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African Journal of Microbiology Research

Full Length Research Paper

Effects of *Lactobacillus* spp. isolated from the sap of palm tree *Elaeis guineensis* (palm wine) on cellular and innate immunity

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The isolation, characterization and effects of *lactobacillus* spp. from fresh palm wine was undertaken in this study. The aim of this study was to isolate and investigate the *in vivo* effects of the isolates on innate and cellular immune system. The result of the phenotypic and genotypic characterizations showed the presence of *Lactobacillus brevis, Lactobacillus paracasei subsp. Tolerans, Lactobacillus paracasei* and *Lactobacillus yonginensis,* which are members of *Lactobacillus* spp., isolated. The effects of the isolates on innate and cellular immunity were investigated, using two models namely: *in vivo* leukocytes mobilization rate (LMR) and delayed type hypersensitivity response (DTHR). The different isolates had very significant effect on cellular immunity as represented by their ability to stimulate delayed type hypersensitivity response in treated rats. They caused a percentage increase of 300, 600, 600, 500 and 650% for *L. brevis, L. paracasei subsp. Tolerans, L. paracasei, L. yonginensis* and positive control respectively when compared with the negative control. Similarly, the various isolates also affected significantly the innate immune system through their marked influence on the total leukocyte count according to the result of the experiment. They were able to produce a percentage increase of 213, 204, 188.6, and 152.5% for *L. paracasei, L. yonginensis, L. paracasei subsp. Tolerans* and *L. brevis* respectively; while the positive control produced 83.8% increase when compared with the negative control. The result of this study showed that the four *lactobacillus* spp. isolated from fresh palm wine significantly affected the innate and cellular component of the immune system positively.

Key words: Delayed type, hypersensitivity response, innate immunity, cellular immunity, leukocyte mobilization.

INTRODUCTION

The popular quotation by Hippocrates adjudged the father of medicine "let food be thy medicine, and let medicine be thy food" hundreds of years ago is now gaining popularity. Thus, in recent time the idea of food having medicinal value has been given the name "functional food". The recent trend of an increasing research in the area of probiotics with demonstrated therapeutic evidence has also given impetus to the

concept of functional food. There is now an increased demand for food/products that have the capacity to enhance health, beyond providing basic nutrition, since humans are now aware of the relationship between diet, lifestyle and good health. This functionality of food has been linked to the presence of certain beneficial bacterial especially lactic acid bacteria (LAB) that are generally recognized as safe (GRAS) bacteria. Besides the nutritional values, ingestion of Lactic acid bacteria (LAB) and their fermented foods has been suggested to confer a range of health benefits including immune system modulation, increased resistance to malignancy, and infectious illness (Soccol et al., 2010). These bacteria have also been linked with improvement in lactose utilization, anti-cholesterol, and production of bacteriocin an effective antimicrobial compound that is of immense benefit to man (Krishnendra et al*.,* 2013; Aween et al*.,* 2012). Some of the benefits of these bacteria have been exploited in its co-administration with antibiotics. They are shown to improve antibiotic therapy as they reduce microbial adhesion and growth by bacteriocins or other inhibitory compounds, possess immunomodulatory properties, and improve intestinal barrier integrity (Reid, 2006). It also promote the recovery of commensal microbiota and increase treatment tolerability in patients on antibiotic therapy (Boyanova and Mitov, 2012). It has become obvious that the need for alternative/ advancement in antibiotic therapy cannot be over emphasized. This is because antibiotics are now losing their effectiveness, particularly due to overuse, misuse and subsequently to the increasing development of antibiotic resistance (D`Souza et al., 2002). These beneficial bacteria have been seen recently as valuable adjunct to antibiotic therapy since continued or excessive use of antibiotics is known to disrupt the normal micro flora of the human body, which they have the capacity to reverse. They are also a veritable tool in the handling of some of the side effects of antibiotics therapy such as antibiotic-associated diarrhoea (AAD) (Cremonini et al., 2002; Armuzzi et al., 2001; Vanderhoof et al., 1999; Arvola et al., 1999).

Palm wine is the collective name for a group of alcoholic beverages, whitish in color and obtained through natural fermentation of the sap of *Elaeis guineensis* (Uzochukwu et al*.,* 1991). Yeast, lactic acid bacteria and acetic acid bacteria are involved in the production of palm wine. The beverage is linked with high content of amino acid, potassium, zinc and iron (Carousel, 2015). It also contains B1, B2, B3, and B6 vitamins and have been linked with increased sperm and breast milk (Mbuagbaw and Noorduyn, 2012). Palm wine is a drink that is common in Southern part of Nigeria because of its central role in most traditional ceremonies. Its use in this ceremonies/social functions is due to its alcohol intoxicating effect. Palm wine also have been linked with somewhat health benefits where amongst some locals, children with suspected cases of measles infection are given fresh palm wine and it's believed to reduce reasonably high fever associated with measles within few hours. It is used extensively as a galactogogue (substances that are capable of stimulating breast milk production) in women newly delivered of their babies. These two common uses of the beverage in our local communities motivated us to seek possible scientific explanation to ascertain the veracity of the claim and to possibly look at the best way to exploit the benefit(s).

MATERIALS AND METHODS

The equipment and instruments used include hot air oven (Genlab thermal engineering, Transhouse lane Widness, Cheshire), autoclave (Shenan LDZX-50FB, England), incubator (Genlab thermal engineering, Transhouse lane Widness, Cheshire), binocular microscope (Olympus), and anaerobic jar (Oxoid, UK). Also included were refrigerator, weighing balance (Adventurer, Ohaus Corp. Pine brook, USA), wire loop, sample containers, micropipette, syringe and needle, surgical gloves, measuring cylinders, Petri dishes, beakers, conical flask, Durham tube, distilled water, Deionized water, 70% ethanol, gentian violet, lugol's iodine, safranin, malachyte green, hydrogen peroxide, glucose, sucrose,lactose, and Linex capsule (lyophilized lactobacillus capsules). Ovalbumin, goat anti-mouse IgG^{Fab} HRP(Southern biotech, USA), Goat anti-mouse $IgG1^{Fab}$ HRP(Southern biotech, USA), Goat anti-mouse $IgG2a^{Fab}$ HRP(Southern biotech, USA), Fat free milk, Tween-20, Phenol red, Immersion oil, Dmso solution, dibasic sodium phosphate, citric acid solution, and TMB substrate tablet (Sigma-Aldrich USA) were also part of the materials used. Sodium hydroxide and Tetraoxosulphate vi acid,animals and organisms used in the study include Sap of the oil palm tree (*E. guinneesis)* palm wine, Wistar albino mice (28-30 g), Wistar albino rat (90-120 g), lactic acid bacteria, modified De Man Rogosa and Sharpe (MRS) Agar (Himedia, India) were also used.

Collection of palm wine

The fresh palm wine samples were collected in the morning around 6:30 am from a local palm wine tapper, Mr Anthony Idoko, in Onicha-Enugu Ezike in Igbo Eze North local Government Area of Enugu state. The samples were kept under cold conditions, using ice pack to reduce the rate of fermentation while being transported to the laboratory.

Preparation of media and culturing of lactic acid bacteria

The MRS agar media used were prepared following manufacturer`s specifications. The test organisms were cultivated from *E. guineensis* sap (Palm wine) using streak plate method. A wire loop was used to collect a loopful of the homogenized Palm wine samples and streaked on the surface of solidified MRS media under aseptic conditions. The inoculated media were incubated

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anaerobically using anaerobic jar at 37°C for 24 to 48 h.

Isolation of lactic acid bacteria

A loopful of distinct colonies formed on the solidified media during culturing were subculture on sterile modified MRS agar plate by quadrant streaking method, under aseptic conditions. After streaking, all the petri dishes were incubated at 37°C for 24 to 48 h. After the incubation, colonies were sub cultured on MRS agar to obtain pure cultures. The purified isolates were streaked on MRS agar slants and stored at 4°C for further use.

Phenotypic characterization of culture

The isolates were presumptively identified based on cultural, morphological and some biochemical characteristics. The parameters investigated included colony morphology, Gram reactions, endospore formation, catalase production, motility, and sugar fermentation. The results were compared to Holt et al. (1994) Bergey's Manual of Determinative Bacteriology.

Genotypic characterization of the Isolate

DNA extraction and PCR amplification of the isolates

DNA Extraction was carried out on test organisms' isolated from Palm wine using the Jena Bioscience Bacteria DNA Preparation Kit (http://www.jenabioscience.com).

Polymerase chain reaction was carried out to identify the suspected lactic acid bacteria isolated from palm wine using the primer pair BSF8 (AGAGTTTGATCCTGGCTCAG) and BSR534 (ATTACCGCGGCTGCTGC) The primer pair are lactic acid specific. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5x concentration to 1X concentration containing 1X Blend Master mix buffer Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25 pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5 µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture.

Thermal cycling was conducted in a Peltier thermal cycler (PTC100) (MJ Research Series) for an initial denaturation of 95°C for 15 min, followed by 35 amplification cycles of 30 s at 95°C; 1 min. at 58°C and 1 min 30 s at 72°C. This was followed by a final extension step of 10 min at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 h 30 min. After electrophoresis, DNA bands were visualized using ethidium bromide staining. 100bp DNA ladder was used as DNA molecular weight standard.

Identification of the isolates

All the isolates were identified using 16S rRNA. All PCR products were purified and sent to Epoch Life science (USA) for Sanger sequencing. The corresponding sequences were identified using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi

Immunological studies

Grouping and dosing of the animals

The animals were grouped into 13 groups of 5 animals in a group. The first 11 groups received 3×10^7 CFU and 9×10^7CFU of each of the isolates determined by 0.5 McFarland standard for experiments involving mice and rats respectively. The 12th and the 13th group represent positive and negative control groups respectively. The positive control receives linex capsule a brand of lyophilized *Lactobacillus* spp. at a dose of 7.2×10^5 and 1.2×10^5 for experiments involving rats and mice respectively.

Studies on delayed type hypersensitivity response (DTHR)

Delayed type hypersensitivity was induced in rat using sheep red blood cells (SRBC) as antigen. Animals were sensitized by subcutaneous injection of 0.02 ml of 1×10^9 cells ml^{-1} SRBC (day 0) in the plantar region of right hind foot paw and challenged on day 5 by subcutaneous injection of same amount of antigen into the left hind paw. The oedema produced by antigenic challenge in the left hind paw was taken as the difference in the paw thickness before and 24 h after the challenge. The paw thickness was measured by utilizing volume displacement of water. LAB isolates were administered 3 days prior to sensitization and continued at a daily dose of 9×10^7 cfu until the challenge (Naved et al., 2005; Shinde et al., 1999).

Study on in vivo leucocyte migration rate

The method of Ribeiro et al., (1991) was utilized in the *in vivo* leucocyte migration study. The *in vivo* leucocyte migration was induced by inflammatory stimulus. One hour after oral administration of the 3×10^7 cfu of the LAB isolates, each mice in the groups received intraperitoneal injections of 0.5 ml of 3% (w/v) agar suspension in normal saline. Four hours later, the mice were sacrificed and the peritoneum washed with 5 ml of a 5% solution of EDTA in phosphate buffered saline (PBS). The peritoneal fluid was recovered and total (TLC) performed on the perfusates. The cells were counted with the help of Atacus 30 haematology autoanalyser

Statistical analysis

The statistical analysis was done using Graph Pad prism version 5.0. One-way ANOVA followed by Post-hoc Dunnet was used to compare mean ± SEM, and values were considered significant at $p < 0.05$.

RESULTS

Morphological and biochemical characteristics of isolated lactic acid bacteria from palm wine

The morphological, cultural and biochemical characteristics of the isolated bacteria from palm wine are as shown in the (Table 1).

Genotypic characterization and blasted sequence results of the isolates

The result of the blasted sequenced results showed the presence of four species of Lactobacillus namely: *L. brevis*, *L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensi.*

Table 1. Morphological and biochemical characteristics of the isolates.

Suspected organism	Gram stain	Endospore test	Catalase	Motility	Glucose fermentation
1	+	-	-	-	+/G $^{-ve}$
2	+	-	-	-	+/G $^{-ve}$
3	+	-	-	-	+/G $^{-ve}$
4	+	-	-	-	+/G $^{-ve}$

(+), positive; (-), negative; +/G $^{+ve}$, gas production= heterofermentation; +/G $^{-ve}$, without gas production= homofermentation.

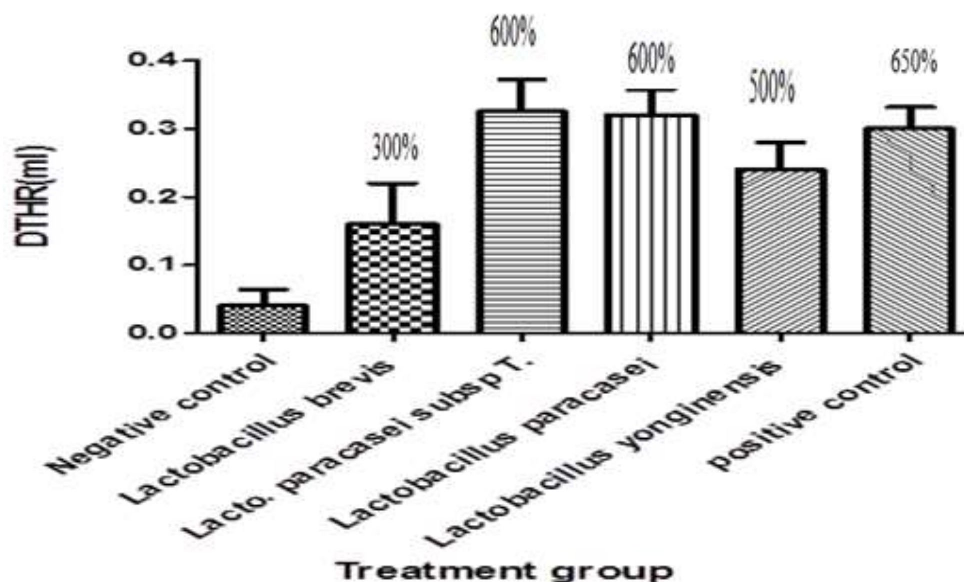

Figure 1. The effect of Lactobacillus spp. on delayed type hypersensitivity response (DTHR) in rats.

The effects of *Lactobacillus* spp. on Delayed type hypersensitivity response in rats (DTHR)

Figure 1 shows the effect of *Lactobacillus* spp. on DTHR in rats. The four *Lactobacillus* spp. produced a significant percentage increase in stimulation of DTHR in rats challenged with sheep red blood cell as antigen when compared with the negative control group. It produced a percentage increase of between 600 to 300% when compared with the negative control.

The effects of *Lactobacillus* spp. on Total Leukocytes count (TLC) on albino mice

The effect of *Lactobacillus* spp. on total leukocyte count (TLC) that mobilized to the site of injury was determined from the peritoneal washout of the sacrificed animals and the TLC determined with the aid of Atacus 30 haematology autoanalyser. The result showed that there was significant percent increase on the amount of Leukocytes mobilized to the site of injury for the *Lactobacillus* spp. isolates (Figure 2).

DISCUSSION

Alcohol consumption has always been a public health problem. Globally, alcohol use is the third leading risk factor for poor health and causes an estimated 2.5 million deaths per year (Mikolajczyk et al., 2016). However, most of the reports on deleterious effect of alcohol on our health are linked to synthetic alcohols and not necessarily from natural alcoholic beverages. This is because in the case of palm wine, when freshly tapped, palm wine has little to no alcoholic content and is said to offer several nutritional benefits to the human body. The main ingredient of the fresh palm sap is sucrose, which is about 12 to 15% by weight with little reducing sugar such as glucose, fructose, maltose and raffinose, in addition to

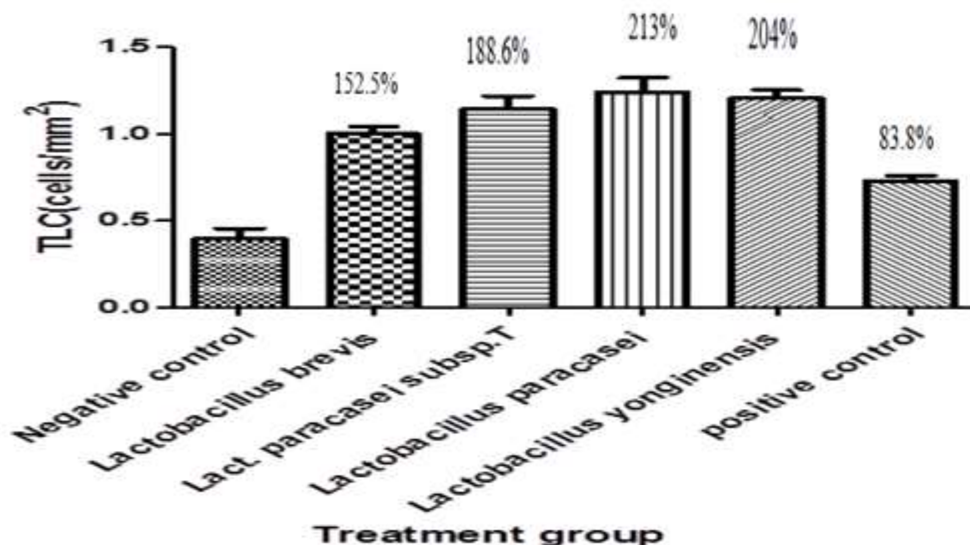

Figure 2. The effects of *Lactobacillus* spp. on Total Leukocytes count (TLC) on albino mice.

sugar, the sap also contains protein, fat and mineral matter (Ezeagu et al., 2003). The sap can be regarded as a safe (GRAS) and beneficial lactic acid bacteria spp.

This study isolated, characterized and investigated the effects of lactobacillus spp. from palm wine on the cellular and innate immune systems. *Lactobacillus brevis, L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensis* were the species of the genera *Lactobacillus* isolated from the sampled Palm wine. Lactobacilli are of significant technological importance as they are involved in the manufacturing of several fermented and non-fermented foods, and have been used as probiotics due to their health-promoting effects. Probiotic lactobacilli have been associated with the prevention and treatment of gastrointestinal disorders, such as rotavirus diarrhea, antibiotic-associated diarrhea, and travelers' diarrhea, and have been suggested as potential therapeutic agents against irritable bowel syndrome and inflammatory bowel disease (Lomax and Calder, 2009). *Lactobacillus* species are the most extensively studied Lactic acid bacteria that have gained importance in the clinics as a therapeutic agent where lyophilized form of the organism is used in the treatment of diarrhea. The ability of lactobacilli to adhere to the gastrointestinal mucosa has been suggested to influence their interaction with the host and the other bacteria present, by affecting the local microbial composition and/or by stimulating the host's immune system (Qin et al., 2009). The effects of the isolates on cellular and innate immune systems were investigated utilizing models that represent the two different components of immunity namely innate immunity (*In vivo* leukocyte mobilization rate); cell mediated immunity (delayed type hypersensitivity response). The isolates *L. brevis, L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensis* produced a percentage stimulation of DTHR by 300, 600, 600 and 500 respectively when compared with the negative control (Figure 1). Delayed hypersensitivity reactions are inflam-matory reactions initiated by mononuclear leukocytes. The term delayed is used to differentiate a secondary cellular response, which appears 48 to 72 h after antigen exposure, from an immediate hypersensitivity response, which generally appears within 12 min of an antigen challenge (Abramson et al., 2018). These reactions are mediated by T cells and monocytes/ macrophages rather than by antibodies. They are also termed type IV hypersensitivity reactions. Delayed hypersensitivity is a major mechanism of defense against various intracellular pathogens, including mycobacteria, fungi, and certain parasites, and it occurs in transplant rejection and tumor immunity (Abramson and Kaliner, 2018). The cellular events that result in delayed hypersensitivity reactions primarily involve T cells and macrophages. First, local immune and inflammatory responses at the site of foreign antigen up-regulate endothelial cell adhesion molecule expression; promoting the accumulation of leukocytes at the tissue site. The antigen is engulfed by macrophages and monocytes and is processed and presented to a T cell that has a specific receptor for that processed antigen (Abramson and Kaliner, 2018).

The various species of lactobacillus isolated from the study showed a significant increase of 152.5, 188.6, 213 and 204% for *L. brevis, L. paracasei subsp. Tolerans, L. paracasei* and *L. yonginensis* respectively for leukocyte that migrated to the site of injury (Figure 2). Circulating blood leukocytes are required to migrate to sites of tissue injury and infection with the principal aim of eliminating the primary inflammatory trigger and contributing to tissue repair. In innate immunity, this process is largely initiated by pathogen-associated molecular patterns (PAMPs),

released by invading microorganisms, and damage-associated molecular patterns (DAMPs), derived from damaged and/or dead-cells, or in response to tissue and/or cellular stress (Medzhitov, 2008). In addition, antigens, largely through activation of resident memory T cells, can trigger recruitment of leukocytes via secretion of various primary inflammatory cytokines. Tissue sentinel cells, including mast cells, macrophages, and dendritic cells (DCs), play a key role in detection of such danger signals and can release a wide range of pro-inflammatory mediators to promote leukocyte recruitment. The high values recorded from the result is in agreement with the postulation that the success of lactobacillus spp. as an immunomodulator is linked to its ability to bind to PRR expressed on immune cells and the cascade of other events that follows (Wells et al*.,* 2010; Abreu, 2010; Kawai and Akira, 2009).

The use of fresh palm wine by local dwellers in the communities to treat children with measles has been an age long practice. Here, children with suspected cases of measles are usually given some amount of fresh palm wine orally and most times the high fever associated with measles subsides immediately. The result of the effect of the isolates on the respective aspects of the immune system may now give us an insight and possible scientific explanation on the use of fresh palm wine in children with measles. The result clearly showed that the isolates had a marked effect on cellular immunity (T-cells) and innate immunity. It is established that measles is an infection involving intra-cellular pathogens (viruses), and intracellular pathogens are absolutely handled by T-lymphocytes. Cell-mediated immunity attributable to T cells is the principal mechanism whereby intracellular organisms are eliminated by macrophages activated by γ-interferon derived from T cells. This beverage use for measles is strongly believed that it is linked to its ability to harbor these beneficial immune stimulatory organisms.

It is also an old practice by locals to administer fresh palm wine to women newly delivered of their babies as lactation enhance. Prolactin the hormone that controls lactation was originally identified as a neuroendocrine hormone of pituitary origin. However, its synthesis is not limited to the hypothesis since numerous extra pituitary tissues also express this protein, including the placenta, ovary, testis, mammary gland, skin, adipose tissue, endothelial cells, and immune cells (macrophages, natural killer cells, T-cells and B- cells) (Harvey et al*.,* 2012). Therefore, it is obvious that the significant stimulation of both the T cell and the innate immune system by these lactic acid bacteria found in this natural beverage may be the scientific explanation of its lactation enhancing potential. All these enumerated health benefits of *Lactobacilus* spp. and many other industrial benefits can be maximally harnessed without the obvious public health challenges of alcohol. This is achievable through moderate intake of sweet non-fermented/slightly fermented *E. guinneesis* sap (Palm wine) since alcoholic fermentation is a process that is time dependent, and the process convert sugar rich beverage by these beneficial bacteria to alcohol and finally to ethanoic acid.

Conclusion

Fresh *E. guinneesis* sap (palm wine) harbor *Lactobacillus* spp. with Immune enhancing property and these enormous benefits can be exploited through moderate intake of the beverage in a fresh and non-fermented state.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Identification of *fli*C and flagella expression in *Salmonella enterica* subspecies *enterica* serovar *Gallinarum biovar* gallinarum

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***Salmonella enterica* subsp. *enterica* ser. *Gallinarum biovar*. gallinarum (*S. gallinarum*) is the causative agent of fowl Typhoid, a bacterial disease that affects domestic and wild birds. For many years, *S.* gallinarum was defined as an aflagellated bacterium, that is, non-motile. However, the closely related strain *Salmonella pullorum* is capable of expressing a flagellum-like filament. Since these bacteria are practically identical, it was determined if *S.* gallinarum expresses flagellum-like filament under different culture conditions in order to increase the basic knowledge of this bacterium. The motility of *S.* gallinarum was evaluated in different culture media (GI, S and nutrient media), and visualized the bacterial filaments using electron microscopy. *fli*C was identified in some of the studied strains; subsequently, sequencing analysis of *fli*C gene shown 98% of identity with that in *S.* Typhimurium. In the present study, the presence of a flagellum-like structure was demonstrate in different strains of *S.* gallinarum using electron microscopy. In addition, the *fli*C gene was amplified, which allowed us to suggest that this bacterium is capable to shows a flagellum under certain culture conditions, similar to that reported for *S.* pullorum.**

Key words: *fli*C, flagella, *S.* gallinarum, motility, medium-dextrose.

INTRODUCTION

Salmonella enterica subsp. enterica ser. Gallinarum biovar. Gallinarum (Grimont and Weill, 2007) causes fowl Typhoid (Barrow and Freitas, 2011). This disease affects poultry, mainly chickens, turkeys, and pheasants, as well as wild birds, which act as natural reservoirs (Barrow and Freitas, 2011; Foley et al., 2011). The differences in susceptibility and resistance to avian typhoid are related to the virulence of the strain and genetic background of the host, the young birds are most susceptible to the disease, whereas adult birds may have asymptomatic infection (Barrow and Freitas, 2011; Fraser and Hughes, 1999).

Due to the importance of fowl typhoid in Mexico, the government implemented the National Campaign

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against Avian Salmonellosis under the NOM-005-ZOO-1993. This campaign has helped to significantly reduce fowl typhoid cases.

The bacteria of the *Salmonella* genus are short, Gram-negative bacilli; they are aerobes or facultative anaerobes, some display a capsule, do not form spores and are generally motile due the presence of peritrichous flagella (Barrow and Freitas, 2011). The bacteria use different mechanisms to colonize and survive within the host (Monack et al., 1996; Fraser and Hughes, 1999; Marimoto and Minamino, 2014). In this regard, it is suggested that *S*. gallinarum release the outer membrane protein A in the presence of heat-labile serum components, as a mechanism to distract the immune system (Vega-Manriquez et al., 2016). To survive, the bacteria have developed various adaptive mechanisms, including motility (Marimoto and Minamino, 2014). This mechanism makes them more efficient for nutrient acquisition, avoidance of toxic substances, and in the case of pathogenic bacteria allows finding optimal colonization sites to establish a symbiotic relationship with the host (Monack et al., 1996; Fraser and Hughes, 1999).

The flagellum is clinically important because it presents antigenic variability (Marimoto and Minamino, 2014); this structure is critical for chemotaxis because it is responsible for bacterial motility (Fraser and Hughes, 1999). The flagellum is a long helical filament, propelled by a rotary engine embedded in the cell envelope (Marimoto and Minamino, 2014). The basic structure of flagellum consists of the extracellular filament, which is a homopolymer formed by thousands of subunits of FliC or flagellin (Marimoto and Minamino, 2014). The filament ends in a hook that connects it with the cell's engine; this hook is flexible, extracellular and is a homopolymer of 120 to 130 copies of the FlgE protein8 (Marimoto and Minamino, 2014). Finally, the basal body is a multiprotein complex embedded in the cell membrane, wherein the flagella motor is located; it consists of a central axis and four ring-shaped complexes (Marimoto and Minamino, 2014).

There are more than 50 genes involved in the processes of assembly, regulation, rotation and movement of flagella (Marimoto and Minamino, 2014). It is known that expression of flagellar operon is regulated by diverse stimulus such as temperature, grade of DNA supercoiling, phospholipids, OmpR proteins (which act in response to osmolarity changes), DnaJ, K, GrpE (corresponding to thermal shock response), cell division, and the catabolite repression through cAMP levels (Iino, 1969; Wang et al., 2005). For example, if the glucose concentration is high, cAMP levels decrease and the master operon is not expressed (Iino, 1969; Wang et al., 2005; Marimoto and Minamino, 2014).

It has been reported that epinephrine plays an important role in stimulating motility in *S*. Typhimurium, so it seems that it is playing a key role in the mechanism of Quorum Sensing. This would trigger the activation of genes related to motility such as fliC owing to the fact that the presence of this neurotransmitter increases its expression 21 times (Conceição et al., 2015).

In strain z66 of S. Typhi, a significant expression of 187 genes has been observed when the bacterium is in contact with Anti-z66, among these genes *fli*C is highlighted as its expression is reduced more than three times (Zhang et al., 2009). There are some controversial results regarding the participation of the flagellum in the interaction with the host cell, as it depends on the serovar studied, the flagellum changes its participation in the infection phase, as shown in the results found in *S*. Typhimurium *fli*C mutated which is not able to induce an inflammatory response. Opposite to this, *S*. Dublin independently if the gene is present or not, does not to participate in the induction of the inflammatory response, so this structure would not be participating in the systemic phase of the serovar (Olsen et al., 2013). Based on these references, suggests that regulation or expression of the flagellum depends on the serovar or may be different according to the serovar or even according to the environment.

S. enterica subsp. *enterica* ser. Gallinarum biovar. pullorum are considered non-motile bacteria. However, in 1993 Kilger and Grimont conducted a study in *S.* gallinarum and *S.* pullorum where they demonstrated the presence of the *fli*C gene (corresponding to the first part of the flagellum) by PCR (Kilger and Grimont, 1993). They suggested that this gene encodes antigens G and M in these serovars (Kilger and Grimont, 1993). Li et al. (1993) identified and sequenced the complete *fli*C gene in *S.* gallinarum and *S.* pullorum (Li et al., 1993). In addition, Holt and Chaubal (1997) reported the development of flagella-like fibrous filaments in *S.* pullorum grow in special culture media; they also determine that this bacterium belonged to the serogroup D of the Kauffmann-White classification.

Non-mobile strains of Salmonella are known that presents the *fli*C gene, but do not form a functional flagella (Poppof and Le Minor, 1997). However, because the gene has a hypervariable central region, that allows, through the PCR-FRLP technique, to characterize and differentiate strains of these two biovarities (Paiva et al., 2009; Cheraghchi et al., 2014). In studies with a motile mutant of *S*. gallinarum, showed that this strain has the ability to induce an inflammatory response, suggesting that has an increased ability to colonize (de Freitas Neto et al., 2013).

In this study the presence of flagella in *Salmonella* gallinarum were demonstrated and their expression induction when the strain in cultured in specific media. It was confirmed the presence of flagella by electron microscopy and sequencing of *fli*C gene. This study contributes to the knowledge about these presence and expression of flagella in this microorganism.

MATERIALS AND METHODS

Bacterial strains

For this study *Escherichia coli*, ATCC1946, wild type *Salmonella* Typhimurium, eight *Salmonella* gallinarum strains (ATCC9184, FVB323, FVB347, FVB383, FVB47, FVB41 and SC) were use. *Salmonella* pullorum ATCC10398 and *S*. pullorum wild type (SP). *Salmonella* Enteritidis ATCC49214, a wild type *Salmonella* Enteritidis and *Staphylococcus aureus* beta-toxin producer.

Culture media

Culture media were used according to methodology reported by Holt et al. (1997). For motility assays tubes and plates of semisolid medium of beef heart infusion with 0.3% agar (Motility GI Medium, Difco, 38800, France) were used; semisolid medium brain and heart infusion with 0.3% agar (Motility Medium S, Difco, 38800, France) and nutrient medium with 0.5% agar (Nutrient Broth, Merck, 64271, Germany). All culture media were used alone or supplemented with 0.5% dextrose.

Motility assay

Strains were inoculated in isolation in plates with agar A (0.7% nutrient broth, 0.1% yeast extract, 0.2% glycerol, 200 mM $K2HPO4$ and 9 mM $KH2PO4$), and were incubated for 24 h at 37 °C. Inoculation in the culture tubes was made using the needle technique; meanwhile, in the agar plates were used the needle technique and by single streak. The cultures were incubated at 37°C; growths were recorded by photographs taken at 24, 48 and 72 h of incubation. After that, 1 to 3 colonies of each strain were inoculated in GI, S, or nutrient medium, alone or supplemented with 0.5% and were incubated at 37 or 42°C for 24, 48 and 72 h. The diameter of the growth halos was measured at 24, 48 and 72 h of incubation. A photographic register also recorded the growth and motility of these cultures.

DNA extraction

All strains were inoculated in 5 ml of broth A and DNA was extracted using the modified guanidine thiocyanate method described by Mozioglu et al. (2014), with minor modifications.

Detection of *fliC* by PCR

The presence of *fliC* gene was determined by PCR using the primers reported by Kilger and Grimont (1993), (forward: 5'-AAGGAAAAGATCATGGCA, reverse: 5'-TTAACGCAGTAAAGAGAG). The PCR was performed in a SelectCycler™ II Thermal Cycler (Select Bio Products, USA) under the following cycling conditions: initial denaturation cycle at 94°C for 5 min, 30 cycles consisting of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, and final extension at 72°C for 7 min. With these conditions, a 1500 bp fragment was obtained. A DNA concentration of 2-3 ng/µl and 0.5 µM primers were use in this technique.

Restriction assay

To verify if the fragment amplified by PCR correspond to *fli*C, a restriction analysis was performed with *Eco*RV and *Kpn*I according to the manufacturer's recommendations. The reaction was incubated at 37°C for 1.30 h. The restriction profile of wild-type *S.* Typhimurium and in *S.* gallinarum ATCC9184 were used as a reference. The resulting fragments were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide. An *in silico* restriction profile prediction was performed using *fli*C of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 (NCBI Reference Sequence: NC_003197.1, 1488 bp) and *Salmonella enterica* subsp. *enterica* serovar Gallinarum str. 287/91 (GenBank: AM933173.1, 1518 bp).

DNA sequencing

To verify the identity of *fli*C of *S.* gallinarum ATCC9184, the amplicon was purified using the QIAquick® Gel Extraction Kit (Qiagen, USA) according to manufacturer recommendations, and the nucleotide sequences were obtained in an automatic sequencer (Sequencing Unit, Institute of Cellular Physiology, UNAM). The sequences obtained were analyzed using Blast® software and compared with the reported sequences of *fliC* in *E. coli* str.K-12 substr. M61655 (NCBI Reference Sequence: NC_000913.2, 1497 pb), *Salmonella enterica* subsp. *enteric* serovar Enteritidis str. SGSC2475 (GenBank: AY649709.1, 1518 pb), *Salmonella enterica* subsp. *enteric* serovar Typhimurium str. LT2 (NCBI Reference Sequence: NC_003197.1, 1488 pb), *Salmonella enterica* subsp. *enteric* serovar Gallinarum str. 287/9 (GenBank: AM933173.1, 1518 pb) and *Salmonella enterica* subsp. enteric serovar Gallinarum str. ST16 incomplete sequence (GenBank: DQ838253.1, 1272 bp).

Electron microscopy

To evidence the presence of flagella by Transmission Electron Microscopy, *S.* Enteritidis ATCC49214, *S.* gallinarum FVB323 and *S.* gallinarum ATCC9184 were growth in GI medium with 0.5% dextrose for 48 h at 37°C. Once the bacterial colonies grew, a swab of each culture was taken, resuspended it in 1ml of sterile distilled water and then stained with 2% uranyl using charcoal grills for visualization. The samples were analyzed in the Transmission Electron Microscopy facility at the Faculty of Veterinary Medicine, UNAM (Mexico City, Mexico), for staining.

RESULTS

Grown in different media

To induce the expression of flagella, all the strains were growth in GI, S, and nutrient media, alone or supplemented with dextrose. It was found that after 48 h of culture in the GI medium supplemented with 0.5% dextrose (GI+D), *Salmonella* gallinarum FVB323 showed wavy expansion and growth effects, characteristic of motile bacteria, compared to the smooth growth of non-motile bacteria. The strongest undulate and irregular growth was observed in plates inoculated by the needle technique while no significant differences were detected in the tubes inoculated by the needle technique (images not showed).

Differences in the growth morphology of the strains cultured in GI medium was observed in comparison with those cultured in GI+D medium. The colonial form of strain growth in GI medium was smooth while in GI+D it was wavy, rhizoidal or lobate (Figures 1 and 2). Following 48 h of incubation in GI medium, an undulated and

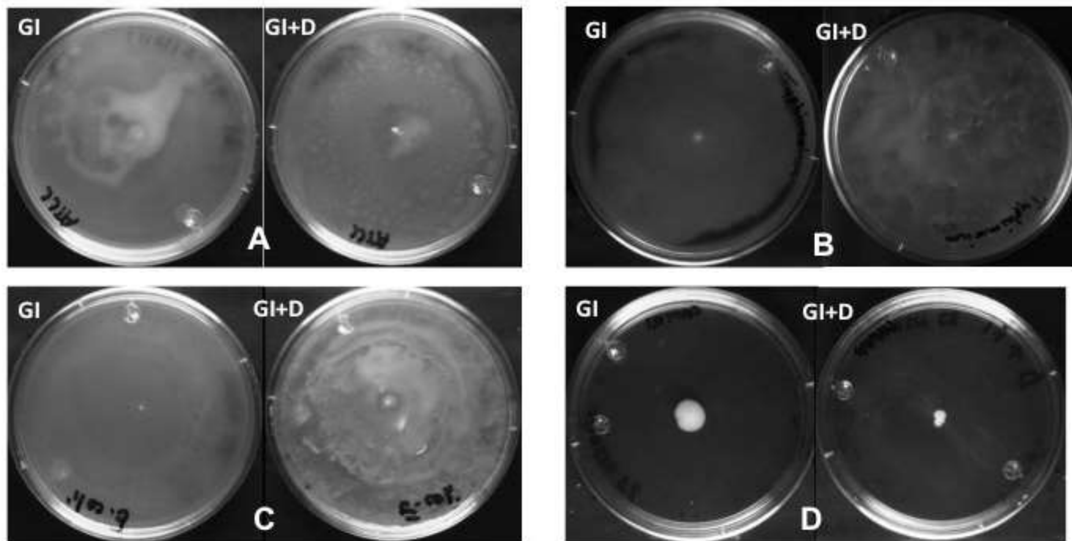

Figure 1. Effect of dextrose on growth and motility of *Salmonella* sp. Strains were cultured in agar plates of GI medium, alone and supplemented with 0.5% dextrose (GI+D) and were incubated for 48 h at 37°C. Panel A shows *Salmonella* Enteritidis ATCC, panel B shows *Salmonella* Typhimurium, panel C shows *Escherichia coli* and panel D shows *Staphylococcus aureus* β-toxin. Strains were inoculated by the needle technique. The effect of wavy growth and expansion of motile bacteria can be better observed, compared with non-motile bacteria in GI medium alone (GI) and supplemented with dextrose (GI+D).

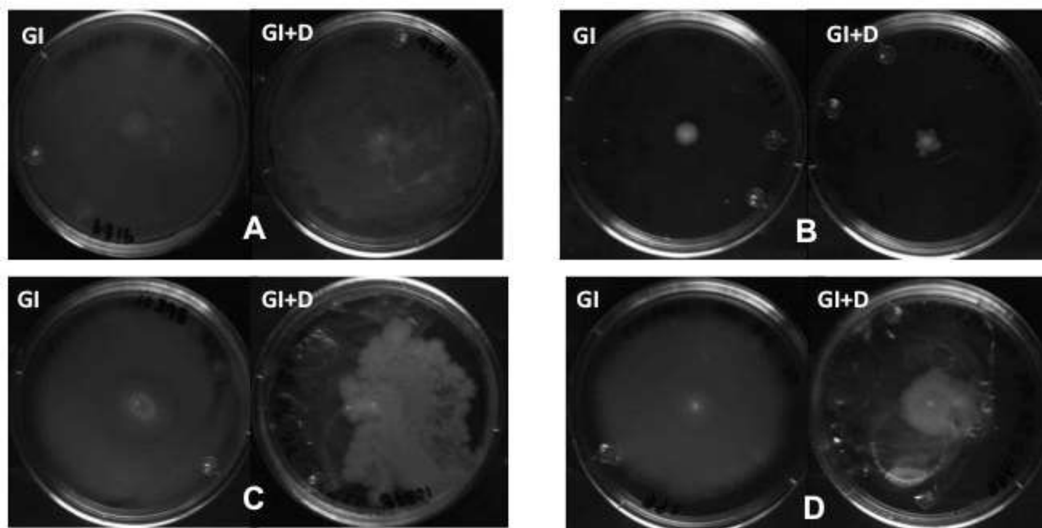

Figure 2. Culture of strains of *Salmonella* gallinarum and *Salmonella* pullorum in GI medium alone and supplemented with 0.5% dextrose in plates at 48 h at 37° C. Panel A shows *Salmonella* gallinarum ATCC9184, panel B *Salmonella* gallinarum FVB323, panel C *Salmonella* pullorum ATCC10398 and panel D *Salmonella* pullorum (SP).

irregular growth pattern at the surface of culture plates in most of the *S*. pullorum and *S*. gallinarum strains was observe, except for the FVB323, FVB383 and FVB47 strains (Figure 3B and D). The temperature had minimal effect on motility since a similar result was observed in the cultures incubated at 37 and 42°C. However, addition of dextrose to GI medium favors the observation of motility (Figures 3 and 4).

The wavy expansion displayed by motile bacteria was evident in the ATCC9184, ATCC10398 and SP strains (Figure 2). The FVB323 strain growth in GI+D

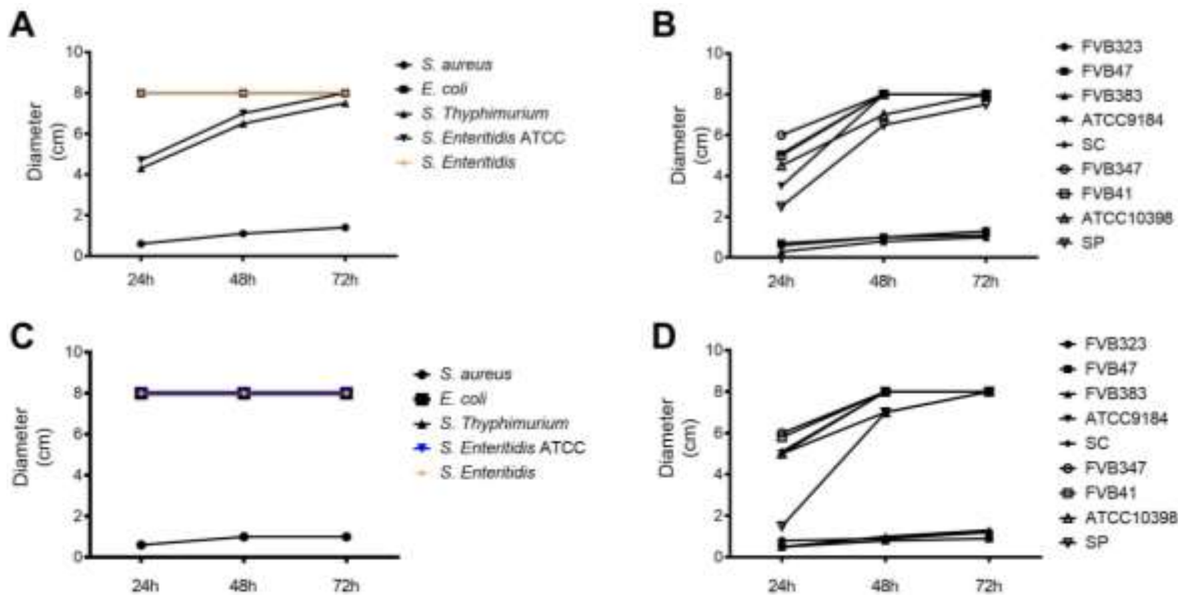

Figure 3. Comparison of halos of growth of *S.* gallinarum and *S.* pullorum. Strains were cultured in GI medium alone (upper panels) or supplemented with 0.5% dextrose (lower panels) at 37°C. Diameter of the halos of growth was measure at 24, 48 and 72 h.

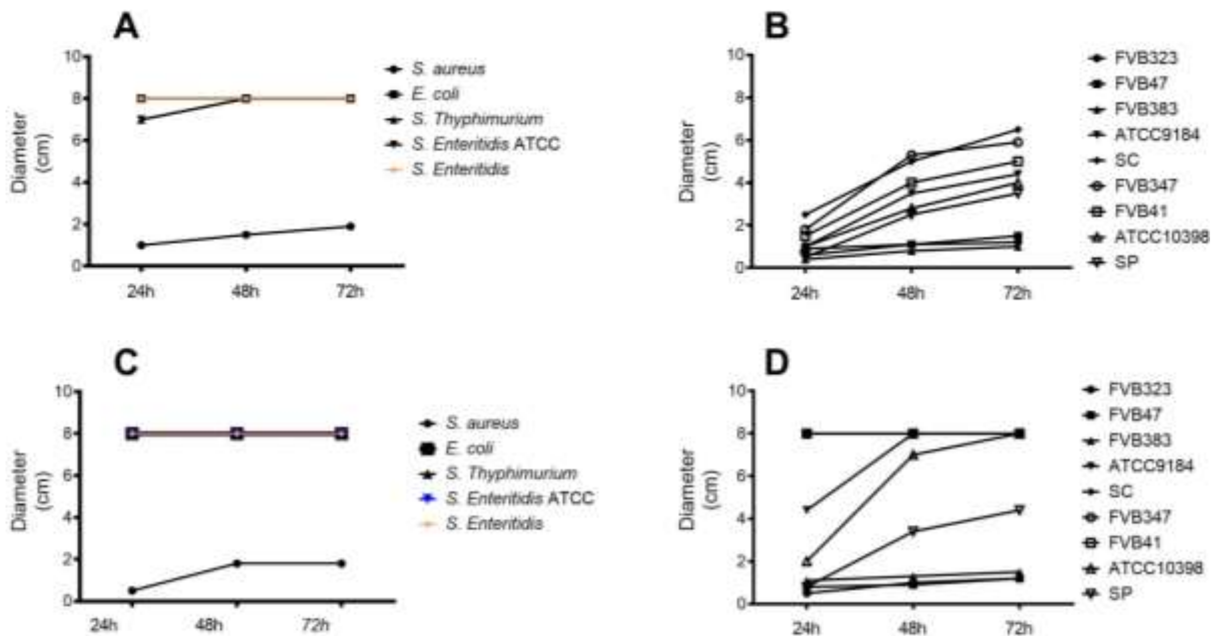

Figure 4. Determination of the diameter of control bacteria and strains of *S.* gallinarum and*S.* pullorum. Strain were cultured in GI medium alone (A and B) and GI medium supplemented with 0.5% dextrose (C and D) at 42°C at 24, 48 and 72 h of incubation.

medium showed the wavy expansion effect observed in motile bacteria (Figure 2B). On the other hand, the growth of FVB323 strain in GI medium was similar to that observed in *S. aureus* (Figure 1D). Additionally, the presence of gas was observed in strains of *S.* pullorum in GI+D medium (Figure 2C and D).

The diameter of halo growth between strains (Figures 3B and 4B) were compared, and observe that almost all *Salmonella* gallinarum strains resemble motile bacteria, except for FVB323, FVB383 and FVB47 strains, which resemble non-motile bacteria (like *S. aureus*). The photographic record clearly illustrates differences in

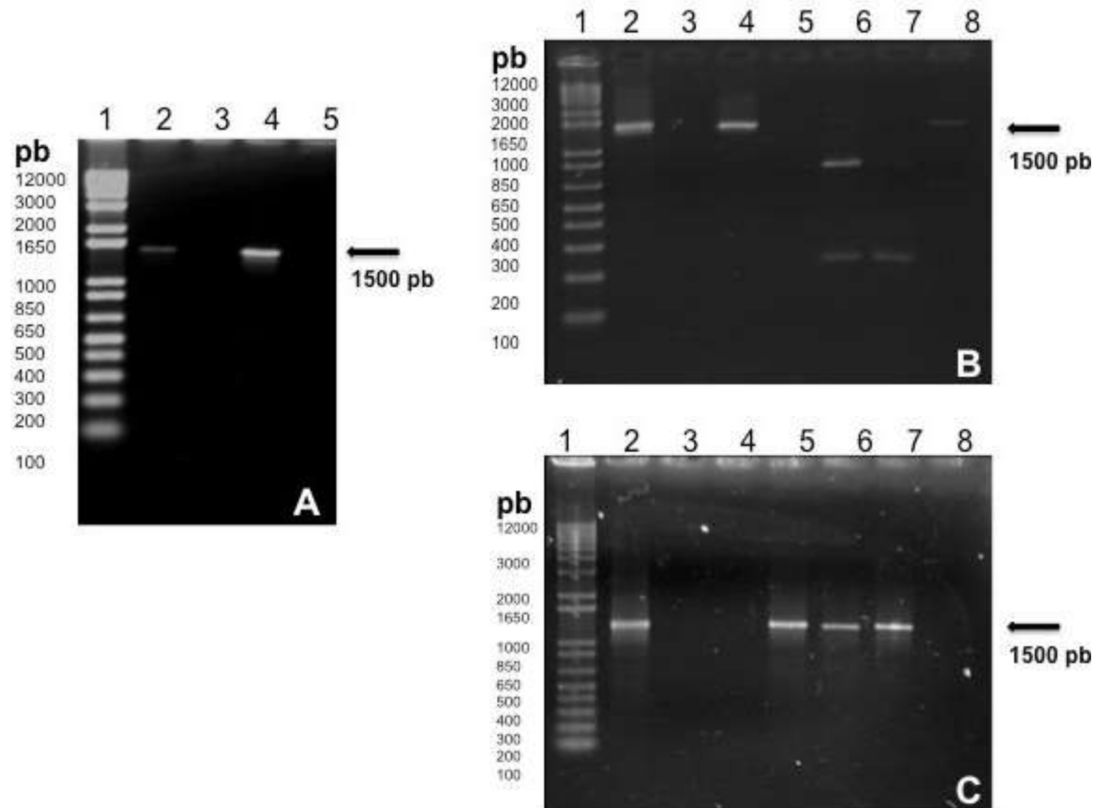

Figure 5. Agarose gels showing the PCR product to amplify *fli*C. Panel A: MWM (lane 1); *S.* Typhimurium (lane 2); *S. aureus* (lane 3); *S.* gallinarum FVB323 (lane 4); and *S.* pullorum ATCC10398 (lane 5). The arrow indicates a 1500 bp amplicon corresponding to *fli*C. Panel B: MWM (lane 1); *S.* Typhimurium (lane 2); *S. aureus* (lane 3); *S.* gallinarum FVB383 (lane 4), *S.* gallinarum FVB347 (lane 5); *E. coli* (lane 6), *S.* Enteritidis wild type (lane 7) and *S.* Enteritidis ATCC (lane 8). Panel C: MWM (lane 1); *S.* Typhimurium (lane 2); *S. aureus* (lane 3); *S.* gallinarum FVB41 (well 4); *S.* gallinarum FVB47 (lane 5); and *S.* pullorum SP (lane 6); *S.* gallinarum ATCC9184 (lane 7); and *S.* gallinarum SC (well 8).

growth morphology between strains cultured in GI medium, with those cultured in medium supplemented with dextrose (Figure 2). The halo of growth of *S.* gallinarum strains was slightly larger in GI+D medium, and the highest growth was observed after 48 h (Figures 2A, B and 3D).

As shown in Figures 3 and 4, the diffusion diameter of the cultures was larger in motile bacteria than in non-motile ones, both in GI and GI+D medium. Motile bacteria showed maximum growth between 24 and 72 h, in contrast with *S. aureus*, which showed the highest growth at 48 h. interestingly, there was no difference between those cultures incubated at 37 or 42°C (Figures 3 and 4A, C). In GI medium, the cultures of *S.* gallinarum FVB323, FVB47 and FVB383 showed a diffusion diameter more like that of *S. aureus* cultures, distinct the other strains, which reassembly that motile bacteria (Figure 3 and 4B, D). The growth morphology of all strains was very similar in GI medium with or without dextrose. The growth of cultures incubated at 42°C was lower to that observed in bacteria incubated at 37°C particularly for SP, ATCC10398, ATCC 9184, FVB41, SC and FVB347 strains (Figure 4B and D). The highest growth was observed in strains cultured for 48 h at 37°C except for the FVB41 strain, which showed maximum growth at 24 h in GI+D medium (Figure 4D).

Identification of *fli*C by PCR and sequencing

Using PCR, a fragment of around 1500 bp was detected in many of the study strains, except for *S. aureus*, *S.* gallinarum strains 347, 41, SC, and *S.* pullorum ATCC10398, in which no amplification was observed. Meanwhile in *E. coli* and *S.* Enteritidis wild type two fragments of approximately 850 and 250 bp were obtained (Figure 5B).

Restriction assay

As was expected, the restriction profile of *fli*C produces

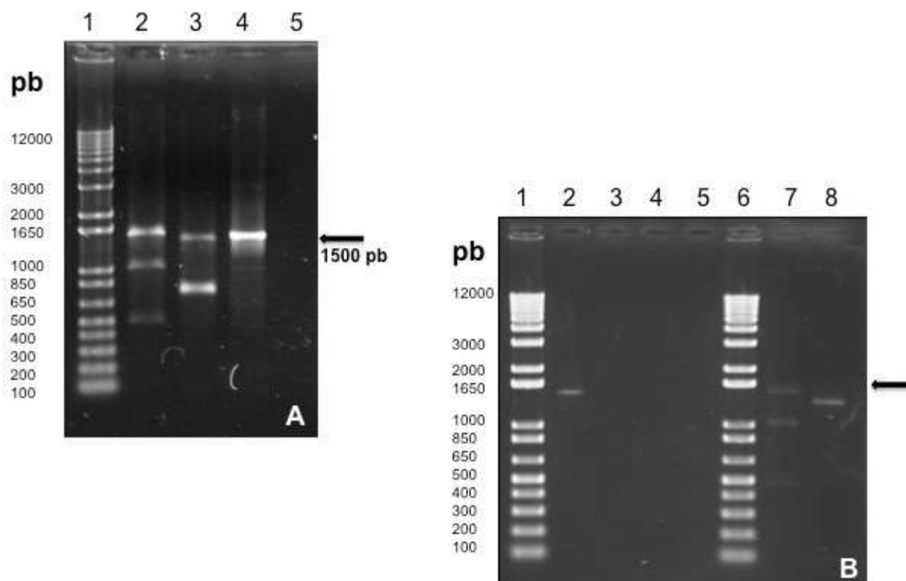

Figure 6. Restriction profile of *fli*C. The *fli*C amplicon of *Salmonella* Typhimurium field strain and *Salmonella* gallinarum ATCC9184 were digested with *Eco*RV or *Kpn*I. Panel A: MWM (lane 1);*S.* Typhimurium wild type (lane 2); *S. Typhimurium* wild type (lane 3); *S.* Typhimurium (lane 4), *fli*C PCR product. Panel B: MWM (lane 1and 6); *S.* gallinarum ATCC9184 (lane 2), the *fli*C PCR product digested with *Eco*RV (lane 7); *fli*C PCR product digested with *Kpn*I (lane 8).

two fragments of 483 and 1017 pb for *Eco*RV and one fragment of 1442 pb for *Kpn*I restriction. The same restriction profile was observed in *S.* gallinarum ATCC9184 and *S.* Typhimurium restricted with *Eco*RV (Figure 6); however, in the case of restriction with *Kpn*I, the expected patrol was only obtained in the *S.* gallinarum ATCC9184 strain (Figure 6B), while in the case of *S.* Typhimurium, restriction assay generates two fragments of approximately 1442 bp and 750 bp (Figure 6A).

Sequencing

A *fli*C PCR product of *Salmonella* gallinarum ATCC9184 (BankIt1674463 SEQ1 KF885733) was sequenced and analyzed using the PSI-Blast® software. The *fli*C genes of *S.* gallinarum ATCC9184 and *S.* Typhimurium showed 99% of identity with *S.* Enteritidis, meanwhile *S.* gallinarum 287/9 and ST showed a 100% identity with *S.* Enteritidis. It is important to note that the sequences of *fli*C of *S.* gallinarum ATCC9184 had a higher identity with *S.* Typhimurium compared with the sequences of *S.* gallinarum (287/9 and ST strains) reported in Genbank, which is identical to *S.* Enteritidis.

Electron microscopy analyzes

To confirm the presence of flagella-like structures in the studied strains, the bacteria cultured in GI+D medium (to stimulate their expression) was analyze by transmission electron microscopy. In *S.* gallinarum ATCC9184 (Figure 7B) filaments similar to the large flagella characteristic of *S.* Enteritis were observed (Figure 7C); interestingly, in *S.* gallinarum FVB323, thick and short filaments were observed, which are different from those observed in *S.* Enteritidis (Figure 7A).

DISCUSSION

This is the first report describing the presence of flagella-like filaments in wild type *Salmonella* gallinarum and ATCC strains. The filaments expression was induced in cultures supplemented with 0.5% dextrose, in which bacterial colony morphology showed a similar growth to that observed in motile bacteria. In addition, the presence of *fliC* gene, involved in flagellar filament formation in *S.* gallinarum FBV323, FBV383, FVB347 and ATCC9184 strains was described.

Motility is an essential property for bacterial colonization; for example, the flagella of *Helicobacter pylori* allow it to move through the gastric mucus to establish itself in the lining of the stomach; in the case of *Salmonella* Enteritidis, adherence to the epithelial surface depends on fimbriae and flagella (Olivares and Gisbert, 2006; de Freitas Neto et al., 2013; Foley et al., 2013; Tomoda et al., 2015). Moreover, flagella cause the activation of proinflammatory cytokines in the host

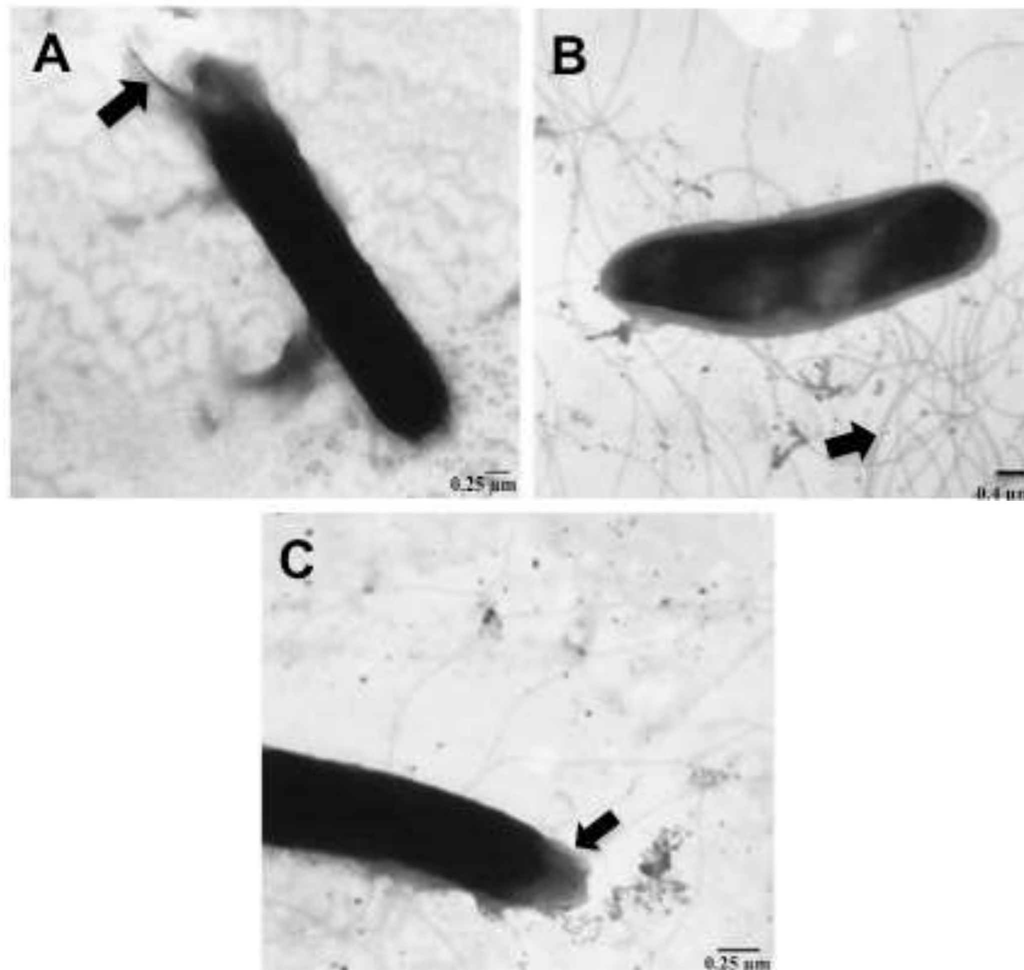

Figure 7. Electron micrograph of *Salmonella* serovars. Strains were stained with negative staining technique contrasted with 2% urany using l30,000X magnification. Panel A: *Salmonella* gallinarum FVB323; panel B: *Salmonella* gallinarum ATCC9184 and panel C: *Salmonella* Enteritidis ATCC49214. The arrows indicate electron-dense filaments emerging from the bacteria (A and C) and loose filaments can be observed in the background (B).

through their recognition by Toll-like receptor 5; however, the bacteria can change their phase and produce a filament with a different protein from that initially recognized by the immune system, and thus evade the immune system, ensuring its establishment in the intestinal epithelium (Santos, 2014; Rogier et al., 2015). In recent studies, participation of the flagellum in the mechanism of respiratory burst in neutrophils was observed in *S*. Typhimurium, because mutated bacteria in *fli*C, *fli*B and *mot*A significantly reduced this event (Westerman et al., 2018). It is also proposed that in order to be unrecognized by the immune system, when under stress signals caused by compounds that act on the cell envelope, such as EDTA or the complement system, it activates *rfl*P transcription and the later, stimulates degradation flagellar proteolytic master regulatory complex $FlhD_4C_2$ by ClpXP protease action. All these events lead to a decrease in the production of the flagellum and loss of motility; this would favor the evasion of the microorganism (Spöring et al., 2018). Study similar to these should be performed in *S*. gallinaurm that presents *fli*C or the complete operon.

In this study, the expression of flagella-like filaments in *Salmonella* gallinarum cultured in a dextrose-rich medium may be related to the regulation of *fli*C. It is known that the regulation of genes by nutrients is an important mechanism of microorganisms to adapt to their nutritional environment, like dextrose, which has been demonstrated, induce the transcription of L-pyruvate kinase genes, fatty acid synthase, acetyl-CoA carboxylase and insulin in humans (Prax and Bestram, 2014). An interesting example of these mechanisms is offered by *V. vulnificus*, who in the presence of glucose, *in vitro*, 87% of the microorganisms do not present flagellum and 13% do develop it but in a short form. In this event, dephosphorylated EIIAGlc is involved and interacts with

FapA, which avoids the biosynthesis of the flagellum and allows the bacteria to remain in the niche rich in glucose; the mechanism is developed through an independent cAMP pathway (Park et al., 2016). On the other hand, it has been reported that in *Saccharomyces cerevisiae*, glucose represses the transcription of a series of genes responsible for respiration, the use of other carbon sources (galactose, maltose, sucrose) and genes encoding enzymes of the gluconeogenic pathway. In contrast, glucose is able to induce genes involved in its own metabolism, especially genes encoding glucose transporters and enzymes of the glycolysis pathway (Meugnier et al., 2007). This work demonstrated that in most strains of *Salmonella* gallinarum, the addition of dextrose to culture medium produces a wavy, rhizoidal or lobate expansion, unlike the media without dextrose in when the bacterial expansion was smooth.

Holt et al. (1997) suggest that the higher growth rate of *S.* gallinarum cultures incubated at 42°C is due to this is the corporal temperature of birds. However, in this study, a decrease in growth rate in cultures incubated at 42°C was observed, contrary to that observed in the motile strains incubated at the same temperature. These discrepancies probably occurred due to the adaptation of the control strains to the *in vitro* culture at this temperature (Foley et al., 2013).

In *S.* gallinarum strain 323, the electron microscopy analyzes confirm the presence of filaments, which appear to be like-fimbriae, since they are small and electron-dense as previously described (Thornley and Horne, 1962). The genome *S.* gallinarum contain 50 genes related to motility and chemotaxis, however, some of them are pseudogenes (de Freitas Neto et al., 2013). By transfecting these genes in wild-type strains, they acquire the ability to produce flagellum and produce a different pathogenesis (Thomson et al., 2008; de Freitas Neto et al., 2013). In this study, the *fliC* gene was analyzed because it is involved in the synthesis of the flagellum, and the protein that encodes forms part of the external structure of flagellar filament. Furthermore, motility was evident in *S*. gallinarum FVB347, FVB41 and SC strains, and in *S.* pullorum ATCC10398 growth in GI and GI+D. However, the *fli*C gene cannot be detected by PCR in these strains; this may be explained by Holt et al. (1997), who argued that the *fli*C gene is not present in all strains of *S*. pullorum; on the other hand Kilger and Grimont (1993) attributed the non-motile phenotype of *S*. Gallinarum-Pullorum to *fliC* gene mutations these could interfere with the detection of the gene by PCR.

Another possibility could be that the *fli*C gene presents phase variation which involves the inversion of a DNA segment from one orientation to another, and a particular gene is expressed when the segment is oriented in one direction, and a different gene is expressed when the fragment is oriented in the opposite direction. Most *Salmonella* strains express both phases while some strains express only one phase (Kilger and Grimont, 1993).

The restriction profile of *fli*C gene of *Salmonella* Typhimurium LT2 digested with *Kpn*I shown an unexpected fragment size, possibly, because the strain analyzed is different to the strain used to predict the restriction profile *in silico*. A previous study reported that the site of the enzymatic cleavage site in might be different according to the bacterial strain (Kilger and Grimont, 1993). Due to the close phylogenetic relationship between different serovars of *Salmonella*, it is not strange that the sequence of *Salmonella* gallinarum ATCC9184 used in this work had greater identity with *Salmonella* Typhimurium. However, it is noteworthy that the sequence of *fli*C obtained in *Salmonella* gallinarum ATCC9184 was incomplete, and represents only half of the sequence reported in *S.* gallinarum 287/9.

Conclusions

This work suggests that *Salmonella* gallinarum develop a motile phenotype under specific culture conditions, and this may be significantly involved in the pathogenesis of the bacteria. In summary, the wavy growth pattern observed in cultures of *Salmonella* gallinarum in media supplemented with dextrose suggests that all *Salmonella* gallinarum strains could present motility under these culture conditions. In addition, the *fli*C gene showed 99% homology with their homolog of *S.* Typhimurium in 4/7 strains of *S.* gallinarum. Finally, our study demonstrated that *S.* gallinarum expressed flagella-like structures after 48h of incubation in media supplemented with dextrose, regards to the temperature of incubation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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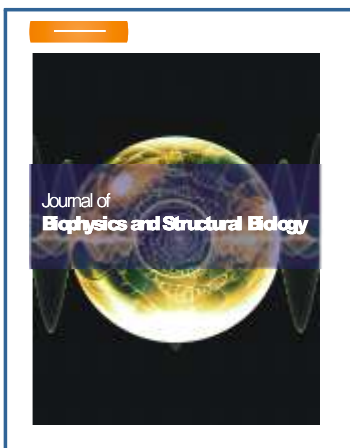

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