A - 124

Bioinformatics Analysis to Improve Automated Molecular Detection of Beer Spoilage Bacteria, Yeast and Hop Resistance Genes

J. Pecone[1], S. Morse[1], D. Bocioaga[1], K. Sweitzer[1], K. Sorensen[1], I.C. McGuire[1], C.H. Higgins[1], P. Trabold[1], G. Spizz[1]

[1] Rheonix, Inc., Ithaca NY

Introduction

Identification of beer spoilage organisms (BSOs) has typically been done using culture-based detection methods, with results obtained up to a week after sample processing begins. Furthermore, traditional methods only detect the presence of a particular species to the genus level and do not necessarily indicate the presence of spoilage genes needed for these organisms to propagate in beer. Nucleic-acid based detection methods, such as those utilizing PCR, have improved the time and efficiency for beer spoilage detection, but development of these methods is time consuming and requires technical laboratory expertise. Moreover, performance of PCR-based methods on a benchtop has a high potential for contamination and thus false positives. These challenges can be overcome by the use of the Rheonix Beer SpoilerAlert™ assay, which is a fully automated, sample-to-results multiplexing molecular detection kit, and the Encompass Optimum™ Workstation.

Development of an all inclusive beer spoilage assay with detection of over 54 species of bacteria, 6 species of wild yeast, and 4 hop resistance genes, has been accomplished through a highly multiplexed assay containing 23 primers divided into two different reaction mixes, Master Mix 1 and Master Mix 2. Through these reactions, 11 genetic loci are targeted that are recognized by at least one of the 21 unique probes on a low density array. Probes are either unique to a specific primer set/target (*S. cerevisiae* variant *diastaticus*, hop resistance genes), combine in a fingerprint to allow for speciation of a genus (*Brettanomyces*), identify all members of a genus without distinction (*Megasphaera*, *Pectinatus*, and *Pediococcus*) or identify individual subsets of a genus (*Lactobacillus*).

The data in this poster outline the results of extensive bioinformatics analysis used to enable this wide-ranging coverage of beer spoilage organisms and targets. The Rheonix Beer SpoilerAlert assay detects 39 species of *Lactobacillus*; 8 species of *Pediococcus*; 3 species of *Pectinatus*, 4 species of *Megasphaera*, 5 species of *Brettanomyces* and *S. cerevisiae* var. *diastaticus.* In addition to detection of the micro-organisms, the assay also detects 4 genes associated with hop resistance in lactic acid bacteria, *horA*, *horC*, *bsrA*, and *bsrB* (Table 1). The Rheonix Beer SpoilerAlert assay (Figure 1) is the only commercially available assay that simultaneously detects such a diverse group of targets within a single test providing actionable results in shorter times compared to traditional microbiology methods (Figure 2).

Table 1: Targets detected with the Beer SpoilerAlert™ Assay

RS	Reference spot; used by imaging software to orient filter
Targets amplified by Master Mix 1	
MM1	Control for PCR Master Mix 1
B1-5	Targets specific to a subset of *Brettanomyces* species
L1-7	Targets specific to a subset of *Lactobacillus* species
MEG	Target found in *Megasphaera* species
PEC	Target found in *Pectinatus* species
DIA	Target found in *Saccharomyces cerevisiae* variant *diastaticus*
BSRB	Hop resistance gene, *bsrB* , found in *P. claussenii*
Targets amplified by Master Mix 2	
MM2	Control for PCR Master Mix 2
PED	Target found in all currently sequenced *Pediococcus* species
LABS	Plasmid biomarker present in strains of various lactic acid bacteria (LAB)
HORA	Hop resistance gene, *horA* , found on plasmids in various LAB
HORC	Hop resistance gene, *horC* , found on plasmids in various LAB
BSRA	Hop resistance gene, *bsrA* , found in *P. claussenii*

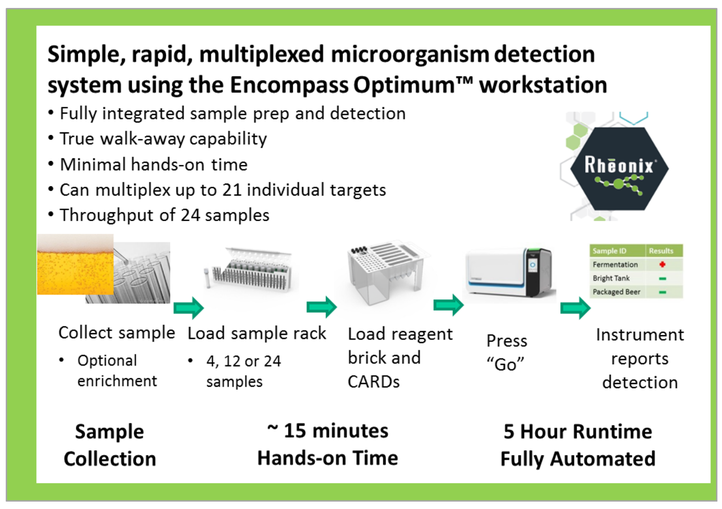

Figure 1. Beer SpoilerAlert Assay Workflow.
Runtime includes dispense of samples to Chemistry and Reagent Device (CARD) cartridge, cell lysis, DNA purification, PCR amplification, reverse dot blot hybridization to capture probes on low density array, and image capture, all performed automatically on the Encompass Optimum workstation. In addition, all data are analyzed and reported in an easy to read sample report.

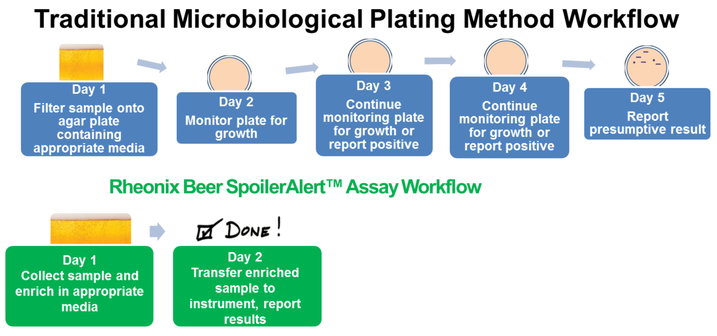

Figure 2. Timeline comparison of traditional microbiological spoilage detection versus Beer SpoilerAlert assay.

Materials & Methods

Strains, Media and Culture Conditions

Microorganisms were obtained from multiple sources including Campden BRI, American Type Tissue Collection, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, National Collection of Yeast Cultures, Agricultural Research Service Culture Collection, and local breweries, maintained as stock cultures in 20% glycerol at -70 ˚C and propagated as per provider instructions. Lactic acid bacteria were grown in B-MRS (DIFCO, MRS supplemented with filtered, clear beer, 18 IBU, 4.1% ABV) and yeast were grown in YM (DIFCO). B-MRS agar plates were prepared by adding filtered beer to autoclaved MRS agar, to a final concentration of 0.5xBeer/0.5xMRS. Lactic acid bacteria were incubated at 30 ˚C under a 10% CO_2 atmosphere, while yeasts were incubated aerobically at 25 ˚C. *Megasphaera cerevisiae* and *Pectinatus cerevisiiphilus* were grown in pre-reduced, anaerobically sterilized PYF and MRS media (Anaerobe systems, Morgan Hill, CA) respectively, at 30 ˚C, under anaerobic conditions (BD GasPak EZ Gas Generating Systems).

Analysis of Samples on the Encompass Optimum Workstation

To validate target specificity of the Rheonix Beer SpoilerAlert assay using the Encompass Optimum workstation, overnight cultures were grown in liquid media under appropriate conditions. Cultures were counted using a Cellometer X2 (Nexcelom), confirmed with plate counts, and diluted accordingly in either media or buffer to desired concentration. Samples were run using the CARD cartridge using supplied reagents on the workstation, and the results were analyzed through an automated software report.

Sequencing/ Bioinformatics

To perform sequencing analysis on the *Brettanomyces* and *Lactobacillus* species/strains utilized in this study, DNA was isolated using the Qiagen Blood and Tissue DNeasy kit followed by amplification to generate a product surrounding the region of interest. Following amplicon purification using the Qiagen PCR purification kit, the DNA was sequenced via Sanger sequencing at the Cornell University (Ithaca, NY) genomics facility. Analyses of the sequences were performed using CLC Main Workbench (Qiagen) and the Basic Local Alignment Search Tool (BLAST) comparing sequencing results to existing sequences in the National Center for Biotechnology Information (NCBI) databases. The new sequence data also informed new probe designs for both *Brettanomyces* and *Lactobacillus* species. In many cases, novel sequencing information was obtained for species that were not well characterized.

Results

	Previous evidience of beer spoilage	Identity confirmed via sequencing	Detection expected with SpoilerAlert assay	Detection confirmed with SpoilerAlert assay	Detected with original *L. brevis based probe*
L. acetotolerans	Y	Y	Y	Y	
L. acidophilus			Y		
L. agilis			Y		
L. amylolyticus			Y		
L. amylovorus			Y		
L. backii	Y	Y	Y	Y	
L. brevis	Y	Y	Y	Y	Y
L. buchneri	Y	Y	Y	Y	
L. casei	Y	Y	Y	Y	
L. collinoides			Y		
L. coryniformis	Y	Y	Y	Y	
L. crispatus			Y		
L. curieae			Y		
L. curvatus			Y		
L. delbrueckii			Y		
L. fermentum		Y	Y	Y	Y
L. gallinarum			Y		
L. gasseri			Y		
L. harbinensis		Y	Y	Y	
L. helveticus			Y		
L. jensenii			Y		
L. johnsonii			Y		
L. lindneri	Y	Y	Y	Y	Y
L. malfermentans	Y	Y	Y	Y	
L. parabuchneri	Y	Y	Y	Y	
L. paracasei	Y	Y	Y	Y	
L. paracollonoides	Y	Y	Y	Y	
L. paraplantarum			Y		Y
L. paucivorans	Y	Y	Y	Y	
L. pentosus			Y		Y
L. perolens	Y	Y	Y	Y	
L. plantarum	Y	Y	Y	Y	Y
L. rennini			Y		
L. reuteri	Y	Y	Y	Y	Y
L. rhamnosus		Y	Y	Y	
L. rossiae	Y	Y	Y	Y	Y
L. ruminis			Y		
L. sakei			Y		
L. salivarus			Y		

Table 2. Outline of the 39 species of *Lactobacillus* analyzed in this study.
When available, the organism was sequenced and tested with the Beer SpoilerAlert assay. Sequence analysis informed the design of 6 probes in addition to a probe previously designed specifically for *L. brevis.* Based on available sequence information, the 7 probes should enable detection of the 39 species indicated in the table. As of this time, only 19 have been available for testing with the assay.

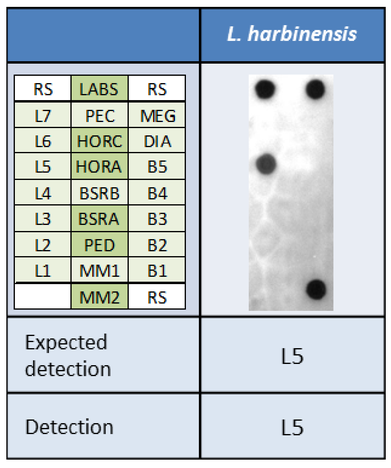

Figure 3. Identification of *L. harbinensis* as a novel beer spoilage organism and detection with the Beer SpoilerAlert assay. To obtain sequencing data on a large variety of *Lactobacillus* species and strains that are capable of beer spoilage, organisms isolated from spoiled beer were obtained from many different sources. Some of these BSOs had previously been characterized based solely on physical and chemical properties. This led to misidentification of some microorganisms, which was confirmed through sequencing. Thus, we can identify *Lactobacillus harbinensis,* which had previously been identified as *L. brevis,* as a potential novel beer spoilage organism through this study.

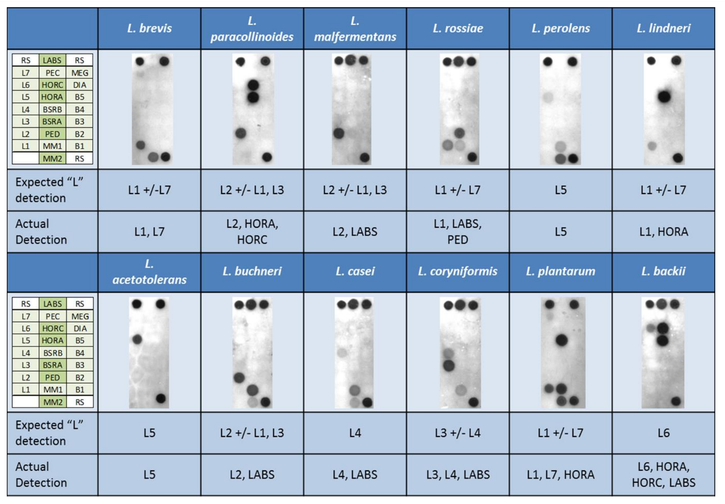

Figure 4. The Beer SpoilerAlert assay detects a wide variety of *Lactobacillus* beer spoilers. A probe originally designed, and based on the known sequence of *L. brevis,* was recognized by 8 species of *Lactobacillus* (Table 2). The addition of 6 new *Lactobacillus* "L" probes expanded this to detection of at least 39 individual species of the genus. The figure shows images of the low density capture arrays following target detection of 12 species of *Lactobacillus* previously shown to be associated with beer spoilage. In addition to *Lactobacillus* specific targets, many of these spoilers also contain spoilage genes present on plasmids. The keys indicate probe location on the array. See **Table 1** for target description.

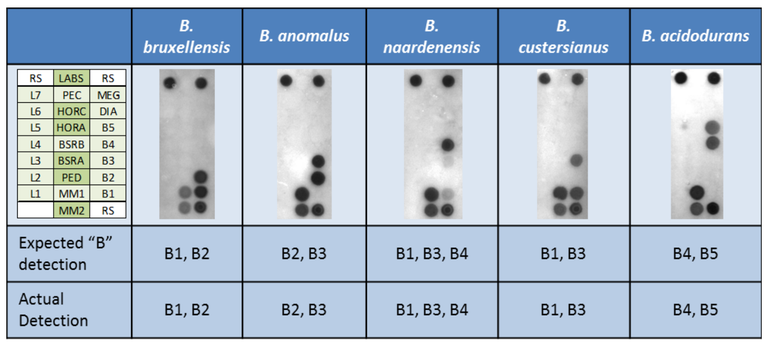

Figure 5. The Beer SpoilerAlert assay detects 5 species of *Brettanomyces*. A probe originally designed based on the known sequence of *B. bruxellensis* was recognized exclusively by *B. bruxellensis.* Sequence analysis of the target region in other species of the genus enabled design of 4 additional probes capable of detecting additional species including *B. anomalus, B. naardenensis, B. custersianus,* and *B. acidodurans*.

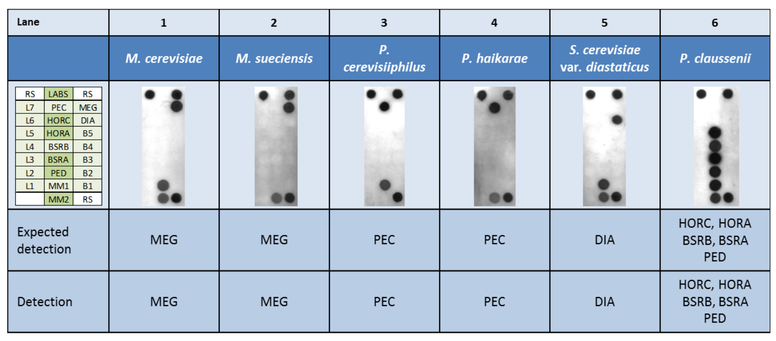

Figure 6. Additional targets included in the Beer SpoilerAlert assay. The figure shows images of low density capture arrays following analysis of representative species of the strict anaerobic bacteria, *Megasphaera* (Lanes 1 and 2), and *Pectinatus* (Lanes 3 and 4). In addition, detection of the wild yeast *S. cerevisiae* var. *diastaticus* (Lane 5) is shown. Finally, the Beer SpoilerAlert assay detects all known species of the lactic acid bacteria, *Pediococcus.* Lane 6 shows results of detection of the known beer spoilage organism *Pediococcus claussenii.* In addition to the marker detected by all *Pediococcus* (PED), *P. claussenii* also contains 2 genes for hop resistance, *bsrA* and *bsrB.* These two genes are present only in *P. claussenii* and no other *Pediococcus* species. However, various strains of *Pediococcus* may contain the plasmid associated hop resistance genes, *horA* and *horC*. The strain of *P. claussenii* used in these studies contains the *horA* gene.

Summary & Conclusions

Target organism/ genes	Effects on Beer	Generation 1 SpoilerAlert	Generation 2 SpoilerAlert
Lactobacillus	Sediment, sour off flavors	*L. brevis* only	**≥39 species of *Lactobacillus***
Pediococcus	Buttery or sour off-flavors; ropiness (slimy)	*P. acidilactici, P. cellicola, P. claussenii, P. damnosus, P. inopinatus, P. parvulus, P. pentosaceus and P. stilesii*	Same (8)
Wild yeast	Off-flavor, exploding bottles	*S. cerevisiae* var. *diastaticus*	Same (1)
		Brettanomyces bruxellensis	**≥4 additional species of *Brettanomyces* (total 5)**
Strict anaerobes	Rotten egg smell	Not included	***Pectinatus* spp., (3) & *Megasphaera* spp (4).**
Hop resistance genes	Allows lactic acid bacteria to grow in the presence of alpha acids generated from hops. In the absence of these genes, alpha acids provide anti-microbial benefits to beer	*horA, horC, bsrA, bsrB*	Same (4)
TOTAL TARGETS SPOILERALERT ASSAY		**Gen 1 total targets: 15**	**Gen 2 total targets: 64**

Table 3. The studies presented here demonstrate expansion of the original Beer SpoilerAlert assay to detect a greater breadth of organisms associated with spoiled beer. The table displays the changes from the original assay to the current assay. Expansion of the assay was made possible via detailed bioinformatics analysis and sequencing of BSOs. The assay was used to investigate the identities of organisms originally isolated from spoiled beer products and has demonstrated that in at least one case, *L. harbinensis,* was originally misidentified as the more common *L. brevis.* These data support the hypothesis that many *Lactobacillus* species are capable of beer spoilage beyond the subset of known spoilage organisms.