante.net).

After these different inoculations, the poultry was weighed again, four weeks after the inoculations, taking into account the incubation period of colibacillosis (1 to 3 weeks). The presence of lactobacilli was sought in the faeces to confirm or not the effectiveness of the latter during their passage through the animal's digestive tract. In addition to this search for lactobacilli in the stool, we also looked for the presence of *E. coli* which was noted.

Fecal samples were cultured in petri dishes containing Rogosa agar base culture medium to isolate potential lactobacilli, while for *E. coli*, their presence was observed in the faeces of hens in the control group that had not been inoculated with lactobacilli strains. The results obtained after these different manipulations and experiments are presented and discussed below.

Results and discussion

Results

Initial poultry weights

The initial weights obtained per species in the two groups are shown in Table 2.

- Average weight (experimental group): $\mu_1 = 822 \pm 11.7$ g;
- Average weight (control group): $\mu_2 = 814 \pm 11.4$ g.

The ten hens were fed under the same conditions for 1 month. Before starting the inoculations, all hens were weighed. There was no significant difference in weight between the two groups because according to the Student's *t*-test (comparison of two observed means on two independent samples) on the difference of two means applied, the t_{cal} was lower than the t_{tab}, i.e., $t_{cal} < t_{tab}$: $1.153 < 2.306$.

Table 2. Initial weight of hens in both groups before inoculations

Group A (experimental)		Group B (control)	
Species	**Weight, (in g)**	**Species**	**Weight, (in g)**
P1GE	810	*P1GT*	800
P2GE	830	*P2GT*	810
P3GE	840	*P3GT*	830
P4GE	820	*P4GT*	810
P5GE	810	*P5GT*	820

Cultivation of fermented dairy products (yoghourt)

After incubation for 72 hours of the samples from the three fermented milk products (yoghourt 1, 2, 3); only in the box where yoghourt 1 (local, industrial) was inoculated that the lactobacilli have grown well, but in an isolated way; in the box of yoghourt 2 (local, artisanal), they did not even grow and in that of yoghourt 3 (imported), they had grown slightly (Table 3).

Table 3. Seeding of yoghourt

Petri dishes	Yoghourt 1 (local, industrial)	Yoghourt 2 (local, artisanal)	Yoghourt 3 (imported)
1	+++	- -	+
2	++	- -	+

Legend: high growth: +++; average growth: ++; low growth: +/-; no growth: - -

Yoghourt 1 (local, industrial) was used for its potential to have *Lactobacillus* strains capable of growing easily in the Rogosa agar base culture medium.

Fig. 1 shows the growth of the colonies of lactobacilli in yoghourt 1 sown in the culture medium Rogosa agar base.

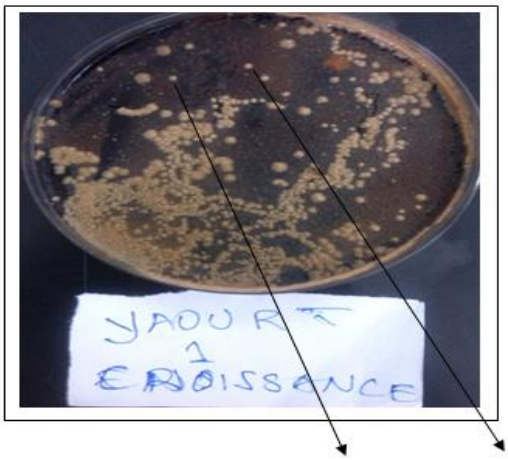

Fig. 1 Growth of the lactobacilli in yoghourt 1

Weight after inoculation with lactobacilli

Final weights after hen inoculations in both groups are shown in Table 4.

- Average weight (experimental group): $\mu_1 = 1280 \pm 83.7$ g; $S_1^2 = 7000$;
- Average weight (control group): $\mu_2 = 1100 \pm 70.7$ g; $S_2^2 = 5000$.

(S_1^2 – variance of Group A; S_2^2 – variance of Group B)

Table 4. Final weights after hen inoculations in both groups

Group A (experimental)		Group B (control)	
species	**Weight, (in g)**	**species**	**Weight, (in g)**
P1GE	1300	*P1GE*	1100
P2GE	1300	*P2GE*	1000
P3GE	1400	*P3GE*	1100
P4GE	1200	*P4GE*	1100
P5GE	1200	*P5GE*	1200

The results of the two groups were subjected to the hypothesis test on the difference of two means to see if this difference was significant or not? For this, the Student's *t*-test showed $t_{cal} > t_{tab}$ indicating a significant difference between the two groups of hens, even though at the beginning the hens had almost the same weight (Student's *t*-test). The increase in weight was due to the administration of lactobacilli.

After the 3 sessions of inoculation of the lactobacilli strains to the experimental species (Group A), a clear improvement in immunity was observed in the poultry of this group, characterized by the absence of diseases (Table 5) compared to the control group (Group B).

Results of fecal swab inoculations in poultry in both groups

From the research of the presence or not of lactobacilli in the poultry stools (indication of the inhibiting effect of lactobacilli against *E. coli* strains) of the two groups and after seeding these samples in petri dishes based on Rogosa agar base, It is clear from the various dishes that only in the dishes containing the stools of the experimental group (Group A) did the lactobacilli grow, whereas in the dishes containing the stools of the control group (Group B), there was no growth (Table 5). This proves that it was the administered strains that resisted passing through the entire digestive tract of the experimental hens.

Table 5. Results of fecal swab inoculations in poultry in both groups

Group A (experimental)		**Group B (control)**	
Species	**Presence of lactobacilli**	**Species**	**Presence of lactobacilli**
P1GE	+	P1GT	- -
P2GE	++	P2GT	- -
P3GE	++	P3GT	- -
P4GE	+	P4GT	- -
P5GE	++	P5GT	- -

Legend: high growth: ++; medium growth: +; no growth: - -

Inoculation of pathogenic E. coli strains to experimental and control species

After the 3 inoculation sessions of *E. coli* strains to the experimental (Group A) and control (Group B) species, the hens were observed for 3 weeks to see the reaction to *E. coli*. During this period, none of the hens in the experimental group (Group A) developed or showed signs of the disease (Table 6), while in the control species (Group B) the following signs were observed: whitish diarrhoea, weakness and drowsiness (Table 6) and the diagnosis of colibacillosis had been made by the veterinary service.

Two of the five hens in Group B died as a result of this colibacillosis and the other three were taken to the laboratory for appropriate treatment.

The Table 6 shows some signs observed in chickens of the reference group after inoculation of the pathogenic *E. coli* strains.

Table 6. Signs observed in Group B poultry after inoculation with *E. coli* strains

Chickens	**Signs**
P1GT	weakness, white diarrhoea, drowsiness, death
P2GT	weakening
P3GT	weakness, white diarrhoea, drowsiness
P4GT	weakness, white diarrhoea, drowsiness, death
P5GT	weakness, drowsiness

Discussion

Much work has been done to highlight the benefits of probiotics on both animal and human health. The present work sought to verify the effects of lactobacilli on the health of poultry by

inoculating them first alone and then combined with *E. coli* strains in the experimental group. The control group was only inoculated with *E. coli* strains.

By enhancing the microbial ecosystem of poultry, Goharrizi et al. [16] have shown that probiotics contribute to immune defense and protect chickens against the consequences of stresses such as vaccination and temperature changes. Improvements in weight gain and feed conversion have been observed following probiotic consumption [9, 36]. This was the case in this work where the hens tested showed significant weight gain.

The role of probiotics is to colonise the gut and thus prevent its colonisation by enteropathogens causing diarrhoea [12]. This effect is probably due to the action of bacterial probiotics on the intestinal mucosa and may be justified in the case of this work, since the addition of lactobacilli to the poultry diet had an effect in preventing colibacillosis from taking hold. It is well known that, particularly in monogastric animals, bacterial probiotics can alter the permeability of the intestinal mucosa, activate immune cells and prevent the adhesion of pathogens to the gut mucosa [4, 10].

For antibiotic-associated diarrhoea, probiotics have been shown to be useful as a preventative treatment, and potentially can be used to alleviate signs and symptoms once antibiotic-induced diarrhoea has occurred [11, 25, 39]. The use of probiotics seems to offer an alternative solution in reducing the use of antibiotics due to their beneficial effects on the health of poultry.

In this experiment, the majority (four) of the hens in Group A showed a higher weight gain than those in Group B (Table 5); after subjecting this difference in average weight to Student's *t*-test, we found that the difference was significant. This difference in weight gain in the experimental group (Group A) reflects the beneficial effect of lactobacilli (probiotics) on the immunity of the poultry, which is generally manifested by a considerable weight gain compared to the control group (Group B), which did not benefit from this immunomodulatory effect of lactobacilli. This has also been asserted in previous work by authors such as [9, 33, 36].

Among the three fermented dairy products (yoghourts) used, only yoghourt 1 (industrial, local) showed a potential to provide strains of lactobacilli that could be used, after culture, as probiotics, whereas the other two showed certain limitations in developing strains of lactobacilli; which amounts to saying that not every fermented dairy product (yoghourt) necessarily has the potential to provide strains of lactobacilli.

The inoculation of the pathogenic *E. coli* strains in the two groups (Group A and Group B) gave different effects: in the experimental group, it was noted that the hens were resistant, with the absence of symptoms and disease until the end of the experiment, despite the extended incubation period. However, in the control group, after the incubation period, disease symptoms were observed and subsequently the death of two hens in this group was recorded. In the experimental group, the inhibitory effect of the lactobacilli on the pathogenic *E. coli* strains worked in favour of the birds.

The efficacy of lactobacilli strains through the digestive tract of the host (animal) should also be demonstrated by the presence of these strains in the faeces of the animal as discussed in the selection criteria for probiotics according to [1, 14, 37, 38]. The results obtained with the faeces showed the presence of lactobacilli in the species of the experimental group while nothing was observed in the species of the control group. Thus, the lactobacilli strains administered in adequate quantities were effective in the experimental group of poultry because they resisted

the acidity along the digestive tract and exerted an inhibitory effect on the pathogenic *E. coli* strains administered to these species, which is why they were found in the feces.

The fact that the control group had to be treated with antibiotics when they fell ill, while the experimental group only benefited from the lactobacilli to resist the disease, shows the ecological role played by lactobacilli (probiotics) in inhibiting the effects of *E. coli* strains to prevent disease. This aspect is very important from an economic point of view in poultry farming, as prevention is cheaper than curative care; even though antibiotics can be used to treat the disease, there is a risk of resistance phenomena and antibiotic by-products in the environment with all the possible consequences.

The results obtained in this work are only preliminary to the actual use of probiotics in healthcare to reduce antibiotic use. The most commonly used probiotics are represented by two genera: *Lactobacillus* and *bifidobacterium* [6, 13, 22, 24, 28], but in the context of this work we were only able to exploit *Lactobacillus.* However, other studies may continue in the future with *bifidobacterium* strains.

Aspects to be developed include serotyping to characterise the various isolated strains of Lactobacillus, the exact quantity of strains to be administered to obtain the expected probiotic effects, the number of CFU per milliliter of inoculum and the quantity required for the beneficial effects of *Lactobacillus* on health. This work has made it possible to highlight *Lactobacillus* and to evaluate their effects on avian health in order to consider their use in the long term in the context of reducing the use of antibiotics.

Conclusion

Through this work, it has been demonstrated that probiotics represent a natural approach to enriching the intestinal flora and competitive exclusion to fight pathogenic bacteria.

This work made it possible to isolate from a fermented dairy product (yoghurt), by means of its culture, strains of lactobacilli capable of being used as probiotics. The effects of the latter on poultry with a view to their use in care to reduce the use of antibiotics yielded results that lead to the following conclusions:

1. The administration of lactobacilli to poultry infected with pathogenic *E. coli* strains results in absolute resistance to colibacillosis, in contrast to the control group of poultry that did not receive lactobacilli.
2. The ingestion of the probiotic *Lactobacillus* strains allows the improvement of the health of the hens by a significant increase in weight.
3. The administered *Lactobacillus* strains that appeared in the stool, prove their probiotic effect sufficiently.
4. Lactobacilli and *bifidobacteria* may be considered for inclusion in yoghourt and other food products to enhance the probiotic effect in consumers.

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