MICA Advance

Alicyclobacillus


# SCIENTIFIC REPORT

DIAMIDEX

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# I. Introduction

MICA Advance *Alicyclobacillus* is a simple and rapid solution that exclusively detects culturable *Alicyclobacillus* guaiacol-positive bacteria, according to the recommendation of IFU standard method no. 12:2019.

MICA Advance *Alicyclobacillus* allows the analysis of raw materials (purees and concentrates) and finished products from the beverage industry within 24 hours. It allows the exclusive detection of guaiacol positive *Alicyclobacillus* microcolonies.

MICA Advance *Alicyclobacillus* enumerates all *Alicyclobacillus* strains positive in the guaiacol test in colony-forming units (CFU), with a LOD of 1 CFU/membrane:

***A. acidoterrestris***
***A. acidiphilus***
***A. herbarius***
***A. hesperidum***
***A. suci***

# II. Principle and procedure

## a. Principle

The principle of the MICA Advance *Alicyclobacillus* solution procedure is the same as that of the IFU standard method no. 12:2019 procedure B (by membrane filtration) for filterable samples and procedure C for non-filterable samples with the main difference that the incubation times are much shorter: 24 hours of incubation instead of 5 days for filterable matrices and 24 hours of enrichment + 24 hours of incubation instead of 5 days + 5 days for IFU MM12.

The detection of guaiacol positive *Alicyclobacillus* using MICA Advance *Alicyclobacillus* does not require either a confirmation step or the use of a guaiacol kit as recommended in IFU MM12.

## b. Procedure for filterable or non-filterable matrices

Two possible protocols depending on the type of matrix (filterable or non-filterable):

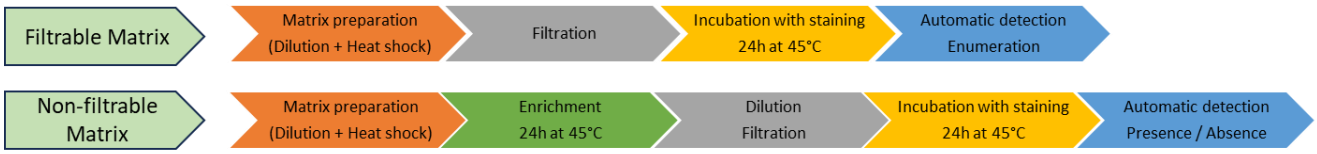



Fig 1: Process for filterable and non-filterable matrices

A matrix is said to be filterable when the entire 1/10 dilution of this matrix (10 ml + 90 ml of culture medium) can be filtered on a membrane with 0,45 µm pore size. Otherwise, the matrix is said to be non-filterable.

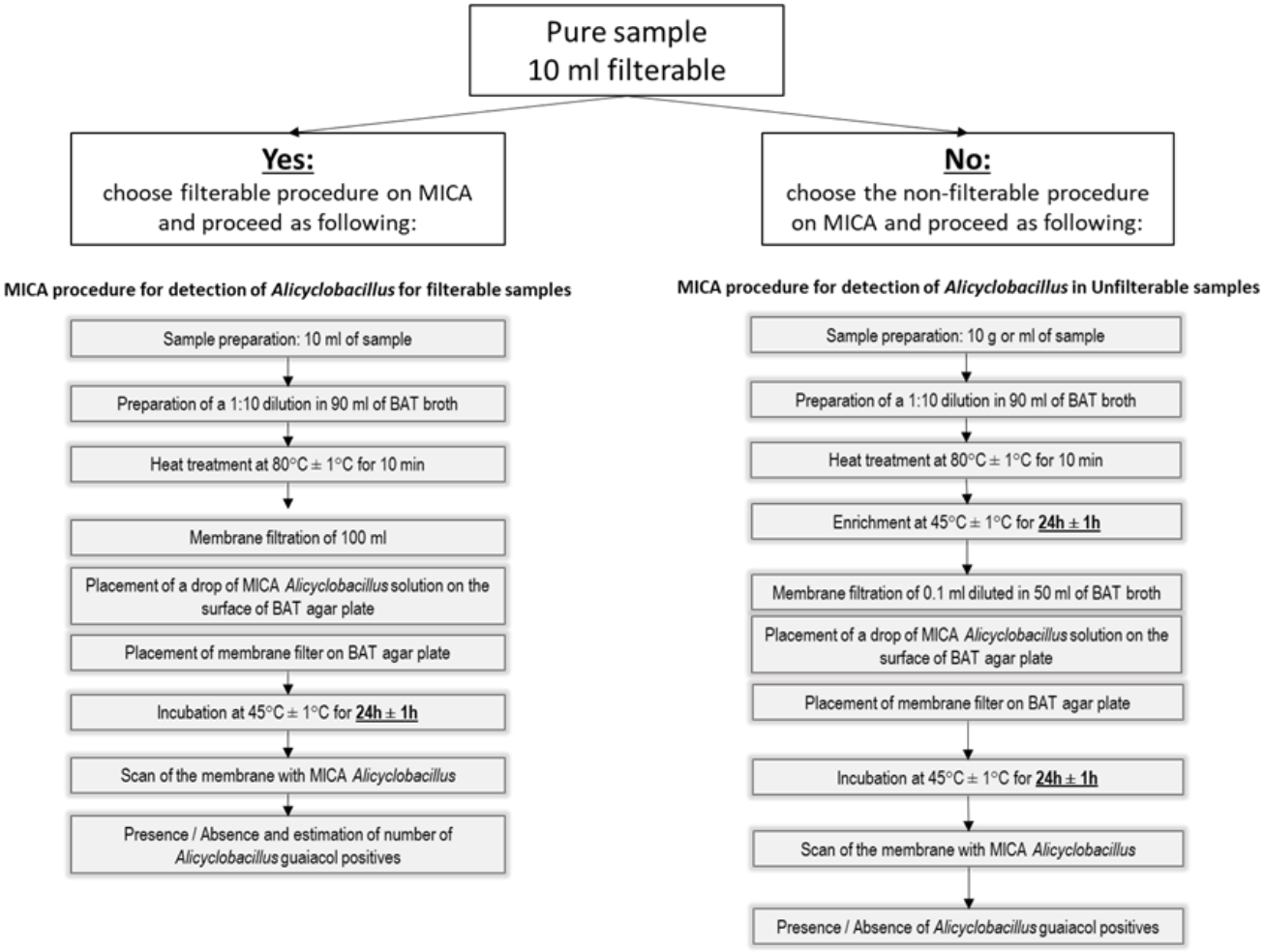



Fig 2: MICA Advance *Alicyclobacillus* detailed procedures for filterable and non-filterable matrices

# III. Performances

## a. Inclusivity / Exclusivity

The majority of *Alicyclobacillus* strains existing in biobanks around the world have been collected and integrated into the Diamidex collection. They come from the collection of microorganisms and cell cultures of the Leibniz Institute in Germany (DSMZ), the Biological Resources Center of the Pasteur Institute in France (CRBIP), the NITE Biological Resources Center in Japan (NRBC) and from the American Type Culture Collection in the United States (ATCC). Many strains have also been isolated

from real matrices from customers or prospects around the world. These strains were all evaluated according to the method developed by Diamidex and the results compared to those obtained with the regulatory method (IFU MM12 by membrane filtration).

### Inclusivity

30 different *Alicyclobacillus* guaiacol positive strains were tested, 23 were collection strains and 7 were environmental strains.

All of those strains previously evaluated as producing guaiacol were diluted in 0.9% NaCl, filtered and incubated on BAT agar for 24 hours for MICA Advance *Alicyclobacillus* according to the procedure for filterable samples, and 5 days for the reference method.

| | Number of tested strains | Origin | MICA *Alicyclobacillus* results in 24h | IFU MM12 results in 8 days* |
|---|---|---|---|---|
| *Alicyclobacillus* guaiacol positive strains | 30 | ***A. acidoterrestris*** DSM 3922, DSM 3923, DSM 3924, DSM 2498, NBRC 106287, NBRC 106288, NBRC 106289, NBRC 106290, NBRC 106291, NBRC 106292, NBRC 106293, NBRC 106294, NBRC 106295, NBRC 106296, NBRC 106297, NBRC 106298, NBRC 106299, NBRC 106300, NBRC 106301, NBRC 106302<br>***A. acidoterrestris* environmental strains** x7<br>***A. acidiphilus*** DSM 14558<br>***A. herbarius*** DSM 13609<br>***A. suci*** DSM 112017 | Positive | Positive |

Fig 3: Results comparison between MICA Advance *Alicyclobacillus* and IFU MM12 for Alicyclobacillus guaiacol positive strains

* 5 days of incubation + 3 days for confirmation and guaiacol test

**Conclusion: For MICA Advance Alicyclobacillus, all of the Alicyclobacillus guaiacol positive strains tested are detected in 24 hours.**

### Exclusivity

59 different strains of *Alicyclobacillus* guaiacol negative strains were tested, 7 were collection strains and 52 were environmental strains. 16 different non Alicyclobacillus strains were tested, 11 are bacteria, 4 are yeasts and 1 is a mold.

*Alicyclobacillus* guaiacol negative strains and non-*Alicyclobacillus* strains were diluted in 0.9% NaCl, filtered and incubated on BAT agar plate for 24 hours for MICA Advance *Alicyclobacillus*, and 5 days for the reference method.

| | Number of tested strains | Origin | MICA *Alicyclobacillus* results in 24h | IFU MM12 results in 8 days* |
|---|---|---|---|---|
| *Alicyclobacillus* guaiacol negative strains | 59 | ***A. acidocaldarius*** DSM 446, DSM 448, DSM 449, DSM 455, NRBC 106287<br>***A. hesperidum*** DSM 12766<br>***A. contaminans*** DSM 17975<br>***A. spp.* environmental strains** x37<br>***A. acidocaldarius*** x9<br>***A. cycloheptanicus*** x2<br>***A. pomorum***<br>***A. hesperidum***<br>***A. acidiphilus***<br>***A. sendaiensis*** | Negative | Negative |

Fig 4 : Results comparison between MICA Advance *Alicyclobacillus* and IFU MM12 for Alicyclobacillus guaiacol negative strains

| | Number of tested strains | Origin | MICA *Alicyclobacillus* results in 24h | IFU MM12 results in 8 days* |
|---|---|---|---|---|
| Non-*Alicyclobacillus* strains | 16 | ***Acetobacter aceti*** ATCC 15973<br>***Aspergillus brasiliensis*** ATCC 16404<br>***Bacillus coagulans*** ATCC 7050<br>***Bacillus subtilis*** ATCC 6633<br>***Candida albicans*** ATCC 10231<br>***Candida krusei*** ATCC 14243<br>***Gluconobacter liquefaciens*** ATCC 14835<br>***Gluconobacter oxydans*** ATCC 19357<br>***Lactobacillus casei*** ATCC 393<br>***Lactobacillus plantarum*** ATCC 8014<br>***Listeria monocytogenes*** ATCC 35152<br>***Micrococcus luteus*** ATCC 10240<br>***Saccharomyces cerevisiae*** ATCC 9763<br>***Salmonella typhimurium*** ATCC 13311<br>***Staphylococcus aureus*** ATCC 6538<br>***Zygosaccharomyces bailii*** DSM 70492 | Negative | Negative |

Fig 5: Results comparison between MICA Advance *Alicyclobacillus* and IFU MM12 for non-Alicyclobacillus strains

* 5 days of incubation + 3 days for confirmation and guaiacol test

**Conclusion: For MICA Advance Alicyclobacillus, none of the exclusivity strains tested are detected in 24 hours.**

## b. Relative limit of detection: RLOD

The relative detection limit (RLOD) of the MICA Advance *Alicyclobacillus* solution was conducted according to ISO 16140 2:2016 on *A. acidoterrestris* ATCC 49025.

The relative detection level of our alternative method is the detection level at P = 0.50 (LOD50) of the alternative method (MICA Advance *Alicyclobacillus*) divided by the relative detection level at P = 0.50 (LOD50) of the reference method (IFU). Microbial Method 12).

The RLOD calculations were carried out using an Excel spreadsheet according to the international standard (ISO 16140), as described in ISO 16140-2.

The procedure was applied to 5 bacteria-free membranes, 5 membranes with a low charge (0.8 cfu / ml) and 5 membranes with a higher charge (5 cfu / ml). The same experimental conditions were carried out by applying the MM12 method for a period of 5 days. These membranes were regrown to verify after 7 days of incubation that the results were consistent with the MM12 procedure.

| Spiking level | Samples Analysed | MICA *Alicyclobacillus* positive results | IFU MM12 positive results | RLOD IFU MM12 / MICA *Alicyclobacillus* | RLOD MICA *Alicyclobacillus* / IFU MM12 |
|---|---|---|---|---|---|
| 0 | 5 | 0 | 0 | 1 | 1 |
| Low Level (0.8 cfu / ml) | 5 | 5 | 5 | | |
| High Level (5 cfu / m) | 5 | 5 | 5 | | |

Fig 6: RLOD results comparison between MICA Advance *Alicyclobacillus* and IFU MM12

It is possible to detect microcolonies of *Alicyclobacillus* even when they are present in very small quantities with an RLOD of 1. In addition, no object is detected in the absence of *Alicyclobacillus* on the membrane. The average counts of the replicates obtained are, for their part, very close to those obtained by applying the timings of the IFU MM12 method.

**Conclusion: The RLOD of MICA Advance *Alicyclobacillus* is 1 cfu / ml.**

## c. Relative limit of detection: RLOD on non-filterable matrices

The procedure was applied to 5 bacteria-free membranes and 5 membranes with a charge of 5 cfu / ml and spiked on some non-filterable matrices (orange and peach concentrates, tomato puree and mango juice). The same experimental conditions were carried out by applying the MM12 method for a period of 5 days. These membranes were regrown to verify after 7 days of incubation that the results were consistent with the MM12 procedure.

| Matrix | Spiking level before enrichment | MICA Alicyclobacillus results | IFU MM No. 12 results | RLOD MICA Alicyclobacillus / IFU MM No. 12 |
|---|---|---|---|---|
| Orange concentrate | 0 | Negative | Negative | 1 |
| | 5 cfu / ml | Positive | Positive | |
| Peach concentrate | 0 | Negative | Negative | |
| | 5 cfu / ml | Positive | Positive | |
| Tomato puree | 0 | Negative | Negative | |
| | 5 cfu / ml | Positive | Positive | |
| Mango juice | 0 | Negative | Negative | |
| | 5 cfu / ml | Positive | Positive | |

Fig 7 : RLOD results comparison between MICA Advance *Alicyclobacillus* and IFU MM12 on non-filterable matrices

**Conclusion: The RLOD of MICA Advance *Alicyclobacillus* on non-filterable matrices is 5 cfu / ml.**

## d. Linearity

Different quantities of *Alicyclobacillus acidoterrestris* were analyzed to determine over what range of values the method can be considered linear by algorithm enumeration. The analyzes were carried out according to the MICA Advance Alicyclobacillus procedure for filterable matrices and the results obtained by the algorithm were compared to those obtained by counting by eye according to the IFU MM12 reference method. 3 membranes were scanned for each concentration.

| Concentration range re-evaluated | Mean values by algorithm | Mean values by eye counting | % algorithm/by-eye counting deviation |
|---|---|---|---|
| **0** | 0 | 0 | 0% |
| **68** | 89 | 77 | 13% |
| **136** | 161 | 132 | 18% |
| **204** | 217 | 196 | 10% |
| **340** | 343 | 315 | 8% |
| **708** | 559 | 708 | -26% |
| **1358** | 773 | 1358 | -75% |

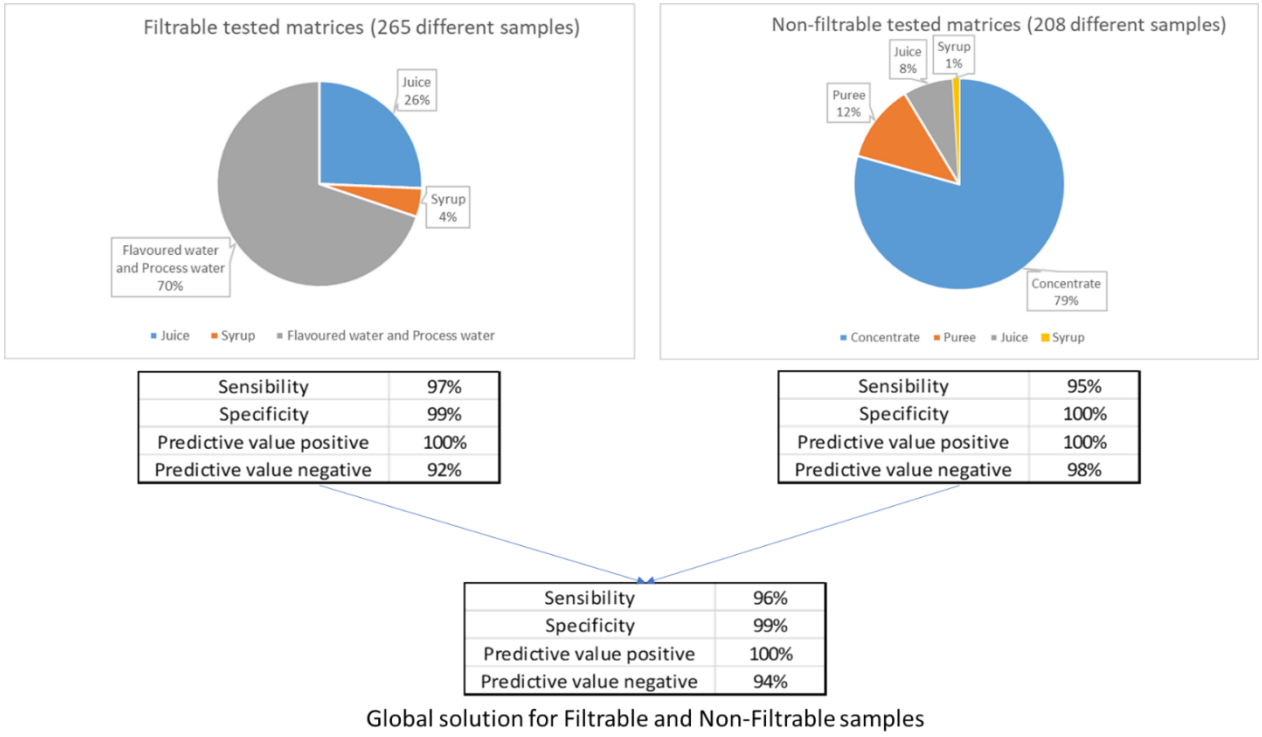
Fig 8 : Counting results by MICA Advance Alicyclobacillus algorithm compared with counting by eye according to IFU MM12

The enumeration, of the microcolonies present on the membrane, given by the algorithm developed for MICA Advance *Alicyclobacillus*, is linear until 340 colonies. Beyond this value, the algorithm greatly underestimates the number of objects present on the membrane. The upper limit up to which reliable linearity is guaranteed with the algorithm in its current version has been set at 250 objects per membrane. For higher counts than the established limit of 250 objects per membrane, the algorithm will give a generic value greater than 250 regardless of the actual number of objects present.

**Conclusion: MICA Advance *Alicyclobacillus* solution is linear from 1 to 250 cfu / membrane**

# IV. Matrices

## a. Listing of filterable and non-filterable matrices tested




| | |
|---|---|
| Sensibility | 97% |
| Specificity | 99% |
| Predictive value positive | 100% |
| Predictive value negative | 92% |

| | |
|---|---|
| Sensibility | 95% |
| Specificity | 100% |
| Predictive value positive | 100% |
| Predictive value negative | 98% |

| | |
|---|---|
| Sensibility | 96% |
| Specificity | 99% |
| Predictive value positive | 100% |
| Predictive value negative | 94% |

Global solution for Filtrable and Non-Filtrable samples

Fig 9: Number and type of tested matrices and MICA Advance *Alicyclobacillus* specifications

**Conclusion: The specifications of the MICA Advance *Alicyclobacillus* solution, whether for filterable matrices or for non-filterable matrices, are compatible with the requirements of the fruit juice industry and more generally beverages.**

## b. Enumeration results for filterable samples

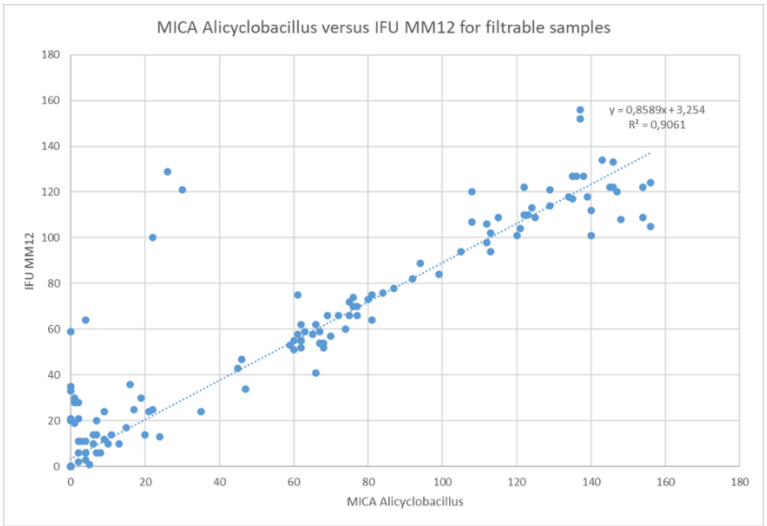



Fig 10 : Comparison of enumeration results for filterable samples between MICA Advance *Alicyclobacillus* and IFU MM12

Knowing that the IFU MM12 standard indicates that plate counting is reliable up to a load of 150 objects per membrane, we have voluntarily established method comparisons only up to this threshold of 150 objects.

**Conclusion: Enumeration results for MICA Advance *Alicyclobacillus* are comparable to those obtained with IFU MM12 standard method.**

## V. Inhibitory matrices

Studies undertaken by Diamidex on different matrices demonstrated that certain juices and experimental conditions led to an inhibition of *Alicyclobacillus* growth.

Some scientific publications deal with the subject of natural inhibitors of *Alicyclobacillus.* Some studies reported the use of natural components for juice preservation.

Yokota et al. (1) reported that *Alicyclobacillus* species were not able to grow in red grape juice due to the presence of polyphenols such as trans-resveratrol or ferulic acid.

Brodbeck et al. (2) showed the influence of polyphenols content on growth of *A. acidoterrestris* in iced tea. The phenol content in iced tea made from infusions of green and black tea had inhibitory influence on growth of *A. acidoterrestris* and iced tea made from soluble extracts with lower levels of phenols did not possess antimicrobial properties.

Dos Anjos et al (3), showed that the enzymes papain and bromelain, derived from papaya and pineapple, respectively, have an antibacterial effect on *A. acidoterrestris*.

On another side, Kapetanakou et al (4) reported that certain parameters such as water activity or the percentage of Brix in a matrix can have an impact on the growth and viability of *Alicyclobacillus* strains.

We also encountered growth delays of *Alicyclobacillus acidoterrestris* in certain matrices such as banana puree or lemon concentrate which have not yet been documented in the scientific literature.

(1) Yokota et al. Alicyclobacillus: Thermophilic Acidophilic Bacilli. (2007) Ed. Springer, p. 2, p. 24 – 35 & p. 117.

(2) Brodbeck et al. Influence of processing related phenol components on growth of Alicyclobacillus acidoterrestris in iced tea. Flüssiges Obst, 2009.

(3) Dos Anjos et al, Antibacterial activity of papain and bromelain on Alicyclobacillus spp. Int J Food Microbiol. 2016 Jan 4:216:121-6. doi: 10.1016/j.ijfoodmicro.2015.10.007. Epub 2015 Oct 8.

(4) Kapetanakou et al, Assessment of Spoilage Potential Posed by Alicyclobacillus spp. in Plant-Based Dairy Beverages Mixed with Fruit Juices during Storage. J Food Prot. 2021 Mar 1;84(3):497-508. doi: 10.4315/JFP-20-298.

## a. Procedure adaptations and example of LOD for inhibitory matrices

Diamidex has therefore adapted its procedure to allow the evaluation of the presence of positive *Alicyclobacillus* guaiacol in some inhibitor matrix.

Two approaches were investigated to permit growth and detection of *Alicyclobacillus*.

A higher dilution of the sample to dilute the inhibitory compounds present natively in these matrices. For some matrices, a 1/50 dilution instead of 1/10 dilution is compatible with a growth of *Alicyclobacillus*. For example, an extended dilution of 1/50 of lemon concentrate permits to detect *Alicyclobacillus* with an initial load before enrichment of 2000 bacteria per milliliter. In the same way, this higher dilution of 1/50 of pineapple concentrate allows the detection of 20 bacteria per milliliter of sample before enrichment.

The second approach consists to an increase in the incubation time during the enrichment phase for non-filterable matrices. For example, 48h of enrichment (instead of 24h for non-inhibitory matrices) permits to detect *Alicyclobacillus* with an initial load before enrichment of 20 bacteria per milliliter in pineapple concentrate.

The 2 approaches show improvements in the reliability of the results for the inhibitory matrices. However, there is no standard procedure to adopt for all these matrices. Therefore, each matrix or environment that can cause inhibition (low water activity, high Brix degree) must be tested to evaluate the most suitable procedure.

**Conclusion: MICA Advance *Alicyclobacillus* solution is made to be adaptable to all type of fruit-based matrices included inhibitory matrices and inhibitory environments.**

# MICA Advance

## Alicyclobacillus


Technical support

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