Isolation and characterization of *Lactobacillus* strains with probiotic potential from dairy products

ABSTRACT

Aims: This research aims to isolate and characterize from dairy products, in particular yogurts, cultivated *Lactobacillus* strains with potential for use as probiotics in poultry farming.

Study design: This research consists of four parts, an introduction with literature review, a description of the materials and methods used, finally the results obtained and their discussion.

Place and Duration of Study: Central Veterinary Laboratory of Kinshasa, specifically in the Bacteriology and Animal Services departments, between April and September 2022.

Methodology: A yogurt was cultured in order to observe the presence of *Lactobacilli* in the MRS (Mans, Rogosa and Sharp) Agar culture medium; this culture revealed the growth of *Lactobacilli*. It was even proceeded to the serial dilution of these strains, the results had also revealed the growth of *Lactobacilli* in the 6 test tubes used.

Results: The results obtained showed a significant increase in weight (positive effect on the immune system) in the poultry of the experimental group and an absence of pathogenicity after an incubation period (inhibitory effect of strains of *Lactobacilli* on those of *Escherichia coli* and *Streptococcus sp.*) whereas in the control group, the poultry showed a lower weight gain compared to the experimental group and developed colibacillosis associated with other effects such as weakness, fever and angina after an incubation period.

Conclusion: The positive results of *Lactobacilli* strains obtained in poultry in the experimental group show the potential of these strains to be used as probiotics in poultry farming to improve poultry health through the inhibitory effect of these strains on pathogenic *Escherichia coli* and *Streptococcus sp*. This opens up safe alternatives to the use of these strains to fight the misuse of antibiotics by reducing their use in human and animal care.

Keywords: probiotics, prebiotics, antibiotics, poultry, yogurt.

1. INTRODUCTION

Lactic acid bacteria by their acidifying, flavouring and texturizing properties are widely used in milk-derived products and their probiotic properties are very useful for health, in fact, they improve digestive functions and have a very positive effect on the intestinal microbiota [1]. Products derived from traditional lactic fermentation have experienced considerable development in recent years thanks to the interest that consumers find on the organoleptic, nutritional, therapeutic and even hygienic level because of their acidity. The biodiversity of these lactic acid bacteria involved in this process is a fundamental factor for the preservation of the typicality and original characteristics of products [2] and without disadvantages for lactose intolerant consumers.

Dairy products, thanks to their rich and diversified composition, have very interesting therapeutic effects and regular consumption of these products can have an anticarcinogenic [3], antimicrobial [4], immunological effect very important [5] thanks to their very low final pH between 4.3 and 4.4 [6,7] without forgetting the beneficial effect on the intestinal tract [8].

In order to improve the quality and avoid the disappearance in the more or less long term [9] of traditional dairy products, the latter deserve a transfer of production on a semi-industrial or industrial scale to be known and marketed on a larger scale. This will make it possible to standardize the various stages of their production process, to better control their quality and to reinforce the added value of their properties.

Hence the idea of isolating lactic acid bacteria from traditional products because the interest of these strains lies in their ability to produce specific metabolites, especially those responsible for flavor, which may be different from those produced by industrial strains [2]. Then use them in a semi-industrial process to obtain other fermented products with better hygienic quality and very advanced therapeutic, especially than in recent years; research has intensified to find probiotic-based therapies for the treatment of various chronic diseases [10].

1.2. Objective

The overall purpose of this research was to isolate and characterize from dairy products, in particular yogurts, cultivated *Lactobacillus* strains with potential for use as probiotics in poultry farming.

2. MATERIALS and METHOD

The field work took place at the Central Veterinary Laboratory in Kinshasa, specifically in the Bacteriology and Animal Services departments. Two valves were fitted out to carry out the experiments on poultry, one had been used for the experimental group with strains of Lactobacilli (GELB) and for the control group (GT). Poultry species were marked as follows: P1GELB, P2GELB, P3GELB, P4GELB, P5GELB, P6GELB & P7GELB (for the experimental group) and P1GT, P2GT, P3GT, P4GT, P5GT, P6GT & P7GT (for the control group). P = Chicken: 1, 2, 3, 4, 5, 6, 7 = number of hens in their respective group; G = Group; E = Experimental and T = Control. During the experiment, these hens, which were of the same breed (Sasouat) and the same age (3 months), were fed poultry feed P1 and P2 with a consumption of 100 g per hen per day, i.e. 1400 g per day for the set of poultry.

2.1 Methodology

The methodology consisted in isolating and characterizing *Lactobacilli* with a dairy product containing potential strains of these microorganisms in order to prepare inoculums on the one hand and prepare strains of pathogenic germs (*Escherichia coli* and *Staphylococcus sp.*) to carry out inoculations in poultry in order to observe the related effects.

The samples of the dairy product (yoghurt) were cultured on MRS Agar, poured into Petri dishes heated to the oven at 37.5 °C for 72 hours. Each sample was read after the incubation period.

2.2 Experimental details

In order to fortify the *Lactobacilli* that grew, the seeded boxes were placed in an anaerobic jar, incubated again at room temperature of 37 °C for 72 hours in a CO_2-enriched atmosphere. Other cultured strains of *Lactobacilli* were subjected to serial dilution to test the effectiveness of this medium (MRS Agar) in order to observe the growth of Lactobacilli.

The different species of *Lactobacillus* strains have been characterized as *Lactobacilli casei* by their origin (dairy product used) through electron microscopic observation. The chickens of the two groups were weighed before the *lactobacillus* and pathogenic germs of *Escherichia coli* and *Staphylococcus sp.* inoculation sessions using a manual scale. In the experimental species, three sessions of inoculation of fortified *Lactobacilli* in peptone water took place within a week, every 72 hours with 2 ml of inoculum per head at each session and administered by the route oral so as to travel through the digestive tract until it reaches the target, that is to say the colon of the poultry where they exert the beneficial effect on health. Inoculation and sampling were done using a sterile single-use syringe.

After having administered the strains of *Lactobacilli* to the experimental species, a series of three sessions of inoculation of the strains of *Escherichia coli* and *Staphylococcus sp.* were carried out in the two groups, in the space of a week, every 72 hours with 2 ml of inoculum per species at each session. To enhance the probiotic effect, honey was also used as a prebiotic during these inoculation sessions at a rate of 2 ml per species in the experimental group. After these different inoculations, the poultry was weighed again, four weeks after the inoculations, taking into account the incubation period for colibacillosis (from 1 to 3 weeks). The presence of *Lactobacilli* was sought in the stools to confirm or not, the effectiveness of the latter during their passage through the digestive tract of the animal. Alongside this search for *Lactobacilli* in the stool, we should also look for the presence of *Escherichia coli* and *Staphylococcus sp.* which was noted.

Stool samples were cultured in Petri dishes containing MRS Agar culture medium to isolate potential *Lactobacilli*, while for *Escherichia coli* and *Staphylococcus sp*. their presence was to be observed in the stools of hens in the control group who have not benefited from the inoculation of *Lactobacillus* strains. The results obtained after these various manipulations and experiments will be presented and discussed below.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. Initial poultry weights

These weights obtained by species in the two groups are shown in the following Table 1:

Table 1. Initial weights of poultry before inoculations

Experimental Lactobacillus Group (GELB)		Control Group (GT)	
Species	Weight (g)	**Species**	Weight (g)
P1GELB	1160	**P1GT**	1405
P2GELB	1190	**P2GT**	945
P3GELB	875	**P3GT**	825
P4GELB	1370	**P4GT**	1365
P5GELB	1270	**P5GT**	1365
P6GELB	1460	**P6GT**	1495
P7GELB	1330	**P7GT**	975

- Average weight (experimental group): $\mu 1 = 1236.4 \pm 189.8$ g; $S^2_1 = 36056.9$ g
- Average weight (control group): $\mu 2 = 1196.4 \pm 270.7$ g; $S^2_2 = 73280.9$ g

  (S^2_1 : variance of experimental group; S^2_2 : variance of control group)

The fourteen hens were fed under the same conditions for two months. Before starting the inoculations, all the hens were weighed. There was no significant difference in weight between the two groups because according to the Student's test on the difference of two means applied, the t_{cal} was lower than the t_{tab}, i.e.: $t_{ca}l < t_{tab}$: 0.320< 2.179. (t_{cal} : calculated test t ; t_{tab} : tabular test t).

3.1.2. Culture of dairy products (yogurt)

The results related to the growth of dairy products are reported in table 2:

Table 2. Inoculation of dairy products

Petri Dishes	Growth
1	+++
2	+++
3	+++
4	++
5	+
6	+
7	+

Legend:
• High growth: +++
• Average growth: ++
• Low growth: +

This dairy product (yogurt) was used for its potential to have strains of *Lactobacilli* capable of growing easily in the culture medium (MRS Agar).

3.1.3. Serial dilution of *Lactobacilli* strains

The results obtained after serial dilution of the sources of *Lactobacilli* are given in table 3:

Table 3. Serial dilution of *Lactobacilli* strains

	Petri Dishes					
	1	**2**	**3**	**4**	**5**	**6**
Peptone water	9 ml	9 ml	9 ml	9 ml	9 ml	9 ml
Diluent	1ml	1 ml	1 ml	1 ml	1 ml	1 ml
Dilution rate	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}

This table shows the serial dilution carried out in 6 test tubes with the addition of 1 ml of diluent (direct sample of *Lactobacillus* strains after inoculation on MRS Agar) in 9 ml of peptone water contained in each of these tubes.

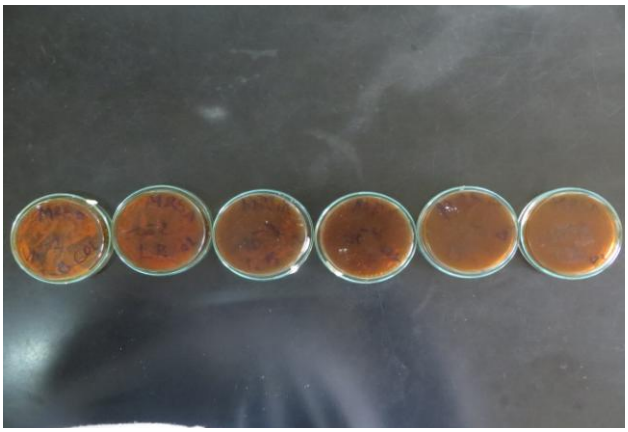

Fig. 1. Growth of *Lactobacilli* in Petri dishes.

This figure (Fig. 2) shows the growth of *Lactobacilli* in inoculated Petri dishes after serial dilution from the direct sample (yogurt). When reading the cultures in these dishes, growth was noted after 48 hours under CO_2 (one decimal dilution):

- Box 1: >300 colonies/box/mm^3 with a dilution rate of 10^{-1};
- Box 2: >300 colonies/box/mm^3 with a dilution rate of 10^{-2};
- Box 3: >250 colonies/box/mm^3 with a dilution rate of 10^{-3};
- Box 4: 120 colonies/box/mm^3 with a dilution rate of 10^{-4};
- Box 5: 37 colonies/box/mm^3 with a dilution rate of 10^{-5};
- Box 6: >3 colonies/box/mm^3 with a dilution rate of 10^{-6}.

3.1.4. Weight after poultry inoculations

These weights obtained by species in the two groups are given in the following table 4:

Table 4. Poultry weight after inoculations.

Experimental *Lactobacillus* Group (GELB)		**Control Group (GT)**	
Species	Weight (g)	**Species**	Weight (g)
P1GELB	2065	**P1GT**	1780
P2GELB	1905	**P2GT**	1410
P3GELB	1945	**P3GT**	1725
P4GELB	1935	**P4GT**	1510
P5GELB	1760	**P5GT**	1415
P6GELB	1700	**P6GT**	1200
P7GELB	1780	**P7GT**	1320

- Average weight (experimental group): $\mu 1 = 1870 \pm 127.9$ g; $S^2_1 = 16366.6$ g
- Average weight (control group): $\mu 2 = 1480 \pm 209.7$g; $S^2_2 = 44008.3$ g

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 (comparison of two means observed on two independent samples) showed $t_{cal} > t_{tab}$ (i.e.: $t_{cal} > t_{tab}$: 4.199 > 2.179) thus indicating a significant difference between the two groups of hens whereas at the start 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 *Lactobacillus* strains to the experimental species (GELB), a marked improvement in the immunity was observed in the poultry of this group, characterized by the absence of disease (**Table 5**) compared to that of the control group (GT).

3.1.5. Seeding of stool collected by swabs from poultry in both groups

From the search for the presence or absence of lactobacilli in poultry droppings (indication of the inhibitory effect of *Lactobacilli* against strains of *Escherichia coli* and *Staphylococcus sp.*) of both groups and after seeding these samples in petri dishes based on MRS Agar, it appears from reading these different dishes that only in the dishes containing the stool of the experimental group (GELB) than *Lactobacilli* grew while in the stool boxes of the control group (GT), there was no growth (**Table 5**). This proves that these are the administered strains that have resisted crossing the entire digestive tract of the experienced hens.

Table 5. Results of inoculation of stools taken by swabs in poultry in the two groups.

Experimental *Lactobacillus* Group (GELB)		**Control Group (GT)**	
Species	Presence of LB	**Species**	Presence of LB
P1GELB	+++	**P1GT**	- - -
P2GELB	+++	**P2GT**	- - -
P3GELB	+++	**P3GT**	- - -
P4GELB	+++	**P4GT**	- - -
P5GELB	+++	**P5GT**	- - -
P6GELB	+++	**P6GT**	- - -
P7GELB	+++	**P7GT**	- - -

Legend: high growth (+++); lack of growth (- - -); LB (Lactobacilli)

Figure 2 below also illustrates the growth of these germs in petri dishes. This figure shows optimal growth of *Lactobacilli* with more than 300 colonies per dish. Inoculation of pathogenic strains of *Escherichia coli* and *Staphylococcus sp*. to experimental and control species.

Fig. 2. *Growth of Lactobacilli colonies in petri dishes inoculated from stools collected by swabs from poultry in the experimental group (GELB)*

Table 6. *Signs observed in poultry of the control group (GT) after inoculation with strains of Escherichia coli.*

GT species	Signs observed
P1GT	Weakness, fever, angina
P2GT	Weakness, whitish diarrhoea, angina
P3GT	Weakness, angina, drowsiness
P4GT	Weakness, white diarrhea, fever
P5GT	Weakness, drowsiness, white diarrhea
P6GT	Weakness, white diarrhea, angina
P7GT	Affaiblissement, diarrhée blanche, somnolence, décès

After the 3 sessions of inoculation of the strains of *Escherichia coli* and *Staphylococcus sp.* to the experimental species (GELB) and to the control species (GT), the hens were observed for three weeks to see the reaction to *E. coli* and *S. sp*.

It appears from this period that no hen in the experimental group (A) developed or showed signs of the disease, whereas 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 established by the veterinary service. One of the seven hens in the control group (GT) had died as a result of this colibacillosis and the six others were treated by the laboratory for the appropriate care.

3.2. Discussion

Lactic acid bacteria make up more than 93% of the intestinal flora and play a key role in our metabolism: they are the sentinels that keep pathogens in small numbers and safe, which allows them to live in symbiosis in our body without harm. For 20 or 30 years, efforts to improve human and animal health have focused on the modulation pathways of the intestinal microbiota by the addition of live microbes now called probiotics. The main probiotics used are lactic acid bacteria, including *Bifidobacteria*, *Lactobacilli* and *Lactococci* but can also be yeasts such as Saccharomyces cerevisiae. The "health benefit claims" related to probiotics are the maintenance of a normal and healthy intestinal flora, protection against infections, alleviation of lactose intolerance, stimulation of the immune system, reduction of blood lipids thus preventing the onset of cardiovascular diseases in human health and improving zootechnical parameters in animal husbandry

In addition, *Bifidobacteria* and *Lactobacilli* can be stimulated by indigestible food ingredients such as oligosaccharides collectively called prebiotics. Pre and probiotics can be combined in a food called a symbiotic. The contribution of probiotics in poultry feed is a favourable alternative to the use of antibiotics as growth factors which is currently contested following the application of multiple resistances. In addition, probiotics contribute effectively to improving livestock performance by maintaining a stable and healthy digestive microbiota [11]. It is for this reason that the present work wanted to verify the effects of lactobacilli on the health of poultry by inoculating them first alone and then associated with the strains of *Escherichia coli* and *Staphylococcus sp.* in the experimental group (GELB).

The control group (GT) received only the inoculation of strains of *Escherichia coli* and *Staphylococcus sp.* (pathogenic strains). By strengthening the microbial ecosystem of poultry, Patterson and Burkholder [12] demonstrated that probiotics contribute to the immune defense and protect chickens against the consequences of stress such as vaccination and temperature changes. Improvements in terms of weight gain and feed efficiency have thus been observed following the consumption of probiotics [13,14]. Such was the case with this work where the hens tested experienced significant weight gain. The role of probiotics is to colonize the intestine and thus prevent its colonization by enteric pathogens causing diarrhea [15].

This effect is probably attributable to the action of bacterial probiotics on the intestinal mucosa and which can be justified in the case of this work, since the addition of lactobacilli in poultry feed had an effect of preventing colibacillosis from settle. It is well known that, especially in monogastrics, bacterial probiotics can modify the permeability of the intestinal mucosa, activate immune cells and prevent adhesion of pathogens to the intestinal mucosa [16, 17]. For antibiotic-associated diarrhea, probiotics have been shown to be useful as a preventative treatment, and potentially, they can be used to alleviate signs and symptoms once antibiotic-induced diarrhea has occurred [18, 19, 20].

The use of probiotics seems to offer an alternative solution in reducing the use of antibiotics thanks to their beneficial effects on the health of poultry. It emerges from this experiment that the majority of hens in the experimental group (GELB) show a greater weight gain than those in the control group; after subjecting this difference in mean weight to Student's t-test, we found that the difference was significant. This difference in weight gain in the experimental group (GELB) reflects the beneficial effect of *Lactobacilli* (probiotics) on the immunity of poultry which is generally manifested by a considerable weight gain compared to the control group (GT) which does not did not benefit from this immunomodulatory effect of *Lactobacilli*. The authors have also affirmed this in previous works, in particular [13, 14, 21].

The fermented milk products (yogurts) used had shown a potential to provide strains of lactobacilli likely to be used, after culture, as probiotics. The inoculation of the pathogenic strains of *Escherichia coli* and *Staphylococcus sp.* in the two groups (GELB & GT) gave different effects: in the experimental group, resistance was noted in the hens translated by the absence of symptoms and the disease until the end of the experiment, despite the prolonged incubation period. On the other hand, in the control group, after the incubation period, symptoms of disease were observed and subsequently, the death of a hen of this group (P1GT) was recorded. In the experimental group, the inhibitory effect of lactobacilli on pathogenic strains of *Escherichia coli* and *Staphylococcus sp.* played in favour of poultry.

The efficacy of *Lactobacilli* strains through the digestive tract of the host (the animal) must also be demonstrated by the presence of these strains in the faeces of the animal as discussed in the probiotic selection criteria according to Klaenhammer and Kullen [22, 23, 24, 25]. The results obtained with the stool 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 strains of *Lactobacilli* administered in adequate quantities demonstrated their effectiveness in poultry of the experimental group because on the one hand, they resisted acidity along the digestive tract and on the other hand, they exerted an inhibitory effect on the pathogenic strains of *Escherichia coli* administered to these species. That's how they ended up in the stool.

The fact that we subsequently had to resort to antibiotics to treat the hens in the control group who had fallen ill, whereas the experimental group had just benefited from *Lactobacilli* to resist the disease clearly shows the ecological role played by *Lactobacilli* (probiotics) to inhibit the effects of strains of *Escherichia coli* and *Staphylococcus sp.* to prevent disease. This aspect is very important economically in poultry farming because prevention costs less than curative care; even if we can treat with antibiotics, we must fear the phenomena of resistance and the by-products of antibiotics in the environment with all the possible consequences.

The results obtained in this work are only preliminary in relation to the actual use of probiotics in care. The most used probiotics are represented by two genera: *Lactobacillus* and *Bifidobacterium* [26, 27, 28, 29, 30] but in the as part of this work, we were only able to exploit the Lactobacillus. However, other studies may continue in the future with *Bifidobacterium* strains. This work has made it possible to highlight *Lactobacillus* and to assess their effects on avian health in order to consider their use in care in the long term with a view to reducing the use of antibiotics.

4. CONCLUSION

This work has made it possible to isolate and characterize from dairy products (yogurts) strains of Lactobacilli with probiotic potential. The results obtained showed that these strains have the ability to give positive effects on poultry health by modulating the immune system through their passage through the animal's digestive tract, characterized by resistance to pathogenic strains (*Escherichia coli* and *Staphylococcus sp.*) on the one hand and weight gain on the other. The addition of fructooligosaccharides, as a prebiotic substance, to the culture medium of these bacteria improves the growth and acidifying power of all lactic strains [31]. It would also be interesting to continue this study on larger numbers of hens, and over a longer period of time in order to be able to better specify the effects of the prebiotic. The interest in the application of probiotic bacteria in the food field has increased considerably in recent years and will certainly continue in the future. Obtaining indigenous probiotic strains remains an important objective in terms of research as well as in terms of applications in human and animal health.

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