

## ***Lactococcus lactis* ssp. *lactis* as Potential Functional Starter Culture**

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Received: July 3, 2014

Accepted: October 20, 2014

### **Summary**

The aim of this study is to identify and characterise potential autochthonous functional starter cultures in homemade horsemeat sausage. The dominant microflora in the samples of horsemeat sausage were lactic acid bacteria (LAB), followed by micrococci. Among the LAB, *Lactococcus lactis* ssp. *lactis* and *Lactobacillus plantarum* were the dominant species, and since the first is not common in fermented sausages, we characterised it as a potential functional starter culture. *Lactococcus lactis* ssp. *lactis* produced a significant amount of lactic acid, displayed good growth capability at 12, 18 and 22 °C, growth in the presence of 5 % NaCl, good viability after lyophilisation and in simulated gastric and small intestinal juice, antimicrobial activity against test pathogens, and good adhesive properties *in vitro*.

*Key words:* horsemeat sausages, *in vitro* adhesion, natural microflora, technological and functional characterisation

### **Introduction**

Lactic acid bacteria (LAB) are natural food preservatives that have been Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA). Their benefits have been known since ancient times, and modern science has confirmed their positive effects on human intestinal microflora, including resistance to infectious diseases, immunomodulatory action, and digestion of lactose (1).

Considering their benefits, LAB have a great potential for application in functional foods (2). In meat preservation and fermentation, LAB are considered technologically fundamental. They decrease pH with lactic acid, produce bacteriocins that prevent the growth of pathogenic and spoilage microorganisms, modify sensory properties (flavour, colour and texture) of raw material, and improve the safety, stability and shelf-life of meat products (2,3).

Naturally fermented sausage products are considered to be of higher quality than sausages whose fermentation is controlled with industrial starters (4). Many authors support the view that indigenous microflora or microorganisms found in traditional sausages originate from raw

materials or the manufacturing environment (5,6). This microbiota is commonly known as the house flora (7).

More and more research is focused on the isolation and identification of autochthonous functional starter cultures with the aim of developing new functional meat products that will be recognised and labelled as autochthonous to the region in which they are produced (8,9).

The aim of this study is to identify and characterise microbial population, LAB in particular, naturally present in homemade horsemeat sausages from Pakrac (Slavonija, eastern Croatia). We also wanted to investigate the technological and functional properties and adhesion ability of the dominant bacterial strain in the intestinal epithelial cells of mice *in vitro* to see its potential as a starter culture for industrial production.

### **Materials and Methods**

All experiments were carried out on five samples of homemade horsemeat sausage. The sausages had been prepared according to a traditional recipe without the use of industrial starters or any additives such as nitrites or

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ascorbic acid. The stuffing consisted of horsemeat (75 %), pork back fat (25 %), and seasoning (kitchen salt, pepper, paprika and garlic). The sausages were smoked for 2–3 days and then left to ripen in the ripening chamber until day 42. Samples were taken at the end of the ripening stage.

#### Isolation and identification of microbial population

To isolate and identify the microbial population in the sausages we used classical microbiological procedures and biochemical analytical profile index (API) methods, as well as random amplification of polymorphic DNA polymerase chain reaction (RAPD PCR) analysis for genetic fingerprinting. A mass of 10 g of each sample was homogenised in 90 mL of sterile 0.5 % NaCl solution and serially diluted before plating on selective and non-selective media as shown in Table 1.

Table 1. Classical microbiological methods for the isolation and identification of natural microbial populations

| Microorganism          | Nutrient medium                              | Incubation condition |
|------------------------|--|----------------------|
| Lactic acid bacteria   | MRS agar (Biolife, Italy)                    | 30 °C/48–72 h        |
| Yeasts                 | Sabouraud agar (Biolife, Italy)              | 25 °C/48–72 h        |
| <i>Micrococcus</i> sp. | Manitol salt agar (MSA) (bioMérieux, France) | 30 °C/48 h           |

One hundred and fifty colonies taken at random from selective plates were identified on the basis of colony morphology, Gram staining, cell morphology and catalase reaction. Genomic DNA was isolated from 1.5 mL of overnight cultures using the method described elsewhere (2,10), which is a modification of the salting-out procedure described by Miller *et al.* (11). The extracted DNA was then used as a template in subsequent PCR reactions, which were performed according to Gardiner *et al.* (12). PCR amplifications were performed in a thermal cycler (Eppendorf Mastercycler® Personal, Hamburg, Germany) using 0.2-mL Eppendorf tubes for a total volume of 50 µL. PCR mixtures contained 1 µL of template DNA, 1× *Taq* polymerase buffer (Fermentas, Pittsburgh, PA, USA), 4 mmol/L of MgCl<sub>2</sub>, 0.2 mmol/L of deoxynucleoside triphosphate (Fermentas), 5 U of *Taq* DNA polymerase (Fermentas), and two primers (5'ACGAGGCAC3' and 5'ACGC-GCCCT3'), each at a concentration of 1 µmol/L. DNA was amplified in 40 cycles using the following temperature profile: denaturation at 94 °C for 30 s, annealing at 36 °C for 30 s, and polymerisation at 72 °C for 2 min. The initial denaturation was performed at 94 °C for 5 min, and the final extension step at 72 °C for 10 min. The PCR products (10 µL of each reaction mixture) were analysed on a 1.5 % (by mass per volume) agarose gel with 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and ethidium bromide staining. The DNA of bacteriophage λ cut with *Hind*III was used as molecular size standard.

#### Storing conditions

The dominant bacterial strain (isolated from horsemeat sausages) was stored at –70 °C in the M17 broth (Difco, Detroit, MI, USA) with 30 % (by volume) glycerol and

kept with the culture collection of the Department of Biochemical Engineering, Laboratory for General Microbiology and Food Microbiology, University of Zagreb, Zagreb, Croatia, until further investigation.

#### Effects of NaCl and temperature on microbial growth and proteolytic activity

The effect of 5 % NaCl on the viability and proteolytic activity of the dominant LAB strain at different incubation temperatures (12, 18 and 22 °C) was tested according to Bonomo *et al.* (13) and Babić *et al.* (8).

#### Antibiotic resistance of lactobacilli

The antibiotic resistance of the dominant LAB was tested on the MRS agar (Biolife, Milan, Italy) using the agar disc diffusion method as described by Babić *et al.* (8). The strains were classified as resistant or sensitive after incubation at 37 °C for 18 h. The following antibiotic discs (BD-Becton, Dickinson and Company, Franklin Lakes, New Jersey, MD, USA) were used: clindamycin (2 µg); methicillin, neomycin and erythromycin (5 µg each); tobramycin, ampicillin and lincomycin (10 µg each); gentamycin, chloramphenicol, rifampicin, vancomycin and tetracycline (30 µg each).

#### Preparation of simulated gastric and small intestinal juice

Gastric and small intestinal juice simulations were prepared according to Kos *et al.* (14) with small modifications.

Simulated gastric juice was prepared by suspending pepsin (3 g/L) in a sterile sodium chloride solution (0.5 %) and adjusting the pH to 2 with concentrated HCl. Pepsin (from porcine stomach mucosa) was obtained from Sigma Chemical Co, St. Louis, MO, USA.

Simulated small intestinal juice was prepared by suspending pancreatin (1 g/L) and bile salts (2 mg/mL of oxgall) in a sterile sodium chloride solution (0.5 %) and adjusting the pH to 8.0 with 0.1 mol/L of NaOH. Pancreatin (from hog pancreas, 165 U/mg) was obtained from Fluka Biochemica, Buchs, Switzerland, and oxgall (dehydrated fresh bile) from Difco Laboratories, Detroit, MI, USA.

#### Survival of the dominant LAB in gastric and small intestinal juice simulations

Washed cell suspensions of the dominant LAB (0.2 mL) were vortexed separately with gastric and small intestinal juice (1 mL) and 0.3 mL of 0.5 % NaCl. Changes in total viable count were monitored during treatment with gastric (2 h) and small intestinal juice (4 h) using the pour plate method. The plates with MRS were incubated at 37 °C for 4 h. The log CFU/mL is expressed as percentage of LAB viability.

#### Determination of lactic acid production by HPLC

Lactic acid produced in the samples was determined using high performance liquid chromatography (HPLC) according to Babić *et al.* (8), and the results were expressed as g/L. Glucose, lactic acid, acetate, and ethanol for stan-

dard solutions were obtained from Sigma-Aldrich (Bellefonte, PA, USA). A solution of  $H_3PO_4$  (85 %, by volume) (Sigma-Aldrich, Hamburg, Germany) was used to prepare the mobile phase ( $H_3PO_4$  0.1 %, by volume), and deionized water with conductivity  $<1 \mu S$  to prepare the mobile phase and standard solutions. Chromatographic analysis was performed using a Shimadzu Class-VP™ LC-10A<sub>VP</sub> system (Shimadzu, Kyoto, Japan). The mobile phase flow rate of the Piston pump (LC-10AD<sub>VP</sub>) was 0.5 mL/min. The substrate and the product were separated on a Supelco<sup>™</sup> C-610H analytical column (30 cm×7.8 mm i.d., 9  $\mu m$  particle size) and a Supelco<sup>™</sup> H guard column (5 cm×4.6mm i.d., 9  $\mu m$  particle size), both supplied by Sigma-Aldrich, Hamburg, Germany, and detected using the refractive index detector (RID-10A).

### Antibacterial activity

Antibacterial activity of the dominant LAB isolate was tested using the turbidimetric method (8) by comparing the growth of test microorganisms in nutrient broth (beef extract 3 %, by mass, peptone 5 %, by mass; Biolife, Milano, Italy) at 37 °C over 72 h with and without the LAB isolate. The results were expressed as growth inhibition of the following test microorganisms: *Staphylococcus aureus* 3048, *Escherichia coli* 3014, *Salmonella* sp. 3064 and *Listeria monocytogenes* ATCC 23074 (Collection of Microorganisms of the Laboratory for General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia).

### In vitro adhesion test

The adhesion test was performed using the method of Marteau *et al.* (15) with some modifications (1). Ileal samples were collected from three-month-old Swiss albino mice obtained from the Rijeka University School of Medicine, Rijeka, Croatia. The tissues were held in phosphate-buffered saline (PBS) at 4 °C for 30 min to loosen the surface mucus and then washed three times with PBS. The adhesion test was performed by incubating tissue samples (1 cm<sup>2</sup>) in bacterial suspensions (10<sup>9</sup> cell/mL of PBS) at 37 °C for 30 min. Samples of ileum were fixed in 10 % formalin, dehydrated with increasing concentrations of ethanol, and embedded in paraffin. Serial sections (5  $\mu m$ ) were cut, mounted on standard microscope slides, and stained for identification of Gram-positive and Gram-negative bacteria according to Frece *et al.* (1). The slides were examined

and photographed using a Nikon Mikrophot-FXA light microscope (Nikon, Tokyo, Japan). All experimental procedures complied with the standards set out in the Guide for the Care and Use of Laboratory Animals of the National Research Council (16). The best of the three photographs is presented in the results.

### Viability of the dominant LAB strain during lyophilisation and storage

The strain was cultivated in MRS broth overnight. The cells were collected by centrifugation (8000×g for 20 min), washed, resuspended in skimmed milk (10 %, by mass per volume), and frozen at -20 °C overnight, then lyophilised in a bench top freeze dryer (model Christ Alpha 1–4, B. Braun Biotech International, Melsungen, Germany). The lyophilised cells were stored at -20 °C for six months. The recovery of viable cells was determined by colony formation on MRS agar using the standard pour plating method. The log CFU/mL is expressed as percentage of viability (17).

### Statistical analysis

All experiments were carried out in triplicate. The results are expressed as mean value±standard deviation (S.D.). Apart from descriptive statistics, no other analysis was done, as it was not necessary for the scope and objective of our study.

## Results and Discussion

Our microbiological analysis showed that the dominant microflora in the horsemeat sausage samples were LAB, followed by micrococci and yeasts (Table 2). Like LAB, micrococci contribute to the colour and flavour of the sausage, but unlike LAB, they are not directly involved in fermentation.

Our finding of the yeasts *Candida zeylanoides* and *Geotrichum candidum* is in agreement with the findings of Nielsen *et al.* (18). Yeasts also play an important role in the maturation of sausages, since they contribute to the development of sensory characteristics of fermented sausages due to their lipolytic and proteolytic activity (19,20).

With API 50 CHL identification kits (bioMérieux, Lyon, France) we were able to classify 100 colonies randomly taken from the MRS agar into four main bacterial

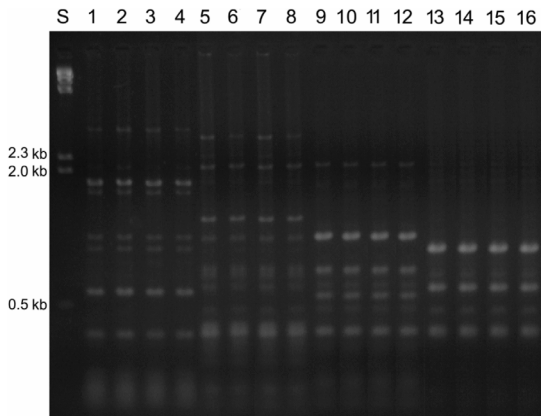
Table 2. Microbiological and biochemical identification of natural microbial populations in horsemeat sausage samples

| Microorganism          | N(sample) | N(microorganism) | API test                            | N(sample) |
|------------------------|-----------|------------------|-------------------------------------|-----------|
|                        |           | log CFU/g        |                                     |           |
| Lactic acid bacteria   | 5         | (7.5–9.3)±1.3    | <i>L. lactis</i> ssp. <i>lactis</i> | 5         |
|                        |           |                  | <i>L. plantarum</i>                 | 4         |
|                        |           |                  | <i>L. delbrueckii</i>               | 1         |
|                        |           |                  | <i>L. acidophilus</i>               | 2         |
| <i>Micrococcus</i> sp. | 5         | (2.7–3.4)±1.5    | <i>Micrococcus</i> sp.              |           |
| Yeasts                 | 5         | (4.4–5.0)±1.3    | <i>Candida zeylanoides</i>          | 5         |
|                        |           |                  | <i>Geotrichum candidum</i>          | 1         |

Results are the mean value±S.D. of triplicate assays

species and 16 strains (the API test precision was 97–99.9 %), and the dominant microflora were LAB, which were then confirmed by the RAPD PCR analysis.

Altogether four species were identified: *Lactococcus lactis* ssp. *lactis*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* and *Lactobacillus acidophilus*, and each consisted of four strains. Each of the four strains of a species had identical RAPD fingerprints (Fig. 1). The most dominant LAB species was *Lactococcus lactis* ssp. *lactis*, as it was isolated from all five sausage samples, followed by *Lactobacillus plantarum*, isolated from four samples (Table 3).



**Fig. 1.** Random amplification of polymorphic DNA (RAPD) fingerprints of lactic acid bacteria. Lanes 1–4: *Lactobacillus acidophilus*, lanes 5–8: *Lactobacillus delbrueckii*, lanes 9–12: *Lactobacillus plantarum*, lanes 13–16: *Lactococcus lactis* ssp. *lactis*, lane S: molecular size standard

**Table 3.** Lactic acid bacteria (LAB) isolates in samples of home-made horsemeat sausage

| N<br>(sample) | N(LAB)<br>log CFU/g                    |                               |                                 |                                 |
|---------------|--|-------------------------------|---------------------------------|---------------------------------|
|               | <i>L. lactis</i><br>ssp. <i>lactis</i> | <i>L.</i><br><i>plantarum</i> | <i>L.</i><br><i>delbrueckii</i> | <i>L.</i><br><i>acidophilus</i> |
| 1             | 8.3±1.1                                | –                             | 1.0±0.8                         | –                               |
| 2             | 5.8±1.2                                | 3.7±1.2                       | –                               | –                               |
| 3             | 4.0±1.1                                | 3.3±1.3                       | –                               | 2.0±1.3                         |
| 4             | 4.3±1.2                                | 3.0±1.1                       | –                               | –                               |
| 5             | 4.5±1.3                                | 2.5±0.9                       | –                               | 1.3±1.4                         |

Results are the mean value±S.D. of triplicate assays

The dominance of *Lactococcus lactis* ssp. *lactis* in fermented sausage has rarely been reported so far. The metabolic properties of the *L. lactis* species have a direct or indirect effect on the organoleptic, nutritional and hygienic quality of fermented products (21). As in a preliminary research (disc diffusion method, data not shown) all *Lactococcus* isolates showed good antimicrobial properties, and our dominant isolate, *Lactococcus lactis* ssp. *lactis* excelled among them, we decided to further characterise it as a starter culture. Table 4 shows its technological and functional properties. Its outstanding ability to grow in the presence of NaCl at a range of concentrations and temperatures used in meat processing industry has already

**Table 4.** Technological and functional characteristics of *Lactococcus lactis* ssp. *lactis*

| Technological characteristics and selection criteria                 | <i>Lactococcus lactis</i><br>ssp. <i>lactis</i> |
|--|---|
| Growth in the presence of 5 % NaCl                                   | +   |
| Growth at 12 °C  | +   |
| Growth at 18 °C  | +   |
| Growth at 22 °C  | +   |
| Proteolytic activity   | +   |
| Homofermentative species   | +   |
| Catalase test  | +   |
| γ(lactic acid)/(g/L)   | 13.83±0.21                                      |
| pH of the medium   | 4.16±1.53                                       |
| Antibiotic resistance  | –   |
| Viability in simulated gastric juice/%                               | 51.24   |
| Viability in simulated small intestinal juice/%                      | 71.23   |
| Viability after lyophilisation (skimmed milk used as lyoprotector)/% | 98.62   |

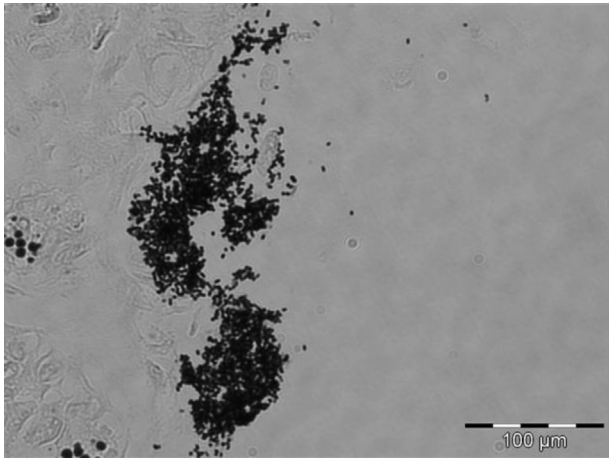
+ = yes, – = no

been reported by Kim *et al.* (22) and Itoi *et al.* (23). In order to act as a probiotic in the gastrointestinal tract, the bacteria must be able to survive the acidic conditions of the stomach and resist the bile acids at the beginning of the small intestine (1,2,24,25). The viability of our *L. lactis* isolate in simulated gastric juice (pH=2, salt content 0.5 %) was 51.24 % (Table 4) after 2 h of exposure. Our results are in agreement with the report by Kimoto-Nira *et al.* (26), who found that *L. lactis* ssp. *lactis* was resistant to low pH and bile salts. In small intestinal juice, the viability of the isolate was 71.23 % (Table 4), which is essential for its probiotic effects.

The production of lactic acid, which results in a decrease in pH, contributes to the texture, colour and acidic taste, prevents the growth of pathogenic and spoilage microorganisms and improves the safety and stability of meat products (27). Our study has shown that *L. lactis* ssp. *lactis* is homofermentative. The concentration of lactic acid produced by homolactic fermentation of glucose was 13.83 g/L, and the medium was acidified to pH=4.16 (28). In contrast, heterofermentative LAB are not suitable for sausage production because they produce large amounts of carbon dioxide that creates holes in the product. In addition, these LAB produce acetic acid, which causes a pungent off-flavour (27).

*L. lactis* ssp. *lactis* isolated in our study was catalase-positive and showed good proteolytic activity (Table 4), which is important in preventing rancidity and preserving meat colour. Another essential property for the probiotic activity of a functional starter culture is the competition with pathogens (29). Our *L. lactis* ssp. *lactis* isolate inhibited the growth of all test pathogens over 72 h of cultivation, as follows (in %): *E. coli* 73.4–85.4, *S. aureus* 51.5–87.0, *Salmonella* spp. 62.7–88.6 and *L. monocytogenes* 61.0–82.4 (28). Antimicrobial properties of *L. lactis* ssp. *lactis* are of great importance for the application of this strain as functional starter culture in fermented meat products.

One of the selection criteria for probiotic strains is the ability to adhere to the intestinal tract of the host. Good adhesion means that a probiotic strain will exert its properties better (1,2). In this respect, our *L. lactis* ssp. *lactis* isolate showed very good adhesion (Fig. 2).



**Fig. 2.** Adhesion of *Lactococcus lactis* ssp. *lactis* to the large intestine of the mouse

Research during the last few decades has shown that starter cultures can develop resistance to clinically relevant antibiotics (8,30). Our isolate, however, showed sensitivity to all 12 tested antibiotics: clindamycin, methicillin, neomycin, erythromycin, tobramycin, ampicillin, lincomycin, gentamycin, chloramphenicol, rifampicin, vancomycin and tetracycline (Table 4).

In industrial application, bacteria are subjected to stress that can lower their viability. Viability can be affected by harsh heat treatment, mechanical processing, storage at room temperature, food matrix, and chemical microenvironment (25). Furthermore, a range of processes has been used to obtain dry formulations with prolonged shelf-life, the most common being freeze drying, vacuum drying, and spray drying. These processes do osmotic damage to probiotic cells, and cell death usually correlates with cell membrane damage (17). The membrane is usually protected by cryoprotectants (against freezing) and lyoprotectants (against lyophilisation). These are small molecules or polymers with osmotic behaviour, which promote the formation of amorphous or glassy solids and reduce ice formation that can be cell-damaging (17). We protected our isolate cells with skimmed milk as lyoprotectant, and, judging by the high survival, they withstood well the stress associated with lyophilisation (Table 4).

## Conclusions

The dominant LAB, *Lactococcus lactis* ssp. *lactis*, isolated from the horsemeat sausage in our study has shown good technological and functional properties important for starter cultures. It has also shown good survival in simulated gastrointestinal environment *in vitro*, desirable adhesive properties in intestinal epithelial cells of mice, and sufficient antimicrobial activity against pathogen microorganisms, which are all important properties for the

selection of a probiotic starter culture. We therefore believe that it could be used for controlled fermentation of meat products.

This work can be considered preliminary, as it was conducted to characterise an autochthonous functional starter culture. Implementation of this culture in small- to medium-sized enterprises and industrial production should improve the quality, uniformity and safety of the final product. Hopefully, the product will meet all marketing regulations yet remain autochthonous and recognisable. Before that happens, further investigation is needed to confirm good adhesive properties and resilience to gastrointestinal environment *in vivo*. Our study is a contribution to an effort to create a bank of traditional and autochthonous functional starter cultures for wider and controlled use in the future.

## Acknowledgement

This study was supported by Ministry of Science, Education and Sports of the Republic of Croatia (grant no. 058-0582184-0432). The authors wish to thank Mr Dado Čakalo for help in editing this article.

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