RESEARCH ARTICLE

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# The Kynurenine Pathway Is Upregulated by Methyl-deficient Diet and Changes Are Averted by Probiotics

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**Scope: Probiotics exert immunomodulatory effects and may influence tryptophan metabolism in the host. Deficiency of nutrients related to C1 metabolism might stimulate inflammation by enhancing the kynurenine pathway. This study used Sprague Dawley rats to investigate whether a methyl-deficient diet (MDD) may influence tryptophan/kynurenine pathways and cytokines and whether probiotics can mitigate these effects.**
**Methods and Results: Rats are fed a control or MDD diet. Animals on the MDD diet received vehicle, probiotics (*L. helveticus* R0052 and *B. longum* R0175), choline, or probiotics + choline for 10 weeks ($n = 10$ per group). Concentrations of plasma kynurenine metabolites and the methylation and inflammatory markers in plasma and liver are measured.**
**Results: MDD animals (vs controls) show upregulation of plasma kynurenine, kynurenic acid, xanthurenic acid, 3-hydroxyxanthranilic acid, quinolinic acid, nicotinic acid, and nicotinamide (all $p < 0.05$). In the MDD rats, the probiotics (vs vehicle) cause lower anthranilic acid and a trend towards lower kynurenic acid and picolinic acid. Compared to probiotics alone, probiotics + choline is associated with a reduced enrichment of the bacterial strains in cecum. The interventions have no effect on inflammatory markers.**
**Conclusions: Probiotics counterbalance the effect of MDD diet and downregulate downstream metabolites of the kynurenine pathway.**

## 1. Introduction

The intestinal microbiota influences nutrient metabolism and systemic and mucosal immune cells in the host by providing metabolic intermediates to the host. For example, the microbiota regulates the degradation of the essential amino acid tryptophan into aromatic organic compounds called indoles that play a role in host immunity[1,2] and inflammatory processes.[3] Several commensal species such as *Bifidobacterium* spp.,[3] *Peptostreptococcus russellii*,[4] and *Lactobacillus* spp.[5,6] have been shown to contribute to tryptophan metabolism. In addition, S-adenosylmethionine is produced by some bacterial strains through fermentation processes.[7] Probiotics induce slight modifications in the gut microbial composition and interfere with tryptophan and methyl group metabolisms in the host, which may alter host immunity.

Tryptophan is used for protein synthesis or converted by hydroxylation in the brain to serotonin and melatonin; by decarboxylation to tryptamine; by transamination indolepyruvic acid; and by conversion to kynurenine. The kynurenine pathway accounts for 95% of tryptophan metabolism[8] and produces biologically active metabolites such as kynurenic acid (antioxidant, modifiers of vascular inflammation),[9,10] anthranilic acid (*N*-methyl-D-aspartate antagonist and neuroprotective), 3-hydroxykynurenine (*N*-methyl-D-aspartate agonist proinflammatory and source of radicals), picolinic acid (neuroprotective), and quinolinic acid (an *N*-methyl-D-aspartate agonist

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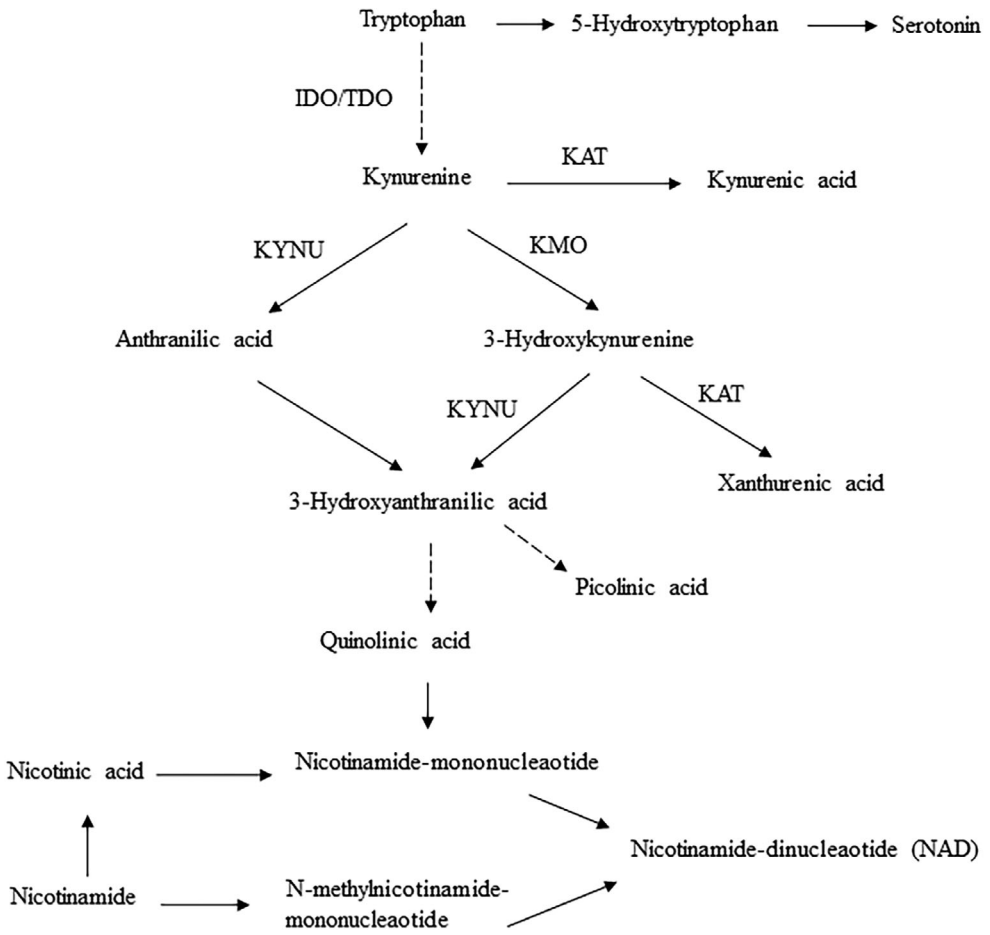


**Figure 1.** Tryptophan metabolic pathways to serotonin and via the kynurenine pathway. IDO, indoleamine 2,3-dioxygenase; KAT, Kynurenine-oxoglutarate transaminase 3; KMO, Kynurenine 3-monooxygenase; KYNU, Kynureninase; TDO, Tryptophan 2,3-dioxygenase.

and proinflammatory metabolite[11,12] (**Figure 1**). Moreover, the kynurenine pathway is the only de novo source of nicotinamide-dinucleotide (NAD) (i.e., CD4+ T-cell differentiation and apoptosis)[13] and thus plays a role in energy metabolism. The enzymes involved in tryptophan metabolism are widely distributed in the intestinal cells, liver, kidney, and immune cells (i.e., macrophages and dendritic cells). The kynurenine pathway has been shown to be upregulated in obesity and diabetes,[14,15] and to be downregulated by caloric restriction and ketogenic diet.[16] A dysregulated kynurenine pathway has been reported in several disease conditions that are associated with inflammation.[10,17,18]

Host tryptophan metabolism is modifiable by probiotics[19] and nutritional cofactors such as pyridoxal 5-phosphate.[20] Pyridoxal 5-phosphate (vitamin B6) is a cofactor for enzymes involved in the tryptophan/kynurenine pathway [i.e., kynurenine-oxoglutarate transaminase 3 (KAT) and kynureninase (KYNU)] and for enzymes operating in the methionine transsulfuration pathway and folate cycle.[21] A diet that is deficient in certain micronutrients can cause metabolic and/or immune dysregulations.[22–24] For example, folate and vitamin B12 deficiency induced liver inflammation and hepatosteatosis in a rat model of colitis.[24] It is not known whether a methyl-deficient diet (MDD) may stimulate tryptophan degradation and increase pro-inflammatory cytokines and whether probiotics can mitigate this effect.

We hypothesized that MDD may upregulate tryptophan metabolism to kynurenine and induce inflammation in rats compared with a control diet (CON, methyl-sufficient diet). Moreover, administration of probiotics (PRO), choline (CHOL) as a methyl donor, or probiotics + choline (PRO+CHOL) to animals on the MDD diet may ameliorate these effects compared with vehicle-treated animals. In a Sprague Dawley rat's model, we investigated 1) whether plasma levels of metabolites of the kynurenine pathway and cytokines are influenced by MDD versus CON diet and 2) whether the intervention with PRO, CHOL, or PRO+CHOL (vs vehicle) can reverse these effects.

## 2. Results

### 2.1. Bodyweight and Food Intake

The experiment started with a 3-week dietary modification phase (CON or $MDD_{100}$) followed by provision of PRO and/or CHOL (Figure S1, Supporting Information). The starting bodyweight did not differ between animals (week −3; mean

± SD; CON: 158 ± 13 g; MDD: 160 ± 20 g; $p = 0.770$; Figure S2A, Supporting Information). Weight gain was lower under the $MDD_{100}$ diet. After 1 week of receiving CON or $MDD_{100}$ diet, CON animals weighed 211 ± 15 g and $MDD_{100}$ animals weighed 128 ± 15 g ($p < 0.001$). $MDD_{100}$ rats lost on average 22.5% of their starting bodyweight after 2 weeks of $MDD_{100}$ diet, whereas CON animals showed rather a linear weight gain during the whole experiment. A 70% methyl-depleted diet ($MDD_{70}$) was provided from week −1 until the end of the whole experiment. The day before starting the intervention with PRO and/or CHOL (hereafter referred to as "baseline"), CON rats weighed [median (IQR) = 280 (28)g], whereas $MDD_{70}$ rats weighed 172 (38) g ($p < 0.001$) (**Table 1**). On decapitation day, CON rats weighed 507 (59) g and VEH-treated $MDD_{70}$ rats weighed 434 (137) g ($p = 0.029$). The four intervention groups (all on $MDD_{70}$ diet) did not differ in bodyweight at baseline ($p = 0.064$) or endpoint (10 weeks; $p = 0.402$) (Figure S3, Supporting Information).

After 1 week of CON or $MDD_{100}$ diet consumption, rats consumed 9.8 ± 0.4 g (CON) versus 3.8 ± 0.8 g food/100 g bodyweight ($MDD_{100}$), $p < 0.001$) (Figure S2B, Supporting Information). After 1 week on CON or $MDD_{70}$ diet (just before the intervention; week 0), CON rats consumed 9.0 ± 3.1 g and $MDD_{70}$ rats consumed 14.7 ± 0.5 g food 100 $g^{-1}$ bodyweight. At the last food measurement (week 9), CON rats consumed 4.9 ± 0.4 100 $g^{-1}$ and VEH-treated $MDD_{70}$ rats consumed 6.0 ± 0.3 g food 100 $g^{-1}$bodyweight ($p = 0.008$). The four $MDD_{70}$ intervention groups did not differ in food intake at baseline. However, the $MDD_{70}$ PRO+CHOL group consumed less food than the $MDD_{70}$VEH group (5.2 ± 0.2 g vs 6.0 ± 0.6 g food/100 g bodyweight; Bonferroni: $p = 0.050$) in week 10 ($p = 0.033$). There were no diet or intervention effects on water intake (mL 100 $g^{-1}$ bodyweight) at baseline or following time points.

### 2.2. C1-related Biomarkers and Liver Function Markers

VEH-treated animals receiving MDD diet had significantly lower plasma concentrations of choline [median (IQR) = 5.0 (1.0) vs 7.1 (1.2) µmol $L^{-1}$, $p < 0.001$] and methionine [57.8 (15.6) vs 76.0 (43) µmol $L^{-1}$; $p = 0.013$] than those on CON diet after 11 weeks of feeding (Table 1). There were no effects of diet on plasma betaine, dimethylglycine, vitamin B12, SAM, or S-adenosylhomocysteine (SAH). Compared to the CON group, the MDD diet caused lower liver SAM [89 (32) vs 52 (35) nmol $g^{-1}$ tissue, $p = 0.001$], a trend toward lower levels of liver SAH [29.8 (7.7) vs 20.7 (16.8) nmol $g^{-1}$ tissue, $p = 0.089$], and a lower liver SAM/SAH ratio [3.1 (1.0) vs 2.4 (0.7), $p = 0.016$] (Table 1). The intervention groups (VEH, PRO, CHOL, and PRO+CHOL) did not differ in concentrations of any of the aforementioned plasma markers or activities of liver enzymes in plasma (**Table 2**).

### 2.3. Kynurenine Pathway

Animals on MDD diet had slightly higher plasma tryptophan than animals on CON diet [190 (66) vs 152 (51) µmol $L^{-1}$; $p = 0.082$]. Rats on the MDD diet showed upregulated tryptophan metabolism to kynurenine compared to those on the CON diet. Rats on the MDD diet had higher kynurenine [3.6 (1.0)

**Table 1.** Concentrations of one-carbon metabolites, liver enzymes, and kynurenine metabolites after 10 weeks of feeding with CON or $MDD_{70\%}$ diet.

| Intervention | VEH | VEH | $p^{a)}$ |
|---|---|---|---|
| Diet | MDD | CON | |
| Body weight [g] | | | |
| Before starting the treatment[b)] | 172 (38)[b)] | 289 (28) | <0.001 |
| At 10 weeks | 434 (137) | 507 (59) | 0.029 |
| Food consumption [g 100 $g^{-1}$ body weight] | | | |
| Before starting the treatment | 14.7 (1.0) | 9.0 (4.7) | 0.008 |
| At 10 weeks | 6.1 (1.3) | 4.9 (0.7) | 0.016 |
| **EDTA plasma biomarkers** | | | |
| Betaine [µmol $L^{-1}$] | 62 (25) | 69 (6) | 0.257 |
| Choline [µmol $L^{-1}$] | 5.0 (1.0) | 7.1 (1.2) | < 0.001 |
| Dimethylglycine [nmol $L^{-1}$] | 1.3 (0.8) | 1.6 (1.2) | 0.940 |
| Vitamin B12 [pg $mL^{-1}$] | 1347 (236) | 1250 (401) | 0.513 |
| Methionine [µmol $L^{-1}$] | 57.8 (15.6) | 76.0 (43) | 0.013 |
| SAH [nmol $L^{-1}$] | 62 (47) | 48 (53) | 0.821 |
| SAM [nmol $L^{-1}$] | 285 (75) | 272 (53) | > 0.999 |
| Serotonin [nmol $L^{-1}$] | 2056 (515) | 1513 (809) | 0.025 |
| AST [U $L^{-1}$] | 123 (74) | 134 (24) | 0.540 |
| ALT [U $L^{-1}$] | 40 (11) | 42 (9) | 0.838 |
| Pyridoxal 5'-phosphate [nmol $L^{-1}$] | 988 (422) | 822 (228) | 0.131 |
| Pyridoxal [nmol $L^{-1}$] | 903 (476) | 675 (364) | 0.174 |
| 4-Pyridoxic acid [nmol $L^{-1}$] | 117 (91) | 93 (42) | 0.520 |
| Pyridoxine [nmol $L^{-1}$] | 1.7 (5.5) | 0.0 (2.8) | 0.267 |
| Riboflavin [nmol $L^{-1}$] | 56.3 (16.2) | 52.3 (17.6) | 0.450 |
| Flavin mononucleotide [nmol $L^{-1}$] | 36.2 (13.7) | 38.1 (10.5) | 0.473 |
| Tryptophan [µmol $L^{-1}$] | 190 (66) | 152 (51) | 0.082 |
| Kynurenine [µmol $L^{-1}$] | 3.6 (1.0) | 2.8 (1.0) | 0.019 |
| Kynurenic acid [nmol $L^{-1}$] | 83.1 (36.5) | 71.2 (25.6) | 0.010 |
| Anthranilic acid [nmol $L^{-1}$] | 48.1 (16.4) | 58.1 (25.7) | 0.473 |
| 3-Hydroxykynurenine [nmol $L^{-1}$] | 19.2 (6.5) | 16.5 (4.3) | 0.220 |
| Xanthurenic acid [nmol $L^{-1}$] | 16.8 (8.3) | 12.5 (3.2) | 0.004 |
| 3-Hydroxyanthranilic acid [nmol $L^{-1}$] | 28.1 (17.7) | 10.7 (5.5) | 0.009 |
| Picolinic acid [nmol $L^{-1}$] | 106 (75) | 100 (66) | 0.545 |
| Quinolinic acid [nmol $L^{-1}$] | 749 (372) | 417 (325) | 0.010 |
| Nicotinic acid [nmol $L^{-1}$] | 28.0 (34.7) | 0.0 (0.0) | 0.013 |
| Nicotinamide [nmol $L^{-1}$] | 4345 (1620) | 2770 (635) | 0.001 |
| N1-methylnicotinamide [nmol $L^{-1}$] | 263 (1761) | 117 (203) | 0.082 |
| Cystathionine [µmol $L^{-1}$] | 1.41 (0.47) | 1.10 (0.31) | 0.034 |
| **Liver extract, nmol $g^{-1}$ tissue** | | | |
| SAH | 20.7 (16.8) | 29.8 (7.7) | 0.089 |
| SAM | 52 (35) | 89 (32) | 0.001 |
| SAM/SAH | 2.4 (0.7) | 3.1 (1.0) | 0.016 |

Data are presented as median (IQR). $n = 10$ in each group. [a)] $p$ values are according to Mann-Whitney test. [b)] before starting the treatment, the MDD rats had received $MDD_{100\%}$ for 2 weeks followed by the $MDD_{70\%}$ for 1 week. Both diet groups received vehicle (xylitol, maize-derived maltodextrin, plum flavor, malic acid). ALT, Alanine transaminase; AST, Aspartate transaminase; CON, Control; MDD, Methyl-Deficient Diet; S-adenosylhomocysteine; SAM, S-adenosylmethionine; VEH, Vehicle.

vs 2.8 (1.0) μmol $L^{-1}$; $p = 0.019$], kynurenic acid [83.1 (36.5) vs 71.2 (25.6) nmol $L^{-1}$; $p = 0.010$], xanthurenic acid [16.8 (8.3) vs 12.5 (3.2) nmol $L^{-1}$; $p = 0.004$], 3-hydroxyxanthranilic acid [28.1 (17.7) vs 10.7 (5.5) nmol $L^{-1}$; $p = 0.009$], quinolinic acid [749 (372) vs 417 (325) nmol $L^{-1}$; $p = 0.010$], nicotinic acid [28.0 (34.7) vs 0.0 (0.0) nmol $L^{-1}$; $p = 0.013$], nicotinamide [4345 (1620) vs 2770 (635) nmol $L^{-1}$; $p = 0.001$], N1-methylnicotinamide [263 (1761) vs 117 (203) nmol $L^{-1}$; $p = 0.082$], and cystathionine (a component of homocysteine transsulfuration pathway) [1.41 (0.47) vs 1.10 (0.31) μmol $L^{-1}$; $p = 0.034$] (Table 1). The diet had no effect on plasma levels of vitamin B6 forms (pyridoxal 5'-phosphate, pyridoxal, 4-pyridoxic acid, and pyridoxine).

The four intervention groups (VEH, PRO, CHOL, PRO+CHOL) differed in the following kynurenine metabolites;

**Table 2.** Concentrations of C1 metabolites, neurotransmitters, liver function markers, and kynurenine metabolites in biospecimens collected after 10 weeks of intervention with VEH, PRO, CHOL, or PRO+CHOL in rats fed on a methyl-deficient diet.

| Intervention | VEH (reference group) | PRO | CHOL | PRO+CHOL | $p$[a)] |
|---|---|---|---|---|---|
| Diet | MDD | MDD | MDD | MDD | |
| Number | 10 | 10 | 10 | 10 | |
| Bodyweight [g] | | | | | |
| Before starting the treatment | 172 (38) | 176 (22) | 174 (33) | 185 (22) | 0.064 |
| At 10 weeks | 434 (137) | 453 (67) | 459 (108) | 479 (32) | 0.402 |
| Food consumption in g $100g^{-1}$ bodyweight, mean (SD) | | | | | |
| Before starting the treatment | 14.7 (0.5) | 14.3 (1.2) | 14.4 (0.7) | 13.5 (0.9) | 0.159 |
| At 10 weeks | 6.0 (0.6) | 5.3 (0.1)[a)] | 5.5 (0.3) | 5.2 (0.2)[l)] | 0.033 |
| Log bacterial strains/g cecum | | | | | |
| *Lactobacillus helveticus* R0052 | 0 | 8.6 (0.8) | 0 | 7.9 (2.5)[a)] | - |
| *Bifidobacterium longum* R0175 | 0 | 7.5 (0.4) | 0 | 6.5 (1.7)[a)] | - |
| Plasma biomarkers | | | | | |
| Betaine [μmol $L^{-1}$] | 62 (25) | 70 (14) | 64 (23) | 77 (19) | 0.085 |
| Choline [μmol $L^{-1}$] | 5.0 (1.0) | 4.3 (1.5) | 5.3 (1.2) | 5.4 (0.9) | 0.369 |
| Dimethylglycine [nmol $L^{-1}$] | 1.3 (0.8) | 1.4 (1.1) | 1.6 (1.0) | 1.9 (1.2) | 0.320 |
| Vitamin B12 [pg $mL^{-1}$] | 1347 (236) | 1175 (188) | 1230 (148) | 1234 (127) | 0.220 |
| Methionine [μmol $L^{-1}$] | 57.8 (15.6) | 64.7 (10.2) | 58.0 (16.6) | 57.9 (9.5) | 0.666 |
| SAH [nmol $L^{-1}$] | 62 (47) | 71 (53) | 52 (47) | 47 (33) | 0.444 |
| SAM [nmol $L^{-1}$] | 285 (75) | 247 (77) | 250 (65) | 274 (36) | 0.457 |
| Serotonin [nmol $L^{-1}$] | 2056 (515) | 1684 (1062) | 2134 (381) | 1896 (780) | 0.296 |
| AST [U $L^{-1}$] | 123 (74) | 134 (38) | 132 (48) | 140 (14) | 0.630 |
| ALT [U $L^{-1}$] | 40 (11) | 46 (13) | 43 (9) | 47 (8) | 0.241 |
| Pyridoxal 5'-phosphate [nmol $L^{-1}$] | 881 (368) | 950 (292) | 967 (320) | 947 (207) | 0.963 |
| Pyridoxal [nmol $L^{-1}$] | 804 (412) | 616 (206) | 900 (427) | 732 (333) | 0.138 |
| 4-Pyridoxic acid [nmol $L^{-1}$] | 111 (51) | 77 (22) | 109 (76) | 88 (79) | 0.090 |
| Pyridoxine [nmol $L^{-1}$] | 1.1 (3.8) | 0.0 (0.9) | 1.3 (3.2) | 0.0 (1.96) | 0.176 |
| Riboflavin [nmol $L^{-1}$] | 55.8 (12.5) | 48.8 (18.1) | 58.6 (7.7) | 49.6 (12.1) | 0.315 |
| Flavin mononucleotide [nmol $L^{-1}$] | 37.5 (9.5) | 37.1 (9.4) | 44.7 (19.3) | 36.6 (11.1) | 0.569 |
| Tryptophan [μmol $L^{-1}$] | 118 (61) | 170 (44) | 191 (35) | 168 (48) | 0.129 |
| Kynurenine [μmol $L^{-1}$] | 3.3 (0.9) | 3.0 (0.5) | 2.8 (0.9)[f)] | 3.0 (0.6) | 0.015 |
| Kynurenic acid [nmol $L^{-1}$] | 77.3 (12.7) | 56.1 (22.5)[c)] | 59.8 (43.7)[g)] | 61.1 (20.0)[k)] | 0.019 |
| Anthranilic acid [nmol $L^{-1}$] | 50.7 (18.5) | 39.4 (6.4)[d)] | 49.1 (17.2)[h)] | 44.0 (12.1) | 0.010 |
| 3-Hydroxykynurenine [nmol $L^{-1}$] | 17.4 (5.4) | 13.9 (6.1) | 18.5 (13.4) | 15.3 (4.6) | 0.075 |
| Xanthurenic acid [nmol $L^{-1}$] | 15.1 (5.0) | 13.0 (1.7) | 17.1 (4.5)[i)] | 14.3 (4.9) | 0.026 |
| 3-Hydroxyanthranilic acid [nmol $L^{-1}$] | 14.4 (20.3) | 17.3 (13.3) | 28.6 (27.0) | 15.7 (7.5)[m)] | 0.024 |
| Picolinic acid [nmol $L^{-1}$] | 102 (76) | 67 (44)[e)] | 118 (58)[j)] | 85 (32) | 0.016 |
| Quinolinic acid [nmol $L^{-1}$] | 608 (342) | 465 (177) | 617 (509) | 501 (155) | 0.040[n)] |
| Nicotinic acid [nmol $L^{-1}$] | 0.0 (28.8) | 25.2 (26.5) | 13.4 (30.8) | 11.9 (33.2) | 0.783 |
| Nicotinamide [nmol $L^{-1}$] | 3400 (1930) | 3780 (535) | 4235 (1608) | 3620 (1525) | 0.285 |
| N1-methylnicotinamide [nmol $L^{-1}$] | 154 (487) | 353 (915) | 242 (1870) | 313 (1688) | 0.993 |
| Cystathionine [μmol $L^{-1}$] | 1.21 (0.41) | 1.27 (0.43) | 1.26 (0.56) | 1.18 (0.34) | 0.720 |

(*Continued*)

**Table 2.** Continued.

| Intervention | VEH (reference group) | PRO | CHOL | PRO+CHOL | $p^{a)}$ |
|---|---|---|---|---|---|
| Diet | MDD | MDD | MDD | MDD | |
| Liver extract (nmol $g^{-1}$ tissue) | | | | | |
| SAH | 20.7 (16.8) | 22.0 (8.5) | 18.9 (12.0) | 19.1 (11.7) | 0.813 |
| SAM | 52 (35) | 60 (18) | 55 (16) | 61 (12) | 0.450 |
| SAM/SAH Ratio | 2.4 (0.7) | 2.4 (1.7) | 2.7 (1.4) | 2.9 (1.9) | 0.579 |

Data are presented as median (IQR) except for food consumption (mean and SD). Rats received VEH, PRO, CHOL, or PRO+CHOL once daily for 10 weeks. VEH intervention consisted of xylitol, maize-derived maltodextrin, plum flavor, and malic acid (i.e., the same excipient as in the other interventions, but without any active substance). [a)] $p$ values are according to independent samples Kruskal-Wallis test. Significance values for paired comparisons have been adjusted by the Bonferroni correction for multiple tests. For the comparisons of PRO versus VEH; [b)] $p = 0.096$, [c)] $p = 0.052$; [d)] $p = 0.016$; [e)] $p = 0.097$. For the comparisons of CHOL versus VEH; [f)] $p = 0.011$; [g)] $p = 0.067$. For the comparisons of CHOL versus POR; [h)] $P = 0.016$; [i)] $P = 0.046$; [j)] $p = 0.034$. For the comparisons of PRO+CHOL versus VEH; [k)] $p = 0.068$; [l)] $p = 0.05$. For the comparisons of CHOL versus POR+CHOL; [m)] $p = 0.017$. [n)] between-groups $p$ values > 0.100 after correction for multiple comparisons. For the comparisons of PRO+CHOL versus PRO; [o)] $p = 0.076$; [p)] $p = 0.028$ (one-way ANOVA test between two groups). ALT, Alanine transaminase; AST, Aspartate transaminase; CHOL, Choline; PRO, Probiotics; S-adenosylhomocysteine; SAM, S-adenosylmethionine; VEH, Vehicle.

kynurenine (independent samples Kruskal-Wallis test $p = 0.015$), kynurenic acid ($p = 0.019$), anthranilic acid ($P = 0.010$), 3-hydroxykynurenine ($p = 0.075$), xanthurenic acid ($p = 0.026$), 3-hydroxyanthranilic acid ($P = 0.024$), picolinic acid ($p = 0.016$), quinolinic acid ($P = 0.040$) (Figure S4, Supporting Information, and Table 2). The between-group comparisons showed that intervention with probiotics suppressed tryptophan degradation to kynurenine as compared to the VEH group. The PRO group showed significantly lower anthranilic acid [39.4 (6.4) vs 50.7 (18.5) nmol $L^{-1}$; $p = 0.016$], and a trend towards lower kynurenic acid [56.1 (22.5) vs 77.3 (12.7) nmol $L^{-1}$; $p = 0.052$] and picolinic acid [67 (44) vs 102 (76) nmol $L^{-1}$; $p = 0.097$] compared to the VEH group. The intervention groups showed no significant differences in plasma concentrations of the cofactor pyridoxal 5'-phosphate or any other vitamin B6 form (Table 2).

The intervention with CHOL caused lower median plasma level of kynurenine [2.8 (0.9) vs 3.3 (0.9) µmol $L^{-1}$; $p = 0.011$] and kynurenic acid [59.8 (43.7) vs 77.3 (12.7); $p = 0.067$] compared to the intervention with VEH. The group that received PRO+CHOL showed slightly lower kynurenic acid [61.1 (20.0) vs 77.3 (12.7); $p = 0.068$] compared to the VEH group.

The intervention with PRO alone showed selective lowering effects on plasma concentrations of anthranilic acid [49.1 vs 39.4 nmol $L^{-1}$; $p = 0.016$], xanthurenic acid [17.1 (4.5) vs 13.0 (1.7) nmol $L^{-1}$; $p = 0.046$], and picolinic acid [118 (589) vs 67 (44) nmol $L^{-1}$; $p = 0.034$] compared to the CHOL group (Table 2). The comparisons of the concentrations of C1 metabolites, neurotransmitters, liver function markers, and kynurenine metabolites between the intervention groups (PRO, CHOL, and PRO+CHOL) fed on a MDD versus the (CON+VEH) group are shown in Table S3 (Supporting Information).

### 2.4. Plasma Serotonin and Plasma and Liver Cytokines

VEH-treated rats on MDD diet had significantly higher plasma serotonin concentrations than those on CON diet [2056 (515) vs 1513 (809) nmol $L^{-1}$; $p = 0.025$] (Table 1). The intervention groups did not differ in plasma serotonin (Table 2).

VEH-treated MDD rats tended to have higher concentrations of RANTES than those on CON diet [701 (381) vs 580 (320) pg $mL^{-1}$; $p = 0.072$]. None of the 22 cytokines that were measured in plasma differed according to the diet (MDD vs CON). Also, none of the plasma cytokines differed according to the intervention (Tables S4 and S5 and Figures S5 and S6, Supporting Information).

### 2.5. Stool Bacterial Population (qPCR) and Behavioral Tests

The qPCR tests showed that *L. helveticus* R0052 and *B. longum* R0175 were not detectable in cecum of VEH-treated rats (CON and MDD diets) or CHOL-treated rats (Tables 1 and 2). The median (IQR) concentrations of *L. helveticus* R0052 were 8.6 (0.8) log bacteria g cecum in the PRO group and 7.9 (2.5) log bacteria/g in the PRO+CHOL group. The concentrations of *B. longum* R0175 were 7.8 (0.5) log bacteria/g in PRO and 6.5 (1.7) log bacteria/g in PRO+CHOL. The PRO group tended to have higher concentrations of both *L. helveticus* R0052 ($p = 0.076$) and *B. longum* R0175 ($p = 0.028$) than the PRO+CHOL group (Table 2).

The intervention groups generally did not differ in several behavioral tests related to cognition (Novel Object Recognition, Y-Maze), anxiety (Elevated Plus Maze), and depression (Forced Swim Test) (Figure S7 and Text, Supporting Information).

## 3. Discussion

We investigated whether feeding a MDD may cause dysregulation in tryptophan metabolism pathways and whether the intervention with probiotics, the methyl donor choline, or probiotics plus choline can modify this effect. The following novel results were found (summarized in **Figure 2**): first, the 70% MDD diet (without choline, methionine, and folate) led to hypomethylation in the animals compared to the CON diet, but did not influence liver function markers (plasma ALT and AST activities) or plasma and liver concentrations of key cytokines. Second, the MDD diet caused higher plasma serotonin and a profound upregulation of the kynurenine pathway in plasma. Third, administration of PRO to animals on the MDD diet ameliorated the effects of the deficient diet on kynurenine metabolites (selectively lowered anthranilic acid, picolinic acid, and 3-hydroxykynurenine).

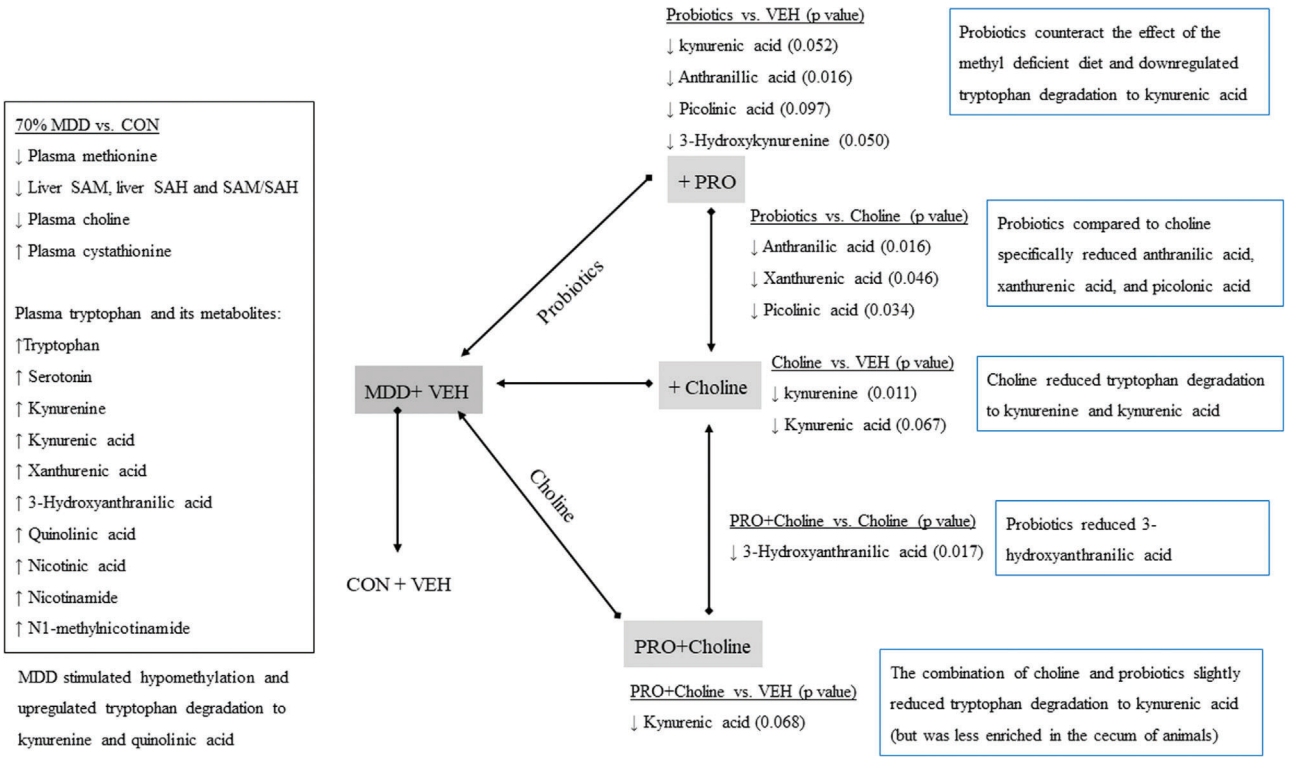


**Figure 2.** Summary of the observed significant differences in the tryptophan and kynurenine metabolic pathways between the methyl sufficient and the methyl deficient diet and according to the intervention. The MDD+VEH versus CON+VEH were compared by Mann-Whitney test. The arrows indicate the direction of statistically significant changes; ↑ higher; ↓ lower. Choline; MDD, methyl-deficient diet; PRO, Probiotics; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; VEH, vehicle.

In contrast, PRO intervention was not associated with significant changes in plasma or liver C1 metabolites, or plasma serotonin compared to animals that received VEH. Administration of CHOL alone was associated with lower plasma kynurenine and slightly lower kynurenic acid, while administration of PRO+CHOL was associated with slightly lower kynurenic acid compared to animals that received VEH. Notably, administering PRO with choline reduced the enrichment of the bacterial strains in the cecum which may explain that the effect of PRO on tryptophan metabolism was weakened when combined with choline.

Supplementation of *Lactobacillus johnsonii* N6.2 (vs placebo) for 8 weeks in healthy individuals caused an increase in serum tryptophan and systemic effects on innate and adaptive immune cell populations (i.e., increased circulating effector Th1 cells and cytotoxic CD8+T cells subset).[29] Compared to the placebo, *Lactobacillus plantarum 299V* has been shown to lower plasma kynurenine while causing no change in plasma IL6, IL-1$\beta$, TNF-$\alpha$, and cortisol in patients with depression.[30] In addition, different probiotic strains have been shown to downregulate the kynurenine pathway in animal models of depression or chronic stress stimuli.[31,32] Therefore, our results on downregulation of the kynurenine pathway after treatment with *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 are in line with earlier studies.

The present study was performed in animals with an upregulated kynurenine metabolism due to the 70% MDD diet. We found no evidence that the dysregulation of the kynurenine pathway is associated with pro-inflammatory or anti-inflammatory cytokines (i.e., in plasma or liver). The liver and immune cells are main sources of kynurenine metabolites. The immunomodulatory role of probiotics could take place in the liver, the kidney, immune cells, and/or the gut mucosal immune system.[33,34] Macrophages express indoleamine 2,3-dioxygenase (IDO), the enzyme that converts tryptophan to kynurenine. IDO1 is induced by pro-inflammatory cytokines (i.e., TNF-$\alpha$, IFN-$\gamma$, IL-1, and IL-6) leading to higher kynurenine production.[35] The source of plasma kynurenine metabolites in our study is not known. The divergent effects of the MDD diet and probiotics on plasma kynurenine without affecting the cytokines suggest that tryptophan dysregulation under MDD diet took place in the intestinal cells rather than in the liver or immune cells. This issue deserves further investigation.

Kynurenine and its downstream metabolites anthranilic acid, picolinic acid, and quinolinic acid represent inflammatory sensors[36] that have been shown to be associated with cardiovascular risk factors such as diabetes.[14,15] The kynurenine pathway delivers NAD+ and shows a tissue specific response to exercise.[37] Feeding a ketogenic diet or a caloric restricted diet to animals caused downregulation of the kynurenine pathway.[16] In our study, the upregulation of the kynurenine pathway in rats on the MDD diet versus CON diet (both received VEH) could reflect a metabolic adaptation to higher NAD+ -demands in the MDD group. Accordingly, the MDD animals had higher food intake compared with the CON towards the end of the experiment,

although they weighted less. Brandacher et al. suggested that activation of the IDO could drive disorders in appetite-satiety regulation and could increase caloric intake.[38] Thus, probiotics may influence food intake and regulation of body weight through downregulation of the kynurenine pathway, but this needs to be further investigated.

The $MDD_{70}$ diet had no marked effects on behavioral tests or liver enzymes, indicating that the diet did not induce brain or liver cell damage, as would be expected by feeding a 100% deficient diet.[39,40] However, previous studies often combined a MDD with a high-fat diet to stimulate hepatosteatosis.

The present study has some limitations. The dietary intervention with $MDD_{100}$ at a young age followed by $MDD_{70}$ could have affected early life epigenetic programming (i.e., weight regulation) that made the rats resilient and resistant to inflammation. The differences between the CON+VEH group and the intervention groups (all on MDD) (Table S3, Supporting Information) should be interpreted with caution since the food intakes and weight gain were different according to the background diets. Moreover, the study was conducted in young male rats. Age and sex differences in C1-metabolism have been reported.[41] In addition, the bacterial enrichment could be minor after using probiotics and could interact with dietary components as was seen in this study in the PRO+CHOL group (i.e., less enrichment than in the PRO group).

Also competing bacterial strains such as those producing trimethylamine from choline may lead to producing trimethylamine N-oxide, a metabolites with potentially atherosclerotic effects.[42]

Overall, our study supports that the MDD induced tryptophan degradation via the kynurenine pathway. Intervention with *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 caused a profound downregulation of the kynurenine pathway especially in the downstream metabolites (i.e., anthranilic acid and 3-hydroxykenurenine). CHOL alone reversed the effect of MDD on plasma kynurenine and kynurenic acid (upstream metabolites). The metabolic effects of the combination of PRO+CHOL were weak, probably due to lower bacterial enrichment in the cecum following the combination of PRO with choline. Future studies may investigate the activities of the enzymes participating in tryptophan metabolism and possible tissues (i.e., liver, intestinal mucosal membrane) where probiotics may target the tryptophan-kynurenine pathway. Our results suggest that tryptophan metabolism is an important molecular mechanism to be investigated in RCTs of probiotics as modifiers of health outcomes in human studies.

## 4. Experimental Section

*Animals and Diet*: Male Sprague Dawley rats ($n = 50$, age 4 weeks) were purchased from Taconic Bioscience A/S (Ry, Denmark) and allowed to acclimatize for 1 week on a standard diet after arrival. The day before the diet modification start (week −3), rats were 5 weeks old and weighed 160 ± 18.3 g (mean ± SD). From week −3 to week −1, they received either a standard control diet (CON) or a customized 100% MDD ($MDD_{100}$) without choline, betaine, methionine, and folic acid for 2 weeks (both diets were obtained from Ssniff Spezialdiäten GmbH, Soest, Germany). The diet specifications are shown in Table S1 (Supporting Information). From week −1 until the end of the experiment (week 10), the degree of methyl deficiency was adjusted to 70% ($MDD_{70}$) as explained below. Rats receiving the same diet and intervention were pair-housed in standard cages (Cage 1291H Eurostandard Type III H, 425 × 266 × 185 mm, Tecniplast, Italy) at 20 ± 2°C and 60 ± 5% relative humidity on a reversed 12 h light/dark cycle (lights on at 2 p.m.), which was introduced in 3-h increments. Rats had access to food and tap water ad libitum. Cages (containing bedding, a tunnel shelter, nesting material, and a wooden stick) were changed once a week. Animal weight and food/water intake (per 100 g bodyweight) were recorded throughout the study.

Rats receiving the $MDD_{100}$ diet ($n = 40$) lost more than 20% of their total body weight within 2 weeks. Thus, the diet was changed from 100% deficiency (received from week −3 to week −1) to 70% deficiency (hereafter referred to as $MDD_{70}$). Rats were allowed to adapt to the $MDD_{70}$ diet for one additional week, and the intervention was started in week 0. The complete experimental timeline is presented in Figure S1 (Supporting Information). The $MDD_{70}$ diet was prepared by grinding and thoroughly mixing the 100% MDD pellets with 30% grinded CON pellets. Tap water (approximately 20% of the total mass) was added to form pellets. Since the consistency of the MDD pellets now changed from dry to wet pellets, the pure CON diet was also grinded and prepared with water in the same manner. Pellets were freshly prepared every 3–4 days from week −1 until week 10. All experiments were approved by the Danish Animal Experiments Inspectorate before initiation of the experiments (approval number: 2012-15-2934-00254) and were conducted in accordance with the European Community Council Directive.

*Intervention*: Rats on $MDD_{70}$ diet were randomly assigned to one of four interventions: VEH, probiotics (PRO), choline (CHOL), or PRO+CHOL. Rats on CON diet received vehicle (VEH) only.

The commercial formulation CEREBIOME (previously known as Probio'Stick, a new name for an existing formula combining *Lactobacillus helveticus* Rosell-52 and *Bifidobacterium longum* Rosell-175, Lallemand Health Solutions Inc., Montreal, QC, Canada) was used. The formulation is a powder mix of probiotics and excipients (xylitol, maize-derived maltodextrin, plum flavor, malic acid). The vehicle consisted of the same excipients (xylitol, maize-derived maltodextrin, plum flavor, malic acid) but without active cultures and was of identical taste to the PRO. The exact composition of the $10^{10}$ CFUs between the two bacterial strains is proprietary information of the company producing the probiotics.

*Dosage Information/Dosage Regimen*: We prepared solutions of the vehicle and probiotic just before administration by dissolving the respective weight (g) of the powder in tap water (mL) 1:1. The intervention dose per rat and day was 0.2 g powder containing either VEH or $10^{10}$ colony-forming units (CFU) dissolved in 0.3 mL water. This amount was administered via syringe-feeding once daily toward the end of the active phase (2 p.m. ± 1 h) over the 10-week intervention period as in our previous study.[25] Syringe-feeding is based on voluntary consumption of a solution directly from a syringe held into the cage, thereby enabling exact and controlled dosing to the animals.[26]

Choline bitartrate (8 mg $rat^{-1}$ $day^{-1}$; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in the same vehicle or probiotic solutions and also administered via syringe-feeding (total volume 0.3 mL $day^{-1}$).

*Sample Collection and Processing*: Rats were decapitated after 10 weeks of starting the intervention. Decapitation took place in a random order between 2 and 6 p.m. over 2 consecutive days. Trunk blood was collected in EDTA-coated tubes and immediately centrifuged at 3000 × *g* for 10 min. Plasma was aliquoted and stored at −80 °C. Plasma for SAM/SAH measurements was added into tubes containing 1 N acetic acid (for a ratio of acetic acid:plasma of 1:10). Liver tissue (from the left lateral lobe) were quickly removed, snap-frozen in pre-cooled isopentane, and stored at −80°C. Tissues were later homogenized in 1 N acetic acid (10 µL $mg^{-1}$ tissue) using a tissue lyser (Mixer Mill MM 400, Retsch GmbH, Haan, Germany; frequency: 30 $s^{-1}$, 2 min, carbide bead) and centrifuged at 10 000 × *g* for 10 min. For monoamine measurements, 20 µL perchloric acid was added to 180 µL plasma followed by centrifugation.

*Biochemical Measurements*: Plasma concentrations of one carbon (C1)-metabolites (betaine, choline, dimethylglycine, SAH, and SAM) and liver SAH and SAM were measured using isotope-labeled internal

standards and an Acquity Ultra Performance LC system coupled to a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (UPLC-MS/MS) (Waters Corporation, Milford, MA, USA) as reported before.[27,28] Plasma alanine transaminase (ALT), aspartate transaminase (AST), and vitamin B12 were measured using routine automated methods (Table S2, Supporting Information). Plasma methionine concentrations were measured at MS-Omics (Frederiksberg, Denmark) using gas chromatography mass spectrometry. Plasma concentrations of kynurenine pathway metabolites were measured at Bevital AS, Norway using LC-MS/MS devices.

Concentrations of plasma serotonin were measured using ultra-high performance liquid chromatography with electrochemical detection (UHPLC-ECD, Dionex Ultimate 3000 UHPLC, Thermo Scientific, Rockford, IL, USA) as described before.[25]

Concentrations of soluble inflammatory protein markers in plasma and liver extracts were analyzed using a fluorescent magnetic bead-based multiplex immunoassay (Luminex-based Bio-Plex 200 Bio-Rad Laboratories Inc., Hercules, CA, #10014905) according to the methods shown in Table S2 (Supporting Information). The following inflammation markers and cytokines were measured; G-CSF, GM-CSF, GRO/KC, IFN-$\gamma$, IL-1$\alpha$, IL-1$\beta$, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, M-CSF, MCP-1, MIP-1$\alpha$, MIP-3$\alpha$, RANTES, TNF-$\alpha$, VEGF.

*Bacterial DNA Extraction and Quantitative Real-time PCR*: Total DNA was isolated from 180 ± 20 g cecal samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions with some modifications (Table S2, Supporting Information). The extracted DNA was used to detect and quantify *L. helveticus* R0052 *and B. longum* R0175 in cecal samples by qPCR using SYBR Select Master Mix (Thermo Fisher) and the primers and cycling conditions shown in Table S2 (Supporting Information).

Behavioral tests for cognition, anxiety, and depression were performed at the time points shown in Figure S1 (Supporting Information) as described in Supplemental Text.

*Data Analysis*: Bodyweight and food consumption of the animals were expressed as mean ± SD and the differences between the groups were tested using one-way ANOVA test and Bonferroni post hoc test when ANOVA was significant. The one-sample Kolmogorov-Smirnov Test with Lilliefors Significance Correction and the quintile-quintile plots were used to study the distribution of the data. The majority of the concentrations of the biomarkers were not normally distributed and the distribution did not improve after ln-transformation. Thus, the biomarkers are shown as median and (interquartile range, IQR). The nonparametric test Mann-Whitney was used to study differences in the concentrations of the markers between the MDD and CON diets. The independent samples Kruskal-Wallis test was used to compare the variables across the intervention groups. When Kruskal-Wallis test was significant for a given variable, pairwise comparisons were performed and the significance values of the subgroup comparisons were adjusted for multiple tests. We used the post hoc Bonferroni test for adjusting the $p$ value to prevent type I error. However, this test is considered rather conservative which may cause higher type II error or missing possible effects. $p$ values below 0.05 were considered statistically significant, while those between 0.05 and 0.1 were interpreted as a trend. Statistical analyses were carried out using IBM SPSS 27.0 (IBM Corp., Armonk, NY, USA).

## Supporting Information

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

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## Conflict of Interest

S.T. declares having received financial research support from Winclove Probiotics (Amsterdam, Netherlands) for an unrelated study. C.W.M. and T.A.T. are employees of Rosell Institute for Microbiome and Probiotics, the research group of Lallemand Health Solutions Inc. The company was not involved in the study design, data analysis or interpretation, manuscript writing, or decision to publish, and only provided financial support in the form of authors' salaries and/or research materials. G.W. declares having received lecture/consultancy fees from H. Lundbeck A/S, Servier SA, Astra Zeneca AB, Eli Lilly A/S, Sun Pharma Pty Ltd, and Pfizer Inc., Shire A/S, HB Pharma A/S, Arla Foods A.m.b.A., Alkermes Inc, and Mundipharma International Ltd., and research funding from the Danish Medical Research Council, Aarhus University Research Foundation (AU-IDEAS initiative (eMOOD)), the Novo Nordisk Foundation, the Lundbeck Foundation, and EU Horizon 2020 (ExEDE). RO received speaker honoraria from Merck-Selbst Medikation, P&G Health Germany, and Worwag Pharma. All other authors report no potential conflicts of interest and have nothing to disclose.

## Author Contributions

S.T. and R.O. designed the study. S.T. performed the animal experiments, tissue collection and extractions, DNA extraction, qPCR, cytokine analysis, supported chromatography sample analysis, analyzed and interpreted the data, and wrote parts of the manuscript. H.M.A. performed chromatography sample analyses. C.W.M. supported tissue extraction and cytokine analysis. D.F.H. and G.T. supported tissue collection. J.G., T.A.T., P.M.U., and G.W. provided critical input to the manuscript. RO measured the concentrations of C1 metabolites and analyzed and interpreted the data and wrote the first draft of the manuscript.

## Data Availability Statement

Data can be made available upon request to the corresponding author.

## Keywords

choline, kynurenine, methyl-deficient diet, probiotics, tryptophan

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