Novel Molecular Assay and Sample Preparation Method for the Detection of *Alicyclobacillus* in Fruit Juice Concentrates and Bottling Process Materials

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Alicyclobacilli are thermophilic, acidophilic and spore-forming bacteria (TAB) capable of surviving extreme conditions during the fruit juice manufacturing and bottling processes. These spoilage organisms can generate strong off-flavors and off-odors, which negatively affect product quality in fruit juices, concentrates and ingredients used in beverage production. Though non-pathogenic, they pose a major economic risk to processors. Rapid detection and identification of TAB spoilage at various identified critical control points of the production process is a valuable tool to facilitate release of high quality products.

The International Fruit Juice Union (IFU) Method 12 is the current testing standard for detecting *Alicyclobacillus* in fruit juice concentrates and bottling process matrices, such as processing water, sugars and other additives. The IFU method requires long enrichment periods and labor-intensive subculturing procedures. In contrast, Pall has developed a fast, sensitive and highly specific molecular detection method, based on qPCR GeneDisc® technology (Figure 1), which is capable of individually identifying four specific *Alicyclobacillus* species most often associated with fruit juice spoilage (*A. acidoterrestris, A. acidophilus, A. cycloheptanicus, A. herbarius*), as well as detecting over 200 *Alicyclobacillus* strains.

Figure 1: GeneDisc Plate for TAB Spoilage

Protocol for Performance Evaluation of the GeneDisc Plate for TAB Spoilage

To evaluate detection performance of this new method, four common juice-spoilage strains of *Alicyclobacillus* were cultured in Yeast-Starch-Glucose (YSG) Broth, pH 3.7, for at least 48 h (filterable matrices) and at least 72 h (unfilterable matrices) at 45 °C. Genomic DNA was extracted using the GeneDisc Extraction Pack for food samples. Several non-targeted *Alicyclobacillus*, non-*Alicyclobacillus* bacteria, yeasts and molds (assay exclusion panel) were also cultured and genomic DNA extracted. The exclusion panel strains were tested at high genomic concentrations, 10^5 genomic units (GU) per well. Assay sensitivity was determined by testing very low genomic concentrations of inclusion strains, 50 GU per well. In addition, 1-10 colony forming units (CFU) of inclusion strains were spiked individually into a variety of test matrices (10 g or mL) and enriched in YSG for at least 48 h for filterable, and 72 h for unfilterable matrices. A 1 mL unfilterable sample enrichment aliquot or a 200 µL filterable sample enrichment aliquot was processed with the Extraction Pack for food samples involving sonication and heating. The processed sample was then loaded onto a GeneDisc Plate for *Alicyclobacillus* and run on the GeneDisc Cycler (Figure 2).

Figure 2: GeneDisc Cycler

Performance of the GeneDisc Plate for TAB Spoilage

1. Limit of Detection

The Limits of Detection (LOD) for individual target assays and the total *Alicyclobacillus* assay were determined to be between 50 to 300 CFU/mL post-enrichment.

2. Assay Specificity

The individual assays (*A. acidoterrestris, A. acidophilus, A. cycloheptanicus, A. herbarius*) achieved 100% specificity against both non-*Alicyclobacillus* organisms and non-targeted *Alicyclobacillus* strains (*e.g. A. hesperidum*). The *Alicyclobacillus* spp. assay achieved 100% specificity, by detecting all 25 *Alicyclobacillus* strains tested (Table 1).

Table 1: Assay Specificity – Inclusivity Panel

Strain	*Alicyclobacillus* spp. assay	*A. acidoterrestris* assay	Strain	*Alicyclobacillus* spp. assay	*A. acidoterrestris* assay
A. acidoterrestris ATCC49025	Presence	Presence	*A. acidophilus* DSM14558	Presence	Absence
A. acidoterrestris ATCC49026	Presence	Presence	*A. herbarius* DSM13609	Presence	Absence
A. acidoterrestris ATCC49027	Presence	Presence	*A. cycloheptanicus* ATCC49029	Presence	Absence
A. acidoterrestris DSM2498	Presence	Presence	*A. cycloheptanicus* DSM4005	Presence	Absence
A. acidoterrestris DSM2498	Presence	Presence	*A. hesperidum* DSM12489	Presence	Absence
A. acidoterrestris DSM3923	Presence	Presence	*A. acidocaldarius* ATCC 43034	Presence	Absence
A. acidoterrestris DSM3924	Presence	Presence	*A. acidocaldarius* ATCC 43031	Presence	Absence
A. acidoterrestris OS 232	Presence	Presence	*A. sendaiensis* ATCC BAA-609	Presence	Absence
A. acidoterrestris OS 247	Presence	Presence	*A. tengchongensis* ATCC BAA-2134	Presence	Absence
A. acidoterrestris OS 249	Presence	Presence	*A. fastidious* ATCC17978	Presence	Absence
A. acidoterrestris OS 258	Presence	Presence	*A. pomorum* ATCC 14955	Presence	Absence
A. acidoterrestris OS 259	Presence	Presence	*A. sacchari* ATCC17974	Presence	Absence
A. acidoterrestris OS N-1155	Presence	Presence			

None of the exclusion panel organisms were detected by any of the GeneDisc Plate assays (Table 2).

Table 2: Assay Specificity – Exclusivity Panel

Strain	PCR results	Strain	PCR results
Bacillus subtilis	Absence	*Lactobacillus brevis*	Absence
Bacillus cereus	Absence	*Lactobacillus casei*	Absence
Bacillus mycoides	Absence	*Lactobacillus collinoides*	Absence
Bacillus megaterium	Absence	*Lactobacillus coryniformis*	Absence
Burkholderia stabillus	Absence	*Lactobacillus lindneri*	Absence
Megasphaera cerevisiae	Absence	*Candida albicans*	Absence
Aspergillus niger	Absence	*Micrococcus naucinus*	Absence
Moorella thermoacetica	Absence		

3. Comprehensive Method Performance Evaluation on Spiked Samples

To evaluate the broad method performance, tests were performed on artificially spiked samples. All samples obtained after enrichment of test matrices in YSG Broth and processed through the GeneDisc Extraction Pack for food samples, were tested positive for the presence of each targeted *Alicyclobacillus* strain as well as for presence of total *Alicyclobacillus*. The non-spiked control samples were all tested negative.

4. Comprehensive Method Performance Evaluation on Naturally Contaminated Samples

In addition, naturally *Alicyclobacillus*-contaminated samples were obtained from fruit juice production sites and tested with the GeneDisc method. All contaminated samples, including a variety of sugars and juice concentrates, were tested positive for the presence of total *Alicyclobacillus*, and identified any of the four specific *Alicyclobacillus* spoiler species present in the sample. Negative control samples were all tested negative.

Enhanced Testing of TAB Spoilage for Industry

In conclusion, the streamlined GeneDisc workflow offers a great advantage over existing culture methods by reducing the time-to-result to 48 or 72 h depending on the matrix tested, while traditional IFU culture method can take as long as 2 weeks. The GeneDisc method for *Alicyclobacillus* provides convenient ease-of-use for laboratory operators combined with high specificity and detection performance in a variety of matrices. This makes the GeneDisc Plate a perfectly fitting tool for routine testing of TAB spoilage.

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