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Manufacture of Bacterial Products from New Species of Lactic Acid Bacteria Isolated from Chicken Intestines

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Abstract:

The present investigation was conducted within the Microbiology Laboratory in the Department of Food Science at the College of Agriculture, Basrah University. This work aims to produce a bacterial preparation using a collection of novel lactic acid bacteria species. These bacteria were obtained from the contents of the jejunum region in the small intestine of adult chickens that were in good condition. The production procedures involved the introduction of the novel bacterial strain into pasteurized skim milk. The substance underwent fermentation, followed by dehydration and pulverization, producing a bacterial formulation including a diverse assemblage of lactic acid bacteria strains. These strains exhibit synergistic behavior and possess resilience against gastrointestinal conditions. The investigation findings revealed that the bacterial product that was created had a minimum concentration of 16×10^9 (cfu/g) of lactic acid bacteria, demonstrating its capacity to endure the circumstances present in the digestive tract. The performed study in laying hens' storage revealed the efficacy of the bacterial preparation as a reliable and suitable product, which can be stored for at least two months from the manufacturing date without compromising its quality. The duration of growth may extend beyond six months when the storage temperature is maintained at 4°C.

Keywords:

Bacterial product, Chicken intestines, poultry, *Lactobacillus*.

Introduction:

The poultry industry is a bio-sector that is undergoing continuous development in response to the increasing need for animal protein, which is driven by the significant expansion of the global population (Al-Fayyad *et al.*, 2011). The rise in demand for poultry meat and egg products necessitates the establishment of additional poultry breeding initiatives, which in turn necessitates the fulfillment of all breeding obligations. Biosecurity is of utmost significance in the context of poultry farming (Al-Ani, 2021).

The popularization of the idea of biosecurity is deemed essential because of its substantial contribution to preventive medicine and its notable economic implications. The phrase "biosecurity" has a wide range of meanings, making it challenging to confine it to a singular definition. However, the development of the subject is contingent upon its utilization. In poultry farming projects, it is common practice to raise poultry in a controlled environment within a house to minimize exposure to harmful microorganisms, with the exception of those acquired through water or feed. These microorganisms often differ from the ones found in the intestines of poultry (Hwang & Singer, 2020). Due to the challenges associated with controlling microorganisms, particularly pathogenic ones, individuals involved in poultry farming projects have resorted to the use of antibiotics as a means to reduce or eliminate pathogenic bacteria and enhance biosecurity. However, this approach has had negative consequences, such as the emergence of antibiotic-resistant bacterial strains like Salmonella. According to Alnajjar&Alemadi (2017), the presence of coliform bacteria is widespread in the majority of poultry farms. This can be attributed to their ability to develop resistance to certain antibiotics. The World Health Organization has implemented restrictions on some antibiotics in chicken production because of concerns over potential transmission to consumers (Agboola *et al.*, 2015). This has prompted several researchers to do diverse investigations on the subject. I have extensively utilized probiotics derived from several sources as an alternative to synthetic antibiotics over an extended period.

According to Markowiak & Śliżewska (2018), it has been noted that around 90% of the microbial population in the intestines is comprised of bacteria that are capable of creating lactic acid. The Lactobacillus genus has significant importance among the group of lactic acid bacteria found in the gastrointestinal system of humans, birds, and animals. The optimal temperature range for the development of the organism spans from 30 to 40 degrees Celsius. Liu *et al.* (2011) have classed it as beneficial bacteria due to its vital functional qualities, as noted by Emara *et al.* (2016). Nevertheless, the utilization of this substance may be deemed harmless and is accompanied by advantageous health outcomes for the human body (Bujňáková&Kmeť, 2012). This is mostly due to its exceptional capacity to stick to the inner surface of the small intestine's cavity. The attribute of adhesion has significance as it plays a role in facilitating the colonization of lactic acid bacteria within the gastrointestinal tract (Mohamed *et al.*, 2019).

Probiotics are incorporated into production processes since they are recognized as functional foods due to their inherent biological qualities that enhance the nutritional composition of food molecules. Furthermore, it has been found to have a significant impact on enhancing sensory attributes such as taste, flavor, and overall acceptability. Additionally, it contributes to the reduction of pH levels. This can be attributed to the presence of lactic acid bacteria, which have been shown to enhance the digestion coefficient and decrease cholesterol levels. These findings have been supported by studies conducted by Ghazal *et al.* (2021), Humam *et al.* (2019), and Nasser *et al.* (2021). Furthermore, it has been observed that this intervention enhances the integrity of the mucosal lining of the gastrointestinal system, hence providing a protective barrier against the colonization and proliferation of harmful bacteria (Mohamed *et al.*, 2019). The utilization of this substance has been found to inhibit food spoiling effectively, prolong the duration of food preservation, and exhibit antioxidant characteristics

(Ozogul&Hamed, 2017; Begunova *et al.*, 2021). Additionally, it has demonstrated resistance to antibiotics in several studies (Yazdi*et al.*, 2017; Reuben *et al.*, 2019).

Building upon prior research, the present study diverged from earlier investigations by introducing a novel concept. Specifically, this study aimed to transfer the intestinal microbiota from adult domestic avian species to other avian counterparts. In order to further the understanding of biosecurity, this study aims to produce a therapeutic bacterial preparation using the contents extracted from the jejunum area of the small intestine in mature chickens.

Materials and methods

This study was conducted in the Microbiology Laboratory of the Department of Food Science at the College of Agriculture at Basrah University from 15/11/2020 to 26/12/2020.Bacterial preparation was prepared from a group of new genera of lactic acid bacteria. Isolated from the contents of the jejunum region in the small intestine of adult chickens. Seven new species of lactic acid bacteria were acquired and registered at the National Center for Biotechnology Information (NCBI) as new local strains, both in Iraq and around the world, as follows:

Lactobacillus gasseri strain Al-Salhi-1,*Lactobacillushelveticus* strain Al-Salhi-2,*Lactiplantibacillus Plantarum*strain Al-Salhi-3,*Limosilactobacillus reuteri* strain AhQuSa-1,*Limosilactobacillus*sp. strain AhQuSa-2,*Ligilactobacillus salivarius strain* AhQuSa-3,*Lactobacillus Johnsonii* strain AhQuSa-4(Al-Salhi *et al.*,2022).

Bacterial preparation steps

1. Pasteurization of the skim milk to eliminate microbes.
2. Inoculating the recovered sorting milk with bacteria and activating them by 10% (10 Starter: 90 skim milk) for three consecutive times (putting a sample of the bacteria secretions present on the surface of the dishes as a starter in the milk, to ensure that it can ferment the milk, after creating the ideal conditions to make the bacterial preparation.
3. Add the active starter to 10% skim milk.
4. Place it in the incubator at 45˚C for 4 hours for a good product.
5. Cool the product and store it in the refrigerator for the next day.
6. Performa whey exudation procedure to get rid of the excess water by using (boring cloth bags) and air-suspending the bags to get rid of as much water as possible.
7. Drying the bacterial preparation in metal trays and placing it in the electric oven at 60 ˚C for 72 hours with continuous stirring.
8. Perform the grinding and softening process of the bacterial preparation. It was placed in sterile bags, closed well and kept in the refrigerator until use.

Culture Media

The culture media used in the study, Nutrient Agar, MacConkey Agar, MRS Agar, and MRS Broth, were prepared according to the Indian manufacturer HIMEDIA (India)instructions and then sterilized by Autoclave at 121°C. At a pressure of 15 pond /inch^{2}for 15 minutes, skim milk was used as a carrier to activate bacterial cultures, and 0.1% Peptone was used to prepare the decadal dilutions (Da Silva *et al.*, 2019).

Biochemical and environmental examinations of isolates manufacture of the bacterial preparation

Some biochemical and environmental tests were carried out, similar to the conditions of the bird's body and related to the physiology of the digestive system of domestic birds. The isolates were subjected to conditions identical to the conditions of the intestines at high and constant levels, such as temperature,

bile salts and pH, as well as a growth test in the medium of litmus milk. To ascertain the resistance of seven species isolated from the intestines of adult chickens to act as a synthetic bacterial preparation.

Growth in different temperature

Activated bacterial isolates were cultured in a dish-casting method. They incubated at a temperature of 35-45˚C for 2-3 days while the growth in the dishes was followed up (Johnson & Case, 2019).

Bile salt resistance test

This test was conducted based on what was mentioned (Cappuccino & Welsh, 2019).

Low pH Resistance Test(pH =4)

This test was conducted based on what was mentioned by (Procop *et al*., 2017).

Growth test in litmus milk

This test was conducted based on what was mentioned (Cappuccino & Welsh, 2019).

Total Bacteria count

The pouring plate method used the microbial counting of total bacteria (Da Silva *et al*., 2019). They took 1g of intestinal contents, transferred them to a test tube containing 9 ml of 0.1% peptone water, and shook it well to obtain a homogeneous bacterial culture, where several decimal dilutions of this diluent were made(Da Silva *et al*., 2019).Then, 1 ml of these series dilutions were transplanted to Petri dishes, and 15-20 ml of medium was added. Nutrient Agar, after stirring and homogenizing, was left to solidify. The plates were incubated under air conditions at 35˚C for 24 -48 hours. The developing colonies were counted employing a colony counter, and the number of bacteria (cfu/g) was calculated by multiplying the average number of colonies for two plates by the inverted dilution.

Lactic acid bacteria count

The same method (Da Silva *et al*., 2019) was adopted to estimate the numbers of lactic acid bacteria Using an MRS Agar culture medium. The dishes were incubated in anaerobic conditions inside the incubator at 37˚C for (48-72) hours.

Coliform Bacteria count

The pouring method mentioned by (Da Silva *et al*., 2019) was adopted to estimate the numbers of coliform bacteria using a MacConkey Agar culture medium, and they were incubated in aerobic conditions inside the incubator at a temperature of 37˚C for 24-48 hours, during which the red or pink colonies were counted.

Feasibility study of the manufactured bacterial preparation

The economic feasibility study for manufacturing the bacterial preparation is the first step that the researcher should consider to infer the cost of manufacturing the preparation, such as whether it is expensive or cheap. On this basis, we can determine the price of the cost that will tell us about the economic feasibility of manufacturing the bacterial preparation, which consists of three basic materials (dried milk, water and a starter made from lactic acid bacteria isolated from the jejunum), which will be calculated based on the production of 1 kg of the bacterial preparation.

The manufacturer's prices are fixed according to the movement of the Iraqi market.

Statistical analysis

The complete random design (CRD) was used to study different treatments' effects on the traits studied. The Duncan Test polynomial compared the significant differences between the means under the significance level 0.05. Moreover, the program (SPSS, 2018) was used in the statistical analysis.

Results and discussion

Microbial count of lactic acid bacteria in the synthetic bacterial preparation

Table (1) indicates the microbial counting of bacterial colonies isolated from the jejunum area after loading them on the skim milk and ending with the production stage of the manufactured bacterial preparation from lactic acid bacteria.

Table 1: Microbial content of the manufactured bacterial preparation (cfu /g)

Type of bacteria	Manufactured bacterial product(cfu/g)	Log
Lactic acid bacteria	16×10^{9}	10.2

Biochemical tests for bacterial isolates of the preparation's manufacture

Table 2 presents the findings of environmental and biochemical assessments conducted on lactic acid bacteria strains that were obtained from the jejunum area, in the study conducted by Al-Salhi*et al.* (2022), a series of ecological and biochemical assessments were conducted to determine the level of resistance exhibited by the seven strains obtained from the gastrointestinal tracts of mature hens. The objective was to evaluate their suitability as potential producers of a bacterial preparation. Nevertheless, these microorganisms were mostly found in the jejunum section of the avian small intestine, indicating their ability to endure the physiological environment of poultry. Nevertheless, emphasis was given to specific biochemical tests pertaining to the physiological aspects of the digestive system in domestic avian species. The isolates were exposed to settings that closely resembled the high and consistent levels seen in the intestines, including internal temperature, pH, and bile salt secretion. This was done in order to ascertain the isolates' maximal tolerance for these specific conditions being investigated.

Furthermore, the results were identical to what is expected in the same Table. Additionally, they were positive in their tolerance of the conditions mentioned above. A growth test was conducted in the middle of the milk of lemmings to know its ability to ferment milk and act as a bacterial preparation. The results were also positive, meaning that it is suitable to work as a starter in Milk fermentation and production of the preparation manufactured in this study.

Table 2: Results of biochemical tests for isolates of bacterial preparations

No.	Confirmed tests for the product	Bacterial Strains						
		1	2	3	4	5	6	7
1	Growth at 35°c	+	+	+	+	+	+	+
2	Growth at 45°c	+	+	+	+	+	+	+
3	Bile salts 0.3%	+	+	+	+	+	+	+
4	Low pH(pH =4)	+	+	+	+	+	+	+
5	Growth in the Litmus Milk	+	+	+	+	+	+	+

A positive sign means good growth of bacterial colonies.

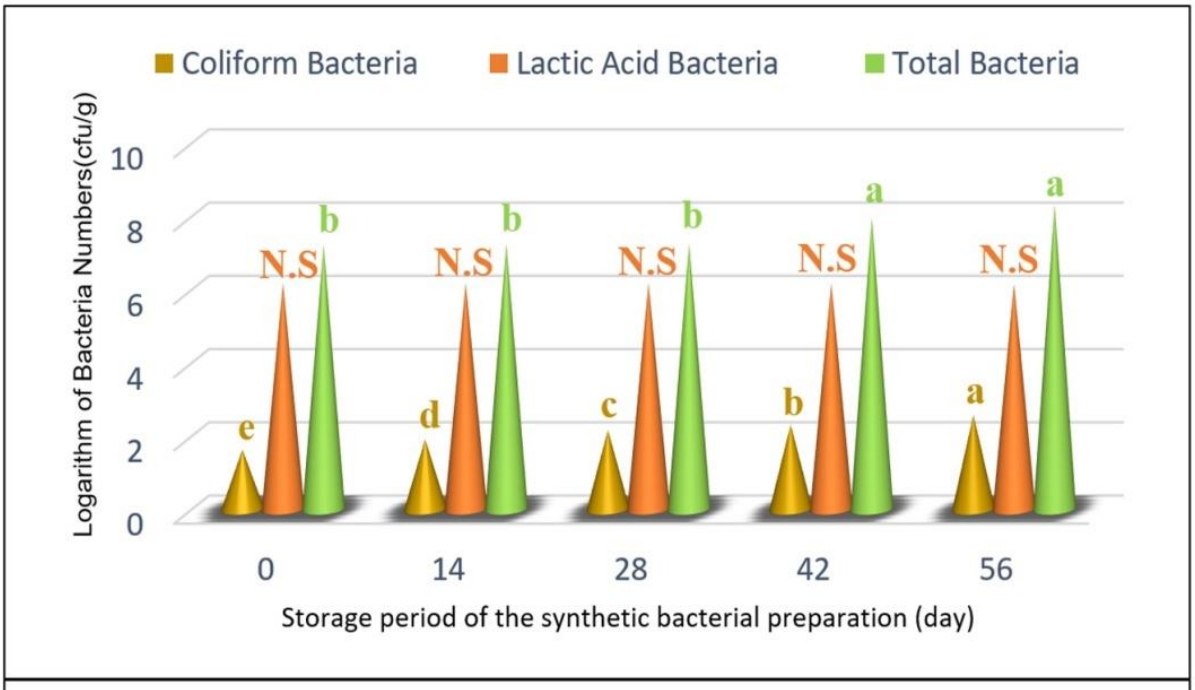
The bacterial isolates can withstand temperatures from 35-45°C, which is close to the body temperature of poultry (Table 2). Thus, it is one of the essential qualities used in manufacturing microbial preparations that enter the bird's body through metabolic reactions and at a temperature ranging between 40-43 °C (chickens body temperature).

The study of lactic acid bacteria is one of the important criteria to identify the tolerance of these bacteria to live in the alimentary canal conditions. pH varies according to parts of the digestive system, as the pH concentration is deficient in the glandular stomach area, reaching up to 2 In the absence of feed. It rises to 4 in the case of the presence of feed (spring, 1997). Since the product will be provided with free-feeding (in the presence of feed and water), the focus has been on pH=4, the

highest concentration recorded by the alimentary canal in the glandular stomach area, and, accordingly, the basis, taking into account. Therefore, it must be accompanied by feed to have its effects by bringing about microbial balance and improving the digestion coefficient (Schneitz*et al.*, 1998). The character of resistance to bile salts is not less than that of lactic acid bacteria; both are equally important because they are among the primary criteria in selecting bacterial isolates to work as a bacterial preparation or probiotic (Jin*et al.*, 1997).

The storage experiment of the manufactured preparation

Figure (1) shows the effect of the storage period (0-56) days on the quality of the manufactured bacterial preparation stored in the laying hens' field during the experiment within the age of 45-52 weeks of laying hen. Significantly, in the total bacteria index compared to time (0), considered a control treatment, the remaining storage period was (42-56) days. The total bacteria index recorded significant differences ($P<0.05$) compared to time (0) when there were no significant differences in the total bacteria index between the two storage periods, 42 and 56 days. While in the coliform bacteria index, a significant increase ($P<0.05$) was observed in the index during the succession of storage periods, and in the lactic acid bacteria index, we noticed no significant differences between the first period (0) and the subsequent periods (14-56) days.



*The letters in the different columns within the same color indicate significant differences between the treatments at the 0.05 level.
N.S. indicates that there are no significant differences between the averages of the transactions.

Figure 1: The effect of the storage period (0-56 days) on the quality of the manufactured bacterial preparation stored in the laying hens' field during the experiment period (45-52 weeks)

The absence of significant differences in the indicator of lactic acid bacteria in (Figure 1) is considered one of the most critical indicators in inferring the quality of the manufactured bacterial preparation (biological activity) after storing it in the conditions of the laying hens' field for two months. This period may exceed six months if kept in the refrigerator at 4° C. This indicates that the quality of the manufactured bacterial preparation did not deteriorate during the storage period.

The economic feasibility study for the synthetic bacterial preparation

After reviewing the production costs of raw materials and their average prices in Iraqi dinars, whose prices are somewhat fixed according to the movement of the Iraqi market and according to (Table 3), we conclude that the production costs per kilogram of the manufactured bacterial preparation are equal to 10,000 Iraqi dinars, which is equivalent to 7 U.S. This price is low for highly biologically adequate bacterial preparation, improving poultry's productivity and physiological and microbial characteristics.

Table 3: costs of the raw materials used in the manufacture of the bacterial preparation in Iraqi dinars

No.	Primary material	The number of units	Price in Iraqi dinars
1	skim milk	2 kg	8,000 dinars
2	drinking water	4 L	250 dinars
3	Bacterial Preparation	320 g	500 dinars
4	Other expenses	-	1250 dinars
Total			10,000 dinars

- Other expenses are represented in (electricity wages, consumption of equipment and some laboratory tools).

The bacterial preparation derived from the natural flora of domestic birds and containing new strains registered for the first time in Iraq and the world (Table) will be a safe and effective product for enhancing the natural intestinal flora, whose numbers are decreasing in birds due to poor management or birds consuming unbalanced diets, which negatively impacts the content. The microbiome of the gastrointestinal tract, in turn, affects the physiological system of the avian and, consequently, the productive performance of poultry.

Conclusions

Based on our findings, it can be inferred that the bacterial preparation produced had a lactic acid bacteria count of less than 16×10^9 colony-forming units per g (cfu/g). Furthermore, this preparation demonstrated resilience in the intestinal environment, suggesting its potential to reach distal regions within the alimentary canal and proliferate to the greatest extent possible when administered via feed or drinking water supplementation. The efficacy of the commercially produced bacterial preparation as a safe and suitable treatment was demonstrated by a storage experiment done in field circumstances with laying hens. The product can be kept for a minimum duration of two months from the time of manufacturing without any discernible impact on its quality.

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