# Validation of the TAAG SP Spoilage kit line in food samples

**Product spoilage has been a problem in the food industry for decades. For fast identification of multiple food spoilage microorganisms, we have developed a Multiplex PCR kit line called TAAG SP Spoilage. These kits are based on a multiplex PCR reaction coupled with advanced artificial intelligence analysis for the detection and identification of over 100 microbial species. In this study we describe the analytical performance of the TAAG SP Spoilage kits in food susceptible to spoil, such as beverages, sauces and milk. Test accuracy and microbial growth were determined using a panel of 106 representative microorganisms. The test showed 100% agreement with all inoculated microorganisms.**

More than 100 microorganisms are known to spoil finished food. Early detection of potential spoilage microorganisms in finished product is key to prevent releasing contaminated food. However, and most importantly, fast identification of the potential spoiler is critical to implement the most appropriate corrective action. Additionally, the identification of the spoiler will allow to do traceability and identification of the root cause of contamination.

However, and since there are around one hundred potential spoilage microorganisms, early detection and identification remains challenging.

Approaches based on next generation sequencing have recently been shown to enable identification of hundreds of microorganisms, but these tests are still too expensive, slow and complex to be useful in the food industry. The ability to comprehensively identify microorganisms causing spoilage in food products remains an unmet food quality need.

Recently, we have developed and validated a new line of kits, called TAAG SP Spoilage. These kits allows detection and identification of multiple spoilage microorganisms in one single PCR reaction coupled with advanced artificial intelligence algorithms for data analysis. This new approach allows the detection and identification of over 50 bacteria and 50 yeast & molds from food samples (click in the following link to see the microorganisms list https://taag-genetics.com/steriplex-mo.php).

Here we describe the analytical validation of two kits, TAAG SP Spoilage Bacteria and TAAG SP Spoilage Yeast & Molds.

## Results

The TAAG SP Spoilage kits are based on a multiplex PCR reaction that allows the simultaneous detection and identification of over 100 spoilage microorganisms.

The TAAG SP Spoilage Bacteria kit detects and identifies over 50 spoilage bacteria, whereas TAAG SP Spoilage Yeast & Molds kit detects and identifies over 50 spoilage yeasts and molds.

Both kits are based on the same protocol from enrichment to results. An overview of this workflow is shown in Fig. 1.

**Analytical validation strategy on 5 finished products.** A reference panel of 54 bacteria (Table 1) and 52 yeast and molds (Table 2) was used to characterize the test performance across 5 different finished products: non-carbonated water, carbonated soft drink, orange juice, ketchup and milk.

The reference panel was selected because, during TAAG's biomapping service, those microorganisms are the most frequently isolated from beverage, dairy and sauces plants.

Prior to inoculation, each reference microbe was adapted to similar conditions found in the final products. Afterwards, 100 µL of a low concentration of each microorganism (<5CFU/100 µL) was inoculated into 10 mL of the final product.

**Microbial growth characterization.** Samples inoculated with bacteria were enriched using the TAAG E12 Universal Bacteria media for 24 hours at 35ºC. Samples spiked with yeast and molds were incubated with TAAG E21 Universal Yeast and Mold for 48 hours at 28ºC. After enrichment, all samples were plated and the microorganisms were quantified using traditional methods.

A summary of the results is shown in Fig. 2. These results demonstrate that the TAAG specific media allows good growth of all bacteria, yeast and molds evaluated.

**Analytical performance of TAAG SP Spoilage kits on 5 finished products.** To assess the analytical performance of the TAAG SP Spoilage kits for detection and identification of the reference panels, we compared the results obtained by TAAG SP Spoilage kits with the expected results according to each inoculum.

DNA extraction, using the TAAG X21 TezBac kit, was performed from the enriched samples. Afterwards, PCR amplification and data analysis was done using the TAAG SP Spoilage Bacteria kit and TAAG SP Spoilage Yeast & Molds kit.

The results obtained confirmed that 100% of the samples were correctly detected and 96.2% of bacteria and 95.8% of yeasts and molds were correctly associated to their corresponding microbial group.

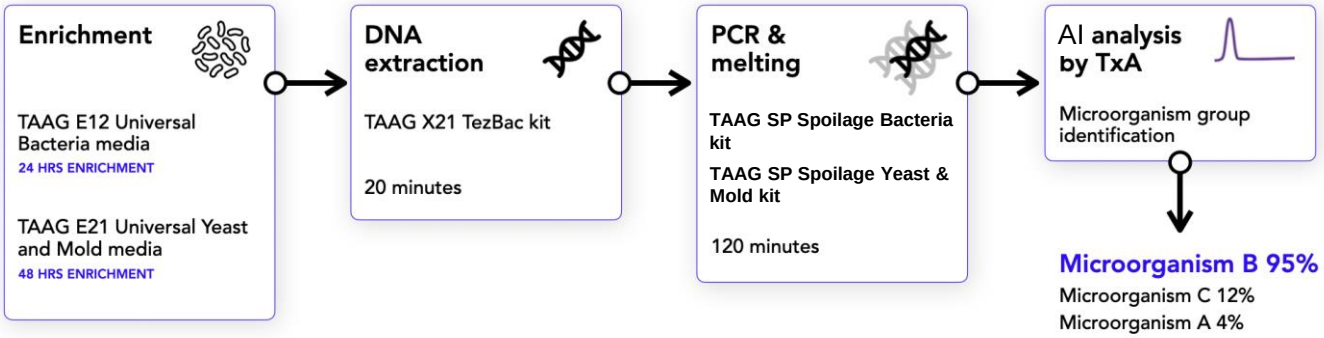


**Fig. 1 | TAAG SP Spoilage workflow.** Depending on the analysis, the food samples are enriched for bacteria or yeasts and molds. Afterwards, DNA is extracted, the corresponding TAAG SP Spoilage kit is used for DNA amplification and the melting curve analysis is performed. For automated data analysis and results, the TAAG Xpert Assistant software (TxA) is used. The TxA incorporates an advanced artificial intelligence algorithm that analyzes the melting curve pattern and links it to a specific microorganism.

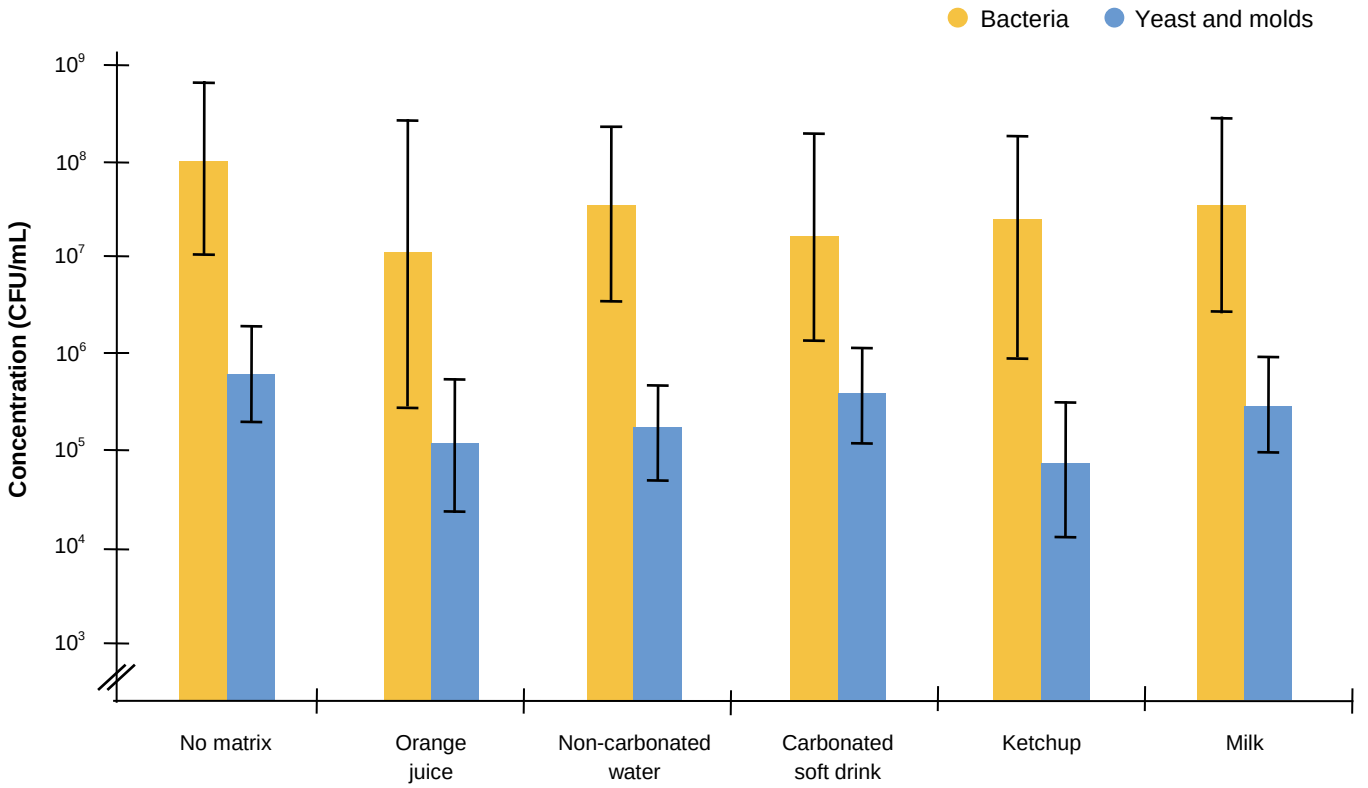


**Fig. 2 | Count of microorganisms after enrichment.** The mean ± s.d. concentration from 54 bacteria and 52 yeasts and molds, inoculated in the finished products is shown. The microorganisms were inoculated in 3 replicate. No matrix (i.e. no orange juice, etc.) represents each microorganism enriched only in its specific enrichment media. Bacteria were incubated using the TAAG E12 Universal Bacteria media by 24 hours at 35ºC. Yeast and molds were incubated using the TAAG E21 Universal Yeast and Mold media by 48 hours at 28ºC.

**Table 1 |** Bacteria inoculated into six finished products

| | | | |
|---|---|---|---|
| 1 | *Acetobacter aceti* | 28 | *Lactobacillus fermentum* |
| 2 | *Aeromonas hydrophila* | 29 | *Lactobacillus helveticus* |
| 3 | *Alicyclobacillus acidoterrestris* | 30 | *Lactobacillus parabuchneri* |
| 4 | *Asaia bogorensis* | 31 | *Lactobacillus paracasei* |
| 5 | *Asaia lannensis* | 32 | *Lactobacillus plantarum* |
| 6 | *Bacillus albus* | 33 | *Lactobacillus rhamnosus* |
| 7 | *Bacillus altitudinis* | 34 | *Lactococcus lactis* |
| 8 | *Bacillus amyloliquefaciens* | 35 | *Leuconostoc citreum* |
| 9 | *Bacillus cereus* | 36 | *Paenibacillus humicus* |
| 10 | *Bacillus coagulans* | 37 | *Paenibacillus macerans* |
| 11 | *Bacillus licheniformis* | 38 | *Paenibacillus motobuensis* |
| 12 | *Bacillus megaterium* | 39 | *Paenibacillus xylanilyticus* |
| 13 | *Bacillus pumilus* | 40 | *Pseudomonas aeruginosa* |
| 14 | *Bacillus subtilis* | 41 | *Pseudomonas alcaligenes* |
| 15 | *Blastomonas natatoria* | 42 | *Pseudomonas mendocina* |
| 16 | *Brochothrix thermosphacta* | 43 | *Pseudomonas plecoglossicida* |
| 17 | *Caulobacter vibrioides* | 44 | *Pseudomonas putida* |
| 18 | *Clostridium perfringens* | 45 | *Salmonella typhimurium* |
| 19 | *Delftia acidovorans* | 46 | *Serratia liquefaciens* |
| 20 | *Enterobacter cloacae* | 47 | *Serratia marcescens* |
| 21 | *Enterococcus faecium* | 48 | *Shewanella baltica* |
| 22 | *Enterococcus hirae* | 49 | *Sphingobium yanoikuyae* |
| 23 | *Escherichia coli* | 50 | *Sphingopyxis terrae* |
| 24 | *Hydrogenophaga pseudoflava* | 51 | *Staphylococcus epidermidis* |
| 25 | *Klebsiella oxytoca* | 52 | *Staphylococcus hominis* |
| 26 | *Lactobacillus alimentarius* | 53 | *Streptococcus pyogenes* |
| 27 | *Lactobacillus brevis* | 54 | *Weissella cibaria* |

**Table 2 |** Yeast and molds inoculated into six finished products

| | | | |
|---|---|---|---|
| 1 | *Alternaria alternata* | 27 | *Lachancea dasiensis* |
| 2 | *Aspergillus niger* | 28 | *Lodderomyces elongisporus* |
| 3 | *Aspergillus versicolor* | 29 | *Penicillium citrinum* |
| 4 | *Barnettozyma californica* | 30 | *Penicillium glabrum* |
| 5 | *Brettanomyces anomalus* | 31 | *Penicillium sp* |
| 6 | *Brettanomyces bruxellensis* | 32 | *Pichia cactophila* |
| 7 | *Candida boidinii* | 33 | *Pichia kudriavzevii* |
| 8 | *Candida devenportii* | 34 | *Rhodosporidiobolus nylandii* |
| 9 | *Candida lactis-condensi* | 35 | *Rhodotorula glutinis* |
| 10 | *Candida magnoliae* | 36 | *Rhodotorula mucilaginosa* |
| 11 | *Candida parapsilosis* | 37 | *Saccharomyces bayanus* |
| 12 | *Candida sojae* | 38 | *Saccharomyces cerevisiae* |
| 13 | *Candida sp* | 39 | *Saccharomyces ludwigii* |
| 14 | *Candida temnochilae* | 40 | *Saccharomyces pastorianus* |
| 15 | *Cladosporium cladosporioides* | 41 | *Talaromyces funiculosus* |
| 16 | *Cladosporium sp* | 42 | *Talaromyces minioluteus* |
| 17 | *Cutaneotrichosporon dermatis* | 43 | *Talaromyces sp* |
| 18 | *Dekkera naardenensis* | 44 | *Trichoderma atroviride* |
| 19 | *Didymella sp* | 45 | *Trichoderma reesei* |
| 20 | *Exophiala oligosperma* | 46 | *Vishniacozyma sp* |
| 21 | *Exophiala sp* | 47 | *Wickerhamomyces anomalus* |
| 22 | *Fusarium equiseti* | 48 | *Zygoascus hellenicus* |
| 23 | *Fusarium oxysporum* | 49 | *Zygosaccharomyces bailii* |
| 24 | *Fusarium solani* | 50 | *Zygosaccharomyces bisporus* |
| 25 | *Geotrichum candidum* | 51 | *Zygosaccharomyces parabailii* |
| 26 | *Kluyveromyces marxianus* | 52 | *Zygosaccharomyces rouxii* |

## Discussion

The results presented here show that both TAAG SP Spoilage kits offer a fast and reliable detection and identification method for a wide variety of microorganisms, which could potentially spoil finished food products.

The results obtained confirmed that 100% of the samples were correctly detected and 96.2% of bacteria and 95.8% of yeasts and molds were correctly associated to their corresponding microbial group.

The inaccurate results were obtained because the melting pattern of theses specific samples, were slightly different from the correct pattern found in the database and therefore the software associated these patterns to a different microbial group. In most cases, the inexact microbial group was phylogenetically similar to the correct microbial group.

The 4% of imprecise microbial identification is presumed to originate from inaccurate aliquoting after enrichment, which originated a much lower amount of microorganisms before DNA extraction, which finally generated a slightly different melting curve pattern. This is the most plausible explanation because the replicates of the same misidentified samples were correctly identified.

We did not observe differences in analytical performance among all 106 microorganisms tested. Even though some microorganisms grew significantly slower than others, all were easily identified. Additionally, the different finished products had no effects on the sensitivity and precision of the method.

The main advantages of implementing TAAG SP Spoilage kits are:

1. **Fast results.** Instead of traditional 3-5 days results, these tests yield results in around 28 hours for bacteria and 52 hours for yeast and molds.
2. **Right corrective actions**. As the microorganism is identified, it is now possible to have different actions according to the spoilage capacity of the microbial.
3. **Traceability**. Once the microorganism is identified, the root cause of the contamination can be tracked down.

## Methods

**Inoculation and enrichment.** *Microorganisms.* The 106 microbial of the reference panels were obtained from either ATCC, DSMZ, finished products or environmental swabbing from different food plants.

Before starting this validation, all strains were refreshed in non-selective growth media and then isolated on agar medium. From each plate, 10 colonies were DNA sequenced in order to confirm the strain and the purity of the cultures.

*Stabilization and inoculation of strains.* Before inoculation in the final products, all strains were stabilized for 72 hours at 4ºC inside the finished products. After the stabilization, all samples were diluted with non-inoculated matrix over 8 0.5-log serial dilutions *a-priori* ranging from 10.000 to 0 CFU/mL per microbe. Five replicates of each dilution were quantified by traditional plate culture methods. For the data analysis and following experiments only the positive dilutions with less than 5 CFU/100 µL were considered. In parallel to traditional quantification, 100 µL of each sample was inoculated into 10 mL of the final product.

The samples spiked with bacteria were enriched for 24 hours at 35ºC using the TAAG E12 Universal Bacteria media and the samples inoculated with yeasts and molds were enriched for 48 hours at 28ºC using the TAAG E21 Universal Yeast and Mold media, both following the user manual instructions.

*Quantification of microbial growth in TAAG media.* After enrichment, 1 mL of each sample was plated. The samples containing bacteria were plated into plate count Agar (PCA) and incubated for 2 days at 35ºC. In the case of samples spiked with yeast and molds, after enrichment 1 mL of each sample was plated in Potato Dextrose Agar (PDA) and incubated for 5 days at 24ºC. Then, in both cases, the colonies were counted and recorded for data analysis.

**TAAG SP Spoilage kits.** *Microorganism database.* A complete list of the microorganisms identified is presented at https://taag-genetics.com/steriplex-mo.php). It is important to mention that this list is continuously updated since we are constantly isolating or acquiring new spoilage microorganisms.

A crucial part of the TAAG SP Spoilage workflow is the microorganisms pattern database. the specific pattern of each microorganism is obtained by melting curve analysis of its PCR products. Some examples of how these patterns look are shown in Fig. 3.

*DNA extraction and DNA amplification.* The DNA extraction and PCR were performed according to TAAG's user manual. 50 µL of the enriched sample is mixed with 450 µL of lysis buffer. This mix is incubated for 20 minutes at 95ºC and the DNA is ready for the next step.

Since the TAAG SP Spoilage kits consists of PCR plates with all PCR reagents already pre-loaded and ready to use, this step is extremely simple. 2 µL of each DNA was added to each well in the PCR plate, the PCR plate was loaded in an AriaMx Real-time PCR equipment (Agilent Technologies), and the PCR and melting protocol were set up according to the user manual.

*Data analysis and results.* For automated data analysis and results, the amplification files were uploaded to the TAAG Xpert Assistant web application (TxA). The artificial intelligence algorithm in the TxA accurately associates the melting curve data pattern with the corresponding microbial group and then it determines the probability of that specific pattern for each microorganism inside that specific group.

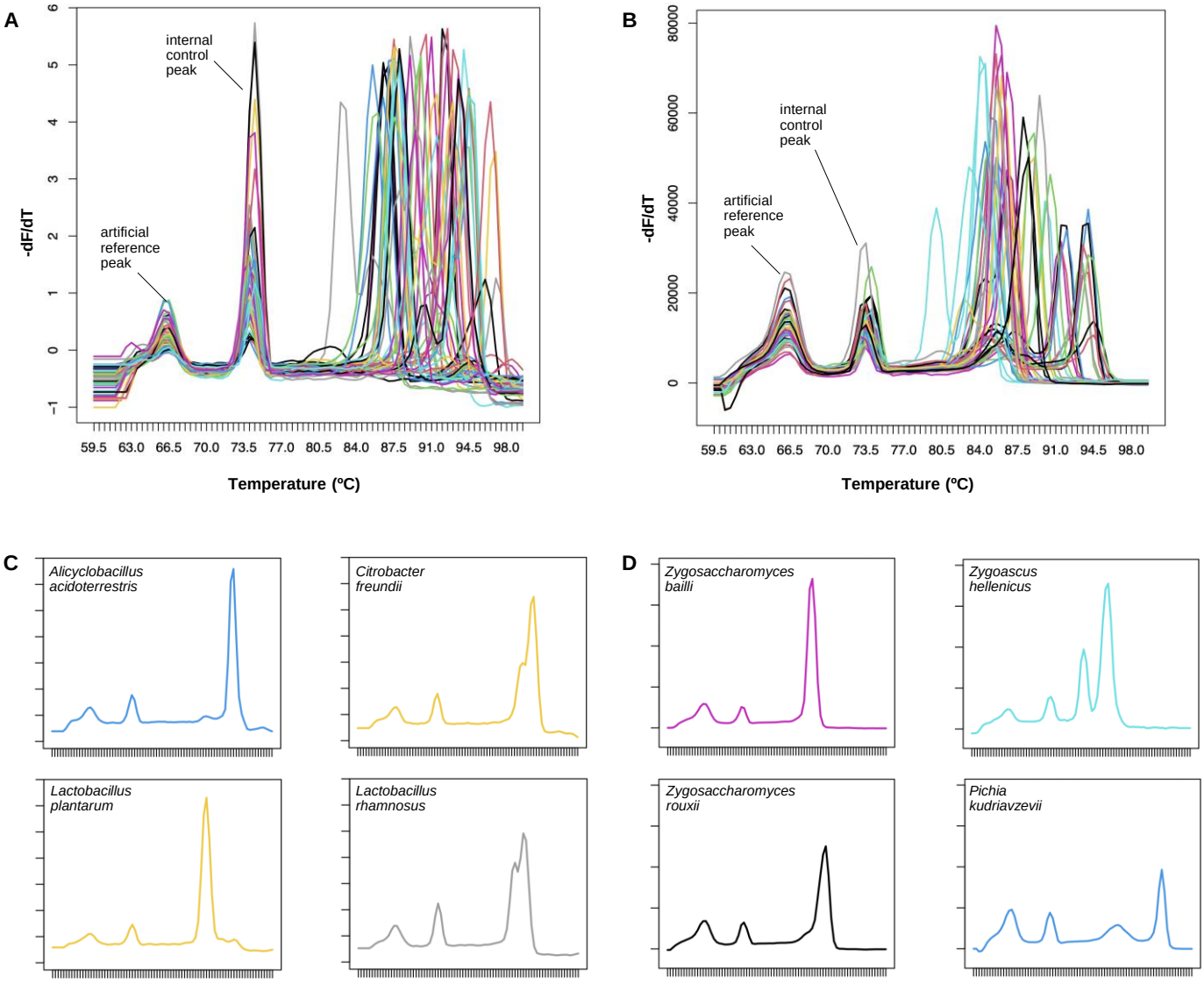


**Fig. 3 | Melting curve patterns analyzed by TxA.** Some examples of melting curve patterns obtained using the TAAG SP Spoilage kits. **A**, shows some patterns from bacteria and **B**, shows some pattern from yeast and molds. The PCR reaction includes an artificial DNA which generates a peak around 66ºC. This peak is used to set a reference inside the curve. Additionally, another artificial DNA is incorporated into the PCR reaction which works as an internal PCR control. This foreign DNA is co-amplified during DNA amplification, producing a peak around 74ºC. If the reaction was successful, this peak will appear. Otherwise, the PCR was not accomplished (i.e. the sample contains PCR inhibitors) and the TxA software will show a warning sign. **C-D**, Examples of four different melting curve patterns from bacteria and yeasts/molds, respectively.