

Beneficial Bacteria Stimulate Youthful Thyroid Gland Activity

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Abstract

Healthful aging with active participation in society are global public health priorities. Slender physique and high productivity levels absent clinical disease are widely recognized features of healthful aging. During studies of obesity in mice, we found that feeding of a purified probiotic microbe, *Lactobacillus reuteri*, forestalled typical old age-associated weight gain and lethargy, and instead conveyed physical features of much younger mice. We hypothesized that these retained features of youth may be related to increased thyroid gland activity. We subsequently discovered elevated levels of serum T4 and larger thyroid glands in slender one-year-old recipients of probiotic microbes, when compared with their age-matched obese control subjects. Oral *L. reuteri* treatment also preserved thyroid follicle epithelial height, a key histologic feature of thyroid gland activity, which relied mechanistically upon bacteria-triggered anti-inflammatory CD25⁺ regulatory T cells. These data from animal models suggest that probiotic microbe supplementation may be used to stimulate beneficial host immune interactions with improved thyroid function and more healthful aging.

Keywords: Probiotics; Obesity; Thyroid gland; Beneficial bacteria

Introduction

Some individuals are able to age in good health and remain active participants in society throughout their lives, while others experience physical and cognitive limitations, and may lose the ability to live independently [1]. Healthful aging is generally associated with host ability to respond to stress and infectious agents with efficient homeostatic balance and minimal pathology [2,3]. In many ways, modernized living conditions predispose to inactivity and excessive accumulation of body fat.

In general, the risk of obesity and inactivity rises substantially with increasing age. Subsequently, obesity contributes to increased mortality and morbidity by predisposing to serious pathological conditions such as type 2 diabetes, cardiovascular disease, fatty liver, arthritis, asthma, and neoplasia [4,5]. Clinical and experimental data suggest that this may be due to the fact that the adipose tissue of obese organisms is in a low-grade, persistent state of chronic inflammation which exerts adverse systemic effects [4,6] creating a downward inflammatory spiral contributing further to obesity [7].

The thyroid gland regulates how the body uses energy for activity or fat storage via thyroid hormones triiodothyronine (T3) and thyroxine (T4) [8], at least in part through interactions with the immune system [9]. These thyroid hormones regulate the body's metabolism, thermodynamics, and haemodynamics [10-12]. Levels of T3 and T4 are controlled by thyroid stimulating hormone (TSH) released by the pituitary gland in response to thyrotropin releasing hormone (TRH) produced in the hypothalamus. Together these hormones also regulate brain growth and rate of function of many different body systems [13,14].

Abnormally decreased thyroid activity, or hypothyroidism, is characterized by an underproduction of hormones T3 and T4. Consequently, the low levels of thyroid hormones fail to meet the metabolic needs of the body [15,16]. Symptoms of hypothyroidism include weight gain, lethargy, slow cognition, depression, menstrual irregularity, dry skin, and hair loss or baldness [15,17]. Hypothyroidism is more common in women than men and its prevalence increases after 45 years of age [18-20]. Hypothyroidism may also lead to congestive heart failure due to the increased systemic vascular resistance and

decreased cardiac contractility [11]. Autoimmune thyroiditis or Graves' disease treatments in humans are leading causes of hypothyroidism in the elderly [20]. To clinically detect hypothyroidism, it is recommended to test free T4 levels [15].

Age-related histological changes in the thyroid gland of mice [21,22] and other rodent species [23,24] include the presence of colloid-depleted follicles, increase of thyroid connective tissue stroma, formation of large coalescing multilocular cystic follicles and flattening of follicular epithelium [21]. The height of epithelial cells and the amount and staining properties of the colloid are major histological indicators of the thyroid follicle secretory activity, which are reduced in the involuting thyroid gland during aging [21]. These changes co-exist with decreased T4 serum concentrations [24, 25]. Thyroid dysfunction of this type occurs in humans and in mice. In humans, it affects mostly elderly women, occurring in up to 10% of women over the age of 60 [22].

It was previously shown that collaborative microbes influence development of obesity by inhibiting age-associated weight gain through microbial restructuring of host immunity [7,26]. Health-protective immune CD4⁺CD25⁺ regulatory T (Treg) cell-mediated events included resilient integument [27] with improved wound healing capability [28]. We hypothesized that immune-mediated thyroid gland activities may be involved in diverse aspects of good health, as well as inhibition of obesity. Knowing hypothyroidism is more common in women, we used female outbred Swiss Webster mice as a tractable experimental system for examining aspects of age associated thyroid functions. *Lactobacillus reuteri* ATCC-PTA-6475, that was originally isolated from human breast milk, served as a model probiotic microbe.

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In these studies, mice were analyzed by evaluating traditional aspects of senescence including weight gains and activity levels, as well as serum T4 levels and thyroid gland histopathology. We found that routine dosing with *L. reuteri* in drinking water not only conveyed external features and activity levels of much younger individuals, but also inhibited typical age-associated thyroid gland deterioration, relying mechanistically upon antigen educated CD25+ host immune cells.

Experimental Procedures

Animals

Genetically outbred CD-1 mice (Charles River; Wilmington, MA) were housed and handled in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facilities with diets, experimental methods, and housing as specifically approved by the Institutional Animal Care and Use Committee. The MIT CAC (IACUC) specifically approved the studies as well as the housing and handling of these animals. Mice were bred in-house to achieve experimental groups. The experimental design was to expose mice to diets starting at age=eight weeks, and then continue the treatment until euthanasia using carbon dioxide at five months or twelve months of age. Each experiment included 5-15 animals per group with two replications (total N=10-30 mice per group). Tissues for thyroid gland weight and histopathology were collected upon necropsy.

Special diets for animals

Mice of 6-8 wks were placed on an experimental control diet AIN-76A (Harlan-Teklad Madison WI) starting at 8 weeks of age until euthanasia. Subgroups were supplemented with a purified preparation of an anti-inflammatory strain of *Lactobacillus reuteri* ATCC PTA 6475 cultivated as described elsewhere [27,29] using a dosage of 3.5×10^5 organisms/mouse/day. Drinking water was replaced twice weekly. Viability of *L. reuteri* organisms in drinking water was assessed as previously described [30].

Home cage baseline activity assessment

To measure baseline locomotor activity levels, cages of mice were viewed for home cage activity for 30 minutes at the same time daily during three consecutive weeks. Initial activity levels were observed to plateau within the first 5 minutes, thus all subsequent data were evaluated using line crossings per minute in 5 minute intervals. Activity was measured from videotape recording, using the line crossings method with manual analysis [31]. Line crossing was defined as number of times a mouse crossed one of the gridlines with all four paws.

Measurements of thyroid hormone

Whole blood was collected terminally by cardiac puncture under general anesthesia to obtain serum. Serum was then tested commercially for T4 levels (*AniLytics*, Inc., Gaithersburg, MD). Euthanasia for mice was performed mid-day for all subjects to minimize variability due to Circadian rhythms.

Depletion of CD25+ cells

Mice were treated with anti-CD25 antibody (clone PC-61; Bio-Express, West Lebanon, NH) at 150 ug per mouse intraperitoneally 3X weekly for 12 weeks. Treated mice were compared to mice receiving sham isotype antibody alone. Depletion of CD25+ cells was confirmed by undetectably low fractions of CD25+ cells in spleens of mice treated with anti-CD25 antibody compared to sham-treated controls using flow cytometry. Depletion was confirmed by absence of Foxp3+ cells in spleen.

Histopathology and immunohistochemistry

For histologic evaluation, formalin-fixed thyroid glands were embedded in paraffin, cut at 4 μm , and stained with hematoxylin and eosin or immunohistochemistry. Four sections from different levels of the thyroid gland, having a distance of 150 μm from each other, were used for quantitative histomorphometry. Images of the whole thyroid gland section profiles taken at x4 magnification were used to assess the (a) average area size of follicle colloid-containing areas (μm^2), (b) the number of thyroid follicles per mm^2 of thyroid gland section profile and (c) total colloid substance per total thyroid gland section profile area ratio. Twenty five x40 magnification images, representing 60-80% of each thyroid gland section, were used to quantify the (d) number of follicular thyrocytes per follicular perimeter ratio and (e) thyroid follicle epithelial height (μm). To determine the mean value of parameters (a), (b) and (c) for each mouse, the thyroid gland section profile area in each section was subscribed and measured with the Image J image processing and analysis program (NIH, Bethesda, MD). Then the colloid areas of thyroid follicles were selected using the magic wand tool of Adobe Photoshop (Adobe Systems Inc.) and extracted from each image. The colloid areas appearing as particles in images were further processed with Image J (NIH, Bethesda, MD) with which the number and average size of particles were automatically determined using the "measure particles" plug-in. Colloid-devoid follicles when evident were counted separately and their number was added to the number of colloid-containing particles in order to determine the total number of thyroid follicles in each section. The mean of the values assessed from the 4 thyroid gland sections used per mouse was then recorded separately for each mouse. Using this morphometric approach the mean value of parameters (a), (b) and (c) resulted in from assessing approximately 650-1000 thyroid follicles per mouse. To determine the mean value of parameter (d) for each mouse the number of thyrocytes contained in each follicle and the perimeter of the same follicle were measured using the cell-counter plug-in and the measure command of Image J respectively. For parameter (e) the distance from the epithelial basal lamina to the apical cell membranes of thyrocytes was measured in the four outermost points (upper, down, left and right) of each follicle to determine its mean epithelial height. A total of 150-200 randomly-selected thyroid follicles from each mouse thyroid were used for this assessment.

Statistical analyses

The Mann-Whitney U test was used for body weight, diet, calorie consumption, and histomorphometry. A p-value<0.05 was statistically significant.

Results

Mice are slimmer after routinely consuming *L. reuteri* in drinking water

It is widely known that human subjects older than 30 years of age gain an average of one-pound-per-year throughout life, with adipose accumulations that predispose to disability and a wide variety of disease conditions. We previously showed that feeding of probiotic yogurt inhibited age associated weight gain in mice [7]. We subsequently examined aging in outbred Swiss female mice to test specific roles for probiotic microbes on maintenance of youthful body weights. Starting at 8 weeks of age, female Swiss mice were routinely fed purified *Lactobacillus reuteri* ATCC-PTA-6475 in regular drinking water and then examined for attitude and body weight at 12 -18 months of age. We found dramatic differences in phenotype and attitudes of

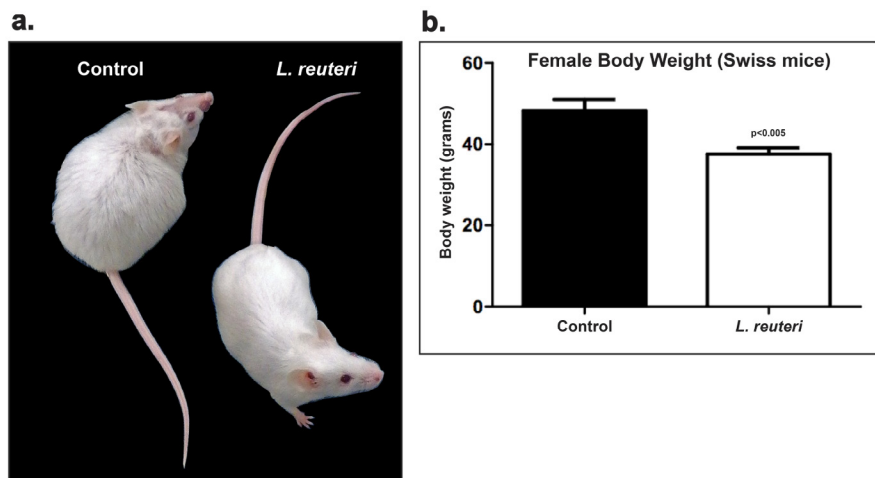


Figure 1: Aged mice eating *L. reuteri* have a youthful phenotype and live longer. (a) Typical phenotypes of female Swiss mice at 18 months of age. Mice fed with *L. reuteri* show clinical signs of good general health such as alert demeanor, shiny fur, and leaner physique in contrast to their aged matched controls. (b) At twelve months of age, the body weight of probiotic-treated mice was significantly lower than that of untreated control mice. The y-axis depicts the mean±SEM of mice body weight in grammars for each experimental group in (b).

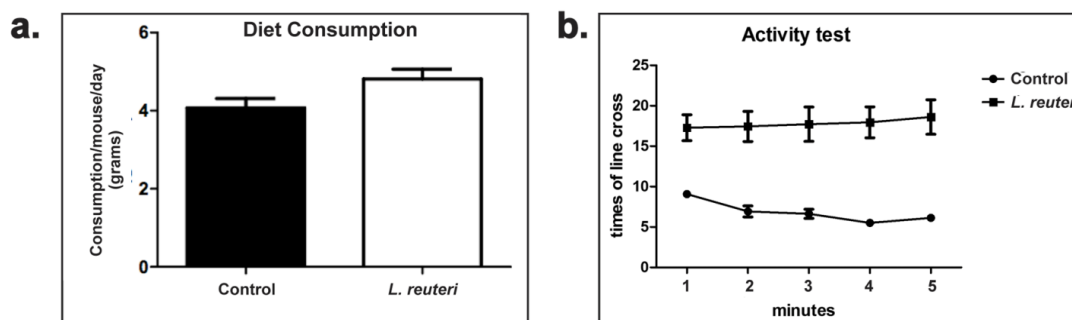


Figure 2: *L. reuteri*-associated effects on feed consumption and physical activity levels. (a) *L. reuteri*-treated and untreated one-year-old female outbred Swiss mice consume comparable amounts of feed daily. (b) Slender mice fed *L. reuteri* show significantly ($P < 0.0001$) increased levels of home cage mobility compared to untreated controls throughout the observation period. The y-axis represents the mean ± SEM of mouse chow consumed daily in grammars for each experimental group (a) or the number of times the mice were crossing standardized home cage landmark lines (b).

senility (Figure 1a) and body weight (Figure 1b) of animals receiving *L. reuteri* supplementation when compared with age-matched untreated control animals. These differences in body weight, skin character, and attitude between treatment groups were evident grossly upon casual physical exam, and have been previously quantified by mechanical and histological evaluation of target tissues [7,27].

Lean mice consuming *L. reuteri* choose similar food intake as obese controls

Given that mice remained lean when consuming *L. reuteri* in their drinking water, we next sought to determine whether *L. reuteri*-fed animals offered *ad libitum* standard mouse chow were thinner due to eating less food per day than their untreated control counterparts. When offered free choice of mouse chow, we discovered there were no significant differences in food consumption between treatment groups (Figure 2a) upon examining grams of control mouse chow eaten daily.

Eating *L. reuteri* increases general activity levels in mice

Considering the similar daily food consumption, the observation

of unusually lean mouse body weight outcomes after eating *L. reuteri* led us to postulate that probiotic fed mice may have increased levels of activity. We selected to monitor activity by videotape in the home cage setting, as this approach reduces handling and stress that can make data interpretation difficult [32]. Using a manual standardized evaluation of home cage activity levels in one-year-old female mice, we determined that mice eating the probiotic microbe were significantly more active than their age-matched untreated control counterparts eating the same *ad libitum* mouse chow diet (Figure 2b). This indicated that feeding of a purified probiotic organism was sufficient to re-direct food energy intake into activity rather than into fat storage.

Dietary *L. reuteri* supplementation up-regulates serum thyroxin (T4) levels

Knowing that serum T4 thyroid hormone levels regulate how the body uses energy for activity or fat storage, we hypothesized that oral *L. reuteri* dosing may be stimulating the production of T4. In order to test whether increased locomotory activity found in mice eating *L. reuteri* may be due to changes in thyroid hormones, we tested levels

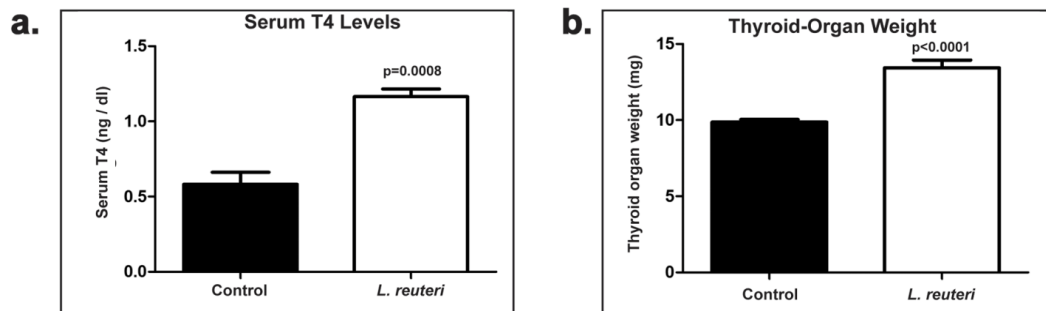


Figure 3: Dietary *L. reuteri* affects circulating T4 levels and thyroid gland weight.

(a) One-year-old female Swiss mice, when treated orally with probiotic bacteria, have significantly increased serum T4 and (b) heavier thyroid glands, when compared to aged-matched untreated controls. Numbers on the y-axis of bar graphs correspond to the mean \pm SEM of T4 ng/dl (a) and thyroid gland weight in milligrams.

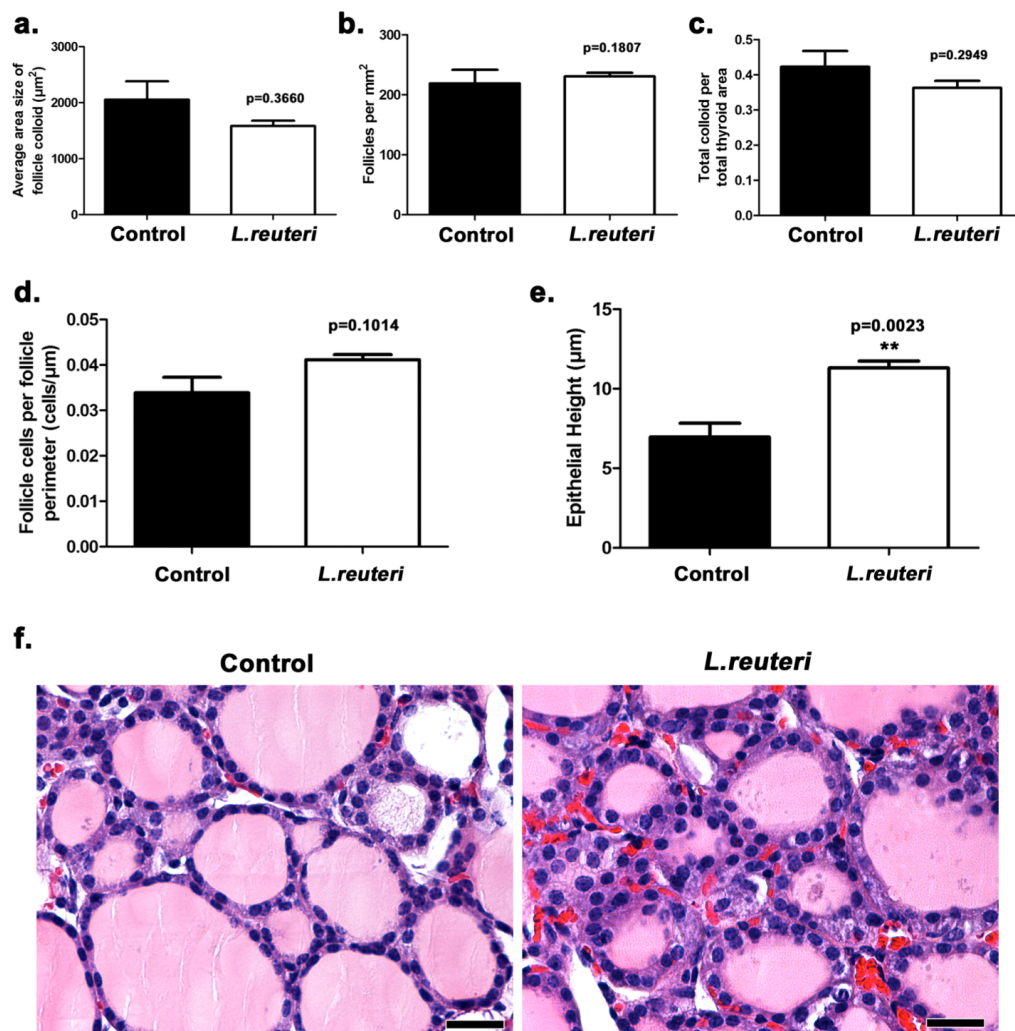


Figure 4: Effects of *L. reuteri* on thyroid gland histology at the age of 1 year (a) the average area size of follicle colloid-containing areas, (b) the number of thyroid follicles per area unit of thyroid gland section profile, (c) the total colloid substance per total thyroid gland section profile area and (d) the number of follicular thyrocytes per follicular perimeter ratio remain unaffected by the dietary supplementation of female swiss mice with *L. reuteri*. (e) However, at the same age the thyroid follicle epithelium is significantly higher in *L. reuteri*-treated mice compared to their controls. (f) Representative histology of the thyroid gland follicles of untreated (left) and *L. reuteri*-treated (right) one-year-old female mice. Compare the low cuboidal thyrocytes lining the follicles of untreated controls with the high columnar thyrocytes that line the follicles of the thyroid gland of probiotic-treated mice. Note the presence of colloid-devoid follicles in the upper right corner of the control mouse thyroid gland image. The y-axis in bar graphs (a to e) depicts the mean \pm SEM of histomorphometric counts in each experimental group. Hematoxylin and Eosin. Bars=25 μm (f).

of a key metabolic indicator, free thyroxin (T4), in mouse serum. As predicted, one-year-old mice drinking *L. reuteri* daily were found to have higher serum T4 levels (Figure 3a) when compared with untreated Swiss female mice of the same age. In our study, one-year-old untreated control mice exhibited underproduction of T4 and classical symptoms of hypothyroidism including obesity, lethargy, and hair loss [15,17].

Thyroid glands are larger in mice after consumption of *L. reuteri*

Thyroid glands typically involute with increasing age. The youthful levels of active thyroid T4 hormone in probiotic-fed mice led us to examine the mass of thyroid gland tissue. We thus weighed the isolated thyroid tissue upon necropsy and found significantly heavier thyroid glands in the slender and active one-year-old female mice routinely consuming *L. reuteri* organisms in their drinking water by comparison with their untreated counterparts (Figure 3b).

Feeding of *L. reuteri* leads to increased height in thyroid glandular epithelia

It is well established that a properly functioning thyroid gland and normal levels of serum T4 are required to maintain a healthy body weight and fat deposition. In order to more accurately probe the effects of dietary probiotics on thyroid gland health, we next performed morphometric measurements on thyroid tissue to identify histologically any detectable effects of *L. reuteri* consumption in thyroid glands. The histological examination of the thyroid gland of untreated control mice at the age of 1 year revealed focal age-related thyroid follicle changes including the presence of occasional colloid-depleted and large coalescing follicles and flattening of follicular epithelium. These lesions were practically undetectable in aged-matched mice fed with *L. reuteri* (Figure S1). The thyroid glands of both experimental groups of mice appeared to have similar proportions of thyroid connective tissue stroma. In order to more accurately probe the effects of dietary probiotics on thyroid gland, we next performed morphometric measurements of selected thyroid gland histomorphological parameters. We found that dietary supplementation with *L. reuteri* had no significant effect on the average area size of follicle colloid-containing areas (Figure 4a), the number of thyroid follicles per mm² of thyroid gland section profile (Figure 4b), the total colloid substance per total thyroid gland section profile area ratio (Figure 4c) and the number of follicular thyrocytes per follicular perimeter ratio (Figure 4d). These results suggested that probiotics did not affect the colloid production and storage capacity of thyroid follicles and thyroid gland. Also, probiotics had no effect on the density of thyroid follicles and the population of thyrocytes that line them. By contrast, we found that probiotic consumption was significantly correlated with a profoundly increased thyroid follicle epithelial height (Figure 4e and Figure 4f), which is a reliable indicator of thyroid follicle metabolic activity [28].

Recognizing that significant thyroid gland differences emerged in older (12 months-of-age) mice, we next sought to determine whether similar thyroid gland changes also occur in younger animals. We found that the thyroid follicle epithelial height was also significantly greater in 5-month-old Swiss female mice consuming the probiotic organism, when compared with their age-matched untreated controls (Figures 5a, 5b and 5e).

Thyroid gland benefits imbued by *L. reuteri* require CD25+ lymphocytes

It was demonstrated previously in animal models of obesity that the immune system plays a critical role in maintaining normal body

weight. Knowing that obesity in Swiss mice is specifically inhibited by *L. reuteri*-induced CD4+CD25+Foxp3+ lymphocytes [7], we performed similar cell depletion experiments to test whether CD25+ cells are required for thyroid gland health in this model. We found that the depletion of CD25+ lymphocytes in 5-month-old mice affected the activation status of their thyroid follicle epithelium as evident by its significantly decreased height (Figures 5c and 5e). The thyroid glands of the prematurely obese mice were also characterized by an increased number of colloid-depleted follicles by comparison with their immunocompetent controls. Interestingly, no evidence of thyroiditis was found in the CD25+ cell-depleted mice. Accumulations of small numbers of lymphocytes and myeloid precursor cells, however, were occasionally seen in the interfollicular connective tissue.

Finally, in order to determine specifically whether the *L. reuteri*-induced increase of thyroid follicle activity depends upon CD25+ cells, we morphometrically measured the thyroid follicle epithelial height in *L. reuteri*-fed CD25+ cell-depleted mice. We found that in the absence of CD25+ cells the beneficial effect of probiotics on epithelial height was entirely abolished (Figures 5d and 5e). This result suggests that the *L. reuteri*-induced increase of thyroid gland activity is mediated by CD25+ lymphocytes. Taken together with earlier work, this led us to propose an integrated model of microbe-induced bidirectional communication between host endocrinology and the immune system (Figure 6).

Discussion

We utilized an animal model to examine microbial mechanisms of modulating obesity and aging in human females. In these studies, the naturally aging genetically outbred female mice mimicked adiposity patterns seen in human females. Age-matched mice eating a purified probiotic microbe displayed: 1) a lean outcome despite eating similar diet, 2) increased homecage activity levels, 3) elevated levels of a key serum thyroid hormone free T4, 4) larger thyroid glands with diminished age-related pathologies and 5) increased thyroid follicle epithelial height typical of active thyroid glands. These gut bacteria-induced features relied mechanistically upon host CD25+ cells. Taken together, these observations led us to propose a circular mechanistic model where GI microbiome composition affects host immunity [33,34], which affects thyroid gland functioning [35-37], which in turn influences host immunity, and so forth. Once initiated, immune feedback loops interconnecting the hypothalamic-pituitary axis with thyroid gland, adrenals, and gonads [3], may then become self-sustaining for healthful aging.

The aging process involves a functional decline in both the immune system and the neuroendocrine system, whereby an impaired relationship causes a homeostatic imbalance with increased risk of death [38,39]. Indeed, it is generally accepted that a bidirectional communication between the nervous and the immune systems is fundamental in good health [40]. The neuroendocrine theory of aging proposes that senility is due to changes in neural and endocrine functions that are crucial for coordination and responsiveness to the external environment, programming physiological responses to environmental stimuli, and maintenance of optimal functioning in reproduction and survival [3]. The regulation of these functions is controlled by hypothalamic hormones interfacing with peripheral endocrine glands (e.g., adrenal cortex, thyroid gland, gonads) [3]. Our recent work has shown that the consumable probiotic microbe *L. reuteri* initiates immune and neuroendocrine events that intervene to halt neuroendocrine aging pathways. Specifically, we have shown that eating probiotic microbes prevents age related testicular atrophy and preserves youthful serum testosterone levels in aged mice [41]. Supplemental

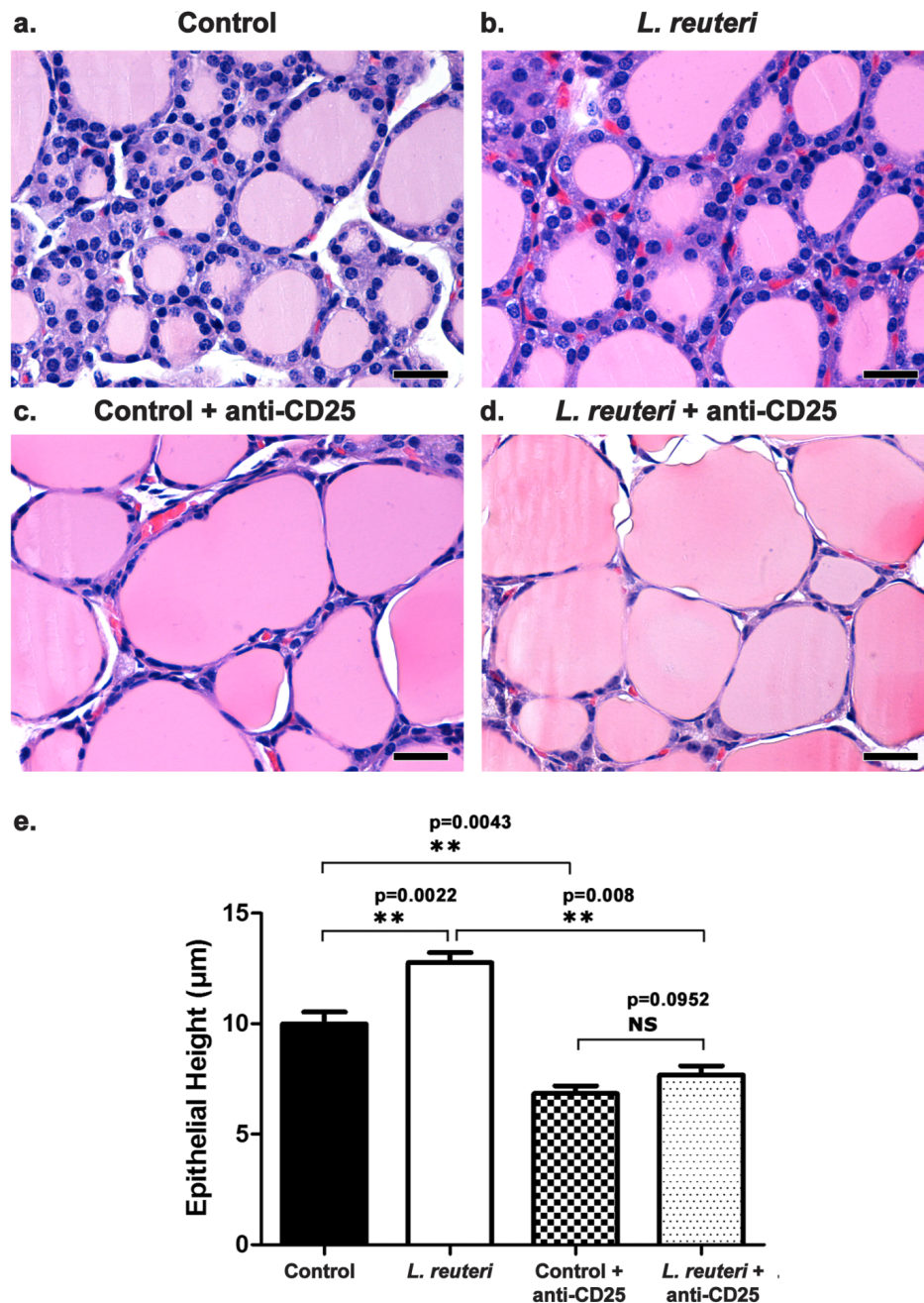
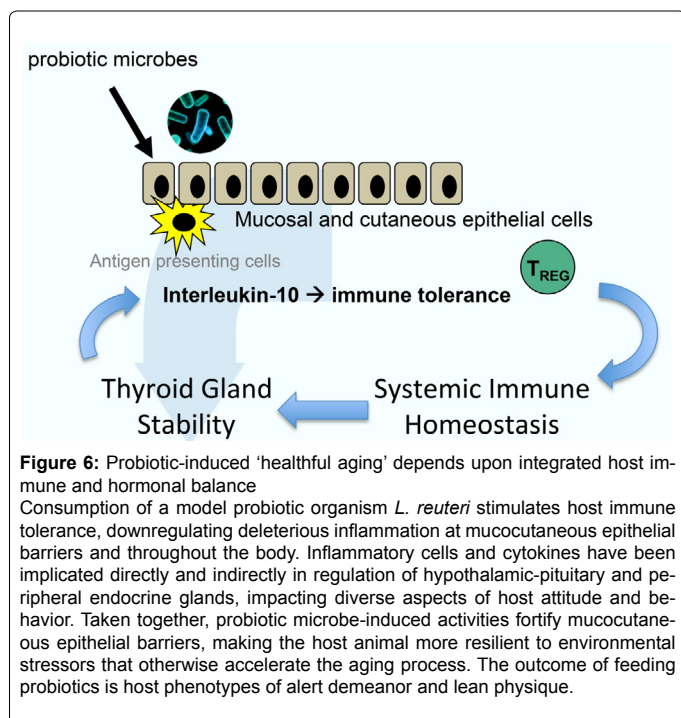


Figure 5: The *L. reuteri* effect on thyroid follicle epithelium height is mediated by CD25+ lymphocytes. Side-by-side comparison of the thyroid follicle epithelium of Swiss female mice at the age of 5 months: (a) control and (b) *L. reuteri*-treated mice. The thyroid follicles of probiotic-treated mice are lined by epithelial cells of increased height. (c) At the same age, the thyroid glands of CD25+ cell-depleted female Swiss mice showed an abundance of inactive, distended follicles lined by a flattened follicular epithelium. (d) Their *L. reuteri*-fed counterparts had thyroid glands with similar histology, suggesting that the *L. reuteri*-associated effect on thyroid gland depends upon the CD25+ cell population. (e) Bar graph of epithelial height measurements in different experimental groups (age=5 months). The mean \pm SEM of epithelial height is demonstrated in the y-axis. Hematoxylin and Eosin. Bars=25 μ m (a to d).

feeding of *L. reuteri* to mice also served to up-regulate plasma oxytocin levels, and, as a result, reprogram host immunity for improved wound repair capability [28]. In each case, systemic benefits of probiotic microbes are transplantable using purified CD4+CD25+Foxp3+ cells [28]. Experimental or natural depletion of CD25+ cells interrupts beneficial immune function and subsequently impairs neuroendocrine health, as well. Thus, probiotic microbes may serve to stimulate immune

balance influencing a hypothalamic-pituitary axis with thyroid gland, adrenals, and gonads, which together impart homeostasis required for healthful longevity. In general, aged human subjects maintaining their immune functions at an exceptionally high level are more likely to have sustained good health and a long life span [42,43].

Upon macroscopic examination, the thyroid glands of *L. reuteri*-treated aged mice were consistently larger when compared to the thyroid



glands of non-treated mice. In order to quantify this observation we weighed the mouse thyroids and found that thyroids of probiotic-treated mice were heavier. The increase of thyroid weight in senile rodents has been described as an age-related pathology that probably occurs due to the increase of interfollicular connective tissue, proliferative epithelial changes and abundance of large cystic, metabolically inactive follicles, the so-called "cold follicles" [23,44]. The histological examination and the histomorphometry of the thyroid glands revealed that the probiotic-fed mice in the present study did not have these lesions. By contrast, thyroid follicle epithelial height was found significantly increased in these mice compared to their aged-matched controls, which suggests increased thyroid follicle activity. The increased size and weight of the thyroid glands taken together with unchanged follicle, colloid and thyrocyte density per tissue unit area in probiotic-fed mice could explain the *L. reuteri*-associated increase of serum T4. Following this reasoning, the increased T4 levels may be due to an increase of total thyroid gland colloid substance productivity. In this case, however, the later should be a consequence of a "physiological" increase of thyroid gland size without follicular or other architectural abnormalities or alterations at the tissue level.

An increase of thyrocyte height may be due to either increased colloid production or the release of T4 [45]. The histomorphometrical analysis in the present study suggests that the increased epithelial height observed in the thyroid follicles of mice consuming *L. reuteri* is not due to increased colloid production in follicles. The logical consequence is that this finding reflects the increased T4 release activity, which matches the increased serum T4 levels found in the probiotic-treated mice.

In the present study, we found that female mice with elevated T4 levels remained slender in spite of their increasing age. Another cause for weight gain in peri-menopausal women is declining estrogen levels [46]. During menopause, ovarian estrogen production declines, producing symptoms similar to hypothyroidism such as thermoregulation, depression, and vaginal dryness [47]. In addition to roles of estrogen in maintaining the reproductive tissues, estrogen also aids

in energy homeostasis, bone remodeling, neuroprotection, and core body temperature [48]. Estrogen is known to have direct effects on thyroid cells [49]. Estrogen participates in a complex thyroid feedback loop by increasing thyroid-binding globin levels, decreasing free T4 levels [50,51] and raising levels of TSH [52,53]. Our unpublished data reveals elevated serum estrogen levels in aging female Swiss mice, an interesting aspect needing further investigation. In fact, thyroid dysfunction is sometimes associated with menopause [54]. Several large cohort studies found hypothyroidism in 7.5% of all women in the Whickman survey [55], 12% of women over 60 in a Framingham Heart Study [20], 16% in women aged 65-74 years and 21% in women aged 75 and over in a Colorado Thyroid Disease Prevalence Study [56]. Likewise, hypothyroidism has been associated with female reproductive disorders such as infertility and abnormal menses with treatment restoring normal cycles and fertility [57-61]. Ovariectomies, a standard research protocol to induce menopause in experimental animals [48], may be used to help determine the interactions between thyroid and estrogen hormone related metabolic changes in our aging female mice.

Knowing that healthful aging is generally associated with host ability to respond to stress and infections with efficient homeostasis and minimal pathology [2,3], we surmise that probiotic-induced 'healthful aging' presently described involves gut microbe-immune interactions culminating in improved Treg functions that make the host more resilient to environmental stressors that otherwise accelerate the aging process. This process appears to involve complex hormonal feedback loops involving interactive immune tolerance and the hypothalamic-pituitary axis with peripheral endocrine glands, conveying features of youth and reproductive fitness together that impart evolutionary success to both the symbiotic bacteria and their mammalian hosts. Ultimately it will be important to test whether human subjects may similarly benefit from eating *L. reuteri* or other probiotic organisms. These data suggest that exposure to probiotic microbes may provide ability to age in good health, remain slender, and be an active participant in society throughout life.

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