Detection of Microbial Contamination in a Flavored, Soy-Based Meal Replacement Drink Using the Innovate™ System

Introduction

Aseptic processing is a widely used method in food and beverage applications. It utilizes ultra-high temperatures to maximize the sterility of long-life shelf-stable products. However, contamination can still occur during manufacturing and production.

To minimize risk, it is vital to test final product for microorganism contamination. The Innovate™ Rapid Microbial Screening System is designed for the rapid detection of microorganisms in a range of products. To detect very low levels of contaminants in these types of products, an enrichment step is required to ensure that there is sufficient ATP present for detection. Typically, a product is incubated in its own packaging to enrich the ATP from any contaminating microbial cells. Pre-established baselines obtained from uncontaminated product are used to determine positive results.

Objective

The goal of this study was to validate the Innovate System using the RapiScreen™ Dairy Kit for the detection of microorganism growth in a soy-based, flavored meal replacement drink to demonstrate equivalence to traditional plate techniques.

Equipment, Supplies and Reagents

- Sterile inoculating loops
- Sterile pipettes and tips
- Incubators capable of 35 ± 2°C
- Alcohol wipes
- RapiScreen Dairy Kit (includes reagents, polypropylene (PP) vials, microtiter plates)
- Sabouraud Dextrose Agar (SDA) Plates
- Potato Dextrose Broth (PDB)
- Tryptic Soy Agar (TSA) Plates
- Tryptic Soy Broth (TSB)
- pH meter and electrodes (i.e., Mettler-Toledo InLab® sensors)
- GasPak™ EZ Anaerobe Gas Generating Pouch System with Indicator
- Syringes, 1 mL and 3 mL
- Dulbecco's Phosphate Buffered Saline - DPBS (1X)
- Ringer's Solution
- Shoe Goo, Clear Shoe Repair and Protective Coating
- Precision Glide Needles, 16 gauge 1 ½"
- ATP Positive Control
- Innovate System instrument

Test Organisms and Products

- Microorganisms tested
  - *Cronobacter sakazakii*, ATCC# 29544
  - *Bacillus cereus* endospores, ATCC# 11778
  - *Clostridium sporogenes*, vegetative, ATCC# 7955 (PA 3679)
  - *Pseudomonas aeruginosa*, ATCC# 9027
  - *Pseudomonas putida*, ATCC# 49128
  - *Staphylococcus aureus*, ATCC# 6538
- Milk product types tested
  - Soy-based meal replacement drink, flavored

Methods

1. Culture preparation

For *Cronobacter sakazakii*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, Quanti-Cult Plus™ cell suspensions were purchased and used for testing. *Clostridium sporogenes*, vegetative and *Pseudomonas putida* were prepared using Culti-Loop® cultures (prepared by Thermo Fisher Scientific and provided by a third-party lab). Rehydration and inoculation of all microorganisms were completed according to the manufacturer's directions.

Pseudomonas putida and *Clostridium sporogenes* were prepared by inoculating an activated-charcoal Culti-Loop into 5 mL of TSB. The broths were then incubated at 37˚C for 24 hours. A ten-fold serial dilution set was then made using Ringer's Solution, and plate counts were prepared on TSA plates to find a concentration of <100 CFU. A GasPak system was used for *Clostridium sporogenes* vegetative plates for incubation to ensure growth. The actual inoculum for all microorganisms used during sample spiking was determined by plating each organism in duplicate onto TSA plates. The plates were incubated at 35 ± 2 °C and counted after 24 hours.

The *Bacillus cereus* endospore suspension was prepared by adding an EZ-Spore™ pellet to 10 mL of 1X DPBS to make a 10^3 CFU/mL concentration. This bacterial pellet suspension was incubated at 37 °C for 30 minutes and then mixed thoroughly to make a homogenous solution. The *B. cereus* suspension was then heated to 80 °C in a water bath for 30 minutes. The sample was serially diluted ten-fold in sterile 1X DPBS and plate counts were prepared on TSA plates to find a concentration of <100 CFU. The plate was incubated at 37 °C for 24 hours.

2. pH Assessments

Products assessed for pH were measured in triplicate to ensure the accuracy of measurements. The pH meter used for measurement of products was calibrated before use. This demonstrated that additional testing was not required to confirm that the RapiScreen Dairy Kit was capable of sufficiently neutralizing the products.

3. Background and Baseline RLU Determination

To determine ATP baseline levels, each product was initially incubated for 24 hours at 37 °C. Samples were mixed thoroughly and 20 mL of each product was transferred to a sterile container for pH and background/ baseline testing.

The background ATP level of each product was determined by running an assay using ATX buffer solution in place of reconstituted ATX reagent. The assay was then repeated using reconstituted ATX to allow for the depletion of the background ATP signal. These results are referred to as the Baseline RLU values. Baseline RLU values should be low and consistent, demonstrating that the background RLU signal has been fully depleted. A stable baseline RLU value allows for setting a threshold (positive/negative) cutoff value for identifying contaminated samples.

- Baseline Protocol: Dispense 60 µL ATX – 10 min shake – follow RapiScreen Dairy instructions for detection
- Background Protocol: Dispense 60 µL ATX buffer – 10 min shake – follow RapiScreen Dairy instructions for detection

Once baselines were established and cultures were prepared, each product type was inoculated in duplicate at <100 CFU per container. The microorganisms were spiked using a syringe through the top of the container and sealed with Shoe Goo glue. A non-inoculated container was incubated with each inoculated set as a negative control. Positive controls were set up by inoculating TSB or PDB with 100 µl of the <100 CFU culture.

Samples were incubated at 35 ± 2 °C for up to seven days. On days 1 - 7, aliquots were taken from each container and tested on the Innovate System using the RapiScreen Dairy Kit. In parallel, 50 µL of each product sample was inoculated onto TSA plates and incubated for 24 hours at 35 ± 2 °C for growth and morphology confirmation. GasPak EZ Anaerobe Gas Generating Pouches were used to incubate confirmation plates streaked with *C. sporogenes* to enable the organism to grow effectively if present. Growth seen on confirmation plates matched the morphology of the microorganism used to spike.

Results

For the initial, uninoculated product, testing demonstrated no unusual properties that may interfere with the study (see Table 1, a, b and c). The product pH was neutral as expected for these drinks and no growth was observed upon plating samples. In addition, the background was low (395) and consistent across 32 individual tests (range of 357 to 481 RLUs). When baseline testing was performed, the RLU results were even lower and again, consistent, with an average of 11 RLUs. As a result, the threshold was set at 32.

In the spiked product testing, *C. sakazakii, P. aeruginosa and P. putida* were all detected on the Innovate System within 24 hours of spiking (Tables 2 and 3). *B. cereus, C. sporogenes and S. aureus* were detected after 48 hours. RLU values were only collected for Days 1-4 for C. *sakazaki*i and *C. sporogenes* because of the overgrowth in this product and excess production of gas making the product containers leak. All positive results on the Innovate System were confirmed by streaking products on TSA. A summary of the results is shown in Table 3.

Table 1. Product Assessments – pH, Background, Baseline and Sterility

a) pH

Product Sample pH			
Read 1	**Read 2**	**Read 3**	**Average**
7.13	7.12	7.12	7.12

b) Background & Baseline Readings

RLU Averages (n=32)		
Background	**Baseline**	**Threshold**
395	11	32

c) Sterility Confirmation

Sterility Plates	
TSA	**SDA**
No Growth	No Growth

Table 2. Detection of Microorganism Growth Over Time in the Soy-based, Flavored Meal Replacement Product

Product Background: 395 RLU		Product Baseline: 11 RLU		Threshold: 32 RLU						
Organism Panel	Target Inoculum Cells or *Spores (CFU)	Actual Inoculum Cells or *Spores (CFU)	Confirmation Plate Result	Days: RLU Value (Avg) 1	2	3	4	5	6	7
Bacillus cereus endospores	<100	28	Growth	10	70	33,144	1,068	2,489	2,782	1,460
Clostridium sporogenes vegetative	<100	20	Growth	18	52,063	89,495	85,773	LEAK	LEAK	LEAK
Cronobacter sakazakii	<100	92	Growth	39,812	56,762	60,615	14,594	LEAK	LEAK	LEAK
Pseudomonas aeruginosa	<100	138	Growth	10,141	2,338	2,590	877	1,555	1,902	1,517
Pseudomonas putida	<100	59	Growth	1,010	6,842	5,232	317	573	37,263	14,846
Staphylococcus aureus	<100	35	Growth	9	461	7,056	892	6,821	20,111	9,441
Blank Panel	**Target Inoculum**	**Actual Inoculum**	**Confirmation Plate Result**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Negative Control	0	0	No Growth	8	12	9	12	8	12	28

***Leak – Gas production from the organism caused the product container to burst or leak.

Table 3. Summary of Detection Time for Microorganism Growth in the Meal Replacement Product

Product Time to Detection in the Innovate System					
B. cereus	***C. sporogenes***	***C. sakazakii***	***P. aeruginosa***	***P. putida***	***S. aureus***
48 hours	48 hours	24 hours	24 hours	24 hours	48 hours

Conclusions

The soy-based, flavored meal replacement product was inoculated with a target of <100 CFU and incubated at 35 ± 2°C, with readings taken on days 1-7. CFU levels were determined to be 28, 20, 92, 138, 59 and 35 for the organisms *B. cereus, C. sporogenes, C. sakazakii, P. aeruginosa, P. putida* and *S. aureus,* respectively.

As shown in the above tables (Tables 2 and 3), *C. sakazakii, P. aeruginosa and P. putida,* were all detected on the Innovate System within 24 hours of spiking. *B. cereus, C. sporogenes and S. aureus* were detected after 48 hours. RLU values for *C. sakazakii* and *C. sporogenes* could only be obtained on Days 1-4 because of overgrowth in the product and excess production of gas making the product containers leak. All positive results on the Innovate System were confirmed by streaking products on TSA.

Summary
Aseptic processing of soy-based products may help reduce the risk of microbial contamination of the products. This is clearly shown in the low baseline values for ATP detection in uninoculated samples. In addition, the baseline RLU values were consistently stable, allowing for the establishment of a positive/negative threshold value. The threshold for this product was set at 32 RLUs. RLU values above this threshold indicated a positive result.

The signal from the growth of all tested organism cultures in this product demonstrated that the signal-to-noise ratio was not an issue. Even when inoculated with low microorganism levels (<100 CFUs), samples tested produced very high RLUs at 24 to 48 hours, thereby validating that the Innovate System would be a useful tool in detecting microbial contamination of flavored, soy-based meal replacement products.

Based on these results, Hygiena® recommends using the Innovate System for the detection of low levels of microbial contamination in soy-based meal replacement products.