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## From *in silico* screening to *in vivo* validation in zebrafish – a framework for reeling in the right psychobiotics†

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The potential of gut bacteria to interact with the nervous system is now well known. Therefore, the characterization of bacterial strains that can modulate signalling pathways of the nervous system is a topic of growing interest, as it represents a potential alternative therapeutic target to treat central nervous system disorders. However, a streamlined screening framework is required to guide the rational identification and selection of such bacteria, known as psychobiotics. In this work, we introduce a framework that integrates *in silico*, *in vitro* and *in vivo* approaches to identify psychobiotic candidates capable of both metabolising prebiotics of interest and producing neuroactive molecules. To prove the effectiveness of the approach, we characterized a bacterial strain, *Lactiplantibacillus plantarum* APC2688, for its capacity to modulate the GABAergic system and alter the stress-related behaviour of zebrafish larvae. In brief, *in silico* analyses of the genomic content of APC2688 identified it as capable of degrading different prebiotics and producing neuroactive compounds known to modulate the stress response in animal models. Then, *in vitro* results confirmed the ability of this strain to produce GABA, tryptophan and acetate, while growing with the candidate prebiotics of interest, fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inositol. *In vivo* experiments demonstrated that the administration of bacterial supernatants induced changes in the expression of *gad1* and *gabra1* in zebrafish larvae, two essential genes in the GABAergic signalling pathway, and altered the anxiety-like behaviour of the larvae. These results highlight the efficiency of our framework in integrating orthogonal approaches to discover and characterise bacteria capable of modulating the microbiome–gut–brain axis.

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## Introduction

The bacteria in the gut microbiome have been identified as a key regulator of the communication between the gut and the

brain.<sup>1</sup> Many carbohydrate-degrading enzymes have been identified in the genomes of gut bacteria.<sup>2,3</sup> This facilitates the degradation of complex prebiotic fibres such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS), and simpler molecules, such as dextrose or myo-inositol, the most abundant isomer of inositol.<sup>4</sup> Once in the gut, some bacteria metabolise the fibres undigested by the host, producing molecules that can modulate the gut–brain axis.<sup>5–7</sup> These and other observations led to the coining of the term “psychobiotic”, including living beneficial bacteria (probiotics) and prebiotics that influence bacteria–brain relationships.<sup>8,9</sup>

It has been proposed that the administration of psychobiotic bacteria represents an alternative avenue to support brain health and the management of disorders such as anxiety and depression.<sup>10–13</sup> Indeed, bacterial strains have been reported to produce molecules involved in the stress response of animal models, such as GABA,<sup>14,15</sup> acetate<sup>16,17</sup> and tryptophan.<sup>18</sup> Some bacterial strains may also modulate the expression of genes involved in the stress response, such as glutamic acid

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decarboxylase 1 (*gad1*) and  $\gamma$ -aminobutyric acid receptor subunit alpha-1 (*gabra1*),<sup>19,20</sup> involved in the GABAergic signalling pathway; the aryl hydrocarbon receptor 2 (*ahr2*),<sup>21</sup> involved in the sensing of metabolites derived from tryptophan; and brain-derived neurotrophic factor (*bdnf*),<sup>20</sup> relevant in neuronal growth and survival. Moreover, administration of psychobiotics has been successfully used to reduce anxiety-like behaviour in animal models<sup>22</sup> and to modulate stress,<sup>23,24</sup> sleep<sup>25</sup> and brain activity<sup>26</sup> in healthy humans.

Although psychobiotics hold the potential to modulate gut-brain signalling, workflows for the rapid and rational screening and identification of promising candidates are still lacking although the potential benefits of their development and use have been highlighted in the literature.<sup>27,28</sup> Current thinking advocates the earlier incorporation of *in silico* predictive tools<sup>29</sup> and increased use of simpler genetic model organisms.<sup>12</sup> This work presents a workflow that integrates *in silico*, *in vitro* and *in vivo* approaches, most often conducted in isolation, to unveil promising new psychobiotic candidates that fit a desired functional profile. This profile consists of defining prebiotics of interest that the bacterial strain can metabolise and neuroactive molecules of interest to be produced by the strain. To test its effectiveness, we defined a profile of interest, which combined the capability to degrade three selected prebiotics of different complexities (FOS, GOS and inositol) with the ability to produce three molecules known to modulate stress response in animals (GABA, acetate and tryptophan). Publicly available genomes were leveraged, and four *Lactiplantibacillus plantarum* strains were found to fit that profile. Therefore, we characterized a novel closely related strain, *L. plantarum* APC2668,<sup>30</sup> isolated in previous studies conducted in our research centre (NCBI assembly accession code: GCA\_040183155.1). *In silico* analysis of its genome demonstrated that the strain also fits the desired profile. *In vitro* experiments confirmed APC2668's potential to degrade the prebiotics of interest while producing the neuroactive molecules of interest. Additional *in vivo* experiments conducted in zebrafish larvae showed that the supernatant of APC2668 grown on GOS, FOS, or inositol can alter the expression of relevant genes (including *gad1* and *gabra1*), and the stress-related behaviour of the model organism.

## Methods

### *In silico* search for candidate bacteria with neuroactive potential and the potential to degrade prebiotics

A manually curated list of enzymes that catalyse catabolic reactions of prebiotics of different complexities was generated by searching the KEGG<sup>47</sup> and MetaCyc<sup>48</sup> databases. The complete list of enzymes and prebiotics can be found in ESI Table 1.† Then, using the R package KEGGREST v.1.40.0, the 6963 bacterial genomes available in the KEGG database were screened to identify bacteria carrying any of those enzymes, as a reflection of their potential to degrade the prebiotics of interest, subsequently discarding genomes lacking them. Next,

genomes were further screened to identify Gut Brain Modules (GBMs), a list of 56 metabolic pathways and their respective enzymes previously defined.<sup>7</sup> These enzymes are involved in synthetic pathways of neuroactive molecules. To determine the presence of GBMs from the enzymes annotated in each genome, the OmixerRpm R package v. 0.3.3 was used.<sup>31</sup> As there are several enzymes in each pathway, the genes for all the enzymes per pathway had to be identified in any given genome to consider the GBM as present. Finally, the list of bacteria with the potential to degrade prebiotics and to synthesise neuroactive molecules was further filtered to only include bacteria previously isolated from human gastrointestinal tracts using an in-house catalogue of bacteria isolated from different studies performed at APC Microbiome Ireland. This final list was then used to determine which species could be of interest to look for new strains with the three desired characteristics: (1) the ability to degrade all three named prebiotics of interest, as they covered a range of different molecular complexities, (2) the ability to produce the three neuroactive molecules of interest, and (3) the ability to live in the human gut. The script used to generate the list of bacteria with the desired characteristics can be accessed from [https://github.com/Benjamin-Valderrama/kegg\\_prebiotics](https://github.com/Benjamin-Valderrama/kegg_prebiotics), and a visual representation of the workflow along with the number of bacterial genomes identified as successful candidates in each step is provided (ESI Fig. 1†).

### Genomic analysis of a new strain from the best psychobiotic candidate species identified (*L. plantarum* APC 2688)

Whole genome sequencing was performed on *L. plantarum* APC2688 as described previously.<sup>30</sup> First, DNA was extracted using the Qiagen PowerFecal Pro DNA kit (Qiagen) according to the manufacturer's guidelines and libraries were prepared using the Nextera XT DNA Library Preparation Kit. Library preparation steps included DNA fragmentation, adapter ligation, amplification, and size selection steps. Sequencing was performed using an Illumina NextSeq2000 (2 × 150 bp). The quality of the raw sequencing data was visualized using FastQC v0.11.9.<sup>32</sup> Shotgun genome sequencing data were then processed through an analysis workflow that utilizes the KneadData v0.9.0 wrapper to obtain quality-controlled reads. Quality filtering was performed using Trimmomatic v0.33<sup>33</sup> with the following parameters: ILLUMINACLIP:/NexteraPE.fa:2:30:10:8:True SLIDINGWINDOW:5:25 MINLEN:110 TRAILING:25 AVGQUAL:25. Genome assembly was performed using Unicycler v0.5.0.<sup>34</sup> The completeness and the contamination percentage of the assembled genome sequence was determined using CheckM v1.1.3,<sup>35</sup> and GTDB-Tk v1.3.0<sup>36</sup> was used for taxonomic classification of the genome. Finally, the annotated genome was queried for the presence of the enzymes involved in the catabolism of the prebiotics of interest and the enzymes in the GBMs pathways.<sup>7</sup> The GBM abundance and coverage analysis was performed using OmixerRpm R package v. 0.3.3 with a coverage cutoff of 1, as the goal of the analysis was to identify complete biosynthetic pathways within the genomes of the bacteria.



### Bacterial growth curves

The growth media used in the experiment consisted of either the basal modified MRS medium with no dextrose (hereafter referred to as mMRS-C) (Condalab MRS Broth w/o Dextrose; catalogue number: 1295), or the same mMRS-C supplemented with 2% (w/v) FOS, GOS, inositol (provided by Reckitt Inc.) or dextrose. Basal mMRS-C used to prepare other media was autoclaved at 121 °C for 5 min prior to the addition of another carbon source (either a prebiotic or dextrose), which was dissolved in dH<sub>2</sub>O and sterilised with 0.2 µm filters before addition to mMRS-C. All media were allowed to deoxygenate and stored under anaerobic conditions for at least 12 h before use.

For growth curves, *Lactiplantibacillus plantarum* APC2688 was grown from a glycerol stock on modified MRS (mMRS) agar for 24–48 h. Single colonies were inoculated into liquid mMRS broth and grown until OD<sub>600</sub> 0.5–0.7. Experimental media were inoculated to an OD<sub>600</sub> of 0.01 in 3 biological replicates. A volume of 200 µL of each biological replicate was added in technical triplicates to a 96 well plate. The OD<sub>600</sub> was monitored in a 96-well automated plate reader (Cerillo™ Stratus) for 36 hours under anaerobic conditions in the PLAS-LABS Simplicity 888 Automatic Atmosphere Chamber (N<sub>2</sub>: 80%, H<sub>2</sub>: 10%, CO<sub>2</sub>: 10%), at 37 °C with shaking at 400 rpm. OD measurements were taken every 10 min. The averages of the technical replicates were used for data analysis and representation.

### Bacterial supernatant harvesting

*L. plantarum* APC2688 was grown from a glycerol stock on mMRS agar for 24–48 h. Single colonies were inoculated into mMRS broth and grown until OD<sub>600</sub> 0.5–0.7, from which experimental media were inoculated to an OD<sub>600</sub> of 0.01 in 3 biological replicates. The OD<sub>600</sub> value was monitored until the supernatant was collected at the late stationary phase of growth (30 h post-inoculation). Bacterial cultures were centrifuged at 5000 rpm for 10 minutes. Thereafter, bacterial cells were removed using 0.2 µm filters and the supernatants were then aliquoted and stored at –80 °C.

### Metabolomics analysis of semi-polar metabolites

Metabolomics analysis of harvested *L. plantarum* APC2688 supernatant samples was performed by MS-Omics (Clinical Microbiomics; Copenhagen, Denmark) as follows. The analysis was carried out using a Thermo Scientific Vanquish LC coupled to an Orbitrap Exploris 240 MS (Thermo Fisher Scientific). An electrospray ionization interface was used as the ionization source. Analysis was performed in positive and negative ionization modes under polarity switching. Ultra-performance liquid chromatography (UPLC) was performed.<sup>37</sup> Peak areas were extracted using Compound Discoverer 3.3 (Thermo Scientific). Identification of compounds was performed at four levels; Level 1: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS

spectra, Level 2a: identification by retention times (compared against in-house authentic standards) and accurate mass (with an accepted deviation of 3 ppm), Level 2b: identification by accurate mass (with an accepted deviation of 3 ppm) and MS/MS spectra, and Level 3: identification by accurate mass alone (with an accepted deviation of 3 ppm). Metabolites annotated at the two highest levels of confidence (levels 1 and 2a) were used for further analysis. The output tables with the abundance of each metabolite per sample were CLR-transformed to account for compositionality<sup>38</sup> using the `clr_c` function from the `Tjazi v. 0.1.0.0` R package. Principal Component Analysis (PCA) was performed using the `prcomp` function from the `stats v. 4.3.0` R package. The PCA plot was prepared using the CLR-transformed count table of the semi-polar metabolites identified.

### Metabolomics analysis of short chain fatty acids

Metabolomics analysis of the harvested *L. plantarum* APC2688 supernatant samples was also carried out by MS-Omics. In this approach, samples were acidified using hydrochloride acid, and deuterium labelled internal standards were added. All samples were analyzed in a randomized order. Analysis was performed using a high polarity column (Zebtron™ ZB-FFAP, GC Cap. Column 30 m × 0.25 mm × 0.25 µm) installed in a GC (7890B, Agilent) coupled with a time-of-flight MS (Pegasus® BT, LECO). The system was controlled using a ChromaTOF® (LECO). Raw data were converted to netCDF format using Chemstation (Agilent), before the data were imported and processed in MATLAB R2021b (MathWorks, Inc.) using the PARADISE software. The statistical analysis of these metabolomics data was done in the same manner as described in the section on semi-polar metabolites.

### Zebrafish hatching and survival analysis

Wild-type AB zebrafish strain maintenance was performed under standard conditions with 14 : 10 light/dark cycles and fed with dry food and brine shrimp,<sup>39</sup> in accordance with the European Directive 2010/63/EC and meeting the requirements of the S.I. No. 543 of 2012. This is in accordance with the Guidelines for Care and Use of Laboratory Animals at University College Cork and approved by the Health Products Regulatory Authority, Ireland. Aliquots of each bacterial supernatant were diluted with egg water (3 g sea salt per 10 L ultra-pure water) in concentrations of 0.01 and 0.02% v/v. Supernatant pH was measured before and after dilution to find the highest administrable concentration of the supernatant to which zebrafish larvae can be exposed without any developmental malformations. Ten fertilized eggs at the same developmental stage were sorted and allocated to each well of a 6 well plate. Each well of the plate had 8 mL of either egg water, as a negative control, or the bacterial supernatant diluted with egg water. Embryos and larvae were incubated at 28 °C with 14 : 10 light/dark cycles for 5 days. From day 1 to 5, fish larvae were visually inspected through a stereomicroscope to assess spinal malformations, the day of hatching and the



proportion of surviving larvae. Dead embryos and larvae were removed under sterile conditions after checking each day.

### Behavioural analysis of zebrafish larvae

At 08:00 AM of day 5 post fertilisation (5 dpf), a single larva was transferred from each well of the 6 well plates to 1 mL of egg water in a 24 well plate. The protocol used was modified from that previously described.<sup>40</sup> Briefly, 4 × 24-well plates were filled, with a total of 4 zebrafish per treatment group in each plate (*i.e.*, zebrafish larvae exposed to egg water, or to the supernatant of *L. plantarum* APC2688 grown in the mMRS-C, FOS, GOS or inositol media described above). Each 24-well plate was placed in a box to avoid light exposure and allowed to habituate in the dark at 28 °C for 2 hours. After that period, the 24-well plate was introduced to the Zantiks MWP system (also kept at 28 °C), and allowed to habituate for 20 min, followed by a 10 minute tracking calibration step, followed by initiation of the behavioural assays.

The larvae spent 10 minutes in darkness, followed by 10 minutes of light exposure (92.07 ± 2.5 Lux), and another 10 minutes of darkness. During the assay, the position of the fish within the well and the time were recorded automatically. Two analyses were performed using these measurements: total distance travelled and thigmotaxis. The total distance travelled measured the distance within each phase of the experiment, or through the entire test. For thigmotaxis, two circular zones of the same total area were computed within each well of the 6-well plate, the inner and outer zone. The position of the fish and the time spent within each zone were determined throughout the experiment. Output data with the total distance travelled and the time spent within each zone were then aggregated into bins of 1 minute. The total distance travelled by the fish during each phase was then calculated, and the delta between the distance travelled during the light phase and the second dark phase was also calculated for each fish. Additionally, the ratio of the time each fish spent in the outer zone over the total length of the second dark phase was also calculated as a proxy of anxiety-like behaviour.

### RT-qPCR with whole larval zebrafish homogenates

An independent cohort of zebrafish larvae not used in behavioural assays was culled on day 5 post fertilisation. Larvae of each well were pooled, so each well of the plate (*i.e.*, 10 larvae) constitutes an *n* of 1. Experimental groups for RT-qPCR are the same as described for behavioural analysis. RNA was extracted from pools of roughly 10 larvae using the Qiagen RNeasy Plus Micro® kit following a modified protocol after disrupting the tissue with an electric homogenizer, in which the initial centrifugation and supernatant removal step was not carried out to increase the final RNA yield. cDNA was prepared using the MultiScribe™ Reverse Transcriptase Thermo Fisher Scientific® kit using 20 µL of RNA at a concentration of 25 ng µL<sup>-1</sup>. The following protocol was performed: 25 °C for 10 minutes, 37 °C for 120 minutes and 85 °C for 5 minutes using the MiniAmp thermal cycler. qPCR was performed using 2 µL of cDNA and 8 µL of PowerUp SYBR Green® Mastermix

from Applied Biosystems in a Lightcycler 480 II (Roche) with the following protocol: 50 °C for 2 minutes (UDG activation), 95 °C for 2 minutes (polymerase activation), and 40 cycles of 95 °C for 15 seconds (denaturation) and 60 °C for 1 minute (annealing). Expression levels were calculated as the average of three technical replicates relative to a stably expressed house-keeper gene,  $\beta$ -actin. The relative mRNA expression was calculated using the  $2^{-\Delta\Delta C_t}$  method.<sup>41</sup> Forward and reverse primers for the *actb1*,<sup>42</sup> *gad1*,<sup>43</sup> *gabra1*,<sup>43</sup> *ahr2*<sup>44</sup> and *bdnf*<sup>45</sup> genes can be found in ESI Table 3.†

### Data analysis and statistics

All statistical analyses of the data were conducted using R version 4.1.1. For data manipulation and visualization, the Tidyverse ecosystem of packages was used.<sup>46</sup> Analysis of the differences in the means between groups was performed using ANOVA for normally distributed data, and the Kruskal–Wallis test for non-normally distributed data. The Shapiro–Wilk test was used to assess the normality of the data. When differences between groups were deemed statistically significant by the corresponding tests, contrast analysis was performed using *t*-tests followed by False Discovery Rate (FDR) correction using the Benjamini–Hochberg method. For metabolomics analysis, the abundance of each metabolite was transformed using the Centred Log Ratios (CLR) transformation as it has been suggested previously for compositional data.<sup>38</sup> The three metabolites of interest, acetate, tryptophan and GABA, were analysed in a targeted manner using ANOVA. When statistically significant differences between groups were identified, pairwise *t*-tests with FDR correction were applied to perform contrasts. For untargeted metabolomics analysis, Generalized Linear Models (GLMs) were implemented for each metabolite to determine differences between means of the groups using the formula “metabolite ~ growth media”, as the growth media was the only relevant explanatory variable in the experimental design. The resulting *p* values obtained were corrected for multiple comparisons using the Benjamini–Hochberg method. Principal Component Analysis (PCA) for an untargeted metabolomics analysis was performed by calculating Aitchison distances between samples (defined as Euclidean distances on CLR-transformed data).

## Results

### *In silico*: Screening of the prebiotic-degradation profile and potential to synthesise neuroactive molecules in bacterial genomes

A list of prebiotic fibres with varying levels of complexity was compiled (ESI Table 1†), and a list of enzymes with experimental evidence of their potential to degrade them was curated from the KEGG<sup>47</sup> and MetaCyc<sup>48</sup> databases. Bacterial genomes in the KEGG database were screened to select only those carrying at least one enzyme from the list. Next, the list of genomes was further filtered to select those in which enzymes involved in synthetic pathways of neuroactive com-



pounds were identified. Finally, the list was filtered again to identify bacterial strains previously isolated from the human intestine (see the Methods section for details on each filtering step). As a result of applying these three filters, a final list of bacteria with three key features was generated: (1) predicted potential to catabolise prebiotics of different complexities, (2) the presence of enzymes required for the synthesis and degradation of neuroactive molecules linked to the modulation of the stress response, and (3) isolation from the human gut. A summary of the *in silico* workflow employed and the number of genomes identified as successful candidates at each step is provided (ESI Fig. 1†).

For further evaluation, bacterial candidates from the KEGG database were aggregated into six genera: *Anaerotruncus*, *Bifidobacterium*, *Coprococcus*, *Dorea*, *Faecalibacterium* and *Lactobacillus*. For each genus, the number of species with genes encoding enzymes required for the degradation of the prebiotics (Fig. 1A) and GBM-associated enzymes (Fig. 1B) was quantified. While prebiotics such as sambubiose and inositol could be degraded by species in all the six candidate genera, fibres such as arabinoxylan oligosaccharides and 3'- and 6'-sialyllactose could only be degraded by species within the genus *Bifidobacterium*. This suggests that species from different genera encode different prebiotic degrading enzymes, depicting genus specific prebiotic-degrading potential. On the other

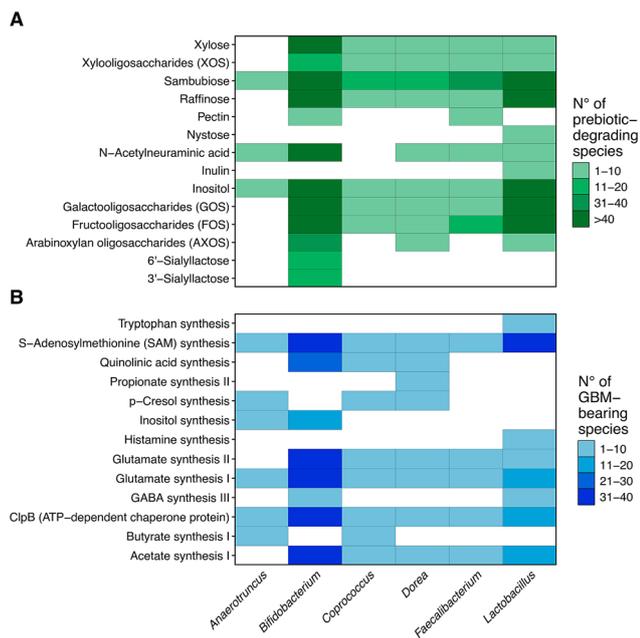
hand, while GBMs such as glutamate synthesis I and *S*-adenosylmethionine synthesis were detected across all the six genera, other GBMs were present only in specific genera, such as tryptophan synthesis that was identified only within the genus *Lactobacillus*. This also suggests genus-specific neuroactive potential.

### *In silico*: *Lactiplantibacillus plantarum* APC2688 was identified as a promising probiotic candidate based on its prebiotic-degradation profile and its potential to synthesize neuroactive molecules

The specific goal of the *in silico* analysis was to identify a bacterial strain fitting a profile of interest: isolated from human stool samples, and degrading prebiotics of different complexities while also producing molecules known to modulate the stress-response in animals. Therefore, bacterial candidates capable of degrading FOS, GOS and inositol and synthesizing GABA, acetate and tryptophan were deemed most relevant for this study. From our results, only the genus *Lactobacillus* showed multiple candidates with the desired profile (Fig. 1A and B). Notably, within that genus, the species *Lactiplantibacillus plantarum* (formerly known as *Lactobacillus plantarum*) was found to have the highest number of strains with the desired profile, with a total of four genomes. Hence, the hypothesis was that a new strain from the same species will maximize the chances of finding a new psychobiotic candidate capable of degrading FOS, GOS and inositol and synthesizing GABA, acetate and tryptophan. Therefore, a previously uncharacterised strain of the species *L. plantarum*, the strain APC2688, was selected from the APC Microbiome Ireland culture collection for further analysis (NCBI assembly accession code: GCA\_040183155.1). The genome of *L. plantarum* APC2688 was analysed, and it showed functional coherence with the profiles of the other four *L. plantarum* genomes available in the KEGG database, supporting our hypothesis (Fig. 2). Additionally, the average nucleotide identity (ANI) between the novel strain and the genomes available in KEGG was calculated (ESI Table 2†).

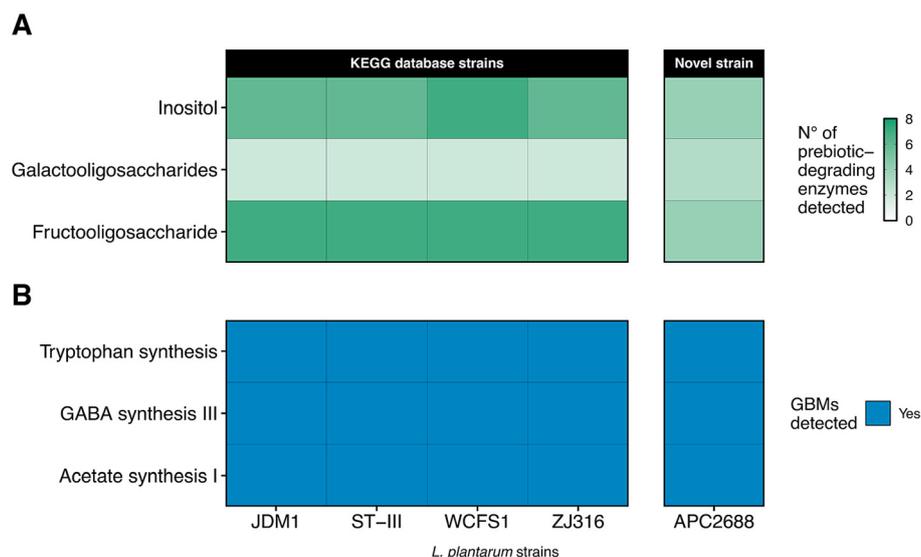
### *In vitro*: *L. plantarum* APC2688 was able to grow in media supplemented with the candidate prebiotics of interest (FOS, GOS, and inositol) and produce the neuroactive molecules of interest (GABA, tryptophan and acetate)

*In vitro* experiments were performed to confirm *in silico* results suggesting that *L. plantarum* APC2688 could grow in the presence of the prebiotics of interest. The growth of the strain was characterized using different media: mMRS with dextrose available at 2% w/v (mMRS group), mMRS without dextrose (MRS-C), and MRS-C supplemented with FOS, GOS, or inositol at 2% w/v referred to as FOS, GOS and inositol, respectively. Although bacterial growth was supported in every media, statistically significant differences were observed among groups ( $F_{(4,10)} = 127.99$ ;  $p < 1.5 \times 10^{-8}$ ). In particular, while the FOS, GOS, and mMRS media showed a higher OD when compared to the mMRS-C group ( $T_{(4)} = -5.04$ ,  $p < 0.05$ ;  $T_{(4)} = -22.3$ ,  $p < 1 \times 10^{-4}$ ; and  $T_{(4)} = -13.6$ ,  $p < 1 \times 10^{-4}$ , respectively), inositol



**Fig. 1** Number of genomes within six genera showing genes encoding for enzymes required for the degradation of carbohydrates of different complexities and involved in the synthesis and degradation of neuroactive molecules. (A) Number of genomes within each genus having at least one gene encoding for a carbohydrate-degrading enzyme. (B) Number of genomes within each genus showing all the genes involved in each GBM, defined as pathways involved in the synthesis of neuroactive molecules.

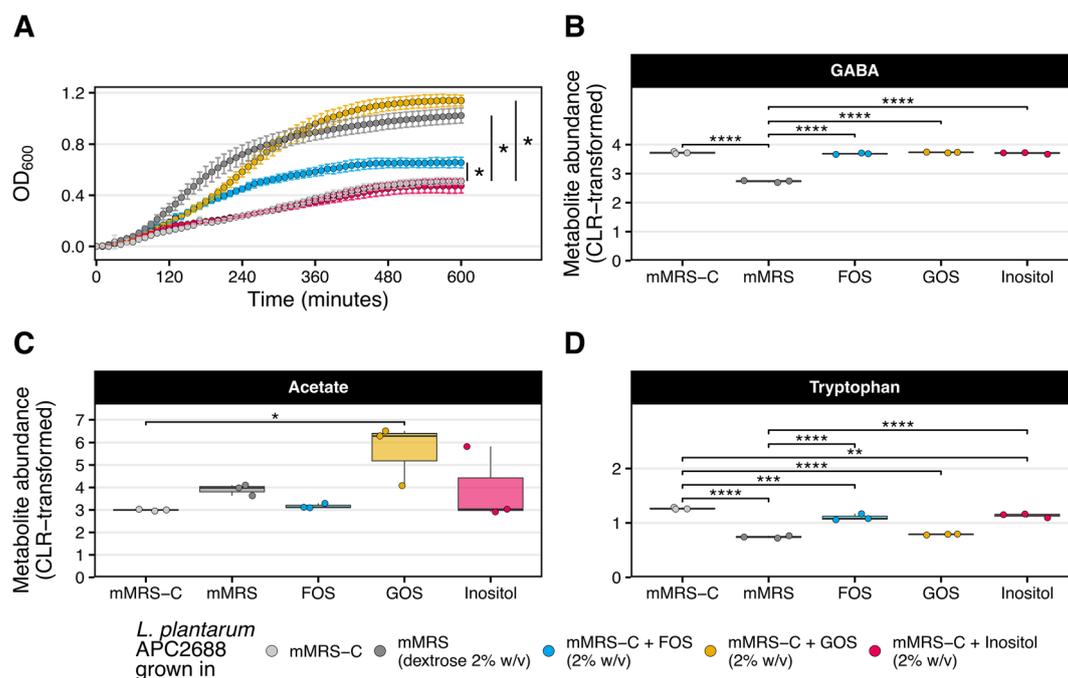




**Fig. 2** Comparison of carbohydrate-degradation potential and neuroactive potential between four strains of *L. plantarum* obtained from the KEGG database and the novel strain APC2688. (A) Number of carbohydrate-degrading enzymes identified for each strain (as a gradient of green). (B) Presence or absence of GBMs, defined as pathways involved in the synthesis or degradation of neuroactive molecules. The presence of carbon-degrading enzymes and enzymes involved in the GBMs was inferred from the genomic analysis of the strains.

supplemented media showed no statistically significant differences when compared to the mMRS-C group ( $T_{(4)} = 1.04$ ;  $p < 0.4$ ) (Fig. 3A).

Next, metabolomics analysis confirmed that *L. plantarum* APC2688 could produce the neuroactive molecules GABA, acetate and tryptophan when grown in every media described



**Fig. 3** *In vitro* validation of *L. plantarum* APC2688 potential to degrade candidate prebiotics of different complexities while producing neuroactive molecules involved in stress modulation. (A) Growth curves of *L. plantarum* APC2688 when growing in different media. An  $N$  of three was used for each growth media. (B), (C) and (D) represent the CLR-transformed abundance of GABA, acetate and tryptophan in the cell-free supernatants of APC2688, respectively. An  $N$  of three was used for each growth medium. Differences between groups were tested by one-way ANOVA. Stars represent contrasts with adjusted  $p$  values below 0.05. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , and \*\*\*\* $p \leq 0.0001$ . Only statistically significant results are shown.



above (Fig. 3B–D). In each experimental group, the CLR-transformed abundance of the metabolite was above 0, suggesting that the metabolites were enriched in the bacterial supernatant. Differences in the abundance of each metabolite were significant according to ANOVA: GABA ( $F_{(4,10)} = 703.99$ ;  $p < 1 \times 10^{-4}$ ), acetate ( $F_{(4,10)} = 3.54$ ;  $p < 0.05$ ), and tryptophan ( $F_{(4,10)} = 139.55$ ;  $p < 1 \times 10^{-4}$ ). Contrasts were conducted to evaluate differences between the mMRS-C and mMRS groups when compared to the prebiotic-treatment groups (see the Methods section). Based on the assessment of the overall metabolic profile of the groups using PCA (see the Methods section), a unique metabolic profile was suggested for the mMRS, FOS and GOS groups, while the mMRS-C and inositol groups were grouped together (ESI Fig. 2†). Finally, untargeted metabolomics analysis was performed to analyse differences in the abundances of every metabolite detected across the different growth media (ESI Table 4†). For this final analysis, GABA, acetate and tryptophan were excluded as they were analysed previously in a targeted manner.

### ***In vivo*: The supernatant of *L. plantarum* APC2688 induced changes in the expression of genes related to the GABAergic signalling pathway and a reduction in anxiety-like behaviour in zebrafish larvae**

After the ability of *L. plantarum* APC2688 to degrade FOS, GOS and inositol while producing GABA, acetate and tryptophan was assessed *in silico* and *in vitro*, the next objective was to test if the psychobiotic candidate could affect the stress-related behaviour in an animal model. We assessed whether the strain supernatant could induce changes in MGB axis signalling pathways or in the anxiety-like behaviour of zebrafish larvae. Therefore, zebrafish embryos were exposed to egg water, or to a cell-free supernatant of *L. plantarum* APC2688 grown in mMRS-C media or mMRS-C media supplemented with FOG, GOS or inositol. The effects of water supplementation with 0.01% and 0.02% v/v of bacterial supernatant were assessed on egg hatching (ESI Fig. 3A†) and larval survival (ESI Fig. 3B†). The results support the selection of the 0.01% v/v concentration, as at higher concentrations a tendency of reduced egg hatching and delayed larval survival was detected.

Zebrafish larvae were exposed to 0.01% v/v of bacterial supernatant for five days and then divided into two experimental groups. One group underwent a molecular analysis and the other behavioural assessments. Expression of genes relevant for GABA signalling (*gad1* and *gabra1*), tryptophan signalling (*ahr2*), and neuronal growth and survival (*bdnf*) was assessed for the first group. The hypothesis was that bacterial-derived metabolites present in the filtered supernatant will induce changes in the larvae's expression of these genes. The Kruskal–Wallis test shows statistically significant differences in the expression of *gad1* ( $X_{(4)}^2 = 10.74$ ,  $p < 0.05$ ) and *gabra1* ( $X_{(4)}^2 = 12.71$ ;  $p < 0.05$ ) but no differences in the expression of *ahr2* ( $X_{(4)}^2 = 0.43$ ;  $p = 0.98$ ) or *bdnf* ( $X_{(4)}^2 = 4.22$ ;  $p = 0.38$ ). Contrasts were performed to compare the egg water treated group against every other group (see the Methods section). The analysis shows that while *gad1* was upregulated in every group

of zebrafish larvae treated with any supernatant, *gabra1* was upregulated in each group except the group treated with the supernatant from APC2688 grown with inositol. Although not significant, our results show a trend of upregulated *bdnf* expression in the three bacterial supernatants supplemented with prebiotics (Fig. 4A).

In parallel, the second group of zebrafish larvae underwent a behavioural assessment to test the hypothesis that bacterial-derived metabolites present in the filtered supernatant will induce changes in anxiety-like behaviours (Fig. 4B). The Kruskal–Wallis tests showed no statistically significant differences in any of the three phases of the experiment (see the Methods section for details of the phases in behavioural assessments): habituation ( $X_{(5)}^2 = 2.88$ ;  $p = 0.81$ ), light ( $X_{(5)}^2 = 6.86$ ;  $p = 0.792$ ), and dark ( $X_{(5)}^2 = 2.26$ ;  $p = 0.81$ ) (Fig. 4C), or in the change in locomotion during the transition from the light to dark phases of the experiment ( $X_{(5)}^2 = 5.17$ ;  $p = 0.79$ ) (Fig. 4D). Interestingly, significant differences between groups were found for the thigmotactic behaviour ( $X_{(5)}^2 = 12.5$ ;  $p < 0.05$ ) (Fig. 4E). Contrasts were performed to compare the egg water-treated group with the groups treated with the bacterial supernatant. The only statistically significant difference was detected for the group of zebrafish larvae treated with the supernatant from APC2688 grown in mMRS-C (Fig. 4E).

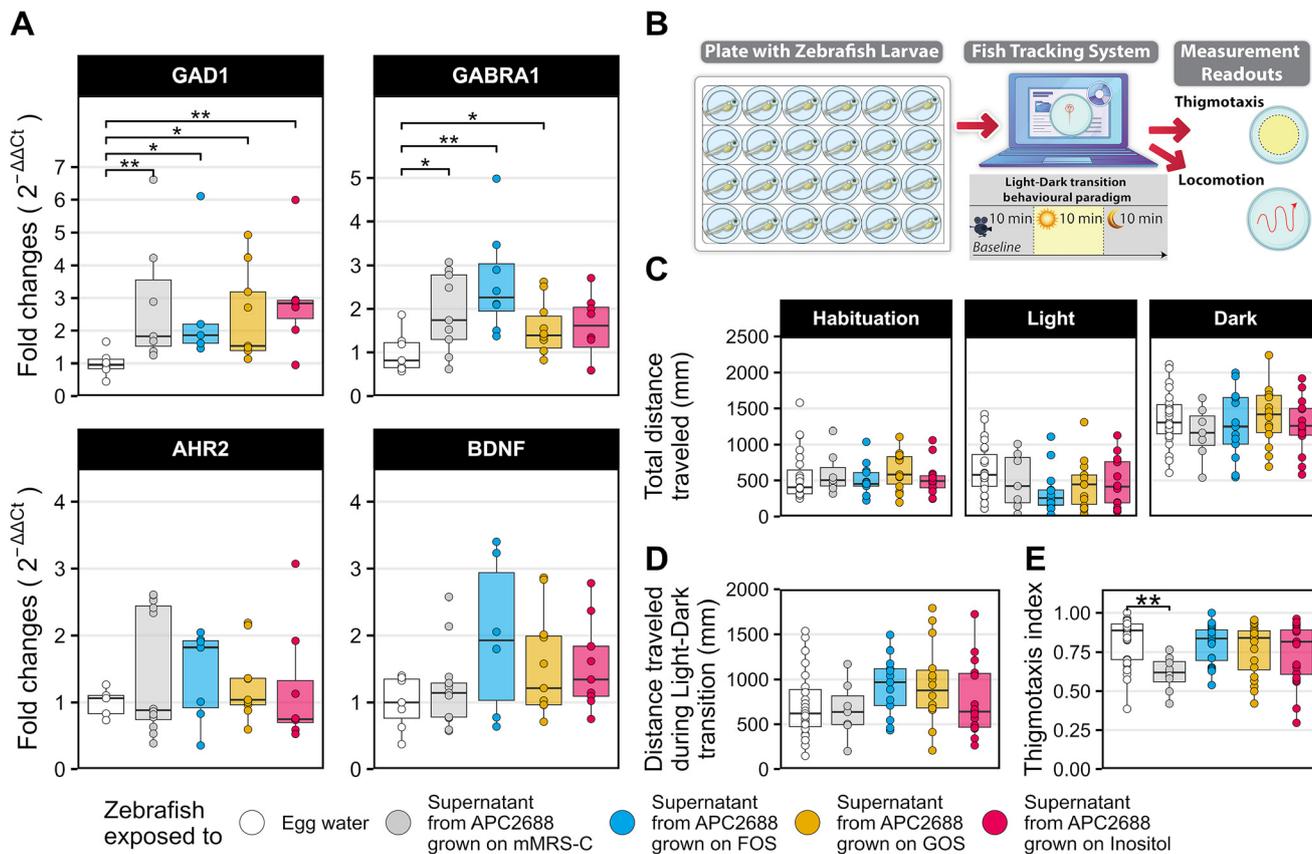
## Discussion

The integrated framework of *in silico*, *in vitro* and *in vivo* approaches allowed us to identify the potential psychobiotic properties of *Lactiplantibacillus plantarum* APC2688. The strain was selected based on its potential to both degrade prebiotics and produce neuroactive molecules previously associated with stress-response modulation in animal models. Additionally, we showed that the APC2688 supernatant can modulate the MGB axis, inducing changes in the expression of *gad1* and *gabra1*, crucial genes within the GABAergic signalling pathway and in the anxiety-like behaviour of the zebrafish larvae. Thus, this study underscores the utility of leveraging public data from databases such as KEGG<sup>47</sup> and MetaCyc<sup>48</sup> for the targeted identification of candidate bacterial strains, offering a more efficient alternative to resource-intensive untargeted screening of numerous candidates.

An overarching strength of this framework lies in the integration of orthogonal approaches, such as *in silico* screening, *in vitro* experiments, metabolomics analysis, gene expression assessments and behavioural tests in model organisms. This allowed the full characterisation of a bacterial strain and its potential psychobiotic effects. The results reported here demonstrate that computational approaches can be used to efficiently identify bacterial strains with psychobiotic potential, materializing previous propositions made to advance the rate of probiotic discovery.<sup>27,28</sup>

Our *in silico* analysis identified *L. plantarum* as a bacterial species from which potentially interesting novel psychobiotic strains may be further characterised. Indeed, through the ana-





**Fig. 4** The supernatant of *L. plantarum* APC2688 grown in media supplemented with the prebiotics induced changes in zebrafish larvae stress response by modulating genes in the GABAergic signalling pathway and altering the larvae behaviour. (A) Expression of genes involved in the anxiety-like behaviour of the zebrafish normalized to the control group (zebrafish treated with egg water). (B) Schematic of the experimental design used in the behavioural paradigm. (C) The total distance travelled during each phase of the behavioural assay. (D) The difference in the total distance travelled during the dark and the light phases used as a measure of anxiety-like behaviour. (E) Thigmotaxis index, which measures the proportion of time each fish spent in the arena's outer zone during the dark phase. Between 5 and 10 samples were used in the gene expression analysis, where a total of 7 to 10 zebrafish larvae were pooled per sample. For behavioural analysis, between 7 and 22 individual fish were used. Differences between groups in panels (A), (C), (D) and (E) were tested using the Kruskal–Wallis test. Contrasts performed in panels (A) and (E) tested differences between the egg water treated group and every other group. Stars represent contrasts with adjusted  $p$  values below 0.05. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ .

lysis of the *L. plantarum* APC2688 genome, we showed a functional overlap in the biochemical profiles of the newly described APC2688 strain and four other strains within the same species (Fig. 2A and B), each previously described as bacterial probiotics.<sup>49–53</sup> Additionally, our *in silico* analysis identified a repertoire of genes across various *L. plantarum* strains that confer their ability to synthesise enzymes capable of degrading prebiotics of different complexities. Previous analyses performed on a wider set of strains of *L. plantarum* also showed a wide repertoire of carbon-utilizing enzymes in different strains,<sup>54</sup> which is consistent with our results (Fig. 2). Furthermore, our results on the growth profile of APC2688 in media supplemented with FOS, GOS and dextrose (Fig. 3A) are consistent with previous data from the *L. plantarum* strain ATCC14917.<sup>55</sup> The distinct overall metabolic profile of the ATCC14917 strain when grown in media supplemented with FOS, GOS and dextrose<sup>55</sup> was similar to that of APC2688 (ESI Fig. 2†). The metabolites identified in the bacterial super-

natant derived from APC2688 are also consistent with previous studies on *L. plantarum* using either *in silico* or *in vitro* approaches. Genes required for the synthesis of tryptophan were identified in *Lactobacillus* (now named *Lactiplantibacillus*) *plantarum* LRCC-5314.<sup>56</sup> Additionally, several *L. plantarum* strains have been previously reported as capable of synthesising acetate<sup>57,58</sup> and GABA.<sup>59–61</sup> Together, this previously reported evidence is consistent with our results and further emphasizes the benefits of incorporating an *in silico* screening step prior to initiating *in vitro* experiments (Fig. 3B–D).

For the *in vivo* validation of the psychobiotic potential of APC2688, zebrafish played a pivotal role. Zebrafish are now a powerful preclinical model for human diseases with conserved physiology, pharmacology and metabolism, which approach (and occasionally surpass) those of rodents.<sup>62,63</sup> Laboratories across academia and industry around the world are leveraging zebrafish as a more cost-effective and scalable alternative to rodents for *in vivo* drug discovery.<sup>64</sup> Numerous drug treatments



that have recently progressed to the clinic or are in clinical trials have their genesis in the zebrafish model providing many aquarium-to-bedside success stories.<sup>62,63,65</sup> In fact, for some drugs, zebrafish recapitulate the effects observed in humans better than mouse models do (e.g. thalidomide).<sup>63</sup> Zebrafish have emerged as a powerful model to study neurobehavioral disorders affecting stress-related and social behaviour due to evolutionary conservation of neuroanatomical and chemical substrates and development of robust behavioural paradigms.<sup>12,66–71</sup> Zebrafish may vary in their exact microbiota composition from humans, but due to the conservation of these functional metabolites and host pathways they interact with, zebrafish can be used as a discovery platform for translational science. For a more detailed discussion on the use and translational relevance of simpler model organisms like zebrafish, we refer the reader to a recent review on this topic.<sup>12</sup>

The *Lactiplantibacillus plantarum* APC2688-derived supernatant showed potential to target the MGB axis of zebrafish larvae (Fig. 4A), consistent with results where another *L. plantarum* strain modulated the gene expression of adult zebrafish when administered alive.<sup>72</sup> Interestingly, while the APC2688 supernatant was sufficient to induce an upregulation of *gabra1* and *gad1*, the administration of a living *L. plantarum* strain was previously reported to upregulate *gabra1* but not *gad1*, which remained unaltered,<sup>72</sup> and conversely, other two *Lactobacillus* species, *L. delbruecki* and *L. casei*, also induced the upregulation of *gad1* but not *gabra1* in the zebrafish brain.<sup>19</sup> Therefore, this result with our administration strategy implies that changes in the expression of these genes may be modulated by metabolites produced by the strains instead of structural molecules. Furthermore, we found larger effect sizes for the differences between the control group and any supernatant-treated group for the two genes involved in the GABAergic signalling pathway, *gad1* and *gabra1*, compared to previous reports.<sup>72</sup> We hypothesise two, potentially complementary, possible explanations for these differences. On the one hand, it could be that early developmental stages are more susceptible to the modulatory effects of *L. plantarum* strains, reflecting the effect of time-sensitive developmental windows. On the other hand, the potential of APC2688 to induce changes in both genes is higher. Further experiments are warranted to understand the basis underpinning these observations.

No changes were detected in the total locomotion of the larvae treated with *L. plantarum* (Fig. 4C), consistent with previous reports using strains from the same species,<sup>72</sup> and from different species from the same genus.<sup>19,73</sup> Additionally, we detected a significant reduction in the thigmotaxis index during the dark phase of the assay in the group of fish treated with the supernatant of APC2688 grown in the media that did not have an additional source of carbon (mMRS-C in figures) (Fig. 4E), suggesting a reduced anxiety-like behaviour in that group. Interestingly, that supernatant showed the highest abundance of tryptophan (Fig. 3D), which was previously linked to the reduction of the stress response in zebrafish adults.<sup>74</sup> Supporting our results showing that APC2688 could

reduce anxiety-like behaviour, other *L. plantarum* strains<sup>72</sup> and other species from the same genus<sup>19,73,75</sup> have shown potential to reduce anxiety-like behaviours in zebrafish in other experimental setups. Similarly, FOS and GOS administration in mice has been shown to elicit a stress-reduction response.<sup>76</sup> Therefore, we anticipated that the supernatant of the APC2688 strain grown in their presence would reduce the anxiety-like behaviour in zebrafish (Fig. 4D and E). However, despite the high abundance of tryptophan in the supernatant from bacterial growth in FOS (Fig. 3D), no statistically significant differences were found between the prebiotic-treated and egg water groups.

Despite the novelty of the approach we have proposed and its potential to identify and validate new psychobiotic candidates, this work is not without its limitations. Our framework relies on the access to reference bacterial genomes to screen and identify genes of interest and allow predictions to be made about the functional properties of candidate bacteria. However, ever-growing compendiums of cultured and uncultured bacterial genomes are available,<sup>77</sup> and many allow querying of genomes filtered according to the environment where they were recovered, which includes the human gut.<sup>78</sup> This opens opportunities to identify new psychobiotic candidates from gut-associated bacterial strains, as well as bacteria from other environmental niches capable of producing neuroactive compounds of interest.<sup>7</sup> Similarly, specialized databases like CAZy<sup>79</sup> allow for thorough annotation of enzymes involved in the degradation of carbohydrates, which may be of interest to researchers interested in applying this framework using less characterised fibres.

In conclusion, this work introduces a framework for the identification and validation of bacteria with a biochemical profile of interest by integrating *in silico*, *in vitro* and *in vivo* approaches. Notably, the framework described guided the successful discovery and characterization of *L. plantarum* APC2688 as a bacterial strain capable of modulating the microbiome-gut-brain axis.

## Author contributions

BV and ID drafted the manuscript. BV performed the bioinformatic analyses. BV and ID conducted the *in vitro* and *in vivo* experiments. BV analysed the data and performed statistical analyses. BV and KOR prepared the figures. GC, JN, JFC, EG, MC, SB, and NP co-designed the study. All authors revised and edited the manuscript. The final version of the manuscript was approved by all authors.

## Data availability

The 6963 genomes used for the *in silico* screening (Fig. 1 and 2) were obtained from the KEGG database, and analysed using the script available in [https://github.com/Benjamin-Valderrama/kegg\\_probiotics](https://github.com/Benjamin-Valderrama/kegg_probiotics). The genome of *Lactiplantibacillus*



*plantarum* APC2688<sup>30</sup> is available in NCBI (assembly accession code: GCA\_040183155.1). Other data from the *in vivo* and *in vitro* sections of this work can be found in the ESI,† available on Zenodo – open data repository (<https://zenodo.org/records/13325351>).

## Conflicts of interest

NP and AS are employed at Reckitt Benckiser Health Limited while MC and SB were previously employed at Reckitt Benckiser Health Limited. JFC has spoken at conferences organized by Mead Johnson, Ordesa, and Yakult and has received research funding from Reckitt, Nutricia, Dupont/IFF, Kerry Group and Nestle. GC has received honoraria from Boehringer Ingelheim, Janssen, Probi, and Apsen, research funding from Pharmavite, Fonterra, Kerry Group, Nestle, Tate and Lyle and Reckitt, and has provided consultancy for Bayer Healthcare, Yakult, Zentiva and Pharmavite. JN has received research funding from Reckitt. APC Microbiome Ireland has received research support from Mead Johnson, Cremo, 4D Pharma, Suntory Wellness and Nutricia. This support neither influenced nor constrained the content of this article.

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