Detection of *Salmonella* in Probiotic Cultures

Benjamin S. Shannon, Josephine D. Greve and J. David Legan*
Covance Food Solutions, Madison, Wisconsin *Corresponding author: David.Legan@Covance.com

Abstract

Probiotics are live microorganisms that provide some health benefit and are becoming increasingly popular with consumers. Exposing probiotics to a "kill step" is counter-productive to the goal of assuring consumption of high levels of live cultures. Hence protecting probiotic consumers from *Salmonella* depends on an effective food safety program supported by robust *Salmonella* detection methods.

The objective of this project was to explore recovery of *Salmonella* from a background of probiotic cultures, a difficult matrix that can apply significant competitive inhibition to any *Salmonella* present. A dehydrated probiotic culture was spiked with known concentrations of *Salmonella abaetetuba*, enriched in different broth media, and then tested using the Roka Atlas® *Salmonella* test protocol. When enriched at a 1 in 10 dilution in buffered peptone water (BPW), *Salmonella* could not be detected from an initial spike level of 30 CFU/25 g test portion. Enrichment at a 1 in 10 dilution in the modified BPW developed by Joosten, Bidlas and Garofalo[1] containing vancomycin, malachite green and non-fat dried milk (NFDM), was equally unsuccessful.

Successful detection of *Salmonella* at 1 CFU/25 g test portion was achieved when enriching at a dilution of 1 in 100 in a differently modified BPW with increased buffering capacity, malachite green and NFDM but no vancomycin. The final protocol is a reliable method for detecting *Salmonella* in dehydrated probiotic cultures. The use of scientifically valid methods for this unique matrix is fundamental to protecting consumers.

Background

Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (WHO).[2] Organisms often seen include species of *Bacillus, Bifidobacterium, Enterococcus, Lactobacillus, Saccharomyces and Streptococcus*.[3] The global probiotics market is large ($35 B USD in 2015) and fast growing (7 % CAGR: category average growth rate), with about 90 % of the market for human food use and 10 % animal feed.[4]

The literature indicates that probiotics may inhibit growth of pathogens, or even kill them,[5,6,7] which is seen as one of the mechanisms by which probiotics provide benefits. However:

- High background counts may inhibit detection of pathogens in probiotic cultures.
- It may be risky to use methods established for food matrices without specific verification or validation for use with probiotics.

Objective

To explore recovery of *Salmonella* from a background of probiotic cultures and demonstrate and validate an effective, sensitive method for detecting *Salmonella* in probiotic cultures to protect public health.

Acknowledgement

The freeze-dried probiotic cultures were provided by BioSource Flavors Inc., Muskego, WI.

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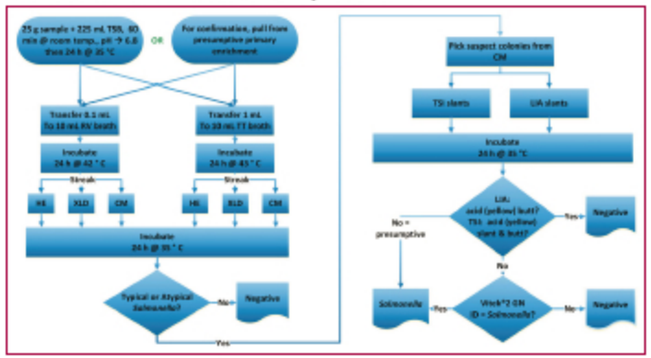
Methods

Reference method: FDA BAM *Salmonella* "dried yeast" variation.[8]



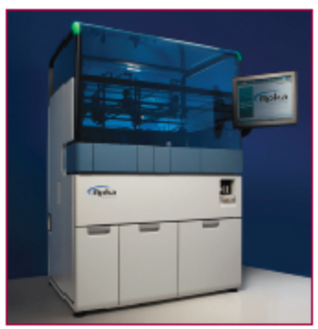
Exploration: Enrichment in multiple different media, followed by Roka® *Salmonella* SEN kit and ATLAS® instrument.

Final rapid detection: Enrichment in double strength buffered peptone water with Non-Fat Dried Milk and Malachite Green (NMD BPW) followed by Roka® *Salmonella* SEN kit and ATLAS® instrument.

Media controls were also included: the positive control was one Biobal® in 2500 mL of broth and the negative control was 2500 mL of uninoculated broth.

Loading transfer tubes into the Atlas. Image from Roka BioSciences, used with permission.

Roka Atlas detection kits. Image from Roka BioSciences, used with permission.



Roka Atlas detection platform, using RNA-based transcription mediated amplification. Image from Roka BioSciences, used with permission.

Confirmation method: The confirmation method was the FDA BAM method above,[8] entered after primary enrichment. Of presumptives from slants, 20% were confirmed for identification via the VITEK®.

Probiotic background count: Probiotic background was enumerated using 3M™ PetriFilm™.

Inoculation: Bioballs® (BioMérieux) containing freeze-dried *Salmonella abaetetuba* at 30 CFU/ Bioball were crushed and distributed into freeze-dried probiotic culture to give desired concentrations.

Comparison: The replicates tested by the Atlas® method and reference method were unpaired, meaning the enrichment conditions differed between the candidate and reference methods

Probiotic: The probiotic culture was a freeze-dried proprietary preparation from BioSource Flavors Inc., Muskego, WI.

Results

Probiotic background count: The probiotic preparation contained 1.4 x 10^{10} CFU/g.

Effectiveness of FDA BAM method: Ineffective, see Table 1.

Exploration of alternative enrichments: There were many failures before finding a combination of broth formulation and enrichment ratio that reliably recovered *Salmonella* (Table 2).

Validation of rapid *Salmonella* assay using modified enrichment: There was 100% agreement between screening and confirmation methods (Table 3) and at 1 CFU/25 g sample, the Probability of Detection (POD) was 0.55 (Table 4), giving an LOD_{50} of < 1 CFU/25 g.

Table 1. Recovery of *Salmonella abaetetuba* inoculated at 30 CFU/25 g Sample of Probiotic Culture Incubated at 35°C for 22-28 h

Sample #	Result
1	Negative
2	Negative
3	Negative
4	Negative
5	Negative

The reference method FDA BAM "dried yeast" variation was used, enriching with 225 mL trypticase soy broth.

Table 2. Recovery of *Salmonella abaetetuba* inoculated at 30 CFU/25 g Sample of Probiotic Culture in Different Enrichment Media Incubated at 42°C for 22-28 h

Sample:broth	Broth #	Medium	Result
1:9	1	Buffered Peptone Water (BPW)	Negative
1:9	2	BPW + Vancomycin + Malachite Green + Non-Fat Dried Milk	Negative
1:99	3	Broth 2	Negative
1:9	4	Broth 2 + Daptomycin at 0.5 µg/mL	Negative
1:9	5	Broth 2 + Daptomycin at 1.0 µg/mL	Negative
1:9	6	Broth 2 + Daptomycin at 2.0 µg/mL	Negative
1:9	7	Broth 2 + Clindamycin at 8.0 µg/mL	Negative
1:9	8	Broth 2 + Erythromycin at 8.0 µg/mL	Negative
1:9	9	Broth 2 + Clindamycin at 8.0 µg/mL + Erythromycin at 8.0 µg/mL	Negative
1:99	10	Broth 8	**Positive**
1:99	11	Double strength BPW + Malachite Green + Non-Fat Dried Milk	**Positive**

Table 3. Detection of *Salmonella* in Inoculated Freeze-Dried Probiotic Powder by Enrichment in NDM BPW at 42°C for 22-28 h

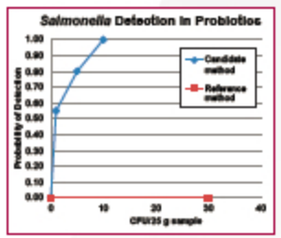
Inoculation Level	Sample	Atlas® Presumptive	Candidate Confirmation: Plates & Slants	Candidate Confirmation: VITEK®
0 CFU/25 g sample	1	-	-	
	2	-	-	
	3	-	-	
	4	-	-	
	5	-	-	
1 CFU/25 g sample	6	-	-	
	7	-	-	
	8	-	-	
	9	+	+	
	10	-	-	
	11	-	-	
	12	+	+	
	13	+	+	*Salmonella* spp.
	14	-	-	
	15	+	+	
	16	+	+	
	17	+	+	
	18	-	-	
	19	+	+	
	20	+	+	*Salmonella* spp.
	21	+	+	
	22	+	+	
	23	+	+	
	24	-	-	
	25	-	-	
5 CFU/25 g sample	26	+	+	
	27	+	+	
	28	-	-	
	29	+	+	*Salmonella* spp.
	30	+	+	
10 CFU/25 g sample	31	+	+	
	32	+	+	
	33	+	+	*Salmonella* spp.
	34	+	+	
	35	+	+	
0 CFU (Negative control)	36	-	-	
30 CFU (Positive control)	37	+	+	

Screening was done by Roka® Atlas® *Salmonella* SEN assay and confirmation by FDA BAM from primary enrichment. There was 100 % agreement between the screening method and the confirmation method.

Table 4. Comparative Results for the Candidate and Reference Methods (Unpaired Samples)

Method	CFU / 25 g	# tested	# positive	POD	LCL	UCL
Candidate: NDM BPW enrichment and Roka® SEN *Salmonella* assay	0	5	0	0.00	0.00	0.43
	1	20	11	0.55	0.34	0.74
	5	5	4	0.80	0.38	0.96
	10	5	5	1.00	0.57	1.00
Reference: FDA BAM	30	5	0	0.00	0.00	0.43

POD = Probability of Detection: LCL and UCL are lower and upper limits of the 95 % Confidence Interval.



Comparison of modified rapid screening assay and FDA BAM: The combination of NDM BPW enrichment and Roka® Atlas® *Salmonella* SEN assay were clearly more sensitive than the FDA BAM culture method (Figure 1).

Figure 1. Comparison of enrichment in NDM BPW for 22-28 h at 42°C followed by Atlas® SEN assay (candidate method) with FDA BAM "dried yeast" variation[8] (reference method).

Discussion

We confirmed that it is hard to detect *Salmonella* in probiotic cultures. The FDA BAM culture method is inadequate for this purpose because the enrichment is unable to inhibit competition from the probiotic culture.

A modified enrichment combined with the Roka® Atlas® *Salmonella* SEN assay was able to recover *Salmonella* with an LOD_{50} of < 1 CFU/25 g from a probiotic culture with a concentration of 1.4 x 10^{10} CFU/g. The final method recovered the pathogen in the face of a challenge that was 10^{8}-times greater than the AOAC recommended challenge with about 100-times the concentration of the pathogen. There was 100% agreement between presumptive and confirmed results from the modified method.

A stand-alone validation showed the method to be suitable for detecting *Salmonella* from freeze-dried probiotic cultures.

- High background counts *do* inhibit detection of pathogens in probiotic cultures.
- It *is* risky to use methods established for food matrices without specific verification or validation for use with probiotics.

Conclusion

The modified enrichment method combined with detection using Roka® Atlas® *Salmonella* SEN assay is suitable for detecting *Salmonella* from freeze-dried probiotic cultures; the FDA BAM *Salmonella* method is not. Care should be taken in selecting a suitable method for this critical testing.

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