

Original Research Article

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Production of Probiotic Biomass (*Lactobacillus rhamnosus* IS9) against *Salmonella* sp for Use as a Feed Supplement in Poultry

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ABSTRACT

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The occurrence of antibiotic resistant *Salmonella* is one of the major threats to the poultry industry. The use of probiotic strains is a promising alternative for the control and eradication of *Salmonella* in poultry. This study aims at producing and assessing the potential of *Lactobacillus rhamnosus* (IS9) strain to reduce *Salmonella enterica* serovar Enteridis and Typhimurium common in poultry. Pathogen-free chicks were orally administered by gavage with 1.0×10^9 CFU / ml of *Lactobacillus rhamnosus* (IS9) suspended in 0.1 ml of sterile water and 24 h later were challenged in separate experiments with *S. Enteritidis* and *S. Typhimurium*. There was a significant reduction ($P < 0.05$) in *S. Enteritidis* and *S. Typhimurium* in the small intestine of infected birds. The action of *L. rhamnosus* (IS9) was more important in 14-days old chicks. *Salmonella* count was nil in duodenum, colon and caeca of 14-days old chicks pre-dosed with a single dosed of *L. rhamnosus* (IS9). Weekly analysis of caeca swabs of chicks pre-dosed with *L. rhamnosus* (IS9) showed significant reduction ($P < 0.05$) in *Salmonella* while the caeca population of lactobacilli increases.

Introduction

Probiotics are microorganisms whose administration in adequate quantities is beneficial to humans and animals (Wang *et al.*, 2012). The probiotic properties are numerous, they can vary from one strain to another and include the inhibition of the proliferation of pathogens in the digestive tract, immunomodulation, blood regulation of sugar and cholesterol, the positive effect against certain cancers, enzymatic activities useful for humans and animals metabolism, such as phytase, amylase etc.

(Zhou *et al.*, 2010; Zuccotti *et al.*, 2015; Zuccotti *et al.*, 2008; Zwolinska-Wcislo *et al.*, 2006). Different types of microorganisms are used as probiotic strains, but strains of lactic acid bacteria are preferable because of their GRAS (Generally Regarded as Safe) status (Zivkovic, 1999). The World Health Organization (WHO) and Food Agricultural organization (FAO) particularly have recommended the use of strains of probiotic lactic acid bacteria in both animal and human nutrition (Amara & Shibl, 2015).

Previous work carried out by La Ragione *et al.*, (2004) and Mappley *et al.*, (2011) reported the potential of some of these strains to reduce or inhibit the proliferation of pathogenic strains such as *Salmonella* sp responsible for salmonellosis in poultry. Studies carried out by Wouafo *et al.*, (2010) and Nzouankeu *et al.*, (2010) in Cameroon revealed a high prevalence of antibiotic resistant *Salmonella* in poultry farming around Yaounde the capital city of Cameroon. In addition, work by Akoachere (2009) reported a significant prevalence of *Salmonella* in foods of animal origin in Buea, the capital city of the South West Region of Cameroon. Moreover, the treatments offered are based on antibiotics. This intensive use of antibiotics led to the occurrence of resistant *Salmonella* strains (Kilonzo-Nthenge *et al.*, 2013). The presence of these *Salmonella* in food products is a major public health problem and is still relevant in Cameroon (Nzouankeu *et al.*, 2010). Very little research has been devoted to the probiotic approach to overcome the occurrence of antibiotic resistant strains in poultry farming in Cameroon. The addition of probiotic strains to animal feeding could be an effective alternative for *Salmonella* control.

The efficacy of a probiotic depend on the microbial strain used and mostly on the number and viability of microorganisms present in the probiotic formulation (Coeuret *et al.*, 2004a). The important production of a viable probiotic biomass is a key step in probiotic formulation. To minimize the cost of fermentation in probiotic production, some cheaper media have been developed such as whey-based medium supplemented with ammonium salt and low level of yeast extract (0.25 g/l) (Mondragon-Parada *et al.*, 2006).

This research work aims at developing a cheaper and efficient method of production of probiotic biomass (*Lactobacillus rhamnosus* IS9) and evaluate its effectiveness in the

proliferation of *Salmonella* sp. during poultry farming in Cameroon.

Materials and Methods

Source of microorganisms

The probiotic strain *Lactobacillus rhamnosus* (IS9) was provided by the microbiology unit of Research foundation for Tropical Diseases and Environment (REFOTDE), Buea, Cameroon. This strain was previously characterized for its ability to considerably inhibit some pathogenic strains (Tatsinkou *et al.*, 2017). This strain was thus selected in accordance with our previous results, showing the probiotic potential of some lactic strains isolated from palm wine. The pathogenic strain *Salmonella enterica* serovar Enteridis and *Salmonella enterica* serovar Typhimurium was provided by the microbiology Unit of REFOTDE. These strains are resistant to chlortetracycline and erythromycin mostly used in poultry in Cameroon.

Preparation of fermenting medium

The medium used for probiotic biomass production was molasses-based medium. The molasses samples were obtained from the sugar production company in Cameroon (SOSUCAM). The crude molasses thus obtained was diluted by adding distilled water to have a final concentration of about 2% (w/v). Then the soybean was added to have a final concentration of 0.5% (w/v) in soybeans. It was subsequently filtered to remove the solid particles by means of a Whatman filter paper (Sigma). In a 1000 ml Erlen Meyer series, 500 ml of the fermentation media thus prepared were introduced and then sterilized at 121 °C for 15 minutes.

Preparation of inoculum

The inoculum was prepared using pure colonies of the *L. rhamnosus* (IS9) strain.

This strain was grown in de Man Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK). The identification of this strain was confirmed by the use of phenotypic methods which include morphological characterization, the use of API 50 CHL (bioMérieux, France) identification kit. The colony PCR based on the amplification of 16 rDNA was also used to confirm the identity of the strain. Pure colonies of the *L. rhamnosus* (IS9) strain were seeded in 100 ml of MRS broth contained in 250 ml conical flasks and incubated in a rotary shaker at 150 rpm for 24 h at 35 ° C. The lactic acid bacteria colonies in the inoculum were counted by pour plate method on the MRS agar (Oxoid, Basingstoke, UK). The viability of the cells in the inoculum was observed by microscopy after staining of the cells with methylene blue. This examination of the viability of the cells was based on the fact that during the staining of cells by methylene blue, the living cells remain colorless, while the dead cells are colored blue.

Fermentation Experiment

The fermentation for the production of probiotic biomass was carried out in a series of conical flasks with a volume of 1000 ml each. To this end, these flasks were previously cleaned and 500 ml of previously prepared fermentation medium were introduced into each, then closed with tissue and aluminum foil and autoclaved at 121 °C for 15 minutes. After cooling the series of conical flasks containing the fermentation media were each inoculated with 10 ml of inoculum containing 10^9 CFU / ml of *Lactobacillus rhamnosus* (IS9). The fermentation was carried out in a batch rotary incubator at 35 °C and 150 rpm for 48 h. To follow up the process of fermentation, 2ml of the fermenting medium were taken every 10 h in order to measure the various kinetic parameters of the fermentation.

Analytical techniques

The biomass was estimated by measuring the absorbance of the fermentation medium as a function of time. The absorbance of the fermentation medium was measured at 580 nm. The values obtained were correlated with the dry mass of probiotic produced during fermentation. The dry mass of probiotic was estimated by the method of (Li & Mira de Orduña, 2010). Sucrose, the source of carbon was determined by the sucrose Assay Kit (MAK013) (Sigma Aldrich, UK).

Effect of *Lactobacillus rhamnosus* (IS9) on the infected one-day old chick model

Chicks not infected with pathogens were obtained from a local farm. They were placed in a wooden cage and were fed according to standard rations. Their watering was done using tap water. Chicks were regularly observed and weighed. A total of 90 uninfected chicks aged one day were arbitrarily divided into 3 groups A, B and C of 30 chicks each. Group A chicks were orally administered by gavage as described by Allen-Vercoe and Woodward (1999) for 24 h with 10^9 CFU / ml of *Lactobacillus rhamnosus* (IS9) suspended in 0.1 ml of sterile water. Whereas those of group B and C were respectively orally dosed by gavage with 10^5 CFU / ml of *Salmonella* Enteridis and *Salmonella* Typhimurium suspended in 0.1 ml of PBS. For the ages of 1, 7, 14 and 35 days post-inoculation, 7 birds were randomly selected from each of the 3 groups. They were slaughtered and the microbiological analysis of their gastrointestinal tract was performed by the enumeration of *Salmonella*.

Effect of *Lactobacillus rhamnosus* (IS9) on the infected 14 days old chick model

A total of 60 uninfected chicks aged 14 days were arbitrarily divided into 3 groups D, E

and F of 20 chicks each. Group D chicks were orally administered by gavage as described by Allen-Vercoe and Woodward (1999) for 24 h with 10^9 CFU / ml of *Lactobacillus rhamnosus* (IS9) suspended in 0.1 ml of sterile water. Whereas those in group E and F were respectively orally dosed by oral gavage with 10^5 CFU / ml of *Salmonella* Enteridis and *Salmonella* Typhimurium suspended in 0.1 ml of PBS buffer. For the ages of 1, 7 14 and 35 days post-inoculation, 7 birds were randomly selected in each of the 3 groups in view of microbiological analyzes.

Enumeration of *Salmonella* in tissues

The birds were slaughtered by cervical dislocation. The liver, duodenum, jejunum, ileum, colon and caeca were removed aseptically from each bird and placed separately in a sterile bottle.

Each organ was homogenized in an appropriate volume of PBS buffer so as to have a dilution factor of 1/10. The enumeration of viable *Salmonella* was carried out by surface seeding on the *Salmonella-Shigella* agar (SS agar) (Liofilchem s.r.l. Bacteriology Products).

Semi-quantitative enumeration of *Salmonella* was carried out using the semi-quantitative approach of (Smith & Tucker, 1980). Caeca swabs were taken at weekly intervals from 24 h after challenge. Swabs were taken from the remaining birds selected at random at each time point. The *Salmonella* were enumerated on *Salmonella-Shigella* (SS) agar.

Statistical analysis

The statistical analysis of the colonization of the different parts of the digestive tract of birds by the *Salmonella* strains was carried out for the birds which received the oral administration of the probiotic and those

which did not receive. Differences were compared in the liver, duodenum etc. using the Mann-Whitney non parametric test.

Results and Discussion

Production of probiotic biomass and control of their viability

The culture of *L. rhamnosus* (IS9) strain in the prepared fermenting medium showed growth materialized in Fig. 1 by three main phases. A logarithmic phase (exponential) characterized by an accelerated growth of the strain. This phase was within the first 18 h of fermentation. During this phase, production of probiotic biomass reached approximately 3.8 g of dry *L. rhamnosus* (IS9) cells per liter of fermenting medium. Viability tests using methylene blue showed that about 98-100% of cells observed after centrifugation and recovery were viable. The production of probiotic biomass was stabilized between 18 and 24 h after the start of the fermentation. Beyond 30 h, there was a decrease in biomass (Fig. 1). Sucrose, the main carbon source used by the strain *L. rhamnosus* (IS9) during its growth, was dosed (Fig. 1). This figure shows that the initial concentration of sucrose decreased as time progressed and finally reached about 4% (w/v) after 60 h of fermentation.

Effect of temperature, pH, rotation rate and inoculum size on the production of probiotic biomass

The study of the effect of variation of temperature on the production of probiotic biomass showed that the production was optimal for temperatures between 35 and 40 °C with a maximum at 37°C. The optimum pH for the production of biomass were ranged between 5.0 and 4.0. The size of the inoculum influenced the production of biomass. The biomass changed as the concentration of the

inoculum increased to a maximum (about 3.8 g / l) when the inoculum concentration was 10^8 CFU / ml (Fig. 2). The biomass also increased with the speed of rotation of the rotary incubator (Fig. 3), it reached an optimal value of 3.7 g/l when the speed was between 150-200 rpm.

***In-vitro* control of the anti-Salmonella activity of the probiotic biomass produced**

The *in-vitro* control tests performed on the ability to inhibit the growth of *Salmonella* strains are shown in the Fig. 4. This result confirms that *L. rhamnosus* (IS9) has the ability to inhibit the growth of several strains of *Salmonella*. The anti-*Salmonella* activity of our *L. rhamnosus* (IS9) was large and comparable to those of some known antibiotics, the inhibition diameters were greater with *S. Typhimurium* compared to *S. Enteridis*.

Effect of predosing birds with *L. rhamnosus* (IS9) on the colonization and persistence of *S. Enteridis* in the one-day-old chick model

Table 1 shows the results of administration of the probiotic strain in infected birds. The animal model used here is the one-day-old chick. These results show the microbial load of the gastrointestinal tract in chicks infected with *S. Enteridis* and those pre-dosed with *L. rhamnosus*. The organs analyzed in this study were: liver, duodenum, jejunum ileum, colon, caeca. The rate of colonization by the pathogenic strain *S. Enteridis* varies from one organ to another and also with the post-inoculation duration.

In the liver, one day after inoculation, there was no statistically significant difference ($P = 0.712$) between chicks infected with *S. Enteridis* and those infected after pre-dosing with *L. rhamnosus* (IS9). On the other hand,

at the end of the seventh day until the 35th day, there was a significant reduction ($P < 0.05$) of *S. Enteridis* in the liver of the pre-assayed chicks with the strain *L. rhamnosus* (IS9). It can also be seen that from the 7th day post-inoculation, the number of salmonellae is almost nil in the liver of the chicks pre-dosed by oral gavage with the probiotic strain, confirming once again the significant suppression of *S. Enteridis* in the liver of chicks given oral gavage of *L. rhamnosus* (IS9).

At the level of the duodenum, the bacterial load is greater than that obtained in the liver. As before, a significant reduction ($P < 0.05$) of *Salmonella* in the duodenum of chicks previously administered the probiotic *L. rhamnosus* strain (IS9) was observed. Further microbiological analyzes of the gastrointestinal tract in infected or uninfected chicks show that the colon compared to other organs is richer in microorganisms. All the same, a significant reduction was observed on day 7 post-inoculation and beyond.

Concerning the effect of the probiotic strain (*L. rhamnosus* IS9) on *S. Typhimurium*, the results (not shown) were similar to those obtained with *S. Enteridis*. But with a higher rate of reduction.

Analysis of weekly caeca swabs of chicks pre-dosed with *L. rhamnosus* (IS9) is presented in Figure 5. A significant reduction in salmonellae ($P < 0.05$) is observed. While the Caecal population of lactobacilli increases.

Probiotics are microbial strains useful to humans and animals. These strains have medical and pharmaceutical interests (Eser *et al.*, 2012; Forssten *et al.*, 2011; Vandenplas *et al.*, 2013; Veldman, 1992; Vyas & Ranganathan, 2012; Wang *et al.*, 2014; Wasilewski *et al.*, 2015).

Table.1 Colonization of 1-day-old chick model by *Salmonella* Enteritidis with and without lactobacilli predose

Days Post-inoculation	Treatment	Tissue Type	Positive tissue	Mean	SD	P-value
1	S.ent. alone	Liver	7/7	1.349	0.021	
1	S. ent +IS9		5/7	1.330	0.410	0.712
7	S.ent. alone		7/7	3.530	0.368	
7	S. ent +IS9		0/7	0	0	0.024
14	S.ent. alone		7/7	2.089	0.046	
14	S. ent +IS9		0/7	0	0	0.031
35	S.ent. alone		7/7	2.292	0.020	
35	S. ent +IS9		0/7	0	0	0.034
1	S.ent. alone	Duodenum	7/7	2.383	0.035	
1	S. ent +IS9		3/7	1.08	0.002	0.100
7	S.ent. alone		7/7	3.99	0.050	
7	S. ent +IS9		0	0	0	0.042
14	S.ent. alone		2/7	2.191	0.124	
14	S. ent +IS9		0	0	0	0.031
35	S.ent. alone		3/7	5.284	0.038	
35	S. ent +IS9		0	0	0	0.023
1	S.ent. alone	Jejunum	7/7	1.385	0.012	
1	S. ent +IS9		1/7	1.108	0.019	0.741
7	S.ent. alone		6/7	4.0466	0.0518	
7	S. ent +IS9		0	0	0	0.025
14	S.ent. alone		7/7	2.168	0.118	
14	S. ent +IS9		0	0	0	0.047
35	S.ent. alone		5/7	2.325	0.023	
35	S. ent +IS9		0	0	0	0.0251
1	S.ent. alone	Ileum	7/7	1.337	0.081	
1	S. ent +IS9		3/7	1.269	0.017	0.056
7	S.ent. alone		7/7	3.957	0.049	
7	S. ent +IS9		1/7	1.052	0.028	0.064
14	S.ent. alone		7/7	3.434	0.070	
14	S. ent +IS9		0/7	0.754	0.0159	0.031
35	S.ent. alone		5/7	4.116	0.080	
35	S. ent +IS9		0/7	0.517	0.005	0.0123
1	S.ent. alone	Colon	7/7	1.725	0.047	
1	S. ent +IS9		3/7	0	0	0.341
7	S.ent. alone		7/7	3.090	0.024	

7	S. ent +IS9		1/7	1.341	0	0.316
14	S.ent. alone		7/7	4.160	0.05	
14	S. ent +IS9		0	0	0	0.017
35	S.ent. alone		7/10	5.261	0.036	
35	S. ent +IS9		0/7	0	0	0.315

Table 1(Continued)

Days Post-inoculation	Treatment	Tissue Type	Positive tissue	Mean	SD	P-value
1	S.ent. alone	Ceaca	7/7	4.384	0.062	
1	S. ent +IS9		6	0.317	0.007	0.023
7	S.ent. alone		7	4.805	0.051	
7	S. ent +IS9		1/7	0.532		0.017
14	S.ent. alone		7/7	4.924	0.084	
14	S. ent +IS9		1/7	0.191	0.006	0.034
35	S.ent. alone		5/7	5.483	0.407	
35	S. ent +IS9		0/7	0.6386	0.037	0.014

S. ent., *Salmonella* Enteridis; IS9, *Lactobacillus rhamnosus*

Fig.1 Time course for the probiotic biomass (*Lactobacillus rhamnosus* IS9) production and sucrose consumption when fermenting medium used was made of 2% (w/v) molasse supplemented with 1% (w/v) soya bean flour. Values are an average of three replicates ± standard deviation

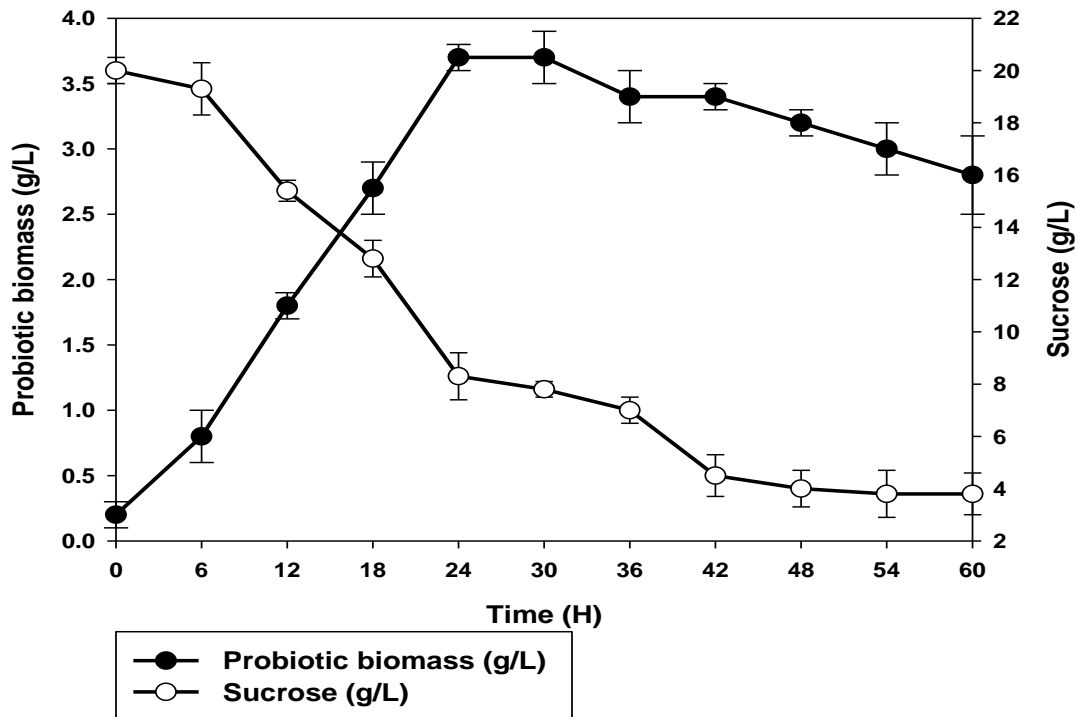


Fig.2 Effect of temperature and pH on probiotic biomass (*Lactobacillus fermentum* IS9) production. Values are an average of three replicates \pm standard deviation

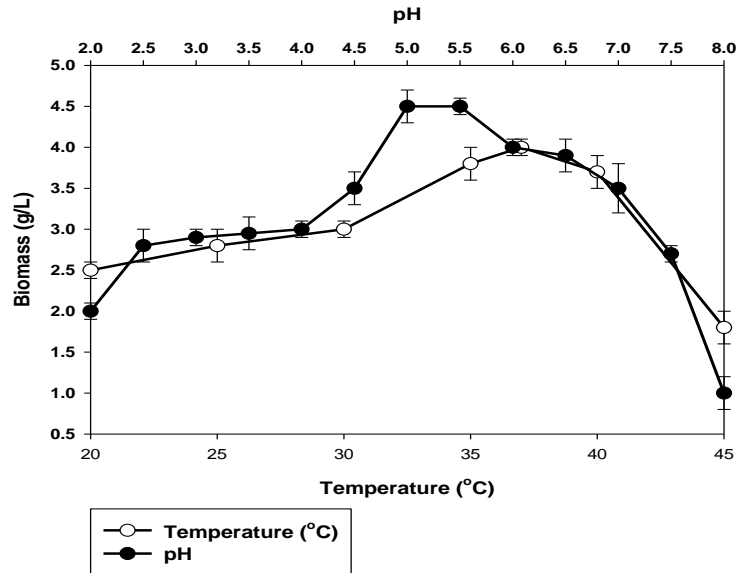


Fig.3 Effect of inoculum concentration and rotation speed of the rotary incubator on probiotic biomass (*Lactobacillus rhamnosus* IS9) production. Values are an average of three replicates \pm standard deviation

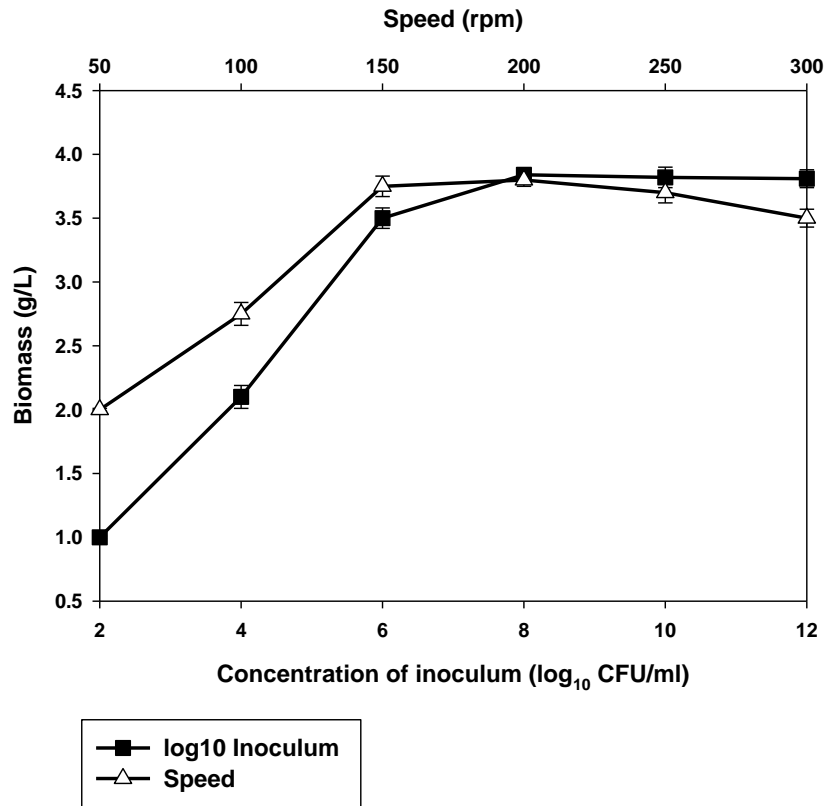


Fig.4 Plate assays for antimicrobial activity of cell free supernatant (CFS) from *Lactobacillus rhamnosus* (IS9) culture against *Salmonella* Enteridis and *Salmonella* Typhimurium after probiotic biomass production and recovery

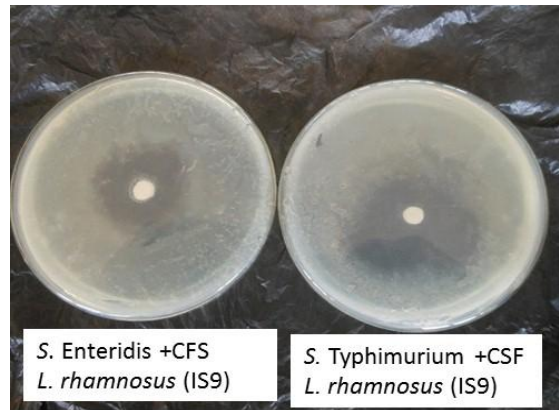
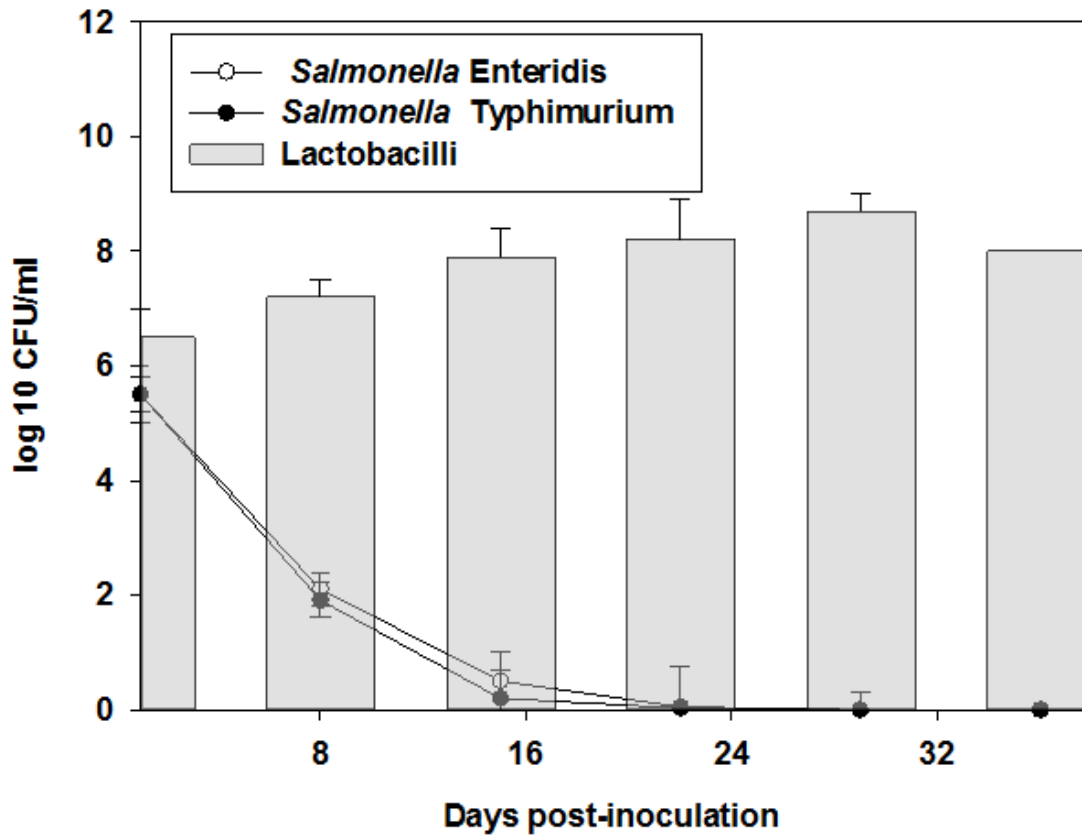


Fig.5 *Salmonella* and lactobacilli count in caeca swabs from chicks pre-dosed with *L. rhamnosus* (IS9)



These reasons explain the interest of many works pursued in this research area. As part of our work, we were interested in producing a large probiotic biomass in order to confront it with the occurrence of *Salmonella* in poultry farming in the South west region of Cameroon. Salmonellosis is one of the most important threats to the poultry industry (Vandeplas *et al.*, 2010). Consumption of raw or uncooked poultry products can induce gastroenteritis (Tsai *et al.*, 2005; Tsiouris, 2016; Vandeplas *et al.*, 2010). The prolonged use of antibiotics in the breeding of poultry generally leads to the development of antibiotic-resistant microbial strains. The use of probiotic microbial strains are now seen as a good approach to the prevention and eradication of salmonella-induced gastroenteritis (Williams *et al.*, 2010; Xie *et al.*, 2015). The effectiveness of a probiotic against a foodborne pathogen depends on the concentration of probiotic germs administered (Salminen *et al.*, 2009; Salminen *et al.*, 2010). The number of viable colonies forming unit (CFU) in a probiotic product is critical for its efficacy against pathogenic strains. Most effective probiotic preparations contain about 10^{10} to 10^{12} CFU / g (Coeuret *et al.*, 2004b). It is therefore important to have a high probiotic biomass in order to make the dosage efficient (Aguirre-Ezkauriatza *et al.*, 2010).

The probiotic biomass of the *L. rhamnosus* (IS9) strain obtained in our study is comparable to that obtained by Aguirre-Ezkauriatza *et al.*, (2010). These authors obtained a probiotic biomass of about 3.2 g/l for the first 20 h in batch fermentation using an inexpensive fermentation medium composed of goat's milk. Studies conducted by (Schiraldi *et al.*, 2003) show a number of colonies forming a unit of about 1.5×10^9 CFU / ml. This value is recommended for probiotic products. Most authors who have worked in this aspect have used milk-based media instead. The peculiarity of our research work

was the production of probiotic biomass from a molasse-based medium. Molasses have the characteristic of being cheap in Cameroon, this is one of the reasons that justify the choice of this substrate to obtain an important biomass of *L. rhamnosus* (IS9). Studies carried out by Salminen and van Loveren (2012), have been consistent with our results. These authors have used low-cost media for the multiplication and production of biomass of bifidobacteria. Kibeom *et al.*, (2013) have developed a cheaper alternative corn and molasses medium for the important growth (biomass production) of *Lactobacillus salivarius* (L29). In studying the effect of prebiotic on production of probiotic biomass, Csutak (2010), demonstrated the importance of the various food components that can be used for the multiplication of *L. acidophilus* (LA-5) and *Bifidobacterium* (BB-12). These few examples show the opportunities to develop a cheaper medium that can be implemented for the industrial production of probiotics.

To return to the anti-*Salmonella* activity of our *L. rhamnosus* (IS9) strain, the *in-vitro* activity is large and comparable to those of some known antibiotics, the inhibition diameters are greater with *S. Typhimurium* compared to *S. Enteridis*.

The strain *L. rhamnosus* (IS9) presented and *in vivo* efficacy against *salmonella* demonstrated by a significant reduction in *Salmonella* count in gastro-intestinal tract of one-day old and 14-days old pre-dosed chick model challenged with *S. Enteridis* and *S. Typhimurium*. Similar observations have been made by La Ragione and Nabad (2004), these authors showed that *L. Johnsonii* (F19785) colonized the gastrointestinal tract of poultry which result in reduction of *S. Enteridis* and significant reduction ($P < 0.01$) of *Clostridium perfringens* also common in poultry. Tsai *et al.*, (2005) showed the

antagonistic effect of *Lactobacillus* strain LAD5 and LF33 against *Salmonella* (S. Typhimurium) invasion to cultured human intestinal cell line Int 407 and to mouse B ALB/c liver and spleen. Laukova *et al.*, (2015) showed that *Enterococcus faecium* AL41 administered orally to ostriches led to a significant reduction in coagulase positive and negative *Staphylococcus*, coliform and pseudomonas like in the gastro intestinal tract of ostriches. Most of the probiotic tested in poultry have been isolated from their gastro intestinal tract (Svetoch *et al.*, 2009) while *L. rhamnosus* (IS9) tested in our study is a palm wine isolate. The used of exogenous or allochthonous bacteria with probiotic properties is not too common in poultry. Whatever their origin, the use of probiotics is very useful in poultry. It could help to reduce the intensive use of antibiotic that cause the development of antibiotic resistant strains. These examples highlighted the possibility of controlling pathogens in poultry using probiotic bacteria.

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