Gut microbiota and fermentation-derived branched chain hydroxy acids mediate health benefits of yogurt consumption in obese mice

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Meta-analyses suggest that yogurt consumption reduces type 2 diabetes incidence in humans, but the molecular basis of these observations remains unknown. Here we show that dietary yogurt intake preserves whole-body glucose homeostasis and prevents hepatic insulin resistance and liver steatosis in a dietary mouse model of obesity-linked type 2 diabetes. Fecal microbiota transplantation studies reveal that these effects are partly linked to the gut microbiota. We further show that yogurt intake impacts the hepatic metabolome, notably maintaining the levels of branched chain hydroxy acids (BCHA) which correlate with improved metabolic parameters. These metabolites are generated upon milk fermentation and concentrated in yogurt. Remarkably, diet-induced obesity reduces plasma and tissue BCHA levels, and this is partly prevented by dietary yogurt intake. We further show that BCHA improve insulin action on glucose metabolism in liver and muscle cells, identifying BCHA as cell-autonomous metabolic regulators and potential mediators of yogurt’s health effects.
The incidence of type 2 diabetes (T2D) is rising to epidemic proportions with recent worldwide figures reaching over 463 million people\(^1\). Poor dietary and lifestyle habits are implicated in T2D development and understanding the contribution of individual food groups is of public health relevance. Dairy products have been suggested to reduce T2D risk\(^2\,3\). Among dairy products, yogurt has received a particular interest since a growing number of prospective studies have reported that yogurt intake is associated with reduced body weight gain\(^4\,5\), reduced non-alcoholic fatty liver disease (NAFLD\(^6\)), and decreased T2D incidence\(^7\,8\). Meta-analyses of prospective cohort studies also reported that yogurt consumption is inversely associated with T2D\(^9\,10\). Moreover, a recent human intervention study showed that 24-weeks consumption of yogurt (220 g/day) improved insulin resistance and reduced liver fat in obese women with NAFLD\(^9\). However, the physiological and molecular mechanisms underlying the beneficial effects of yogurt consumption remain unknown.

It has been proposed that yogurt intake might reduce T2D risk through its nutrient-rich profile\(^3\,9,10\) and/or by the presence of specific compounds derived from milk fermentation by Streptococcus salivarius subsp. thermophilus (S. thermophilus) and Lactobacillus delbrueckii subsp. bulgaricus (L. bulgaricus) starters or the bacteria itself\(^7\,9,21\). Whether these effects are related to changes in the composition of the gut microbiota, metabolic inflammation, and/or linked to stimulation of specific metabolic pathways remain to be explored.

The aim of this study was therefore to determine the effect of yogurt intake on the development of whole-body and tissue-specific insulin resistance in a mouse model of obesity-linked T2D and to determine the potential role of the gut microbiota and hepatic metabolic pathways. We found that lyophilized yogurt intake (equivalent to two servings of yogurt) reduced the development of high-fat diet-induced insulin resistance, which was linked to its effects on liver metabolism and the gut microbiota. These beneficial metabolic effects of yogurt intake were associated with an increase of branched-chain hydroxy acids (BCHA) in the liver of these animals. We further show that BCHA are specifically present in yogurt and generated upon milk fermentation. Remarkably, BCHA modulated glucose metabolism in both hepatocytes and myocytes in vitro providing evidence for a cell-autonomous control of liver and muscle metabolism by these yogurt-specific metabolites. Thus, our work identifies BCHA as new milk fermentation-derived molecules that potentially contribute to the beneficial effects of yogurt intake on T2D and related metabolic disturbances.

Results

Yogurt consumption preserves glucose homeostasis and insulin sensitivity. Three individual studies were performed to explore the effect of a lyophilized yogurt product (Y) on diet-induced obesity and associated metabolic impairments using a high-fat high-sucrose (H) diet whose protein source was not limited to casein but was a mixed source of protein to be more representative of those consumed by humans\(^22\) (Table 1). The yogurt was administered in the lyophilized form to reach the equivalent of two servings per day, hence replacing 7.6% of the daily energy intake. The lyophilized yogurt was incorporated in the H diet every day and all the diets were changed daily. The first two studies (Study 1, \(n = 18–24\) per group and Study 2, \(n = 14–24\) per group) were carried out to explore the overall impact of yogurt treatment on body weight, hepatic metabolism, and indices of glucose homeostasis and insulin resistance after 12 and 15 weeks of dietary treatments, respectively. The third study (Study 3, \(n = 36\) per group) was designed to investigate the impact of yogurt treatment on the whole body, hepatic and peripheral insulin sensitivity using the gold standard hyperinsulinemic-euglycemic clamp (HIEC) method. The experimental designs are detailed in Supplementary Fig. 1, and parameters measured in the three studies are recapitulated in Supplementary Data 1.

As expected, 12 weeks of H diet feeding induced obesity when compared to the low-fat low-sucrose control diet (C) in all three studies (Fig. 1a and Supplementary Fig. 2A, B). The yogurt treatment slightly and transiently reduced body weight gain in H-fed animals in the first study (from week 6 to 9) (Fig. 1a). While this was not significant in the two others (Supplementary Fig. 2A, B), a pooled analysis of the three studies showed a small 2.9% decrease in body weight of H-fed animals after 12 weeks of yogurt treatment (Fig. 1b). Interestingly, energy intake was slightly but significantly lower (\(P<0.05\)) in yogurt-treated group in each of the three individual studies (Fig. 1c and Supplementary Fig. 2C, D), as also revealed by the pooled analysis (Fig. 1d). While total relative fat mass was not affected by dietary yogurt treatment, we observed a lower inguinal adipose tissue weight (IWAT) in Y-fed animals as compared to H mice (Supplementary Data 1). Yogurt treatment significantly increased relative lean mass in Study 1 (Supplementary Data 1). There was no significant effect of yogurt treatment on energy excretion, the weight of other fat depots or on the plasma lipid profile (Supplementary Data 1).

We next measured the impact of yogurt treatment on obesity-related metabolic phenotypes. Pooled analysis of the three studies showed that fasting glycemia, fasting insulinemia, and insulin resistance, estimated by the HOMA-IR calculation were lower in Y-fed mice after 12 weeks of treatment (Fig. 1e–g). Oral glucose tolerance test (oGTT) was performed at the end of Study 1 and revealed that Y-treated mice displayed similar glucose excursion, but lower glucose-stimulated insulin response (GSIS) as compared to H-fed mice, further indicating a higher insulin sensitivity after yogurt treatment (Fig. 1h, i). We also observed a numerical decrease in C-peptide levels during the oGTT (Fig. 1j), suggesting that lower GSIS may be related to decreased insulin secretion by the beta-cells. Yogurt treatment also modestly but not significantly displayed better fasting insulin, HOMA-IR, and GSIS after 15 weeks of treatment of H-fed mice (Supplementary Fig. 2E–I) suggesting that a greater sample size rather than longer duration of treatment may be necessary to detect a significant effect. Accordingly, increasing the number of animals from 24 to 36 in Study 3 allowed us to detect a significant effect of yogurt on fasting glycemia and insulinemia (Supplementary Fig. 2G–I). We also performed tracer-coupled HIEC studies in this latter group of mice to thoroughly examine whole-body insulin sensitivity and determine the potential site(s) of better insulin action in yogurt-treated H-fed mice. These results confirmed that H-fed mice were extremely resistant to insulin as revealed by the marked decrease in whole-body glucose infusion rate (GIR) during insulin infusion when compared to C-fed mice (Fig. 1k). This was linked to both reduced insulin suppression of hepatic glucose production (Ra) and lack of insulin-induced glucose disposal (Rd) in H-fed clamped animals (Fig. 1l, m). Yogurt treatment significantly prevented insulin resistance of H-fed mice as shown by higher GIR values in the Y group (\(p<0.05\)), which was associated with a trend for higher insulin-suppressed hepatic glucose production (Ra) (\(p = 0.06\)) (Fig. 1l). Although yogurt-treated H-fed mice did not show higher Rd values as compared to their H-fed counterparts during the clamp, it is important to note that yogurt treatment significantly preserved insulin response as compared to basal Rd values (Fig. 1m).

Yogurt consumption prevents hepatic steatosis and dysfunction. Dietary yogurt treatment for 12 weeks prevented H-fed
induced liver steatosis in Study 1 as revealed by significantly lower relative liver weight and hepatic triglyceride levels (Fig. 2a, b), which was also apparent from histological examination of the livers (Fig. 2c). Qualitative histopathological analyses of liver sections showed that yogurt-treated animals displayed a generally healthier liver phenotype as revealed by reduced hepatic steatosis (Fig. 2c). Qualitative histopathological analyses of liver sections showed that yogurt-treated animals displayed a generally healthier liver phenotype as revealed by reduced hepatic steatosis (Fig. 2c).

Next, we explored the hepatic expression of genes involved in fatty acid uptake, de novo lipogenesis, and fatty acid oxidation. We found that livers of mice treated with yogurt in Study 1 displayed lower expression of *Cd36* gene expression (Fig. 2g) while *Srebf1* and *Srebf2* were not changed (Supplementary Fig. 3A). Multiplex analyses further suggested that yogurt intake blunted hepatic inflammation as shown by small but consistent reduction of several chemokines and cytokines in the liver of yogurt-treated H-fed animals although these effects failed to reach statistical significance (Supplementary Fig. 3B). Taken together with the clamp data, these findings demonstrate that the beneficial effects of yogurt intake on glucose homeostasis and insulin resistance are linked to a healthier liver metabolism in H-fed mice.

Prolonging dietary yogurt treatment for 15 weeks in Study 2 did not further prevent liver steatosis (Supplementary Fig. 3G, H) and did not prevent H-induced changes in hepatic gene expression and inflammation (Supplementary Fig. 3I, J) but it should be noted that these mice were fasted for 6 h instead of 4 h in Study 1 which resulted in higher TG levels, which may explain the lack of significant yogurt effect in this setting.

### Role of the gut microbiota in the beneficial effect of yogurt intake

We next explored whether the metabolic effects of yogurt intake could be linked to changes in the gut microbiota. Yogurt treatment of H-fed mice was associated with inconsistent changes in α-diversity in between studies (Fig. 3a). Dietary yogurt treatment was also globally associated with a shift of β-diversity in H-fed mice in Study 1 as depicted by the Bray-Curtis dissimilarity (p = 0.03) and unweighted UniFrac distance (p = 0.04) (Fig. 3b). The DESeq log 2 fold changes analysis at the genus level revealed that yogurt treatment led to an enrichment of *Streptococcus* and a depletion of an unknown *Peptostreptococcaceae* genus relative abundance in the fecal microbiota of mice from all studies compared to untreated H-fed controls (Fig. 3c). The *S. thermophilus* CNCM I-1630 was quantified in fecal material and total cell counts reached 9 log_{10} g in both feces and caecum after yogurt feeding (Supplementary Fig. 4).

The effect of yogurt intake on the fecal microbiota composition was not associated with a reduction in metabolic endotoxemia as revealed by the lack of changes in either LPS or LBP circulating levels (Supplementary Data 1). Interestingly, yogurt treatment

### Table 1 Diet composition.

| Ingredient                          | C                  | H                  | Y                  |
|-------------------------------------|--------------------|--------------------|--------------------|
| Lyophilized yogurt product          | 0.0                | 0.0                | 8.3                |
| Protein                             | 0.0                | 0.0                | 8.3                |
| Carbohydrate                        | 0.0                | 0.0                | 8.5                |
| Fat                                 | 0.0                | 0.0                | 9.1                |
| Protein mixa                        | 21.0               | 17.2               | 1.5                |
| Carbohydrates                       | 0.1                | 0.7                | 0.2                |
| Protein                             | 0.0                | 0.0                | 0.0                |
| Carbohydrates                       | 0.1                | 0.7                | 0.2                |
| L-cystine                           | 0.3                | 0.3                | 0.2                |
| Corn starchb                        | 56.9               | 56.8               | 6.5                |
| Sucrose                             | 9.0                | 9.0                | 3.7                |
| Cellulose (fiber)                   | 5.0                | 5.0                | 4.1                |
| Lard                                | 0.5                | 2.9                | 0.9                |
| Mineral mixc                        | 3.5                | 3.5                | 1.0                |
| Vitamin mixd                        | 1.5                | 1.5                | 1.0                |
| Choline bitartrate                  | 0.3                | 0.3                | 1.0                |
| BHTe                                | 0.03               | 0.03               | 0.03               |
| Total                               | 100.0              | 100.0              | 100.0              |

*a* Home-made protein mix, formulated with animal (26.6% beef, 23.1% pork, 22.0% chicken, 6.8% white egg, 10.3% casein) and vegetal (11.2% soy) proteins.

*b* Tert-butylhydroxytoluene.

*c* MP Biomedical animal mix AIN-76A.

*d* MP Biomedical mineral mixture 76-12 of sucrose.

*e* Tecklad Vitamin mixture AIN-76A. 98% of sucrose.

*f* Tecklad Vitamin mixture AIN-76A. 98% of sucrose.

| Macronutrient | C | Study 1 | Study 2 | Study 3 | H | Study 1 | Study 2 | Study 3 | Y | Study 1 | Study 2 | Study 3 |
|---------------|---|---------|---------|---------|---|---------|---------|---------|---|---------|---------|---------|
| Protein       | % kcal | 19.2 | 19.2 | 19.2 | 15.1 | 15.0 | 15.1 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Carbohydrate  | % kcal | 68.1 | 68.0 | 68.0 | 34.9 | 35.0 | 35.0 | 35.0 | 35.0 | 35.0 | 35.0 | 35.0 |
| Fat           | % kcal | 12.7 | 12.8 | 12.8 | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 |
| SAT:PUFA ratio | kcal/g | 1.0 | 1.3 | 0.9 | 0.9 | 1.3 | 0.9 | 0.9 | 1.3 | 0.9 | 0.9 | 0.9 |
| L-cystine     | % kcal | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Corn starch   | % kcal | 56.9 | 56.8 | 56.9 | 6.5 | 6.5 | 6.5 | 6.5 | 6.6 | 6.5 | 6.5 | 6.5 |
| Sucrose       | % kcal | 9.0 | 9.0 | 9.0 | 34.0 | 34.0 | 34.1 | 29.6 | 29.6 | 29.6 | 29.6 | 29.6 |
| Cellulose     | % kcal | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| Lard          | % kcal | 0.5 | 2.9 | 1.7 | 13.5 | 19.4 | 15.9 | 13.1 | 17.5 | 13.0 | 13.0 | 13.0 |
| Mineral mix   | % kcal | 3.5 | 3.5 | 3.5 | 6.7 | 6.7 | 6.7 | 6.8 | 6.7 | 6.7 | 6.7 | 6.7 |
| Vitamin mix   | % kcal | 1.5 | 1.5 | 1.5 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 |
| Choline bitartrate | % kcal | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| BHTe           | % kcal | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 |
| Total          | % kcal | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

SAT:PUFA ratio and kcal/g values are shown as: SAT:PUFA ratio kcal/g.

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*NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-29005-0* | ARTICLE

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*NATURE COMMUNICATIONS* | https://doi.org/10.1038/s41467-022-29005-0 | www.nature.com/naturecommunications
Fig. 1 Yogurt consumption preserves glucose homeostasis and insulin sensitivity. Metabolic results of mice in the three studies (see also Supplementary Fig. 1 for the experimental design, Supplementary Data 1 and Supplementary Fig. 2). a Body weight throughout the Study 1. b Pooled analysis of the three studies showing mice final body weight after 12 weeks of dietary treatment. c Energy intake was recorded from week 4 to week 12 in Study 1. d Pooled analysis of the three studies showing mice daily energy intake. e-g Pooled analysis of fasting glucose, insulin, and corresponding HOMA-IR index after 12 weeks of treatment. h Glucose tolerance test at week 11 in Study 1. i Insulin response following the oral glucose challenge in Study 1. j C-peptide response following the oral glucose challenge in Study 1. k-m In Study 3, mice underwent an hyperinsulinemic-euglycemic clamp at week 12. k Glucose infusion rate (GIR). l Glucose rate of appearance (Ra) depicting hepatic glucose production. m Whole-body glucose rate disappearance (Rd) depicting glucose disposal. Data are expressed as mean ± SEM. Study 1: n = 18–24 except for panel (l) (n = 9–12) and (j) (n = 6–10); Study 3: n = 23–27; Pooled analysis: n = 66–84 biologically independent mice. H versus C: *p < 0.05, **p < 0.01, ***p < 0.001. Y versus H: #p < 0.05, ##p < 0.01. For clamps data, basal versus clamped condition: @p < 0.01, @@p < 0.001. C: low-fat low-sucrose control diet (C: soft gray, C1: white, C3: red); H: high-fat high-sucrose diet with a protein mixture replacing casein (H: soft red, H1: pink, H3: red); Y: lyophilized yogurt incorporated in H diet (Y: soft blue, Y1: light blue, Y3: blue). Numbers (1, 2, 3) refer to the study affiliation. Two-way repeated-measures ANOVA or Mann–Whitney tests depending on data distributions with (a, h–j) or without (b–g, k–m) baseline adjustment. T-test at baseline (j). All tests were two-sided.
preserved claudin-3 gene expression in the ileum of H-fed animals whereas neither mucin-2 nor occludin gene expression was affected (Supplementary Fig. 3C). No effect of yogurt intake was observed on ileum inflammation- and bile acid metabolism-related gene expression (Supplementary Fig. 3D, E). However, we found an increase of the 6α-hydroxylated acid, hyodeoxycholic acid (HDCA) in fecal content of mice fed yogurt for 3 weeks in Studies 1 and 2 (Fig. 3d), and this effect was further visually amplified after 12 weeks of yogurt feeding in Study 3 indicating altered intestinal bacteria bile metabolism (Supplementary Fig. 3F and Data 1). No differences were found in cecal short-chain fatty acid content between the Y- and H-fed groups (Supplementary Data 1).

To explore the role of the gut microbiota in the beneficial effects of yogurt treatment in H-fed mice, we next performed fecal microbiota transplantation (FMT) studies where germ-free (GF) mice were inoculated with fecal slurries recovered from H-fed or Y-treated animals from Study 1 and fed H diet, denominated as H1-T and Y1-T mice. FMT of the microbiota from yogurt-treated mice reproduced the healthier metabolic

**Fig. 2 Yogurt preserves hepatic steatosis and function.** Liver state in mice from Study 1. a Liver weight. b Hepatic triglyceride content. c Representative liver sections (hematoxylin and eosin staining) of n = 16–24 sections from independent mice. d, e Pathological analyses of liver sections. d Hepatic steatosis. e Global histologic diagnosis. f Fibrosis score. g Liver expression of genes involved in fatty acid uptake, de novo lipogenesis and fatty acid oxidation. Data are expressed as mean ± SEM. n = 18–24 biologically independent mice, except for panel (g) (n = 8 for C1 group, n = 20 for H1 and Y1 groups). H versus C: **p < 0.01, ***p < 0.001. Y versus H: #p < 0.05, ##p < 0.01. For panel (g) expressed in percentage, C group is considered as a reference and represented by a dash line. C: low-fat low-sucrose control diet (C1: white); H: high-fat high-sucrose diet with a protein mixture replacing casein (H1: pink); Y: lyophilized yogurt incorporated in H diet (Y1: light blue). Number (1) refers to the study affiliation. Mann-Whitney tests or T-test depending on data distributions (a, b). One-way ANOVAs and Benjamini-Hochberg adjustment for multiple testing on the number of variables (g). All tests were two-sided.
phenotypes observed in animals consuming yogurt compared to controls submitted to FMT from H-fed donors as revealed by lower fasting insulinemia and HOMA-IR values ($p < 0.05$) (Fig. 3e) as well as reduced GSIS during the oGTT (Fig. 3f, g). However, no differences were observed on liver weight whereas a numerical but not significant reduction was seen on hepatic triglyceride levels (Fig. 3h, i). The beneficial effect of FMT from donor Y1-treated mice has not observed in a group of gnotobiotic mice fed a chow diet (Supplementary Fig. 5A, B), suggesting that a H-mediated compromised metabolic trait is needed to observe the beneficial impact of the yogurt-associated fecal microbiota. Altogether, these studies are consistent with the implication of changes in the gut microbiota composition and function in the preserved insulin sensitivity of yogurt-treated mice.
Metabolomics identify BCHA as common liver and yogurt metabolites. Since 12-week yogurt treatment prevented hepatic steatosis in Study 1, we next examined the liver metabolome to explore potential mechanisms that could explain the protective effects of yogurt on liver metabolism. Based on epidemiological evidence and previous meta-analyses, we also reasoned that the beneficial effects of yogurt may come from metabolites derived from milk fermentation and therefore focused on liver metabolites that would also be selectively overrepresented in yogurt vs non-fermented milk.

We performed targeted lipidomics (CLP platform) analysis to quantify eleven lipid classes including the complete fatty acid composition of each lipid. Among the 999 lipid species detected in liver by the CLP platform, 712 were differentially abundant between the C-fed lean and H-fed obese mice and 20 of them were also differentially modulated by yogurt treatment (q-value ≤ 0.05) (Supplementary Data 2). However, none of these hepatic lipids was enriched or depleted upon yogurt fermentation when comparing the lipidomic profile of the lyophilized yogurt and milk, suggesting that the beneficial effect of yogurt is linked to another class of metabolites (Supplementary Data 3). We next performed untargeted metabolomics (HD4 platform) analysis to quantify chemically and structurally diverse metabolites involved in amino acid, carbohydrate, cofactor, vitamin, energy, lipid, nucleotide, peptide, and xenobiotics metabolism. The HD4 platform profiling of the liver metabolome showed that 227 out of the 563 metabolites identified in the liver of obese H-fed mice were significantly differentially abundant as compared to lean C-fed mice (q-value ≤ 0.05) (Supplementary Data 4). In this set, no detectable metabolite was significantly different between H and Y-fed groups (q-value ≤ 0.05) but reducing the statistical threshold (p-value ≤ 0.05) allowed us to identify a total of 35 metabolites that were differentially abundant in the liver of yogurt-treated H-fed mice compared to H-fed controls (Supplementary Data 4). We thus compared this set of metabolites with those significantly different between yogurt and milk products (Supplementary Data 3) and found six metabolites that were increased in yogurt compared to milk, and overrepresented in the liver of yogurt-treated H-fed mice: N-acetylglucine, ornithine, N-acetylserin, alpha-hydroxyisocaproate (HICA), 2-hydroxy-3-methylvalerate (HMVA), and alpha-hydroxyisovalerate (HIVA) (Supplementary Data 3 and 4). The last three metabolites (HICA, HMVA, and HIVA) are BCHA and were of particular interest since they were elevated in the liver of yogurt-treated mice and lean C-fed controls compared to H-fed obese mice (Fig. 4a, Supplementary Data 4). They were also markedly concentrated (by 96, 119, and 257-fold, respectively) in the yogurt product as compared to the non-fermented lyophilized milk (Fig. 4b, Supplementary Data 3).

Levels of fermentation-derived BCHA correlate with the metabolic benefits of yogurt intake. Following metabolomic analysis, we next used nuclear magnetic resonance (NMR) to quantify BCHA levels. This confirmed that the three BCHA are present in high quantity in the yogurt product whereas their levels were below the limit of detection in lyophilized milk (<1.2 μM for HICA and HMVA, <2.5 μM for HMVA) (Fig. 4c). HICA and HMVA were over-represented compared to HMVA, resulting in a HICA: HMVA: HIVA ratio of 1:0.5:1.

To confirm that BCHA are produced upon milk fermentation, we also quantified them in several dairy products such as non-fermented and fermented milks, non-lyophilized yogurt product used in the study, and three other yogurts, or skyr (Fig. 4d). Remarkably, we could not detect them in non-fermented milks but did find them at varying concentrations in all tested commercially available fermented products (Fig. 4d and Supplementary Fig. 6A, B). To explore the biological role of BCHA in the preventive effect of yogurt treatment, we next correlated their hepatic abundance with relevant metabolic parameters (Study 1 and 2). We found in Study 1 an inverse correlation between BCHA hepatic levels and fasting glucose (HICA r² = 0.237, p = 0.035; HMVA r² = 0.370, p = 0.016; HIVA r² = 0.327, p = 0.016), as well as hepatic triglyceride content (HICA r² = 0.210, p = 0.042; HMVA r² = 0.485, p = 0.002; HIVA r² = 0.451; p = 0.002) (Fig. 4e). This latter association was confirmed in Study 2 (Supplementary Fig. 7). Interestingly, HICA, HMVA, and HIVA fall within the metabolism of branched-chain amino acids (BCAA) (Fig. 4f) as they are derived from leucine, isoleucine, and valine, respectively. Impaired BCAA metabolism is implicated in insulin resistance and a BCAA metabolic signature has been reported to predict insulin resistance and T2D risk in humans33–35, but the potential metabolic roles of their BCHA derivatives remain unknown. We have thus directly measured their levels by targeted LC-MS/MS in plasma, liver, and muscle tissues from mice treated with dietary yogurt for 12 weeks (Study 1). Mean plasma levels of BCHA ranged between 0.19 and 3.86 μM (in line with human range26), and mean liver content ranged between 0.02 and 0.70 ng/mg in liver and 0.04–0.57 ng/mg in muscle tissues, with HIVA levels being about one order of magnitude higher than HMVA and HICA (Fig. 4g–i). Importantly, the data further show that HICA, HMVA, and HIVA levels are all decreased in plasma and liver of H-fed obese mice (and to a lesser extent in the muscle) as compared to C-fed lean controls. Remarkably, yogurt treatment of H-fed mice partially prevented the loss of BCHA in both the plasma and liver of H-fed mice (Fig. 4g, h) and a similar trend was observed for HIVA and HMVA in skeletal muscle (Fig. 4i). HIVA levels increased more than HICA in yogurt-treated mice although...
their levels were similar in the yogurt product, and that HMVA was also clearly enhanced in these animals despite representing only ~20% of the BCHA found in yogurt product, suggesting that the systemic and tissue levels of these metabolites are not only reflecting their nutritional exposure but may also be linked to some BCHA type-specific absorption and/or metabolism (Fig. 4c, g–i). These quantitative LC-MS/MS results confirm the liver metabolome data and further demonstrate that diet-induced obesity causes a dysregulation of BCAA metabolism that specifically leads to loss of BCHA production in both the circulation and in metabolic tissues, and that this defect can be partly prevented by yogurt consumption.
**BCHA are cell-autonomous modulators of hepatic and muscle glucose metabolism.** Since yogurt-treated H mice displayed greater whole-body and tissue insulin sensitivity, we next questioned whether BCHA are merely a biomarker of the preventive effect of yogurt or whether they could be directly involved in these beneficial metabolic phenotypes. Given that we found BCHA to be reduced in H-fed obese mice and that this decrease was partially prevented by yogurt treatment in the liver and to a lesser extent in skeletal muscle of H-fed animals, we elected to test the hypothesis that BCHA could exert direct effects in relevant cellular models. First using FAO hepatic and L6 muscle cells, we investigated the effect of BCHA on glucose production and glucose uptake, respectively. We found that a mixture of HICA:HDCA, as validated by targeted NMR, increased glucose uptake in L6 myocytes (Fig. 5e, f), and more recently we found that the BCHA mixture used at 0.1–4% of the daily energy intake, prevents insulin resistance and hepatic steatosis in diet-induced obese mice. Consistent with one of our hypotheses, we showed that the beneficial impact of yogurt on insulin sensitivity is partly driven by changes in gut microbiota composition and function (HDCA), as validated by fecal material transplantation in GF mice. In addition, we discovered that three BCHA, derived from LAB-driven metabolism of BCAA during milk fermentation, are abundantly found in yogurt and other fermented milk products but not detectable in milk. BCHA levels were positively associated with healthier metabolic parameters in obese mice and interestingly, these BCHA hydroxyl metabolites are also detected in metabolic tissues, even in non-yogurt fed mice. Remarkably, both systemic and tissue levels of BCHA are reduced in obese and insulin-resistant mice and their levels were partially maintained upon yogurt treatment, indicating that BCHA are metabolically regulated and not merely reflecting yogurt intake. We further show that BCHA can directly regulate glucose metabolism in insulin target tissues, as demonstrated by their ability to blunt glucose production and stimulate glucose uptake in liver and muscle cells, respectively.

Previous human studies reported negative associations between yogurt intake and body weight gain suggesting the yogurt consumption may reduce the prevalence of obesity, although this remains to be demonstrated in randomized control trials. Here using a preclinical model of obesity, we did find a slightly lower body weight (~2.9%), result which was significant upon pooling analysis from three independent studies, and which most likely resulted from the 4% lower energy intake that was observed in yogurt-treated mice. However, it is unlikely that this minor effect on body weight can explain the better insulin sensitivity and hepatic health of yogurt-fed obese mice, especially considering that the slightly lower body weight was linked to a lower subcutaneous inguinal fat in these animals, with no changes in the most metabolically harmful visceral fat depots.

Few studies have tested the potential effect of yogurt intake on metabolic phenotypes in animal models. Lasker et al. previously reported an improvement of glucose handling following a glucose bolus in yogurt-fed obese rats, while Johnson et al. found no difference between groups of mice fed a control diet or a diet added with yogurt during an insulin tolerance test. However, these studies were less powered, did not address the potential sites of improved glucose disposal and/or did not explore the underlying mechanisms in play. Here we observed a significant difference of fasting glucose, fasting insulin, HOMA-IR index, and the insulin response during a glucose tolerance test (GSI) providing several indices of greater glucose homeostasis and insulin sensitivity in yogurt-treated H-fed obese mice. We have directly established the insulin-sensitizing effect of yogurt treatment by performing clamp studies, which not only confirmed the higher insulin sensitivity at the whole-body level but further indicated that both the liver and skeletal muscle contributed to
better glucose handling during insulin infusion of yogurt-fed obese mice.

Our finding that liver is a key target of yogurt action is in line with the recent finding that high consumption of yogurt (>4 times/week) was inversely associated with the prevalence of newly diagnosed NAFLD in the general population. Chen et al. further showed that when compared to milk, yogurt improved insulin sensitivity and markers of steatosis in obese women with metabolic syndrome and NAFLD. Accordingly, we also observed that yogurt consumption not only preserved liver insulin sensitivity but also prevented hepatic triglyceride accumulation in H-fed mice, which was linked to lower gene expression of hepatic enzymes responsible for fatty acid metabolism. These observations were associated with slightly lower liver inflammation, NAFLD, and fibrosis scores.

The gut microbiota is increasingly recognized as a key driver of both obesity-linked T2D and hepatic diseases and changes in gut microbiota composition were associated with yogurt intake and the increase in LAB strains were consistently observed. Here, we report that yogurt treatment of H-fed mice induced a slight reproducible shift in the gut microbiota in all three studies with an enrichment of *Streptococcus* and a depletion of an unknown *Peptostreptococcaceae*. We further observed a few study-specific changes in bacteria from the Firmicutes phylum. In Studies 1 and 3, where we saw more robust effects of the yogurt treatment, the metabolic improvements were further associated with a lower relative abundance of bacteria from *Peptococcaceae* or *Lachnospiraceae Incertae Sedis* families, as compared to H-fed controls. Interestingly, Clarke et al. have shown a relative increase in the abundance of bacteria from *Lachnospiraceae* in diet-induced obese versus lean mice, whereas colonization of GF mice with *Lachnospiraceae* bacterium was reported to impair glucose tolerance and increase the weight of liver and adipose tissues. Furthermore, bacteria from *Lachnospiraceae Incertae Sedis* family were reported to be increased in the gut microbiota of patients with NAFLD.

Bile acids (BA) are key players in the gut-liver axis, impacting both glucose homeostasis and liver metabolism through undergoing deconjugation, dehydrogenation, and dehydroxylation in the gut altering their physico-chemical and physiological...
properties\textsuperscript{41,45,47–53}. Interestingly, we found that fecal levels of HDCA were consistently higher in H-fed mice treated with yogurt. HDCA treatment reduced hepatic triglyceride levels in mice\textsuperscript{54} and decreased blood glucose in LDL receptor KO mice\textsuperscript{55}, while a decrease in HDCA was reported as the main BA alteration in a rat model of hypertensive NAFLD\textsuperscript{56}. Collectively, the changes in the gut microbiota composition together with an increase in HDCA found in yogurt-treated obese mice likely contribute to the healthier metabolic phenotypes.

FMT studies in GF mice further established the causal role of the gut microbiota changes in some of the beneficial effects of yogurt treatment in obese mice. Indeed, transplantation of fecal slurries from yogurt-treated mice to GF mice promoted lower fasting insulinemia and HOMA-IR, as well as lower insulin levels during the glucose tolerance test. However, FMT studies failed to reduce liver weight and hepatic steatosis in GF mice, suggesting that the healthier liver phenotypes may not be driven by the shift in the gut microbiota. Alternatively, the microbial species responsible for the hepatic benefits of yogurt intake may have been lost during processing or the transfer of microbial communities to GF mice, such as those species involved in HDCA production. Indeed, the colonization of GF mice remains a reductionist approach, whereby only a fraction of the donor microbiota is expected to survive sample collection, fecal preparation and storage, and passage through the recipient’s stomach to find a niche in the gut.

In the search for potential liver metabolites that may be associated with the health benefits of yogurt intake, we found that yogurt administration preserved hepatic levels of three hydroxyacids (HIVA, HICA, and HMVA) that are derived from BCAA catabolism. Indeed, HIVA, HICA, and HMVA are generated following the catabolism of BCAA and reduction of their cognate ketoacids to hydroxyacid intermediates, a process that is well conserved and common in yeasts, fungi, and bacteria including LAB\textsuperscript{57}. BCAA levels did not differ between diets (3.5 vs 3.4 g/100 g of H diet and Y diet, respectively), further suggesting that the difference in BCHA in the experimental groups may come from yogurt starter activity, and/or from different microbial and/or host metabolism of BCAAs. Bioinformatics search revealed that both \textit{L. bulgaricus} CNCM I-1519 and \textit{S. thermophilus} CNCM I-1630 strains harbor gene with identity to hydroxyacid-dehydrogenases. Although LAB activity on yogurt BCAA catabolism explains the enrichment of BCHA in yogurt versus milk, it is also of major interest that these metabolites are readily detected in the circulation, liver and skeletal muscle of non-yogurt exposed lean mice and significantly reduced upon H-fed diet-induced obesity. To the best of our knowledge, this is the first study reporting that obesity is linked to reduced levels of all three BCHA in the plasma and metabolic tissues. The levels of these metabolites were, in addition, negatively associated with plasma glucose as well as liver triglycerides, suggesting that they are novel biomarkers of metabolic and liver health. Importantly, we also found that yogurt administration of H-fed obese mice partially preserved the levels of BCHA in both the plasma and tissues of these animals. Since the dose of yogurt used in these studies corresponds to two servings of yogurt per day in humans, it can be assumed that the resulting BCHA levels are metabolically relevant. Interestingly, BCAA catabolism by LAB yields flavor compounds, carboxylic acids and aldehydes, and also BCHA that are devoid of aroma\textsuperscript{58}. In cheese, limiting hydroxyacid dehydrogenase activity is, however, preferred to enhance cheese aroma and this could underlie lower BCHA levels and contribute to the epidemiological differences in T2D risk reduction of fermented dairy\textsuperscript{1,2,17}. Further and importantly, we showed that BCHA can directly modulate glucose metabolism in liver and muscle cells and that even very low concentrations of these metabolites, corresponding to their levels measured in the circulation, are able to increase glucose uptake by myocytes. Although higher levels of BCHA were needed to blunt glucose production by hepatocytes it is speculated that higher levels of these BCAA metabolites are released in the portal circulation to impact on liver metabolism. These data thus provide evidence that BCHA are cell-autonomous modulators of glucose metabolism and may be involved in the beneficial metabolic effects of yogurt treatment through their action on both liver and muscle tissues.

Future studies will be needed to determine the mechanisms underlying our findings of reduced BCHA levels in obesity. In contrast to BCAA metabolism by LAB and other microorganisms, very little is known about BCHA production or metabolism by commensal gut microbes and host mammalian tissues. It will be important to determine whether some of the bacterial taxa found to be altered by obesity and yogurt treatment express the enzymes required to metabolize BCAA to BCHA and whether this could contribute to the yogurt effect. It is also possible that alterations in liver and muscle BCAA metabolism in obesity contribute to the lower production of their BCHA congeners. Indeed, maple syrup urine disease (MSUD) caused by deficient activity of the branched-chain alpha-ketoacid dehydrogenase complex (BCKDH) is characterized by increased plasma concentrations of BCAA but also of their keto-intermediates (2-ketoisovaleric acid, 2-ketoisocaproic, and 2-keto-3-methylvaleric acid) and BCHA\textsuperscript{59}. Although MSUD is an extreme example of mammalian BCHA metabolism, it shows that the enzymatic machinery to catabolize BCAA to BCHA exists in humans. In line with this, the lactate dehydrogenase LDHA was shown to produce the L-form of HIVA in vitro\textsuperscript{60} and elevated D-forms of HIVA and HICA were observed in patients with missense variants in LDHHD gene\textsuperscript{61}. Considering that altered BCAA metabolism is a pathognomonic feature of insulin resistance and T2D, the BCAA to BCHA conversion could possibly be harnessed for therapeutic applications\textsuperscript{23,62–68}. Further work is necessary to unravel the host-microbial mechanisms driving the BCAA metabolism to BCHA and whether targeting the enzymatic machinery involved in the production of these hydroxyl BCAA congeners could improve insulin sensitivity and liver diseases in obesity. BCAA catabolism to BCHA is well documented in bacteria but poorly characterized in mammals and thus represents a major gap in our understanding of BCHA role and regulation by mammalian orthologs of these key BCAA catabolic enzymes. However, our findings show that yogurt intake over only three months can partially prevent BCHA reduction in obese animals, suggesting that nutritional interventions with yogurts, and possibly other fermented dairy products, should be explored in clinical trials to determine whether they can supply exogenous BCHA and compensate for their impaired endogenous production, and thus alleviate insulin resistance and NAFLD in obesity.

**Methods**

**Lyophilized yogurt product and diet preparation**

Lyophilized yogurt product. The yogurt (1.9% fat, 4.7% protein, 6.6% carbohydrate (g/100 g of product), \textit{L. bulgaricus} CNCM I-1519, \textit{S. thermophilus} CNCM I-1630) was used due to its representative macronutrient composition (un-supplemented with calcium, vitamin D, starch, no fruits or flavors, protocoooperation of the two strains) and freeze-dried. For all studies, the yogurt was produced at the Danone Nutricia Research site (Study 1 and 2 multiple productions, Study 3 single production) following bovine milk, cream, milk powder hydration, and pasteurization at 95 °C for 5 min. The yogurt was fermented using the industrial (Hansen) cultures of \textit{L. bulgaricus} CNCM I-1519 and \textit{S. thermophilus} CNCM I-1630 (ratio 5:95) at 39 °C for 9 h with breaking at pH ~ 4.65. Following smoothing at 3000 RPM, the yogurt was stored at 4 °C for 3 days before lyophilization (Studies 1 and 2). Briefly, yogurt freezing (~42 °C for 17 h) was followed by two steps of sublimation (~20 °C for 30 h30 and −10 °C for 17 h) and bound-linked water was...
removed by desorption (20 °C for 24 h), which resulted in the lyophilized yogurt product (Studies 1 and 2). In Study 3, one batch of 43 kg of yogurt was conditioned, shipped, and stored at 4 °C for 2 days before lyophilized at Lysol, France with a Usifroid SML 150 lyophilizer. The stability of yogurt and lyophilized product was monitored throughout protocol: until day 50 (Study 1), day 80 day (Study 2) and day 230 (Study 3). The L. bulgaricus CNCC 1-1519 count was 7.1 ± 0.1 log CFU/g on day 50 (Study 1), 7.0 ± 0.2 log CFU/g on day 80 (Study 2) and 6.9 log CFU/g on day 230 (Study 3). The S. thermophilus CNCC 1-1630 count was 9.2 ± 0.2 log CFU/g on day 50 (Study 1), 8.8 ± 0.2 log CFU/g on day 80 (Study 2) and 9.5 log CFU/g on day 230 (Study 3). In Study 1, lyophilized yogurt was consumed by mice between day 13 and 42 post-production and nine productions were needed to cover the study. In Study 2, lyophilized yogurt was consumed by mice between day 29 and 80 post-production and five productions were needed. A single batch of lyophilized yogurt was consumed by mice through the Study 3. The lyophilized yogurt was stored at 4 °C and the Y diet was formulated daily using the pre-conditioned sealed bags. Nutritional and microbiological profiles (Enterobacter aerogenes, yeast, and mold ≤10 CFU/g; NF ISO 215 28; 2, ISO 6611) of different lyophilized products were analyzed and checked whether well concentrated versus the initial yogurt. Nutritional composition (ISHA and ITERG, France) and microbiological profile (plate count method ISO 7899:2003 IDF 127, accuracy of ±0.5, mean of productions over time) of lyophilized product is available in Table 2. Some other dairy products were also used compared to the studied yogurt product and their nutritional profile and species used for fermentation are detailed in Supplementary Fig. 6.

**Dietary treatments.** Mice were fed with either a low-fat low-sucrose (C) or a high-fat high-sucrose diet containing a protein mixture representative of the United States diet and a high-fat high-sucrose diet that contained a protein mixture representative of the US diet in which lyophilized yogurt was incorporated (Y). The protein sources for mixture incorporation in diets consisted of pre-conditioned sealed bags. Nutritional and microbiological profiles (Enterobacter aerogenes, yeast, and mold ≤10 CFU/g; NF ISO 215 28; 2, ISO 6611) of different lyophilized products were analyzed and checked whether well concentrated versus the initial yogurt. Nutritional composition (ISHA and ITERG, France) and microbiological profile (plate count method ISO 7899:2003 IDF 127, accuracy of ±0.5, mean of productions over time) of lyophilized product is available in Table 2. Some other dairy products were also used compared to the studied yogurt product and their nutritional profile and species used for fermentation are detailed in Supplementary Fig. 6.

**Microbiological profile (log CFU per 100 g)**

| Microbiological profile (log CFU per 100 g) | Study 1 | Study 2 | Study 3 |
|-------------------------------------------|---------|---------|---------|
| L. bulgaricus CNCC 1-1519                 | 7.1     | 7.1     | 6.9     |
| S. thermophilus CNCC 1-1630              | 9.1     | 9.1     | 9.4     |

**Table 2** Nutritional composition of Y lyophilized product.

| Nutritional composition (g per 100 g of product) | Study 1 | Study 2 | Study 3 |
|-------------------------------------------------|---------|---------|---------|
| Fat                                             | 12.3    | 13.1    | 13.2    |
| Carbohydrate                                    | 45.0    | 43.8    | 43.5    |
| Protein                                         | 31.3    | 31.2    | 31.7    |
| Val. Cal. (Kcal)                                | 415.7   | 417.9   | 419.6   |
| Fructose                                        | <0.2    | <0.2    | <0.2    |
| Glucose                                         | 0.4     | <0.2    | <0.2    |
| Lactose                                         | 35.5    | 33.5    | 33.6    |
| Maltose                                         | <0.2    | <0.2    | <0.2    |
| Sucrose                                         | <0.2    | <0.2    | <0.2    |
| Galactose                                       | 4.4     | 5.4     | 4.6     |
| Calcium                                         | 1.2     | 1.2     | 1.1     |
| Sodium                                          | 0.4     | 0.4     | 0.3     |

**Germ-free study.** One week before fecal material transplantation and for all the 9-week duration of the protocol, GF mice were fed with H diet previously supplemented with 50% more vitamins and irradiated twice (29.1–41.6 kGy, Nordion, Laval, QC).

**Animals.** All procedures were previously approved by the Laval University Animal Ethics Committee (Québec, Canada), referred to the 2016-004-21 protocol and followed the internal Guide for the care and use of laboratory animals.

**Conventional animals.** Six-week-old male C57Bl/6 mice (Jackson, USA) were single-housed in the animal facility of the Quebec Heart and Lung Institute (Québec, Canada) in a controlled environment (12 h daylight cycles, lights on 6:00 PM, 22 °C) with food and water ad libitum for all studies. After two weeks of acclimatization on C diet, baseline metabolic measurements were assessed. Animals were then allocated to treatment groups matching for body weight, and dietary challenged for 12, 15, and 12 weeks respectively in Study 1 (n = 18–24 per group), 2 (n = 14–24 per group) and 3 (n = 36 per group). Food intake and body weight were measured daily and weekly, respectively. Body composition was analyzed via quantitative NMR spectroscopy (Bruker minispec LF90) before and at 12 and 15 weeks of diet challenge in Studies 1 and 2, respectively.

**Germ-free animals.** Male Swiss Webster mice (TacSw genetic background), were bred and housed in the GF animal facility of the IUCPQ Research Center with free access to water and irradiated chow diet. At eight-week old, animals were fed with H diet. At nine-week old, mice were allocated to treatment groups taking care of litter and dam and matching for their body weight. Four groups of 11–12 animals were formed, each constituted of three isolates of n = 3–4 mice. For FMT, feces were collected at week 12 in Study 1 from mice fed with H or Y diets. Three pools of feces for each treatment group were formed and resuspended with sterile saline (1:10 dilution). The resultant solution was homogenized, spin-down and 200 μL were gavaged to the receivers housed in the same cage. The remaining pellet of the solution was spread on mice nests. Mice were gavaged only once with fecal slurries as it was previously shown to be sufficient to establish microbiota in isograde system conditions. Mice were maintained in the GF facility and fed the H diet for nine weeks. A separate FMT study was also performed with another cohort of GF mice that were kept on a chow diet after fecal microbiota transfer from H1 and Y1-fed donors. Body weight was recorded weekly and food intake was changed and recorded twice a week.

Mice were euthanized under isoflurane anesthesia by cardiac puncture. In Studies 1 and 2, mice underwent a blood collection after 4 and 6 h fasting, respectively, before being euthanized. In Study 3, mice were euthanized right after the clamp experiment. In the GF Study, mice were euthanized after undergoing an OGTT (4 h fasting, 2 h of test). Blood was drawn and immediately centrifuged, and plasma was collected and stored at −80 °C. Relevant tissues were harvested, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C for further analysis.

**Glucose homeostasis.** All tests requiring blood samples were performed via saphenous vein puncture after 4 h fasting for Study 1, 3, and GF, or 6 h fasting for Study 2. For fasting glucose and insulin measurements, blood samples were withdrawn before and at the end of the treatment, except for the GF experiment. An OGTT was performed at week 11 in Studies 1 and 2, and at week 9 for the GF study. Glycemia was recorded before (0 min) and after (15, 30, 60, 90, 120 min)
after a glucose challenge administrated by gavage (1 g/kg body weight). Insulin response was measured in plasma of half of the animals (time 0, 15, 30, 60, and 120 min) in Study 1, and all of the animals in studies 2, 3, and GF. The C-peptide was measured in animals for which remaining plasma was available in Study 1.

**Hyperinsulinemic-euglycemic clamps (HIEC).** After 12 weeks of dietary intervention in Study 3, HIEC were performed in conscious unrestrained mice as previously described71. The left common carotid artery and the right jugular vein were catheterized 3 days before the experiment for blood sampling and infusions, respectively. A blood sample was collected for measuring basal glucose and insulin levels 90 min prior to the clamp start. Then, the [3-H]-glucose (Perkin Elmer, Boston, MA) was prime-infused at 0.23 μCi/min during 90 min for equilibration (90 to 0 min). The insulin clamp started upon primed infusion of insulin (2.5 mU/min/kg; Humulin R; Eli Lilly Canada Inc., Scarborough, ON) and the [3-H]-glucose infusion was increased to 0.67 μCi/min (t = 0 to 120 min). Euglycemia was maintained at ± 0.5 mM level by infusing 20% dextrose as needed. Blood samples (5 μL) were collected every 10 min from 0 to 120 min to measure blood glucose and adjust dextrose infusion in consequence. Blood samples (30–50 μL) were collected every 10 min from 30 min to 0 min and from 90 to 120 min to calculate basal and clipped Ra and Rd, respectively. Clamp insulin levels were determined from samples obtained at 120 min. A saline-washed erythrocytes solution from mice donors was infused (0.1 mL/hr) throughout the HIEC to prevent a decrease in the hematocrit.

**Analytical methods.** Blood glucose was recorded using a One Touch glucometer UltraMini® and values higher than the upper limit of glucose determiner were measured by an Ampex® red glucose/glucose oxidase assay kit. Plasma insulin and C-peptide were measured by Mouse ELISA kits (ALPCO Ultr specific Insulin and Crystal Chem Inc. C-peptide). Homeostasis model assessment of insulin resistance was calculated using the formula: fasting insulin (microU/mL) × fasting glucose (mmol/L) /22.5. Liver steatosis and inflammation were measured using the Histoscore scoring system of fourokit and histologic changes were calculated as NAFLD and the association of both steatosis and inflammation in more than 0.5 μl per field was classified as NASH. Other paraffin-embedded sections (6-μm thick) were deparaffinized through toluene and a graded alcohol series to assess liver fibrosis. Sections were stained for 8 min with Weigert Hematoxyline and rinsed for 5 min in water. Subsequently, the sections were stained for 60 min with Sirius red (0.1% of Sirius red in saturated aqueous picric acid) for collagen bundle staining. Sections were then rinsed with acetic acid 0.05% and mounted for observation under polarized light microscopy (Zeiss AXIOPLAN; Oberkochen, Germany). Fibrosis was scored according to the procedure used by Kleiner et al. A score of 1 was awarded when fibrosis was found in perisinusoidal or periporal regions, 2 when fibrosis was present in both, 3 with bridging fibrosis, and 4 with cirrhosis.

**Targeted lipids and untargeted metabolites profiling.** The targeted lipomics (CLP platform) and untargeted metabolomics (HD4 platform) analysis were performed by High-Throughput Lipidomics (HTL) in Toronto. Liver biopsy samples were soaked overnight in MeOH in a 2:1 V/V ratio. Milk powder samples were reconstituted with a 50:1 V/V ratio of water to render liquid samples. For liquid samples, including QC samples, 100 μL of material was extracted with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) to precipitate protein and dissociate small molecules bound to protein or trapped in the precipitated protein matrix, followed by centrifugation to recover chemically diverse metabolites. The resulting extracts were divided into multiple fractions: two for analysis by two separate reverse-phase ultra-high-performance liquid chromatography-tandem mass spectrometry (RP/UPLC-MS/MS method using positive ion mode electrospray ionization (ESI)), one for analysis by RP/UPLC-MS/MS using negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS using negative ion mode ESI, and others reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

**Sample preparation CLP.** Liver samples were soaked overnight in DCM:MeOH in a 30:1 V/V ratio. 240 μL of the resultant extract was further processed with a modified Bligh-Dyer extraction76,77 using methanol/water/dichloromethane in the presence of deuterated internal standards (Supplementary Data 7). Milk powder samples were reconstituted with a 50:1 V/V ratio in water and 50 μL was extracted using the same modified Bligh-Dyer extraction. The extracts were dried under nitrogen prior to instrumental analysis.

**Sample preparation HD4.** Detailed description of sample preparation, gradients, standards, and instrumental parameters have been published78. Briefly, methods utilized a Waters ACQUITY UPLC, and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a high-performance ion trap (HESII-ESI) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The dried sample extract was reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient-eluted from a C18 column (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluorooctanoic acid (PFPA) and 0.1% formic acid. A second aliquot was also analyzed using positive ion mode electrospray ionization (ESI), chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the C18 column using methanol, acetonitrile, water, 0.05% PFPA, and 0.01% formic acid, and was operated at an overall higher organic content. A third aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The gradient-eluted from the column using methanol and water, however, with 6.5 mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, pH 10.8. The MS analysis consisted of between MS and data-dependent MS® scans using dynamic exclusion. The scan range varied slightly between methods, but covered ~70–1000 m/z.

**CLP FIA-DMs-MRM.** The CLP methodology is a flow injection analysis (FIA), differential mobility separation (DMS), and multiple reaction monitoring (MRM) method. We used the CLP assay targeting 34 lipid species covering 14 different lipid classes (Supplementary Data 8) and 29 fatty acid constituents. The CLP extracts were reconstituted solvent is 1:1 DCM:MeOH with 10 mM NH4Ac were transferred to glass-lined plates for infusion-MS analysis, performed on a Shimadzu LC with nano PEEK tubing and the Sciex Selexion-5500 QTRAP. The CLP samples were analyzed via both positive and negative mode electrospray as appropriate for each lipid class (Supplementary Data 8). The lipid extract was continuously infused into the mass spectrometer without the use of any chromatographic column at 8 μL/min. A uniform sample was analyzed throughout the infusion (~6 min), allowing 17 replicate measurements per lipid species for more robust and reproducible acquisitions. After ionization, 9 different ions were monitored, 7 of which were introduced into the DMS cell. The DMS cell acted as a liquid filter or gate that permitted a specific lipid class to pass into the mass spectrometer at a time, while the other lipid classes were filtered out. The DMS cell cycled through the different lipid classes over the course of a single infusion, sequentially passing each
lipid class into the mass spectrometer for MRM analysis. When applicable the DMS functionality was utilized to allow unambiguous lipid identification. In the MRM analysis, the first quadrupole (Q1) filtered based on the m/z of a characteristic fragment of that same lipid species, such as one of the fatty acid side chains (Supplementary Data 8).

**HDM data extraction and compound identification.** Raw data were extracted, peak-identified, and quality control processed using Metabolon’s hardware and software. These systems were built on a web-service platform utilizing Microsoft’s.NET technologies and compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library of authentic standards or recurrent unknown entities. Metabolon maintains a library of standards that contain the retention time/index (RI), mass, and accurate mass match to the library ±10 ppm, and the MS/MS fragmentation pattern. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in each of the technical replicate samples. Experimental samples were randomized across the platform run, with quality control processed using Metabolon’s.NET hardware and software. The 135 HD4 method compounds were quantified by area-under-the-curve detector ion counts and raw data were scaled for purposes of data visualization. For CLP compounds, individual lipid species were quantified by taking the ratio of the signal intensity of each target compound to that of its assigned internal standard, then multiplying by the concentration of internal standard added to the sample. CLP used a range of internal lipid standards (Supplementary Data 7) for which most classes covered both carbon length and number of double bonds. Experimental lipids were matched to specific standards based on the combination of carbon length and number of double bonds. Lipid class concentrations were calculated from the sum of all molecular species within a class, and fatty acid compositions were determined by calculating the proportion of each class comprised by individual fatty acids.

**Compound quantification.** HDM method compounds were quantified as area-under-the-curve detector ion counts and raw data were scaled for purposes of data visualization. For CLP compounds, individual lipid species were quantified by taking the ratio of the signal intensity of each target compound to that of its assigned internal standard, then multiplying by the concentration of internal standard added to the sample. CLP used a range of internal lipid standards (Supplementary Data 7) for which most classes covered both carbon length and number of double bonds. Experimental lipids were matched to specific standards based on the combination of carbon length and number of double bonds. Lipid class concentrations were calculated from the sum of all molecular species within a class, and fatty acid compositions were determined by calculating the proportion of each class comprised by individual fatty acids.

**Data quality control.** Several types of quality control measures were used in concert with the experimental samples. These included: (1) technical replicate samples generated by combining a small portion of each experimental sample spaced evenly among experimental sets; (2) extracted water samples (process blanks) and unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral).

**Statistical analyses** (significance tests and classification analysis) were performed by Metabolon. Missing values were imputed with the observed minimum for each compound. Statistical analyses were performed on log-transformed data using two-way ANOVA with Study, Group (diet or product) and their interaction, separately for metabolomic HD4 and lipidomic CLP datasets. Multiple testing correction was done using FDR (p-value) comparison-wise. Analyses were performed using ArrayStudio.

**NMR targeted analysis.** The sample preparation and the NMR analysis were performed by Bioaster (Lyon, France). The lyophilized milk (100 mg, −80 °C storage) was rehydrated (100 mg), then lyophilized to 10 mg, 20% v/v of MiliQ water (20 mL, n = 1 of 0% fat milk, n = 1 of 2% fat milk, n = 3 of whole fat milk), non-lyophilized yogurt (−80 °C storage), yogurt 1 to 3, fermented milk 1 and 2 and skim (1 mL each) were shipped to Bioaster (n = 5 per each sample and storage at 4°C unless stated otherwise). The chemical standards were HMVA, HIVA, and HICA (Sigma Aldrich Ref. 80529, Ref. 219835, and Ref. 219827, respectively).

**Sample preparation of lyophilized products.** Hundred milligrams of lyophilized products were solubilized in 1 mL of MiliQ water, vortexed shortly, sonicated for 10 min, and then centrifuged 10 min (10 °C, 10,000 × g). Recovered 200 μL of clean supernatants were additionally ultra-filtered using 10 kDa cut-off filtration tubes for 10 min (at 10 °C and 10,000 × g centrifugation speed).

The 135 ultra-filtered of slurries were mixed with 45 μL of 2 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, Eurisotop) internal standard solution containing 1 M phosphate buffer (pH = 7.4) and D2O for the signal lock in 60:40 v/v ratio. The final concentration of the DSS was 0.5 mM. The supernatants were centrifuged in 3 mm Samplelet NMR tubes. In addition to samples analysis, blank samples were also prepared using the same sample preparation protocol, but for which the milk-derived product was replaced by MiliQ water.

**Sample preparation of fermented products.** Fermented milk products were shocked 10 min using tube rotorator (Stuart) with 15 mL Falcon adapter. In addition, before sampling, all tubes were vortexed 15 s and then 1 mL of samples were transferred in 1.5 mL Eppendorf tubes. The tubes were centrifuged for 10 min at 10 °C and 10,000 × g speed in order to obtain a clean supernatant. Next, 200 μL of the supernatants were ultra-filtered with 10 kDa cut-off filtration tubes and then the filtrates were processed in the same way as described above for lyophilized products.

**Sample preparation for accuracy and precision evaluation.** To 330 μL of milk, fermented products or suspended lyophilized products in MiliQ water at a rate of 100 mg to 1 mL was spiked with 20 μL stock solutions containing hydroxy metabolites at medium, low, and high concentrations and the background samples was spiked with MiliQ water. All spiked samples prepared in 1.5 mL Eppendorf tubes (n = 3) were vortexed 15 s and mixed on the Thermoshaker (Eppendorf) during 10 min at 10 °C and 1000 rpm.

**NMR analysis and processing.** The sample analysis was performed using Ascend 600 MHz NMR spectrometer from Bruker Biospin (Lyon, France) equipped with cryogenically cooled 5 mm QCI (1H/13C/15N/31P) probe head. All samples were stored at 4–6 °C in Samplelet autosampler before analysis. For each sample, a one-dimensional proton acquisition was performed using nopyrin1D sequence, which contains a pre-saturation block for water attenuation during relaxation delay. The spectral width of proton acquisition was 12 ppm using 32 k data points during 2.3 s acquisition time and 4 s relaxation delay. The mixing time delay was 80 ms. All spectra contained 128 scans for about 14 min total acquisition time. The free induction decay raw data were processed by Fourier transformation using 0.3 Hz exponential apodization function within 32 k data points.

**Metabolite quantification.** The resulting spectra were phased, and baseline corrected. The spectra alignment was done according to internal standard DSS chemical shift. Finally, the metabolite quantification was performed using Chenomx NMR suite 8.3. The metabolites library database was developed with the help of Compound Builder module using experimental proton spectra of a known concentration. The limit of detection was 1.2 μM for HICA (0.915 ppm, dD, CH3), 1.2 μM for HIVA (0.825 ppm, dD, CH3), and 2.5 μM for HMVA (0.78 ppm, dD, CH3). The data are reported as a mean and standard deviations.

**Targeted LC-MS/MS analysis.** The samples preparation and the targeted LC-MS/MS analysis were performed by Bioaster (Lyon, France). The plasma, liver, and gastrocnemius tissues sample (4–5 from group C; 8 from group H; 8–9 from Group Y) were used to measure HICA, HIVA, and HMVA quantity. Flash-frozen samples (>30 μL of plasma, >100 mg of liver biopsy, >200 mg of gastrocnemius biopsy in liquid nitrogen were stored at −80 °C and shipped to Bioaster on dry ice. The chemical standards were HMVA, HIVA and HICA (as above) and the internal standard was 2-hydroxy-2-methylbutyric acid 98% (Sigma Aldrich Ref. H40009).

**Sample preparation.** The plasma samples (30 μL) were thawed and mixed with ice cold methanol (105 μL) for proteins precipitation during 10 min at 1000 rpm and 4°C. Next, for each sample 100 μL of supernatant was recovered by centrifugation and transferred in a clean tube for evaporation. The dry residue was suspended in 100 μL methanol/water solution (85:15 v/v) for further LC-MS/MS analysis.

**NMR targeted analysis.** The sample preparation and the NMR analysis were performed by Bioaster (Lyon, France). The lyophilized milk (100 mg, −80 °C storage) was rehydrated and lyophilized to 10 mg, 20% v/v of MiliQ water (20 mL, n = 1 of 0% fat milk, n = 1 of 2% fat milk, n = 3 of whole fat milk), non-lyophilized yogurt (−80 °C storage), yogurt 1 to 3, fermented milk 1 and 2 and skim (1 mL each) were shipped to Bioaster (n = 5 per each sample and storage at 4°C unless stated otherwise). The chemical standards were HMVA, HIVA, and HICA (Sigma Aldrich Ref. 80529, Ref. 219835, and Ref. 219827, respectively).
same protocol. The new extract was combined with the first one, for a total of 1.1 mL. The phase separation was carried out by the addition of 200 μL of water and 200 μL of chloroform to extract and the biphase fractions were collected and dried under nitrogen stream. The residues were suspended in 85 μL of water and 15 μL of methanol for further LC-MS/MS analysis.

LC-MS/MS. The metabolites quantitation was based on the protocol developed by Ehling and Reddy 78,79. Thus, the analysis was performed using an HPLC (Ultimate 3000, Thermo Scientific) coupled to a Triple Quadrupole Mass spectrometer (LC/MS/MS, Ultra Thermo). The chromatographic separation of the metabolites was carried out on a Waters Aquity BEH C18 1.7 μm 2.1 x 100 mm column thermostated at 40 °C and gradient eluted with water containing 0.1% formic acid as mobile phase A and the methanol with 0.1% formic acid as mobile phase B. Thus, the solvents flow rate was 300 μL/min and the gradients were as follows: it starts with 10% B; then 0–2 min the B increased to 15% and remain constant 8 min; from 10 to 10.5 min the B increased to 100% and remain constant for 2 min; from 12.5 to 13.5 the mobile phase B was returned back to 10% and maintained for equilibration 3 min. The sample injection volume was 10 μL. The MS analysis was performed in negative electrospray ionization mode (ESI). Source settings were as follows: the spray voltage 3 kV; the vaporizer temperature 100 °C; the sheath and auxiliary gas pressure were 15 and 10 respectively; the capillary temperature 350 °C and the tube lens and skimmer offset were 46 and 10 V respectively.

BCH detection and quantification. The standards for each metabolite were detected in MRM mode and well separated, with distinct retention times: HICVA (117 * 71 m/z, 13 V (CE)), 3.6 min; HMAVA metabolite was detected under two diastereomeric forms HMAVA1 (131* 85 m/z, 16 V (CE)) and 7.9 min & HMAVA2 (131* 85 m/z, 16 V (CE)), 8.1 min; HICA (131* 85 m/z, 16 V (CE)), 8.6 min. The recovery and accuracy were determined during the feasibility step and limits of quantifications (precisions < 15%) were as follows: HMAVA – 0.05 μM; HICVA – 0.15 μM; HICA – 0.04 μM. All samples were analyzed in three different batches and, to eliminate the impact of signal attenuation in mass spectrometry, all samples were randomized before analysis. For each batch, blank samples and metabolite standard solutions were injected together with the samples (the calibration curve was carried out at the beginning, the middle and the end of the analytical sequence. The resulting calibration curves gave good linearity with a R2 > 0.99).

Cell culture and glucose metabolism determinations. FAO rat hepatocytes were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. Cells were maintained in this medium 48 h before treatment. They were then serum-deprived overnight for 16 h with or without insulin (1 nM) and with the indicated treatments. The resulting calibration curves gave good linearity with a R2 > 0.99).

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parametric Mann and adjusted for baseline value. If the hypothesis of normality was not met, non-parametric equivalence Kruskal–Wallis followed by Dunnett post hoc test. If data did not respect normality and homoscedasticity postulates, the non-parametric equivalent Mann–Whitney tests were used on variations vs. baseline.

For bile acids, cytokines/chemokines, and liver qPCR, P-values were corrected for multiple testing using a Benjamini–Hochberg adjustment. For HIVA, HICA, and HMVA, T-tests were performed for each tissue independently, to address (1) and (2), but (2) was assessed, even if (1) was not significant, in an exploratory way. P-values were adjusted for multiple testing considering all comparisons performed for one tissue using the Benjamini–Hochberg procedure. Pearson correlations were assessed between BCHA (HIVA, HICA, HMVA) using log-transformed values of metabolomics intensity and hepatic triglycerides, fasting insulin, and fasting glucose for Study 1 and 2. P-values were adjusted for multiple testing for each parameter using the Benjamini–Hochberg procedure.

For the GE Study, a t-test or the corresponding nonparametric Mann–Whitney test was performed, while significant differences for repeated measures were detected using a mixed model followed by a Tukey post hoc test (GraphPrism v8.0.1). For cells experiments, a one-way analysis variance (ANOVA) was performed, followed by Dunnett post hoc test. If data did not respect normality and homoscedasticity postulates, the non-parametric equivalent Kruskal–Wallis followed by Dunn’s post hoc test was applied. For quantitative parameters measured at more than one-time point (HGP of FAO cells treated with two concentrations of HICA, leucine and their combination), data were analyzed using a two-way ANOVA with fixed factors group, dose, and group*dose, followed by Dunnett post hoc test (GraphPrism v8.0.1).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request; the raw data of 16S rRNA sequence for the project through the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under accession number PRJEB47834 and the raw metabolomics data from MetaboLights (https://www.ebi.ac.uk/metabolights/) under study number MTBLS442

Code availability
No custom code was used in this work.

Received: 19 July 2021; Accepted: 22 February 2022; Published online: 15 March 2022

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Acknowledgements

We gratefully thank Christine Dion, Valérie Dumas, Christine Dallaire, Joanne Dupont-Morissette, Jacinthe Julien, Jenny Rancourt, Cinthia Faubert and Alexandre Pleau for their help with animal care, and Perrine Feutry for SCFA measurement. We also thank Agnes Cartier Meheust for her input on the nutritional profile of diet, and François Faure and Christian Chevret for their input on microbial metabolic pathways. Research relating to this study was funded through a research collaboration agreement by Danone Nutricia Research Palaiseau, France and the IUCPQ institute-Laval University, Quebec, Canada. Part of the research was also funded through a Canadian Institute of Health Research (CIHR) grant to A.M. (FDN-143247).

Author contributions

N.D., H.K., and A.M. designed the diets and animal studies. A.M. and H.K. designed the in vitro studies. H.K. and A.M. supervised the research. T.T.T. contributed to the conduct and interpretation of the first two animal studies. N.D. and R.N. carried out animal studies and interpreted the results. A.O. and B.M. carried out the in vitro studies. M.J.D. performed the qPCR analysis and interpreted the resulting results. P.S.P. and R.N. contributed to the design and interpretation of the clamp study. A.G. and P.I. performed live histological analyses. J.T. and O.B. performed B.A. analyses and interpreted the data. H.K., A.C., and L.Q. designed the targeted lipidsomics and untargeted metabolomics profiling study and H.K., M.P. interpreted data. H.K. designed and interpreted the targeted lipidsomics and LC-MS/MS study. Statistical analyses were handled by A.C. and L.Q. for animal studies; M.P., M.S., and T.V. for the fecal microbiota analyses; N.D. and R.N. for in vitro studies supervised by L.Q.; M.P. for LC-MS/MS and M.P. validated the targeted...
lipidomics and metabolomics profiling. N.D., R.N., H.K., and A.M. wrote the manuscript. All authors edited the manuscript.

**Competing interests**
A.C., L.Q., H.K., and T.T.T. were employees of Danone Nutricia Research (DNR), Palaiseau, France. At the time of the study, M.P. was an employee of IT&M Innovation on behalf of DNR, Palaiseau, France, and M.S. was an employee of Soladis on behalf of DNR, Palaiseau, France. DNR provided the test yogurt product and has led patent applications based on the enclosed findings. The remaining authors declare no competing interest.

**Additional information**
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-29005-0.

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Peer review information Nature Communications thanks Maria Marco, and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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