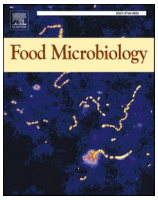
Food Microbiology 118 (2024) 104418

Contents lists available at ScienceDirect

Food Microbiology

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



Lactic acid bacteria naturally associated with ready-to-eat rocket salad can survive the human gastrointestinal transit

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A B S T R A C T

It was theorized that modernization and the decline in harmless microbial populations associated with food have altered the gut microbiota, impacting host metabolism and immunity. Western dietary patterns, characterized by processed foods and preservation methods, may significantly reduce the microbial population associated with food. To mitigate the consequences of bacterial deprivation, the integration of these diets with fermented foods is commonly proposed. Nonetheless, non-fermented food consumed raw may also be an important source of viable microbial cells for the human microbiome. This study investigates whether salad-associated LAB can survive the gastrointestinal transit (GIT) and contribute to the gut microbiota. LAB strains were quantified and isolated from rocket salad (*Eruca vesicaria* subsp. *sativa*), and their survival through GIT was assessed via intervention trials in healthy adults and in vitro. Moreover, bacterial communities in fecal samples were analyzed after three days of rocket salad consumption. Washing with a sodium hypochlorite solution drastically reduced total bacterial load and eliminated viable LAB. The quantity of LAB introduced through salads did not significantly alter the gut microbiota composition. Rocket salads harbored *Weissella* and *Leuconostoc* species. A significant increase in *Weissella* spp. but not in *Leuconostoc* spp. was observed after the consumption of rocket salad. Simulated GIT experiments suggested that the food matrix and the initial number of ingested viable bacteria may have been important in determining survival. These findings propose that plant products could serve as sources of live LAB for the human gut. Further research with diverse vegetables and longer interventions is needed, encouraging studies on raw, non-fermented foods and their impact on the human intestinal microbiome.

1. Introduction

The human gut microbiota plays a crucial role in maintaining human health, and its composition can be influenced by various factors such as lifestyle, diet, and medication. Recent studies suggest that with industrialization, we also experienced a drastically reduced harmless microbial population associated with food. This reduction also led to an altered gut microbiota community structure, resulting in harmful consequences to the host's metabolism and immune system. In particular, western-type dietary patterns, characterized by the widespread consumption of processed food and the extensive use of various methods of preservation and sanitization are relevant in this context (Scudellari, 2017; Sonnenburg and Sonnenburg, 2019). The integration of Western-type dietary patterns with fermented foods has been suggested as a potentially effective strategy to counteract the negative consequences of bacterial deprivation in the environment and in food. Fermented foods are a natural source of beneficial microorganisms, mainly lactic acid bacteria (LAB) (Chen, 2021; Mantegazza et al., 2023a; Rosa et al., 2017), which are the most studied and recognized microorganisms for their beneficial health properties. For example, some of them can produce vitamins, antagonize harmful microbes, and improve intestinal immune homeostasis (Cristofori et al., 2021; Hati et al., 2019; Li et al., 2017; Taverniti and Guglielmetti, 2012). Nonetheless, non-fermented foods that are consumed raw, although they may contain fewer microbial cells than fermented foods, can still provide LAB to the human gastrointestinal tract, as well as many other microorganisms with a broad taxonomic representation (Mantegazza et al., 2023b).

Given the importance of the gut microbiota in human health and the health-promoting potential of LAB, it is crucial to understand how food-associated microbes, notably LAB, can survive the human gastrointestinal transit (GIT) and potentially contribute to the community structure of the gut microbiota. While the ability of some strains of LAB to survive the GIT is already well known, most existing studies have focused on probiotic products (Arioli et al., 2018; Taverniti et al., 2019). These products provide LAB in large quantities (generally higher than one billion up to 100 billion) and may contain compounds that create a

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https://doi.org/10.1016/j.fm.2023.104418
Received 11 August 2023; Received in revised form 3 October 2023; Accepted 28 October 2023
Available online 31 October 2023

protective environment for the bacteria during GIT (Han et al., 2021). However, to our knowledge, very few studies have evaluated whether microbes naturally associated with food can survive the GIT and contribute to the gut microbiota (Pasolli et al., 2020).

The World Health Organization (WHO) advised a daily intake of fruit and vegetable products exceeding 400 g per capita. However, many people cannot meet this recommendation due to limited availability, cost, and convenience. The sector of ready-to-eat products can facilitate the daily intake of fruits and vegetables, providing fresh and minimally processed products. Among these products, rocket salad (*Eruca vesicaria* subsp. *sativa*), which is a vegetable rich in biologically active compounds (e.g., ascorbic acid, carotenoids, fiber, polyphenols, and glucosinolates), is becoming increasingly economically interesting for its use in ready-to-use salads.

With this in mind, we recently conducted a study that demonstrated that ready-to-eat rocket salads contain a significant number of viable LAB cells (Mantegazza et al., 2023b). Contextually, the present study aimed to investigate whether salad-associated LAB can survive GIT and contribute to the gut microbiota. To this end, we isolated and taxonomically identified the LAB strains associated with the rocket salad, and we tested their specific ability to survive GIT through in vitro tests and human intervention trials. Finally, we performed taxonomic profiling of the bacterial communities in the fecal samples collected before and after rocket salad consumption.

2. Material and methods

2.1. Products utilized in the study

This research was conducted using commercially available ready-to-eat rocket salad packaged in 100 g plastic bags, purchased in a local supermarket after a period of 10 days. The bags were sourced from only two production batches (rocket salads R1 and R2) from a single distributor. Rocket salad was cultivated and processed in northern Italy. Half of the salad was used as it was, while the other half was washed with a 0.02% sodium hypochlorite solution for 5 min at room temperature and then thoroughly rinsed with tap water. Subsequently, the rocket salad was dried using a household salad spinner and repackaged in 100 g portions in plastic bags.

2.2. Analysis of bacteria associated with rocket salads

The viable enumeration of total mesophilic bacteria and LAB was carried out following the standard protocols ISO 4833-1:2013 and ISO 15214:1998. Briefly, 10 g of salad was homogenized with 90 g of Maximum Recovery Diluent (MRD; Scharlab, Milan, Italy) using a Stomacher 3500 peristaltic homogenizer (Seward, West Sussex, United Kingdom) for 2 min. The resulting suspension was then subjected to decimal serial dilutions. Subsequently, after decimal serial dilutions, 1 ml of the suspensions was inoculated by pour plating onto plate count agar (PCA) for total bacterial counts (Scharlab, Riozzo di Cerro al Lambro, Milan, Italy), while de Man-Rogosa-Sharpe (MRS) agar (Difco, Detroit, MI, USA) at pH 5.7 was used for LAB counts. The plates were incubated at 30 °C for 72 h aerobically.

To determine the taxonomy of the LAB associated with the salads used in the intervention, twenty colonies were isolated from each of the two batches of rocket salads (R1 and R2), collected from the highest dilutions on MRS agar plates. DNA was extracted from each colony grown in MRS broth using the DNeasy UltraClean Microbial kit (Qiagen). Subsequently, the extracted DNA served as a template in an end-point PCR reaction with the pan-bacterial primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′) as in Guglielmetti et al. (2010). The obtained amplicons were sequenced, and the resulting sequences were used for species-level taxonomic identification through a search in the rRNA/ITS database using BLASTN. Furthermore, the DNA isolated from the twenty colonies was employed in molecular fingerprinting experiments using RAPD-PCR with primers OPI02mod (5′- GCTCGGAGGAGAGG-3′) and BOXA1R (5′-CTACGGCAAGGCGACGCTGACG -3′) as previously described (Guglielmetti et al., 2010; Koirala et al., 2014).

2.3. Human intervention trials

Two identical intervention trials were conducted, administering commercial ready-to-eat rocket salads as described below. The study scheme is illustrated in Fig. 1.

2.3.1. Study population

Healthy (non-diseased) adult volunteers of both sexes, aged 18–65 years, with regular bowel movements (between 1 and 3 evacuations per day with a consistency equal to 3 or 4 based on the Bristol Stool Scale). Volunteers provided signed informed consent of their participation in the study. Characteristics of the volunteers participating in the intervention trials are reported in Supplementary Table S1.

2.3.2. Exclusion criteria

(i) Antibiotic consumption in the month preceding the start of the trial; (ii) consumption of antacids or prokinetic gastrointestinal drugs; (iii) chronic inflammatory bowel diseases; (iv) intestinal diseases of infectious origin; (v) episodes of viral or bacterial enteritis in the 2 months prior to the study; (vi) episodes of gastric or duodenal ulcers in the previous 5 years; (vii) pregnancy or breast-feeding; (viii) recent history of alcohol abuse or suspected drug use; (ix) any severe disease that may interfere with treatment (x) diagnosis of irritable bowel syndrome (IBS) in active phase; (xi) Inadequate reliability or the presence of conditions that may determine a patient's non-compliance/adherence to the protocol; and (xii) allergy or intolerance to the product in study.

2.3.3. Study protocol

During the initial visit, volunteers provided signed informed consent and received training on the entire procedure. The study consisted of a 1-week run-in phase, where participants adhered to their regular while avoiding probiotic, prebiotic, and synbiotic foods and supplements, Brassicaceae vegetables, Brassicaceae-containing food products, and fermented foods (e.g., kimchi, pickles, and sauerkraut). Following this period, volunteers were instructed to consume 100 g of rocket salad daily for 3 days. The salad was consumed without the addition of salt, vinegar, or garlic. Following the 3-day consumption period, the volunteers underwent a 1-week follow-up, which was identical to the run-in week (Fig. 1).

2.3.4. Sample collection

At the start of the study, volunteers were trained to collect and deliver fecal samples as follows: each stool specimen (minimum 2 g) was collected in sterile containers, stored at +4 °C, and delivered to the laboratory within 24 h.

Ethical statement

The study protocol was approved by the Research Ethics Committee of the Università degli Studi di Milano (opinion no. 20/22, February 25, 2022). Written informed consent was obtained from all subjects before recruitment.

2.4. Quantification of LAB, Leuconostoc spp. and Weissella spp. in fecal samples

Upon delivery, fecal samples were immediately subjected to viable counts. For this purpose, 1 g of the sample was diluted in MRD, homogenized in a sterile Stomacher bag, plated on MRS at pH 5.7, and incubated anaerobically with the use of Anaerocult A (Merck) at 30 °C for 72 h. In addition, we enumerated viable *Leuconostoc* spp. and *Weissella* spp. in fecal samples as follows. DNA was extracted from all

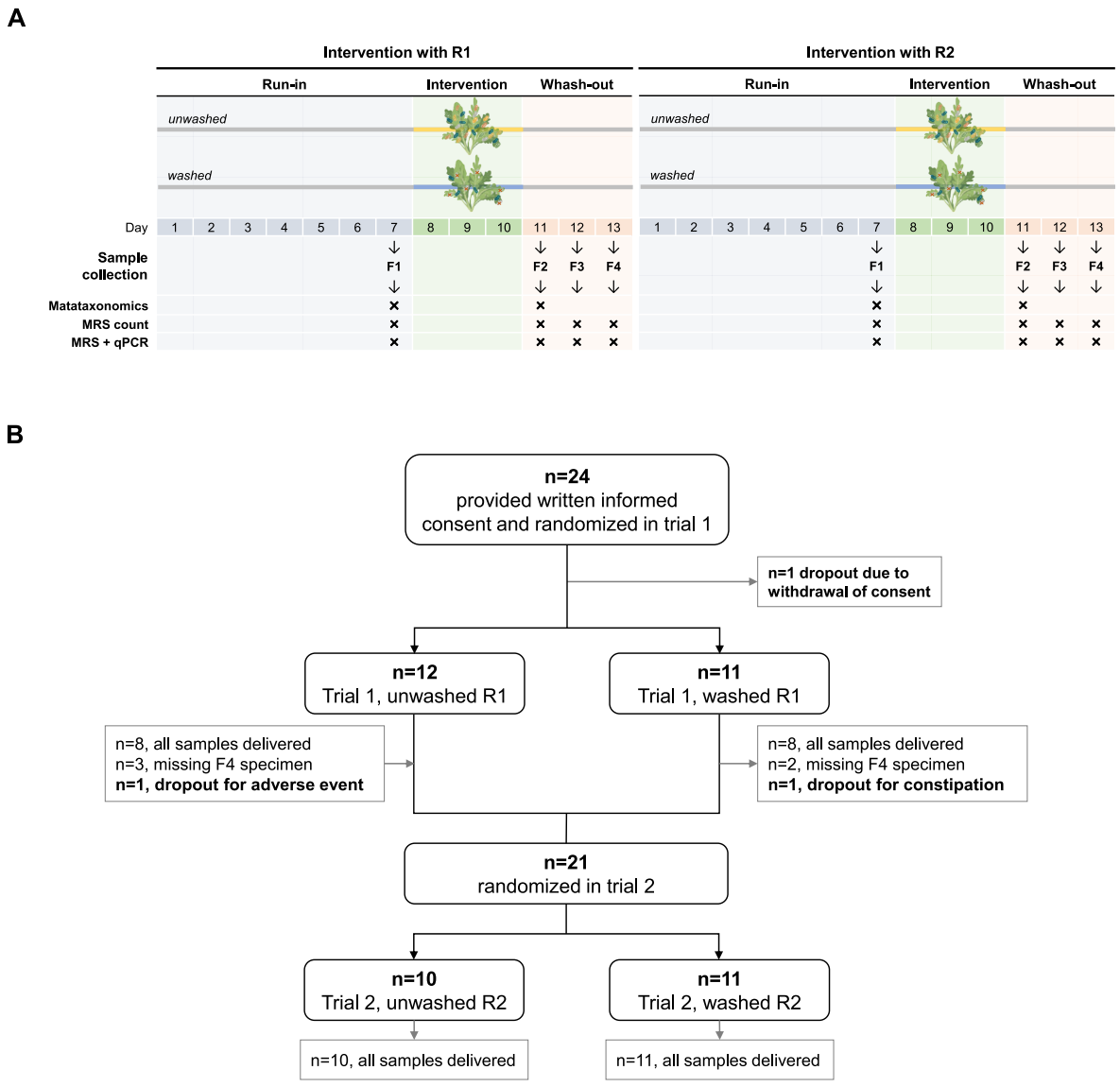

Fig. 1. Study scheme (panel A) and flow chart describing patient numbers (B) for the two intervention trials with commercial ready-to-eat rocket salads R1 and R2. F1 to F4, fecal sample collections.

colonies grown on MRS agar using the QIAsymphony PowerFecal Pro Kit (Qiagen, Hilden, Germany), according to the protocol illustrated in Supplementary Fig. S1. Specifically, after incubation, all the colonies from each dilution plate were collected separately by adding the CD1 solution of the kit directly to the surface of the agar plates. Then, colonies were detached from agar using a sterile 10 μl loop, and the resulting suspension was recovered using a pipette. Extraction protocol was applied to the resulting colony biomass according to the manufacturer's instructions. Subsequently, real-time quantitative PCR (qPCR) was adopted for the identification of *Weissella* and *Leuconostoc* in the DNA extracted from the colony biomass, using the following primers sets. Primers rplB-F (5′-RTA CAA GCC AAC CTC TAA CGG-3′) and rplB-R (5′-TGT GGR CGT TGA CCC TTC C-3′) targeting the 50S ribosomal protein L2 (*rplB*) were used for *Weissella* spp. Primers tuf-F (5′-TGG ACA CGT CGA TCA YGG AA-3′), and tuf-R (5′-CCA ACT TGA CGT GCC AAC AA-3′) targeting the elongation factor Tu (*tuf*) genes were used for *Leuconostoc* spp. qPCR reactions were carried out in a final volume of 15 μl containing 7.5 μl of EvaGreen® Supermix (Bio-Rad Laboratories, Segrate, Italy) and 0.5 μM of each primer. 10 ng of template DNA was used in each reaction. Thermal conditions were as follows: initial hold at 98 °C for 30 s, followed by 40 cycles of 96 °C for 2 s and 60 °C for 5 s. The fluorescent products were detected at the last step of each cycle. After amplification, a melting curve analysis was made to distinguish the targeted PCR product from the nontargeted one. The highest dilution giving a positive signal in qPCR and the obtained Cq value were used to calculate the "estimated minimum CFU number" (eCFU) for each investigated bacterium.

2.5. Metataxonomic analysis of fecal samples

After the viable count analysis, fecal samples were stored at −80 °C until DNA extraction. To this purpose, feces were thawed at +4 °C and vigorously mixed for 2–3 min with a sterile spatula. Subsequently, 150 mg of feces were weighed and processed using the QIAsymphony PowerFecal Pro Kit (Qiagen), following the manufacturer's instructions. The extracted DNA was quantified through the Qubit Broad Range kit (Thermo Fisher Scientific, Waltham, MA, USA) and used for metataxonomic analysis through 16S rRNA gene profiling. The NovaSeq 6000 platform with 2 × 250bp sequencing (NovaSeq 6000 SP Reagent Kit, 500 cycles) was used to sequence the 16S rRNA gene amplicons, which encompassed the V3 and V4 variable regions. These amplicons were obtained using primers 341F (5′-CCT ACG GGN GGC WGC AG-3′) and

805R (5′-GAC TAC HVG GGT ATC TAA TCC-3′) (LC Sciences, Houston, TX). The obtained sequencing reads were processed using the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) 2 version 2022.2 through the Divisive Amplicon Denoising Algorithm (DADA2 (Callahan et al., 2016);) adopting the Silva Database version 138 for taxonomic assignment to amplicon sequence variants (ASVs). To minimize technological bias, the 16S rRNA gene profiling analysis has been carried out simultaneously for all fecal samples considered in this study. Metataxonomic raw sequencing data are available as FASTQ files in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under the accession code PRJEB66456.

2.6. In vitro assessment of bacterial survival during gastrointestinal transit

The ability of *Weissella* and *Leuconostoc* isolates to survive the gastrointestinal transit (GIT) was tested in vitro using the "INFOGEST static in vitro simulation of gastrointestinal food digestion" (INFOGEST 2.0) protocol (Brodkorb et al., 2019), with slight modifications. Briefly, 100 g of rocket salad were washed with 0.02% sodium hypochlorite solution as described above. Subsequently, 5 g of washed rocket salad were homogenized with 5 ml of Simulated Salivary Fluid (SSF) without α-amylase, and 5×10^9 active fluorescent units (AFUs, as determined via flow cytometry with SYTO™ 24 labeling) of each tested strain. For the gastric phase, we used 28 U/ml of rabbit gastric lipase (Lipolytech, Marseille, France) and 940 U/ml of pepsin. The intestinal phase was performed as described in (Brodkorb et al., 2019). At the end of the intestinal phase, the final volume was adjusted to 50 ml and diluted in MRD. Serial dilutions in MRD were inoculated by pour plating onto MRS agar at pH 5.7 with the addition of 1.4 g/l of sorbic acid to inhibit mold and yeast growth. Each strain was tested in three independent experiments conducted in duplicate.

2.7. Statistics

Statistical calculations were performed using the R programming language (version 3.4.2). For paired/unpaired comparisons, we utilized either the paired/unpaired Student's t-test or the Mann-Whitney *U* test, and the Wilcoxon signed-rank test based on the assessment of normal distribution through the Shapiro-Francia test. To identify significantly different taxa, the Wilcoxon-Mann-Whitney test was carried out on read abundances that underwent centered log ratio (CLR) transformation.

3. Results

3.1. Microbiological characterization of rocket salads

The bacterial viable count on PCA plates revealed a similar bacterial cell load between rocket salads R1 and R2 (6.1 and 6.5 log CFU/g, respectively). Conversely, viable count on MRS agar plates evidenced a higher amount of LAB in R1 (4.1 log CFU/g) compared to R2 (2.3 log CFU/g) (Table 1). The washing with sodium hypochlorite solution completely removed viable LAB (i.e., less than 10 CFU/g), whereas the mesophilic bacterial count was reduced by 1.9 log in R1 (4.3 log CFU/g, about 99% reduction) and 1.3 log in R2 (5.2 log CFU/g, about 95% reduction) (Table 1).

Table 1
Viable counts of bacteria associated with rocket salads R1 and R2. Data are reported as log CFU/g of fresh salad. Analyses have been performed on the salads before and after washing with a 0.02% sodium hypochlorite solution. PCA, plate count agar, used for the counts of mesophilic bacteria; MRS, de Man Rogosa Sharpe agar medium at pH 5.7, used for the counts of lactic acid bacteria. n.d., not detected (*i.e.*, <1.0 log CFU/g).

Culture medium	R1		R2	
	unwashed	washed	unwashed	washed
PCA	6.1	4.3	6.5	5.2
MRS	4.1	n.d.	2.3	n.d.

Subsequently, we isolated 20 colonies from MRS plates for R1 and 20 for R2. Taxonomic identification using 16S rRNA gene sequences revealed that all colonies from R1 belonged to the genus *Weissella*, with 15 isolates of *W. cibaria*, 4 isolates of *W. confusa*, and 1 isolate of *W. hellenica*. On the other hand, all 20 colonies isolated from rocket salad R2 were identified as belonging to the species *Leuconostoc lactis*. In addition, molecular fingerprinting analysis revealed two distinct phenotypes within the species *W. cibaria* (n = 9 and n = 6) and within the species *L. lactis* (n = 19 and n = 1) (Supplementary Fig. S2).

3.2. Intervention trials with rocket salads

We recruited twenty-four healthy adult volunteers (mean age 27.9 ± 5.6; 46% females) to participate to two distinct intervention trials with rocket salads R1 and R2, according to the scheme in Fig. 1A. One volunteer dropped out due to withdrawal of consent before the first trial, and two volunteers dropped out after trial 1 due to adverse event (nausea) and due to constipation (no evacuation during the study) (Fig. 1B). All the other volunteers did not report adverse events and the food product was well tolerated. The remaining 21 volunteers concluded the study, 16 of whom delivered all fecal samples according to the protocol (i.e., 8 specimens). The other 5 volunteers delivered 7 fecal samples due to the absence of an evacuation in a day during the trial (Fig. 1B).

3.3. Impact of rocket salads on fecal bacterial communities

Metataxonomics based on 16S rRNA gene profiling was employed to investigate the bacterial community structure of fecal samples collected the day before (sample collection F1) and the day after (F2) the three-day rocket administration phase. The analysis of α-diversity did not indicate any significant changes in either the richness or evenness of the fecal bacterial communities following the short-term consumption of rocket (Supplementary Fig. S3). The analysis of β-diversity, utilizing both weighted and unweighted UniFrac algorithms, showed a shift in the bacterial community structure for each subject across various time points (Supplementary Fig. S3). However, these changes were generally more minor than the variability observed between different subjects (Supplementary Fig. S3) and cannot be directly attributed to either the consumption or washing of rocket salads.

Subsequently, we performed a statistical analysis of the metataxonomic data to determine whether the consumption of rocket salad and/or the washing of rocket salad could have influenced the abundance of individual bacterial taxa. Specifically, three levels of analysis were conducted: (i) a comparison of all fecal samples collected before rocket salad consumption and all fecal samples collected afterward, irrespective of washing and the type of rocket salad; (ii) a comparison of fecal samples collected before and after rocket salad consumption separately for each of the two interventions (with R1 and R2), independent of washing; (iii) a comparison of fecal samples collected before and after rocket salad consumption, differentiating between individuals who consumed unwashed rocket salad and those who consumed washed rocket salad, and conducted separately for each of the two interventions with R1 or R2 rocket salads.

The initial analysis, designed to determine the impact of rocket salad consumption on the fecal microbiota regardless of the microorganisms hosted by salads, revealed modifications in 13 bacterial taxa, with 10 of them belonging to the class Clostridia (Fig. 2A). Specifically, the bacterial groups that exhibited an increase included *Acinetobacter schindleri*, which is known as a plant endophyte (Yuan et al., 2022), an unidentified species from the family Christensenellaceae, as well as the genera *Flavonifractor* and *Methanobrevibacter*.

When the analysis was performed separately for the data obtained

A

F1	F2	Taxonomy (n=42)	*P*	F1	F2
		d_Archaea;p_Euryarchaeota;c_Methanobacteria;o_Methanobacteriales;f_Methanobacteriaceae;g_Methanobrevibacter	0.039	-1.31	-1.15
		p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae	0.013	3.60	3.39
		*p_Firmicutes;c_Clostridia;o_Christensenellales;f_Christensenellaceae;g_Christensenellaceae_* R-7 group; Unknown species	0.008	-1.46	-1.33
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_:Eubacterium ventriosum group	0.033	2.13	2.04
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Coprococcus	0.024	3.46	3.25
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Coprococcus;s_* Unknown species	0.009	2.61	2.13
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia	0.007	2.97	2.33
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia;s_* Unknown species	0.001	1.85	-1.12
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Butyricicoccaceae;g_Butyricicoccus	0.045	2.98	2.80
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Oscillospiraceae;g_Flavonifractor	0.012	-1.35	-1.12
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Ruminococcus	0.003	3.51	3.32
		*p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Ruminococcus;s_* Unknown species	0.009	3.46	3.26
		p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter;s_schindleri	0.010	-1.35	-1.12

B

F1	F2	Intervention with R1 (n=21)	*P*	F1	F2
		p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Muribaculaceae;g_Muribaculaceae	0.042	-1.15	-1.08
		p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella	0.019	-1.15	-1.47
		p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae;g_Enterococcus	0.026	-1.38	-0.42
		p_Firmicutes;c_Bacilli;o_Mycoplasmatales;f_Mycoplasmataceae;g_Ureaplasma	0.008	-1.25	-1.47
		p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae	0.046	2.48	2.86
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_[Eubacterium] hallii group;s_* Unknown species	0.029	3.20	3.03
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Dorea	0.046	3.50	3.02
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia;s_* Unknown species	0.046	0.84	-1.11
		p_Firmicutes;c_Clostridia;o_Oscillospirales	0.042	5.64	5.40
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Oscillospiraceae;g_Flavonifractor	0.024	-1.38	-1.08
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae	0.046	5.22	4.83
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Ruminococcus	0.010	3.37	3.25
		*p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Ruminococcus;s_* Unknown species	0.011	3.27	3.08
		p_Proteobacteria;c_Alphaproteobacteria	0.035	2.52	2.85
		p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales	0.032	2.86	3.27
		p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Enterobacteriaceae	0.046	2.45	3.16
		p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Hafniaceae;g_Hafnia/Obesumbacterium	0.042	-1.31	1.63

C

F1	F2	Intervention with R2 (n=21)	*P*	F1	F2
		d_Archaeap_Euryarchaeota;c_Methanobacteria;o_Methanobacteriales;f_Methanobacteriaceae;g_Methanobrevibacter	0.019	-1.30	-1.13
		p_Actinobacteriota;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Bifidobacterium;s_bifidum	0.026	-1.26	-1.13
		p_Actinobacteriota;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Gardnerella;s_vaginalis	0.014	-1.26	-1.13
		p_Actinobacteriota;c_Actinobacteria;o_Corynebacteriales;f_Corynebacteriaceae	0.006	-1.55	-1.24
		p_Actinobacteriota;c_Coriobacteriia;o_Coriobacteriales;f_Eggerthellaceae;g_Adlercreutzia	0.004	1.71	2.49
		p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Paraprevotella	0.024	-1.30	-1.13
		p_Cyanobacteria;c_Vampirivibrionia;o_Gastranaerophilales;f_Gastranaerophilales;g_Gastranaerophilales	0.024	-1.45	-1.37
		p_Desulfobacterota;c_Desulfovibrionia;o_Desulfovibrionales;f_Desulfovibrionaceae;g_Desulfovibrio; Unknown species	0.046	-1.30	-1.26
		p_Firmicutes;c_Bacilli;o_Erysipelotrichales	0.002	3.66	3.98
		p_Firmicutes;c_Bacilli;o_Erysipelotrichales;f_Erysipelatoclostridiaceae;g_Catenibacterium	0.046	-1.31	-1.24
		p_Firmicutes;c_Bacilli;o_Erysipelotrichales;f_Erysipelatoclostridiaceae;g_Erysipelatoclostridium; Unknown species	0.024	-1.30	-1.24
		*p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus;s_* Uncultured species	0.032	-1.30	-1.24
		*p_Firmicutes;c_Clostridia;o_Christensenellales;f_Christensenellaceae;g_Christensenellaceae_* R-7 group; Unknown species	0.002	-1.45	-1.16
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Coprococcus	0.042	3.57	3.21
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Lachnospiraceae_* FCS020 group	0.026	2.43	-0.73
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia	0.001	3.21	2.21
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia;s_* Unknown species	0.011	2.24	-1.13
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_CAG-352	0.038	-1.45	-1.37
		*p_Firmicutes;c_Clostridia;o_Peptostreptococcales;Tissierellales;f_Anaerovoracaceae;g_* Family-XIII UCG-001	0.046	-1.12	0.67
		p_Firmicutes;c_Clostridia;o_Peptostreptococcales;Tissierellales;f_Peptostreptococcales;Tissierellales	0.026	-1.55	-1.26
		p_Firmicutes;c_Negativicutes;o_Acidaminococcales;f_Acidaminococcaceae;g_Phascolarctobacterium; Unknown species	0.026	-1.45	-1.26
		p_Proteobacteria	0.019	5.13	4.93
		p_Proteobacteria;c_Gammaproteobacteria	0.026	5.00	4.88
		p_Proteobacteria;c_Gammaproteobacteria;o_Burkholderiales	0.003	4.34	4.13
		p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter;s_schindleri	0.004	-1.30	-0.98
		p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Enhydrobacter	0.006	-1.30	-0.76

Fig. 2. Bacterial taxa in fecal samples exhibiting a significantly different abundance between before and after rocket salad intake. Statistical significances have been determined through the Wilcoxon signed-rank test carried out with CLR-transformed bacterial abundances (indicated on the right). Higher and lower abundances for each taxon are reported with a red and cyan background, respectively. The green-yellow-red heatmap represents the median CLR-transformed abundances of the reported taxonomic units. The taxonomic lineage of each taxon is shown: p, phylum; c, class; o, order; f, family; g, genus; s, species. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

D

F1	F2	Intervention with R1, Unwashed (n=11)	*P*	F1	F2
		p_Firmicutes;c_Bacilli;o_Erysipelotrichales;f_Erysipelotrichaceae	0.032	3.08	2.42
		p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae;g_Enterococcus	0.014	-1.47	1.45
		p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Enterococcaceae;g_Enterococcus; Unknown species	0.042	-1.47	-1.27
		p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Leuconostocaceae	0.032	-1.55	-1.43
		p_Firmicutes;c_Bacilli;o_Mycoplasmatales;f_Mycoplasmataceae;g_Ureaplasma	0.042	-1.31	-1.54
		p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales	0.032	-1.15	-1.55
		*p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas;s_* Unknown species	0.042	-1.31	-1.55

E

F1	F2	Intervention with R1, Washed (n=10)	*P*	F1	F2
		p_Actinobacteriota;c_Actinobacteria;o_Micrococcales	0.020	-0.99	2.06
		*p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_* Unknown species	0.049	3.95	2.83
		p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella	0.027	-1.01	-1.47
		p_Firmicutes	0.027	7.20	6.86
		p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus	0.049	3.77	3.16
		p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus; Unknown species	0.010	2.91	-1.03
		p_Firmicutes;c_Clostridia	0.027	6.97	6.55
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae	0.006	6.43	5.82
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_[Eubacterium] hallii group	0.037	3.81	3.15
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_[Eubacterium] hallii group;s_* Unknown species	0.020	3.49	2.72
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Blautia; Unknown species	0.027	4.26	3.69
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Dorea	0.014	3.66	2.89
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Lachnospiraceae_* NK4A136 group	0.002	2.64	-1.00
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Lachnospiraceae_* UCG-001	0.014	-1.11	-1.29
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia	0.020	2.74	1.17
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia;s_* Unknown species	0.014	1.75	-1.10
		p_Firmicutes;c_Clostridia;o_Oscillospirales	0.049	5.76	5.24
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Oscillospiraceae;g_Flavonifractor	0.020	-1.31	1.31
		*p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Oscillospiraceae;g_* UCG-005	0.049	2.42	0.19
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Oscillospiraceae;g_uncultured	0.027	3.09	2.63
		*p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Oscillospiraceae;g_uncultured;s_* Unknown species	0.037	3.04	2.19
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae	0.049	5.35	4.59
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_[Eubacterium] siraeum group	0.020	1.79	0.22
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Faecalibacterium	0.010	4.14	3.14
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Negativibacillus	0.027	0.73	-1.05
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Ruminococcus	0.037	3.53	2.52
		*p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Ruminococcaceae;g_Ruminococcus;s_* Unknown species	0.037	3.24	2.52
		p_Proteobacteria;c_Alphaproteobacteria	0.027	0.58	2.79
		p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales	0.004	-1.20	1.94
		p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Beijerinckiaceae;g_Bosea	0.049	-1.31	0.42
		p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Xanthobacteraceae	0.014	-1.20	1.38
		p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Xanthobacteraceae;_;_	0.037	-1.31	0.13
		p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae	0.049	-1.20	-0.003
		p_Proteobacteria;c_Gammaproteobacteria;o_Burkholderiales;f_Comamonadaceae	0.004	-0.03	2.26
		p_Proteobacteria;c_Gammaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Comamonas	0.027	-1.20	1.90
		p_Proteobacteria;c_Gammaproteobacteria;o_Burkholderiales;f_Rhodocyclaceae	0.049	-1.31	-0.96
		p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacterales;f_Hafniaceae;g_Hafnia /Obesumbacterium	0.014	-1.42	1.77
		*p_Proteobacteria;c_Gammaproteobacteria;o_Pasteurellales;f_Pasteurellaceae;g_Haemophilus;s_* Unknown species	0.049	-0.99	-1.29

Fig. 2. (*continued*).

during the interventions with R1 and R2 rockets, a larger number of significantly altered bacterial taxa was observed. Specifically, 17 bacterial taxa were identified for R1 (Fig. 2B), and 26 were identified for R2 (Fig. 2C). In both interventions, most modified taxa belonged to the class Clostridia. However, the only alteration common to both interventions was the reduction of an unidentified species belonging to the genus *Roseburia*.

The statistical analysis of bacterial abundances before and after rocket salad consumption was finally conducted for each intervention, separating those who had consumed washed rocket from those who had consumed unwashed rocket salad. The results of this analysis showed a significant modification of only 7 bacterial taxa after consumption of unwashed and 38 after consumption of washed R1 rocket salads (Fig. 2D and E). In the trial with rocket salad R2, 18 and 14 significantly changed bacterial taxa were identified for unwashed and washed rocket, respectively (Fig. 2F and G). No changes were found in common between the two interventions with unwashed rocket or between those with washed rocket, with the sole exception of the genus *Roseburia*, which decreased after consumption of washed rocket for both the R1 and R2 interventions.

Overall, the metataxonomic analysis of fecal bacteria showed that the consumption of 100 g of rocket per day for three days did not change the bacterial community structure in a way that could be attributed to the treatments.

3.4. Effect of rocket consumption on LAB levels in fecal samples

Fecal samples collected during the two intervention studies were used for quantification of LAB through dilution and plating on MRS agar medium. The amount of LAB in the feces ranged from 9.0 to 5.2 (median 7.1) log CFU/g. During the two interventions, the viable count of LAB did not significantly change in any of the subgroups considered (Fig. 3).

Subsequently, we conducted quantitative PCR using specific probes for the dominant genera of LAB in the two rocket salads under study,

F

F1	F2	Intervention with R2, Unwashed (n=10)	*P*	F1	F2
		p_Actinobacteriota;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Gardnerella;s_vaginalis	0.027	-1.24	-0.75
		p_Cyanobacteria;c_Vampirivibrionia;o_Gastranaerophilales;f_Gastranaerophilales;g_Gastranaerophilales	0.049	-1.38	-1.26
		p_Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Bacillus; Unknown species	0.049	-1.24	0.54
		p_Firmicutes;c_Bacilli;o_Erysipelotrichales	0.002	3.61	4.01
		p_Firmicutes;c_Bacilli;o_Erysipelotrichales;f_Erysipelatoclostridiaceae	0.049	3.15	3.36
		p_Firmicutes;c_Bacilli;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Holdemanella	0.037	-1.30	-1.07
		p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus;s_reuteri	0.037	-1.24	-0.87
		p_Firmicutes;c_Bacilli;o_Mycoplasmatales;f_Mycoplasmataceae;g_Ureaplasma	0.037	-1.38	-1.07
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Anaerostipes	0.037	3.01	3.41
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia	0.037	3.41	1.35
		*p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia;s_* Unknown species	0.027	2.92	-1.07
		p_Firmicutes;c_Clostridia;o_Oscillospirales;f_Butyricicoccaceae;g_UCG-009	0.037	-1.38	-0.87
		p_Firmicutes;c_Clostridia;o_Peptostreptococcales;Tissierellales	0.020	4.06	4.51
		p_Firmicutes;c_Clostridia;o_Peptostreptococcales;Tissierellales;f_Peptostreptococcaceae	0.010	3.56	4.39
		p_Proteobacteria	0.010	5.20	4.32
		p_Proteobacteria;c_Gammaproteobacteria	0.014	5.02	4.26
		p_Proteobacteria;c_Gammaproteobacteria;o_Burkholderiales	0.010	4.12	3.66
		p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter;s_schindleri	0.020	-1.24	-0.75

G

F1	F2	*Intervention with* R2, Washed (n=11)	*P*	F1	F2
		p_Actinobacteriota;c_Actinobacteria;o_Actinomycetales;f_Actinomycetaceae	0.024	-0.61	1.44
		p_Actinobacteriota;c_Actinobacteria;o_Actinomycetales;f_Actinomycetaceae;g_Actinomyces	0.024	-0.82	1.44
		p_Actinobacteriota;c_Actinobacteria;o_Corynebacteriales	0.032	-1.26	0.50
		p_Actinobacteriota;c_Actinobacteria;o_Corynebacteriales;f_Corynebacteriaceae	0.019	-1.63	-1.24
		p_Actinobacteriota;c_Coriobacteriia;o_Coriobacteriales;f_Eggerthellaceae;g_Adlercreutzia	0.019	-1.17	1.86
		p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Marinifilaceae;g_Odoribacter;s_splanchnicus	0.010	-1.26	-1.13
		p_Bacteroidota;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Paraprevotella	0.042	-1.30	-1.13
		*p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus;s_* Unknown species	0.014	-1.63	-0.96
		p_Firmicutes;c_Clostridia;o_Christensenellales;f_Christensenellaceae;g_Christensenellaceae_R-7 group; Unknown species	0.010	-1.63	-1.26
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Coprococcus;s_uncultured organism	0.032	2.57	1.80
		p_Firmicutes;c_Clostridia;o_Lachnospirales;f_Lachnospiraceae;g_Roseburia	0.024	2.80	2.44
		*p_Firmicutes;c_Clostridia;o_Peptostreptococcales;Tissierellales;f_Peptostreptococcaceae;g_Romboutsia;s_* Unknown species	0.042	-1.63	-1.29
		p_Firmicutes;c_Negativicutes;o_Acidaminococcales;f_Acidaminococcaceae;g_Phascolarctobacterium; Unknown species	0.042	-1.58	-1.13
		p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Enhydrobacter	0.032	-1.58	0.57

MIN MAX

Median of CLR-transformed abundances

>

Median of CLR-transformed abundances

Fig. 2. (*continued*).

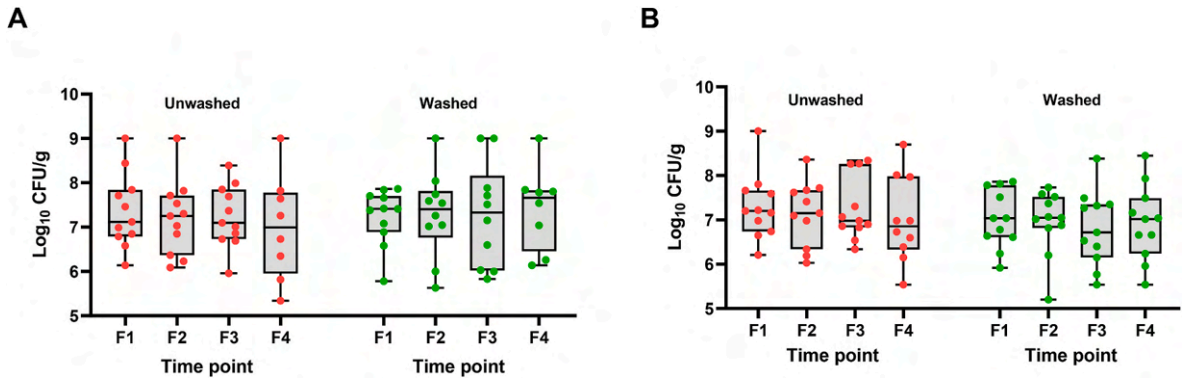

Fig. 3. Tukey boxplot of colony counts on MRS plates (pH 5.7) cultivated from the fecal samples collected during the intervention trials with rocket salads. Time points refer to the study scheme of Fig. 1. No statistically significant differences were detected. A, intervention with rocket salad R1. B. intervention with rocket salad R2.

namely *Leuconostoc* and *Weissella*. Specifically, qPCR assays were performed using as a template the DNA extracted from colonies collected from the MRS agar plates (see Supplementary Fig. 3 for a schematic representation of the experiment) to obtain information on the quantity of viable cells of the target bacterial taxa. The results showed that, for the *Leuconostoc* spp., the estimated number of viable cells in the feces did

not significantly change with either unwashed or washed rocket intervention. However, for the genus *Weissella*, a significant increase was observed following consumption of unwashed salad, but not washed salad (Fig. 4). In particular, the increase in *Weissella* spp. was statistically significant in the second fecal sample collected after the end of rocket consumption (F3) but was no longer evident in the subsequent sample (F4) (Fig. 4).

3.5. Survival of rocket salad-associated LAB during simulated gastrointestinal transit

We utilized the INFOGEST static in vitro simulation of gastrointestinal digestion to assess the survival capability of strains isolated from rocket salad under digestive tract conditions. Two isolates, *Weissella cibaria* (from rocket salad R1) and *Leuconostoc lactis* (from rocket salad R2), were examined. The results showed that both strains demonstrated similar abilities to survive simulated gastrointestinal transit, and the presence of rocket salad significantly enhanced their survival. Specifically, starting from approximately ten billion CFUs, *W. cibaria* and *L. lactis* displayed a 5.1 and 5.2 log CFUs reduction, respectively, when tested alone, whereas the reduction of viable cells was only 1.2 and 1.4 log CFUs when the cells of the two isolates were tested in the presence of rocket salad (Fig. 5).

4. Discussion

This experimental study aimed to investigate the indigenous bacteria in commercially available ready-to-eat rocket salad, in line with the hypothesis that naturally occurring LAB associated with raw foods may play a role in shaping the human gut microbiota and may directly interact with the host. The study pursued with a dual objective: (i) evaluating the impact on fecal microbiota and (ii) assessing the survival of rocket-associated LAB during gastrointestinal transit. To this aim, we carried out two intervention studies involving healthy adult individuals, testing two different batches of rocket salad with or without washing with a sodium hypochlorite solution.

The results of viable microbial count revealed that unwashed rocket salad had a significantly higher total aerobic bacterial number by at least one order of magnitude than salad manually washed with a diluted solution of sodium hypochlorite; nonetheless, washing did not eliminate the bacteria associated with the rocket salad. This finding is consistent with previous studies where similar washing with a diluted solution of sodium hypochlorite reduced total aerobic bacteria within the range of 1–2 log units in fresh-cut iceberg lettuce (López-Gálvez et al., 2010) and fresh jalapeno peppers (Adler et al., 2016).

Although washed rocket salad did not harbor any associated LAB, no significant difference in LAB quantity was observed in the feces of volunteers following interventions with washed or unwashed rocket salad. This result can be explained by the fact that ready-to-eat rocket salad may provide an insufficient amount of LAB to significantly alter the abundance of resident LAB in the human gut, which we found to be, on average, in the range of 6.8–7.4 log CFU per gram of feces at each time point for each group on volunteers. In our study, the LAB quantities in the two rocket salads investigated were 4.1 log CFU/g for the first intervention (R1 rocket salad) and 2.3 log CFU/g for the second intervention (R2). Thus, even considering a daily intake of 100 g repeated for three consecutive days, the number of LAB cells introduced with rocket salad in our interventions is at least two log units lower than the LAB already present in volunteers' feces in this study (estimation done considering an average amount of 150 g of feces per defecation).

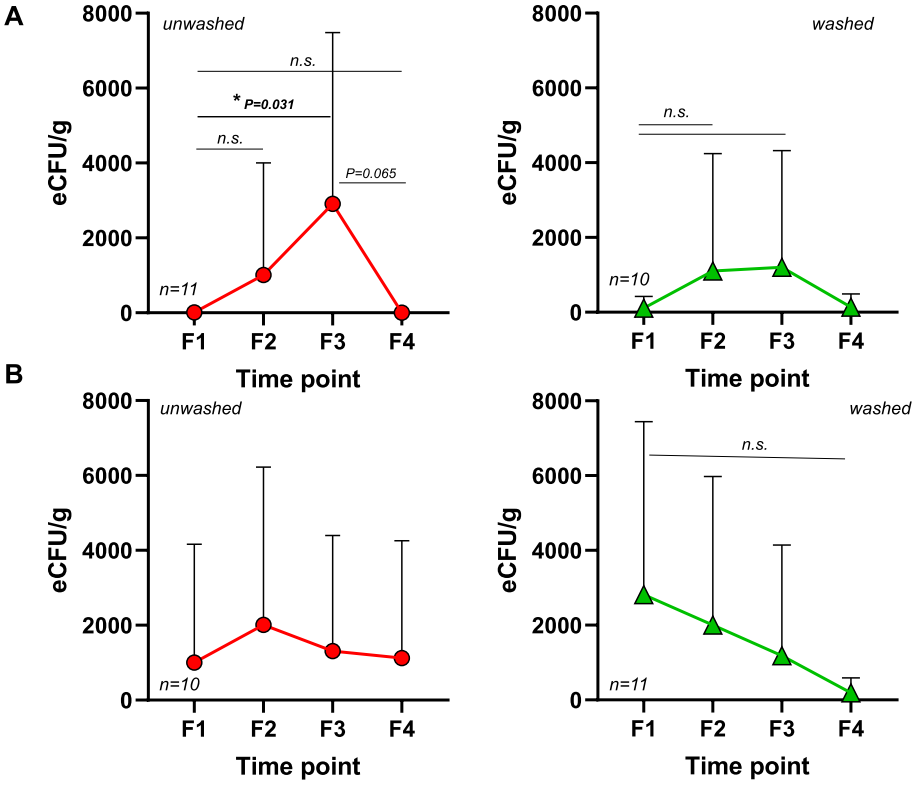

Fig. 4. Recovery on MRS agar (pH 6.7) of viable cells of the dominant lactic acid bacterial species from rocket salads in feces collected at the end of the three-day intervention trials (see Material and Methods and Supplementary Fig. S1 for a detailed description of the methodology). Panel A: Intervention with rocket salad R1 with (on the right, in green) or without (on the left, in red) washing using a sodium hypochlorite solution. Panel B: intervention with washed (on the right, in green) and unwashed (on the left, in red) rocket salad R2. Data are presented as the estimated minimum CFU count (eCFU; see the Methods section for details). Statistical analysis was performed using a Wilcoxon signed-rank test; *, $p < 0.05$; n.s., not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

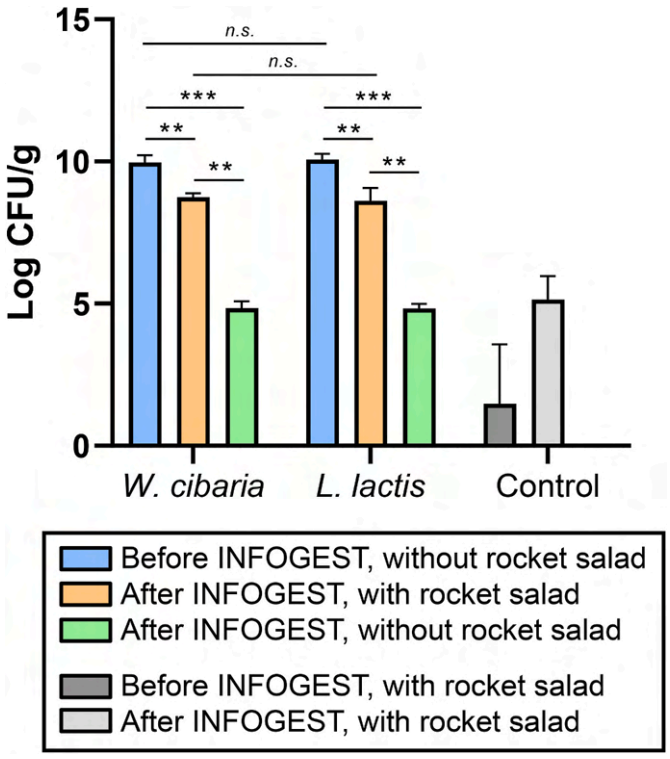

Fig. 5. Survival of lactic acid bacterial strains isolated from rocket salad R1 (*Weissella cibaria*) and R2 (*Leuconostoc lactis*) in the in vitro static simulated gastrointestinal digestion (INFOGEST protocol). Control, experiment performed with rocket salad without the addition of bacterial cells. Data are derived from three independent experiments. Statistical analysis was performed using an unpaired Student's t-test; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant.

Following rocket consumption, the results of metataxonomic analysis of fecal bacteria indicated that the consumption of 100 g of salad per day for three days did not lead to significant changes in the bacterial community structure attributable to the treatments. This result can be explained by the limited number of volunteers in the study and the wide inter-individual variability of the human gut microbiota (Healey et al., 2017). Although, reportedly, the composition of the human gut microbiota undergoes changes within a few hours (David et al., 2014), the very short duration of the intervention could also have been a determinant factor for the observed results. We failed to find any other study in scientific literature where rocket salad intake's effect on the gut microbiota was assessed.

We found that most LAB isolated from rocket R1 belonged to the species *Weissella cibaria*, while isolates from rocket salad R2 belonged to the species *Leuconostoc lactis*. These findings are consistent with our previous study that has identified members of the bacterial genera *Leuconostoc* and *Weissella* among the primary LAB associated with ready-to-eat fresh salads (Fessard and Remize, 2019; Mantegazza et al., 2023b; Zhou et al., 2020). However, rocket salad R1 harbored a significantly higher quantity of LAB (almost two orders of magnitude higher) compared to R2. This result aligns with our previous study that showed a wide variability in the quantity of LAB associated with commercial ready-to-eat rocket salad, ranging from the absence of LAB to approximately 5.0 log CFU per gram (Mantegazza et al., 2023b). The notable difference in LAB content between R1 and R2 salads may explain the results of the qPCR experiments performed on the DNA purified from the bacterial colonies isolated on MRS from feces, which revealed a significant increase of *Weissella* spp. exclusively in the feces of volunteers who consumed unwashed salad R1, whereas the abundance of *Leuconostoc* spp. did not change significantly in the feces of volunteers consuming rocket salad R2. This finding is also consistent with the results of 16S rRNA gene profiling, which showed a significant increase in the family Leuconostocaceae only in the group of volunteers who consumed unwashed rocket salad R1 (i.e., the salad harboring a larger amount of LAB cells; Fig. 2D). Recently, the family Leuconostocaceae was reclassified and included into the family Lactobacillaceae (Zheng et al., 2020). Nonetheless, the database we used for the metataxonomic analysis (Silva Database version 138) was based on the previous taxonomic classification, where the family Leuconostocaceae included the genera *Weissella* and *Leuconostoc* (Chelo et al., 2007). These results allow us to hypothesize that ready-to-eat rocket salad can carry LAB capable of surviving gastrointestinal transit only if the naturally occurring quantity of these bacteria associated with the product is sufficiently high. Another possible explanation is related to the fact that the rocket salads used in the two studies hosted two different taxonomic groups of LAB (*Weissella* spp. and *Leuconostoc* spp.), which may have a significantly different predisposition to survive gastrointestinal transit. It has been reported that the *Weissella* genus is frequently found in fecal samples of healthy adults (Fusco et al., 2015), unlike the *Leuconostoc* genus, which is not usually considered part of the autochthonous intestinal microbiota (Hemme and Foucaud-Scheunemann, 2004). We tested this hypothesis by conducting a simulated gastrointestinal transit experiment using a *W. cibaria* strain isolated from rocket salad R1 and a *L. lactis* strain isolated from R2. However, the obtained results did not evidence any significant difference between *W. cibaria* and *L. lactis* in the ability to survive the gastrointestinal transit, supporting the hypothesis that cell abundance in the original rocket salad is critical to permit the recovery of viable LAB cells in feces after salad intake. Furthermore, in vitro simulated gastrointestinal digestion evidenced that rocket salad significantly preserved bacterial cell viability in the gastrointestinal tract, as already demonstrated for other food matrices (Karimi et al., 2011; Mattila-Sandholm et al., 2002).

5. Conclusion

This study provides a better understanding of the survival of LAB naturally associated with the indigenous microbiota of rocket salad during gastrointestinal transit. It should be noted that the rocket samples used in this study were limited to fresh-cut, ready-to-eat commercial rocket salad, and the results may vary with other varieties of rocket salad or other vegetables. Furthermore, the short duration of salad intake in this study may not be sufficient to highlight the beneficial effects of naturally occurring LAB in rocket on the participants' gut microbiota. Therefore, further research with larger sample sizes and longer intervention periods are necessary. Nonetheless, to the best of our knowledge, this is the first intervention study that provides data supporting the idea that fresh plant products consumed raw can be a source of live LAB cells for the human gut. More generally, the presented results encourage the implementation of intervention studies focusing on the microbial component of raw, non-fermented foods and their potential impact on the human intestinal microbiome.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used the AI-powered language model ChatGPT-3.5 (https://chat.openai.com/) in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

None.

Acknowledgements

This work was completed as part of the project MIND FoodS HUB (Milano Innovation District Food System Hub): Innovative Concept for the Eco-intensification of Agricultural Production and for the Promotion of Dietary Patterns for Human Health and Longevity through the Creation in MIND of a Digital Food System Hub, cofounded by POR FESR 2014–2020_BANDO Call HUB Ricerca e Innovazione, Regione Lombardia.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104418.

References

Adler, J.M., Cain-Helfrich, E.D., Shen, C., 2016. Reductions in natural microbial flora, nonpathogenic *Escherichia coli*, and pathogenic salmonella on jalapeno peppers processed in a commercial antimicrobial cabinet: a pilot plant trial. J. Food Protect. 79, 1854–1859. https://doi.org/10.4315/0362-028X.JFP-16-222.

Arioli, S., Koirala, R., Taverniti, V., Fiore, W., Guglielmetti, S., 2018. Quantitative recovery of viable *Lactobacillus paracasei* CNCM I-1572 (L. casei DG®) after gastrointestinal passage in healthy adults. Front. Microbiol. 9. https://www.frontiersin.org/articles/10.3389/fmicb.2018.01720.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A.R., Martins, C., Marze, S., McClements, D.J., Ménard, O., Minekus, M., Portmann, R., Santos, C.N., Souchon, I., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Recio, I., 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 14, 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Chelo, I.M., Zé-Zé, L., Tenreiro, R., 2007. Congruence of evolutionary relationships inside the *Leuconostoc–Oenococcus–Weissella* clade assessed by phylogenetic analysis of the 16S rRNA gene, dnaA, gyrB, rpoC and dnaK. nt. J. Syst. Evol. Microbiol. 57, 276–286. https://doi.org/10.1099/ijs.0.64468-0.

Chen, P., 2021. Chapter 24 - lactic acid bacteria in fermented food. In: Dhanasekaran, D., Sankaranarayanan, A. (Eds.), Advances in Probiotics. Academic Press, pp. 397–416.

Cristofori, F., Dargenio, V.N., Dargenio, C., Miniello, V.L., Barone, M., Francavilla, R., 2021. Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: a door to the body. Front. Immunol. 12. https://www.frontiersin.org/articles/10.3389/fimmu.2021.578386.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y., Fischbach, M.A., Biddinger, S.B., Dutton, R.J., Turnbaugh, P.J., 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505, 559–563. https://doi.org/10.1038/nature12820.

Fessard, A., Remize, F., 2019. Genetic and technological characterization of lactic acid bacteria isolated from tropically grown fruits and vegetables. Int. J. Food Microbiol. 301, 61–72. https://doi.org/10.1016/j.ijfoodmicro.2019.05.003.

Fusco, V., Quero, G.M., Cho, G.-S., Kabisch, J., Meske, D., Neve, H., Bockelmann, W., Franz, C.M.A.P., 2015. The genus Weissella: taxonomy, ecology and biotechnological potential. Front. Microbiol. 6. https://www.frontiersin.org/articles/10.3389/fmicb.2015.00155.

Guglielmetti, S., Taverniti, V., Minuzzo, M., Arioli, S., Stuknyte, M., Karp, M., Mora, D., 2010. Oral bacteria as potential probiotics for the pharyngeal mucosa. Appl. Environ. Microbiol. 76, 3948–3958. https://doi.org/10.1128/AEM.00109-10.

Han, S., Lu, Y., Xie, J., Fei, Y., Zheng, G., Wang, Z., Liu, J., Lv, L., Ling, Z., Berglund, B., Yao, M., Li, L., 2021. Probiotic gastrointestinal transit and colonization after oral administration: a long journey. Front. Cell. Infect. Microbiol. 11. https://www.frontiersin.org/articles/10.3389/fcimb.2021.609722.

Hati, S., Patel, M., Mishra, B.K., Das, S., 2019. Short-chain fatty acid and vitamin production potentials of Lactobacillus isolated from fermented foods of Khasi Tribes, Meghalaya, India. Ann. Microbiol. 69, 1191–1199. https://doi.org/10.1007/s13213-019-01500-8.

Healey, G.R., Murphy, R., Brough, L., Butts, C.A., Coad, J., 2017. Interindividual variability in gut microbiota and host response to dietary interventions. Nutr. Rev. 75, 1059–1080. https://doi.org/10.1093/nutrit/nux062.

Hemme, D., Foucaud-Scheunemann, C., 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int. Dairy J. 14, 467–494. https://doi.org/10.1016/j.idairyj.2003.10.005.

Karimi, R., Mortazavian, A.M., Da Cruz, A.G., 2011. Viability of probiotic microorganisms in cheese during production and storage: a review. Dairy Sci. Technol. 91, 283–308. https://doi.org/10.1007/s13594-011-0005-x.

Koirala, R., Ricci, G., Taverniti, V., Ferrario, C., Malla, R., Shrestha, S., Fortina, M.G., Guglielmetti, S., 2014. Isolation and molecular characterization of lactobacilli from traditional fermented Dahi produced at different altitudes in Nepal. Dairy Sci. Technol. 94, 397–408. https://doi.org/10.1007/s13594-014-0167-4.

Li, P., Gu, Q., Yang, L., Yu, Y., Wang, Y., 2017. Characterization of extracellular vitamin B12 producing *Lactobacillus plantarum* strains and assessment of the probiotic potentials. Food Chem. 234, 494–501. https://doi.org/10.1016/j.foodchem.2017.05.037.

López-Gálvez, F., Allende, A., Truchado, P., Martínez-Sánchez, A., Tudela, J.A., Selma, M.V., Gil, M.I., 2010. Suitability of aqueous chlorine dioxide versus sodium hypochlorite as an effective sanitizer for preserving quality of fresh-cut lettuce while avoiding by-product formation. Postharvest Biol. Technol. 55, 53–60. https://doi.org/10.1016/j.postharvbio.2009.08.001.

Mantegazza, G., Dalla Via, A., Licata, A., Duncan, R., Gardana, C., Gargari, G., Alamprese, C., Arioli, S., Taverniti, V., Karp, M., Guglielmetti, S., 2023a. Use of kefir-derived lactic acid bacteria for the preparation of a fermented soy drink with increased estrogenic activity. Food Res. Int. 164, 112322 https://doi.org/10.1016/j.foodres.2022.112322.

Mantegazza, G., Gargari, G., Duncan, R., Consalez, F., Taverniti, V., Riso, P., Guglielmetti, S., 2023b. Ready-to-eat rocket salads as potential reservoir of bacteria for the human microbiome. Microbiol. Spectr. 11, e02970-02922 https://doi.org/10.1128/spectrum.02970-22.

Mattila-Sandholm, T., Myllärinen, P., Crittenden, R., Mogensen, G., Fondén, R., Saarela, M., 2002. Technological challenges for future probiotic foods. Int. Dairy J. 12, 173–182. https://doi.org/10.1016/S0958-6946(01)00099-1.

Pasolli, E., De Filippis, F., Mauriello, I.E., Cumbo, F., Walsh, A.M., Leech, J., Cotter, P.D., Segata, N., Ercolini, D., 2020. Large-scale genome-wide analysis links lactic acid bacteria from food with the gut microbiome. Nat. Commun. 11, 2610. https://doi.org/10.1038/s41467-020-16438-8.

Rosa, D.D., Dias, M.M.S., Grześkowiak, Ł.M., Reis, S.A., Conceição, L.L., Peluzio, M.d.C.G., 2017. Milk kefir: nutritional, microbiological and health benefits. Nutr. Res. Rev. 30, 82–96. https://doi.org/10.1017/S0954422416000275.

Scudellari, M., 2017. News feature: cleaning up the hygiene hypothesis. Proc. Natl. Acad. Sci. U.S.A. 114, 1433–1436. https://doi.org/10.1073/pnas.1700688114.

Sonnenburg, J.L., Sonnenburg, E.D., 2019. Vulnerability of the industrialized microbiota. Science 366, eaaw9255. https://doi.org/10.1126/science.aaw9255.

Taverniti, V., Guglielmetti, S., 2012. Health-promoting properties of *Lactobacillus helveticus*. Front. Microbiol. 3 https://doi.org/10.3389/fmicb.2012.00392.

Taverniti, V., Koirala, R., Dalla Via, A., Gargari, G., Leonardis, E., Arioli, S., Guglielmetti, S., 2019. Effect of cell concentration on the persistence in the human intestine of four probiotic strains administered through a multispecies formulation. Nutrients 11, 285. https://doi.org/10.3390/nu11020285.

Yuan, Q.S., Deng, T., Gao, Y., Jiang, W., Ou, X., Wang, Y., Guo, L., Zhou, T., 2022. Genome resource for *Acinetobacter schindleri* H4-3-C1: an endophyte of *Pseudostellaria heterophylla* with degradation activity to toxins produced by fungal pathogens. Mol. Plant Microbe Interact. 35, 1124–1126. https://doi.org/10.1094/MPMI-04-22-0088-A.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. Int. J. Syst. Evol. Microbiol. 70, 2782–2858. https://doi.org/10.1099/ijsem.0.004107.

Zhou, S.-Y.-D., Wei, M.-Y., Giles, M., Neilson, R., Zheng, F., Zhang, Q., Zhu, Y.-G., Yang, X.-R., 2020. Prevalence of antibiotic resistome in ready-to-eat salad. Front. Public Health 8. https://www.frontiersin.org/articles/10.3389/fpubh.2020.00092.