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RESEARCH ARTICLE

Selection, characterisation and safety of *Limosilactobacillus reuteri* DSM 32846, an evolved version of DSM 17938

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Abstract

Predicting probiotic strain properties that translate into beneficial effects in the host is challenging and could be considered the holy grail in developing new probiotics. One approach is to selectively evolve well-studied probiotic strains known to elicit beneficial effects in clinical studies. In this study, *Limosilactobacillus reuteri* DSM 17938, among the world's most studied probiotic strains, constituted the origin from which the novel strain *L. reuteri* BG-R46® (DSM 32846) was obtained by exposure to bile, a major stressor of the gastrointestinal tract. The bioactivity of DSM 32846 in preclinical models is documented elsewhere; this work adds crucial strain characteristics, genomic and phenotypic safety profiles, and safety and tolerability in a randomised human study. DSM 32846 was compared to DSM 17938 in terms of tolerance to bile and synthetic gastric juice, with DSM 32846 showing increased bile tolerance and slightly reduced tolerance to gastric pH. Morphological examination by scanning electron microscopy revealed high abundance of extracellular membrane vesicles on the surface of DSM 32846, a previously described bioactive feature of the strain. In addition, the activity of 5′-nucleotidase, an adenosine-producing enzyme expressed on the bacterial cell surface and membrane vesicles, was increased in large-scale production batches of DSM 32846 compared to DSM 17938. Phenotypic safety assessment showed that DSM 32846 does not produce any of the evaluated biogenic amines, produces D- and L-lactate with a ratio typical for the species, and lacks haemolytic activity. The minimum inhibitory concentration profile for antibiotics did not raise any safety concerns. A genome safety assessment revealed no antibiotic resistance and virulence genes. DSM 32846 was found safe for human consumption with no differences from the control group in any of the evaluated parameters. In conclusion, *L. reuteri* DSM 32846 is a safe strain that shows interesting differences to its parent strain DSM 17938.

Keywords

Limosilactobacillus reuteri – DSM 32846 – BG-R46® – probiotics – bile tolerant

1 Introduction

The basis for probiotic products is the included bacterial strains, and there are several requirements that must be met for a strain to be called probiotic (Hill *et al.*, 2014). Binda and colleagues further clarified criteria required to use the terminology correctly stating that probiotic strains must be sufficiently characterised, safe for the intended use, supported by at least one positive human clinical trial conducted according to generally accepted scientific standards, and alive in the product at an efficacious dose throughout shelf life (Binda *et al.*, 2020). Further, Dunne and colleagues have described criteria to assist in the selection of probiotic strains (Dunne *et al.*, 2001). Within the latter criteria are tolerance to processing and stress related to passage through the gastrointestinal tract, adherence in the intestine, influence and modulation of immune responses. In addition to these general probiotic characteristics, there are species- and strain-specific properties that are important for the beneficial effects elicited by the probiotics. *Limosilactobacillus reuteri* DSM 17938 has been shown to carry the adenosine-producing enzyme 5′-nucleotidase, which likely plays an important role in the anti-inflammatory properties of the strain (He *et al.*, 2017). Further, DSM 17938 affects gut motility, improves mucosal integrity and barrier function, inhibits pain signalling, and exhibits antimicrobial properties through the secretion of 3-hydroxipropionaldehyde (commonly known as reuterin) (Burgos *et al.*, 2015; Chung *et al.*, 1989; Karimi *et al.*, 2018). Although selection of strains with improved activity towards any of the bioactivity traits is difficult, traits related to tolerance can be used as tools for generation of adapted strains. By exposing strains to stressful environments related to the mentioned traits, one can obtain naturally adapted mutants with improved properties (Foster, 2007).

L. reuteri DSM 17938 is among the most clinically well-studied probiotic strains in the world and has, for example, been shown to alleviate infantile colic (Gutiérrez-Castrellón *et al.*, 2017; Reis Buzzo Zermiani *et al.*, 2021; Szajewska *et al.*, 2014; Sung *et al.*, 2017) and reduce diarrhoeal symptoms (Mu *et al.*, 2018; Sun *et al.*, 2023). DSM 17938 has many beneficial properties and is well-documented both clinically and preclinically but has a lower bile tolerance compared to other *L. reuteri* strains (Sendelius *et al.*, 2023). A high tolerance to the stressful conditions in the upper GI tract could potentially be linked to increased activity and probiotic activity, and thus we speculated that a strain with the properties of DSM 17938 and having an improved bile tolerance could have an even greater probiotic efficacy. By exposing the strain to bile, a new strain named DSM 32846 was obtained (Pang *et al.*, 2022). Changes such as improved bile tolerance rarely occur in isolation and Galhardo and colleagues emphasise how microbial evolution is achieved through stressful environments, and that the initial stress response in cells that are poorly adapted to the environment is the occurrence of random mutations (Galhardo *et al.*, 2007). Mutations and possible alterations in the regulation of the genome may explain why the two strains differ. We have previously described that extracellular membrane vesicles from DSM 32846 have increased bioactivity in several models (Pang *et al.*, 2022) and the strain increased both adenosine and inosine levels in the cecum of scurfy mice (Liu *et al.*, 2023). However, the basic properties and safety of DSM 32846 have not been described previously. Therefore, this paper aimed to describe the characteristics of DSM 32846 in depth and elucidate whether any other basic property of the strain has been altered in the strain development procedure. Furthermore, we aimed to evaluate the safety of the strain in a human safety study.

2 Materials and methods

Bacterial strains and culture conditions

Limosilactobacillus reuteri strain DSM 32846 (commercial name *L. reuteri* BG-R46®, a trademark of BioGaia AB) is an evolved strain obtained by repeated bile stress of the well-characterised probiotic strain *L. reuteri* DSM 17938 (Pang *et al.*, 2022). DSM 17938 was previously obtained after curation of antibiotic resistance-carrying plasmids from *L. reuteri* ATCC 55730, a strain derived from breast milk (Rosander *et al.*, 2008; Wolf *et al.*, 1995). DSM 32846, DSM 17938, as well as *L. reuteri* strains ATCC PTA 4659 (Sendelius *et al.*, 2023), ATCC PTA 5289 and ATCC PTA 6475 were used in this study and were all kind gifts from BioGaia (Stockholm, Sweden).

Genomic analysis

Whole-genome sequencing

The genome of DSM 32846 was obtained by hybrid assembly of Nanopore and Illumina reads. The Unicycler pipeline v0.4.8 in hybrid mode was used to obtain *de novo* assemblies. All dependencies for Unicycler were installed in a Conda environment. The dependency programs include SPAdes v3.13.0, racon v1.4.1, bowtie2 v2.3.5.1, and pilon v1.23. The hybrid assemblies were annotated using Prokka v1.14.5 (https://github.com

/tseemann/prokka). Total genomic DNA was extracted from an overnight culture in DeMan Rogosa and Sharpe (MRS, Sigma Aldrich, Saint Louis, MO, USA) broth. The culture was centrifugated for 10 min at 4,500 rpm at 4 °C, after which the pellet was washed once with phosphate buffered saline pH 7.4 (PBS) to remove carry-over contaminants. DNA for Illumina short-reads sequencing was extracted using the NucleoSpin Soil kit (740780.50, Macherey-Nagel) as described by the manufacturer with SL2 lysis buffer and Sx enhancer. Cells were bead beated at 5.5 m/s for 60 s twice in a FastPrep-24 Instrument (MP Biomedicals), with incubation on ice for 5 min in between the two bead-beating bursts. DNA quality was evaluated using Tapestation 4200 with Genomic DNA ScreenTape and reagents (Agilent), and quantification was performed using Quant-iT dsDNA BR Assay Kit (ThermoFisher Scientific). Libraries for sequencing were prepared using Covaris S220 Focused-ultrasonicator (Covaris), fragmented to average 550-bp insert size, and the TruSeq DNA PCR-free Library Preparation kit (20015963 and 20015949, Illumina). Libraries were quantified using Quant-iT dsDNA HS Assay Kit (ThermoFisher Scientific) and sequenced on an Illumina Miseq instrument using MiSeq Reagent Kit v3, 600 cycles.

Large amounts of high-quality DNA for Nanopore long-reads sequencing were obtained with a modified version of the Marmur procedure (Marmur, 1961). Cells were suspended in Tris-EDTA buffer (20 mM Tris HCl pH 8.0, 2 mM EDTA) and lysed with lysozyme (20 mg/ml) and SDS (2%, w/v) in the presence of proteinase K (80-200 µl/ml, Qiagen, Hilden, Germany). The extracted total DNA was purified by repeated extraction in phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and chloroform:isoamyl alcohol (24:1, v/v), followed by precipitation by addition of 2 volumes of cold ethanol (99.5%, v/v) and spooling of the DNA on a glass rod. The DNA was washed with 70% (v/v) ethanol, dried at room temperature and resuspended in water overnight at 4 °C. DNA integrity and concentration were evaluated using Tapestation 4150 with Genomic DNA ScreenTape and reagents (Agilent) and Qubit 3.0 Fluorometer and Qubit dsDNA BR assay kit (ThermoFisher Scientific). Isolated DNA was prepared using Rapid barcoding kit (SQK-RBK004) following the manufacturer's instructions (ONT) and sequenced on a ONT MinION device on a R9.4.1 flow cell (FLO-MIN106D). Base-calling was performed using ONT Guppy v. 4.2.2.

The genome sequence was deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank) and has the accession number CP169042 (NCBI RefSeq: GCF_041888795.1). The genome of the parental strain DSM 17938 (generated with the above-described method) was deposited with the accession number CP169045 (NCBI RefSeq: GCF_041888805.1). Both strains carry two plasmids, pLR580 and pLR584, and the sequences were deposited with the accession numbers CP169047 and CP169046 for DSM 17938, and CP169044 and CP169043 for DSM 32846.

The average nucleotide identity (ANI) value was calculated based on MUMmer using the JSpeciesWS ANI calculator (Richter *et al.*, 2016).

Genome annotation and risk analysis

The genome annotations of the coding sequences (CDS) of the strains DSM 32846, DSM 17938 and ATCC PTA 6475 (NCBI RefSeq: GCF_000159475.2 (MM4-1a)) were obtained by subjecting the *de novo* assembled genomic DNA sequences in FASTA format to NCBI's 'Prokaryotic Genome Annotation Pipeline (PGAP)', version 2024-04-27.build7426 (https://github.com/ncbi/pgap). The genome annotations of the *L. reuteri* strains JCM 1112 and SD2112 (same strain as ATCC 55730) are publicly available with NCBI accession numbers GCF_000010005.1 and GCF_000159455.2, respectively. To make the functional annotation comparable between the different strains, the CDSs and the genomic DNA sequences were further annotated by using eggnog-mapper (version 2.1.12, https://github.com/eggnogdb/eggnog-mapper) based on their ortholog and functional cluster database – EggNOG (v5). The mapping mode used for the analysis was 'MMseqs2' and the taxonomic level was set to 'Bacteria', while other parameters for eggnog-mapper were unchanged. The Clusters of Orthologous Genes (COG) identified by eggnog-mapper were compared across the different strains, with a special consideration for the 'COG V' category, namely 'defence mechanisms'.

Detection of antibiotic resistance genes, genomic islands and virulence factors

To determine whether any antibiotic resistance genes are present in the strains, Resistance Gene Identifier (RGI v6.0.3, https://card.mcmaster.ca/analyze/rgi), developed based on the Comprehensive Antibiotic Resistance Database (CARD, version 3.2.9), was used with the selection criteria 'Perfect and Strict hits only'. As a complement to CARD, ResFinder (v4.5.0, http://genepi.food.dtu.dk/resfinder) was used to identify potential antibiotic resistance genes.

IslandViewer (v4, http://www.pathogenomics.sfu.ca/islandviewer/) and MobileElementFinder (v1.0.3,

https://cge.food.dtu.dk/services/MobileElementFinder/) were used to detect genomic islands (GI)/mobile genetic elements (MGEs) on the genomes. IslandViewer uses a set of different tools and databases to identify GIs as well as relevant antibiotic resistance genes and genes encoding virulence factors that may be located on the GIs. MobileElementFinder is another tool for identifying MGEs using a curated database and it can also assess antimicrobial resistance genes and virulence genes.

The above-mentioned assessments were conducted in June-July 2024 and the analysis was performed separately on the chromosomal and plasmid DNAs.

Phenotypic safety assessment

Determination of minimum inhibitory concentrations (MICs)

DSM 32846 was assessed for susceptibility to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, and tetracycline using a broth microdilution method according to guidelines from the European Food Safety Authority (EFSA) (EFSA FEEDAP Panel *et al.*, 2018). The tests were performed at RISE Research Institutes of Sweden AB (Borås, Sweden) following ISO 10932:2010 (British Standards Institute Staff, 2010). Two-fold dilutions of the antibiotics were prepared, and bacterial cells were diluted in LSM broth (Klare *et al.*, 2005). Fifty µl of the bacterial cells were added to each well (approximately 5×10^5 CFU/ml) and the microwell plates were incubated anaerobically at 37 °C for 48 h. *Lacticaseibacillus paracasei* ATCC 334 and *Lactiplantibacillus plantarum* ATCC 14917 were tested in tandem with DSM 32846 to verify the methodology, and they exhibited MICs within accepted ranges according to ISO 10932:2010 (British Standards Institute Staff, 2010). MIC values were compared with the cut-off values for *L. reuteri* strains defined by EFSA (EFSA FEEDAP Panel *et al.*, 2018).

In addition, MIC measurement for chloramphenicol was performed using the Etest strip method (bioMérieux, Lyon, France) according to the manufacturer's instructions. The antibiotic concentration gradient of the Etest strips was 0.016 to 256 µg/ml chloramphenicol. DSM 32846 was cultivated anaerobically at 37 °C on LSM agar supplemented with Tween 80 (1 g/l) to ensure optimal growth. Colonies from overnight cultures (22 to 26 h) were suspended in saline solution (0.9% NaCl, wt/vol). The cell suspension (3×10^8 CFU/ml) soaked in a sterile cotton swab was streaked with a plate rotation 60 degrees three times on LSM agar supplemented with Tween 80 (1 g/l). After drying the plate surface, Etest strips were applied to the agar medium. The Etest agar plates were incubated anaerobically at 37 °C for 48 h.

If the MIC value for an antibiotic was above the cut-off value, the genome sequence of DSM 32846 was manually investigated to find potential explanations. This was performed by comparisons with known determinants of elevated MICs as well as by review of the background information used to define the cut-off values (EFSA FEEDAP Panel *et al.*, 2018).

Biogenic amines

Production of the biogenic amines histamine, tyramine, cadaverine and putrescine was investigated for DSM 17938 and DSM 32846. The analyses were performed at RISE Research Institutes of Sweden (Borås, Sweden). After culturing bacteria overnight in MRS broth (Merck, Darmstadt, Germany) at 37 °C, the culture was centrifuged at 3,000 × *g* for 15 min. The pellet was resuspended and diluted in PBS to an optical density (OD) of 1.0 measured at 600 nm, which was used for inoculation (10%) of both MRS broth and MRS broth supplemented with L-histidine (0.25% w/v), L-lysine (0.25% w/v), L-ornithine (0.25% w/v) or L-tyrosine (0.25% w/v). After incubation at 30 °C for 48 h, the suspensions were centrifuged at 3,000 × *g* for 15 min. The supernatants were filtered through a 3 kDa filter. Potential remaining polymeric materials were precipitated by the addition of 1% formic acid in acetonitrile. The samples were thereafter derivatised by AccQ Tag and analysed using liquid chromatography/mass spectrometry (LC/MS, iClass Acquity UPLC, Xevo G2-s QToF), according to the instructions of the AccQ Tag Ultra derivatisation kit (Waters, Waltham, MA, USA). The four biogenic amines were quantified against external standards.

Production of D- and L-lactate

The production of D- and L-lactate was analysed at RISE Research Institutes of Sweden (Borås, Sweden). Supernatants from overnight cultures in MRS broth at 37 °C were obtained after centrifugation at 3,000×*g* for 15 min. The supernatants were filtered through a 3 kDa filter and potential remaining polymeric materials were precipitated by the addition of 1% formic acid in acetonitrile. The solvents were evaporated from the samples and the D- and L-lactate were quantified following the procedure reported by Scheijen and colleagues with adaptions (Scheijen *et al.*, 2012). The samples were resolubilised in 50 mg/ml (+)-O,O′-diacyl-L-tartaric anhydride (DATAN) in 4:1 dichloromethane:formic acid and incubated 30 min at 75 °C for derivatisation. The samples were again dried by evaporation and resolu-

bilised in 1:2 acetonitrile:water before analysis by LC/MS (iClass Acquity UPLC, Xevo G2-s QToF). The D- and L-lactates were separated using a UPLC BEH C18 column (100×2.1 mm, 1.7, Waters) at 31 °C. The flow rate was 0.5 ml/min of a gradient from 99.5% solvent A (1.5 mM ammonium formate) and 0.5% solvent B (acetonitrile) to 3% solvent A after 3 min. Electrospray ionisation in negative ion mode and high accurate mass was used for detection. The labelled lactates were quantified against external standards.

Haemolytic activity

Assessment of haemolytic activity was performed by cultivation on Brain Heart Infusion (BHI, Merck) agar supplemented with 5% sheep blood. DSM 32846 was inoculated onto the agar plates from frozen stocks by gently tapping the inoculation loop onto the agar. *Staphylococcus aureus* S151:2 (α-haemolytic) (Rosengren *et al.*, 2010) and *Streptococcus sanguis* Kx126C2 (β-haemolytic, human origin, strain collection at Department of Molecular Sciences, Swedish University of Agricultural Sciences) were used as positive controls. Plates containing DSM 32846 were incubated anaerobically at 37 °C for 72 h and plates containing *S. aureus* and *S. sanguis* were incubated aerobically at 37 °C for 24 h.

Physiological characterisation

Morphology assessment by scanning electron microscopy

The morphological appearance of DSM 32846 was observed using scanning electron microscopy. The analysis was performed at the Center for Cellular Imaging, Core Facilities, Sahlgrenska Academy, University of Gothenburg. Bacteria were cultivated, lyophilised and visualised as described by Pang *et al.* (2022) and Ermann Lundberg *et al.* (2024). Briefly, lyophilised bacteria were rehydrated in PBS for 20 min, coverslips coated with 0.1% poly-L-lysine for 10 min followed by drying for 5 min, then incubated in cell suspension for 30 min at room temperature. The cells were washed and incubated in 2.5% glutaraldehyde in 0.1 M PIPES for 30 min at room temperature. After post-fixation in 1% osmium tetroxide in 0.1 M PIPES for 1 h at 4 °C in the dark, the samples were dehydrated successively in a series of ethanol solutions of increasing ethanol concentration (35-100%). The samples were suspended in Hexamethyldisilazane solution, air dried and sputtered with gold (Emitech, Taunusstein, Germany) before visualisation with a Zeiss Gemini 450 II scanning electron microscope (Carl Zeiss, Toronto, ON, Canada).

Carbohydrate utilisation

To determine the ability to utilise carbohydrates, the API 50 CHL kit (bioMérieux) was used according to the manufacturer's instructions. Bacterial colonies on MRS agar (Oxoid) were suspended in API 50 CHL medium to a cell density corresponding to McFarland Standard No. 2 (bioMérieux). The suspended cells were inoculated into a well-type plate provided by the manufacturer and then incubated at 37 °C for 48 h. The ability to utilise carbohydrates was assessed by determining colour changes.

API ZYM

The enzymatic activity profile was determined using the API ZYM kit (bioMérieux) according to the manufacturer's protocol. Briefly, DSM 17938 and DSM 32846 were grown overnight at 37 °C in MRS broth (Merck). The bacteria were harvested by centrifugation and the pellet was resuspended in sterile saline to a turbidity of 5.0-6.0 McFarland. The resuspended cells were added to a well-type plate provided by the manufacturer and the plate was incubated at 37 °C for 4 h. Then, one drop of each of ZYM-A and ZYM-B reagents (bioMérieux) was added to each well. After 5 min at room temperature, the colour changes were assessed according to the manufacturer's instructions and used to obtain an enzyme activity profile.

Reuterin production

The production of reuterin was analysed by using a colorimetric method that allows the produced reuterin (3-hydroxy-propinoaldehyde) to react with the reagent 2,4-dinitrophenylhydrazine (Rosander *et al.*, 2008). After the bacteria had grown on MRS agar (Oxoid) for 48 h, the plates were overlaid with 500 mM glycerol (1% agar) followed by an incubation for 30 min at 37 °C. Then 5 ml 2,4-dinitrophenylhydrazine (0.1% in 2 M HCl) was added and the plates were incubated for 3 min at room temperature. Thereafter the solution was poured off and 5 ml of 5 M KOH was added. The production of reuterin resulted in reddish-brown zones around the colonies with sizes that reflected the magnitude of the production.

5′-nucleotidase activity

5′-nucleotidase (5′NT) activity of freeze-dried large-scale batches of DSM 17938 and DSM 32846 was measured using the 5′-nucleotidase Assay kit (CrystalChem, Elk Grove Village, IL, USA). Briefly, 0.2 g of freeze-dried bacteria (obtained by BioGaia AB) was first suspended in 10 ml water. After incubation at room temperature for

20 min, the suspensions were centrifuged at 4,000 *g* for 10 min. 10 µl of the supernatants were added to a 96-well plate containing 180 µl of reagent CC1 per well. After 5 min incubation at 37 °C, 90 µl of reagent CC2 was added and the quinone dye was measured at 550 nm after 3, 5, 9 and 13 min using a plate reader. The change in absorbency values was used for calculations of 5′NT activities according to the manufacturer's instructions.

Acid tolerance

Survival at pH 2.0 in synthetic gastric fluid without digestive enzymes was analysed according to Wall *et al.* (2007). Duplicate samples were taken after 50 and 90 min. The survival rate was calculated as the percentage of bacteria surviving acid stress relative to the number of bacteria at the start. The assay was performed in biological triplicates.

Bile tolerance

The bile tolerance was analysed in two different ways. (i) Both DSM 32846 and DSM 17938 were grown in MRS broth (Oxoid) for 16-20 h and centrifuged for 10 min at 3,500 × *g*. Supernatants were removed, and the bacterial pellets were resuspended and aliquoted in MRS containing 0.5% (w/v) porcine bile (Sigma, Saint Louis, MO, USA). The bacterial suspensions were incubated at 37 °C and samples were taken at 0 and 180 min. Samples were serially diluted and plated on MRS agar (Merck, Darmstadt, Germany) plates, which were incubated anaerobically for 24 h at 37 °C, after which colonies were counted and survival rate calculated. (ii) In addition, the impact of growth-limiting stress caused by bovine bile was investigated through culturing the strains in a microtiter plate at 37 °C for 27-30 h in MRS broth (Merck) with and without the addition of 0.2% bovine bile (B3883, Sigma Aldrich) using a short shaking step every 30 min. OD_{620} was measured using a Multiskan FC Microplate Photometer (Thermo Fisher Scientific). Bile tolerance was expressed as the ratio between OD at the end of a cultivation in the presence of bovine bile and OD at the end of a cultivation without bile.

Bile salt hydrolase assay

Bile salt hydrolase (BSH) activity was analysed by adding 10 µl of overnight cultures in MRS broth (Merck) on MRS agar plates (Merck) supplemented with 0.5% (w/v) sodium salt of glycodeoxycholic acid (GDCA; Sigma-Aldrich) and 0.37 g/l $CaCl_2$. The plates were incubated anaerobically at 37 °C for 72 h. Bacterial spots surrounded by precipitation zones (opaque halos) were considered BSH positive (Hernández-Gómez *et al.*, 2021). The diameter of the zones was measured. MRS agar plates without supplementation were used as controls.

Adhesion to mucus

The mucus attachment assay is a modification to the original method described by Roos *et al.* (2000). The modified method has been described in full in Ermann Lundberg *et al.* (2024).

Briefly, cells were removed from porcine small intestine mucus by centrifugated at 11,000 × *g* for 10 min, after which remaining particulate matter was removed by centrifugation 26,000 × *g* for 15 min. The crude mucus was diluted to A_{280} 0.1 in PBS after which it was added to the Nunc Maxisorb plate wells (Nalgene-Nunc, Thermo Fisher Scientific, Rochester, NY, USA). The plate was incubated at 4 °C overnight with slow rotation, after which the wells containing mucus were washed three times with PBS (pH 6.0) supplemented with 0.05% Tween 20 (PBST). The wells were then blocked for 60 min with PBS (pH 6.0) supplemented with 1% Tween 20. Bacterial suspensions were diluted to OD_{600} 0.5 followed by two washes with PBST before addition to the wells (suspended in PBST). A reference was taken before bacteria were added to the wells. Plates were incubated for 4 h at 37 °C with slow agitation and then the wells were washed four times with PBST. Trypsin (0.25%) EDTA (1.0 mM) was added for 30 min at 37 °C to detach adherent bacteria from the wells, followed by serial dilutions and plating on MRS plates. The plates were incubated anaerobically for 48 h at 37 °C before viable counts were assessed.

Clinical safety and tolerability

A randomised, double-blind, placebo-controlled clinical study was performed on healthy volunteers. The study had a parallel-group design, and the objective was to evaluate safety and tolerability of *L. reuteri* DSM 32846. The study was carried out by Clinical Trial Consultants AB in Uppsala, Sweden in October to December 2021. The study was approved by the independent Swedish Ethical Review Authority (Dnr 2021-02923) and registered at ClinicalTrials.gov (NCT05044949) prior start of the study. The study was conducted in accordance with ethical principles originating in the Declaration of Helsinki and was compliant with International Conference of Harmonization (ICH)/Good Clinical Practice (GCP) E6 (R2) guidance. Applicable parts of the European Union Clinical Trials Directive and local regulatory requirements were followed. All participants gave verbal and written informed consent before being included

in the study and study-specific assessments were performed. Inclusion and exclusion criteria are provided in Supplementary Tables S1 and S2.

The study was comprised of one screening visit and four study visits: at baseline (day 1), day 7, day 14 and day 28, and a final follow-up telephone call was performed 14 days after day 28 to collect adverse events and use of concomitant medications (Supplementary Figure S1).

36 healthy subjects (28 females) with a mean age of 33.9 ± 13.6 years and a mean BMI of 23.7 ± 2.2 kg/m^2, were included in the study. Subjects were randomly assigned at a ratio of 1:1:1, to receive low dose (1×10^8 CFU), high dose (1×10^{10} CFU) or placebo product (n = 12/group) for a total of 28 days. The study products were vegan, and gluten free white capsules filled with freeze-dried DSM 32846, maltodextrin (filler) and calcium stearate (lubricant). The placebo capsules contained maltodextrin instead of DSM 32846. The study products were similar in appearance. The bacterial content of the study products was analysed and shown to be stable during storage for the duration of the study.

The subjects were instructed to consume the study product orally once a day in the morning together with at least one glass of water, milk or yogurt, but not juice or carbonated soft drinks. To be included in the per protocol set, >80% of the study product should have been consumed according to instructions without any major deviations that were judged to compromise the analysis of the data. All protocol violations were classified as major or minor prior to database lock.

Vital signs were measured in terms of systolic and diastolic blood pressure and pulse rate.

Physical examination included general appearance including skin, auscultations of lungs and heart, and by abdomen palpation of liver and spleen. Blood samples were collected through venous puncture and *in vitro* assessments of clinical safety were evaluated. This included measurement of chemical parameters such as alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, bilirubin, creatinine and non-fasting glucose. Haematological parameters including haematocrit, haemoglobin (Hb), platelet count, and white blood cell count with differential count including leukocytes, lymphocytes, monocytes, neutrophils, eosinophils, and basophils, were also evaluated. Follicle stimulating hormone (FSH), serum and urine pregnancy tests were conducted in females. To evaluate GI symptoms the gastrointestinal symptom rating scale (GSRS) was used, which is comprised of 15 items that cluster into five symptom clusters: reflux, abdominal pain, indigestion, diarrhoea and constipation (Svedlund *et al.*, 1988). The absolute changes in GSRS survey items, subscores for each symptom cluster, and total scores between baseline and final visit were analysed using Wilcoxon Rank Sum test to investigate any differences between the study arms.

Stool samples were collected at baseline and after the treatment period on day 28 for analysis of the presence of viable DSM 32846. The samples, still blinded, were analysed for viable DSM 32846 by using the following method: Samples were thawed at room temperature and 0.1 g was suspended in 0.9 ml PBS and then added to 9 ml MRS broth. After incubation at 37 °C for 24 h, 100 µl of the enrichment culture was streaked onto Rogosa agar (Merck) supplemented with 50 µg/ml vancomycin and 2 µg/ml ampicillin (Romani Vestman *et al.*, 2013). The plates were incubated anaerobically at 37 °C for 24 h before further analysis. A mix of bacterial colonies on the plate was collected with a 1-µl loop and thereafter suspended in 100 µl sterile water. The presence of DSM 32846 was examined by PCR analysis using DreamTaq Green PCR Master Mix (Thermo Fisher) and strain-specific primers FwdR46p and RevR46p (see below; 0.4 µM of each). Bacterial suspension (0.5 µl) was added to the PCR mix and the PCR reaction was performed by running the program 95 °C for 7 min; 35 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. The PCR products were separated and visualised by standard agarose gel electrophoresis. A pure culture of DSM 32846 was used as positive control and *L. reuteri* DSM 17938 and ATCC PTA 6475 were used as negative controls.

The primers FwdR46p (CACAAACGAAATACTATTCCACGG) and RevR46p (TTGCGATGAGTGGCTTTGG) were designed to amplify parts of the unique region of one of the duplicated insertion sequence elements in DSM 32846. The optimization procedure showed that the primers resulted in a PCR product (224 bp) from DSM 32846 but no product from *L. reuteri* strains DSM 17938, ATCC PTA 4659, ATCC PTA 5289 and ATCC PTA 6475 (Supplementary Figure S2).

3 Results

Whole genome sequence analysis

Limosilactobacillus reuteri ATCC 55730 (also designated SD2112), the parental strain of DSM 17938, has been identified as *L. reuteri* subsp. *kinnaridis* (Li *et al.*, 2021). The genome of *L. reuteri* DSM 32846 was sequenced, resulting in one contig with a total size of 2,225,488 base pairs. Analysis of the genome sequence identified

TABLE 1 Minimum inhibitory concentrations of antibiotics for DSM 32846

Antibiotics	MIC (μg/ml)[1]	EFSA MIC resistance threshold (μg/ml)[2]
Ampicillin	>16	2
Chloramphenicol	8 (4[3])	4
Clindamycin	0.0625	4
Erythromycin	0.5	1
Gentamicin	2	8
Kanamycin	32	64
Streptomycin	16	64
Tetracycline	>64	32

1 Results from a broth microdilution method.
2 European Food Safety Authority (EFSA FEEDAP Panel *et al.*, 2018).
3 Results from Etest analysis.

the strain as *L. reuteri* subsp. *kinnaridis* with an ANI value of 98.90% between DSM 32846 and the strain *L. reuteri* AP3, which is described as the type-strain for *L. reuteri* subsp. *kinnaridis* (Li *et al.*, 2021). The ANI values between DSM 32846 and the ancestor strains ATCC 55730 and DSM 17938 were both 99.99% and ANI values between DSM 32846 and some *L. reuteri* subsp. *reuteri* strains were above 96.00% (Supplementary Table S3). A comparative genomic assessment between DSM 32846 and DSM 17938 revealed that there are two insertion sequence elements that have been duplicated and inserted in the genome of DSM 32846 (positions 1102637-1103717 and 1809646-1810722).

Genomic risk assessment

The annotation from the PGAP pipeline revealed that, on the chromosome, DSM 32846 has a total number of CDS of 2115, which is in the same range as DSM 17938, ATCC PTA 6475, JCM 1112, and SD2112, all of which range from 2004-2181 CDSs on the chromosome. The CDSs identified on the plasmids were 18 on pLR584 and 5 on pLR580, which are the same as on the plasmids of DSM 17938. The eggnog-mapper analysis showed that the overall COG composition of DSM 32846 was very similar to the other related strains on both the chromosomes and plasmids. The COG category 'V', which is a functional cluster of orthologs with defence mechanisms, was also found to be very similar to the other strains (Supplementary Table S4). No category 'V' COG was identified on the plasmids. In conclusion, from a functional composition point of view, there is no safety concern for DSM 32846.

Analysis conducted using RGI (CARD database) revealed a match to resistance gene 'vanT gene in vanG cluster', but only with 33.6% alignment, and this was not further identified by ResFinder, suggesting an extremely low possibility of being linked to antibiotic resistance. IslandViewer identified 26 GIs from DSM 32846, but no antibiotic resistance genes or genes encoding virulence factors were found within or outside the GI regions. MobileElementFinder identified 33 MGEs (12 with high prediction quality fulfilling three criteria: alignment coverage > 95%, sequence identity > 90%, and truncation < 30 nt) with no resistance genes or genes encoding virulence factors within or outside the MGEs. In conclusion, the genomic risk analysis showed that DSM 32846 had no safety concerns when used as a probiotic.

Determination of and explanations of minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MIC) of the antibiotics ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, and tetracycline were tested by broth microdilution for DSM 32846 (Table 1). The MICs for clindamycin, erythromycin, gentamicin, kanamycin, and streptomycin were all below or equal to the cut-off values, but the MICs for ampicillin, chloramphenicol, and tetracycline were above the cut-off values defined by EFSA. However, using the Etest strip method, the MIC value for chloramphenicol was determined to be 4 μg/ml, which is equal to the cut-off value. As described above, no antibiotic resistance genes could be found in the genome sequence and to explain the elevated MIC values, the genome sequence of DSM 32846 was manually investigated. Point mutations in the penicillin-binding proteins of DSM 17938 have previously been linked to the elevated MIC for ampicillin (Rosander *et al.*, 2008) and the same point mutations were found in DSM 32846 (PBP1A – GenBank accession number XGD31112;

PBP2X – accession number XGD30840). In the search for an explanation for the elevated MIC for tetracycline, the *rpsJ* gene encoding ribosomal protein S10 was identified. The genes of both DSM 32846 (XGD29732) and DSM 17938 (XGD31779) differ from sensitive strains such as ATCC PTA 6475 at two adjacent positions. These result in a change from amino acid residues KF (positions 57-58) in ATCC PTA 6475 (in GenBank designated MM4-1A; Accession EGC14332) to IS in DSM 32846 and DSM 17938. A similar change of *rpsJ* in *Neisseria gonorrhoeae* results in increased tolerance to tetracycline (Hu *et al.*, 2005). This strongly suggests that the exchange of amino acid residues in ribosomal protein S10 in DSM 32846 and DSM 17938 is the cause of the increased tolerance of tetracycline. Both *rpsJ* and the genes encoding the penicillin-binding proteins are housekeeping genes located on the chromosome without adjacent mobile genetic elements. The risk for gene transfer is therefore negligible.

No potential explanation for the elevated MIC for chloramphenicol could be identified in the genome of DSM 32846. However, a review of the background information used for defining the EFSA cut-off values revealed that the value for chloramphenicol is likely to be incorrectly defined. The background is that EUCAST used the information from (Egervärn *et al.*, 2007) to build its MIC database for *L. reuteri* (https://mic.eucast.org/search/, accessed March 4, 2025). This database has further been used for defining the cut-off values in the document Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA FEEDAP Panel, 2012). This document clearly states that a microdilution method should be used for the MIC analysis. However, the EUCAST MIC database contains a mix of values obtained by both microdilution and Etest, and the value for chloramphenicol has been obtained from Egervärn *et al.* (2007) that used Etest for chloramphenicol. Based on our analysis of the cut-off value using both microdilution (8 μg/ml) and Etest (4 μg/ml) a correct cut-off value probably should be 8 μg/ml.

Analysis of biogenic amines and D,L isomers of lactate

Biogenic amine production was evaluated because the production of the biogenic amines histamine, tyramine, cadaverine, and putrescine may be a potential health concern (Ruiz-Capillas and Herranz, 2019). According to HPLC analysis, neither DSM 32846 nor DSM 17938 produced any of the biogenic amines histamine, tyramine, cadaverine, and putrescine neither in MRS broth nor in MRS broth supplemented with L-histidine, L-lysine, L-ornithine or L-tyrosine (Supplementary Table S5).

Many lactic acid bacteria produce both the L- and D-isomers of lactate (Hammes and Hertel, 2006). D-Lactate is not a highly toxic compound, but it is a metabolite that under specific circumstances can cause health problems (Petersen, 2005). Quantitative analysis of lactic acid production showed that DSM 32846 produced both L-lactate (69%) and D-lactate (31%). The ratio of L- to D-lactate is consistent with the results for DSM 17938 (L-lactate: 71% and D-lactate: 29%) a strain with a solid safety profile (Fatheree *et al.*, 2017; Mu *et al.*, 2018; Urbańska and Szajewska, 2014) and has in addition been shown not to cause an increase in urinary D-lactate after 28 days of administration to healthy infants (Papagaroufalis *et al.*, 2014). Furthermore, no elevation of D-lactate was detected in serum of healthy infants given *L. reuteri* ATCC 55730 (the parental strain of DSM 17938) for 12 months (Connolly *et al.*, 2005).

Analysis of haemolytic and enzymatic activities

Haemolytic activity results in lysis of red blood cells and thus destruction of haemoglobin. Beta-haemolytic activity is a representative virulence factor, and the absence of this activity is a criterion for the selection of novel probiotic strains. The ability of DSM 32846 to cause haemolysis was therefore evaluated and the result showed that the strain did not exhibit haemolytic activity. DSM 32846 did not show clear zones on BHI agar containing sheep blood, while the positive controls *S. aureus* S151:2 and *S. sanguis* Kx126C2 showed beta- and alpha-haemolysis, respectively (Supplementary Table S6).

A semiquantitative assessment of enzymatic activities was performed using the API ZYM kit. The results are presented in Supplementary Table S7. The following enzymatic activities were detected for DSM 32846 and DSM 17938: esterase, leucine arylamidase, acid phosphatase, naphthol-AS-BI phosphohydrolase, α-galactosidase, β-galactosidase and α-glucosidase. The enzymatic profile was similar to other *L. reuteri* strains (Ali *et al.*, 2023; Lee *et al.*, 2021). An example of a potential enzymatic activity that may cause concern and that would need to be further investigated are glucuronidases, which can release toxins and mutagens and thus increase the risk of cancer (Humblot *et al.*, 2007; Kim and Jin, 2001). However, no production of β-glucuronidase by DSM 32846 or DSM 17938 was detected.

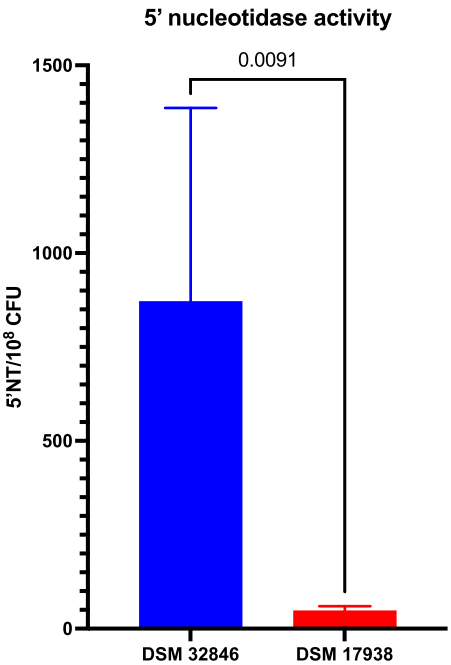

FIGURE 1 Secreted 5′-nucleotidase activity in supernatants from industrially produced batches of DSM 32846 (n = 3) and DSM 17938 (n = 6) measured using a colorimetric method. Data is presented as median with interquartile range. Unpaired *t*-test was used for statistical comparison.

Physiological characterisation

Carbohydrate utilisation, reuterin production and 5′-nucleotidase activity

The ability of both DSM 32846 and DSM 17938 to ferment different carbohydrates was analysed (Supplementary Table S8), and the strains were found to utilise ten of the 50 substrates evaluated. The fermentation profile was consistent with that of other *L. reuteri* subsp. *kinnaridis* strains (Li *et al.*, 2021).

Furthermore, the production of reuterin, a potent antimicrobial substance, was evaluated. DSM 32846 produced reuterin of the same order of magnitude (14 mm zones) as DSM 17938.

5′-nucleotidase activity has been described as a major contributor to the bioactivity of DSM 32846 and DSM 17938 and has previously been shown to be higher in the former strain (Liu *et al.*, 2023; Pang *et al.*, 2022). In this study, we evaluated supernatants of industrially produced freeze-dried culture powders with DSM 32846 or DSM 17938 and in agreement with previously reported elevated 5′NT activity in DSM 32846, the industrially produced DSM 32846 supernatant expressed higher activity compared to DSM 17938 (P = 0.0091, Figure 1).

Scanning electron microscopy

SEM images showed that DSM 32846 is rod-shaped with an estimated size of 1.2-2.2 μm (Figure 2A). Attached on the surface and in the surrounding area extracellular membrane vesicles of varying size were present.

Acid tolerance

Probiotic strains taken orally should preferably survive passage through the stomach with its acidic pH. The survival of DSM 32846 and DSM 17938 in synthetic gastric juice with pH 2.0 was therefore analysed (Figure 2B). DSM 32846 had a slightly lower survival rate after 50 min of exposure compared to DSM 17938, but the two strains were in the same range after 90 min. Compared to most probiotics, both strains survived relatively well, as many species are very sensitive to pH 2 (Ko *et al.*, 2022).

Mucus adhesion

Adhesion to mucus is considered an important property of probiotic strains as it allows the bacteria to get closer to the underlying intestinal host cells and is also a prerequisite for colonisation (long-term or transient). Therefore, the mucus-adhesion capacity of DSM 32846 was compared with DSM 17938. There was no difference in the binding capacity of the two strains, and the average number of adhered bacteria was 4.9×10^4 cells/cm^2 for DSM 17938 and 6.1×10^4 cells/cm^2 for DSM 32846 (Figure 2C).

Bile tolerance

L. reuteri DSM 32846 was selected by exposure to bile and the strain was found to be more tolerant to bile (0.5% porcine) compared to the parental strain DSM 17938. The average Δ log loss after 180 min of bile exposure was 1.8 for DSM 32846 and 3.1 for DSM 17938 (Figure 2D), showing that DSM 32846 tolerates exposure to bile to a greater extent. The bile tolerant phenotype was stable for more than 10 passages. However, methodological variance in bile tolerance assays has a major impact on bacterial survival, and to broaden the concept of bile tolerance, we also evaluated growth of DSM 32846 and DSM 17938 in MRS supplemented with bile. The strains were not shown to differ in this assay (Figure 2E).

Analysis of BSH activity

Bile salt hydrolases (BSH) catalyse the degradation of bile salts. BSH activity was analysed for DSM 32846 and the three well-known probiotic *L. reuteri* strains DSM 17938, ATCC PTA 5289 and ATCC PTA 6475. The two strains ATCC PTA 5289 and ATCC PTA 6475 showed

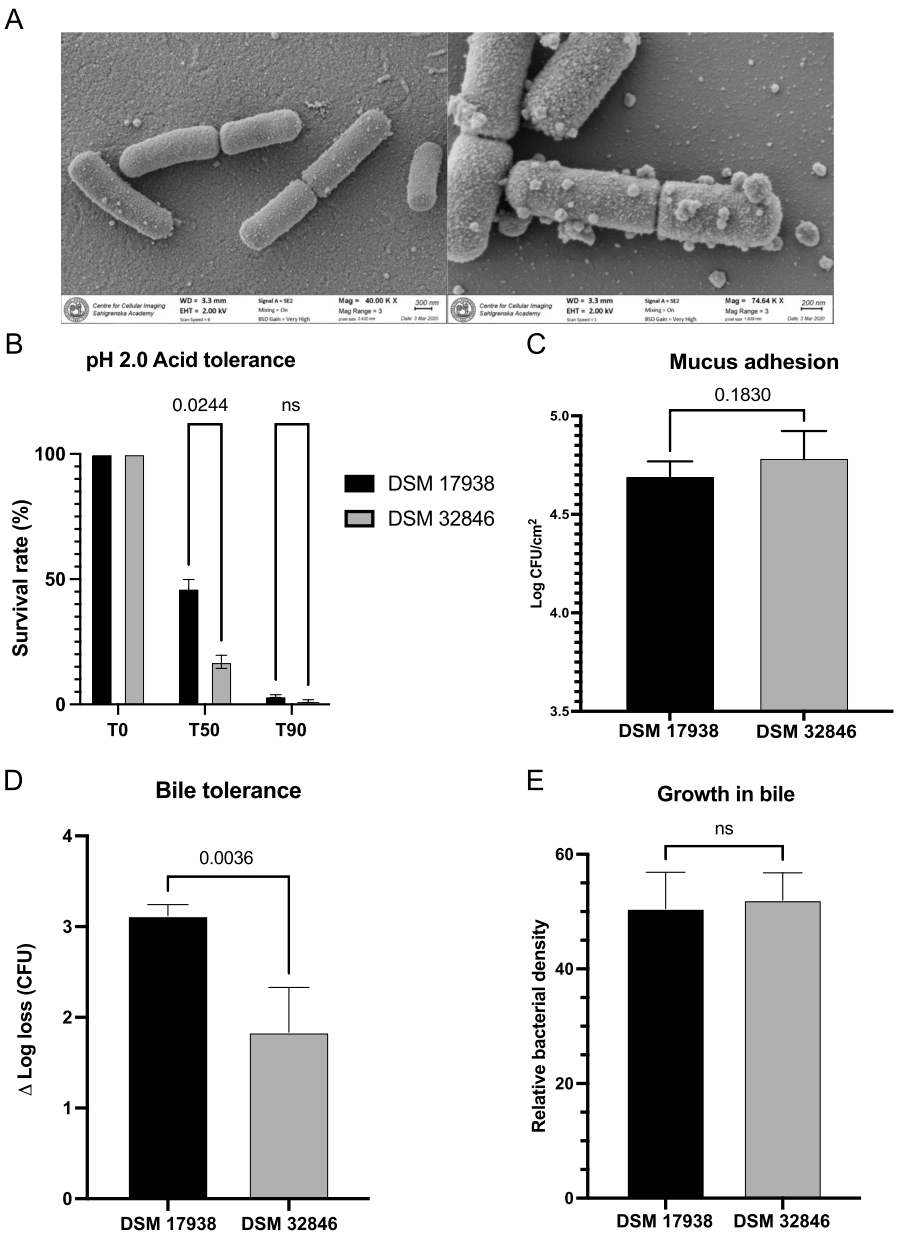

FIGURE 2 (A) scanning electron microscopy imaging of DSM 32846 was performed on freeze-dried culture powder. The bacteria were fixed on 0.1% poly-L-lysine coated coverslips, gold sputtered and imaged with a Zeiss Gemini 450 II scanning electron microscope. (B) acid tolerance at pH 2.0 was evaluated for DSM 32846 and DSM 17938. The bacteria were suspended in synthetic gastric juice at pH 2.0 and then incubated at 37 °C. Samples were taken at 0, 50 and 90 min. Survival rates were calculated after performing viable count analysis of the samples. (C) mucus adhesion was evaluated for *L. reuteri* DSM 32846 and compared to DSM 17938. The strains were allowed to adhere to mucus-coated microtiter wells, after which they were released by trypsin treatment and CFU determined by plate count analysis. (D) bile tolerance of *L. reuteri* DSM 32846 was assessed and compared with DSM 17938. The bacteria were suspended in MRS supplemented with 0.5% (w/v) porcine bile, incubated at 37 °C and samples were taken at 0 and 180 min. The survival rate was calculated after performing viable count analysis of the samples. (E) growth in bile was evaluated for DSM 17938 and DSM 32846 where MRS broth supplemented with 0.2% bovine bile was used as substrate as the strains were grown in a microtiter plate and OD was measured. All data is presented as mean with SD and *t*-tests were used for statistical comparisons.

activity against glycodeoxycholic acid (GDCA; precipitation zone diameters in Supplementary Table S9). DSM 32846 and DSM 17938 did not grow on MRS agar plates supplemented with GDCA, whereas they grew well on MRS agar plates without supplementation, demonstrating that DSM 32846 does not express BSH activity.

TABLE 2 Overview of adverse events

	L. reuteri 1×10^8 CFU (N = 12)		*L. reuteri* 1×10^{10} CFU (N = 12)		Placebo (N = 12)		Total (N = 36)	
	n	*m*	n	*m*	n	*m*	n	*m*
Any AE	9	15	7	16	8	11	24	42
Any SAE	0	0	0	0	0	0	0	0
Any AE leading to withdrawal	0	0	0	0	0	0	0	0
Causality								
Unlikely related	7	12	6	14	8	11	21	37
Possibly related	3	3	1	1	0	0	4	4
Probably related	0	0	1	1	0	0	1	1
Severity								
Mild	9	15	7	16	7	10	23	41
Moderate	0	0	0	0	1	1	1	1
Severe	0	0	0	0	0	0	0	0

n, number of subjects; *m*, number of events; AE, adverse event; SAE, serious adverse event.

Clinical safety and tolerability

The safety and tolerability of *L. reuteri* DSM 32846 was evaluated in a clinical study. 35 subjects completed the study, of whom one subject was lost to follow-up. Gender, ethnicity, age and body mass index were equally distributed across the three study arms. Of the 35 subjects who completed the study, 27 had 100% compliance, meaning that the study product was consumed according to the instructions every day. Five of the 27 subjects took one dose extra. Six subjects missed one dose each, one subject missed two doses, and one subject missed one dose and then took two doses too many.

There were no clinically significant changes from baseline to Day 28 in the primary endpoints vital signs and physical examination. There were no deaths, serious adverse events, or other significant adverse events (AE), or withdrawals due to AE during the study. Most reported AEs were mild and unlikely to be related to the study product (Table 2). Constipation, flatulence, gastroesophageal reflux disease and dyspepsia were reported as single events by four separate subjects, and these AEs were assessed as possibly related. Flatulence was reported by two subjects in the high-dose arm and was assessed as mild in intensity. The only GI AE assessed as likely related to the use of the study product was one of the two instances of flatulence. Apart from the slight occurrence of flatulence in the DSM 32846 high-dose arm, there was no trend with regards to AE type between the high-dose, low-dose and placebo arms of the study.

There were no clinically significant changes in clinical chemistry or haematology parameters from baseline to Day 28, overall or for any of the study products used. There were no individual values assessed as abnormal. Subjective tolerability, as measured by GSRS, indicated low levels of GI discomfort across the three study arms. The GSRS scores did not show any obvious differences between the study arms at any timepoint, and DSM 32846 did not increase any of the symptom clusters or total GSRS scores compared to placebo. The presence of viable DSM 32846 in stools was measured at baseline and Day 28. At baseline, none of the participants had detectable levels of DSM 32846 in faeces, and the same result was obtained for the Day 28 samples from participants receiving placebo. At Day 28, viable DSM 32846 was detected in 73% (8 out of 11) of the samples from both the high- and low-dose groups.

In conclusion, *L. reuteri* DSM 32846 at a dose of up to 10^{10} CFU/day was safe and well tolerated in a healthy male and female population as assessed by AEs, vital signs, clinical laboratory parameters, physical examinations, and subjective GI tolerability assessment during the study.

4 Discussion

This paper describes the development, characterisation and demonstration of safety and tolerability of the novel strain *Limosilactobacillus reuteri* DSM 32846. Like *L. reuteri* DSM 17938, DSM 32846 was shown to be safe

from a genomic, phenotypic and clinical perspective, which adds a safety perspective to the already existing preclinical data for DSM 32846.

Common strategies to increase efficacy of probiotics are to provide the bacteria with a substrate, e.g. a prebiotic fibre that in combination with the probiotics can be the components of a so-called synbiotic (Swanson *et al.*, 2020), or by combining probiotic strains that together provide synergistic effects (Dias *et al.*, 2022). Microbial strain development is another strategy for improving probiotics (Wang *et al.*, 2023) and a suitable target for the selection may be a trait that is known or thought to contribute directly to the bioactivity of the strain. Selecting strains with improved tolerance to a stressor is a relatively straightforward task, but selecting strains with increased bioactivity is more challenging. For example, *L. reuteri* DSM 17938 is known to have calming effects on a stressed intestine, normalising intestinal motility (Burgos *et al.*, 2015), but selecting for an improvement in this function is complicated. Instead, we hypothesised that a strain with increased robustness under stressful conditions relevant for the GI-tract may result in higher metabolic activity and increased likelihood of beneficial interactions with host cells. In this work, we have applied microbial strain development to modulate the properties of *L. reuteri* DSM 17938, and DSM 32846 was obtained after exposure of the parental strain to bile. DSM 32846 was found to be more tolerant to bile exposure, suggesting an adaptation to conditions in the small intestine. However, the strain did not show increased growth rate in a bile-rich substrate. Probiotic bacteria rarely colonise the GI-tract but are considered transient colonisers, and bile contributes to this microbial equilibrium and to the colonisation resistance of the GI tract (Larabi *et al.*, 2023). In line with this, it is reasonable to believe that DSM 32846 overcomes the passage of the bile-rich upper small intestine better than the parental strain and thereby obtains an increased metabolic activity in the ileum. Interestingly, previous studies have shown that the strain has altered properties that extend beyond increased bile tolerance (Liu *et al.*, 2023; Pang *et al.*, 2022). This is in line with what has been described for various forms of adaptive mutagenesis in *Escherichia coli* (Foster, 2007), which describes adaptive mutation as 'The appearance among cells under selection of mutations that relieve the selective pressure whether or not other nonselected mutations are also produced'. The development of DSM 32846 with its bile-tolerant phenotype is indeed a result of stress, but the underlying mechanisms of its properties remain unknown and are a topic for future studies. The results of this study, together with the previous preclinical studies showing that DSM 32846 and DSM 17938 exhibit different bioactivities (Liu *et al.*, 2023; Pang *et al.*, 2022), indicate that the bile-tolerant phenotype has come with other properties. Clinical studies addressing the probiotic effects of DSM 32846 are warranted.

Another interesting difference between DSM 32846 and DSM 17938 is the increased secretion of 5′-nucleotidase (5′NT) in DSM 32846. The role of the enzyme is to convert AMP to adenosine, a potent signalling molecule involved in the regulation of many host functions such as gastrointestinal motility, intestinal epithelial barrier function and immune homeostasis (Antonioli *et al.*, 2008; Haskó and Cronstein, 2004; Liu and Xia, 2015; Stepanova and Aherne, 2024). The increased production has been previously described (Liu *et al.*, 2023; Pang *et al.*, 2022) and to this we now add that the increased secreted activity of DSM 32846 is also retained during industrial-scale production of freeze-dried bacterial culture powder. It has been shown that extracellular membrane vesicles (MV) of DSM 32846 express higher 5′NT activity than MV from DSM 17938 (Pang *et al.*, 2022). Furthermore, He and colleagues have shown that DSM 17938 rescues scurfy mice from multiorgan inflammation and that adenosine receptor 2A is required for this effect (He *et al.*, 2017). Later, the same group reported increased adenosine and inosine in the cecum of DSM 32846-administered scurfy mice, an observation not seen with DSM 17938, confirming that DSM 32846 produces adenosine and inosine *in vivo* (Liu *et al.*, 2023). Showing that the strain retains the increased adenosine production capacity in culture powders that can be used for the production of probiotic products strengthens the notion that the strain may exhibit potentiated bioactivity.

Limosilactobacillus reuteri was already 2007 evaluated by the European Food Safety Authority (EFSA) and has since been listed as a Qualified Presumption of Safety (QPS) species. In addition, there is a need to evaluate the safety of new probiotic strains at three different levels: genotypic, phenotypic and clinical. The genome safety assessment showed that there are no safety concerns with DSM 32846. However, the phenotypic assessment of antibiotic resistance showed that the MIC values for ampicillin, tetracycline and chloramphenicol all exceeded the breakpoints defined by EFSA. The resistance to ampicillin can be explained by point mutations in genes encoding penicillin-binding proteins, similar to what was previously described for DSM 17938 (Rosander *et al.*, 2008). Both DSM 32846 and DSM 17938 lack the

tetracycline resistance gene *tet*(W), which is found in their common ancestor *L. reuteri* ATCC 55730 (Rosander *et al.*, 2008). DSM 17938 was shown in the same study to have an MIC value for tetracycline below the cut-off value. In that study, the Etest method was used, but when using the methods defined in later guidelines (broth microdilution), both DSM 17938 and DSM 32846 got MIC's above the cut-off values. By analysing the genome sequences, point mutations of *rpsJ* were identified that result in an exchange of amino acid residues in the ribosomal protein S10. Both Hu *et al.* (2005) and Lupien *et al.* (2015) have described corresponding point mutations in the homologous *rpsJ* in *Neisseria* and *Streptococcus pneumoniae*, respectively, resulting in increased tolerance to tetracycline. Most likely, the substitution of amino acid residues in S10 leads to a decreased affinity of tetracycline to its target, the 30S subunit of the ribosome, which increases tolerance to the antibiotics. The *rpsJ* gene as well as the genes encoding the penicillin binding proteins are all housekeeping genes located on the chromosomes of DSM 32846 and DSM 17938 and furthermore have no adjacent mobile genetic element. This type of genes is considered to pose a low risk of transmission (Devirgiliis *et al.*, 2011; van Reenen and Dicks, 2011) and is therefore considered as generally acceptable by EFSA (EFSA FEEDAP Panel, 2012). Regarding the elevated MIC for chloramphenicol, our study suggests that it can be explained by an incorrectly defined cut-off value. EFSA should only use data generated with a microdilution method but for *L. reuteri* and chloramphenicol they have based the cut-off value on Etest results (EFSA FEEDAP Panel, 2012; Egervärn *et al.*, 2007) (https://mic.eucast.org/search/, accessed 4 March 2025).

In the clinical safety study, we demonstrated that *L. reuteri* DSM 32846 is safe and well tolerated in healthy adults. In addition, no significant changes were found in any of the study groups high-dose DSM 32846, low-dose DSM 32846, or placebo in physical examination, vital signs, biochemical markers, AE or GSRS scores. Subjective tolerability as measured by GSRS indicated low levels of GI discomfort. In addition, viable DSM 32846 was detected in faeces from 73% of subjects receiving the strain, which is consistent with a previous study with *L. reuteri* DSM 17938 (Rosander *et al.*, 2008). In summary, no safety concerns were identified and there was no trend between the three study arms with regards to the types of AEs reported, AE reporting frequency, intensity or relatedness to the study products. Additionally, subjective evaluation of GI tolerability, as evaluated by GSRS, showed no clear differences between the study arms, and the use of active study product did not result in increased GI discomfort or less tolerance compared to placebo.

5 Conclusions

Limosilactobacillus reuteri DSM 32846, a novel strain derived from *L. reuteri* DSM 17938, exhibits increased bile tolerance and increased secretion of 5′-nucleotidase involved in the production of the signalling molecule adenosine. Genomic, phenotypic and clinical safety assessments show that the strain is safe for human consumption at a concentration up to 10^{10} CFU. Altogether, DSM 32846 is an interesting strain that differs from its parental strain and is interesting for further evaluation in clinical studies.

Supplementary materials

Data is available on https://doi.org/10.1163/18762891-bja00101 under Supplementary Materials.

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Authors' contribution

Conceptualisation, L.E.L., MS and S.R.; Methodology, L.E.L., M.S., AP., P.L., PPM, and S.R.; Software, L.E.L., P.L.; Validation, C.L., S.R.; Formal analysis, L.E.L., M.S., A.P., P.L. and P.P.M.; Investigation, L.E.L., M.S., A.P., S.R. and

P.P.M.; Resources, G.G. and S.R.; Writing – original draft, L.E.L., M.S., C.L., and S.R.; Writing – review & editing, L.E.L., M.S., C.L., G.G., and S.R.; Visualisation, L.E.L., M.S., C.L. and S.R.; Supervision, S.R.; Project administration, L.E.L, M.S. and S.R.; Funding acquisition, G.G. and S.R. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

L.E.L., M.S., C.L., G.G. and S.R. are employees of BioGaia AB.

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