Detection of *Cronobacter* spp. Using the Innovate™ System and RapiScreen™ Dairy Kit

Introduction

Cronobacter species are gram-negative bacteria that are found naturally in the environment. *Cronobacter* can survive in dry foods, like powdered infant formula, powdered milk, herbal teas and starches, even throughout the desiccation process. They are known to cause severe and often-life threatening infections in infants.

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, including milk and infant formula. 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 *Cronobacter muytjensii* and *Cronobacter sakazakii* in various oat milk products to demonstrate equivalence to traditional plate techniques.

Equipment, Supplies and Reagents

- Sterile inoculating loops
- Sterile pipettes and tips
- Incubators capable of 30 or 35 ± 2°C
- Alcohol wipes
- RapiScreen Dairy Kit (includes reagents, polypropylene (PP) vials, microtiter plates)
- Potato Dextrose Agar (PDA)
- Tryptic Soy Agar (TSA) Plates
- Tryptic Soy Broth (TSB)
- pH meter and electrodes (i.e., Mettler-Toledo InLab® sensors)
- Gas Pak™ EZ Anaerobe Gas Generating Pouch System with Indicator
- Syringes, 1 mL and 3 mL
- Dulbecco's Phosphate Buffered Saline - DPBS (1X)
- 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
  - *Cronobacter muytjensii*, ATCC # 51329
  - *Cronobacter sakazakii*, ATCC# 29544
- Milk product types tested
  - ESL Chocolate Milk
  - ESL Low-fat Milk
  - ESL Original Milk
  - UHT Barista Milk
  - UHT chocolate Milk
  - UHT Original Milk

Methods

Cronobacter cultures were prepared by rehydrating the ATCC pellets, streaking them onto TSA plates and culturing for 24 hours at the appropriate temperature (30 ± 1 °C and 35 ± 1 °C, respectively, for *C. muytjensii* and *C. sakazakii*). Colonies were selected and a series of 10-fold dilutions were prepared in DPBS and plate counts were prepared to identify a concentration of <100 CFU.

To determine ATP baseline levels, each milk product was initially incubated for 48 hours at 32 °C. Samples were mixed and 25 mL of each product was transferred to a sterile container for testing. Both pH and background/baseline testing using the RapiScreen Dairy Kit were completed.

Once baselines were established and cultures were prepared, each product type was inoculated in triplicate at <100 CFU per container (High spike levels were also inoculated for comparison). The microorganisms were spiked using a syringe through the top of the container and sealed with Shoe Goo. A non-inoculated container was incubated with each inoculated set as a negative control. Positive controls were set up by inoculating tryptic soy broth. Samples for *C. muytjensii* were incubated at 30 ± 1 °C while samples for *C. sakazakii* were incubated at 35 ± 1 °C for a total of two days. On both days 1 and 2, aliquots were taken from each container and tested on the Innovate System using the RapiScreen Dairy Kit. In parallel, 10 µL of each product sample was inoculated onto TSA plates and incubated for 24 hours for growth and morphology confirmation.

Results

In all milk types tested, growth of both high and low spike levels of both *Cronobacter* species was detected after 24 hours of incubation using the Innovate System and the RapiScreen Dairy Kit (Tables 1 and 2). Typical RLU values fell between 40,000 and 175,000, demonstrating robust growth and ATP production, even when spiked at <100 CFUs. The uninoculated controls had baseline values between 6 and 25 CFUs, typical for properly processed UHT and ESL products.

In contrast, the detection of *Cronobacter* on plates was not observed until after 48 hours of incubation. All positive samples on the Innovate System were confirmed to grow by culture plating, even those at low spike levels (<100 CFUs). Even high spiked cultures were not confirmed until 48 hours. This standard method did not confirm contamination at 24 hours in any sample type – even though the Innovate System and RapiScreen Dairy Kit could.

Table 1. Detection of *Cronobacter muytjensii* in Various Oat Milk Product Types

***C. muytjensii* Detection (hours)**				
Product Type	**Innovate System**		**Plating**	
	High CFU Spike	**Low CFU Spike**	**High CFU Spike**	**Low CFU Spike**
ESL Original	24 h	24 h	48 h	48 h
UHT Original	24 h	24 h	48 h	48 h
ESL Chocolate	ND	24 h	ND	48 h
ESL Low Fat	ND	24 h	ND	48 h
UHT Barista	ND	24 h	ND	48 h
UHT Chocolate	ND	24 h	ND	48 h
Uninoculated	-	-	-	-

Table 2. Detection of *Cronobacter sakazakii* in Various Oat Milk Product Types

C. sakazakii Detection (hours)				
Product Type	Innovate System		Plating	
	High CFU Spike	Low CFU Spike	High CFU Spike	Low CFU Spike
ESL Original	24 h	24 h	48 h	48 h
UHT Original	24 h	24 h	48 h	48 h
ESL Chocolate	ND	24 h	ND	48 h
ESL Low Fat	ND	24 h	ND	48 h
UHT Barista	ND	24 h	ND	48 h
UHT Chocolate	ND	24 h	ND	48 h
Uninoculated	-	-	-	-

Conclusions

As shown in the above tables (Tables 1 and 2), both *C. muytjensii* and *C. sakazakii* were detected using the Innovate System after 24 h incubation in all products spiked with the organisms. As for the traditional plating method, positive results were obtained after 48 h for all samples tested.

Results for growth on day 3 and beyond were not completed due to the excessive growth of both organisms in all the products. By day 2, all the product containers were bloated. Due to this, the containers were disposed of after day 2 to prevent the risk of the products bursting inside the incubator. Similarly, only the low spikes for the ESL Chocolate, ESL Low Fat, UHT Barista, and UHT Chocolate Milk types were tested due to the fast growth of both organisms in all the Oat milk products.

Summary

Aseptic processing of oat milk products may help reduce risk of microbial contamination of the products. This is clearly shown in the low baseline values for ATP detection in uninoculated samples. In addition, *Cronobacter* contamination at low levels (<100 CFU per container) could be detected at 24 hours using the Innovate System and RapiScreen Dairy Kit. This was 24 hours faster than detection using standard plating methods, verifying that Innovate Rapid Microbial Screening System outperformed the reference method when used for *Cronobacter* detection.

Based on these results, Hygiena® recommends using the Innovate System for the detection of low levels of *Cronobacter* species in UHT and ESL milk products.