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Evaluation of a Targeted Amplicon Sequencing Method for Detection of Contaminating Microorganisms in a Probiotic Product

Patel, Isha; Mammel, Mark; Gangiredla, Jayanthi; Mukherjee, Amit; Tartera, Carmen.
Division of Molecular Biology, Office of Applied Safety and Research Assessment, Center for Food Safety and Applied Nutrition

Introduction

In recent years there has been an increase in consumption of live microbial dietary supplements or probiotics. One of the challenges in maintaining the safety of probiotic products is the detection of low levels of contaminating microbes and/or pathogens in the presence of large number of beneficial microbes. The use of Whole Genome Sequencing (WGS) for pathogen detection has increased the accuracy and reduced the time for traceback and source attribution in foodborne outbreaks. Whole metagenomic sequencing (WMS) and target amplicon sequencing (TAS) have proven to be robust methods for detection of pathogens in food matrices. Here we evaluate and compare how accurate and sensitive these two sequencing methods are in detecting low levels of microbial contaminants in a probiotic matrix.

Materials and Methods

Probiotic products which contained combinations of varying bacterial species were spiked with the foodborne pathogen, *L. monocytogenes* (Lm) at different levels ranging from 20 to $2x10^9$ CFUs. Probiotic 1 contained only one bacterial species, *L. rhamnosus*, probiotic 2 contained four species *L. crispatus*, *L. rhamnosus*, *L. gasseri*, *L. jensenii* and probiotic 3 contained fifteen species, *L. plantarum, L. fermentum*, *L. acidophilus*, *B. infantis*, *L. casei*, *B. longum*, *L. rhamnosus*, *B. lactis*, *L. reuteri*, *L. salivarius*, *L. paracasei*, *L. gasseri*, *B. bifidum*, *B. breve*, *S. thermophilus*. The sensitivity of detection using TAS was evaluated against WMS. Target amplification was done using a custom primer panel designed in collaboration with Swift Biosciences (Ann Arbor, MI). The panel targets 135 pathogens which includes ten *L. monocytogenes* genes and eight *L. monocytogenes* virulence genes. Primer3 software was used to design primers from alignments of multiple sequences of ten core genes for each of the 135 pathogens. The desired amplicon size was set to approximately 600bp. For the TAS library preparation, amplicons generated after the multiplex PCR step were then used for library preparation for the MiSeq sequencer using the manufacturers protocol as shown in Figure 1. For the WMS library preparation, NexteraXT library preparation kit was used following manufacturers protocol. Ampure XP beads were used to cleanup the respective reactions followed by indexing and final elution. The library was then quantified, and library size and quality were determined using Agilent Tapestation. The indexed amplicon and whole metagenome libraries (12 picomolar) were sequenced using Illumina MiSeq Platform.

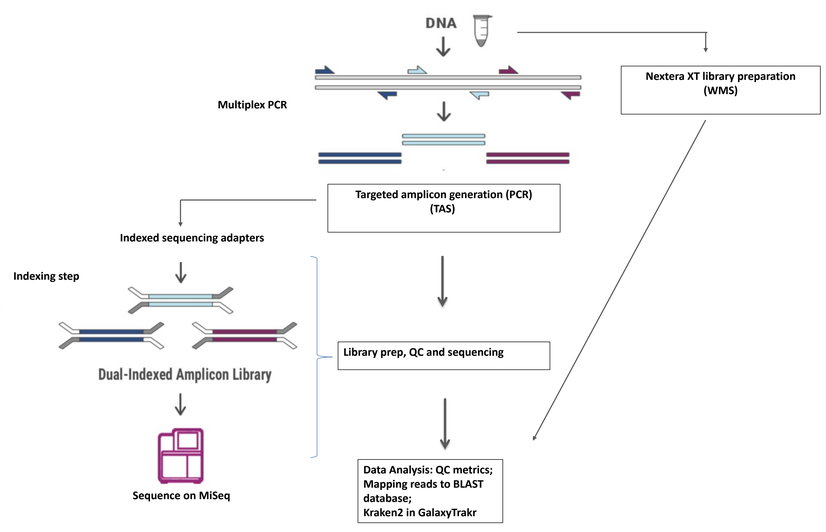

Figure 1. Library preparation workflow

Materials and Methods

Sequence Analysis: GalaxyTrakr and BLAST matching of the amplicons were used for data analysis. Kraken2 was used in GalaxyTrakr to identify and additionally, our in-house bioinformatic pipeline was used for identification and quantification of the targeted organisms from the sequence reads datasets. To quantify the number of genes present from each species, all reads were matched by BLAST to a database of MLST genes for each species. The database contains multiple sequence types for each of the 10 genes for each species. The top BLAST match for each read was taken, and the number of matching bases in that read was added to a tally for that gene. The tally for each gene was then normalized by dividing by the amplicon length that was represented in the database. A total count of genes present in each species was obtained by summing the tally for all genes belonging to that species. The reads were normalized to 1 million.

Results

Probiotic Product Sample Name	Mapped Read Count TAS /WMS	Total Reads TAS /WMS	Virulence Genes TAS /WMS
Product1	-/-	1789816/1887298	nd/nd
Product1+Lm $2x10^9$	46130/47	1497204/1794237	+/+
Product1+Lm $2x10^8$	3736/4	1093935/1632844	+/+
Product1+Lm $2x10^7$	236/1	1836597/2284282	+/-
Product1+Lm $2x10^6$	11/-	1048214/1883897	+/-
Product1+Lm $2x10^5$	1/-	1180193/2227549	-/-
Product2	-/-	334408/3841214	nd/nd
Product2+Lm $2x10^9$	27071/124	854121/541092	+/+
Product2+Lm $2x10^8$	1099/8	917028/356899	+/-
Product2+Lm $2x10^7$	16/1	1403670/820032	+/-
Product2+Lm $2x10^6$	7/1	266773/1977035	+/-
Product2+Lm $2x10^5$	1/-	932476/1759476	-/-
Product3	-/-	1139694/319837	nd/nd
Product3+Lm $2x10^9$	192/-	1327437/926731	+/+
Product3+Lm $2x10^8$	4/-	1114482/964638	+/-
Product3+Lm $2x10^7$	10/3	995380/614749	+/-
Product3+Lm $2x10^6$	21/-	629097/508959	+/-
Product3+Lm $2x10^5$	7/-	2078330/1741336	+/-
Product3+Lm $2x10^4$	4/-	936125/496212	+/-
Product3+Lm $2x10^3$	8/-	983366/703144	+/-
Product3+Lm 200	46/-	864283/585384	+/-
Product3+Lm 20	38/-	1117328/448009	+/-

Table 1. Sequencing results from TAS and WMS of three probiotic products spiked with Lm at various CFUs.
*not detected

Conclusion

Here we show that compared with WMS, TAS is a more sensitive method that can detect spiked Lm in all three probiotic products to as low as $2x10^6$ CFU but in probiotic product 3, Lm can be detected to as low as 20 CFU. Whereas WMS can only detect Lm at a high CFFU of $2x10^9$ in all three probiotic products but in probiotic product 1 it can be detected at a CFU of $2x10^8$. Currently we are working on conditions that will allow TAS to detect Lm at CFUs lower than $2x10^6$ in all three probiotic products. We show that TAS is a sensitive method for detection of low-level contaminants that may be present in probiotic products thus enhancing public health safety.

FDA Mission Relevance

The use of a targeted approach for detecting low number of bacterial pathogens without the need of sample enrichment may provide an efficient and effective tool for the FDA to identify foodborne pathogens such *Listeria monocytogenes*. Using an approach like TAS has potential to significantly reduce labor and time for compliance testing of samples to assure a safe food supply chain particularly for products with a short shelf-life.