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Original article

Gut microbiota modulation in Alzheimer's disease: Focus on lipid metabolism

Laura Bonfili [a, *], Massimiliano Cuccioloni [a], Chunmei Gong [a], Valentina Cecarini [a], Michele Spina [b], Yadong Zheng [a], Mauro Angeletti [a], Anna Maria Eleuteri [a]

[a] School of Biosciences and Veterinary Medicine, University of Camerino, Via Gentile III da Varano, 62032 Camerino MC, Italy
[b] Laboratorio Analisi Dr. Fioroni, Viale A. de Gasperi, 19, 63074, San Benedetto Del Tronto AP, Italy

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SUMMARY

Background & aims: Alzheimer's disease (AD) and age-related dementias represent a major and increasing global health challenge. Unhealthy diet and lifestyle can unbalance the intestinal microbiota composition and, consequently energy metabolism, contributing to AD pathogenesis. Impairment of cerebral cholesterol metabolism occurs in both aging and AD, and lipid-lowering agents have been associated to a lower risk of neurodegenerative diseases, but the link between blood lipid profile and AD remains a matter of debate. Recently, probiotics have emerged as a promising and safe strategy to manipulate gut microbiota composition and increase the host health status through a multi-level mechanism that is currently under investigation. Specifically, oral supplementation with a multi-strain probiotic formulation (SLAB51) reduced amyloid beta aggregates and brain damages in a triple transgenic mouse model of AD (3xTg-AD). Treated mice showed improved cognitive functions in response to an enrichment of gut anti-inflammatory metabolites, increased plasma concentrations of neuroprotective gut hormones, and ameliorated glucose uptake and metabolism.
Methods: This work focuses on the evaluation of the effects of SLAB51 chronic administration on lipid metabolism in 3xTg-AD mice and the respective wild-type counterpart. On this purpose, 8 weeks old mice were orally administered with SLAB51 for 4 and 12 months to analyze the plasma lipid profile (using lipidomic analyses and enzymatic colorimetric assays), along with the cerebral and hepatic expression levels of key regulators of cholesterol metabolism (through Western blotting and ELISA).
Results: Upon probiotics administration, cholesterol biosynthesis was inhibited in AD mice with a process involving sterol regulatory element binding protein 1c and liver X receptors mediated pathways. Decreased plasma and brain concentration of 27-hydroxycholesterol and increased brain expression of cholesterol 24S-hydroxylase indicated that alternative pathways of bile acid synthesis are influenced. The plasmatic increase of arachidonic acid in treated AD mice reflects dynamic interactions among several actors of a complex inflammatory response, in which polyunsaturated fatty acids can compete each other and simultaneously co-operate in the resolution of inflammation.
Conclusions: These evidence, together with the hypocholesterolemic effects, the ameliorated fatty acids profile and the decreased omega 6/omega 3 ratio successfully demonstrated that microbiota modulation through probiotics can positively change lipid composition in AD mice, with arachidonic acid representing one important hub metabolite in the interactions among probiotic-induced lipid profile changes, insulin sensitivity, and inflammation.

1. Background

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that afflicts millions of adults worldwide and represents the main cause of late-life dementia. AD is characterized by neuronal cell loss associated with memory and cognitive decline,

* Corresponding author.
E-mail address: laura.bonfili@unicam.it (L. Bonfili).

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due to formation of extracellular aggregates of insoluble amyloid-β (Aβ) protein and intracellular neurofibrillary tangles of the microtubule-associated tau protein [1,2]. Although AD-associated cognitive dysfunction is strongly correlated with the accumulation of Aβ and the severity of tau pathology, the molecular mechanisms involved in AD development are not fully explained. Among AD modifiable risk factors, diabetes, obesity, hypertension and hypercholesterolemia can be managed through healthy diet, regular physical activity and cognitive training. In particular, a diet rich in saturated fats and simple carbohydrates increases the risk of dementia and a suboptimal diet is associated with a more severely impaired cognition in AD [3], whereas Mediterranean diet correlates with better cognitive status in healthy people with reduced risk of AD [4]. Experimental and clinical evidences indicate that neurodegenerative disorders are often associated with metabolic dysfunction [5], which may worsen neurological symptoms [6]. Impairment of cerebral cholesterol metabolism occurs in both aging and AD [7,8] and lipid-lowering agents have been associated to a lower risk of developing AD [9–11], but the link between blood lipid profile and AD continues to be a matter of debate [12].

In this context, gut microbiota–host interactions have a key role in controlling oxidative status, neuroinflammation, glucose and lipid metabolism. Modulation of the gut microbiota composition by food-based therapy or by probiotic supplementation represents a promising preventive and therapeutic opportunity for AD [13,14]. Preclinical studies indicate that changes in the gut bacterial profile can reduce amyloid plaques in AD animal models, improving memory and lowering inflammation [15], which is a crucial pathogenic hallmark in neurological disorders [16]. Interestingly, polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can exert pro/anti-inflammatory effects and neuronal protective functions [17]. *Hjorth* et al. reported that DHA and EPA can be beneficial in AD by enhancing the removal of Aβ, increasing the production of growth factors like neurotrophin, and decreasing the secretion of pro-inflammatory cytokines [18]. Moreover, treatment with EPA and DHA markedly reduced the release of pro-inflammatory cytokines in peripheral blood mononuclear cells from AD patients [19].

Recently, new insights into the pathways controlling the levels of lipids, sterols and their metabolites indicated their important implications in AD. Specifically, cholesterol levels are sensed and regulated by two distinct transcription factor pathways, namely the sterol regulatory element-binding proteins (SREBPs) [20] and the liver X receptors α and β (LXRα and LXRβ) [21]. In detail, sterol response element binding protein 1c (SREBP-1c) can be modulated in different tissues by LXR α/β in response to altered sterol and oxysterols levels, consequently regulating the expression of genes that coordinate cholesterol and fatty acids metabolism [22].

Interestingly, SREBPs regulate 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), which is the rate-limiting enzyme in cholesterol biosynthesis, but the exact mechanism of action of cholesterol lowering drugs and their implication in reducing susceptibility to AD are not exactly defined [11,23,24]. Additionally, LXRs represent attractive therapeutic opportunities in AD because their activation controls the expression of genes involved in cholesterol uptake, efflux, transport, and excretion in multiple tissues, counteracts inflammation by modulating innate and adaptive immune responses, positively impacts glucose homeostasis, and decreases Aβ production by modulating amyloid precursor protein processing [25,26].

Upon increased cellular sterol levels, LXRs can enhance the expression of the inducible degrader of LDL receptor (IDOL), an E3 ubiquitin ligase involved in the ubiquitin-proteasome mediated degradation of important substrates including LDL receptor [27] thus regulating the structural and functional plasticity of synapses and neural circuits [28,29]. However, the contribution of LXR-IDOL pathway to feedback inhibition of cholesterol uptake and the complementary role of SREBP pathway in regulating lipid homeostasis in AD need to be further explored.

Differently from lipoprotein-bound cholesterol, oxidized cholesterol metabolites can cross the blood brain barrier (BBB) [30]. Increased levels of 27-hydroxycholesterol (27-OHCE) were observed in the brain of AD patients [31] due to hypercholesterolemia, increased BBB permeability and reduced neuronal metabolism of 27-OHCE by oxysterol 7 α-hydroxylase, a cytochrome p450 enzyme responsible for the first and rate limiting step in bile acid synthesis [32]. Abnormal levels of oxysterols in the brain can increase Aβ production [33] and worsen neuroinflammation [34]. In detail, oxysterols are endogenous activators of LXRs, which in turn play a role in the regulation of Aβ in the brain by mechanisms involving cholesterol transporters [35]. Moreover, cerebral cholesterol turnover is mainly performed by cholesterol 24-hydroxylase (CYP46A1), a cytochrome p450 enzyme that is mainly expressed in hippocampal and cortical neurons [36]. Several works demonstrated that increased CYP46A1 activity improved memory [37], reduced Aβ plaques and restored spatial memory performances in mice [38].

Current AD therapeutic approaches target Aβ production and clearance, Aβ and tau aggregation, neuroprotective protocols and symptomatic cognitive enhancers, causing a number of side effects [39]. Recent studies have identified the modulation of gut–brain axis as a successful opportunity to counteract age-related decline and AD [13,40]. It was previously demonstrated that the oral administration of SLAB51 probiotic formulation reduced Aβ aggregates and brain damages, and partially restored the impaired neuronal proteolysis in 3xTg-AD mice. The increased gut content of anti-inflammatory short-chain fatty acids and the increased plasma concentrations of neuroprotective peptide hormones partially explained the improved learning and memory and the reduced inflammation in treated AD mice [15]. In addition, SLAB51 mitigated cerebral oxidative stress through sirtuin-1-dependent mechanisms [41] and ameliorated glucose uptake and metabolism [42] in AD mice.

Several studies described the cholesterol-lowering effects of probiotics in both animals and humans [43,44]. Considering the key role of impaired cerebral lipid metabolism in AD cognitive dysfunction and histopathological alterations, the amelioration of lipid metabolism represents a promising approach in AD therapy and deserves more attention than it has received until now. In this perspective, the present work has focused on exploring the effects of SLAB51 oral administration on lipid metabolism in 3xTg-AD mice and their wild-type (wt) counterpart. For this purpose, 8-week-old mice were orally administered with SLAB51 for 4 and 12 months to investigate the hypocholesterolemic effects and the ability to qualitatively ameliorate plasma lipid profile. The brain expression level of LXRs, SREBPs, IDOL, and the liver expression level of HMGCR, LXRs and SREBPs, were evaluated to demonstrate that cholesterol biosynthesis is influenced, following the activation LXRs and SREBPs dependent pathways. Moreover, the plasma and cerebral amounts of the oxidative metabolite 27-OHCE and the brain expression of CYP46A1 enzyme were monitored to explore the ability of the probiotics to influence alternative bile acids biosynthetic pathways, maintain cholesterol turnover and homeostasis and support neuronal function, with the aim of elucidating the mechanisms through which the probiotic formulation counteracts AD progression.

2. Results

2.1. Probiotic administration ameliorates blood lipid profile in AD mice

Cholesterol is involved in APP processing and high levels of cholesterol correlate with increased risk of AD [45]. Considering that cholesterol lowering therapies improve cognitive performance in AD subjects, the ability of SLAB51 to ameliorate blood lipid profile was investigated. As expected, cholesterol plasma concentration in 3xTg-AD mice was significantly higher than that of age-matched wt mice (Fig. 1). Interestingly, upon probiotic administration, a significant decrease of total cholesterol concentration was detected in the plasma of 56-week-old AD mice (Fig. 1). Conversely, no significant variations were observed in wt animals.

Moreover, old AD mice showed decreased levels of high-density lipoprotein cholesterol (HDL-C) and increased levels of low-density lipoprotein cholesterol (LDL-C) with respect to age matched wt mice. Probiotics oral administration induced a significant increase of HDL-C and a significant decrease of LDL-C plasma concentrations, indicating a positive effect on blood lipid profile and suggesting an amelioration of AD pathology.

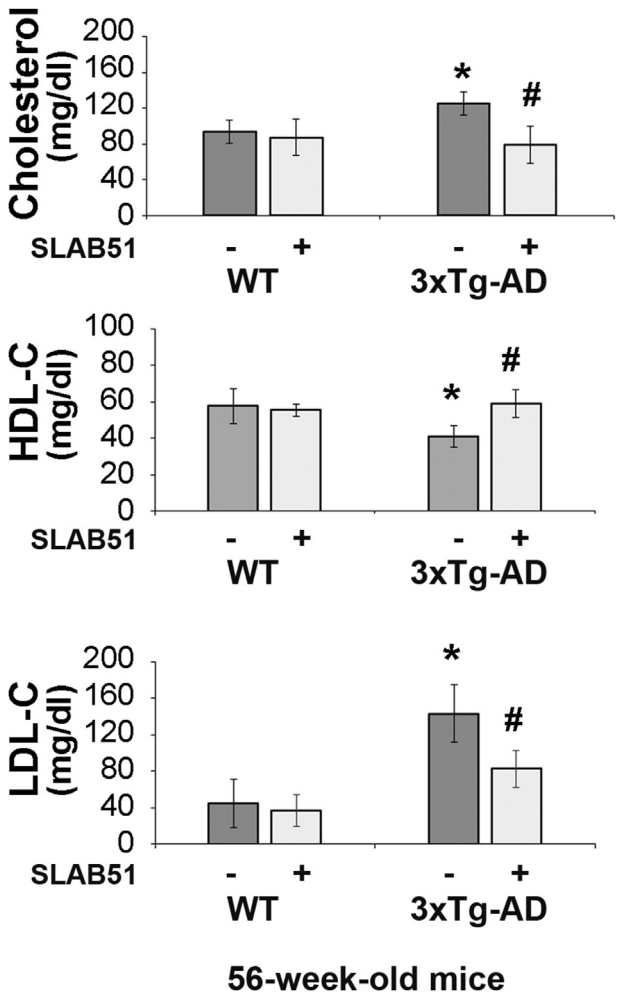

Fig. 1. Blood lipid profile. Plasma concentrations (mg/dL) of total cholesterol, HDL-C and LDL-C in 56-week-old wild type and AD mice treated (light grey) or not (dark grey) with SLAB51 probiotic formulation (*p > 0.05 compared with untreated wild type mice; #p > 0.05 compared with untreated age-matched mice of the same group).

2.2. SLAB51 oral administration influences cholesterol biosynthesis and metabolism

Then we explored the ability of probiotics to modulate the functionality of HMGCR, the rate-limiting step of cholesterol biosynthesis. In detail, the activity of HMGCR was measured in liver microsomes of mice administered with SLAB51 or water using a previously described HPLC based method [46].—

HMGCR activity was significantly lower in 56-week-old AD mice orally administered with SLAB51 for 12 months, as indicated by the decreased area of the CoA peak (Fig. 2). No significant differences were observed in wt mice (data not shown).

Liver homogenates were analyzed through western blotting in order to evaluate the expression levels of HMGCR enzyme. 56-week-old untreated AD mice showed a significant increased expression of HMGCR with respect to young animals (Fig. 3). Interestingly, HMGCR levels significantly decreased in the liver of 56-week-old treated mice, indicating that the cholesterol lowering effect (Fig. 1) was due to both decreased HMGCR activity (Fig. 2) and reduced expression of the protein (Fig. 3).

Amelioration of blood lipid profile can also be explained by the modulated expression of SREBP1c and LXRs in probiotic-treated 3xTg-AD mice. In detail, increased levels of SREBP1c were observed in both liver and brain of untreated AD mice (Fig. 4, upper panels) indicating an increased cholesterol synthesis and in agreement with the enhanced expression and activity of HMGCR (Fig. 3). Interestingly, cerebral and hepatic protein expression of SREBP1c significantly decreased upon 12 months treatment with probiotics (Fig. 4, upper panels), suggesting that HMGCR inhibition can be also modulated through SREBP1c dependent pathways.

Additionally, SLAB51 oral administration significantly upregulated LXRs expression in the brain and the liver of 3xTg-AD mice, at 24- and 56- weeks of age (Fig. 4, lower panels), in line with the previously documented anti-inflammatory effects [41] and cognitive improvement [15], and consistently with the ameliorated blood lipid profile (Fig. 1). This is of significant importance considering that LXRs also regulate the expression of genes involved in cholesterol absorption, transport, efflux, excretion and conversion to bile acids, finally affecting the reverse cholesterol transport [47].

In turn, LXRs can induce the expression of the inducible degrader of LDL receptor (IDOL) which is a E3 ubiquitin ligase that targets lipoprotein receptors for proteasome-mediated degradation and with a key role in metabolism and AD. Figure 5 shows that IDOL increased in the brain of SLAB51-treated mice, in agreement

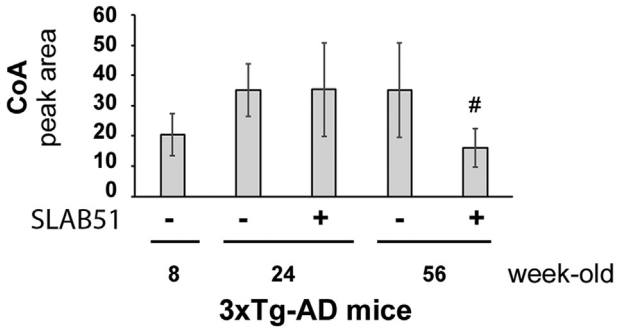

Fig. 2. Inhibition of HMGCR activity in 3xTg-AD mice treated with SLAB51. Mevalonate production calculated from changes in CoA peak area (mevalonate/CoA 1:1 stoichiometric ratio) in 8, 24 and 56-week-old mice orally administered with SLAB51. Data points marked with a hashtag are statistically significant compared to age-matched untreated mice (#p < 0.05).

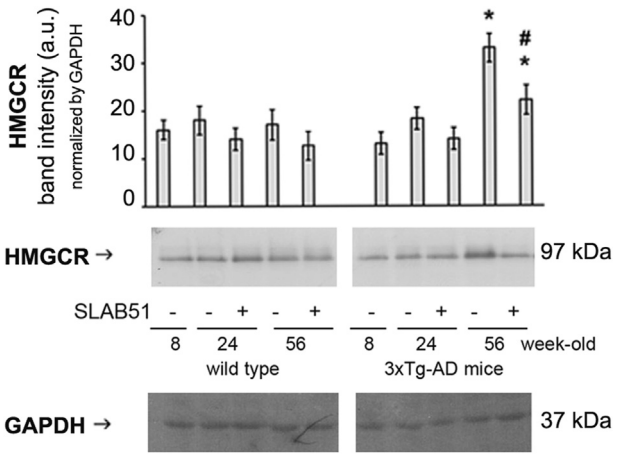

Fig. 3. Effect of SLAB51 on HMGCR expression levels. HMGCR expression levels were detected in 8-week-old mice treated for 4 and 12 months with SLAB51 probiotic formulation. Representative immunoblots of both wt (right) and 3xTg-AD (left) mice is reported and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody and normalized expression of the target protein is reported as arbitrary units (a.u.). Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype (*$p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).

with the ability of probiotics to improve neuronal proteolysis in AD mice [15].

2.3. Probiotic supplementation modifies plasma fatty acids composition

The determination of the fatty acid composition of plasma glycerophospholipids was performed to investigate the neuroprotective effects of chronic SLAB51 treatment attributable to the modulation of lipid metabolic pathway.

Plasma lipidomic analyses confirmed the probiotics anti-inflammatory effects as indicated by the significant decrease of cis-linoleic acid (Fig. 7) and the decreased ratio of cis-linoleic acid/(EPA + DHA) in 3xTg-AD mice supplemented with SLAB51 for 12 months (Fig. 6). In fact, the ratio of omega-6 to omega-3 is a recognized indicator of the health status and a lower ratio of n-6 to n-3 fatty acids is associated with reduction in inflammation [48,49].

Data reported in Fig. 7 reveal that SLAB51-modified intestinal microbial status can affect plasma fatty acids composition. Although a slight but significant decrease of the omega-3 fatty acids α-linolenic acid and eicosapentaenoic acid was observed in 56-week-old AD mice upon SLAB51 supplementation (Fig. 7), the important reduction of the omega-6 cis-linoleic acid definitively determine the anti-inflammatory effect, by decreasing the omega6/omega-3 ratio (Fig. 6).

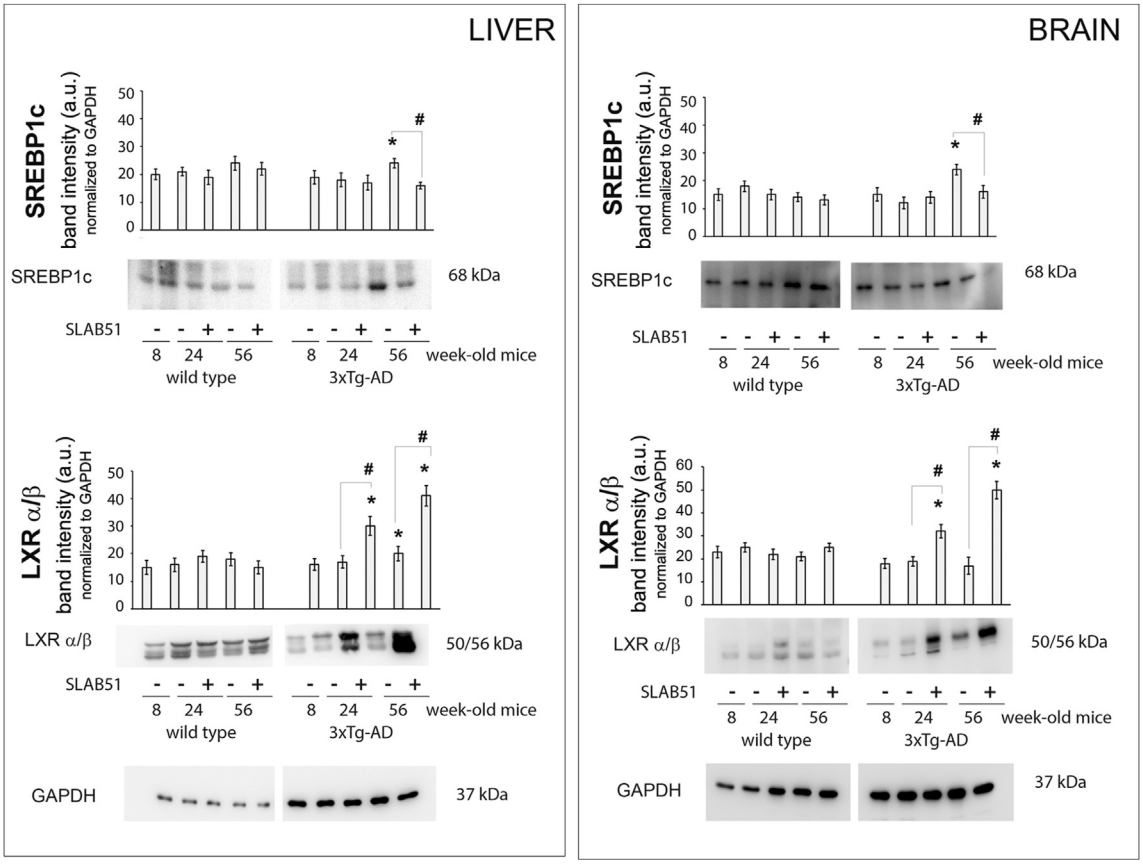

Fig. 4. SREBP1c and LXRs expression levels in wt and AD mice orally administered with SLAB51 for 4 and 12 months. Representative immunoblots and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody and normalized expression of the target protein is reported as arbitrary units (a.u.). Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype (*$p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).

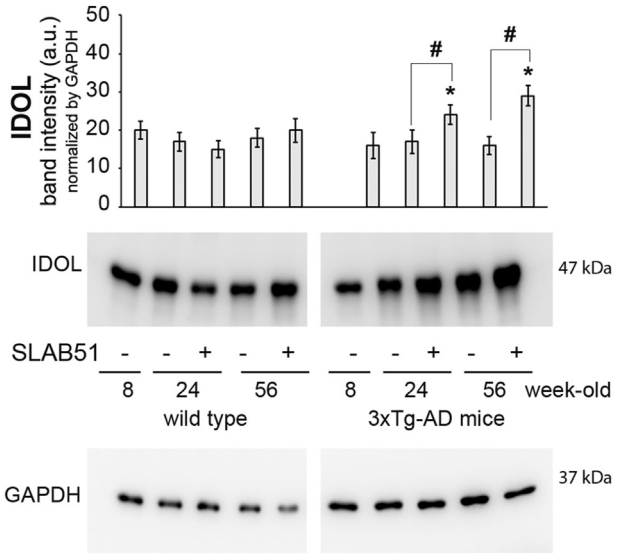

Fig. 5. IDOL expression levels in wt and AD mice orally administered with SLAB51 for 4 and 12 months. Representative immunoblots and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody and normalized expression of the target protein is reported as arbitrary units (a.u.). Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype (*$p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).

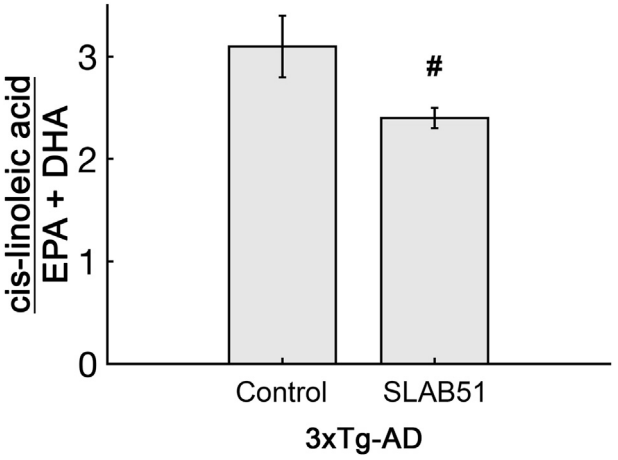

Fig. 6. Decreased omega6/omega 3 polyunsaturated fatty acids ratio in treated AD mice. cis-linoleic acid/(EPA + DHA) ratio in the plasma of 56-week-old AD mice chronically administered with water (control) or with SLAB51 for 12 months. Data points marked with an asterisk are statistically significant compared to control (#$p < 0.05$).

Interestingly, SLAB51 treated AD mice displayed increased plasma levels of stearic acid, which was previously demonstrated to exert hypocholesterolemic effects [50]. Moreover, probiotic treated AD mice showed higher levels of heptadecanoic acid, which is inversely associated with insulin resistance and type 2 diabetes [51], in line with a correlation with the amelioration of glucose metabolism in the same mice upon SLAB51 chronic administration [42].

Altered composition of plasma fatty acids observed in AD mice was significantly modulated by probiotics (Fig. 7), indicating dynamic effects of the shifted gut microbiota on the plasma lipid profile of AD mice. In fact, gut microbiota of AD mice has a significantly different structure with respect to wild type mice, causing disequilibrium in energy homeostasis. No significant differences were observed in SLAB51-treated wt mice, confirming the safety of the probiotic-based approach, most likely due to the higher stability of wt gut microbiota [15].

2.4. Decrease of 27-hydroxycholesterol upon probiotic treatment in AD mice

The neurotoxic oxysterol 27-hydroxycholesterol (27-OHCE) can cross BBB thus mediating the effects of hypercholesterolemia on the brain [31]. Interestingly, upon SLAB51 chronic administration, 27-OHCE concentration significantly decreased in both the plasma and the brain of 56-week-old AD mice compared to age-matched controls (Fig. 8).

2.5. SLAB51 increased the brain expression of CYP46A1

CYP46A1 is the rate limiting enzyme in cholesterol degradation and its cerebral restoration implicates neuroprotective effects. The increased expression of CYP46A1 enzyme in the brain of 24- and 56- week-old AD mice chronically administered with SLAB51 suggested the probiotic-dependent induction of cerebral cholesterol turnover [36]. No significant differences were observed in wild type animals (Fig. 9).

3. Discussion

The incidence of Alzheimer's disease is rapidly growing in developed countries with no definitive remedy available. One of the main global challenges for health and social care is to identify new strategies aimed at preventing or delaying AD onset and development. Potentially modifiable risk factors received increasing attention, with gut microbiota representing an attractive preventative/therapeutic target because of its role in regulating multiple neurochemical pathways through the gut–brain axis [52]. Deregulation of lipid homeostasis substantially contributes to AD onset and progression and cholesterol lowering compounds (like statins), were demonstrated to reduce the risk of dementia. However, mechanistic insights into the link between abnormal lipid metabolism and Aβ aggregation and clearance are still unavailable. The ability of statins to improve metabolism can be explained through gut microbiota altered composition [53]. In this context, dietary interventions, including probiotics, which are able to modulate microbiota composition, have been studied for their ability to improve energy homeostasis and immune system in neurodegenerative diseases [13,54].

Chronic dietary supplementation with SLAB51 probiotic formulation modified gut microbiota in 3xTg-AD mice, ameliorated glucose metabolism, inflammatory and oxidative status and partially recovered the impaired neuronal proteolysis, finally decreasing Aβ and tau aggregates and improving cognitive abilities, with consequent delay of AD progression [15,41,42].

Considering the contradictory data on the role of deregulated lipid homeostasis in AD and in light of the promising effects of probiotics on energy metabolism, 3xTg-AD mice and their wild type counterpart were chronically treated with SLAB51 with the aim of deeply dissecting the role of microbiota modulation in ameliorating AD pathology by affecting lipid homeostasis.

High levels of total cholesterol and LDL-C and low levels of HDL-C are associated with vascular dementia and, indirectly, with AD [55]. As expected, dyslipidemia was observed in older 3xTg-AD mice but not in wild type animals (Fig. 1). Interestingly, the probiotic mixture exerted hypocholesterolemic effects in AD mice, qualitatively ameliorating plasma lipid composition (Fig. 1), with a consequent reduction of total cholesterol/HDL-C and LDL-C/HDL-C

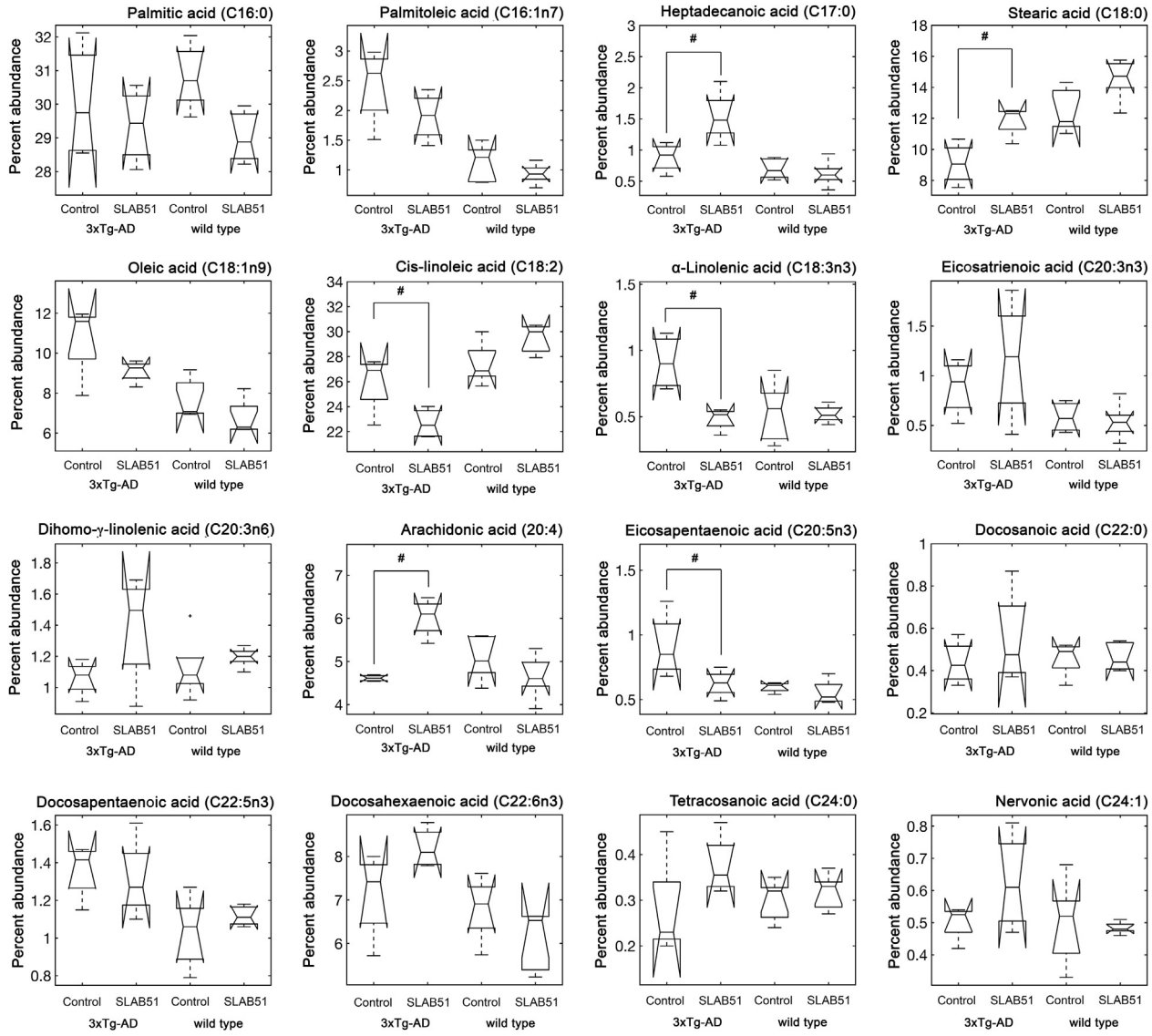

Fig. 7. SLAB51 affects plasma lipid profile. Fatty acid composition of plasma glycerophospholipids in 56-week-old wt and 3xTg-AD mice treated with water (control) or SLAB51 for 12 months. Results were analyzed using the post hoc Tukey's test and are expressed as percent abundance. Data points marked with an asterisk are statistically significant compared to age-matched control mice of the same genotype (#p < 0.05).

ratios. These results can be partially explained with the significant decrease in HMGCR concentration and activity observed in hepatic microsomes, indicating that this multi-strain probiotic mixture can inhibit cholesterol biosynthesis, consistently with other studies [56,57]. The present data are in agreement with the increased fecal content of short chain fatty acids induced by SLAB51 in the same animal model [15], particularly for the ability of propionate to inhibit hepatic lipogenesis and cholesterol synthesis, finally alleviating metabolic disorders [58].

Additionally, the increased hepatic expression of the lipogenic SREBP1c in untreated AD mice (Fig. 4, upper panels) indicates an age-dependent increase in cholesterol synthesis, in line with the enhanced expression and activity of HMGCR (Fig. 3). Interestingly, cerebral and hepatic protein expression of SREBP1c significantly decreased upon 12 months treatment with probiotics (Fig. 4, upper panels), suggesting a probiotic-dependent effect on fatty acid and triglyceride synthesis [59] and indicating that HMGCR levels/activity can be regulated through SREBP1c dependent pathways. Moreover, SREBP1c decreased expression in both the brain and the liver of treated AD mice is in agreement with the ability of SLAB51 to counteract insulin resistance in the same animal model [42], since SREBP1c emerged as a metabolic mediator between insulin/glucose signaling and lipogenesis [60]. In fact, other studies indicated that hyperinsulinemia could mediate the induction of SREBP-1c that, in turn, triggers lipogenesis, suggesting inhibition of SREBP-1c as a therapeutic approach in dyslipidemia-associated disorders [61,62].

In parallel, SLAB51 improved blood lipid profile and cholesterol homeostasis also by triggering LXRs-mediated pathways. In fact, these receptors can induce genes that positively regulate bile acid synthesis and apolipoprotein metabolism, and that decrease Aβ production, exerting neuroprotection [63], as previously reported [15]. Considering the recognized anti-inflammatory effects of LXRs [41], their increased levels upon treatment (Fig. 4, lower panels) better explain the ameliorated inflammatory status in SLAB51 treated AD mice [15]. Additionally, SLAB51 anti-inflammatory properties were confirmed by the decreased ratio of cis-linoleic/(EPA + DHA) in the plasma of 3xTg-AD mice (Fig. 6). This is a crucial

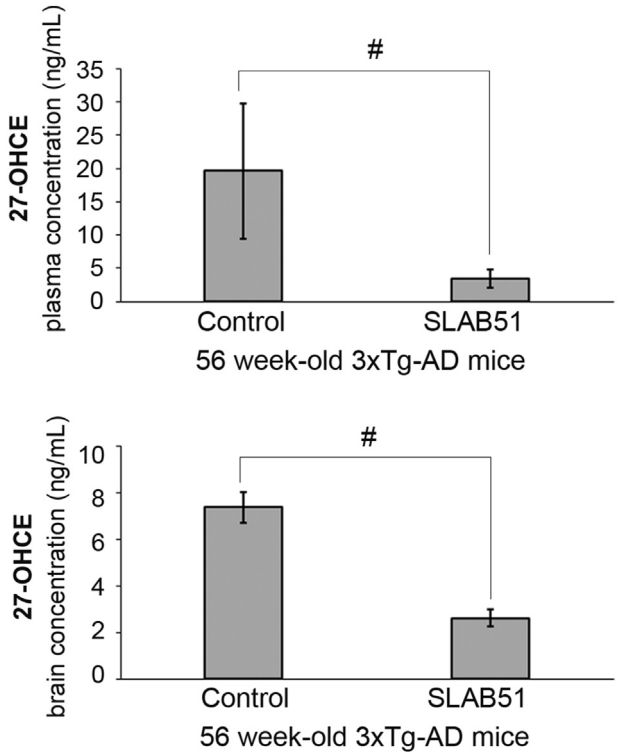

Fig. 8. 27-Hydroxycholesterol plasma and brain levels. ELISA determination of 27-Hydroxycholesterol (27-OHCE) in the plasma (upper panel) and the brain (lower panel) of control and treated 56-week-old 3xTg-AD mice. 27-OHCE concentrations are expressed as ng/mL ± SE. Data marked with a hashtag are significantly different with respect to age-matched controls ($^{\#}p < 0.05$).

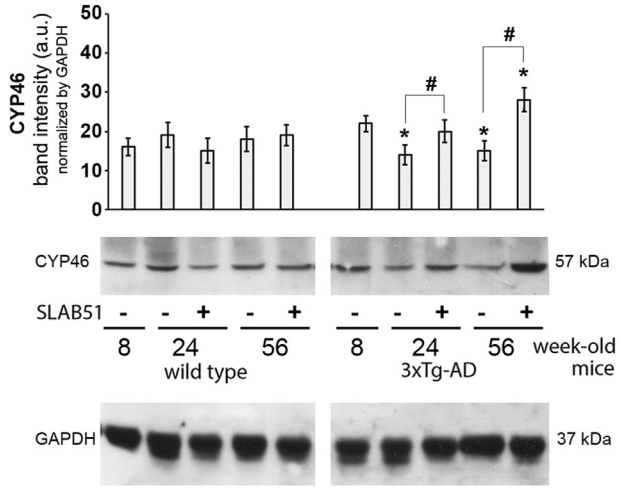

Fig. 9. Brain CYP46 expression levels in wt and AD mice orally administered with SLAB51 for 4 and 12 months. Representative immunoblots and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody. Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype ($^{*}p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).

information, because the ratio of omega-6 to omega-3 has been previously described as a fundamental indicator of human health and a lower ratio of omega-6 to omega-3 fatty acids is associated with reduction in inflammation [49]. Inflammation-associated diseases, such as diabetes, obesity, mental disorders, cardiovascular and autoimmune diseases are associated to an increased omega-6/omega-3 ratio leading to the production of pro-inflammatory molecules [48,64]. Consequently, the ameliorated omega6/omega 3 balance in AD mice chronically supplemented with SLAB51 can represent one possible mechanism through which oral bacteriotherapy can modulate inflammatory processes.

Interestingly, the augmented expression of LXRs in the brain of treated mice contribute to elucidate the partial restored functionality of the ubiquitin-proteasome system (UPS) observed in the same mice upon SLAB51 treatment [15]. In fact, LXRs can trigger the expression of the E3 ubiquitin ligase IDOL, promoting the lipoprotein receptors proteolysis, affecting the structural and functional plasticity of synapses and neural circuits with effects on memory and behavior [28]. Moreover, they can also limit cholesterol uptake, complementary to SREBP mediated effects [29]. Improved cognitive abilities are also confirmed by the significant decrease of EPA plasma concentrations in treated AD mice, in line with a recent study suggesting that this n-3 polyunsaturated fatty acid contributed to impair memory and learning in animal models [65].

Other significant variations in plasma fatty acid composition were detected upon SLAB51 chronic supplementation. For example, heptadecanoic acid increased in probiotic-treated AD mice, suggesting a correlation with the improved glucose homeostasis and decreased insulin resistance in the same animal model chronically supplemented with SLAB51 for 12 months [42], since heptadecanoic acid is inversely associated with insulin resistance and type 2 diabetes mellitus [51].

On the other hand, the slight but significant reduction of α-linolenic acid and the increase of arachidonic acid detected in the plasma of 56-week-old treated AD mice are apparently in contrast with the previously documented decrease of pro-inflammatory cytokines in the plasma of SLAB51 treated mice at 24 weeks of age [15]. Actually, these variations highlight the dynamic interactions among numerous actors of a complex inflammatory response, in which polyunsaturated fatty acids can compete each other [66] and simultaneously co-operate in resolution of inflammation. In particular, the role of arachidonic acid in neurodegenerative conditions is controversial [67]; importantly, it is essential for the development of the brain, as it regulates cell membrane fluidity and ion channels activity and guarantees optimal cognitive functions, in agreement with the ameliorated behavioral performances observed in 3xTgAD mice chronically administered with SLAB51 [15]. Additionally, it stimulates type 2 immune response [68] and possesses anti-diabetic properties [69] in agreement with the previously documented effects of SLAB51 on glucose uptake and metabolism in 3xTgAD mice [42]. Consequently, arachidonic acid represents one of the key mediators in the interactions among probiotic-induced lipid profile changes, insulin sensitivity and inflammation.

An important linker among hypercholesterolemia, oxidative stress and neuropathology of AD in the brain is the cholesterol oxidation product 27-hydroxycholesterol [31], which is an intermediate of the alternative pathway of bile acids biosynthesis [70] and a recognized biomarker for impaired glucose homeostasis [5]. As expected, 27-OHCE was significantly increased in both the plasma and the brain of AD mice (Fig. 8, upper panel), confirming a correlation between circulating 27-OHCE and cholesterol [71] and in agreement with the altered BBB permeability, enhanced oxidative status and reduced glucose uptake in the brain of 3xTg-AD mice with respect to wild type animals [41,42]. Upon SLAB51 supplementation, decreased concentrations of the oxysterol were measured both in the plasma and the brain of transgenic mice confirming the probiotics antioxidant efficacy [41] and the ability to restore glucose homeostasis [42]. Decreased 27-OHCE, together with the increased levels of CYP46A1 enzyme in the brain of

SLAB51 treated AD mice (Fig. 8) indicated an effect on alternative pathways of bile acid production and confirm the activation of LXRs dependent pathways that maintain cholesterol turnover and homeostasis and support neuronal function [70,72]. Considering the role of CYP46A1 in regulating memory functions [37] and Aβ deposition [38], its increase in SLAB51 treated AD mice contribute to elucidate the restored cognitive abilities and the reduced number of Aβ plaques previously observed in younger animals [15].

Collectively, these data contribute to address the unclear microbiota-influenced changes in the plasma lipids and demonstrate that probiotics can significantly affect the gut microbial composition with positive impact on the host lipid profile.

4. Conclusion

Our studies are part of the enormous effort that scientists are making to identify a definitive preventative and/or therapeutic approach for the uncurable, progressive and persistent dementia associated to AD. There is still no global consensus on the etiology of this multifactorial disease, in which proteinopathies, oxidative stress, inflammation, metabolic disorder, and other factors coexist. The link between the unbalanced gut microbiota and AD pathology has been demonstrated in both preclinical [15] and human [73] studies, but how metabolic pathways are involved is not yet clearly understood. Our evidence contributes to define the currently unclear mechanisms through which gut microbiota manipulation can ameliorate specific AD characteristics, and suggest that by identifying a successful combination of probiotic strains it is possible to strategically shift gut microbiota composition with positive consequences on the brain oxidative and inflammatory status, by regulating plasma lipid profile, cholesterol biosynthesis, and bile acid synthesis.

Concluding, our data definitely demonstrate that using a safe, non-invasive and not-toxic probiotic-based approach it is possible to ameliorate lipid metabolism in this preclinical model of Alzheimer's disease, mimicking effects exerted by statins, demonstrating which pathways are involved.

The effective prevention of AD through the fine regulation of the associated risk factors will remain a constant goal of research and present findings should be inevitably considered in future preventative and therapeutic AD protocols.

4.1. Material and methods

4.1.1. Reagents and chemicals

SLAB51 probiotic formulation was provided by Ormendes SA (Jouxtens-Mézery, Switzerland, https://agimixx.net). SLAB51 contains eight different live bacterial strains: *Streptococcus thermophilus* DSM 32245, Bifidobacterium lactis DSM 32246, Bifidobacterium lactis DSM 32247, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helveticus* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, *Lactobacillus brevis* DSM 27961.

Enzymatic colorimetric kits for total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) determination were kindly provided by Chema Diagnostica (Monsano, AN, Italy). HMGCR, HMG-CoA, NADPH, $NADP^+$, CoA, potassium phosphate, sodium phosphate, magnesium sulfate, phenyl-methanesulfonyl-fluoride, tosyl-phenyl-alanyl-chloromethyl-ketone, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) were all purchased from Sigma–Aldrich. HPLC grade methanol was obtained from JT Baker. All solvents and reagents were of the highest purity available.

The reverse phase Luna C18 column (5 μm particle size, 250 × 4.6 mm, equipped with a 5 mm guard column) was purchased from Phenomenex S.r.l. (Bologna, Italy).

Membranes and reagents for western blotting analyses were purchased from Merck KGaA, (Darmstadt, Germany). The rabbit polyclonal anti IDOL (ab-74562) and the rabbit monoclonal anti-HMGCR [EPR1685(N)] (ab174830) antibodies were from AbCam (Milano, Italy). The mouse monoclonal antibodies anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti CYP461A antibody, anti-LXRα/β antibody and anti-SREBP-1c were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

4.1.2. Animal model

AD triple-transgenic mice, B6;129-Psen1^{tm1Mpm} Tg (APPSwe, tauP301L)1Lfa/J (named 3xTg-AD) and the wild type B6129SF2 mice, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). 3xTg-AD mice contain three mutations associated with frontotemporal dementia or familial AD (amyloid precursor protein [APP]Swe, tau MAPT P301L, and presenilin-1 M146V). This reliable model of human AD displays both plaque and tangle pathology, with Aβ intracellular immunoreactivity detectable at three months of age and hyperphosphorylation of tau protein occurring by 12–15 months of age [74]. Consequently, in 12–15 months it is possible to reliably reproduce traits similar to those observed in the entire life of Alzheimer's disease patients.

Experiments complied with the ARRIVE guidelines, in accordance with the EU Directive 2010/63/EU for animal experiments and with a protocol approved by the Italian Ministry of Health (518/2018-PR). Mice were housed in plastic cages (Makrolon, Covestro A.G., Filago, Italy) in a temperature-controlled room (21 ± 5 °C) and 60% humidity on 12-h light/dark reversed cycle (light was switched on at 8:00 p.m.) and maintained on standard laboratory diet (Mucedola, Italy) and water ad libitum. Appropriate measures minimized pain and discomfort in experimental animals.

4.1.3. Experimental design

Eight-week-old AD male mice (n = 48) were organized in two groups and were treated with SLAB51 dissolved in water (n = 24) or with water (control group, n = 24). At the same time, 48 age-matched wild type (wt) mice were organized into wt control (n = 24) and wt-treated (n = 24) groups. Based on the body surface area principle the dosage of SLAB51 2×10^{11} bacteria/kg/day was calculated. The body weight was monitored during the treatment to ensure single-housed animals received the proper intake of the probiotic. Preliminary studies were performed to evaluate both viability and stability of the probiotic formulation as previously described [42] and probiotic drinking solution was freshly prepared every day. With the aim to start treatment before the deposition of protein aggregates, and based on preliminary experiments, 8, 24 and 56 weeks of age were selected as timepoints, in order to evaluate the preventative and therapeutic properties of chronic supplementation with probiotics. Eight mice per group were euthanized by CO_2 overdose at 8, 24 and 56 weeks of age, and the tissues were properly collected for biochemical analyses. In detail, murine brains and livers were quickly removed and placed on an ice-cold glass plate. Plasma samples and tissue homogenates were promptly supplemented with protease inhibitors (1 mM tosyl phenylalanyl chloromethyl ketone (TPCK) and Pefabloc).

4.1.4. Plasma lipid analysis

Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined enzymatically, using commercially available colorimetric kits (Chema Diagnostica, Italy) according to the manufacturer's instructions. Data are expressed as mg/dL ± SE.

4.1.5. Preparation of brain and liver homogenates

Upon sacrifice, tissues were homogenized in 50 mM Tris buffer, 150 mM KCl, 2 mM EDTA, pH 7.5 (1:5 weight/volume of buffer). Homogenates were immediately centrifuged at 13,000 × g for 20 min at 4 °C and the supernatants were used. Protein concentration was measured with the Bradford protein assay [75].

4.1.6. Liver microsomes preparation

Liver microsomes were prepared as previously reported [76]. In detail, liver tissue samples (0,1 g) were added to 1 mL of cold homogenization buffer (50 mM Tris−HCl buffer, 0,3 M sucrose, 10 mM EDTA, 10 mM DTT and 50 mM NaCl at pH 7.4 in the presence of protease inhibitors and homogenized using a bench-top Ultra-Turrax TP 18/10 homogenizer (Janke and Kunkel; Staufen, Germany). The homogenate was centrifuged at 20,000 × g for 15 min at 4 °C. Supernatant was collected and (ultra)centrifuged at 100,000 × g for 60 min at 4 °C. Microsomal pellet was finally resuspended in the activity buffer (100 mM phosphate buffer, 10 mM DTT, 1 mM EDTA, 2% DMSO at pH 6.8). Total protein concentration was determined with the Bradford assay.

4.1.7. High performance liquid chromatography (HPLC) analysis

The Amersham Biosciences AKTA basic HPLC system equipped with a UV/VIS detector was used for the analysis. Reaction mixture consisting of ultracentrifuged liver homogenates, NADPH and HMG-CoA was incubated at 37 °C (aliquots were withdrawn at indicated times and separated by HPLC). Each species was injected and separated with Phenomenex Luna C18 reverse-phase-HPLC column (5 μm particle size, 250 × 4.6 mm) with the following linear gradient of 100 mM potassium phosphate (solvent A) and methanol (solvent B): 10%−30% B in 3 column volumes, 30%−30% B in 1 column volume and 30%−10% B in 1 column volume at flow rate of 0.6 mL/min, UV/VIS detector set at 260 nm. After each chromatographic elution, column was regenerated with two column volumes of 60% methanol. HMG-CoA, NADPH, and $NADP^+$ were directly monitored, whereas mevalonate was determined by monitoring CoA production (mevalonate/CoA 1:1 stoichiometric ratio) [46].

Liver microsomes dissolved in the activity buffer (100 mM sodium phosphate buffer containing 1 mM EDTA, 10 mM DTT, 2% DMSO, and 1 mM magnesium sulfate, pH 6.8) were incubated with substrate and NADPH for 60 min at 37 °C. The resulting mixture (10 μL) was separated with the above-described Luna C18 column thermostatted at 26 ± 0.1 °C.

4.1.8. Western blotting

The expression levels of liver microsomal HMGCR were analyzed through Western blot assay with the aim to verify a possible change upon probiotics treatment. In detail, for each time point (8-week-old, 24-week-old and 56-week-old) microsomes (20 μg of total proteins) were resolved on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred on polyvinylidene fluoride (PVDF) membranes. Successively, upon incubation with specific antibodies, the immunoblot detection was carried out with an enhanced chemiluminescence (ECL) Western Blotting ChemiDoc™ System (Biorad, Milano, Italy). Molecular weight markers were included in the gel. With the same approach LXRs (α/β) and SREBP1c expression levels were measured.

GAPDH was used to check equal protein loading and to normalize Western blot data. The densitometric analysis was conducted as previously described [77]. Briefly, ChemiDoc acquired images or scanned autoradiographs (16-bit gray scale) were processed through Image J (NIH) to calculate the background mean value and its standard deviation. The background-free image was then obtained subtracting the background intensity mean value from the original digital data. The integrated densitometric value associated with each band was then calculated as the sum of the density values over all the pixels belonging to the considered band having a density value higher than the background standard deviation. The band densitometric value was then normalized to the relative GAPDH signal intensity. The ratios of band intensities were calculated within the same Western blot. All the calculations were carried out using the Matlab environment (The MathWorks Inc., Natick, MA, USA).

4.1.9. Plasma fatty acid profile

The determination of the fatty acid composition of plasma glycerophospholipids was obtained using a previously described method [78], with the aim to verify the ability of the treatment to shift the lipid metabolic pathway exerting a neuroprotective effect. Briefly, 100 μL of plasma and 0.6 mL methanol (precooled at 5 °C) were combined in glass tubes and shaken for 30 s. The precipitated proteins were separated from the methanolic phase by centrifugation at 900 × g for 5 min. The methanolic supernatant was transferred into another glass tube. 25 μL of sodium methoxide solution were added to the supernatant, then the tubes were shaken at room temperature during selective synthesis of methyl esters. The reaction was stopped after 3 min with 75 μL of methanolic HCl. Fatty acid methyl esters (FAME) were extracted by adding 300 μL hexane and shaking the tubes for 30 s. The upper hexane phase was transferred into a 2 mL vial. The extraction was repeated, and combined extracts were dried under nitrogen flow at RT. The dry residue was taken up in 50 μL hexane (containing 2 g/L tert-buthyl-hydroxy-toluene) for GC analysis.

FAME were quantified using gas chromatography by a standard procedure using a capillary column ZB-FAME, 30 m × 0.25 mm, film thickness 0.20 μm (Phenomenex, USA) in a 7820 A GC System (Agilent Technologies, Santa Clara, California, EUA). The FAME was evaluated by calculating each FA as a percentage over the total FA cluster (relative %). GC peaks were identified as 97% of the total peaks present in the GC analysis by comparison with commercially available standards. Results were interpreted using the post hoc Tukey's test and are expressed as percent abundance.

4.1.10. ELISA determination of 27-hydroxycholesterol

27-Hydroxycholesterol (27-OHCE) was measured in the plasma and the brain of control and treated 56-week-old AD mice using the mouse 27-hydroxycholesterol solid-phase ELISA kit (My BioSource, San Diego, California, USA) following the manufacturer's instructions. Data are expressed as ng/mL ±SE.

4.1.11. Statistical analysis

Biochemical data are expressed as mean values ± SE. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni and Tukey test using Sigma-Stat 3.1 software (SPSS, Chicago, IL, USA). Statistical significance of treated mice compared to untreated 8-week-old mice of the same genotype is indicated with asterisks ($*p < 0.05$). To describe the effect of SLAB51 treatment, statistical significance of treated mice compared to age-matched untreated mice of the same genotype is indicated with hashtags ($^{\#}p < 0.05$).

Ethics approval and consent to participate

Experiments on mice complied with the ARRIVE guidelines, in accordance with the EU Directive 2010/63/EU for animal experiments and with a protocol approved by the Italian Ministry of Health (518/2018-PR).

Consent for publication

Not applicable.

Availability of data and materials

All data used and analyzed for the current study are available from the corresponding author on reasonable request.

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Author contributions

AME: conceptualization, project administration, funding acquisition, resources, methodology, supervision; LB: methodology, investigation, data curation, formal analysis, visualization, writing; MC: methodology, data curation, formal analysis, validation, visualization; CG, YZ, MS: investigation, data curation; MA: formal analysis, validation, supervision; VC: writing, review and editing. The authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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