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Probiotic Abilities of *Lactobacillus spp.* Isolated from Algerian Fermented Wheat 'Hamoum' Intended to Use in Broiler Production

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ABSTRACT

Background: The use of probiotics has become popular in the broiler poultry industry due to its positive results on the health of animals, as well as the safety of consumers. In general, probiotics are isolated and selected *In vitro* from several sources. Despite this, only a few studies focused on isolating probiotics from cereals, which are the main natural food source for poultry. This study aimed to evaluate and identify an autochthonous *Lactobacillus spp.* isolated from Algerian fermented wheat "Hamoum" as potential probiotics for use in broiler feed.

Methods: This research was conducted over the period between 2019-2022. Five bacterial isolates of the genus *Lactobacillus* were tested for their hemolysis activity, exopolysaccharides production, the ability to withstand the simulated hostile conditions of the chicken's gut and their capacity to acidify the concentrate feed beverage. Finally, molecular identification was performed using the REP-PCR technique.

Result: These isolates were found to produce exopolysaccharides and have no hemolytic activity. Also, could tolerate simulated gastric acidity. However, only three isolates were able to survive in the presence of bile salts and were found to have a high capacity for acidifying medium in a relatively short time. These isolates were identified as *Lactobacillus acidophilus* and *Lactobacillus plantarum subsp plantarum*. Those strains can be considered safe candidate probiotics for use in broiler feeds feed. Further studies are needed to investigate and evaluate the effectiveness of these isolates through an *in vivo* experiment on broiler chickens.

Key words: Acidification ability, Acid resistance, Broilers feed, *Lactobacillus,* Probiotic.

INTRODUCTION

Algeria's poultry sector is important to the country's economy and a key source of protein for the population. However, the increasing costs of compound feed ingredients pose achallenge for the main objective of this industry, which is to produce the maximum amount of output with the minimum inputs (Jadhav *et al.,* 2015). Therefore, great emphasis has been placedon improving the effectiveness of poultry feed, and probiotics have been included as one of the solutions to promote growth, production and immunity, by contributing to gut health and nutrient use and providing beneficial properties to the host (Abd El-Hack *et al.,* 2020).

Probiotics have been reported to modify the gut microbiota, increase gut barrier function and boost broiler immunological response. They also have the ability to increase feed conversion efficiency and minimize the environmental effect of broiler production. However, the selection and administration of probiotics in broilers necessitates careful assessment of the probiotic strain, its mode of action, as well as its safety and efficacy. Probiotic preparations use various species of microorganisms. However, Lactobacilli, Bifidobacteria and Streptococci are the most common bacterial treatments used in the production of probiotics (Divya *et al.,* 2019; Jadhav *et al.,* 2015; Manjari Divya *et al.,* 2018). During selection of a probiotic strain, it is necessary to have a platform of basic information about the origin, genetic makeup and growth characteristics of a candidate strain. Additionally, an evaluation of the safety and benefit-risk ratio associated with their use is required (Shewale *et al.,* 2014; Markowiak and Slizewska, 2018).

Fermentation techniques have been used to produce safe products that have specific nutritional and functional attributes, either to produce a large number of microbial cells or to produce extracellular microbial products compounds (Tsafrakidou *et al.,* 2020). As a model, "Hamoum" is an Algerian popular fermented food that is traditionally made from, it is also a rich source of lactic acid bacteria with potential probiotic properties. It is prepared by storing the

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grains in an artisanal underground granary called "Matmora" for more than one year (Tahlaiti *et al.,* 2017).

This study aims to evaluate and identify auto chthonous *Lactobacillus spp.* Isolated from fermented wheat "Hamoum" as potential probiotic candidates, for use in broiler poultry. The study employed a multi-faceted approach, including technological properties testing and molecular identification, in order to select the best performing isolates. The technological properties were presented by exopolysaccharides production, hemolytic activity, acidity and bile salts tolerance and ability of the isolates to acidify a medium through the production of organic acids. Finally, molecular identification using REP-PCR technique was used to identify the selected isolates.

MATERIALS AND METHODS

This research was conducted over the period between 2019-2022, at the Laboratory of Animal Production Sciences and Techniques, University of Abdelhamid Ibn Badis Mostaganem, Algeria.

In this study, lactobacilli were carried from the Laboratory of Animal Production Sciences and Techniques collection, which were isolated from Algerian fermented wheat known as "Hamoum" and previously characterized using the classical techniques of microbiology. The purity of isolates was assured three times on MRS agar with pH 6.5 at 37°C for 48 hour. Microscopic observation was assessed, then bacteria that were rod-shaped, were tested for gram-positive and catalase-negative to considerate a bacteria of the genus *Lactobacillus* (Mohammad *et al.,* 2018). Totally five isolates named CX-02, CX-10, CX-13, CX-17 and CX-22 were selected to test *in-vitro.*

The production of exopolysaccharides by the isolates was evaluated using the method described by (Bounaama *et al.,* 2022; Ketrouci *et al.,* 2021). This involved observing the emergence of mucoid properties for colonies. However, in this study, the exopolysaccharides production was tested using a composed agar-concentrate feed beverage which is prepared by dissolving 42.25 g of commercial concentrated feed fortified with vitamins (E.P.E Poultry Group of the West, Unit of Mostaganem, Algeria), as well as 10 g of glucose and 10 g of agar, in approximately 1 liter of distilled water, boiled for 30 minutes at 100°C filtered using a 0.1 mm strainer and sterilized at 121°C for 20 min.

The hemolytic activity was performed by culturing the isolates on Columbia agar plates supplemented with 5% sheep blood and incubating at 37°C for 48 h. The plates were then examined for the presence of hemolysis, isolates that displayed γ-hemolysis (no zones around colonies) were considered safe for use as probiotics (Royan *et al.,* 2021).

The acid tolerance were evaluated using a mixture of the protocols described by (Nazila *et al.,* 2016; Jin *et al.,* 1998; Hassanzadazar *et al.,* 2012 ; Mohammad *et al.,* 2018). The acid resistance of the fiveisolates was evaluated by testing their tolerance to pH 3 in an MRS medium adjusted with HCl.The isolates were incubated in MRS broth at pH 6.5 for 18 h at 37°C, then 100 µl of each isolate was transferred to 9.9 ml of MRS broth at pH 3 and control tubes at pH 6.5 incubated at 37°C for 120 minutes. Then, 100 µl from each tube (pH 3.0 and 6.5) of each isolate was transferred to new tubes containing 10 ml of MRS broth at pH 6.5 and incubated under the same conditions for 6 hours, cells were harvested by centrifugation (4300 g for 10 min at 4°C) and washed three times in sterile saline solution (0.85% NaCl). The absorbance at 600 nm (OD600) was measured for the tested tubes at pH 3.0 and compared with the control tubes at pH 6.5. The difference in growth was considered as the rate of delay in the growth of the isolates due to acidity.

Bile salts resistance was tested using the same methodology, but at a concentration of 0.3% (pH 6.5) for a period of 240 min.

The acidification ability of the isolates was determined by measuring the degree of acidity at 0, 2, 4, 6 and 24 h using a 0.1 mol NaOH titration method and measuring pH values (Fawzi *et al.,* 2022)., Test tubes containing 10 ml of a liquid concentrate feed beverage (without agar) were inoculated with 50 µl of fresh bacterial cultures and incubated at 37°C. The titratable acidity was calculated based on lactate molecular weight, which is the main organic acid, using the equation provided by (Matela *et al.,* 2019).

$$\text{Titrable acidity} = \frac{\text{Vol NaOH(ml)} \times \text{0.1N NaOH} \times \text{melliequivalent factor}}{\text{Vol of simple (ml)}}$$

90.08 g / mol are the lactate molecular weight.

The molecular identification by the REP-PCR technique, developed by the BIO-RAD laboratories and adopted by (Gevers *et al.,* 2001; Dahou, 2017) was applicated. According to the manufacturer's instructions, a genomic DNA extraction of bacterial isolates was performed using the (Kit DNA Extraction IQ-Check BIO-RAD). DNA concentration and purity were controlled with (Thermo Scientific™ 840-210600 spectrophotometer). The amplification of the DNA fragments obtained from the extraction was carried out on a Biorad cycler (REP-PCR: The 96-well T100 thermal cycler. Biorad, USA), using a specific primers (Qbiogène Research Service Germany) and (IQ-Check PCR Detection BIO-RAD Kit) which allowed amplifying the DNA fragment encoding the 16SRNA region, with the amplification operation described by Bounaama *et al.,* (2022). Only isolates those having the potential probiotic properties were identified. The primers which used in this study were 5′-GTAAATCTGTTGG TTCCGCT-3′3′ATGGCTGCTCGCGTCTTTAA-5′for *Lactobacillus acidophilus* and 5′-TCTAATGTAAATCAT GATGG-3′3′-GCCCATCTTTAAGACCATCG-5′for *Lactobacillus plantarum*. Also, the reference strains used were *Lactobacillus plantarum* subsp *plantarum* ATCC 14917 and *Lactobacillus acidophilus* ATCC 4356.

Graphical representations were performed using Microsoft Office Excel. Results represented in the graphs were calculated using the average of three repetitions of tests.

RESULTS AND DISCUSSION

All isolates were capable of producing exopolysaccharides. And none of the tested isolates displayed hemolytic activity.

Acidity resistance

The graph in Fig 1 illustrates the acid tolerance of the five selected isolates at pH 3 for 120 minutes compared to the control at pH 6.5. Spectrophotometer readings at optical density OD 600 nm indicate that all control isolates recorded similar readings, with an estimated OD600nm of 1. Isolates CX-02 and CX-10 exhibited partial tolerance, with average spectrophotometer readings of 0.35 and 0.38 respectively, indicating lower survival rates than the control. In contrast, isolates CX-13, CX-17 and CX-22 displayed superior tolerance, as they recorded higher readings of OD 600nm = 1, 1.32 and 1.02 respectively, indicating higher survival rates than the control.

Bile salts resistance

In terms of tolerance to bile salts, the graph in Fig 2 illustrates partial tolerance of isolates CX-13, CX-02 and CX-10. Spectrophotometer readings recorded average OD values of 0.38 for CX-13, 0.24 and 0.25 forCX-02 and CX-10 respectively. IsolatesCX-17 and CX-22 were unable to tolerate 0.3% bile salts for 240 min.

Acidification ability

The ability of selected isolates to acidify the concentrate feed beverage over time is shown in the graph on Fig 3. The isolates CX-02, CX-10 and CX-13 demonstrate similar acidification abilities. In the first two hours, there is little effect on the pH. However, between two and four hours, the pH declines rapidly, reaching values of 5.31, 5.29 and 5.55 for CX-02, CX-10 and CX-13, respectively. This acceleration continues between four and six hours, reaching pH values of 4.7, 4.7 and 4.86. Over the next 18 hours, the isolates continue to decrease the pH, but at a slower rate, reaching final values of 3.96, 4.04 and 3.39.

The production of lactic acid

In the concentrate feed medium over time is shown in the graph on Fig 4. Isolates CX-02 and CX-10 have similar abilities to produce lactic acid, with levels increasing from 0.33 and 0.39 to 0.57 and 0.6 within two hours, then to 0.78 and 0.72 in four hours, and rapidly increasing to 1.29 and 1.23 at six hours, finally reaching 2.19 and 2.13 after 24 hours. In contrast, isolateCX-13 has a slower lactic acid production, remaining unchanged at 0.48 during the first two hours, then increasing belatedly to 0.6 in four hours, 1.02 in six hours and 2.22 after 24 hours.

Molecular identification

Using rep-PCR genomic fingerprinting, the three *Lactobacillus* isolates were identified by comparing their extracted DNA profiles to those of reference strainsand confirming the results using the NCBI Blast database. As shown in Fig 5 and Fig 6 the DNA extracted from CX-02 and CX-10 isolates was matched with the reference strain *Lactobacillus plantarum subsp plantarum* ATCC 14917, while the DNA extracted from CX-13 was matched with the reference strain *Lactobacillus acidophilus* ATCC 43.

In this study, the five tested isolates had no haemolytic activity (γ-haemolysis), which is similar to the results obtained by other studies (Kaktcham *et al.,* 2012; Halder *et al.,* 2017). Generally, *Lactobacillus spp*. is considered safe for human or animal consumption (FAO, 2002; Magdalena *et al.,* 2018; Abhyankar *et al.,* 2022). But it is recommended to use a bacterial strain as a probiotic supplement only if it is deemed safe and does not have any negative effect on the host (Dumitru *et al.,* 2021). Isolates that have β or α-haemolysis can be unsafe (Royan *et al.,* 2021).

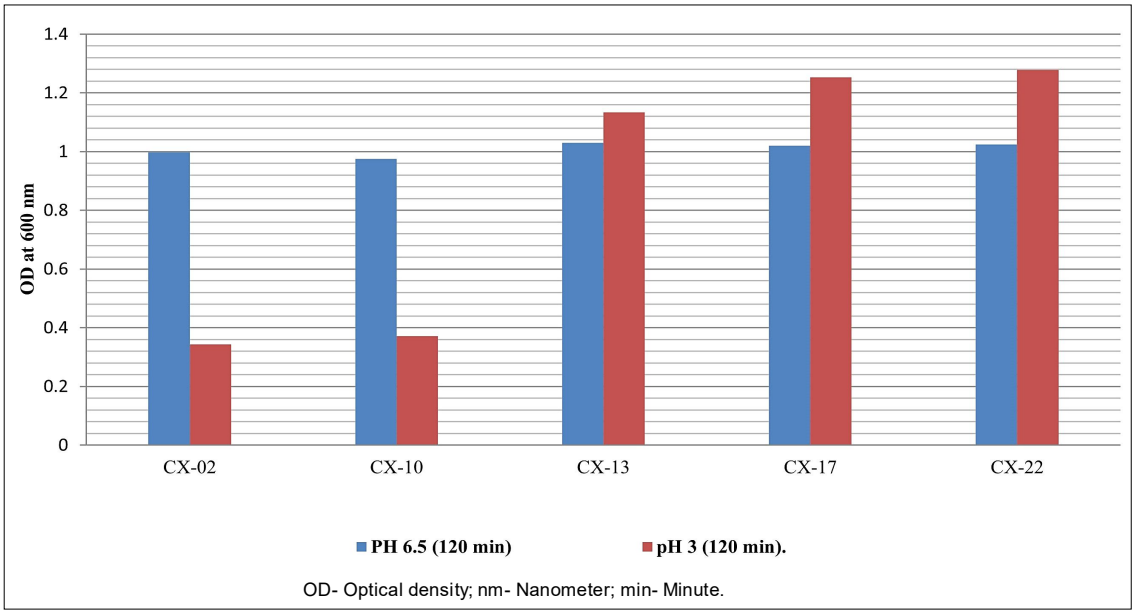

Fig 1: Acidity tolerance of selected isolates of the genus *Lactobacillus spp.*

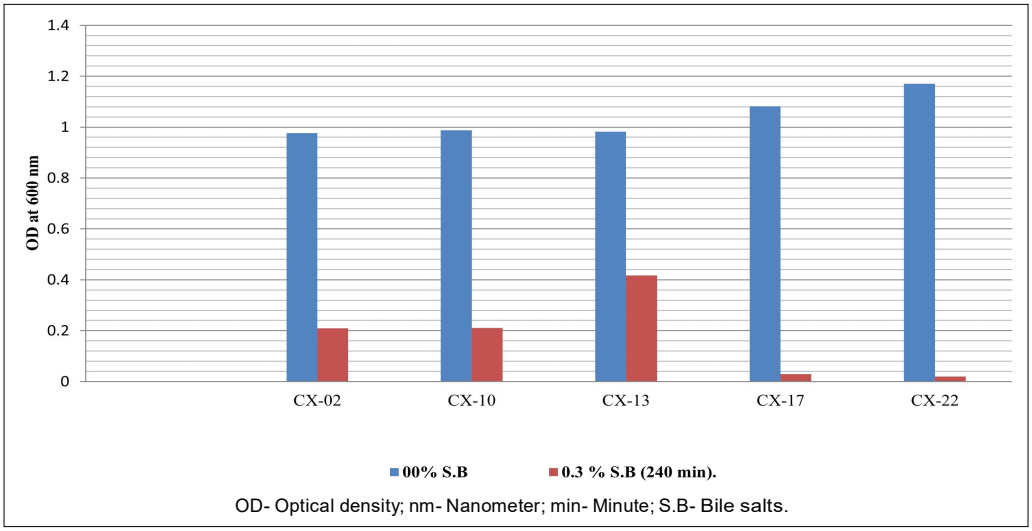

Fig 2: Bile salts tolerance of selected *Lactobacillus* isolates.

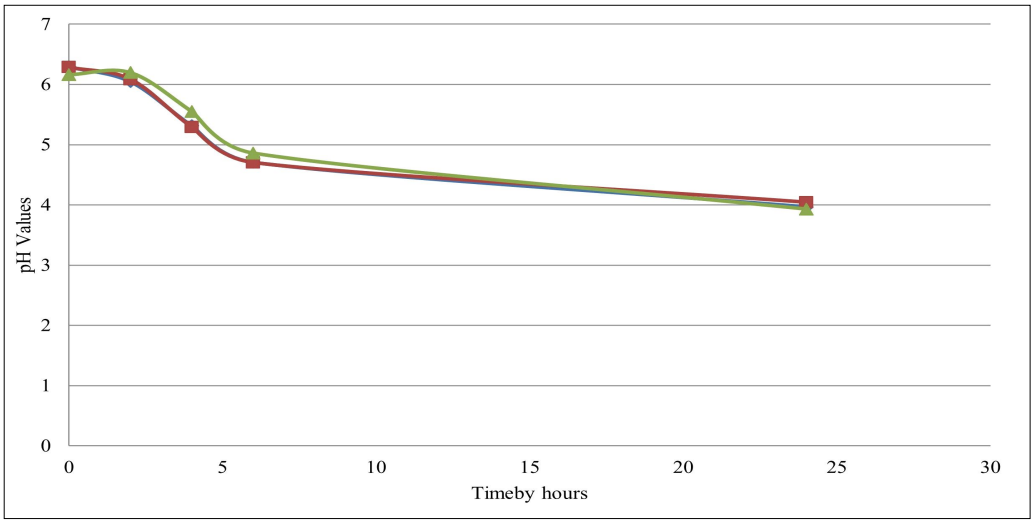

Fig 3: Acidification potential of the selected isolates in the concentrate feed beverage.

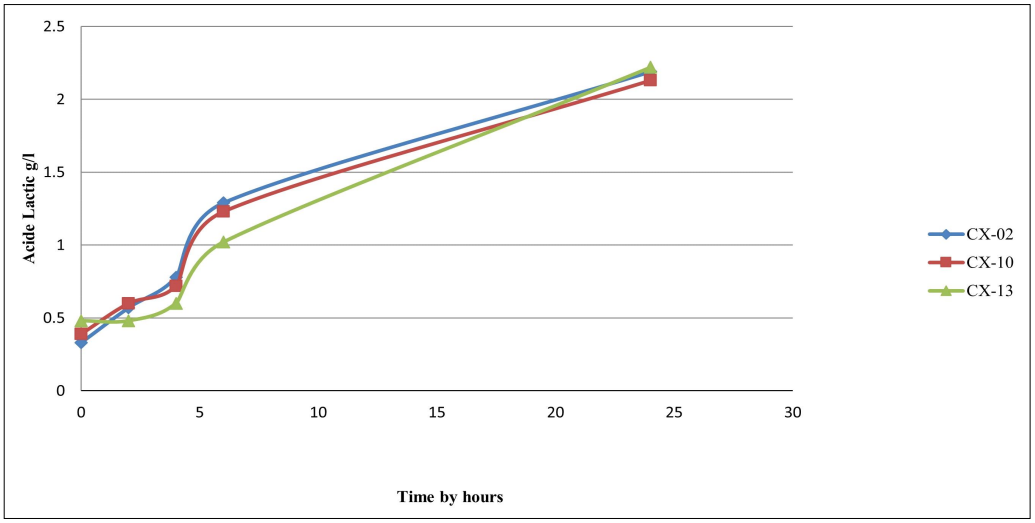

Fig 4: Lactic acid production in the concentrate feed beverage by selected isolates over time.

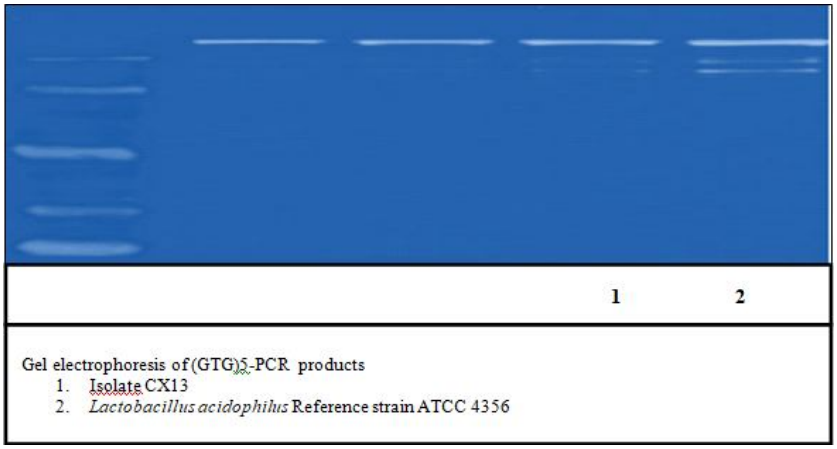

Fig 5: Gel electrophoresis (GTG)5- PCR products for isolate CX-13.

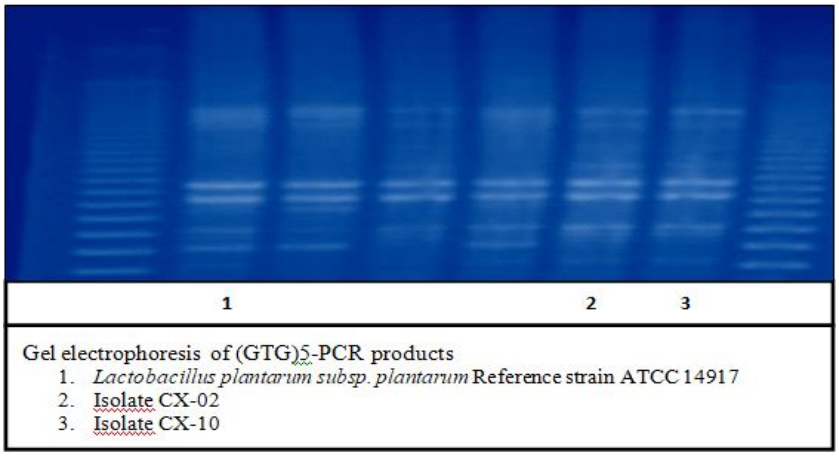

Fig 6: Gel electrophoresis (GTG)5-PCR products for isolates CX-02 and CX-10.

All isolates were able to grow quickly and survive on the agar - concentrate feed beverage as the only source of nutrition and also able to produce exopolysaccharides (EPS) from the same medium, which represent according to Magdalena *et al.* (2018) a functional food ingredients which can confer health. Also, considered a prebiotics that stimulates probiotic microorganisms (Rajasekhar *et al.,* 2022).

Only isolates CX-02, CX-10 and CX-13 were able to tolerate acidity and bile salts. Despite their high acid resistance, CX-17 and CX-22 were unable to resist bile salts for 240 min and were thus eliminated from further tests. *Lactobacillus* species can survive in the presence of bile and low pH (Drissi *et al.,* 2017). These bacteria are known to be acid resistant and can survive ingestion (Goldstein *et al.,* 2015). Can colonize both the proventriculus and gizzard, due to their acid resistance (Rychlik, 2020). Previous studies of Wang *et al.* (2018) were reported the resistance of certain *Lactobacillus* strains to low pH values. Also, Isolates of *Lactobacillus spp.* Isolated from "Hamoum" have been found to resist low pH values for extended periods of time (Tahlaiti *et al.,* 2017). According to Rajasekhar *et al.* (2022), acid and bile tolerance indicates the probiotic nature of lactic acid bacteria. This primary selection criterion allows us to identify, from the tested isolates, the isolates which can survive the harsh conditions of the chicken's gut.

The obtained results of the three isolates showed that isolates CX-02 and CX-10 had a faster rate of organic acid production during the first 6 h, while isolate CX-13 had a slower rate but ultimately produced more than the other two isolates after 24 h of incubation. It is important to note that the ability of these isolates to acidify the medium through organic acid production can have a significant impact on the gut microbiota of broiler chickens. Probiotics can aid in decreasing the pH of the gut, creating a more favorable environment for certain microorganisms and reducing pathogen colonization (Abd El-Hack *et al.,*2020). Also, in a similar research, Dumitru *et al.* (2021) was suggested a *L. acidophilus* Strain as a suitable candidate for amylase and cellulase production, which can improve digestion in animal nutrition and raw materials fermentation.

Using rep-PCR genomic fingerprinting technique, the isolates were identified as *Lactobacillus plantarum subsp plantarum* for CX-02 and CX-10, and *Lactobacillus acidophilus* for CX-13. *L. plantarum* is known for its robustness and competitiveness against other lactic acid

bacteria, and is commonly found in fermented vegetables, fruits, and cereal flours, as well as the ability to grow on plant materials high in polyphenolic compounds (Shori, 2016). Similar results were reported by Kaktcham *et al.,* (2012), using the same identification technique, has been identified *Lactobacillus* strains such as *Lactobacillus plantarum* from a maize-based fermented beverage. Additionally, Tahlaiti *et al.* (2017) previously identified the presence of these two bacterial species in fermented wheat (Hamoum).

CONCLUSION

The obtained results lead us to conclude that the three autochthonous isolates witch identified by the molecular technique rep-PCR were found to match the reference isolates, *Lactobacillus acidophilus* for isolate CX-13 and *Lactobacillus plantarum subsp plantarum* for isolates CX-02 and CX-10. These strains possess properties that make them suitable for *in vivo* testing, they were able to grow on the concentrate feed beverage which can represent a favorable carrier for these strains, also, can produce exopolysaccharides on the same medium and are considered safe due to the absence of hemolytic activity. Additionally, they could reduce the pH values of the composed beverage in a relatively short period by producing organic acids. Furthermore, the three strains have also demonstrated the ability to tolerate harsh gut conditions represented by simulated gastric acidity and the presence of bile salts. Those three strains can contribute to the digestion of chicken food influencing the gut microbiota by excluding pathogens through a competitive exclusion for nutrients and organic acids production.

Finally, it would be important to investigate the impact of these isolates on the gut health of broiler chickens, by measuring the changes in the gut microbiota, specifically the growth of pathogens and the levels of organic acids.

Conflict of interest

All authors declared that there is no conflict of interest.

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