


Cecal microbial transplantation attenuates hyperthyroid-induced thermogenesis in Mongolian gerbils

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Summary

Endothermic mammals have a high energy cost to maintain a stable and high body temperature (T_b , around 37°C). Thyroid hormones are a major regulator for energy metabolism and T_b . The gut microbiota is involved in modulating host energy metabolism. However, whether the interaction between the gut microbiota and thyroid hormones is involved in metabolic and thermal regulations is unclear. We hypothesized that thyroid hormones via an interaction with gut microbiota orchestrate host thermogenesis and T_b . L-thyroxine-induced hyperthyroid Mongolian gerbils (*Meriones unguiculatus*) increased resting metabolic rate (RMR) and T_b , whereas Methimazole-induced hypothyroid animals decreased RMR. Both hypothyroid and hyperthyroid animals differed significantly in faecal bacterial community. Hyperthyroidism increased the relative abundance of pathogenic bacteria, such as *Helicobacter* and *Rikenella*, and decreased abundance of beneficial bacteria *Butyrimonas* and *Parabacteroides*, accompanied by reduced total bile acids and short-chain fatty acids. Furthermore, the hyperthyroid gerbils transplanted

with the microbiota from control donors increased type 2 deiodinase (DIO2) expression in the liver and showed a greater rate of decline of both serum T3 and T4 levels and, consequently, a more rapid recovery of normal RMR and T_b . These findings indicate that thyroid hormones regulate thermogenesis depending on gut microbiota and colonization with normal microbiota by caecal microbial transplantation attenuates hyperthyroid-induced thermogenesis. This work reveals the functional consequences of the gut microbiota-thyroid axis in controlling host metabolic physiology and T_b in endotherms.

Introduction

Endothermic mammals have a high cost to maintain a relatively stable and high body temperature (T_b , around 37°C). Thyroid hormones play a key role in regulating energy metabolism and thermoregulation in endothermic mammals (Silva, 2003; Mullur *et al.*, 2014). The active 3,5,3'-tri-iodothyronine (T3) is usually derived from inactive thyroxine (T4) by type 2 deiodinase (DIO2) which is localized widely in the hypothalamus and peripheral tissues (Mullur *et al.*, 2014). T3, acting via its nuclear receptors, modifies gene expression related to metabolism and development. In humans, serum thyroid hormone levels are clinically important indicators of thyroid diseases, such as hyperthyroidism and hypothyroidism. The factors influencing the conversion from T4 to T3 are diverse, but one of the important mechanisms may rely on the gastrointestinal tract and commensal microbiota (de Herder *et al.*, 1989).

The gastrointestinal tract of a man of standard body weight is colonized by 3.9×10^{13} bacteria, along with bacteriophages, archaea, viruses and fungi (Sender *et al.*, 2016). This huge community of gut bacteria plays crucial functions not only in digestion but also in hormonal, immunological and metabolic homeostasis (Tremaroli and Backhed, 2012; Fetissov, 2017). Research in microbial endocrinology indicates that gut microbiota can produce the same neuroactive compounds as the host and possess cognate receptors, which enable bi-directional communications between the host and the gut microbiota (Lyte, 2014). For example, certain bacteria like *Bacteroides* and *Lactobacillus* can produce neurochemicals such as

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γ -aminobutyric acid that are major inhibitory neurotransmitters of both the vertebrates and microbes, and regulate physiological functions (Barrett *et al.*, 2012; Strandwitz *et al.*, 2019). The deiodinases are widely distributed in the gastrointestinal tract and this may contribute substantially to the whole body T3 pool, due to the large gut surface, and the gut microbiota can hydrolyse iodothyronine conjugates (Nguyen *et al.*, 1993). Thus, the gut microbiota may affect deiodinase activity and alter the availability of T3 and T4, and thus may modulate hormone recycling and thyroid homeostasis (Fröhlich and Wahl, 2019; Shin *et al.*, 2020). In addition, thyroid function may affect gut motility and bacterial growth (Lauritano *et al.*, 2007). Bacterial overgrowth in the small intestine was found in a high percentage of humans with a history of hypothyroidism, presumed to be due to slower transit time (Lauritano *et al.*, 2007), whereas hyperthyroidism was associated with significantly accelerated intestinal transit compared to controls (Wegener *et al.*, 1992). Although phenotypic data support a possible role of microbiota in the peripheral metabolism of thyroid hormones and thyroid function (Köhling *et al.*, 2017; Virili and Centanni, 2017; Feng *et al.*, 2019; Knezevic *et al.*, 2020), the interaction between gut microbiota and thyroid homeostasis in regulating metabolic rate and T_b is still unclear.

Wild Mongolian gerbils inhabit semi-arid steppes, desert grasslands, and agricultural fields of northern China, Mongolia, and Russia (Wilson and Reeder,). They show seasonal changes in body mass, resting metabolic rate (RMR), and nonshivering thermogenesis (NST) (Zhang and Wang, 2007), and exhibit plastic responses in body mass, body composition, morphology of digestive tract, energy intake, and serum leptin level to cope with variations in temperature, photoperiod and food availability (Li and Wang, 2005; Zhao and Wang, 2006, 2009). Thyroid hormones are involved in regulating metabolic rate at different temperatures (Khakisahneh *et al.*, 2019). Our recent study showed that alterations in gut microbiota mediated huddling- or cold-induced metabolic and thermal homeostasis (Zhang *et al.*, 2018). However, it is not known whether the interaction between gut microbiota and thyroid hormones is involved in metabolic and thermal regulations. We first investigated the changes in RMR, T_b , and gut microbiota composition in drug-induced hypothyroid or hyperthyroid gerbils and then examined whether the gut microbiota could attenuate hyperthyroid- or hypothyroid-induced metabolic phenotypes. We hypothesized that thyroid hormones via an interaction with gut microbiota orchestrate host metabolism and thermogenesis. We demonstrate that hyperthyroid-induced increases in RMR and T_b were associated with a disturbed gut microbial community, and colonization with normal microbiota could attenuate hyperthyroid-induced thermogenesis.

Results

Hyperthyroid and hypothyroid states alter metabolic phenotypes and bacterial metabolites

To test the role of thyroid hormones in regulating metabolic phenotypes and bacterial metabolites, the gerbils drank tap water with 0.0036% L-thyroxin (Hyper), 0.04% Methylimazol (to inhibit thyroid hormone synthesis, Hypo) or just water (control) for 4 weeks (more details about the experimental design in the method section). There was no change in body mass among groups or with time (Fig. S1A). Food intake in the Hyper group was 67% higher than in the control ($P < 0.001$; Fig. 1A). At the end of experiment, both RMR and maximum NST (NST_{max}) were higher in the hyperthyroid (by 167% in RMR and by 72% in NST_{max}), and were lower in the hypothyroid gerbils (by 38% in RMR and by 36% in NST_{max}) than controls ($P < 0.001$; Fig. 1B). The regulatory NST (NST_{reg}) showed a significant difference only between the Hyper and Hypo groups ($P < 0.001$; Fig. 1B). Hyper gerbils had a higher T_b (by 0.4–0.7°C) from the 15th day after T4 treatment till the end than the other two groups ($P < 0.05$; Fig. 1C), and showed a slight but not significant increase in activity in the first week of treatment (Fig. 1D). DIO2 expression in the liver was lower in the Hyper group than in the control (Fig. 1E and F). The Hyper gerbils had lower levels of serum total bile acids compared to the control and Hypo groups ($P = 0.007$; Fig. 1G). There was no difference in serum tumour necrosis factor alpha (TNF- α) among groups (Fig. S1B). The concentrations of faecal total short-chain fatty acids (SCFAs) ($P = 0.007$), particularly acetic acid, butyric acid and valeric acid, were significantly lower in the hyperthyroid gerbils (Fig. 1H and I, S1C), whereas the concentration of isobutyric acid was higher in the hypothyroid than in the control group ($P = 0.022$; Fig. 1I).

Hyperthyroid and hypothyroid states alter the diversity and composition of faecal microbiota

To determine whether thyroid status affected gut microbiota, we analysed 16S rRNA gene sequencing in faecal samples in Control, Hyper and Hypo groups. The sequencing resulted in a total of 3 210 280 valid reads which were identified into 739 907 unique OTUs at a threshold of 97% sequence identity. The rarefaction curve of Goods coverage for the samples reached saturation (Fig. S1D), indicating that most bacteria were identified in the study. The Hypo gerbils showed higher phylogenetic diversity of the microbial community than the control and Hyper groups, but the Hyper group did not differ from the control (α -diversity, Table S1). The β -diversity indicated by Principal Coordinate Analysis

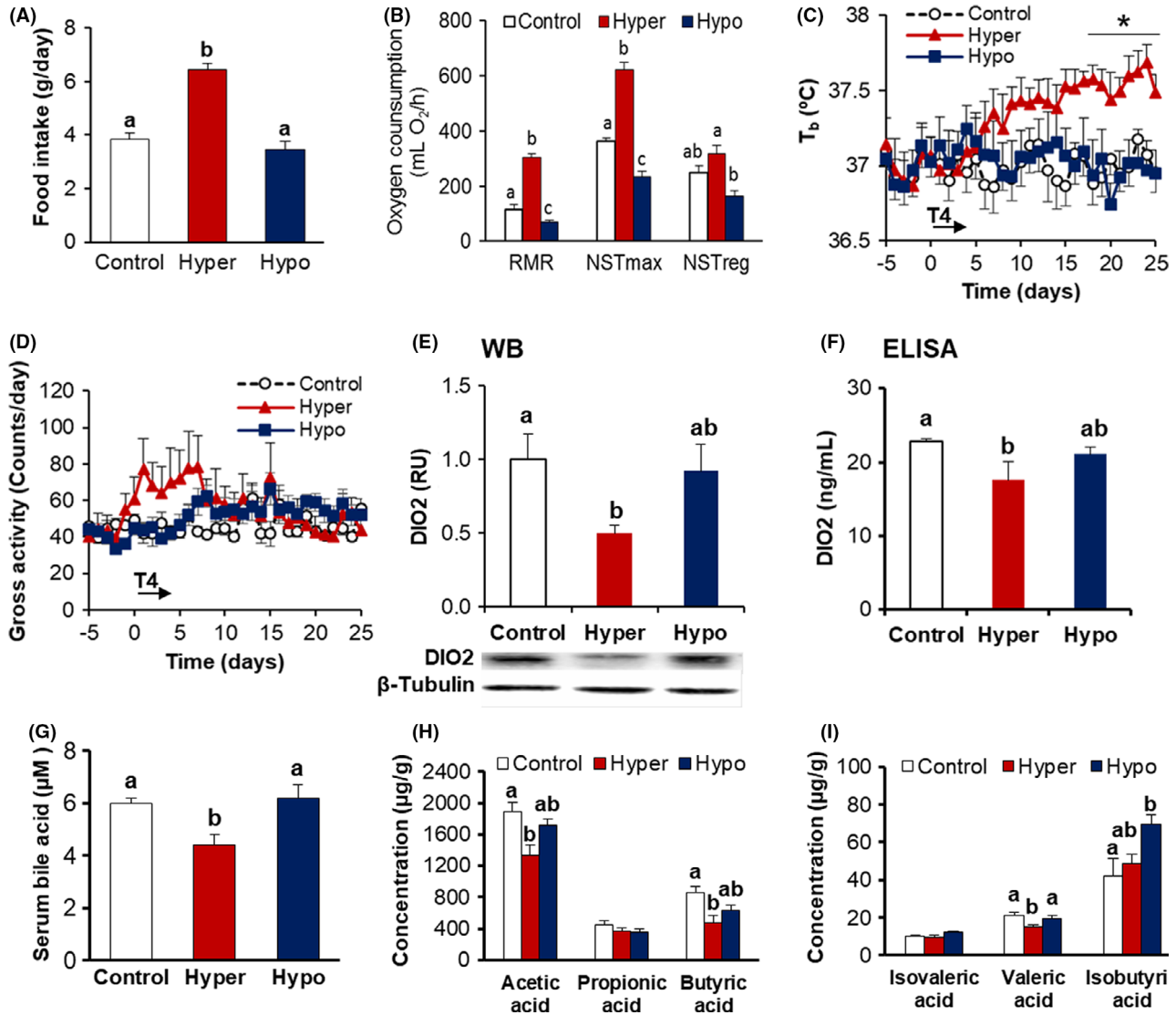
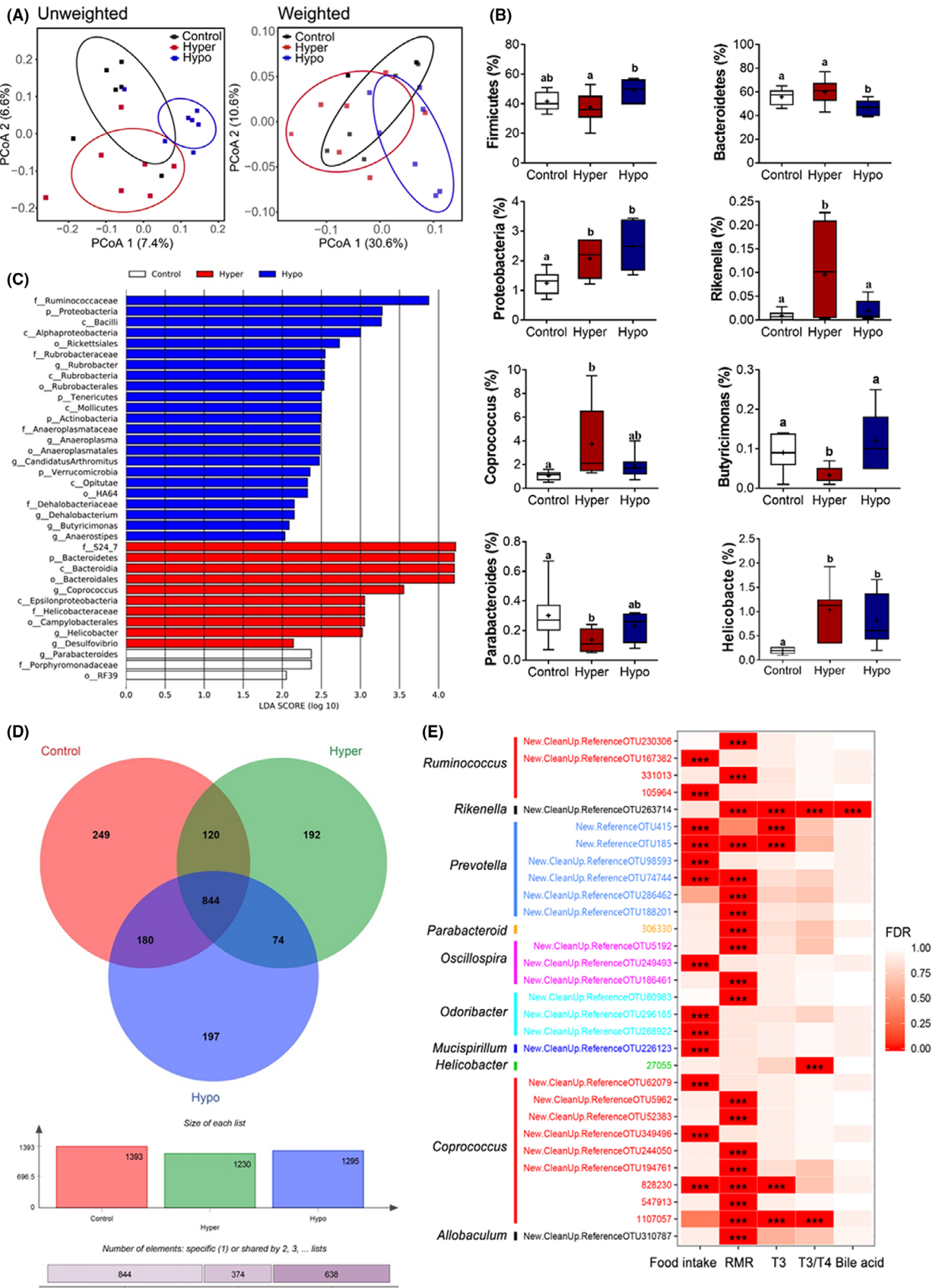


Fig. 1. Metabolic phenotypes and metabolites in the hyperthyroid (Hyper), hypothyroid (Hypo) and control gerbils. A. Food intake. B. Resting metabolic rate (RMR), maximum nonshivering thermogenesis (NST_{max}) and regulatory NST (NST_{reg}). C, D. Average core body temperature (T_b) and activity during the experiment ($n = 5$ per group). E, F. The expression of type 2 deiodinase (DIO2) in the liver, measured by Western blot (WB) and ELISA. G. The levels of total bile acids in serum. H, I. The concentrations of faecal short-chain fatty acids (SCFAs). Data are presented as means \pm SEM ($n = 7$ –8 per group, except T_b and activity). * $P < 0.05$, Hyper vs Control. Different letters above columns indicate significant differences among groups ($P < 0.05$).

(PCoA) based on unweighted (ANOSIM, $R = 0.319$, $P = 0.001$) and weighted UniFrac distances (ANOSIM, $R = 0.212$, $P = 0.011$) showed that the structure of the microbial communities differed from each other (Fig. 2A).

The dominating bacterial phyla and families are shown in Fig. S1E, F. At the phylum level, the relative abundance of Bacteroidetes tended to increase in Hyper gerbils and decreased in the Hypo group compared to the control, and Firmicutes showed inverse patterns (Fig. 2B). The proportion of Proteobacteria was significantly higher in the Hyper and Hypo groups than in the

control group (One-way ANOVA, $F_{2,19} = 8.734$, $P = 0.002$; *post hoc* tests, $P < 0.05$; Fig. 2B). At the genus level (Fig. 2B, Table S2), the Hyper gerbils showed increased proportions of *Rikenella* ($F_{2,19} = 5.335$, $P = 0.014$), *Helicobacter* ($F_{2,19} = 7.968$, $P = 0.003$), and *Coprococcus* ($F_{2,19} = 4.162$, $P = 0.032$), but decreased proportions of *Butyricimonas* ($F_{2,19} = 4.994$, $P = 0.018$) and *Parabacteroides* ($F_{2,19} = 3.046$, $P = 0.071$) when compared with the control group. To assess differences in microbial communities affected by thyroid hormone treatment, we applied



the linear discriminant analysis (LDA) effect size (LEfSe) method and identified four distinctive bacteria in the control gerbils, 10 in the Hyper, and 23 in the Hypo gerbils [LDA score ($\log_{10} > 2$] (Fig. 2C; Fig. S1G).

The Venn diagram identified the specific genus in the OTUs of control as *Devosia*, specific genera of the Hyper as *Staphylococcus* and *Veillonella*, and specific genera of the Hypo as *Rubrobacter*, *Syntrophomonas*, *Luteimonas* and *Mycoplasma*, and there were 52 common genera belonging to all groups (Fig. 2D). Pearson correlation analyses revealed that both food intake and RMR were correlated with OTUs of *Coprococcus*, *Odoribacter*, *Oscillospira*, *Prevotella* and *Ruminococcus*, and serum T3 and/or T3/T4 levels were correlated with OTUs of *Coprococcus*, *Helicobacter* and *Rikenella* (Fig. 2E; Table S3).

Caecal microbiota transplantation attenuates hyperthyroid thermogenesis

To test the role of gut microbiota in regulating thyroid hormones and metabolism, we performed caecal microbiota transplantation (CMT) in the control and Hyper gerbils (Fig. 3A). The Hyper gerbils were colonized via an intragastric gavage with the microbiota from the control donors (Hyper-Con) or with sterile saline (Hyper, sham CMT); while the control gerbils were colonized with microbiota from the Hyper animals (Con-Hyper) or with sterile saline (Con, sham CMT). Food intake both in the Hyper and Hyper-Con gerbils increased dramatically compared to that in the control group by the end of the T4 treatment ($F_{3,15} = 20.875$, $P < 0.001$; Fig. 3B). After CMT, food intake in the Hyper-Con group decreased significantly and showed no difference from the control group in the third week after CMT ($P = 0.117$, Fig. 3B), whereas food intake in the Hyper gerbils remained higher than both the control and Hyper-Con gerbils till the end of experiment (the 6th week) ($P < 0.05$, Fig. 3B). All Hyper animals showed increased RMR by the end of the T4 treatment when compared with the control ($F_{3,14} = 27.768$, $P < 0.001$; Fig. 3C). The RMR in Hyper-Con gerbils decreased markedly and returned to the control level by the 6th week after CMT ($P = 0.212$). In contrast, the Hyper gerbils showed a steady higher RMR even in the 6th week after termination of the T4 treatment ($P = 0.011$). At the end of the T4

treatment (0th week), all the Hyper gerbils showed significantly higher T_b compared with their control counterparts ($F_{2,11} = 8.713$, $P = 0.005$; Fig. 3D). The Hyper-Con gerbils started to decrease T_b immediately following the CMT and did not differ from the control group by 5 weeks after the CMT ($P = 0.649$), while T_b in the Hyper group remained higher than in the control gerbils till the end ($P = 0.02$). Both T3 and T4 were at higher levels after T4 treatment (week 0, Fig. 3E and F), and then started to decline after termination of drug treatment. The Hyper gerbils still showed higher T3 and T4 levels 6 weeks after drug treatment, whereas the Hyper-Con gerbils returned to the control level by the first week after CMT (Fig. 3E and F). The DIO2 expression in the liver was higher in the Hyper-Con group than in the Hyper group ($P < 0.075$; Fig. 3G and H).

One week after CMT, the α -diversity of microbial community showed a difference between Hyper and Con-Hyper groups, indicated by observed OTUs and PD whole tree (Table S4, Fig. 4A). The β -diversity showed separation only between Hyper-Con and control measured by unweighted UniFrac distances (ANOSIM, $R = 0.181$, $P = 0.005$), but did not separate among groups indicated by weighted UniFrac distances (ANOSIM, $R = 0.083$, $P = 0.817$; Fig. 4B). Microbiota transplantation led to an alteration in the microbial composition of recipients in the Hyper-Con gerbils with a 21% increase in the Firmicutes compared to the Hyper group (Fig. 4C and D), and the Hyper-Con gerbils increased the proportions of *Allobaculum* ($F_{3,16} = 6.221$, $P = 0.005$) and *Lactobacillus* ($F_{3,16} = 8.186$, $P = 0.002$; Fig. 4D) one week after CMT, when compared with the Hyper group. We also observed that the Con-Hyper gerbils showed a higher abundance of *Roseburia* compared to the control group ($F_{3,16} = 4.046$, $P = 0.026$; Fig. 4D). The genus *Anaeroplasm* maintained a relatively higher proportion even 6 weeks after CMT (Fig. 4E). The LEfSe method identified distinctive microbial biomarkers in each group (Fig. S2A and B).

Microbiota transplantation has no effects in the hypothyroid gerbils

To explore whether gut microbiota could modulate the hypothyroid-induced reduction in metabolic rate, we

Fig. 2. Thyroid hormone treatments shape bacterial composition.

A. Principal coordinate analyses (PCoA) plots based on unweighted and weighted UniFrac distances in faecal microbiota of different groups (unweighted, ANOSIM, $R = 0.319$, $P = 0.001$; weighted, ANOSIM, $R = 0.212$, $P = 0.011$). The ellipses cover 68% of the data for each group.

B. Relative abundance of different bacteria at the phylum and genus levels ('+' indicates the mean of data).

C. Differential bacterial taxonomy selected by LEfSe analysis with LDA score > 2 in the faecal microbiota community (Differences between groups are represented by the colour of the most abundant class).

D. Venn diagram based on OTUs distribution between groups.

E. Heatmap of correlation between specific OTUs and physiological parameters. Data are presented as means \pm SEM ($n = 7-8$ per group). Different letters above columns indicate significant differences among groups ($P < 0.05$). Hyper, hyperthyroid; Hypo, hypothyroid.

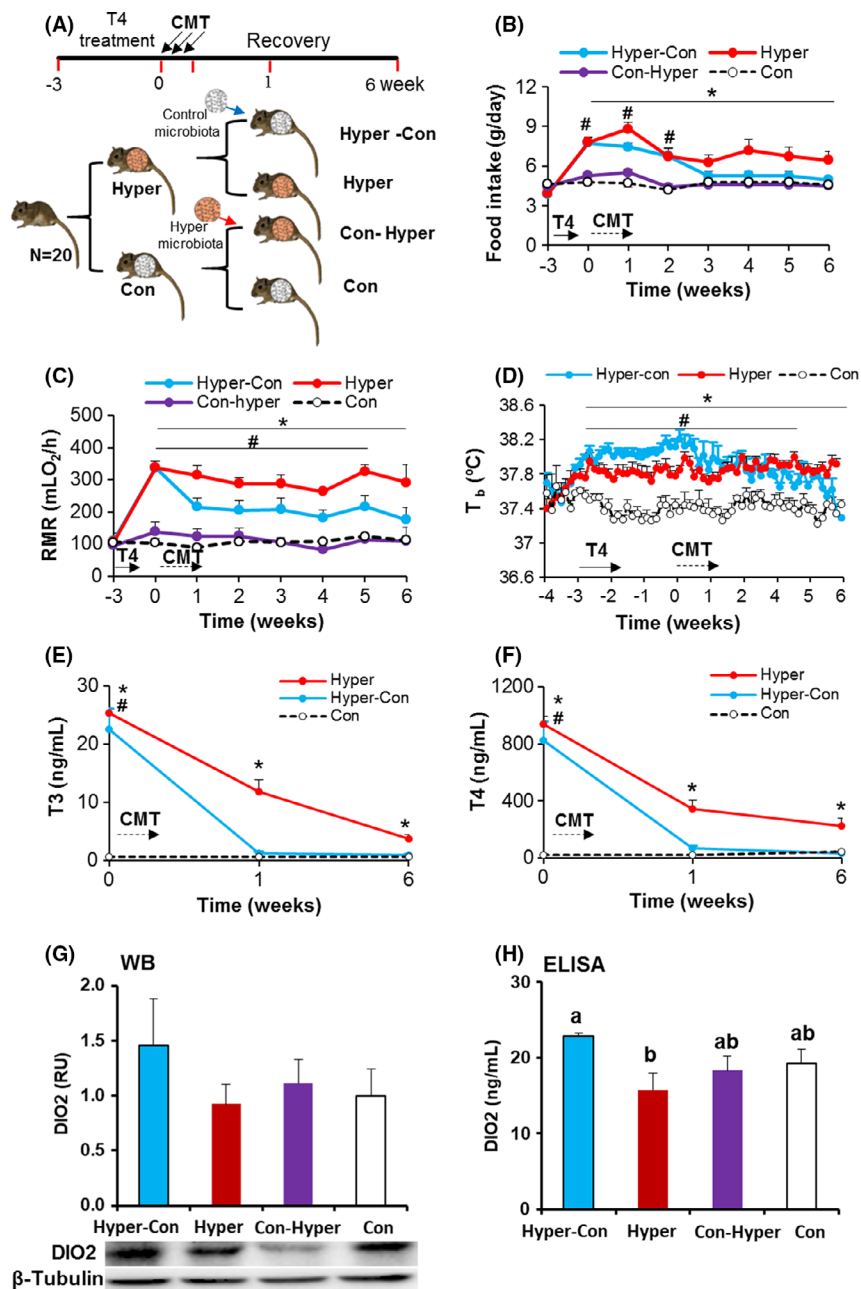


Fig. 3. Gut microbiota buffer hyperthyroidism-induced hyperphagia and hypermetabolism.

A. Schematic overview of experimental design.

B. Food intake in different groups during the experiment.

C. Resting metabolic rate (RMR) of every group during the experimental period.

D. Average core body temperature (T_b) of gerbils from different groups.

E, F. Serum tri-iodothyronine (T₃) and thyroxine (T₄) levels.

G, H. Type 2 deiodinase (DIO2) by Western blot (WB) and ELISA. Values are means \pm SEM ($n = 5$ per group). * $P < 0.05$, Hyper versus Con.

$P < 0.05$, Hyper-Con versus Hyper. CMT, caecal microbiota transplantation; Hyper-Con, Hyper gerbils were colonized with control microbiota; Hyper, Hyper gerbils with sterile saline; Con-Hyper, control gerbils were colonized with Hyper microbiota; Con, control gerbils with sterile saline.

induced hypothyroidism in Mongolian gerbils with administered methimazole in the drinking water (Hypo), and transplanted caecal microbiota from the control donors to hypo gerbils or from hypo donors to control gerbils

(for more details see follow in method section) (Fig. 5A). Neither body mass nor food intake was influenced by CMT among groups ($P > 0.05$) or with time ($P > 0.05$; Fig. 5B and C). RMR also showed no difference among

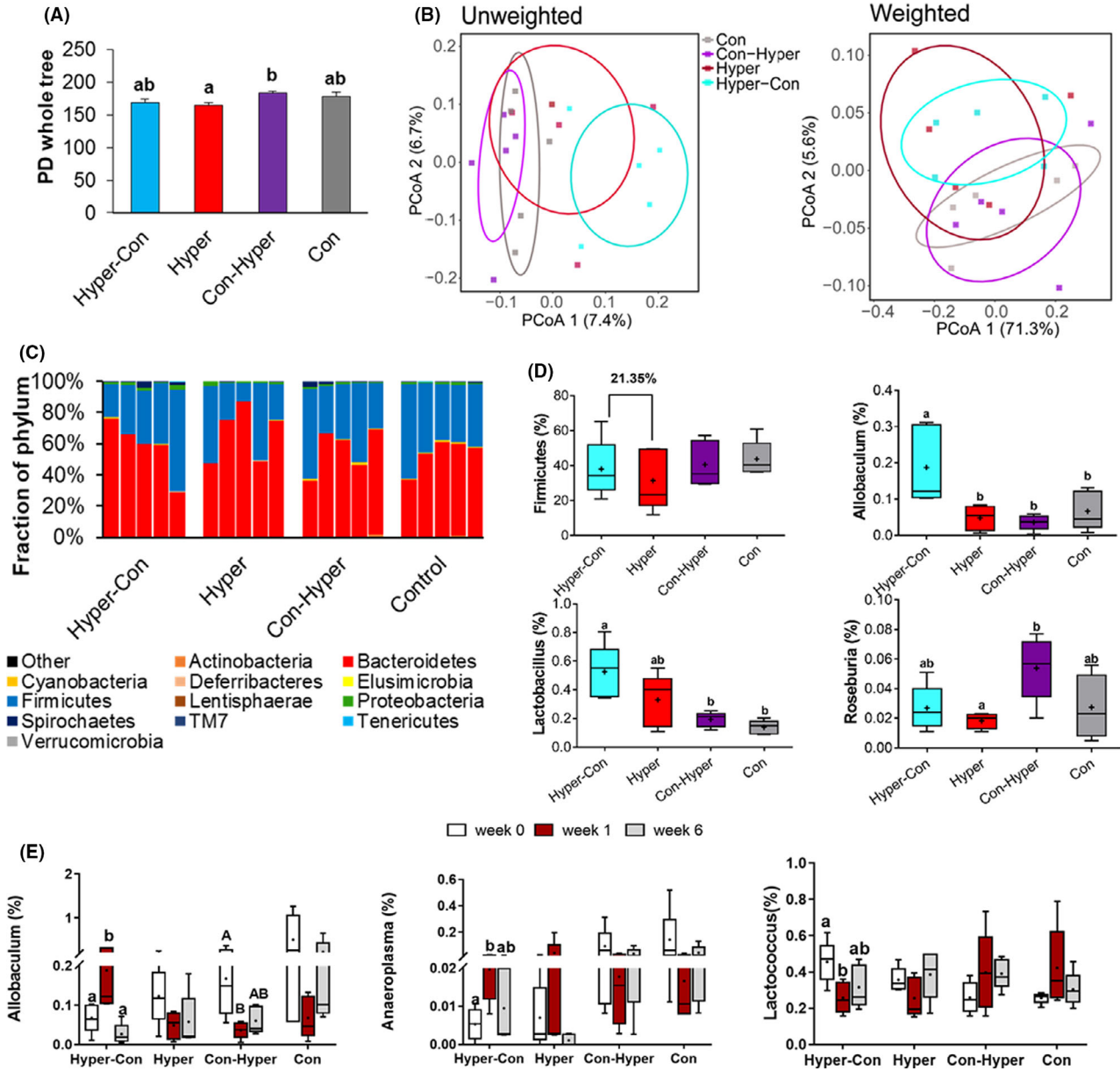


Fig. 4. Caecal microbiota transplantation (CMT) alters faecal microbiota composition in hyperthyroid gerbils.

A. Phylogenetic diversity (PD)—whole tree analysis for the samples.

B. Principal coordinate analyses (PCoA) plots based on unweighted and weighted UniFrac distances in faecal microbiota of different groups (unweighted, ANOSIM, $R = 0.181$, $P = 0.005$; weighted, ANOSIM, $R = 0.083$, $P = 0.817$).

C. Relative abundance at the phylum levels in faecal microbiota community one week after CMT in each individual animal of the four groups.

D. The relative abundance of different bacteria at the phylum and genus levels in different experimental groups at week 1 after CMT.

E. The relative abundance of some specific bacteria in different experimental groups before (week 0) and after CMT (weeks 1 and 6). Data are presented as means \pm SEM ($n = 5$ per group). Different letters above columns indicate significant differences among groups ($P < 0.05$). Hyper-Con, Hyper gerbils were colonized with control microbiota; Hyper, Hyper gerbils with sterile saline; Con-hyper, control gerbils were colonized with hyper microbiota; Con, control gerbils with sterile saline.

groups ($F_{3,17} = 1.328$, $P = 0.298$; Fig. 5D). Methimazole treatment led to a significant decrease in serum T3 levels in the gerbils before CMT ($F_{3,17} = 5.547$, $P = 0.007$; Fig. 5E), but one week after CMT till the last week of experiment no significant difference was observed in serum T3 levels among groups (week 1,

$F_{3,17} = 1.448$, $P = 0.257$; week 6, $F_{3,17} = 3.055$, $P = 0.051$; Fig. 5E).

Microbiota transplantation did not affect the α -diversity of the microbial community in the recipients (Fig. S3A). The β -diversity based on unweighted UniFrac distances showed clear separation between the control and Hypo

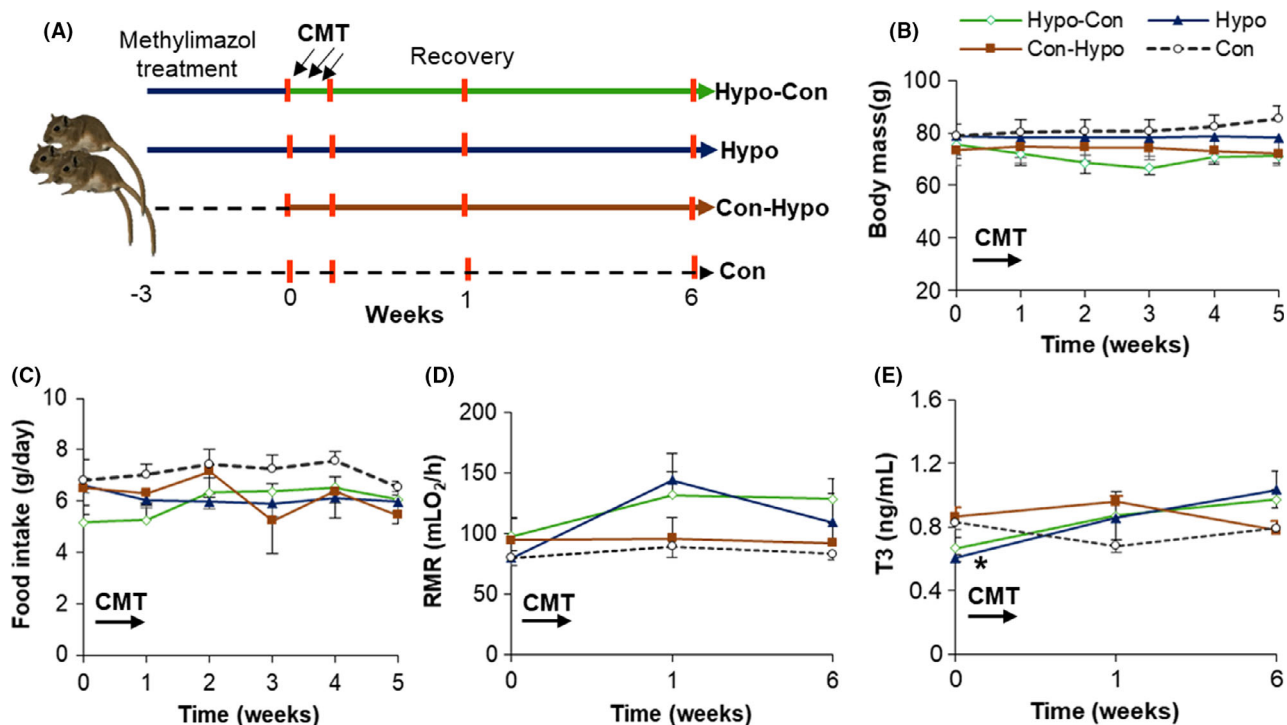


Fig. 5. Caecal microbiota transplantation (CMT) did not affect metabolic traits in hypothyroid or control gerbils.

A. Schematic overview of experimental design.

B. Body mass.

C. Food intake.

D. Resting metabolic rate (RMR).

E. Serum tri-iodothyronine (T3) levels. Values are means \pm SEM ($n = 5$ per group). * $P < 0.05$, Hypo versus Con. Hypo-Con, Hypo gerbils were colonized with control microbiota; Hypo, Hypo gerbils with sterile saline; Con-hypo, control gerbils were colonized with hypo microbiota; Con, control gerbils with sterile saline.

groups (ANOSIM, $R = 0.275$, $P = 0.001$), but no separation between CMT and sham CMT (Fig. S3B). The β -diversity based on weighted UniFrac distances showed no separation among groups (ANOSIM, $R = 0.070$, $P = 0.995$; Fig S3B). The specific bacteria in each group were identified by LEfSe method (Fig. S3C and D).

Discussion

Increasing evidence indicates that the gut microbial community is crucial for metabolic homeostasis (Tremaroli and Backhed, 2012). To understand the relationship between gut microbiota, thyroid hormones and metabolism, we determined the changes in gut microbiota diversity in the hyper and hypothyroid gerbils, and further evaluated the role of the gut microbiota in regulating thyroid hormones and metabolic phenotypes in Mongolian gerbils. We observed, as expected, that the hyperthyroid gerbils had higher RMR and T_b compared with the control and hypothyroid groups. Both hyper- and hypothyroid animals showed significant alterations in the bacterial composition. Interestingly, CMT of control bacteria to the hyperthyroid gerbils had a profound effect in reducing

RMR, T_b and thyroid hormone levels. These results indicated that the interaction between thyroid hormones and gut microbiota contributes to regulating host thermogenesis.

Thyroid hormones shaped metabolic phenotype and microbiota diversity

It is conventionally thought that thyroid hormones regulate not only basal metabolic rate, but also the thermogenesis from brown adipose tissue (BAT) (Mullur *et al.*, 2014). As expected, our data showed that thyroid hormone levels have direct effects in controlling metabolism and T_b . The hyperthyroid gerbils had higher RMR (by 167%), NST_{max} (by 72%), and T_b (by 0.4–0.7°C) compared to the control. However, the regulatory NST (directly from BAT thermogenesis) both in the hyper and hypothyroid gerbils did not differ significantly from the control, although there was a difference between the hyper and hypothyroid groups. Our data are supported by evidence that thyroid thermogenesis is independent of uncoupling protein 1 (UCP1, a thermogenic protein found in the mitochondrial inner membrane of BAT)

(Cannon and Nedergaard, 2004; Dittner *et al.*, 2019; Johann *et al.*, 2019; Mittag, 2020). The higher energy expenditure in hyperthyroid gerbils was compensated by increased food intake (by 67%) and these gerbils could maintain body mass stable. Diet drives quick changes in gut microbial community in humans and animals (Gentile and Weir, 2018; Zmora *et al.*, 2019). Hyperthyroid-induced changes in gut microbiota might be induced by increased food consumption and transit time. We did not restrict food intake of animals to exclude this possibility. Our previous study, via a paired-feeding protocol, supported that the alteration in diversity and composition of gut microbiota in cold-acclimated animals can be induced by ambient temperature, not due to increased food intake (Bo *et al.*, 2019). Gut microbes produce a variety of neuroendocrine hormones and possess cognate host receptors (Lenard, 1992; Roshchina, 2010; Lyte, 2014). Therefore, changes in host thyroid hormones could induce corresponding responses both in the host and gut microbiota. No significant difference in serum TNF- α (one inflammatory factor) was observed in the different groups, implying that the higher T_b in the hyperthyroid gerbils was probably not due to an inflammatory response. We observed an initial increase followed by a decrease to the control levels in the activity of hyperthyroid gerbils. The change in activity curves could be interpreted to suggest that the hypothyroid gerbils increase activity to stay warm and the hyperthyroid animals decrease activity to counteract body temperature increases. These data indicate that hyperthyroidism induces increases in thermogenesis, which leads to the elevated T_b .

The gastrointestinal tract is the largest endocrine organ and also a metabolic organ, with a wide range of enzymes, hormones and neurotransmitters being expressed in the intestinal epithelium, and thus it can interact with host cells and contribute to the regulation of metabolism (Rastelli *et al.*, 2019). Besides genetics, the diets as well as host hormones or neurotransmitters, can affect gut microbial diversity of the hosts (Koren *et al.*, 2012). Studies in humans showed that hyperthyroid patients had significant enrichments in pathogenic bacteria such as *Enterobacteriaceae* and *Clostridium* in the gut in comparison with a healthy group (Zhou *et al.*, 2014), while hypothyroidism was accompanied by a modest intestinal bacterial overgrowth (Lauritano *et al.*, 2007; Zhao *et al.*, 2018). Both rat (Shin *et al.*, 2020) and gerbil models (in the present study) of thyroid dysfunction revealed that profile of gut microbiota varied with different thyroid functional status. We also observed that the hyperthyroid gerbils showed an increase in pathogenic bacteria, such as *Rikenella*, *Helicobacter*, *Staphylococcus* and *Veillonella*, which have been reported as causes of serious infections and inflammation (Alkadhi

et al., 2014). Moreover, the hyperthyroid gerbils had a lower abundance in beneficial SCFA-producing bacteria such as *Butyricimonas* and *Parabacteroides*. Furthermore, the correlation analyses support the concept that these specific bacteria such as *Allobaculum*, *Coproccoccus*, *Odoribacter*, *Oscillospira*, *Parabacteroid*, *Prevotella*, *Rikenella* and *Ruminococcus* were involved in the regulation of host RMR and food intake. These data indicate that thyroid hormones shift the gut microbiota, which may affect host thermogenesis.

The alteration of the gut microbial community leads to changes in SCFAs, bacterial fermentation products. Bile acid metabolism is also related to gut microbiota, which via bile salt hydrolases enzymes deconjugate the glycine or taurine from the sterol core of the primary bile acids. And subsequently, it undergoes a variety of microbiota-encoded transformations like 7 α /beta-dehydroxylation that generate secondary bile acids, which can have a wide effects on the host metabolism (Ridlon *et al.*, 2006; Yao *et al.*, 2018; Foley *et al.*, 2019). Bile acids have a critical role in regulating lipid metabolism and adaptive thermogenesis (Watanabe *et al.*, 2006; Worthmann *et al.*, 2017). Both SCFAs and bile acids exert systemic impacts on host appetite, lipid metabolism and inflammation (Smith *et al.*, 2013; Foley *et al.*, 2019). We observed decreases both in SCFAs and total bile acids in the hyperthyroid animals, which may lead to increased feeding and diet-induced thermogenesis. In support of our results, a study by Ellis showed that treatment of hepatocytes with T3 decreased total bile acid formation to 44% of controls (Ellis, 2006), and another study showed that the decrease in thyroid hormones was closely related to the increase in plasma bile acid levels (Ockenga *et al.*, 2012). The decreases seen in total SCFAs and bile acids would also promote the expression of virulent genes in the bacteria, and affect the diversity and structure of the gut microbiota (Worthmann *et al.*, 2017; Lin *et al.*, 2018). Consequently, the changes in gut microbiota and bacterial metabolites would influence host metabolism and thermal homeostasis. The thyroid-microbiota linkage will be further verified in non-orally-manipulated models, since the administration of thyroxine and/or methimazole orally may cause intestinal microbiota to interact artificially with these components even before absorption.

Normal microbiota attenuates hyperthyroid-induced thermogenesis of the host

Gut microbiota-host interaction is crucial in maintaining hormonal and metabolic homeostasis. The hyperthyroid gerbils still had elevated serum T3 and T4 levels in the 6th week after termination of the T4 treatment. We measured the enzyme DIO2, which can mediate T4-to-T3

conversion, and found that DIO2 expression in the liver was reduced with T4 treatment and still tended to decrease even after T4 treatment stopped. The study by (Watanabe *et al.* (2006) also showed a hyperthyroid-associated reduction in DIO2. The other enzyme, type 1 deiodinase (DIO1), may also contribute to changes in thyroid hormone levels, since it was upregulated in the hyperthyroid state (Worthmann *et al.*, 2017). This indicates that T4 treatment exerted a long-lasting effect on thyroid metabolism and the conversion of extra T4 was delayed in these animals.

We observed a greater rate of decline of both T4 and T3 in the hyperthyroid gerbils receiving microbiota from the control donors, which was accompanied by the return of both RMR and T_b to control levels. Hyper gerbils with the CMT showed an increase of DIO2 expression in the liver compared to sham CMT. Hyper-Con gerbils had a reduced Firmicutes/Bacteroidetes ratio and increased the abundance of SCFA-producing bacteria such as *Allobaculum* and *Lactobacillus* compared to sham CMT. The greater rate of decline of both T4 and T3 in the Hyper-Con group demonstrates an effect of the CMT on the concentrations of both T4 and T3 and this may be what affects thermogenesis. In contrast, the CMT from hyperthyroid to control did not have an effect. One possibility is that the microbes from hyperthyroid gerbils failed to colonize control gerbils. The gut microbiome via their metabolites, SCFAs and bile acids may regulate the expression of both DIO1 and DIO2 and thus affect serum T3 and T4 levels (Ito *et al.*, 2011; Virili and Centanni, 2017). Hyperthyroidism was associated with reduced SCFAs and bile acids, and thus reduced DIO2. It is also possible that the changes in gut microbiome can affect enterohepatic metabolism of thyroid hormones (Virili and Centanni, 2017), further contributing to the faster rate of decline in T4 and T3 in the Hyper-Con group. Therefore, gut microbiota may regulate the metabolism of thyroid hormones by modulating the deiodinase activity and the enterohepatic circulation, and the changes in thyroid hormone levels may contribute to the changes in RMR and T_b . Besides thyroid hormones, other endogenous regulators such as catecholamines and glucocorticoids can also mediate the changes in metabolic rate and T_b (Cannon and Nedergaard, 2004; Luijten *et al.*, 2019), and these regulators may interact to control thermogenesis in endothermic species (Ribeiro *et al.*, 2001; Mullur *et al.*, 2014).

Although the hyperthyroid animals receiving control microbiota could reduce metabolic rate and T_b , we observed that CMT of hyperthyroid microbiota to control recipients did not cause any changes in metabolic traits. These results support the idea that the changes in metabolism and T_b may be the consequence of interactions among a number of bacterial species and depend on

both the diversity of the microbial community and their host. We did not observe any effects of CMT in hypothyroid to control recipients or control to hypothyroid recipients on metabolic traits, bacterial metabolites or the bacteria in different genera. Whether normal gut microbiota can also regulate hypothyroidism should be further investigated.

Conclusion

The current study illustrates the interaction between gut microbiota and thyroid hormones in regulating host thermogenesis. We observed that hyperthyroid-induced increases in metabolic rate and T_b were associated with altered gut microbial composition and metabolites, such as SCFAs and bile acids. We then confirmed for the first time that normal microbiota could regain normal conditions in a hyperthyroid animal by regulation of DIO2 expression in the liver and buffer hyperthyroid-induced thermogenesis. We demonstrate that thyroid hormones regulate thermogenesis depending on gut microbiota and CMT attenuates hyperthyroid-induced thermogenesis. These findings emphasize the functional and evolutionary consequences of the thyroid-microbiota interface in controlling host metabolic physiology and T_b in endotherms.

Experimental procedures

Animals and housing

The breeding colonies of Mongolian gerbils were outbred and rejuvenated with wild populations from the desert grasslands of Inner Mongolia or with gerbils from other laboratories every 3 years. Female Mongolian gerbils between 6 months and one year of age were housed individually in plastic cages (30 × 15 × 20 cm) that contained sawdust bedding in the laboratory at the Institute of Zoology, the Chinese Academy of Sciences (CAS). All animals were maintained under a 16L: 8D photoperiod with lights on at 0400 h at a room temperature of 23 ± 1°C. The gerbils were fed a standard rat pellet chow (Beijing KeAo Bioscience Co., Beijing, China) and were provided with water *ad libitum*. All procedures on animals in this study were approved under the Animal Care and Use Committee of the Institute of Zoology, CAS.

Experimental designs

Experiment 1 examined the role of thyroid hormones in regulating metabolic rate, T_b , and gut microbiota. A total of 24 adult Mongolian gerbils were randomly divided into three groups ($n = 8$ per group) for 4 weeks. In the hyperthyroid (Hyper) group, the gerbils drank tap water with

0.0036% L-thyroxine (T₄, weight per volume, w/v) (Ahmed *et al.*, 2012; Jena *et al.*, 2013). In the control group, the animals drank only tap water. In the hypothyroid (Hypo) group, the animals drank tap water with 0.04% methimazole (w/v) to inhibit thyroid hormone synthesis (Ahmed *et al.*, 2012). Fresh faeces were collected, frozen in liquid nitrogen and then stored at -80°C for later DNA extraction. The detailed methods for measuring RMR, NST, T_b and activity are available in the supplementary materials.

Experiment 2 examined the role of gut microbiota colonization in buffering hyperthyroid-induced changes in metabolic phenotypes. This experiment included three processes, T₄ treatment for 3 weeks, and then caecal microbiota transplantation (CMT) for 3 days and then recovery for 6 weeks (Fig. 3A). In the T₄ treatment phase, 26 Mongolian gerbils were induced to become hyperthyroid with 0.0036% L-thyroxine (T₄, weight per volume, w/v) in the drinking water or drank only tap water as the Control group ($n = 13$ per group). Three animals in each group were donors for CMT. After 3 weeks, T₄ treatment was stopped. The hyperthyroid gerbils were colonized via intragastric gavage with the microbiota from the control donors (Hyper-Con) or sterile saline (Hyper, sham CMT) for 3 days (once every day, five gerbils per group), and then kept for 6 weeks. The control gerbils were colonized with hyperthyroid microbiota (Con-Hyper) or sterile saline (Con, sham CMT) at the same time. Blood was collected from the infraorbital vein of animals in the last week before CMT and the first week after CMT, and also at the end of treatment (the 6th week after CMT). Fresh faeces were collected before CMT and also in the 1st and 6th weeks after CMT, frozen in liquid nitrogen and then stored at -80°C for later DNA extraction.

In experiment 3 we investigated the role of gut microbiota in buffering hypothyroid-induced changes in metabolic phenotypes. In the treatment phase, one set of 26 adult Mongolian gerbils were induced to become hypothyroid with 0.04% methimazole (w/v) in the drinking water for 3 weeks or drank only tap water as the control ($n = 13$ per group) (Fig. 5A). For the subsequent 6 weeks in the CMT phase (five gerbils per group), the hypothyroid gerbils were colonized with control microbiota (Hypo-Con) or sterile saline (Hypo) for 3 days (once every day). The control gerbils were colonized with hypothyroid microbiota (Con-Hypo) or sterile saline (Con). Blood was collected at the same time points (before CMT, the 1st and 6th weeks after CMT) with the same method as experiment 2.

Body temperature, activity and metabolic trials

Core T_b and activity were recorded during the treatment period through transponders (G2 Emitter, to $\pm 0.1^{\circ}\text{C}$,

STARR life sciences). Animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium (1%) with a dose of 50 mg kg^{-1} and the Emitter was implanted in the abdomen of the gerbils. After surgery, the animals were allowed 10 days to recover. All receivers for collecting data were connected to a computer with the Vital View software.

An open-flow respirometry system (TSE LabMaster, Bad Homburg, Germany) was used to determine metabolic rates, as described previously (Zhang *et al.*, 2018). The temperature of the metabolic chamber was maintained at $30 \pm 0.5^{\circ}\text{C}$ (within the thermal neutral zone). For each individual, the average of three consecutive and minimum readings of oxygen consumption was taken as the RMR after at least 1 h acclimation in the metabolic chamber. NST was measured one day after the RMR measurement. Norepinephrine (NE), with a dosage calculated according to the equation, $\text{NE} (\text{mg kg}^{-1}) = 6.6W^{-0.458}$ (W is body mass in gram), was subcutaneously injected to every individual gerbil to induce the maximum NST (NST_{max}) (Zhang and Wang, 2007). Oxygen consumption was recorded for 60 min at 5 min intervals and the temperature was maintained at $25 \pm 1^{\circ}\text{C}$. The three highest consecutive readings of oxygen consumption 15–20 min post-injection were taken to calculate the maximum NST during the 1-h measurement. The regulatory NST, (NST_{reg}, which is from brown adipose tissue, BAT), was calculated as NST_{max} minus the RMR determined the previous day. The detailed methods are available in the supplementary materials.

DIO2 measurement by Western blot

After the experiment, the gerbils were sacrificed by CO₂ asphyxiation between 0900–1100 h. Blood was collected from the superior vena cava to get the serum for measurement of thyroid hormones, and the liver was collected and frozen in liquid nitrogen. About 0.1 g of liver tissue was homogenized in RIPA buffer (10 mM Tris (pH 7.0), 158 mM NaCl, 1% TritonX-100, 5 mM EDTA, 1 mM DTT, 1 mM PMSF (P7626-5G; Sigma-Aldrich Corp., St. Louis, MO, USA), and PIC (P8340, 1:1000; Sigma-Aldrich Corp., St. Louis, MO, USA), and centrifuged at 14 000 rpm at 4°C for 10 min to collect the supernatant. Protein concentration was measured by the Folin phenol method.

Total liver protein was separated in a discontinuous sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE), and was blotted to a PVDF membrane (IPVH00010; Merck Millipore Ltd., Darmstadt, Germany) (270 mA, 2 h). Membranes were blocked in 5% non-fat dry milk and incubated with rabbit anti-DIO2 antibody (1:3000; PAC903Ra01; Cloud-Clone Corp., Wuhan, China) and rabbit anti- β -Tubulin (30302ES60,

Shanghai Yisheng Biotechnology Co., Shanghai, China) overnight at 4°C. Peroxidase-conjugated goat anti-rabbit IgG (1:5000; 111-035-003; Jackson ImmunoResearch, West Grove, PA, USA) was used as the secondary antibody. Blots were quantified with Quantity One Software (BioRad Laboratories, Hercules, CA, USA, Ver.4.4.0) and analysed using Image Lab™ Software (Bio-Rad Laboratories), and normalized to β -Tubulin (a protein marker) as relative units (RU).

DIO2 measurement by ELISA

The DIO2 level in the liver was also measured using an ELISA kit (SEC903Ra, Cloud-Clone Corp., Wuhan, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm against a blank using an ELISA reader (RayBiotech, Canada). The minimum detectable value of the kit was 0.059 ng ml⁻¹. The intra- and inter-assay coefficients of variation (CV) for DIO2 were 10% and 12%, respectively. Assay results were expressed as ng ml⁻¹.

Serum total thyroid hormones assays

Serum total T3 and T4 levels were quantified with a radioimmunoassay using RIA kits (China Institute of Atomic Energy, Beijing, China), according to the instructions as previously described (Khakisahneh *et al.*, 2019). The lower limit of the assay when using a 50 μ l sample was 0.25 ng ml⁻¹ for T3 and 3.96 ng ml⁻¹ for T4. Intra- and inter-assay coefficients of variation were 2.4% and 8.8% for T3, and 4.3% and 7.6% for T4, respectively.

Serum total bile acid concentration

Serum concentrations of total bile acids were measured using Total Bile Acids Assay Kit (Colorimetric) (ab239702; Abcam, Cambridge, UK). According to the manufacturer's instructions, the standards and the serum (25 μ l) were added to a 96-well plate and adjusted to 50 μ l with ddH₂O. After that, the probe mix (100 μ l) was added to the wells of each sample and standard, and the plate was incubated at 37°C for 10 min. Finally, 50 μ l of reaction mix was added to the wells and absorbance was measured at 405 nm in a kinetic mode at 37°C for 60 min and protected from light.

Serum TNF- α levels

Serum TNF- α levels were detected using an ELISA kit (SEA133Ra, Cloud-Clone Corp., Wuhan, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm against a blank using an ELISA

reader (RayBiotech, Canada). Assay results were expressed as pg ml⁻¹.

Short-chain fatty acids (SCFAs)

The faecal pellets (0.2 g) were extracted with ddH₂O (200 ml) and centrifuged at the speed of 13 000 rpm at 4°C for 20 min. H₃PO₄ (25%, 6 μ l) was added to the supernatant (54 μ l) with the ratio 1:9 of each sample and the supernatant was then filtered through a centrifugal filter (0.22 μ m) for measurement. The identification and quantification of six SCFAs, acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate were carried out by gas chromatography (GC, Agilent7890A; Agilent Technologies, Palo Alto, CA, USA) with a GC autosampler and a FID system (Zhang *et al.*, 2018). The GC was equipped with a DB-WAX column [Polyethylene Glycol 20000, 30 m \times 0.25 mm (ID), film thickness 0.25 μ m, Agilent Technologies]. Helium (> 99.999%) was used as carrier gas at a constant flow rate of 1 ml min⁻¹. The system was operated at a maximum temperature of 250°C. A 0.5 μ l sample was injected using a splitless injection and the injection temperature was 230°C. The temperature of the oven was programmed from 60°C for 1 min then increased at 5°C min⁻¹ to 200°C, and then at 10°C min⁻¹ to 230°C, and the total running time lasted for 32 min for each sample. The FID detector was set at 300°C, H₂ flow rate was 40 ml min⁻¹, air flow rate was 400 ml min⁻¹, and make-up N₂ flow rate was 25 ml min⁻¹. The SCFAs were identified by comparing their retention times with those of authentic reference compounds and quantified by the abundance relative to that of the standards.

CMT

In this study, the strategy for CMT was developed based on wild rodents and without use of antibiotics. Caecal contents were collected and mixed from three donors of the Control, Hyper and Hypo groups, respectively. The caecal contents (200 mg) were diluted in 2 ml sterile saline (0.9% sodium chloride), and then a 200 μ l suspension was delivered by intragastric gavage to the recipient gerbils for 3 days (once every day) with a modified method (Zhang *et al.*, 2018). For sham CMT, the recipient gerbils received an intragastric gavage of 200 μ l sterile saline on the same 3 days.

16S rRNA gene amplicon sequencing analysis

Total DNA was extracted from 180 to 220 mg faecal pellets using QIAamp® fast DNA stool kit (Germany) according to the manufacturer's protocols. The V3–V4 hypervariable regions of the 16S rRNA gene were

amplified using two universal primers (341F – 805R) (Table S5) (Zhang *et al.*, 2018). The sequencing was performed on an Illumina HiSeq 2500. The 16S sequence paired-end data set was joined and quality was filtered using the FLASH method (Magoc and Salzberg, 2011). We performed all sequences analysis using QIIME (version 1.9.1) software suite, according to the Qiime tutorial (<http://qiime.org/>) with some modified methods (Caporaso *et al.*, 2010). Chimeric sequences were removed using usearch61 with *de novo* models (Edgar, 2010). Sequences were clustered against the 2013 Greengenes (13_8 release) ribosomal database's 97% reference dataset. Sequences which did not match any entries in this reference were subsequently clustered into *de novo* operation taxonomic units (OTUs) at 97% similarity with UCLUST. Taxonomy was assigned to all OTUs using the RDP classifier within QIIME and the Greengenes reference data set. We calculated the rarefaction and rank abundance curves from OTU tables using α diversity and rank abundance scripts within the QIIME pipeline. The hierarchical clustering based on population profiles of the most common and abundant taxa was performed through UPGMA clustering (Unweighted Pair Group Method with Arithmetic Mean, also known as average linkage) on the distance matrix of OTU abundance and a Newick formatted tree was obtained using the QIIME package.

Statistical analysis

The data of RMR, NST_{max}, NST_{reg} and food intake were analysed by one-way or two-way ANCOVA or by repeated measure ANCOVA with body mass as a covariate. T_b , activity, DIO2, serum hormones and others were analysed by one-way or two-way ANOVA. Significant group differences were further evaluated using Tukey's *post hoc* tests. The software SPSS 17.0 was used for statistical analyses. Results are presented as mean \pm SEM, and $P < 0.05$ was considered to be statistically significant.

Gut microbial diversity was analysed using α diversity and β diversity. The α diversity was evaluated by calculating the Chao1, number of observed OTUs, Shannon, Simpson's, and PD whole tree. Comparing the differences of α diversity between groups was analysed by parametric *t*-test using the *t*-distribution at rarefaction depth 24 000. The β diversity was shown by PCoA based on unweighted and weighted UniFrac distances using evenly sampled OTU abundances at rarefaction depth 24 000, and statistical significances between groups were analysed by ANOSIM by 999 permutations. The LEfSe method was used to assess differences in microbial communities using an LDA score threshold of 2 (Segata *et al.*, 2011). The significant group differences of relative

abundances of specific bacteria were analysed by one-way ANOVA followed by Tukey's *post hoc* tests where required. Venn diagrams were made by jvenn (Bardou *et al.*, 2014). The correlation between specific OTUs and metabolic traits was based on Bootstrapping (–permutations 1000) and calculated via Pearson correlation. The level of statistical significance was set at $P < 0.05$ (False Discovery Rate (FDR)-corrected).

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Conflict of interest

The authors disclose no conflicts.

Authors' contributions

X.Y.Z. and D.H.W. conceived the study and design the experiments. S.K. performed most of the experiments. Z.N. performed the measurements by Western blot and ELISA kits. X.Y.Z. and S.K. analysed the data and wrote the manuscript. All authors have read and critically revised the manuscript.

Data Availability Statement

Raw sequence data are deposited in the NCBI Sequence Read Archive under accession PRJNA557008, PRJNA557014 and PRJNA557010.

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