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Short communication

Isolation and identification of spoilage microorganisms using food-based media combined with rDNA sequencing: Ranch dressing as a model food

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ABSTRACT

Investigating microbial spoilage of food is hampered by the lack of suitable growth media and protocols to characterize the causative agents. Microbial spoilage of salad dressing is sporadic and relatively unpredictable, thus processors struggle to develop strategies to minimize or prevent spoilage of this product. The objectives of this study were to (i) induce and characterize spoilage events in ranch-style dressing as a model food, and (ii) isolate and identify the causative microorganisms using traditional and food-based media, coupled with rDNA sequence analysis. Ranch dressing (pH 4.4) was prepared and stored at 25 °C for 14 d and microbial populations were recovered on MRS agar and ranch dressing agar (RDA), a newly formulated food-based medium. When isolates suspected as the spoilage agents were inoculated into ranch dressing and held at 25 °C for 9–10 d, three unique spoilage events were characterized. Using rDNA sequence comparisons, spoilage organisms were identified as *Lactobacillus brevis*, *Pediococcus acidilactici*, and *Torulaspora delbrueckii*. *P. acidilactici* produced flat-sour spoilage, whereas *Lb. brevis* resulted in product acidification and moderate gas production. The RDA medium allowed for optimum recovery of the excessive gas-producing spoilage yeast, *T. delbrueckii*. The isolation and identification strategy utilized in this work should assist in the characterization of spoilage organisms in other food systems.

1. Introduction

Microbial spoilage of food leads to considerable economic losses worldwide (Huis in't Veld, 1996). To minimize the financial impact of food spoilage, manufacturers aspire to implement strategies to control or eliminate the organisms responsible for product degradation. However, spoilage of commercial food is often sporadic and little is known about the microorganisms responsible for causing the deterioration. The difficulties associated with predicting food spoilage are compounded by the lack of appropriate selective microbiological media and reliable protocols to characterize the causative microbial agents. Numerous media and molecular-based methods are developed for the detection and identification of foodborne pathogens, but little effort is directed toward spoilage microorganisms.

Mayonnaise-based dressings, including ranch-style dressing, are complex food systems containing a variety of nutrients that support the growth of acid-tolerant microorganisms, leading to spoilage and product failure (Smittle, 1977; Vargo, 1989). Failure of these products has been associated with sensory defects, including gas production (often described as "bubbling over"), change in color, rancid odor, decrease in pH, increase in volatile acidity, and emulsion separation (Fabian and Wethington, 1950; Iszard, 1927; Pederson, 1930). These changes occur singularly or in combinations and product failure is described as sporadic. Sources of spoilage microorganisms are believed to be raw ingredients or environmental contaminants (Fabian and Wethington, 1950).

For more than half a century, researchers have attempted to identify spoilage-causing agents in salad dressings and mayonnaise with some success, primarily relying on traditional non-selective [e.g., plate count agar (PCA)] and selective [e.g., de Man, Rogosa and Sharpe (MRS) agar] media. Initially, Gram-positive spore-forming bacteria (*Bacillus* spp.) were implicated as the primary spoilage organisms, however these were later identified as part of the natural microbiota of raw ingredients and determined to be incapable of growth in the finished product. Lactobacilli and yeasts were recognized as primary spoilage organisms of defined salad dressings and mayonnaise (Fabian and Wethington, 1950; Iszard, 1927; Pederson, 1930). *Lactobacillus fructivorans*, *Lb. buchneri*, *Lb. fermentum*, *Lb. brevis*, *Lb. plantarum* and *Lb. cellobiosus* have been specifically recognized as spoilage agents in these products (Kurtzman et al., 1971; Smittle and

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Cirigliano, 1992). *Zygosaccharomyces* spp. (most commonly *Z. bailii*) have been isolated from numerous mayonnaise-based products and their presence is often associated with gas production (Fabian and Wethington, 1950; Smittle and Flowers, 1982). Other yeasts have been associated with spoilage of mayonnaise and salad dressings, including *Pichia membranifaciens*, *Issatchenkia orientalis*, *Candida inconspicua*, *C. parapsilosis*, *Saccharomyces cerevisiae*, *S. exiguous*, *S. dairensis*, *Debaryomyces hansenii*, *Rhodotorula mucilaginosa*, and *Torulopsis cutaneum* (Smittle, 1977; Tornai-Lehoczki et al., 2003).

Ranch-style dressing, which has no standard of identity in the US Code of Federal Regulations, differs significantly from defined mayonnaise and other salad dressings in formulation. Scientific literature is almost void of knowledge about causes of spoilage of this type of product. Manufacturers provided this laboratory with microorganisms isolated from spoiled ranch dressing, but upon inoculation, these cultures failed to induce spoilage in freshly prepared product. Temperature abuse of commercially available products did not result in product spoilage. Further discussion with manufacturers revealed the sporadic nature of ranch dressing spoilage and relatively little information about the causative microorganisms. The objectives of this study were to (i) induce and define ranch dressing-specific spoilage events, (ii) isolate microorganisms associated with these spoilage events using traditional microbiological techniques with recovery on standard and food-based growth media, and (iii) identify the isolated spoilage microorganisms using rDNA sequencing methods.

2. Methods

2.1. Formulation of ranch dressing

Ranch dressing was prepared using the following recipe as suggested by a national industrial ranch dressing manufacturer: 2800 g mayonnaise (Kraft Real Mayonnaise, Kraft Foods Global, Inc., Northfield, IL), 1258 g buttermilk made with whole milk (Kroger, The Kroger Co., Cincinnati, OH), 170 g dried chopped onions (Spice Classics, Por Han-Dee Pak, Inc., Cockeysville, MD), 13 g canned minced garlic (Goya, Goya Foods, Inc., Secaucus, NJ), 3.5 g dried parsley flakes (McCormick, McCormick and Co., Inc. Hunt Valley, MD), 1.5 g iodized salt (Morton, Morton International, Inc., Chicago, IL), 1.2 g dried ground thyme (McCormick), 1.0 g xanthan gum (Bob's Red Mill, Newburg, OR), 0.75 g ground black pepper (McCormick), and 0.75 g monosodium glutamate (Kroger). All ingredients were transferred to a stainless steel mixing bowl and mixed by hand with a whisk to achieve a uniform consistency. The dressing was covered with plastic wrap and held at room temperature for 30–60 min to stabilize, then pH of the dressing was adjusted to 4.4 with 5 M NaOH; original pH was 4.2.

2.2. Product storage and analyses

Aliquots of the laboratory-prepared ranch dressing (50 g each) were transferred to sterile polyethylene bags (Fisher Scientific, Pittsburgh, PA) or sterile 50-ml conical bottom polystyrene tubes (BD Biosciences, Bedford, MA). Bags were heat-sealed with minimum air present inside. Tubes were completely filled to minimize headspace prior to sealing. Both types of packages were stored at 25 °C for 14 d to induce a spoilage event. Visual observations (i.e., emulsion separation, color change, and gas production) were recorded and the pH of the product was measured periodically. Preparation and storage of ranch dressing to induce spoilage events was repeated on seven separate occasions.

2.3. Formulation of ranch dressing agar (RDA)

Ranch dressing, formulated and prepared as described earlier, was treated with ultra-high pressure (UHP) at 600 MPa for 5 min. UHP processing was performed using a hydrostatic food processor (Quintus QFP6, Flow Pressure Systems, Kent, WA) containing 1:1 (vol/vol) glycol/water pressure transmitting fluid (Houghto-Safe 620 TY, Houghton International Inc., Valley Forge, PA). The pressure-treated dressing was aseptically diluted (1:1 weight/volume) with a sterile molten 3% agar solution containing 0.1% FD&C Blue No. 2. Addition of Blue No. 2 aided in the recognition of colonies by improving the color contrast between the medium and the colonies. Other colorants (i.e., FD&C Blue No. 1 and FD&C Green No. 3) produced satisfactory results and could be used in lieu of Blue No. 2 in the recovery media. The product was mixed using a magnetic stirrer until a uniform consistency was obtained. The resulting medium (RDA) was poured into Petri dishes and held at 25 °C for 24–48 h before use.

2.4. Enumeration and isolation of spoilage microorganisms in ranch dressing

Freshly prepared and temperature-abused (stored at 25 °C for 14 d) ranch dressing, was analyzed for microbial counts. Three packages per time-point were analyzed every 2 d throughout the 14-d storage period, regardless of product spoilage status. For microbial analysis, ranch dressing was decimally diluted in 0.1% peptone water and dilutions were spread-plated onto MRS agar and RDA. Microorganisms were enumerated after incubation of plates at 30 °C for 3–5 d. Microorganisms were recovered from all samples and examined for colony morphology. Various colony morphologies were observed and representative plates were stored at 4 °C to compare with the morphologies and counts recovered with subsequent samplings. Isolates with distinct colony morphologies were recovered from MRS agar and RDA plates with populations $\geq 10^5$ cfu/g. These isolates were examined microscopically for cellular morphology and bacterial isolates were analyzed for Gram reaction. Frozen stock cultures were prepared for five isolates with distinct colony and cellular morphologies and stored at −80 °C.

2.5. Identification of spoilage-causing isolates by rDNA sequence analysis

Bacterial isolates were identified by sequencing their 16S rDNA genes using primers fD1 and rD1 as previously described (Weisburg et al., 1991). Briefly, isolates were grown overnight in MRS broth and DNA was isolated using the QIAquick DNeasy protocol for Gram-positive bacteria (Qiagen, Valencia, CA). Polymerase chain reaction (PCR) mixture was composed of 1× buffer containing magnesium chloride (Qiagen), 25 μM dNTPs, 2.5 U *Taq* polymerase (Qiagen), 50 μg/ml primer fD1 (5′-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT TGG CTC AG-3′), 50 μg/ml primer rD1 (5′-CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC-3′). Reaction mixture (25 μl) was aliquoted into sterile PCR tubes. DNA (1 μl) isolated from suspected spoilage bacteria was added to the reaction mixture. PCR was performed using the following program in the thermocycler (Bio-Rad MJ Mini, Bio-Rad Hercules, CA): initial denaturation at 95 °C for 3 min; 30 cycles of 95 °C for 1 min, 52 °C for 30 s, 72 °C for 2 min; followed by final extension at 72 °C for 10 min. The PCR product was sequenced by The Ohio State University Plant and Microbe Sequencing Facility. Data were compared to published sequences in the NCBI database using the BLAST function (www.ncbi.nlm.nih.gov). Species identification was considered to be confirmed if sequence similarity was ≥97% (Stackebrandt and Goebel, 1994).