Scientific Research Publishing

Food and Nutrition Sciences, 2020, 11, 98-112
https://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

# Use of *Lactococcus lactis* Subsp. *Lactis* Strains to Inhibit the Development of Pathogens

**Abir Maalaoui[1,2], Abdesselem Trimeche[2], Pierre Guy Marnet[3], Yann Demarigny[4*]**

[1]Institut Supérieur Agronomique de Chott Mariem, Université de Sousse, Sousse, Tunisie
[2]Laboratoire de gestion de la santé et de la qualité des productions animales, Ecole Nationale de Médecine Vétérinaire de Sidi Thabet, Université de La Manouba, Manouba
[3]Department of Animal Science and Production, AGROCAMPUS OUEST, Rennes, France
[4]Bioengineering and Microbial Dynamic at Food Interfaces (Associated team n˚3733 (BioDyMIA), University of Lyon 1-ISARA Lyon), Isara-Lyon, Agrapole-23 rue Jean Baldassini, F-69364 Lyon Cedex 07, France
Email: *ydemarigny@isara.fr

**How to cite this paper:** Maalaoui, A., Trimeche, A., Marnet, P.G. and Demarigny, Y. (2020) Use of *Lactococcus lactis* Subsp. *Lactis* Strains to Inhibit the Development of Pathogens. *Food and Nutrition Sciences*, **11**, 98-112.
https://doi.org/10.4236/fns.2020.112009

**Received:** January 12, 2020
**Accepted:** February 24, 2020
**Published:** February 27, 2020

Copyright © 2020 by author(s) and Scientific Research Publishing Inc.
This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).
http://creativecommons.org/licenses/by/4.0/

Open Access

## Abstract

Bovine mastitis affects the udder health and thus causing significant economic losses. Probiotic products based on the use of lactic acid bacteria (LAB) to limit pathogens multiplication and pre-infection risks can be an interesting alternative to post infection allopathic treatment with antibiotics. *Lactococcus lactis* is one of the most important bacteria used in dairy technology. In this work, a total of 21 *Lactococcus lactis subsp. lactis* strains, 20 from goat milk whey and one strain from cow milk were used to evaluate their antibacterial activity against four pathogenic germs responsible for mastitis: *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus agalactiae*. The nisin-producing cow milk strain was active against *St. uberis* and *Str. agalactiae* using the well diffusion method. For the strains isolated from goat milk whey, no antimicrobial effect was observed against these pathogens. However, a different approach based on the growth of pathogenic bacteria interacting with the *Lactococcus lactis* strains in a minimum medium was used to study the barrier effect of LAB. The *Lactococcus lactis* strains S1 and S2 from goat milk whey depleted the growth of *Sa. aureus*, *St. uberis* and *E. coli* during 8 h and stopped the development of *St. agalactiae*.

## Keywords

*Lactococcus lactis*, Minimal Medium, Pathogenic Bacteria, Antibacterial Activity, Barrier Effect

## 1. Introduction

Mastitis is the most frequent and costly health problem for dairy cattle. These

losses are explained by the decrease in milk production and milk discarded [1], the deterioration of the milk quality and the problems in the process of milk transformation [2], the reform of cows at an early age [1] and the costs of the mastitis treatment [3]. Mastitis results from an infection of the udder by exogenous pathogen bacteria such as *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae* or *Escherichia coli*. This disease is generally healed by the use of antibiotics. However, these substances could disrupt the technological quality of milk and later the process of milk transformation. In addition, antibiotic residues in milk are a problem for animal and human health: it may cause food poisoning and subsequent bacterial resistances [4] [5]. Consequently, there is a growing expectation for alternative approaches to treat mastitis. More attention needs to be bestowed to new and effective prevention methods, for instance the use of lactic acid bacteria (LAB) as preservatives. LAB are Gram-positive bacteria that can display rod or cocci forms. They are immobile and non-sporulating. Following the species, they can exhibit a facultative aerobic metabolism; they never produce catalase. LAB share the ability to ferment sugars to produce organic acids, mainly lactic acid [6], which causes a reduction of the pH and the redox potential. It contributes to inhibit many microorganisms [7].

LAB are often found naturally in food such as meat, milk, grains, plants and vegetables. They can be divided into two groups according to their optimal growth temperature: mesophilic—with an optimum growth temperature between 20˚C and 37˚C—and thermophilic bacteria—between 40˚C and 45˚C [8]. The main LAB genera include *Lactobacillus*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Lactosphora*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* [9].

Hence, close to their organoleptic influence, LAB used in food also prevent contaminations from pathogenic or spoiling germs. This capacity is related to the synthesis of antibacterial compounds such as organic acids, bacteriocins and other compounds (hydrogen peroxide, diacetyl …) [10] [11]. The production of bacteriocins by some LAB strains to deplete pathogenic microorganisms has been studied for many decades [12]. Bacteriocins mainly influence microorganisms by destabilizing the bacterial membrane [13]. Among bacteriocins, nisin is a peptide produced by some *Lactococcus lactis* subsp. *lactis* strains; for instance, it is known to inhibit *Listeria monocytogenes*. Nisin can be used as a biocontrol agent in veterinary and health care.

The use of LAB is not restricted to the food field. Nowadays, other applications are investigated in the medical and veterinary fields. The food microbiology lab of ISARA has worked for many years on food fermentations and on the use of LAB: production of exopolysaccharides, retreatment of food wastes, etc. The gathering of LAB strains from many environments allows expanding their use to other contexts. This is why we decided to check the possibility of using these bacteria as preventing treatment against mastitis. This work aimed at the evaluation of the antimicrobial activity of caprine *Lactococcus lactis* strains towards some frequent pathogens of the udder, using direct and indirect tests. The

direct tests explored the possible production of inhibiting substances by LAB against pathogens (antagonism test) while the indirect way evaluated the barrier effect exerted by LAB in harsh conditions.

## 2. Materials and Methods

### 2.1. Bacterial Strains and Growth Media

In this study, we initially followed the growth of pathogenic bacteria to determine the growth kinetics of each strain. Then we tested the direct effect of LAB against these pathogens by the agar well diffusion method to determine the inhibitory effect of LAB. Afterwards, we evaluated the barrier effect exerted by LAB in limited conditions by the use of a minimum medium. We tried to be as close as possible to the reality to mimic the characteristics of the udder skin. After having tested the effect of LAB, we evaluated the autolytic capacity of these strains under boundary conditions. We evaluated their ability to survive between two milking steps.

### 2.2. Lactic Acid Bacteria Strains

Twenty *Lactococcus lactis* strains from goat milk whey were purchased from the food microbiology laboratory of ISARA. These strains were reactivated in M17 broth (Biokar) and grown over night at 30˚C.

The nisin-producing strain *Lc lactis* subsp. *lactis* LMG 07930 was obtained from the Microbiology Laboratory of the University of Gent (Belgium); this strain is originating from cow milk. It was reactivated according to the producer recommendations, in MRS broth (Biokar) and grown overnight at 30˚C. During the course of this study, this strain was used as a positive control against the pathogen strains tested.

### 2.3. Pathogenic Strains

The following pathogens were tested during the course of this study. *Escherichia coli* and *Staphylococcus aureus* came from the microbiology lab of ISARA. *Streptococcus uberis* (LMG14371) and *Streptococcus agalactiae* (LMG14694) which originate from cow milk were obtained from the Microbiology Laboratory of the University of Gent.

These pathogenic strains were reactivated in brain heart infusion broth (BHI, Biokar) and grown overnight at 37˚C.

### 2.4. Tracking the Growth of the Pathogens

The optical density at 600 nm ($OD_{600}$) was measured over time to determine the kinetic of bacterial growths in liquid medium.

All the strains were stored at −80˚C. To re-activate the pathogenic germs, 50 µL of each strain were inoculated in a 9mL BHI broth previously sterilized at 121˚C for 15 min. Incubations were performed at 37˚C for one night.

The day after, 100 µL of the previous culture were added in a 200 mL Erlen-

meyer flask containing 100 mL of BHI broth. The Erlenmeyer flasks were placed in a water bath at 37˚C. Every half-an-hour, 1 mL was sampled to measure $OD_{600}$.

Bacterial counts were made at initial time (T0) and at the end of the culture (Tf, after 16 h). Samples were poured on solid BHI medium after subsequent dilutions in tryptone-salt diluent (Biokar). Colony counts encompassing between 30 and 300 colonies were kept for further calculations.

## 2.5. Antagonism Tests

### 2.5.1. Preparation of Bacterial Pre-Cultures

Each pathogenic strain was inoculated in a test tube containing 9 mL of BHI broth. The tube was incubated at 37˚C for 18 h. The different strains of *Lc lactis* were inoculated in 5 mL of their favorite culture medium: either M17 or MRS broth. Tubes were incubated at 30˚C for 18 h.

### 2.5.2. Agar Well Diffusion Method

The antibacterial activity of *Lc lactis* was evaluated by the well diffusion method in BHI agar. LAB and pathogen cultures were prepared according to the procedure detailed above. LAB supernatants were first gathered by centrifugation at 20,000 g for 2 min at 20˚C. They were then filter-sterilized (0.22 μm, Millipore). Agar plates were inoculated in depth by a volume of the pathogen inoculum, to obtain approximately a concentration of 6 log(cfu)/mL of medium. Afterwards, a 5 mm diameter well was punched aseptically with a sterile tip; 100 μL of the LAB supernatant were poured into the well. A well including sterile broth was systematically reserved as negative control. Plates were then left at 4˚C for 1 h to enable the diffusion of the antibacterial substances into the medium. Finally, plates were incubated at 37˚C for 24 h in aerobic conditions, except for *St agalactiae* (anaerobic conditions). An inhibition was checked when a transparent zone surrounded the well.

Inhibition zones (IZ) were reported in millimeters. The diameters of the well and the translucent zone around the well were measured. Results were expressed according to the following formula:

$$IZ = \text{Inhibition zone diameter} - \text{Well diameter}$$

The inhibition was considered significant if IZ were larger than 2 mm [14]. Antibacterial activity tests were carried out four times. The average values were calculated.

## 2.6. Development of a Minimum Medium to Mimic the Characteristics of the Udder Skin

### 2.6.1. Tracking the Survival Rate of *Lactococcus lactis* in a Minimum Medium

A minimum medium (MMD) was prepared to mimic the physicochemical conditions that prevail at the surface of the skin of the udder. The composition of this medium was the following: $K_2HPO_4$ (0.1M, Sigma), peptone (0.5%, Biokar),

glucose (0.5%, Sigma), Agar (15 g/l, Biokar), pH 7.

A *Lc lactis* culture was obtained after one night of incubation at 30˚C in M17 broth. Two milliliters from this culture were poured in duplicate at the surface of a MMD petri dish. The excess of liquid was re-aspirated. The two agar plates were dried at room temperature. The whole medium was then removed from the first plate to be diluted ten times in tryptone salt diluent. The bacterial concentration was evaluated by colony counting (on M17 or MRS). The result (N0) was expressed in cfu/$cm^2$. The second plate was incubated at 30˚C for 8 h. This duration was chosen to mimic the lapse between two milking steps. The procedure followed to evaluate the microbial rate after 8 h (N8) was the same as detailed above. The survival rate was expressed as the ratio N8/N0 between the level at 8 h and the level at initial time. A value above 1 indicated a growth whereas a value below 1 meant a decrease.

#### 2.6.2. Tracking the Survival Rate of the Pathogen Germs in a Minimum Medium

The same methodology was followed to study the survival rate of the pathogens. However, colony count media differed. The VRBG, Chapman and Blood media (all purchased from Biokar) were respectively used to count *E coli*, *Sa aureus*, and *St uberis* and *St agalactiae* colonies.

#### 2.6.3. The Barrier Effect Exerted by *Lactococcus lactis*

A *Lc lactis* strain was cultured for 24 h in M17 broth (30˚C). Two milliliters were spread at the surface of a MMD plate. The excess of liquid was sucked up and the plate let dry for few minutes. Then, 2 mL of the germ to be tested were spread on the plates. The objective levels were respectively, 2, 3 and 6 log(cfu)/plate for *E coli* and around 6 log(cfu)/plate for *Sa aureus*, *St agalactiae* and *St uberis*. As previously stated, the excess of this second culture was re-aspirated; the plates were then dried. Four plates were prepared for each pathogen. The same procedure was followed to measure the concentrations of each microbial population at initial (T0) and final time (T8) as described above.

#### 2.6.4. Autolytic Capacity of *Lactococcus lactis* under Minimal Conditions

To test the ability of *Lc lactis* strains to survive in a limited medium, we used a $K_2HPO_4$ buffer (0.1M). First, LAB strains were cultured in a liquid broth (M17 or MRS) at 30˚C for 18 h. Then, cultures were centrifuged at 20.000 g for 2 min. Supernatants were removed and the pellet washed twice with $K_2HPO_4$ buffer (0.1M). Pellets were finally diluted in 1 mL of the same buffer. 100 µL of this suspension were put in a vial containing 20 mL of $K_2HPO_4$ (0.1M). The optical density ($OD_{600}$) of the solution was followed over time at two temperatures (20˚C - 22˚C and 4˚C). OD measures were made twice a day for one week.

## 3. Results

### 3.1. Tracking the Growth of Pathogens

The growth curves of the different pathogenic strains are displayed on **Figure 1**.

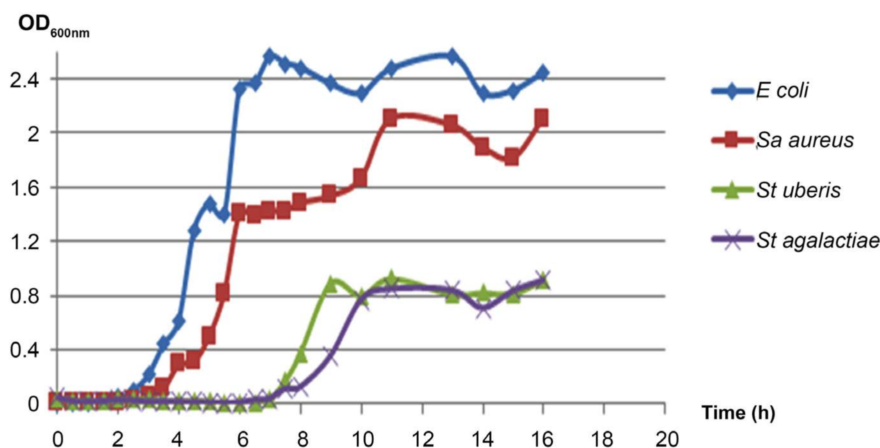


**Figure 1.** Growth of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus agalactiae* in BHI broth under standard conditions (pH 7.4, 37˚C).

In standard conditions (37˚C, pH 7.4), the curve shapes were similar. But the growth levels obtained were different. These differences are explained by the lag phase duration, linked with the inoculation levels which varied from $3.4 \times 10^7$ cfu/ml for *E coli* to $6.2 \times 10^6$ cfu/ml for *Sa aureus*, $1.97 \times 10^6$ cfu/ml for *St uberis* and $2.55 \times 10^5$ cfu/ml for *St agalactiae.* Final counts were similar. They were equal to $2.69 \times 10^9$ cfu/ml, $1.73 \times 10^9$ cfu/ml, $1.70 \times 10^9$ cfu/ml and $1.64 \times 10^9$ cfu/ml for *E coli*, *Sa aureus*, *St uberis* and *St agalactiae* respectively.

## 3.2. Screening of the *Lactococcus lactis* Strains for Their Antimicrobial Activity

The 20 strains of *Lc lactis* from the lab and the nisin-producing LMG 07930 strain were tested for their antimicrobial activity against *E coli*, *Sa aureus*, *St uberis* and *St agalactiae.* Results are summarized in Table 1. In this study, the strain LMG 07930 was used as a positive control against pathogens, knowing that our goal preferentially aimed at the selection of one or two strains from the lab collection.

Among the 20 strains *Lc lactis* from goat milk whey, no antagonistic activity against the germs tested was observed (Table 1). This methodology allows to test the direct effect of substances released against a target bacterium. It does not concern any other effect, such as nutrient competition between microbes.

The nisin-producing *Lc lactis* LMG 07930 strain seemed to be inactive against *E coli* and *Sa aureus*. Concerning *St uberis* and *St agalactiae*, the presence of an inhibition zone surrounding the well was visible, with an average diameter respectively equal to 5.75 mm and 6.75 mm (Figure 2).

## 3.3. Utilization of a Minimum Model Medium to Test the Behavior of the Microorganisms

A minimum model medium (MMD) was used to mimic the growth at the surface of the udder skin.

**Table 1.** Diameter of the inhibition zones (IZ) obtained with the *Lactococcus lactis* cell-free supernatants on the pathogenic strains tested: *E coli*, *Sa aureus*, *St uberis* and *St agalactiae.* Each value, expressed in mm, is the mean of 4 repetitions.

| Pathogenic germs | *Lactococcuslactis* strains | Diameter of the IZ (mm) Mean ± standard deviation |
|---|---|---|
| *Sa aureus* | Control cow strain LMG07930 | 0.0 ± 0.0 |
| | Goat *Lclactis* strains 1 to 20 | 0.0 ± 0.0 |
| *E coli* | Control cow strain LMG07930 | 0.0 ± 0.0 |
| | Goat *Lclactis* strains 1 to 20 | 0.0 ± 0.0 |
| *Stuberis* | Control cow strain LMG07930 | 5.75 ± 3.59 |
| | Goat *Lclactis* strains 1 to 20 | 0.0 ± 0.0 |
| *Stagalactiae* | Control cow strain LMG07930 | 6.75 ± 0.5 |
| | Goat *Lclactis* strains 1 to 20 | 0.0 ± 0.0 |

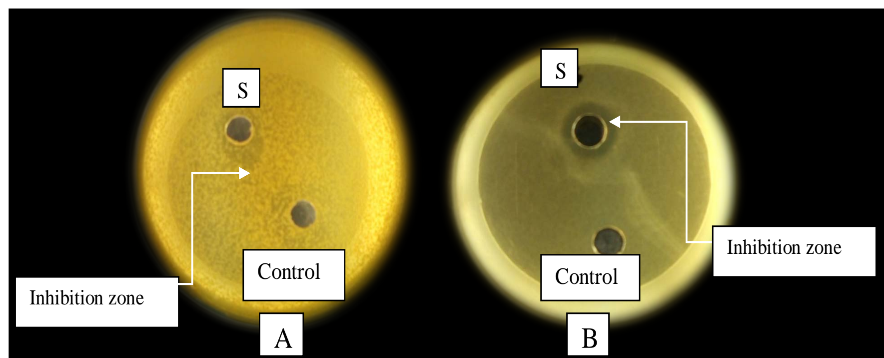


**Figure 2.** Antagonistic activities of the nisin-producing *Lc lactis* LMG 07930 strain cell-free supernatant (S) against, A: *St uberis* and B: *St agalactiae*; a negative control (MRS broth) was systematically present.

Among the twenty *Lc lactis* from the lab, two strains, S1 and S2, were selected for further tests. The behavior of these two strains and of the four pathogenic germs was evaluated in the minimum medium between two steps: initial time (N0) and after a 8 h delay of culture at 30˚C (N8).

Between the initial time (N0) and 8 h (N8), the level of the two strains S1 and S2 was multiplied by approximatively 13. Hence, the two LAB were able to survive in a minimal context, and even grow (Table 2).

The pathogenic strains strongly increased: +4.08, +2.88, +2.10 log(cfu)/cm$^2$ for *Sa aureus*, *St agalactiae*, *St uberis* respectively, and between +0.99 and +2.71 log(cfu)/cm$^2$ for *E coli* (Table 3). As noted for the two LAB, the minimum medium allowed the development of the bacteria tested.

Interactions between each strain of *Lc lactis* and each pathogenic germ were then evaluated (Table 4).

The LAB levels in the medium were set at 9 log(cfu)/plate. Whereas for pathogens, the objective levels were adjusted to 6 log(cfu)/plate for *Sa aureus*, *St agalactiae*, *St uberis* and to 2, 3 or 6 log(cfu)/plate for *E coli.* In details, the adjustment of each initial level proved to be difficult.

**Table 2.** Behavior of the two *Lactococcus lactis* strains S1 and S2 after 8h (N8) of culture at 30˚C in a minimal medium.

| | S1 | S2 |
|---|---|---|
| N0 (log(cfu)/cm$^2$) | 5.10 | 5.13 |
| N8 (log(cfu)/cm$^2$) | 6.24 | 6.25 |
| Multiplication rate between N8 and N0 | 13.70 | 13.30 |

**Table 3.** Growth of the pathogenic germs *Sa aureus*, *E coli*, St agalactiae and *St uberis* cultured in a minimum medium for 8h delay at 30˚C.

| | *Sa aureus* at 6 log(cfu)/plate | *E coli* at 3 log(cfu)/plate | *E coli* at 6 log(cfu)/plate | *St agalactiae* at 6 log(cfu)/plate | *St uberis* at 6 log(cfu)/plate |
|---|---|---|---|---|---|
| N0(log(cfu)/cm$^2$) | 1.25 | 1.02 | 0.93 | 1.55 | 0.28 |
| N8(log(cfu)/cm$^2$) | 5.33 | 2.01 | 3.64 | 4.43 | 2.38 |
| Multiplication rate between N8 and N0 | 1.22 $10^4$ | 9.7 | 5.12 $10^2$ | 7.67·$10^2$ | 1.26·$10^2$ |

**Table 4.** Influence of the *Lc lactis* strains S1 and S2 on the survival of the pathogenic germs *Sa aureus*, *St agalactiae*, *E coli* and *St uberis*. N corresponds with the initial target level of the strains S1 and S2. ND: not determined.

| N | Strains | *Sa aureus* at 6log(cfu)/plate | *St agalactiae* at 6 log(cfu)/plate | *E coli* at | | | *St uberis* at 6 log(cfu)/plate |
|---|---|---|---|---|---|---|---|
| | | | | **3 log(cfu)/plate** | **2 log(cfu)/plate** | **6 log(cfu)/plate** | |
| | **N0 (log(cfu)/cm$^2$)** | 0.64 | 4.08 | 0.72 | 0.34 | 0.9 | 0.63 |
| S1 | **N8h (log(cfu)/cm$^2$)** | 2.30 | 4.43 | 1.53 | 1.48 | 2.97 | 2.26 |
| | **Multiplication rate** | 45.5 | 2.2 | 6.4 | 13.6 | 1.16 $10^2$ | 43 |
| | **N0 (log(cfu)/cm$^2$)** | 0.72 | 3.15 | <0.7 | <0.7 | 0.68 | 0.72 |
| S2 | **N8h (log(cfu)/cm$^2$)** | 2.23 | 3.30 | 2.18 | 1.57 | 2.93 | 2.3 |
| | **Multiplication rate** | 32.1 | 1.4 | 0 | 0 | 1.79 $10^2$ | 37.4 |

At T0, the number of Sa aureus was lower than expected, irrespective of the LAB strain considered: 0.64 log(cfu)/cm$^2$ for S1 and 0.72 log(cfu)/cm$^2$ for S2. After a 8h incubation delay, the population increased slightly and was multiplied by 45.5 and 32.1 in the presence of S1 and S2 respectively. The growth rate of *Sa aureus* was close to 0.2 log(cfu)/h, which was very slow. We can so infer that *Sa aureus* was partially affected by the presence of the LAB. For *St uberis*, results were almost similar to Sa aureus. The level of this pathogen increased by a factor equal to 43 and 37.4 in presence of S1 and S2 respectively.

Concerning *St agalactiae*, the inoculated level of this population was higher. However, it did not change significantly between the two steps: the difference between N8 and N0 was inferior to 0.5 log(cfu)/cm$^2$.

The starting level of *E coli* (intended to be at 2, 3 or 6 log(cfu)/plate) was actually set between <0.2 and 0.9 log(cfu)/cm$^2$. If the growth was significant, the number of bacteria did not strongly increase; the multiplication rates were slight whatever the initial level or the LAB strain used. Compared with *Sa aureus*, the

inhibition exerted by S1 and S2 on the growth of E. coli were nearly identical.

The OD600 nm of the strains S1 and S2 was followed in a minimal medium ($K_2HPO_4$, 0.1M) during seven days. We sought to evaluate the survival rate of each bacterium in harsh nutritional conditions. On **Figure 3** are shown the evolution curves of the strain S1 at two different temperatures, knowing that the behavior or the strain S2 was identical. The curves obtained with the control strain LMG 07930 are also displayed. As one can notice, the strains remained stable whatever the temperature. For instance, at ambient temperature, the OD600 nm of the S1 strain decreased from 0.283 to 0.217 in seven days. Consequently, the LAB selected seemed quite resistant to limited conditions.

## 4. Discussion

Two different strategies can be put forward to find solutions against mastitis: the use of chemical products spread at the surface of the udder skin is certainly efficient. But close to the eradication of the pathogens, it leads also to the destruction of the saprophytic microbial populations able to exert a barrier effect. Moreover, antimicrobial substances can injure the skin. Another strategy relies on the settlement of beneficial bacteria (*i.e.* lactic acid bacteria, LAB) to colonize the udder skin. This approach could be interesting for two reasons: LAB could avoid the pathogen contamination; and furthermore their growth. It can even lead to their destruction. LAB being known as positive technological agents, it could lead to the seeding of the milk with "good" bacteria. In this work, we decided to select two *Lactococcus lactis* strains originating from whey goat milk whey to test them against different pathogens involved in mastitis disturbance. In a further work, we intend to test them directly in dairy cattle.

The twenty strains of *Lc lactis* from goat milk whey that we tested exerted no direct inhibitory effect on the different pathogenic germs studied, meaning that they did not seem to produce any active antimicrobial substance. This assertion was confirmed by the observation of the behavior of the control nisin-producing strain LMG 07930. In this latter case, an inhibition was clearly recorded on *St uberis* and *St agalactiae*.

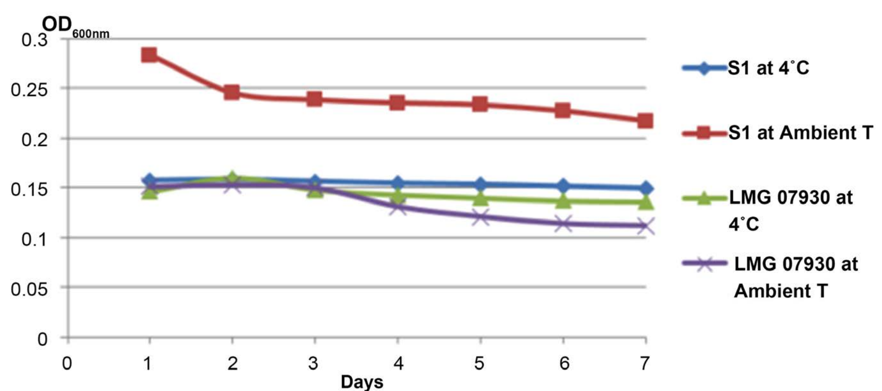


**Figure 3.** Evolution of the optical density (OD600 nm) of the *Lactococcus lactis* strains S1and LMG 07930 in a $K_2HPO_4$ buffer (0.1 M) at 4˚C and at ambient temperature (T).

In contrast, Aziz *et al.* [15] found that *Lc lactis* strains isolated from cow milk, presented a strong activity against Gram-positive (*Bacillus subtilis*, *Sa aureus* and *Clostridium perfringens*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *E coli*). They reported that 86% and 73% of the isolates were active respectively against *E coli* and *Sa aureus*. This observation is in agreement with our own experience. Among the fifty wild Lc lactis strains tested from different dairy products, more than 80% of the isolates exhibited an inhibitory effect against lactic acid bacteria (Demarigny, personal data). Using the disk methodology, Metlef and Dilmi-Bouras [16] showed that most of the *Lc lactis* subsp. *lactis* strains were active against *E coli*. According to Tenea *et al.* [17], three isolates of *Lc lactis* subsp. *lactis* from plant origin presented antimicrobial activities against four foodborne pathogens, *E coli* ATCC 25922, *E coli* UTN Ec1, *Salmonella enterica* ATCC 51741 and *Salmonella* UTN Sm2. Perin and Nero [18] showed that *Lactococcus* strains isolated from raw goat milk had an antimicrobial activity against *Listeria monocytogenes*.

The antimicrobial activity of LAB is generally explained by the production of organic acid, diacetyl, hydrogen peroxide, bacteriocins or specific substances [19] [20]. Our contradictory results can be partially explained by the origin of the *Lc lactis* strains, the type of inhibitory compounds produced by these strains and the susceptibility of the target bacteria used. To illustrate this assertion, Yerlikaya [21] showed that among eight *Lc lactis* strains isolated from raw cow milk, only three exerted an antimicrobial activity against *Sa aureus* ATCC12600. Three isolates were active against *E. coli* ATCC 25922; but none showed any effect on *E coli* CECT 4267.The authors also tested four *Lactococcus* isolates from raw goat milk: three were active against *Sa aureus* ATCC 12600, three against *E coli* ATCC 25922, and all inhibited *E coli* CECT4267. However, the strains isolated from kefir grains had no effect against the three pathogens previously mentioned, albeit they showed some interesting properties against other germs (*Enterobacter aerogenes*, *Listeria monocytogenes* and *Salmonella enteria* subsp. *enterica*). Likewise, Sharaf and Al Harbi [22] studied the antimicrobial activity of five *Lc lactis* strains from different origins against *E coli* and *Sa aureus*. Two strains from camel milk were active against *E coli* and four against *Sa aureus*, whereas strains from cow milk proved to be inactive.

In our study, we observed an antibacterial activity of the cell-free supernatants of a *Lc lactis* nisin producer strain against *St uberis* and *St agalactiae*. According to Guerra and Pastrana [23], this effect could be explained by the synergistic effect of nisin and other antimicrobial compounds produced by the bacterium (*i.e.* organic acids). Gram-negative bacteria such as *E coli*, *Ps aeruginosa*, *Salmonella typhimurium* and *Serratia marcescens* are known to be resistant to lantibiotics, in particular to nisin. This resistance is related to the outer membrane of Gram-negative bacteria rich in lipopolysaccharides [24] [25]. However, some other bacteriocins can inhibit Gram-negative bacteria, for instance bozacin and lacticin [26] [27].

Like any toxic substances, repeated exposure of some Gram-positive bacteria to increasing nisin concentrations can lead to resistances towards this peptide among the pathogens targeted [28] [29]. For instance, resistance to nisin has been observed among several species of bacteria such as *St bovis* [30], *L monocytogenes* [28] [29] [30] [31], *L innocua* [32], Sa aureus [33] and *Cl botulinum* [34]. Mechanisms for acquiring nisin resistance are complex and can differ from one strain to another [35]. They involve structural and physiological changes in the bacterial cell [36]. For all these reasons, we preferred to forbid the settlement of pathogens by the competition exerted by a LAB strain present at the surface of the udder skin.

During the course of this project, we proposed to use a minimum solid medium to mimic the nutritional composition of the surface skin. The skin is known to exhibit particular features: the pH is generally acid (±5.5), salty and characterized by the succession of dry and wet periods. As a consequence, water activity can vary a lot. In this work, we just focused on the nutritional aspects, knowing that the physico-chemical parameters are also important. We will improve our model in a later work. It is noteworthy that such an approach was not made in the past. Some models were developed, for instance by Charkoudian [37] or Forshind [38]. But they aimed at human health concerns. Otherwise, some articles were published on thermodynamic purposes to model the heat exchanges at the surface of the skin [39]. But, to our knowledge, no work specifically tried to propose a skin model for the study of bacterial interactions.

Based on the use of this medium, we observed that the two strains of *Lc lactis* S1 and S2 slowed down the growth of *Sa aureus* during 8 hours. This pathogen multiplied its initial level by 45.5 (+0.208 log(cfu)/cm$^2$/h) and 32.1 (+0.189 log(cfu)/cm$^2$/h) in presence of S1 and S2 respectively. At 30˚C, in a rich medium, the growth rate of Sa aureus in exponential phase is generally close to 0.4-0.5 log(cfu)/cm$^2$/h. Concerning *E coli*, the results led to the same conclusions as *Sa aureus*. The development of St agalactiae was stopped, the difference between T0 and T8h being non-significant. This germ seemed to be disturbed by the presence of the two LAB strains. Concerning St uberis, the two strains S1 and S2 slowed down the growth of this pathogen during the 8 h duration of the incubation. These results show that LAB can inhibit the growth of pathogenic microorganisms by their own presence due to a competition for nutrients and space. The competition for nutrients selects the most appropriate microorganisms to limited compounds (minerals, amino acids, sugars) [40]. Because of their important nutritional requirements, LAB probably overran the environment and limited the multiplication of other bacteria [41]. This type of competition for nutrients and space was observed between LAB and *Listeria* spp [42] [43] and between LAB and *Sa aureus* [44].

Our model seems to be suitable to study interactions between microbes. Right now, the protocol requires several improvements such as the incubation at different temperatures (the surface temperature of the udder can change), pH and

water activities to evaluate the influence of these parameters on the survival of LAB and pathogens.

The use of LAB strains to colonize the skin surface requires to test their ability to survive in limited conditions, in particular during the shelve-life of the LAB solution. The behavior of the Lc lactis strains was followed in a K2HPO4 (0.1M) buffer for one week. OD600nm did not change significantly during the seven days of the test. It allowed us to propose the storage of the LAB mixture during this duration.

Based on all these results, the two strains S1 and S2 can be used to dip the teats after milking. According to Guerra *et al.* [23], supernatants of *Lc lactis* can be used to treat surfaces in contact with food to prevent the adhesion of unwanted bacteria, such as *L. monocytogenes*.

## 5. Conclusions

The potential use of LAB strains to colonize the surface of the udder skin led to different results. The well diffusion test was done using a rich culture medium; it allowed the pathogenic bacteria to grow easily, a situation which proved to be far from reality by comparison with the udder surface. The 20 strains of *Lc lactis* originating from goat milk whey were ineffective against the four pathogenic germs tested in this rich medium. The direct interaction between LAB previously spread at the surface of a minimum medium and a pathogen led to different results; in particular, it depended on the pathogens used. But generally, their growth was slowed down (sometimes stopped) because of the presence of the LAB.

These encouraging results need to be deepened by taking into account the physico-chemical structure of the skin in our model. Thereafter, we manage ourselves to test the settlement of the LAB in a dairy herd and its consequences on cow health and on the quality of the raw milk.

## Conflicts of Interest

The authors declare no conflict of interest.

## References

[1] Seegers, H., Fourichon, C. and Beaudeau, F. (2003) Production Effects Related to Mastitis and Mastitis Economics in Dairy Cattle Herds. *Veterinary Research*, **34**, 475-491. https://doi.org/10.1051/vetres:2003027

[2] Le Maréchal, C., Thiéry, R., Vautor, E. and Le Loir, Y. (2011) Mastitis Impact on Technological Properties of Milk and Quality of Milk Products: A Review. *Dairy Science and Technology*, **91**, 247-282. https://doi.org/10.1007/s13594-011-0009-6

[3] Barkema, H.W., Schukken, Y.H. and Zadoks, R.N. (2006) Invited Review: The Role of Cow, Pathogen, and Treatment Regimen in the Therapeutic Success of Bovine *Staphylococcus aureus* Mastitis. *Journal of Dairy Science*, **89**, 1877-1895. https://doi.org/10.3168/jds.S0022-0302(06)72256-1

[4] Rodionov, G.V. (2008) Control of Inhibitory Substances in Milk. *Dairy Industry*, **2**,

25.

[5] Klimov, N.T. and Pershin, S.S. (2012) Modern View on the Problem of Mastitis in Cows. *Proceedings of International Scientific-Practical Conference*, Voronezh, 237-242.

[6] Fooks, L.J. and Gibson, G.R. (2002) Probiotics as Modulators of the Gut Flora. *British Journal of Nutrition*, **88**, 39-49. https://doi.org/10.1079/BJN2002628

[7] Schnürer, J. and Magnusson, J. (2005) Antifungal Lactic Acid Bacteria as Biopreservatives. *Trends in Food Science and Technology*, **16**, 70-78. https://doi.org/10.1016/j.tifs.2004.02.014

[8] Jozola, A.F., de Lencastre Novaes, L.C., Cholewa, O., Moraes, D. and Penna, T.C.V. (2005) Increase of Nisin Production by *Lactococcus lactis* in Different Media. *African Journal of Biotechnology*, **4**, 262-265.

[9] Dortu, C. and Thonart, P. (2009) Bacteriocins from Lactic Acid Bacteria: Interest for Food Products Biopreservation. *Biotechnologie Agronomie Societé et Environnement*, **13**, 143-154.

[10] Oyetayo, V.O., Adetuyi, F.C. and Akinyosoye, F.A. (2003) Safety and Protective Effect of *Lactobacillus acidophilus* and *Lactobacillus casei* Used as Probiotic Agent *in Vivo*. *African Journal of Biotechnology*, **2**, 448-452. https://doi.org/10.5897/AJB2003.000-1090

[11] Deegan, L.H., Cotter, P.D, Hill, C. and Ross, P. (2006) Bacteriocins: Biological Tools for Bio-Preservation and Shelf-Life Extension. *International Dairy Journal*, **16**, 1058-1071. https://doi.org/10.1016/j.idairyj.2005.10.026

[12] Moreno, I., Lerayer, A.L.S. and Baldini, V.L.S. (2000) Characterization of Bacteriocins Produced by *Lactococcus lactis* Strains. *Brazilian Journal of Microbiology*, **31**, 184-192. https://doi.org/10.1590/S1517-83822000000300007

[13] Hancock, R.E. and Diamond, W. (2000) Cationic Antimicrobial Peptides towards Clinical Applications. *Expert Opinion on Investigational Drugs*, **9**, 1723-1729. https://doi.org/10.1517/13543784.9.8.1723

[14] Thompson, J.K., Collins, M.A. and Mercer, W.D. (1996) Characterisation of a Proteinaceous Antimicrobial Produced by *Lactobacillus helveticus* CNRZ 450. *Journal of Applied Bacteriology*, **80**, 338-348. https://doi.org/10.1111/j.1365-2672.1996.tb03229.x

[15] Aziz, E.K., Abdulraman, Z.F.A. and Essa, R.H. (2015) Antibiotic Resistance Pattern and Effect of Some Growth Condition on *Lactococcus lactis* subsp. *lactis* Isolated from Cow Milk. *International Journal of Current Microbiology and Applied Sciences*, **4**, 388-405.

[16] Metlef, S. and Dilmi-Bouras, A. (2009) Effet antagoniste de *Lactococcus lactis*, souches extrêmophiles locales, sur des espèces de la flore intestinale résidente. *Revue Nature et Technologie*, **1**, 33-44.

[17] Tenea, G.N., Hurtado, H. and Ortega, C. (2018) Inhibitory Effect of Substances Produced by Native *Lactococcus lactis* Strains of Tropical Fruits towards Food Pathogens. *Preventive Nutrition and Food Science*, **23**, 260-268. https://doi.org/10.3746/pnf.2018.23.3.260

[18] Perin, L.M. and Nero, L.A. (2014) Antagonistic Lactic Acid Bacteria Isolated from Goat Milk and Identification of a Novel Nisin Variant *Lactococcus lactis*. *BMC Microbiology*, **14**, Article No. 36. https://doi.org/10.1186/1471-2180-14-36

[19] Fan, L. and Song, J. (2013) Antimicrobial Microbes-Bacteriocin Producing Lactic Acid Bacteria. In: Mendez-Vilas, A., Ed., Microbial Pathogens and Strategies for

Combating Them: Science, Technology and Education, Formatex Research Center, Badajoz, 899-909.

[20] Viana de Souza, J. and Silva Dias, F. (2017) Protective, Technological, and Functional Properties of Select Autochthonous Lactic Acid Bacteria from Goat Dairy Products. *Current Opinion in Food Science*, **13**, 1-9. https://doi.org/10.1016/j.cofs.2017.01.003

[21] Yerlikaya, O. (2019) Probiotic Potential and Biochemical and Technological Properties of *Lactococcus lactis* ssp. *lactis* Strains Isolated from Raw Milk and Kefir Grains. *Journal of Dairy Science*, **102**, 124-134. https://doi.org/10.3168/jds.2018-14983

[22] Sharaf, E.F. and Al Harbi, R.M. (2011) Isolation, Identification and Antimicrobial Activity of Some Local Isolates of Lactic Acid Bacteria. *Research Journal of Microbiology*, **6**, 826-838. https://doi.org/10.3923/jm.2011.826.838

[23] Guerra, N.P., Araujo, A.B., Barrera, A.M., Torrado, A., López, C., Carballo, J. and Pastrana, L. (2005) Antimicrobial Activity of Nisin Adsorbed to Surfaces Commonly Used in the Food Industry. *Journal of Food Protection*, **68**, 1012-1019. https://doi.org/10.4315/0362-028X-68.5.1012

[24] Chatterjee, C., Miller, L.M., Leung, Y.L., Xie, L., Yi, M., Kelleher, N.L. and van der Donk, W.A. (2005) Lacticin 481 Synthetase Phosphorylates Its Substrate during Lantibiotic Production. *Journal of the American Chemical Society*, **127**, 15332-15333. https://doi.org/10.1021/ja0543043

[25] Gänzle, M.G., Hertel, C. and Hammes, W.P. (1999) Resistance of *Escherichia coli* and *Salmonella* against Nisin and Curvacin A. *International Journal of Food Microbiology*, **48**, 37-50. https://doi.org/10.1016/S0168-1605(99)00026-4

[26] Lee, N.K. and Paik, H.D. (2001) Partial Characterization of Lacticin NK24, a Newly Identified Bacteriocin of *Lactococcus lactis* NK24 Isolated from Jeot-Gal. *Food Microbiology*, **18**, 17-24. https://doi.org/10.1006/fmic.2000.0368

[27] Todorov, S.D. and Dicks, L.M.T. (2007) Bacteriocin Production by *Lactobacillus pentosus* ST712BZ Isolated from Boza. *Brazilian Journal of Microbiology*, **38**, 166-172. https://doi.org/10.1590/S1517-83822007000100034

[28] Mazzotta, A.S. and Montville, T.J. (1997) Nisin Induces Changes in Membrane Fatty Acid Composition of *Listeria monocytogenes* Nisin Resistant Strains at 10 ˚C and 30 ˚C. *Journal of Applied Microbiology*, **82**, 32-38. https://doi.org/10.1111/j.1365-2672.1997.tb03294.x

[29] Garde, S., Ávila, M., Medina, M. and Nuñez, M. (2004) Fast Induction of Nisin Resistance in *Streptococcus thermophilus* INIA 463 during Growth in Milk. *International Journal of Food Microbiology*, **96**, 165-172. https://doi.org/10.1016/j.ijfoodmicro.2004.03.023

[30] Mantovani, H. and Russell, J.B. (2001) Nisin Resistance of *Streptococcus bovis*. *Applied and Environmental Microbiology*, **67**, 808-813. https://doi.org/10.1128/AEM.67.2.808-813.2001

[31] Collins, B., Curtis, N., Cotter, P.D., Hill, C. and Ross, R.P. (2010) The ABC Transporter AnrAB Contributes to the Innate Resistance of *Listeria monocytogenes* to Nisin, Bacitracin, and Various Lactam Antibiotics. *Antimicrobial Agents Chemotherapy*, **54**, 4416-4420. https://doi.org/10.1128/AAC.00503-10

[32] Maisnier-Patin, S. and Richard, J. (1996) Cell Wall Changes in Nisin-Resistant Variants of *Listeria innocua* Grown in the Presence of High Nisin Concentrations. *FEMS Microbiology Letters*, **140**, 29-35. https://doi.org/10.1111/j.1574-6968.1996.tb08310.x

[33] Blake, K.L., Randall, C.P. and O'Neill, A.J. (2011) *In Vitro* Studies Indicate a High Resistance Potential for the Lantibiotic Nisin in *Staphylococcus aureus* and Define a Genetic Basis for Nisin Resistance. *Antimicrobial Agents Chemotherapy*, **55**, 2362-2368. https://doi.org/10.1128/AAC.01077-10

[34] Mazzotta, A.S. and Montville, T.J. (1999) Characterization of Fatty Acid Composition, Spore Germination, and Thermal Resistance in a Nisin Resistant Mutant of *Clostridium botulinum* 169B and in the Wild Type Strain. *Applied and Environmental Microbiology*, **65**, 659-664. https://doi.org/10.1128/AEM.65.2.659-664.1999

[35] Zhou, H., Fang, J., Tian, Y. and Lu, X.Y. (2013) Mechanisms of Nisin Resistance in Gram-Positive Bacteria. *Annals of Microbiology*, **64**, 413-420. https://doi.org/10.1007/s13213-013-0679-9

[36] Wang, G., Mishra, B., Lau, K., Lushnikova, T., Golla, R. and Wang, X. (2015) Antimicrobial Peptides in 2014. *Pharmaceuticals*, **8**, 123-150. https://doi.org/10.3390/ph8010123

[37] Charkoudian, J.C. (1988) A Model Skin Surface for Testing Adhesion to Skin. *Journal of the Society of Cosmetic Chemists*, **39**, 225-234.

[38] Forslind, B. (1994) A Domain Mosaic Model of the Skin Barrier. *Acta Dermato- Venereologica*, **74**, 1-6.

[39] Gebremedhin, K.G. and Wu, B. (2001) A Model of Evaporative Cooling of Wet Skin Surface and Fur Layer. *Journal of Thermal Biology*, **26**, 537-545. https://doi.org/10.1016/S0306-4565(00)00048-6

[40] Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B. and Givskov, M. (2002) Food Spoilage-Interactions between Food Spoilage Bacteria. *International Journal of Food Microbiology*, **78**, 79-97. https://doi.org/10.1016/S0168-1605(02)00233-7

[41] Castellano, P., Belfiore, C., Fadda, S. and Vignolo, G. (2008) A Review of Bacteriocinogenic Lactic Acid Bacteria Used as Bioprotective Cultures in Fresh Meat Produced in Argentina. *Meat Science*, **39**, 225-234. https://doi.org/10.1016/j.meatsci.2007.10.009

[42] Nilsson, L., Gram, L. and Huss, H.H. (1999) Growth Control of *Listeria monocytogenes* on Cold Smoked Salmon Using a Competitive Lactic Acid Bacteria Flora. *Journal of Food Protection*, **62**, 336-342. https://doi.org/10.4315/0362-028X-62.4.336

[43] Buchanan, R.L. and Bagi, L.K. (1997) Microbial Competition: Effect of Culture Conditions on the Suppression of *Listeria monocytogenes* Scott a by *Carnobacterium piscicola*. *Journal of Food Protection*, **60**, 254-261. https://doi.org/10.4315/0362-028X-60.3.254

[44] Charlier, C., Cretenet, M., Even, S. and Le Loir, Y. (2009) Interactions between *Staphylococcus aureus* and Lactic Acid Bacteria: An Old Story with New Perspectives. *International Journal of Food Microbiology*, **131**, 30-39. https://doi.org/10.1016/j.ijfoodmicro.2008.06.032