Generation of germ-free common marmosets

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Generation of germ-free common marmosets

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Summary

Recent studies using germ-free mice have demonstrated that microbiota have functional roles in host homeostasis[1,2]. However, the phylogenetic distance between rodents and humans translates into differences in their metabolism, immune response, neural function and microbiota colonisation abilities. Hence, translational research using nonhuman primates (NHPs) is important for bridging the gap between rodent studies and human medicine[3]. Although several attempts to produce germ-free NHPs were made more than 50 years ago[4,5], currently none are available. Here, we generated germ-free common marmosets suitable for rearing and handling under sterile conditions and maintained them with no culturable bacteria/fungi, for 22 months. The faecal microbiota composition and metabolome in conventional marmosets are more similar to those in humans than to those in mice. The transplantation of a bacterial consortium isolated from humans[6] into marmosets and mice resulted in a significantly steadier bacterial colonisation in the former than that in the latter. Germ-free marmosets exhibited low levels of faecal short-chain fatty acids, bile acid metabolites, plasma and faecal immunoglobulins, and enlarged caecum in contrast-enhanced X-ray. These stable germ-free marmosets can serve as novel models that enable the development of therapeutics that target gut microbiota and elucidation of their interaction with higher-order brain function.

Introduction

Germ-free (GF) animals, born and raised under sterile conditions, have contributed immensely to microbiota research. The absence of commensal microbiota in these animals enables the understanding the role played by commensal microbiota in hosts. Recent studies using gnotobiotic mice – obtained by colonising known bacteria or bacterial flora into GF mice – have demonstrated that intestinal bacteria have functional roles in host homeostasis (e.g., modulating the host's immune system and influencing host development and physiology, including that of the central nervous system[1,2,7]). However, extrapolating the findings of these mouse studies to human medicine is hampered by the evolutionary distance between rodents and primates, and the differences in terms of metabolism, immune responses, neural functions, and colonisation ability of microbiota[8].

Non-human primates (NHPs) are critical for translational research to bridge such gaps and are sometimes the only relevant animal models because of their close genetic, physiological, and behavioural similarities with humans[3,9]. Recent research involving NHPs has enhanced our understanding of the host-microbiota interactions in humans[10,11]. However, currently, no GF NHPs are available. More than 50 years ago, a few reports catalogued the acquiring and rearing of GF NHPs, i.e., rhesus (*Macaca mulatta*)[4,12] and cynomolgus (*Macaca fascicularis*)[5] monkeys. These preliminary records mention a few individuals who remained GF for short spans of time but do not mention whether the GF status was monitored continuously; thus, it remains controversial whether these primates were indeed born and raised under GF conditions.

The common marmoset (*Callithrix jacchus*) is an important NHP used in biomedical research and in preclinical studies aimed at drug development[14-16]. This New World monkey species has some advantages as laboratory models, such as small body size, high fecundity and relatively short life cycle. These advantages make the species relatively easy to breed and handle in a sterile isolator. Furthermore, the marmoset has been developed as a model animal for research in the field of neuroscience[17] and has been used as a system to model various human disease,

especially those reported to be associated with imbalances in intestinal bacterial flora[18,19] such as Parkinson's[20], multiple sclerosis[21], obesity[22] and age-related diseases[23]. Recent progress in transgenic and genome editing technology has further expanded the use of this NHP in research[24-26]. Thus, if GF marmosets are available, they can serve as novel NHP models in microbiota research, i.e., investigations of brain-gut microbiota interactions and preclinical studies on microbiota-based therapeutics.

The purpose of this study was (i) to investigate the characteristics of the marmoset as an NHP model for microbiota research by investigating the faecal bacterial flora, faecal and plasma metabolomes and susceptibility to colonisation by a bacterial consortium isolated from human faeces[6], and (ii) to produce GF marmosets.

Profiling conventional marmosets

Preliminarily, faecal bacterial compositions in marmosets maintained under conventional conditions were compared with those in humans and in two groups of specific-pathogen free (SPF) mice maintained on different diets (normal rodent food or marmoset food). The relative abundance of phylum-level identification by 16S ribosomal RNA gene analysis showed that Firmicutes and Bacteroidetes were the major bacteria in all three species (Fig. 1a). Actinobacteria, although present in marmosets and humans, were rarely detected in mice, e.g. *Bifidobacterium*, although detected in marmosets and humans, was absent in mice (Fig. 1a). Principal coordinate analysis (PCoA) using operational taxonomic unit (OTU) data sets showed that the composition of the faecal flora in marmosets was similar to that in humans than to that in mice (Fig. 1a).

Next, metabolome analysis for primary metabolites, short chain fatty acids (SCFAs) and oxylipins was conducted on faecal and plasma samples collected from the individuals mentioned above. Principal component analysis (PCA) of data sets from 235 metabolites in faeces and 182 metabolites in plasma revealed that the faecal and plasma clusters corresponding

113 to human and marmoset metabolomes were separated from those of the mouse groups along the
114 PC1 axis, although those of the faecal metabolome were in proximity for marmosets and mice
115 fed the marmoset diet (Fig. 1b). Among the faecal metabolites, the bile acid composition
116 differed between mice and marmosets/humans, with muricholic acids (MCAs) being primarily
117 detected in mice (Supplementary Table 1). With respect to plasma metabolites, marmosets and
118 humans shared higher levels of amino acids, such as proline, histidine and alanine, and lower
119 levels of polyunsaturated fatty acids compared to those in mice (Supplementary Table 2).
120 In addition, we investigated how intestinal bacterial strains from humans colonised in the
121 marmoset intestines. The 11-strains mixture, which was isolated from healthy human faeces and
122 found to induce interferon-γ-producing CD8 T cells in the intestine[6], was orally inoculated into
123 marmosets and mice. Quantitative PCR (qPCR) analysis of the relative amounts of bacteria in
124 faecal samples detected all 11 strains steadily for 3 weeks post-inoculation in marmosets,
125 whereas some strains decreased over time in mice (Fig. 1c). These results showed the usability
126 of marmosets as NHPs for microbiota research. Based on this knowledge, we attempted to
127 generate GF marmosets.
128
129 **Obtaining GF marmosets**
130 A total of 18 impregnated females were operated upon for obtaining GF newborns (Fig. 2a).
131 First, as with acquiring GF mice or rats, on day 142 post determined ovulation, we performed
132 hysterectomy on a marmoset impregnated with embryo transfers (ET) and delivered a newborn
133 inside a sterile flexible film isolator. Next, we prepared a dedicated isolator to establish a
134 surgical procedure for aseptically obtaining marmoset neonates through caesarean section,
135 which was minimally invasive to dams (Fig. 2b, c, Extended Data Fig. 1). A total of 17
136 caesarean sections were performed on 12 ET-impregnated and 5 naturally impregnated females.
137 Operations were conducted between the 136th and 142nd day post expected ovulation in ET
138 cases. The foetal biparietal diameter, measured using ultrasonography, was found to be 17.4-

20.3 mm 10 days prior to surgery (Extended Data Table 1). Of the 25 newborn marmosets obtained from 18 operations, we successfully resuscitated 23. The time from the induction of anaesthesia to delivery ranged from 13 to 30 min in the resuscitated cases, whereas it was 35 min in the non-resuscitated cases. All operated females recovered without apparent problems after surgery.

Of the 23 resuscitated marmosets, 7 neonates were weaned by hand-rearing in sterile isolators (Fig. 2a); all weaned animals were singletons and the four newborns from the latest operation cases were weaned by improving hand-rearing procedures such as modifying milk formula and careful maintenance of body temperature (Supplementary Table 3). The remaining 16 individuals showed varied clinical signs such as severe diarrhoea, marked weight loss and immature birth, and died 1–12 d after birth (Extended Data Table 2). In 14 of the 1 caesarean cases (82%), culture tests detected no bacteria or fungi from faecal and isolator swabs until weaning or death. Of the three remaining cases, *Paenibacillus taichungensis* and *Bacillus licheniformis* were detected in two weaned cases, respectively, between 1 and 4 weeks of age, and *Staphylococcus warneri* was detected in one dead individual (Extended Data Table 2).

Thus, two GF (culture negative) females, three GF males, and two mono-colonised (MC) males were obtained and maintained in sterile isolators (Fig. 3a). Monthly culture tests of their faecal and isolator swabs were negative for up to 22 months (Fig. 3b); *Staphylococcus aureus* was detected in the animal (881M) that had the longest GF state at 22 months old when pinholes were observed on the gloves of the isolator. A male-female pair (939M and 795F) were maintained culture-negative for more than 15 months prior to manuscript submission (Fig. 3b). Furthermore, daily administration of a sensitive antibiotic, kanamycin, caused MC animals (905M, 926M, 792F and 947M) to be negative for culture tests (Fig. 3b). All the marmosets reared under sterile conditions grew without apparent problems and gained body weights similar to those in conventionally reared animals (Fig. 3c).

Characteristics of GF and MC marmosets

Faecal metabolome analysis revealed that GF and MC marmosets had as few metabolites involved in intestinal bacteria as did GF mice; SCFA concentrations in GF/MC marmoset faeces were significantly lower ($P < 0.001$ or $P < 0.05$) than those in conventional marmosets, similar to those in GF mice when compared to those in SPF mice (Fig. 4a). Only conjugated and no deconjugated primary/secondary bile acids were detected in GF/MC marmoset faecal samples, similar to those in GF mice (Fig. 4a). However, muricholic acid, which is considered to be rodent-specific, was not detected in marmosets.

Assay of secretory immunoglobulin A (sIgA) in faeces as a marker of intestinal immune activation showed that its values in GF and MC marmosets were significantly lower ($P < 0.01$) than those in conventional marmosets (Fig. 4b). In addition, proteome analysis of faecal samples showed downregulation of IGHA1 (IgA1) in GF/MC marmosets as one of the differentially expressed proteins (DEPs) compared to that in conventional marmosets (Fig. 4c, Supplementary Table 4). In this analysis, downregulation of the LYPD8 protein, which was reported to mediate segregation of intestinal bacteria and epithelial cells in the colon[27], was also observed. Furthermore, proteome analysis of plasma samples showed downregulation of immunoglobulins, including IGHA1 and IGHM (IgM), complement system proteins and several proteins related to the immune response in GF/MC marmosets as DEPs (Fig. 4c, Supplementary Table 5).

To verify whether the caecal enlargement in GF rodents was also observed in the obtained GF marmosets, contrast-enhanced radiography acceptable for live animals was performed on the animals in sterile isolators and showed enlargement of the caecum in a GF marmoset compared to that in conventional marmosets (Fig. 4d). The caecal diameter measured from the X-ray images of GF state marmosets, including those from culture-negative individuals after antibiotic administration (906M, 926M and 939M), was significantly larger than that measured in conventional marmosets ($P < 0.05$).

Discussion

In this study, we succeeded in obtaining GF marmosets in which no viable bacteria and fungi were detected in culture and maintained them for a long period of up to 22 months (Fig. 3b). Reyniers and Trexler (1943), in the first record of obtaining GF primates, reared GF-rhesus monkeys for 4 months[4]. Wolfe et al. (1966) reportedly raised cynomolgus monkeys under GF conditions for up to 10 months[5]. Barnes et al. (1969) reported that a human infant, delivered by caesarean section, was maintained in a sterile isolation unit in the absence of bacteria for 6 days for the treatment of a potential case of immune deficiency[13]. In another case, a human patient with severe combined immunodeficiency was reported to have lived under isolated conditions for 12 years, but several bacteria were detected in the patient since the first month after birth[28,29]. These preliminary reports showed that primates could live under GF conditions for short spans of time but there was no mention of continued negative culture results over 1 month. Here, we showed that primates can grow under sterile conditions, as monitored by monthly culture tests, for up to 22 months when reaching sexual maturity.

The comparison of faecal bacterial composition in this study indicated that intestinal bacterial flora in conventional marmosets is similar to that in humans, such as *Bifidobacterium* colonisation, which did not occur in SPF mice. Spontaneous *Bifidobacterium* colonisation was also consistently observed in marmosets from other facilities[30,31]. Furthermore, the inoculation of the intestinal bacterial strains obtained from humans showed that marmosets underwent significantly steady colonisation by all these strains, suggesting similar colonisation milieu for intestinal bacteria with humans. Faecal and plasma metabolome analyses also showed that the metabolic profile of marmosets was more similar to that in humans than that in mice fed marmoset diet. Phylogenetic relationships in metabolome profiles of plasma reported previously[32] were consistent with those detected in the present study. These results demonstrate

that marmosets can serve as suitable NHP models to bridge the gap between mouse studies and human medicine in microbiota research.

In this study, we developed a series of techniques for GF-marmoset production including preparation of full-term pregnant animals, aseptic caesarean section that was safe for dams and neonates (Fig. 2, Extended data Fig. 1), hand-rearing of neonates and long-term maintenance of animals under sterile conditions (Fig. 3). Hobbs et al. (1977) reported that aseptic caesarean section for producing SPF marmosets resulted in resuscitation of 40% of the neonates[33]. Our results showed that the resuscitation rate of neonates in caesarean section was more than 90%, indicating the more accurate timing and method of our operations (Fig. 2a). However, hand-reared neonates often died of diarrhoea, suffered body weight loss within 2 weeks of birth and had 30% weaning rates. Hand-rearing under conventional conditions was previously performed to rear triplets or more marmoset neonates[15]whose resuscitation rate was reported to be 80% or more[34,35]. The lower survival of neonates reared under sterile conditions may be related to bacterial colonisation that contributes to postnatal gut development[1,36]. Nevertheless, arranged rearing protocols such as feeding for preventing diarrhoea and maintaining the body temperature in the latter four neonates led to successful weaning in the present study (Extended Data Table 2, Supplementary Table 3). These results demonstrate that GF marmosets can be reproducibly produced.

In the marmosets reared under sterile conditions, phenomena associated with the absence of microbiota such as lack of the metabolites derived from gut microbiota, inactivation of the immune system and enlargement of the caecum appeared (Fig. 4), thus demonstrating their characteristics as GF animals. Furthermore, our results from stable biomaterial sampling and live imaging that could be performed while maintaining sterile conditions indicate that gnotobiotic experiments using GF marmosets are practicable even in NHPs. Future gnotobiotic marmoset studies will have a great potential to explore unknown fields in microbiota research. Their physiological similarities to humans, including susceptibility to colonisation by

242 microbiota and metabolome profiles as observed in this study, can contribute toward high
243 predictability in preclinical research for therapeutics targeting gut microbiota. Moreover,
244 marmosets and humans share core features of brain architecture and function, and the complex
245 social and cognitive behaviours typical of primates[37]. Recent progress in research using GF
246 mice has been revealing the mechanisms by which the gut microbiota and its metabolites
247 influence the host central nervous system, including neurogenesis, neuronal activity,
248 neuroinflammation and host behaviour[38-40]. The GF marmosets generated in this study can
249 become a powerful resource to clarify the unknown phenomena of interactions between
250 microbiota and its primate host including the microbiota-gut-brain axis.
251

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Figure legends

Figure 1. Gut microbiota and metabolome profiles of common marmosets compared with those in mice and humans. (a) Average relative abundance (%) of phylum and genus level bacterial identification in faecal samples of specific-pathogen free (SPF) mice (n = 6), marmosets (n = 6) and humans (n = 25) using 16S ribosomal RNA analysis. **(b)** Score plots of principal component analysis (PCA) of faecal and plasma metabolomic profiles in SPF mice fed normal rodent food (n = 3), SPF mice fed marmoset food (n = 3), marmosets (n = 6) and humans (n = 25). **(c)** Gut colonisation by 11-strains mixture isolated from healthy human faeces and inoculated into both marmosets (n = 6) and mice (n = 5). * $P<0.05$, ** $P<0.01$ (two-tailed Mann-Whitney test, Day 21). Data are mean±SD.

Figure 2. Production of germ-free (GF) marmosets

(a) Summary of results in trials of obtaining GF marmosets. **(b)** Flexible film isolator developed for caesarean delivery. A schema of the caesarean section delivery is shown in Extended data Fig. 1. **(c)** Pregnant dam's abdomen attached to the bottom film of the isolator.

Figure 3. Germ-free (GF) marmosets.

(a) Photographs of GF marmosets. **1,** Hand rearing; **2,** grown GF marmoset (881M); **3,** rearing marmosets in sterile isolators (939F and 795F); **4,** full-suite type isolator for maintaining marmosets. **(b)** Culture test results of faeces and isolator samples obtained from GF and mono-colonised (MC) marmosets. No bacteria and fungi were detected for up to 22 months in 881M. We detected *Staphylococcus aureus* (Sa) in 23-month-old (881M), *Paenibacillus taichungensis* (Pt) in 1-month-old (905M) and *Bacillus licheniformis* (Bl) in 1-week-old (926M), 9-month-old (792F) and 3-month-old (947M) individuals. Antibiotic administration turned samples from mono-colonised animals culture-negative (905M, 926M, 792F and 947M). (**c)** GF/MC-

361 marmoset body weights (n = 7) compared with those of conventional marmosets (n = 54). Data
362 are mean±SD.
363
364 **Figure 4. Characteristics of germ-free (GF) and mono-colonised (MC) marmosets.**
365 **(a)** Faecal metabolite profiles in GF and specific-pathogen free (SPF) mice, GF/MC marmosets
366 and conventional marmosets. Short-chain fatty acid (SCFA) concentration in GF/MC marmoset
367 faeces was significantly lower, similar to that in GF mice, than that in SPF mice and
368 conventional marmosets (upper). Only conjugated bile acids and no deconjugated
369 primary/secondary bile acids were detected in GF/MC marmosets, similar to those in GF mice
370 (lower). Muricholic acids were not detected in marmosets. Data are mean±SD. (**b)** Faecal
371 secretory IgA was significantly low in GF and MC marmosets compared to that in conventional
372 marmosets. (**c)** Proteome analysis of faecal samples showed downregulation of IGHA1 (IgA1)
373 and LYPD8 protein in GF/MC marmosets as differentially expressed proteins (DEPs) compared
374 to that in conventional marmosets. Proteome analysis of plasma samples showed
375 downregulation of immunoglobins including IGHA1 (IgA) and IGHM (IgM) and complement
376 system proteins in GF/MC marmosets as DEPs. **(d**) Contrast-enhanced X-ray images show
377 enlargement of the caecum and upper colon (allow head) in a GF marmoset (939M) compared
378 to that in a conventional marmoset. The caecal diameter in GF state marmosets was
379 significantly larger than that in conventional ones. TCA, taurocholic acid; CA, cholic acid;
380 DCA, deoxycholic acid; TCDCA, taurochenodeoxycholic acid; CDCA, chenodeoxycholic acid;
381 LCA, lithocholic acids; and TMCA, tauromuricholic acid. * $P<0.05$, ** $P<0.01$, *** $P<0.001$,
382 one-way ANOVA followed by Tukey's multiple comparison test (a, b) or two-tailed unpaired
383 Welch’s t-test (d).
384

Methods

Animals. All animal-experiment protocols were approved by the Institutional Animal Care and Use Committee of the Central Institute for Experimental Animals (CIEA) (approval no. 17046A and 20079A) and by the Keio University Institutional Animal Care and Use Committee (approval no. 15072-(4)). Common marmosets obtained from CLEA Japan, Inc. (Tokyo, Japan) or bred in CIEA, and SPF and GF mice (C57BL/6N) obtained from CLEA Japan were used in this study. The animals were cared for and used in accordance with the Guidelines for Proper Conduct of Animal Experiments prescribed by the Science Council of Japan (2006).

Faecal and plasma sample collection. Faeces and plasma obtained from three male conventional marmosets were sampled in duplicate (28-29 months old; 314–363 g body weight) at a month-long interval. The marmosets had been fed only a New World primate diet (CMS-1M; CLEA Japan) for a month prior to sampling. Faeces and plasma were sampled from two groups of three female SPF mice (16 weeks old) – one group was fed a normal rodent diet (CL-2, CLEA Japan) and the other a New World Primate diet (CMS-1M) similar to that fed to marmosets. Human faecal and plasma samples were collected from 25 and 24 individuals, respectively, at Keio University according to the study protocol approved by the institutional review boards. Informed consent was obtained from each subject.

Faecal microbiota analysis using 16S ribosomal RNA gene sequencing. Faecal samples were suspended in a 10-fold volume of phosphate buffer saline containing 20% glycerol and 10 mM ethylenediaminetetraacetic acid (EDTA) and stored at -80 ºC until use. After thawing, 100 µL of faecal suspension was added to 800 µL TE10 (10 mM Tris-HCl, 10 mM EDTA) buffer containing RNase A (final concentration of 100 µg/mL, Invitrogen, CA, USA) and lysozyme (final concentration of 15 mg/mL, Sigma, MO, USA) and incubated for 1 h at 37 ºC. Purified

411 achromopeptidase (final concentration of 2,000 U/mL; FUJIFILM Wako Chemicals, VA, USA)
412 was added and further incubated for 30 min at 37 ºC. Sodium dodecyl sulphate (final
413 concentration of 1%) and proteinase K (final concentration of 1 mg/mL, Roche, Basel,
414 Switzerland) was further added to the mixture and incubated for 1 h at 55 ºC. High molecular
415 weight DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1 at pH 7.9),
416 precipitated with isopropanol (equal volume to the aqueous phase), washed with 1 mL of 70%
417 ethanol, and gently resuspended in 200 µL of TE buffer. The 16S rRNA sequencing was
418 performed using MiSeq according to the Illumina protocol. The V1–V2 region of the 16S rRNA
419 gene was PCR-amplified using 27Fmod 5′-AGRGTTTGATYMTGGCTCAG-3′ and 338R 5′-
420 TGCTGCCTCCCGTAGGAGT-3′. Amplicons generated from each sample (~330 bp) were
421 purified using the AMPure XP magnetic beads (Beckman Coulter, CA, USA). DNA was
422 quantified using a Quant-iT Picogreen dsDNA assay kit (Invitrogen) and Infinite M Plex plate
423 reader (Tecan, Männedorf, Switzerland), and then stored at 4 ºC. The pooled amplicon library
424 was sequenced using a MiSeq Reagent Kit v2 (500 cycles) and Miseq sequencer (2 x 250 bp
425 paired-end reads, Illumina, CA, USA). Two paired-end reads were merged using the fastq-join
426 program based on overlapping sequences. Reads with an average quality value of <25 and
427 inexact matches to both universal primers were filtered out. Both primer sequences were
428 trimmed off and 3,000 quality filter-passed reads were rearranged in descending order according
429 to the quality value and then clustered into OTUs with a 97% pairwise-identity cutoff using the
430 UCLUST program v5.2.32. Taxonomic assignment of each OTU was made via searching by
431 similarity against the Ribosomal Database Project (RDP) and the National Center for
432 Biotechnology Information (NCBI) genome database using the Global/Local (GL) SEARCH
433 program.
434
435 **Metabolome analyses**

436 Frozen faeces were homogenized as previously described[41]. Oxylipins were extracted from 1.0
437 mg-equivalent faeces and 25 µL of plasma spiked with deuterated standards as described in a
438 previous study[41]. Plasma samples were additionally mixed with 3 µL of acetic acid, vortexed
439 and incubated for 30 min on ice for deproteination. After the addition of 150 µL of water,
440 vortexing and centrifugation at 17,000 × g for 5 min at 4 ºC, the supernatant was added to the
441 Monospin C18-AX (GL Sciences Inc., Tokyo, Japan) column preconditioned with 300 µL of
442 methanol and 300 µL of water, and then washed twice by applying 300 µL of water and once
443 with 300 µL of methanol/water [50:50 (vol/vol)]. Oxylipins were eluted using 100 µL of
444 methanol/water/acetic acid [90:8:2 (vol/vol/vol)] and served for LC-MS/MS analysis as
445 described[42].
446 SCFAs were extracted from 5.0 mg-equivalent faeces and 20 µL of plasma, then analysed by
447 GC-MS as described previously[41]. Water soluble primary metabolites and amino acids were
448 extracted from 1.0 mg-equivalent faecal homogenate in 200 µL of methanol and mixed with
449 200 µL of chloroform, 70 µL of water and 10 µL of internal standard mix (100 µM of
450 cycloleucine, 500 µM of citric acid-d4 and 1.0 mM of ornithine-d7, Cambridge Isotope
451 Laboratories, Inc., MA, USA). After vortexing for 1 min and centrifuging at 15,000 × *g* for 5
452 min at 4 ℃, 100 µL of supernatant was evaporated to dryness. Next, 20 µL of plasma was
453 mixed with 400 µL of acetonitrile, 70 µL of water and 10 µL of internal standard mix and
454 incubated for 2 h at –30 ℃. After vigorous mixing at 1,250 rpm for 10 min at room temperature
455 and centrifugation at 16,000 × *g* for 3 min at 4 ℃, 200 µL of supernatant was evaporated to
456 dryness. The dried samples were derivatized via methoxiamination and trimethylsilylation or
457 *tert*-Butyldimethylsilylation, then analysed by GC-MS/MS using Smart Metabolite
458 DatabaseTM (Shimadzu, Tokyo, Japan) or GC-MS operated in selected ion monitoring mode,
459 respectively, as previously described[43]. Bile acids were extracted from 1.0 mg-equivalent faeces
460 spiked with deuterium-labelled internal standard mix (1.0 µM of cholic acid-d4, 1.0 µM of
461 lithocholic acid-d4, 1.0 µM of deoxycholic acid-d4, 1.0 µM of taurocholic acid-d4 and 1.0 µM

of glycocholic acid-d4; Cayman Chemical, MI, USA) and applied it to the Monospin C18 column (GL Sciences Inc.) and washed twice with 300 μL of water and once with 300 μL of hexane, respectively. Bile acids were eluted with 100 μL of methanol, then served for LC-MS/MS analysis using an UPLC I class (Waters Corporation, MA, USA) with a linear ion-trap quadrupole mass spectrometer (QTRAP 6500; AB SCIEX, Singapore) equipped with an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm; Waters Corporation). Samples were eluted with mobile phases consisting of water/methanol/acetonitrile [14:3:3 (vol/vol/vol)] and acetonitrile, both containing 5 mM ammonium acetate under the following gradients: 100:0 for 4 min; increased to 40:60 after 12 min, increased to 5:95 after 2 min, and held for 2 min; with a flow rate of 300 μL/min. Multiple-reaction monitoring in negative mode was operated by measuring product ions corresponding to $[M-H]^-$, taurine (*m/z*, 124) and glycine (*m/z*, 74) generated from precursor ion $[M-H]^-$ for deconjugated, taurine-conjugated, and glycine-conjugated bile acids, respectively. MS/MS settings were as follows: ion source, turbo spray; curtain gas, 30 psi; collision gas, 9 psi; ion spray voltage, -4500 V; source temperature, 600 ºC; ion source gas 1, 50 psi; ion source gas 2, 60 psi.

Colonisation of a consortium of bacteria strains isolated from humans. The 11-strains mix, which was isolated from healthy human faeces and was found to induce interferon-γ-producing CD8 T cells in the intestine[6], was administered orally to six male marmosets (8–12 months old; 221–280 g) and five male SPF mice (C57BL/6N, 8-weeks old; 23-24 g). The day before inoculation, animals were administered vancomycin orally (50 mg/head, marmosets; 5 mg/head, mouse). For bacterial inoculation, the 11 strains were grown separately for 2 or 3 d in modified GAM broth (Nissui), reinforced clostridial medium (Oxoid) or EG broth, and equal volumes of bacterial suspensions were mixed. The suspension of 11-strains mix was inoculated intragastrically into each animal (2 mL for marmosets, 200 uL for mice). Faeces were collected and faecal DNA was extracted as a part of the previously mentioned 16S rRNA gene

pyrosequencing. Confirmation of colonisation was achieved by qPCR using the following specific primers for each strain: 81A6 (5′-TGCACTGTTGGATTTTCTAAAAAGG-3′, 5′-ACTTTGGGCATGCTAAACCA-3′); 81B11 (5′-CGCCGGATGCATATACAAGA-3′, 5′-TCTCGCCAATGATGTCCAAA-3′); 81C1 (5′-CCGCACAAGAGAAATAAACGCCA-3′, 5′-TGGCAAATTCAAAGGTGAGCGAA-3′); 81E7 (5′-GGGAATAAAGCTGTTCCGATATGC-3′, 5′-TCATGCAACATTCTTTCGTTGG-3′); 81H9 (5′-TTCACCTTCTACGGCTACTACTACG-3′, 5′-ACATAACGATCAAGGGTGCTGAAG-3′); 82A6 (5′-GCTCTTTTTAGCCTGTATCCGGT-3′, 5′-ATACGATACGAACGACCAACCT-3′); 82B1 (5′-TACCAATGCAAAGCGACCAA-3′, 5′-CGGTTTTGTTGCCGAACTCT-3′); 82F11 (5′-GCACAGATTCTACACTCCCCT-3′, 5′-AGCAACGAAACAACCTGTGA-3′); 82G1 (5′-TCCATGCTGAAGCGTTGAAG-3′, 5′-GGACCGAACATCCCAATCAC-3′); 82G5 (5′-CTGCTTCCGACAGCACACAT-3′, 5′-AGCTTGGCGGAGAGCCTATT-3′); and 82G9 (5′-AGCGCGTAAACTTAGTCAAGGA-3′, 5′-TAGGGCCAAAACCTGCATTA-3′).

Production of GF marmosets

Pregnant marmosets. Pregnant marmosets were prepared by natural mating and ET for obtaining GF marmosets; the animals are listed in the Extended data Table 1. Embryo collection and transfer were performed as previously described[44,45]. The recipients of ET were monitored for ovulation and pregnancy by measuring their plasma progesterone until the pregnancies. The foetuses in the pregnant marmosets were monitored using transabdominal ultrasonography (ProSound Alpha 7 ultrasound system; Hitachi Aloka Medical, Tokyo, Japan) every 2 weeks. Surgical operations for delivery were performed between the 139 and 142 d after ovulation and/or when dam's activity was presumed as a sign of labour onset as observed by video monitoring in the 2 h after turning the lights off.

Perioperative procedures. The abdominal hair of pregnant marmosets were removed with depilatory cream and the animals were administered pre-anaesthetic medication as follows: 0.15 mg/kg lidocaine (Xylocaine; Aspen Japan, Tokyo, Japan) at the incision site as local anaesthetic, 1.2 mg/kg ketoprofen (Capisten; Kissei Pharmaceutical, Matsumoto, Japan) as analgesic, 15 mg/kg ampicillin (Viccillin; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) as antibiotic, 17 mg/kg tranexamic acid (Transamin; Daiichi Sankyo Co., Ltd., Tokyo, Japan), 0.5 mg/kg carbazochrome sodium sulfonate (Adona; Nipro, Osaka, Japan) as haemostatic and 5 mL/head subcutaneous infusion (KN No.1 injection; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). Next, the animals were anaesthetised with isoflurane (Pfizer Japan, Tokyo, Japan) and aseptically operated on a heating mat by monitoring the oxygen saturation of peripheral artery and pulse rate (Oxypal R; Nihon Kohden, Tokyo, Japan). Another analgesic, 0.03 mg/kg butorphanol (Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan), which may cause respiratory depression in foetuses, was administered after the deliveries. After surgery, the animals were kept warm in an intensive care unit chamber (Fukuda ME, Tokyo, Japan) until recovery. In cases where delay in uterine contraction was observed, 1–2 unit/kg oxytocin (Atonin-O; Aska Pharmaceutical Co., Ltd., Tokyo, Japan) was administered. Postoperative medication including analgesics, antibiotics and haemostatics was administered once daily for more than two consecutive days.

Hysterectomy. A median incision was applied on the abdomen of anaesthetised pregnant marmosets and the uterus was excised by clamping the utero cervix. The uterus was transferred into a flexible film isolator (JV-1300; Jic, Tokyo, Japan), which was sterilised using chlorine dioxide sterilant (Exspor; Ecolab G.K., Tokyo, Japan) through a germicidal trap filled with a quaternary ammonium compound solution (Mikro Quat; Ecolab G.K., Tokyo, Japan). Neonates were removed from the uterus with an incision while inside the isolator and resuscitated by

wiping and stimulating their mouth and body. At the same time, the surgical incision of the dam was sutured and closed.

Caesarean section. A schema of the caesarean section is described in Extended data Fig. 1. A flexible isolator designed for aseptic caesarean section (Jic) was sterilised as described previously and connected to another sterilised isolator for resuscitation of neonates (JV-1000S, Jic). An incise-film drape (Ioban, 3M, Japan, Tokyo) and a double-sided adhesive film (Medical Tape 1513, 3M) were stuck on the abdomen of anaesthetised pregnant marmosets following hair removal and disinfection with povidone iodine. The abdomen of the animals was adhered to the bottom film of the isolator for caesarean section. The abdominal skin was incised along with adhered films and the subsequent peritoneal incision exposed the uterus within the isolator. The uterus and amnion were incised to remove the foetuses and the umbilical cord was cut after clipping. The obtained neonates were transferred to another connected isolator and resuscitated by wiping and stimulating their mouths and bodies, whereas the uterus was manually compressed for haemostasis following the removal of the placenta. After splitting the connector between the isolators, the dam's abdomen was unstuck from the isolator, and the surgical incision was sutured and closed.

Rearing marmosets under sterile conditions. Resuscitated neonates were transferred to a sterilised isolator (JV-1000, Jic) and hand-reared. The equipment needed for rearing, including cages, water, feeding materials, electric power cables, heating devices, humidifiers, thermometers sterilised with autoclave, formaldehyde sterilizer, 30 kGy gamma irradiation or chlorine dioxide sterilant (Exspor) were prepared in the isolators using sterilizing cylinders (JV-1059, Jic). Human infant formula (Hohoemi; Meiji, Tokyo, Japan)[35], elemental liquid diet (Elental P; EA Pharma, Tokyo, Japan) and/or powdered goat milk (Meyenberg, Turlock, USA)[36] sterilised with 30 kGy gamma irradiation were used as neonate food; the feeding

protocols are shown in Supplementary Table 3. The environment around the animals was controlled at 30–34 ºC and 50%–75% humidity for the first 2 weeks after birth and gradually changed to 27–29 ºC and 35%–45% humidity. A humidifier within the isolator, a panel heater and a sheet heater on the outside of the isolator were used for environmental control. A mobile hand warmer wrapped in an artificial fur pouch was used as a hug pillow for keeping neonates' body temperature instead of a stuffed animal from the 13th neonate (the 11th operation) onwards. The infants were fed solid food, CMS-1M (CLEA Japan), sterilised with 30 kGy gamma irradiation from the 7th to 11th week after birth and weaning.

Culture test for GF status. Microbial culture tests were performed for evaluation of the GF status. The surface swabs inside isolators were tested before use. The faeces, foods and surface swabs inside isolators were tested in the first, second and fourth week after birth of the animals and monthly thereafter. Thioglycolate broth (Eiken Chemical, Tokyo, Japan) and potato dextrose agar (Eiken Chemical, Tokyo, Japan) were used for all culture tests. Cooked meat broth (Becton; Dickinson and Company, NJ, USA), heart infusion broth (Becton; Dickinson and Company, NJ, USA) and SensiMedia (MicroBio, Sendai, Japan) were also used for the tests in the second month after birth in animals that had previously shown negative culture tests. All cultures were incubated for 14 d at both 37 °C and room temperature in thioglycolate broth, cooked meat broth and heart infusion, at 37 °C in SensiMedia, and at room temperature in potato dextrose agar. In cases with positive culture tests, the bacterial species was identified using the MALDI Biotyper (Bruker Daltonik GmBH, Bremen, Germany).

Analyses of SCFAs and bile acids in faecal samples. Three faecal samples from marmosets reared under sterile conditions, two samples from a GF marmoset (I881M) at 16 and 21 months of age and one from a 10-month-old MC marmoset (I905M), along with faecal samples from five female GF mice (16 weeks old) were used for analysis. SCFAs and bile acids in these

590 samples were analysed by the methods described above and compared to the above metabolome
591 analysis data in conventional marmosets and SPF mice.
592
593 **Assay of faecal sIgA.** Faeces from 7 marmosets reared under sterile conditions and 11
594 conventional marmosets (4–70 months old) were delicately sampled with 2 weeks more interval
595 and stocked at -80 ºC. These samples were assayed for sIgA using an ELISA kit (IDK sIgA
596 ELISA; Immundiagnostik AG, Bensheim, Germany).
597
598 **Proteome analysis.** Faeces and plasma were sampled in duplicate from GF marmosets (I881M)
599 at 16 and 21 months of age and from an MC-state marmoset (I905M) at 10 and 11 months of
600 age. These samples and the ones used in the previous metabolome analysis from conventional
601 marmosets duplicated from three male conventional marmosets (28–29 months old; 314–363 g
602 body weight) were also used for analysis. Host proteins in faeces were extracted by pipetting
603 and invert after incubating at 30 min on ice in TBS-T with protease inhibitors (cOmplete,
604 ULTRA, Mini, EDTA-free, EASYpack Roche, Germany). After centrifugation at 15,000 × g for
605 15 min at 4 °C to remove insoluble matter, the supernatant was transferred to a new tube. The
606 transferred sample was added to an equal volume of 25% trichloroacetic acid and incubated for
607 30 min on ice. After centrifugation at 15,000 × g for 15 min at 4 °C to remove the supernatant,
608 the precipitate was washed with acetone and dried through an opened lid. The dried sample was
609 redissolved in 0.5% sodium dodecanoate and 100 mM Tris-HCl (pH 8.5) using a water-bath-
610 type sonicator (Bioruptor UCD-200, SonicBio Corporation, Kanagawa, Japan). Plasma sample
611 was diluted 100-fold in 0.5% sodium dodecanoate and 100 mM Tris-HCl (pH 8.5), of which 20
612 μL was used. The pre-treatment for shotgun proteome analysis was performed as reported
613 previously[46].
614 Peptides were directly injected onto a 75 μm × 20 cm PicoFrit emitter (New Objective, Woburn,
615 MA, USA) packed in-house with C18 core-shell particles (CAPCELL CORE MP 2.7 μm, 160

616 Å material; Osaka Soda Co., Ltd., Osaka, Japan) at 50 °C and then separated with a 90 min
617 gradient at a flow rate of 100 nL/min using an UltiMate 3000 RSLCnano LC system (Thermo
618 Fisher Scientific, Waltham, MA, USA). Peptides eluting from the column were analysed on a Q
619 Exactive HF-X (Thermo Fisher Scientific) for overlapping window DIA-MS[46]. MS1 spectra
620 were collected in the range of 495 to 785 m/z at 30,000 resolution to set an automatic gain
621 control target of 3e6 and maximum injection time of 55. MS2 spectra were collected in the
622 range of more than 200 m/z at 30,000 resolution to set an automatic gain control target of 3e6,
623 maximum injection time of "auto", and stepped normalized collision energy of 24, 26, and 28%.
624 An isolation width for MS2 was set to 4 m/z and overlapping window patterns in 500-780 m/z
625 were used that were optimized by Skyline v19.1.
626 MS files were searched against a callithrix spectral library using Scaffold DIA v2.1 (Proteome
627 Software, Inc., Portland, OR). The callithrix spectral library was generated from the callithrix
628 protein sequence database (UniProt id UP 000008225) by Prosit[47,48]. The Scaffold DIA search
629 parameters were as follows: experimental data search enzyme, trypsin; maximum missed
630 cleavage sites, 1; precursor mass tolerance, 8 ppm; fragment mass tolerance, 10 ppm; static
631 modification, cysteine carbamidomethylation. The protein identification threshold was set to
632 both peptide and protein false discovery rates of less than 1%. Protein quantification was
633 estimated from the summed peptide quantification.
634 The quantitative results were normalised such that the total expression value of each sample was
635 1.0×10^9. Principal component analysis (PCA) was performed using the standardised
636 expression values for each protein. Two tailed Welch's t-test was performed to detect DEPs,
637 and proteins with p-values less than 0.01 were designated as DEPs.
638
639 **Contrast-enhanced radiography.** Three male marmosets reared under sterile conditions (28-
640 month-old I905M, 19-month-old 926M and 17-month-old 939M), who were negative for
641 culture test in X-ray imaging, and three male conventional marmosets (25-28-month-old) were

used. After fasting for 20 h, the animals were anaesthetised using an intramuscular injection of 8–10 mg/kg alfaxalone (Alfaxan, Meiji Seika Pharma) and transanal administration of 10 mL of two-fold diluted Gastrografin (Bayer, Leverkusen, Germany). Abdominal radiography was performed directly (conventional marmosets) or through a flexible film isolator using a digital radiography system (Carestream Vita CR system; Carestream Health, NY, USA) with 55 kV and 1.2 mAs at 75 cm distance or 100 kV and 2.0 mAs at 130 cm distance. The maximum length of short axis of the cecum in the X-ray images was measured as the caecal diameter using the OsiriX MD (Pixemo, Bernex, Switzerland).

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, CA, USA) and R v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Two-tailed Mann-Whitney test, one-way ANOVA with Tukey's multiple comparison test or two-tailed unpaired Welch's t-test were used to analyse the data.

Data availability statement

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions

T. I., N. Okahara, N. Okahashi, M. Ueda, K. A., T. N., M. A., K. H. and E. S. contributed to the study conception and design. T. I., N. Okahara, K. S., C. L., Y. Kurotaki, T. M., T. Y. and E. S. contributed to produce germfree marmosets; N. Okahashi, M. Ueda, K. I., M. A. contributed to metabolome analysis; K. A., J. I. and Y. A. contributed to microbiota analysis; Y. Kawashima and Y. A. contributed to proteome analysis; M. Ueno and R. N. contributed to microbiological examinations in germfree marmosets. All authors have read and approved the final manuscript.

Competing interest declaration

K.H. is a scientific advisory board member of Vedanta Biosciences and 4BIO CAPITAL.

Additional Information

Supplementary Information is available for this paper.

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Extended Data Legends

Extended Data Fig. 1. Schema of caesarean delivery of germ free (GF) marmosets. Germ-free marmoset neonates were delivered from their anaesthetised dam's uterus inside a flexible film isolator while the dam's abdomen was attached to the bottom film of the isolator, and then moved to a connected isolator to be resuscitated.

Extended Data Table 1. Operations for acquiring germ-free (GF) marmosets.

Extended Data Table 2. Hand-rearing of marmoset neonates in sterile isolators.

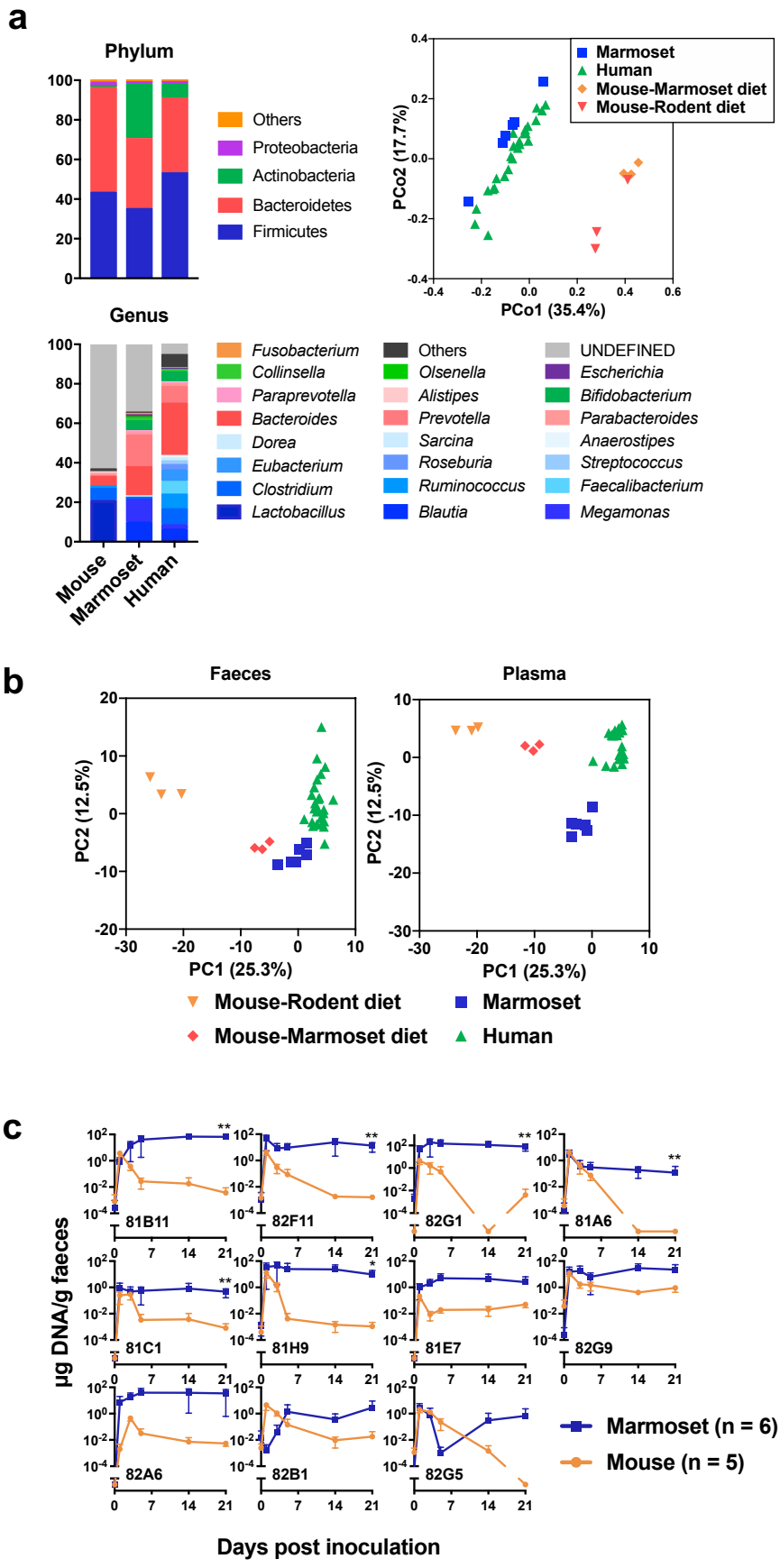


Figure 1. Gut microbiota and metabolome profiles of common marmosets compared with those in mice and humans.

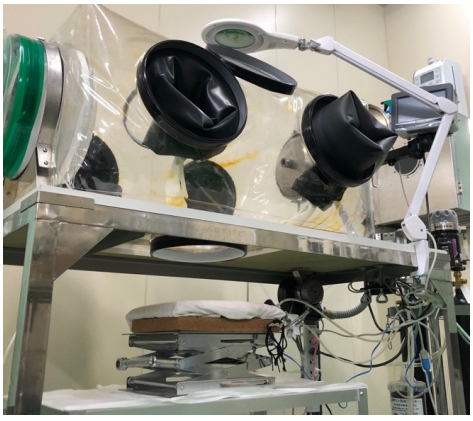
(a) Average relative abundance (%) of phylum and genus level bacterial identification in faecal samples of specific-pathogen free (SPF) mice (n = 6), marmosets (n = 6) and humans (n = 25) using 16S ribosomal RNA analysis. **(b)** Score plots of principal component analysis (PCA) of faecal and plasma metabolomic profiles in SPF mice fed normal rodent food (n = 3), SPF mice fed marmoset food (n = 3), marmosets (n = 6) and humans (n = 25). **(c)** Gut colonisation by 11-strains mixture isolated from healthy human faeces and inoculated into both marmosets (n = 6) and mice (n = 5). * P<0.05, ** P<0.01 (two-tailed Mann-Whitney test, Day 21). Data are mean ± SD.

a

Pregnant dams				Delivered neonates		
Pregnancy	Number of operation	Operation method	Recovered post surgery	Resuscitation (%)	Weaning (%)	Germ-free* (%)
Embryo transfer	13	Hysterectomy	1/1	1/1 (100)	1/1 (100)	1/1 (100)
		Caesarean section	12/12	14/16 (88)	4/14 (29)	2/4 (50)
Natural mating	5	Caesarean section	5/5	8/8 (100)	2/8 (25)	2/2 (100)
Total	18	-	18/18	23/25 (92)	7/23 (30)	5/7 (71)

*Germ-free, culture test-negative for 8 weeks more.

b



c

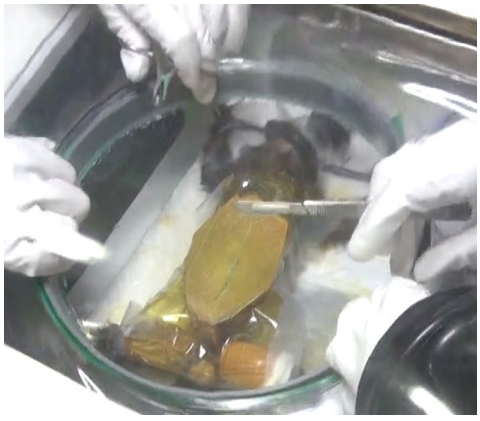


Figure 2. Production of germ-free (GF) marmosets

(a) Summary of results in trials of obtaining GF marmosets. **(b)** Flexible film isolator developed for caesarean delivery. A schema of the caesarean section delivery is shown in Extended data Fig. 1. **(c)** Pregnant dam's abdomen attached to the bottom film of the isolator.

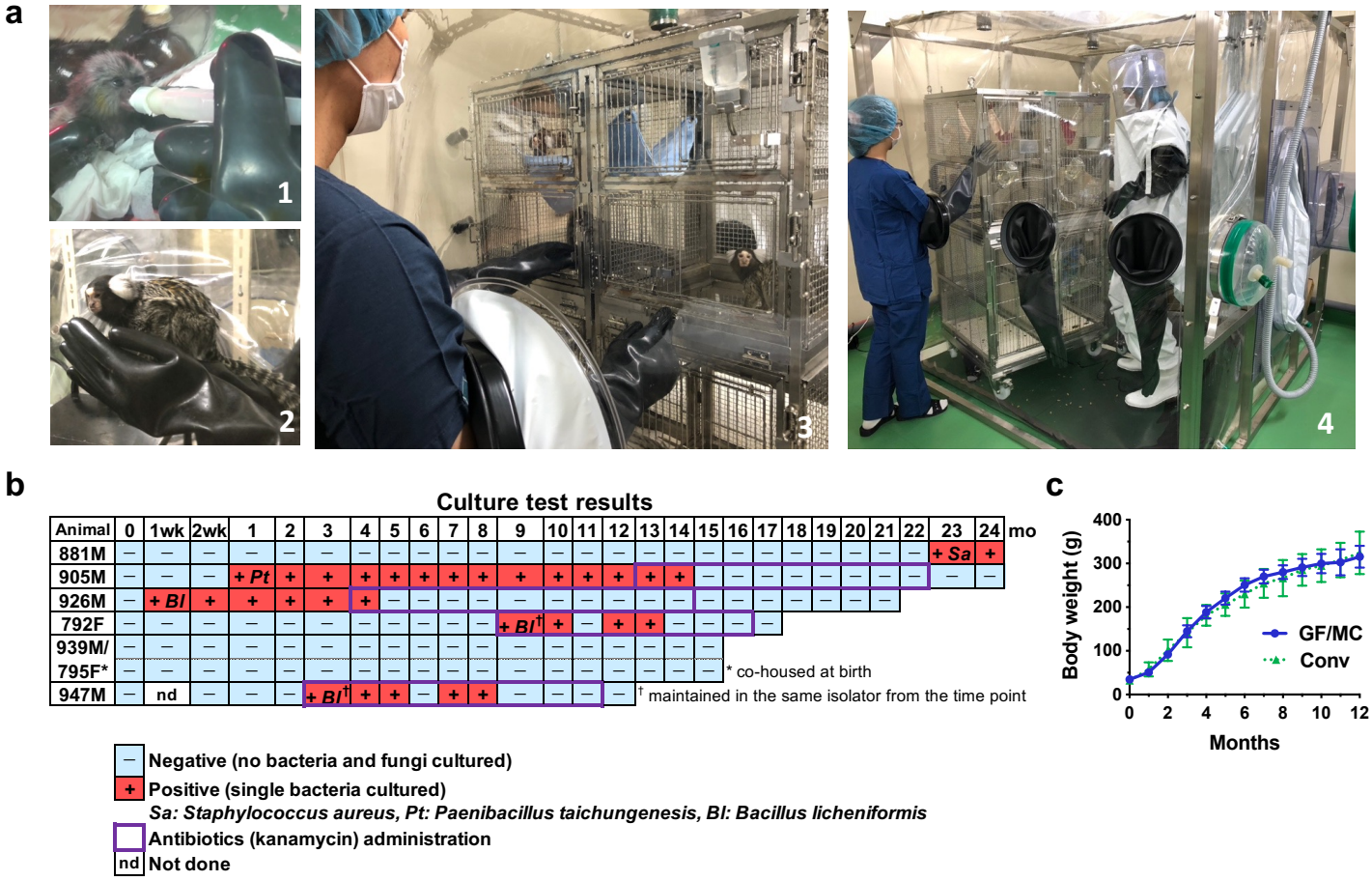


Figure 3. Germ-free (GF) marmosets.

(a) Photographs of GF marmosets. 1, Hand rearing; 2, grown GF marmoset (881M); 3, rearing marmosets in sterile isolators (939F and 795F); 4, full-suite type isolator for maintaining marmosets. **(b)** Culture test results of faeces and isolator samples obtained from GF and mono-colonised (MC) marmosets. No bacteria and fungi were detected for up to 22 months in 881M. We detected Staphylococcus aureus (Sa) in 23-month-old (881M), *Paenibacillus taichungensis* (Pt) in 1-month-old (905M) and *Bacillus licheniformis* (Bl) in 1-week-old (926M), 9-month-old (792F) and 3-month-old (947M) individuals. Antibiotic administration turned samples from mono-colonised animals culture-negative (905M, 926M, 792F and 947M). **(c)** GF/MC marmoset body weights (n = 7) compared with those of conventional marmosets (n = 54). Data are mean ± SD.

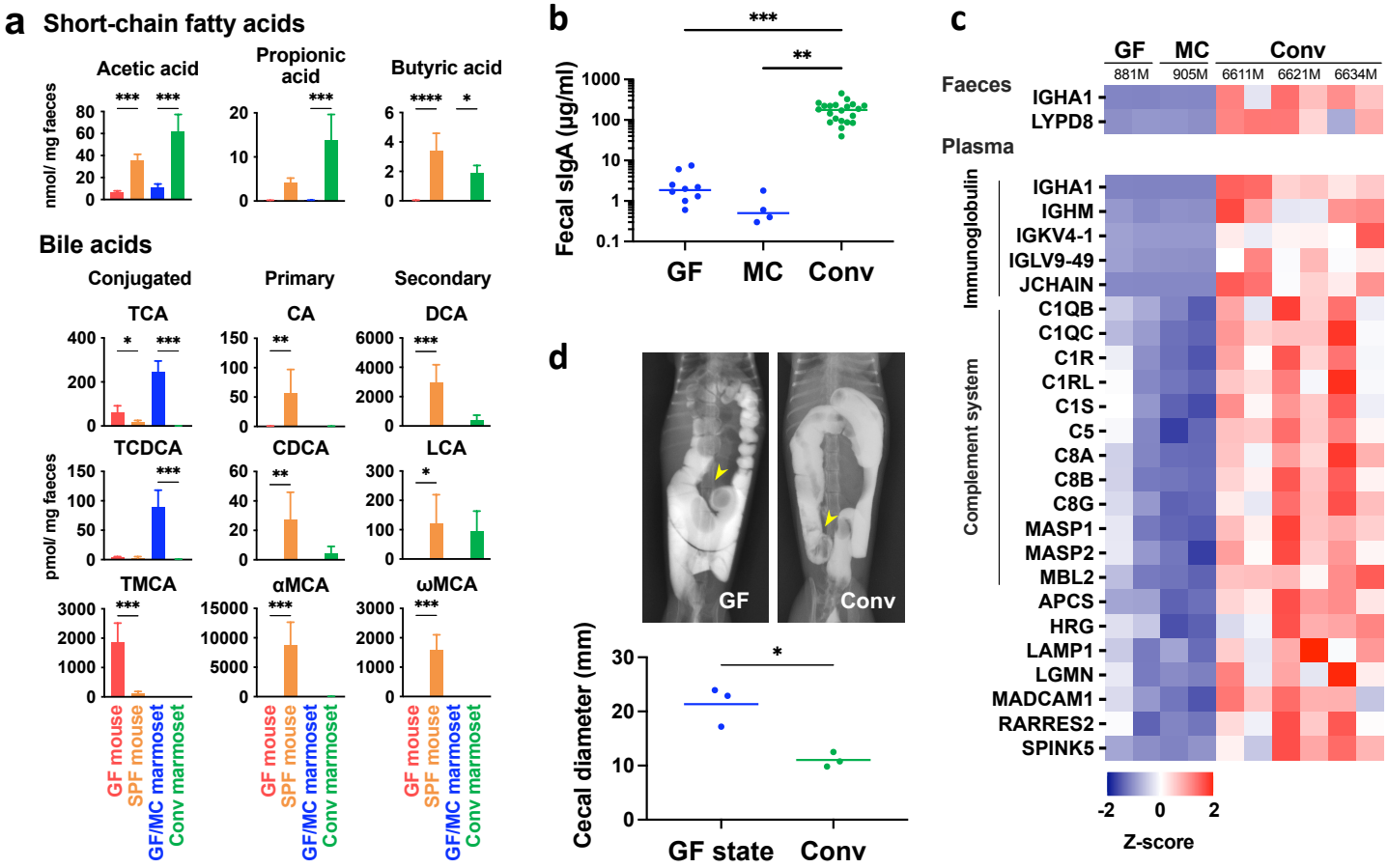


Figure 4. Characteristics of germ-free (GF) and mono-colonised (MC) marmosets.

(a) Faecal metabolite profiles in GF and specific-pathogen free (SPF) mice, GF/MC marmosets and conventional marmosets. Short-chain fatty acid (SCFA) concentration in GF/MC marmoset faeces was significantly lower, similar to that in GF mice, than that in SPF mice and conventional marmosets (upper). Only conjugated bile acids and no deconjugated primary/secondary bile acids were detected in GF/MC marmosets, similar to those in GF mice (lower). Muricholic acids were not detected in marmosets. Data are mean ± SD. **(b)** Faecal secretory IgA was significantly low in GF and MC marmosets compared to that in conventional marmosets. **(c)** Proteome analysis of faecal samples showed downregulation of IGHA1 (IgA1) and LYPD8 protein in GF/MC marmosets as differentially expressed proteins (DEPs) compared to that in conventional marmosets. Proteome analysis of plasma samples showed downregulation of immunoglobins including IGHA1 (IgA) and IGHM (IgM) and complement system proteins in GF/MC marmosets as DEPs. **(d)** Contrast-enhanced X-ray images show enlargement of the caecum and upper colon (allow head) in a GF marmoset (939M) compared to that in a conventional marmoset. The caecal diameter in GF state marmosets was significantly larger than that in conventional ones. TCA, taurocholic acid; CA, cholic acid; DCA, deoxycholic acid; TCDCA, taurochenodeoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acids; and TMCA, tauromuricholic acid. * P<0.05, ** P<0.01, *** P<0.001, one-way ANOVA followed by Tukey's multiple comparison test (a, b) or two-tailed unpaired Welch's t-test (d).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SIGuide210416.docx
- SupplementaryTables210416.xlsx
- ExtendedData.pdf