Detection of *Lactobacilli*, Yeast and Molds in Kraft Heinz Tomato Ketchup Using Hygiena's Innovate™ System

Bernard Linke[1], Gabriela Tarlowska[1], Mat Lovesmith[1], Alison Bennett[2], Rachel Bayliss[2]
1. Hygiena®, Guildford, United Kingdom
2. Kraft Heinz, Wigan, United Kingdom

Innovate™ | Innovate™ AUTOSAMPLER III

INTRODUCTION:

Lactic acid bacteria, yeast, and molds are common contaminants of low pH condiments otherwise considered shelf-stable. The growth of these organisms often leads to severe spoilage and bursts containers. The initially low contamination levels coupled with a difficult growth environment can mean that traditional detection methods may miss the spoilage.
Hygiena's Innovate™ System with RapiScreen™ reagent technology is a high-throughput microbial screening system designed to meet the needs of today's manufacturers of diverse food and beverage products. The robust ATP bioluminescence detection system overcomes issues with inhibition in difficult food matrices when deployed in conjunction with a product-specific enrichment, often producing a faster time-to-result than traditional plating methods.
In the experiments outlined below, Hygiena looked at optimizing growth of lactic acid bacteria (*Lactobacillus brevis* and *Lactobacillus casei*) and yeast/mold (*Zygosaccharomyces bailii*) contaminants during the enrichment step with the use of larger product dilutions. The optimal dilution ratio for Tomato Ketchup (TK) was then applied in a spike study designed to replicate the type of contamination events seen in a manufacturing setting.

PURPOSE:

- Rapidly detect lactic acid bacteria (LAB) and low-pH tolerant yeast and molds in Kraft Heinz Tomato Ketchup.
- Optimize growth of contaminants during the enrichment step by using larger product dilutions.
- Improve the dynamic range by removing signal inhibition seen in neat product samples.

REGISTERED TRADEMARKS:

METHOD:

- LAB were cultured in MRS Broth at 25 °C for 5 days. Yeast organisms *were* cultured in Yeast Growth Broth at 25 °C for 5 days.
- The LAB cultures were diluted to <10, <5, <2.5 and <1.25 per 25 mL of diluted sample. LoD were estimated using 3 replicates at each dilution series.
- The TK was aseptically decanted from the original packaging, diluted 1:4 in MRS broth for LAB detection and 1:4 in Yeast Growth broth for yeast detection.
- After 96 hours incubation samples were drawn from each bottle and tested for ATP content using the Innovate System and plated on MRSA and PDA. The confirmation plates were grown for another 5 days.
- The eLoD calculations were performed using *Microbiology of the food chain - Method validation - Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory.*

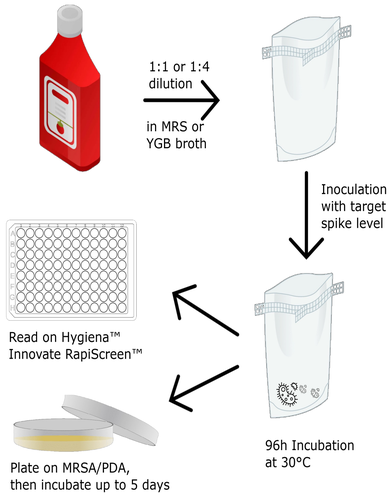

Figure 1. A simplified depiction of the protocol used.

RESULTS:

		LAB detection - MRS broth dilutions						Yeast detection - YG broth dilutions					
		Ketchup 1:1			Ketchup 1:4			Ketchup 1:1			Ketchup 1:4		
		Innovate		Plating	Innovate		Plating	Innovate		Plating	Innovate		Plating
	CFU Added	RLU	Result		RLU	Result		RLU	Result		RLU	Result	
Results from the Innovate System & Plate Methods	<10	1,543	+	+	116,155	+	+	19,667	+	+	69,554	+	+
		493	+	+	135,517	+	+	N/D			N/D		
		848	+	+	114,168	+	+						
		292	+	+	133,604	+	+						
	<5	364	+	+	147,186	+	+	23,800	+	+	66,202	+	+
		4,572	+	+	146,019	+	+	34,393	+	+	46,934	+	+
		779	+	+	140,194	+	+	33,575	+	+	36,589	+	+
		5261	+	+	146,691	+	+	26,181	+	+	67,541	+	+
	<2.5	237	+	-	143,401	+	+	5,321	+	+	69,871	+	+
		1,301	+	+	183,195	+	+	6,765	+	+	54,237	+	+
		92	-	-	150,570	+	+	366	-	-	31	-	-
		170	+	-	300	-	-	19,657	+	+	48,828	+	+
	<1.25	49	-	-	1,405	-	-	17	-	-	54,751	+	+
		155	-	-	165,629	+	+	33	-	-	35,941	+	+
		285	+	+	748	-	-	16	-	-	23	-	-
		1,095	+	-	102,552	+	+	866	+	-	67,441	+	+
Statistical Analysis	eLoD CFU	0.7		1.3	0.7		0.7	0.7		0.7	0.7		0.7
		0.6		1.7	0.6		0.6	0.9		1.2	0.5		0.5
	TP	10		10	13		13	11		11	14		14
	TN	3		6	3		3	5		5	2		2
	FP	3		0	0		0	1		0	0		0
	FN	0		0	0		0	0		0	0		0
	Sensitivity	100%		100%	100%		100%	100%		100%	100%		100%
	Specificity	50%		100%	100%		100%	100%		100%	100%		100%
	Mean Positives	1,574			140,375			21,170			56,172		
	SD	1,818			21,362			10,850			12,890		
	Mean Negatives	300		N/A	818		N/A	260		N/A	27		N/A
	SD	395			556			370			6		
	S/N	5.3			171.7			81.5			2,080.4		
	Z Score	3.2			251.1			56.5			9,925.1		

Table 1. Summarizes the results and statistical analysis of the Ketchup spike study. Positives obtained from the Innovate System reads and the reference plating method are shown side by side. The lower portion of the table shows statistical analyses of both methods, as well as metrics concerning the RLU dynamic range, such as Z-score and signal-to-noise ratio (S/N). RLU Fail Thresholds: MRS 1:1 = 165, MRS 1:4 = 2,574 , YG 1:1 = 414, YG 1:4 = 5,073

DISCUSSION:

The data sets were analyzed to produce **Table 1** comparing the efficacy of the two methodologies and establish the optimal product dilution level. The plate count data is taken as the gold standard for calculating the sensitivity and specificity. The RLU thresholds were tailored to each product-broth combination by performing calculations using RLU values from sterile products. The average negative RLU is then multiplied by three to give a robust fail threshold. The Z-score and Signal-to-noise ratio are two important metrics when deciding on the most optimal enrichment parameters and assessing robustness of the assay. Both parameters should be greater than 10 as a minimum, with higher values demonstrating a more optimal assay.

At 96 hours, the Innovate System detected all tested dilutions of LABs and yeast, though not always across both 1:1 and 1:4 product dilutions. The lower spike levels down at <1.25 CFU were fractionally detected, meaning that 1 or 0 CFU were inoculated or have grown in each 25 mL TK dilution. For the 1:1 TK:MRS dilution the mean RLU for positive growth was 1,574 RLU and for negative growth was 300 RLU, whereas for the 1:4 dilution, the mean RLU for positive growth was 140,375 RLU and for negative growth was 818 RLU. The latter dilution gives a Z-score of 251 which is an overwhelming positive detection. For *Zygosaccharomyces*, the 1:1 YGB dilution yielded a positive RLU of 21,170 and 260 RLU for negative growth. In the 1:4 dilution of TK:YGB, the mean positive RLU was 56,172 and mean negative RLU was 27, resulting in a very robust Z-score of 9,925.

Overall, the results showed that the 1:1 TK:MRS dilution does not resolve the growth of contaminants well in Tomato Ketchup. There is significant inhibition of ATP signal from the Tomato Ketchup in the 1:1 dilution, which makes the resolution of positivity and negativity difficult. The depression in overall bioluminescence can be seen in the Z-score, which is a 3.2 for 1:1 TK:MRS, this is low for bioluminescence assays and indicates a depressed dynamic range. The 1:4 TK:MRS dilution seems to improve the growth of the contaminants, whilst also eliminating the intrinsic ATP signal inhibition. The improvement is made obvious by the Z-Score of 251 and translates into a large dynamic range for the test. Although the 1:1 TK:YGB dilution resolves the growth of *Zygosaccharomyces* well, there is also a pronounced improvement in the Signal to Noise and Z-score metrics from the 1:4 dilution TK:YGB. Subsequent testing on further organisms (*Candida albicans*, *Lactobacillus fructivorans*, *Lactobacillus plantarum, and Saccharomyces cerevisiae*) as well as other condiments confirmed the findings and further substantiated the benefit of using the Innovate System in conjunction with a 1:4 dilution for detection of contaminants in low-pH condiments.

SIGNIFICANCE:

- Early and significant detection of lactic acid bacteria and yeast in Tomato Ketchup using an out-of-pack dilution in selective media using ATP is resolved faster than plating assays.
- Use of a 1:4 product dilution recovers bioluminescent signal and greatly increase S/N.
- The increased resolution between positive and negative results allows a quicker release of products and shorter hold times.

REFERENCES:

1. *Microbiology of the food chain - Method validation - Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory.*