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Application Note
Version 2

Probiotic Strain Level Identification

Using qPCR

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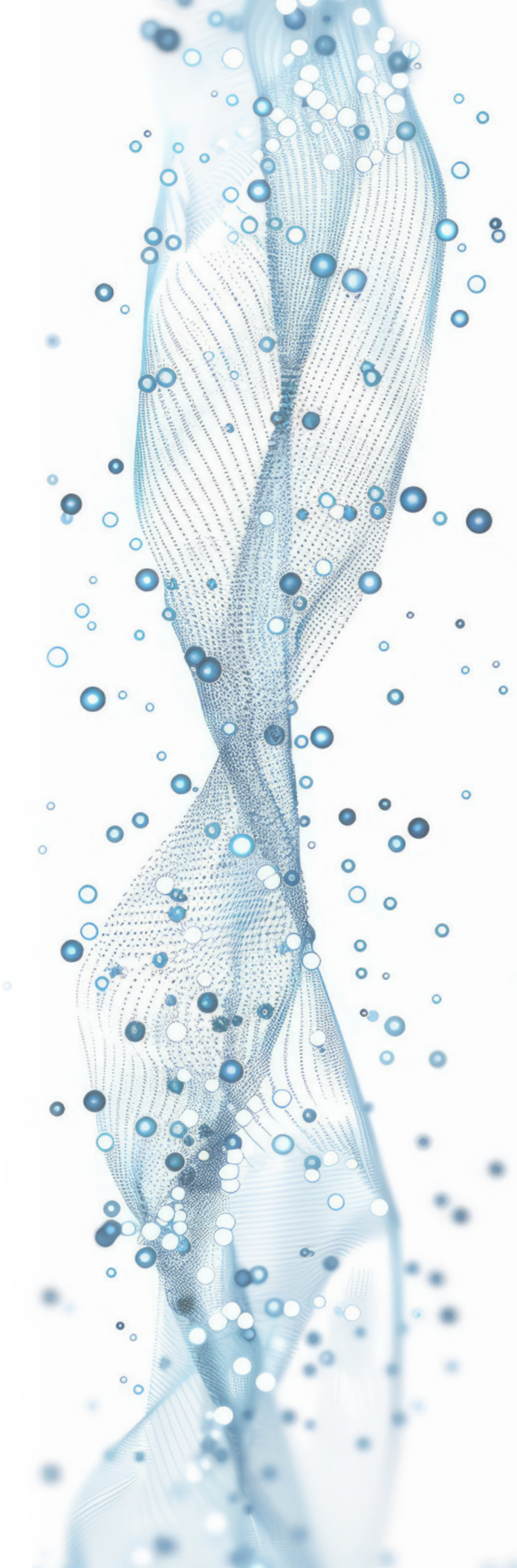
Background

Recent years have witnessed a rapid increase in the global probiotic market size which was valued at USD 58.17 billion in 2021 and is expected to reach USD 111.21 billion in 2030 (Grand-View-Research-Inc, 2022). This rapid growth was accompanied by reports on non-compliance and fraud in probiotic products (Morovic et al., 2016; Patro et al., 2016; Kolaček et al., 2017; Shehata and Newmaster, 2020b; Shehata and Newmaster, 2020a). For example, a probiotic product may fail to meet label claims of strain contents which can be in the form of strain substitution, missing strains or presence of undeclared strains (Shehata and Newmaster, 2020a). The importance of proper probiotic identification to strain level is becoming increasingly recognized since probiotic health benefits are strain specific (Klein et al., 2010; McFarland et al., 2018). Thus, reliable, and specific strain identification methods are an important component in probiotic authentication and quality assessment.

DNA based methods are the most commonly used method for probiotic identification. qPCR based methods are widely used in diagnostics because they are sensitive, accurate, fast, simple, allow reaction monitoring in real time, and eliminate the need for post-PCR processing (Wilhelm and Pingoud, 2003; Shehata et al., 2021b). However, designing strain specific qPCR assays can be challenging especially when the target strain belongs to a highly isogenic taxon such as *Bifidobacterium animalis* subsp. *lactis* (Milani et al., 2013). Here, we present the development and validation of a qPCR method for the strain specific identification of a clinically important probiotic strain; *Bifidobacterium animalis* subsp. *lactis Bi-07*™.



Materials and Methods

To design a strain-specific qPCR assay, nucleotide variations were identified using multiple bioinformatic tools in the genome of strain *Bifidobacterium animalis* subsp. *lactis Bi-07* compared to closely related strains. The assay was validated following the guidelines for validation of qualitative real-time PCR methods for molecular diagnostic identification of probiotics (Shehata et al., 2019), which includes validation for specificity, sensitivity, and precision (Broeders et al., 2014;Shehata et al., 2019).

Assay specificity was first evaluated in silico then evaluated experimentally using 25 reference samples of *Bifidobacterium animalis* subsp. *lactis* strain Bi-07, and 22 non-target reference samples. To confirm strain level specificity, closely related *Bifidobacterium animalis* subsp. *lactis* strains (DSM 15954, HN019, Bl-04, B420, UABla-12, and HA-194) were included as non-targets. To determine reaction sensitivity, three standard curves were established. The assay was evaluated for precision using five samples tested at three different DNA concentrations. The applicability of the developed assay for strain detection in finished dietary supplements and food products was also evaluated.

Results and Discussion

Bioinformatic analyses identified a unique single nucleotide polymorphism (SNP) in the genome of *Bifidobacterium animalis* subsp. *lactis Bi-07*. A strain specific qPCR assay was designed to target the identified SNP. The target region for *Bifidobacterium animalis* subsp. *lactis Bi-07* assay codes for a glycosyltransferase (Shehata et al., 2021a). The SNP identified in *Bifidobacterium animalis* subsp. *lactis Bi-07* is unique to strain Bi-07 compared to all other *Bifidobacterium animalis* subsp. lactis strains deposited in GenBank, as of August 2021 (**Figure 1**).

Sequence ID	66 68 70 72 74 76 78 80 82 84 86 88 90 92	Organism	Identity
Query_225... (+)	T T G G A C C T C G T G C C A G C G C C G A C G C A G		100.00
CP003498.1 (+)	C	Bifidobacterium animalis subsp. lactis Bi-07	100.00
CP047190.1 (+)	C	Bifidobacterium animalis	99.19
CP042940.1 (+)	C	Bifidobacterium animalis	99.19
CP028460.1 (+)	C	Bifidobacterium animalis subsp. animalis	99.19
CP031154.1 (+)	C	Bifidobacterium animalis subsp. lactis	99.19
CP031703.1 (–)	C	Bifidobacterium animalis subsp. lactis	99.19
CP035497.1 (+)	C	Bifidobacterium animalis	99.19
CP022724.1 (+)	C	Bifidobacterium animalis subsp. lactis	99.19
CP017098.1 (+)	C	Bifidobacterium animalis	99.19
CP009045.1 (+)	C	Bifidobacterium animalis subsp. lactis	99.19
LR699002.1 (+)	C	Bifidobacterium animalis	99.19
CP007522.1 (+)	C	Bifidobacterium animalis subsp. lactis KLDS2.0603	99.19
CP007755.1 (+)	C	Bifidobacterium animalis	99.19
CP080571.1 (+)	C	Bifidobacterium animalis subsp. lactis	99.19
CP003941.1 (+)	C	Bifidobacterium animalis subsp. lactis ATCC 27673	99.19
CP004053.1 (+)	C	Bifidobacterium animalis subsp. lactis BI12	99.19
CP069249.1 (+)	C	Bifidobacterium animalis subsp. lactis	99.19
CP069248.1 (+)	C	Bifidobacterium animalis subsp. lactis	99.19
CP003039.2 (+)	C	Bifidobacterium animalis subsp. lactis BLC1	99.19
CP001853.2 (+)	C	Bifidobacterium animalis subsp. lactis BB-12	99.19
CP045589.1 (+)	C	Bifidobacterium animalis	99.19
CP003497.1 (+)	C	Bifidobacterium animalis subsp. lactis B420	99.19
CP002567.1 (+)	C	Bifidobacterium animalis subsp. animalis ATCC 25527	99.19
CP002915.1 (+)	C	Bifidobacterium animalis subsp. lactis CNCM I-2494	99.19
CP001892.1 (+)	C	Bifidobacterium animalis subsp. lactis V9	99.19
CP001606.1 (+)	C	Bifidobacterium animalis subsp. lactis DSM 10140	99.19
CP001515.1 (+)	C	Bifidobacterium animalis subsp. lactis BI-04	99.19
CP001213.1 (+)	C	Bifidobacterium animalis subsp. lactis AD011	99.19
CP015407.2 (–)	C	Bifidobacterium animalis subsp. animalis	98.37
CP065311.1 (–)	C	Bifidobacterium animalis	98.37

Figure 1: Multiple Sequence Alignment from NCBI Multiple Sequence Alignment Viewer 1.20.1 showing the single nucleotide polymorphism (SNP) identified and targeted in *Bifidobacterium animalis* subsp. lactis Bi-07 assay. The SNP identified was unique to strain Bi-07 compared to all other *Bifidobacterium animalis* subsp. *lactis* strains deposited in GenBank, as of August 2021.

Evaluating the specificity of *B. animalis* subsp. *lactis* Bi-07 specific assay in qPCR showed that all 25 target samples successfully amplified with a mean Cq of 24.41 (**Figure 2**). None of the non-target samples amplified in this assay, including the closely related *Bifidobacterium animalis* subsp. *lactis* strains (DSM 15954, HN019, Bl-04, B420, UABla-12, and HA-194) which confirms strain level specificity (**Figure 2**). The limit of detection (the minimum amount of target that can be detected by the assay) was 0.5 picograms of DNA, indicating that the assay is highly sensitive which is advantageous when detecting target strains that exist at low levels in multi-strain products. This limit of detection allows the identification of strain *B. animalis* subsp. *lactis* Bi-07 represented by as low as 0.05% of a blend.

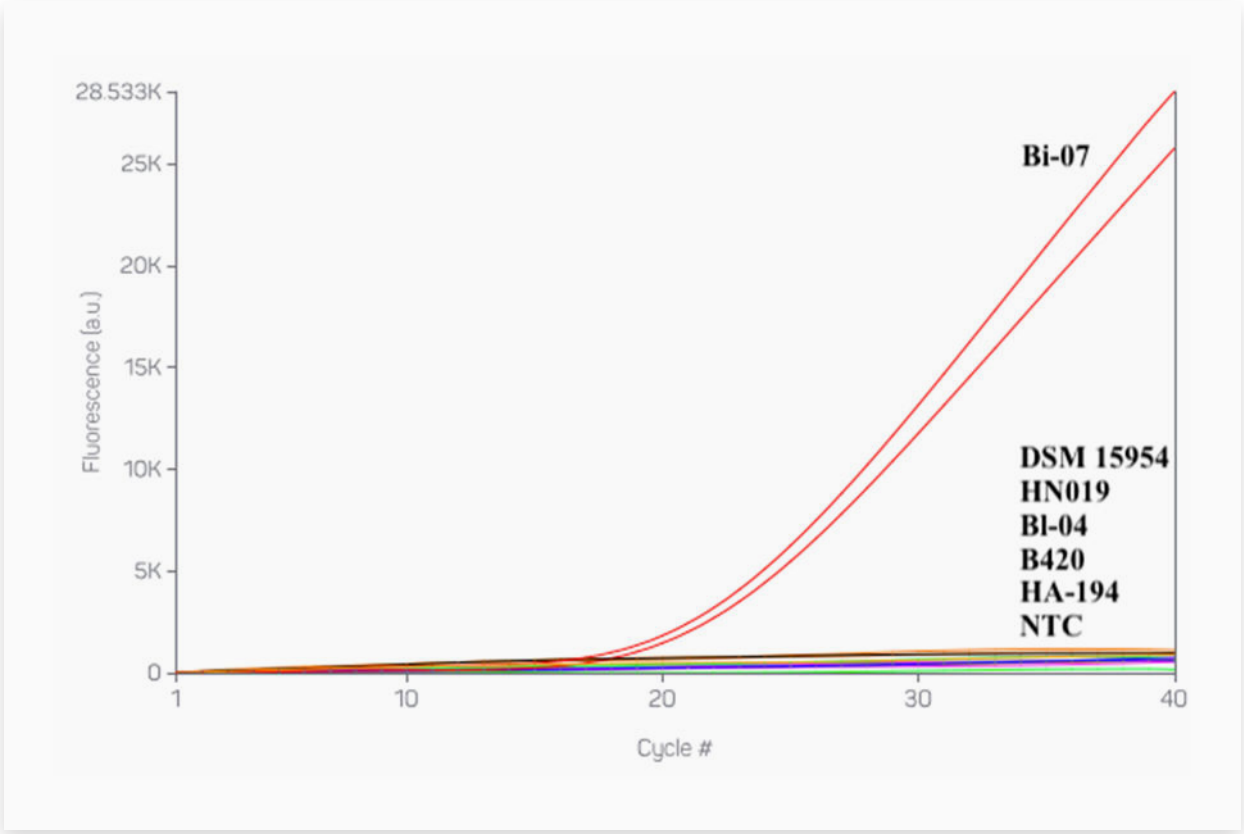


Figure 2: Evaluating the specificity of the assay developed for the strain-specific identification of *Bifidobacterium animalis* subsp. *lactis* Bi-07. Target samples successfully amplified while none of the non-target samples amplified, including the closely related *Bifidobacterium animalis* subsp. *lactis* strains (DSM 15954, HN019, BI-04, B420, and HA-194) which confirms strain level specificity. NTC is the No Template Control.

The assay was evaluated for precision and proved to be highly repeatable and reproducible with minimal variation when repeated on different days or on different qPCR platforms (Hyris bCUBE and QuantStudio 5). Evaluating the matrix effect of food and pharmaceutical ingredients on the assay performance to assess the applicability of the assay for food products and for finished pharmaceutical forms showed that all samples added to food (milk, juice, yogurt, chocolate, kombucha or protein powders) or mixed with multiple active and non-active ingredients (such as cellulose, silica, gum, salts, sugars, dietary fibres, or vitamins) successfully amplified indicating no PCR inhibitory effect. Thus, the assay proved to be applicable to samples in various matrices of foods or dietary supplement ingredients.

Conclusions

In a rapidly growing probiotic market, availability of methods that enable strain level identification is vital to facilitate authentication for probiotic researchers and probiotic manufacturers. The assay developed for the specific identification of strain *Bifidobacterium animalis* subsp. *lactis Bi-07* is a qPCR-based method with high specificity, sensitivity, and precision. The assay is applicable to mono-strain and multi-strain samples and to samples in various food matrices or mixed with active and non-active ingredients. The assay can be used on a standard qPCR platform such as QuantStudio 5 Real-Time PCR System or on a portable qPCR platform such as bCUBE for on-site testing. Such strain-specific identification methods demonstrating outstanding performance and wide applicability to matrix types and qPCR platforms are extremely valuable for strain level authentication to achieve compliance requirements in probiotic products and to ensure probiotic efficacy.

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