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In vivo evaluation of the therapeutic effect of *Streptococcus thermophilus* isolated from camel milk on intestinal disorders

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Abstract. This study aimed to isolate, select, and evaluate lactic acid bacteria possessing probiotic properties. Isolates obtained from camel milk from El-Oued region in Algeria were investigated for their potential effect on intestinal disorders in Wistar rats. The results relating to the selection of probiotic strains confirm that one strain, identified as *Streptococcus thermophilus* exhibited the best probiotic activity, with an important tolerance to different degrees of pH and to bile salts, and a remarkable antibacterial activity and resistance to antibiotics. During *in vivo* studies, the administration of isolated lactic acid bacteria was evaluated after inducing intestinal disorders in rats. The microscopic observations of the histological section of the intestine showed an almost complete disappearance of the damages in the intestinal structure. The haematological parameters were in agreement with the results of the histological sections.

Keywords: camel milk, intestinal disorders, lactic acid bacteria, probiotics, pathogenic bacteria.

Introduction

Lactic acid bacteria (LAB) are among the most widely used bacteria in food fermentations thanks to the production of a wide range of metabolites. Lactic acid bacteria are found in abundance in fermented milk (Mokoena, 2017; Ruiz Rodriguez *et al*., 2017). These bacteria have been used for a long time in the food industry and allow, through their metabolism, to increase the nutritional quality, organoleptic and shelf life of food (Bouguerra, 2021).

Due to their health benefits, some bacteria are widely used as probiotics, such as *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, if these live probiotic bacteria are administered in sufficient quantities, they improve human or animal health (Amrouche, 2005). Probiotics are available in fermented foods such as yogurt, or as nutritional supplements that contain live bacteria for the constitution of the intestinal microbiota (Mokoena, 2017). The beneficial effects of probiotics on the health of the host are, in theory, numerous, but the scientific evidence confirming these claims requires additional investigations. Several clinical studies have already demonstrated the effectiveness of certain probiotics in the treatment of systemic and infectious diseases (Villeger, 2014).

To prove the efficacy of a probiotic strain or product, testing must be performed using increasingly complex systems, ranging from in vitro studies to in vivo animal and human studies. This work aims to highlight the importance of camel milk as a biological source of preferment probiotic autochtone strains. Similarly, this research concerns proving the therapeutic effect of these strains in intestinal disorders in Wistar rats.

Materials and Methods

Besides, pathogenic strains used in the detection of the antibacterial activity come from the Pasteur Institute in Algiers, namely: *Staphylococcus aureus* ATCC 44300; *Pseudomonas aeruginosa* ATCC 9027; *Escherichia coli* ATCC 25922.

Isolation and purification of lactic acid bacteria

Samples of raw milk were taken from a healthy female approximately three or four years old, in the region of the Wilaya of El Megheir (Setil) during February 2022. Camel milking was done in the evening under aseptic conditions to avoid contamination. The lactic acid bacteria were selectively isolated by culture on several media according to the method described by the International Milk Federation in 1999 (Badis *et al.,* 2005). The shape of the bacteria, their Gram type as well as their cell arrangement were determined after Gram staining and only Gram-positive bacteria were selected. The production of catalase by

all lactic acid isolates was detected by the addition of hydrogen peroxide at 10 V (Tabak, 2007). Temperature test makes it possible to distinguish mesophilic lactic acid bacteria from thermophilic lactic acid bacteria (Boullouf, 2016). After inoculation of the M17 broth with the pure cultures, the tubes are incubated at different temperatures (Badis *et al.,* 2005).

The cultures to be tested were inoculated on hypersaline broths containing 2%,4% and 6% NaCl for all strains. After incubation at 30 ° C. for 24 to 72 hours, bacterial growth resulted in turbidity of the culture medium (Guiraud et Galzy, 1980; Badis *et al.,* 2005).

The test of Gallery API S10 was used for biochemical identification of the lactic acid strain. The micro-tubes were inoculated with bacterial suspensions. Following anaerobic incubation at 36°C ± 2°C for 18-24 hours. positive/negative reactions were read and interpreted based on the list of identification profiles (Mehtar and Afsha, 1983).

Selection of probiotic strains

Several *in vitro* and *in vivo* tests were applied for the determination of the probiotic potential of the lactic acid strains.

Acidity tolerance. pH 3 resistance is often used in *in vitro* assays to determine stomach pH resistance. As the food stays for 3 hours, this time limit has been taken into account (Prasad *et al.,* 1998). For this purpose, active cultures (incubated for 16-18 h in M17 broth) were used. The cells were harvested by centrifugation for 10 min. At 2500 rpm, the viable microorganisms were counted after exposure to the acid state for 0.3 h of incubation at 37°C. This process is repeated three times. LAB counts were expressed in log colony-forming units per millilitre (log CFU/ml) (Benyoucef, 2019).

Bile salt tolerance. To exert their beneficial effects in the digestive tract, LAB must resist the toxicity of bile salts which was tested by following the steps of the protocol described by Ruiz *et al.* (2019). For this purpose, to estimate bile tolerance, bile salt was used to perform the bile salt tests at different percentages. The cell pellets were harvested by centrifugation, washed twice and resuspended in phosphate buffer saline (PBS at pH 8) supplemented with 0.3%, 0.5% and 1% bile salts and incubated at 37°C (Hosseini *et al.,* 2009). LAB counts were expressed in (log CFU/ml) (Benyoucef, 2019).

Antibacterial activity. The antibacterial activity of lactic acid strains against selected pathogenic strains was determined using the agar disc diffusion method (Labioui, 2009). After aerobic incubation at 36°C ± 2°C for 18-24 hours, the Petri plates were observed for a zone of inhibition around the discs (Achemchem and Abrini, 2005).

The antimicrobial susceptibility of each LAB was determined using the disk diffusion method described by Zhang *et al.* (2016) against certain antibiotics, including gentamicin (10μg/ml), ofloxacin (5μg/ml), erythromycin (15μg/ml), amoxicillin (25μg/ml), penicillin (10 μg/ml), vancomycin (30 μg/ml) and aztreonam (10μg/ml). The zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21 mm); intermediate, I (16–20 mm) and resistance, R (≤15 mm) (Guesh *et al.,* 2019).

In vivo evaluation of probiotic properties. The 25 white Wistar rats used in this experiment were supplied from the Pasteur Institute in Algiers, as adult males of rats, with an average body weight of 250 ± 25g at the start of the experiment. In the 15 days of the adaptation phase, all rats received a normal diet. After this phase, the diet of the four target groups was modified by fasting them for 16 hours each day before intragastric gavage for one week. The rats were divided into 5 Lots of on each 5 rats, which were:

Lot 01: uninfected and untreated healthy control.

Lot 02: target rats with a disease of bacterial origin at a dose of 0.5 ml of *E. coli* diluted in a quantity of physiological water, for one week.

Lot 03: target rats with a disease of biochemical origin at a dose of 0.5mL of castor oil each day by force-feeding, for one week.

Lot 04: treatment of rats, indicated for disease of bacterial origin, with a dose of 0.5mL of a probiotic lactic acid bacteria diluted in 5ml of physiological water, for one week.

Lot 05: treatment of rats, indicated for the disease of biochemical origin, with a dose of 0.5mL of a probiotic lactic acid bacterium diluted in a quantity of physiological water (5ml), for one week.

The blood sample was taken when the rats were sacrificed (the rats were fasted for 24 hours before being sacrificed), and the blood taken from each rat was collected in EDTA tubes, and transported in a cooler in the laboratory. The blood was then used for the biochemical parameter assay, consisting in the coplete blood counts (CBC).

The realization of the histological sections of the intestines of the rats was carried out in the laboratory of the faculty and with the assistance of the laboratory of pathological anatomy of the hospital BIN OMAR JILANI of El-Oued. Organ sampling (the intestine) was performed at the end of the sacrifice of the rats after washing the organ with physiological water (NaCl 0.9 %) and then preserved in an appropriate medium (Formal 10%).

Results

Pre-identification of isolates

Macroscopic and microscopic examination. The morphology of lactic acid bacteria is an important criterion for their identification. According to the results of isolation and identification of lactic acid bacteria, their contents revealed results shown in Table 1.

Biochemical and physiological tests. The biochemical and physiological tests make it possible to better list our strains towards the appropriate genera. The results relating to the biochemical and physiological tests are represented the Table 2 and Figure 1.

Table 1. Macroscopic and microscopic observation of isolates

Code	Macromorphology (appearance of colonies)	Micromorphology (bacterial forms)	Gram staining	Assembling mode
S_1	Whitish and cream Rounded Very small	Coccus	+	Chains
S_2	Whitish Rounded Small	Coccus	+	Isolated or diplococcus
S_3	Whitish Rounded Small	Coccus	+	Diplococcus and tetracoccus
S_4	Whitish and cream Rounded or lenticular Variable sizes	Coccus	+	Bacillus

Table 2. Physiological and biochemical criteria of lactic acid bacteria strains

Parameter		S_1	S_2	S_3	S_4
Temp	10 °C	+	+	+	+
	40 °C	+	+	+	+
NACL	2 %	+	+	+	+
	4 %	+	+	+	+
	6%	+	+	+	+
Catalase test		+	+	-	-
ONPG		Yellow (+)	Yellow(+)	Yellow (+)	Yellow (+)
GLU		Yellow(+)	Yellow(+)	Yellow (+)	Yellow (+)
ARA		Yellow (+)	Yellow(+)	Yellow(+)	Yellow (+)
LDC		Yellow (-)	Orang (+)	Orang (+)	Red (+)

Parameter	S_1	S_2	S_3	S_4
ODC	Yellow (-)	Red (+)	Red (+)	Red (+)
CIT	Yellow (-)	Bleu (+)	Glass blue (+)	Glass blue (+)
H_2S	Colourless (-)	Black (+)	Black (+)	Black (+)
URE	Yellow (-)	Red (+)	Red (+)	Red (+)
TDA	Yellow (-)	Brown (+)	Brown (+)	Brown (+)

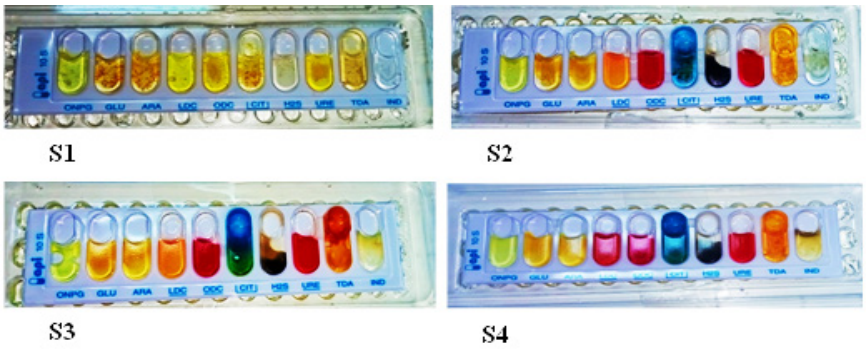

Figure1. API S10 test results.

Selection of strains with probiotic properties

Acidity tolerance. The acid resistance analysis study was carried out under acidic conditions similar to those of the stomach by exposing our strains to different pH: 2, 3 and 4 for 3 hours. The results obtained are presented in Table 3.

Table 3. Effect of acid pH on the viability of lactic acid bacteria (log CFU/ml)

Strain	pH 2		pH 3		pH 4	
	0h	3h	0h	3h	0h	3h
S_1	8.16±0.06	8.38±0.05	8.46±0.02	8.17±0.14	9.97±0.03	10.89±0.07
S_2	9.60±0.03	6.00±0.92[a]	9.31±0.1	7.92±0.16	9.15±0.4	8.42±0.1
S_3	8.54±0.08	8.70±0.09	8.32±0.04	8.77±0.9	8.68±0.01	8.97±0.14
S_4	8.47±0.17	8.47±0.02	9.15±0.22	9.73±0.08	8.85±0.02	9.97±0.03

Mean values (n=3) ± standard deviation (SD). a: highly significant difference($p<0.01$).

There has been little study on probiotic activity since it is generally assumed that *Lactococcus* do not survive during passage through the digestive tract, this is due to the low pH of the stomach, however, several recent works have suggested that *Lactococcus* can survive to reach the human or animal gastrointestinal tract (Kimoto-Nira *et al.,* 2013).

Bile salt tolerance. Bile salts are one of the barriers that probiotic bacteria must cross to gain their site of action. The results for resistance to bile salts are shown in Table 4. Bile tolerance is a determining criterion for the selection of probiotic bacteria, thus allowing survival during passage through the gastrointestinal tract and colonization of the intestinal environment (Marteau and Shamahon, 1998).

Table 4. Effect of different bile salt concentrations on the viability of lactic acid bacteria (log CFU/ml)

Strain	**Bile salts**					
	0.3%		**0.9%**		**1.5%**	
	0h	**3h**	**0h**	**3h**	**0h**	**3h**
S_1	0.95	0.80	1.07	0.20	1.22	1.04
S_2	1.06	1.22	1.20	0.80	1.26	1.10
S_3	1.29	1.23	0.10	0.99	0.50	0.70
S_4	0.58	1.31	1.25	1.10	1.12	1.24

Antibacterial activity. Antimicrobial activity is a very important property in the selection of probiotics, thus allowing the preservation of food and the prevention of gastrointestinal infections (Champomier-Verges *et al.,* 2010; Azat *et al.*, 2016).

The measurement of inhibition zones of isolated strains against selected bacteria by the method of diffusion on an agar disk has been illustrated in Figure 2.

Antibiotic sensitivity. Antibiotic sensitivity tests can be performed using various phenotypic methods. In our study, the selected lactic acid strains were tested using the standardized agar diffusion method (Charteris et *al.*, 1998). For this, eight antibiotics were used, and the results of resistance and sensitivities to the various antibiotics used in this study are presented in Table 5.

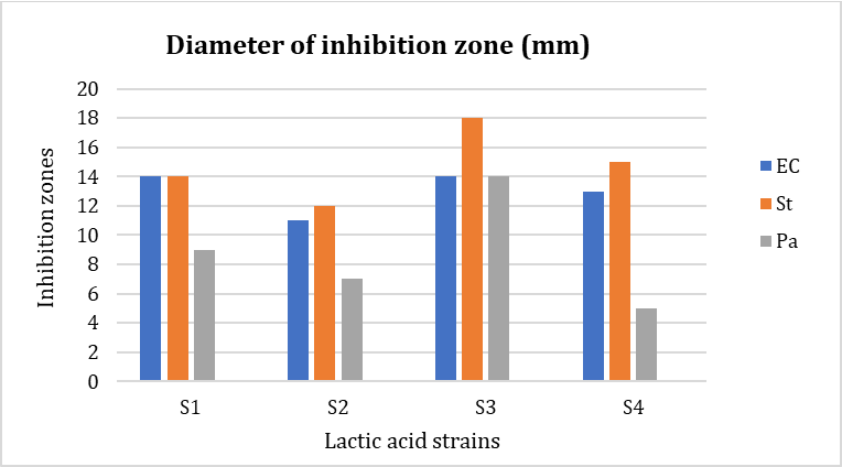

Figure 2. Diameters of inhibition zones obtained by lactic acid bacteria strains against certain pathogenic bacteria (*EC: Escherichia coli; St: Staphylococcus aureus; Pa: Pseudomonas aeruginosa*)

Table 5. Antibiogram of the selected lactic acid strains(mm)

Antibiotic	**Dose (µg)**	**S_1**	**S_2**	**S_3**	**S_4**
Amoxicillin	10	R	R	R	S
Erythromycin	30	R	R	R	R
Vancomycin	30	S	S	I	S
Gentamicin	10	S	R	S	I
Oxacillin	01	R	R	R	R
Penicillin G	10	R	R	R	R
Ofloxacin	05	S	S	S	S
Erythromycin	15	I	R	I	I

R: resistant S: sensitive I: intermediate resistant.

In vivo evaluation of the therapeutic effect on intestinal disorders

Microscopic observations. During the period of infection, we noticed the emergence of several factors, the most important of which are: underweight and diarrheal (a condition indicating that the intestine is irritated).

The histological sections of the organs (small intestines), observed by a microscope with a camera, (Optika, Italy) (objective ×10), are shown in Figure 3.

The observation of the histological section of the intestine in **Lot 1** shows a healthy structure accompanied by a healthy intestinal wall composed of the

mucosa, with villi in a healthy and normal state also a total absence of inflammation and no tissue/cell damage, with an absence of necrosis, so the histological section shows a more or less regular tissue appearance.

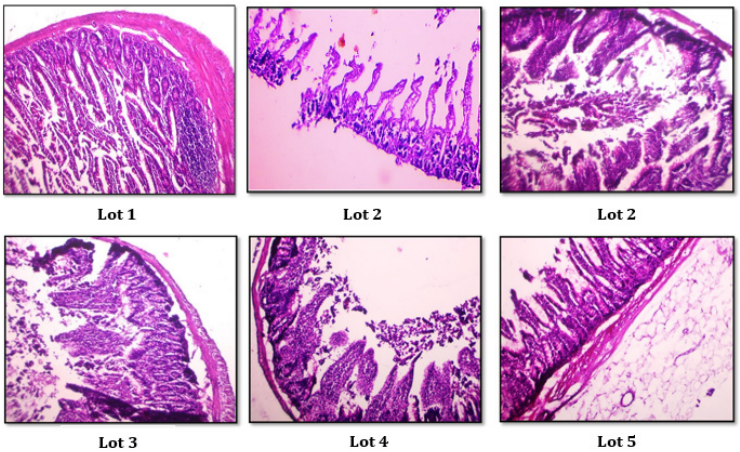

Figure 3. Microscopic observations of the histological small intestine of the rats (100X).

Likewise, microscopic observation of the histological section of the intestine of the rats of **Lot 2** and **3** shows a damaged intestinal structure and some symptoms of irritation including a decrease in the height of the villi and the presence of others destroyed, inflammation (grouping of white blood cells), some villi are necrotized in appearance. In addition to the almost total disappearance of mucous cells.

In the rats in **Lot 4** and **5** and after dissection, the signs of infection were less severe, microscopic observations of the histological sections of the small intestine of the rats show a less affected intestinal structure, and the mucosa seems to be less affected, with a decrease in the rate of inflammation, with a partial return of the height of the villi accompanied by the absence of necrosis.

We also noted a decrease in the severity of inflammation and the absence of necrotic cells and also found the healthy intestinal wall structure with their components, the microscopic study shows that the histological section of the intestine is healthy and has a normal structure compared to the damaged one.

During the period of infection, all rats received a daily dose of the pathogens for a week, the results obtained reveal a harmful effect and damage to the intestines accompanied by structural modification of intestinal behaviours. The results obtained after the treatment period of the rats confirm the beneficial and/or protective effect exerted by *Streptococcus thermophilus* against infection or contamination by whatever the origin of infection.

Haematological parameters. It was observed that the target groups (infected) ***2*** and ***3*** have an increase in the level of red blood cells (6.53 and 7.15 10^3 cel/µl) compared to the other groups (2.61 at 3.5 10^3 cel/µl). This is a symptom of an infection in the body, regardless of the cause (either a bacterial infection or the presence of inflammation). Similarly, it turned out that the third group had an increase in hematocrit levels (37.65%), which corresponded to the concentration of red blood cells in the blood.

It has been observed that the two infected groups recorded the highest values compared to the other groups regarding the number of platelets. The results were probably due to a reaction to an infection, or an inflammatory disease. It has also been observed that the two infected batches showed the highest values of white blood cells (4.95 and 5.1 10^3 cel/µl) compared to the other groups (1.5 to 3.5 10^3 cel/µl). An increase in the number of white blood cells is often due to the body's fight response against an infection. A high white blood cell count may indicate that the immune system is working well to kill pathogens.

The CBC analysis allows ennumeration of red blood cells, white blood cells, HGB, platelets count, and haematocrit measurement. It is applied for diagnosisi of inflammation, infection, and anaemia. The results are illustrated in Figure 4.

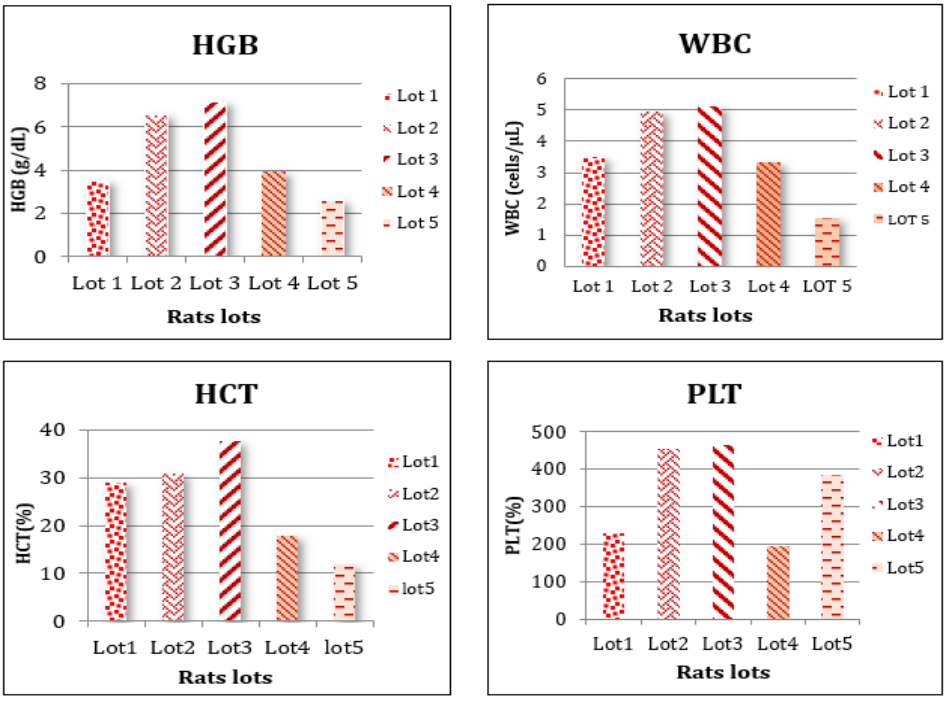

Figure 4. Average values of haematological parameter of rats in experimental groups.

Discussion

Based on morphological, microscopic and biochemical criteria; our isolates can probably be listed in the following species: S1: *Lactococcus lactis,* S2: *Pediococcus acidilactici* S3 and S4: *Streptococcus thermophilus.*

Microscopic observation after Gram staining carried out on the isolated strains shows the following characteristics: all isolates are Gram-positive, in the form of coccus or bacillus, the grouping of cells into chains or isolated or into diplococci and tetrads. Our results were similar and consistent with those obtained by Salhi *et al*., (2020) in terms of macroscopic and microscopic observations.

A significant increase in bacterial growth was noticed for most strains at a temperature of 40 °C. This confirms that lactic acid bacteria are capable of multiplication at high temperatures (Salhi *et al.,* 2020). Regarding the saline tolerance, good growth was observed at a concentration of 2% NaCl in most strains, normal growth in 4%, and little growth in 6% compared to 2% and 4%. The results indicate that probiotic bacteria have a good tolerance to physiological saline concentrations, consistent with the study by Hadj *et al.,* (2013).

The catalase test revealed a negative reaction (no gas bubbles) in the strains S3 and S4, then it is devoid of catalase, this indicates that it is a streptococcus because this bacterium does not produce the catalase enzyme. A positive catalase reaction (presence of gas bubbles) was produced by strains S1 and S2. The enzyme production acts to avoid the toxicity of H_2O_2 by breaking the water and oxygen bond.

In our study, all strains showed a strong resistance to acidity (pH 2, pH 3 and pH 4) after three hours of incubation, these results are in agreement with the results of (Bouguerra, 2021). Our results confirm that lactic acid bacteria can survive and resist deadly acid concentrations, this is consistent with the work of Mathara et *al*.;(2008). It seems that pH 3 does not affect the viability of most of the lactic acid strains tested, they are considered acid-tolerant (Muller *et al.,* 2009); Azat *et al.,* 2016).

According to our results, it turns out that all strains have a tolerance to 0.3%, 0.9% and 1.5% bile salts. Strain S1 was the most affected by the increase in the concentration of bile salts, showing an improvement in the survival rate of bile salts to 1.5%. The results are consistent with the findings of Noriega *et al.,* (2004), which confirmed that several strains of lactic acid bacteria have been stably adapted to salts bile ducts, through a gradual adaptation after growth in bile salt extracts in increasing concentration.

Regarding the antibacterial activity against indicator bacteria, the best inhibition was obtained against *Staphylococcus aureus* with an average inhibion zones of 18 mm, while lactic acid bacteria weakly inhibited *Pseudomonas aeruginosa* with an average of inhibition zones of 5 mm (Davati et *al*. 2015). The inhibitory

properties of LAB are mainly attributed to the production of organic acids, in particular lactic and acetic acids, responsible for the decrease in pH, they also affect the integrity of the cell membrane compromising the viability of the cells and leading in many cases to their lysis. The inhibition of certain pathogenic bacteria can also be associated with the exopolysaccharides secreted by the producing strains, in addition, are capable of inhibiting aerobic pathogenic bacteria by producing CO_2 which creates an anaerobic environment (Denkova et *al.*, 2017).

Most of the strains were sensitive to antibiotics, except for penicillin G, oxacillin and erythromycin, which all strains have been resistant. Several studies have shown the natural resistance of a large range of lactic acid bacteria to antibiotics (Botes et *al*.,2008). According to the results obtained, most of the tasted lactic acid strains are resistant to most antibiotics and very resistant to oxacillin, similar results were obtained by Morandi *et al*., (2013).

The results of all analyses that we obtained led us to choose the best *Streptococcus thermophilus* strain with probiotic properties, to be applied in the in vivo study.

Manjarrez-Hernandez *et al.* (2000) have shown that enteropathogenic *E. coli* (ECEP) could induce lesions (fixation /erasure) in the intestinal epithelium. However, after infection (7 days and after dissection), the almost total disappearance of the villi and the destruction of intestinal behaviours were observed (group 2), with a colour change accompanied by a bad odour (macroscopic observations).

Guergour (2011) in his study of "toxicity of *Ricinus communis* oil, mentioned the signs of poisoning by castor oil and according to the route of administration the oral route is more toxic compared to other routes, the signs are nonspecific (anuria; diarrheal; gastric haemorrhages). This is what we observed after the 3rd group received an oral injection (intragastric gavage) of castor oil. *In vitro* and *in vivo* studies show that taking probiotics reduces the colonization of the digestive tract by pathogenic bacteria and stimulates the specific immune defence response of the host by activating lymphocytes, stimulating anti-tumour activity and reducing infections (Amrouche, 2005).

Compared to the control group, the analysis of CBC confirmed the diagnosis of infection in rats as caused by both methods (infection with castor oil; by *E. coli*). This can also be due to atrophy of the intestinal villi resulting from cell damage. The parameter values of RBC, HCT, PLT and WBC in groups 2 and 3 had higher values compared to the control group. The increase in the level of RBC in the body is a symptom of an infection. The interest of this analysis is to detect possible diseases, in particular haematological, infectious and inflammatory.

The high percentage of HCT analysis may indicate the presence of a bacterial infection in the body, and the higher the analysis, the more severe the infection will be (group 3). This analysis makes it possible to find out if the disease is caused by a bacterial infection. An increase in the level of platelets (PLT) means the presence of infectious diseases, inflammatory diseases, and massive haemorrhage (groups 2 and 3).The *in vitro* and *in vivo* studies show that the selected strain of our study *Streptococcus thermophilus* has a beneficial effect on the regeneration and protection of the intestine, this is confirmed by the results of the blood analysis (CBC) of the treated groups (lot 4 and 5).

Conclusions

Probiotics are very benign microorganisms used as nutritional and medicinal supplements that exert beneficial effects on human and animal health. Certain strains of probiotics have proven long-term safety and efficacy.

From the Eimegheir region, a sample of camel milk was taken under rigorous conditions, the objective of which was to isolate and select lactic strains with probiotic properties, after carrying out identification and isolation tests of the lactic strains and evaluating their properties in vitro, followed by an in vivo study. The four strains isolated were characterized by their form of forms, gram-positive, and catalase-negative. These four isolates are retained and have undergone physiological and biochemical tests for the identification of the species. The species revealed are *Lactococcus lactis, Pediococcus acidilactici., and Streptococcus thermophilus.*

The results obtained through this study show a total disappearance of symptoms of infection and inflammation at the intestinal level after treatment with *Streptococcus thermophilus.* The results of the haematological analysis of the infected groups are in agreement with the results of the histological sections, where the results of the blood count indicated values that the infected rats present symptoms of inflammation on the other hand the treated rats presented values following the standards. Due to these results.

From these results, we conclude this work with the importance of lactic acid bacteria, which have the property of probiotics and their ability to restore the small intestine after damage, as well as their ability to resist pathogenic foreign elements inside the intestinal system.

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El-Oued–Algeria. The Algerian Ministry of HigherEducation and the General Directorate of Scientific Research and Technological Development supported the D01N01UN390120230004 research project.

Ethics approval. The study protocol approach for laboratory animals followed ethical principles specified in the Declaration of Helsinki and The Council for International Organizations of Medical Sciences (CIOMS). In accordance with ethical health research standards outlined in the Algerian Executive Directive (No 10–90 JORA, dated 18 March 2004), and in compliance with the regulations of Law No. 88 – 08 issued on 26 January 1988, which addresses veterinary medicine activities and the protection of animal health (No JORA: 004 of 27-01-1988), approval for these protocols was granted.

Conflict of interest. The authors declare that they have no conflicts of interest.

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