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Metabolism, bioenergetics and thermal physiology: influences of the human intestinal microbiota

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Abstract

The micro-organisms which inhabit the human gut (i.e. the intestinal microbiota) influence numerous human biochemical pathways and physiological functions. The present review focuses on two questions, 'Are intestinal microbiota effects measurable and meaningful?' and 'What research methods and variables are influenced by intestinal microbiota effects?'. These questions are considered with respect to doubly labelled water measurements of energy expenditure, heat balance calculations and models, measurements of RMR via indirect calorimetry, and diet-induced energy expenditure. Several lines of evidence suggest that the intestinal microbiota introduces measurement variability and measurement errors which have been overlooked in research studies involving nutrition, bioenergetics, physiology and temperature regulation. Therefore, we recommend that present conceptual models and research techniques be updated via future experiments, to account for the metabolic processes and regulatory influences of the intestinal microbiota.

Key words: **Bacteria: Fermentation: Energy expenditure: Doubly labelled water: RMR**

Introduction

The human intestinal microbiota (IM) contains a diverse array of micro-organisms that inhabit the surface and contents of the gastrointestinal tract[1]. Among more than 1000 species[2], Bacteroidetes (genera *Bacteroides* and *Prevotella*) and the Firmicutes (genera *Clostridium*, *Eubacterium* and *Ruminococcus*) account for more than 90 % of the IM population[3]. The collective genome of this diverse gut ecosystem[4–6] contains at least $9{\cdot}8 \times 10^6$ genes and is >490 times larger than the 20 000 protein-coding genes in the human genome[4]. The IM communicates with the host, consumes, stores and redistributes energy and nutrients, mediates important chemical transformations, and replicates to maintain and repair itself. Indeed, host–IM interactions have important evolutionary significance[7] because natural selection acts upon the integrated host–IM organism known as the holobiont (i.e. consisting of interactive biomolecular networks), and its collective genome known as the hologenome (i.e. consisting of the nuclear genome, organelles and microbiome). Microbes may be acquired from the environment, can be constant or inconstant in the host, and holobiont phenotypes can change in time and space as microbes move into and out of the holobiont[8]. Considering the numerous IM–host interactions, the primary purpose of the present review is to describe research methods that may be influenced by the IM, during measurements of metabolism, energy expenditure and temperature regulation. It is relevant that some of these IM effects are sufficiently large to have a measurable impact on physiological responses and research data. Because few published investigations have considered or acknowledged these effects, the IM represents an uncontrolled, unmeasured factor in the design of many human experiments.

Host and intestinal microbiota co-metabolism

Along approximately 200 m^2 of intestinal surface area[9], the metabolic processes of the IM vary, depending on dietary substrates and intermediate metabolites formed[10,11]. For example, 10–20 % of dietary carbohydrates are resistant to digestion in the human small intestine, including forms of resistant starch and NSP (i.e. cereals, raw banana, potato, pectin, cellulose) that are not degraded by amylase (i.e. present in saliva and produced by the pancreas). These carbohydrates pass to the colon, where bacterial fermentation converts them to SCFA (for example, acetate, propionate, butyrate), lactate, and gases such as CO_2, H_2 and CH_4[12]. Proteins are degraded to peptides and amino acids, whose fermentation also results in the formation of SCFA, CO_2 and H_2[13]. In addition to SCFA, microbes produce other metabolites including secondary bile acids, amino acid derivatives and vitamins[14]. Via these products, the gut microbiota can influence host whole-body metabolism[15,16]; inflammation and gene expression[17]; diurnal rhythms of the host[18,19]; absorption of electrolytes and minerals[20]; adipose tissue[21]; as well as the renal, cardiovascular[22], musculoskeletal[23] and neuroendocrine[24,25] systems.

Abbreviations: DEE, diet-induced energy expenditure; DLW, doubly labelled water; IM, intestinal microbiota; $_rCO_2$, rate of CO_2 production.

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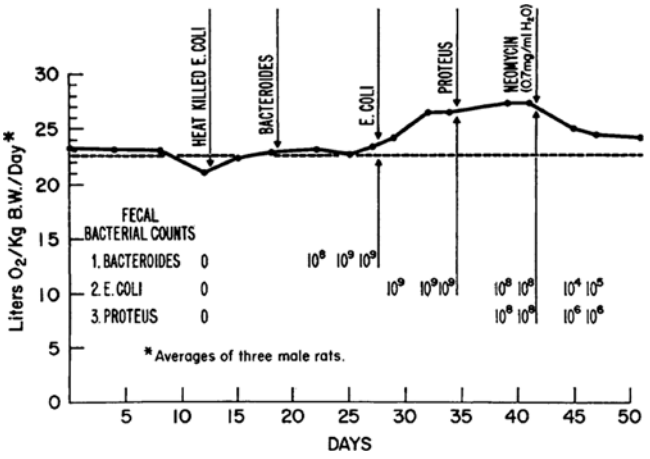

Fig. 1. Oxygen consumption of germ-free rats during sequential feedings of heat-killed *Escherichia coli* and *Bacteroides*, followed by feedings of live *E. coli* and *Proteus* (both Gram-negative bacteria). Neomycin (0·7 mg/ml drinking water per 24 h) was administered for 7 d. Faecal counts are expressed as viable bacteria per g of faeces. B.W., body weight. Reprinted with permission from Levenson *et al.*[34].

Evidence also suggests that interactions occur between the IM and host energy balance, nutrient absorption[26–28] and processing of carbohydrates[29] and complex dietary lipids[30]. Three research studies exemplify these interactions. First, an in-patient energy balance study demonstrated that altered nutrient load (2400 *v.* 3400 kcal (10 042 *v.* 14 225 kJ) diets) induced rapid changes in the bacterial composition of the human gut microbiota, and that these changes correlated well with the stool energy loss of lean individuals[31]. Increased Firmicutes and reduced Bacteroidetes counts were associated with an increased energy harvest of about 150 kcal (628 kJ) per 3 d. Second, Vrieze *et al.*[32] conducted a randomised clinical trial to study the effects of infusing IM (i.e. from lean donors into nine male recipients with the metabolic syndrome) on glucose metabolism and IM composition. At 6 weeks after the faecal transplant, insulin sensitivity of the recipients increased (glucose disappearance before, 26·2 *v.* post, 45·3 mol/kg per min; $P<0·05$), as did the abundance of butyrate-producing IM (*Roseburia intestinalis* in faecal samples and *Eubacterium hallii* in intestinal biopsy samples). Because butyrate is produced both in the large and small intestines for energy and signalling purposes, and because orally administrated butyrate has direct effects on glucose metabolism, these findings suggest a regulating role for butyrate that is derived from gut microbial metabolism, leading to improved insulin sensitivity. Third, to investigate the effects of cold air exposure on energy homeostasis, Chevalier *et al.*[33] transplanted faecal microbiota from cold-exposed or control mice into germ-free animals. Following cold air exposure, comparison of phylum-level proportions in faeces showed that Firmicutes abundance increased (18·6 to 60·5 %) and Bacteroidetes decreased (72·6 to 35·2 %). Interestingly, both processes (i.e. cold air exposure at 6°C for 30 d and transplantation of faecal matter from cold-exposed mice into germ-free mice) increased insulin sensitivity and caused browning of white adipose tissue. This suggested that infusing the IM of cold-exposed mice was sufficient to transfer part of this phenotype, including increased energy expenditure and lower body fat content[33].

Levenson *et al.*[34] reported that small animal metabolic rate increased following administration of selected strains of intestinal bacteria. Fig. 1 illustrates the changes of O_2 consumption (litres O_2/kg) and faecal bacterial counts across 50 d, during sequential oral feedings of heat-killed *Escherichia coli* (facultative anaerobes), live *Bacteroides* (obligate anaerobes), live *E. coli* and live *Proteus* (facultative anaerobes). Also, after addition of the antibiotic neomycin to rat drinking water (0·7 mg/ml; day 42 in Fig. 1), the number of live organisms per g of faeces dropped 5–7 d later, concurrent with a decrease in O_2 consumption and CO_2 production. Levenson *et al.*[35] believed that intestinal facultative anaerobes, such as those described in Table 1, were responsible for the increased O_2 consumption (day 28 to day 43 in Fig. 1), probably because the rapid rate of increase following administration of live *E. coli* (day 28 to 32 in Fig. 1) suggested an exponential bacterial growth phase[36,37]. Employing a different research design[35], the overnight fasting O_2 uptake of both conventionalised rats (i.e. littermates of the germ-free rats contaminated with caecal contents of open-animal-room rats on the day after weaning) and germ-free animals receiving faecal transplants was 15–20 % greater than that of germ-free counterparts, with no between-group difference of RQ (V_{CO2}/V_{O2}). However, neither immune responses nor macrophage metabolism/growth curves were measured in either of these studies[34,35]. This is important because, during controlled laboratory incubations, the O_2 consumption rate of isolated human bronchial macrophage cells was 0·49 μmol $O_2/5 \times 10^6$ cells per h[38]; similar values (0·17–0·20 μmol $O_2/10^6$ cells per h) were reported for rabbit alveolar macrophages[39] and isolated IM bacteria (Table 2). Thus, the increased energetic cost of an inflammatory response (i.e. following oral intake or faecal transplant) may include the sum of bacterial metabolism (Table 2), the metabolism of macrophages and other innate immune cells, plus host adaptive immune bioenergetic responses (see the section below titled 'Indirect calorimetry of RMR'). Until new methods allow measurement of the *in vivo* energy metabolism of innate and adaptive immune cells, the precise contribution of macrophage cells will remain unknown.

Doubly labelled water measurements of energy expenditure

Tables 1 and 2 demonstrate that IM bacteria utilise aerobic and/or anaerobic metabolism, along the course of the human intestine. We propose that the metabolic processes of the IM influence the doubly labelled water (DLW) method of measuring energy expenditure; however, no mention of IM effects appear in the human scientific literature. The DLW method is theoretically based on the differential turnover kinetics of the stable isotopes of oxygen (^{18}O) and hydrogen (^{2}H). After drinking a known mass of DLW ($^{2}H_2{}^{18}O$), ^{2}H is eliminated from body water as H_2O whereas ^{18}O is eliminated as H_2O and CO_2[40]. The difference between these two elimination rates is proportional to the rate of CO_2 production ($_rCO_2$) over time, and therefore energy expenditure. This non-invasive technique, utilised when direct calorimetry measurements are not possible or feasible (i.e. field studies, across weeks), is considered by many investigators to be the most accurate available[41–43]. However, the assumptions

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Table 1. Number of obligate anaerobic, facultative anaerobic and obligate aerobic bacteria in the human intestine

Anatomical site	Obligate anaerobes*	Facultative anaerobes†	Obligate aerobes‡	Number of subjects or samples	Reference
Jejunum	5–6§	4–5§	‖	7	Nelson & Mata[152]
Colon	6·4–8·0§	7·3–8·5§	5·5–8·0§	6	
Jejunum	3·7–4·3§	2·3–3·8§	3·3–3·8§	4	Peach *et al.*[153]
Terminal ileum	2·6–3·7§	0·6–4·9§	2·4–4·9§	10	
Colon	3·1–6·3§	3·4–6·0§	4·9–6·0§	6	
Rectum	6·0–6·2§	3·0–5·7§	5·6–5·8§	2	
Caecum	7·2–8·3¶	<2·9–6·1¶	4·0–7·4¶	4	Croucher *et al.*[154]
Ascending colon	7·0–8·4¶	2·8–6·3¶	3·6–6·5¶	4	
Transverse colon	6·2–8·2¶	<2·8–5·9¶	3·6–6·6¶	4	
Sigmoid colon	6·5–8·1¶	<2·9–7·3¶	3·8–7·6¶	3 or 4	
Large intestine**	7·8††	2·2–6·6††	4·9††	6	Langlands *et al.*[155]
Rectum‡‡	2·3–7·0§§	1·9–6·5§§	‖	10	Macfarlane *et al.*[156]

* These bacteria are poisoned by O_2 and metabolise energy via anaerobic respiration or fermentation.
† Grow with and without O_2.
‡ Require O_2 because they cannot perform anaerobic fermentation or respiration.
§ Log_{10} organisms per g of biopsied tissue.
‖ None was observed, not reported.
¶ Log_{10} organisms per g wet weight of colon wall, sudden death cadaver dissection.
** Colonoscopy biopsies sampled at the caecum, transverse colon, descending colon and rectum.
†† Mean log_{10} colony-forming units observed on agar.
‡‡ Endoscopy biopsies sampled at the rectum.
§§ Log_{10} organisms per cm^2 of biopsied tissue.

inherent in the DLW method have been challenged by multiple authors[44–49], using various lines of reasoning, without mentioning gut bacteria.

We propose that the characteristics and metabolism of the human IM directly influence DLW measurements of energy expenditure in three ways. First, as noted above, the DLW method relies on the elimination of ^2H and ^{18}O isotopes as water[40]. However, numerous IM metabolic reactions produce water, in addition to the water shown in equation 1[50–53]. This synthesised water may or may not alter the calculation of $_rCO_2$, depending on whether IM bacteria have internalised ^2H and ^{18}O isotopes; evidence[54] demonstrates that bacteria assimilate heavy water (deuterium oxide, D_2O), but the rate of uptake depends on the phase of growth or maintenance and the distinctive functional or genomic characteristics of each bacterial species. Second, direct measurements of colonic gases suggest that significant amounts of CO_2 and H_2 exist in the colon[55,56]. As these gases combine, they form CH_4 plus water[57]:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (1)$$

Because the DLW method relies on the differential kinetics of ^2H and ^{18}O (see above), the bacterial biosynthesis of CH_4 in the colon influences accuracy, via the loss of ^2H as C^2H_4. Recognising that this biochemical conversion causes underestimation of $_rCO_2$ (the error in ruminants ranged from –3·27 to –6·54 %), Midwood *et al.*[47] recommended that the calculation of $_rCO_2$ include a factor that corrects for IM CH_4 production during fermentation. Interestingly, the incidence of CH_4 production in healthy Scandinavian adults, determined by a single midday breath sample, was reported to be 41 %[58] and 44 %[59], respectively, of those who participated in separate investigations. This suggests that humans may be either methanogenic or non-methanogenic, depending on the existence of H_2-utilising gut bacteria. Relevant to this, Bjørneklett & Jenssen[59] reported that breath testing of methanogenic adults typically showed either high excretion of H_2 and low excretion of CH_4 or *vice versa*, suggesting an inverse relationship between H_2S and CH_4 production. Other relevant biochemical reactions may include H_2 production and acetogenesis which utilises CO_2 to produce acetate and acts as an H sink, depending on the abundance of specific IM species, diet composition, and the amount and type of resistant or undigested carbohydrates consumed[60]. The complex interactions of these factors are difficult to unravel *in vivo* and with present-day research methods; thus the magnitude of effects due to one or all pathways are unknown in humans. Third, the DLW model assumes that the stable isotopes ^2H and ^{18}O exist in pools which are homogeneous and constant[45,61]. To the contrary, the growth and biosynthetic reactions of IM bacteria cause fractionation of stable isotopes in the gut[62]; this affects the relative abundance of isotopes ($^1H/^2H$ and $^{16}O/^{18}O$) within bacterial cells, in relation to the total body water pool. For example, up to 70 % of intracellular water in growth-phase *E. coli* can be derived from metabolism, and can be isotopically distinct from the ^2H and ^{18}O isotope dilution spaces[63]. This probably occurs because the metabolic production of water exceeds the rate of isotopic equilibrium across cell membranes[64]. This bacterial pool, which consists of trillions of bacterial cells in the human intestine[6], influences, in unknown ways, the ingested DLW dose ($^2H_2{}^{18}O$) during water absorption from the intestine into blood. This perspective of the human intestine (i.e. that IM cells constitute an unmeasured isotope dilution space) supports a published model[64] which proposes that the isotopic composition of body water may represent a heterogeneous mosaic of local body water pools. Although the influence of bacterial fractionation probably is small, we are not aware of any published research that acknowledges this effect as part of DLW models or data analyses.

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Table 2. Oxygen consumption (mmol oxygen/l culture medium per h) and carbon dioxide evolution (mmol carbon dioxide/l culture medium per h) rates of intestinal bacteria, measured during controlled laboratory incubations

Bacterial species*	Median	Maximum	Duration (h)	Bacterial cell count (per litre culture medium)	Culture medium	Incubation conditions	Reference
	O_2 consumed†						
Escherichia coli	22 ml O_2/min per ml‡	24 ml O_2/min per ml‡	3·5–4·3	$4·2 \times 10^9$	§	37·5°C, pH 7·5	Martin[36]
E. coli	42‡	52‡	‖	‖	¶	37°C, pH 7·0	Cooney *et al.*[68]
Bacillus subtilis	48‡	64‡	‖	‖	¶	37°C, pH 7·0	Cooney *et al.*[68]
E. coli	20‡	83‡	10–16	1–8 g DW	**	37°C, pH 7·0	Luong & Volesky[37]
	CO_2 produced†						
E. coli	40‡	68‡	‖	‖	¶	37°C, pH 7·0	Cooney *et al.*[68]
B. subtilis	35‡	82‡	‖	‖	¶	37°C, pH 7·0	Cooney *et al.*[68]

DW, dry weight of bacterial mass; IM, intestinal microbiome.
* All are facultative anaerobes which reside in the human IM.
† Dependent on species, metabolic substrate in culture medium, temperature, pH and incubation apparatus employed in each investigation.
‡ Data derived from a graph in the original publication.
§ Peptone water + 0·5 % NaCl.
‖ Not reported.
¶ Glucose + trace mixed salt solution.
** Glucose + lactose.

The average mass of faecal contents in the human colon is 285 g wet weight, at a single point in time[65]. We calculate that the mass of faecal bacteria contains 46 g DM (see section below titled 'Intestinal microbiota habitats') and 112 g water[66]. Thus, the water in faecal bacteria is small (<1 %) relative to the total body water of a 70 kg male (43 litres). In the section below, we also note that bacteria inhabiting the intestinal mucosa represent an unknown and underappreciated factor in microbial biology. Future research that clarifies their number and biomass also will allow calculation of their effect on the DLW technique. However, even if the total mucosal bacteria biomass and water content equals, or exceeds that of faecal bacteria by 2- to 4-fold, the overall effect of the IM on DLW measurements probably will be small.

Models of human heat balance

Heat is an inevitable by-product of bacterial growth and the formation of intermediate metabolites[67]. Utilising enclosed laboratory apparatus, investigators have observed that bacterial O_2 consumption and CO_2 production are directly proportional to heat production[36,37,68]. However, the rate of heat production varies (Table 3), depending on the bacterial species, concentration and pulsing of substrates[37,69,70], and intestinal transit time[31]. Because estimates of the total number of bacterial cells in a 70 kg reference adult range from 10^{13} to 10^{14}[6,71], and because micro-organisms produce more heat per unit of mass than any other organism[72], it is relevant to ask, 'What is the magnitude of heat production by the human IM?', 'Does this quantity influence experimental measurements of human heat balance and temperature regulation?' and 'What is the inter-individual variation in heat balance?'.

In the text below, we estimate that the dry weight of faecal bacteria in the human colon is 46 g (see section titled 'Intestinal microbiota inhabitants'). In Table 3, we present the median heat production of *Lactobacillus helveticus*[73] during anaerobic fermentation of glucose (800 mW/g dry weight) as an example. Calculating the product of these two values, we estimate that the rate of IM heat production in the human colon is 32 kcal/h (134 kJ/h) for faecal, but not mucosal, bacteria. This rate of IM heat production is considerable, when compared with both the resting energy expenditure of men (42 % of 76 kcal/h (318 kJ/h)) and women (52 % of 62 kcal/h (259 kJ/h)), as well as the total energy expenditure of men (23 % of 140 kcal/h (586 kJ/h)) and women (34 % of 94 kcal/h (393 kJ/h))[74]. Also in the text below, we describe the bacterial inhabitants of the intestinal mucosa and note that their numbers, biomass and metabolic heat production may approach or equal the bacteria that inhabit faecal samples; this IM contribution has not been acknowledged in previous publications (see section below titled 'Heat balance calculations'). Thus, we believe that thermal balance research which does not consider the IM (Table 3) has omitted, or incorrectly attributed (for example, to the metabolic heat produced by host tissues), an important component of heat balance. Two animal investigations, conducted at the University of Michigan Medical School, support this proposition. These studies demonstrated that the presence of Gram-negative and Gram-positive bacteria in the gastrointestinal tract of rats and mice caused a continuous low-grade elevation (0·4–0·9°C) of the thermoregulatory set point without influencing the normal circadian rhythm of body temperature[75], that non-absorbable antibiotics (i.e. which remained in the intestine) lowered resting body temperature, and that these effects occurred during day and night hours independent of activity level[76].

In the human colon, fermentation is the predominant metabolic process[77], due to the fact that the partial pressure of O_2 in the intestinal lumen progressively decreases from the gastric fundus (77 mmHg) to the sigmoid colon (39 mmHg) and rectum (<1 mmHg)[78]. This type of anaerobic energy metabolism is similar to that which occurs in the rumen and colon of goats, sheep and cows[79]. The exact contribution of fermentation to the overall energy balance of an organism is unknown, but fermentative energy which evolves as heat in the colon has been calculated as 7 %[79] to 10 %[80] of normal daily energy metabolism (i.e. RMR plus energy expenditure during activities) in human subjects, and 5–6 % in sheep[81–83]. This percentage of

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Table 3. Metabolic heat production of intestinal bacteria, measured during controlled calorimetry experiments

Bacterial species*	Heat produced (per litre culture medium per h)†		Duration (h)	Bacterial content (per litre culture medium)	Culture medium	Incubation conditions	Reference
	Median/steady rate	Maximum					
Bacillus subtilis	6 kcal (25 kJ)‡	9 kcal (38 kJ)‡	§	§	‖	37°C, pH 7·0	Cooney *et al.*[68]
Escherichia coli	6 kcal (25 kJ)‡	8 kcal (33 kJ)‡	§	§	‖	37°C, pH 7·0	Cooney *et al.*[68]
E. coli	22 kJ (5 kcal)‡	43 kJ (10 kcal)‡	10–16	1–8 g DW	¶	37°C, pH 7·0	Luong & Volesky[37]
Lactobacillus helveticus	800 mW/g DW‡	1000 mW/g DW‡	11	0·1–1·8 g DW‡	**	42°C, pH 5·9	Liu *et al.*[73]

DW, dry weight of bacterial mass; IM, intestinal microbiota.
* All species are facultative anaerobes that reside in the human IM.
† Dependent on species, metabolic substrate in culture medium, temperature, pH and incubation apparatus employed in each investigation.
‡ Data derived from a graph in the original publication.
§ Not reported.
‖ (Glucose or molasses) + trace mixed salt solution.
¶ Glucose or (glucose + lactate).
** Glucose + yeast + various mineral salts.

daily energy metabolism is likely to be greater among individuals who consume only plant-based diets (for example, 100 g/d of fermentable carbohydrate[84]) because the metabolism of 1 g of plant material generates 2·8–3·7 kcal (11·7–15·5 kJ) of heat[85].

Under certain conditions, the composition of dietary nutrients in the gut causes the rate of bacterial heat production to increase greatly. Known as bacterial spilling or futile cycling, this process involves uncoupling of bacterial respiration from ATP synthesis, during which excess ATP energy is dissipated as heat[86]. This metabolic inefficiency (i.e. wasting ATP) was measured via calorimetry to be 24 %[87] and 39 %[70] in two separate studies. Both the type (for example, glucose, citrate, K, SCFA) and the availability (for example, limited *v.* excessive glucose) of substrate influence the degree of inefficiency and the amount of heat produced by the mixed microbial community of the intestine. Present models of human heat balance do not acknowledge energy utilisation, energy spilling or heat production by the IM.

Heat balance calculations

In studies of human and animal temperature regulation, the calculation of E_{req} (required evaporative heat loss to offset metabolic heat production) involves the following equation[88]:

$$E_{req} = (M - W) - H_D, \quad (2)$$

where M is the rate of transformation of chemical energy to heat within the body (metabolic rate measured via indirect calorimetry), W is externally released energy in the form of external work, the quantity (M – W) represents human metabolic heat production, and the term H_D refers to the rate of dry heat loss from skin. All terms in this equation are expressed as watts[89]. Regarding the calculation of E_{req} (equation 2), we recommend that future research recognise the heat produced by the IM, by adding an additional factor (M_{IM}), and representing human metabolic heat production (metabolic rate measured via indirect calorimetry) with the term M_H:

$$E_{req} = (M_H + M_{IM} - W) - H_D \quad (3)$$

We also recommend modifying the widely recognised heat balance equation similarly:

$$S = (M_H + M_{IM}) - W - C - K - R - E, \quad (4)$$

where S is the storage of heat within the human body, M_H and M_{IM} are described in equation 3, W is the work rate (useful mechanical power) accomplished, C is convective heat loss to the environment, K is conductive heat loss to the environment, R is radiant heat loss to the environment, and E is evaporative heat loss from skin to the environment[89]. Since the IM contributes to heat production, future research should seek to identify mechanisms by which the IM dissipates heat into the body, as well as the inter-individual differences in IM and host heat balance interaction[90]. To our knowledge, no previous publication has acknowledged IM heat production in human heat balance calculations, or proposed modifications to present models and techniques that account for the influence of metabolic heat generated by the IM.

Assuming that IM heat production represents 47 % of RMR (based on the 46 g dry weight of faecal bacteria in the human colon, IM median heat production of 800 mW/g dry weight, and resting whole-body energy expenditure of 76 kcal/h (318 kJ/h) for men; see above), Table 4 illustrates the potential magnitude of differences that could result from introducing bacterial heat production into heat balance calculations. These data suggest that E_{req} (equation 3) and S (equation 4) are not altered by introducing the term M_{IM} (see Table 4), regardless of the experimental protocol or the ambient temperature. However, measurements of human metabolic heat production (M_H) could be altered greatly by introducing the term M_{IM}. Further, Table 4 suggests that resting experimental protocols (47 % difference; see Table 4) will be affected more than those protocols involving exercise. This results from the fact that indirect calorimetry does not distinguish between human and IM heat production or energy expenditure, and because the total human energy expenditure is smaller during resting protocols.

Indirect calorimetry measurements of RMR

Dietitians utilise empirically based formulas to determine RMR (also known as resting energy expenditure), energy needs, and protein requirements of healthy and ill adults[91,92]. These prediction formulas incorporate personal characteristics such

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Table 4. Potential differences in heat balance calculations when accounting for intestinal microbiome (IM) metabolic activity (column 4)*

Equation	Scenario (units)	M_H	M_{IM}†	W	H_D	E_{req}	Difference (%)‡ E_{req}	Difference (%)‡ M_H
$(M_H + M_{IM} - W) - H_D = E_{req}$ (equation 3)	Semi-recumbent cycling, T_{amb} 25°C§	+285	0	–105	–5	+175	0	13
	(watts/m^2)	+247	+38	–105	–5	+175		
	Seated upright cycling, T_{amb} 30°C‖	+360	0	–200	–60	+100	0	16
	(watts)	+301	+59	–200	–60	+100		
	Semi-recumbent cycling, T_{amb} 45°C¶	+290	0	–90	+200	+400	0	20
	(watts)	+231	+59	–90	+200	+400		

		M_H	M_{IM}†	W	(C, K, R)	E	S	Difference (%)‡ S	Difference (%)‡ M_H
$(M_H + M_{IM}) - W - C - K - R - E = S$ (equation 4)	At rest, T_{amb} 18°C**	+40	0	0	–85	–10	–55	0	47
	(kcal/m^2 per h)	+21	+19	0	–85	–10	–55		
	At rest, T_{amb} 28°C††	+40	0	0	–15	–30	–5	0	47
	(kcal/m^2 per h)	+21	+19	0	–15	–30	–5		
	At rest, T_{amb} 48°C††	+40	0	0	+45	–65	+20	0	47
	(kcal/m^2 per h)	+21	+19	0	+45	–65	+20		
	Treadmill running, T_{amb} 10°C‡‡	+620	0	–30	–300	–250	+40	0	5
	(watts/m^2)	+587	+33	–30	–300	–250	+40		
	Treadmill running, T_{amb} 35°C‡‡	+620	0	–30	+75	–660	+5	0	5
	(watts/m^2)	+587	+33	–30	+75	–660	+5		

M_H, human metabolic heat production; M_{IM}, intestinal microbiome heat production; W, externally released energy, in the form of external work; H_D, dry heat loss from skin via conduction, convection and radiation; E_{req}, required evaporation for thermal balance; T_{amb}, ambient dry bulb temperature; E, wet heat loss from skin via evaporation; (C, K, R), dry heat loss from skin via conduction, convection and radiation (equivalent to the term H_D); S, heat storage in bodily organs; IM, intestinal microbiota.

* Numerical values are representative approximations, based on six published research studies.

† Values for M_{IM} in resting experiments are based on an IM biomass of 46 g faecal dry weight (see text) which represents 47 % of all metabolic heat produced by a 70 kg adult at rest[74,94], whereas values for M_{IM} in exercise experiments are estimated using a resting metabolic heat production of 70 watts/m^2[157].

‡ Difference due to inclusion of M_{IM} (column 4).

§ Cramer *et al.*[158].

‖ Gagnon *et al.*[159].

¶ Meade & Kenny[88].

** Hardy & Stolwijk[160].

†† Stolwijk & Hardy[161].

‡‡ Adams *et al.*[162].

as age, height, body mass and sex. Physiologists and clinicians measure RMR by employing a metabolic cart (i.e. indirect calorimetry) to determine O_2 consumption, CO_2 production and minute ventilation. RMR is typically defined as the amount of energy expended when the individual is awake, in a post-absorptive state, free from emotional stress, and familiar with the apparatus[93]. In contrast, BMR is usually measured in the morning, soon after waking, after an overnight fast, with no exercise or strenuous activity for the previous 24 h[94]. Best practices for the measurement of awake RMR include consuming no alcohol or nicotine before testing[95], and resting quietly for 10–20 min before the measurement begins. A 10-min test duration, with the first 5 min of data discarded, will give an accurate measure of RMR with a CV < 10 %. Relevant to this laboratory analysis, human nutrient processing in the gut includes the metabolic activity of the IM[16]. This colonic fermentation generates CO_2[13,55,56], which is readily absorbed into the circulation[57,96] and can be expired into the air. However, multiple fates for CO_2 exist, including reduction to CH_4 and the production of acetate by acetogenic bacteria[97]. Thus, not all microbially produced CO_2 is expired as a respiratory gas, and its volume in indirect calorimetry measurements is unknown.

Similarly, the effects of bacterial O_2 consumption (Tables 1 and 2) affect RMR measurements to an unknown degree. Although previously published research studies have not considered the IM as a factor which influences RMR[98], laboratory measurements of O_2 consumption in closed cell culture bioreactors allow comparisons of the metabolic activity of bacteria *v.* mammalian cells. For example, mouse or rat hybridoma cells utilise O_2 at rates (0·1–0·3 mmol O_2/l per h) which are approximately 1 %[99] of the bacterial O_2 consumption rates shown in Table 2[37,68]. The fact that the number of bacterial cells in the human gut (10^{13}–10^{14}) is similar to the number of cells in the entire human body[6,71] may mean that IM metabolism equals or exceeds the resting metabolism of all human tissues and organs. However, the following paragraph suggests otherwise. The most abundant gut bacteria phyla (i.e. Bacteroidetes and Firmicutes) convert primary bile acids, which initially are produced in the host liver from cholesterol, and convert them to secondary bile acids[14]. These secondary metabolites promote the conversion of inactive thyroxine (T_4) into active thyroid hormone (T_3)[100], and regulate metabolism and heat production because RMR is unambiguously dependent on thyroid hormones[101,102]. Although additional research is necessary to quantify these effects on RMR, authorities have identified mechanisms by which secondary bile acids influence thyroid gland function, energy metabolism and O_2 consumption in healthy adults[101,103–105]. These effects are accomplished by a variety of microbial enzymes (for example, hydroxysteroid dehydrogenases) during bile acid metabolism in the intestine and regulation of host bile acid homeostasis[106]. The bacteria *Eggerthella lenta* is particularly relevant to RMR measurements because they not only affect bile acid metabolism but also influence CO_2 and H_2 dynamics[97].

The median total dietary fibre intakes of men and women (aged 31–50 years) in the USA have been reported as 17·9 and 13·1 g/d, respectively, with 5th percentile values of 9·3 and 6·5 g/d, and 95th percentile values of 31·6 and 23·3 g/d[107]. Similar values have been published by other authors for men[108,109] and women[110]. Considerable debate exists regarding the definition of dietary fibre and its constituents. For example, Tungland & Meyer[111] cited twenty-two different definitions for dietary fibre and ten analytical methods that were published between 1953 and 2002. In the majority of publications, dietary fibre refers to the edible parts of plants or analogous carbohydrates (for example, oligosaccharides, lignin, NSP such as cellulose and pectin) which are resistant to digestion and absorption in the human small intestine, but which are completely or partially fermented in the large intestine. Depending on the analytical method used, resistant and undigested starches may or may not be included in total fibre determinations[112]. However, IM fermentation in the colon utilises undigested carbohydrate that escapes digestion in the small intestine. If we consider undigested and resistant starches as the only substrate for fermentation, then about 20 g/d is metabolised in the colons of adults eating a typical Western diet[79]. At present, it is not possible to determine precise energy values for the undigested complex carbohydrates in all or even many foods, but general values can be assigned. In most foods, 2–3 kcal (8–13 kJ) of energy per g of starch becomes available via fermentation in the large intestine[60,107,112,113]. If we also assume 70 % digestibility for unavailable complex carbohydrates[60,112] (for example, apple and mixed diets, 70 %; cabbage, 80 %), then the energy salvage from SCFA represents only 28–42 kcal per d (117–176 kJ per d)[79], 1·4–2·1 % of a 2000 kcal (8368 kJ) diet. As a comparison, measurements of resting energy expenditure average 7594 (SD 1201) kJ/d (1815 (SD 287) kcal/d) for men aged <52 years and 6197 (SD 1000) kJ/d (1481 (SD 239) kcal/d) for women aged <52 years, whereas total daily energy expenditure values average 14 092 (SD 2598) kJ/d (3368 (SD 621) kcal/d) for men aged <52 years and 10 694 (SD 1900) kJ/d (2556 (SD 454) kcal/d) for women aged <52 years[74]. Based on the above values, daily IM fermentation in the colon represents only 1·5–2·3 % of resting energy expenditure in men and 1·9–2·8 % in women; of a similar magnitude, IM fermentation represents 0·8–1·2 % of total daily energy expenditure in men and 1·1–1·6 % in women. The impact of these IM energy contributions will depend on the measurement precision and reliability required.

Although not part of the RMR in healthy individuals, bacterial effects on host immune responses represent another means by which the IM can influence the rates of whole-body energy utilisation and heat production[114]. The IM modulates host innate and adaptive immune responses at the mucosal surface of the intestinal epithelium, during infection and inflammation[115]. The energetic cost of an inflammatory response is substantial. The most striking example involves sepsis, a whole-body inflammatory state that increases RMR 30–60 %[116]. However, even mild or subclinical immune responses can elicit increased energy expenditure at rest. Fever, for example, results in a 10–15 % increase of RMR for every 1°C rise of internal body temperature[117], and a respiratory tract infection with no fever can potentiate RMR 8–14 %[118]. In addition, gut microbes produces exogenous pyrogens which can further contribute to fever[119]. Thus, it is important that investigators screen test participants carefully for minor infections or subclinical illnesses that may induce immune responses and increase RMR unintentionally.

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Diet-induced energy expenditure

Total daily energy expenditure is partitioned into three components: maintenance RMR (see previous section), activity-induced energy expenditure and diet-induced energy expenditure (DEE)[120]. The latter quantity (i.e. also named the thermic effect of foods and diet-induced thermogenesis) is defined as the energy required for intestinal absorption of nutrients, the initial steps of nutrient metabolism, and the storage of the absorbed nutrients which are not immediately metabolised during the postprandial period. The mean resting energy expenditure and DEE of seventeen women and twenty-three men were reported by a Dutch research team[121]. Following each of three meals, DEE caused RMR to increase during postprandial hours[122]. After the final meal of the day, for example, RMR did not return to the morning baseline level (i.e. measured upon waking) for approximately 8 h, because of DEE. The energy utilised and heat produced by gut bacteria was not considered in this study[121], but probably contributed to DEE during the hours after dinner. For example, during controlled fermentation in a respiratory calorimeter, Hershberger & Hartsook[81] observed that bacterial heat production peaked at 4–5 h and continued >20 h. This suggests that bacterial energy consumption in the human colon, after one high-fibre meal, could span several hours because average intestinal transit lasts 15·9–19·3 h[31].

Westerterp[122] summarised eleven previously published studies (n 257) in which diets contained 15–80 % carbohydrate, 8–32 % protein, 2–67 % fat and 0–23 % alcohol (range, expressed as the percentage of 1900–3799 kJ (454–908 kcal) energy consumed during the observation period of 4·0–5·5 h). The DEE values of these men and women ranged from 4·0 to 9·0 % of total daily energy expenditure. To our knowledge, none of these studies attributed any portion of DEE to energy consumption or conversion by the IM. This is noteworthy because 24 h heat production by the IM is estimated to be 47 % of all heat generated by a 70 kg male at rest (see section above titled 'Models of human heat balance'). Further, multiple controlled laboratory investigations, measuring fermentation in closed apparatus during pulsed substrate addition, have shown that bacterial cells increase their rate of glucose uptake rapidly (i.e. > 7-fold in 2 min[123]) across a 1 h time span, the glucose consumption rate increased 15-fold and the bacterial growth rate increased 8-fold[124]. Although this experimental methodology may not simulate colonic metabolism exactly, these data suggest that a rapid metabolic increase is possible when substrate becomes available. Because the physiological, biochemical and genetic mechanism(s) which modulate DEE have not been clearly delineated[98,125], the rapid changes of DEE across 2 h[121] could be, in part or in majority, due to both host and IM metabolism[31].

Intestinal microbiota habitats

The vast majority of IM biomass and number estimates have considered faecal bacteria but have overlooked bacteria that inhabit the intestinal mucosa. The average mass of wet faecal content in the human colon is 285 g at a single point in time[65], dry faecal matter (29 % of the total mass[66]) weighs 84 g, and the rate of excretion averages 130 g/24 h[126]. To estimate the mass of unattached bacteria inhabiting faeces, most investigators have utilised visual microscopic counts and converted these to a weight by assuming an average mass for the bacteria. Because recent estimates (5 pg[6]) and direct measurements (0·1 pg[127,128]) of the wet weight of a single bacterium vary greatly, the unique method of Stephen & Cummings[66] assessed bacterial mass by separating the microbial fraction from other faecal material (i.e. three components: bacteria, undigested fibre and soluble substances) and weighing it. Their experiments indicated that bacterial weight is 55 % of faecal dry solids. Using the 84 g weight of faecal DM (above), we calculate that the dry mass of unattached bacteria in the human colon to be 46 g. Further, because the number of bacteria in stool samples is $0{\cdot}3–1{\cdot}5 \times 10^{10}$/g dry weight[129], we calculate that the total number of faecal bacteria ranges from 13·8 to $69{\cdot}0 \times 10^{10}$.

Unfortunately, few published estimates of IM biomass, number or metabolic rate include organisms residing in the extensive mucosa overlying the luminal surface of the gut. This dual-layer mucus gel (biofilm) overlies the epithelium, contributes to structural and functional stability, and fortifies host defences[130]. Bacteria normally are not observed in the thin inner layer, but inhabit the outer layer which is four to five times thicker (for example, several hundred micrometres in humans)[131–133]. Adhesion of bacteria to this biofilm may be one of the factors involved in the ability of IM organisms to colonise and persist[134]. Research teams have surveyed IM bacteria in tissue specimens taken from healthy adults during colonoscopic examinations. In one such study, Macfarlane & Macfarlane[129] compared the number of IM bacteria in mucosal biopsies and faecal samples of fifteen adults. Counts of aerobes and facultative aerobes (twenty to thirty-one specimens) attached to mucosal biofilm ranged from $4{\cdot}1$ to $5{\cdot}8 \times 10^{10}$, whereas non-adherent bacteria in faeces ranged from 1·0 to $5{\cdot}3 \times 10^{10}$/g wet weight. Anaerobic bacteria counts (sixty-eight specimens) ranged from 1·0 to $5{\cdot}6 \times 10^{10}$ in mucosal gel and 1·0 to $5{\cdot}4 \times 10^{10}$ in faeces. Zoetendal *et al.*[135] assessed bacteria in mucosal and faecal tissues donated by ten healthy adults. Biopsy samples from ascending, transverse and descending colon segments contained 10^5 to 10^6 bacterial cells; faecal sample counts were at least 10^3 times greater. Similarly, Hartley *et al.*[134] observed mucosal bacteria across the entire length of the intestine; numbers ranging from 10^3 to 10^9 (mean, 10^6; fourteen healthy adults; forty-three specimens) per g of biopsied wet tissue. In total, these findings suggest that most published values for IM abundance and biomass in the colon underestimate the ecosystem, perhaps by as much as 50 %, because they were derived only from faecal sample counts[6] and did not include microbes inhabiting the intestinal biofilm at the luminal surface.

Summary: dynamic and complex interactions

The preceding paragraphs describe ways that gut bacteria may introduce unrecognised variability or error into experimental measurements of O_2 consumption, CO_2 production, RMR, DEE, energy expenditure using the DLW method, and heat balance. These IM effects are transmitted through a vast array of intermediate metabolites and signalling pathways to the host gut

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epithelium, liver, muscle and brain[26,136]. In addition, IM research is complicated by the sheer number of bacterial species (>1000) residing in the healthy human gut[2], the metabolic diversity of closely related bacterial species[15,137], IM organisms[27,138] other than bacteria (for example, viruses, fungi), the vast and dynamic IM genome[3,4], temporal changes of the IM community in response to numerous environmental and lifestyle factors (for example, antibiotics, meals, disease[139,140]), IM diurnal rhythms[18,19,141], and the large inter-individual variability of human thermal and metabolic responses[102,142–144].

We believe that the variability or error due to the IM has been overlooked in experiments involving nutrition, physiology, medicine, metabolism, temperature regulation, energy expenditure and exercise. One published analysis assessed the variance in BMR measurements[145], partitioned into within- and between-subject effects (i.e. fat-free mass, fat mass, bone mineral content, sex, age, plasma leptin and plasma thyroid hormones). Only 2 % of the observed variance in BMR was attributable to within-subject effects, of which 0·5 % was analytic error. Of the remaining variance, which reflected between-subject effects, 63 % was explained by fat-free mass, 6 % by fat mass and 2 % by age. A total of 26 % of BMR variance remained unexplained, yet no mention was made of the possible variance due to the gut microbiota. Until methods are developed to control IM influences during the conduct of human experiments, researchers should acknowledge this as a research limitation. Researchers also should control those factors which strongly and rapidly affect the IM community (for example, exercise, antibiotics, probiotics[136]). Not surprisingly, diet exerts a great influence[146]. Altered nutrient load induces measurable, readily reversible[147] and rapid changes of IM species diversity and functions[31,147]; these changes occur within 24 h of initiating high-fat/low-fibre or low-fat/high-fibre diets[148]. Therefore, at a minimum, we recommend that strict dietary controls be implemented in future IM studies which measure O_2 consumption, CO_2 production, DEE, RMR, DLW energy expenditure and heat balance. We also recommend that *in vitro* incubations of known IM abundance (as shown in Tables 2 and 3) and controlled nutrient concentrations be observed and compared with human whole-body values (as performed in the section above titled 'Indirect calorimetry measurements of RMR'), to determine the magnitudes and relative contributions of the IM. It is unlikely that profiling the faecal microbiota will be informative, considering the large taxonomic and metabolic variation of the faecal community[3,149].

During the period 1855–1870, yeasts were established as microbes and responsible for alcoholic fermentation; this led to the study of bacterial pathogenicity. The subsequent research of Pasteur, Koch, Schwann, Fischer and Metchnikoff laid the foundation for our present understanding of IM–host interactions[150,151]. Indeed, the publications indexed in the PubMed online database (United States National Library of Medicine, National Institutes of Health) demonstrate a resurgence of interest that began less than 20 years ago but now is widespread. Using the search term 'intestinal microbiota', we identified the following publication totals: 2000–2004, 163; 2005–2009, 956; 2010–2014, 4537; and 2015–2018 (4 years), 12 889. This exponential 21st-century growth has been encouraged by technological advances (for example, high-throughput sequencing techniques in medical research) which allow us to study the IM more effectively and efficiently. Concurrently, news media reports regarding the profound influences which the IM ecosystem has on long-term health prospects[30] stimulate public interest[138]. This awareness generates new perceptions about ourselves and carries new expectations. Therefore, we believe that the time is right for physiologists, nutritionists, microbiologists and clinicians to explore the premises of this article. Specifically, investigators should ask, 'Are IM effects measurable and meaningful?' and 'What research methods and variables are influenced by IM effects?' because the present review has demonstrated that IM effects are measurable and that some methods are affected. Details regarding ways to modify conceptual models and laboratory techniques await future investigation, to account for IM metabolic processes and regulatory influences.

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