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Contaminants in shrimp probiotics - a potential emerging threat to food security

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Abstract

Probiotics are used widely in shrimp aquaculture to improve growth and prevent disease; however, for the most part their safety and efficacy remains largely unknown. Shrimp aquaculture may be vulnerable to the negative effects of unsafe and ineffective commercial products, such as the transfer of shrimp and human pathogens and antibiotic resistance genes. Shrimp health may also be adversely affected by the use of these products, potentially leading to crop yield losses. Here, we used 16S amplicon sequencing to identify the bacterial genera present in commercial shrimp probiotics and assess this against their listed product content. We identified the presence of additional genera to the labelled contents including *Escherichia/Shigella* and *Enterococcus* that may pose a risk of disease to animals and potentially humans that come into contact with these products, as well as potentially acting as carriers for virulence and antimicrobial resistance genes. Our results suggest that some commercial shrimp probiotics may be misleading to consumers and potentially unsafe for shrimp, people and the environment. We highlight the risks that contaminated commercial probiotics may pose to food security and present a series of safety and efficacy considerations to support the sustainable use of commercial shrimp probiotics.

Key words: sustainable shrimp aquaculture, food security, microbial contaminants, food safety, commercial probiotic safety, probiotic efficacy

1. Introduction

Shrimp are one of the world's most valuable aquaculture species and play an important role in providing food and economic security (Azra *et al*., 2021). Probiotics, 'live microorganisms that, when administered in adequate amounts, confer a health

benefit on the host' (Hill *et al*., 2014), are widely used in shrimp aquaculture to improve performance parameters such as growth, survival, and resistance to disease. Probiotic effects have been demonstrated for over 20 bacterial genera in shrimp, however, their mechanisms of action, safety, and efficacy remain largely unknown (Knipe *et al.,* 2021). Furthermore, there is currently considerable variation in the global regulation of animal probiotics (Leistikow *et al.,* 2022). At one end of the spectrum, the EU has strict regulation (Regulation (EC) No 1831/2003) for animal probiotics, classified as zootechnical additives [gut flora stabilisers], with only *Bacillus subtilis* C-3102 (DSM 15544) currently authorised for use in ornamental fish aquaculture (European Commission, 2023). In contrast, at the other end of the spectrum, animal probiotic regulation is still developing in global regions in low and middle income countries (LIMC), such as India and Bangladesh, where the Food Safety and Standards Authority of India (FSSAI) and Department of Livestock Services (DLS), respectively, are responsible for ensuring the safety and efficacy of commercial animal probiotics. Regardless of the country of origin and intended host, there remains significant issues with the quality, consistency, and safety of commercial probiotics (Fusco *et al.,* 2022; Kruasuwan *et al.,* 2023; Merenstein *et al.,* 2023). These products may contain microbial species that have not undergone a safety assessment, nor demonstrated a probiotic effect in their intended host. Without thorough product testing, some food animal sectors, including shrimp aquaculture, are potentially vulnerable to hazardous microbial contaminants and the transfer of antimicrobial resistance (AMR) (Fu *et al.,* 2020; Rokon-Uz-Zaman *et al.,* 2023).

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There are growing concerns that contaminated commercial animal feed probiotics may present an emerging threat to public health. For example, Cui *et al.,* (2020) isolated 65 Bacillus strains from probiotics intended for use in humans, animals, plants, aquaculture, and the environment in China, nearly half of which were capable of producing hazardous toxins and harboured multiple AMR genes coupled with mobile genetic elements. Similarly, Xu *et al.,* (2021) found that 88 enterococcal isolates from human and animal probiotics harboured virulence genes, AMR (including 77 that were highly resistant to gentamicin) and mobile genetic elements. Furthermore, these authors demonstrated that representative isolates were toxic in both *in vitro* (human intestinal epithelial cells) and *in vivo* (*Galleria mellonella*) infection models, causing cell and larval death respectively. Fu *et al.,* (2020) also found that more than one-third of animal-use probiotic products in China contained contaminating pathogens and antimicrobial resistance (AMR). In that work these authors also identified an anthrax toxin–positive *Bacillus cereu*s strain at a chicken farm and, using genomic surveillance techniques, showed transmission of this strain into the groundwater and to a nearby fish farm. A retrospective analysis of surveillance data confirmed the transmission of *B. cereus* from farm to humans (exhibiting intestinal anthrax symptoms). Anthrax toxin–positive strains were detected across 3 provinces, highlighting the widespread significance of animal-use probiotics. Their genomic analysis of shrimp aquaculture probiotics also confirmed the transmission of an emerging fish pathogen, *Acinetobacter pittii*, from a shrimp farm to the environment (groundwater and the Liaohe River (Xu *et al.,* 2021)). These studies not only suggest that contaminated commercial shrimp probiotics may facilitate the transfer of human pathogens and AMR genes, but also broader animal and environmental health and food security risks. For example, the use of probiotics

contaminated with shrimp pathogens may cause disease in shrimp, leading to crop yield losses.

For probiotics to be considered safe for use in the food chain, they need to be taxonomically well characterised and absent of potential functional traits of concern, including pathogenicity and virulence factors (e.g., toxins, invasion, and adhesion factors) and AMR. Furthermore, *in vivo* efficacy studies are required to demonstrate that there are no adverse effects in the host animal and that probiotic claims are substantiated. Importantly, product labelling must be accurate and accessible to the consumer, including strain level identification and clear safety instructions to ensure consumer protection. There has been an increasing number of recent reports, however, of commercial probiotic labelling inconsistencies, as well as calls for improved quality controls and regulation (Fusco *et al.,* 2022; Kruasuwan *et al.,* 2023; Roe *et al.,* 2022). Despite the widespread use of culture-based methods for probiotic identification and characterisation, their selectivity limits their ability to provide a comprehensive representation of microbial diversity in commercial products.

In response to the need for more accurate and reliable methods, we employed a culture-independent 16S amplicon sequencing approach to characterise the bacterial genera present in commercial shrimp probiotics. Our study aims to provide insight into the safety and efficacy of these products for use in the food chain, including a comparison of our results with the product labelling. We highlight the risks that contaminated commercial probiotics may pose to food security and present a series of safety and efficacy considerations to support the sustainable use of commercial shrimp probiotics.

2. Materials and methods

2.1 Commercial shrimp probiotics

We obtained 17 products commonly used (Table 1) in India (samples P01-P04 [n=4], purchased in 2016) and Bangladesh (purchased in 2016 (P05-P13) and 2019 (P14-P17) [n=13]). Probiotics from India were transferred into 50mL Falcon tubes before being transported to the UK, whereas probiotics from Bangladesh were received in their original packaging. All products were powders and stored at room temperature according to the manufacturer's instructions.

2.2 DNA extractions and 16S amplicon sequencing

Genomic DNA was extracted from approximately 0.1g of the commercial shrimp probiotics using a CTAB-based protocol (Chaput, 2021). To check successful extraction of DNA and amplification, a PCR was performed with the 515fB/806rB primer pair (Walters *et al.,* 2015), which targets the V4 hypervariable region of the bacterial 16S rRNA small subunit, and visualised by gel electrophoresis. The DNA extracts were quantified using a Qubit Fluorometer (ThermoFisher Scientific, Massachusetts, USA) following the manufacturers protocol and diluted to 2ng/ul with 10mM Tris-HCl pH 8.5. Gene libraries were constructed using a custom dual-index 1-step PCR (Kozich *et al.,* 2013) with adapted 515fB/806rB primers (Walters *et al*., 2015) and sequenced across three sequencing runs. For the PCR reactions, 1 µL genomic DNA (2 ng/ µL) was combined with 19 µL PCR grade water, 25 µL

NEBNext high-fidelity PCR master mix (New England Biolabs, Ipswich, MA, USA), 2.5 µL of the 10 µM barcoded forward primer, and 2.5 µL of the 10 µM reverse primer. The PCR block was pre-heated to 98 °C before inserting the 96-well indexed plates and an initial denaturation step was performed at 98 °C for 30 s, followed by 30 cycles of denaturing at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 2 min; holding the samples at 10 °C. To check DNA amplification, PCR products were visualised on an agarose gel. The PCR products were then purified using a magnetic bead clean-up developed by Jolivet and Foley (2015). The Promega QuantiFluor ONE dsDNA kit (Cat. No. E4871, Promega, Hampshire, UK) was used to quantify each cleaned PCR product. To check quality and average nucleotide length, we used a TapeStation instrument with High Sensitivity D1000 ScreenTape and reagents (Agilent, Santa Clara, CA, US). The PCR products [N=96] were pooled and the TapeStation was used again to ensure sample purity. Finally, the samples were submitted to the Exeter Sequencing Service for sequencing on the Illumina MiSeq platform with v2 chemistry (PE250) and 2x250 paired-end format. We multiplexed 96 samples per lane, however, the probiotic samples only made up a small proportion of each run. The first run contained a sample from probiotics P01, P03, P04, P05, P07, P09 and P13, as well as two replicates of a mock community (ZymoBIOMICS™ Microbial Community DNA Standard, Zymo Research, California, USA) and four negative controls [n=13]. The second run included probiotics P02, P06, P08, P10, P11, and P12, as well as four mock community positive controls and four negative controls [n=14]. Probiotics P14, P15, P16 and P17 were sequenced on the third run, along with 8 negative controls and four mock community samples [n=16]. Negative controls included both extraction blanks and PCR negatives for each run.

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2.3 Bioinformatics

To characterise the bacterial taxa detected in the commercial shrimp probiotics, the assembly and analysis of V4 16S rRNA amplicons was conducted in RStudio (v1.3.959) using R version 4.0.2 (R Core Team, 2020). Reads were filtered, trimmed (truncLen = c(200, 100)) and amplicon sequence variants (ASVs) were inferred for each MiSeq run independently, following the standard DADA2 workflow (Callahan *et al.,* 2016). ASVs with an unexpected length (outside of 250-256 bp) were removed to reduce the presence of sequencing errors. The runs were merged (minOverlap=40) and chimeras removed before assigning taxonomy with the SILVA (Yilmaz *et al*., 2014) (v138) reference database (at minBoot = 80). The 'addSpecies' function was used to make species level assignments based on exact matching (100% identity) between ASVs and the reference sequence and 'allowMultiple=TRUE' was specified to return multiple identifications. The resulting ASV table and sample metadata were combined in Phyloseq (McMurdie and Holmes, 2013). The Decontam package (Davis *et al.,* 2018) was used to detect and remove contaminant sequences present in the negative controls, using the "prevalence" method (presence/absence across samples) at a threshold of 0.5, which identifies sequences that are more prevalent in negative controls than in positive samples. Non-target sequences (eukaryotes or sequences unclassified at the Domain level, mitochondria and chloroplasts), samples with fewer than 200 reads and low abundance ASVs (with fewer than 5 counts in total) were removed from the final dataset. Mock community reference sequences were used to validate the accuracy of the resulting ASVs.

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As the overall aim of our study was to detect the bacterial taxa present in commercial shrimp probiotics and compare our findings to the product labels, that claim to contain only a few specific species, read normalisation (i.e., rarefying) was not necessary. The Phyloseq function 'tax_glom' was used to agglomerate the ASVs at both the phylum and genus levels, specifying 'NArm = FALSE' to keep the NA taxonomic assignments (McMurdie and Holmes, 2013). Relative abundance was calculated using the 'transform_sample_counts' function in Phyloseq (McMurdie and Holmes, 2013) and, to further improve the accuracy and reproducibility of this analysis (Cao *et al.,* 2021), only those groups with a mean relative abundance greater than 0.1% were kept. Results were visualised using ggplot2 (Hadley Wickham, 2016) and tidyverse (Wickham *et al.,* 2019). The package 'viridis' (Garnier *et al*., 2021) was used to make the plots colour-blind-friendly.

2.4 Safety and efficacy of shrimp probiotics

To make an assessment on the likely safety and efficacy of commercial shrimp probiotics for use in the food chain, the samples were subset according to which genera (or phyla, in the case of Cyanobacteria in P15) were labelled on the probiotic product description, unlabelled (contaminants), have members with Qualified Presumption of Safety (QPS) status (EFSA BIOHAZ, 2023) and/or have shown a probiotic effect in shrimp (Knipe *et al.,* 2021). All ASVs belonging to genera that were not listed on the product packaging were considered bacterial contaminants. Similarly, for products that did not list any taxonomic information, all ASVs were considered potential contaminants.

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The QPS list contains species that have undergone a risk assessment and are taxonomically well defined, lack pathogenic properties and AMR genes, do not produce antibiotics of clinical significance and, in review of the available literature, are considered safe for use in the food chain. There are currently 86 bacterial species across 38 genera on the QPS list (EFSA BIOHAZ, 2023).

The World Health Organisation (WHO) has published a list of antibiotic-resistant priority pathogens (WHO, 2017), which have been divided into three categories (critical, high and medium priority) according to the urgency for new antibiotics (Priority 1 (critical): *Acinetobacter baumannii, Pseudomonas aeruginosa*, Enterobacteriaceae, Priority 2 (high): *Enterococcus faecium, Staphylococcus aureus, Helicobacter pylori, Campylobacter* spp., *Salmonellae*, *Neisseria gonorrhoeae*, Priority 3 (medium): *Streptococcus pneumoniae, Haemophilus influenzae, Shigella* spp.). This list was used to identify ASVs belonging to, at the genus or family level, pathogens that pose the greatest threat to human health. The genera *Enterobacter* (Enterobacteriaceae), *Enterococcus* and *Pseudomonas* have also shown a probiotic effect in shrimp (supplementary SFig1), while members of the genus *Streptococcus* have additionally been awarded QPS status.

To further assess the safety of the probiotic products, we identified ASVs belonging to major shrimp and fish pathogens; *Vibrio* (Ina-Salwany *et al.,* 2019), *Aeromonas* (Vignesh *et al.,* 2022), *Pseudomonas* (Algammal *et al.,* 2020) and flavobacteria (Chen *et al.,* 2017).

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3. Results

3.1 Commercial shrimp probiotics

Three of the 17 probiotic products (P02, P08, P09) did not include any taxonomic information about the species contained (Table 1). Of those products with labelled taxa [n=14], 9 (64.2%) were missing at least one species name (stating only genera), and one product (P15) only stated that it contained members of the phylum Cyanobacteria. Notably, none of the labels stated a bacterial strain identification. Spelling mistakes on the packaging have been corrected in Table 1 and the labelling of *Lactobacillus lactis* in probiotic P11 is presumed to refer to *Lactococcus lactis*.

The probiotic products collectively claimed to contain 22 bacterial and 4 fungal species across 18 genera (15 bacterial and 3 fungal; *Aspergillus*, *Trichoderma* and *Saccharomyces*), as well as members of the phylum Cyanobacteria (Table 1). The most diverse genus labelled across all products was *Bacillus*, with 8 different species (and an additional unclassified *Bacillus* sp.; supplementary SFig 2a), followed by *Lactobacillus* and *Pseudomonas* (4 species each). *Bacillus*, present in 93% of products, was also the most common genus labelled as a shrimp probiotic, followed by *Lactobacillus* (labelled on 50%), *Nitrobacter* and *Nitrosomonas* (labelled on 36% of products each; supplementary SFig 2b). The most popular bacterial species was *Bacillus subtilis,* labelled on 79% of products, followed by *Bacillus licheniformis* (50%) and *Lactobacillus acidophilus* (36%; supplementary SFig3).

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Of the labelled probiotics, 12 (86%) claimed to contain at least one bacterial species with QPS status that has been shown to have a probiotic effect in shrimp, highlighted in Table 1. However, only two probiotics (P01 and P16) reported exclusively containing species that have QPS status and an established probiotic effect in shrimp. Over half of the products claimed to contain at least one bacterial species that does not have QPS status (Table 1). Additionally, whilst the bacteria labelled on probiotics P05, P06 and P07 (*B. subtilis* and *L. acidophilus*) have both QPS status and an established probiotic effect in shrimp, they also claim to contain the filamentous fungi *Aspergillus oryzae*; which has been excluded from the QPS list. A further 3 products (P11, P12, P15) also claimed to contain *A. oryzae,* with P11 additionally claiming to contain *A. niger* (excluded from the QPS list; Table 1).

3.2 16S amplicon sequencing & bioinformatics

With the exception of probiotics P08, P11 and P12, DNA was successfully extracted from all products [N=14] at the required concentration for sequencing. Nevertheless, we processed and sequenced the DNA extracts for probiotics P08, P11 and P12 to confirm that no biological material was in those products.

In total, there were 3,337,941 reads processed across the three runs, with an average of 7,340 reads generated per probiotic sample (ranging from 42 to 14513). After generating ASVs for each run separately, merging datasets and removing chimeric sequences, a total of 503 ASVs were detected across all samples [n=43]. Of these, the Decontam package (Davis *et al*., 2018) was able to detect and remove sequences belonging to 12 contaminant ASVs, present in the negative PCR and

sequencing controls. Mock community samples all contained 9 ASVs, which were exact matches to the reference sequences provided by the manufacturer, ensuring accuracy of the taxonomic assignment. Sequencing failed to generate enough high-quality reads for three probiotic samples (P08, P11 and P12) and so they were excluded from further analysis [N=14].

The ASV table, having removed non-target and low abundance sequences and samples, contained 357 ASVs across 14 probiotic samples, with an average sequencing depth of 6,336 high-quality reads (ranging from 587 to 12,439) per product. Taxonomic assignments were successfully made at the genus level for 212 (59.38% of) ASVs, representing 124 different genera and 16 phyla. Unambiguous species level assignments were made for only 31 (8.68% of) ASVs.

In the final ASV table, after removing rare taxa (with a mean relative abundance of less than 0.1%), however, there were 93 ASVs with 65 genus level assignments (69.89%) and 9 unambiguous species assignments (9.68%). We therefore used genus level taxonomic assignments to confirm the presence of labelled genera and whether members of the genus have shown to have a probiotic effect in shrimp. In total, there were 61 genera with a mean relative abundance of less than 0.1% across the dataset, 37.7% of which occurred in only one probiotic sample.

All products contained genera that could not be taxonomically assigned, representing 1.74 to 74.53% of the reads in each probiotic sample, with an average of 21.64% (Table 1).

We found that Firmicutes and Proteobacteria were the most common phyla amongst the probiotic products, except for P14 and P17 (unlabelled) where the most

abundant phyla were Cyanobacteria (Fig 2). The Cyanobacteria present in samples P14, P15 (labelled) and P17 was identified as Arthrospira_PCC-7345. Unassigned genera were most abundant overall across the whole dataset, followed by *Bacillus*, *Arthrospira_PCC-7345, Acinetobacter, Escherichia/Shigella* and *Lactobacillus* (Fig 1). However, *Acinetobacter* was the most prevalent amongst the samples (detected in 78.57% of the probiotics), followed by three unassigned genera (belonging to the orders Burkholderiales and Bacillales), *Brevibacillus* (64.29%), *Weissella* (50%) and *Escherichia/Shigella* (42.86%).

3.3 Accuracy of product labelling

Overall, we found that only five (35.71%) of the products (P01, P04, P05, P07 and P16) contained all of the labelled bacterial genera (Table 1), whereas the majority (57.14%) of the products were missing at least one of the labelled genera. We did not detect any of the genera labelled on probiotic P06. The proportion of reads assigned to labelled genera was, on average, only 25.6% (ranging from 0 to 75.95%), with the large majority belonging to the genus *Bacillus* (Fig 1). Probiotics P05, P06 and P07 all claimed to contain the same probiotic species; however, we detected several genera present exclusive to each of these products (Fig 1).

3.4 Safety and efficacy of shrimp probiotics

Out of the 61 genera identified in the commercial shrimp probiotics, only 6 contain species that have been awarded QPS status (*Bacillus, Lactobacillus, Lysinibacillus, Paenibacillus, Pediococcus* and *Streptococcus*; Fig 2). The genus *Xanthomonas*,

detected in probiotics P05 and P07, is included in the QPS list, however, for production purposes only and therefore viable cells must be absent i.e., it cannot be considered a probiotic. We identified 7 genera in the products that have shown probiotic effects in shrimp (*Bacillus, Enterococcus, Lactobacillus, Paenibacillus, Pediococcus, Pseudomonas* and *Streptococcus*; Knipe *et al*., 2021).

The proportion of reads assigned to genera with QPS ranged from 0 to 75.94% per probiotic and 0.79 to 75.94% of the reads were assigned to genera with probiotic effects (Table 1). We did not detect any genera with QPS status in probiotic P06 and in probiotic P14 the proportion was very low (0.79%). Similarly, these samples contained very low numbers of reads belonging to genera that have shown a probiotic effect in shrimp (3.92% and 0.79% respectively, Table 1). On average, per probiotics product, 38.8% of the reads belonged to genera with QPS status and 39.7% belonged to those that showed a probiotic effect in shrimp. The genus *Bacillus* was by far the most prevalent of all the genera shown to have a probiotic effect and have QPS status (Fig 1). Of the 10 ASVs that were unambiguously assigned at the species level, only *Bacillus amyloliquefaciens* has been awarded QPS status and has been shown to have a probiotic effect in shrimp.

Nine (64.29%) of the products tested contained genera that have shown a probiotic effect in shrimp and have been awarded QPS status (P01, P02, P03, P05, P07, P09, P10, P15, P16; Fig 1) that did not appear on the product labelling.

3.5 Bacterial contaminants and potential pathogens

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All of the commercial shrimp probiotics contained unlabelled, contaminant genera, ranging from 24.06% to 98.13% of the reads per sample (Table 1, Fig 2). On average, 70.52% of the reads obtained from commercial shrimp probiotics were assigned to taxa that were not listed on the product label and therefore considered contaminants. This includes the genera for which no taxonomic assignments are made. Probiotic P10 contained a very large proportion of reads (74.53%) that could not be assigned at genus level, belonging to the orders Enterobacterales and Bacillales. Contaminant reads accounted for more than 50% of the reads present in 10 (71.43%) of the probiotics, with over 90% of reads in 4 products (P02, P06, P09 and P14) belonging to unlabelled bacterial contaminants.

There were no ASVs belonging to the genus *Vibrio* or *Aeromonas* (containing shrimp pathogens), however the presence of flavobacteria was detected in P14, P16 and P17 at low relative abundance (0.56%, 0.06% and 1.33% per sample, respectively. Furthermore, *Pseudomonas* spp. was found in 8 of the probiotics (Fig 1).

The species *P. aeruginosa* is also in the Priority 1 critical category of the WHO Priority pathogens list, along with *Acinetobacter baumannii* and the family *Enterobacteriaceae.* Members of the genus *Acinetobacter* were detected in 11 (78.57% of) probiotic products, with one unambiguous assignment of *Acinetobacter lwoffii* present in two products (P06 and P17). The family Enterobacteriaceae was detected in 10 (71.43%) of the commercial shrimp probiotics, with the genus *Escherichia/Shigella* (also in the Priority 3 category) detected in six of them (42.86%). Of the Priority 2 (high) category of the WHO Priority pathogens list, we were detected the genera *Enterococcus* in probiotics P01 and P16. The only other

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genera of this category detected was *Staphylococcus*, that was present in two of the products (P03 and P15). Out of the Priority 3 (medium) category, we detected *Streptococcus* in 7 products.

On average, 9.80% of the reads generated from each commercial shrimp probiotic was assigned to a genus listed as a WHO Priority pathogen (Table 1, Fig 1). There were no ASVs belonging to priority pathogens in probiotic P14, whereas all other products contained reads assigned to these genera (ranging from 0.15 to 25.36%; Table 1).

4. Discussion

Accurate labelling is an important aspect of probiotic safety, allowing consumers to make informed decisions and take appropriate actions to mitigate the potential risks associated with the product use. We found multiple issues with commercial shrimp probiotic labelling, however, including spelling mistakes and missing taxonomic information (species names and strain identification). This creates a significant problem, since strains of the same species do not necessarily share the same functional traits and so probiotic effects and safety cannot be assumed (Fu *et al*., 2020). This not only creates an obvious limitation for the interpretation of our results, (discussed later), but highlights the importance of including strain level identifiers (on all copy, i.e., websites) to avoid misleading consumers, such as shrimp farmers. This is particularly true for multi-strain/species products for which the combinatory effect of individual taxa is completely unknown and cannot be predicted (Knipe *et al*., 2021), highlighting the urgent need for further research to support probiotic claims.

Similarly, misuse of the term ‘probiotic’ on commercial products may lead consumers to wrongly believe that the product will not only be safe but confer a beneficial effect on shrimp. We found a number of listings that have not shown a probiotic effect in shrimp, nor awarded QPS status, suggesting that these products (even without further analysis) are potentially unsafe and ineffective. For example, as well as being an opportunistic human pathogen (Balajee *et al*., 2009), *A. niger* causes collar rot disease in the economically important crop species *Arachis hypogaea* (groundnut). Asia, predominantly China and India, produces approximately 70% of the world groundnut (Kumari *et al*., 2017) and there is potentially a risk of collar rot infection from this probiotic product if it contaminates the soil surrounding shrimp farms with potentially far-reaching consequences for animal, human and environmental health and food security for these regions. The presence of this filamentous fungi was not confirmed in this study, nevertheless these results suggest that some manufacturers may actively be misleading consumers. To protect animal, human and environmental health, closer links between policymakers, scientists and users of the probiotic products would help to better ensure that probiotic manufacturers list appropriate strain level identifiers (in an accessible way, mindful of the languages spoken by target consumers) and that these strains are indeed safe and effective probiotics. Even without additional testing, educating shrimp farmers, probiotic manufacturers and distributors, and screening product labels are likely to be a highly cost-effective way of reducing the potentially harmful impacts of these products.

Culture based methods have traditionally been employed to identify and assess the safety of commercial probiotic products. For example, Noor Uddin *et al*., (2015) found that commercial shrimp probiotics used in Vietnam contained additional

Bacillus species when compared to product labelling and some specified *Bacillus* species in the product were in fact absent. A major drawback of culture-based methods is, however, that they may be unable to detect the full range of bacteria present. Nevertheless, the authors also found a number of bacteria that indicated contamination during the probiotic manufacturing process. Using culture-independent 16S rRNA gene amplicon sequencing, we show that our results are consistent the culture-based work of Noor Uddin *et al*. (2015). None of the commercial shrimp probiotics tested in this study were accurately labelled and all products contained bacterial contaminants, including the few products in which we detected all of the bacterial genera listed. Furthermore, Noor Uddin *et al*., (2015) found several bacterial isolates that were resistant to multiple (in some cases 4) antimicrobials of clinical significance. We were unable to confirm the presence of AMR in this study, but our results suggest a high likelihood that the commercial shrimp probiotics tested are carriers of multiple AMR genes (as well as other genetic elements of concern such as virulence factors); given the diverse nature of the microbial contaminants, of which a large proportion lack QPS status and are potentially pathogenic or completely unknown (unassigned). Our results suggest reasonable likelihood for commercial shrimp probiotics to contribute to the global AMR burden. The species contained in animal probiotic products can transfer between food systems and their use may impact multiple industries, emphasising further the potential problem. Commercial shrimp probiotics may, if not thoroughly assessed for safety, act as vectors for disease and AMR genes. In a recent study, it was found that processed shrimp (i.e., cooked) had an increased abundance of AMR genes present in the gut microbiome when compared with raw shrimp, suggesting that thermal stresses can induce cross-adaptation (selection) for AMR (Giacometti

and Shirzad-aski, 2021) and that the problems presented by the use of these products may be amplified downstream in the food chain (Sharma *et al*., 2021). The most accurate way to taxonomically identify the species (strain) and demonstrate a lack of pathogenic properties (virulence factors) and AMR genes, as well as confirming that the species does not produce toxins or antibiotics of clinical significance, is whole genome sequencing. To reduce the potentially negative impacts of commercial shrimp probiotics on food security, reference quality genomes need to be established and made available for all commercial shrimp probiotics on the market.

We did not detect any *Vibrio* spp., (consistent with the findings of Noor Uddin *et al*., (2015)), however, we did find a number of ASVs belonging to genera such as *Flavobacterium* and *Pseudomonas* that may be pathogenic to fish and shrimp (Chen *et al*., 2017). It is also possible that these products contain species that do not directly cause disease, but rather destabilise the microbial community dynamics of the shrimp gut (or pond), leading to dysbiosis and an environment which favours opportunistic pathogens. In turn, shrimp health could therefore be negatively affected by the use of these products, leading to crop yield losses and directly impacting food security. Consistent with our findings, Vargas-Albores *et al*., (2016) also found that Firmicutes and Proteobacteria were the most abundant bacterial phyla in a contaminated commercial shrimp probiotic mixture. They further showed that in combination with *B. subtilis*, the contaminant bacteria had an immunostimulatory effect on the shrimp studied (*Litopenaeus vannamei*) and resulted in better survival. This suggests that there may still be some beneficial short-term effects on shrimp

health. The impact of probiotic application on the gut microbiome and disease susceptibility of shrimp, however, is beyond the scope of this study.

The widespread detection of genera that potentially contain WHO priority human pathogens (*Pseudomonas, Acinetobacter, Escherichia/Shigella, Enterococcus, Staphylococcus, Streptococcus* and the family *Enterobacteriaceae*) in commercial shrimp probiotics emphasises the need to more thoroughly assess these products for their safety, especially in environments without proper safety equipment, as is common in many shrimp aquaculture regions. None of the products instructed users to do so, potentially putting shrimp farmers at personal health risks. The presence of the genus *Pseudomonas* and *Shewanella* suggests that these commercial products may contain specific spoilage organisms (SSOs). This in turn can lead to spoiled shrimp (Fan *et al*., 2022) and a loss of profit. However, further research is required to fully assess the impact of these products on food security.

We detected the presence of the genus *Xanthomonas*, which contains species that can cause disease in over 400 different plants (including rice, wheat and bean; Timilsina *et al*., 2020), raising further concerns for the wider impact of these products on environmental health and food security. Commercial animal feed probiotics often contain additional ingredients such as plant material. This may introduce contaminants, and more specifically plant pathogens, to the product during the manufacturing process. It is therefore advisable that all ingredients are appropriately assessed for quality assurance before adding them to the final product, which again should be screened for microbiological, chemical and botanical hazards before entering the market. Our results indicate that at least two of these products (P09 and

P16) may be contaminated with faecal matter, as we detected the indicator genus *Proteus* (Drzewiecka, 2016). Further testing is required to assess the batch variability in these products, however, as Noor Uddin *et al*., (2015) found that they could not isolate the same strains from different batches of the same, contaminated, commercial shrimp probiotic.

Species level identification based on 16S rRNA gene amplicon sequencing is often limited by the ambiguity of the region shared between members of the same genus and the availability of reference sequences, leading to a large proportion of unassigned ASVs. The optimal identity threshold for sequencing the V4 hypervariable region of the 16S rRNA gene is 100% (Edgar, 2018), ensuring that species assignments are made unambiguously. It was therefore necessary to use genus level assignments, for which large variability in functional traits exists, to more broadly assess the safety and efficacy of these products. A major limiting factor in this study is, therefore, that we were largely unable to confirm the presence nor viability of specific bacterial species. It is entirely possible that the bacteria present in commercial shrimp probiotics may not be viable. A combination of shotgun metagenomic sequencing and bacterial cell enumeration by flow cytometry (Lugli *et al*., 2022), however, would allow for a more accurate picture of commercial shrimp probiotic safety and efficacy. Nevertheless, there may still be significant issues associated with the administration of dead bacteria, particularly from uncharacterised and contaminant taxa, such as through overstimulating the host immune system (with microbial fragments such as surface proteins, for example) in diseased and/or stressed animals and the transfer of genetic material (such as virulence factors and AMR (De Simone, 2017, Kittredge *et al*., 2022, Merenstein *et al*., 2023)).

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Whilst a lack of QPS status raises significant safety concerns that warrant further investigation, it also highlights the growing need to assess the safety of more microorganisms for use in the food chain. Research practises in the probiotics field are often limited by their reliance on species that are already considered safe by regulatory authorities, for example members of the QPS list. However, this is not necessarily reflective of the broad spectrum of microbial species that could be considered probiotic. This is particularly important to consider with the recent advances in next generation probiotic (NGP) selection, as it is unlikely that the QPS list currently reflects the species most likely to be selected as shrimp probiotics in the future. Therefore, researchers should provide evidence of safety to support probiotic claims, rather than rely on pre-assessed species. To avoid inaccurate results, we recommend that researchers validate the presence of labelled species when using commercial shrimp probiotics in their studies. This will also aid feed probiotic regulators, as safety assessments largely rely on the available body of scientific knowledge.

5. Conclusion

Stentiford *et al*., (2020) used a One Health approach (by considering that the health of humans, animals, and the environment are interconnected) to define a series of success metrics to achieve more productive and sustainable aquaculture. Given the potential far-reaching effects that contaminated commercial shrimp probiotics may have on animal, human and environmental health and food security, a One Health approach to probiotic use in animal feeds could significantly improve food security.

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Probiotic	Genus	Species	Proportion of reads assigned to genus (%)	Proportion of reads assigned to labelled genera (%)	Proportion of reads assigned to genera with QPS (%)	Proportion of reads assigned to genera with probiotic effect (%)	Proportion of contaminant reads (%) [inc unlabelled]	Number of contaminant genera detected [inc NA]	Proportion of contaminant reads unassigned at genus level (%)	Proportion of reads assigned to WHO pathogen genera (%)

Our results suggest that contaminated commercial shrimp probiotic products may limit progress towards sustainable aquaculture and improved food security by acting as vectors for pathogenic species, AMR genes and/or species that promote environmental conditions that increase disease susceptibility. Commercial shrimp probiotics may be misleading to consumers and potentially unsafe for shrimp, people and the environment. Our results suggest that contaminated shrimp probiotics may pose an emerging threat to food security and scientists, policymakers and stakeholders within and between probiotic subsectors need to collaborate to design and implement strategies to improve methods for the assessment of probiotic safety and efficacy.

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7. Data availability

The raw-read datasets were deposited in The European Nucleotide Archive (ENA) under accession no. PRJEB58029.

8. Table

P01	Lactobacillus	plantarum*	10.2	11.74	43.70	44.16	88.06	11	5.7	25.36
	Pediococcus	acidilactici*	1.54							
P02	NA	NA	0	0	70.44	70.59	95.72	11	9.03	0.15
P03	Bacillus	NA	24.88	24.88	32.80	31.2	69.75	19	37.32	8.77
	Streptococcus	NA	0							
P04	Bacillus	subtilis*	75.01	75.95	75.94	75.94	24.06	7	18.52	1.81
		licheniformis*								
	Lactobacillus	acidophilus*	0.62							
		sporogenes								
	Saccharomyces	NA	NA							
	Streptococcus	Lactis	0.32							
P05	Aspergillus	oryzae	NA	43.32	43.47	44.45	46.46	19	16.39	13.75
	Bacillus	subtilis*	42.29							
	Lactobacillus	acidophilus*	1.03							
	Saccharomyces	cerevisiae	NA							
P06	Aspergillus	oryzae	NA	0	0	3.92	98.13	15	2.39	20.61
	Bacillus	subtilis*	0							
	Lactobacillus	acidophilus*	0							
	Saccharomyces	cerevisiae	NA							
P07	Aspergillus	oryzae	NA	20.96	21.47	22.97	62.09	23	26.59	11.27
	Bacillus	subtilis*	20.75							
	Lactobacillus	acidophilus*	0.21							
	Saccharomyces	cerevisiae	NA							
P08	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
P09	NA	NA	0	0	60.89	60.89	99.14	12	25.48	9.96
P10	Bacillus	subtilis*	6.41	8.67	15.65	17.91	87.87	12	74.53	5.47
		megaterium*								
	Nitrococcus	NA	0							
	Pseudomonas	florecium	2.26							
	Thiothrix	NA	0							
P11	Aspergillus	oryzae	NA	NA	NA	NA	NA	NA	NA	NA
		niger								
	Bacillus	subtilis*	NA							
		licheniformis*								
		polymyxa								
		megaterium*								
		pumilus*								
	Cellulomonas	uda	NA							
	Lactococcus	lactis*	NA							
	Lactobacillus	helveticus								
	Nitrosomonas	NA	NA							
	Nitrobacter	NA	NA							
	Pseudomonas	denitrificans	NA							
		putida								
	Saccharomyces	cerevisiae	NA							
	Thiobacillus	thiooxidans	NA							
P12	Aspergillus	oryzae	NA	NA	NA	NA	NA	NA	NA	NA
	Bacillus	subtilis*	NA							
		licheniformis*								
		mensentericus								
	Lactobacillus	acidophilus*	NA							
	Nitrosomonas	NA	NA							
	Nitrobacter	NA	NA							
	Saccharomyces	cerevisiae	NA							
P13	Bacillus	NA	59.38	59.38	59.38	59.38	40.29	5	30.84	0.24
	Pediococcus	NA	0							
P14	Bacillus	subtillus*	0.79	0.79	0.79	0.79	94.21	10	1.74	0
		linchcniformis*								
		polymyxin								
	Nitrobacter	NA	0							
	Nitrosomonas	NA	0							
	Pseudomonas	denitnificans	0							
P15	Aspergillus	oryzae	NA	37.41	38.71	38.86	62.44	13	38.79	22.55
	Bacillus	subtillus*	37.16							
		linchcniformis*								
	Cyanobacteria	NA	0.10							
	Nitrobacter	winogradskyi	0							
	Nitrosomonas	europea	0							
	Pseudomonas	denitrificans	0.15							
		oxalaticus								
	Rhodococcus	NA	0							
	Rhodospirillum	ubrum	0							
	Trichoderma	viride	NA							
P16	Bacillus	Subtilis*	62.24	62.24	66.66	71.75	35.66	15	13.51	9.72
		licheniformis*								
		megaterium*								
		amyloliquefaciens*								
P17	Aerobactor	NA	0	13.01	13.01	13.01	83.37	12	2.17	7.59
	Bacillus	subtilis*	13.01							

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		*licheniformis**							
	Nitrobacter	*NA*	*0*						
	Nitrosomonas	*NA*	*0*						
Average [N=14]				*25.60*	*38.78*	*39.70*	*70.52*	*13.14*	*21.64*	*9.80*

Table 1. Commercial shrimp probiotics. Taxonomic information provided on the product packaging by the manufacturer. Bacterial genera were detected by amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene.

** Bacterial species with QPS status and reported probiotic effect in shrimp. NA Data not available due to no DNA.*

Figure 1. Relative abundance (%) of labelled and contaminant genera present in commercial shrimp probiotics. Genera in bold include members that have been awarded Qualified Presumption of Safety (QPS) status, whilst genera in italics contain species that have demonstrated a probiotic effect in shrimp. Genera that share members with the World Health Organisation (WHO) Priority Pathogen list are also highlighted. Taxa were identified by amplicon sequencing of the V4 hypervariable region of the 16s rRNA gene. Genera are ordered in decreasing order of overall abundance across the whole data set.

Figure 2. Relative abundance (%) of the bacterial phyla detected in commercial shrimp probiotics. Bacteria were identified by amplicon sequencing the V4 hypervariable region of the 16S rRNA gene.

9. Supplementary material

Supplementary results include original and filtered ASV table, relative abundance of ASVs grouped by genus and their corresponding QPS status, WHO priority

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pathogen group and presence in the literature. Supplementary figures 1-3 are accompanied by descriptive captions.

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Hazel Knipe: Conceptualization, Data curation, Formal Analysis, Visualization, Writing – original draft, investigation. **Dominique Chaput**: Supervision, Data curation, Writing – review & editing. **Siddhwartha Kumar Basak**: Resources, Investigation, Writing – review & editing. **Anke Lange**: Supervision, Writing – review & editing. **Charles R. Tyler**: Conceptualization, Funding acquisition, Investigation, Writing – review & editing, Supervision.

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- Commercial shrimp probiotics were found to contain contaminant bacterial DNA
- Contaminated commercial shrimp probiotics may negatively impact food security
- Improved probiotic regulation is required for sustainable shrimp aquaculture

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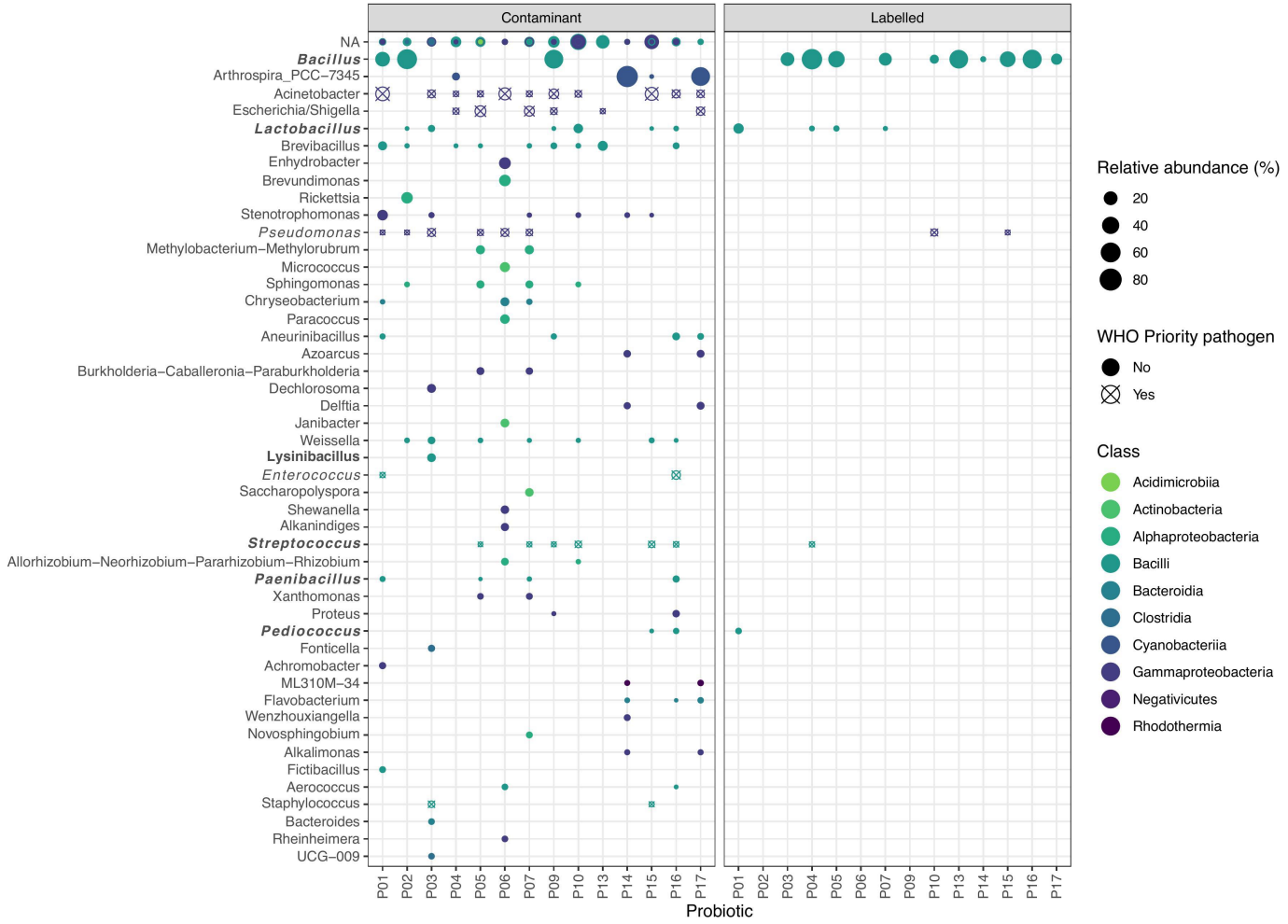
Contaminant
Labelled
NA
Bacillus
Arthrospira_PCC−7345
Acinetobacter
Escherichia/Shigella
Lactobacillus
Brevibacillus
Enhydrobacter
Brevundimonas
Rickettsia
Stenotrophomonas
Pseudomonas
Methylobacterium−Methylorubrum
Micrococcus
Sphingomonas
Chryseobacterium
Paracoccus
Aneurinibacillus
Azoarcus
Burkholderia−Caballeronia−Paraburkholderia
Dechlorosoma
Delftia
Janibacter
Weissella
Lysinibacillus
Enterococcus
Saccharopolyspora
Shewanella
Alkanindiges
Streptococcus
Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium
Paenibacillus
Xanthomonas
Proteus
Pediococcus
Fonticella
Achromobacter
ML310M−34
Flavobacterium
Wenzhouxiangella
Novosphingobium
Alkalimonas
Fictibacillus
Aerococcus
Staphylococcus
Bacteroides
Rheinheimera
UCG−009
P01
P02
P03
P04
P05
P06
P07
P09
P10
P13
P14
P15
P16
P17
Probiotic
Relative abundance (%)
20
40
60
80
WHO Priority pathogen
No
Yes
Class
Acidimicrobiia
Actinobacteria
Alphaproteobacteria
Bacilli
Bacteroidia
Clostridia
Cyanobacteriia
Gammaproteobacteria
Negativicutes
Rhodothermia

Figure 1

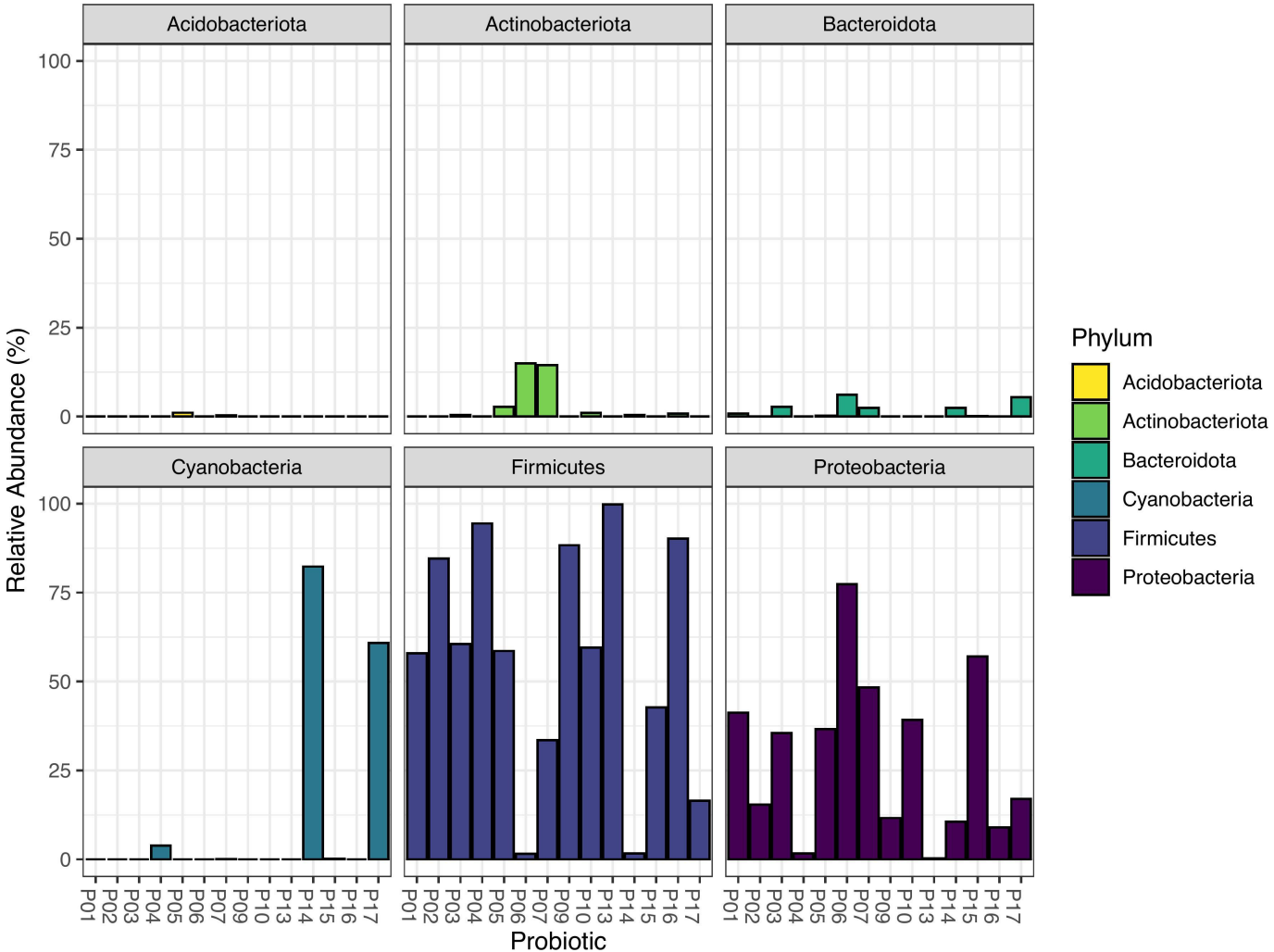
Acidobacteriota
Actinobacteriota
Bacteroidota
Cyanobacteria
Firmicutes
Proteobacteria
Relative Abundance (%)
100
75
50
25
0
P01
P02
P03
P04
P05
P06
P07
P09
P10
P13
P14
P15
P16
P17
Probiotic
Phylum
Acidobacteriota
Actinobacteriota
Bacteroidota
Cyanobacteria
Firmicutes
Proteobacteria

Figure 2