Lactobacillus fructivorans Spoilage of Tomato Ketchup

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ABSTRACT

Spoilage characterized by bulging as a result of gas formation in bottled ketchup was studied. Samples produced microbial growth on MRS and Rogosa selective *Lactobacillus* agar. Seventy randomly selected isolates typed by using restriction endonuclease (*Cla*I, *Eco*RI, *Hin*dIII) analysis were found to have identical DNA fragment patterns in gel electrophoresis. The strain was identified as *Lactobacillus fructivorans* using morphological, physiological and biochemical characteristics, combined with the information obtained from ribotyping. Factors affecting growth and survival of this *L. fructivorans* strain in ketchup production were also studied. An *L. fructivorans* count of 10^5 CFU/g resulted in spoilage of inoculated ketchup samples. Spoilage occurred only in samples incubated at 15 to 30°C. The *L. fructivorans* implicated in causing spoilage demonstrated heat resistance with a *D* value of 1.2 min at 65°C. The strain did not show resistance to alkaline active chloride-containing detergent sanitizer; alkyldimethylbenzylammonium chloride and alkyldimethylethylbenzylammonium chloride-containing sanitizer were also found to be effective antimicrobial agents.

Key words: *Lactobacillus fructivorans*, lactic acid bacteria, spoilage, tomato ketchup, characterization, molecular typing

Tomato ketchup is considered to be a shelf-stable product. Low pH, addition of sodium benzoate and heating of the mixture during the manufacturing process contribute to the shelf stability of ketchup. In acidic (below pH 4.0) foods like ketchup spoilage microbes are usually found to be restricted to non-spore-forming bacteria (lactic acid bacteria), or yeasts (*Saccharomyces* spp. *Candida* spp.) or molds (*Byssochlamus fulva*) (2, 12, 14, 16, 19, 41, 43). *Bacillus coagulans, Clostridium pasterianum,* and the *Bacillus macerans-B. polymyxa* group can also cause spoilage at pH 4.0 to 3.8 (6, 19), but growth is retarded. Heterofermentative lactobacilli (42, 43) have been shown to cause gas formation in ketchup but further species identification of the spoilage strains was not performed (43) or the species characterized (42) do not exist in current taxonomy.

Despite the factors used for controlling microbial growth in ketchup, in-plant spoilage of ketchup occurred inflicting considerable economic hardship on the manufacturer. Spoilage was indicated by bulging of plastic ketchup bottles as a result of gas formation. The expected shelf life of this product is 8 months. Bulging occurred after 8 weeks in bottles from several product lots. No increase in total aerobic bacteriological count or yeasts were detected in routine quality-control tests.

In this study, the isolation and identification of the spoilage strain of *Lactobacillus fructivorans* found in the bottled ketchup is described. This organism was first isolated from spoiled salad dressing (9) and has been reported to spoil acidic food or ethanol-containing sources, mainly mayonnaise, salad dressings, vinegar preserves, sake, dessert wines, and aperitifs (12, 18, 27). *L. fructivorans* can be difficult to isolate (9, 37) and identification of a species of the genus *Lactobacillus* using classical methods of physiological testing is not always sufficient (18). Traditional microbiological methods together with rDNA typing techniques were used for characterization. Factors affecting the growth and survival of this *L. fructivorans* strain in the production plant environment were also studied.

MATERIALS AND METHODS

Ketchup samples

Eight bulging plastic bottles of ketchup, one from each of eight different manufacturing lots, were sampled. The ingredients of the ketchup were tomato puree, spices, sugar, starch, sodium benzoate, acetic acid, ascorbic acid, emulsifiers, and coloring substances. The pH of each lot was 3.8 ± 0.1. After mixing of the ingredients the lot was steam heated for 10 min at 85°C. The temperature was reduced and maintained at 80°C for 10 to 20 min. Excess air was removed after heat treatment. The ketchup was cooled to 15°C prior to bottling. The bottling line was not enclosed. During bottling, caps and empty and full bottles were briefly exposed to ambient air.

Reference strains

Type strains used for the identification of the *L. fructivorans* spoilage strain were: *Lactobacillus brevis* ATCC 14869, *Lactobacillus buchneri* ATCC 4005, *Lactobacillus collinoides* ATCC 27612, *Lactobacillus fructivorans* ATCC 8288, *Lactobacillus hilgardii* ATCC 8290, *Weissella confusa* ATCC 10881, *Weissella halotolerans* ATCC 35410, *Weissella viridescens* ATCC 12706.

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Microbiological analyses

Total plate counts of the ketchup samples were determined by using pour plates with plate count agar (Difco Laboratories, Detroit, MI, USA). Parallel plates were made for aerobic and anaerobic enumeration. The anaerobic environment was created using an anaerobic jar with an H_2-plus CO_2-generating kit (Oxoid Ltd, Basingstoke, UK). Parallel plates with blood agar (Columbia agar base) (GIBCO BRL, Paisley, UK) were also inoculated for aerobic and anaerobic determination. Detection of lactic acid bacteria was performed by using MRS agar (Oxoid Ltd) and *Lactobacillus* enumeration using Rogosa selective *Lactobacillus* (SL) agar (Orion Diagnostica, Espoo, Finland). MRS and Rogosa SL agar were incubated anaerobically. All plates were incubated at 30°C. Growth was inspected after 2 days. Plates with no growth were incubated for an additional 5 days (i.e., for an incubation period of 7 days) and were inspected daily for growth.

Ten- to twenty-gram samples were also enriched for 2 days at 30°C in meat extract broth (Orion Diagnostica), Robertson's medium *(32)*, and MRS broth (Difco). Meat extract broth was incubated under aerobic conditions and Robertson's and MRS broths anaerobically. If growth was not observed in 2 days incubation was continued for up to 7 days. After the enrichment the procedure described by NCFA *(32)* was followed. Seventy colonies from MRS and Rogosa SL agar, 35 colonies from each agar type, were randomly isolated and streaked to purity. Isolates cultured from the 8 bottles were included.

Isolation of DNA, restriction endonuclease analysis (REA) and determination of rRNA-coding gene restriction patterns (ribotyping)

DNA was isolated according to the guanidium thiocyanate method of Pitcher et al. *(33)* modified by Björkroth and Korkeala *(3)* with combined mutanolysin (Sigma Chemical Company, St. Louis, MO, USA) and lysozyme (Sigma) treatment. Restriction endonuclease digestion of 5 µg of DNA was done according to the manufacturer's instructions with *Cla*I, *Hin*dIII, *Eco*RI, *Sma*I and *Sac*II (New England Biolabs, Beverly, MA, USA). The REA, genomic blots, and the rDNA probe for rRNA-coding gene restriction patterns (ribotypes) *(17)* were prepared as described by Björkroth and Korkeala *(3)*.

Determination of morphological, physiological, and biochemical characteristics

General morphological, physiological, and biochemical characteristics were determined according to Sharpe *(35, 36)* for five isolates. Growth at 4, 15, and 45°C was tested as well as survival at 65°C for 10 min in MRS broth. Growth in solutions with pH values of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.2, with ethanol concentrations of 5%, 10%, and 15%, and NaCl concentrations of 8%, 10%, and 12% were also tested in MRS broth. Carbohydrate fermentation patterns were obtained with the API 50 CH *Lactobacillus* identification system (Biomerieux, Marcy l'Etoile, France). The production of ammonia from arginine was observed using the method of Briggs *(8)*. Gas production was detected in MRS broth containing Durham tubes *(37)*.

Production of spoilage in ketchup

Sterile ketchup samples were inoculated to determine the ability of the suspected spoilage strain to cause spoilage in ketchup. Tests were performed with two *L. fructivorans* isolates. Overnight 100 ml MRS broth cultures were used to inoculate 50-g ketchup samples. The inoculated samples were incubated for 22 days at 5, 10, 15, 20, 25, 30, 37, and 42°C. Sensory evaluation, preformed by 5 judges as described by Korkeala et al. *(22)*, and microbial counts on MRS agar were performed immediately and after 2, 5, 12, and 22 days.

European suspension test

The European suspension test was performed for two of the *L. fructivorans* isolates according to the procedure of Council of Europe *(11)*, with modifications concerning agar media as described by Mäkelä et al. *(26)*. The commercial detergent sanitizer and sanitizer used in the plant were tested. Detergent sanitizer was a foam-forming, alkaline (buffered to pH 12), and 12 to 13% active chloride-containing concentrate. The plant sanitizer contained antimicrobial compounds of 4.5% alkyldimethylbenzylammonium chloride and 4.5% alkyldimethylethylbenzylammonium chloride. Both the detergent sanitizer and the sanitizer were diluted for use into 1% (vol/vol) solutions.

Determination of D values

D values were determined for one spoilage isolate and for one *Lactobacillus fructivorans* type strain (ATCC 8288) in sterile ketchup at 65°C and at 72°C using the open-tube-method *(41)*. REA was performed for 40 randomly selected colonies from the plates inoculated during heat inactivation testing to ensure that the growing bacteria were the same as the inoculated strain.

RESULTS

All 8 bottles showed strong gas formation and had a pungent odor. The typical pH value of this product is 3.8 ± 0.1. The 8 sample bottles had pH values of 3.3 to 3.4. Growth was not detected on directly cultured PCA plates or blood agar, in enriched meat extract or Robertson's broths. Colonies were detected on directly cultured MRS and Rogosa SL agar media from the ketchup of all 8 bottles. The parallel enriched MRS broths showed growth as well. Directly cultured MRS plates showed growth of 3×10^5 to 9×10^6 CFU/g and on Rogosa SL plates 2×10^5 to 1×10^7 CFU/g. Microbiological counts of all the ketchup samples were very similar on parallel MRS and Rogosa SL agar media, suggesting *Lactobacillus* spp. growth.

The *Cla*I, *Eco*RI and *Hin*dIII digests of all 70 isolates were identical. REAs generated by *Sma*I and *Sac*II digests did not produce suitable patterns for characterization of these strains. The general morphological, physiological, and biochemical characteristics of the isolates were: gram-positive, thin rods, strong gas production from glucose, and able to use a very limited spectrum of carbohydrates: only ribose, D-fructose, D-glucose, and gluconate were fermented. Growth was observed at 15°C but not at 4 or 45°C. Survival tests were positive for 10 min at 65°C. Ammonia was produced from arginine and growth was observed in 8% NaCl concentration, but not in 10% NaCl-containing broth. Optimal pH values favorable for growth of the spoilage strain were 4.5 to 5.5; growth was not detected below pH 3.5. Isolates grew weakly on MRS agar and in MRS broth (pH 6). Subcultivation of the clones resulted in better growth. Isolates and the *L. fructivorans* type strain grew in MRS broth containing 5%, 10%, and 15% ethanol.

An *L. fructivorans* strain was suspected to be the cause of spoilage resulting in the bulged ketchup bottles. The identification was confirmed by comparing the ribotypes of

the spoilage strain with corresponding patterns of heterofermentative *Lactobacillus* type strains that are biochemically or genetically close to *L. fructivorans*. These results (Fig. 1a, lanes 1, 2, 10, and 11; Fig. 1b lanes 1 and 2) showed that the ketchup isolates and the *L. fructivorans* type strain had very similar ribotypes generated by *Cla*I, *Eco*RI, and *Hin*dIII digests. Only *Cla*I digests generated different patterns between the type strain and ketchup isolates (Fig. 1a, lanes 1 and 2). *L. fructivorans* REA patterns were clearly distinguished from the patterns of the other type strains studied.

The *L. fructivorans* isolates used for inoculation produced characteristic spoilage changes in 3 weeks when incubated at 15 to 25°C. Test samples incubated at 30°C already showed spoilage changes after 2 weeks. Samples were deemed unfit for human consumption when bacterial counts reached 10^5 CFU/g on MRS agar. Spoilage was not detected in test samples incubated at 5 and 10°C. Samples incubated at 42°C showed 90% reduction of colonies after 3

days. The same reduction was observed after 5 days at 37°C. In the European suspension test both the detergent sanitizer and the plant sanitizer were found to be very effective (microbicidal effect, ME, was 8) on *L. fructivorans* strain causing spoilage.

Heat-inactivation kinetics of the spoilage strain and the type strain resembled each other. For the spoilage isolate, a D_{65} of 1.2 min and a D_{72} of 0.4 min were obtained in ketchup. Corresponding values for the *L. fructivorans* ATCC 8288 were D_{65}, 0.9 min and D_{72}, 0.4 min. These values were calculated from the log-linear parts of the inactivation patterns. However, a clear tail was noticed in all inactivation curves when testing was continued. In the beginning of the test there were 10^8 to 10^9 CFU/g of inoculated ketchup. At 65°C tailing started after reduction of 3 to 4 log cycles and at 72°C the same effect was noticed after reduction of 4 to 5 log cycles. Forty strains analyzed by REA all shared the same pattern as the inoculant.

DISCUSSION

L. fructivorans strains are fastidious on primary isolation *(18, 27)*, resulting in detection difficulties when PCA or blood-agar media are used. Depending on the source of isolation, mevalonic acid, tomato juice, and/or ethanol are required for growth *(18)*. Modified MRS medium or other media designed for heterofermentative lactobacilli may provide better growth conditions *(15, 40, 44)*. Strains from nonalcoholic sources are known to become less fastidious during laboratory transfers and to start growing well in basic MRS broth *(18)*. This phenomenon was also noticed with the original strains isolated from ketchup. High preincubation temperatures used in the quality control of preserved food prior to microbiological analyses also contribute to difficulties in detection of *L. fructivorans*. For fully preserved canned foods, preincubation at 30°C for 14 days has been recommended *(32)*. Bulging was not present in test bottles incubated over 8 weeks at 37°C at the manufacturing plant. In our inoculation test, a 5-day incubation period at 37°C reduced 90% of inoculated *L. fructivorans* cells.

Lactic acid bacteria are not generally considered to be heat resistant. In meat-processing plants, lactic acid bacteria contamination leading to spoilage problems, with the exception of that caused by *Weissella viridescens* (7, 28, 30, 31), is usually considered a post-heat-treatment issue *(7, 13, 21, 23, 24, 25, 29, 30)*. Due to the layout of the ketchup-processing line, post-heat-treatment contamination during cooling and packaging was possible. *L. fructivorans* has been reported to be more heat resistant than other lactic acid bacteria tested *(38, 39)*. Splittstoesser et al. *(39)* established a D value of 1.7 min at 60°C for an *L. fructivorans* strain in 12% ethanol wine originally isolated from a spoiled, premixed Bloody Mary cocktail. This D value is close to the 1.2-min D_{65} value of the ketchup spoilage strain determined in this study. Due to the strong tailing effect noticed in D_{65} and D_{72} plots, the D value evaluation must be considered an estimation. Biphasic destruction of *L. fructivorans* has also been observed before *(38)*. D values for the second phase, following a reduction of about 4 log cycles, ranged from 4 to 6 min at 60°C. It is
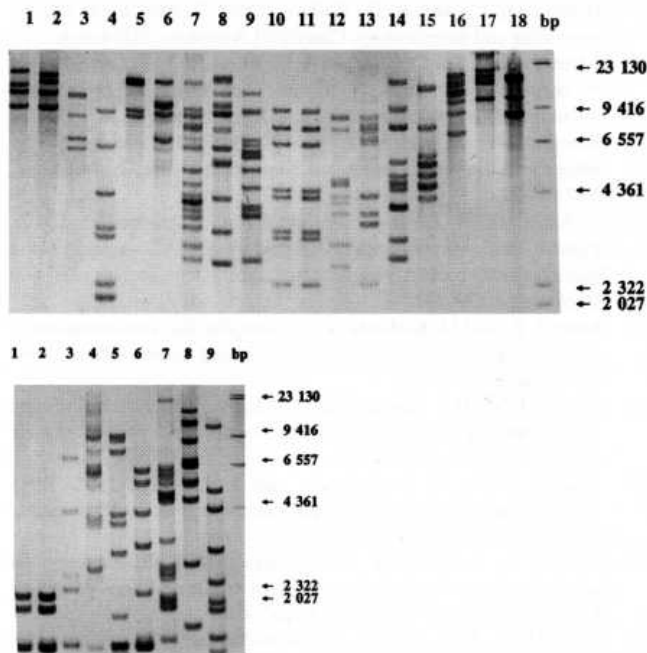

FIGURE 1a. *Ribotypes obtained by ClaI (lanes 1 to 9) and EcoRI (lanes 10 to 18) digests of the spoilage strain and the reference strains. Phage lambda DNA cleaved with HindIII was used as a fragment size marker. Lanes 1 and 10, the ketchup-spoilage strain; lanes 2 and 11,* Lactobacillus fructivorans *ATCC 8288; lanes 3 and 12,* Lactobacillus buchneri *ATCC 4005; lanes 4 and 13,* Lactobacillus brevis *ATCC 14869; lanes 5 and 14,* Lactobacillus collinoides *ATCC 27612; lanes 6 and 15,* Lactobacillus hilgardii *ATCC 8290; lanes 7 and 16,* Weissella confusa *ATCC 10881; lanes 8 and 17,* Weissella halotolerans *ATCC 35410; lanes 9 and 18,* Weissella viridescens *ATCC 12706.* FIGURE 1b. *Ribotypes obtained by HindIII digests of the spoilage strain and the reference strains. Phage lambda DNA cleaved with HindIII was used as a fragment size marker. Lane 1, the ketchup-spoilage strain; lane 2,* Lactobacillus fructivorans *ATCC 8288; lane 3,* Lactobacillus buchneri *ATCC 4005; lane 4,* Lactobacillus brevis *ATCC 14869; lane 5,* Lactobacillus collinoides *ATCC 27612; lane 6,* Lactobacillus hilgardii *ATCC 8290; lane 7,* Weissella confusa *ATCC 10881; lane 8,* Weissella halotolerans *ATCC 35410; lane 9,* Weissella viridescens *ATCC 12706.*

difficult to determine if the heat treatment used in the ketchup processing entirely destroys the spoilage-causing *L. fructivorans* strain, especially when the level of initial contamination was unknown. Tomato juice is also known to have factors associated with heat resistance of lactic acid bacteria *(20)*. Pectins were found to be the main tomato juice constituents protecting *Lactobacillus fermentum* cells against heat *(20)*.

Species identification of *L. fructivorans* is considered to be difficult using traditional phenotypic markers *(1, 15, 18)*. High tolerance to ethanol, a limited spectrum of fermentable carbohydrates and pronounced acidophily are characteristics typical for both *L. fructivorans* and *Lactobacillus hilgardii*. *Lactobacillus suebicus* is very tolerant to ethanol and low pH and ferments a broad spectrum of carbohydrates; it is genotypically closely related to *Lactobacillus vaccinosterus* *(10)*. Ribotyping can be of great help in species identification *(4, 17, 34)*. Molecular typing techniques such as REA and ribotyping can also be excellent tools for industrial contamination analysis *(3, 4, 5)*. These methods can provide information concerning spoilage by revealing the nature of the contamination. The spoilage described here was eradicated by increased sanitizing of the production line using the substances found to be effective against the causative *L. fructivorans* strain.

ACKNOWLEDGMENTS

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