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Short communication: Pseudomonas azotoformans causes gray discoloration in HTST fluid milk

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ABSTRACT

Pseudomonas species are well recognized as dairy product spoilage organisms, particularly due to their ability to grow at refrigeration temperatures. Although *Pseudomonas*-related spoilage usually manifests itself in flavor, odor, and texture defects, which are typically due to production of bacterial enzymes, *Pseudomonas* is also reported to cause color defects. Because of consumer complaints, a commercial dairy company shipped 4 samples of high temperature, short time (HTST)-pasteurized milk with distinctly gray colors to our laboratory. Bacterial isolates from all 4 samples were identified as *Pseudomonas azotoformans*. All isolates shared the same partial 16S rDNA sequence and showed black pigmentation on Dichloran Rose Bengal Chloramphenicol agar. Inoculation of one pigment-producing *P. azotoformans* isolate into HTST-pasteurized fluid milk led to development of gray milk after 14 d of storage at 6°C, but only in containers that had half of the total volume filled with milk (~500 mL of milk in ~1,000-mL bottles). We conclusively demonstrate that *Pseudomonas* can cause a color defect in fluid milk that manifests in gray discoloration, adding to the palette of color defects known to be caused by *Pseudomonas*. This information is of considerable interest to the dairy industry, because dairy processors and others may not typically associate black or gray colors in fluid milk with the presence of microbial contaminants but rather with product tampering (e.g., addition of ink) or other inadvertent chemical contamination.

Key words: gray pigment, spoilage, *Pseudomonas*, milk

Short Communication

Gram-negative *Pseudomonas* spp. are commonly associated with post-pasteurization contamination of milk (Dogan and Boor, 2003; Stellato et al., 2017). *Pseudomonas* spp., which grow well at refrigeration temperatures, can cause dairy product spoilage through a variety of mechanisms (Martin et al., 2011; Stellato et al., 2017). For example, many *Pseudomonas* spp. produce proteases and lipases, which can create sensory defects in food products (Dogan and Boor, 2003). Dogan and Boor (2003) reported that among 338 *Pseudomonas* isolates collected from raw milk, pasteurized milk, and dairy processing plant environments, 51% were protease positive, 47% were lecithinase positive, and 67% were lipase positive. Some *Pseudomonas* spp. also produce several different pigments (Palleroni, 1984), which can cause discoloration of dairy products and other foods (Cantoni et al., 2003). Of particular relevance to dairy products, *Pseudomonas fluorescens* biovar IV produces both a blue nondiffusible pigment as well as soluble pyoverdin (Palleroni, 1984). Contamination of a commercial fresh, low-acid cheese with *P. fluorescens* biovar IV generated alarming dark blue and fluorescent pigments on the surface of the cheese (Martin et al., 2011); this color defect precipitated at least one product recall. This represents a major business risk, particularly in the modern era of social media, because consumers easily notice color defects. Images of products with color defects can be shared widely and quickly, rapidly fueling consumer backlash against a product or company.

As part of the routine services offered by Cornell University's Milk Quality Improvement Program (MQIP), we received 4 samples (in 60-mL dairy vials) of fluid milk following consumer complaints of a "gray color defect." Samples were from whole, 2%, and 1% HTST-processed milk, and the gray discoloration had been an ongoing problem for the plant for several weeks before they contacted the MQIP. Microbiological analyses revealed large numbers of black-pigmented colonies on Dichloran Rose-Bengal Chloramphenicol (DRBC) agar; bacterial numbers were not assessed due to the unknown temperature history of the samples upon receipt. The same milk samples plated on SPC agar yielded gray colonies that produced pigment that dif-

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fused into the agar. Based on these initial observations, we hypothesized that the predominant dark-pigmented colonies isolated from the original milk samples were responsible for the observed gray color defect in the fluid milk. One pigmented isolate from each milk sample was characterized by partial 16S rDNA sequencing using previously described methods (Huck et al., 2007); all 4 isolates showed 100% identity over 669 nucleotides of partial 16S rDNA sequence data, suggesting that these isolates were clonal. Analysis of full-length 16S rDNA sequences for one isolate (FSL E2-0548), which was selected for further characterization, classified this isolate as *Pseudomonas azotoformans*. Among the six 16S rRNA operon sequences obtained from the FSL E2-0548 genome sequence, 3 showed a top match (99.6% identity) with the 16S rRNA sequence of the *Pseudomonas azotoformans* type strain included in the Ribosomal Database Project (RDP) Release 11 (https://rdp.cme.msu.edu). The FSL E2-0548 16S rRNA operon sequences also clustered with the 16S rRNA sequence of the *Pseudomonas azotoformans* type strain on a maximum likelihood phylogeny constructed with 16S rDNA type strain sequences obtained from RDP. Therefore, we conclude that the predominant contaminant of the original milk samples was *P. azotoformans*. All bacterial isolates are maintained as frozen stocks and are available for future studies through the Cornell University Food Safety Laboratory collection (http://www.foodmicrobetracker.com). Further characterization of these isolates with whole-genome sequencing will provide additional information, including confirmation that the same strain was isolated from all samples, as well as potential information on pathways responsible for the formation of the molecules responsible for the gray color.

We selected a single dark pigment–producing *P. azotoformans* isolate (FSL E2-0548) for inoculation studies into HTST-pasteurized fluid milk to determine if this strain could cause the gray discoloration defect that was initially reported in the commercial fluid milk samples. Briefly, isolate FSL E2-0548 was streaked onto brain heart infusion (**BHI**) agar from a frozen glycerol stock and incubated for 48 h at 32°C. A single colony was suspended in 5 mL of sterile BHI broth, followed by incubation for 18 h at 32°C. After stationary incubation, 1 mL of the 10^8 cfu/mL BHI suspension was placed in a 1.5-mL microcentrifuge tube and centrifuged at 21,000 × *g* for 1 min. The pellet was resuspended in 1 mL of sterile PBS. Serial dilutions of the bacterial suspension were prepared in PBS and used to inoculate containers of homogenized and pasteurized 2% fat milk (Cornell University Dairy, Ithaca, NY) to a final concentration of approximately 10 cfu/mL. Treatments groups included (1) inoculated "full" quart-size containers with original milk volume, (2) inoculated "half-full" quart-size containers with approximately half of the milk volume removed (to create headspace to simulate consumer usage in the home), as well as corresponding "full" and "half-full" uninoculated controls (3 and 4); each treatment was performed in 2 biological replicates. Five containers of each of the above treatments were prepared, one for each day of testing. Our goals were to ensure similar headspace and fluid milk volume for each sampling day and to prevent inadvertent bacterial contamination during subsequent handling.

The milk samples were stored at 6°C. One container from each of the 4 treatment groups was tested at 0, 3, 7, 10, and 14 d after inoculation. Before analysis, each sample was mixed by 25 inversions, according to *Standard Methods for the Examination of Dairy Products* procedures for preparing fluid milk samples for microbiological analyses (Laird et al., 2004). Samples were spiral plated in duplicate, on the test days stated above, onto 100-mm SPC agar plates using the 50-μL exponential setting (Autoplate 5000, Advanced Instruments Inc., Norwood, MA). The plates were incubated for 48 h at 32°C, and colonies were enumerated on the Color Q-Count (model 530, Advanced Instruments Inc.). To monitor possible color changes in the milk, L*a*b* values were measured on each testing day for each sample using the D65/10 setting on a colorimeter (HunterLab ColorQuest XE, Hunter Associates Laboratory Inc., Reston, VA), and Euclidean distance d_w from white was calculated by $d_w = \sqrt{\left(L^* - 100\right)^2 + \left(a^*\right)^2 + \left(b^*\right)^2}$, where L* represents lightness (L* = 0 is black; L* = 100 is a diffuse white); a* measures the sample's position between red/magenta and green (negative values are green; positive values are magenta); b* measures the sample's position between yellow and blue (negative values are blue; positive values are yellow) (Yam and Papadakis, 2004). A linear model was fit to model d_w by time, headspace, and inoculation, including all interactions, in R (R Core Team, 2016). The lsmeans package (Lenth, 2016) was used to compare the fitted d_w on d 14 at different values of headspace or inoculation. Plots were generated with the ggplot package (Wickham, 2009).

"Half-full" fluid milk samples inoculated with *P. azotoformans* (FSL E2-0548) developed a gray color after 14 d of incubation at 6°C (Figure 1), whereas none of the uninoculated samples, with or without extra headspace, showed visibly detectable color development. Statistical analyses showed that the color (i.e., d_w) in the half-full inoculated bottles was significantly less white than the half-full controls ($P < 0.0001$). Additionally, the color