www.genaqua.org
ISSN 2459-1831
Genetics of Aquatic Organisms 2: 29-33 (2018) DOI: 10.4194/2459-1831-v2_1_05

SHORT PAPER

Identification of Lactic Acid Bacteria from Spoiled Marinated Anchovy (*Engraulis encrasicolus*) Using 16S rRNA Gene Sequence Analysis

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Received 01 January 2018
Accepted 12 February 2018

Abstract

Cold marinated foods are semi-preserve and not fully fermented products. However, when the fermented products such as vinegar are added to the process, lactic acid bacteria may develop in the medium. The aim of the study was to determine the presence of lactic acid bacteria associated with the spoiled vinegar-marinated anchovy. A total of 12 bacterial strains were isolated from this product. They were identified to species level using 16S rRNA gene sequence analysis. *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus curvatus* were the two main species determined. This is the first report on the isolation of the lactic acid bacteria in marinated anchovy prepared with persimmon vinegar.

Keywords: Marinated anchovy, lactic acid bacteria, sequence analysis.

Introduction

Marination is a process in which foods are treated with a solution containing organic acids and salts for a certain period of time. Marinades are mostly classified into 3 groups, cold, cooked, and fried marinades. Cold marinated products are also defined as semi-preserve marinades. This type of marinades has restricted shelf-life since they are not cooked and, are herewith not sterile sufficiently. Most of the commercial marinades are not fully fermented products. But when the fermented marinating sources like natural vinegar were used, some lactic acid bacteria (LAB) may grow in these food products (Lyhs, Korkeala, & Björkroth, 2002; Gokoglu, Topuz, & Yerlikaya, 2009).

The LAB is a very diverse group of bacteria which include six families and many genera. *Lactobacillus, Aerococcus, Alcalibacterium, Carnobacterium, Enterococcus, Lactococcus, Leuconostoc, Streptococcus* and, *Vagococcus* are the most described genus among these bacteria group. They have significant economic value in fermented products industry. The fermentation of food products is generally catalyzed by interactions of different LAB species. These bacteria may ferment the carbon sources available in food materials and produce other metabolites such as antibacterial and aroma compounds, vitamins, short-chain fatty acids, and sugars. Hence, they improve the safety and storage quality of foods by preventing the growth of pathogenic microorganisms (Ruiz-Rodriguez, Bleckwedel, Ortiz, Pescuma & Mozzi, 2017). On the other hand, facultatively heterofermentative LAB such as *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus curvatus* may produce CO_2 and other by-products only under certain conditions or from specific substrates. During the storage of marinated products, a complex bacterial flora associated with spoilage may be developed. Identification of these bacteria is important not only in basic research but also applied studies (Cocolin, Manzano, Cantoni, & Comi, 2000; Vodnar, Paucean, Dulf, & Socaciu, 2010).

In the present study, it is aimed to determine the presence of LAB associated with the spoiled vinegar-marinated anchovy (*Engraulis encrasicholus*). *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus curvatus* strains were isolated from this product using DNA sequencing of their 16S rRNA genes. This is the first LAB identification from spoiled persimmon vinegar-marinated anchovy. Results will allow for further investigations into the mechanisms of spoilage by these organisms on this type of marinated fish products.

Materials and Methods

Marination Process

Persimmon vinegar was produced from fresh persimmon fruits in laboratory conditions. The brine

solution was prepared with 20% persimmon vinegar and 10 % NaCl. 1500 g of anchovy fillets were covered with brine solution and kept at 4°C for 72 h. Marinated anchovies were put into the plastic containers and filled with sunflower oil. The samples were stored at 4 °C for 6 months for bacterial growth.

Chemical Analyses

The pH value of marinades was measured using a pH-meter (Mettler-Toledo FG2, Schwerzenbach, Switzerland) with a glass electrode. The acidity of the samples was determined by titration method (AOAC, 1990) and Mohr method was performed to detect the salt content of the marinated anchovies (Fuselli, Casales, Fritz, & Yeannes, 1994). All tests were carried out in triplicate.

Samples Preparation and Microbiological Analysis

The 10 g marinated anchovy was homogenized with 90 ml of 0.1% peptone-water containing 0.9% NaCl using the stomacher apparatus (BagMixer CC, Interscience, Paris, France). Five-fold serial dilutions of marinated samples were prepared and streaked on de Man, Rogosa and Sharpe (MRS, Merck, 1.10660) and Plate Count Agar (PCA, Merck, 1.05463) for the count of the LAB and total aerobic mesophilic bacteria (TAMB) respectively. Petri dishes were allowed to incubate at 30ºC for 48 hours under aerobic conditions for TAMB. LAB also was incubated at 30°C for 48h in anaerobic jars (Merck, 116387). At the end of incubation, the total number of TAMB and LAB were calculated by counting the colony-forming units (Harrigan & McCance, 1976; Didinen, Onuk, Metin & Cayli, 2017). Then, presumptive colonies were selected and purified by streaking onto the same medium for detection of the LAB. Pure colonies were biochemically characterized by following biochemical tests: Gram staining, cytochrome oxidase and catalase.

DNA Isolation and PCR Amplification

Isolation of genomic DNA from LAB was performed as a template for the PCR assay by QIAamp DNA mini kit (Qiagen), according to its instructions. The optical density and concentrations of DNA were measured by RNA/DNA calculator (ND 8000 spectrophotometer, Thermo Fisher Scientific). Average, A260/280 value and DNA concentrations were 1,89 and 28 ng/µl respectively. The LAB was identified by a partial DNA sequencing of their 16S rRNA genes. The forward and reverse primer oligonucleotides, fDl (AGAGTTTGATCCTGGCTCAG) and rP2 (ACGGCTACCTTGTTACGACTT) were used for PCR amplification respectively (Weisburg, Barns, Pelletier & Lane, 1991). The universal primers were synthesized for a less conserved region of the small subunit 16S rRNA gene sequence of all bacteria. DNA amplification was performed with AmpliTag Gold 360 Master Mix (Thermo Fisher Scientific) in a thermocycler (Applied Biosystems) according to the manufacturer's recommendations. Analysis of PCR products was performed using electrophoresis in 1.5% (w/v) agarose gel with 1×TBE (Tris-Borate-EDTA) buffer containing SYBR Green. The DNA bands were viewed by UV transillumination. DNA fragment length was determined with the migration of 100-bp DNA ladder (Bio Basic).

16S rRNA Gene Sequence and Phylogenetic Analysis

Sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. ABI PRISM 3500 Genetic analyzer and POP-7 polymer were used as the separation machine and matrices. The sequence data were analyzed by ABI Prism DNA Sequencing Analysis Software v5.1. The quality value score (QV20) was used as an indicator. The derived nucleotide sequences were analyzed and aligned with the BioEdit Sequence Alignment Editor. The obtained consensus sequences were compared with previously published data in GenBank. Neighbor-joining (NJ) method was used because it is more appropriate to estimate the phylogenetic relationships of the species (Didinen *et al.*, 2017).

Results

Chemical and microbiological analysis results of the marinated fish samples were shown in Table 1. pH, acidity and saltiness of spoiled marinades were determined as 4.62, 1.34%, 3.19% respectively. It has been shown that the TAMB count was 7.40 log CFU/g and the LAB count was 3.65 log CFU/g.

A total of 12 bacterial strains were isolated from spoiled marinated anchovy. The strains were considered as a LAB because dominant colonies grew on MRS agar. They also were Gram-positive and oxidase and catalase-negative. All bacteria strains were further identified by DNA sequencing of their 16S rRNA genes. PCR products from the 16S rRNA gene amplicons generated about 1.5 kb fragments (Figure 1). Two main bacteria isolates were identified to species level. These bacteria species are *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus curvatus.* Two species appeared to belong to the genus *Lactobacillus* according to phylogenetic analyses based on 16S rRNA gene sequences (Figure 2). 16S rRNA gene sequences of *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus curvatus* strains were demonstrated to have ≥99% similarity with reference strains from Genbank (GenBank Acc. No: NZ AP012541.1 and NZ CP022474.1). These sequence date of

Table 1. Chemical and micobiological properties of the marinated anchovies

Property	Level
pH	5,26±0,04
Acidity (% Acetic acid)	1,14±0,05
Salt content (%)	3,19±0,07
TAMB (log CFU/gl)	7,40
LAB (log CFU/g)	3,65

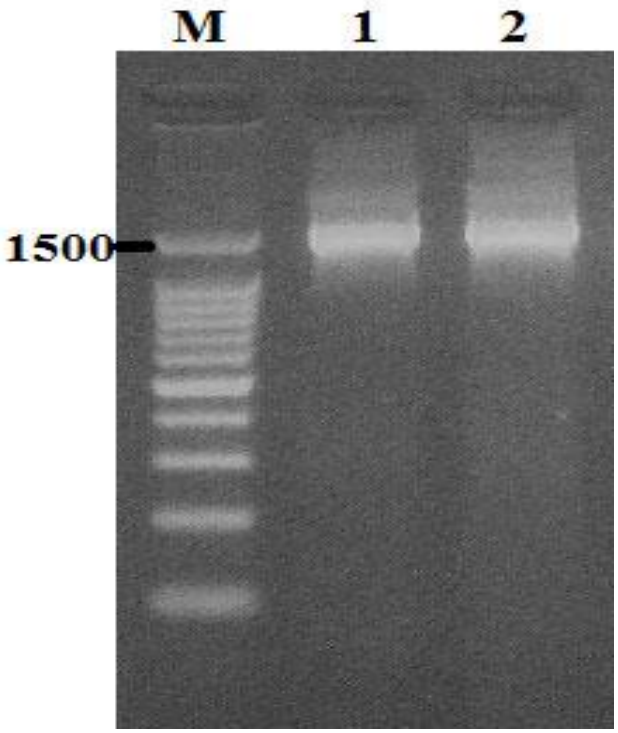

Figure 1. Gel electrophoresis image of PCR product of LAB isolates. M: 100 bp DNA marker, 1 and 2: All bacterial isolates have the expected 1500-bp PCR amplification product.

Lactobacillus paracasei subsp. *paracasei* and *Lactobacillus curvatus* isolates have also been deposited in GenBank databases under the accession numbers MG770030 and MG768976 respectively.

Discussion

Previous studies have shown that the commercial formulations prepared with acetic acid generally may a shelf life of more than 6 months. (Bilir, 2011; Pons-Sánchez-Cascado, Vidal-Carou, Mariné-Font, & Veciana-Nogués, 2005; Giuffrida, Ziino, Orlando, & Panebianco, 2007). It has been reported a spoilage type called 'protein swell' in cold marinated fish products. According to the theory, proteolytic enzyme activity causes protein degredation, as a result, decarboxylation products and CO_2 have been released. LAB having the gas-forming ability may also decompose the glucose homofermentatively. Ammonia, which is the result of all these mechanisms, decreases the acidity of the product (Schillinger, Holzapfel, Björkroth, & Blackburn, C.d.W. 2006; Lyhs & Björkroth, 2008). In this study, persimmon vinegar was first used as an acidifier compared with vinegar-marinated fish studies. Given that, the acidity of the marinades is not sufficiently elevated and, the spoilage has occurred. The point to be noted in this study is that what kind of lactic acid bacteria have developed in this marination. For this reason, it is important to identify possible LAB in such a food product that has not been previously studied.

The studies on the detection and characterization of LAB associated with spoiled or not-spoiled marinated products were usually performed by culture methods in the past. However, exact LAB identification based on traditional biochemical methods is very difficult and time-consuming. Recently developed molecular techniques including DNA sequencing and ribotyping have been described

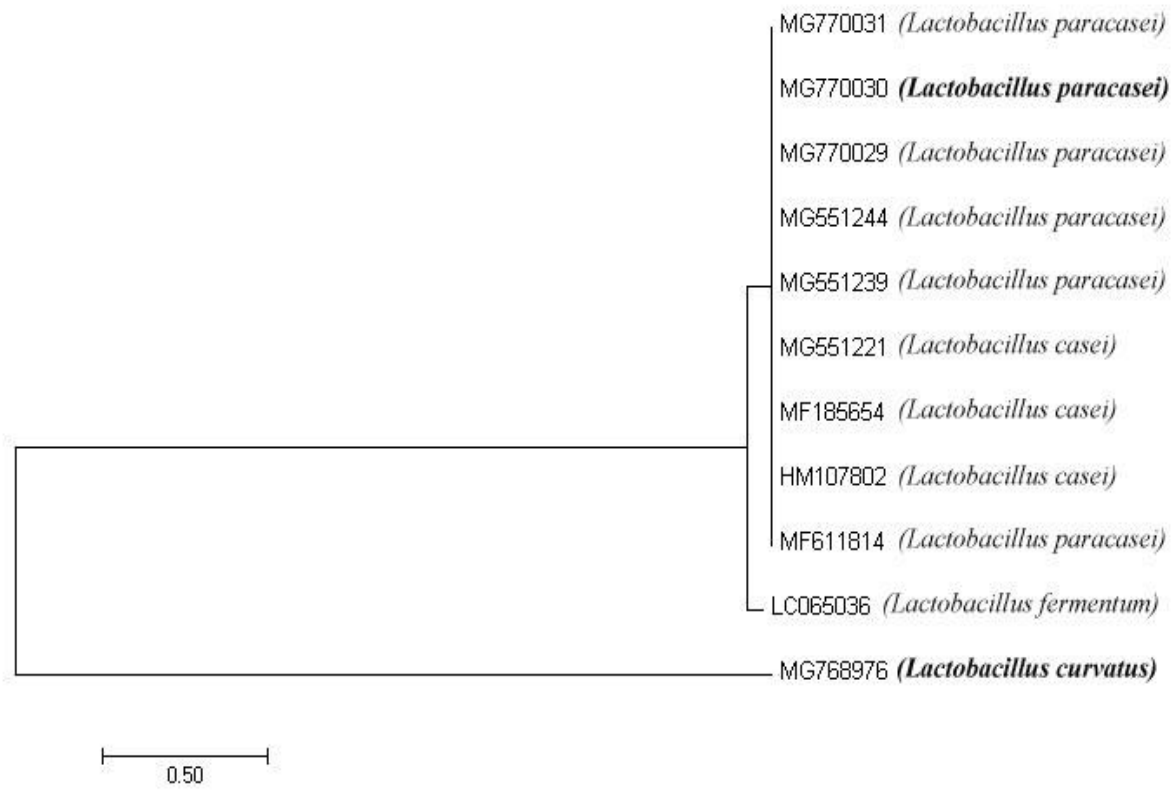

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences, obtained with the neighbor-joining algorithm, showing the LAB strain with related taxa. The identified strains were shown with bold charachter.

as useful tools for accurate identification of LAB species (Lyhs *et al*., 2002; Chenoll, Macian, Elizaquivel & Aznar, 2006). In the current study, we identified the LAB in marinated anchovy with spoilage symptoms using culture-dependent 16S rRNA gene analysis by PCR. This is the first report on the isolation of LAB in marinated anchovy prepared with persimmon vinegar. *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus curvatus* strains were isolated from marinated anchovy. This study provides some new data about LAB community present in marinated fish.

Lactobacillus curvatus is one of the most prevalent LAB found in fermented meat environments. It is a lactic acid bacterium commonly associated with fermented products including refrigerated meat, vacuum-packaged meat, fish and poultry products (Hebert *et al.* 2012). In a previous study, a number of *L. curvatus* strains were isolated from spoiled, vacuum-packaged rainbow trout and identified using the molecular approach (Lyhs, *et al.*, 2002). In our study, 3 of 12 LAB were identified as *L. curvatus*.

Lactobacillus paracasei subsp. *paracasei* is an important member of the LAB as a starter culture. At the same time, the active substance produced by *L. paracasei* subsp. *paracasei* strains displayed antibacterial and antifungal activities against many bacterial and yeast strains (Atanassova *et al*. 2003). However, in the present study, among LAB strains in spoiled marinated anchovy, *L. paracasei* subsp. *paracasei* was the most prevalent.

This research showed that Lactobacillus genus was dominant bacterial flora in the spoiled vinegar-marinated fish product. Our results were in agreement with many previous studies that examined in marinated meat product (Schirmer, Heir & Langsrud, 2008; Chenoll *et al*., 2006). *L. paracasei* subsp. *paracasei* and *L. curvatus* strains were isolated from spoiled marinated anchovy. For understanding the mechanisms of spoilage further studies are required on the presence and growth of LAB in marinated fish products.

Acknowledgements

This research was carried out as additional output of a project supported by the General Directorate of Agricultural Research and Policies (TAGEM/HSGYAD/16/A05/P01/110) (Republic of Turkey Ministry of Food, Agriculture and Livestock, Turkey). The authors would like to thank Central Fisheries Research Institute (Trabzon, Turkey) for providing the equipment and for financial support.

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