Genomics 113 (2021) 4028–4038

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

A genome guided evaluation of the Lab4 probiotic consortium

L.M. Baker [a,1], T.S. Webberley [b,1], G. Masetti [b,2], T.R. Hughes [c], J.R. Marchesi [d], A.A. Jack [b], T.S. C. Joyce [b], M.D. Allen [b], S.F. Plummer [b], D.R. Michael [b], G. Ramanathan [e], R. Del Sol [a], P. D. Facey [a,*]

[a] Swansea University Medical School, Swansea University, Singleton Park Campus, Swansea SA2 8PP, United Kingdom
[b] Cultech Limited, Unit 2 Christchurch Road, Baglan Industrial Park, Port Talbot SA12 7BZ, United Kingdom
[c] Systems Immunity Research Institute, Henry Welcome Building, Cardiff University, CF14 4XN, United Kingdom
[d] Division of Digestive Diseases, Department of Metabolism, Digestion and Reproduction, Faculty of Medicine, Imperial College London, London, United Kingdom
[e] Pharmacology based Clinical Trials, Pennington Biomedical Research Center, 6400 Perkins Rd, Baton Rouge, LA 70808, USA

ARTICLE INFO

Keywords:
Probiotic
Lactobacillus
Bifidobacterium
Lab4
Probiogenomics
Safety

ABSTRACT

Draft genome sequences of the Lab4 probiotic consortium were deposited in Genbank: *Bifidobacterium animalis* subsp lactis CUL34 (PRJNA482550), *Bifidobacterium bifidum* CUL20 (PRJNA559984), *Lactobacillus acidophilus* CUL60 (PRJNA482335), *Lactobacillus acidophilus* CUL21 (PRJNA482434). Probiogenomic analyses confirmed existing taxonomies and identified putative gene sequences that were functionally related to the performance of each organism during in vitro assessments of bile and acid tolerability, adherence to enterocytes and susceptibility to antibiotics. Genomic stability predictions identified no significant risk of gene acquisition of both antibiotic resistance and virulence genes. These observations were supported by acute phase and repeat dose tolerability studies in Wistar rats. High doses of Lab4 did not result in mortalities, clinical/histopathological abnormalities nor systemic toxicity. Increased faecal numbers of Lab4 in supplemented rats implied survival through the gastrointestinal tract and/or impact the intestinal microbiota composition. In summary, this study provides multifaceted support for probiotic functionality and the safety of the Lab4 consortium.

1. Introduction

Facultatively anaerobic lactic acid bacteria and species of the anaerobic bifidobacteria are included in many probiotic products which are defined as 'live microorganisms that when administered in adequate amounts confer a health benefit on the host' [1]. These organisms, particularly the lactobacilli, show significant genomic size variation that drives the large heterogeneity observed in both genotype and phenotype at species level [2]. A number of traits are considered essential for probiotic function including tolerance of the harsh conditions of the gastrointestinal tract (GIT), the ability to adhere to the intestinal epithelium and/or mucosa and the absence of virulence factors including transferable antibiotic resistance genes (ARGs) [3].

As access to genome sequencing technology improves, it enables more accurate *in silico* taxonomical classifications of probiotic bacteria and assessments of safety and potential functionality on the basis of their gene composition [4]. The demonstrable heterogeneity of many probiotic rich genre together with inconsistent classification of these organisms renders bioprospecting of probiotic traits difficult. Thus, in depth, genomic guided evaluations based on a robust phylogenetic framework are needed to determine the scope of the traits. Functionality of the genes detected by analysis *in silico* must be confirmed using *in vitro* models of the GIT and progressing to *in vivo* safety studies.

The aim of this study was to report the draft genomes of each of the organisms included in the Lab4 probiotic consortium and interrogate the genomic sequences to confirm the taxonomical classification and the presence of genes relating to a number of potential probiotic traits. The Lab4 probiotic consortium is composed of *Lactobacillus acidophilus* CUL21 (NCIMB 30156), *Lactobacillus acidophilus* CUL60 (NCIMB 30157), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis* subsp. *lactis* CUL34 (NCIMB 30172). Acid and bile tolerances, the ability to adhere to enterocytes and antibiotic sensitivity

* Corresponding author.
E-mail address: P.Facey@swansea.ac.uk (P.D. Facey).
[1] Authors contributed equally to the study.
[2] Current address: Department of Cellular Computational and Integrative Biology, University of Trento, Povo, Italy.

https://doi.org/10.1016/j.ygeno.2021.08.007
Received 22 February 2021; Received in revised form 16 July 2021; Accepted 10 August 2021
Available online 13 August 2021

profiles for each organism were assessed *in vitro* and the outcomes were aligned with genomic findings. The element of safety of the Lab4 consortium was assessed *in vivo* by means of both a short-term acute phase oral toxicity study and a longer-term oral toxicity study.

2. Materials and methods

All reagents were purchased from Sigma Aldrich (Poole, UK) and all media were supplied by Oxoid Ltd. (Basingstoke, UK) unless otherwise stated.

2.1. Culture conditions for Lab4 consortium

The Lab4 consortium consists of *Lactobacillus acidophilus* CUL21 (NCIMB 30156), *Lactobacillus acidophilus* CUL60 (NCIMB 30157), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis* subsp. *lactis* CUL34 (NCIMB 30172) that were provided by Cultech Ltd. Lactobacilli were growth on DeMan Rogosa Sharpe (MRS) medium while bifidobacteria were grown on modified MRS (MRSX) containing lithium chloride (1 g/L), sodium propionate (1.5 g/L) and L-cysteine hydrochloride (0.25 g/L). All cultures were grown anaerobically (10% carbon dioxide, 10% hydrogen and 80% nitrogen) at 37 °C in an AW400SG Anaerobic Workstation (Elektrotek, United Kingdom).

2.2. Whole genome sequencing and annotation

Genomic DNA (gDNA) was isolated from mid-logarithmic, anaerobically grown pure cultures of each strain using the DNeasy Blood and Tissue DNA isolation kit (Qiagen, Manchester, UK) with the following modifications: harvested bacteria were washed three times with NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH = 8) [5] and lysed enzymatically using 20 mg/mL lysozyme for 2 h at 37 °C. gDNA was quantified using a Qubit fluorometric analyser (Thermo Fisher Scientific, Gloucester, UK) in accordance with the manufacturer's instructions. Whole genome sequencing of intact DNA (quality checked by the presence of a discrete high molecular weight band of approximately 20 kb on 0.7% *w/v* agarose, not shown) was performed at LGC (Berlin, Germany) or Microbes NG (Birmingham, UK). Genome assembly was performed in SPAdes v. 3.5.0 [6], annotated in PGAP [7] and deposited into GenBank [8]. Genome metrics were retrieved from RAST and compared with median values for closely related species (retrieved from Genbank). Gene annotations for Lab4 were retrieved from RAST [9]. Presence of plasmids in Lab4 was assessed by aligning raw reads (FASTQ) to known plasmid sequences. In brief, FASTA sequences of two L. *acidophilus* plasmids (pLA103 and pLA106) and pB80 from *B. bifidum* were used as references to map reads for CUL21, CUL60 and CUL20 respectively using the Burrows-Wheeler Alignment tool [10]. The *B. animalis* complex is known to be depleted in plasmids (and no plasmid sequences have been deposited in GenBank), thus this analysis was not performed for CUL34.

2.3. Confirmation of Lab4 phylogenetic classification

Phylogenetic classification of the Lab4 strains was undertaken using a multifaceted approach. Initially, identities were confirmed by single-colony PCR. Primers for amplification of the 16S rRNA genes were (for lactobacilli) 27F (5′ – AGAGTTTGATCCTGGCTCAG –3′) and 1492R (5′- GGTTACCTTGTTACGACTT –3′) and (for bifidobacteria) U1 (5′-ACGCGTCGACAGAGTTTGATCCTGGCT –3′) and U1R (5'-GGACTAC-CAGGGTATCTAAT-3′). Amplicons were Sanger sequenced in both directions by Eurofins Genomics (Ebersberg, Germany) and their identity confirmed as the top BLASTN hit (data not shown). In addition, to enhance robustness, identity was also validated through reconstruction of both *Lactobacillus* and *Bifidobacterium* 16S rRNA gene type strain phylogenies (not shown). Maximum-Likelihood (ML) phylogenies were created using a multi-locus phylogeny in PhyloPhlAn [11]. Briefly, whole genome sequences (WGS) were autonomously retrieved for (*i*) all lactobacilli, pediococci and lactococci and (*ii*) all *Bifidobacteria* and *Aeriscardovia aeriphila* LMG 21773 from the GenBank FTP site using a WGET script (database last accessed August 2019). WGS were annotated in PROKKA [12] with the translated coding sequences used for the identification, alignment and concatenation of 400-core protein sequences in Phylophlan (using the "-u" command). The ML phylogeny was reconstructed from the concatenated alignments in FastTree MP [13] (JTT + CAT) implemented in the Cipres Science Gateway Server [14]. The robustness of the phylogeny was assessed using 1000 bootstrap pseudoreplicates. Final rooted trees (using *Lactococcus* as an outgroup for *Lactobacillus* and *Aeriscardovia* for *Bifidobacterium* phylogenies) were rendered and annotated as circular phylogenies in iTOL (https://itol.embl.de/). Construction of the species specific SNP phylogenies were performed in Parsnp [15].

2.4. Probiogenomic analysis of Lab4

The prediction of phage elements was undertaken using PHAST [16]. Genomic islands (GI) were predicted using IslandViewer4 [17] and manually curated to remove islands consisting of ribosomal genes as these are often considered erroneous predictions of GIs [18]. GIs were analysed by comparing the genomic neighbourhoods among closely related species. Reference strains used for GI prediction were *B. animalis* subsp. *lactis* BL-04 (SD5219), *B. bifidum* (ATCC 29521) and L. *acidophilus* NCFM (ATCC700396) for CUL34, CUL20 and CUL21/CUL60 respectively. Probiogenomic analysis was performed to highlight genes encoding proteins that may impart beneficial traits by the organisms of the Lab4 consortium. Genes were selected based on an in-depth scrutiny of the literature and included those encoding proteins that aid in persistence and survivability within the GIT, metabolism of prebiotic compounds, immunomodulation and production of exopolysaccharides. In addition, metabolic pathway prediction was undertaken using gapseq [19]. Pathway predictions were performed with default parameter (bitscore >200 and a coverage of at least 75%). Outputs from gapseq were filtered so that only pathways that contained all genes (100% completeness) in at least one strain were retained. Carbohydrate active enzyme profiles (CAZy) were predicted using the dbCAN2 meta server [20]. For CAZyme prediction, translated coding sequence was submitted to the dbCAN2 online server with results filtered for enzymes predicted by three methods (HMMer, Hotpep and DIAMOND).

Putative antimicrobial resistance genes (ARGS) were predicted in the genomes according to Campedelli and colleagues [21]. Briefly, all translated CDS from genomes were retrieved from RAST and used firstly to query the online Comprehensive Antibiotic Resistance Database [22] for strict hits. In parallel, all ARGS (4807 protein sequences) retrieved from the CARD database were used to create a *de novo* BLASTP database with translated coding sequences from each Lab4 genome used as queries. In this case, ARGS were identified as the top BLASTP hits with an amino acid sequence identity of >30% and a query coverage of >70%. The resultant hit list was filtered to remove duplicates and singleton results manually re-annotated by querying the NCBI nr protein database using "*Lactobacillus*" or "*Bifidobacterium*" as a hit filter. Heatmaps were reconstructed in R using both ggplots and the Heatmap plus package.

2.5. Assessment of acid and bile tolerance

Overnight cultures of each of the Lab4 organisms were grown anaerobically in MRS broth and then inoculated in duplicate into flasks containing Phosphate Buffered Saline (PBS) with 3 g/L pepsin. One series of flasks was adjusted to pH 2.5 and the other was at pH 7. Viable numbers were determined after 90 min incubation anaerobically at 37 °C.Bile tolerance was assessed using 2-fold dilutions of OxGall-bile included in MRS agar from 6.9 to 0.43 mM. Briefly, an overnight culture of each strain was pelleted by centrifugation (4500 rcf for 5 min), washed once and resuspended to 0.1 OD_{600} (approximately 1 McFarland

standard) in fresh MRS. 5 µl of diluted culture was then used to spot inoculate MRS plates containing the OxGall-bile with a control plate of MRS alone. Plates were incubated anaerobically for 48 h at 37 °C. Survivability under each bile concentration was determined by the presence (+) or absence (−) of growth in 3 independent experiments.

2.6. Adherence to enterocytes

Caco-2 cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 4500 mg/L glucose, 1% (*v*/v) non-essential amino acids, 10% (v/v) heat inactivated fetal bovine serum (Labtech, Sussex, UK), penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C in 5% CO_2 and 95% humidity. Cells were seeded at 2×10^5 cells/cm^2 into tissue culture coated 12 well plates (Costar, Cambridge, UK) and allowed to grow until confluence. Confluent monolayers were maintained for 15 days to facilitate differentiation of the Caco-2 cells into a physiologically representative phenotype [23]. Prior to exposure to the probiotic organisms the Caco-2 cells were incubated with supplemented DMEM without penicillin and streptomycin for 1 h.

Freeze-dried preparations of the Lab4 consortium were diluted to 2×10^8 cfu/ml with supplemented DMEM (without penicillin and streptomycin) and the numbers of viable lactobacilli and bifidobacteria enumerated as previously described. 0.5 mL of the bacterial suspensions were added to the confluent monolayers of Caco-2 (approximately 5×10^5 cells/well) to achieve multiplicity of infection of approximately 200. After 1 h incubation at 37 °C in 5% CO_2, bacterial suspensions were removed and discarded and the Caco-2 cells washed 3 times in sterile PBS (37 °C). Cell lysates were collected by incubation with 1 mL of DMEM containing 10% tryspin/EDTA until all cells had detached from the plate and the content of viable bacteria enumerated. The number of viable adhered bacteria was expressed as a percentage of the total number of viable bacteria added.

2.7. Antibiotic susceptibility testing

The Minimum Inhibition Concentration (MIC) of ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol was determined for each strain using a modified version of the micro-broth dilution methods recommended by the Clinical and Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.Eucast.org) and ISO standards (ISO 10932/IDF 223, 2010). Briefly, overnight cultures grown in MRS media were pelleted by centrifugation and adjusted to 0.2 OD_{600} in *Lactobacillus* susceptibility test medium, LSM broth (90% MRS with 10% Iso-sensitest plus the addition of 0.05% *wt/vol* L-cysteine for bifidobacteria strains). The suspension was further diluted 1:1000 (in LSM broth) into a 96 well plate containing 2-fold dilutions of antibiotic stock. Plates were incubated anaerobically between 24-48hr depending on strain requirements and read at 600nm. Dilutions used were determined empirically to provide consistent growth across experimental replicates. *L. acidophilus* ATCC 4356 was used a control strain in all experiments. MIC breakpoints were initially determined visually and then confirmed by reading absorbance using a 96-well plate reader. To further support the assessment of AST we performed disc diffusion assays where resistance to antibiotics was seen above the EFSA breakpoints using the microbroth dilution method.

2.8. Maintenance of animals and administration of Lab4 probiotics

All animal work was completed within the test facility of Intox Pvt. Ltd. (Maharashtra, India) and was performed in compliance with the OECD Principles of Good Laboratory Practice (OECD, 1998). A preliminary acute phase toxicity study was performed on three 9 to 10 week old female rats that were housed in a specific pathogen-free ventilated cage in a light- and temperature-controlled facility (12 h light, 12 h dark, 19 to 25 °C). Initially, a single rat was exposed to two doses of the Lab4 consortium (5×10^{11} cfu/kg) administered 2 h apart by oral gavage (in 10 mL PBS). The remaining 2 rats received similar dosing 4 days later. Dosing was calculated using body weights on the day of probiotic administration. Each rat was then monitored for mortality and clinical signs of toxicity for 14 days post supplementation before termination and histopathological analysis. Throughout the study, rats were given *ad libitum* access to standard diet (Altromin 1320, Spezialfutter GmbH & Co. KG, Germany) and sterilised water.

The acute phase toxicity study was followed by a repeated dose 90-day oral toxicity study in which one hundred 6 to 7-week-old Wistar rats (50 male and 50 female) were housed in specific pathogen-free ventilated cages (up to 2 rats of similar sex per cage) in a light- and temperature-controlled facility (12 h light, 12 h dark, 19 to 25 °C). These rats were assigned into 4 groups per sex that received a daily gavage of PBS alone (control, 15 rats) or PBS with 1×10^{11} cfu/kg/day (dose 1, 10 rats), 2×10^{11} cfu/kg/day (dose 2, 10 rats) or 5×10^{11} cfu/kg/day (dose 3, 15 rats) of the Lab4 consortium. Dosing was calculated using body weights at the start of the study that were 0.22 ± 0.015 kg for males and 0.17 ± 0.010 kg for females. Throughout the study, rats were given *ad libitum* access to standard diet (Altromin 1320, Spezialfutter GmbH & Co. KG, Germany) and sterilised water. Body weights and food consumption were monitored weekly. At the end of the feeding period (day 90), 10 rats from each group were terminated for sample collection and the remaining 5 rats in dose 3 and control groups entered a 28-day probiotic free wash-out period before termination on study day 118.

2.9. Clinical observations during repeat dose 90-day oral toxicity study

Daily examinations of mortality (plus any moribund animals) and changes in anthropometry, posture/movement, respiration, palpebral closure, lacrimation, salivation, skin and hair coat were performed at the cage side. Detailed weekly examinations of changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as, pilo-erection, pupil size, respiratory pattern, gait, posture, response to handling, presence of clonic or tonic movements, stereotypies or unusual behaviour were performed in a standard enclosure. During weeks 11 and 12 of the 90-day feeding study, locomotor activity was assessed by tracking each animal for 10 min in an open field enclosure (50 cm by 50 cm by 38 cm (width:length:height)) using the ANYMAZE® video tracking system (Stoelting Co, IL, USA).

After termination, organs were extracted, weighed and fixed in 10% *v*/v neutral buffered formalin (lungs were inflated with fixative prior to immersion), embedded in paraffin wax, sectioned (5 µm thickness) and stained with haematoxylin and eosin for microscopic examination.

2.10. Analysis of faeces during repeat dose 90-day oral toxicity study

Faecal samples were collected from control rats at 90 days and high dose male rats at days 90 and 118 days and stored at -80 °C under anaerobic conditions. Prior to analysis, the faecal samples were thawed under anaerobic conditions and 10-fold dilution series were set up in Maximum Recovery Medium (MRD). Numbers of viable lactobacilli and bifidobacteria were determined on MRS and MRSX respectively and incubated anaerobically at 37 °C. Total aerobes were enumerated on horse blood agar, staphylococci on Baird Parker agar and yeasts on DRBC agar under aerobic conditions for 48 h at 37 °C. *Clostridioides* spp. were enumerated on *C. difficile* agar following heat shock treatment of the faecal samples and incubated anaerobically at 37 °C for 48 h. Total anaerobes were enumerated on pre-reduced anaerobic agar and incubated under anaerobic conditions for 48 h at 37 °C. Identification of bacteria was performed by Gram staining, colony morphology and by analytical profile index (API, BioMerieux, Marcy-l'Étoile, France). Viable bacterial cell counts were recorded as the numbers of $\log_{10}$ cfu per gram of sample.

2.11. Statistical analysis

All data are presented as the mean ± standard deviation (SD) of the assigned number of independent experiments or rats. All data were subject to Levene's test for homogeneity and the Kolmogorov-Smirnov and Shapiro-Wilk tests for normality of distribution. Log or Box Cox transformations were performed as required. For multiple comparisons, values of p were calculated using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison of means test where the data was normally distributed and with homogeneous intra-group variances or Kruskal Wallis one-way analysis of variance on rank followed by Mann-Whitney U Test or Dunn's test with Bonferroni correction (as necessary) where data was non-parametric or with heterogeneous intra-group variances. For single comparisons, values of p were calculated using Independent t-Test. Statistical analysis was performed using IBM SPSS Statistical Software (version 23) or GraphPad PRISM (version 8.2.1, California, USA) and values of p were considered significant when <0.05.

3. Results & discussion

3.1. Genome metrics and taxonomical classification of the Lab4 strains

The whole genome sequence for each organism in the Lab4 consortium has been deposited in the DDBJ/EMBL/GenBank under the following BioProjects: PRJNA559984 (*B. bifidum* CUL20), PRJNA482335 (*Lactobacillus acidophilus* CUL60), PRJNA482434 (*Lactobacillus acidophilus* CUL21) and PRJNA482550 (*Bifidobacterium animalis* subsp. *lactis* CUL34). The versions presented in this paper are the first versions and represent a set of contigs. Estimated genome sizes and percentage GC content of the organisms (Table 1) are consistent with their membership in their respective clades and suggest that near complete genomes have been retrieved. The topologies of the multilocus ML phylogenies (Fig. 1) further support the standing nomenclature of the strains. Of note is that species of *Pediococcus* were positioned as a subclade within the genus *Lactobacillus* (Fig. 1A) – a paraphyly that has been described previously [24]. Given the agreement of our topology with previous studies, we were able to ascribe a high-level of confidence in our taxonomic identity of the individual Lab4 strains. The SNP phylogeny of the L. *acidophilus* clade (Supplementary Fig. S1), used to further differentiate CUL21 and CUL60, indicated that CUL21 and CUL60 were most closely related to L. *acidophilus* KLDS 10901 and L. *acidophilus* La14 respectively. CUL34 and CUL20 were grouped within the *B. animalis* and *B. bifidum* clades respectively (Fig. 1C–E).

3.2. Survival, colonisation and persistence of Lab4 within the gastrointestinal tract

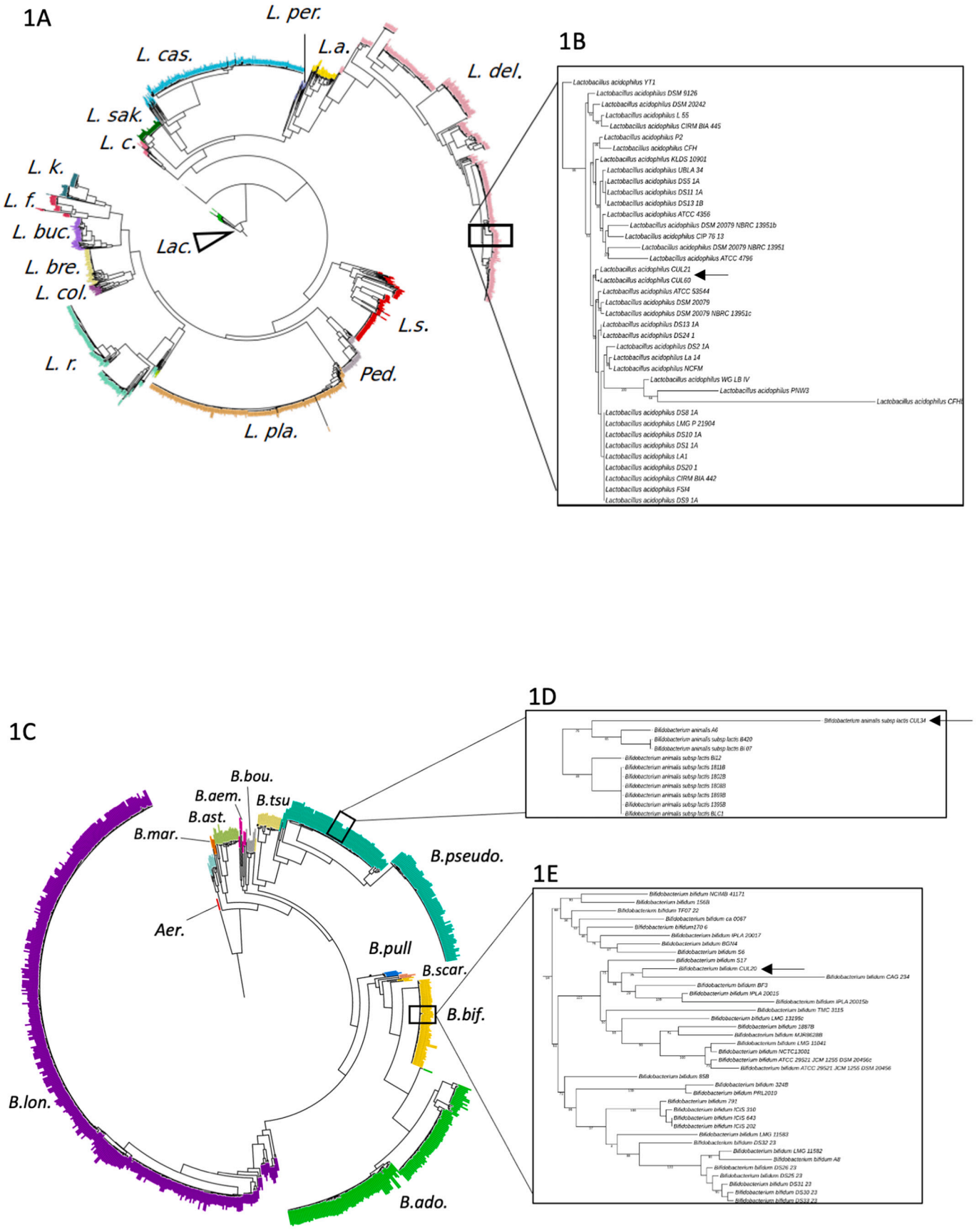
The ability to survive in the harsh conditions of the GIT and adhere to the intestinal mucosa are considered essential requirements conforming with the definition of a probiotic organism [3]. We mined the genomes of each strain to identify putative genes involved in these processes and all strains were found to possess repertoires of genes involved in acid and bile tolerance, adherence to the intestinal epithelium and biofilm formation capabilities evidencing the presence of the requisite probiotic characteristics in the organisms in the Lab4 consortium (Table 2 and **Supplementary Material 1**).

An acidic environment is maintained in the GIT to aid digestion and to prevent the growth of harmful bacteria and viruses [25]. Highest acidity occurs in the empty stomach and that can fluctuate between pH 2 during fasting and pH 5 after food [26]. The viability of all strains was unaffected by 90 min exposure to pH 2.5 (Fig. 2A) suggesting an ability to survive transit through the stomach and into the intestines. In support of these results *in vitro*, all Lab4 strains possessed genes encoding for L-lactate dehydrogenase (L-LDH) with CUL21 and CUL60 possessing an additional gene encoding for the optical isomer, D-Lactate dehydrogenase (D-LDH). LDH genes have been shown to be upregulated under acidic conditions and convert pyruvate to lactate enabling the removal of acidic compounds from the cell [27]. The bifidobacteria (CUL34 and CUL20) possess multiple genes coding for F0F1-type ATP synthase and a gene encoding the Na+/H+ NhaA antiporter which act similarly to LDH [27].

Upon entry to the intestines, the probiotic bacteria are exposed to bile acids that can be highly toxic to microorganisms that are not adapted to survive under these conditions [28]. Bile acid concentrations in the duodenum can range between 4 mM (preprandial) to 14 mM (postprandial) [29,30] and we show that CUL21, CUL60 and CUL20 were viable in total bile acid concentrations exceeding 6.9 mM (Fig. 2B) suggesting an ability to survive within the physiological bile range. CUL34 was found to be less tolerant (up to 0.8 mM). The genetic basis for these findings appears to be the presence of at least one copy of a bile salt hydrolase (BSH) gene (choloylglycine hydrolase) in all Lab4 strains (two copies in CUL21 and CUL60) that is thought to aid bile tolerance through the deconjugation and precipitation of toxic bile acids [31], although the exact mechanisms are yet to be resolved. BSH activity is associated with the reduction of circulating cholesterol levels in the host [32–34] and CUL21, CUL60 and CUL20 (not CUL34) possess copies of a gene coding for glucosamine-6-phosphate deaminase which is thought to impart protection during bile stress by liberating glucosamine for use as an additional energy source [35]. A bile inducible operon that confers bile tolerance in L. *acidophilus* NCFM (LBA1425 to LBA1432 [36]) was also present in both CUL21 and CUL60. The genomes of both CUL21 and CUL60 encoded for Dps proteins that are stress regulating proteins implicated in the protection of DNA under a wide variety of conditions (including osmotic, heat [37,38] and oxidative stress [39]).These proteins appear poorly studied in lactobacilli (not at all in L. *acidophilus*), but have been shown to be upregulated in *Lactobacillus plantarum* in the presence of bile [40].

The adherence of probiotic bacteria to the intestinal epithelium/mucosa supports transient colonisation thus prolonging exposure to and interaction with the host facilitating beneficial effects such as immunemodulation [41]. Each of the Lab4 strains was applied to Caco-2 enterocytes (Fig. 2C) and showed an ability to adhere. Interestingly, highest adherence was observed for *B. bifidum* (~8%) that might have been expected to demonstrate the poorest adherence ability on the basis of our predicted gene analysis (Table 2). However, there appeared to be no significant difference between the strains in their ability to adhere to Caco-2 enterocytes ($p = 0.084$). This highlights the possibility of the presence of unidentified adherence mechanisms or that the presence of adherence related genes is not a good indicator of function. Both strains of lactobacilli (CUL21 and CUL60) encode for enolase that is capable of binding to laminin present in the basal lamina in the host gastrointestinal tract [42], mutliple MucBP domain containing proteins that promote mucus adhesion [43] and for two surface layer proteins (homologous to products of the gene *slpA* in L. *acidophilus* NCFM) that aid adhesion [44]. Additionally, our CAZYome predictions for all Lab4 strains indicate an overrepresentation of glycoside hydrolase (GH) genes in all genomes (**Supplementary Material 2**) and these have been implicated in binding to mucin [45]. Both lactobacilli also contained genes for fibronectin-binding protein that binds to fibronectin in the extracellular matrix of epithelial cells [46].

It is hypothesised that the successful colonisation of the intestinal epithelium can lead to the formation of biofilms that enhance resistance to the environmental conditions of the GIT thus prolonging survival [47]. There is an indication that Lab4 has the ability to form biofilms *in vitro* (Supplementary Fig. S2) and we propose that this ability may be mediated, at least in part, by the presence of a complete gene cluster that is homologous to the *wzy/wzx* exopolysaccharide gene cluster in CUL21 and CUL60. Presence of this cluster has not only been shown to enhance biofilm formation, but also supports adherence to the intestinal epithelium and immunomodulation [48]. Symptomatic improvements were observed in a human cohort of irritable bowel syndrome sufferers



(caption on next page)

Fig. 1. Maximum-Likelihood Multi Locus *Lactobacillus and Bifidobacterium* phylogenies.
(A) Maximum-Likelihood multi-locus phylogeny of the genus *Lactobacillus*. Phylogeny was created using a concatenation of 400 core proteins from each genome. Phylogeny is rooted along the lineage leading to *Lactococcus* (open arrow + *Lac.*). Phylogeny includes the paraphyletic clade *Pediococcus* (*Ped.*). Phylogeny was created in PhyloPhlAn and rendered in iTOL. **(B)** Position of CUL20 and CUL60 indicated on subclade image with a black arrow. Clade colours represent species phylogroups. Genus name = *Lactobacillus,* unless stated otherwise. *L.s* = *Ligilactobacillus salivarius*; *ped* = *Pediococcus* spp.; *L. pla.* = *Lactiplantibacillus plantarum*; *L.r.* = *Limosilactobacillus reuteri*; *L. col.* = *L. collinoides*; *L. bre.* = *Levilactobacilus brevis*; *L. buc* = *L. buchneri*; *L.f.* = *L. fructivorans*; *L.k.* = *L. kunkeei*; *L.c.* = *L. coryniformis*; *L. sak.* = *L. sakei*; *L. cas.* = *Lacticaseibacillus casei; L. per* = *L. perolens; L.a.* = *L. alimentarius; L. del* = *L. delbrueckii.* **(C)** Maximum-Likelihood multi-locus phylogeny of the genus *Bifidobacterium.* Phylogeny was created using a concatenation of 400 core proteins from each genome. Phylogeny is rooted along the lineage leading to *Aeriscardovia* (*Aer*). Phylogeny was created in PhyloPhlAn and rendered in iTOL. Coloured regions represent homogenous clades. *Aer* = *Aeriscardovia; B. mar* = *B. margollesii; B. ast* = *B. asteroides; B. aem* = *B. aemilianum; B. bou* = *B. boum; B. tsu* = *B. tsurumiense*; *B. pseudo* = *B. pseudolongum; B. pull* = *B. pullorum; B.scar* = *B. scardovii; B. bif* = *B. bifidum; B. ado* = *B. adolescentis; B. lon* = *B. longum*. Positions of CUL34 **(D)** and CUL20 **(E)** indicated with black arrows on subclade images. Numbers at nodes in panels (B,D and E) represent percentage bootstrap values from 1000 pseudoreplicates.

Table 1
Genome metrics of Lab4 genomes and a comparison with strains from the same genus.

Species	Strain	Contig GC content (%)	Genome Size (Mb)	Median genome size (Mb)*	Median GC content (%)*
L. acidophilus	CUL21	34.6	1.97	1.99	34.7
L. acidophilus	CUL60	34.6	1.98	1.99	34.7
B. animalis	CUL34	60.4	1.93	1.93	60.5
B. bifidum	CUL20	62.6	2.20	2.21	62.7

* median genome size of bacterial species retrieved from Genbank. Abbreviations: Mb, megabases; G, guanine; C, cytosine.

in response to supplementation with Lab4 suggesting an anti-inflammatory capability [49].

The survival of all Lab4 strains in the GIT maybe supported by functional complementarity between strains. Comparative analysis of metabolic pathways (Supplementary Fig. S3) indicated differences in the amino acid producing capabilities and it is of particular interest that the lactobacilli (CUL21 and CUL60) possess complete pathways for the synthesis of L-cysteine whereas the bifidobacteria (CUL20 and CUL34) do not. L-cysteine auxotrophy has been observed in bifidobacteria [50] and our findings highlight the potential of CUL21 and CUL60 to support the survival of the CUL20 and CUL34 and other autochthonous bifidobacteria *via* the synthesis of L-cysteine. In addition, it is worth noting that, collectively, the Lab4 strains possess complete pathways encoding for the synthesis of 5 of the 9 essential amino acids and 10 of the 12 non-essential amino acids that support tissue growth, immune function and hormone synthesis in humans [51].

3.3. Lab4 antibiotic resistome and genomic stability

The World Health Organisation (WHO) has described antibiotic resistant bacteria as 'one of the biggest threats to human health' and regulatory agencies governing food safety such as the European Food Standards Authority (EFSA) require an assessment of the antibiotic susceptibility and any potential genomic risk factors for all probiotic organisms [52]. Such genomic risk factors include the presence of intrinsic antibiotic resistance genes (ARG), acquired genomic islands (GI) or any plasmids containing ARG that may enable horizontal gene transfer.

Antibiotic sensitivity testing using the EFSA recommended micro-broth dilution method (Table 3) indicated that CUL21 and CUL20 were susceptible to all antibiotics tested. However, CUL60 was resistant to

Table 2
Genes in the Lab4 consortium annotated by RAST that contribute to probiotic traits, genomic risk factors and key safety subcategories.

Category/Role	EC Number*	CUL21	CUL60	CUL34	CUL20	Proposed relevance
L-Lactate dehydrogenase	1.1.1.27	✓	✓			Acid tolerance
	1.1.2.3	✓	✓	✓	✓	
D-Lactate dehydrogenase	1.1.1.28	✓	✓			Acid tolerance
F0F1-type ATP synthase:						
α-chain				✓	✓	
β-chain				✓	✓	
γ-chain				✓	✓	
δ-chain				✓	✓	
ε-chain				✓	✓	
F0 sector subunit a				✓	✓	
F0 sector subunit b				✓	✓	
F0 sector subunit c					✓	Acid tolerance
Na+/H+ NhaA antiporter				✓	✓	Acid tolerance
Choloylglycine hydrolase	3.5.1.24	✓	✓	✓	✓	Bile tolerance
Glucosamine-6-phosphate deaminase	3.5.99.6	✓	✓		✓	Bile tolerance
Non-specific DNA-binding protein Dps		✓	✓			DNA binding/Stress response
Enolase	4.2.1.11	✓	✓	✓		Adherence
Fibronectin-binding protein		✓	✓			Adherence
Mycobacterium virulence operons:						
SSU ribosomal protein S7p (S5e)		✓	✓	✓	✓	
SSU ribosomal protein S12p (S23e)		✓	✓	✓	✓	
LSU ribosomal protein L35p			✓			
LSU ribosomal protein L20p			✓			
DNA-directed RNA polymerase β-subunit	2.7.7.6		✓			
Translation initiation factor 3			✓			
Translation elongation factor Tu		✓	✓	✓	✓	Virulence
Translation elongation factor G		✓	✓	✓	✓	Virulence/Antibiotic resistance
Ribosome protection-type tetracycline resistance related proteins		✓	✓	✓	✓	Antibiotic resistance
Tetracycline Resistance (*tetW*)				✓		Antibiotic resistance
Phage portal protein		✓	✓			Mobile element

* Enzyme commission number assigned by the annotation committee of the International Union of Biochemistry and Molecular Biology.

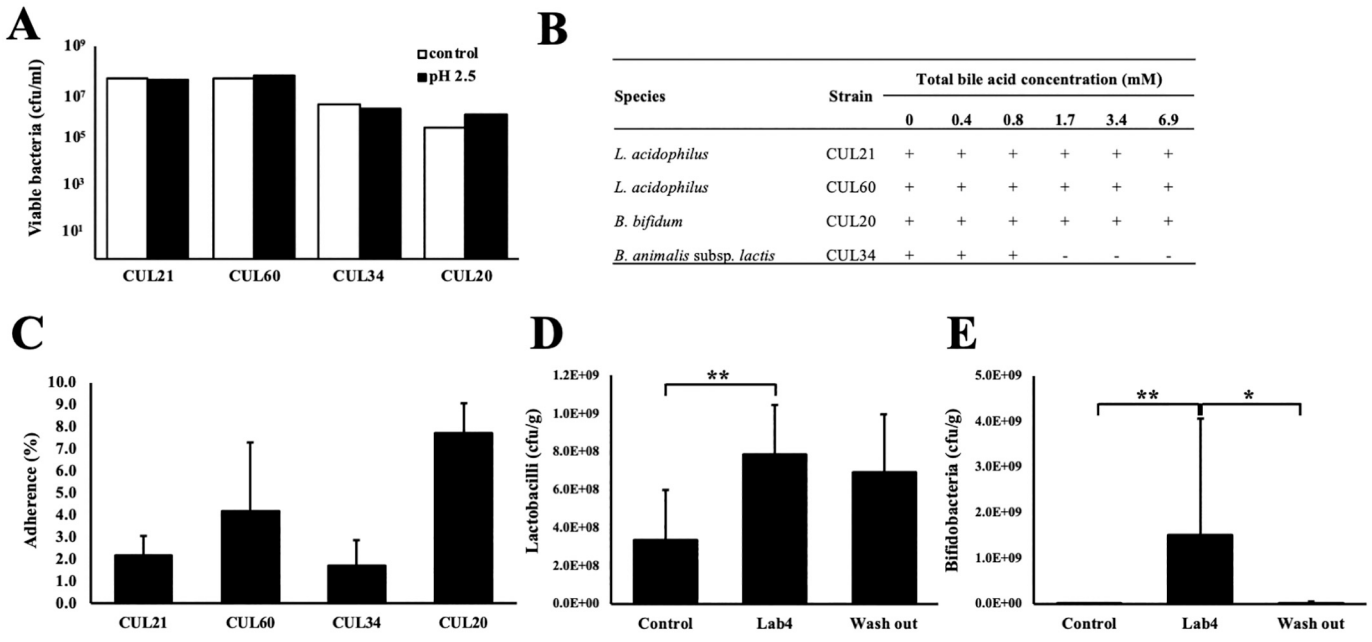


Fig. 2. *In vitro* assessment of probiotic traits.
(A) Total viable numbers of the Lab4 strains after exposure to PBS (control, empty bars) or PBS at pH 2.5 (filled bars) for 90 min. **(B)** Survival of the Lab4 strains in the presence of bile where + or – indicate the presence of growth or no growth, respectively, in 3 independent experiments. **(C)** Adherence of the Lab4 strains to Caco-2 enterocytes. Data is presented as the means of triplicate samples from a single experiment (panel A) or as the mean ± standard deviation (SD) of three independent experiments (panels B and C). Values of p were determined using Kruskal Wallis one-way analysis of variance on rank and were considered significant when <0.05 (Fig. C). Viable numbers of **(D)** lactobacilli and **(E)** bifidobacteria in the faeces of male Wistar rats supplemented for 90 days with the highest dose of Lab4 (dose 3, 10 rats) and after the 28 day probiotic-free wash-out period (5 rats). Data is present as mean ± standard deviation (SD) and values of p were determined on box-cox transformed data using the Kruskal Wallis one-way analysis of variance on ranks followed by Dunn's test with Bonferroni correction for multiple comparisons where $*p < 0.05$ and $** p < 0.01$.

ampicillin and chloramphenicol (4 mg/L), while CUL34 showed resistance to tetracycline (32 mg/L). Comparison of the resistance profiles using the disc diffusion method somewhat supported these data (**Supplementary table S1**) in that CUL60 was resistant to ampicillin at concentrations > 1 mg but resistance to chloramphenicol was < 10 mg. For CUL34, resistance to tetracycline was < 10 mg. Whilst there is some congruence between the antibiotic resistance profiles determined between these two methods, with the disc diffusion method giving lower resistance profiles, care should be taken when interpreting data from this method as it is not supported by EFSA. Thus for this study, we use values obtained by the microbroth dilution method. These observations were confirmed by the presence of a number of antibiotic resistance genes (Table 2) including beta-lactamase genes and tetracycline resistance (*tet*(W)) genes in CUL34. Of further interest is the sensitivity of both L. *acidophilus* isolates to vancomycin that is in contrast to the intrinsic resistance to vancomycin displayed by the many lactobacilli [53]. However, further genomic analysis indicated that the sensitivity to vancomycin correlated with the presence of a tyrosine (Y) residue located within the active site of the D-alanyl-d -alanine ligase (Ddl) protein; which is consistent with reports elsewhere [54]. Predictions *in silico* of the resistome (Fig. 3A) showed that the genomes of the Lab4 strains possessed genes relating to macrolide, chloramphenicol, tetracycline, vancomycin, fosfomycin, bacitracin, trimethoprim and kanamycin resistance as well as possessing genes encoding for efflux pumps and transporters. Interestingly, and in agreement with previous studies [21], our analysis indicated there was very little correlation between gene presence and demonstrable resistance to specific antibiotics (Fig. 3B). In fact, aside from the intrinsic resistance to vancomycin, we only identified two cases - the presence of *tet*(W) in CUL34 and the presence of *tetA*60/*tetBP* in CUL60 - where the resistance could be attributable to the presence of specific resistance genes. That said we appreciate that the presence of a number of broad-spectrum efflux pumps and transporters may also be mediating the resistance profiles identified.

The presence of putative plasmid genes was investigated by mapping raw sequencing reads to known, common plasmids. None of the reads

Table 3
Antibiotic resistance profiles for Lab4 strains.

	Minimum inhibitory concentration (mg/L)								
	Ampicillin	Vancomycin	Gentamicin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
EFSA Breakpoints for L. *acidophilus*†	**1**	**2**	**16**	**64**	**16**	**1**	**1**	**4**	**4**
L. acidophilus CUL21	1	0.5	8	32	8	0.25	0.25	4	1
L. acidophilus CUL60	4	1	4	32	8	0.25	0.25	16	4
EFSA Breakpoints for *Bifidobacterium*†	**2**	**2**	**64**	**nr**	**128**	**1**	**1**	**8**	**4**
B. bifidum CUL20	0.125	0.5	8	nr	16	0.125	0.5	1	2
B. animalis subsp. *lactis* CUL34	1	1	16	nr	125	0.125	1	32	1

†According to *Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance*, EFSA journal 2012; 10(6):2740. nr = not required.

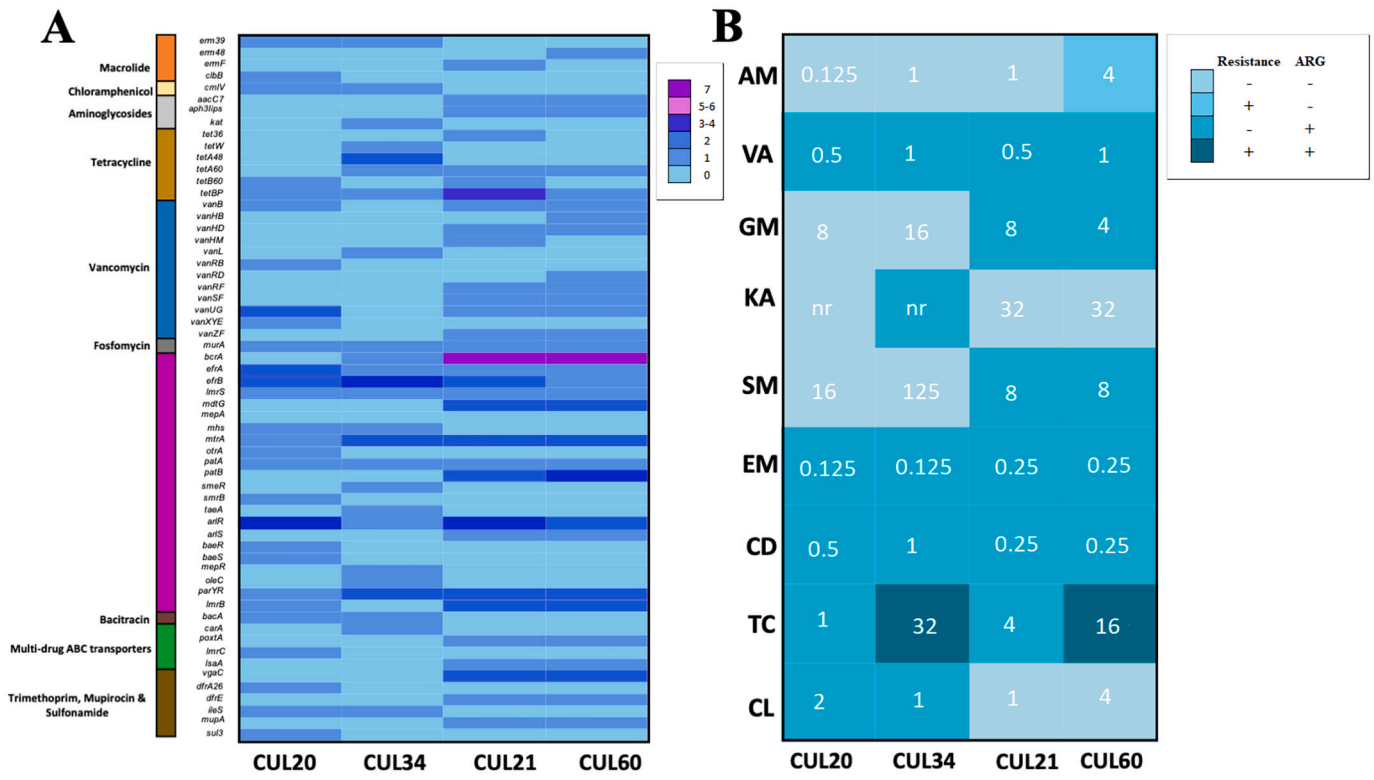


Fig. 3. *In silico* predicted Lab4 resistome and heatmap correlation of antibiotic phenotypic resistance with predicted resistance genes in Lab4.
(A) Heatmap schematic representation of the predicted *In silico* Lab4 resistome. Prediction was performed using BLASTP against a *de novo* BLAST database of ARGS downloaded from the CARD database. Drug class is indicated on the left side of the heatmap. Colours indicate number of gene hits. **(B)** Heatmap correlating resistance phenotype with genes related to specific antibiotic drug classes. Colours indicate presence or absence of resistance with the presence or absence of ARGS. AM = Ampicillin, VA = Vancomycin, GM = Gentamycin, KA = Kanamycin, SM = Streptomycin, EM = Erythromycin, CD = Clindamycin, TC = Tetracycline, CL = Clindamycin.

mapped to the references pLA103 and pLA106 (*L. acidophilus* strains) nor pB80 (*B.bifidum*) suggesting a lack of plasmids in the Lab4 organisms and supporting an absence of acquired extraneous, mobile genetic material.

Genomic islands were predicted using Islandviewer 4.0 with the genomes of CUL21, CUL60, CUL34 and CUL20 containing 6, 7, 9 and 11 genomic islands respectively (**Supplementary Material 3**). Scrutiny of the gene composition of the predicted GIs showed that they were mainly composed of hypothetical proteins, recombinases, proteins related to transport, glycotransferases and endopeptidases although GI#1 of CUL34 was found to harbour a tetracycline resistance (*tet*(W)) gene. Further analysis of this GI showed that *tet* related genes appear to be ubiquitous in *B. animalis* subsp. *lactis* [55] and the genomic region presented here is homologous to that of other strains of bifidobacteria [56]. It is likely that this genomic region is a false prediction of an acquired island and we consider that this GI would be of low risk. Attempts at transferring *tet* genes from bifidobacteria to other bacterial species have usually been unsuccessful [57] meaning that *tet*(W) is generally not considered to be easily transmissible [56,58].

ARGs can be acquired *via* bacteriophages and bacterial conjugation [59–61] and genomes of the Lab4 organisms were screened for relevant genes and phage-related DNA sequences have been observed in strains of lactobacilli and bifidobacteria [4,62,63]. In our analysis, we identified potential phage encoding genes in CUL21 and CUL60 through our RAST annotation (Table 2) relating to a phage portal protein that functions as a conduit for the entry of viral DNA into bacterial cells. The presence of phage elements was also assessed using PHAST that confirmed the presence of these genes, but importantly, identified them as part of incomplete phage genomes. Indeed, BLASTP comparison against phage proteins showed that CUL21 had sequences that identified as the integrase, CL-repressor, DUTPase and Lysin of the *Lactococcus* phage 50,101 but the percentage identities were less than 40% and were not present within a contiguous region of the CUL21 genome. CUL60 possessed the lysin, integrase, repressor, cro repressor and a further putative protein from the *Lactobacillus* phage Lj965, but again, these were not located within the same genomic region and had low sequence identity (~50–60%). RAST analysis did not identify any phage-related genes in the bifidobacteria CUL34 and CUL20. In addition, we did not identify any genes relating the Type-4 secretion system (T4SS) machinery, implying that these species are unable to undergo conjugation.

3.4. Safety assessment of the Lab4 consortium

The potential for probiotic bacteria to negatively impact the host *via* the production of toxins is another area of concern and some strains of *Lactobacillus* have been shown to express histidine and tyramine carboxylase enzymes that generate the biogenic amines, histamine and tyramine [64]. RAST analysis did not detect any genes that have been directly linked to toxic effects among the organisms in the Lab4 consortium. This analysis was supported by the absence of any adverse effects observed when Wistar rats received repeated high doses of the Lab4 consortium. More specifically, no mortalities, clinical/histopathological abnormalities nor indications of systemic toxicity were observed throughout either the acute phase or repeat dose toxicity studies. A number of statistically significant changes in locomotor actively, organ weight, plasma biochemistry and haematology were observed in male and/or female rats during the repeat dose study, but all changes were within normal physiological ranges for these animals and were deemed inconsequential (Table 4). Based on these findings, it might be feasible to assign a no-observed-adverse-effect level (NOAEL) of at least 5×10^{11}

cfu/body weight(kg)/day to the Lab4 probiotic consortium.

Faecal samples were collected from males at the end of the feeding (control and high dose rats) and wash-out periods (high dose rats). At the end of the intervention there were significantly higher numbers of lactobacilli detected in the supplemented rats compared to the controls ($p = 0.0055$) and during the wash-out period the numbers in the supplemented rats started to decline ($p = 0.0993$, Fig. 2D). With regard to the bifidobacteria (Fig. 2D), these were only detectable in 40% of the control rats at 90 days, but all of the supplemented rats were positive for bifidobacteria (with counts in excess of 10^7 cfu/g, $p = 0.0013$) suggesting that supplementation with Lab4 significantly impacted the intestinal microbiota composition. During the washout period, the numbers of bifidobacteria decreased significantly ($p = 0.0106$) with only 40% of the rats testing positive indicating the colonisation was transient and that regular supplementation was required to maintain their presence (**Supplementary Table S1**). The numbers of total aerobes were higher in the supplemented rats at the end of the intervention period and the numbers remained stable during washout. No significant differences were detected in the numbers of anaerobes at the end of the intervention nor following wash-out. Numbers of staphylococci, yeasts and *Clostridioides* spp. were below the levels of detection for all samples tested.

3.5. Limitations of the study

In this study, we report draft genome sequences for each of the Lab4 strains which may mean that the genome annotations here should be considered partial. However, given that sequenced genome sizes reported here are commensurate with those reported for complete genome sequences for closely related strains, it is unlikely that we are missing significant portions of these genomes. Nevertheless, future completion of the Lab4 genomes would facilitate a more comprehensive analysis of gene composition. Our analysis of adherence of Lab4 considers only the use of Caco-2 enterocytes; whilst this cell line has been used extensively to study adherence in other studies, it is likely that the use of a higher mucin producing cell line (such as HT29-MTX) would bring about a higher percentage adherence and provide a better representation of the gastrointestinal epithelium.

3.6. Summary and conclusions

In this study, both the individual organisms and the Lab4 probiotic consortium were assessed using a combination of methodologies *in silico*, *in vitro* and *in vivo*. Draft genome sequences were deposited for each of the bacterial strains within Lab4 (*Lactobacillus acidophilus* CUL21 (NCIMB 30156), *Lactobacillus acidophilus* CUL60 (NCIMB 30157), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis* subsp. *lactis* CUL34 (NCIMB 30172)) and phylogenetic analysis confirmed their taxonomical classifications. Testing *in vitro* of individual strains and/or the complete Lab4 consortium showed the ability to tolerate physiological acid and bile levels, adhere to the intestinal epithelium and form biofilms and these findings aligned well with the presence of functionally related genes. In addition, we found that the antibiotic sensitivity profiles did not exceed accepted levels despite the presence of genes associated with antibiotic resistance in all isolates and, further, that the potential for horizontal gene transfer was very low. We also observed that the genomes of the Lab4 strains were void of toxicity/virulence-related genes and the tolerability and safety of the complete Lab4 consortium was confirmed through acute and repeat dose feeding studies in Wistar rats, also providing evidence of an ability to modulate the gut microbiota. In conclusion, this study supports the presence of probiotic traits in the Lab4 consortium and demonstrates tolerability and safety in toxicity studies. Future work should involve safety testing

Table 4
Statistically significant alterations observed in Lab4 fed Wistar rats.

	90-days feeding				28-days wash-out		Normal Range‡
	Control	Dose 1	Dose 2	Dose 3	Control	Dose 3	
MALES							
Locomotor							
Total distance travelled (m)	11.47 ± 4.66	18.40 ± 3.62*	17.22 ± 2.82*	14.01 ± 4.17	NM	NM	U
Average speed (m/s)	0.02 ± 0.01	0.03 ± 0.01*	0.03 ± 0.00*	0.02 ± 0.01	NM	NM	U
Clockwise rotation of body	3.9 ± 1.79	8.89 ± 2.67*	7.40 ± 3.10*	7.90 ± 2.47*	NM	NM	U
Liver (%)	2.52 ± 0.15	2.69 ± 0.20	2.49 ± 0.09*	2.69 ± 0.13	2.65 ± 0.31	2.62 ± 0.20	2.02 to 3.25
Haematology							
MCH (fl)	18.09 ± 0.33	18.00 ± 0.35	18.02 ± 0.48	18.15 ± 0.51	18.50 ± 0.33	17.94 ± 0.25#	16.43 to 19.34
MCV (pg)	51.48 ± 0.96	51.30 ± 1.03	51.33 ± 1.50	51.30 ± 1.62	52.80 ± 1.13	51.04 ± 0.83#	45.32 to 55.19
Total WBCC (x10^3/cmm)	3.79 ± 0.96	4.00 ± 0.87	3.91 ± 0.82	4.28 ± 1.07	4.68 ± 0.32	5.82 ± 0.88#	2.84 to 8.69
Plasma							
Total Protein (g/dL)	6.73 ± 0.25	7.18 ± 0.33*	7.12 ± 0.25*	7.15 ± 0.30*	7.24 ± 0.28	6.62 ± 0.26	5.33 to 8.12
Total Bilirubin (mg/dL)	0.1 ± 0.05	0.08 ± 0.04	0.05 ± 0.05*	0.09 ± 0.06	0.12 ± 0.04	0.10 ± 0.00	0.08 to 0.17
Glucose (mg/dL)	79.8 ± 8.00	94.6 ± 9.12*	90.40 ± 7.40*	94.80 ± 9.87*	81.60 ± 5.18	89.20 ± 7.4	55.51 to 131.73
Creatinine (mg/dL)	0.62 ± 0.07	0.66 ± 0.12	0.74 ± 0.09*	0.71 ± 0.05	0.56 ± 0.06	0.52 ± 0.04	0.42 to 0.77
GGT (IU/L)	5.90 ± 0.32	6.40 ± 0.52*	6.60 ± 0.70*	6.60 ± 0.70*	5.80 ± 1.10	5.80 ± 0.45	3.89 to 6.78
Calcium (mg/dL)	10.47 ± 0.20	11.00 ± 0.25*	10.75 ± 0.56	10.88 ± 0.15*	10.22 ± 0.22	10.24 ± 0.17	9.24 to 12.15
Globulin (g/dL)	5.38 ± 0.22	5.77 ± 0.29*	5.77 ± 0.24*	5.75 ± 0.28*	5.20 ± 0.34	5.36 ± 0.17	4.34 to 6.53
FEMALES							
Organ Weight							
Ovaries (%)	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.05 ± 0.01*	0.05 ± 0.01	0.06 ± 0.01	0.03 to 0.07
Haematology							
MCHC (g/dL)	35.52 ± 0.48	35.43 ± 0.47	35.49 ± 0.50	35.65 ± 0.31	35.42 ± 0.24	34.88 ± 0.35#	33.14 to 37.69
Monocytes (%)	0.62 ± 0.21	0.74 ± 0.34	0.41 ± 0.21*	0.56 ± 0.23	0.49 ± 0.25	0.48 ± 0.41	0.00 to 1.37
APTT (Seconds)	20.77 ± 6.55	18.93 ± 3.73	20.42 ± 7.66	21.96 ± 7.35	14.10 ± 0.97	20.60 ± 4.61#	10.47 to 20.44
Plasma							
Sodium	152.60 ± 7.35	154.20 ± 6.78	159.90 ± 17.80	154.60 ± 6.17	146.00 ± 1.58	150.20 ± 3.11#	139.22 to 156.47

Data represent the means ± SD of 10 rats (90-days) or 5 rats (wash-out) per group. Values of p were determined using one-way analysis of variance with Dunnett's multiple comparison of means test or Kruskal Wallis one-way analysis of variance on rank with Mann-Whitney U Test or Dunn's test with Bonferroni correction. Independent t-Test was used to compare groups after washout. *$p < 0.05$ compared to the 90-day control group, #$p < 0.05$ compared to the wash-out control group. Abbreviations: MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; WBCC, white blood cell count; GGT, gamma-glutamyl transpeptidase; APTT, activated partial thromboplastin time; NM, not measured; U, unavailable. †assessed during weeks 11 and 12, ‡from historical data at test facility.

in a human cohort to support the animal studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.08.007.

Author contribution

PDF, SFP, DRM and AAJ were responsible for the design of the study. Experiments were performed by LMB, TSW, MDA and TSCJ. Data analysis was performed by LMB and TSD with all statistical analyses performed by GM. TRH, JRM, GR and RDS provided assistance and knowledge that was vital to the completion of the manuscript. LMB and PDF prepared the manuscript and all authors contributed to the review of the manuscript.

Declaration of Competing Interest

LMB is a PhD student funded by the Knowledge Economy Skills Scholarships (KESS2) programme and is part funded by Cultech Ltd. TSW, DRM, GM, AAJ, MDA, TSCJ and SFP are/were employees of Cultech Ltd.

Acknowledgments

We would like to acknowledge Dr. Narendra Deshmukh of Intox Pvt. Ltd. for providing administrative and technical support during the project. The Division of Digestive Diseases at Imperial College London and JRM receive financial support from the NIHR Imperial Biomedical Research Centre (BRC) based at Imperial College Healthcare NHS Trust and Imperial College London. The views expressed in this publication are those of the authors and not necessarily those of the NHS, NIHR, or the Department of Health.

References

[1] Food and Agriculture Organization of the United Nations, World Health Organization, eds., Probiotics in food: health and nutritional properties and guidelines for evaluation, Food and Agriculture Organization of the United Nations, World Health Organization, Rome, 2006.

[2] Z. Sun, H.M.B. Harris, A. McCann, C. Guo, S. Argimón, W. Zhang, X. Yang, I. B. Jeffery, J.C. Cooney, T.F. Kagawa, W. Liu, Y. Song, E. Salvetti, A. Wrobel, P. Rasinkangas, J. Parkhill, M.C. Rea, O. O'Sullivan, J. Ritari, F.P. Douillard, R. Paul Ross, R. Yang, A.E. Briner, G.E. Felis, W.M. de Vos, R. Barrangou, T. R. Klaenhammer, P.W. Caufield, Y. Cui, H. Zhang, P.W. O'Toole, Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera, Nat. Commun. 6 (2015) 8322, https://doi.org/10.1038/ncomms9322.

[3] R. George Kerry, J.K. Patra, S. Gouda, Y. Park, H.-S. Shin, G. Das, Benefaction of probiotics for human health: a review, J. Food Drug Anal. 26 (2018) 927–939, https://doi.org/10.1016/j.jfda.2018.01.002.

[4] B. Lehri, A.M. Seddon, A.V. Karlyshev, Potential probiotic-associated traits revealed from completed high quality genome sequence of Lactobacillus fermentum 3872, Stand. Genomic Sci. 12 (2017) 19, https://doi.org/10.1186/s40793-017-0228-4.

[5] M. Alimolaei, M. Golchin, A comparison of methods for extracting plasmids from a difficult to lyse bacterium: Lactobacillus casei, Biologicals. 45 (2017) 47–51, https://doi.org/10.1016/j.biologicals.2016.10.001.

[6] A. Bankevich, S. Nurk, D. Antipov, A.A. Gurevich, M. Dvorkin, A.S. Kulikov, V. M. Lesin, S.I. Nikolenko, S. Pham, A.D. Prjibelski, A.V. Pyshkin, A.V. Sirotkin, N. Vyahhi, G. Tesler, M.A. Alekseyev, P.A. Pevzner, SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing, J. Comput. Biol. 19 (2012) 455–477, https://doi.org/10.1089/cmb.2012.0021.

[7] T. Tatusova, M. DiCuccio, A. Badretdin, V. Chetvernin, E.P. Nawrocki, L. Zaslavsky, A. Lomsadze, K.D. Pruitt, M. Borodovsky, J. Ostell, NCBI prokaryotic genome annotation pipeline, Nucleic Acids Res. 44 (2016) 6614–6624, https://doi.org/10.1093/nar/gkw569.

[8] D.A. Benson, M. Cavanaugh, K. Clark, I. Karsch-Mizrachi, J. Ostell, K.D. Pruitt, E. W. Sayers, GenBank, Nucleic Acids Res. 46 (2018) D41–D47, https://doi.org/10.1093/nar/gkx1094.

[9] R.K. Aziz, D. Bartels, A.A. Best, M. DeJongh, T. Disz, R.A. Edwards, K. Formsma, S. Gerdes, E.M. Glass, M. Kubal, F. Meyer, G.J. Olsen, R. Olson, A.L. Osterman, R. A. Overbeek, L.K. McNeil, D. Paarmann, T. Paczian, B. Parrello, G.D. Pusch, C. Reich, R. Stevens, O. Vassieva, V. Vonstein, A. Wilke, O. Zagnitko, The RAST Server: rapid annotations using subsystems technology, BMC Genomics 9 (2008) 75, https://doi.org/10.1186/1471-2164-9-75.

[10] H. Li, R. Durbin, Fast and accurate short read alignment with Burrows-Wheeler transform, Bioinformatics. 25 (2009) 1754–1760, https://doi.org/10.1093/bioinformatics/btp324.

[11] N. Segata, D. Börnigen, X.C. Morgan, C. Huttenhower, PhyloPhlAn is a new method for improved phylogenetic and taxonomic placement of microbes, Nat. Commun. 4 (2013) 2304, https://doi.org/10.1038/ncomms3304.

[12] T. Seemann, Prokka: rapid prokaryotic genome annotation, Bioinformatics. 30 (2014) 2068–2069, https://doi.org/10.1093/bioinformatics/btu153.

[13] M.N. Price, P.S. Dehal, A.P. Arkin, FastTree 2–approximately maximum-likelihood trees for large alignments, PLoS One 5 (2010), e9490, https://doi.org/10.1371/journal.pone.0009490.

[14] M.A. Miller, W. Pfeiffer, T. Schwartz, Creating the CIPRES Science Gateway for inference of large phylogenetic trees, in: 2010 Gateway Computing Environments Workshop (GCE), 2010, pp. 1–8, https://doi.org/10.1109/GCE.2010.5676129.

[15] T.J. Treangen, B.D. Ondov, S. Koren, A.M. Phillippy, The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes, Genome Biol. 15 (2014) 524, https://doi.org/10.1186/s13059-014-0524-x.

[16] Y. Zhou, Y. Liang, K.H. Lynch, J.J. Dennis, D.S. Wishart, PHAST: a fast phage search tool, Nucleic Acids Res. 39 (2011) W347–W352, https://doi.org/10.1093/nar/gkr485.

[17] C. Bertelli, M.R. Laird, K.P. Williams, Simon Fraser University Research Computing Group, B.Y. Lau, G. Hoad, G.L. Winsor, F.S.L. Brinkman, IslandViewer 4: expanded prediction of genomic islands for larger-scale datasets, Nucleic Acids Res. 45 (2017) W30–W35, https://doi.org/10.1093/nar/gkx343.

[18] X. Zhang, C. Peng, G. Zhang, F. Gao, Comparative analysis of essential genes in prokaryotic genomic islands, Sci. Rep. 5 (2015) 12561, https://doi.org/10.1038/srep12561.

[19] J. Zimmermann, C. Kaleta, S. Waschina, gapseq: Informed prediction of bacterial metabolic pathways and reconstruction of accurate metabolic models, BioRxiv. (2020), https://doi.org/10.1101/2020.03.20.000737.

[20] H. Zhang, T. Yohe, L. Huang, S. Entwistle, P. Wu, Z. Yang, P.K. Busk, Y. Xu, Y. Yin, et al., Nucleic Acids Res. 46 (2018) W95–W101, https://doi.org/10.1093/nar/gky418.

[21] I. Campedelli, H. Mathur, E. Salvetti, S. Clarke, M.C. Rea, S. Torriani, R.P. Ross, C. Hill, P.W. O'Toole, Genus-wide assessment of antibiotic resistance in Lactobacillus spp, Appl. Environ. Microbiol. 85 (2019), https://doi.org/10.1128/AEM.01738-18.

[22] B.P. Alcock, A.R. Raphenya, T.T.Y. Lau, K.K. Tsang, M. Bouchard, A. Edalatmand, W. Huynh, A.-L.V. Nguyen, A.A. Cheng, S. Liu, S.Y. Min, A. Miroshnichenko, H.-K. Tran, R.E. Werfalli, J.A. Nasir, M. Oloni, D.J. Speicher, A. Florescu, B. Singh, M. Faltyn, A. Hernandez-Koutoucheva, A.N. Sharma, E. Bordeleau, A. C. Pawlowski, H.L. Zubyk, D. Dooley, E. Griffiths, F. Maguire, G.L. Winsor, R. G. Beiko, F.S.L. Brinkman, W.W.L. Hsiao, G.V. Domselaar, A.G. McArthur, CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database, Nucleic Acids Res. 48 (2020) D517–D525, https://doi.org/10.1093/nar/gkz935.

[23] M. Natoli, B.D. Leoni, I. D'Agnano, F. Zucco, A. Felsani, Good Caco-2 cell culture practices, Toxicol. in Vitro 26 (2012) 1243–1246, https://doi.org/10.1016/j.tiv.2012.03.009.

[24] E. Salvetti, H.M.B. Harris, G.E. Felis, P.W. O'Toole, Comparative genomics of the genus lactobacillus reveals robust phylogroups that provide the basis for reclassification, Appl. Environ. Microbiol. 84 (2018), https://doi.org/10.1128/AEM.00993-18.

[25] J.L. Smith, The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions, J. Food Prot. 66 (2003) 1292–1303, https://doi.org/10.4315/0362-028x-66.7.1292.

[26] L. Ovesen, F. Bendtsen, U. Tage-Jensen, N.T. Pedersen, B.R. Gram, S.J. Rune, Intraluminal pH in the stomach, duodenum, and proximal jejunum in normal subjects and patients with exocrine pancreatic insufficiency, Gastroenterology. 90 (1986) 958–962, https://doi.org/10.1016/0016-5085(86)90873-5.

[27] N. Desriac, V. Broussolle, F. Postollec, A.-G. Mathot, D. Sohier, L. Coroller, I. Leguerinel, Bacillus cereus cell response upon exposure to acid environment: toward the identification of potential biomarkers, Front. Microbiol. 4 (2013) 284, https://doi.org/10.3389/fmicb.2013.00284.

[28] M. Begley, C.G.M. Gahan, C. Hill, The interaction between bacteria and bile, FEMS Microbiol. Rev. 29 (2005) 625–651, https://doi.org/10.1016/j.femsre.2004.09.003.

[29] L. Humbert, D. Rainteau, N. Tuvignon, C. Wolf, P. Seksik, R. Laugier, F. Carrière, Postprandial bile acid levels in intestine and plasma reveal altered biliary circulation in chronic pancreatitis patients, J. Lipid Res. 59 (2018) 2202–2213, https://doi.org/10.1194/jlr.M084830.

[30] T.C. Northfield, I. McColl, Postprandial concentrations of free and conjugated bile acids down the length of the normal human small intestine, Gut. 14 (1973) 513–518, https://doi.org/10.1136/gut.14.7.513.

[31] L. Ruiz, A. Margolles, B. Sánchez, Bile resistance mechanisms in Lactobacillus and Bifidobacterium, Front. Microbiol. 4 (2013) 396, https://doi.org/10.3389/fmicb.2013.00396.

[32] D.R. Michael, T.S. Davies, J.W.E. Moss, D.L. Calvente, D.P. Ramji, J.R. Marchesi, A. Pechlivanis, S.F. Plummer, T.R. Hughes, The anti-cholesterolaemic effect of a consortium of probiotics: An acute study in C57BL/6J mice, Sci. Rep. 7 (2017) 2883, https://doi.org/10.1038/s41598-017-02889-5.

[33] C.-C. Tsai, P.-P. Lin, Y.-M. Hsieh, Z. Zhang, H.-C. Wu, C.-C. Huang, Cholesterol-lowering potentials of lactic acid bacteria based on bile-salt hydrolase activity and effect of potent strains on cholesterol metabolism in vitro and in vivo,

ScientificWorldJournal. 2014 (2014) 690752, https://doi.org/10.1155/2014/690752.
[34] D.R. Michael, J.W.E. Moss, D.L. Calvente, I. Garaiova, S.F. Plummer, D.P. Ramji, Lactobacillus plantarum CUL66 can impact cholesterol homeostasis in Caco-2 enterocytes, Benefic. Microbes 7 (2016) 443–451, https://doi.org/10.3920/BM2015.0146.
[35] K. Koskenniemi, K. Laakso, J. Koponen, M. Kankainen, D. Greco, P. Auvinen, K. Savijoki, T.A. Nyman, A. Surakka, T. Salusjärvi, W.M. de Vos, S. Tynkkynen, N. Kalkkinen, P. Varmanen, Proteomics and transcriptomics characterization of bile stress response in probiotic Lactobacillus rhamnosus GG, Mol. Cell. Proteomics 10 (2011), https://doi.org/10.1074/mcp.M110.002741. M110.002741.
[36] E.A. Pfeiler, M.A. Azcarate-Peril, T.R. Klaenhammer, Characterization of a novel bile-inducible operon encoding a two-component regulatory system in Lactobacillus acidophilus, J. Bacteriol. 189 (2007) 4624–4634, https://doi.org/10.1128/JB.00337-07.
[37] P.D. Facey, B. Sevcikova, R. Novakova, M.D. Hitchings, J.C. Crack, J. Kormanec, P. J. Dyson, R. Del Sol, The dpsA gene of Streptomyces coelicolor: induction of expression from a single promoter in response to environmental stress or during development, PLoS One 6 (2011), e25593, https://doi.org/10.1371/journal.pone.0025593.
[38] P.D. Facey, M.D. Hitchings, P. Saavedra-Garcia, L. Fernandez-Martinez, P.J. Dyson, R. Del Sol, Streptomyces coelicolor Dps-like proteins: differential dual roles in response to stress during vegetative growth and in nucleoid condensation during reproductive cell division, Mol. Microbiol. 73 (2009) 1186–1202, https://doi.org/10.1111/j.1365-2958.2009.06848.x.
[39] N.R. de Alcântara, F.M. de Oliveira, W. Garcia, O.A.L. Dos Santos, A.P. Junqueira-Kipnis, A. Kipnis, Dps protein is related to resistance of Mycobacterium abscessus subsp. massiliense against stressful conditions, Appl. Microbiol. Biotechnol. 104 (2020) 5065–5080, https://doi.org/10.1007/s00253-020-10586-z.
[40] E. Hamon, P. Horvatovich, E. Izquierdo, F. Bringel, E. Marchioni, D. Aoudé-Werner, S. Ennahar, Comparative proteomic analysis of Lactobacillus plantarum for the identification of key proteins in bile tolerance, BMC Microbiol. 11 (2011) 63, https://doi.org/10.1186/1471-2180-11-63.
[41] A. Monteagudo-Mera, R.A. Rastall, G.R. Gibson, D. Charalampopoulos, A. Chatzifragkou, Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health, Appl. Microbiol. Biotechnol. 103 (2019) 6463–6472, https://doi.org/10.1007/s00253-019-09978-7.
[42] C.R.W. Carneiro, E. Postol, R. Nomizo, L.F.L. Reis, R.R. Brentani, Identification of enolase as a laminin-binding protein on the surface of Staphylococcus aureus, Microbes Infect. 6 (2004) 604–608, https://doi.org/10.1016/j.micinf.2004.02.003.
[43] M.L. Van Tassell, M.J. Miller, Lactobacillus adhesion to mucus, Nutrients. 3 (2011) 613–636, https://doi.org/10.3390/nu3050613.
[44] M. Jakava-Viljanen, S. Avall-Jääskeläinen, P. Messner, U.B. Sleytr, A. Palva, Isolation of three new surface layer protein genes (slp) from Lactobacillus brevis ATCC 14869 and characterization of the change in their expression under aerated and anaerobic conditions, J. Bacteriol. 184 (2002) 6786–6795, https://doi.org/10.1128/jb.184.24.6786-6795.2002.
[45] L.E. Tailford, E.H. Crost, D. Kavanaugh, N. Juge, Mucin glycan foraging in the human gut microbiome, Front. Genet. 6 (2015) 81, https://doi.org/10.3389/fgene.2015.00081.
[46] J.P. Hymes, B.R. Johnson, R. Barrangou, T.R. Klaenhammer, Functional analysis of an S-layer-associated fibronectin-binding protein in lactobacillus acidophilus NCFM, Appl. Environ. Microbiol. 82 (2016) 2676–2685, https://doi.org/10.1128/AEM.00024-16.
[47] Z. Deng, X.M. Luo, J. Liu, H. Wang, Quorum sensing, biofilm, and intestinal mucosal barrier: involvement the role of probiotic, Front. Cell. Infect. Microbiol. 10 (2020), https://doi.org/10.3389/fcimb.2020.538077.
[48] G. Caggianiello, M. Kleerebezem, G. Spano, Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms, Appl. Microbiol. Biotechnol. 100 (2016) 3877–3886, https://doi.org/10.1007/s00253-016-7471-2.
[49] E.A. Williams, J. Stimpson, D. Wang, S. Plummer, I. Garaiova, M.E. Barker, B. M. Corfe, Clinical trial: a multistrain probiotic preparation significantly reduces symptoms of irritable bowel syndrome in a double-blind placebo-controlled study, Aliment. Pharmacol. Ther. 29 (2009) 97–103, https://doi.org/10.1111/j.1365-2036.2008.03848.x.
[50] C. Ferrario, S. Duranti, C. Milani, L. Mancabelli, G.A. Lugli, F. Turroni, M. Mangifesta, A. Viappiani, M.C. Ossiprandi, D. van Sinderen, M. Ventura, Exploring amino acid auxotrophy in bifidobacterium bifidum PRL2010, Front. Microbiol. 6 (2015), https://doi.org/10.3389/fmicb.2015.01331.
[51] Y. Hou, G. Wu, Nutritionally essential amino acids, Adv. Nutr. 9 (2018) 849–851, https://doi.org/10.1093/advances/nmy054.
[52] EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Guidance on thr assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, EFSA J. 10 (n.d.) 2740–2750. https://doi.org/10.2903/j.efsa.2012.2740.
[53] M. Gueimonde, B. Sánchez, C.G. de Los Reyes-Gavilán, A. Margolles, Antibiotic resistance in probiotic bacteria, Front. Microbiol. 4 (2013) 202, https://doi.org/10.3389/fmicb.2013.00202.
[54] S. Zhang, J.-H. Oh, L.M. Alexander, M. Özçam, J.-P. van Pijkeren, d-Alanyl-d-Alanine ligase as a broad-host-range counterselection marker in vancomycin-resistant lactic acid bacteria, J. Bacteriol. 200 (2018), https://doi.org/10.1128/JB.00607-17.
[55] J. Aires, F. Doucet-Populaire, M.J. Butel, Tetracycline resistance mediated by tet (W), tet(M), and tet(O) genes of Bifidobacterium isolates from humans, Appl. Environ. Microbiol. 73 (2007) 2751–2754, https://doi.org/10.1128/AEM.02459-06.
[56] M. Gueimonde, A.B. Flórez, A.H.A.M. van Hoek, B. Stuer-Lauridsen, P. Strøman, C. G. de los Reyes-Gavilán, A. Margolles, Genetic basis of tetracycline resistance in Bifidobacterium animalis subsp. lactis, Appl. Environ. Microbiol. 76 (2010) 3364–3369, https://doi.org/10.1128/AEM.03096-09.
[57] A. Polit, H. Yang, D. Amund, Investigating the transmissibility of tet(W) in bifidobacteria exposed to acid and bile stress, Biosci. Microbiota. Food Health. 37 (2018) 39–43, https://doi.org/10.12938/bmfh.17-017.
[58] M. Bennedsen, B. Stuer-Lauridsen, M. Danielsen, E. Johansen, Screening for antimicrobial resistance genes and virulence factors via genome sequencing, Appl. Environ. Microbiol. 77 (2011) 2785–2787, https://doi.org/10.1128/AEM.02493-10.
[59] F. Enault, A. Briet, L. Bouteille, S. Roux, M.B. Sullivan, M.-A. Petit, Phages rarely encode antibiotic resistance genes: a cautionary tale for virome analyses, ISME J. 11 (2017) 237–247, https://doi.org/10.1038/ismej.2016.90.
[60] M. Muniesa, M. Colomer-Lluch, J. Jofre, Could bacteriophages transfer antibiotic resistance genes from environmental bacteria to human-body associated bacterial populations? Mob. Genet. Elem. 3 (2013), e25847 https://doi.org/10.4161/mge.25847.
[61] D. Sun, Pull in and push out: mechanisms of horizontal gene transfer in bacteria, Front. Microbiol. 9 (2018), https://doi.org/10.3389/fmicb.2018.02154.
[62] W. Morovic, J.M. Roper, A.B. Smith, P. Mukerji, B. Stahl, J.C. Rae, A.C. Ouwehand, Safety evaluation of HOWARU® Restore (Lactobacillus acidophilus NCFM, Lactobacillus paracasei Lpc-37, Bifidobacterium animalis subsp. lactis Bl-04 and B. lactis Bi-07) for antibiotic resistance, genomic risk factors, and acute toxicity, Food Chem. Toxicol. 110 (2017) 316–324, https://doi.org/10.1016/j.fct.2017.10.037.
[63] M. Ventura, J.-H. Lee, C. Canchaya, R. Zink, S. Leahy, J.A. Moreno-Munoz, M. O'Connell-Motherway, D. Higgins, G.F. Fitzgerald, D.J. O'Sullivan, D. van Sinderen, Prophage-like elements in bifidobacteria: insights from genomics, transcription, integration, distribution, and phylogenetic analysis, Appl. Environ. Microbiol. 71 (2005) 8692–8705, https://doi.org/10.1128/AEM.71.12.8692-8705.2005.
[64] D. Proyadarshani, S.K. Rakshit, Screening selected strains of probiotic lactic acid bacteria for their ability to produce biogenic amines (histamine and tyramine), Int. J. Food Sci. Technol. 46 (2011) 2062–2069, https://doi.org/10.1111/j.1365-2621.2011.02717.x.