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Identification of guaiacol producing *Alicyclobacillus* recovered from commercial orange juices distributed in Greek markets

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ORIGINAL ARTICLE

Abstract

The objective of this work was the isolation and characterization of *Alicyclobacillus acidoterrestris* strains from orange juice. In all, 72 isolates were recovered from commercial orange juice bought from the Greek market. The isolates were subjected to polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) to discriminate them at strain level and *A. acidoterrestris* types I and II. The restriction patterns of *16S rRNA* gene revealed that isolates were categorized in different groups depending on the restriction endonucleases. Moreover, the digestion of the part of *vdc* operon gene with *Hph*I restriction enzyme successfully differentiated between *A. acidoterrestris* types I and II. In conclusion, RFLP succeeded in differentiating *A. acidoterrestris* isolates at strain and type levels.

Keywords: *Alicyclobacillus acidoterrestris*; orange juice; PCR-RFLP; *vdc* gene

Introduction

In recent years, the fruit juice industry has suffered financial loss because of the reported fruit juice contaminations (Centers for Disease Control and Prevention [CDC], 1999; Griffiths, 2000). The most serious threat to fruit juice producers is the presence of *Alicyclobacillus,* which has increased considerably in a variety of fruit juices, including the orange juice (Eguchi *et al.*, 1999; Eiroa *et al*., 1999; Pinhatti *et al.*, 1997; Sourri *et al.*, 2022). *Alicyclobacillus* spp. are non-pathogenic thermoacidophilic, spore-forming bacteria that can survive pasteurization conditions and subsequently germinate under favorable conditions, and therefore limit the shelf life of products (Cacho *et al.*, 2011; Molva and Baysal, 2015; Silva and Gibbs, 2001). Spoilage because of the presence of *Alicyclobacillus* is not visible during storage or retailing because neither gas production nor swelling of the container takes place (flat sour-type spoilage). Only after consumption, one can describe flavors as "smoky", "antiseptic", or "disinfectant", and possible increased turbidity and sediment formation can lead to the conclusion of spoilage of the juice (Lusardi *et al.*, 2000; Smit *et al.*, 2011; Uchida and Silva, 2017). Although the predominant taint compound responsible for these defects is 2-methoxyphenol (guaiacol), the presence of halophenols

2,6 dibromothenol and 2,6 dichlorophenol can also lead to spoilage of the juice (Hartyáni *et al.*, 2013; Molva and Baysal, 2015). *Alicyclobacillus acidoterrestris* is linked to the majority of spoiling, although not all *Alicyclobacillus* spp. are able to produce guaiacol (Molva and Baysal, 2015). Hence, in order to optimize the pasteurization process, *Alicyclobacillus acidoterrestris* is regarded as the target microorganism by fruit juice manufacturers (Silva *et al.*, 2000; Vieira *et al.*, 2002).

In the present study, a survey was conducted involving Greek commercial orange juices by using the IFU method No. 12 (International Federation of Fruit Juice Producers, 2007). This is recognized as the most effective microbiological method for detection of *Alicyclobacillus* (Witthuhn *et al.*, 2011). The aim of this survey, besides examining the occurrence of the bacterium in orange juice, was to identify different isolates of *Alicyclobacillus* spp., and in addition compare the relative presence of *Alicyclobacillus acidoterrestris* with other *Alicyclobacillus* spp. Another goal of the survey was to determine whether the isolates had the ability to produce guaiacol and thus lead to spoilage issues.

Numerous polymerase chain reaction (PCR) techniques, including polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis of *16S ribosomal RNA* (*16S rRNA*), *rpoB*, and *valine decarboxylase* (*vdc*) genes, were successfully used over the years for differentiation and/or identification of *Alicyclobacillus* (Chen *et al.*, 2006; Dekowska *et al.*, 2018; Roth *et al.*, 2021; Sourri *et al.*, 2019; Wang *et al.*, 2021). Vermicon Identification Technology (VIT) is another technique that was used for *Alicyclobacillus* differentiation, because *A. acidoterrestris* glows in different colors (Thelen, 2003). Distinction between guaiacol-producing and non-producing species was accomplished with the denaturing gradient gel electrophoresis (DGGE) method (Osopale *et al.*, 2016). Three restriction enzymes were employed in PCR-RFLP to discriminate and characterize *Alicyclobacillus* isolates. The classification of *Alicyclobacillus* isolates into clusters, and their association with guaiacol production, is of major interest and significance for juice manufacturers to prevent incidents of spoilage.

Material and Methods

Bacterial strains

In all, 72 *Alicyclobacillus* isolates were recovered from four different commercial Greek orange juice brands (JA, JL, JH, and JP) with the use of IFU method No. 12 developed by the Working Group on Microbiology of the International Federation of Fruit Juice Producers (2007). In addition, a culture collection of *A. acidoterrestris* DSMZ 2498 obtained from DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkuturen, Braunschweig, Germany) was used as a reference strain.

DNA extraction from pure cultures

All isolates were cultured in yeast extract starch glucose (YSG) broth (2 g/L yeast extract [Biolife Italiana]; 1 g/L glucose [LAB M Limited]; and 2 g/L soluble starch [NeoLab Migge Laborbedarf-Vertiebs GmbH], pH adjusted to 3.7 with 1-N HCl) and incubated at 45°C for 48 h. Bacterial cells at the exponential phase, 10 mL, were collected by centrifugation (8,000 rpm for 5 min at 4°C) and treated with 20-mg/mL lysozyme in lysis buffer (20 mmol/L Tris-HCl, pH = 8.0, 2 mmol/L ethylenediaminetetraacetic acid [EDTA], and 1% Triton ×100) at 37°C for 60 min for lysis of *Alicyclobacillus* cells. To extract genomic DNA from *Alicyclobacillus* cultures, nucleospin tissue (Macherey-Nagel, Düren, Nordrhein-Westfalen, Germany) was used according to the manufacturer's instructions. DNA concentration was measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher, IL, US).

Detection of polymorphism by PCR-RFLP targeting *16S rRNA* gene

Genomic DNA of all isolates was subjected to PCR using the universal primers: P1: 5′-AGCAGTAGGGAATCTTCCA-3′ and P2: 5′-TTCCCCACGCGTTACTCACC-3′ for the amplification of *16S rDNA* gene (Klijn *et al.*, 1991). PCR amplification was performed in a total volume of 25 μL containing 1.25 U of thermostable (Taq) DNA polymerase (Kapa Biosystems), 2.5-μL Taq buffer, 0.8-mM deoxynucleotide triphosphate (dNTP), 0.2 μM of each primer, and 2-mM total $MgCl_2$. Amplification was performed in a Bio-Rad thermocycler, with the following PCR conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 30 s, primer extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Then 5 U of three different restriction endonucleases (REs) *Hha*I, *RSa*I, and *HiNF*I (New England Biolabs [NEB], Ipswich, MA, USA)) were used to digest 5 μL of each PCR product after incubation at 37°C for 2 h. The restriction profiles of the isolates were separated by gel electrophoresis on 3% (w/v) agarose for 2 h. After staining with ethidium bromide, the restriction fragments were detected under ultraviolet (UV) light (Gel Doc, Biorad, Hercules, CA, USA). A molecular weight marker (NEB) of 50 bp was used to estimate the size of fragments. Dendrogram construction and normalization were performed using the Dice coefficient

and the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis (optimization: 0.5 and tolerance: 1.0) via the BioNumerics software version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium).

Bacterial identification by targeting *16s rRNA*, *rpoB*, and *vdc* genes

In all, 13 representative isolates grouped in major clusters (at a similarity level of 70%; Figure 1) were subjected to DNA extraction using a DNeasy Power Food Microbial kit (Qiagen) according to the manufacturer's instructions. A Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific,Waltham, MA, USA) was used to estimate DNA concentration. The method used by Dekowska *et al.* (2018) was applied to characterize *Alicyclobacillus* isolates by amplification of *16S rRNA* gene (primers 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1512R:5′-ACGGCTACCTTGTTACGACT-3′), *rpoB* gene (primers Gru5:5′-CGCGACGTACACTATTCGCACTA-3′; Gru6: 5′-GCCCAAACCTCCATCTCACCAAA-3′) and part *vdc* operon (primers 5′-Bur5: GCCGACGTGAT GCTCAARGAGCGCA-3′; Bur6:5′-GTSGCRTCGAG AATCATCTTGTG-3′). DreamTaq Green PCR Master Mix (Thermo Fisher) was used for DNA amplification in 50 μL PCR reaction. The amplicons were checked for purity in 1% agarose gel using a 1-kB ladder. Restriction enzymes *Bsu*RI (*Hae*III), *Hph*I, and *Hin6*I (*HinP*1I) were used to digest amplicons at 37°C according to the manufacturer's (NEB) recommendations. Following the inactivation of enzymes at 65°C (*Hph*I and *Hin6*I) and 80°C (*Bsu*RI) for 20 min, the RFLP patterns were analyzed on 3% agarose gel. A 100-bp ladder was included as a molecular marker. The following eight isolates belonging to the Culture Collection of Industrial Microorganisms–Microbiological Resource Center IAFB (Warsaw, Poland) culture collection were included as reference strains for *16S rRNA* and *rpoB* genes: *Alicyclobacillus acidoterrestris* type I DSM 2498, *A. acidoterrestris* type II ATCC 49025, *A. acidiphilus* DSM 14558, *A. herbarius* DSM 13609, *A. hesperidum* DSM 12489, *A. acidocaldarius* DSM 446, *Bacillussubtilis* ATCC 6655, and *Geobacillus stearothermophilus* ATCC 7953. In addition, *Alicyclobacillus acidoterrestris* B41 and *A. hesperidum* 6G belonging to the Culture Collection of Institute of Technology of Agricultural Products (ITAP, Athens, Greece) were included for *16S rRNA* and *rpoB* genes analysis. Similarly, the following four isolates belonging to IAFB were included as reference strains for *vdc* operon: *Alicyclobacillus acidoterrestris* type I DSM 2498, *A. acidoterrestris* type II ATCC 49025, *A. acidiphilus* DSM 14558, and *A. herbarius* DSM 13609. In addition, *Alicyclobacillus acidoterrestris* B41 belonging to ITAP was included for *vdc* operon analysis. Syngene gel documentation was used for the visualization of amplicons and RFLP patterns. Sequencing of *vdc* gene of representative isolates was performed. In addition, sequencing of *16S rRNA* and *rpoB* genes was performed for non-*A. acidoterrestris* isolates.

Test of the guaiacol producing potential of *Alicyclobacillus* strains

Alicyclobacillus isolates were also checked for their potential of producing guaiacol with the Peroxidase Enzyme Colorimetric Assay (PECA) method. A commercial Guaiacol detection kit (Dohler DMD; Microsafety Design, Darmstadt, Germany) was used for this purpose according to the manufacturer's instructions. *Alicyclobacillus acidoterrestris* B41, *A. acidoterrestris* 4a, and *A. hesperidum* 6G belonging to the Culture Collection of Institute of Technology of Agricultural Products (ITAP, Athens, Greece) were included as positive (*A. acidoterrestris*) and negative (*A. hesperidum*) controls.

Results and Discussion

Differentiation of *Alicyclobacillus* isolates

The occurrence of spoilage caused by *Alicyclobacillus* has been extensively documented in several fruit juices, including orange juice (Gocmen *et al.*, 2005; Osopale *et al.*, 2016). Furthermore, there are reports of isolation from many other fruit juices, such as mango (Gouws *et al.*, 2005), passion (McKnight *et al.*, 2010), and kiwi (Zhang *et al.*, 2013). In addition, Yamazaki *et al.* (1996) successfully isolated *Alicyclobacillus* strains from fruit juice blends, while Van Luong *et al.* (2019) achieved the same outcome from a range of fruit juices. Isolations for each specific type of juice are presented in Table 1. All isolates were amplified using the PCR-RFLP technique, followed by restriction digestion using three endonucleases *Hha*I, *RSa*I, and *HiNF*I. Figure 1 displays the dendrogram derived from the restriction pattern cluster analysis.

The clustering process yielded eight groups at a similarity level of 80% and 16 clusters at a similarity level of 90%. It was observed that at the similarity level of 80%, 16 (referring to group VI) out of 72 isolates (A14, A26, A28, A29, A30, A31, B1, B24, B26, B27, B32, B34, B35, B38, B42, and B43) obtained from orange juice exhibited comparable restriction patterns with the reference strain *A. acidoterrestis* DSMZ 2498 as shown in Figure 1. Group IV, including 15 isolates (A1, A2, A3, A4, A5, A6, A9, A12, A13, A15, A17, A18, A19, B22, and B44) along with the former group, were the larger clusters as indicated in the dendrogram. The remaining groups comprised

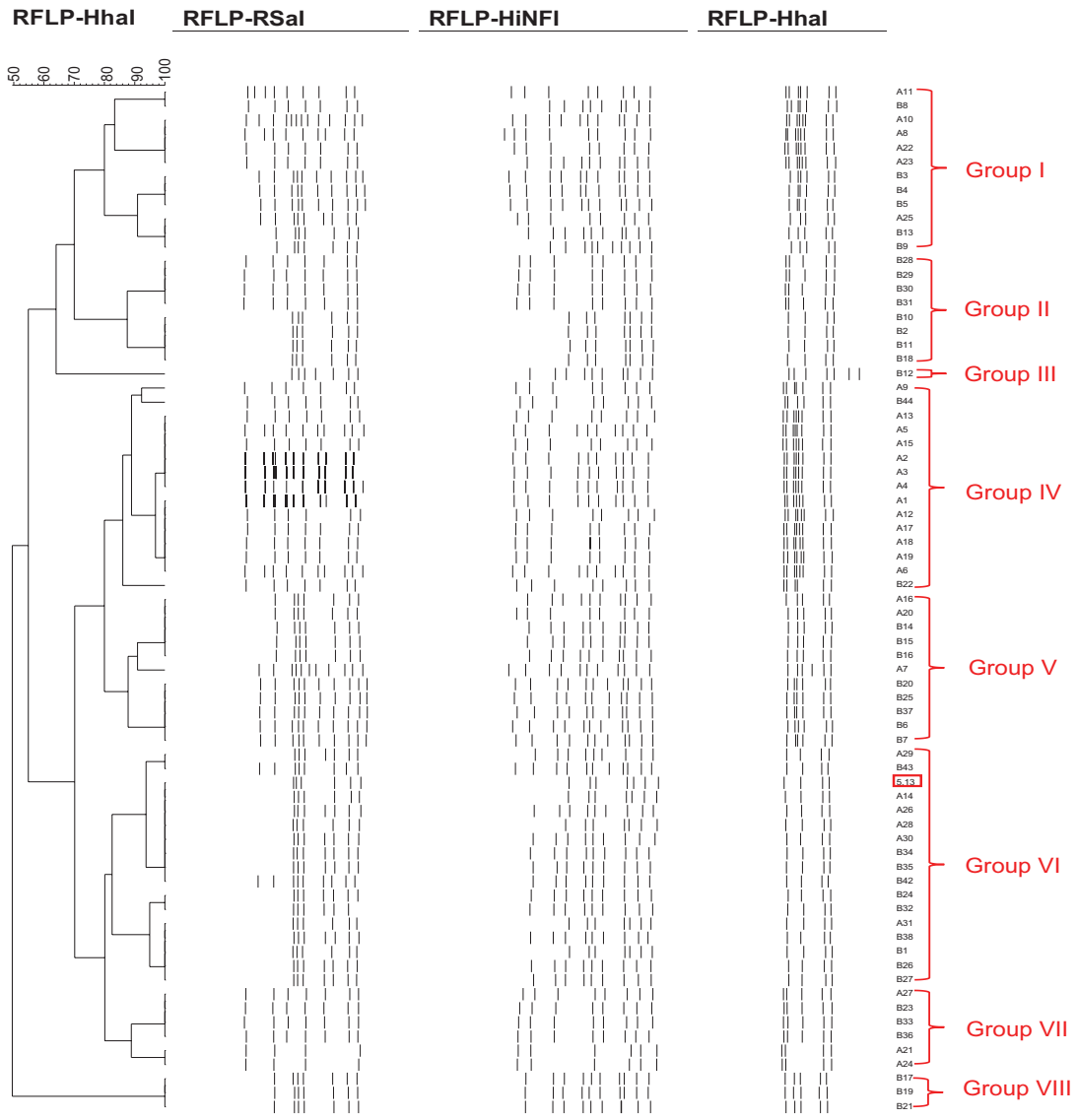

Figure 1. Dendrogram of *16S rRNA* PCR-RFLP with restriction endonucleases *Hha*I, *RSa*I, and *HiNF*I of *Alicyclobacillus* isolates from commercial orange juices. The reference strain *A. acidoterrestris* DSMZ 2498 is marked as 5.13 in red box.

Table 1. *Alicyclobacillus* isolates from four types of commercial orange juices.

Juice type	*Alicyclobacillus* isolates
JA (juice)	A1-A31
JL (juice)	B1-B10, B21-B38, and B42-B44
JH (concentrated)	B11-B16
JP (juice)	B17-B20

12 isolates (A8, A10, A11, A22, A23, A25, B3, B4, B5, B8, B9, and B13) classified as group I, 11 isolates (A7, A16, A20, B6, B7, B14, B15, B16, B20, B25, and B37) that composed group V, 8 isolates that comprised group II (B2, B10, B11, B18, B28, B29, B30, and B31), 6 isolates comprising group VII (A21, A24, A27, B23, B33, and B36), and 3 isolates of group VIII (B17, B19, and B21). One particular isolate (B12) with a distinctive pattern comprised group III. Table 2 summarizes the distribution of *Alicyclobacillus* isolates into eight distinct groups based on their restriction patterns and the type of orange juice from which they were obtained.

The findings provided in Table 2 demonstrate that *Alicyclobacillus* isolates obtained from each juice were categorized into distinct groups, indicating potential variations among isolates from the same juice. Furthermore, the dendrogram revealed that not all groups were present in all juices. Even the isolates that were grouped in the largest clusters (III and V) were found exclusively in the juices with a high number of recovered colonies (JA and JL).

Table 2. Distribution of *Alicyclobacillus* isolates from different orange juices.

Juice	Total isolates	Groups[a]							
		I	II	III	IV	V	VI	VII	VIII
JA	31	6			13	3	6	3	
JL	31	5	6		2	4	10	3	1
JH	6	1	1	1		3			
JP	4		1			1			2
Total	72	12	8	1	15	11	16	6	3

[a]According to the Dendrogram given in Figure 1.

It should also be highlighted that juices (JH and JP) from which a lower number of colonies were retrieved exhibited isolates that were spread throughout distinct groups.

Furthermore, the PECA method was used to detect guaiacol synthesis in all isolates. This approach relies on the oxidation of guaiacol by peroxidase enzymes in the presence of H_2O_2, resulting in the production of a brown molecule, known as 3,3′-dimethoxy-4,4′-biphenoquinone (Doerge *et al.*, 1997). In accordance with the instructions provided by the commercial detection kit, the color change was detected for all isolates, with the presence of brown color, indicating guaiacol production. The results indicated that all isolates were capable of producing guaiacol. The isolates have been arranged in ascending order of brown color intensity, as depicted in Figure S1, while the negative and positive control samples are on the left and right sides, respectively. Figures S2 and S3 show representative isolates which produce guaiacol based on the development of brown color. *Alicyclobacillus acidoterrestris* B41 and *A. acidoterrestris* 4a were included as positive control strains (known guaiacol producers; Table 3), while *Alicyclobacillus hesperidum* 6G was used as negative control strain (data not shown). The isolates were visually classified into six groups based on the varying degrees of the brown color as displayed in Table 3. However, there does not appear to be any correlation between the groups separated in the dendrogram on the basis of the restriction patterns with those distinguished using the PECA method (Figure 1).

Alicyclobacillus identification targeting *16S RNA*, *rpoB*, and *vdc* genes

In all, 13 representative isolates from major clusters (Figure 1; similarity level 70%) were subjected to species identification based on the protocol described by Dekowska *et al.* (2018). According to the obtained results, differentiation of the isolates at the species level was achieved based on the restriction profile of the *16S rRNA* gene of *Alicyclobacillus* isolates obtained with restriction enzyme *HinP1*I (Figure 2). All isolates were characterized as *Alicyclobacillus acidoterrestris*. Moreover, the discrimination of *A. acidoterrestris, A. acidiphilus, A. herbarius, A. hesperidum*, and *A. acidocaldarius* was achieved by the application of *HinP1*I (Figure 2a). On the other hand, inability of the restriction profile of *16S rRNA* gene of *Alicyclobacillus* isolates obtained with restriction enzymes *Hph*I and *Hae*III to distinguish *A. acidoterrestris* and *A. hesperidum* was shown in Figures 2b and 2c, respectively. However, the discrimination of *A. herbarius* and *A. acidocaldarius* from *A. acidoterrestris*, *A. acidophilus*, and *A. hesperidum* was achieved following the observation of restriction patterns obtained after the application of *Hph*I. Similarly, the application of *Hae*III was able to discriminate *A. herbarius* and

Table 3. Distribution of *Alicyclobacillus* isolates according to the PECA method.

Groups	Color intensity	Isolates	Total
I	Very light brown	B1, B6, B10	3
II	Light brown	A16, B20, B44, **B41***	3
III	Pale brown	A5, A6, A7, A15, A17, A25, A26, A31, B8, B9, B15, B19, B22, B30, B31, B32, B42, B43	18
IV	Brown	A4, A14, A18, A24, A28, A29, A30, B4, B7, B13, B17, B21, B23, B29, B33, B34, **4a**	16
V	Dark brown	A3, A8, A9, A13, A19, A23, B11, B12, B16, B18, B24, B26, B27, B28, B35	15
VI	Very dark brown	A1, A2, A10, A11, A12, A20, A21, A22, A27, B2, B3, B5, B14, B25, B36, B37, B38	17

*Reference strains: known guaiacol producers are marked in bold font.

A. acidocaldarius from the pattern of *A. acidophilus*, *A. herbarius*, and *A. acidocaldarius* from the pattern of *A. acidoterrestris* and *A. hesperidum* (*Hae*III). In another study, discrimination of *A. acidoterrestris* by other *Alicyclobacillus* spp. was also achieved by RFLP-PCR (Sourri *et al.*, 2019). The sequencing analysis of *16S rRNA* gene of *A. hesperidum* 6G strain confirmed the 99.78% identity to *A. hesperidum* DSM 12489.

The ability to discriminate *A. acidoterrestris* from the other tested *Alicyclobacillus* spp. based on the restriction profiles of *rpoB* gene using any of the used restriction enzymes was shown. In addition, the differentiation of *A. acidoterrestris* in type I and type II was achieved (Figure 3). It was shown that the isolates A16, A28, A30, B1, B6, B19, and B20 (*A. acidoterrestris* type I) were discerned from A5, A17, A27, B30, and B31 (*A. acidoterrestris* type II). The *A. acidoterrestris* B41 was also assigned to *A. acidoterrestris* type I. In previous studies, similar grouping of *A. acidoterrestris* isolates was achieved by RAPD and sequencing analysis of *16S rRNA* gene (Durak *et al.*, 2010; Osopale *et al.*, 2016). The sequencing analysis of *rpoB* gene of *A. hesperidum* 6G strain confirmed the 99.63% identity to *A. hesperidum* DSM 12489.

The digestion of partial *vdc* operon with the restriction enzyme *Hph*I (Figure 4) succeeded in discerning the *A. acidoterrestris* type I from *A. acidoterrestris* type II (Dekowska *et al.*, 2018) according to the results obtained from the digestion of *rpoB* gene with the three enzymes. On the other hand, the results obtained after the digestion of *vdc* operon gene with restriction enzymes *Hae*III and *HinP1*I showed the inability of 14 isolates to be discerned in type I and II *A. acidoterrestris*, which was not in accordance to the previously published results (Dekowska *et al.*, 2018) and the results obtained from *rpoB* gene. Following the differentiation of 14 *A. acidoterrestris* isolates in type I and type II according to the *rpoB* gene restriction profiles, sequencing analysis of partial *vdc* operon gene of two representative isolates (A16 and A5) was performed. According to the obtained results, the sequence of *vdc* operon gene of the A5 isolate was found to be 99.93% similar to the sequence of reference strain DSM 2498 (reference strain AATI) and 94.31% similar to ATCC 49025 (reference strain AATII). Similarly, the sequence of *vdc* gene of A16 isolate was found to be 99.73% similar to the sequence of reference strain DSM 2498 (reference strain AATI) and 94.11% similar to ATCC 49025 (reference strain AATII). In addition, the sequences of *vdc* gene fragments of A5 and A16 isolates were 99.8% identical.

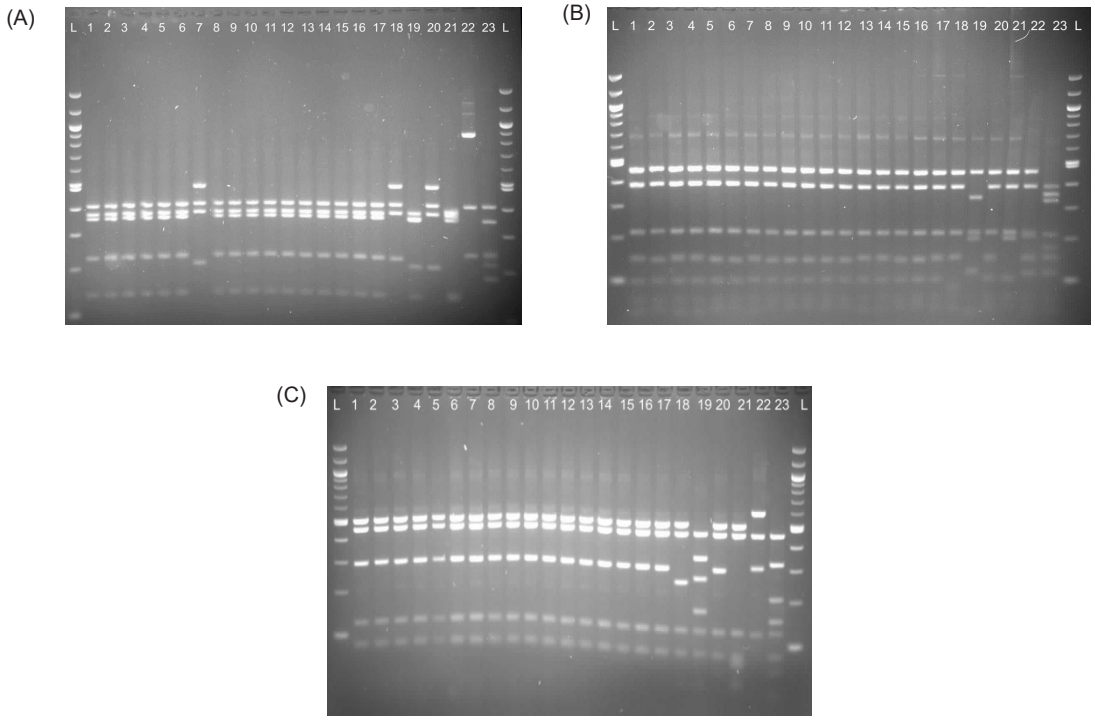

Figure 2. Restriction profile of *16S rRNA* gene of *Alicyclobacillus* isolates obtained with (A) *HinP1*I, (B) *Hph*I, and (C) *Hae*III. L: 100-bp DNA ladder; isolates 1–6: A5, A16, A17, A27, A28, A30; isolate 7: *A. hesperidum* 6G, isolates 8–14: B1, B6, B18, B19, B20, B30, B31; isolate 15: *A. acidoterrestris* B41; isolate 16: *A. acidoterrestris* type IDSM 2498; isolate 17: *A. acidoterrestris* type IIATCC 49025; isolate 18: *A. acidiphilus* DSM 14558; isolate 19: *A. herbarius* DSM 13609; isolate 20: *A. hesperidum* DSM 12489; isolate 21: *A. acidocaldarius* DSM 446; isolate 22: *Bacillussubtilis* ATCC 6655; and isolate 23: *Geobacillus stearothermophilus* ATCC 7953.

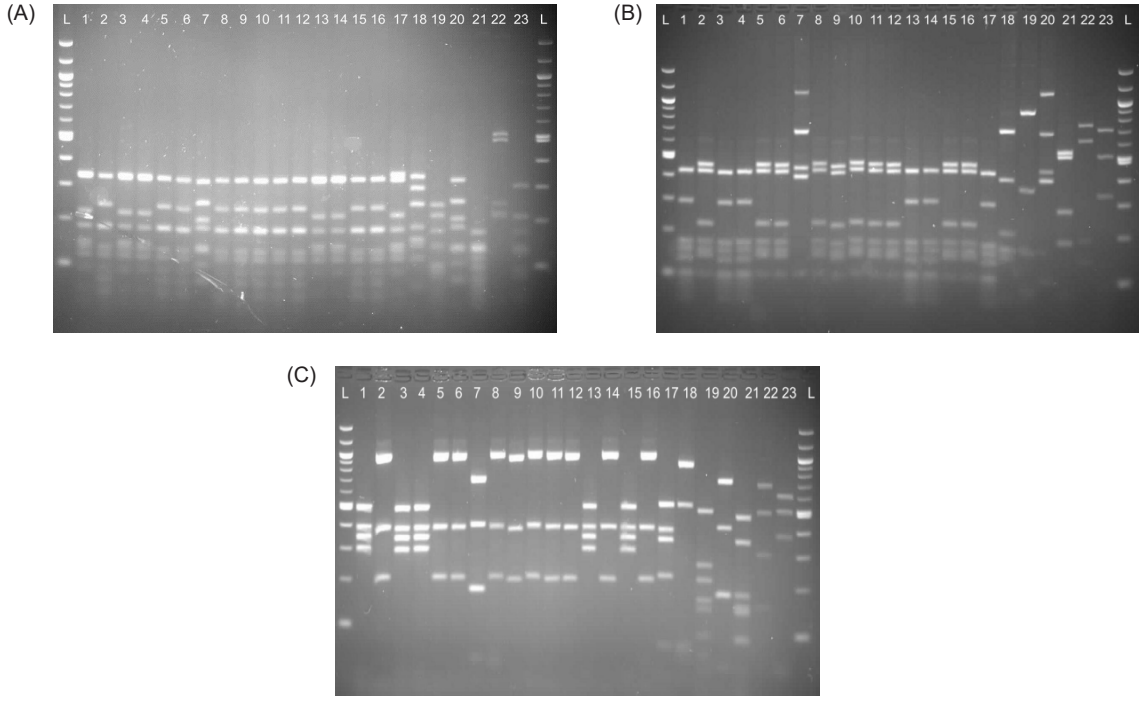

Figure 3. Restriction profile of *rpoB* gene of *Alicyclobacillus* isolates obtained with (A) *HinP1*I, (B) *Hph*I, and (C) *Hae*III. L: 100-bp DNA ladder; isolates 1–6: A5, A16, A17, A27, A28, A30; isolate 7: *A.hesperidum* 6G, isolates 8–14: B1, B6, B18, B19, B20, B30, B31; isolate 15: *A. acidoterrestris* B41; isolate 16: *A. acidoterrestris* type I DSM 2498; isolate 17: *A. acidoterrestris* type II ATCC 49025; isolate 18: *A. acidiphilus* DSM 14558*;* isolate 19: *A. herbarius* DSM 13609; isolate 20: *A. hesperidum* DSM 12489; isolate 21: *A. acidocaldarius* DSM 446; isolate 22: *Bacillussubtilis* ATCC 6655; and isolate 23: *Geobacillus stearothermophilus* ATCC 7953.

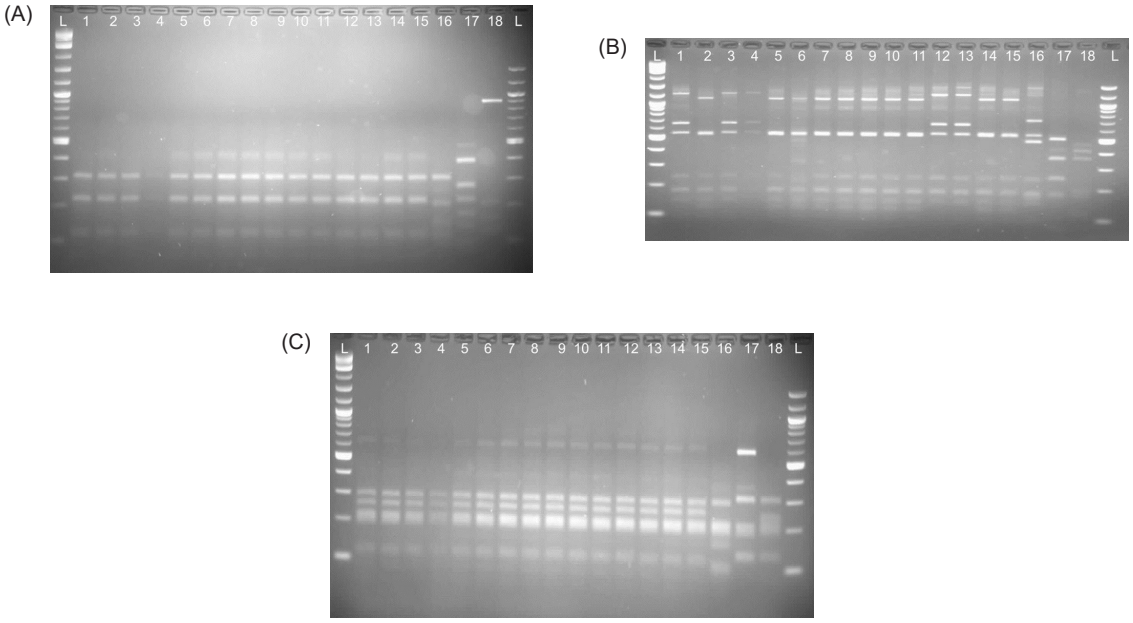

Figure 4. Restriction profile of partial *vdc* operon of *Alicyclobacillus* isolates obtained with (A) *HinP1*I, (B) *Hph*I, and (C) *Hae*III. L: 100-bp DNA ladder; isolates 1–13: A5, A16, A17, A27, A28, A30, B1, B6, B18, B19, B20, B30, B31; isolate 14: *A. acidoterrestris* B41; isolate 15: *A. acidoterrestris* type IDSM 2498; isolate 16: *A. acidoterrestris* type IIATCC 49025; isolate 17: *A. acidiphilus* DSM 14558; isolate 18: *A. herbarius* DSM 13609.

Conclusion

The *Alicyclobacillus* isolates obtained from various commercially available orange juices exhibited distinct patterns, including those derived from the same juice. The successful utilization of PCR-RFLP, employing the restriction endonucleases *Hha*I, *RSa*I, and *HiNF*I, clustered the isolates into eight distinct groups at a similarity level of 80%. The employed methodology for distinguishing the isolates demonstrated a wide range of distinct isolates, suggesting the presence of several strains, subgroups, or even distinct species in orange juice. Furthermore, it is worth noting that the isolates capable of producing guaiacol could lead to the spoilage of the juice under favorable conditions. The presence of *Alicyclobacillus*, therefore, has emerged as a serious concern for fruit juice manufacturing. Additionally, since *Alicyclobacillus acidoterrestris* is considered as the primary spoiler because of guaiacol production, it is critical to determine whether the isolates pertain to this species.

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Author Contributions

Conceptualization & Methodology: AID; Validation & Formal analysis AID; Investigation: PS, AD & AID; Writing - Original Draft: PS; Writing - Review & Editing: AID, PS, AD, JB-K, G-JEN &CT; Visualization: Investigation: PS & AID; Supervision & Project administration: AID & CT; Funding acquisition: CT & JB-K.

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Supplementary

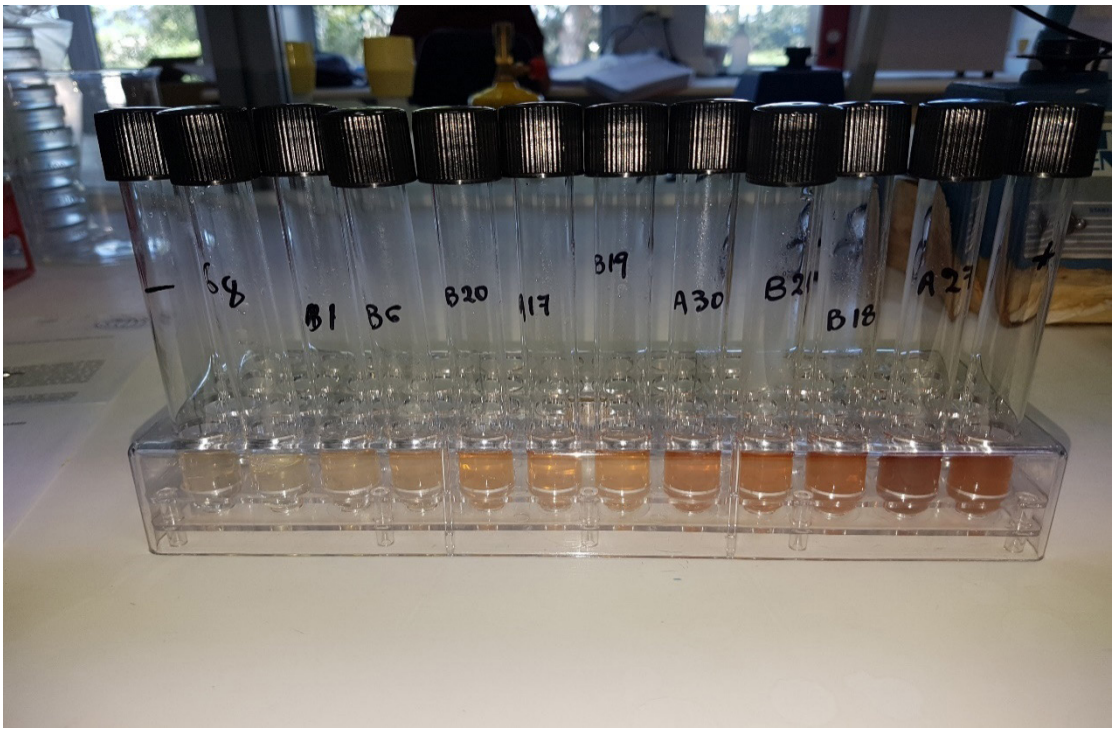

Figure S1. Color gradient of *Alicyclobacillus* isolates after incubation for the detection of guaiacol production with Dohler DMD kit.

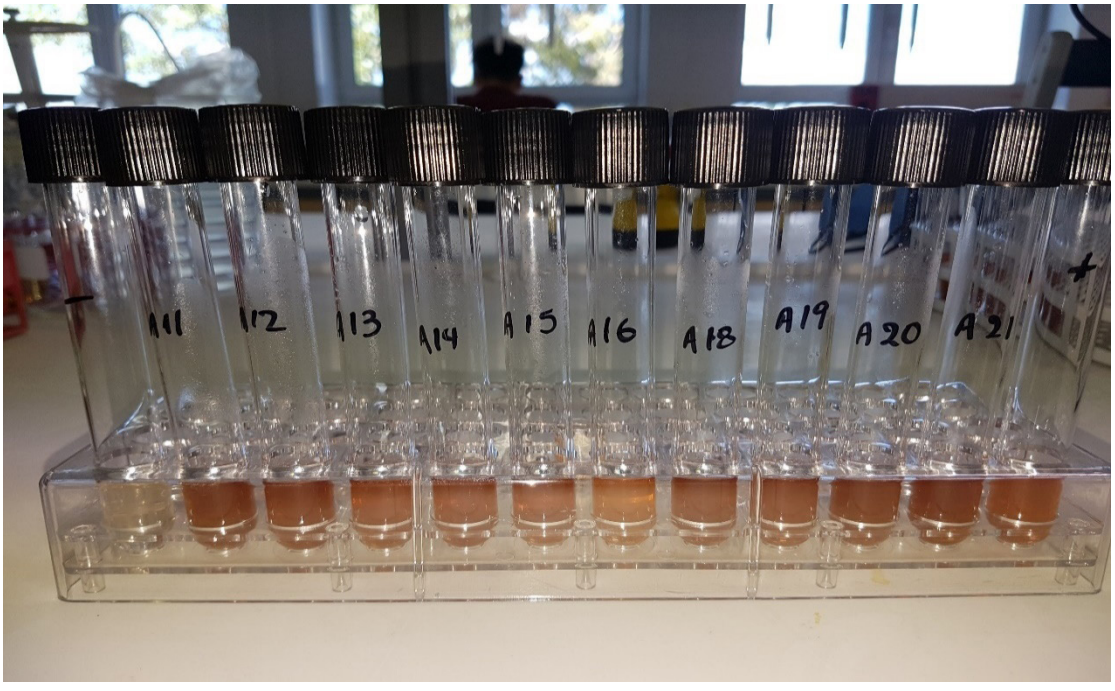

Figure S2. Representative isolates of *Alicyclobacillus* for the detection of guaiacol production with the Dohler DMD kit.

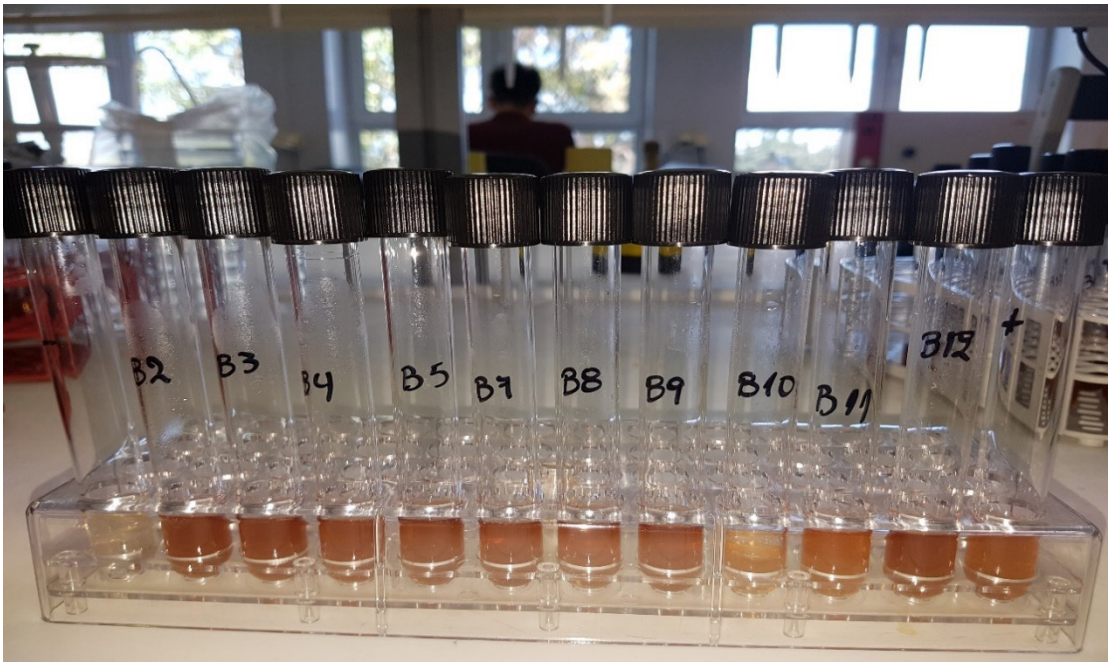

Figure S3. Representative isolates of *Alicyclobacillus* for the detection of guaiacol production with the Dohler DMD kit.