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Fermentation of *Pleurotus ostreatus* and *Ganoderma lucidum* mushrooms and their extracts by the gut microbiota of healthy and osteopenic women: potential prebiotic effect and impact of mushroom fermentation products on human osteoblasts†

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Recent data have highlighted the role of the gut microbiota and its several metabolites in maintaining bone health. Thus, gut microbiota manipulation, *e.g.*, by prebiotics, might offer a plausible target in the fight against bone degenerative diseases. This study aimed (a) to investigate the *in vitro* prebiotic potential of *Ganoderma lucidum* and *Pleurotus ostreatus* mushrooms in healthy and osteopenic women and (b) to explore the impact of mushroom fermentation products on human osteoblasts. *G. lucidum* LGAM 9720 and *P. ostreatus* IK 1123 lyophilized mushroom-powders (2% w/v) and their hot-water extracts (1% w/v) were fermented in a 24 h static batch culture model by using faecal inocula from healthy (n = 3) or osteopenic (n = 3) donors. Gut microbiota analysis (qPCR) and measurement of short chain fatty acids (SCFAs) were performed during fermentation, and 24 h-prebiotic indexes were calculated. Evaluation of the effects of fermentation products on bone metabolism parameters (OPG: osteoprotegerin; and RANKL: receptor activator of nuclear factor kappa B ligand) in osteoblast cultures was also performed. Our data suggest that the origin of the gut microbiota inoculum plays a major role in the viability of osteoblasts. The treatments using *P. ostreatus* mushroom-powder and *G. lucidum* mushroom-extract had positive effects based on gut microbiota and SCFA analyses. Both mushrooms exhibited lower RANKL levels compared to controls, whereas their extracts tended to enhance the osteoblastic activity. In conclusion, mushrooms that are rich in beta-glucans may exert beneficial *in vitro* effects on bone physiology by alterations in the gut microbiota and/or SCFA production.

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1 Introduction

The human body is the natural habitat of a large number and variety of microorganisms including bacteria, eukaryotes, archaea and viruses, widely known as "gut microbiota". While the composition of an individual's gut microbiota is unique, it usually remains relatively stable during adult life and it may be altered by various factors including host immune status, gender-specific hormones, age, diet and use of antibiotics.[1,2] The most dominant phyla detected in the gut microbiota are *Firmicutes* and *Bacteroidetes*, whereas *Proteobacteria*, and *Actinobacteria* are traced in minor proportions.[3] Dysbiosis of the gut microbiota is indicative of various chronic and pathological conditions, such as obesity,[4] inflammatory bowel disease,[5] irritable bowel syndrome,[6] allergies, cancer,[7] non-intestinal autoimmune diseases,[8] cardiovascular diseases[9] and neurological disorders.[10] Female postmenopausal osteopenia and osteoporosis are common skeletal diseases leading to fractures and disability, underlying the impact of estrogen

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deficiency on the skeleton. Declining estrogen levels result in a potent stimulation of bone resorption/osteoclastogenesis combined with a lower rate of bone formation/osteoblastogenesis leading to a period of rapid bone loss.[11] There is no single cause of osteoporosis and multiple mechanisms are involved in the pathogenesis of osteopenia. Decreased bone density has been recently correlated with gut microbiota dysregulation whereas several mechanisms have been described to support a gut–bone axis.[12] Research data have highlighted the role of the gut microbiota and its several metabolites (*e.g.* SCFAs) in maintaining bone health and bone mass through mechanisms including immune regulation, nutrient acquisition (calcium and phosphate), effects on gut serotonin or estrogen-like molecule production and alterations in gut barrier integrity and permeability.[13–15] "osteomicrobiology" combines bone physiology, gastroenterology, immunology and microbiology in order to define the connection of the gut microbiota and bone beyond simply facilitating the absorption of minerals that are important for bone health.[16]

Diet is one of the major factors that affects the gut microbiota composition.[17] According to the 2017 ISAPP consensus statement about the definition and scope of prebiotics, "a prebiotic is a substrate that is selectively utilized by host microorganisms conferring a health benefit".[17] The main fermentation products are short chain fatty acids (SCFAs), which have an important role in host's metabolism, beyond the gut environment.[17] Recent accumulating data from animal and human trials have underlined the beneficial role of prebiotics on bone mass density, and/or calcium and magnesium absorption, and/or impact of bone turnover markers.[15,18] Increased calcium absorption has been noticed in adolescents[19–24] and postmenopausal women[25–27] after consumption of prebiotics. In other studies, supplementation with calcium and prebiotics affected the bone turnover markers while it did not change the bone mineral density.[28–30] Recently, research has been focused on finding new alternative sources of prebiotics, with evidence-based effects on bone health.[31] So far mushrooms have demonstrated such a great potential, due to their high content of beta-glucans.[32,33] Particularly, mushrooms produced by *Ganoderma* and *Pleurotus* species are well known for their health-beneficial properties, due to their antioxidant, immunoregulatory, anti-hypertensive, anti-inflammatory, hypocholesterolemic, anti-diabetic and prebiotic activities.[34,35] Thus, gut microbiota manipulation by prebiotics of fungal origin might offer a plausible target in the fight against bone degenerative diseases by focusing on the bone remodeling cycle.

In this context, we aimed to investigate the *in vitro* prebiotic impact of *Ganoderma lucidum* and *Pleurotus ostreatus* mushrooms and their extracts on the gut microbiota of healthy and osteopenic women. Moreover the effects of the *in vitro* batch-culture fermentation products of *G. lucidum* and *P. ostreatus* on human osteoblasts were assessed by using the two important and highly interesting bone turnover markers osteoprotegerin (OPG) and human receptor activator of nuclear factor kappa-B ligand (RANKL).

2 Materials and methods

2.1 Characteristics of faecal donors

This study was conducted in Athens, Greece and the faecal donors were 6 postmenopausal women (3 healthy and 3 osteopenic) aged 55 to 65 years who met the following inclusion criteria: (a) normal weight [Body Mass Index (BMI) < 30 kg (m^{-2})] under no weight-loss program, (b) no consumption of antibiotics in the last two months prior to the study, (c) no consumption of dietary supplements related to bone metabolism (calcium, vitamin D, and cod oil) in the last six months prior to the study, (d) no history of chronic or autoimmune diseases (*e.g.* Idiopathic Inflammatory Bowel Disease, Irritable Bowel Syndrome, any form of constipation or diarrhea, kidney disease, liver disease, cardiovascular disease, cancer or any form of parathyroid disease) and (e) no consumption of dietary supplements or fortified foods that could affect the intestinal microbiota such as probiotics, prebiotics and symbiotics. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Bioethics Committee of Harokopio University. Written informed consent was obtained from all subjects before their inclusion in the study (Approval Number: 58/10-11-2017). A flowchart of the experiments performed in the frame of this study is presented in the ESI (Fig. S1†).

2.2 Demographic, anthropometric, dietary and physical assessment

The faecal donors completed questionnaires (in the presence of an expert) related to sociodemographic parameters (including age, sex, marital status and education level), smoking habits, medical history and gastrointestinal symptomatology for the 7 days preceding stool collection. Their body weight and height were self-reported and the Body Mass Index (BMI) was calculated by dividing the weight (kg) by the squared height (m^2). At the baseline, individual habitual energy intakes of the participants were assessed by a dietitian using 3-d food diaries, and their physical activity levels were assessed using the International Physical Activity Questionnaire-Short Form.[36] The duration of sedentary activity (sitting or resting) expressed as 'h $week^{-1}$' was also recorded.

2.3 Biochemical and bone density measurements

Blood samples were obtained after a 12 h overnight fasting. Blood serum analysis included the assessment of the lipidemic profile [*i.e.* Total Cholesterol (TC), Low-Density Lipoprotein Cholesterol (LDL-C), High-Density Lipoprotein Cholesterol (HDL-C), and Triglycerides (TG)], glucose metabolism [*i.e.* Fasting Blood Glucose (FBG) and insulin] and bone biomarkers [*i.e.* serum calcium (Ca), bone specific alkaline phosphatase (BALP), osteocalcin and 25-OH vitamin D]. HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) was calculated using the formula: [fasting insulin (uIU mL^{-1}) × fasting glucose (mg dL^{-1})]/22.5.[37] DXA (dual energy X-ray absorptiometry) was used for the examin-

ation of bone health and diagnosis of osteopenia, based on *T*-score, *Z*-score and BMD (Bone Mass Density) (gr cm^{-2}) evaluation.

2.4 Fungal strains, mushroom cultivation and determination of glucan contents

Ganoderma lucidum strain LGAM9720 and *Pleurotus ostreatus* strain LGAM1123 (maintained in the fungal culture collection of the Laboratory of General and Agricultural Microbiology, Agricultural University of Athens) were cultivated on a beech-sawdust based substrate and on a wheat-straw based substrate, respectively, as previously described.[38] Mushrooms were freeze-dried in a Telstar Cryodos apparatus and milled to fine powder, which was further used either untreated (GLBS and POWS treatments for *G. lucidum* and *P. ostreatus*, respectively) or after a stage of polysaccharide extraction.

For polysaccharide extraction, the suspension of the mushroom powder in distilled H_2O (1 : 40 w/v; initial volume: 400 ml) was maintained at 95 °C for 50 h. After thorough mixing and centrifugation (10 000*g* for 15 min at 4 °C), the supernatants were collected and subjected to condensation in a rotary evaporator to obtain a final volume of 30–40 ml and were stored at 4 °C for 24 h. Then the polysaccharides were precipitated by adding ice-cold ethanol to the samples (1 : 1 v/v), followed by stirring (200 rpm for 30 min at 4 °C) and centrifugation (10 000*g* for 5 min at 4 °C).[39–42] The precipitate was freeze-dried for 24 h, milled to fine powder and used for the additional two treatments (GLBSE and POWSE for *G. lucidum* and *P. ostreatus*, respectively). The total glucan and alpha-glucan contents were estimated using a Mushroom and Yeast Assay Kit (Megazyme Int. Ireland). The beta-glucan content was calculated by subtracting the alpha-glucan content from the total glucan content.

2.5 Faecal sample collection and *in vitro* static batch culture fermentation

Stool collection was performed according to the method described by Mitsou *et al.*[43] and the *in vitro* static batch culture fermentation procedure was based on the protocol of Olano-Martin *et al.*[44] and Rycroft *et al.*[45] with slight modifications, as previously described.[46] In order to minimize the potential cytotoxicity of the basal medium, we further modified the composition by reducing or excluding some evidence-based ingredients with a cytotoxic effect (*e.g.* reduction of the amount of hemin and Tween® 80 and exclusion of resazurin from the recipe).[47,48] The modified basal medium had comparable results with the classic basal medium in terms of *in vitro* fermentation capacity and microbial modifications, based on previous testing (data not shown).

In detail, the modified basal medium consisted of the following ingredients (g l^{-1}): peptone water (Merck KGaA, Darmstadt, Germany), 2.0; yeast extract (Merck KGaA), 2; NaCl, 0.10; K_2HPO_4, 0.04; KH_2PO_4, 0.04; $MgSO_4{\cdot}7H_2O$, 0.01; $CaCl_2{\cdot}2H_2O$, 0.01; $NaHCO_3$, 2.0; L-cysteine HCl (Merck KGaA), 0.50; dehydrated bile (OxgallTM, BD and Company, Sparks, MD, USA), 0.50; hemin (dissolved in some drops of 1.0 M NaOH) (Fluka, Sigma-Aldrich, Netherlands), 0.005;[48] Tween® 80 (Panreac Quimica SA, Barcelona, Spain), 0.2 ml l^{-1} (ref. 47) and vitamin K1 (Fluka, Sigma-Aldrich, Switzerland), 10 µl l^{-1}. The medium was adjusted to pH 7.0 with 1.0 M HCl, aliquoted in appropriate volumes into glass bottles (45 ml), sterilized at 121 °C for 15 min and transferred into an anaerobic chamber for overnight pre-reduction (BACTRON™ 1.5 Anaerobic Environmental Chamber, SHELLAB, Cornelius, Oregon). On the day of the *in vitro* experiment, we added 2% (w/v) untreated mushroom powders (POWS and GLBS) or 1% (w/v) mushroom hot-water extracts (POWSE and GLBSE) to the basal medium aliquots. Positive controls with already proven prebiotic effect were used (inulin by Orafti® GR, BENEO-Orafti, Oreye, Belgium), *i.e.*, 1% w/v inulin (INU1) for POWSE and GLBSE, and 2% w/v inulin (INU2) for POWS and GLBS. A negative control (NC; a basal medium with no carbohydrate source) was also included in the experiment.

On the day of the *in vitro* experiment we prepared a faecal slurry (20%w/v) in PBS, pH 7.3 (8.0 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.15 g l^{-1} Na_2HPO_4, and 0.2 g l^{-1} KH_2PO_4).[49] From this slurry, 10% (v/v) inocula were transferred into the pre-reduced basal medium aliquots of the substrates or the controls. The static batch cultures were incubated for 24 h under anaerobic conditions at 37 °C. The samples were collected at the baseline (0 h), and after 8 h and 24 h of fermentation and stored at −80 °C until further analysis (gut microbiota and SCFA profiling). In addition, the samples were collected, centrifuged, filtered (0.22 µm) and stored at −20 °C at 0 h and 24 h for MG-63 cell line experiments, as described in the following sections.

2.6 Gut microbiota analysis

Enumeration of the total bacterial load and selected members of the gut microbiota (*e.g. Bacteroides* spp., *Clostridium perfringens* group, *Bifidobacterium* spp., *Lactobacillus* group, *Roseburia* spp.–*Eubacterium rectale*, and *Faecalibacterium prausnitzii*) at the baseline (0 h) and after 24 h of fermentation was performed by real-time quantitative PCR (qPCR), as previously described.[43,46] In detail, genomic DNA was extracted from the frozen sample (1 ml, −80 °C) according to the method described by Salonen *et al.*[50,51] using the QIAamp® DNA Mini Kit (QIAGEN GmbH). Quantitative real-time PCR based on SYBR Green I detection chemistry was used to characterize the gut microbiota using species-, genus- and group-specific primers targeting 16S rRNA genes of different bacterial groups and the KAPA SYBR® Fast Master Mix (2×) Universal Kit (Kapa Biosystems Inc.) (ESI, Table S1†). PCR amplification and detection were performed using a LightCycler® 2.0 Real-Time PCR System (Roche Diagnostics GmbH). Microbial quantification was performed based on the standard curves for genomic DNA from the reference strains using the LightCycler® software version 4.1 (Roche Diagnostics GmbH). Data were expressed as $\log_{10}$ copies of 16S rRNA gene per ml of sample.

2.7 Prebiotic indexes

The prebiotic potential of the tested substrates was qualitatively evaluated based on the calculation of prebiotic indexes

(PIs) after 24 h of fermentation, as previously described.[46] PI is a useful tool for the comparison of prebiotic efficiency[52] and is calculated using the following equation,[53] based on quantification of bacteria (copies of 16S rRNA gene per ml of sample):

$$\mathrm{PI} = (\mathrm{Bif}/\mathrm{Total}) - (\mathrm{Bac}/\mathrm{Total}) + (\mathrm{Lac}/\mathrm{Total}) - (\mathrm{Clos}/\mathrm{Total})$$

where Bif refers to *Bifidobacterium* spp. numbers after 24 h of fermentation (t = 24 h)/numbers at inoculation (t = 0 h), Bac refers to *Bacteroides* spp. numbers after 24 h of fermentation (t = 24 h)/numbers at inoculation (t = 0 h), Lac refers to *Lactobacillus* group numbers after 24 h of fermentation (t = 24 h)/numbers at inoculation (t = 0 h), Clos refers to *Clostridium perfringens* group numbers after 24 h of fermentation (t = 24 h)/numbers at inoculation (t = 0 h) and Total refers to total bacteria numbers after 24 h of fermentation (t = 24 h)/numbers at inoculation (t = 0 h). According to the prebiotic index equation, an increase in the population of bifidobacteria and/or lactobacilli is assumed as a positive effect and an increase in bacteroides and/or clostridia is assumed as a negative effect.[53] This prebiotic index equation offers the advantage of normalizing the microbial population changes in relation to the initial bacterial counts, accounting for the physiological variability that characterizes the experimental process of the *in vitro* fermentation.[53]

2.8 Measurement of SCFAs

Short-chain fatty acid (SCFA) concentrations of the *in vitro* static batch cultures were determined using capillary gas chromatography (GC), as previously described[54] by Mountzouris *et al.*[55] The samples (1 ml) were centrifuged at 13 000*g* for 15 min at 4 °C and 300 μl of the supernatants were transferred into fresh, sterile microcentrifuge tubes and stored at −80 °C until analysis. On the day of analysis, the supernatants (300 μl) were vortexed and centrifuged at 13 000*g* for 5 min at room temperature. Subsequently, 85 μl of each supernatant was mixed with 10 μl of 2-ethyl-butyrate (20 mM, internal standard) (2-ethyl butyric acid 99%, Sigma-Aldrich C., USA) and 5 μl of hydrochloric acid (HCl, 1 M). 1 μl of samples were injected into a gas chromatograph (Agilent 6890GC System, Agilent Technologies) using a Supelco Nukol™ Capillary GC Column (size × I.D. 30 m × 0.25 mm, df 0.25 μm) (Sigma-Aldrich C., USA). Chromatography was performed with an injection split ratio of 1 : 25, isothermal at 185 °C with injector and detector temperatures set to 200 °C and 220 °C, respectively. The concentrations of SCFAs were computed based on instrument calibration with the SCFA standard mixture (Supelco Volatile Acid Standard mix, Sigma-Aldrich C., USA). The total volatile fatty acid (VFA) and SCFA concentrations were expressed as μmol ml^{-1} of sample and the molar ratios of acetate, propionate, butyrate, branched-chain SCFAs (iso-butyrate, iso-valerate, and iso-caproic acid) and other SCFAs (valerate, caproic acid and heptanoic acid) were also calculated. The differences (Δ) in the concentrations (μmol mL^{-1}) of total VFAs, major SCFAs (acetate, propionate, and butyrate) and minor SCFAs (BSCFAs and other SCFAs) after 8 h of fermentation (ΔCt8-0) and 24 h of fermentation (ΔCt24-0) in comparison with the baseline were further calculated as previously described.[46]

2.9 Viability of MG-63 cell line and quantification of bone metabolism parameters

MG-63 is an osteosarcoma cell line with osteoblastic phenotype, consisting of oval-spindle shape cells without branching extensions, having a doubling rate of about 24 h, which was obtained from the American Type Culture Collection (ATCC, Bethesda, MD). This cell line was grown in 75 cm^2 culture flasks at 37 °C under 5% CO_2 using Roswell Park Memorial Institute-1640 Medium (RPMI-1640, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA).

The fermentation supernatants that were collected initially (0 h) and after 24 h of fermentation were diluted 1 : 8 for the treatment of human MG-63 cells.[56–59] Cells were seeded at a density of 1500 per well into a 96-well plate. After 24 h, the cells were treated with the fermentation supernatants for 48 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma M-5655) was added at a concentration of 5 mg mL^{-1} directly to each well for 4 h at 37 °C. The medium was aspirated and the blue MTT formazan precipitate was dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm using a Powerwave microplate spectrophotometer (Biotek Instruments, Inc., Winooski, Vermont, USA) and the cell viability results are presented as the percent of OD in the treated wells *versus* the controls.[60]

The ELISA method was used for the quantitative determination of human OPG (Osteoprotegerin) (Human OPG ELISA Kit, ref. EA100335) and human RANKL (Receptor activator of Nuclear factor kappa-B ligand) (Human TNFSF11/RANKL ELISA Kit, ref. EA100531) (OriGene Technologies Inc., MD, USA) in MG-63 cells' supernatant, in order to identify the effect of the processed fermentation products on these parameters according to the manufacturer's protocol. The 96-well microplates filled with the cell culture medium DMEM-10% FBS were treated with the fermentation supernatants of GLBS, GLBSE, POWS and POWSE in a dilution of 1 : 8 and were incubated for 48 h, while the cell culture medium (control) was used as the negative control and inulin (INU2) as the positive control. The absorbance was measured at 450 nm using a Powerwave microplate spectrophotometer (Biotek Instruments, Inc.).

2.10 Statistical analysis

Continuous variables are presented as mean values ± standard deviation or median and interquartile range (Q1–Q3). Categorical variables are presented as frequencies. Associations between the categorical variables were tested using the chi-square test. Comparison of the tested variables (*e.g.* bacterial levels and SCFAs) was performed for the whole six-plicate experiment and according to the bone health status (normal, n = 3; osteopenic, n = 3), respectively. The normality of the distribution of variables was tested by the Shapiro–Wilk test. The bacterial levels (t = 0 h and 24 h) and SCFA characteristics (t = 0 h, 8 h and 24 h) were compared prospectively by

Repeated Measures ANOVA (RM-ANOVA) for parametric data and by the Friedman test for non-parametric data with *post hoc* (Tukey's HSD test) and parameter estimates analyses. Comparisons of the bacterial levels and SCFA characteristics after each treatment (NC, INU1, INU2, POWS, POWSE, GLBS, and GLBSE) for different time periods (0 h, 8 h, and 24 h) were performed by the paired-sample *t* test for parametric data and by the Wilcoxon signed rank test for non-parametric data. For prebiotic indexes, the Kruskal Wallis and Mann–Whitney tests were performed. For differences in the SCFA concentrations (ΔCt8-0 or ΔCt24-0), parametric and non-parametric tests were performed (the Kruskal Wallis test, *t*-test, and Mann–Whitney test). The software program IBM® SPSS® Statistics version 21 was used for the statistical analysis of the results and the significance threshold was set at 5% ($p < 0.05$).

3 Results and discussion

Based on glucan analysis, it was found that both powdered mushrooms and their extracts were high in beta-glucan content. Specifically, the beta-glucan content of GLBS was 35.83 ± 2.05% w/w of dry weight, whereas higher levels were detected in the extract (the beta-glucan content of GLBSE: 47.70 ± 0.91% w/w of dry weight). Regarding POWS and POWSE, a similar beta-glucan content was detected in both cases (POWS: 30.64 ± 2.45% w/w of dry weight; and POWSE: 28.80% ± 0.67% w/w of dry weight), indicating that the extraction methodology was not effective at purifying beta-glucans of *P. ostreatus*.[39–42]

3.1 Descriptive characteristics of faecal donors

Healthy subjects and participants with osteopenia (faecal donors) were comparable in terms of their baseline characteristics, including sociodemographic factors, anthropometric indices, biochemical measurements, physical activity levels and dietary intake. Higher protein intake (%) in healthy subjects was probably due to seasonal differences of dietary intake. *Z*-Scores between the two groups were significantly different as expected ($p = 0.048$), even though *T*-scores showed no difference ($p = 0.114$) since the subjects with osteopenia were just diagnosed in their annual check-up (Table 1). Significantly higher levels of insulin and consequently HOMA-IR were also detected in osteopenic subjects (ESI, Table S2†).

3.2 Effect of mushroom fermentation on the microbiota composition

Overall, no significant differences were detected among different treatments in terms of tested bacteria based on the main effects (Table 2 and Tables S3a, S3b†) and *post-hoc* analysis, though a trend was detected in the case of bacteroides in all subjects ($p = 0.059$) (Table 2) and bifidobacteria in osteopenia ($p = 0.096$) (Table S3b†). Time had a significant effect in the case of lactobacilli ($p < 0.001$), *Roseburia* spp.–*E. rectale* ($p < 0.001$) and likely *C. leptum* group ($p = 0.080$), whereas a significant time × treatment interaction was detected in the case of *Bifidobacterium* spp. ($p = 0.006$) and likely *Bacteroides* spp. ($p = 0.055$), lactobacilli ($p = 0.068$) and *Roseburia* spp.–*E. rectale* ($p = 0.099$). In healthy subjects, a significant time effect was detected in the case of lactobacilli and *Roseburia* spp.–*E. rectale*, with a trend for time × treatment interaction in the case of lactobacilli ($p = 0.093$). In osteopenic subjects time had significant effects on bifidobacteria, lactobacilli, *C. leptum* group and *C. perfringens* ($p = 0.052$), with significant time × treatment interactions in the case of total bacteria, bifidobacteria and likely bacteroides ($p = 0.099$).

POWS fermentation resulted in an increase of total bacteria, *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* counts after 24 h of fermentation compared to the baseline (Table 2). Based on the parameter estimates (Table 2), the butyrate producer *F. prausnitzii* increased after POWS fermentation relatively to NC. In addition, the levels of *Bacteroides* spp. after 24 h fermentation of POWS and counts of *Bifidobacterium* spp. and *Lactobacillus* group after 24 h fermentation of GLBS were significantly lower than INU2 in all volunteers (Table 2). The positive controls INU1 and INU2 exhibited increased the counts of total bacteria, *Lactobacillus* group, *Bifidobacterium* spp. and *Bacteroides* spp. after 24 h of fermentation compared to the NC (Table 2).

In healthy subjects, 24 h fermentation of POWS increased the level of total bacteria compared to the NC ($p = 0.012$) and decreased the count of *Bacteroides* spp. compared to INU1 ($p = 0.034$) and INU2 ($p = 0.034$) (ESI, Table S3a†).

In the osteopenic group, POWS fermentation ended in higher levels of total bacteria, *Bifidobacterium* spp. ($p = 0.004$), and *F. prausnitzii* ($p = 0.010$) than in the NC group. *F. prausnitzii* counts were also increased after 24 h fermentation of POWS compared to INU1 ($p = 0.014$) and INU2 ($p = 0.034$). Moreover, the fermentation of POWSE demonstrated higher populations of total bacteria, *F. prausnitzii* and *Bacteroides* spp. than the NC.

Bacteroides spp. increased after 24 h of fermentation compared to the baseline in this substrate, while *Roseburia* spp. – *E. rectale* decreased. GLBS fermentation resulted in the lower levels of *Bifidobacterium* spp. ($p = 0.025$) than INU2. In addition, the use of GLBSE exhibited higher populations of *Bifidobacterium* spp. ($p = 0.047$) and *Bacteroides* spp. ($p = 0.019$) than the NC at 24 h. *Roseburia* spp. – *E. rectale* decreased after 24 h of fermentation compared to the baseline in both substrates ($P_{GLBS} = 0.005$ and $P_{GLBSE} = 0.043$) (ESI, Table S3b†). Our results were in agreement with those of previous studies regarding bacterial changes after fermentation of inulin.[61]

In vitro studies have proved the prebiotic potential of inulin-type fructans after fermentation by human cecal or faecal inoculum, leading to a significant increase of *Bifidobacterium* spp. and *Lactobacillus* spp.[62] Nevertheless, several members of the *Lachnospiraceae* and *Ruminococcaceae* families in the phylum *Firmicutes* also increased after inulin fermentation.[63] These two families include most of the known butyrate producers,[64] such as *Eubacterium rectale*, *Roseburia*

Table 1 Descriptive characteristics of faecal donors

	Total (n = 6)	Normal bone health (n = 3)	Osteopenia (n = 3)	p-value
Anthropometric measurements				
Age (years)	56.17 ± 3.65	58.33 ± 3.51	54.00 ± 2.65	0.163
Body weight (kg)	63.17 ± 7.39	65.00 ± 10.82	61.33 ± 3.06	0.602
Height (m)	1.64 ± 0.05	1.62 ± 0.02	1.65 ± 0.07	0.432
BMI (kg m^{-2})	23.66 ± 2.86	24.80 ± 3.40	22.52 ± 2.22	0.384
T-Score	−0.98 ± 1.83	0.20 ± 1.47	−2.17 ± 1.41	0.114
Z-Score	0.03 ± 1.66	1.27 ± 1.07	−1.20 ± 1.08	0.048*
BMD	0.93 ± 0.20	1.08 ± 0.18	0.78 ± 0.72	0.055
Nutritional parameters				
Total energy Intake (kcal d^{-1})	2036.1 ± 718.34	1624.55 ± 551.64	2447.83 ± 690.99	0.182
Protein intake (%)	13.20 (11.98–16.15)	16.00 (15.10–16.30)	12.30 (11.70–12.30)	0.046*
Protein intake (g d^{-1})	68.89 ± 17.15	63.67 ± 16.55	74.11 ± 19.48	0.518
Carbohydrate (% of energy)	43.80 ± 7.82	40.77 ± 9.31	46.83 ± 6.20	0.401
Carbohydrate (g d^{-1})	227.82 ± 93.17	162.34 ± 25.80	293.29 ± 90.41	0.073
Dietary fiber intake (g d^{-1})	24.24 ± 9.07	20.94 ± 8.30	27.54 ± 10.21	0.434
Fat intake (%)	40.62 ± 6.07	40.30 ± 7.39	40.93 ± 6.11	0.914
Fat intake (g d^{-1})	96.02 ± 40.69	77.65 ± 40.35	114.39 ± 38.73	0.319
Calcium intake (mg d^{-1})	822.85 ± 530.98	773.65 ± 597.90	872.05 ± 583.19	0.848
Vitamin D intake (μg d^{-1})	0.92 (0.61–5.05)	1.09 (0.85–10.12)	0.75 (0.44–2.90)	0.513
Biochemical measurements				
Ca serum (mg dL^{-1})	9.40 ± 0.25	9.47 ± 0.32	9.33 ± 0.21	0.579
BALP serum (μg dL^{-1})	15.10 ± 2.30	16.30 ± 1.05	13.87 ± 2.75	0.220
Osteocalcin serum (ng mL^{-1})	29.20 ± 5.38	32.20 ± 5.90	26.20 ± 3.27	0.198
Vitamin D serum (ng mL^{-1})	18.95 ± 6.06	18.53 ± 6.15	19.37 ± 8.42	0.897

BMI: Body Mass Index; BMD: Bone Mass Density; Ca: serum calcium; BALP: Bone specific Alkaline Phosphatase; values are expressed as mean and standard deviation (SD) for parametric or median and interquartile range (Q1–Q3) for non-parametric data. * significantly different ($p < 0.05$).

spp. and *F. prausnitzii*, all of which are capable of producing butyrate from inulin fermentation.[63,65] In our study, both positive controls showed powerful bifidogenic and lactogenic effects, especially INU2, which was also accompanied by higher *F. prausnitzii* levels compared to the NC.

With respect to different mushrooms, our data support that POWS fermentation increases the bifidobacteria and *F. prausnitzii* levels, a phenomenon that is more evident in osteopenic women. Mitsou *et al.* have recently examined the *in vitro* fermentation of different edible mushrooms using the same methodology while they used different faecal donors (men and women over 65 years old); increase of the same microbial populations has been also noticed after the *in vitro* fermentation of two different strains of *P. ostreatus*.[46] In another paper where the *in vitro* fermentation of edible mushrooms was also studied, the authors highlighted the different impacts of various mushroom strains on the gut microbial populations.[66] *P. ostreatus* and *P. eryngii* mushrooms promoted the growth of *Bifidobacterium* spp. compared to the control group, while they did not favor the growth of *Lactobacillus* spp., as it was also noticed in the present study. Despite the similar research approach adopted by both studies involving *in vitro* fermentation of *Pleurotus* mushroom samples, the type of participants (young healthy faecal donors) and the methodology used (16SrRNA sequencing for microbial analysis) may explain the differences observed in the growth of other microbial groups (*i.e.*, *F. prausnitzii*).[66] The POWSE treatment appears to significantly impact *F. prausnitzii* and *Bacteroides* spp., but only in the group of osteopenic women. According to these results, the *P. ostreatus* mushroom powder has a more beneficial effect on the gut microbial populations than the respective mushroom-extract. In another relevant study where polysaccharide extracts from *P. ostreatus* and *P. eryngii* mushrooms were studied for their impact on the gut microbiota *in vitro*, a significant increase of *Lactobacillus* and *Bifidobacterium* species as well as *Enterococcus faecium* was observed.[42] Furthermore, the consumption of *P. eryngii* polysaccharide extracts by mice strongly influenced their gut microbiota and increased the abundance of *Porphyromonadaceae*, *Rikenellaceae*, *Bacteroidaceae* and *Lactobacillaceae*.[67] Even though the methodological approach was different from that used in the present study, the results were rather similar since *Bacteroides* spp. are the main members of the family *Bacteroidaceae* in the gut.

GLBS fermentation had no significant effect on the initial levels of bifidobacteria and lactobacilli (Table 3). However, in the osteopenic group the fermentation of GLBSE (t = 24 h) exhibited significantly higher bifidobacterial counts than in the NC group. Based on the results concerning the effect of *G. lucidum*, the mushroom-extract exhibits a more favorable effect on the gut microbial populations than the mushroom powder. Our results were in accordance with the outcome of previous studies that reported the promising prebiotic activity of *Ganoderma* mushroom-extracts.[35,68] Recently Khan *et al.* has

Table 2 Faecal microbial quantification (qPCR; $\log_{10}$ copies of 16S rRNA gene per mL of sample) at the baseline and after 24 h fermentation of *Pleurotus ostreatus* IK1123 and *Ganoderma lucidum* LGAM 9720 in all volunteers

	TOTAL (n = 6)														
	Baseline (t = 0 h)							After fermentation (t = 24 h)							
overall	NC	INU1	INU2	POWS	GLBS	POWSE	GLBSE	NC	INU1	INU2	POWS	GLBS	POWSE	GLBSE	P
Total bacteria	10.08 (9.86–10.35)	10.10 (9.93–10.32)	10.05 (9.92–10.33)	9.97 (9.89–10.30)	9.96 (9.89–10.25)	10.11 (9.95–10.40)	10.08 (9.82–10.33)	10.00 (9.88–10.11) †, ‡	10.33 (10.06–10.54) *	10.38 (10.11–10.47) *	10.45 (10.23–10.49) *[a]	10.13 (9.99–10.17)	10.21 (10.07–10.35)	10.16 (9.91–10.23)	0.407
Bifidobacterium spp.	8.69 (7.52–9.10)	8.69 (7.52–9.16)	8.64 (7.49–9.00)	8.60 (7.51–9.07)	8.65 (7.51–9.14)	8.67 (7.52–9.17)	8.68 (7.42–9.20)	8.81 (7.75–9.15) †, ‡	9.75 (9.729.96)*[a]	9.94 (9.82–10.21) *[a]	9.75 (8.56–9.95)[a]	9.25 (7.33–9.57)†	9.20 (7.59–9.51)†	9.33 (7.81–9.94)	0.875
Lactobacillus group	6.28 ± 0.97	6.27 ± 0.88	6.23 ± 0.96	6.20 ± 0.91	6.03 ± 0.90	6.36 ± 0.90	6.24 ± 0.94	6.34 ± 0.93 †	7.35 ± 0.83[a]	7.60 ± 1.04*[a]	6.90 ± 1.19	6.16 ± 1.23‡	6.64 ± 1.00	7.07 ± 1.01	0.734
Clostridium perfrigens group	5.90 (5.71–7.01)	6.07 (5.77–7.03)	6.04 (5.77–7.05)	6.27 (6.05–7.01)	5.42 (4.93–6.69)	6.08 (5.85–7.09)	6.21 (5.79–7.03)	6.10 (5.73–6.94)	6.03 (5.53–7.17)	6.08 (5.64–7.35)	6.47 (5.93–7.21)	6.08 (5.31–6.90)	6.51 (5.68–7.32)	5.98 (5.41–7.28)	0.810
Bacteroides spp.	9.43 ± 0.23	9.43 ± 0.16	9.43 ± 0.25	9.45 ± 0.23	9.39 ± 0.23	9.49 ± 0.20	9.43 ± 0.24	9.21 ± 0.29 †, ‡	9.60 ± 0.27*	9.63 ± 0.35*	9.19 ± 0.30†, ‡	9.45 ± 0.27	9.75 ± 0.15*[a]	9.44 ± 0.20	0.059
Clostridium leptum group	9.20 ± 0.43	9.22 ± 0.38	9.20 ± 0.44	9.18 ± 0.39	9.14 ± 0.47	9.32 ± 0.40	9.27 ± 0.45	9.12 ± 0.43	9.03 ± 0.54	9.13 ± 0.53	9.31 ± 0.47	9.11 ± 0.44	9.22 ± 0.41	9.04 ± 0.39	0.995
Faecalibacterium prausnitzii	8.52 (7.66–9.03)	8.56 (7.80–8.89)	8.51 (7.84–9.00)	8.61 (7.81–8.97)	8.58 (7.61–8.95)	8.60 (7.86–8.97)	8.54 (7.78–8.98)	8.12 (7.53–8.40) ‡	8.81 (7.99 8.87)	8.86 (8.32–8.94)*	8.92 (8.70–9.24)*[a]	8.65 (7.67–8.73)	8.80 (8.40–8.94)	8.48 (7.80–8.74)	0.763
Roseburia spp.–*Eubacterium rectale*	8.39 ± 0.58	8.35 ± 0.58	8.39 ± 0.60	8.35 ± 0.51	8.43 ± 0.59	8.44 ± 0.54	8.4 ± 0.60	7.62 ± 0.42[a]	8.11 ± 0.83	8.10 ± 0.78	8.02 ± 0.76	7.67 ± 0.44[a]	7.79 ± 0.43[a]	7.91 ± 0.69[a]	0.989

Values are expressed as mean value and SD for parametric and median and interquartile range (Q1–Q3) for non-parametric data; *: significantly different compared to [NC] at t = 24 h ($p < 0.05$); †: significantly different compared to [INU1] at t = 24 h ($p < 0.05$); ‡: significantly different compared to [INU2] at t = 24 h ($p < 0.05$); a: significantly different compared to the baseline ($p < 0.05$) (paired-sample t-test or Wilcoxon signed-rank test); p-overall refers to the tests of between-subjects effects and symbols *,†, and ‡ refer to parameter estimates at t = 0 h and 24 h (repeated-measures ANOVA or Friedman test; NC: Negative control; INU1: Inulin 1% (w/v); INU2: Inulin 2% (w/v); POWS: *P. ostreatus* untreated mushroom powder; POWSE: *P. ostreatus* mushroom-extract; GLBS: *G. lucidum* untreated mushroom powder; GLBSE: *G. lucidum* mushroom-extract.

Table 3 Total volatile fatty acid (VFAs) concentrations (μmol mL^{-1} of sample) and molar ratios (%) of SCFAs at the baseline and after 8 h and 24 h of fermentation in all subjects

Total (n = 6)

Baseline (t = 0 h)

		Molar ratios of SCFAs			Molar ratios of branched-chain SCFAs (%)†				Molar ratios of other SCFAs (%)‡			
	Total VFAs (μmol mL^{-1} of sample)	Acetate (%)	Propionate (%)	Butyrate (%)	Branched-chain SCFA (%)‡	Iso-butyrate (%)	Iso-valerate (%)	Iso-caproic acid (%)	Other SCFA (%) ¥	Valerate (%)	Caproic acid (%)	Heptanoic acid (%)
NC	4.38 †, ‡ (3.96–4.34)	34.83 (31.08–48.48)	12.94 (10.75–14.16)	30.03‡ (20.61–43.17)	7.91 (5.60–12.18)	2.04 (1.04–3.21)	3.01 (2.08–6.08)	2.85 (1.18–4.56)	5.94 (9.55–12.13)	4.02 (2.54–5.47)	0.96 (0.42–2.78)	0.87 (0.39–1.77)
INU1	2.41* (1.89–2.71)	37.44 (34.06–43.43)	13.62 (11.91–17.05)	31.29‡ (24.97–39.47)	9.21 (5.71–9.96)	1.95 (1.56–2.46)	2.89 (1.94–3.60)	3.67 (1.06–5.04)	6.50 (4.26–9.63)	3.89 (2.70–4.51)	1.02 (0.83–2.99)	0.80 (0.71–1.00)
INU2	2.58* (2.21–3.14)	31.30† (28.69–33.45)	11.74 (9.05–15.46)	45.34*,† (38.13–48.67)	6.33 (5.15–7.71)	1.50 (0.99–2.39)	2.31 (1.88–3.43)	2.47‡ (0.92–3.50)	4.72 (4.44–7.74)	3.04 (2.91–3.16)	0.88 (0.78–2.28)	0.72 (0.65–0.87)
POWS	2.93†(2.79–3.84)	32.48 (30.78–34.53)	8.36*,† (5.85–11.77)	47.90*,† (46.50–52.88)	5.25*,† (4.36–5.70)	1.07*,† (0.90–1.45)	2.62 (2.34–3.00)	0.69 *,†,‡ (0.62–1.7)	3.79 (3.55–5.35)	2.42*,†,‡ (2.25–2.47)	0.70 (0.62–1.62)	0.58 (0.56–0.69)
GLBS	2.74 † (2.51–3.79)	32.93 (27.40–36.00)	9.84*,† (7.02–11.39)	48.85*,†,‡ (45.17–56.76)	4.60*,†,‡ (4.08–5.38)	1.90 (1.21–2.14)	2.10 (1.79–2.25)	0.69*,†,‡ (0.61–0.89)	3.64*,† (3.37–4.39)	2.09*,†,‡ (1.69–2.28)	0.73 (0.67–1.36)	0.62 (0.60–0.69)
POWSE	2.64 (2.41–3.07)	35.35 (30.75–37.30)	10.64† (9.69–11.78)	45.58*,† (40.76–48.99)	4.68*,† (3.82–6.02)	1.60 (1.09–1.99)	2.51 (1.91–3.36)	0.76*,†,‡ (0.57–0.82)	4.56‡ (4.08–5.38)	2.73 (2.50–3.28)	0.81 (0.72–0.83)	0.74 (0.66–0.75)
GLBSE	2.16* (2.12–2.68)	35.95‡ (33.84–37.21)	11.42 (9.52–13.85)	44.08*,† (40.01–46.74)	4.12*,†,‡ (3.54–6.25)	1.52 (1.27–2.00)	1.75 (1.36–3.52)	0.84*,† (0.65–0.95)	4.82 (4.62–5.76)	3.08 (2.71–3.62)	0.90 (0.81–0.98)	0.76 (0.63–0.87)

After fermentation (t = 8 h)

		Molar ratios of SCFAs			Molar ratios of branched-chain SCFAs (%)†				Molar ratios of other SCFAs (%)‡			
	Total VFAs (μmol mL^{-1} of sample)	Acetate (%)	Propionate (%)	Butyrate (%)	Branched-chain SCFA (%)‡	Iso-butyrate (%)	Iso-valerate (%)	Iso-caproic acid (%)	Other SCFA (%) ¥	Valerate (%)	Caproic acid (%)	Heptanoic acid (%)
NC	16.59†,‡[a] (11.34–17.32)	47.65 (41.65–48.52)	16.00 (15.18–21.52)[a]	25.90 (24.40–27.42)	5.60†, ‡ (4.28–9.36)	1.47 (1.10–2.64)	4.12†, ‡ (2.98–5.91)	0.15†,‡[a] (0.13–0.26)	5.94 †, ‡ (3.48–9.33)	3.80†, ‡ (2.43–4.06)	0.38† (0.12–1.00)	0.14†,‡[a] (0.11–0.22)
INU1	38.11*[a] (30.13–44.58)	50.94[a] (46.59–54.68)	14.47 (10.54–30.14)	33.23 (11.79–33.51)	1.46*[a] (0.77–2.10)	0.94[a] (0.39–1.65)	0.39*[a] (0.37–0.43)	0.05*[a] (0.04–0.06)	0.86*[a] (0.75–1.01)	0.72*[a] (0.51–0.85)	0.05*[a] (0.04–0.23)	0.04*[a] (0.04–0.06)
INU2	37.28*[a] (25.43–43.55)	50.09[a] (44.37–69.40)	14.3 (11.01–16.29)	23.71[a] (17.55–36.65)	1.67*[a] (1.15–2.26)	1.19 (0.81–1.90)	0.34*,‡[a] (0.29–0.41)	0.05*,‡[a] (0.05–0.07)	0.77*[a] (0.68–0.85)	0.61*[a] (0.43–0.76)	0.06[a] (0.05–0.23)	0.05*[a] (0.04–0.06)
POWS	47.80*[a] (35.11–55.79)	41.72[a] (39.45–58.56)	18.07[a] (14.90–28.72)	27.67[a] (15.44–41.97)	1.57*[a] (1.04–2.73)	1.07 (0.57–2.12)	0.34*[a] (0.33–0.49)	0.05*[a] (0.04–0.19)	0.64*[a] (0.49–0.99)	0.55*[a] (0.35–0.91)	0.04*[a] (0.03–0.05)	0.04*[a] (0.03–0.05)
GLBS	30.20*[a] (26.21–39.32)	42.55†[a] (41.20–46.19)	23.08‡[a] (19.56–25.58)	27.18[a] (22.66–32.44)	2.85†,‡ (2.26–5.19)	1.17 (0.75–1.63)	2.09†, ‡ (0.86–3.48)	0.06[a] (0.05–0.07)	2.46†, ‡ (1.50–3.22)	2.24†, ‡ (1.31–3.07)	0.11[a] (0.05–0.31)	0.05*[a] (0.05–0.06)
POWSE	42.66*[a] (33.91–48.68)	46.48[a] (38.71–49.42)	22.17‡[a] (19.69–33.78)	25.29[a] (23.40–27.90)	2.08*,† (1.71–2.90)	1.17 (0.83–1.60)	0.82†,‡ (0.66–1.13)	0.07*[a] (0.04–0.17)	1.65*,†,‡[a] (0.96–2.08)	1.48*,‡[a] (0.80–1.99)	0.05*[a] (0.04–0.23)	0.04*[a] (0.03–0.05)
GLBSE	39.93*[a] (30.44–42.28)	48.32[a] (46.86–50.24)	20.48[a] (15.34–23.16)	26.23[a] (17.00–37.19)	1.59*[a] (1.25–2.84)	0.93 (0.57–1.25)	0.50*,†[a] (0.42–0.68)	0.15†[a] (0.05–0.03)	1.09*[a] (0.73–2.00)	0.98*[a] (0.58–1.90)	0.05[a] (0.05–0.06)	0.05*[a] (0.04–0.06)

After fermentation (t = 24 h)

		Molar ratios of SCFAs			Molar ratios of branched-chain SCFAs (%)†				Molar ratios of other SCFAs (%)‡			
	Total VFAs (μmol mL^{-1} of sample)	Acetate (%)	Propionate (%)	Butyrate (%)	Branched-chain SCFA (%)‡	Iso-butyrate (%)	Iso-valerate (%)	Iso-caproic acid (%)	Other SCFA (%) ¥	Valerate (%)	Caproic acid (%)	Heptanoic acid (%)
NC	23.68†,‡^{b,c} (19.52–26.24)	44.52‡ (38.89–47.41)	14.85 (12.64–18.65)	25.35 (22.19–26.25)	10.09 †,‡^{b} (9.55–10.13)	3.44 †,‡^{b} (3.41–3.52)	6.24†,‡^{b,c} (6.15–6.57)	0.45 (0.07–0.76)	4.70†,‡ (2.73–8.99)	3.76†,‡ (3.13–4.18)	0.87†, ‡ (0.39–1.77)	0.24 †, ‡, c (0.07–0.71)
INU1	43.11*^{b,c} (38.38–61.90)	50.12 (46.60–60.75)	16.00 (10.93–19.12)	28.23 (10.53–37.19)	1.48*^{c} (1.21–2.05)	0.93*^{c} (0.44–1.46)	0.44*^{c} (0.38–0.50)	0.21^{c} (0.03–0.32)	0.75*^{c} (0.61–0.88)	0.63*^{c} (0.52–0.71)	0.04*^{c} (0.03–0.06)	0.04*^{c} (0.03–0.06)
INU2	36.88*^{c} (31.58–61.07)	57.04*^{c} (47.10–69.42)	13.28 (10.69–18.59)	20.39^{b,c} (12.55–33.02)	1.00*^{b,c} (0.80–1.56)	0.57*^{b,c} (0.26–1.18)	0.31*,†,‡^{c} (0.20–0.37)	0.16^{c} (0.04–0.38)	4.72*^{c} (4.44–7.74)	0.53*,‡^{c} (0.37–0.64)	0.05*^{c} (0.04–0.06)	0.05*^{c} (0.03–0.06)
POWS	76.06*,†,‡^{b,c} (69.36–81.49)	37.28*,†,‡^{c} (35.91–38.61)	15.55^{c} (14.81–19.45)	43.47*,†,‡^{b,c} (40.02–46.55)	1.15*^{b,c} (0.79–1.23)	0.78*^{b} (0.29–1.24)	0.38*^{c} (0.20–0.51)	0.11 *^{c} (0.03–0.11)	0.52*^{c} (0.41–1.00)	0.43*^{c} (0.32–0.95)	0.03*^{c} (0.03–0.11)	0.02*,†,‡^{b,c} (0.02–0.02)
GLBS	49.61*^{b,c} (44.25–54.03)	39.64†,‡^{c} (36.14–42.54)	20.50‡^{c} (18.71–22.18)	27.52^{c} (25.32–29.17)	7.91†, ‡^{b,c} (6.91–8.19)	2.47‡^{b} (1.85–3.09)	4.98*,†,‡^{b,c} (4.76–5.33)	0.17^{c} (0.04–0.22)	4.14†,‡,^{b} (2.74–6.28)	3.21†,‡^{c} (2.67–4.33)	0.78 (0.04–1.53)	0.16^{c} (0.03–0.42)
POWSE	60.27*,†,‡^{b,c} (57.11–62.20)	39.27†,‡,^{b} (34.14–42.84)	24.46*,†,‡^{c} (22.41–28.46)	30.31^{b,c} (28.08–33.26)	3.10*,†,‡^{c} (2.24–3.83)	0.98*,† (0.70–1.58)	1.66*,†,‡^{b} (1.13–2.22)	0.24^{c} (0.06–0.39)	2.17†,‡^{c} (1.58–3.10)	2.01†,‡^{c} (1.52–2.65)	0.11^{c} (0.04–0.21)	0.05*^{c} (0.03–0.11)
GLBSE	47.06*^{b,c} (45.46–48.21)	44.1 (41.60–48.71)	18.44^{c} (14.23–21.09)	29.77^{b,c} (19.34–39.36)	2.40*,‡ (1.03–4.57)	1.51* (0.42–2.30)	0.74*,‡ (0.38–1.17)	0.19^{c} (0.05–0.27)	1.15*,†,‡ (0.75–2.06)	0.96*,† (0.68–1.97)	0.05 (0.05–0.23)	0.04*^{c} (0.04–0.06)
P overall	<0.001	0.008	0.525	0.054	<0.001	0.016	<0.001	0.002	<0.001	<0.001	0.066	<0.001

Values are expressed as median and interquartile range (Q1–Q3) for non-parametric data; ‡ Sum of iso-butyrate, iso-valerate and iso-caproic acid; ¥ Sum of valerate, caproic acid and heptanoic acid; *: significantly different compared to [NC] at t = 0 h, 8 h or 24 h (p < 0.05); †: significantly different compared to [INU1] at t = 0 h, 8 h or 24 h (p < 0.05); ‡: significantly different compared to [INU2] at t = 0 h, 8 h or 24 h (p < 0.05); a: significantly different from the baseline compared to after 8 h of fermentation (p < 0.05) (paired sample t-test or Wilcoxon for non-parametric); b: significantly different from after 8 h of fermentation compared to after 24 h of fermentation (p < 0.05) (paired sample t-test or Wilcoxon for non-parametric); c: significantly different from the baseline compared to after 24 h of fermentation (p < 0.05) (paired sample t-test or Wilcoxon for non-parametric); p-overall refers to the tests of between-subjects effects and symbols *,†, and ‡ refer to parameter estimates at t = 0 h, 8 h and 24 h (repeated-measures ANOVA or the Friedman test); NC: Negative control; INU1: Inulin 1% (w/v); INU2: Inulin 2% (w/v); POWS: *P. ostreatus* untreated mushroom powder; POWSE: *P. ostreatus* mushroom-extract; GLBS: *G. lucidum* untreated mushroom powder; GLBSE: *G. lucidum* mushroom-extract.

observed high levels of beneficial bacteria (*e.g. Bifidobacterium choerinum*, *Lactobacillus johnsonii*, and *Lactococcus lactis*) in the mice group, which was fed with *G. lucidum* mushroom-extract, compared to the control group.[69] Similar results in the growth of bacteria of the genera *Bifidobacterium*, *Lactobacillus* and *Bacteroides* were also reported after 24 h fermentation of GLBSE using faecal material from healthy donors.[70] However, Chang *et al.*, who tested water extracts from *G. lucidum* mycelia in mice, did not notice an increase in the *Bifidobacterium* spp. levels, but noticed an enhancement in the growth of *Clostridium* clusters IV, XVIII and XIVa.[71]

3.3 Prebiotic effect of the mushroom substrates after 24 h of fermentation

The prebiotic indexes of INU1, INU2, POWS and GLBSE increased significantly in all volunteers after 24 h of fermentation compared to the NC (Fig. 1). Furthermore, no significant differences were detected in the PI values of GLBSE with respect to the positive controls (INU1 and INU2) (Fig. 1). POWS and GLBSE were characterized by positive prebiotic indexes in both groups of bone health status, while GLBSE demonstrated the highest PI values in both total and bone health groups (Fig. 1 and ESI, Fig. S2A, B†). When these results are associated with bacterial compositional analysis, our data suggest that the high counts of *Bifidobacterium* after POWS and GLBSE treatments clearly demonstrated an advantageous prebiotic effect, especially in the osteopenic group.

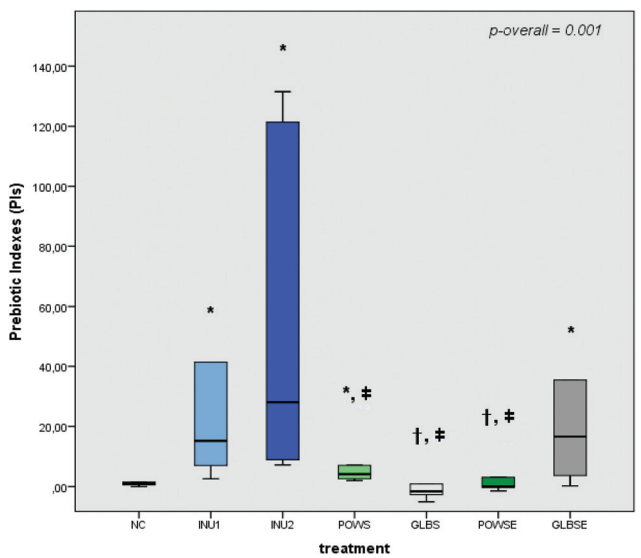

Fig. 1 Prebiotic indexes (PIs) for all volunteers. NC: Negative control; INU1: Inulin 1% (w/v); INU2: Inulin 2% (w/v); POWS: *P. ostreatus* untreated mushroom powder; POWSE: *P. ostreatus* mushroom-extract; GLBS: *G. lucidum* untreated mushroom powder; GLBSE: *G. lucidum* mushroom-extract; *: significantly different compared to [NC] ($p < 0.05$) (Mann–Whitney test); †: significantly different compared to [INU1] ($p < 0.05$) (Mann–Whitney test); ‡: significantly different compared to [INU2] ($p < 0.05$) (Mann–Whitney test); data are presented as boxplots, with the horizontal line representing the median and the whiskers the minimum and maximum values.

3.4 Impact of mushroom substrates on faecal SCFAs

Although at the baseline (0 h) the total concentration of SCFAs was quite low in all cases examined (approximately 2–4 μmol mL^{-1} culture), after 8 h and particularly after 24 h of fermentation all substrates were characterised by higher SCFA concentration changes ($p < 0.05$) compared to the negative control.

Overall, the treatments resulted in significant differences in the total concentration and molar ratios of SCFAs in all subjects, except in the case of propionate (Table 3). Nevertheless, overall analysis in the osteopenic group indicated differences among treatments in cases of propionate ($p = 0.078$), butyrate ($p = 0.083$) and iso-butyrate ($p = 0.015$), a fact not evident in healthy subjects (Tables S4a and S4b†). Time had significant effects on the osteopenic group (priopionate, valerate, and BSCFAs) and on healthy subjects (iso-caproic acid). Furthermore, significant time × treatment interactions were detected in total SCFAs (all subjects and healthy group), propionate (all subjects), butyrate (all subjects and osteopenic group), iso-butyrate (all subjects), iso-valerate (all subjects and healthy group), iso-caproic acid (all subjects), BSCFAs (all subjects and osteopenic group), valerate (all subjects, and healthy and osteopenic groups) and other SCFAs (all subjects and healthy group) ($p<0.05$ for all).

Post hoc analysis indicated higher SCFA concentration after POWS treatment compared to the NC in all cases and when compared to the rest of the treatments (except POWSE) in all subjects and the healthy group. The POWSE substrate also demonstrated higher levels of SCFAs than the NC in all subjects and the osteopenic group. POWS and GLBS had a lower molar ratio of acetate than INU1, whereas POWS had a higher molar ratio of butyrate than the NC in all subjects. In general, the NC has a higher molar ratio of BSCFAs than INU1, INU2, POWS, POWSE (all subjects and osteopenic group) and GLBSE (all subjects and subgroups of bone health). Analysis of individual BSCFA indicated that POWS exhibited lower molar ratios of iso-butyrate (all subjects and osteopenic group), isovalerate (all subjects and subgroups of bone health) and isocaproic acid (all subjects) than the NC. Higher molar ratios of other SCFAs were detected after NC treatment compared to INU1, INU2, POWS, GLBSE (all subjects and healthy group) and INU2, POWS (osteopenic group). POWS had lower molar ratios of other SCFAs and valerate than the NC, POWSE, GLBS and GLBSE in all subjects and the healthy group. Furthermore, the POWS treatment induced a lower molar ratio of caproic acid than the NC in all subjects and lower levels of heptanoic acid than the NC and the rest of the treatments in all subjects ($p <0.05$ for all).

Based on parameter estimates the POWS and POWSE substrates demonstrated the highest 24 h SCFA concentrations, which were significantly higher than those of the negative and positive controls in all volunteers (Table 3).

Our results are in concordance with those of Mitsou *et al.*, who reported that 24 h fermentation of *P. ostreatus* untreated mushroom powder exhibited significantly higher SCFA concentration levels compared than the negative control and

inulin (2% w/v) in elderly healthy subjects.[46] In the case of *G. lucidum* substrates, total SCFAs after 24 h of fermentation were similar to those of the positive controls and higher than the NC values (Table 3 and Fig. 2a). Similar results were obtained in both bone health groups (ESI, Tables S4a and S4b†). The main sources of SCFAs are dietary carbohydrates fermented by gut bacteria and due to the high content of glucans in mushrooms[17] the total SCFA concentration was enhanced in all mushroom-based substrates in the present study. Primary bacterial degraders depolymerize specific polysaccharides to mono-, di-, and oligosaccharides that can be fermented to acidic end products such as acetate or lactate; such intermediate fermentation products are metabolized by secondary fermenters such as *F. prausnitzii*, *Eubacterium rectale* and *Roseburia* spp. into new molecules, including butyrate.[62] In our study, both positive controls exhibited the highest molar ratio of acetate as expected, since they also showed increased levels of *Bifidobacterium* and *Lactobacillus* spp. It has been well established by studies performed in humans and in rodents,[72,73] that inulin and oligofructose stimulate the growth of bifidobacteria, which lead to acetic and lactic acid production.[61] Mushroom-based substrates significantly increased the acetate concentration after 24 h of fermentation, which is particularly notable in comparisons *versus* NC (Fig. 2b).

Similar results have been recently reported after 24 h *in vitro* fermentation of POWS; the mushroom-based substrate substantially increased the acetate concentration compared to the NC and INU2, whereas its molar ratio was significantly lower than both negative and positive controls.[46]

Propionate and butyrate are produced by distinct subsets of the gut bacteria.[74,75] Butyrate is well known as the main energy source for intestinal epithelial cells and both of these preceding SCFAs have been associated with many beneficial effects on host health by activating not fully recognized signaling pathways,[76,77] those associated with anti-lipogenic, anti-inflammatory and anti-carcinogenic activities[74,78,79] or the enhancement of epithelial barrier integrity.[80]

SCFAs have been suggested to exert inhibitory effects on bone resorption and inflammation, especially by suppressing osteoclast formation, and to promote osteoblast differentiation by enhancing the production of bone sialoprotein and osteopontin in degenerative bone diseases such as osteoporosis.[81–83]

Regarding propionate, it reached the highest ratios after 8 h of fermentation in all treatments (POWS, POWSE, GLBS, and GLBSE) compared to the baseline, and remained stable after 24 h in all volunteers. In addition, fermentation of POWS and POWSE for 24 h resulted in the highest concentrations of propionate compared to the NC, INU1 and INU2 (Fig. 2c). Furthermore, a significant increase in the molar ratio of propionate was detected especially for POWSE and GLBS compared to the baseline and positive controls in all volunteers (Table 3).

In the healthy group, POWSE fermentation for 24 h resulted also in a significantly higher molar ratio compared to the base-

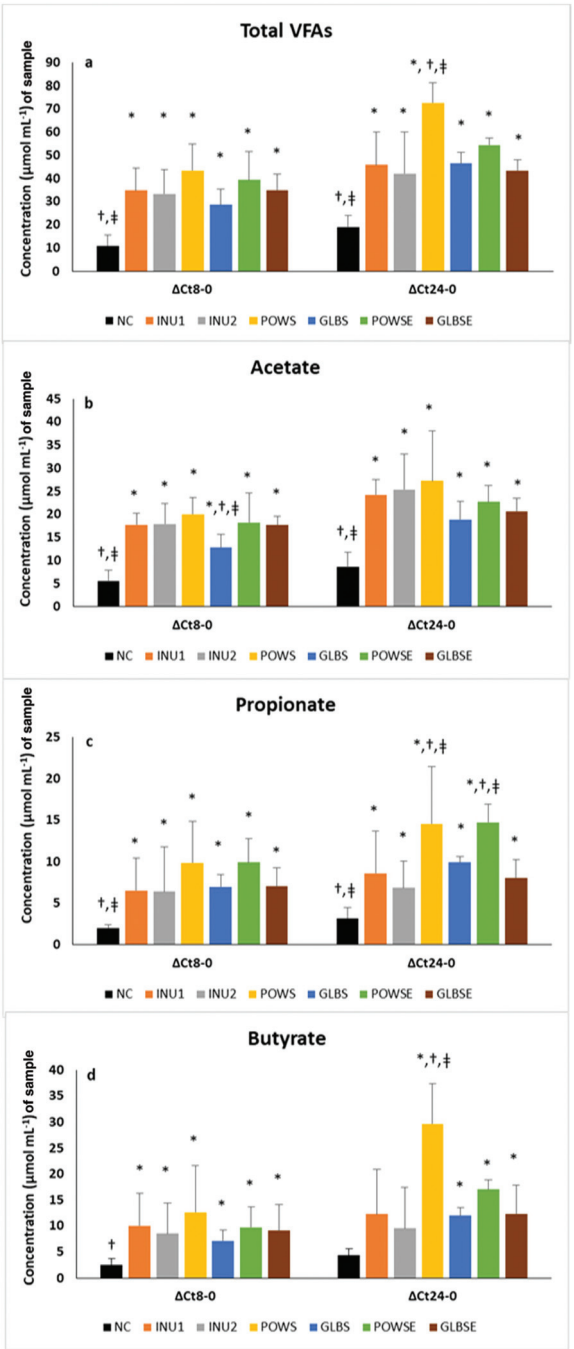

Fig. 2 Differences (Δ) in the concentrations (μmol mL^{-1}) of (a) total volatile fatty acids (VFAs), (b) acetate, (c) propionate and (d) butyrate after 8 h of fermentation (ΔCt8-0) and 24 h of fermentation (ΔCt24-0) compared to the baseline; values are expressed as mean and SD, where ΔCt8-0 is defined as 'concentration t = 8 h minus concentration t = 0 h' and ΔCt24-0 is defined as 'concentration t = 24 h minus concentration t = 0 h'; *: significantly different compared to [NC] ($p < 0.05$) (Mann–Whitney test or t-test); †: significantly different compared to [INU1] ($p < 0.05$) (Mann–Whitney test or t-test); ‡: significantly different compared to [INU2] ($p < 0.05$) (Mann–Whitney test or t-test); NC: Negative control; INU1: Inulin 1% (w/v); INU2: Inulin 2% (w/v); POWS: *P. ostreatus* untreated mushroom powder; POWSE: *P. ostreatus* mushroom-extract; GLBS: *G. lucidum* untreated mushroom powder; GLBSE: *G. lucidum* mushroom-extract.

line. In the osteopenic group, the molar ratio of propionate was higher compared to the baseline for POWS, POWSE and GLBS, while for POWSE it was also higher than the negative and positive controls (ESI, Tables S4a and S4b†). In our study, the POWSE treatment induced a significant increase of *Bacteroides* spp., which is probably associated with the propionate outcome. Previous studies have reported that Bacteroidetes were able to produce several SCFAs, such as acetic and propionic acids based on a broad range of glycoside hydrolases and the carbohydrate metabolic pathways.[4] Furthermore, Bacteroidetes are considered as the major producers of propionate from dietary carbohydrates.[17]

With respect to GLBSE, our results were in agreement with those of Ding *et al.*, who reported that *in vivo* and *in vitro* fermentation of *Ganoderma atrum* polysaccharides resulted in increased concentrations of total SCFAs, and acetic and propionic acids.[84] Butyrate concentrations were found to be high especially in the case of POWS after 24 h of fermentation compared to both negative and positive controls (Fig. 2d); similar results were obtained when the molar ratio was measured in all volunteers (Table 3), particularly in the osteopenic group (ESI, Table S4b†). This fact could be partially explained by the increased presence of *F. prausnitzii* after fermentation of the POWS substrate. Moreover, the butyrate molar ratio increased significantly after 24 h of POWSE and GLBSE fermentation (compared to 8 h) in all volunteers. Our results were in agreement with those of Mitsou *et al.*, demonstrating the same effect after 24 h fermentation of POWS on butyrate and propionate concentrations and molar ratios when compared to NC.[46]

It is well established that the degradation of branched-chain amino acids (valine, leucine, and isoleucine) leads to the production of isobutyrate, isovalerate and 2-methyl butyrate acid, respectively, which are known as branched-chain SCFAs (BCSCFAs).[85,86] The production of BCSCFAs after 24 h fermentation of NC was significantly increased when compared to 8 h fermentation, which is probably due to the proteolytic activity of the faecal microbiota, a phenomenon that was reflected also in the iso-butyrate and iso-valerate molar ratios (Fig. 3b and Table 3).

The concentration levels of BCSCFAs and other SCFAs were significantly higher after 24 h fermentation of POWSE and GLBS compared to the positive controls (Fig. 3a and b). The molar ratio of total BCSCFAs diminished substantially in all substrates (except the GLBS) after 24 h of fermentation compared to the negative control in all volunteers.

Indeed, the molar ratio of total BCSCFAs for GLBS increased compared to the baseline and 8 h of fermentation. The same pattern was observed for iso-butyrate and iso-valerate (Table 3). Analysis by bone health status also indicated an increase in the molar ratios of BCSCFAs after 24 h fermentation of NC and GLBS in both groups (ESI, Tables S4a and S4b†).

Although both mushrooms exhibited a relatively high protein content,[38,87,88] enhanced molar ratios of BCSCFAs were noticed only after fermentation of the substrates based on *G. lucidum*.

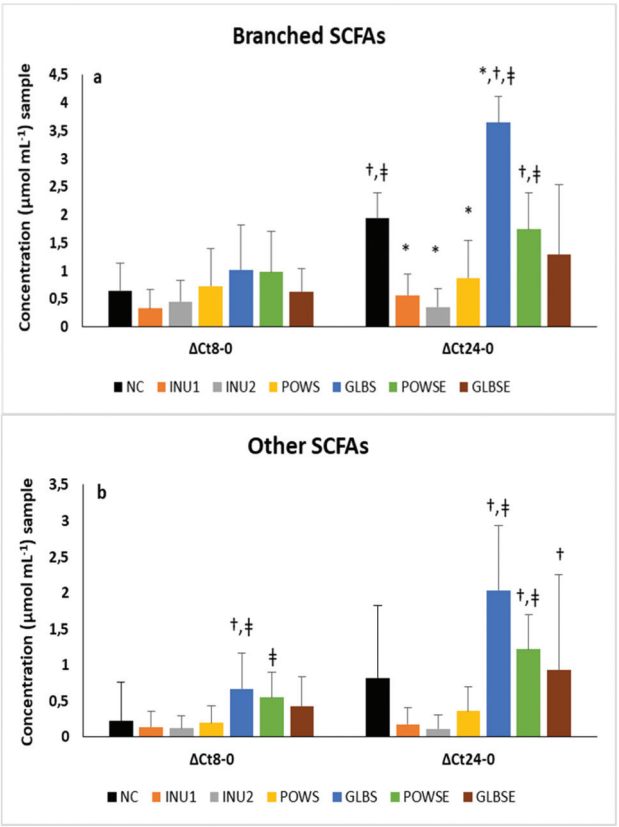

Fig. 3 Differences (Δ) in the concentrations (μmol mL^{-1}) of (a) branched SCFAs (BCSFAs) and (b) other SCFAs after 8 h of fermentation (ΔCt8-0) and 24 h of fermentation (ΔCt24-0) compared to the baseline; values are expressed as mean and SD, where ΔCt8-0 is defined as 'concentration t = 8 h minus concentration t = 0 h' and ΔCt24-0 is defined as 'concentration t = 24 h minus concentration t = 0 h'; *: significantly different compared to [NC] ($p < 0.05$) (Mann–Whitney test or t-test); †: significantly different compared to [INU1] ($p < 0.05$) (Mann–Whitney test or t-test); ‡: significantly different compared to [INU2] ($p < 0.05$) (Mann–Whitney test or t-test); NC: Negative control; INU1: Inulin 1% (w/v); INU2: Inulin 2% (w/v); POWS: *P. ostreatus* untreated mushroom powder; POWSE: *P. ostreatus* mushroom-extract; GLBS: *G. lucidum* untreated mushroom powder; GLBSE: *G. lucidum* mushroom-extract.

This result could imply different structures of mushroom macronutrients and/or the presence of complex compounds affecting protein fermentation. In general, the end-products of protein fermentation in the colon (*e.g.* ammonia, H_2S and phenols) are associated with important injuries of the epithelium which may lead to inflammatory bowel diseases and colon cancer;[86] however, the role of BCSCFAs is still poorly known. Otherwise, BCSCFAs such as isobutyric and isovaleric acid have been proposed to interfere with adipocyte lipid and glucose metabolism *in vitro* and may contribute to improved insulin sensitivity in metabolically burdened individuals.[85]

3.5 Cytotoxicity of fecal inocula on MG-63 cell line

As revealed by the results of the present study, individual's intestinal microbiota has a distinct impact on the viability of osteoblast cells (Fig. 4A). Despite the fact that the viability of

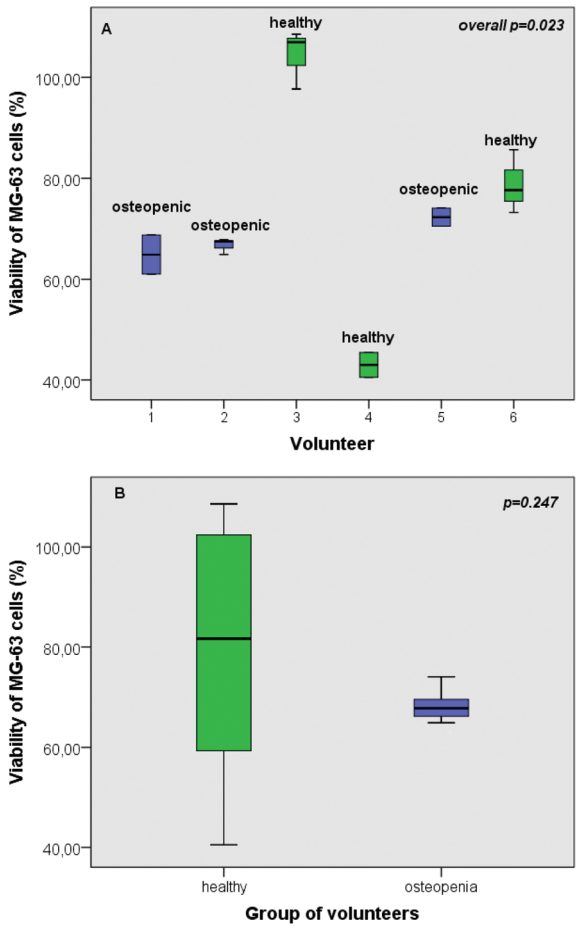

Fig. 4 (A and B) Viability (%) of MG-63 cells in negative control treatments after 24 h of fermentation with faecal inocula from six volunteers (A) and by grouping volunteers according to their bone health (B); data are presented as boxplots, with the horizontal line representing the median and the whiskers the minimum and maximum values.

MG-63 cells was significantly affected by interindividual variability of the fermentation procedure with different faecal inocula (p = 0.023) (Fig. 4A), this discrepancy was not verified by the group-specific analysis (p = 0.247) (Fig. 4B).

3.6 Impact of mushroom treatments on OPG and RANKL

The osteoblastic activity of OPG was induced in similar levels by the inulin (INU2) fermentation supernatant and the culture control treatment, both in all- and group-specific analysis (Fig. 5A–C). Similar results were also reported in the case of the osteoclastic effect of RANKL (Fig. 5D–F) in all groups (p for all >0.05). The POWSE treatment induced a drastic decrease in OPG levels compared to both the control (p_{total} = 0.002, $p_{healthy}$ < 0.001, $p_{osteopenic}$ < 0.001) and INU2 (p_{total} < 0.001, $p_{healthy}$ < 0.001, $p_{osteopenic}$ = 0.001) in all groups (Fig. 5A–C). *G. lucidum* based treatments also caused a significant reduction in OPG levels in all volunteers and according to the bone health status compared to INU2 ($p_{GLBS\text{-}total}$ < 0.001, $p_{GLBS\text{-}healthy}$ < 0.001, $p_{GLBS\text{-}osteopenic}$ = 0.001, $p_{GLBSE\text{-}total}$ < 0.001, $p_{GLBSE\text{-}healthy}$ = 0.005, $p_{GLBSE\text{-}osteopenic}$ = 0.026) and the culture control ($p_{GLBS\text{-}total}$ < 0.001, $p_{GLBS\text{-}healthy}$ < 0.001, $p_{GLBS\text{-}osteopenic}$ < 0.001, $p_{GLBSE\text{-}total}$ = 0.007, $p_{GLBSE\text{-}healthy}$ = 0.013, $p_{GLBSE\text{-}osteopenic}$ = 0.017) (Fig. 5A–C).

Moreover, the POWS treatment resulted in lower RANKL levels than INU2 in all volunteers and osteopenic women. The hot-water extract of the same mushroom (POWSE) showed lower levels of RANKL than INU2 (p = 0.013) or the control (p < 0.001) in all volunteers and different subgroups (Fig. 5D–F). The RANKL levels after the GLBS treatment decreased when compared to INU2 in all groups, and the same effect was observed when GLBS was compared to the control in the healthy group only (p = 0.045) (Fig. 5E). In addition, GLBSE resulted in significantly lower RANKL levels than the control in the osteopenic group (p = 0.030) and INU2 in all volunteers (p = 0.011). Bone health specific analysis revealed also a lower RANKL concentration in GLBSE than in INU2 for both healthy (p = 0.098) and osteopenic (p = 0.076) women (Fig. 5D–F). In our study, INU2 induced no significant changes in parameters related to bone metabolism, such as OPG and RANKL levels. The dynamic osteoblastic potential of POWS could be attributed to the effect of butyrate and at a lesser extent to the effect of propionate.

Previous studies have highlighted the anti-resorptive properties of butyrate and propionate. Hence, Lucas *et al.* have recently proposed that propionate or butyrate increased the bone volume of healthy mice following diets rich in fermentable, indigestible fibers; no consistent effects of SCFA were reported on osteoblasts and bone formation, whereas propionate and butyrate strongly suppressed osteoclast differentiation.[82]

Previous *in vitro* studies based on administration of butyrate as a histone deacetylase inhibitor of bone cell metabolism reported suppression of osteoclastic activity, with incoherent effects on osteoblastic activity.[71,89–91] In contrast to pre-existing evidence, Chang *et al.* supported that butyrate can stimulate RANKL, but decreases OPG expression and secretion from osteoblasts, within 24 h of exposure; however, exposure to lower concentrations of butyrate (<8 mM) for 72 h was demonstrated to stimulate OPG secretion.[92] Due to the wide heterogeneity of results, caused by methodological discrepancies (*e.g.* different cell types, differentiation stage, butyrate concentration, and exposure time), no concluding statements could be made on the role of butyrate in bone metabolism.

The beneficial role of *G. lucidum* in RANKL inhibition could be attributed to its beta-glucan content and other equally bioactive compounds, such as triterpenoids. Among triterpenoids isolated from *G. lucidum*, ganoderic acid DM and its structurally related ganoderic acid F show inhibitory activity against osteoclastic differentiation.[93]

Miyamoto *et al.* proposed that the ethanol extracts of *G. lucidum* exhibit a bone-protective effect in ovariectomized rats, without substantially affecting the uterus. Ganoderic acid DM in particular suppressed the expression of c-Fos and NFATc1, which consequently regulated DC-STAMP expression and reduced osteoclast fusion.[94] In addition, Tran *et al.* reported that *Ganomycin* I, which is a meroterpenoid com-

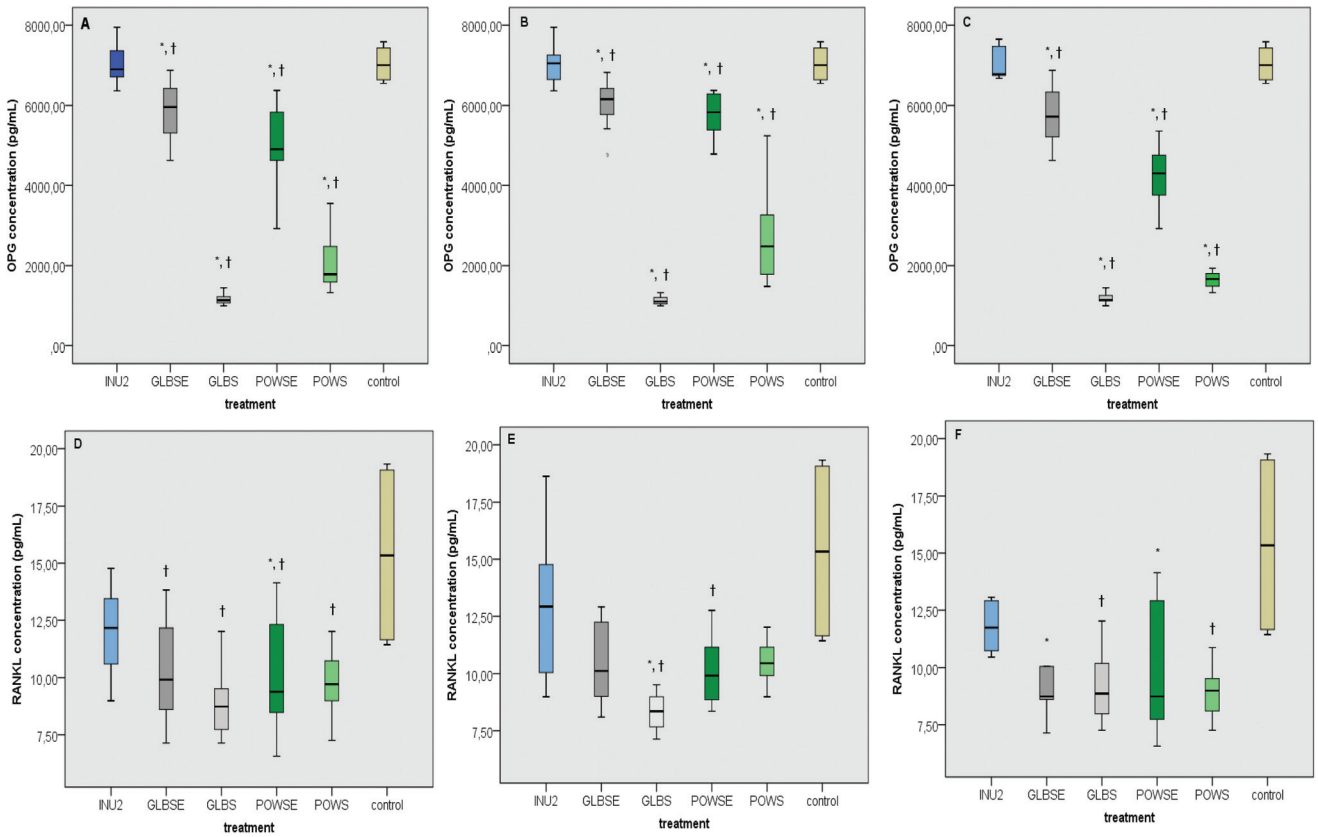

Fig. 5 (A–F) Bone metabolism parameters of MG-63 cell cultures [OPG (A, B and C) and RANKL (D, E and F)] after treatment with 24 h fermentation supernatants from all subjects (A and D), healthy subjects (B and E) or subjects with osteopenia (C and F); *: significantly different compared to the control ($p < 0.05$); †: significantly different compared to INU2 ($p < 0.05$); INU2: Inulin 2% (w/v); POWS: *P. ostreatus* untreated mushroom powder; POWSE: *P. ostreatus* mushroom-extract; GLBS: *G. lucidum* untreated mushroom powder; GLBSE: *G. lucidum* mushroom-extract; control: MG-63 culture supernatant. Boxplots show OPG or RANKL concentration; data are presented as boxplots, with the horizontal line representing the median and the whiskers the minimum and maximum values.

pound isolated from *G. lucidum*, withheld RANKL-induced osteoclast differentiation, actin-ring formation, and resorption pit formation, while it also repressed the activation of MAPKs and their downstream transcription factor c-Fos expression.[95]

4. Conclusions

Recent data demonstrated that gut microbiota manipulation could be a promising strategy in the prevention or/and adjuvant treatment of chronic metabolic diseases, including bone metabolism disorders. Edible mushrooms are inexpensive, safe food choices and their potential positive effects on bone physiology have been currently highlighted, offering new alternatives in pharmacotherapy choices. The present work aimed to investigate two mushroom species, namely the 'reishi mushroom' (*G. lucidum*) and the 'oyster mushroom' (*P. ostreatus*), as potential novel prebiotics with possible beneficial effects on bone metabolism. In the present work an *in vitro* fermentation study was performed by using fresh faecal inocula from healthy and osteopenic women and different lyophilized mushrooms (rich in beta-glucans) as substrates. Instead of focusing only on a single category of potentially bioactive substances (*e.g.* beta-glucans), mushrooms were treated as suitable food in order to explore the possible synergistic effects of their constituents. In addition and in accordance with our experimental design, we hypothesized that the fermentation supernatant contains the microbial metabolic products that can be transferred from the gut environment to exert their biological action. Therefore, we used the fermentation products, which resulted from different gut microbiota inocula, to study their impact on the osteoblasts; in the past, most of the *in vitro* studies used single isolated products (*e.g.* SCFAs).[71,89]

Our data suggested that high counts of *Bifidobacterium* spp., after fermentation of POWS and GLBSE, demonstrated an advantageous prebiotic effect especially in the osteopenic group. Furthermore, fermentation of POWS significantly enhanced the growth of *F. prausnitzii* and was accompanied by a substantial increase in butyrate production. Our results also indicated that the gut microbiota of each volunteer influences in a unique way the viability of osteoblastic cells. The fermentation products of mushrooms and extracts induced a drastic decrease in OPG and RANKL levels, compared to inulin, in all volunteers. In subjects with osteopenia, fermentation of

GLBSE and POWSE resulted in lower RANKL levels than the control. Hence, human studies of larger scale are necessary to elucidate the effects of edible mushrooms on bone health (based on the current human skeletal status) and bone turnover mechanisms in order to optimize osteopenia/osteoporosis prevention strategies, generate effective interventions for individuals belonging to high-risk groups and offer novel targeted therapeutics.

Conflicts of interest

The authors have no conflicts of interest to declare.

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