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*Research article*

# Unveiling the probiotic potential of *L. rhamnosus* strain 044AE by genomic and phenotypic characterization

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## Supplementary

### 1. Library preparation for short read (Illumina) sequencing

Illumina library preparation was performed using QIASeq FX DNA Library Preparation protocol (Cat#180475) (Figure S1). Briefly, 50 ng of Qubit quantified DNA was enzymatically fragmented using the nuclease-enzyme cocktail supplied in-line with the library preparation kit. The fragmentation protocol entailed incubation of the template DNA with the enzyme mix at 32 ℃ for 16 min (this yields fragmented DNA of size in the range 200–300 bp) followed by 30 min incubation at 65 ℃ (and infinite hold at 4 ℃). Fragmented DNA was end-repaired and A-tailed in a one-tube reaction using the FX Enzyme/ligation Mix provided in the QIASeq FX DNA kit. The end-repaired and adenylated fragments were subjected to adapter ligation, whereby index-incorporated Illumina adapter was ligated, to generate sequencing library. This library was subjected to 6 cycles of Indexing-PCR (Initial Denaturation at 98 ℃ for 20 sec, cycling (98 ℃ for 20 sec, 60 ℃ for 30 sec, 72 ℃ for 30 sec) and final extension at 72 ℃ for 1 min) to enrich the adapter-tagged fragments. Finally, the amplified library was purified using JetSeq Magnetic Beads (Bio, # 68031).

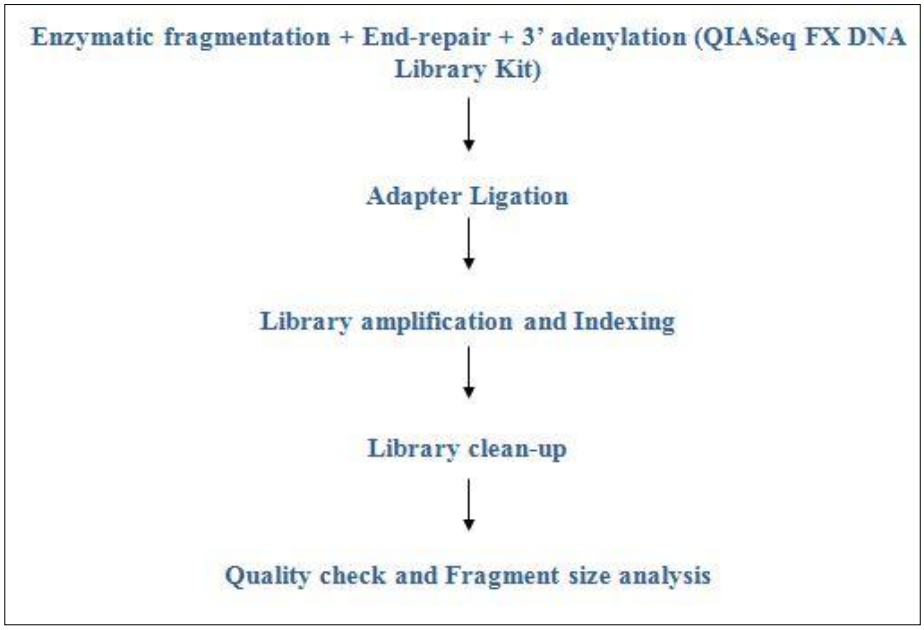


**Figure S1.** QIASeq FX DNA Library Preparation Protocol (HB-2015-002_1102703, May 2016).

The Illumina-compatible sequencing library was quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA) and the fragment size distribution was analysed on Agilent TapeStation (Figure S2).

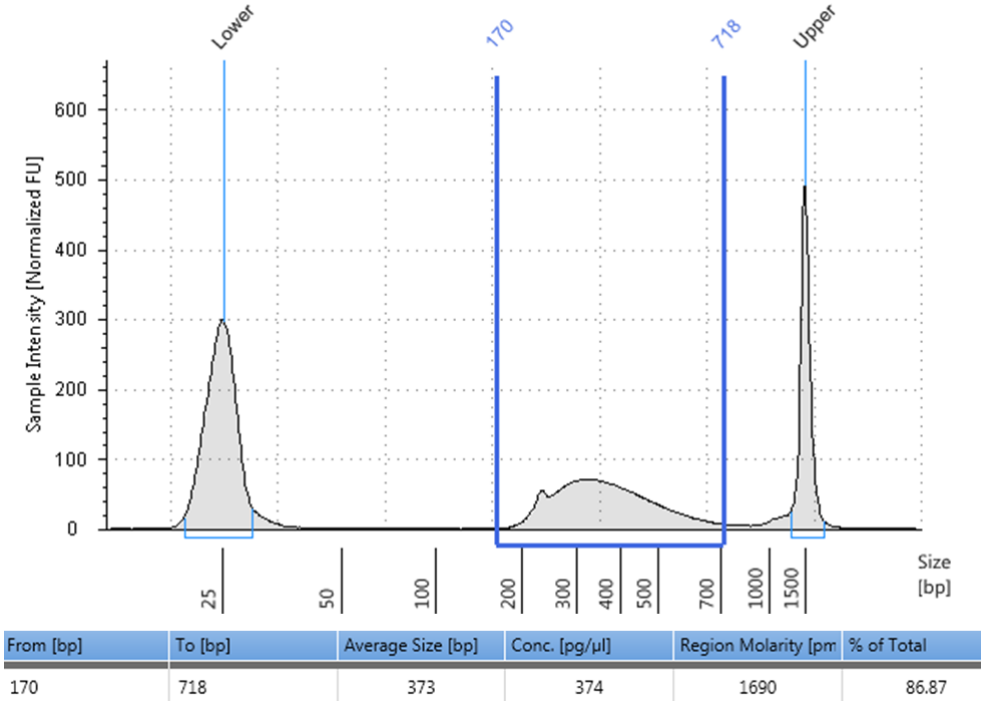


**Figure S2.** Tape Station profile of the Illumina library of *L. rhamnosus* 044AE.

The Illumina-compatible sequencing library showed average fragment sizes ranging between 170 bp and 718 bp, with enrichment around 373 bp. Given a combined adaptor size of approximately 120 bp, the effective user-defined insert size was estimated for the sample to be in the range 80 bp and 880 bp. The library thus constructed showed optimal concentration and qualified as suitable for Illumina sequencing to get the desired amount of sequencing data. Further, the library was molar-normalised and pooled to enable multiplex sequencing. Table S1 outlines the index used for barcoding the library.

**Table S1.** Barcodes used for Illumina sequencing

| Sl.No. | Sample ID | Qubit Conc. (ng/uL) | Vol (uL) | Yield (ng) | Barcode 1 | Index Sequence 1 | Barcode 2 | Index Sequence 2 |
|---|---|---|---|---|---|---|---|---|
| 1 | *L. rhamnosus* 044AE | 19.7 | 10 | 197 | D712 | AGCGATAG | D508 | GTACTGAC |

## 2. Library preparation for long read (Nanopore) sequencing

A total of 600 ng of purified total DNA from the sample was end-repaired (NEBNext Ultra II end repair kit, New England Biolabs, MA, USA); cleaned up with 1x AMPure beads (Beckmann Coulter, USA). Native barcode ligation was performed with NEB blunt/TA ligase (New England Biolabs, MA, USA) using EXP-NBD114 (ONT) and cleaned with 1xAmPure beads. Barcode sequence is detailed in Table S2.

**Table S2.** Barcode used for Nanopore sequencing.

| Sample ID | Barcode name | Sequence |
|---|---|---|
| *L. rhamnosus* 044AE | NB40 | TAGTTTGGATGACCAAGGATAGCC |

Qubit quantified barcode ligated DNA sample was Adapter ligated for 15 minutes using NEBnext Quick Ligation Module (New England Biolabs, MA, USA). The library was cleaned up using 0.6X AmPure beads (Beckmann Coulter, USA), eluted in 15 µL of elution buffer and used for sequencing. Figure S3 illustrates the overview of native barcode library preparation.

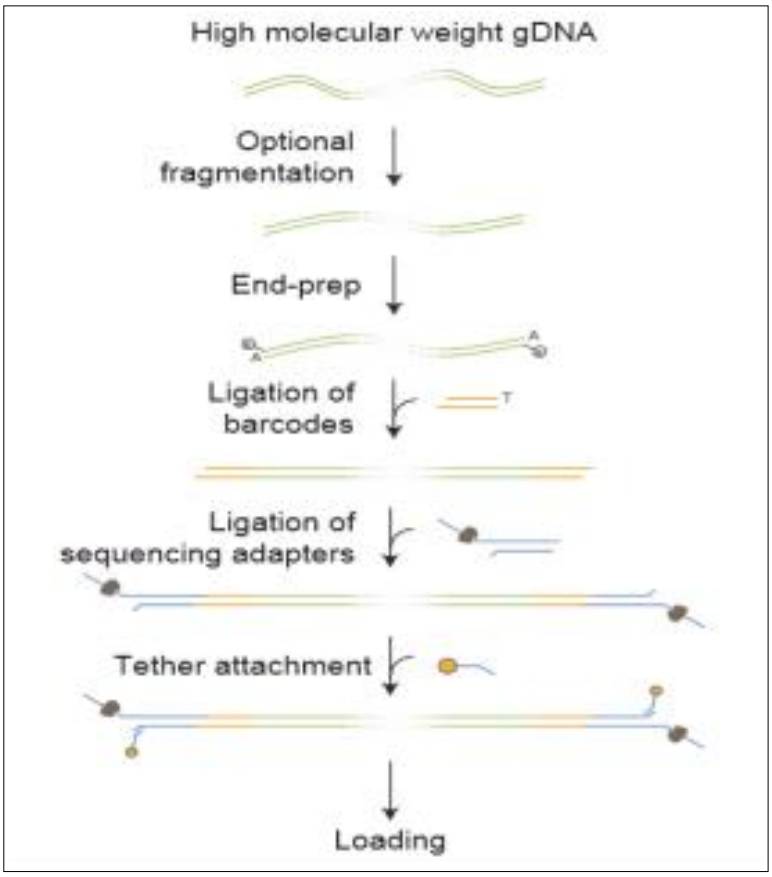


**Figure S3.** overview of native barcoding library preparation.

## 3. Sequencing for long read using Nanopore

Sequencing was performed on GridION X5 (Oxford Nanopore Technologies, Oxford, UK) using SpotONflow cell (R9.4) in about 15hrs using sequencing protocol on MinKNOW 2.1 v18.08.3 with default parameters (quality score > 7, Output file format selected: Fast5 and fastq fast5 bin size: 4000 files, fastq file bin size 4000 files). Nanopore raw reads (‘fast5’ format) were basecalled (‘fastq’ format) and demultiplexed using Guppy v2.3.4 with default parameters (Minimum phred score of 7, path for fast5 files, number of threads). After base calling, reads with a phred score (Q-Score) greater than 7 were retained as high-quality reads which were used for downstream analysis.

**Table S3**. Nanopore read statistics.

| Data type | Rawdata | Processed |
|---|---|---|
| Reads Generated | 452380 | 366807 |
| Maximum Read Length | 63321 | 60056 |
| Minimum Read Length | 102 | 9 |
| Average Read Length | 2277.7 | 2180.9 |
| Median Read Length | 12516 | 2168 |
| Total Reads Length | 1030397966 | 799961376 |
| Total Number of Non-ATGC Characters | 0 | 0 |
| Percentage of Non-ATGC Characters | 0 | 0 |
| Reads >= 100 bp | 452380 | 366291 |
| Reads >= 200 bp | 451602 | 352436 |
| Reads >= 500 bp | 385053 | 297091 |
| Reads >= 1 Kbp | 285631 | 219682 |
| Reads >= 10 Kbp | 11399 | 8863 |
| Sequencing Coverage (X) | 343 | 267 |

## 4. Sequencing for short reads using Illumina technology

Around 2.82 million reads were generated from Illumina NovaSeq platform with 501X coverage for sample *L. rhamnosus* 044AE (Table S4). Raw data was pre-processed using Trim Galore version 0.4.0 with different parameters (Minimum phred score of 30, minimum read length of 20, default universal adapter removal, number of base pairs before trimming is 150 and after trimming is between 20 to 150) and quality was calculated using FastQC (version 0.11.9). Phred quality score of pre-processed data was Q30.

**Table S4.** Illumina read statistics.

| Sample Name | AETL63 |
|---|---|
| Raw Reads | 5013434 (5.0 Mb) |
| Pre-processed | 4987013 (4.9 Mb) |
| Sequencing Coverage (X) | 501 |

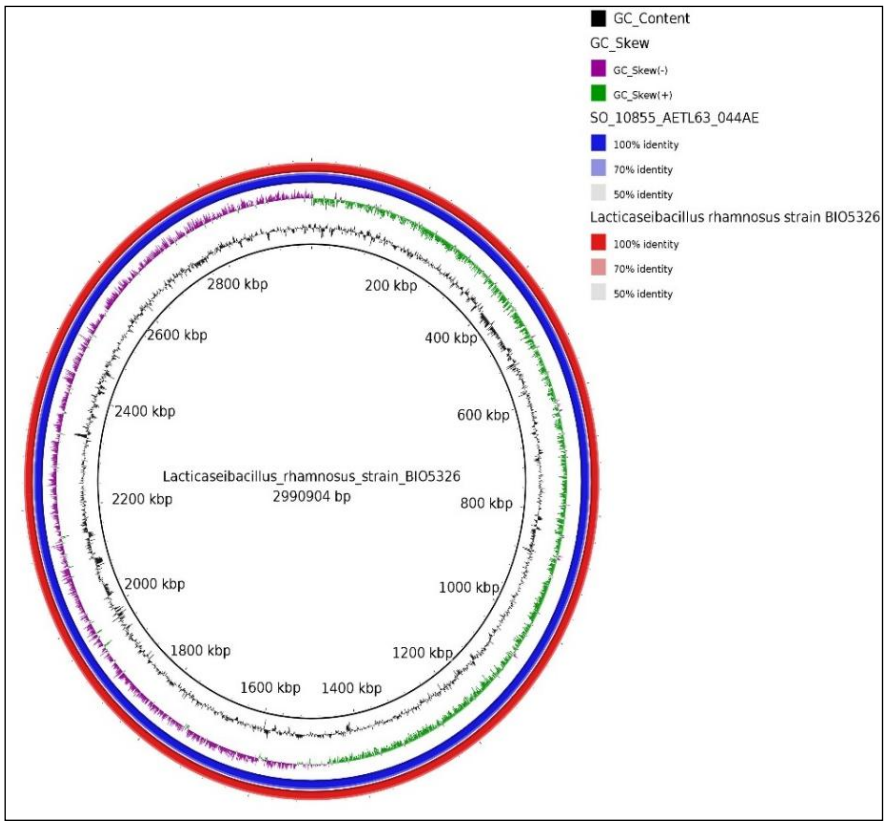


**Figure S4.** Circos plot comparison of *L. rhamnosus* strain 044AE (size of genome is 4204670 bp) with *L. rhamnosus* strain BIO5326.

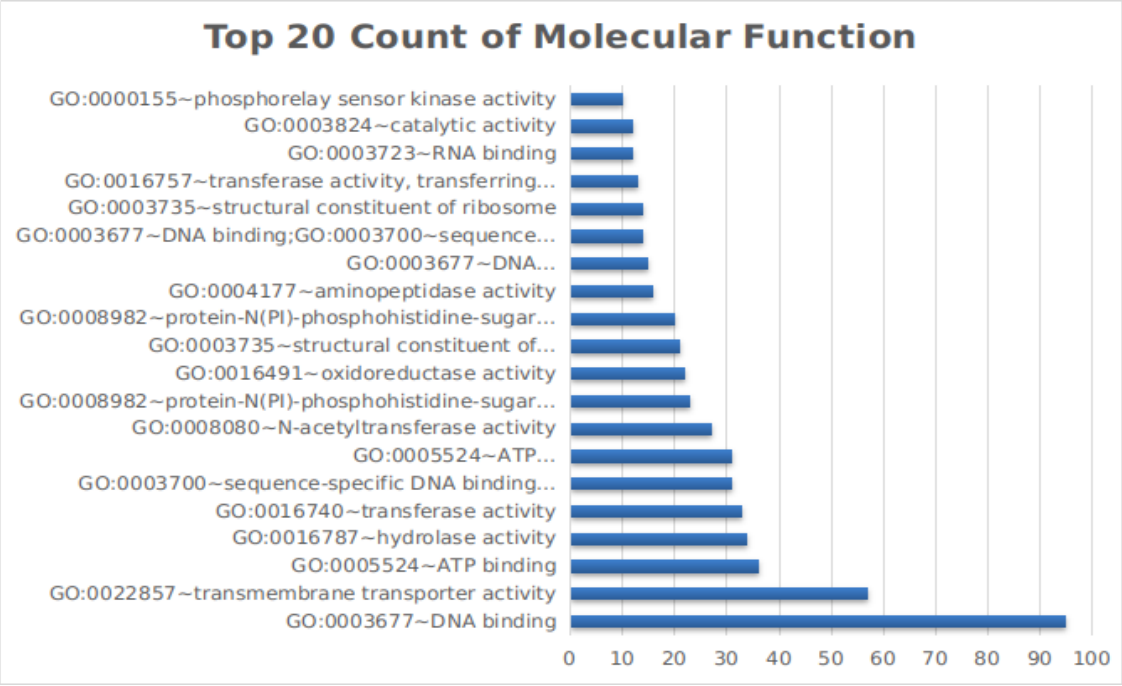


**Figure S5.** Top 20 Gene Ontology (GO) functions involved in molecular function for *L. rhamnosus* 044AE.

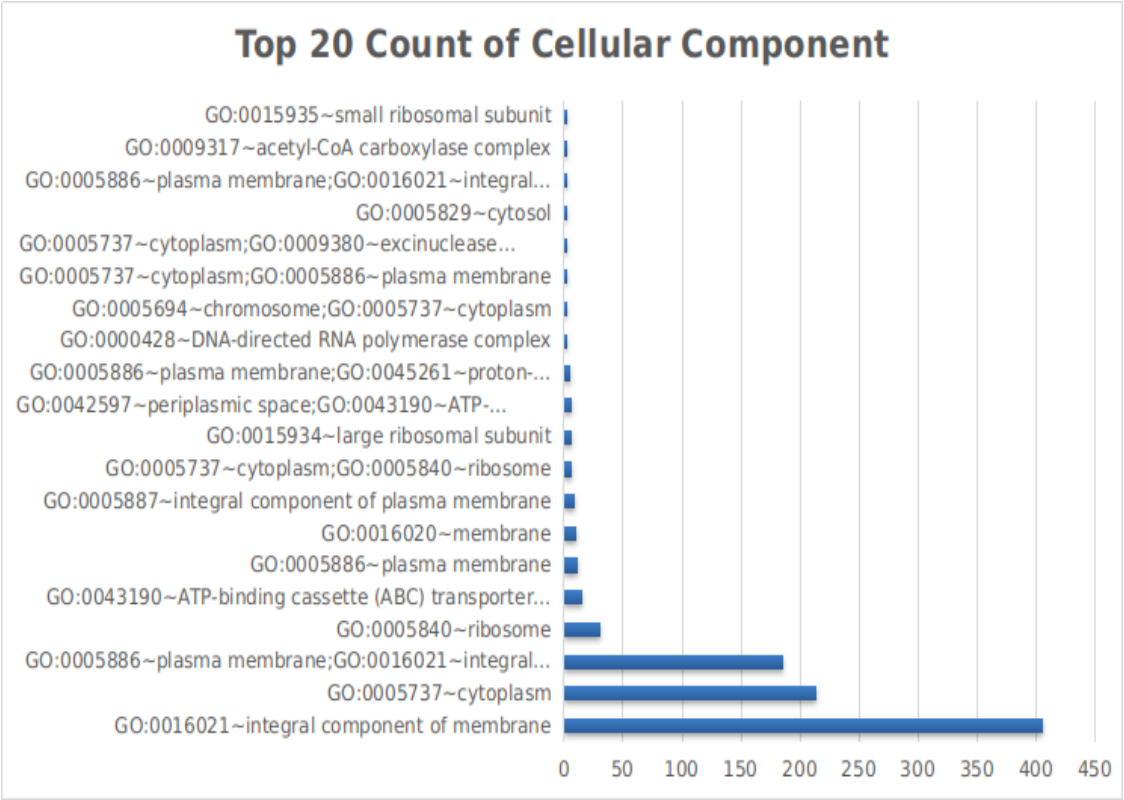


**Figure S6.** Top 20 Gene Ontology (GO) functions involved in cellular component for *L. rhamnosus* 044AE.

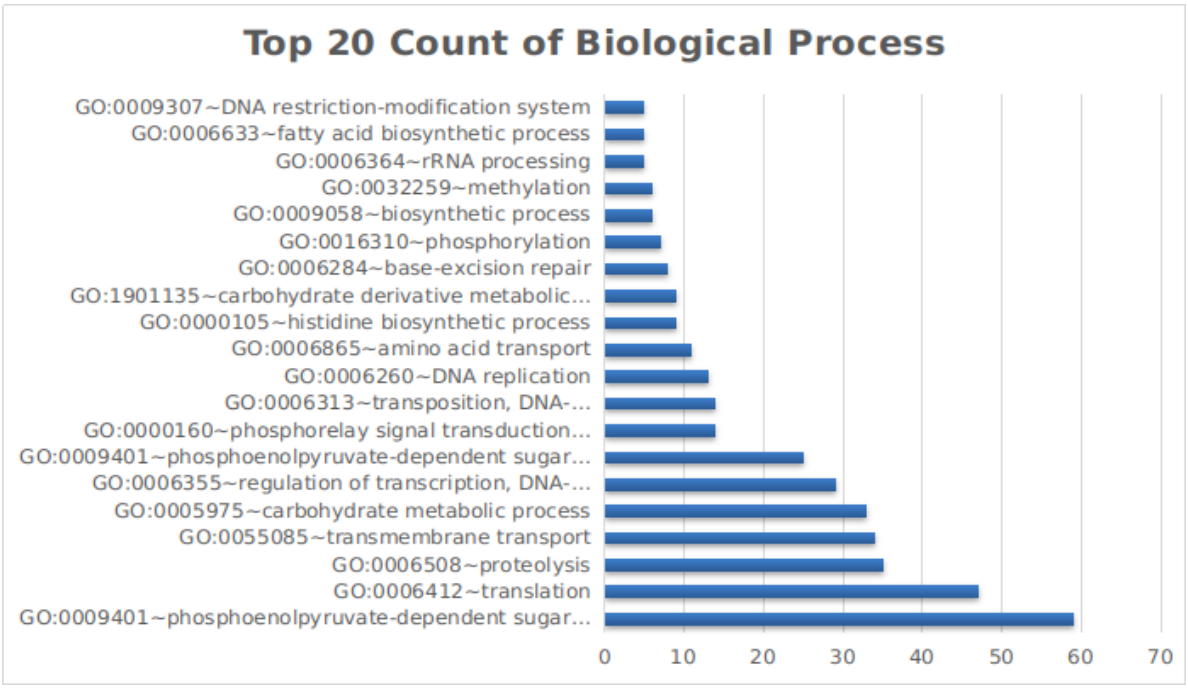


**Figure S7**. Top 20 Gene Ontology (GO) functions involved in biological process for *L. rhamnosus* 044AE.

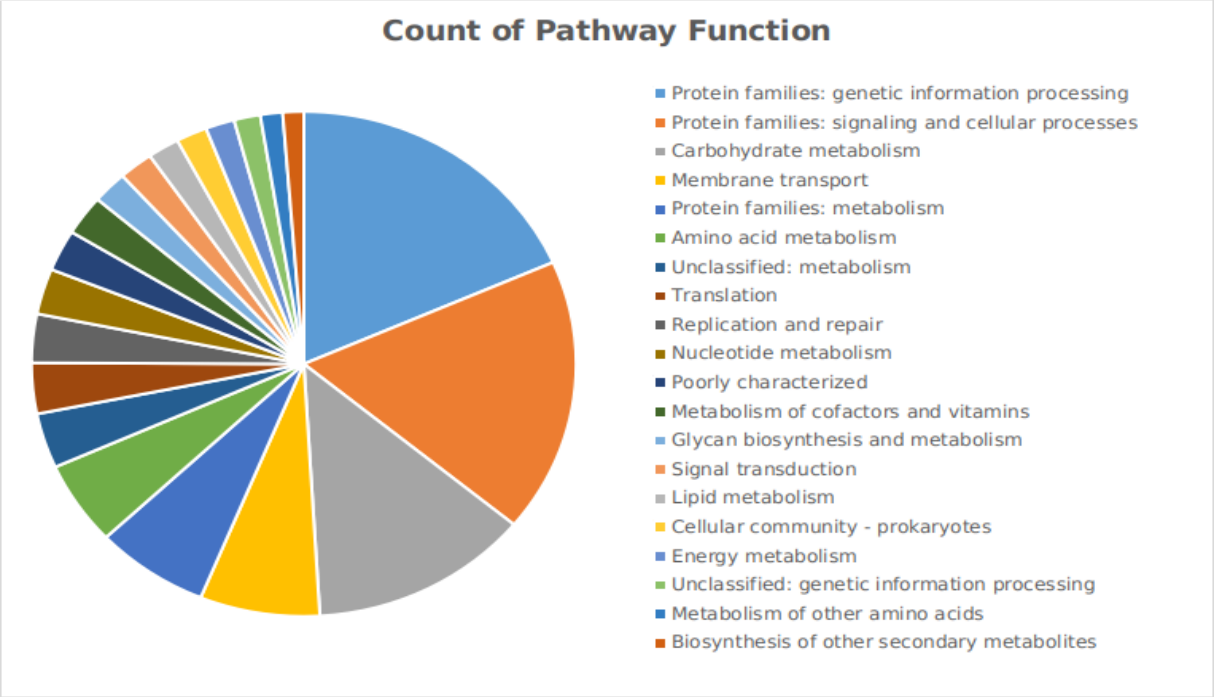


**Figure S8.** Pathway function associated with predicted proteins for *L. rhamnosus* 044AE.

## 5. Assays for *in vitro* acid, bile and temperature stability

*L rhamnosus* 044AE cells ($2 \times 10^9$ CFU/mL) were exposed to bile salt solutions of 0.01, 0.1, 0.2, 0.3, 0.5, 0.7, and 1.0% at 37 °C, as well as pH 1.5, 2.5, 3.0, 5.0, and 7.0. Up to five hours, 1 millilitre of sample was taken out of each set every hour. Using the pour plate technique, viable activity was assessed.

*L rhamnosus* 044AE cell suspension ($2 \times 10^9$ CFU/mL) was prepared and tested for stability at various temperatures (0, 40, 60, 80, 90 °C) for 6 h. Samples were taken every hour, immediately cooled in ice-cold water and analysed for viable activity.

**Table S5.** Composition of simulated digestive fluids.

| Sr. No. | Electrolyte solution | Stock conc. (M) | Stock (mL) to be added to prepare 400 mL of | | |
|---|---|---|---|---|---|
| | | | SSF | SGF | SIF |
| 1 | KCl | 0.50 | 7.550 | 3.450 | 6.800 |
| 2 | $KH_2PO_4$ | 0.50 | 1.850 | 0.450 | 0.800 |
| 3 | $NaHCO_3$ | 1.00 | 3.400 | 6.250 | 42.500 |
| 4 | NaCl | 2.00 | 0.000 | 5.900 | 9.600 |
| 5 | $MgCl_2(H_2O)_6$ | 0.15 | 0.250 | 0.200 | 1.100 |
| 6 | $(NH_4)_2CO_3$ | 0.50 | 0.030 | 0.250 | 0.000 |
| 7 | HCl | 6.00 | 0.045 | 0.650 | 0.700 |
| 8 | Mili-Q water | | 186.875 | 182.850 | 338.500 |

**Table S6.** Oral master mix.

| | | | |
|---|---|---|---|
| 1. | Simulated salivary fluid (SSF) | 8 | mL |
| 2. | 0.3 M $CaCl_2$ | 50 | µL |
| 3. | Water | (As per pH adjustment) | mL |
| 4. | 6 M HCl/6M NaOH | (As per pH adjustment) | µL |
| | Total volume | 10 | mL |

**Table S7.** Gastric master mix.

| | | | |
|---|---|---|---|
| 1. | Simulated gastric fluid (SGF) | 14.2 | mL |
| 2. | 0.3 M $CaCl_2$ | 8.9 | µL |
| 3. | 6 M HCl/6 M NaOH | (As per pH adjustment) | µL |
| 4. | Water | (As per pH adjustment) | mL |
| | Total volume | 16 | mL |

For each 8 mL of master mix, add 0.4 mL separately of pepsin stock prepared as per the Template for the harmonized *in vitro* digestion method from COST Infogest, available at: http://www.proteomics.ch/IVD/.

**Table S8.** Intestinal master mix.

| | | | |
|---|---|---|---|
| 1. | Simulated intestinal fluid (SIF) | 11.95 | mL |
| 2. | 0.3 M $CaCl_2$ | 56.28 | µL |
| 3. | Water | (As per pH adjustment) | mL |
| 4. | Pancreatin solution | 7 | mL |
| 5. | Bile solution | 3.51 | mL |
| 6. | 6 M HCl/6 M NaOH | (As per pH adjustment) | µL |
| | Total volume | 28 | mL |

## 6. Cell surface properties of *L. rhamnosus* 044AE

Hydrophobicity—Microbial adhesion to hydrocarbons (MATH) was employed to quantify the hydrophobicity of *L rhamnosus* 044AE cell surface. Overnight grown culture was centrifuged, and the washed pellet was reconstituted in PBS (Phosphate Buffer Saline) at pH of 7.4. Optical density was adjusted to approximately 1.0 ($A_0$) at 600 nm. Equal volumes of organic solvents with varying polarities, such as xylene, ethyl acetate, and toluene, were mixed with the culture suspension, vortexed for five minutes at 1800 rpm (Labquest, Borosil, MT V012). The aqueous and organic phases were allowed to separate for 30 min at 37 °C. Optical density ($A_1$) at 600 nm was measured for the aqueous layer. Percentage cell surface hydrophobicity of the bacterial cells adhering to solvents was calculated using the following equation (Eq 1) [7].

$$\text{Cell Surface Hydrophobicity } = \left[1 - \frac{A_1}{A_0} \times 100\right] \quad (1)$$

Auto-aggregation—To perform the auto-aggregation test, *L rhamnosus* 044AE cells were centrifuged from an overnight culture (cultured in MRS broth at 37 °C) at 120 rpm. The pellet was washed and re-suspended in PBS to achieve an ($A_0$) $OD_{600}$ of 0.3 ± 0.05. The suspension was incubated at 37 °C for 6 hours, mixed for 10 seconds, and the $OD_{600}$ of the samples was determined ($A_6$) [7,8]. The auto-aggregation percentage was calculated with the following equation (Eq 2):

$$\text{Auto} - \text{aggregation}\ (\%)\ = \frac{A_0 - A_6}{A_0} \times 100 \quad (2)$$

where ($A_6$) represents the absorbance at 6 h, and ($A_0$) represents the initial absorbance.

Co-aggregation—To obtain the pellet, overnight developed cultures of pathogenic bacteria and *L rhamnosus* 044AE were centrifuged at 3500 for 15 minutes, much like autoaggregation. After two PBS washes $OD_{600}$ was adjusted to 0.7 ± 0.05. *L rhamnosus* 044AE and pathogen cell suspensions were combined in equal volumes, and the cell suspension was incubated at 37 ℃ in a static environment. At 0 and 6 hours, $OD_{600}$ was measured [8,9]. The following equation (Eq 3) was used to calculate co-aggregation (%):

$$Co - aggregation\ (\%) = \frac{\left(\frac{Apat + Aprobio}{2} - Amix\right)}{\left(\frac{Apat + Aprobio}{2}\right)} \times 100 \quad (3)$$

Apat, Aprobio = the absorbance of the pathogen and the probiotic strain at time t, Amix = the absorbance of the mixed culture at time t.

## 7. Mucin adhesion assay

Overnight of *L rhamnosus* 044AE were prepared in 2 stages to obtain an $OD_{600}$ of approximately 1.5 (Pellets twice washed and reconstituted with sterile PBS). 120 µL of mucin agar (pH 6.8) was placed in each of the 96 well plates (Nunc® Edge 2.0, Sigma) together with 100 µL of the suspensions ($OD_{600}$ ~1.5). The agar control was a suspension cultured of 1% (w/v) bacteriological agar. The plates were incubated at 50 rpm at 37 ℃. Following a 90-minute incubation period, the liquid phase was removed and the wells were washed twice with 100 µL of PBS to remove any loosely adhering cells. Using a sterile spatula, solidified mucin and bacteriological agar were removed and homogenized in 5 millilitres of peptone saline. The material was tested for cell viability by pour plate [10]. Viable activities were determined in terms of CFU/well for both agar control and mucin test well.

## 8. Adhesion to Caco-2 cell lines

Caco-2 cells were cultured in MEM media with 20% fetal bovine serum at 37 ℃ and 5% $CO_2$. Media was refreshed every 2–3 days. Caco-2 cells ($1 \times 10^5$ cells/mL) were seeded in six-well plates and incubated at 37 ℃ and 5% $CO_2$. Medium was replaced every 48 hours until cells reached 80% confluency. Spent medium was replaced with MEM (antibiotic-free) and incubated at 37 ℃ for 30 min. Cells were washed twice with PBS (pH 7.4). 1 mL MEM (serum and antibiotic-free) was added and incubation was done at 37 ℃ for 30 min. Bacterial isolates ($1 \times 10^9$ CFU in 1 mL MEM) were added to wells and plates were incubated at 37 ℃, 5% $CO_2$ for 2 hours. Monolayer was washed five times with PBS to remove non-adherent bacteria. Cells were fixed with 2 mL methanol for 10 min and stained with 3 mL Giemsa (1:20 in PBS) for 20 min. After rinsing with distilled water, monolayer was air dried and examined under a 40X microscope. Bacteria were counted in 20 random fields and adhesion was classified as non-adhesive (≤40), adhesive (41–100), or strongly adhesive (>100).

Fir the calculation of percent adhesion, monolayer was washed five times with PBS to remove non-adherent bacteria. Cells were deattached using 1 mL 0.25% trypsin–EDT and incubated for 15 min.

Cell-bacteria suspension was serially diluted in saline and plated on MRS agar. Viable bacteria were enumerated after incubation. Adhesion was calculated as follows (Eq 4).

$$\text{Percent adhesion} = \frac{B1}{B0} \times 100 \quad (4)$$

where B0 and B1 are initial and final CFU counts.

**Table S9.** Gene prediction statistics.

| Summary | Number |
|---|---|
| Genes (total) | 2870 |
| CDS (total) | 2754 |
| tmRNAs | 1 |
| tRNAs | 61 |
| miscRNA | 39 |
| rRNA | 15 |

**Table S10.** List of some of the important AMR and virulence genes which were absent in the genome of *L. rhamnosus* 044AE.

| Important AMR genes | | | | |
|---|---|---|---|---|
| Accession | AMR gene family | Resistance Mechanism | Identity | Remark |
| ARO:3003438 | elfamycin resistant EF-Tu | antibiotic target alteration | 73.49 | Absent |
| ARO:3003078 | daptomycin resistant liaR | antibiotic efflux;antibiotic target alteration | 75.51 | Absent |
| ARO:3003735 | antibiotic resistant fusA | antibiotic target alteration | 71.42 | Absent |
| ARO:3000024 | ATP-binding cassette (ABC) antibiotic efflux pump | antibiotic efflux | 52.83 | Absent |
| ARO:3003778 | antibiotic resistant ndh | antibiotic target alteration | 29.279 | Absent |
| Important virulence genes | | | | |
| VFDB ID | Gene | Product | Identity | Remark |
| VFG041185 (gi:269139781) | evpH | type VI secretion system protein EvpH [EVP (E. tarda virulent protein) (SS189)] [*Edwardsiella tarda* EIB202] | 38.61 | Absent |
| VFG006826 (gi:16803417) | lisR | two-component response regulator [LisR/LisK (CVF253)] [*Listeria monocytogenes* EGD-e] | 30.18 | Absent |
| VFG002165 (gb\|NP_815739) | efaA | endocarditis specific antigen [EfaA (VF0354)] [*Enterococcus faecalis* V583] | 29.41 | Absent |
| VFG019048 (gi:76787756) | psaA | manganese ABC transporter, manganese-binding adhesion liprotein [Pneumococcal surface antigen A/Metal binding protein SloC (CVF181)] [*Streptococcus pneumoniae* CGSP14] | 52.22 | Absent |
| VFG044147(gi:16766086) | iroC | ABC transporter protein [Salmochelin (IA021)] [*Salmonella enterica subsp. enterica serovar Typhimurium* str. LT2] | 26.23 | Absent |

**Table S11.** Cell surface properties shown by *Lactobacillus rhamnosus* 044AE.

| Cell surface Properties | | |
|---|---|---|
| | Adhesion to | % Adhesion |
| Adhesion to non-polar solvent | Xylene | 1.5 ± 0.47 |
| | Toluene | 7.83 ± 1.01 |
| | Chloroform | 13.03 ± 1.16 |
| | Ethyl acetate | 24.86 ± 3.12 |
| Autoaggregation | 044AE | 7.34 ± 0.94 |
| Co aggregation | 044AE + *C. perfringens* | 45.4 ± 1.18 |
| | 044AE + *S. enterica* | 16.38 ± 9.29 |

**Table S12.** Antioxidant activity of *Lactobacillus rhamnosus* 044AE.

| Sample | % Radical scavenging activity |
|---|---|
| 100 µg/mL std. Ascorbic acid | 39.45 |
| 044AE | 16.82 ± 4.64 |

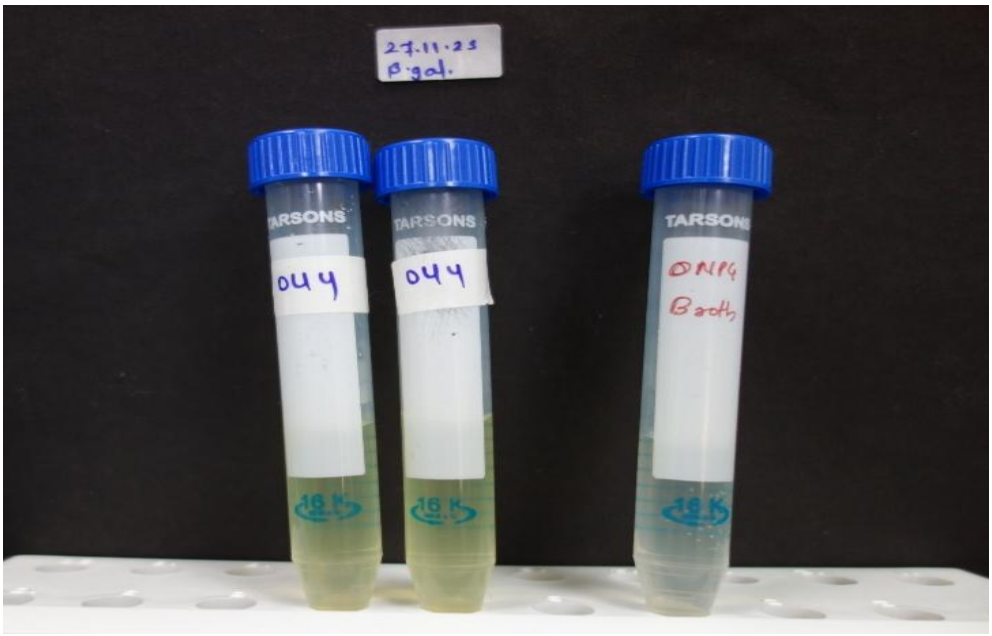


**Figure S9.** Yellow coloration of ONPG broth due to production of β-galactosidase by *L rhamnosus* 044AE.

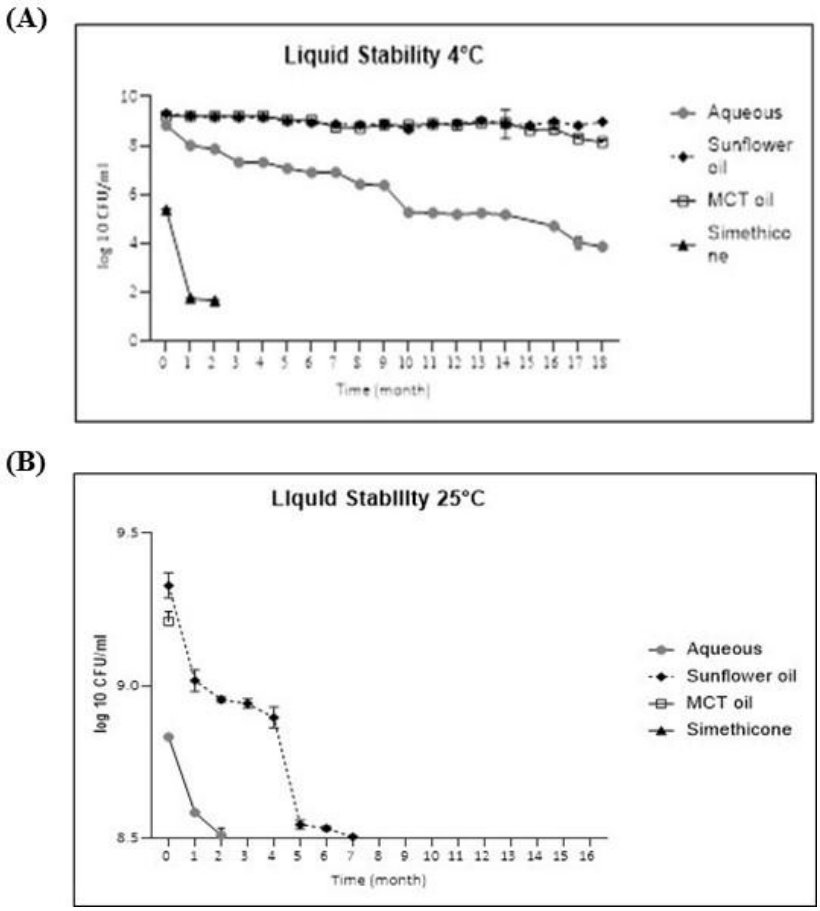


**Figure S10.** Viability of *L rhamnosus* 044AE cells in different matrices for 1, 2, 3, 6 and 9 months at 4°C (A) and 25°C (B).