

Impact of personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota e The "RISTOMED project": Randomized controlled trial in healthy older people

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Randomized control trials

Impact of personalized diet and probiotic supplementation on inflammation, nutritional parameters and intestinal microbiota – The "RISTOMED project": Randomized controlled trial in healthy older people

Luzia Valentini [a, *], Alessandro Pinto [b], Isabelle Bourdel-Marchasson [c, d, e], Rita Ostan [f], Patrizia Brigidi [g], Silvia Turroni [g], Silvana Hrelia [h], Patrizia Hrelia [g], Stefan Bereswill [n], André Fischer [n], Emanuela Leoncini [h], Marco Malaguti [h], Christéle Blanc-Bisson [l], Jessica Durrieu [l], Liana Spazzafumo [i], Fabio Buccolini [j], Florence Pryen [k], Lorenzo Maria Donini [b], Claudio Franceschi [f], Herbert Lochs [b, m]

[a] Dept Gastroenterology and Hepatology, Charité-Universitätsmedizin Berlin, CCM, Charitéplatz 1, 10117 Berlin, Germany
[b] Experimental Medicine Department, Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
[c] CHU Bordeaux, Clinical Gerontology, France
[d] CNRS, RMSB, UMR 5536, France
[e] University Bordeaux, RMSB, UMR 5536, Bordeaux, France
[f] Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Via S. Giacomo 12, 40126 Bologna, Italy
[g] Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy
[h] Dept Life Quality Studies, Alma Mater Studiorum University of Bologna, Via Irnerio 48, 40126 Bologna, Italy
[i] Biostatistical Center INRCA, Via S. Margherita 5, 60100 Ancona, Italy
[j] R&D, VoxNet CEO, Via Giovanni Paisiello 32, 00198 Rome, Italy
[k] Actial Farmaceutica Lda, Praca Severiano Ferraz 258, 09000 082 Funchal, Portugal
[l] CHU Bordeaux, Clinical Gerontology, 33000 Bordeaux, France
[m] Medical University of Innsbruck, Innrain 52, 6020 Innsbruck, Austria
[n] Dept Microbiology and Hygiene, Charité-Universitätsmedizin Berlin, CBF, Hindenburgdamm 27, 12203 Berlin, Germany

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SUMMARY

Objectives: To assess the impact of a personalized diet, with or without addition of VSL#3 preparation, on biomarkers of inflammation, nutrition, oxidative stress and intestinal microbiota in 62 healthy persons aged 65–85 years.
Design: Open label, randomized, multicenter study. Primary endpoint: High-sensitivity C-reactive protein.
Setting: Community.
Interventions: Eight week web-based dietary advice (RISTOMED platform) alone or with supplementation of VSL#3 (2 capsules per day). The RISTOMED diet was optimized to reduce inflammation and oxidative stress.
Measurements: Blood and stool samples were collected on days 1 and 56.
Results: Diet alone reduced ESR ($p = 0.02$), plasma levels of cholesterol ($p < 0.01$) and glucose ($p = 0.03$). Addition of VSL#3 reduced ESR ($p = 0.05$) and improved folate ($p = 0.007$), vitamin B12 ($p = 0.001$) and homocysteine ($p < 0.001$) plasma levels. Neither intervention demonstrated any further effects on inflammation. Subgroup analysis showed 40 participants without signs of low-grade inflammation (hsCRP<3 mg/l, subgroup 1) and 21 participants with low-grade inflammation at baseline (hsCRP≥3 mg/l, subgroup 2). In subgroup 2 addition of VSL#3 increased bifidobacteria ($p = 0.005$) in more participants and improved both folate ($p = 0.015$) and vitamin B12 ($p = 0.035$) levels compared with subgroup 1. The increases were positively correlated to the change in the bifidobacteria concentration for folate ($p = 0.023$) and vitamin B12 ($p = 0.001$). As expected change in homocysteine correlated negatively to change in folate ($r = -0.629$, $p = 0.002$) and vitamin B12 ($r = -0.482$, $p = 0.026$).

* Corresponding author. Current address: University of Applied Sciences Neubrandenburg, Department of Agriculture and Food Technology, Section of Dietetics, Brodaer Str. 2, 17033 Neubrandenburg, Germany. Tel.: +49 395 5693 2512.
E-mail addresses: valentini@hs-nb.de, luzia.valentini@gmx.net (L. Valentini).

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Conclusions: Addition of VSL#3 increased bifidobacteria and supported adequate folate and vitamin B12 concentrations in subjects with low-grade inflammation. Decrease in homocysteine with VSL#3 was clinically relevant. suggesting protective potentials for aging-associated conditions, e.g. cardiovascular or neurological diseases.
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1. Introduction

Aging is associated with a general increase in inflammation, as measured by an increase in C-reactive protein and pro-inflammatory cytokines [1,2]. This phenomenon has been termed 'inflamm-aging', and is characterized by a chronic, low-grade pro-inflammatory condition leading to long-term tissue damage [3]. Increased inflammatory markers are associated with frailty and mortality [1,4–6]. Reducing low-grade inflammation may be a way to prevent or reduce the onset and the severity of some age-related diseases, such as Alzheimer's disease, Parkinson's disease, atherosclerosis, type 2 diabetes, osteoporosis, cognitive decline and general frailty. Oxidative stress, caused by accumulated cellular damage from reactive oxygen species, plays an important role in the aging process and is one of the primary causal factors in producing a chronic state of inflammation [2].

Aging deeply affects the homeostasis of the gut microbiota through changes in diet and lifestyle and age-related changes in gut physiology. Gut microbiota can directly influence host wellbeing by providing nutritional, metabolic and immunological benefits [7], such as the synthesis of folate and vitamin B12 [8], two critical vitamins in aging people [9]. It has been proposed that age-related changes in gut microbiota, associated with an increase in systemic inflammation [10], may also contribute to the progression of disease and frailty in older people [11].

Evidence exists that inflammation [12], oxidative stress [13] and gut microbiota [7,14] are influenced by diet, and it may therefore be possible to reduce or delay the effects of age-related changes in these parameters through appropriate dietary intervention and/or use of nutraceutical dietary supplements.

The RISTOMED project aimed to use diet as a means to improve health-related quality of life for older people and to prevent aging-related diseases. It promotes an optimized diet that delivers the recommended daily requirement of nutrients, vitamins and minerals for older people recommended by WHO, and is designed to reduce inflammation and oxidative stress by promoting a healthy gut microbiota. The initiative is also investigating the utility of specific nutraceutical supplements in improving the above-mentioned parameters. RISTOMED operates via an internet-based platform known as E-Health Dietary Services. The diet was designed based on data from the literature indicating foods and nutrients known or suspected to modulate oxidative stress, inflammatory activity and gut microbiota composition in older subjects. For a review and full details regarding the RISTOMED project, see www.ristomed.eu.

In this paper, we report specifically on the effect of VSL#3 taken concomitantly with the RISTOMED diet. VSL#3 has been marketed for many years as a food supplement or medical food in Europe and North America. Supplementation with VSL#3 has been associated with symptomatic improvement in ulcerative colitis [15] and irritable bowel syndrome [16], and with a reduction in inflammatory cytokines and measures of oxidative stress in a pilot study in patients with chronic liver disease [17]. Recently, two large studies have shown a reduced incidence of, or better recovery from, hepatic encephalopathy in cirrhotic patients after 3-month intervention with VSL#3 [18,19].

The primary objective of the study was to investigate whether an optimized diet alone or in conjunction with VSL#3 can decrease high-sensitivity C-reactive protein (hsCRP) plasma concentration in older people. HsCRP was chosen as internationally recognized biomarker of low-grade inflammation and cardiovascular risk [20]. Secondary objectives included the interventional effects on gut microbiota composition, oxidative stress and nutritional parameters. Based on the secondary hypothesis that dietary interventions might be more effective in stress situations compared to the physiological state we additionally performed subgroup evaluations in participants with existing low-grade inflammation.

2. Patients and methods

2.1. RISTOMED study design

RISTOMED was an open label, randomized, controlled trial with four parallel arms, conducted at three centers in France (CHU, Bordeaux), Germany (Charité, Berlin) and Italy (La Sapienza, Roma). Participants were enrolled between May 2009 and April 2010 and were randomized to one of four study arms: 'Optimal diet for the elderly' alone; or 'Optimal diet' in conjunction with argan oil, VSL#3 bacterial blend, or AISA 5203-L fruit extract terpene, respectively. The randomization was performed in Bologna by a centralized computer-generated random number list, and stratified by recruitment center and gender. Each recruitment center received a list with consecutive participants codes for men and women (Italy: M101-M124 and F101-F124; Bordeaux:M201-M224 and F201-F224; Berlin: M301-M324 and F301-F324) together with closed envelopes containing the random allocation for each participant code. The participants were enrolled by the principal investigator at each site. The study was conducted in accordance with International Good Clinical Practice guidelines (ICH, May 1977), the Declaration of Helsinki, and national laws and regulations. The study protocol was approved by the ethics committee at each recruiting institution. The participants gave written informed consent at enrollment. The study was conducted under the 7th Framework Programme of the European Commission (FP7-SME-2007-1, grant agreement 222230), and is registered at ClinicalTrials.gov, identifiers: NCT01069445 (Bordeaux) and NCT01179789 (Berlin).

This report presents the results of the study with respect to the comparison of the RISTOMED diet alone (Arm A) or combined with VSL#3 bacterial blend (Arm B). The data relating to argan oil and AISA 5203-L fruit extract terpene will be reported separately.

2.2. Participants

Eligible participants were healthy individuals aged 65–85 years, male or female, with BMI 22–30 kg/m^2 and Eastern Cooperative Oncology Group Performance Status (ECOG) performance status 0–2, able to use a computer and with access to the internet, by themselves or with help. Exclusion criteria were assessed through

an interview and were based on current physical status and history of conditions including anorexia, chronic severe diseases (cardiac, lung, renal or neurological) and cancers, gastrointestinal diseases requiring treatment, diabetes mellitus, current infection and antibiotic treatment, or anti-inflammatory drugs within the previous four months, and any diseases or medications that could interfere with study outcome measures. Intake of conventional yoghurt, probiotics, prebiotics and symbiotics or other supposed functional foods (vitamin- or mineral supplementation, nutritional supplements in general) within the previous three weeks was disallowed. Participants were withdrawn from the study if they had more than one day per week or 4 days per month of non-compliance to the assigned diet, used disallowed food/nutraceutical supplementation, or experienced concomitant diseases requiring treatment with antibiotics or anti-inflammatory drugs. Those completing the study were counted as the per-protocol population.

2.3. Dietary interventions

Each participant received a personal login to the RISTOMED web platform, and was trained by a dietitian to use the personalized diet platform. The platform organized the diet for participants, tailoring a complete menu with recipes throughout the treatment period and adapting and personalizing the diet individually. The technical organization of the platform is shown in Fig. S1. The diet alone or the diet plus VSL#3 supplementation was administered for 8 weeks (56 ± 2 days).

'Optimized diet': All participants received a personalized diet plan formulated by a registered dietitian via the RISTOMED internet platform. The diet varied between the three centers to reflect local (country-specific) food customs and preferences, and also took into account participants' personal preferences. After comparing the main population reference intakes of macronutrients, vitamins and minerals in older persons published by national and international scientific organizations it was decided to use the reference values for older people set out by the WHO (http://whqlibdoc.who.int/publications/9241562102.pdf) for a joint census between the three participating countries. Thereby, the diet for all countries specified an intake of vitamins E and C, carotenoids, selenium and zinc 30–150% higher than recommended levels, an intake of polyphenols >400 mg/day, an n6:n3 polyunsaturated fatty acid ratio of 3:1, and a fiber intake of 35 g/day with a ratio of soluble to insoluble fiber of 1:1. Weekly intake frequencies for each food group were recommended. The energy requirement of each participant was estimated according to the individual Total Daily Energy Expenditure (TDEE) to maintain the actual body weight using two different methods: if the weight was steady (variation < 5%) in the last 3 months, TDEE was obtained from the habitual dietary energy intake, while if the weight had changed (variation > 5%) the TDEE was calculated by multiplying the resting energy expenditure estimated by Harris-Benedict equation by the Physical Activity Level (PAL) estimated by the International Physical Activity Questionnaire (IPAC, www.ipaq.ki.se/downloads.htm). Weight was monitored weekly and the diet was amended when variations were observed. Compliance with the diet was controlled by the self-reported intake on the RISTOMED web platform.

2.3.1. VSL#3 bacterial blend (VSL#3®, supplied by ACTIAL Farmaceutica Lda, Funchal, Portugal)

VSL#3 is formulated in vegetable capsules containing 112 billion lyophilized bacteria consisting of the following strains: *Bifidobacterium infantis* DSM 24737, *Bifidobacterium longum* DSM 24736, *Bifidobacterium breve* DSM 24732, *Lactobacillus acidophilus* DSM 24735, *Lactobacillus delbrückii* ssp. bulgaricus DSM 24734, *Lactobacillus paracasei* DSM 24733, *Lactobacillus plantarum* DSM 24730, and *Streptococcus thermophilus* DSM 24731, in defined ratios. Excipients include microcrystalline cellulose, stearic acid, magnesium stearate, silicon dioxide and coloring agent. VSL#3 was administered orally, 2 capsules daily on an empty stomach (at least 30 min before lunch and dinner).

2.4. Study outcomes

The aim of this study was to investigate the effect of a "healthy diet" with or without a probiotic preparation on inflammation and several nutritional parameters in clinically healthy older persons. The primary outcome measure was the change from baseline in hsCRP (high-sensitivity C-reactive protein) levels in each study arm. HsCRP was selected as the primary outcome because it is an internationally recognized marker of low-grade inflammation and cardiovascular risk [20] Secondary outcomes included measures of oxidative stress (including homocysteine and related parameters), inflammatory status, and gut microbiota composition.

A range of clinical, anthropometric and laboratory parameters were recorded at clinical visits at baseline and study end (56 days). Compliance with the diet and VSL#3 supplementation was recorded by each participant every day via the web platform, and assessed via telephone interview on days 14 and 42 and at each visit. Weight was self-reported weekly. Oxidative stress and inflammatory status were measured using specific markers in blood samples [21]. Plasma was obtained from blood within 30 min of venipuncture by centrifugation at $2000\times$ *g* for 20 min. It was rapidly frozen and stored at −80 °C. Plasma levels of IL-6, IL-10, and TNF-α trimer were measured by multiplex sandwich ELISA technology (SearchLight, Aushon Biosystems, Billerica, MA) according to the manufacturer's instructions. Samples, standards, and reagents were dispensed in the plates using a standardized technique employing a robotic liquid handling system with 16 channels (Microlab® STAR, Hamilton Robotics, Reno, NV). Since changes in the gut microbiota composition may be connected with "inflammaging", the fecal abundances of *Clostridium* cluster IV and bifidobacteria were assessed using 16S rDNA gene-targeted qPCR. All analyses were carried out using established protocols [20,22,23].

2.5. Statistical analysis

It was a priori determined that a sample size of 30 participants per intervention group has a 90.3% power to detect a statistically significant result for hsCRP at the 0.05 level of alpha in a two-tailed test. Assuming a dropout rate of 20%, 36 participants were recruited per arm. Comparison of response rates between groups was performed using a Chi-squared test and Mann–Whitney *U*-test for biochemical parameters. The Wilcoxon signed rank test was used to compare the difference before and after dietary interventions of all parameters for each diet.

All analyses were performed on the per-protocol population. Since a reduction of inflammatory parameters could not be expected in persons with no inflammation at the beginning, a sensitivity analysis was performed after the main analysis in which the study groups were then divided into a subgroup without vs. a subgroup with low-grade inflammation, based on the hsCRP at the beginning of the study. A cut-off of hsCRP $\geq$3 mg/l was used according to previously published data [20]. Differences of inflammatory parameters before and after dietary interventions were analyzed by 2-way analysis of variance (ANOVA) to investigate the effects on inflammation. Two-way analysis of covariance (ANCOVA) was used to correct for baseline difference in inflammatory parameters to compare the intervention arms. Data analysis was performed using the SPSS/Win program version 20 (Spss Inc., Chicago, IL). The level of statistical significance was defined by a two-tailed p value <0.05.

3. Results

3.1. Baseline data

A total of 69 participants were randomized, 24 at each center in Italy and Germany (12 men and 12 women) and 21 in France (10 men and 11 women). Sixty-two participants completed the study (18/24 in Italy, 20/21 in France and 24/24 in Germany), 29 males and 33 females (the per-protocol population). Seven participants discontinued, due to non-compliance ($n = 2$) or onset of diseases and/or pharmacological treatment not allowed during the study ($n = 5$). Among the per-protocol population, 31 subjects were randomized to diet alone (Arm A) and 31 subjects to diet plus VSL#3 (Arm B) (Fig. 1). Gut microbiota analyses were performed on 60 participants only, 30 subjects for Arm A and 30 subjects for Arm B. No clinically relevant adverse effects which required intervention or termination of the study were reported (Fig. 1). Compliance with the intake of VSL#3 as controlled by pill counting was between 87 and 95% in the different centers.

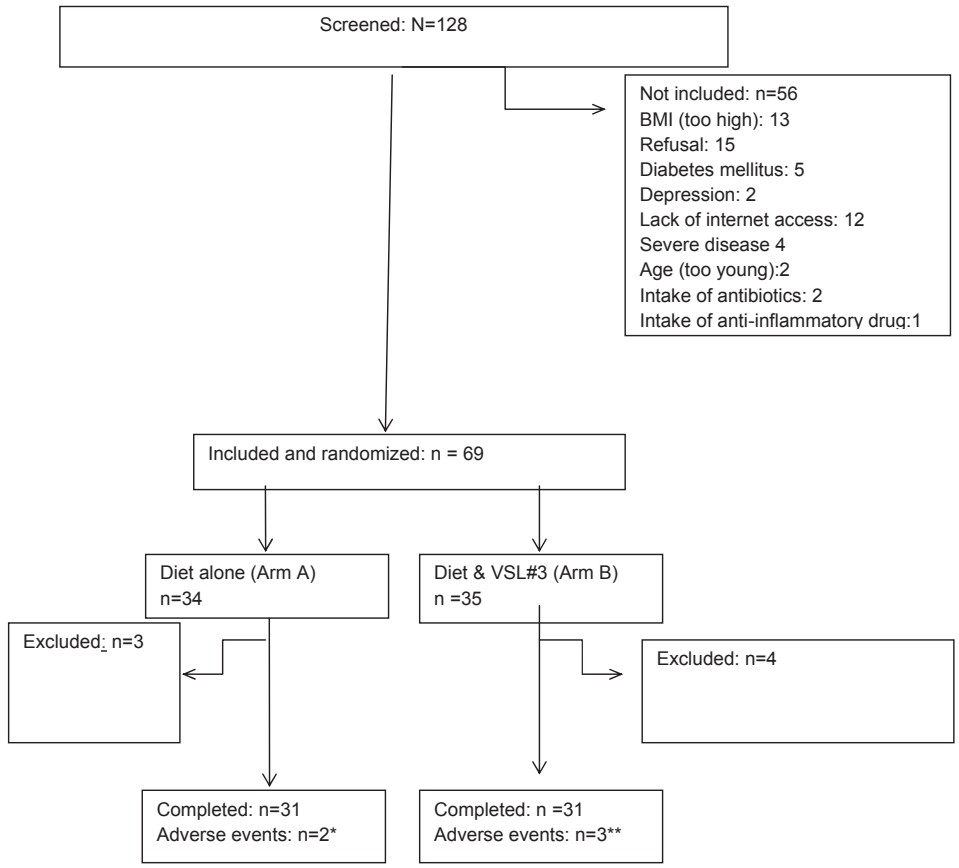
Mean (±SD) age of participants was 70.1 ± 3.9 years, and mean BMI was 26.8 ± 3.59 kg/m^2, with no significant differences in age, BMI, sex or country distribution between study arms. Although the diet was calculated for weight maintenance, participation in the study induced minor before-and-after weight differences both in Arm A (−1.05 ± 1.89 kg, $p = 0.004$) and in Arm B (−0.57 ± 1.5 kg, $p = 0.014$). Interestingly, despite being clinically healthy, the majority of participants ($n = 21$ in Arm A vs. $n = 22$ in Arm B) had a hsCRP value slightly increased above the normal range (1 mg/l) and 23 participants ($n = 12$ in Arm A vs. $n = 11$ in Arm B) had moderately increased homocysteine concentrations (>12−30 μmol/l) at the beginning of the study (Table 1). Otherwise only minor deviations from normal values were seen.

3.2. Changes of nutritional and inflammatory markers

Parameters analyzed from blood samples at baseline and study end are shown in Table 1 for Arm A and Arm B. End-of-study values are not directly comparable between arms because of baseline differences. Arm A was associated with significant decreases in erythrocyte sedimentation rate (ESR) ($p = 0.02$), total cholesterol ($p < 0.01$) and blood glucose ($p = 0.03$). Arm B did not significantly affect cholesterol or glucose, however it reduced ESR ($p = 0.05$) and was associated with significant increases in serum folate ($p = 0.007$) and serum vitamin B12 ($p = 0.001$), and a decrease in plasma homocysteine ($p < 0.001$). The decrease in homocysteine resulted in a significantly lower number of participants with moderately elevated homocysteine concentrations in Arm B compared to Arm A at study end ($n = 3$ vs. $n = 11$, chi = 5.599, $p = 0.018$). Apart from ESR, neither intervention showed effects on inflammatory parameters, including the primary endpoint hsCRP, even when ANCOVA was used to correct for baseline differences (not shown).

3.3. Biomarkers of oxidative stress

Baseline value distributions of oxidative stress markers were scattered. No significant changes from baseline in total antioxidant



* Dental infection (antibiotics) or breast oppression drug for more than 3 days
** Sleep disturbance, stomach pain, inflammatory state (anti-inflammatory drug)

Fig. 1. Flow chart of participants.

Table 1
Analysis of nutritional and inflammatory markers at baseline and study end (day 56).

	Normal range	ARM A (diet)			ARM B (diet plus VLS#3)		
		Baseline	Day 56	*P*	Baseline	Day 56	*P*
Nutritional markers							
Hemoglobin (g/dl)	12–17.5	14.2 (0.2)	14.2 (0.3)	ns	14.5 (0.2)	14.3 (0.2)	ns
Total cholesterol (mg/dl)	<200	215 (7.5)	202 (7.1)	<0.01	227 (7.9)	221 (8.4)	ns
Triglycerides (mg/dl)	<180	120 (11.5)	116 (11.5)	ns	113 (8.7)	112 (8.7)	ns
Glucose (mg/dl)	55–110	94.5 (4.1)	91.5 (3.9)	0.03	92.5 (2.3)	95.1 (3.2)	ns
Insulin (μU/ml)	2.6–24.9	9.0 (1.1)	9.8 (1.6)	ns	8.9 (1.3)	8.1 (1.3)	ns
Folate (ng/ml)	4.6–18.7	9.0 (0.7)	9.4 (0.6)	ns	8.5 (0.6)	9.9 (0.6)	0.007
Vitamin B12 (ng/l)	195–865	417 (38.1)	426 (35.8)	ns	403 (27.2)	437 (0.6)	0.001
Homocysteine (μmol/l)	<10	11.5 (0.8)	11.8 (1.0)	ns	11.2 (0.9)	9.5 (0.6)	<0.001
Inflammatory markers							
High sensitivity CRP (mg/l)	<1.0	3.6 (0.6)	3.8 (4.5)	ns	2.9 (0.7)	2.4 (0.4)	ns
White blood cells (10*9/l)	4–10	6.1 (0.24)	5.9 (0.25)	ns	6.0 (0.26)	6.2 (0.31)	ns
ESR (mm/h)	20–30	24.9 (3.4)	18.9 (3.1)	0.02	23.3 (3.9)	17.8 (3.0)	0.05
Fibrinogen (mg/dl)	150–450	377 (17.3)	382 (13.4)	ns	392 (19.3)	383 (22.1)	ns
IL-6 (pg/ml)	<20	29.9 (9.1)	34.7 (10.9)	ns	12.3 (1.8)	17.7 (2.5)	ns
TNF-α (pg/ml)	<8.1	60.2 (27.7)	64.6 (28.0)	ns	6.9 (3.3)	9.5 (4.2)	ns
IL-10 (pg/ml)	n.d.	5.3 (1.5)	7.6 (2.8)	ns	3.5 (0.8)	3.5 (0.6)	ns

Mean (SD). P = pre-post difference with Wilcoxon signed rank test. n.d. not defined. ESR = erythrocyte sedimentation rate.

activity (as measured by mM Trolox activity), or reduced glutathione (GSH), catalase, glutathione reductase, glutathione peroxidase activity were observed in either study arm. Both diet alone and diet plus VSL#3 were associated with an increase in glutathione-S-transferase activity. A significant increase in superoxide dismutase activity was seen only in Arm A (Table S1, see online supplement).

3.4. Gut microbiota

The baseline levels of both *Clostridium* cluster IV and *Bifidobacterium* spp. were not significantly different between the two intervention arms. A high interindividual variability was evident, with baseline levels ranging from 1.35×10^3 to 1.77×10^7 16S rDNA copy number/ng stool DNA for clostridia and from 2.52 to 5.82×10^5 16S rDNA copy number/ng stool DNA for bifidobacteria (Table S2, see online supplement). Overall no significant changes in the fecal abundances of either *Clostridium* cluster IV or *Bifidobacterium* spp. were observed as a result of both interventions.

However, the proportion of subjects with increased/decreased bifidobacterial concentration after intervention tended to be different between the two study arms (Chi square, $p = 0.071$). In particular, bifidobacteria increased in 18/30 (60%) participants in Arm B, with a mean 11.6-fold ratio. An opposite trend, with decreasing values, was observed in 19/30 (63%) participants in Arm A (Fig. 2, Fig. S2).

3.5. Subgroup analysis

In order to evaluate the response to the dietary interventions according to inflammation status, participants were divided into two groups according to baseline hsCRP. With a cut-off of hsCRP ≥3 mg/l we identified 40 individuals with no inflammation and 21 with low-grade inflammation at baseline. The two groups were statistically significantly different for hsCRP (p = <0.001), ESR ($p = 0.017$), fibrinogen ($p = 0.002$) and IL-6 ($p = 0.010$), but not for TNF-α or IL-10 at baseline.

We thus compared the two subgroups and found a significantly higher decrease in hsCRP ($p = 0.024$), but no significant difference in all other inflammatory parameters, in the subgroup with low-grade inflammation (see Table 2). There was no treatment effect on inflammatory markers within the inflammation subgroups.

3.6. Low-grade inflammation and gut microbiota

We further analyzed the effect of diet alone and diet with VSL#3 on gut microbiota separately in the two inflammation subgroups. In the group without inflammation, no consistent changes in the concentration of bifidobacteria were seen. However, in the group with low-grade inflammation all participants in Arm B (receiving VSL#3) showed an increase in bifidobacteria, in contrast to participants in Arm A (Fig. 2).

We further evaluated the mean change of the absolute gut microbiota concentration and confirmed the significant increase of bifidobacteria in the low-grade inflammation diet plus VSL#3 group. Furthermore, we observed statistical trends toward increased *Clostridium* populations and decreased clostridium/bifidobacteria (C/B) ratio (Table 3).

3.7. Correlation between low-grade inflammation, bifidobacteria and folate, vitamin B12, and homocysteine

As known from the literature, bifidobacteria produce folate and vitamin B12 [8]. Therefore, we evaluated the possible correlation between changes in bifidobacteria and the two vitamins depending

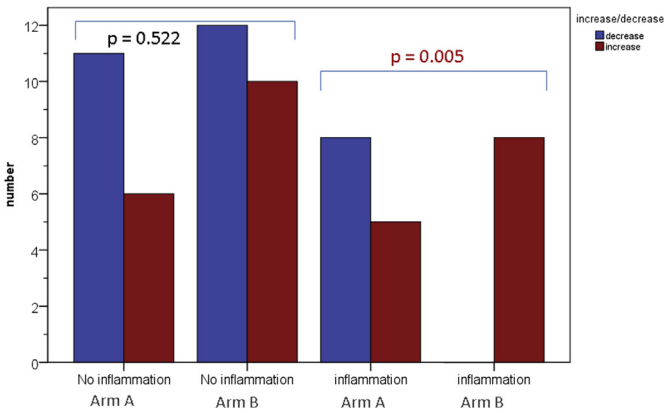


Fig. 2. Number of participants with increase/decrease of bifidobacteria concentration after both interventions. In the subgroup without inflammation, bifidobacteria increased and decreased in similar numbers of participants. In participants with low-grade inflammation, however, addition of VSL#3 (Arm B) was associated with an increase of bifidobacteria in all participants, in contrast to the diet only arm.

Table 2
Change in inflammatory parameters.

		hsCRP_diff. (mg/l)	ESR_diff. (mm/h)	Fibr_diff. (mg/dl)	IL-6_diff. (pg/ml)	TNFα_diff. (pg/ml)
No inflammation (hsCRP <3 mg/l)	Arm A Diet	1.42 (5.0)	−4.9 (11.3)	18.5 (58)	6.3 (16.8)	−4.1 (21.2)
	Arm B Diet+VSL#3	0.28 (1.4)	−4.3 (13.7)	−13,6 (133)	4.2 (13.7)	0.61 (3.9)
Low-grade inflammation (hsCRP = 3–20 mg/l)	Arm A Diet	−1.40 (4.6)	−7.7 (14.7)	−13.2 (83)	2.6 (18.9)	17.0 (53.5)
	Arm B Diet+VSL#3	−2.70 (7.06)	−8.8 (16.7)	2.6 (85)	8.8 (15.4)	8.1 (20.4)
	F-test	6.019	0.864	0.081	0.010	3.490
	Sig	0.017	0.357	0.777	0.919	0.067

Mean before-after differences of inflammatory parameters in the subgroups without and with low-grade inflammation at baseline. Arm A: diet alone, Arm B: diet + VSL#3, mean (SD).

on the study arm as well as presence and absence of low-grade inflammation.

Figure 3 shows that before-after change in Arm A (diet alone) for folate, vitamin B12 and homocysteine (Hcy) was similar in both inflammation groups, however, in Arm B folate and vitamin B12 increased significantly more in the low-grade inflammation group. The decrease in homocysteine concentration did not correlate with inflammation. However, diet with VSL#3 supplementation was associated with more pronounced improvements (decreases) in homocysteine compared to diet alone ($p = 0.007$).

Figure 4 associates the before-after changes in folate, vitamin B12 and homocysteine with changes in the concentration of bifidobacteria. No correlation was observed in participants without inflammation. However, in participants with low-grade inflammation the changes in folate and vitamin B12 concentration were positively associated with the change in the bifidobacteria concentration.

Additionally (and not shown in Fig. 4), homocysteine was negatively associated with folate ($r = -0.629$, $p = 0.002$) and vitamin B12 serum concentrations ($r = -0.486$, $p = 0.026$) in the low-grade inflammation group, suggesting a potential causal relationship between increased folate or vitamin B12 and decreased homocysteine, as commonly described in the literature.

Diet plus VSL#3 showed a significantly greater improvement of homocysteine concentration compared to diet alone ($p = 0.026$) in the no inflammation group. In the low-grade inflammation group the increase in folate ($p = 0.010$) and vitamin B12 ($p = 0.016$) was significantly higher with VSL#3 compared with diet alone, whereas the change in homocysteine was similar in both study arms ($p = 0.140$).

Table 3
Mean change in gut microbiota concentration (delta day 56 − day 1) in all participants and in the subgroups with no inflammation (hsCRP < 3 mg/L) and low-grade inflammation (hsCRP ≥3 mg/L) at baseline.

	Number total (arm A/Arm B)	Arm A (diet)	Arm B (diet + VSL#3)	*P*-value
Bifidobacteria (log10 16S rDNA copy number/ng stool)				
All	60 (30/30)	−0.051 (0.782)	0.102 (0.772)	0.188
hsCRP < 3 mg/L	39 (17/22)	−0.149 (0.750)	−0.030 (0.837)	0.392
hsCRP = 3–20 mg/L	21 (13/8)	0.078 (0.834)	0.467 (0.397)	0.020
***Clostridium* cluster IV** (log10 16S rDNA copy number/ng stool)				
All	60 (39/21)	0.073 (0.643)	0.008 (0.430)	0.723
hsCRP < 3 mg/L	39 (17/22)	0.105 (0.708)	−0.062 (0.455)	0.769
hsCRP = 3–20 mg/L	21 (13/8)	0.030 (0.571)	0.200 (0.296)	0.089
C/B ratio				
All	60 (39/21)	0.123 (0.751)	−0.095 (0.814)	0.101
hsCRP < 3 mg/L	39 (17/22)	0.254 (0.749)	−0.032 (0.935)	0.292
hsCRP = 3–20 mg/L	21 (13/8)	−0.047 (0.748)	−0.267 (0.280)	0.053

Gut microbiota results were log10 transformed for calculation. Results are depicted as mean (SD). Two participants could not be analyzed: One in Arm B lacked hsCRP values and one in Arm A lacked gut microbiota results.

4. Discussion

This study demonstrated several interesting findings. First it confirmed previous epidemiologic evidence [24]. demonstrating that a significant proportion of healthy older people have elevated hsCRP concentrations (≥3 mg/l). This threshold is defined as severe cardiovascular risk by the American Heart Association [20] and predicts a two-fold increased risk for myocardial infarction or stroke. In absence of a generally agreed consensus, we chose this hsCRP threshold to define low-grade inflammation.

Secondly, the study showed that eight weeks of the RISTOMED web platform dietary intervention, with and without addition of VSL#3, was able to modify several nutritional parameters like total cholesterol, glucose and ESR. These are encouraging data suggesting the opportunity to further develop such web-based diets, even for older persons. However, the results for the primary outcome parameter may appear disappointing at first impression. Neither intervention changed hsCRP concentration or any other analyzed inflammatory parameter apart from ESR. This might not be surprising considering that about two thirds of participants did not have elevated inflammatory parameters at the beginning of the study.

Therefore we performed a subgroup analysis to investigate the effects of dietary intervention with or without probiotic supplementation in participants with low-grade inflammation. Interestingly, the RISTOMED diet alone did not show significant effects, despite previous investigations showing that diet can improve hsCRP concentration in similar populations [25]. It is still unclear which dietary compounds contribute most to a reduction of inflammatory parameters, therefore the composition of the RISTOMED diet has to be reconsidered for future investigations.

With respect to oxidative stress, the RISTOMED diet improved superoxide dismutase activity, indicating a better capacity to scavenge superoxide anions. The addition of VSL#3 increased GST activity without any other effect on oxidative stress [17]. The lack of a significant modification in total antioxidant activity is not surprising, because this parameter reflects the plasma redox state to which many nutritional, genetic and environmental factors contribute, often counterbalancing each other. In particular, the activity of antioxidant enzymes may be affected by specific genetic polymorphisms, and consequently, total antioxidant activity may be modulated by a combination of these polymorphisms. Notwithstanding the lack of alteration in total antioxidant activity, the effect of VSL#3 on glutathione-S-tranferase activity globally contributes to ameliorating the oxidative status of the subjects.

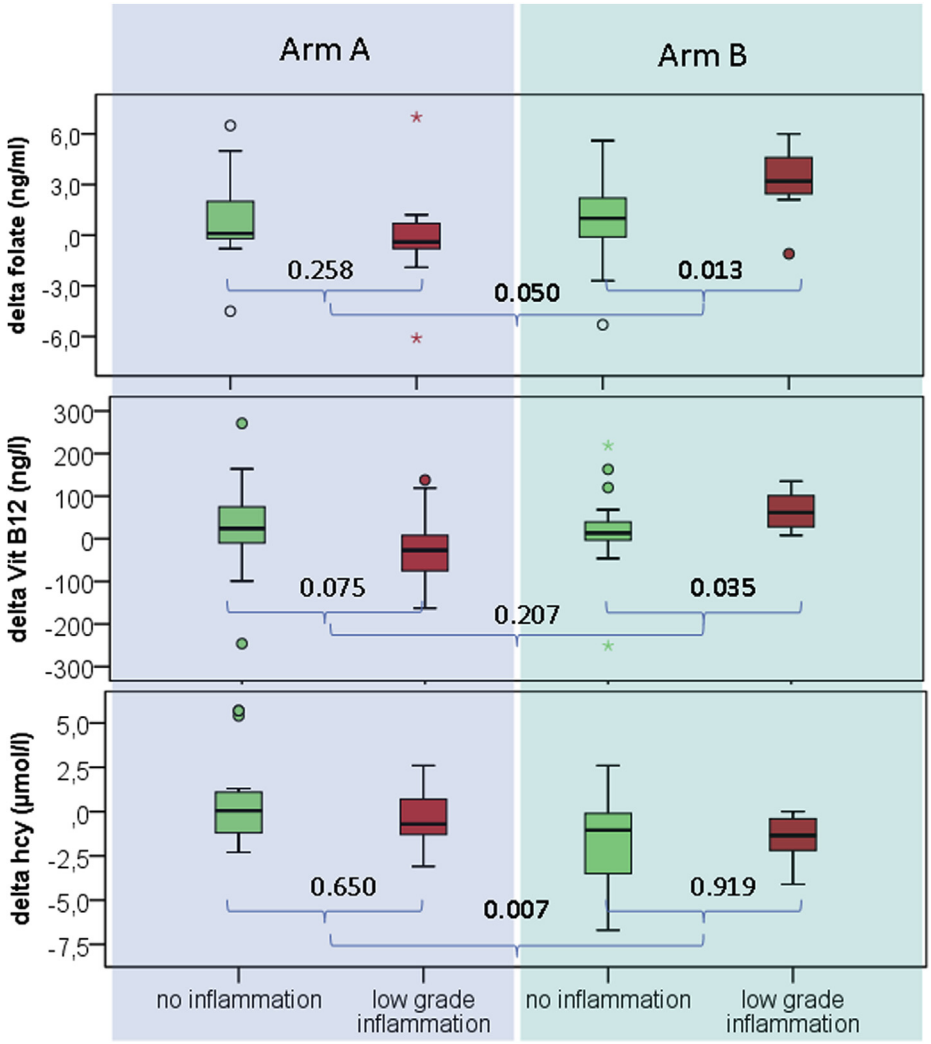


Fig. 3. Pre-post change of folate, vitamin B12 and homocysteine with and without low-grade inflammation. Low grade inflammation: hsCRP ≥3 mg/l; no inflammation: hsCRP <3 mg/L at baseline. Numbers indicate *p*-values between respective groups (Mann Whitney *U* test). Hcy = homocysteine.

The composition of fecal microbiota varied tremendously between individuals, and no correlations with the inflammatory status of the participants could be shown. In contrast to some previous publications, no clear pattern of a composition was seen. Concentrations of clostridia and bifidobacteria ranged from 10^3 to 10^7 per ng/stool. The two interventions did not significantly affect the fecal abundances of either *Clostridium* cluster IV or *Bifidobacterium* spp. in the total group. Of note, to date conflicting data have been published with respect to the proportions of bifidobacteria and *Clostridium* cluster IV in the gut microbiota of older persons, as well as to the efficacy of dietary interventions in modulating microbiota composition and activity [26–28]. However, most feeding trials with pre- and probiotics consistently indicated an increase in fecal *Bifidobacterium* levels [26,28]. In the present investigations, interestingly, all participants with low-grade inflammation showed an increased bifidobacteria population after VSL#3 intervention, and the mean increase was significantly higher compared with diet alone. These observations were not made in participants without low-grade inflammation.

It must be noted that the study assessment did not include a search for the specific bacterial strains found in VSL#3, so other changes in colonization may have occurred but not been measured. In addition, colonization is not the only parameter for evaluating the clinical impact of probiotics, as they may exert their health benefits through mechanisms of action different from alteration of microbiota composition [26–28].

Interestingly, in the study population, diet plus VSL#3, but not diet alone, was associated with a clinically relevant reduction in homocysteine levels, and increased levels of folate and vitamin B12. Both vitamins increased significantly more in the subgroup with low-grade inflammation. Raised homocysteine levels have been identified in many epidemiological studies as an independent risk factor for cardiovascular disease [29,30], and for stroke in patients with pre-existing coronary heart disease [31]. High plasma levels of homocysteine contribute approximately 10% of the thrombotic risk in the general population [32], most probably in combination with others risk factors, such as smoking and high blood pressure [33]. Thus, the reduction of homocysteine could indicate a potential role for VSL#3 in reducing cardiovascular risk. Folate is involved in the metabolism of homocysteine to methionine, and increased folate levels are associated with homocysteine lowering [34]. Increased folate was also associated with decreased homocysteine in the present report, albeit only in the subgroup with low-grade inflammation. Clinical studies have confirmed the close relationship between plasma homocysteine and folate [35–37], so that the presence of hyperhomocysteinemia should be considered as an index of folate deficiency from inadequate intake or absorption. Inadequate intake is due to the low consumption of raw vegetables,

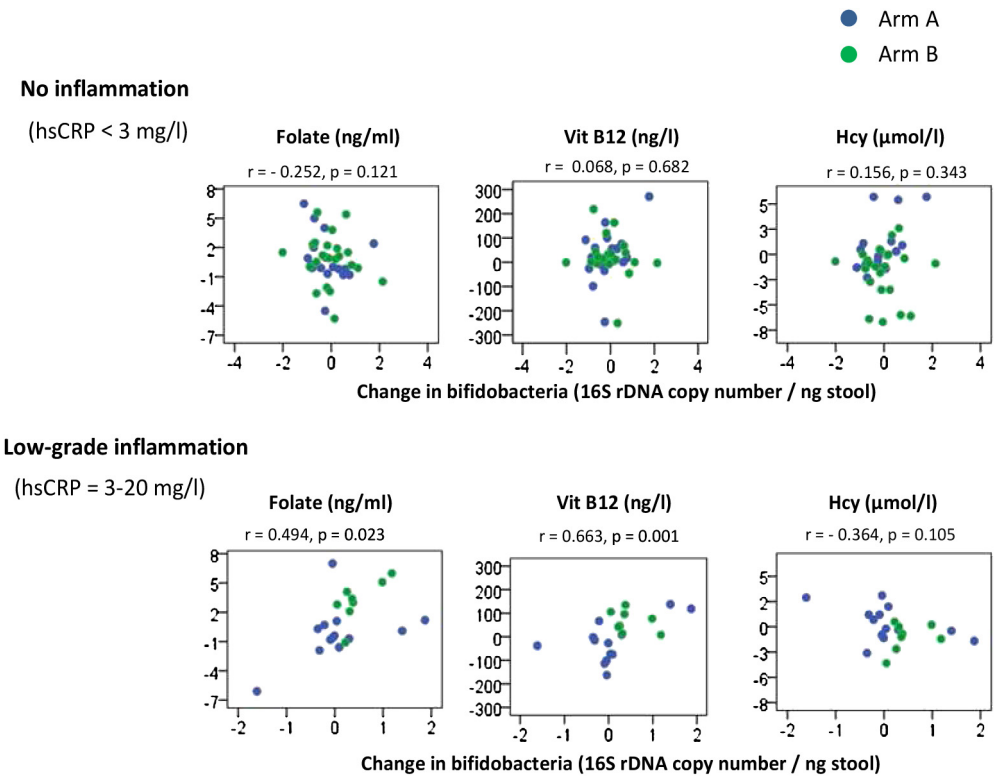


Fig. 4. Association between before-after changes in folate, vitamin B12 and homocysteine (Hcy) and changes in the amount of bifidobacteria. Stratified for participants without inflammation (hsCRP < 3 mg/l)) and participants with existing low-grade inflammation (hsCRP 3–20 mg/l). Blue circles: group A (diet only); green circles: group B (diet + VSL#3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

particularly common in older persons. The average European diet provides only about 100 µg per day of folic acid from non-vegetable sources. However, about 300 µg/day are necessary to meet the needs of healthy subjects, and thus folic acid has to be integrated into the diet. Some degree of folate deficiency is very likely in older persons due to reduced dietary intake and/or poor absorption.

Experimental studies suggest that it may be possible to use certain strains of bifidobacteria to increase folate uptake, either through their use in foods, such as fermented dairy products, or in the intestine by administration as live bacterial supplements [38]. Several strains of bifidobacteria have been shown to produce folate in the gut of rats, leading to increased plasma folate levels [39,40]. In a pilot study, three strains, belonging to *Bifidobacterium adolescentis* and *Bifidobacterium pseudocatenulatum* species, were also shown to synthesize and secrete folate in the human intestine [40]. Also, some strains of lactic acid bacteria are able to synthesize B vitamins, and these could be used in fermentation to enhance the vitamin content of cereal-based foodstuffs [41]. According to this, the administration of *Bifidobacterium* and *Lactobacillus* spp. included in VSL#3, along with the increase in the bifidobacterial community detected at study end, may have contributed to provide a complementary endogenous source of folate, leading to the increase of serum folate levels in the diet plus VSL#3 study arm. Currently we are unable to explain why relevant increases occurred in the subgroup with low-grade inflammation only.

Vitamin B12 also has a homocysteine lowering effect. Subclinical vitamin B12 deficiency is more prevalent in older persons than in the general population. The age-related decline in the production of gastric acids results in decreased release of vitamin B12 from protein in the food and thus decreased bioavailability [42]. Subclinical vitamin B12 deficiency has been associated with increased risk of frailty in several epidemiological studies [43,44]. Supplementation to increase levels of folate and vitamin B12 is also associated with reduced risk of age-related macular degeneration [45]. There is some evidence suggesting a possible association between low vitamin B12 levels and cognitive decline, although more work is needed to confirm this [34,46,47]. Combined folate and vitamin B12 supplementation has recently been shown to improve cognitive functioning in older people with depression [48].

This study has two possible methodological limitations. Firstly, it was an open label study without a placebo arm; secondly, it used biological instead of clinical endpoints. We still believe that the results are valuable since it is very unlikely that endpoints like intestinal microbiota or inflammatory parameters might be influenced by the knowledge of the composition of the supplement. Furthermore these biological endpoints, although they may be considered surrogate endpoints, are generally accepted indicators for clinical endpoints, which might only be observed with a much longer follow-up time. Nevertheless, this study gives important information about new approaches to improve the diet for older persons.

Another limitation might be seen in not analyzing biomarkers of intake, such as serum concentrations of vitamins E or C, carotenoids, selenium, zinc or PUFAs, although they could have been useful to verify the compliance to RISTOMED diet. Nevertheless, dietary compliance was monitored and strengthened through nutritional counseling by weekly phone calls and every 4 weeks by ambulatory follow-ups; moreover the subjects were encouraged to record on the web-platform any changes about the prescribed diet.

We can not exclude that stricter dietary recommendations for higher folate and B12 intake can result in increased concentrations of both vitamins independent of VSL#3 intake. Nevertheless, both arms followed the same RISTOMED diet; so the difference seen in

the current report emerges from the addition of VSL#3. To our knowledge and suggested by a recent review [49] this is the first time that increased vitamin serum concentrations by intake of a bacterial blend was observed in humans.

In conclusion, the RISTOMED web platform dietary intervention in older adults is feasible, but dietary approaches that can be useful to reduce low-grade inflammation must be reassessed for future investigations. The reduction in homocysteine concentration and the increase in folate and vitamin B12 seen with VSL#3 supplementation in existing low-grade inflammation could potentially reduce the risk of a range of age-related conditions. Long-term studies are warranted to investigate whether VSL#3 can improve or slow the decline of physical or cognitive function in older people, and reduce cardiovascular risk.

Author contributions

Conception and design of the study: AP, IBM, CF, HL, LV, RO, FB, PB, SH, PH, FP, FB, LMD. Acquisition of data: LV, AP, IBM, CBB, MM, EL JD, LMD, CF, HL. Analysis and interpretation of data: LS, RO, PB, ST, SH, PH, SB, AF, EL, MM. Drafting of the article or revising it critically for important intellectual content: HL, LV, FP, LS, IBM, RO, PB, ST, AP, SH, PH, LMD, CF. Final approval of the manuscript: all authors.

Conflict of interest

Fabio Buccolini is owner of the RISTOMED platform and Florence Pryen was employed by Actial Farmaceutica at the time of study implementation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2014.09.023.

References

[1] Li H, Manwani B, Leng SX. Frailty, inflammation, and immunity. Aging Dis 2011;2(6):466–73.
[2] Kregel KC, Zhang HJ. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. Am J Physiol Regul Integr Comp Physiol 2007;292(1):R18–36.
[3] Franceschi C, Bonafe M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci 2000;908:244–54.
[4] Harris TB, Ferrucci L, Tracy RP, Corti MC, Wacholder S, Ettinger Jr WH, et al. Associations of elevated interleukin-6 and C-reactive protein levels with mortality in the elderly. Am J Med 1999;106(5):506–12.
[5] Reuben DB, Cheh AI, Harris TB, Ferrucci L, Rowe JW, Tracy RP, et al. Peripheral blood markers of inflammation predict mortality and functional decline in high-functioning community-dwelling older persons. J Am Geriatr Soc 2002;50(4):638–44.
[6] Carriere I, Dupuy AM, Lacroux A, Cristol JP, Delcourt C. Biomarkers of inflammation and malnutrition associated with early death in healthy elderly people. J Am Geriatr Soc 2008;56(5):840–6.
[7] Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol 2011;12(1):5–9.
[8] Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. Sci (New York, NY) 2012;336(6086):1262–7.
[9] Clarke R, Grimley Evans J, Schneede J, Nexo E, Bates C, Fletcher A, et al. Vitamin B12 and folate deficiency in later life. Age Ageing 2004;33(1):34–41.
[10] Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57(6):1470–81.
[11] Biagi E, Nylund L, Candela M, Ostan R, Bucci L, Pini E, et al. Through ageing, and beyond: gut microbiota and inflammatory status in Seniors and Centenarians. PLoS ONE 2010;5(5):e10667.
[12] Chrysohoou C, Panagiotakos DB, Pitsavos C, Das UN, Stefanadis C. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adultsThe Attica study. J Am Coll Cardiol 2004;44(1):152–8.
[13] Sies H, Stahl W, Sevanian A. Nutritional, dietary and postprandial oxidative stress. J Nutr 2005;135(5):969–72.
[14] De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci 2010;107(33):14691–6.
[15] Sood A, Midha V, Makharia GK, Ahuja V, Singal D, Goswami P, et al. The probiotic preparation, VSL#3 induces remission in patients with mild-to-moderately active ulcerative colitis. Clin Gastroenterol Hepatol 2009;7(11).1202-9, 9 e1.
[16] Kim HJ, Camilleri M, McKinzie S, Lempke MB, Burton DD, Thomforde GM, et al. A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. Aliment Pharmacol Ther 2003;17(7):895–904.
[17] Loguercio C, Federico A, Tuccillo C, Terracciano F, D'Auria MV, De Simone C, et al. Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases. J Clin Gastroenterol 2005;39(6):540–3.
[18] Agrawal A, Sharma BC, Sharma P, Sarin SK. Secondary prophylaxis of hepatic encephalopathy in cirrhosis: an open-label, randomized controlled trial of lactulose, probiotics, and no therapy. Am J Gastroenterol 2012;107(7):1043–50.
[19] Mittal VV, Sharma BC, Sharma P, Sarin SK. A randomized controlled trial comparing lactulose, probiotics, and L-ornithine L-aspartate in treatment of minimal hepatic encephalopathy. Eur J Gastroenterol Hepatol 2011;23(8):725–32.
[20] Pearson TA, Mensah GA, Alexander RW, Anderson JL, Cannon 3rd RO, Criqui M, et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice: a statement for healthcare professionals from the Centers for Disease Control and Prevention and the American Heart Association. Circulation 2003;107(3):499–511.
[21] Maffei F, Angeloni C, Malaguti M, Moraga JM, Pasqui F, Poli C, et al. Plasma antioxidant enzymes and clastogenic factors as possible biomarkers of colorectal cancer risk. Mutat Res 2011;714(1–2):88–92.
[22] Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, et al. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 2002;68(11):5445–51.
[23] Ballantyne CM, Nambi V. Markers of inflammation and their clinical significance. Atheroscler Suppl 2005;6(2):21–9.
[24] Imhof A, Frohlich M, Loewel H, Helbecque N, Woodward M, Amouyel P, et al. Distributions of C-reactive protein measured by high-sensitivity assays in apparently healthy men and women from different populations in Europe. Clin Chem 2003;49(4):669–72.
[25] de Mello VD, Schwab U, Kolehmainen M, Koenig W, Siloaho M, Poutanen K, et al. A diet high in fatty fish, bilberries and wholegrain products improves markers of endothelial function and inflammation in individuals with impaired glucose metabolism in a randomised controlled trial: the Sysdimet study. Diabetologia 2011;54(11):2755–67.
[26] Biagi E, Candela M, Turroni S, Garagnani P, Franceschi C, Brigidi P. Ageing and gut microbes: perspectives for health maintenance and longevity. Pharmacol Res 2012;69(1):11–20.
[27] Michail S, Kenche H. Gut microbiota is not modified by Randomized, Double-blind, placebo-controlled Trial of VSL#3 in diarrhea-predominant irritable bowel syndrome. Probiotics Antimicrob Proteins 2011;3(1):1–7.
[28] Rampelli S, Candela M, Severgnini M, Biagi E, Turroni S, Roselli M, et al. A probiotics-containing biscuit modulates the intestinal microbiota in the elderly. J Nutr Health Aging 2013;17(2):166–72.
[29] Humphrey LL, Fu R, Rogers K, Freeman M, Helfand M. Homocysteine level and coronary heart disease incidence: a systematic review and meta-analysis. Mayo Clin Proc 2008;83(11):1203–12.
[30] Antoniades C, Antonopoulos AS, Tousoulis D, Marinou K, Stefanadis C. Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. Eur Heart J 2009;30(1):6–15.
[31] Tanne D, Haim M, Goldbourt U, Boyko V, Doolman R, Adler Y, et al. Prospective study of serum homocysteine and risk of ischemic stroke among patients with preexisting coronary heart disease. Stroke 2003;34(3):632–6.
[32] Motulsky AG. Nutritional ecogenetics: homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid. Am J Hum Genet 1996;58(1):17–20.
[33] Graham IM, Daly LE, Refsum HM, Robinson K, Brattstrom LE, Ueland PM, et al. Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. JAMA 1997;277(22):1775–81.
[34] Brattstrom LE, Israelsson B, Jeppsson JO, Hultberg BL. Folic acid–an innocuous means to reduce plasma homocysteine. Scand J Clin Lab Invest 1988;48(3):215–21.
[35] de Franchis R, Fermo I, Mazzola G, Sebastio G, Di Minno G, Coppola A, et al. Contribution of the cystathionine beta-synthase gene (844ins68) polymorphism to the risk of early-onset venous and arterial occlusive disease and of fasting hyperhomocysteinemia. Thromb Haemost 2000;84(4):576–82.

[36] Guttormsen AB, Ueland PM, Nesthus I, Nygard O, Schneede J, Vollset SE, et al. Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia (> or = 40 micromol/liter). The Hordaland Homocysteine Study. J Clin Invest 1996;98(9):2174–83.
[37] Kluijtmans LA, Young IS, Boreham CA, Murray L, McMaster D, McNulty H, et al. Genetic and nutritional factors contributing to hyperhomocysteinemia in young adults. Blood 2003;101(7):2483–8.
[38] D'Aimmo MR, Mattarelli P, Biavati B, Carlsson NG, Andlid T. The potential of bifidobacteria as a source of natural folate. J Appl Microbiol 2012;112(5):975–84.
[39] Rossi M, Amaretti A, Raimondi S. Folate production by probiotic bacteria. Nutrients 2011;3(1):118–34.
[40] Strozzi GP, Mogna L. Quantification of folic acid in human feces after administration of bifidobacterium probiotic strains. J Clin Gastroenterology 2008;42. S179-S84 10.1097/MCG.0b013e31818087d8.
[41] Capozzi V, Russo P, Duenas MT, Lopez P, Spano G. Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. Appl Microbiol Biotechnol 2012;96(6):1383–94.
[42] O'Leary F, Samman S. Vitamin B12 in health and disease. Nutrients 2010;2(3):299–316.
[43] Matteini AM, Walston JD, Fallin MD, Bandeen-Roche K, Kao WH, Semba RD, et al. Markers of B-vitamin deficiency and frailty in older women. J Nutr Health Aging 2008;12(5):303–8.
[44] Bartali B, Semba RD, Frongillo EA, Varadhan R, Ricks MO, Blaum CS, et al. Low micronutrient levels as a predictor of incident disability in older women. Arch Intern Med 2006;166(21):2335–40.
[45] Christen WG, Glynn RJ, Chew EY, Albert CM, Manson JE. Folic acid, pyridoxine, and cyanocobalamin combination treatment and age-related macular degeneration in women: the Women's antioxidant and Folic Acid Cardiovascular Study. Arch Intern Med 2009;169(4):335–41.
[46] de Jager CA, Oulhaj A, Jacoby R, Refsum H, Smith AD. Cognitive and clinical outcomes of homocysteine-lowering B-vitamin treatment in mild cognitive impairment: a randomized controlled trial. Int J Geriatric Psychiatry 2012;27(6):592–600.
[47] Smith AD, Refsum H. Vitamin B-12 and cognition in the elderly. Am J Clin Nutr 2009;89(2):707S–11S.
[48] Walker JG, Batterham PJ, Mackinnon AJ, Jorm AF, Hickie I, Fenech M, et al. Oral folic acid and vitamin B-12 supplementation to prevent cognitive decline in community-dwelling older adults with depressive symptoms–the beyond Ageing Project: a randomized controlled trial. Am J Clin Nutr 2012;95(1):194–203.
[49] LeBlanc GJ, Milan C, Savoy de Giori G, Sesma F, Van Sinderen D, Ventura M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr Op Biotechnol 2013;24:160–8.