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Lactic acid bacteria from chicken carcasses with inhibitory activity against *Salmonella* spp. and *Listeria monocytogenes*

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ABSTRACT

This study was conducted to isolate psychrotrophic lactic acid bacteria (LAB) from chicken carcasses with inhibitory activity against strains of *Salmonella* spp. and *Listeria monocytogenes*. A total of 100 broiler samples were examined for the presence of LAB. Ninety-two LAB isolates that showed antimicrobial effects against *Salmonella* spp. and *L. monocytogenes* were further analysed to examine their LAB (Gram-positive, catalase negative, oxidase negative) and psychrotrophic characteristics (ability to grow at 7 °C). Fifty isolates were further selected and identified initially using standard biochemical tests in miniature (Micro-kits API CH 50) and then by sequencing of the 16S–23S rRNA gene boundary region (Intergenic Spacer Region). By molecular identification, these isolates were classified into 5 different LAB species: *Lactobacillus salivarius*, *Lactobacillus reuteri*, *Lactobacillus johnsonii*, *Pediococcus acidilactici*, and *Lactobacillus paralimentarius*. None of the isolates produced tyramine or histamine.

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1. Introduction

Poultry meat is one of the foods most often implicated in food borne infections, especially infections caused by *Salmonella* spp. and *Campylobacter* spp. Although poultry meat is rarely implicated in *Listeria* outbreaks, the relatively high prevalence of *Listeria monocytogenes* in chicken carcasses presents a potential risk [1]. The contamination of raw chicken with these pathogens can be minimized by good manufacturing practices, but their complete elimination is difficult. Many methods have been developed in order to further decrease contamination levels, one being biopreservation, a method that has lately attracted increased attention.

Biopreservation is an innovative method for extending the shelf life of food products and reducing the microbial risks and consists of the inoculation of food products with selected bacterial strains able to inhibit the growth of undesirable bacteria [2]. Antagonistic microorganisms added to food products to inhibit pathogens or to prolong shelf life, while affecting the sensoric properties as little as possible, are termed protective cultures [3]. Lactic acid bacteria (LAB) should be considered as the ideal choice for application as protective cultures as they are frequently naturally present in food products, have a long history of safe use and form part of the gut

microflora of humans and animals [4]. Their antagonism refers to inhibition through competition for nutrients or to production of one or more antimicrobial active metabolites such as organic (lactic and acetic) acids, hydrogen peroxide, diacetyl, antimicrobial enzymes, bacteriocins and reuterin [5,6]. The basic requirements for LAB strains to be used as protective cultures include the following: 1) they should be generally recognized as safe (GRAS), 2) they should exhibit antagonistic activity against pathogenic bacteria, 3) they should not cause any detrimental effects on the sensory, chemical and physical properties of the target food, 4) they should be able to survive in a hostile environment and 5) they should be able to retain their inhibitory properties against pathogenic and spoilage bacteria at refrigeration temperatures [4,7–9].

LAB have been extensively used for preserving fermented and cooked meat products over the last few decades and a variety of strains have been found to be effective against pathogens and spoilage organisms related with those products [9–11]. However, there has been relatively few reports on biopreservation of fresh red meat [12–14] and even fewer on fresh poultry meat [4,15].

Certain parameters may complicate the biopreservation of fresh meat [16]; first, the presence of proteolytic enzymes, which degrade bacteriocins thus reducing the antimicrobial effect of the bioprotective culture; second, the fact that fresh meat is often contaminated by the growth of Gram-negative bacteria which are generally not affected by the bacteriocins produced by LAB. Finally, fresh meat contains a large number of diverse bacteria with which

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LAB should be capable of competing in order to become the dominant bacterial flora throughout the entire storage period.

To overcome these factors, some researchers have used lactic acid as a means of controlling or reducing the microbial growth on fresh poultry [17–19].

The aim of this study was to isolate LAB originating from the natural microflora of poultry carcasses that demonstrate antagonistic activity against strains of *Salmonella* spp. and *L. monocytogenes* isolated from poultry in our previous studies [20,21]. The biochemical and molecular identification of these LAB isolates was carried out employing API CH 50 test strips and sequencing of the 16S–23S rRNA gene boundary region, respectively. Finally, their ability to produce biogenic amines was examined to ensure their safety for use in poultry and poultry products.

2. Materials and methods

2.1. Sample collection

The samples were pieces of neck skin weighing approximately 10 g from poultry carcasses, taken immediately following the slaughtering process in 4 different poultry slaughterhouses of northern Greece. The neck skin samples from three carcasses were pooled before examination to form a 25 g final sample. A total of 100 samples (from 300 carcasses) were examined for the presence of LAB.

The samples were transported to the laboratory within an hour after collection in coolers with ice and were processed immediately.

2.2. Isolation procedure

LAB were isolated using the method described by Matamoros et al. [8], slightly modified. Each poultry sample (25 g) was weighed into peptone dilution water (225 ml) and blended for 2 min in a Stomacher 400 – laboratory blender (Seward Medical, London, UK). After 30 min of revivification, four decimal dilutions were made in peptone dilution water and 0.1 ml from each dilution was spread on MRS agar (de Man, Rogosa and Sharpe) (Merck, KGaA, Darmstadt, Germany). For each sample, 7 plates of each dilution were made (total: 35 plates per sample). Plates were then incubated in anaerobic conditions at 7 °C for 10–15 days. Plates presenting between 10 and 30 colonies were selected for a double layer inhibition test.

2.3. Double layer inhibition test

Six different strains of *Salmonella* were selected (*S. Blockley*, *S. Paratyphi B*, *S. Bredeney*, *S. Neftenbach*, *S. Hadar* and *S. Thompson*) and they formed a culture. A pool of *L. monocytogenes* isolates was also used. One strain of *L. monocytogenes* was chosen from each of the 10 subclades that were found by a RAPD analysis [22] and they also formed a culture. Both *Salmonella* and *L. monocytogenes* strains used were isolated from poultry carcasses in previous studies [21,22]. These strains were pre-cultivated at 37 °C for 48 h in Brain-Heart Infusion (BHI) (BD BBL, Franklin Lakes, NJ, USA) broth before being diluted to previously determined concentrations. Three decimal dilutions were made and 1 ml of each dilution was added to 15 ml of molten soft BHI agar (37 g l⁻¹ BHI, 10 g l⁻¹ agar) and then spread on an isolation plate showing growth of 10–30 colonies of LAB. Plates were then incubated at 37 °C for 48 h in order to obtain a regular lawn. The presence of an inhibition zone was checked visually. Based on the size and the appearance of the inhibition halo, 92 colonies were picked and cultivated in MRS broth (Merck, KGaA, Darmstadt, Germany) at 15 °C. The selected isolates were streaked twice on MRS agar plates at 15 °C and stored in microbanks (PRO-LAB Diagnostics, Richmond Hill, ON, Canada) at –80 °C.

2.4. Psychrotrophic and LAB characteristics

The selected isolates were cultivated at 7 °C in MRS broth in order to check their psychrotrophic characteristics. Growth was monitored visually after two weeks of culture. The isolates were also tested for three characteristics: Gram, catalase and oxidase tests. Only presumptive LAB i.e. Gram-positive, catalase- and oxidase negative with psychrotrophic characteristics were selected for further studies [8].

2.5. Biochemical identification

The 50 most efficient strains of presumptive LAB were selected for biochemical identification, taking into account their overall inhibitory properties and psychrotrophic characteristics. Evaluation of their inhibitory properties was based on the diameter of the inhibition halo (0.5 cm > halo, 0.5 cm < halo < 1.0 cm, 1.0 cm < halo), the concentration level of both LABs and pathogens (the lowest concentration of LABs that could inhibit the highest concentration of pathogen) and their ability to inhibit both *Salmonella* spp. and *L. monocytogenes*. The selected isolates were tested using an API 50 CH biochemical test strip (bioMérieux SA, Marcy l'Etoile, France). Using the API CHL medium (bioMérieux SA, Marcy l'Etoile, France) as a base, the fermentation of 49 different carbohydrates was studied. Species identification was done using a computerized database program provided by the manufacturer.

2.6. DNA extraction

DNA was extracted from 7 isolates using a modified in-house protocol [22]. This method is based on the lytic and nuclease-inactivating properties of a chaotropic agent, guanidinium hydrochloride (GuHCl), together with the use of silica particles as the affinity matrix. In brief, 700 µl lysis buffer [8 M GuHCl, 25 mM EDTA, 1% Sarcosyl, 2% Triton X-100, 25 mM sodium citrate, 0.2 M sodium acetate, pH adjusted to 5.2 with acetic acid] were added to the suspensions of each isolate on 140 µl of sterilized distilled water and samples were incubated at 70 °C for 10 min. The lysate solution was added to a 2 ml tube containing 300 µl chloroform. The mixture was vigorously rotated for 5 min. After centrifugation at 16,000 g for 10 min, 400 µl of the aqueous phase was transferred to a tube containing 200 µl ethanol and the mixture was passed through a silica column (FT-2.0 Filter-Tube Spin-Column System, G. Kisker GbR, Steinfurt, Germany) by centrifugation at 8000× g. The DNA bound to the silica was washed sequentially by centrifugation, once with 500 µl of “wash-1” buffer (4 M GuHCl, 25 mM Tris–HCl, pH 6.6, and 50% ethanol) and twice with “wash-2” buffer (2 mM Tris–HCl, pH 7.0, 20 mM NaCl, and 80% ethanol), using 900 and 500 µl respectively. DNA was finally resuspended in 100 µl of preheated (80 °C) nuclease-free elution buffer (10 mM Tris–HCl, pH 8.0).

2.7. PCR amplification

The 16S–23S intergenic spacer region (ISR) from these seven isolates was amplified using primers that annealed to conserved regions of the 16S and 23S genes. These primers were 16S/p2 (5'-CTTGACACACCGCCGTC-3') and 23S/p10 (5'-CCTTCCCTCACGG-TACTG-3') which anneal to positions 1392 to 1410 of the 16S rRNA gene and to positions 713 to 731 of the 23S rRNA gene (*Lactobacillus salivarius*, GenBank accession number CP002034), respectively. The resultant PCR product corresponded to the complete 16S–23S ribosomal ISR and parts of the flanking rDNAs (ca. 136 bp of 16S rDNA and 551 bp of 23S rDNA). PCR mixtures contained 2 µl of purified DNA, 3 µl of 10× polymerase buffer (Boehringer Mannheim GmbH, Mannheim, Germany), 200 µM each deoxynucleoside

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