Environmental Microbiome

METHODOLOGY

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Standardizing microbiome research: interlaboratory validation of SOPs for sample preparation and DNA extraction from food and environmental ecosystems

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Abstract

Background Microbiome research has expanded rapidly, however, lack of standardized and validated protocols for microbiome sampling and DNA extraction has hindered the reproducibility and comparability of studies. The SUS-MIRRI.IT project aimed to prepare and validate Standard Operating Procedures (SOPs) for microbiome analysis across diverse ecosystems, including fermented foods, soils, waters, and more. To validate these protocols, 15 Italian research units (RUs) participated in an interlaboratory trial on 120 samples (liquid and solid fermented foods, waters, and soils). Metataxonomic sequencing was performed using 16S rRNA gene amplicon sequencing to assess the reproducibility of the protocols. The interlaboratory trial involved distributing homogenized samples to participating RUs and evaluating performance both between and within RUs. This was done by comparing results obtained from DNA extraction and amplicon-based sequencing.

Results The results demonstrated high reproducibility of the procedures suggested in the SOPs across different sample types, with no significant differences in microbial diversity or composition between biological replicates or research units. DNA recovery was generally consistent, with minor variations observed in solid samples.

Conclusions This study underlines the importance of standardized protocols in microbiome research. The validated Standard Operating Procedures developed by the SUS-MIRRI.IT project demonstrate robustness and reproducibility across diverse ecosystems, providing a foundation for future microbiome studies. The adoption of these protocols will enhance data comparability and support large-scale meta-analyses in food systems microbiome research.

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Keywords Microbiota Standard Operation Procedures (SOPs), Food Microbiota, Environmental Microbiota, Metataxonomic sequencing

Background

Microbiome science is driven by the advances in sequencing platforms, user-friendly data analysis platform tools, decreasing cost per sample, and the need for more in depth tools to discover the composition and function of the microbes in the ecosystems. Microbiome study has attracted attention from industry, policy makers, citizens and researchers and has the potential to beneficially impact our planet [15]. Since 2007, when the Human Microbiome Project (NIH HUMAN MICROBIOME https://hmpdacc.org/) was launched, researchers recognized the essential need for harmonized methods in metadata collection, sampling procedure, wet lab steps, and bioinformatic tools for microbiomes analysis. To ensure long-term value and reproducibility, microbiome studies must adopt the FAIR principles: "*Findable, Accessible, Interoperable and Reusable*" [40].

Large national and international coordinated efforts (e.g. METASUB https://metasub.org/; EARTHMICROBIOME http://www.earthmicrobiome.org; MICROBE https://www.microbeproject.eu/; MASTER https://www.master-h2020.eu/; MICROBIOMESUPPORT ASSOCIATION https://www.microbiomesupport.eu/, EMBRC https://www.embrc.eu/) have been established to make the implementation of FAIR principles for microbiome study more efficient. All these actions take into account: metadata standard collection [8], wet lab procedures for microbiome collection and storage [32], nucleic acid extraction protocols [3], sequencing approach and data analysis [1] in different fields of research. Despite major advances, the lack of validated and ecosystem-specific protocols has limited comparability between studies. Standard operation procedures (SOPs) are not always validated and datasets available in public repositories do not take into account this crucial information. Even if some examples are available for a few ecosystems (e.g. humans, soil/rhizosphere, environmental samples from manufacturing environments), to the best of our knowledge there are no available SOPs for fermented foods. With harmonized, controlled and validated methods, microbiome data can become interoperable and easily used for global scale meta-analyses. In 2022, the project "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy" SUS-MIRRI.IT was launched with the aim of creating an infrastructure for the preservation, systematic investigation, provision and valorisation of microbial resources and biodiversity. One of the specific aims of the project was to contribute to the definition of Standard Operating Procedures (SOPs) for microbiome sampling from different ecosystems (solid and liquid fermented foods, human and canine gastrointestinal samples, soil/rhizosphere/plant, insect gut, water and geothermal/sediments) which were subsequently validated by an interlaboratory trial. A total of 15 SOPs were created, specifically 8 for microbiome sampling and 7 for DNA extraction [9], and 15 Italian research units (RUs), expert on different microbiome research fields, participated in an interlaboratory trial to validate 8 out 15 developed SOPs for microbiome sampling and DNA extraction, including solid and liquid fermented foods, waters and soils. Amplicon metataxonomic sequencing based on 16S rRNA genes was chosen to assess variability between and within RUs.

Methods

Samples collection

Based on Standard Operating Procedures (SOPs) for sample preparation and DNA extraction [9], a total of 120 samples were analysed for interlaboratory comparison using 16S rRNA gene amplicon sequencing (15 RUs, 4 biological samples categories [liquid fermented foods n = 2; solid fermented foods n = 2; waters n = 2 and soils n = 2] and 3 commercial DNA purification kits; Table 1).

a) Fermented foods

For the interlaboratory comparison of SOPs for fermented foods, 5 RUs participated in the trial (Table 1 and supplementary Fig. 1). A commercial yoghurt and a laboratory-scale prepared kombucha were used as representatives of liquid fermented foods. A single batch (1 L) of commercial yoghurt was purchased from a local market by a single RUs. After homogenization with a sterile spoon, three aliquots of 50 g each, were taken, placed in sterile plastic containers, and shipped at 4 °C to the four collaborating RUs (Table 1 and supplementary Fig. 1A). Kombucha tea was prepared by a single RUs (UNIMORE) according to previous protocols [25]. Briefly, sucrose 10% (w/v) was added to demineralized water and allowed to boil. Then, 25 g of white tea was added and allowed to infuse for about 5 min. Once the sucrose–tea had cooled at room temperature, it was filtered through 0.45 and 0.2 µm filters (Nalgene™ Rapid-Flow™ Filter Unit PES, Thermo Scientific) and 10% (v/v) of the previous kombucha preparation, obtained from a homebrewer propagating kombucha for several years, was added. The jars were covered with a sterile gauze and fixed with an elastic band. Fermentations were carried out for 12 days at 28 °C. Then, after homogenization with a sterile spoon, three aliquots of 25 ml each were taken, placed in sterile plastic tubes, and shipped at 4 °C to the

other RUs (Table 1 and supplementary Fig. 1B). Regarding solid fermented foods a single batch of a traditional mid- ripened sheep cheese from Sicily and an end-of-ripening Apulian fermented sausages were purchased from selected producers by a single RUs (ISPA). After homogenization with sterile tools, three aliquots from each solid fermented product were taken, placed in sterile plastic containers, and shipped at 4 °C to the other collaborating RUs (Table 1 and supplementary Fig. 1C and 1D).

b) Waters

For the interlaboratory comparison of SOPs for waters, 4 RUs participated in the trial (Table 1 and supplementary Fig. 2A). Two types of water samples (wastewater (L) and surface water (R)), representing anthropogenic and natural aquatic environments, were collected by a single RUs (UNIPG). Wastewater was collected from the Bresso wastewater treatment plant (located in Milan, Italy), downstream of primary sedimentation, using a peristaltic pump, and then distributed in three sterile 250 mL bottles per RUs. Samples were immediately stored at 4 °C and shipped to the other RUs using freezer packs to maintain temperature during transport. Surface water was collected from Trasimeno Lake (Umbria, Italy) by directly immersing a 20 L sterile tank in the lake. The water was aseptically distributed into twelve 1 L bottles (three bottles allocated to each RUs, Table 1 and supplementary Fig. 2A). Samples were stored at 4 °C and shipped with freezer packs to ensure appropriate temperature conditions during transit. Upon arrival at each RUs, the samples were kept at room temperature until all laboratories had received them. Subsequently, they were stored at 4 °C until filtration. The filtration of all the samples was then conducted simultaneously across all RUs (Table 1 and supplementary Fig. 2).

c) Soils

For the interlaboratory comparison of SOPs for soils, 6 RUs participated in the trial (Table 1 and supplementary Fig. 2B). For soils we decided to investigate the composition of the bulk soil microbiota of grapevine. Two plants of the "Moscato Bianco" cultivar, grafted on "Kober 5BB" rootstocks, were selected from an experimental vineyard located in Grugliasco (Torino, Italy). For each plant (namely A and B), 1 kg of fresh soil was sampled according to the previously defined sampling SOPs [9]. In short, five bulk soil aliquots were recovered after litter removal in a star shaped pattern around each plant using a soil corer. Bulk soil from each plant was pooled and passed through a fine mesh sieve to uniform the granularity, then mixed with a sterile spoon to homogenize it. From this soil, three aliquots of 50 g were collected in 50 ml sterile Falcon tubes and sent to the five collaborating RUs at −80 °C in dry ice, with an additional aliquot kept at −80 °C for house testing (Table 1 and supplementary Fig. 2B).

Standard operating procedures for sample preparation from liquid fermented foods

Before DNA extraction, the procedure for sample preparation described in the SOPs protocol developed within SUS-MIRRI.IT [9] were used by the participating RUs. Three independent aliquots, as biological replicates, were taken from the same homogenized sample and processed separately through the entire workflow by each RUs (supplementary Fig. 1). Briefly, the protocols for liquid fermented foods (performed by 5 RUs, Table 1) are shown in Figs. 1A and 1B for yoghurt and kombucha, respectively. An aliquot of 10 mL of yoghurt (Fig. 1A) was homogenized with 90 mL of 2% (v/v) sodium citrate in a stomacher bag. A volume of 18 mL of the mixture was centrifuged at 10,000 × g for 5 min at 4 °C. The resulting pellet was washed three times with 1.8 mL of STE buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 8), followed by centrifugation at 12,000 × g for 5 min at 4 °C. For kombucha (Fig. 1B): A 3.6 mL aliquot of kombucha was first centrifuged at 2,500 × g for 20 min at 4 °C. The resulting pellet was then subjected to a second centrifugation at 13,000 × g for 3 min at room temperature to remove residual supernatant.

Standard operating procedures for sample preparation from solid fermented foods

The protocols for solid fermented foods (performed by 5 RUs, Table 1 and supplementary Fig. 1) are shown in Figs. 1C and 1D for cheese and fermented sausages, respectively. Three independent aliquots, as biological replicates, were taken from the same homogenized sample and processed separately through the entire workflow by each RUs (supplementary Fig. 1). 10 g of cheese were homogenized in a Stomacher for 3 min at room temperature with 90 mL of 2% (v/v) sodium citrate (Fig. 1C). A volume of 18 mL of the homogenate was centrifuged at 10,000 × g for 5 min at 4 °C. The pellet was washed three times with 1.8 mL of STE buffer (100 mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 8), followed by centrifugation at 12,000 × g for 5 min at 4 °C. For fermented sausages (Fig. 1D), 10 g of sample was homogenized in a Stomacher for 3 min at room temperature with 90 mL of Ringer's solution (Oxoid, Milan, Italy). A 1.8 mL aliquot of the homogenate was centrifuged at 13,000 × g for 10 min at room temperature. The resulting pellet was then subjected to a second centrifugation at 13,000 × g for 1 min at 4 °C.

Table 1 Table reporting the interlaboratory variability of DNA extraction and sequencing

RU	Institute	Samples categories	Sample type	DNA purification kit	mean DNA concentrations (ng/µuL)	SD	total yield (µug)	260/280 ratio	mean n°reads	SD n°reads
ISA	Institute of Food Sciences	Food	Cheese	Qiagen DNeasy Power Food	29,3	4,9	1,5	2,0	40,417,0	43,698,8
ISPA	Institute of sciences of food production	Food	Cheese	Qiagen DNeasy Power Food	169,6	39,1	8,5	1,8	107,179,3	26,145,1
UNIBAS	University of Basilicata	Food	Cheese	Qiagen DNeasy Power Food	56,0	8,2	2,8	1,8	73,824,0	4396,1
UNISS	University of Sassari	Food	Cheese	Qiagen DNeasy Power Food	102,8	9,1	5,1	1,7	61,645,0	45,352,8
UNITO	University of Torino	Food	Cheese	Qiagen DNeasy Power Food	42,5	7,5	2,1	1,9	106,296,0	18,693,9
ISA	Institute of Food Sciences	Food	Kombucha	Qiagen DNeasy Power Food	8,8	6,1	0,4	1,2	41,592,0	14,952,7
ISPA	Institute of sciences of food production	Food	Kombucha	Qiagen DNeasy Power Food	21,5	12,7	1,1	1,7	25,773,0	24,578,5
UNIBAS	University of Basilicata	Food	Kombucha	Qiagen DNeasy Power Food	9,6	0,8	0,5	1,7	21,420,0	7493,0
UNISS	University of Sassari	Food	Kombucha	Qiagen DNeasy Power Food	10,8	1,3	0,5	1,7	47,814,3	19,261,2
UNITO	University of Torino	Food	Kombucha	Qiagen DNeasy Power Food	5,8	1,0	0,3	1,2	78,308,7	33,706,4
ISA	Institute of Food Sciences	Food	Sausages	Qiagen DNeasy Power Food	65,0	18,5	3,3	1,8	60,763,0	48,846,7
ISPA	Institute of sciences of food production	Food	Sausages	Qiagen DNeasy Power Food	18,4	3,6	0,9	1,6	51,867,0	39,495,0
UNIBAS	University of Basilicata	Food	Sausages	Qiagen DNeasy Power Food	18,4	4,1	0,9	1,8	71,701,3	9983,8
UNISS	University of Sassari	Food	Sausages	Qiagen DNeasy Power Food	36,1	1,2	1,8	1,8	12,576,0	2024,3
UNITO	University of Torino	Food	Sausages	Qiagen DNeasy Power Food	43,5	15,0	2,2	1,9	68,610,7	2361,3
ISA	Institute of Food Sciences	Food	Yogurt	Qiagen DNeasy Power Food	216,8	9,2	10,8	1,8	80,505,7	26,143,7
ISPA	Institute of sciences of food production	Food	Yogurt	Qiagen DNeasy Power Food	69,3	12,2	3,5	1,9	116,964,7	26,905,7
UNIBAS	University of Basilicata	Food	Yogurt	Qiagen DNeasy Power Food	140,0	46,3	7,0	1,9	102,127,3	23,472,0
UNISS	University of Sassari	Food	Yogurt	Qiagen DNeasy Power Food	154,7	27,8	7,7	1,7	57,473,3	11,237,2
UNITO	University of Torino	Food	Yogurt	Qiagen DNeasy Power Food	90,7	6,6	4,5	1,9	114,427,7	6645,4
UNIMIB	Universita of Milano-Bicocca	Water	Water_L	Qiagen DNeasy Power Water	63,0	9,1	6,3	1,9	67,979,7	12,632,7
UNIPA	University of Palermo	Water	Water_L	Qiagen DNeasy Power Water	58,6	14,5	5,9	1,8	55,024,0	2295,8
UNIPG	University of Perugia	Water	Water_L	Qiagen DNeasy Power Water	79,4	4,6	7,9	1,9	45,186,0	17,631,4
UNIVR	University of Verona	Water	Water_L	Qiagen DNeasy Power Water	103,0	3,3	10,3	1,9	43,243,0	15,243,8
UNIMIB	University of Milan	Water	Water_R	Qiagen DNeasy Power Water	81,8	9,5	8,2	1,9	48,312,0	4248,8
UNIPA	University of Palermo	Water	Water_R	Qiagen DNeasy Power Water	30,9	6,6	3,1	1,8	62,596,7	27,865,5
UNIPG	University of Perugia	Water	Water_R	Qiagen DNeasy Power Water	48,8	10,4	4,9	2,0	43,551,3	17,570,9

Table 1 (continued)

RU	Institute	Samples categories	Sample type	DNA purification kit	mean DNA concentrations (ng/µuL)	SD	total yield (µug)	260/280 ratio	mean n°reads	SD n°reads
UNIVR	University of Verona	Water	Water_R	Qiagen DNeasy Power Water	49,1	12,5	4,9	1,9	44,349,3	13,438,7
ENEA	Italian National Agency for New Technologies, Energy and Sustainable Economic Development	Soil	Soil_A	Qiagen Power Soil DNA Pro	18,9	6,9	1,9	1,8	41,689,7	15,176,4
UNIMIB	Universita of Milano-Bicocca	Soil	Soil_A	Qiagen Power Soil DNA Pro	34,2	7,4	3,4	1,9	20,741,3	1766,6
UNIPA	University of Palermo	Soil	Soil_A	Qiagen Power Soil DNA Pro	57,1	9,0	5,7	1,7	35,969,0	19,583,9
UNIPG	University of Perugia	Soil	Soil_A	Qiagen Power Soil DNA Pro	51,5	8,3	5,2	2,0	50,585,0	6058,2
UNITO	University of Torino	Soil	Soil_A	Qiagen Power Soil DNA Pro	24,3	8,7	2,4	1,8	50,268,3	8639,5
UNIVR	University of Verona	Soil	Soil_A	Qiagen Power Soil DNA Pro	10,9	3,6	1,1	1,6	38,204,0	7764,5
ENEA	Italian National Agency for New Technologies, Energy and Sustainable Economic Development	Soil	Soil_B	Qiagen Power Soil DNA Pro	9,0	2,7	0,9	1,7	24,677,3	11,403,9
UNIMIB	Universita of Milano-Bicocca	Soil	Soil_B	Qiagen Power Soil DNA Pro	25,5	2,4	2,6	1,8	50,348,0	8461,3
UNIPA	University of Palermo	Soil	Soil_B	Qiagen Power Soil DNA Pro	35,6	2,2	3,6	1,8	64,650,7	6359,8
UNIPG	University of Perugia	Soil	Soil_B	Qiagen Power Soil DNA Pro	59,6	2,3	6,0	1,9	77,958,3	117,298,8
UNITO	University of Torino	Soil	Soil_B	Qiagen Power Soil DNA Pro	21,2	5,0	2,1	1,8	29,392,7	14,554,9
UNIVR	University of Verona	Soil	Soil_B	Qiagen Power Soil DNA Pro	11,8	3,1	1,2	1,8	24,001,0	10,810,6
ISA	Institute of Food Sciences	Mock	Mock	Qiagen DNeasy Power Food	69,6	8,7	7,0	1,9	98,120,0	17,105,8
ISPA	Institute of sciences of food production	Mock	Mock	Qiagen DNeasy Power Food	29,0	1,0	2,9	1,6	102,818,0	13,903,0
UNIBAS	University of Basilicata	Mock	Mock	Qiagen DNeasy Power Food	35,3	3,1	3,5	1,8	49,996,7	22,058,3
UNISS	University of Sassari	Mock	Mock	Qiagen DNeasy Power Food	36,1	3,5	3,6	1,8	30,219,3	29,070,8
UNITO	University of Torino	Mock	Mock	Qiagen DNeasy Power Food	28,8	2,2	2,9	1,8	80,771,3	10,308,9
ENEA	Italian National Agency for New Technologies, Energy and Sustainable Economic Development	Mock	Mock	Qiagen Power Soil DNA Pro	37,8	1,8	3,8	1,8	29,454,0	150,3
UNIMIB	Universita of Milano-Bicocca	Mock	Mock	Qiagen Power Soil DNA Pro	22,8	2,7	2,3	1,7	43,347,0	438,9
UNIPA	University of Palermo	Mock	Mock	Qiagen Power Soil DNA Pro	26,6	4,0	2,7	1,7	51,739,0	3428,5

Table 1 (continued)

RU	Institute	Samples categories	Sample type	DNA purification kit	mean DNA concentrations (ng/µuL)	SD	total yield (µug) 260/280 ratio		mean n°reads	SD n°reads
UNIPG	University of Perugia	Mock	Mock	Qiagen Power Soil DNA Pro	29,5	1,0	3,0	1,9	16,760,0	203,1
UNITO	University of Torino	Mock	Mock	Qiagen Power Soil DNA Pro	18,5	3,0	1,8	2,0	36,531,3	269,5
UNIVR	University of Verona	Mock	Mock	Qiagen Power Soil DNA Pro	29,9	3,0	3,0	1,7	19,847,7	165,6
UNIMIB	Universita of Milano-Bicocca	Mock	Mock	Qiagen DNeasy Power Water	19,8	1,4	2,0	1,9	47,992,3	10,572,0
UNIPA	University of Palermo	Mock	Mock	Qiagen DNeasy Power Water	41,9	8,5	4,2	1,9	45,161,0	18,231,4
UNIPG	University of Perugia	Mock	Mock	Qiagen DNeasy Power Water	18,8	3,1	1,9	1,9	58,818,7	35,177,0
UNIVR	University of Verona	Mock	Mock	Qiagen DNeasy Power Water	24,4	1,7	2,4	1,9	318,276,7	179,251,4

The table presents data from different RUs, detailing the sample categories, sample types, DNA purification kits used, mean DNA concentrations (ng/µL), standard deviations (SD), total DNA yield (ng), mean number of reads, and standard deviations (SD) of reads for each sample set. Mean and standard deviation (SD) of DNA concentration (ng/µL) and number of sequencing reads are reported for each RU and sample type

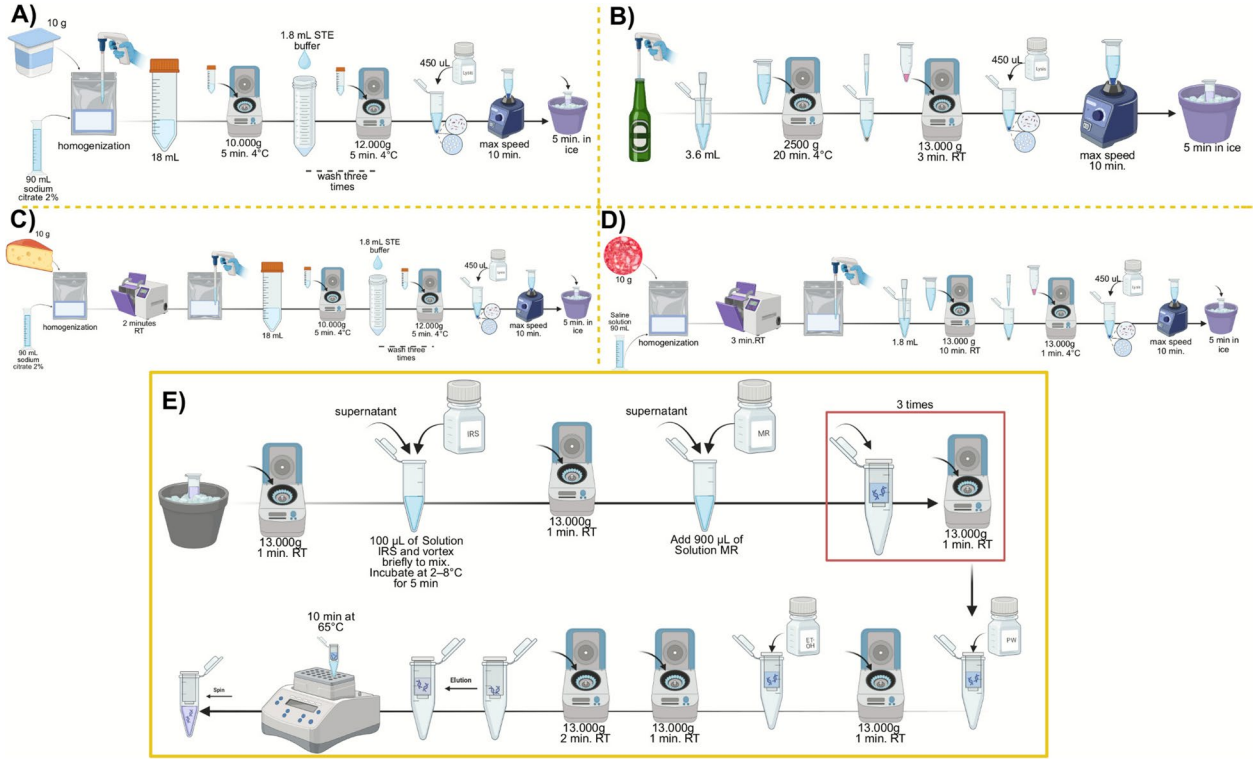

Fig. 1 Workflow for sampling of yoghurt (plot **A**), kombucha (plot **B**), cheese (plot **C**) and fermented sausages (plot **D**). DNA is extracted from the cell pellet by using the protocol based on the DNeasy PowerFood Kit (plot **E**). Created in https://BioRender.com

Standard operating procedures for DNA extraction from solid and liquid fermented foods

For all fermented food samples, cell lysis was carried out using a combination of mechanical and chemical methods (Fig. 1E). Mechanical lysis was performed via bead beating using the Qiagen's PowerBead Pro Tubes (Qiagen, Milan, Italy) for 10 min at maximum speed using a Vortex-Genie2 mixer (Fisher Scientific, Milan, Italy). Chemical lysis was conducted using a lysis buffer from the DNeasy PowerFood Kit (Qiagen). After lysis, samples were cooled on ice for 10 min. To maximize the recovery of the total microbial DNA from all fermented

food samples, the final step of the DNeasy PowerFood Kit protocol was modified. Specifically, 50 µL of Solution EB was applied to the center of the white filter membrane and incubated at 65 °C for 10 min prior to centrifugation at 13,000 × g for 5 min. ZymoBIOMICS Microbial Community Standard (Zymo Research, Milan, Italy) was extracted in triplicate by each RUs using 75 µl per prep and following the DNeasy PowerFood Kit protocol.

Standard operating procedures for sample preparation and DNA extraction from waters

The commercial DNeasy PowerWater Kit (Qiagen) was chosen for the DNA extraction from water (performed by 4 RUs, Table 1 and supplementary Fig. 2). Three independent aliquots, as biological replicates, were taken from the same homogenized sample and processed separately through the entire workflow by each RUs (supplementary Fig. 2A). Prior extraction, water samples were filtered in triplicate using a reusable filter funnel connected to a vacuum pump, equipped with 0.2 µm filter membranes. A volume of 30 mL was filtered for wastewater and 150 mL for surface lake water, respectively (Fig. 2A). The filters were then placed into a 5 mL PowerWater Bead Tube. DNA extraction was performed according to Qiagen protocol as described in the SOPs developed within SUS-MIRRI.IT project. ZymoBIOMICS Microbial Community Standard was extracted in triplicate by each RUs using 75 µl per prep and following the DNeasy PowerWater Kit protocol.

Standard operating procedures for sample preparation and DNA extraction from soils

The commercial DNeasy PowerSoil Pro Kit (Qiagen) was chosen for the DNA extraction from microbial cells in soils (performed by 6 RUs, Table 1 and supplementary Fig. 2) (Fig. 2B). Three independent aliquots, as biological replicates, were taken from the same homogenized sample and processed separately through the entire workflow by each RUs (supplementary Fig. 2). Briefly 250 mg of sieved soils were transferred in a clean 2 mL PowerBead Tube, together with 800 µl of solution CD1. All tubes were vortexed at full speed for 10 min, then centrifuged at 15,000 g for 1 min at room temperature. The supernatant was transferred in a clean, 2 mL microcentrifuge tube and

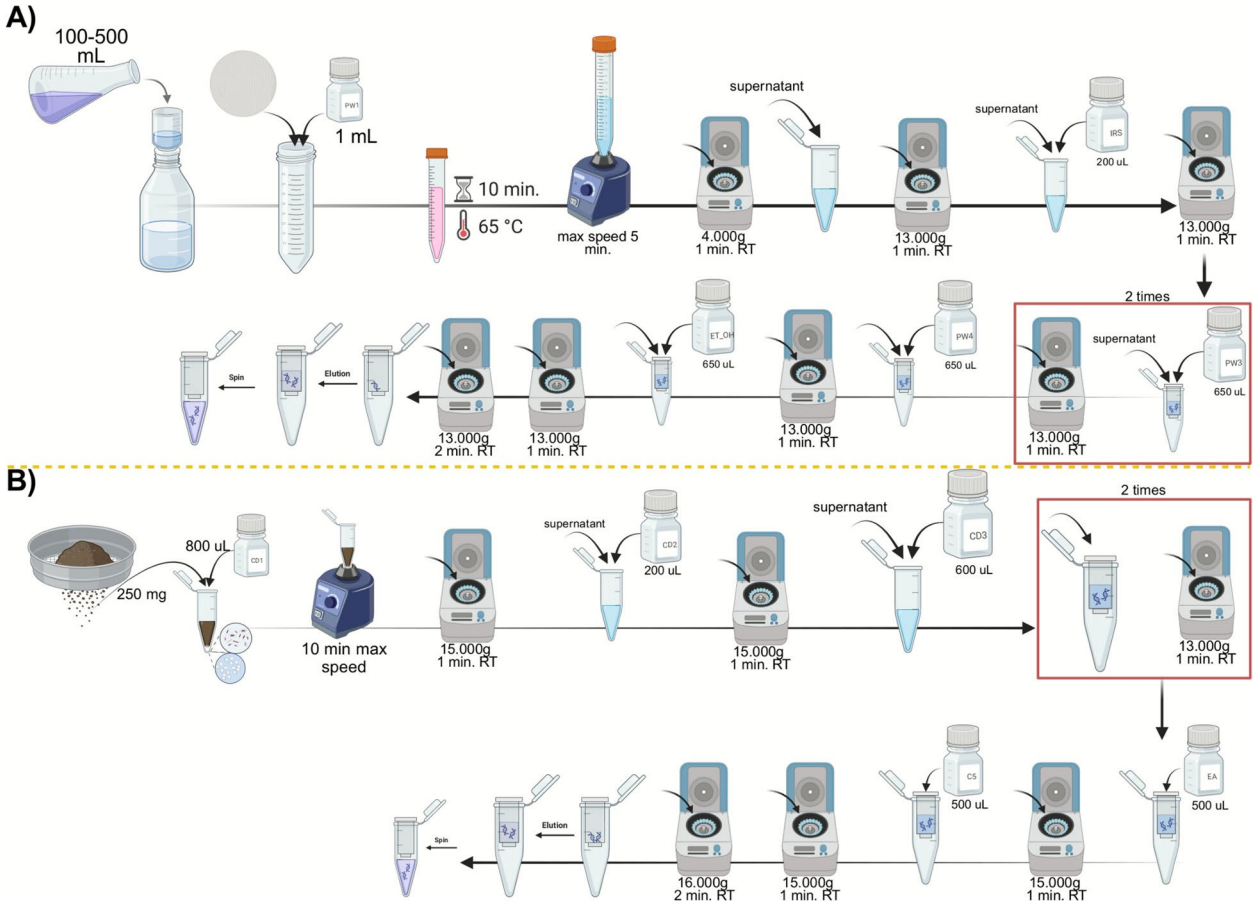

Fig. 2 Workflow for sampling and DNA extraction by using the protocol based on the DNeasy PowerWater Kit of water (plot **A**) and soil (plot **B**) by using the DNeasy PowerSoil Pro Kit. Created in https://BioRender.com

added with 200 µl of solution CD2. The tubes were centrifuged at 15,000 g for 1 min at room temperature and up to 700 µl of the resulting supernatant transferred to a new, clean 2 mL microcentrifuge tube, in which 600 µl of solution CD3 were also added. The resulting solution was loaded in 650 µl aliquots onto a MB spin column, where it was centrifuged at 15,000 g to bind the DNA to the column and discard the rest. The spin column was first washed with 500 of solution EA and then with 500 of solution C5, each time centrifuging at 15,000 g for 1 min and discarding the flow-through. In order to remove any possible trace of the previous washes, the columns were then centrifuged empty at 16,000 g for 2 min. Finally, the column was transferred into a 1.5 mL elution tube and 80 µl of buffer C6 were added. After 5 min of incubation at room temperature, columns were centrifuged one last time at 15,000 g for 1 min to collect the DNA adsorbed on the column. ZymoBIOMICS Microbial Community Standard was extracted in triplicate by each RUs using 75 µl per prep and following the DNeasy PowerSoil Pro Kit protocol.

Amplicon sequencing, bioinformatic and statistical analysis

All DNA samples were sent to a single RUs for PCR preparation and bioinformatic analysis to reduce variation. DNA was first quantified with a Qubit fluorometer by using the Qubit high-sensitivity double-stranded DNA (dsDNA) quantification kit (Thermo, Milan Italy) and 25 ng of DNA was used to amplify the V3-V4 region of the 16S rRNA gene using the primers 16SF 5'-CCTACGGGNGGCWGCAG-3' and 16SR 5'-GACTACHVGGGTATCTAATCC-3 [23], according to the Illumina 16S sequencing library preparation protocol. Each of the three individual DNA replicates for each biological sample from each RUs was subjected to three separate amplifications and the PCR products were then pooled (supplementary Fig. 1 and 2). ZymoBIOMICS Microbial Community DNA Standard was used to avoid bias arising from sequencing library preparation and subsequent processes according to the manufactured instruction. Additionally, the mock sample was amplified six times, and the pools were created by combining two amplifications at a time. Amplicons were then sent for sequencing by Novogene company generating 250 bp paired-end reads. The.*fastq* data were processed using QIIME2 v. 2022.2.0 [4]. Primer sequences were trimmed using the qiime cutadapt trim-paired script v. 2022.2.0 with default parameters. Denoising of the paired-end reads was performed jointly for all sequencing run using qiime dada2 denoise-paired v. 2022.2.0 [6], where reads were truncated at 233 bp for the forward and 229 bp for the reverse reads, with additional filtering parameters such as quality thresholds (*p*-trunc-q 20). The taxonomic classification of Amplicon Sequence Variants (ASVs) was performed using the qiime feature-classifier classify-consensus-vsearch command against the reference GreenGenes2 database (version 2024.1) for taxonomic assignment with default parameters. The feature table was filtered to exclude sequences classified as mitochondria, chloroplasts, or unassigned taxa at phylum level using the qiime taxa filter-table command. A rarefaction step at the lowest n° of reads/sample was then performed to standardize the sequencing depth across samples. The *decontam* package of R was used to identify and remove contaminants based on DNA concentration, ensuring that only relevant biological sequences were retained for downstream analysis since the negative control failed to library preparation and sequencing. The negative control showed no detectable DNA after Qubit quantification and therefore failed library preparation and sequencing. DNA concentration data were analyzed using a one-way ANOVA, using the *multcompView* package of R, to assess differences between RUs. Post-hoc comparisons were performed using Tukey's Honest Significant Difference (HSD) test. The alpha diversity index (including Shannon, sample coverage and richness), were calculated using the *vegan* package of R [12]. To evaluate agreement within RUs, Bland–Altman plots were generated for Shannon diversity indices, and differences along with 95% confidence intervals were calculated for each RUs to reflect variability among replicates. Bias was defined as the mean difference between measurements of each replicate and the mean across all RUs for the same sample type, effectively using the multi-laboratory consensus as a reference. Additionally, the reproducibility of alpha diversity measurements across different RUs was assessed using the Mock sample. Effect sizes were quantified using Hedges' g, with confidence intervals, to measure the magnitude of differences, and Cliff's delta to assess non-parametric effects. Statistical significance of the observed differences was evaluated using the Wilcoxon rank-sum test. The divergence between RUs was assessed using the Bray–Curtis dissimilarity index using the ZymoBIOMICS Microbial Community DNA Standard or a reference RUs as appropriate. For each sample type we chose as a reference, the RUs showing the lowest standard deviation among its biological replicates, assuming that this RUs likely exhibited the smallest variation between replicates. Comparisons of divergence values between RUs were conducted using Wilcoxon rank-sum tests with Bonferroni correction for multiple comparisons. Bray–Curtis distance matrix was used to perform PERMANOVA by the "vegan" package in R environment. The Mean Interquartile Quality (MIQ) score was used to check how much the observed ASVs frequency of each species in the ZymoBIOMICS Microbial Community DNA Standard or a reference RUs deviate from what is expected. Each

species was expected to be 100%, and any measurement outside ± 15% of this value was considered a deviation that cannot be explained by the standard itself. The 15% tolerance limit was chosen based on the manufacturer's instructions for the ZymoBIOMICS Microbial Community DNA Standard, which recommend this threshold to evaluate potential deviations and biases in observed frequency. To calculate MIQ, we first measure the Root Mean Square Error (RMSE) of the deviations outside the ± 15% tolerance. RMSE is a standard way to quantify how far values are from a reference. The MIQ score was then calculated as 100 minus the RMSE. Higher MIQ values indicate that the observed frequency were close to the expected values. The frequency of each ASVs between RUs was summarized by calculating the mean, standard deviation, and 95% confidence intervals (CI). To assess the statistical significance of differences between observed frequency and reference values, a Wilcoxon test was performed for each ASV. Bar charts and box/scatter plots were generated using the *ggplot2* whereas radar plots were obtained through *ggradar* package of R. The complete R code used for all analyses is accessible on Zenodo (https://doi.org/10.5281/zenodo.17532698).

Results

Development of standard operating procedures (SOPs) for microbiome sampling and DNA extraction

Within the Project SUS-MIRRI.IT "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy", in a joint effort to standardize procedures and protocols in microbiome research, 15 Italian research units, expert on different microbiome fields, developed SOPs for sampling and DNA extraction from samples of different origin. In order to do so a thorough survey was conducted with the aim of collecting relevant information. A total of 82 studies including 2785 sets of sequences spanning from food (7%), soils and rhizosphere (26%), wastewater and water (18%), insect (1%), plant (34%), animal (6%), human (5%) others (3%) were collected. The exercise included 1239 datasets for microbiota, 1138 for mycobiota and 408 for microbiomes, of which 1248 were not deposited or were private, 956 were public and 221 unpublished. About 99% of the study used DNA as a target molecule and applied a metataxonomic approach (16S rRNA sequencing in 44% of cases and ITS sequencing in 38% of the cases). Commercial kits were used in 68% of the studies, while commercial kits with custom modification were included in 27% of the experimental plans. Among brands, the Qiagen kits were the preferred option in 73% of the studies. For sequencing technology and bioinformatic analysis, the Illumina platform and QIIME2 software were used for 96% and 53% of the studies respectively. Based on the inputs collected a total of 15 SOPs were created, specifically 8 for microbiome sampling and 7 for DNA extraction [9] and among them those related to liquid fermented foods, solid fermented foods, waters and soils were selected for further interlaboratory validation.

DNA extraction performance

Interlaboratory variability obtained with a mock microbial community

In order to evaluate the interlaboratory variability, the ZymoBIOMICS Microbial Community Standard was used by each RUs while the ZymoBIOMICS Microbial Community DNA Standard was included in the library preparation workflow to assess bias from sequencing library preparation and subsequent processes.

Subjecting the Microbial Community Standard samples to DNA extraction using different kits, we observe varying levels of DNA concentrations, with the highest quantity obtained using for the Qiagen DNeasy Power Food and Qiagen Power Soil DNA Pro kits (Table 1). Most of the RUs reached the expected extraction yield (approximately 2 μg), indicating an efficient isolation of DNA, as indicated in the product instructions.

After DNA extraction and sequencing of the Microbial Community Standard, an average of 74,408 reads/samples (Table 1) were obtained (sample coverage > 99%). After sequencing a relative frequency deviation in the average of less than 15% indicated a highly accurate process, in accordance with the manufacturer's recommendations. Bland Altman analysis performed on Shannon index showed that for each group of RUs working with soils, foods and waters, the Shannon index was not affected by the interlaboratory variability (Supplementary Fig. 3). No significant differences between the confidence interval (CI) within RUs was observed (ranging from 1.65 to 2 with an average mean between biological replicates of 1.8) suggests that if a systematic differenceoccurred those were uniform across the RUs. Effect size analyses comparing the performance of each RUs using the MOCK sample reveal few differences in alpha diversity across RUs working on water, soil, and food matrices (Supplementary Fig. 4). For Shannon diversity, most RUs show negative Hedges' g values and negative Cliff's delta, indicating lower diversity than the theoretical MOCK values, with the largest deviations observed in soil samples. Simpson diversity shows smaller and more variable deviations. Although effect sizes suggest measurable differences between RUs, Wilcoxon p-values indicate that these differences were not statistically significant ($P > 0.05$, Supplementary Fig. 4).

The Bray–Curtis dissimilarity index was then used to assess the divergence (dissimilarity) between RUs that extract the Microbial Community Standard and the Microbial Community DNA Standard (Supplementary Fig. 3). Each RUs displayed a good similarity (values

between 0.15 and 0.25) with no significant differences (Wilcoxon Test $P > 0.05$) confirmed by PERMANOVA ($P > 0.05$). Notably, only one biological sample from water exceeded the upper limit (Supplementary Fig. 3E).

The ASVs frequency of the Microbial Community Standard between RUs was analysed by comparing the observed frequency to the theoretical values of the Microbial Community DNA Standard (Supplementary Fig. 5). The Mean Index of Quality (MIQ) was calculated using the Root Mean Squared Error (RMSE) for each RUs vs. the theoretical Microbial Community values, with tolerance limits of ± 15% set around the expected Microbial Community DNA Standard values. The analysis showed that all RUs displayed a highest similarity to the theoretical Microbial Community, with MIQ values > 90%, indicating that the observed frequencies were within the established tolerance range (Supplementary Fig. 5). Notably, for each ASV no significant deviations within RUs outside the tolerance limits were found, suggesting that the microbial abundance data was unbiased, reliable and reproducible (Supplementary Fig. 5).

Interlaboratory variability of extracted DNA from matrices

Regarding liquid fermented food samples, DNA concentration in yoghurts was 134.32 ± 57.63 ng/µL on average, with three RUs showing the highest values (ANOVA, $P < 0.05$; Supplementary Fig. 6A). For kombucha, the concentration was 11.32 ± 7.70 ng/µL on average, with no significant differences between the RUs (Supplementary Fig. 6B). For solid fermented foods: in cheeses, the concentration was 80.02 ± 55.29 ng/µL in average, with ISPA and UNISS having the highest values (ANOVA, $P < 0.05$; Supplementary Fig. 6C); in fermented sausages, it was 36.27 ± 20.25 ng/µL in average, with ISA and UNITO showing the highest values (ANOVA, $P < 0.05$; Supplementary Fig. 6D); The mean DNA concentration obtained from the RUs working on water L and R was 76.00 ± 17.75 ng/µL and 52.6 ± 20.98 ng/µL in average, respectively, with the highest values observed in UNIVR (for water L) and UNIMIB (for water R) (ANOVA, $P < 0.05$; Supplementary Figs. 6E and 6F). No significant differences were found between the other RUs. The DNA concentration obtained from RUs working on soil A and on soil B was 32.83 ± 18.40 ng/µL and 27.12 ± 17.75 ng/µL in average respectively with the highest value obtained from UNIPA and UNIPG while the other RUs showed comparable differences (ANOVA, $P < 0.05$; Supplementary Figs. 6G and 6H). In general, all DNA samples were likely pure with minimal contamination (Table 1).

Interlaboratory variability of liquid fermented foods samples: yoghurt

A total of 1,414,496 reads were used for downstream analysis (mean of 94,299 reads/sample) with a coverage > 99%. Species richness (calculated using *specnumber*) was consistent across the RUs, indicating no variation in species richness between RUs.

The Bland Altman analysis performed on Shannon index showed no difference between RUs (Fig. 3A). Within RUs, ISA and UNIBAS showed similar values, but their CI, which includes negative values, suggest greater variation between biological replicates. ISPA and UNISS have similar mean to UNITO, but their broader CI, especially for UNISS (0.0176 – 0.437), indicate slightly higher variability (Fig. 3A).

UNITO exhibits the lowest mean value (0.210) and the lowest CI (0.000448 – 0.420) between biological replicates, making it the most precise RUs to be used as a reference. The divergence analysis of Bray–Curtis dissimilarity between RUs vs. UNITO showed no statistical differences (Fig. 3B). MIQ values for *Lactobacillus* and *Streptococcus* were > 97% (Fig. 3C) and no significant differences in the relative frequency of each ASV between the RUs were observed (Fig. 3D). *Lactobacillus* displayed a larger CI if compared to *Streptococcus*, but no significant differences were found based on the Wilcoxon test (Fig. 3E, p-value > 0.05).

Interlaboratory variability of liquid fermented foods samples: kombucha

A total of 644,724 reads were used for downstream analysis (mean of 42,981 reads/sample) with a coverage > 99% and no significant variation in species richness between RUs was observed.

The Bland–Altman analysis showed no statistical difference between RUs (Fig. 4A). Within the RUs, ISA shows the lowest mean value (0.0338) with a wide CI (– 0.176 to 0.244), indicating some variability between biological replicates. ISPA follows with a higher value (0.0697) and a broad CI (–0.140 to 0.280). UNIBAS and UNISS have similar value, with CI ranging from –0.0945 to 0.328 and –0.103 to 0.318, respectively. UNITO displays the lower CI (–0.131 to 0.290) between biological replicates and was used as a reference RUs. The divergence analysis between RUs vs. UNITO showed no significant differences (Fig. 4B, p-value > 0.05). MIQ values for ASVs detected were near 100% for all taxa, indicating no differences between RUs (Fig. 4C). No significant differences were observed in term of relative frequency (Fig. 4D); however, *Acetobacter* and *Streptococcus* displayed the highest CI between biological replicates within RUs (Fig. 4E).

Interlaboratory variability of solid fermented foods samples: cheese

A total of 1,168,084 reads were used for downstream analysis (mean of 77,872 reads/sample) with a

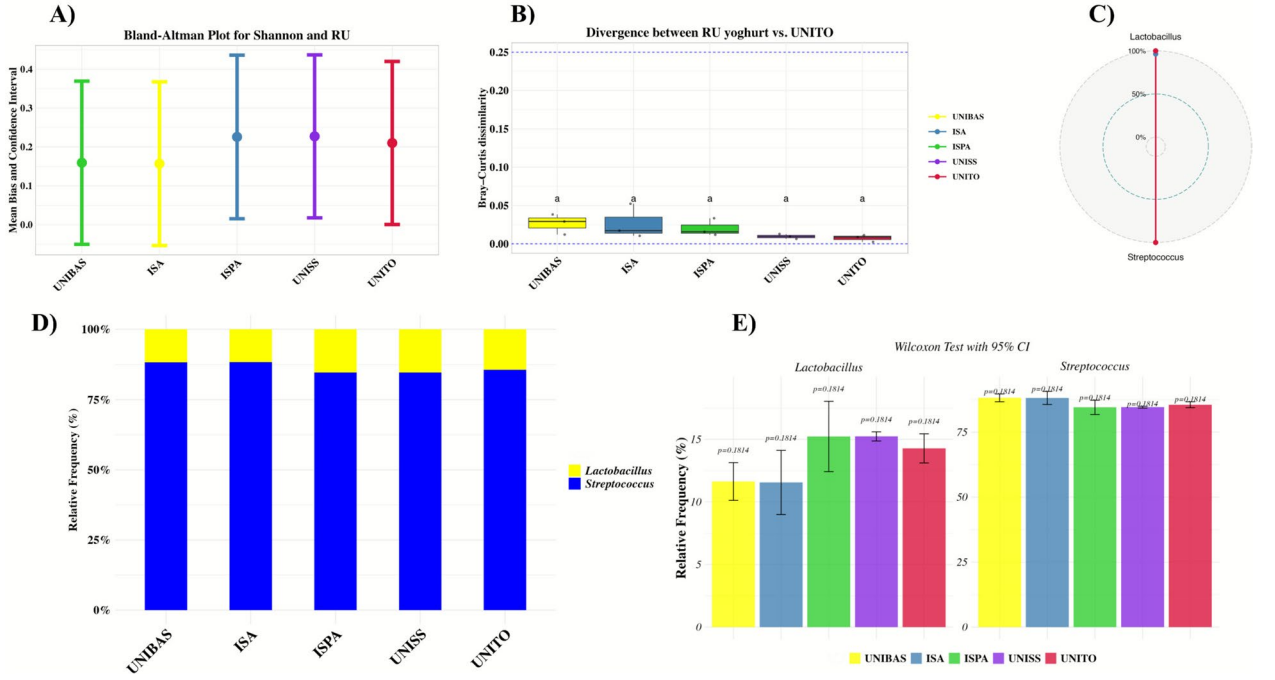

Fig. 3 Plot A shows the Bland–Altman plot comparing the Shannon diversity index obtained from the analysis of yoghurt samples. Each point represents the mean Shannon index, and the lines denote the 95% confidence interval between replicates. Plot B displays the divergence in Bray–Curtis dissimilarity between each RUs and UNITO, which was chosen as the reference RUs. Statistical differences between RUs were assessed using pairwise Wilcoxon tests and are indicated by different lowercase letters. Dashed blue lines represent predefined Bray–Curtis dissimilarity thresholds (upper and lower limits) used for quality assessment. Plot C presents radar charts of the MIQ (Microbial Index Quality) scores, calculated based on deviations from the values obtained by UNITO (reference RUs). Plot D shows stacked bar plots illustrating the mean relative frequency of the most abundant ASVs (Amplicon Sequence Variants) in yoghurt samples (relative frequency > 0.5% in at least three samples) across RUs. Plot E displays the relative frequency of individual microbial taxa identified in yoghurt samples. For each ASV, bars represent relative abundance across RUs, with colored segments corresponding to specific microbial taxa. 95% confidence intervals and statistical differences (assessed using pairwise Wilcoxon tests) are also shown

coverage > 99%, and no significant variation in species richness between RUs was observed.

The Bland–Altman analysis showed that the mean values were very similar between the RUs, ranging from 1.07 to 1.10, indicating no significant differences (Fig. 5A, *p*-value > 0.05). The standard deviation was also consistent within each RUs, with values around 0.416–0.419, suggesting nearly identical value between biological replicates (Fig. 5A, *p*-value > 0.05). However, UNISS showed a lower CI (0.863–1.28), indicating highest concordance between biological replicates. The divergence analysis between RUs vs. UNISS showed no significant differences (Fig. 5B, *p*-value > 0.05). MIQ values for ASVs detected in cheeses were near 100% for all taxa, indicating no bias in the analysis between the RUs (Fig. 5C). No significant differences were observed in terms of relative frequency (Fig. 5D). *Lacticaseibacillus*, *Lentilactobacillus*, and *Propionibacterium* displayed the highest CI within biological replicates across RUs (Fig. 5E).

Interlaboratory variability of solid fermented foods samples: fermented sausages

A total of 796,554 reads were used for downstream analysis (mean of 53,103 reads/sample) with a coverage > 99% and no significant variation in species richness between RUs was observed. The Bland–Altman analysis showed no significant differences between RUs (Fig. 6A, *p*-value > 0.05). Within RUs, ISA has the highest mean bias (0.283), followed by ISPA (0.225, CI from 0.0142 to 0.435). UNIBAS has a mean bias of 0.284, with a CI from 0.0739 to 0.493, indicating lowest variability between biological replicates. UNISS showed the lowest mean bias (0.201) and a CI from − 0.0102 to 0.411, suggesting more consistent results. UNITO has the lowest mean value (0.142), with a lower CI from − 0.0695 to 0.353 indicating the highest reproducibility among biological replicates and was used as a reference RUs. (Fig. 6A, *p*-value > 0.05).

No significant differences in the divergence analysis between RUs vs. UNITO were observed (Fig. 6B). MIQ values for ASVs detected were > 97%, indicating no differences in the analysis between RUs (Fig. 6C). No significant differences between RUs was observed in terms of relative frequency for all the ASV. (Fig. 6D, *p*-value > 0.05). *Secundilactobacillus* displayed the highest confidence intervals between biological replicates within RUs (Fig. 6E).

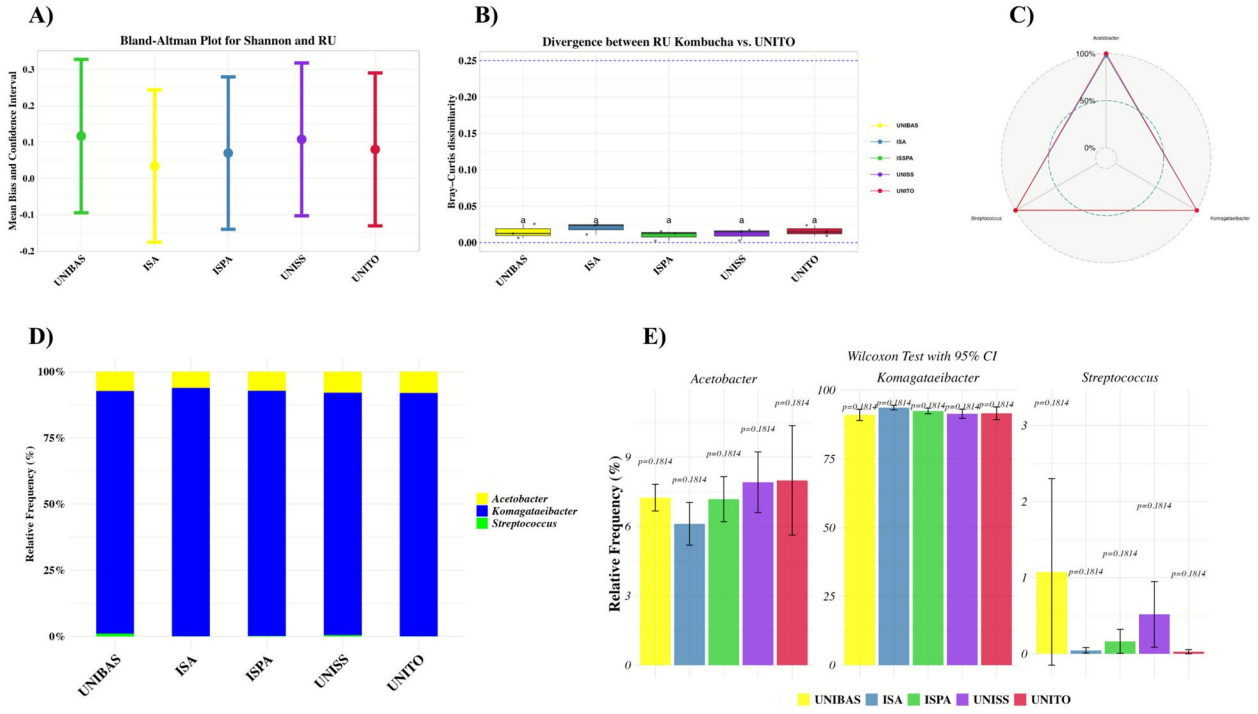

Fig. 4 Plot A shows the Bland–Altman plot comparing the Shannon diversity index obtained from the analysis of kombucha samples. Each point represents the mean Shannon index, and the lines denote the 95% confidence interval between replicates. Plot B displays the divergence in Bray–Curtis dissimilarity between each RUs and UNITO, which was chosen as the reference RUs. Statistical differences between RUs were assessed using pairwise Wilcoxon tests and are indicated by different lowercase letters. Dashed blue lines represent predefined Bray–Curtis dissimilarity thresholds (upper and lower limits) used for quality assessment. Plot C presents radar charts of the MIQ (Microbial Index Quality) scores, calculated based on deviations from the values obtained by UNITO (reference RUs). Plot D shows stacked bar plots illustrating the mean relative frequency of the most abundant ASVs (Amplicon Sequence Variants) in kombucha samples (relative frequency > 0.5% in at least three samples) across RUs. Plot E displays the relative frequency of individual microbial taxa identified in kombucha samples. For each ASV, bars represent relative abundance across RUs, with colored segments corresponding to specific microbial taxa. 95% confidence intervals and statistical differences (assessed using pairwise Wilcoxon tests) are also shown

Interlaboratory variability of water samples

A total of 1,230,726 reads were used for downstream analysis (mean of 51,280 reads/sample) with a coverage > 97% and no significant variation in species richness between RUs was observed. Regarding water L, the Bland–Altman analysis showed that the mean values between RUs were very similar, ranging from 1.46 to 1.47. (Supplementary Fig. 7A, *p*-value > 0.05). The standard deviation within RUs was also comparable, indicating similar variability between biological replicates. The CI was closely aligned, with UNIMIB, UNIPA, and UNIPG having intervals between 1.21 and 1.72, while UNIVR had a slightly wider interval (1.21 to 1.73). UNIPG showed the smallest CI between biological replicates and was used as a reference RUs. Divergence analysis between UNIPG and RUs showed no significant difference. (Supplementary Fig. 7B, *p*-value > 0.05). The MIQ score for all the ASVs was > 99% and no differeneces were observed in the ASVs relative frequency. (Supplementary Fig. 7C, *p*-value > 0.05). For water R, the Bland–Altman analysis showed no differences between RUs. (Supplementary Fig. 8A, *p*-value > 0.05). The CI between the biological replicates within RUs was also quite similar. UNIPG displayed a slightly lower mean bias and the narrowest interval and was used as a reference RUs. Divergence analysis between RUs vs. UNIPG showed no differences (Supplementary Fig. 8B). The MIQ score for all the ASVs was > 99% and no differences were observed in the ASVs relative frequency (Supplementary Fig. 8C).

Interlaboratory variability of soil samples

A total of 1,255,456 reads were used for downstream analysis (mean of 42,373 reads/sample) with a coverage > 94% and no significant variation in species richness between RUs was observed. For soil A, the Bland–Altman analysis showed no significant difference between RUs. The lowest CI was observed for ENEA, suggesting a more precise result between biological replicates and was used as a reference RUs (Supplementary Fig. 9A, p-value > 0.05). Divergence analysis between RUs vs. ENEA showed no differences in terms of dissimilarity (Supplementary Fig. 9B, *p*-value > 0.05). The MIQ score for all the ASVs was > 99% with no significant differences in the ASVs relative frequency (Supplementary Fig. 9C). For soil B, no significant differences were observed based on Bland–Altman analysis (Supplementary Fig. 10A).

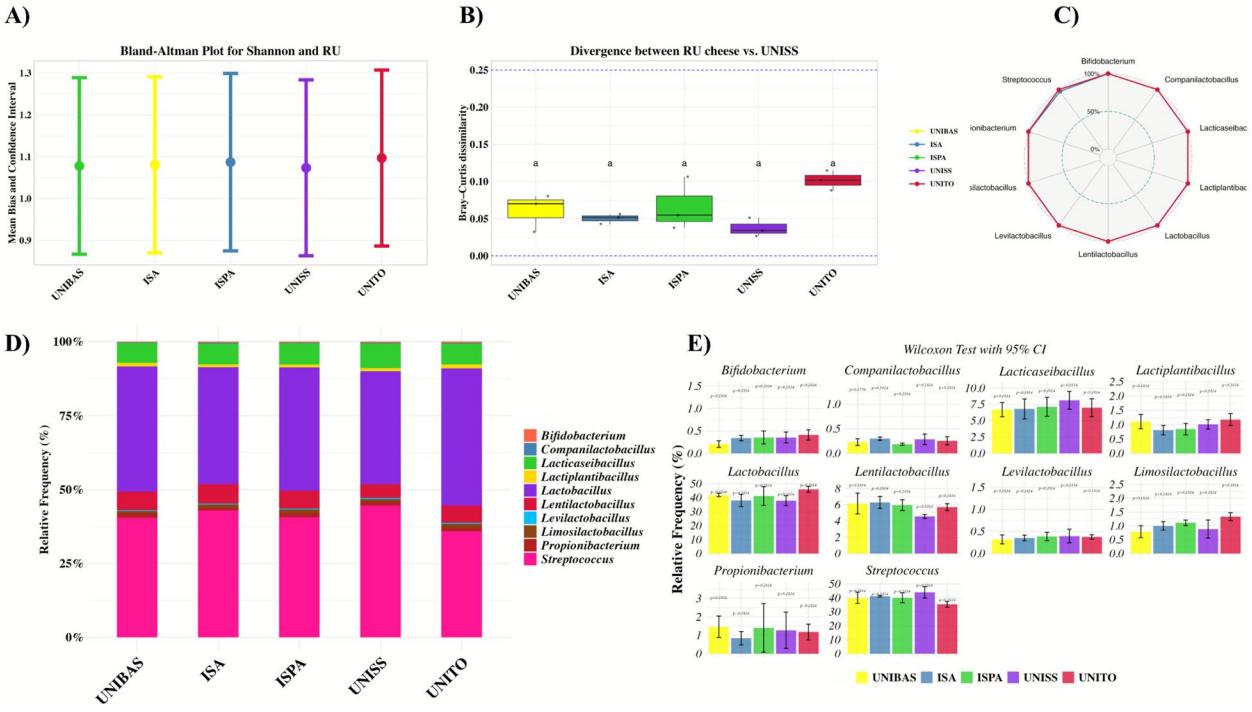

Fig. 5 Plot A shows the Bland–Altman plot comparing the Shannon diversity index obtained from the analysis of cheese samples. Each point represents the mean Shannon index, and the lines denote the 95% confidence interval between replicates. Plot B displays the divergence in Bray–Curtis dissimilarity between each RUs and UNISS, which was chosen as the reference RUs. Statistical differences between RUs were assessed using pairwise Wilcoxon tests and are indicated by different lowercase letters. Dashed blue lines represent predefined Bray–Curtis dissimilarity thresholds (upper and lower limits) used for quality assessment. Plot C presents radar charts of the MIQ (Microbial Index Quality) scores, calculated based on deviations from the values obtained by UNISS (reference RUs). Plot D shows stacked bar plots illustrating the mean relative frequency of the most abundant ASVs (Amplicon Sequence Variants) in cheese samples (relative frequency > 0.5% in at least three samples) across RUs. Plot E displays the relative frequency of individual microbial taxa identified in cheese samples. For each ASV, bars represent relative abundance across RUs, with colored segments corresponding to specific microbial taxa. 95% confidence intervals and statistical differences (assessed using pairwise Wilcoxon tests) are also shown

ENEA displayed the lowest CI. Divergence analysis between RUs vs. ENEA showed no differences in terms of dissimilarity (Supplementary Fig. 10B, p-value > 0.05). The MIQ score for all the ASVs was > 99% with no differences in the ASVs relative frequency (Supplementary Fig. 10C).

Discussion

Microbiome research has provided valuable insights into various biological systems, and the huge amount of available microbiome datasets holds significant potential for data reuse (e.g. for meta-analysis or if a novel bioinformatic tool wants to be used) [26]. However FAIR principles [8] along with standard operating procedures (SOPs) are necessary to ensure reproducibility [1]. Several SOPs have become recently available in the literature with different objectives, mainly to develop standardized sampling procedures for low-biomass food processing environments [3], mass-transit systems [11], environmental [34], water [5], air [19], soil [13], plant [14] and stool [28]. Others considered different DNA extraction methods (Lim et al., 2018,sample pre-treatment, and the choice of marker genes [18]. However, to our best knowledge none of them were validated testing the variability between laboratories. Only a collaborative effort was done in human microbiome study, including 21 labs from 11 countries where an interlaboratory comparison of DNA extraction methods was performed [10]. In our study we evaluated the performance of different RUs that developed SOPs for sample preparation and DNA extraction and purification. The primary objective of this study was to verify the consistency of sample preparation between RUs by sending uniform and homogenized samples, avoiding in this way bias in sampling and or preservation steps. Each RUs performed three DNA extractions, and the nucleic acid was sent to a single sequencing center to minimize sources of variation during the downstream workflow. The same approach was recently adopted to evaluate SOPs for human gut microbiome analysis [10]. The second objective was to evaluate the reliability and reproducibility between biological replicates within and between RUs. We decided to use DNA as a target molecule and amplicon sequencing technique to validate the SOPs based on the results of the survey within the Project SUS-MIRRI.IT. For the selected sample categories (liquid fermented foods, solid fermented

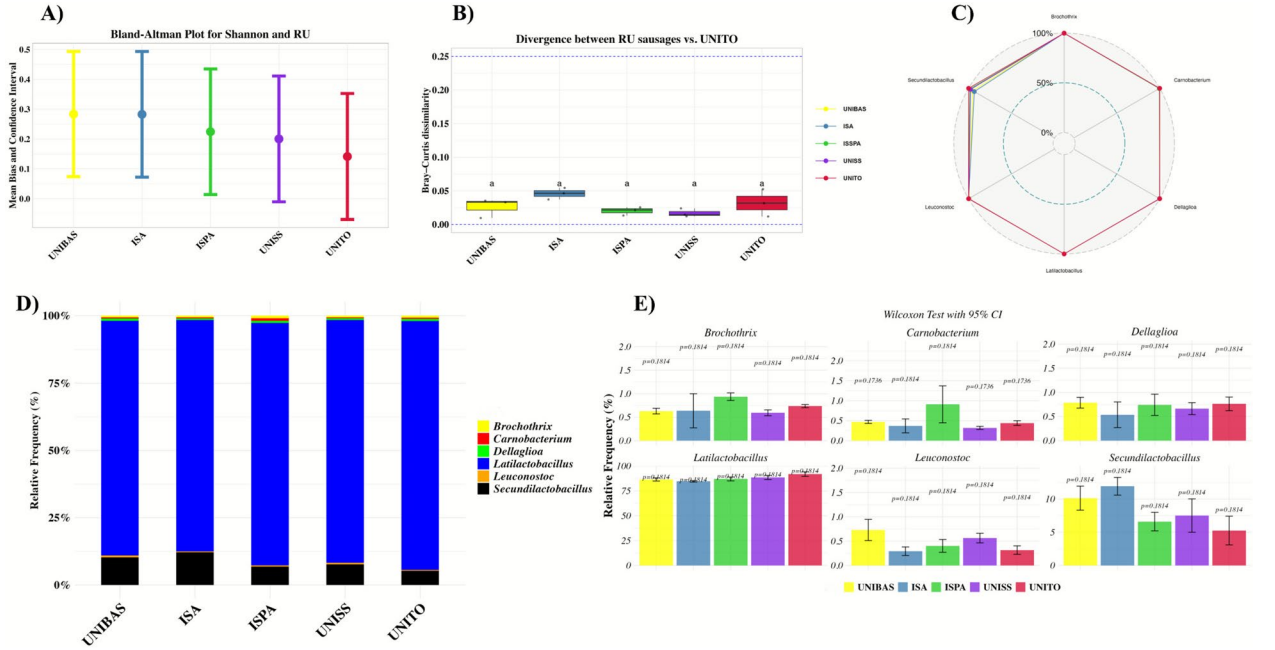

Fig. 6 Plot A shows the Bland–Altman plot comparing the Shannon diversity index obtained from the analysis of sausages samples. Each point represents the mean Shannon index, and the lines denote the 95% confidence interval between replicates. Plot B displays the divergence in Bray–Curtis dissimilarity between each RUs and UNITO, which was chosen as the reference RUs. Statistical differences between RUs were assessed using pairwise Wilcoxon tests and are indicated by different lowercase letters. Dashed blue lines represent predefined Bray–Curtis dissimilarity thresholds (upper and lower limits) used for quality assessment. Plot C presents radar charts of the MIQ (Microbial Index Quality) scores, calculated based on deviations from the values obtained by UNITO (reference RUs). Plot D shows stacked bar plots illustrating the mean relative frequency of the most abundant ASVs (Amplicon Sequence Variants) in sausages samples (relative frequency > 0.5% in at least three samples) across RUs. Plot E displays the relative frequency of individual microbial taxa identified in sausages samples. For each ASV, bars represent relative abundance across RUs, with colored segments corresponding to specific microbial taxa. 95% confidence intervals and statistical differences (assessed using pairwise Wilcoxon tests) are also shown

foods, waters and soils) we observed a high degree of intra-center reproducibility. One of the main aspects to pay attention to in microbiome studies is the sample lysis (enzymatical, chemical, thermal or mechanical or their combinations) [18, 21, 33] and homogenization step (frequency and time) that can mainly affect nucleic acid recovery and differentially affect the recovery of Gram-positive bacteria [3], Lim et al., 2018). Thereby, the lysis step is considered a crucial one for the proper standardization procedure, as already assessed in stool samples [33]. We then observed the highest variation in frequency (even if not statistically different) among the detection of Gram-positive in fermented foods. Each RUs was compliant regarding this key step, however small differences in DNA concentration were observed. Those differences can be attributed to the microbial distribution especially for solid samples. Even if the samples sent to each RUs were uniform and homogenized, an equal spatial distribution of microbes could not be ensured. This situation was less evident in liquid samples (eg. kombucha, water) where a few differences were observed in DNA recovery. The inclusion of the mock standard in our trial allowed to take into account all the biases arising from this procedure including: nucleic acid extraction methods [2, 24, 41–43], extraction efficiency between gram-positive and gram-negative [42], PCR amplification [31, 36], choice of the marker gene region [17, 39], library preparation [28, 37], sequencing platform [7, 22] and bioinformatic step [30, 38]. Those biases, already well discussed by several authors, were consistent across the RUs. The analysis suggested that the microbial abundance data obtained by the three groups of RUs was unbiased, reliable and reproducible. We observed few differences in each ASV relative frequency but the deviations within and between RUs were in line with the tolerance limits. Our study offers valuable insights into the reproducibility of microbiome workflows across different research groups RUs focusing on several biological models. Interesting to highlight the strong alignment of the results obtained in this study with those targeting only one type of biological samples (e.g. specimen) [10], Henderson et al., 2013; [20, 29, 33, 35].

Limitations of the study

However, certain limitations should be acknowledged. First, although the samples were collected by one RUs and homogenized before distribution, the possibility of microbial heterogeneity, especially in solid samples, may have influenced DNA yield and microbial composition, leading to variability which is not caused by the method

utilized. Second, the study focused exclusively on DNA as a target molecule without taking into the account the possibility that the performance of our SOPs cannot be directly extended to RNA studies. Third, we used amplicon sequencing targeting the 16S rRNA gene without taking into account variability associated with other marker genes (like ITS or 18S rRNA gene for yeast and filamentous fungi) that may affect the performance of our SOPs.

The data analysis was performed by a single sequencing platform and a bioinformatic pipeline, potentially limiting the generalizability of the findings to other technologies or software tools. In addition, the current validation was restricted to DNA-based 16S rRNA amplicon sequencing workflows using the Greengenes2 as reference database. Therefore, the applicability of these SOPs to other genes (e.g., ITS or 18S rRNA), shotgun metagenomics, or metatranscriptomic studies has not yet been established. However, it was already assessed that different next-generation sequencing library preparations showed significant differences in taxonomy profiling [20], thereby comparison between them can introduce biases which are not related to the method used for the DNA extraction. Finally, it should be noted that amplicon sequencing based on relative frequency does not allow accurate assessment of microbial load. Absolute quantification would require alternative approaches, such as qPCR or digital PCR targeting total 16S or specific taxa. Nevertheless, the primary aim of this study was to standardize DNA extraction procedures, rather than to quantify absolute microbial biomass. Future work integrating absolute quantification methods could complement and extend the findings reported here. Moreover, while user-friendly bioinformatics pipelines such as EasyAmplicon [44] and platforms like Wekemo Bioincloud [16] facilitate reproducible analysis of high-throughput microbiome datasets, the reliability of these tools ultimately depends on consistent upstream sample processing and DNA extraction. Similarly, curated databases such as FoodMicroDB [27] emphasize the importance of standardized protocols to enable cross-study comparability, particularly for food microbiome research. Addressing these limitations in future studies will be crucial to further refine SOPs and strengthen reproducibility in microbiome research across broader contexts and technologies including shotgun metagenomics, metatranscriptomics, or metabolomics.

Conclusion and future perspectives

This study underlines the critical role of standardized protocols in the field of microbiome research taking into the account the procedures for DNA isolation and purifications. Through a large-scale inter-laboratory trial under the SUS-MIRRI.IT project, we validated 8 SOPs. To our best knowledge none had developed SOPs for microbiome analysis in fermented foods samples and none have performed a validation trial on foods, soils and waters. The consistent results from mock communities used as a standard to evaluate the operator's variability support the robustness of the adopted SOPs. The results demonstrated a high degree of reproducibility both within and between RUs using complex biological matrices. The adoption of common procedures across research infrastructures will enhance reproducibility, support tool benchmarking, and foster data reuse.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40793-025-00833-z.

Supplementary file 1.

Acknowledgements

The research was was granted by the European Commission – NextGenerationEU, Project SUS-MIRRI.IT "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy", code n. IR0000005. Furthermore, we acknowledge the contribution and support from the Italian national Node (MIRRi-IT) of the European Research Infrastructure MIRRI-ERIC

Author contributions

Conceptualization: LC, IF; data curation: IF; formal analysis: IF, MFa, MAn, RAl, FAl, ABe, EBr, RCo, LDe, TDi, AFr, RGa, GGa, MGu, RGu, MGi, SLa, GMu, PQu, ARe, FSb, CSa, DSp, VTa, AVi, GZa, BT, MG, VC, TZ, MPA; funding acquisition: GCV; methodology: IF, BT, MG, VC, TZ, MPA, GCV, LC; resources: GCV, LC; software: IF; supervision: LC, IF, BT, MG, GCV; visualization: IF; writing—original draft: IF; writing—review & editing: IF, BT, MG, VC, TZ, MPA, GCV, LC

Funding

The research was was granted by the European Commission – NextGenerationEU, Project SUS-MIRRI.IT "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy", code n. IR0000005. Furthermore, we acknowledge the contribution and support from the Italian national Node (MIRRi-IT) of the European Research Infrastructure MIRRI-ERIC.

Data availability

Sequences data were deposited on NCBI database under the bioproject PRJNA1266140.

Declarations

Competing interests

The authors declare no competing interests.

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Received: 22 August 2025 / Accepted: 29 November 2025
Published online: 12 December 2025

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