

# Natural antagonists for control of *Listeria monocytogenes* in Moroccan Dromedary camel meat

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## Abstract :

The growth and survival of *Listeria monocytogenes* ( $10^3$  CFU/g) on whole pieces of camel meat was investigated throughout the storage period at 4°C and 20°C during 10 days. A strain of *Lactobacillus plantarum* was previously isolated from fresh camel meat and selected for their antimicrobial activity against *L. monocytogenes*. Cell suspension of this lactic acid bacteria ( $6.8 \times 10^6$  CFU/g ) was tested in packages of fresh camel meat stored at 4°C and at abusive temperature (20°C) for their ability to reduce the viability of *L. monocytogenes* (initial inoculum of ~4 log CFU/g) inoculated during storage. Samples were analysed for *L. monocytogenes* survivors and lactic acid bacteria on 1 to 10 days. Significant growth ( $p < 0.05$ ) of *L. monocytogenes* was observed in meat samples. *Lactobacillus* reduced the viability *L. monocytogenes* by the end of the storage period at both temperatures of 4 °C and 20°C. Towards the end of vacuum storage, the counts of *L. monocytogenes* were lower than those in the control.

**Key words:** Camel meat, antagonistic activity, *Lactobacillus plantarum*, *Listeria monocytogenes*, Antibacterial activity, Biopreservation.

## Résumé :

La croissance et la survie de *Listeria monocytogenes* inoculé à environ  $10^3$  CFU/g sur des morceaux de viande de dromadaire a été étudiée durant 10 jours de stockage à 4°C et à 20°C. Une souche de *Lactobacillus plantarum* ( $6.8 \cdot 10^6$  CFU/g) a été inoculée et étudiée pour sa capacité à réduire la croissance de *listeria monocytogenes* (taux initial d'inoculation  $\sim 4$  log CFU/g) dans la viande fraîche de dromadaire stockée à 4°C et 20°C. Ces bactéries lactiques ont été sélectionnées pour leur pouvoir antibactérien contre *L. monocytogenes*. Le suivi de la croissance de *Listeria* et des bactéries lactiques a été effectué du premier au dixième jour de stockage. Les résultats ont montré que les lactobacilles ont réduit la viabilité de *L. monocytogenes* jusqu'à la fin de la période de stockage à 4°C et 20°C. Le nombre de *L. monocytogenes* dans la viande inoculée, était beaucoup plus faible en comparaison avec le témoin non inoculé avec les bactéries lactiques.

**Mots clés :** Viande du dromadaire, Activité antagoniste, *Lactobacillus plantarum*, *Listeria monocytogenes*, Activité antibactérienne, Bioconservation.

## Introduction :

Food borne illness resulting from consumption of food contaminated with pathogenic bacteria has been of concern to public health. *Listeria monocytogenes* is a Gram positive bacterium responsible for the severe food-borne illness, listeriosis, and has become a major concern to the food-processing industry and to health authorities over the last decades. Despite the efforts made worldwide to eradicate the organism from foods, *L. monocytogenes* contamination continues to occur. It is a common bacterium in environment and animals, and may be transferred to food and human gastrointestinal tract via raw milk and other dairy products. This organism may cause meningitis, sepsis or abortion, but in practice pregnant women and people with immune defects

are at a high risk (Nester *et al.*,1998).

*L. monocytogenes* can contaminate meat and meat products during slaughter, processing and production and the microorganism can persist and grow at low and high pH values, at low water activity and at refrigeration temperatures (Lahti *et al.*, 2001). Many countries do not tolerate the presence of *L.monocytogenes* in 25 g of ready-to-eat foods (Jemmi *et al.*, 2002). However, the International Commission on Microbiological Specifications for Foods considers that if the organism does not exceed 100 CFU/ g of food at the point of consumption, the food is acceptable for individuals who are not at risk (Szabo *et al.*, 2003).

Due to the economical impacts of spoiled foods and the consumer's concerns over the safety of foods containing synthetic chemicals, increased attention has been paid to naturally derived compounds or natural products (Alzoreky *et al.*, 2003). However, in literature the most promising group of micro-organisms for meat preservation are lactic acid bacteria (LAB). Lactic acid bacteria have been widely used for dairy, meat and vegetable fermentations (Stiles, 1996). In addition to the contribution to the typical sensory characteristics of these foods, LAB exert a strong antagonistic activity against many food contaminating microorganisms as a result of the production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and bacteriocins (Piard et Desmazeaud, 1991).

In non-fermented products like vacuum-packaged meat products, LAB becomes the dominant microflora during storage (Shillinger et Lücke, (1987) ; Bredholt *et al.*, (1999)). However, LAB have also been reported to contribute keeping the fresh sensory attributes of meat product during storage (Blixt et Broch (2002)). Meat products are among the most frequent sources for LAB of importance to biopreservation. Many strains of LAB were shown to inhibit *L. monocytogenes* in meat products suggesting their potential as protective cultures (Bredholt *et al.*, (1999)).

The aim of this study was therefore to investigate the growth and survival of *L. monocytogenes* at an initial levels  $10^3$  CFU/g (the  $10^3$  is for the first experiment and the 4 log units it is for the second experiment) on whole pieces of camel meat stored at 4°C and at 20°C; the growth of native microflora of raw meat (Lactic acid bacteria and *Enterobacteriaceae* and aerobic mesophilic counts) and pH were also monitored. The present study was also undertaken to determine the effectiveness of a *Lactobacillus plantarum* strain as bioprotective strain on inactivation of pathogenic *L. monocytogenes* at an initial levels 4 log units in fresh camel meat stored at 4°C and at 20°C.

### **Material and methods:**

#### **Growth and survival of *L. monocytogenes* onto camel meat stored at 4°C and 20°C :**

##### **Preparation and storage of meat samples :**

Lean camel was obtained from traditional butchers in Casablanca city, 5h after slaughtering. Samples were transported to the laboratory in sterile plastic bags within one hour. Small meat pieces weighing approximately 25 g were aseptically prepared.

##### **Bacterial strains and growth conditions :**

Strains of *L. monocytogenes* were maintained at -20°C in Bacto Brain Heart Infusion broth (BHI, Difco) containing 20% (v/v) glycerol (Bacto Glycerol, Difco). Working stock cultures were prepared by incubating overnight in BHI at 37°C without shaking. A volume of 50 µl of culture suspension was inoculated into 20 ml aliquots of BHI at 37°C for 18h to achieve viable cell population of  $\sim 9 \log_{10}$  CFU/ml.

##### **Inoculation and packaging of the camel meat :**

An inoculum of *L. monocytogenes* was prepared by diluting 1 ml of the suspension with 1000 ml of sterile 0.1% (w/v) peptone (Merck, Darmstadt, Germany) water. Concentration of the resulting cultures of *L. monocytogenes* was determined as 6-7  $\log_{10}$  CFU/ml by serial dilutions and viable counts by surface plating on PALCAM agar (Difco). Half of each meat cubes were inoculated with 3  $\log_{10}$  CFU/g as follow:

Using sterile forceps, each meat piece was immersed for one min. into sterile dishes containing the prepared suspensions of *L. monocytogenes* at room temperature (20°C), excess culture was allowed to drip from the cubes, which were held one hour at 4°C to allow optimum adherence of bacteria to the meat. Immediately after draining, inoculated meat pieces (each meat pieces weighing approximately 25 g) and thereafter were gathered in lots of  $250 \pm 5$  g, inoculation two meat slices from each treatment were placed into sterile polystyrene stomacher bags, closed hermetically and divided into two portions: one was stored at 10 °C and the other stored at 20°C. During storage, enumerations of *L. monocytogenes*, Lactic acid bacteria and total viable count were performed at regular intervals. A non-inoculated sample (neither with *Lb. plantarum* nor with *L. monocytogenes*) was prepared and tested in the same conditions to serve as a negative control to exclude the presence of *L. monocytogenes* untreated camel meat. This experimental procedure was performed in two independent trials, and the results presented are a mean of two replicates.

#### **Bacterial enumeration :**

After preparation and inoculation of beef samples at day 1 and the 10 subsequent days of storage, meat samples were taken at random to enumerate *L. monocytogenes*, Lactic acid bacteria, *Enterobacteriaceae* and Total Aerobic Counts.

Portions of 10 g of each meat sample were weighed and homogenized in a plastic bag, with 0.1 % peptone solution in the Stomacher for 1 min, in order to have decimal dilutions from 10<sup>-1</sup> to 10<sup>-12</sup>. Total aerobic counts were determined using Plate Count Agar (PCA; Difco Laboratories) incubated aerobically at 30°C for 2 days. *L. monocytogenes* was enumerated by using the standard plate count on PALCAM agar (Difco) after incubation of 48 h at 30°C. Lactic acid bacteria counts were determined using de Man Rogosa Sharpe (MRS) agar (Merck; Darmstadt, Germany). Inoculated MRS plates were incubated at 30°C for 48h. *Enterobacteriaceae* were enumerated on Sorbitole MacConkey agar (SMCA, Merck, Darmstadt, Germany), using the pour plate method and incubated at 37°C for 18-24 h. Red colonies were counted and the results expressed as means ( $\pm$  standard deviation).

### **pH determinations :**

The pH was measured in slurry made of 10 g of meat blended with 100 ml of distilled water for 2 min in a Stomacher according to the procedure described by Koniecko (1985). Readings were taken using pH/Temp meter (Model 8000, VMR Scientific product) according manufacturer's instructions.

### **Statistical analyses :**

Each experiment was repeated four times. Bacterial counts were means of two replicate, the numbers of total aerobic counts, *L monocytogenes*, *Enterobacteriaceae* and LAB were transformed with logarithm to the base Data and were analyzed using the Statistical Analysis System software program (SAS Institute, Cary, NC). Microbiological and pH data were analyzed by the general linear models (glm) procedure and duncan's multiple range tests with examination for significant differences ( $p < 0.05$ ).

### ***Antagonistic action of biopreservatives Lactobacillus plantarum strain on L. monocytogenes in fresh camel meat stored at 4°C and at 20°C :***

#### **Preparation of cultures for treatments:**

*Lactobacillus plantarum* were grown for 24h at 30°C in MRS broth (Difco). These cultures were centrifuged at 10 000 x g for 10 min at 4°C, washed three times and re-suspended in sterile saline water to give a cell concentration of  $10^6$  CFU/ml. Freshly prepared cultures of *L. monocytogenes* were harvested from 18 h in BHI (Difco, USA) culture by centrifugation at 10 000 x g for 10 min at 4°C, re-suspended in 10 ml of sterile saline water, and diluted to a final concentration of  $10^4$  CFU/ml.

#### **Fresh meat inoculation and storage:**

Fresh camel meat was divided into 100 g portions, placed in individual plastic bags for further treatments and inoculations. The control sample was inoculated with *L. monocytogenes* alone to an initial inoculum of approximately  $\sim 4$  log CFU/ml. Test

samples were inoculated with both *L. plantarum* to the levels of  $6.0 \log_{10}$  CFU/ml and *L. monocytogenes* at the same concentration as the control. Inoculated meat samples were thoroughly mixed with a sterile spatula followed by shaking the bag by hand. A control sample was immediately analyzed. The remaining bags were vacuum packed and stored at 4°C and at 20°C for 10 days, and the counts of *L. monocytogenes* and LAB were monitored as described above.

## **Results and discussion :**

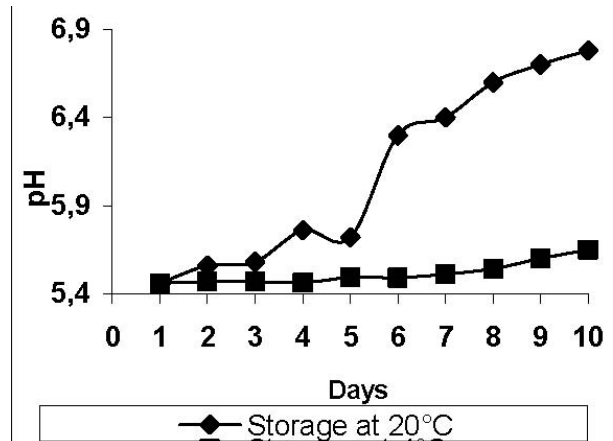
### **Growth and survival of *L. monocytogenes* onto camel meat stored at 4°C and 20°C:**

The initial pH of non-inoculated camel meat was 5.46, while the pH of inoculated samples has increased significant ( $P < 0.05$ ) during the storage period at both temperatures (i.e., 4°C and at 20°C) (Fig 1).

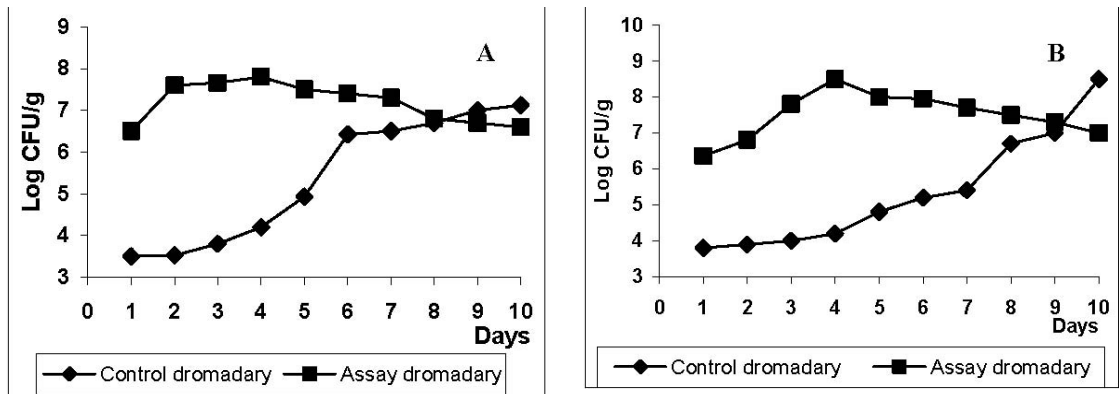
However, within the first 6 days of storage, only slight changes were observed, and the pH increased by approximately 0.03 to 0.2 units in meat samples stored at 4°C. However, at 20°C, the pH increased rapidly starting from day 3. By the end of storage at 4°C (day 10), the pH increased to a final value of 5.55. Such increase did not represent a significant difference ( $p > 0.05$ ) compared to the initial pH of meat samples. However, at the end of storage at 20°C, the pH of camel meat has increased rapidly to reach a value of 6.78. The pH increased rapidly presumably as a result of the production amines during storage (Tamplin, 2002).

In the present study, *L. monocytogenes* was not detected in non-inoculated camel meat suggesting the absence the pathogen of the meat samples analysed. Table 1 shows the Results of the microbiological analysis carried out on camel meat samples inoculated with *L. monocytogenes* during storage at 4°C and at 20 °C for 10 days. A slight increase in the counts of *L. monocytogenes* and TAC was observed during the first 3 days

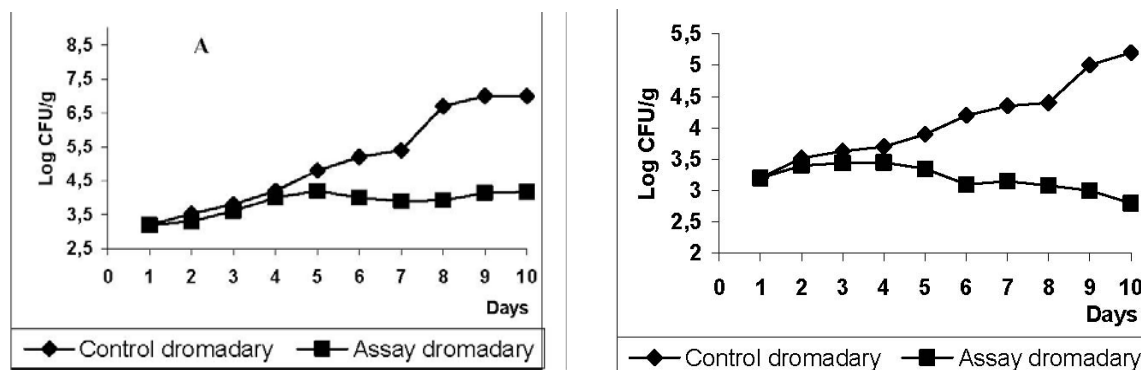
of cold storage (~ 4°C). After 4 and 3 days of storage at 4°C and 20°C respectively, significant difference in the growth of the *L. monocytogenes* was observed.



**Fig 1: Changes of pH values on camel meat pieces inoculated with *L. monocytogenes* during storage at 4°C and 20°C for 10 days**



**Fig 2: Changes in numbers of presumptive LAB on camel meat inoculated with *L. monocytogenes* for test control, and supplemented with *Listeria* and *L. plantarum*  $10^6 - 10^7$  CFU/g during 10 days of storage at 4°C (A) and at 20°C (B).**



**Fig 4: Inhibition of *L. monocytogenes* on camel meat supplemented with *L. plantarum*  $10^6$  –  $10^7$  CFU/g stored at 4°C (A) and at 20°C (B) for 10 days.**

Reducing the redox potential by vacuum-packaging and storage at refrigerated temperatures are two of the factors that enhance growth of LAB. Growth of LAB was observed at both temperatures; 9.1 Log CFU/g at 20°C, and 10.4 Log CFU/g at 4°C. However, LAB counts were influenced by factors such as levels of initial contamination. In our work, counts on MRS constituted the majority of the background microflora in meats during storage. Studies on refrigerated vacuum-packed meat products carried out by other authors have demonstrated a similar dominance of this microbial group (Blixt and Borch, 2002).

The initial level of *Enterobacteriaceae* was approximately  $2.2 \log_{10}$  CFU/g was observed in both temperatures of storage. Growth of the *Enterobacteriaceae* was initiated in the first 4 days and 3 days at 4°C and 20°C, respectively. At the end of storage, *Enterobacteriaceae* counts at 4°C and 20°C have reached their highest levels of  $4.22 \log_{10}$  CFU/g and  $5.33 \log_{10}$  CFU/g, respectively.

Microbial spoilage of Food occurs when total aerobic counts and/or *Enterobacteriaceae* counts reach  $7 \log_{10}$  CFU/g and when lactic acid bacteria reach  $7$

$\text{Log}_{10} \text{CFU/cm}^2$  (Nortjé and Shaw, 1989). In the present study none of the dromedary samples with  $>7 \log_{10} \text{CFU/g}$  had any off-odours or color changes, even when higher than  $8 \log_{10} \text{CFU/g}$  total aerobic count were detected. These results, are in agreement with those of the growth and survival of *P.aeruginosa* and *E.coli* inoculated onto ground raw dromedary stored at  $10^\circ\text{C}$  founds by Kalalou (2005).

The consequence of growth of *L. monocytogenes* on meat is particularly serious. In this study we used a storage temperature of  $20^\circ\text{C}$  as a “worst case” of inadequate refrigeration. Several studies have predicted the growth of pathogens based in various conditions of temperature, pH, and in presence or presence of additives, such as salt and sodium nitrite (Tamplin, 2002). Hygiene conditions, temperature and storage time influence the survival and growth of *L. monocytogenes*. In this experiment, we simulated the worst possible storage conditions (10 days at  $20^\circ\text{C}$ ).

#### **Antagonistic activity of protective culture of *Lactobacillus plantarum* against *L. monocytogenes* in fresh camel meat stored at 4 or $20^\circ\text{C}$ :**

In the present work, the initial count of LAB in the control samples(non-inoculated fresh camel meat), was  $3.5 \log_{10} \text{CFU/g}$  which increased to  $7.12 \log_{10} \text{CFU/g}$  and  $8.5 \log_{10} \text{CFU/g}$  at the end of storage at  $4^\circ\text{C}$  or  $20^\circ\text{C}$ , respectively (fig 2). In meat samples inoculated with cell suspensions of *Lb. plantarum* at  $6.5 \log_{10} \text{CFU/g}$ , the counts have increased to  $7.6 \log_{10} \text{CFU/g}$  at the second day 2 and to  $8.5 \log_{10} \text{CFU/g}$  at day 4 of the storage at  $4^\circ\text{C}$  and at  $20^\circ\text{C}$ , respectively. It should be noted that all LAB, either inoculated or naturally occurring in meat were enumerated in the treated samples. The subsequent increase in LAB counts of the treated and vacuum packaged sample indicated the development of spontaneous lactic flora. The results are in general agreement with those reported by Smith and Palumbo (1983) on mesophilic LAB.

Vold et al., (2000) showed the importance of the natural background flora in meat consisted mainly of LAB of which approximately 80 % were *Lb. sakei* for inhibition of growth of *E.coli* O157:H7.

Viable counts of *L. monocytogenes* from the *Lb. plantarum* treated cubes were 2.4 log units and 2.83 less compared with count from untreated cubes at 10 day at 4 °C and 20 °C (fig 4). Similar extent of inhibition was reported by Greer et al. (1995) who have studied the inhibitory effect of *Brochothrix campestris* against *L. monocytogenes* in pork adipose tissue discs suggesting that reduction of the viability of *L. monocytogenes* during storage at 4°C was about 2–3 log units as compared with the non-inoculated samples. Alves et al., (2003) found that *L. monocytogenes* was significantly inhibited in the presence of a bacteriocin-producing *Lb. sakei* in comparison to the control (*L. monocytogenes* alone) on cooked ham stored at 8°C for 10 days.

Antimicrobial activity of lactic acid against *L. monocytogenes* has been reported by many researchers (Dubal et al., 2004). 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 (1972) observed that the exposure of vacuum packed beef in air changed the microflora to resemble that of aerobically packed meat. Shillinger and Lucke (1987) inoculated psychrotrophic LAB in beef and pork at low concentrations (Log 3-4/g) before vacuum packaging and observed that *Lb. sakei* 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 psychrotrophic LAB onto sterile beef slices and observed that the aerobic deterioration of meat quality was faster with increased time of storage under vacuum.

Previous workers have used mesophilic LAB such as *Lb. plantarum* (Raccach et al., (1979)) to inhibit the growth of spoilage and pathogenic organisms under aerobic storage and concluded that very high cell concentrations were required to affect the

growth of spoilage microorganisms growth (Ahn *et al.*, (1990)). Also, Murthy *et al.*, (1997) observed an antagonistic effect of LAB under aerobic storage only when initial contamination of meat with psychrotrophs was low (log 2.8/g). The shelf life of vacuum packed fresh meat is highly variable depending upon several factors such as pH(Rousset and Renerre, (1991), initial contamination and storage temperature (Bell and Garout, (1994)) and ultimately spoiled due to discoloration, off-odours and purge loss (Borch and Agerhem, (1992)).

**Table I : Evolution of microbiological counts (log CFU/g) as function of time in camel meat artificially contaminated with *L. monocytogenes* during storage at 4° or 20 °C.**

	Storage period at 4°C (days)									
	1	2	3	4	5	6	7	8	9	10
<i>L. m</i>	3.02±	3.50 ±	3.90 ±	4.20 ±	4.27±	4.60±	4.80±	5.00 ±	5.23 ±	5.74±0.28
	0.04	0.07	0.36	0.32	0.19	0.00	0.24	0.16	0.19	
TCA	4.62±	4.70 ±	4.73 ±	5.60 ±	6.75±	7.20 ±	7.60±	8.10 ±	8.00 ±	8.25
	0.17	0.02	0.14	0.38	0.15	0.12	0.54	0.05	0.41	±0.01
<i>E</i>	2.21 ±	2.52 ±	2.73 ±	2.73 ±	2.89±	3.62 ±	3.90±	4.21 ±	4.23 ±	4.22
	0.17	0.16	0.66	0.73	0.09	0.10	0.28	0.41	0.26	±0.38
LAB	3.90 ±	4.05 ±	3.97 ±	5.42 ±	6.61±	7.39 ±	8.56±	8.77±	9.20 ±	8.87
	0.14	0.17	0.02	0.15	0.41	0.50	0.54	0.13	0.24	±0.12

*L.m* = *L. monocytogenes* ; *E* = *Enterobacteriaceae*

	Storage period at 20 °C (days)									
	1	2	3	4	5	6	7	8	9	10
<i>L. m</i>	3.27 ±	3.39 ±	3.72 ±	4.55 ±	5.62 ±	5.71 ±	4.89±	7.14 ±	6.63 ±	7.52 ±
	0.04	0.36	0.10	0.15	0.73	0.45	0.05	0.32	0.82	0.28
TCA	4.62 ±	4.67 ±	4.63 ±	4.90 ±	5.35 ±	5.70 ±	7.02±	5.12 ±	7.50 ±	9.45 ±
	0.18	0.35	0.12	1.01	0.12	0.16	0.20	0.32	0.01	0.17
<i>E</i>	2.20 ±	2.43 ±	3.50 ±	4.79 ±	4.81 ±	4.90 ±	5.10±	9.48 ±	5.30 ±	5.33 ±
	1.07	0.25	0.40	0.09	0.17	0.22	0.38	0.12	0.06	0.01
LAB	3.90 ±	3.96 ±	4.12 ±	4.75 ±	4.86 ±	5.72 ±	7.65±	5.97 ±	9.90 ±	10.41 ±
	0.19	0.52	0.03	0.16	0.06	0.28	0.42	0.70	0.04	0.07

Values are expressed in log<sub>10</sub> colony forming unit (CFU)/g ± SD (Standard deviation).

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