Rapid and Reliable Detection of Guaiacol-Producing *Alicyclobacillus* in Fruit Juices, Fruit Concentrates and Other Beverages by Real-Time PCR

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INTRODUCTION:

Alicyclobacillus spp. are gram-positive, thermophilic, acidophilic, spore-forming bacteria known for their resistance to the pasteurization process and ability to cause off flavors in acidic beverages, particularly fruit juices, by the production of guaiacol. Their growth characteristics and the ubiquity of their spores, introduced via contaminated fruit, pose significant challenges to the beverage industry. Due to the ubiquitous occurrence and production of guaiacol, *Alicyclobacillus* contamination can quickly lead to the spoilage of beverages during production. Therefore, a rapid and reliable screening test, with differentiation for guaiacol and non-guaiacol producing *Alicyclobacillus,* is of high advantage.

To ensure product safety, Hygiena® offers the beverage industry the foodproof® *Alicyclobacillus* Detection Lyokit, a real-time PCR method that detects both guaiacol-producing and non-guaiacol-producing *Alicyclobacillus* strains in a single enrichment and PCR reaction.

PURPOSE:

This study evaluated the foodproof® *Alicyclobacillus* Detection LyoKit, a real-time PCR assay for rapid screening for *Alicyclobacillus* spp. and differentiation between guaiacol-producing and non-guaiacol-producing strains in enrichment cultures of juices, juice concentrates and other beverages.

REGISTERED TRADEMARKS

DNA Extraction: foodproof® StarPrep Two Kit (KIT230177)
foodproof® Magnetic Preparation KIT VI (KIT230190)
PCR: foodproof® *Alicyclobacillus* Detection LyoKit (KIT230151/KIT230152/KIT230153)

METHOD:

For the evaluation of the foodproof® *Alicyclobacillus* Detection LyoKit, inclusivity, exclusivity and sensitivity tests as well as a method comparison study were conducted. The PCR assay detects guaiacol-producing *Alicyclobacillus*, *Alicyclobacillus* spp. and an internal control in separate channels. For testing, 63 target strains of 20 species, including guaiacol-producing strains like *A. acidoterrestris*, *A. herbarius*, *A. dauci*, *A. acidiphilus*, *A. fastidiosus* and *A. suci*, as well as 46 relevant non-target strains of 20 species, were cultured for the inclusivity and exclusivity study. A Limit of Detection (LoD) determination was carried out using RNA-free DNA in a concentration of 0.75 to 100 genomic equivalents per reaction. Different *Alicyclobacillus* strains, including *A. acidoterrestris*, *A. fastidiosus*, *A. herbarius*, *A. dauci* and *A. acidocaldarius,* were tested. For the sensitivity study, different juices and concentrates were spiked post-enrichment with 3 CFU/mL, 7 CFU/mL and 70 CFU/mL of *A. acidoterrestris* and DNA extraction was performed with the foodproof® StarPrep® Two Kit (manual) and the foodproof® Magnetic Preparation Kit VI (automated). PCR was performed on the Dualo 32® (Hygiena Diagnostics) instrument. A paired method comparison study with the reference method, International Fruit and Vegetable Juice Association (IFU) No. 12:2019, was conducted in beverage juice and concentrate samples. The samples were spiked with two different concentrations of either *A. acidoterrestris* or *A. acidocaldarius* and detection was examined after 24 to 72 hours of enrichment at 45 °C.

INCLUSIVITY & EXCLUSIVITY STUDY:

19 target strains from six guaiacol-producing *Alicyclobacillus* species and 44 target strains from 14 *Alicyclobacillus* spp. species were used for inclusivity testing. For exclusivity testing, 46 relevant non-target strains belonging to 20 different species were selected due to phylogenetic relation to *Alicyclobacillus* spp., frequent occurrence in juice and other beverages, resistance to pasteurization or the ability to form guaiacol.

For inclusivity and exclusivity testing, DNA was extracted from single colonies either with the foodproof® StarPrep Two Kit and diluted prior to PCR or as a purified RNA-free extract in a concentration of 100 to 1000 genomic equivalents per reaction for inclusivity and 10^5 to 10^6 genomic equivalents per reaction for exclusivity. PCR was performed on the AriaMx (Agilent Technologies) or LightCycler® 480 (Roche).

Data from the specificity studies show 100% inclusivity for all relevant guaiacol-producing and non-guaiacol-producing *Alicyclobacillus* spp. strains and 100% exclusivity for all 46 relevant non-target strains for the foodproof® *Alicyclobacillus* Detection LyoKit.

METHOD COMPARISON STUDY:

A paired method comparison study with the reference method, IFU No. 12:2019, was conducted in orange concentrate and tomato puree. 10 mL of beverage samples were diluted in BAT broth in a concentration of 1:10 and spiked with pre-enrichments containing either *A. acidoterrestris* or *A. acidocaldarius* at two different concentrations. After enrichment for 24, 48 and 72 hours at 45 °C, in comparison to IFU No. 12:2019 (120 hours of enrichment at 45 °C), samples were extracted with the foodproof® StarPrep Two Kit and detection of *Alicyclobacillus* was examined with the foodproof® *Alicyclobacillus* Detection LyoKit. PCR was performed on the Dualo 32® instrument.

Orange juice concentrates inoculated prior to enrichment with 30 CFU/mL of *A. acidoterrestris* lead to three positive samples out of four replicates after 24 hours of enrichment at 45 °C. An inoculation level of 2.7 CFU/mL of *A. acidoterrestris* resulted in one positive sample out of four replicates (Table 1). A longer enrichment time up to 72 hours (Table 1) and even up to 120 hours (data not shown) did not lead to a higher proportion of positive replicates. Four out of four replicates of tomato puree inoculated with 6 CFU/mL or 20 CFU/mL of non-guaiacol-producing *A. acidocaldarius* could be detected after incubation for 24 hours at 45 °C using the alternative method (Table 2). The results for *A. acidocaldarius* show positive results for the HEX channel (*Alicyclobacillus* spp.) but negative results in the FAM channel (guaiacol-producing strains). This data underscores the precise differentiation of the foodproof® *Alicyclobacillus* Detection LyoKit between guaiacol and non-guaiacol-producing strains.

The results of the fractional spiking study obtained with the foodproof® StarPrep Two Kit and foodproof® *Alicyclobacillus* Detection LyoKit are in 100% agreement with the IFU No. 12:2019 reference method, which needs 120 hours of enrichment (Tables 1 and 2) and is therefore comparable in sensitivity.

Table 1: Method Comparison Study for Orange Concentrate Spiked with *Alicyclobacillus acidoterrestris* and Enriched for 24, 48 and 72 hours at 45 °C in Comparison to IFU No. 12:2019

A. acidoterrestris (BCD 16102)													
Method		**foodproof® StarPrep Two + foodproof® *Alicyclobacillus* Detection LyoKit**										**IFU No. 12:2019**	
Incubation		**24 hrs**			**48 hrs**			**72 hrs**			**120 hrs**		
Matrix	Inoculation CFU/sample	Pos/Rep PCR	FAM Cq-value	HEX Cq-value	Pos/Rep PCR	FAM Cq Value	HEX Cq Value	Pos/Rep PCR	FAM Cq Value	HEX Cq Value	Pos/Rep IFU No. 12	Microbiology
Orange Concentrate	30	3/4	0.00 24.16 19.91 33.45	0.00 24.48 19.61 34.00	3/4	0.00 14.66 12.36 18.72	0.00 15.00 13.22 19.16	3/4	0.00 12.01 11.44 11.70	0.00 12.35 11.89 12.44	3/4	neg pos pos pos
	2.7	1/4	0.00 32.81 0.00 0.00	0.00 34.00 0.00 0.00	1/4	0.00 22.38 0.00 0.00	0.00 22.93 0.00 0.00	1/4	0.00 12.65 0.00 0.00	0.00 13.21 0.00 0.00	1/4	neg pos neg neg
	0	0/1	0.00	0.00	0/1	0.00	0.00	0/1	0.00	0.00	0/1	neg
BAT	/	0/1	0.00	0.00	0/1	0.00	0.00	0/1	0.00	0.00	0/1	neg

Table 2: Method Comparison Study for Tomato Puree Spiked with *Alicyclobacillus acidocaldarius* and Enriched for 24, 48 and 72 hours at 45 °C in comparison to IFU No. 12:2019

A. acidocaldarius (BCD 16140)													
Method		**foodproof® StarPrep Two + foodproof® *Alicyclobacillus* Detection LyoKit**										**IFU No. 12:2019**	
Incubation		**24 hrs**			**48 hrs**			**72 hrs**			**120 hrs**		
Matrix	Inoculation CFU/sample	Pos/Rep PCR	FAM Cq Value	HEX Cq Value	Pos/Rep PCR	FAM Cq Value	HEX Cq Value	Pos/Rep PCR	FAM Cq Value	HEX Cq Value	Pos/Rep IFU No. 12	Microbiology
Tomato Puree	20	4/4	0.00 0.00 0.00 0.00	27.44 26.85 27.51 27.14	4/4	0.00 0.00 0.00 0.00	17.77 17.89 18.49 18.02	4/4	0.00 0.00 0.00 0.00	16.01 16.22 15.39 15.37	4/4	pos pos pos pos
	6	4/4	0.00 0.00 0.00 0.00	27.94 31.66 26.13 30.43	4/4	0.00 0.00 0.00 0.00	17.62 17.17 17.53 17.27	4/4	0.00 0.00 0.00 0.00	15.20 14.96 14.66 14.91	4/4	pos pos pos pos
	0	0/1	0.00	0.00	0/1	0.00	0.00	0/1	0.00	0.00	0/1	neg
BAT	0	0/1	0.00	0.00	0/1	0.00	0.00	0/1	0.00	0.00	0/1	neg

SENSITIVITY STUDY:

To determine the Limit of Detection (LoD), RNA-free DNA in the concentrations of 0.75 to 100 genomic equivalents per reaction of different *Alicyclobacillus* strains was tested. PCR was performed on an AriaMx (Agilent Technologies) instrument. Results demonstrate the high sensitivity of the foodproof® *Alicyclobacillus* Detection LyoKit, with a LoD of 1.25 genomic equivalents per reaction for all tested *Alicyclobacillus* strains (Table 3 for *Alicyclobacillus herbarius,* data for other *Alicyclobacillus* strains not shown).

Matrix compatibility was examined in different fruit juices and juice concentrates. 10 mL of beverage samples were enriched at 45 °C for 72 hours in BAT broth in a dilution of 1:10. Samples were spiked post-enrichment to a final concentration of 3 CFU/mL, 7 CFU/mL and 70 CFU/mL. For DNA extraction from spiked enrichment cultures, the foodproof® StarPrep® Two Kit (manual) and the foodproof® Magnetic Preparation Kit VI (automated) were tested.

A concentration of 3 CFU/mL of *Alicyclobacillus acidoterrestris* is detectable in all tested fruit juices, juice concentrates and other beverages with the foodproof® *Alicyclobacillus* Detection LyoKit (Table 4). Three out of three replicates of the tested samples were analyzed as positive in FAM and HEX channels, which demonstrates a correct detection of guaiacol-producing organisms even at low concentration in enrichment culture, regardless of a manual or automation-based DNA isolation method.

Table 3: LoD Determination with the foodproof® *Alicyclobacillus* Detection LyoKit for *Alicyclobacillus herbarius*

A. herbarius (DSM 3922)				
Genomic Equivalents per Reaction	FAM		HEX	
	Positive	Mean Cq ± CV%	Positive	Mean Cq ± CV%
100	2 of 2	30.74 ± 0.00	2 of 2	29.92 ± 0.02
10	2 of 2	33.50 ± 0.39	2 of 2	32.59 ± 0.89
5	2 of 2	34.06 ± 0.56	2 of 2	33.48 ± 0.67
2.5	2 of 2	35.09 ± 0.53	2 of 2	34.85 ± 0.10
1.25	2 of 2	37.69 ± 0.65	2 of 2	35.06 ± 1.71
0.75	2 of 2	37.21 ± 2.20	2 of 2	36.05 ± 0.97

Table 4: Sensitivity Study with Enriched Juice and Juice Concentrates Spiked with *Alicyclobacillus acidoterrestris*

A. acidoterrestris (BCD 16102)					
foodproof® Star Prep Two for *Alicyclobacillus* Detection					
Matrix	Spiking CFU/mL	FAM		HEX	
		Positive	Mean Cq ± CV%	Positive	Mean Cq ± CV%
Black Currant Juice	70	3 of 3	28.22 ± 1.37	3 of 3	28.54 ± 1.03
	7	3 of 3	31.02 ± 0.36	3 of 3	31.88 ± 0.69
	3	3 of 3	32.74 ± 0.51	3 of 3	34.01 ± 0.99
Cranberry Juice	70	3 of 3	28.09 ± 0.71	3 of 3	28.50 ± 0.91
	7	3 of 3	31.22 ± 0.38	3 of 3	31.94 ± 0.46
	3	3 of 3	32.34 ± 0.10	3 of 3	33.49 ± 0.52
Raspberry Sirup	70	3 of 3	27.76 ± 0.40	3 of 3	28.13 ± 0.60
	7	3 of 3	31.28 ± 0.34	3 of 3	31.87 ± 0.22
	3	3 of 3	32.64 ± 0.47	3 of 3	33.31 ± 0.51
Apple Concentrate	70	3 of 3	28.32 ± 0.14	3 of 3	28.80 ± 0.17
	7	3 of 3	31.37 ± 0.32	3 of 3	32.31 ± 0.90
	3	3 of 3	32.65 ± 0.26	3 of 3	33.94 ± 0.20
Apple-Orange-Carot Concentrate	70	3 of 3	30.15 ± 0.41	3 of 3	30.49 ± 0.70
	7	3 of 3	32.96 ± 0.10	3 of 3	34.01 ± 0.52
	3	3 of 3	34.36 ± 1.37	3 of 3	35.58 ± 0.62
Orange Concentrate, clear	70	3 of 3	28.37 ± 0.40	3 of 3	28.97 ± 0.30
	7	3 of 3	31.25 ± 0.26	3 of 3	32.33 ± 0.19
	3	3 of 3	32.33 ± 0.89	3 of 3	33.81 ± 0.44
Orange Concentrate, cloudy	70	3 of 3	31.10 ± 0.26	3 of 3	31.93 ± 0.56
	7	3 of 3	33.53 ± 0.54	3 of 3	35.16 ± 0.77
	3	3 of 3	34.58 ± 1.10	3 of 3	36.52 ± 0.93
foodproof® Magnetic Preparation Kit VI					
Matrix	Spiking CFU/mL	FAM		HEX	
		Positive	Mean Cq ± CV%	Positive	Mean Cq ± CV%
Black Currant Juice	70	3 of 3	29.52 ± 0.37	3 of 3	30.37 ± 0.17
	7	3 of 3	33.14 ± 0.94	3 of 3	34.69 ± 1.97
	3	3 of 3	35.45 ± 1.87	3 of 3	36.39 ± 0.68
Cranberry Juice	70	3 of 3	32.11 ± 0.83	3 of 3	32.82 ± 1.09
	7	3 of 3	34.38 ± 0.74	3 of 3	35.65 ± 0.32
	3	3 of 3	33.93 ± 0.45	3 of 3	35.17 ± 0.52
Raspberry Sirup	70	3 of 3	29.99 ± 0.49	3 of 3	30.73 ± 0.11
	7	3 of 3	32.78 ± 0.78	3 of 3	33.98 ± 1.05
	3	3 of 3	34.54 ± 1.11	3 of 3	35.77 ± 1.61

SIGNIFICANCE:

The foodproof® *Alicyclobacillus* Detection LyoKit offers beverage industries a rapid, reliable and easy-to-use PCR-based technology for the detection of *Alicyclobacillus* spp. with differentiation between guaiacol-producing and non-guaiacol-producing strains. This method provides a safe way to detect and differentiate guaiacol and non-guaiacol-producing *Alicyclobacillus* spp. in beverages, with prevention of false-negative results with the internal PCR control and prevention of carry-over contamination due to integrated Uracil-N-Glycosylase. Our technique offers a high sensitivity and fast detection of guaiacol-producing and non-guaiacol-producing *Alicyclobacillus* spp. for all tested organisms, with savings of up to three to five days compared to the IFU No. 12:2019. The alternative method can be combined with different extraction methods, such as foodproof® StarPrep Two (KIT230177) for manual processing or the foodproof® Magnetic Preparation Kit VI (KIT230190) for automated processing.