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Effect of Mastiha supplementation on NAFLD: The MAST4HEALTH Randomised, Controlled Trial

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Scope: Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease with poor therapeutic strategies. Mastiha possesses antioxidant/anti-inflammatory and lipid-lowering properties. The authors investigate the effectiveness of Mastiha as a nonpharmacological intervention in NAFLD.

Methods and Results: Ninety-eight patients with NAFLD in three countries (Greece, Italy, Serbia) are randomly allocated to either Mastiha or Placebo for 6 months, as part of a multicenter, randomized, double-blind, placebo-controlled, parallel-group clinical trial. The authors assess NAFLD severity via magnetic resonance imaging (MRI) scanning and LiverMultiScan technique and evaluate the effectiveness of Mastiha through medical, anthropometric, biochemical, metabolomic, and microbiota assessment. Mastiha is not superior to Placebo on changes in iron-corrected T1 (cT1) and Liver Inflammation Fibrosis score (LIF) in entire patient population; however, after BMI stratification (BMI $\leq$ 35 kg m^{-2} and BMI $>$ 35 kg m^{-2}), severely obese patients show an improvement in cT1 and LIF in Mastiha versus Placebo. Mastiha increases dissimilarity of gut microbiota, as shown by the Bray-Curtis index, downregulates *Flavonifractor*, a known inflammatory taxon and decreases Lysophosphatidylcholines-(LysoPC) 18:1, Lysophosphatidylethanolamines-(LysoPE) 18:1, and cholic acid compared to Placebo.

Conclusion: Mastiha supplementation improves microbiota dysbiosis and lipid metabolite levels in patients with NAFLD, although it reduces parameters of liver inflammation/fibrosis only in severely obese patients.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease, characterized by excessive fat accumulation in liver, not caused by alcohol consumption.[1] It ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) and is associated with obesity, dyslipidemia, insulin resistance, and high cardiometabolic risk.[2]

Liver biopsy is considered the gold standard in diagnosis and prognosis of NASH, but it is expensive, invasive, with variable results and procedural complications. NAFLD diagnosis can be relied on imaging techniques of which magnetic resonance imaging (MRI) is the gold standard.[1] Repetition of liver biopsy to monitor progression of the disease might be unfeasible and changes in liver fat alone are not predictive of histological changes.[3] There is an urgent need for reliable, accurate, and non- or minimally invasive methods like imaging and biomarker panels.[4] Noninvasive predictive algorithms for hepatic fibrosis are based on routine, inexpensive clinical and biochemical parameters,[2] but lack diagnostic accuracy. MRI offers good

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sensitivity and specificity in detecting histologically confirmed steatosis, ranging from 76.7% to 90.0% and 87.1% to 91%, respectively.[5] LiverMultiScan (Perspectum Ltd, UK) is a recent multiparametric MRI technique for the quantification of fibrosis and inflammation[6] and has been successfully used to detect and stage liver disease in clinical trials.[7,8]

Microbiome dysbiosis is frequently detected in NAFLD and is associated with increased intestinal permeability, leading to bacterial translocation and contributing to hepatic inflammation.[9] Several studies have identified bacterial genera, families, and phyla that differ significantly in NAFLD and are implicated in the disease pathogenesis through increased intestinal permeability (i.e., Lachnospiraceae), decreased short chain fatty acids (SCFA) production (i.e., *Faecalibacterium*), and elevated serum endotoxin production (i.e., *Bacteroides*, Enterobacteriaceae).[10,11]

While lifestyle modification remains the main mode of therapy,[1] still there is an unmet need for new treatments, with natural products being explored as candidates.[12] Mastiha is a natural nutritional supplement based on the dried resinous exudate from stems and branches of the tree *Pistacia lentiscus*. The resin consists of several bioactive compounds, such as terpenes, the poly-β-myrcene (approximately 20%), phytosterols, and phenolic compounds.[13,14] It possesses anti-bacterial[15], antioxidant, and anti-inflammatory activities[13,16,17] and modulates biochemical parameters, e.g., total cholesterol, lipoprotein (a), γ-glutamyltransferase (γ-GT), aspartate transaminase (AST), and alanine transaminase (ALT).[18] In mice with diet-induced obesity, NASH, and fibrosis, Mastiha reduced plasma ALT, hepatic steatosis, and histological activity score.[19]

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Mastiha Treatment for Obese with NAFLD Diagnosis (MAST4HEALTH) is an EU-funded project designed to explore the effectiveness of Mastiha as a nonpharmacological intervention in NAFLD. We hereby report the results of the multicenter, randomized, double-blinded, and placebo-controlled clinical trial where the effect of Mastiha on liver inflammation and fibrosis was investigated through MRI, biochemical, and multi-omic analyses.

2. Experimental Section

MAST4HEALTH was conducted in three clinical trial sites (Department of Dietetics and Nutritional Science, Harokopio University, Athens, Greece (HUA), Consiglio Nazionale delle Ricerche Institute of Clinical Physiology, Milano section at Niguarda Hospital Italy, (CNR) and Faculty of Medicine, University of Novi Sad, Serbia (UNS)). The Consortium consists of 13 partners (seven academic, six nonacademic) from eight European countries (Bosnia Herzegovina, France, Germany, Greece, Italy, Serbia, Spain, United Kingdom) under the coordination of HUA.

2.1. Ethics

Ethics Committees approvals were obtained from all centers, HUA (Bioethics Committee 49/29-10-2015), CNR (Ethical Clearance by Commissione per l'Etica e l'Integrità nella Ricerca, February 2016), and Niguarda Hospital Ethics Committee 230–052017 (Comitato Etico Milano Area 3–11.05.2017), UNS (Faculty of Medicine Novi Sad, The Human Research Ethics Commission No. 01–39/58/1-27.06.2016). The trial was conducted following the Helsinki declaration and the Data Protection Act 1998, and was registered with ClinicalTrials.gov (Identifier: NCT03135873). All volunteers gave their informed consent after given a detailed information leaflet. Also, participant consent for genotyping was obtained. "Ample time" was given for consideration and participants were free to leave the study at any time. A case report form was created for reporting adverse events. All authors had access to the study data and reviewed and approved the final manuscript.

2.2. Study population

Recruitment took place from 2017 to 2019 following specific inclusion and exclusion criteria: The study group comprised of men and women with established NAFLD/NASH based on the sensitive LiverMultiScan technique (Perspectum Ltd, UK), aged 18–67 years and with BMI >30 kg m^{-2}. Exclusion criteria included hepatotoxic medication, concomitant liver disease, decompensated diabetes mellitus [diabetes mellitus type 1, uncontrolled diabetes mellitus type 2 (HbA1c ≥ 7.5%), thyroid disease, hypopituitarism, Cushing syndrome, alcohol abuse (>20 g day^{-1} (women), >30 g day^{-1} (men), EASL Guidelines][1] or drug addiction, systemic diseases, pregnancy, lactation, vegan or lacto- and ovo-lacto-vegetarianism, psychiatric or mental disorder, recent loss in body weight or current diet, any use of antioxidant-phytochemical rich supplement, pre- or pro-biotics, changes in drug treatment, antibiotic treatment during, or prior to screening.

After completing the baseline assessment, patients were randomly allocated to either the Mastiha or the Placebo group. To avoid allocation bias, randomization was carried out by a computer-generated random number list (Supporting Information). Both groups received nutritional counseling to allow for body weight regulation up to 5% of initial body weight within 6 months. Nutritional counselling was focused on the distribution of nutrients in relation to total caloric value as follows: 30% of total energy as fat (<10% as SFAs, ~10% as MUFAs, and ~10% as PUFAs), 20% as protein, 50% as carbohydrate, 300 mg day^{-1} as dietary cholesterol, and 20–30 g fiber day^{-1}. Daily energy requirements were calculated based on the Harris–Benedict equation of basic metabolic rate and sedentary lifestyle. The un-blinding took place on the completion of the study, once all statistical analyses were completed. Compliance and side effects were monitored bi-weekly through phone calls. No side effect or any discomfort was reported.

2.3. Dosage Information/Dosage Regimen

Mastiha (100% natural) or matching placebo (corn starch) capsules weighing 0.35 g each were given in three equal doses daily (total of 2.1 g). The dose was chosen based on its effectiveness towards inflammation in a pilot study while exhibiting no side effects.[16,17] Placebo was identical in physical form, sensory perception, packaging, and labeling, with no pharmaceutical activity.

2.4. Medical, Anthropometric, and Lifestyle Assessment

Detailed medical history was obtained including personal/family medical history and medication. Body weight was measured to the nearest 0.1 kg. Height was measured to the nearest millimeter and BMI was computed as weight (kg) / height $(m)^2$. Total diabetes risk was assessed using the validated Finnish Diabetic Risk Score (FINDRISK) questionnaire.[20] Dietary intake was assessed using a 24-h recall record (three random days) and data was analyzed using Nutritionist Pro™ (Axxya Systems) software for the estimation of caloric intake. Physical activity level was evaluated via the International Physical Activity Questionnaire (IPAQ)[21] and Metabolic Equivalent Task minutes per week (MET-min wk^{-1}) were derived according to the IPAQ scoring protocol. The sum of all METs has been considered as a total physical activity score.

2.5. Trial Outcomes

The primary outcome was the improvement in Liver Inflammation Fibrosis score (LIF) assessed by MRI scanning and the sensitive LiverMultiScan.[22] LIF is based on iron-corrected (cT1) that correlates with liver fibro-inflammation and NAFLD activity score (NAS).[23] LIF has been superseded by cT1 to reflect improved algorithm correction, ensuring cross-scanner and field strength reproducibility, as well as repeatability for this metric.[24]

Table 1. Selected baseline characteristics for Mastiha and Placebo groups.

Baseline Characteristics	All		Placebo		Mastiha		p^*
	n	Mean (SD) or *n*	*n*	Mean (SD) or *n*	*n*	Mean (SD) or *n*	
Age (years)	98	48.83 (9.36)	57	48.95 (9.04)	41	48.66 (9.89)	0.929[$]
Sex (M/F)	98	68/30	57	42/15	41	26/15	0.386
Centre (GR/IT/SR)	98	38/30/30	57	23/17/17	41	15/13/13	0.931
Statin (Y/N)	98	12/86	57	8/49	41	4/37	0.636
T2D (Y/N)	98	4/94	57	2/55	41	2/39	1.000
Total Physical Activity Score	91	3622.17 (5128.18)	52	3536.78 (5345.85)	39	3736.04 (4889.48)	0.921
BMI (kg m^{-2})	98	34.44 (4.41)	57	34.66 (5.05)	41	34.14 (3.38)	0.513
Glucose (mg dL^{-1})	93	102.44 (15.64)	53	102.89 (14.38)	40	101.84 (17.33)	0.893
120 min-OGTT glucose (mg dL^{-1})	87	131.57 (47.47)	47	126.88 (41.86)	40	137.08 (53.33)	0.260
Insulin (μU mL^{-1})	94	18.94 (9.79)	54	18.63 (10.46)	40	19.38 (8.92)	0.586
HOMA_IR	90	4.88 (2.6)	51	4.83 (2.69)	39	4.95 (2.52)	0.654
FINDRISK_score	96	13.67 (3.77)	56	13.32 (3.69)	40	14.15 (3.89)	0.217
TC (mg dL^{-1})	98	201.75 (37.43)	57	202.91 (37.61)	41	200.14 (37.59)	0.528
LDL (mg dL^{-1})	97	127.14 (34.64)	56	129.69 (37.31)	41	123.65 (30.75)	0.276
Triglycerides (mg dL^{-1})	98	148.21 (65.08)	57	141.96 59.35)	41	156.91 (72.15)	0.331
HDL (mg dL^{-1})	98	44.49 (10.35)	57	44.31 (9.91)	41	44.75 (11.04)	0.980
ALT (IU L^{-1})	95	37.78 (20.45)	56	36.7 (21.67)	39	39.33 (18.71)	0.253
AST (IU L^{-1})	95	25.29 (11.12)	56	24.39 (11.72)	39	26.59 (10.19)	0.198
AST/ALT	95	0.74 (0.24)	56	0.74 (0.25)	39	0.74 (0.22)	0.477
γ-GT (U L^{-1})	97	55.12 (60.22)	57	49.63 (54.97)	40	62.95 (66.94)	0.305
NFS_score	97	−1.97 (1.39)	57	−2.01 (1.53)	40	−1.91 (1.2)	0.713
NASH_score	88	−1.24 (0.94)	50	−1.32 (0.94)	38	−1.14 (0.94)	0.218
LIF	95	2.26 (0.62)	55	2.25 (0.68)	40	2.29 (0.55)	0.667
Hepatic_Iron (mg g^{-1})	98	1.25 (0.22)	57	1.24 (0.24)	41	1.26 (0.2)	0.451
cT1 (ms)	95	878.36 (79.49)	55	879.88 (92.12)	40	876.26 (58.93)	0.876
PDFF (%)	96	16.47 (11.98)	57	16.09 (13.31)	39	17.02 (9.87)	0.547

Results are presented as mean (SD) for continuous variables and counts for categorical ones. $^*p$ value for the difference between Placebo and Mastiha groups was assessed with ANCOVA (adjusted for age, sex, and center) for the continuous variables and with Chi-square for the categorical ones; [$] Adjusted only for sex and center.

Other outcomes accessed were: changes in proton density fat fraction (PDFF), hepatic iron content, BMI, total diabetes risk, liver function enzymes, lipid profile, insulin resistance, NAFLD fibrosis score (NFS),[25] NASH score,[26] plasma metabolites, and gut microbiota composition (details on samples collection and biomarkers in Supporting Information).

2.6. Sequencing and Analysis of 16S rRNA Amplicons

DNA from fecal samples was extracted using the MagNA Pure LC DNA isolation kit II (Roche Life Science, Basel, Switzerland). The V3-V4 region of the 16S rRNA gene was amplified to construct amplicon libraries that were sequenced using the Reagent Kit v3 (2 × 300 cycles) in a MiSeq platform (Illumina, San Diego, CA, USA). The authors used the DADA2 pipeline[27] (R package) to generate amplicon sequence variants (ASV) from raw sequences. Chimeras and sequences matching the human genome were filtered out to generate the final ASV abundance tables. The taxonomic information of the ASVs was obtained by comparison with the SILVA reference database (v.132).[28] The sequences have been deposited in the European Nucleotide Archive under accession number PRJEB40538.

2.7. Liquid Chromatography–High Resolution Mass Spectrometry (LC-HRMS) Based Metabolomics

LC-HRMS analysis was performed as described in the Supporting Information. All samples were analyzed in duplicate, in a random order. QC pooled samples were used and mass accuracy was maintained ≤5 ppm. Raw data file preprocessing was achieved using the MZMine 2.53 software.[29] A generic streamline was employed, including mass detection, chromatogram building, chromatogram deconvolution, isotopic peak grouping, spectral alignment, and gap filling to generate the peak list. Metabolite annotation was performed comparing the recorded HRMS and HRMS/MS spectra with online databases such as METLIN[30] and the Human Metabolome Database (HMDB).[31] Further details in Supporting information.

Table 2. Significant associations between taxon balances and MRI variables, in both groups, at baseline.

Balance	Bacterial genera or genus-level groups		LIF		cT1		PDFF		Hepatic Iron	
		n	Beta	*p*	Beta	*p*	Beta	*p*	Beta	*p*
Balance 1	*Faecalibacterium*[a], *Fusobacterium*[b], *Prevotella 9*[b]	89	−0.210	0.178	−0.002	0.098	−0.034	6.4e-08	−0.252	0.556
Balance 2	*Veillonella*[b]	89	−0.466	1.5e-02	−0.006	5.9e-05	−0.029	1.3e-02	0.231	0.895
Balance 3	Ruminococcaceae UCG-014[a]	89	0.618	1.4e-03	0.004	9.2e-03	0.011	0.250	−0.160	0.996
Balance 4	*Coprobacter*[b]	89	0.262	1.7e-03	0.002	1.2e-02	0.005	0.242	−0.002	0.993

[a] The bacterial group decreases with the associated MRI variables; [b] The bacterial group increases with the associated MRI variables.

2.8. Statistical Analysis

Primary and secondary outcomes, selected metabolomics, and microbiota markers

Twenty anthropometric, biochemical and liver MRI outcomes (as listed in **Table 1**, starting with BMI), were considered. Apart for the triglyceride levels (log transformed), the population normal distribution for the rest was assumed. Furthermore, the proportions of 11 bacterial taxa were selected to be included in the main analysis, based on their significant association with liver MRI outcomes at baseline (**Table 2**) or their previously reported associations with dysbiosis in NAFLD[11,32] (Supporting Information). Three indexes, namely the Chao1 richness index, the Shannon diversity index and the Bray-Curtis dissimilarity index, were also computed to evaluate the overall change in gut microbiota composition and diversity (Supporting Information). The authors obtained levels for 65 annotated metabolites and calculated the mean values per metabolite per sample per time point, based on duplicate measurements. Baseline and post-treatment metabolite levels have been transformed and normalized, via the "bestNormalise" package in R, prior to the analysis. A list of all metabolites and the transformation applied is shown in Table S1, Supporting Information. As part of the exploratory analysis, a PCA including all 65 metabolites at baseline and post-treatment was performed, but we did not observe any significant clustering between the intervention groups (Fig. S1, Supporting Information). In order to prioritize metabolites for further statistical analysis, all metabolites with a log2fold change >1.5 between the Mastiha and Placebo groups post-treatment have been selected. These included 24 metabolites, as shown in Fig. S2, Supporting Information. The selection of the microbiota and metabolites variables is described in more detail in the Supporting Information.

In total, 58 variables under this group of outcomes have been considered, including anthropometric, biochemical, liver MRI (20 variables), microbiota (14 variables), and metabolites (24 variables). To avoid overcorrection for multiple testing, the Pearson's coefficient for all pairwise correlations among these 58 variables have been calculated (Fig S3, Supporting Information). Then, the effective number of tests,[33] as estimated via the "meff" function of the "poolr" package in R, has been ascertained. The authors performed 34 independent tests and accordingly set the multiple testing threshold of significance at 0.0015. Additionally, findings at nominal level of significance ($p \leq 0.05$) were also reported. To assess the effect of the Mastiha treatment on each of these 58 variables, post-treatment mean levels between the Mastiha and the Placebo have been compared, via analysis of covariance (ANCOVA) models. The models have been adjusted for the corresponding baseline levels of the tested outcome (apart from the Bray Curtis index), age, sex, and the center of recruitment. Furthermore, a number of sensitivity analyses have been performed with a sequential adjustment for the baseline BMI levels, the baseline physical activity levels, the difference in the caloric intake between post-treatment and baseline or the difference in the BMI level between post-treatment and baseline. Based on the latter sensitivity analyses, a further stratification of the study samples into two categories has been made, in order to carefully investigate the effect of Mastiha on the MRI outcomes (namely LIF, cT1, PDFF, and hepatic iron): Class I obesity (BMI$\leq$ 35 kg m^{-2}, $N = 65$) and Class II or III obesity (BMI > 35 kg m^{-2}, $N = 33$). In the stratified analysis, ANCOVA models (adjusted for age, sex, and center) have been applied to assess differences in the mean values of the MRI outcomes, separately for each BMI category (i.e., BMI $\leq$ 35 kg m^{-2} or BMI > 35 kg m^{-2}). All ANCOVA models were implemented in R. The "lsmeans" package in R was also used, in combination with the ANCOVA models, to calculate adjusted means of selected outcomes post-treatment. In order to assess the improvement within individual treatment for the MRI outcomes, the mean difference between post-treatment and baseline was also estimated and tested for significance between the Mastiha and the Placebo group, per BMI category, through ANCOVA models (adjusted for age, sex, and center). Even though the body weight has not considered as an outcome, the changes between baseline and the end of trial, separately in each group, via paired t-tests in R, have been assessed. Power calculations and analysis of differential abundance of gut bacteria are presented in the Supporting Information.

3. Results

Ninety-eight patients were randomized to Mastiha ($N = 41$) or Placebo ($N = 57$) for 6 months (CONSORT Flow diagram in Fig. S4, Supporting Information). Baseline characteristics are presented in Table 1 and Table S2, Supporting Information. Overall, there were no significant differences between Mastiha and Placebo. Patients had moderate liver disease at baseline: mean LIF of 2.3 (SD = 0.6), cT1 of 878.4 (79.5) ms, PDFF of 16.5 (12.00) %, and hepatic iron 1.2 (0.2) mg g^{-1} (Table 1). Out of the 98 volunteers who participated in the trial, 87 completed the

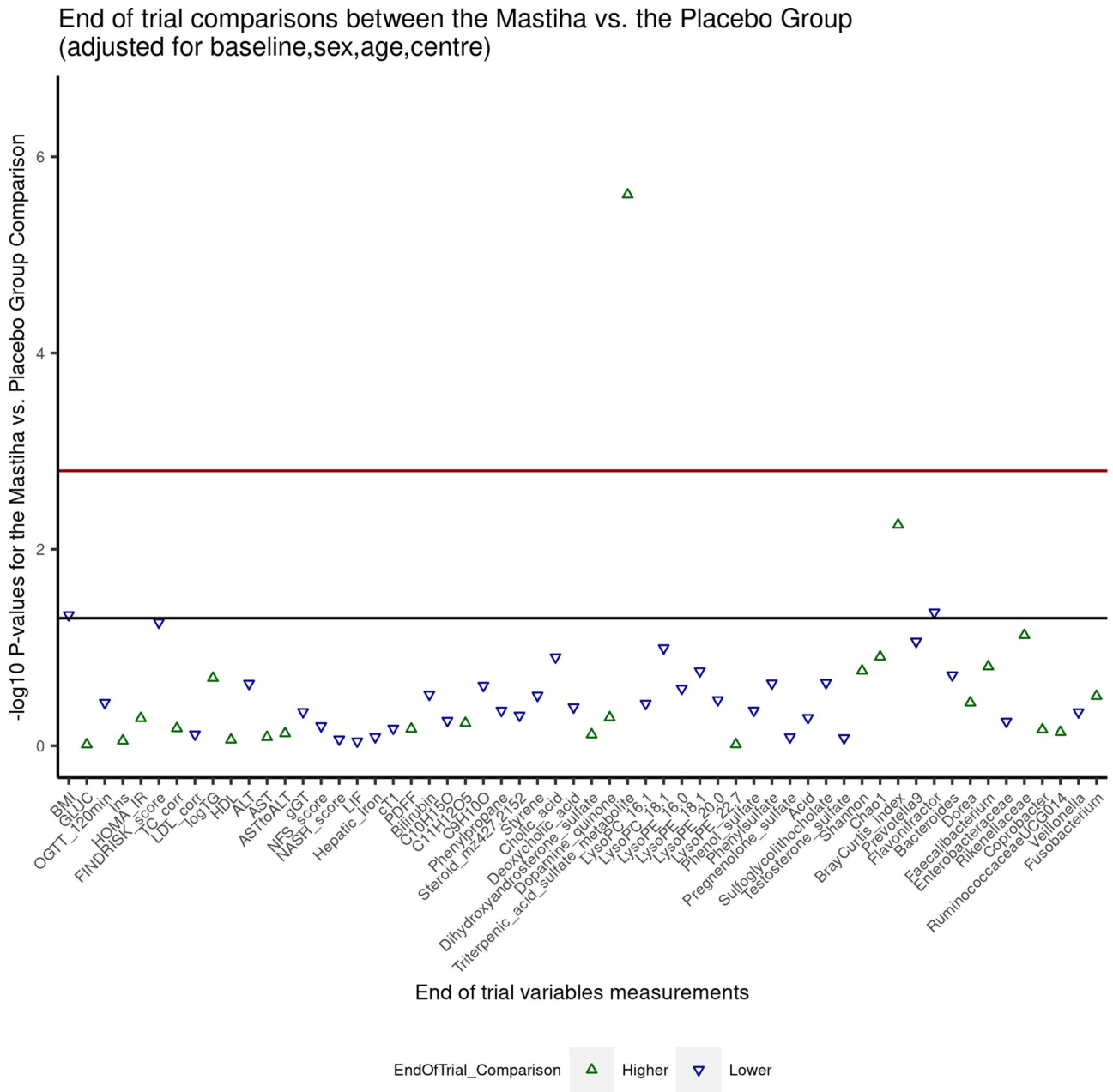

Figure 1. Differences in all outcomes assessed post treatment between the Mastiha and Placebo groups. Comparisons were performed using ANCOVA and adjusted for the corresponding baseline levels for each outcome, age, sex, and center (post-treatment outcome ~ mastiha versus placebo group + baseline outcome + age + sex + center). Triangles indicate the *p* value (-log10 transformed) for the comparison. Blue descending triangles indicate lower mean values in the Mastiha group compared to the Placebo, while green ascending triangles indicate the opposite. The black horizontal line is marking nominal significance level ($p = 0.05$) and the red line the multiple testing significance level ($p = 0.0015$).

intervention. Mean baseline and end-of-trial values for body weight in the Mastiha group were 100.80 kg (±15.84) and 100.23 kg (±15.00) respectively, (paired t-test $p = 0.02$). In Placebo, body weight was 105.13 kg (±19.75) and 104.05 kg (±20.62), respectively, (paired t-test $p = 0.79$). Significant microbiota markers of dysbiosis were associated with liver MRI measurements at baseline (Table 2). The most significant association was between decreased *Faecalibacterium* and increased *Fusobacterium* and *Prevotella 9* and the levels of PDFF ($p = 6.4e-08$).

Post-treatment levels between Mastiha and Placebo for the 58 outcomes have been compared. The findings are summarized in **Figure 1** and Table S3. Sensitivity analyses are presented in the different panels of Fig. S6, Supporting Information.

A very significant post treatment increase in a Mastiha derived metabolite (sulpho-conjugated) (Fig. S5, Supporting Information) within the Mastiha group compared to the Placebo ($p = 2.43e-06$, Table S3, Supporting Information) has been detected and remained robust across the sensitivity analysis

Table 3. Post-treatment MRI outcomes showing significant difference between the Mastiha and Placebo groups, stratified by BMI category.

Post-treatment MRI outcome	Placebo		Mastiha		Post-treatment differences in relation to the Mastiha group	
	n	Mean (SD)	*n*	Mean (SD)	Beta (SE)	*p*
	BMI< = 35 kg m^{-2}					
cT1 (ms)	30	860.13 (52.06)	20	877.2 (58.9)	16.177 (13.691)	0.244
LIF	32	2.08 (0.57)	22	2.32 (0.53)	0.177 (0.124)	0.159
PDFF (%)	33	13.45 (9.63)	22	19.32 (9.75)	2.676 (2.049)	0.198
Hepatic Iron (mg g^{-1})	33	1.21 (0.29)	23	1.24 (0.13)	0.027 (0.032)	0.410
	BMI>35 kg m^{-2}					
cT1 (ms)	13	904.43 (74.71)	12	865.19 (71.32)	−53.526 (23.168)	0.033
LIF	13	2.51 (0.62)	12	2.3 (0.65)	−0.496 (0.235)	0.049
PDFF (%)	15	16.26 (9.32)	12	11.06 (8.44)	−4.903 (3.327)	0.155
Hepatic Iron (mg g^{-1})	15	1.2 (0.17)	12	1.19 (0.21)	−0.059 (0.067)	0.388

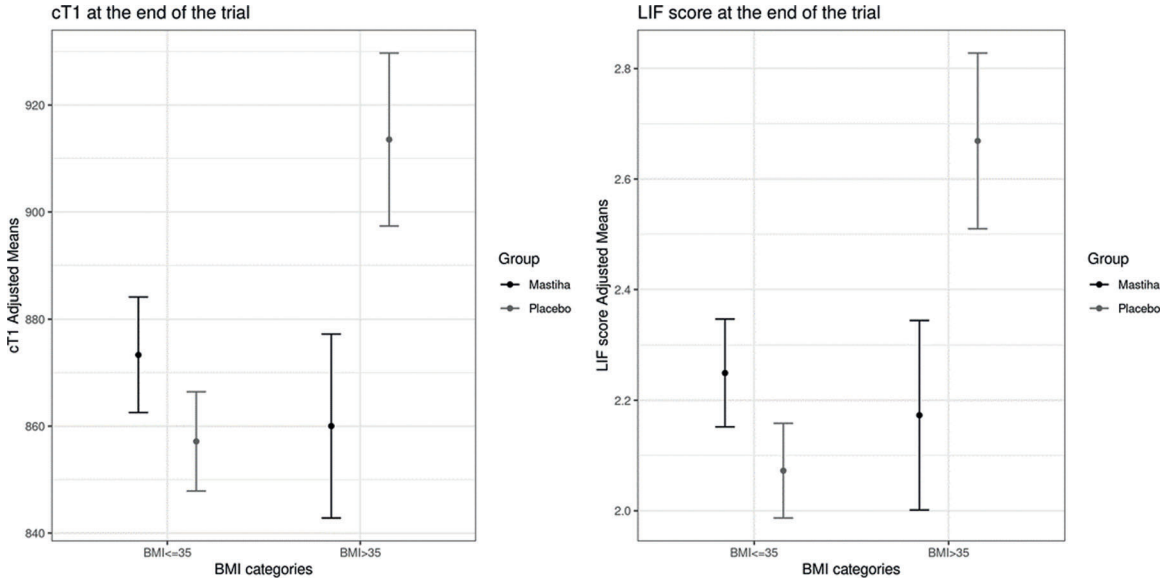

Figure 2. Differences in adjusted means for post-treatment cT1 and LIF levels, between the Mastiha and Placebo groups, stratified by BMI category. Adjustments were performed for baseline levels of cT1 and LIF respectively, age, sex, and center.

(Figure 1, Fig S6, Supporting Information), indicative of compliance to treatment protocol. None of the other outcomes met the multiple testing threshold of significance for an association with the Mastiha treatment (Figure 1). However, for the MRI parameters (LIF, cT1, PDFF, and Hepatic Iron), an increase in the signal magnitude and a change in the direction of effect have been found, after adjusting for the difference in BMI levels between post-treatment and baseline (Fig. S6B, Supporting Information). Based on this, a further investigation of the MRI parameters has been assessed, after stratifying by BMI category (baseline characteristics after stratification are presented in Table S4, Supporting Information). In BMI>35 kg m^{-2}, mean baseline values for LIF and cT1 in the Mastiha group were 2.52 (±0.46) and 890.46 ms (±36.79), respectively, and in the Placebo group 2.5 (±0.68) and 915.65 ms (±112.89), respectively.

Post-treatment levels of both cT1 and LIF were lower ($p = 0.033$ and 0.049, respectively) in Mastiha compared to the Placebo in BMI>35 kg m^{-2} (**Table 3**). Differences among the adjusted means are illustrated in **Figure 2**. The stratified analysis showed a change in the direction of the association effect for all MRI parameters between BMI categories. In BMI >35 kg m^{-2}, the mean post-treatment levels for cT1 and LIF were lower in Mastiha compared to Placebo (Table 3 and Fig. S7, Supporting Information). Finally, a pronounced reduction in both cT1 and LIF values was detected only in the Mastiha group with BMI>35 kg m^{-2} (mean, SD: −29.61, 57.86 and −0.30, 0.54, for cT1 and LIF respectively) (Table S5, Supporting Information).

In the un-stratified analysis, several associations at a nominal level of significance were identified. The Bray-Curtis dissimilarity index between baseline and post-treatment bacterial communities was larger in Mastiha versus Placebo ($p = 0.006$,

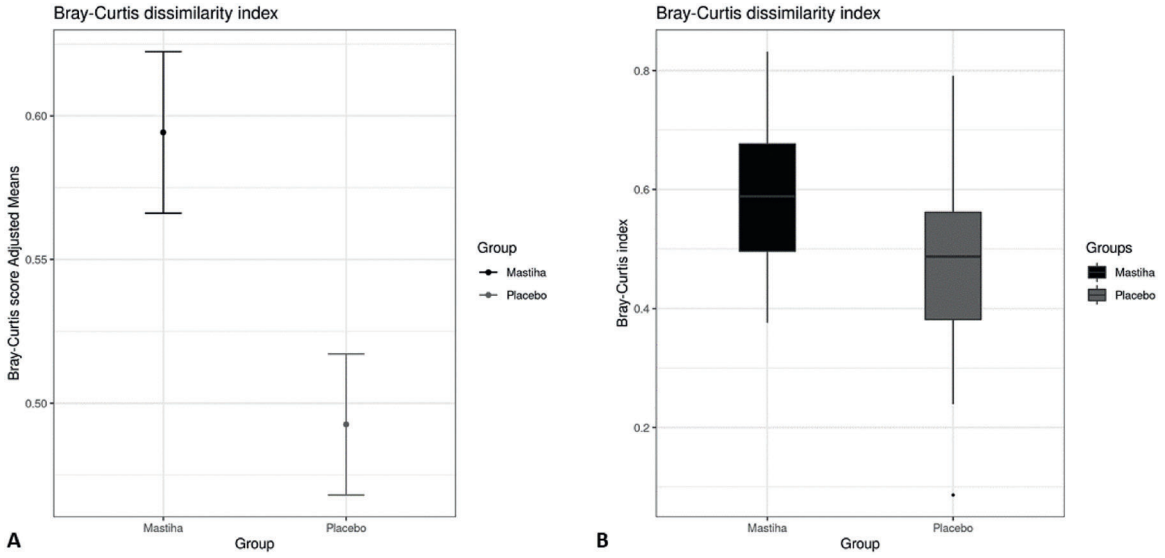

Figure 3. Differences in the Bray-Curtis dissimilarity index between the Mastiha and Placebo groups. A) Adjusted (for age, sex, and center) means of post-treatment versus baseline dissimilarities in the Mastiha and the Placebo group. B) Boxplots plots of unadjusted values in the two groups.

adjusted for age, sex, and center, **Figure 3**, Table S3, Supporting Information). This difference was not attenuated when adjusted for baseline BMI levels (Fig S6A, Supporting Information) or the difference in caloric intake between post-treatment and baseline (Fig S6C, Supporting Information). The post-treatment relative abundance of *Flavonifractor* was lower in the Mastiha group compared to the Placebo (Table S3; Fig. S6, Supporting Information). The association was more significant after adjusting for the difference in caloric intake ($p = 0.036$). A nominally significant decrease in post-treatment BMI in Mastiha group compared to Placebo ($p = 0.047$) (Table S3, Supporting Information) was detected, but the effect was attenuated after adjustments for baseline levels of physical activity (Fig. S6D, Supporting Information) or the difference in caloric intake (Fig. S6C, Supporting Information). Metabolites showed no differences post-treatment between the two groups in the main model (adjusted for the baseline metabolite level, age, sex, and center) apart from triterpenic acid sulphate (Figure 1). However, several metabolite levels significantly decreased in Mastiha compared to Placebo (Lysophosphatidylcholines-(LysoPC) 18:1, $p = 0.030$, and Lysophosphatidylethanolamines-(LysoPE) 18:1, $p = 0.015$), when adjusting for the corresponding baseline metabolites, age, sex, center, and differences in caloric intake (Fig. S6C, Supporting Information). Similarly, in Mastiha group, cholic acid decreased significantly compared to Placebo, after adjusting for baseline cholic acid, age, sex, center, and physical activity (Fig. S6D, Supporting Information).

4. Discussion

Dietary natural products in NAFLD have been investigated in several human studies.[34,35] A study on the effect of a natural supplement in NAFLD integrating biochemical, MRI imaging, metabolome and microbiome data is reported herein for first time.

No significant changes in primary outcomes were detected in the main analysis of the study. When stratifying the samples by BMI category, some interesting results were found. Specifically, lower post-treatment levels of cT1 and LIF in Mastiha compared to Placebo, only in Class II or III obesity (BMI>35 kg m^{-2}) have been detected. cT1 and LIF have been previously shown to strongly correlate with increasing liver fibrosis, as assessed by Ishak stage[6], and especially cT1 has been suggested as a useful tool in the monitoring of longitudinal changes in patients with NASH. When stratifying cT1 into groups (<840 , 840–990 , >990 ms), the risk of clinical events is increasing with increasing cT1.[36] Similarly, in a biopsy-confirmed mouse model of advanced NASH, hepatic pathology improved and NAFLD activity and expression of collagen genes (Col1a1 and Col4a1) were reduced upon Mastiha intake.[19]

In our study, we showed that the Bray-Curtis dissimilarity index was significantly greater among patients with NAFLD that received the Mastiha, compared to the Placebo. As intestinal microbiota dysbiosis is well-established in NAFLD pathogenesis, modification in microbiota composition is important in the resolution of the disease. The bidirectional communication between gut microbiota and bile acid metabolism has a substantial role in NAFLD.[37] The gut microbiota is involved in the conversion of primary bile acids into secondary bile acids in the intestine. We found decreased cholic acid levels only in the Mastiha group suggesting a potential effect of Mastiha on the interaction between gut microbiota and bile acid metabolism. The observed effect on microbiota composition may be associated with increased secondary bile acids synthesis, and decreased cholic acid levels, thus contributing to the regulation of lipid and energy metabolism.[38]

Herein, the Mastiha group had lower proportion of *Flavonifractor* compared to the Placebo. Data on the *Flavonifractor* signature in NAFLD is contradictory, with either increased or decreased levels compared to healthy.[32,39] *Flavonifractor* is involved in the catabolism of quercetin, a flavonoid with antioxidant and

anti-inflammatory properties and is considered a potentially proinflammatory species.[40,41] The downregulation of *Flavonifractor* and *Prevotella* (we detected a positive association between *Prevotella* and PDFF at baseline and a trend towards a lower abundance of *Prevotella* post-treatment in the Mastiha group) may be related to the anti-inflammatory activity of Mastiha. Weak trends of change in the relative abundance of other important bacterial taxa, previously associated with NAFLD, were also found in the Mastiha group, namely a decrease in Enterobacteriaceae and *Bacteroides* and an increase in *Faecalibacterium*. Overall, Mastiha's beneficial effect on patients' microbiota was likely through the decrease of inflammatory and endotoxin-producing bacteria and the increase of anti-inflammatory ones. In agreement with our findings, an alteration on fecal microbiome not paralleled with an improvement in liver histopathology in patients under symbiotic treatment was reported by Scorletti et al.[42]

The metabolomic analysis showed a significant reduction of LysoPCs and LysoPEs only in the Mastiha group suggesting that Mastiha exhibits a beneficial effect in phospholipid homeostasis. Phospholipid metabolism is strongly linked to NAFLD/NASH pathogenesis and these compounds are associated with increased risk of liver injury and oxidation.[43,44] Patients suffering from different grades of hepatic fat accumulation have significantly higher concentrations of these metabolite groups.[43,44,45] This data confirm Mastiha's lipid lowering properties, in line with previous results in overweight and obese patients.[46]

While this study has some interesting results, it is subject to a number of limitations, such as the absence of confirmatory biopsies as part of the trial, and the relatively small sample size of the Mastiha and Placebo groups. Furthermore, the duration of the trial might not have been sufficient for significant changes in the investigated parameters. Additionally, we observed a trend for body weight reduction only within the Mastiha group, albeit not large enough to have biased the results.

In conclusion, after six months of Mastiha supplementation, we observed a significant improvement on microbiota dysbiosis and lipid metabolite levels in patients with NAFLD. Although no significant effect of the Mastiha on the primary outcomes was identified in the un-stratified analysis, an improvement of the liver fibrosis as assessed via MRI has been observed in severely obese patients. Mastiha improved microbiota dysbiosis mainly through decreasing the abundance of inflammatory taxa. The beneficial effect on the microbiota parallel with a decrease in plasma cholic acid and phospholipids, may be attributed to the bioavailable triterpenic acids of Mastiha. Overall, Mastiha could be considered an emerging nonpharmacological agent in NAFLD. More clinical trials are required to replicate and further investigate these initial findings.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

C.A., S.K., and A.C.K. shared co-first authorship. A.C.K., G.V.D. designed the study. C.A., A.C.K., M.B., A.G., S.S., M.H., I.V., N.M., M.M.S., L.C., J.C., A.G., P.D., M.G.T., M.P.F., G.V.D. oversaw patient recruitment and trial procedures, oversaw collection and analysis of biological samples, and finalized the dataset. G.D'A., M.J.G., M.H., E.V.M., M.P.F. analyzed and interpreted the data. C.A., S.K., A.C.K., P.D., M.P.F. drafted the manuscript. A.C.K., S.M., M.G.S., B.S., M.H., A.K., J.L., C.L., F.J.R., S.V.S., M.V., N.M., M.M.S., A.G., P.D., M.G.T., M.P.F., G.V.D. revised the manuscript for important intellectual content. S.K. conducted the statistical analysis. A.C.K., R.B., A.K., J.L., C.L., F.M., M.M., I.S., S.V.S., M.V., M.M.S., A.G., P.D., M.P.F., G.V.D. obtained funding. G.V.D. supervised the study. All authors read and approved the final manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

Mastiha, metabolomics, microbiota dysbiosis, MRI, NAFLD/NASH

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