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Antagonistic Action of Biopreservative *Lactobacillus plantarum* Strain on Pathogenic *E. coli* O157:H7 in Fresh Camel Meat Stored at 10°C

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Abstract: Cell suspension of lactic acid bacteria was tested in packages of fresh camel meat stored at a mildly abusive temperature (10°C) for their ability to reduce the viability of *Escherichia coli* O157:H7 during storage. The lactic acid bacteria, a strain of *Lactobacillus plantarum*, was previously isolated from fresh camel meat and selected for their antimicrobial activity against some hazardous microorganisms including *Escherichia coli* O157:H7. Fresh camel meat was inoculated with 4 log CFU/g of the enterohemorrhagic *E. coli*. The trial sample was treated with *L. plantarum*, per kg of meat at a level of 6.8 10^6 CFU/g and stored at 10°C for 4 days in plastic bags. Samples were analyzed for *E. coli* O157:H7 survivors and lactic acid bacteria on days 1 to 4. The lactic counts of the inoculated samples dropped followed by the development of spontaneous lactic flora. There were increases in lactic counts in both treated and control samples during aerobic storage after refrigeration storage of 4 days. *L. plantarum* reduced the viability *E. coli* O157:H7 (2 log CFU/g) by the end of the storage period. Towards the end of vacuum storage, *E. coli* numbers were lower than those in the control.

Key words: Camel · Meat · *E. coli* O157:H7 · Bioprotective · *Lactobacillus plantarum* · Storage

INTRODUCTION

Escherichia coli O157:H7 has become a significant worldwide cause of food-borne illness since its discovery in the early 1980's. *E. coli* O157:H7 has been isolated in numerous worldwide outbreaks of hemorrhagic colitis and hemolytic uremic syndrome. In the United States, it has been documented as the source of over 100 outbreaks and estimated to cause more than 20,000 infections each year, with up to 250 deaths [1, 2]. Also, *E. coli* O157 is the STEC (Shiga toxin-producing *E. coli*) serotype most commonly associated with severe human infections within the EU [3]. Both young children and the elderly are most vulnerable to the severe consequences of infection.

Because of the severity of these illnesses and the apparent low infective dose (□100 cells) [4], *E. coli* O157:H7 is considered as one of the most serious known food borne pathogens [5,6].

E. coli O157:H7 has been identified as a serotype of an enterohemorrhagic *E. coli* that causes abdominal cramps, bloody diarrhea and in some cases hemolytic uremic syndrome [7-11]. This enteric organism is able to secrete a Shiga toxin distinguishing it from other pathogenic strains of *E. coli*. In Morocco, occurrence of Shiga toxin-producing *E. coli* O157 in selected dairy and meat products marketed in the city of Rabat has been reported by Benkerroum, et *al.* [12]. It has been implicated as the source of numerous outbreaks and sporadic cases predominantly from consumption of meat products. Illness and death associated with meat containing *E. coli* O157:H7 have prompted the meat industry to enhance its efforts to lower levels of *E. coli* O157:H7 and other pathogens in meat by monitoring food production more closely [13, 14].

Lactobacilli are known to have an inhibitory effect against the growth of a wide range of foodborne pathogens [15 - 17]. Short chain organic acids, such as acetic acid and lactic acid, which are the major metabolites of lactic acid bacteria have been reported to be responsible for their antimicrobial activity against *E. coli*

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[18]. Several works reported the *in vitro* inhibitory effect of lactic acid bacteria on the growth of Shiga toxin-producing *Escherichia coli* O157:H7 [18-22], but to our knowledge few are those who studied the antagonistic effect of lactobacilli strains against enterohemorrhagic *E. coli* in stored fresh meat [23]. Especially since LAB behavior in laboratory media may not necessarily be reproducible in the foods [24 - 26].

The present study was undertaken to determine the effectiveness of selected LAB strains identified as *Lactobacillus plantarum* as bioprotective strain on inactivation of pathogenic *E. coli* O157:H7 in fresh camel meat stored at 10°C.

MATERIALS AND METHODS

LAB Isolation: Ten samples of camel meat were collected from different butchers in Morocco and transported to the laboratory using a refrigerated box (4°C) and analyzed on arrival.

Preparation of Initial Suspension and Decimal Dilutions: An amount of 10 g of each sample was added to 90 ml of saline water (8.5 g/l) to make the initial dilution (10^{-1}). This suspension was homogenized and immediately used for making suitable serial dilutions up to 10^{-8} by incorporating a 1 ml into 9 ml of sterile saline water in tubes.

Isolation of Lactic Acid Bacteria: Isolation of lactic acid bacteria was determined using various elective media (Table 1). After incubation, two to five colonies were picked from plates at higher dilutions and transferred in 10 ml of appropriate broth. The selected colonies were purified by repeated streaking on the appropriate agar media. All pure culture were stored at -80°C in MRS broth (Difco Laboratories, Detroit, MI) containing 20% glycerol.

***In Vitro* Inhibition:** LAB strains isolated from camel meat were selected on the basis of their inhibitory activities on various antibioresistant bacteria to select the most efficient strain to be used in meat preservation.

The antimicrobial activity of the isolated strains on *E. coli* O157:H7 (4 strains), *Pseudomonas aeruginosa* (4 strains), *Klebsiella pneumoniae* (4 strains), *Staphylococcus aureus* (4 strains), *Citrobacter freundii* (2 strains), *Bacillus subtilis* (1 strain), *Bacillus megaterium* (1 strain), *Bacillus cereus* (3 strains), *Listeria monocytogenes* (2 strains) were determined by the well diffusion assay [25]. For the detection of antibacterial activity of the LAB strains, ten ml of MRS(Difco, USA) broth was inoculated with each LAB strains and were incubated at 30°C for 48 h. After incubation, a cell-free solution was obtained by centrifuging (6000×g for 15 min) the culture, followed by filtration of the supernatant through a 0.2 µm pore size filter. The pathogenic test bacteria were incubated in Brain Heart Infusion (BHI) broth (Difco, USA) at appropriate temperature for 24 h. Petri dishes with 20 ml of Muller Hinton agar were prepared, previously inoculated with 0.1 ml of a 24 h broth culture of pathogen microorganisms. Once solidified, the dishes were stored for 2 h in a refrigerator. Four wells (ø6 mm) were then made and filled using 100 µl of cell-free filtrate and incubated for 24 h at 37°C. The antimicrobial activity was determined by measuring the clear zone around the colonies (Table 2).

Antibiotic Sensivity of the Used Pathogens: Antibiotic sensitivity of bacterial isolates was performed by the disc diffusion method [27]. The bacterial suspension (100 µl) was inoculated onto Muller Hinton Agar plates (Biocar, France) and allowed for 15 to 20 minutes to solidify. The antibiotic (Safoni Diagnostic Pasteur, France) discs were placed aseptically in the plates and incubated at 37°C for 24 h. Diameter of the inhibitory zone around the discs was measured and recorded in millimeters (Table 3, Figure 1, Figure 2).

Characterization and Identification of the Selected Strain: One of the most efficient strains was chosen to carry out this study. Prior to use, the isolate was activated in MRS broth at 30°C for 24 h and subcultured in MRS agar at 30°C for 24h. For the selected pure culture phenotypic and biochemical tests were performed to identify the selected isolate according to the criteria of the Bergey's Manual of Systematic Bacteriology [28]. Selected isolate were also confirmed by using API 50 CH strips according to manufacturer's instructions (API system, Bio-Merieux, France). The selected strain identified as *L. plantarum* was maintained by subculturing in deMan Rogosa Sharpe (MRS) broth (Difco, USA) with 1% inoculum and incubation at 37°C for 24h.

E. coli O157:H7 strain was obtained from the bacteriology laboratory of the National Institute of Hygiene (Rabat, Morocco). They were maintained by subculturing on Trypticase Soy Bro th (Difco, USA) and incubation at 37°C for 18h. Lactic acid bacteria and *E. coli* strains were stored respectively on MRS broth and TSB containing 20% (v/v) glycerol (Glycerol, Merck) at -80°C.

Table 1: Media and conditions for isolating lactic acid bacteria

Microorganisms	Media	T (°C)	Duration (h)	Incubation
Lactococci	Elliker	30	48	Aerobiosis
Leuconostocs	MSE	21	72-144	Aerobiosis
Pediococci	APT	30	48	Aerobiosis
Mesophlic lactobacilli	MRS	30	24-48	Aerobiosis

Table 2: Antibacterial activity detected in supernatant fluid of MRS broth from *Lactobacillus plantarum* by agar well diffusion assay

	Strains		Zone diameter (mm)
Gram negative bacteria	*Escherichia coli* O157:H7	1	1.1±0.02
		2	1±0.01
		3	1.1±0.02
		4	1.5±0.01
	Pseudomonas aeruginosa	1	0.9±0.02
		2	1.1±0.03
		3	0.9±0.02
		4	1.1±0.02
	Klebsiella pneumoniae	1	1.1±0.01
		2	1.1±0.02
		3	1.9±0.02
		4	1±0.01
	Citrobacter freundii	1	0.9±0.01
		2	0.5±0.02
Gram positive bacteriaGram + bacteria	*Listeria monocytogenes*	1	2.0±0.02
		2	2.1±0.02
	Staphylococcus aureus	1	1.5±0.02
		2	0.9±0.01
		3	1±0.01
		4	1.5±0.01
	Bacillus subtilis	1	1±0.02
	Bacillus megaterium	2	0.9±0.01
	Bacillus cereus 1	3	1±0.02
	Bacillus cereus 2	4	1.1±0.01
	Bacillus cereus 3	5	1.1±0.01

Table 3: Antibiotic sensitivity of the strains used in the antimicrobial activity of the selected LAB strains

	E	ST	AMX	D	DA	CE	AM	TM	CP
S. aureus 1	R	R	R	R	R	R	R	R	R
S. aureus 2	R	R	R	R	R	R	R	R	R
B. subitilis 1	R	R	R	S :13	R	R	R	R	R
B. megaterium	R	R	R	S :12	R	13	R	S:22	S:26
P. aeruginosa 1	S: 11	R	R	R	R	R	R	R	R
P. aeruginosa 2	S :08	R	R	S :20	R	14	R	R	R
E. coli O157:H7 1	R	R	R	S :24	R	R	R	R	R
E. coli O157:H7 4	R	R	R	S :21	R	R	R	R	R

E: Erythromycin 15 µg; ST: Sulfathiazol 0,25mg; AMX: Amoxycillin 25µg; D: Doxycillin 30µg; DA: Clindamycin 2mg; CE: Cefalothin 30µg; AM: Ampicillin 10µg; TM: Tobramycin 10µg; CP: Ciprofloxacin 5 µg; R: Resistant; S: Sensitive

Preparation of Cultures for Treatments: Bacteria were grown in MRS broth. *L. plantarum* was incubated at 30°C for 24 h. The cultures were centrifuged at 4°C and 7000 rpm for 15 min, washed three times and resuspended in sterile saline water to give a cell concentration of 10^6 per ml.

Freshly prepared cultures of *E. coli* O157:H7 were harvested from 18 h BHI (Difco, USA) culture by centrifugation at 4000 rpm for 15 min, resuspended in 10 ml of sterile saline water and diluted to a final concentration of 10^4 CFU/ml.

Raw Material: Camel meat was obtained from a local abattoir (Temara city) from animal slaughtered 6-10 h before. No sex, breed, or pre-slaughtering conditions were recorded. Samples were taken from the *Longissmus dorsi* muscle and immediately transported to the laboratory. Sample was then cut into 2×2×2 cm portions and inoculated with the studied strain.

Fresh Meat Inoculation and Storage: Fresh camel meat was divided into 100 g portions and placed in individual plastic bags and allowed to treatment or control.

Fig. 1: Photograph representing the antibiotic sensitivity of the *E. coli* O157: H7 N°4 comparatively to the inhibitory activity of the *Lactobacillus plantarum* toward the same strains (in the center).

Fig. 2: Photograph representing the antibiotic sensitivity of the *P. aeruginosa* N°1 comparatively to the inhibitory activity of the *Lactobacillus plantarum* toward the same strains (in the center)

The control sample was inoculated with *E. coli* O157:H7 alone to make the initial inoculum level of approximately 4 log CFU/ml. The treated meat portions received levels of 6.0 log CFU/ml of *Lactobacillus plantarum* cells in addition to *E. coli* at the same concentration as the control. Meat was thoroughly mixed with a sterile spatula and also by gently shaking the bag by hand. One control sample was immediately analyzed. The remaining bags were vacuum packed manually and stored at 10°C for 4 days.

Microbiological Analyses: Samples (10 g), were cut into small pieces and blended in 90 ml of saline water (8.5g NaCl /l) to make the initial dilution (10^{-1}). Serial dilutions up to 10^{-6} were then prepared. The microbial growth of *E. coli* O157:H7 and Lactic Acid Bacteria were followed, respectively, on Eosin Methylene Blue (EMB, Difco, USA) agar and MRS agar plates at 0, 1, 2, 3 and 4 days. The plates were incubated at 30°C for 48h for Lactic Acid Bacteria and at 37°C for *E. coli* O157:H7 for 24h.

RESULTS AND DISCUSSION

In the present work, the initial LAB count of the control fresh camel meat was 4.8 10^4 CFU/g which increased to a cell-density of 10^6 CFU/g at the end of storage at 10°C. In meat samples inoculated with cell suspensions of *Lactobacillus plantarum* at 6.8 10^6 CFU/g, the counts increased to 7 10^7 at 4 days of the storage. It should be noted that all lactic acid bacteria, both inoculated and spontaneous lactic flora, were counted in the treated samples. The subsequent increase in LAB count of the treated sample during vacuum storage indicated the development of spontaneous lactic flora (Figure 3). The results are in general agreement with those of Smith and Palumbo [29] using mesophilic LAB and Schillinger and Lucke [30] employing psychrotrophic lactics at low concentrations. Vold, et *al.* [31] showed the importance of the natural background flora in meat consisted mainly of lactic acid bacteria of which approximately 80% were *Lactobacillus sakei* for inhibition of growth of *E. coli* O157:H7.

In this trial, at the 4th day of storage, the *L. plantarum* inoculated samples showed very low increase in the counts of *E. coli* from 3.8 10^4 CFU/g to 9 10^4 when compared with those in the control which increased from 5 10^4 to 5.5 10^6 (Figure 4). Viable counts of *E. coli* O157:H7 from the *L. plantarum* -treated cubes were 2 logs less compared with counts from untreated control cubes at day 4 at 10°C. The same inhibitory effect was reported by Muthukumarasamy, *et al.* [23] who tested *Lactobacillus reuteri* in packages of refrigerated ground beef for their ability to reduce the viability of *E. coli* O157:H7 during storage for its action against a cocktail of five strains of *E. coli* O157:H7 in ground beef held at 4 degrees C for 25 days. *L. reuteri* killed *E. coli* O157:H7 at both inoculated levels (6 log CFU/g and 3 log CFU/g) during refrigerated storage (4 degrees C) in modified atmosphere packages before day 20.

Few studies have been carried out on meat preservation with LAB and stored under vacuum and the effect of subsequent aerobic environment on microbial and sensory changes in meat under retail display conditions. Roth and Clark [32] observed that the exposure of vacuum packed beef in air changed

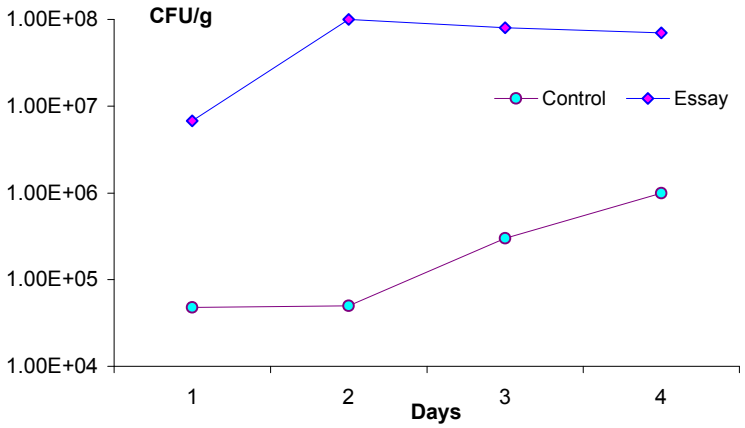

Fig. 3: Profiles of lactic acid bacteria in camel meat supplemented with an initial level of 10^6-10^7 CFU/g of *L. plantarum* strains and the control one during storage at 10 °C for 4 days

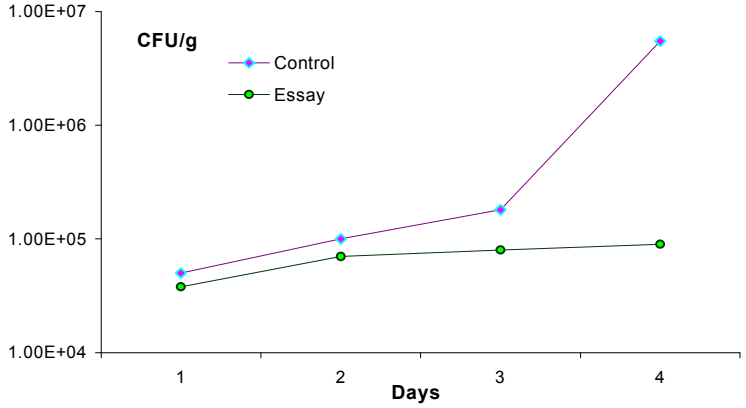

Fig. 4: Inhibition of *E. coli* on packaged meat supplemented with 10^6-10^7CFU/g of *L. plantarum* stored at 10°C for 4 days

the microflora to resemble that of aerobically packed meat. Schillinger and Lucke [30] inoculated psychrotrophic LAB in beef and pork at low concentrations (log 3-4/g) before vacuum packaging and observed that *Lactobacillus sake* and *Lactococcus raffinolactis* were highly competitive. These authors further observed that a greater influence of LAB could possibly be achieved with higher pH, higher initial counts and at a higher storage temperature. Leisner, et *al.* [33] inoculated *Bacteriocinogenic sychrotrophic* LAB on to sterile beef slices and observed that the aerobic deterioration of meat quality was faster with increased time of storage under vacuum.

Previous workers have employed mesophilic LAB such as *Lactobacillus plantarum* [34] for inhibiting the growth of spoilage and pathogenic organisms under aerobic storage and concluded that very high cell concentrations were required for affecting microbial inhibitions [35]. Also, Murthy, et *al.* [36] observed an antagonistic effect of LAB under aerobic storage only when initial contamination of meat with psychrotrophs was very low (log 2.8/g).

Lactic acid bacteria are generally recognized as safe and less deleterious for product quality than many other food-spoilage bacteria. Castellano, *at al.* [16], reported that LAB do not produce dramatic change in sensory characteristics of meat compared to changes that take place in milk and vegetable fermentation due to a low carbohydrate content and strong buffering capacity of meat. Even better, the finding of Fadda, *et al.* [37] showed a positively impact of *Lactobacillus* on beef meat taste and aroma. They grow relatively slowly leading to a longer shelf-life of meat when the microflora is dominated by LAB as in vacuum packaged meats [38]. The shelf life of vacuum packed fresh meat is highly variable depending upon several factors such as film permeability [39], pH [40, 41], initial contamination and storage temperature [42] and ultimately spoiled due to discoloration, off-odours and purge loss [43, 44].

In conclusion, with its inhibitory effect, the studied *L. plantarum* strain is well suited for application as protective cultures in fresh meat and other meat products. However, further tests must be conducted to control the microbiological quality of the refrigerated camel meat (psychotrophs microorganisms) and to study the sensorial quality of the end product.

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