INTESTINAL MICROBIOTA CHANGES IN MICE LACKING PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP) – BIFIDOBACTERIA MAKE THE DIFFERENCE

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Pituitary adenylate cyclase activating polypetide (PACAP) constitutes a neuropeptide that is widely distributed in the host exerting essential cytoprotective properties, whereas PACAP$^{-/-}$ mice display increased susceptibility to distinct immunopathological conditions. The orchestrated interplay between the gut microbiota and the host is pivotal in immune homeostasis and resistance to disease. Potential pertubations of the intestinal microbiota in PACAP$^{-/-}$ mice, however, have not been addressed so far. For the first time, we performed a comprehensive survey of the intestinal microbiota composition in PACAP$^{-/-}$ and wildtype (WT) mice starting 2 weeks postpartum until 18 months of age applying quantitative culture-independent techniques. Fecal enterobacteria and enterococci were lower in PACAP$^{-/-}$ than WT mice aged 1 month and ≥6 months, respectively. Whereas *Mouse Intestinal Bacteroides* were slightly higher in PACAP$^{-/-}$ versus WT mice aged 1 and 6 months, this later in life held true for *Bacteroides/Prevotella* spp. (≥12 months) and lactobacilli (>15 months of age). Strikingly, health-beneficial bifidobacteria were virtually absent in the intestines of PACAP$^{-/-}$ mice, even when still breastfed. In conclusion, PACAP deficiency is accompanied by distinct changes in fecal microbiota composition with virtually absent bifidobacteria as a major hallmark that might be linked to increased susceptibility to disease.

Keywords: intestinal microbiota dynamics, pituitary adenylate cyclase activating polypeptide (PACAP), bifidobacteria, intestinal ecology, gut–brain axis, predisposition to immunopathology, probiotic immunomodulation

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was initially identified as a neuropeptide in the hypothalamus stimulating adenylate cyclase activity in the pituitary gland [1]. Beyond the nervous system, PACAP is widely expressed in peripheral organs of the endocrine, respiratory, reproductive, and digestive system as well as in lymphoid organs including immune cells [2]. PACAP can bind to VPAC1, VPAC2, and PAC1 receptors that are present on innate immune cell subsets such as lym-

phocytes and macrophages, for instance [3–5]. In the context of its virtual ubiquitous distribution, PACAP exerts a broad mode of biological actions with cytoprotective including anti-inflammatory and anti-apoptotic properties in particular [5, 6]. Hence, PACAP-deficient (PACAP$^{-/-}$) mice display several morphological, biochemical, and behavioral abnormalities that are characterized by decreased fertility but higher mortalitity rates due to compromised respiratory and metabolic functions, in additon to hyperactivity, decreased anxiety, depression-like behavior, and abnormal stress responses as reviewed by Reglodi and

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colleagues [6]. PACAP$^{-/-}$ mice have been shown to be more vulnerable to harmful stimuli to the central nervous system, but also peripheral organs including peripheral nerves, kidney, and the intestinal tract [6]. For instance, applying the acute dextran sodium sulfate (DSS)-induced colitis model, PACAP$^{-/-}$ mice displayed 50% higher mortality rates as compared to wildtype (WT) mice [7]. In addition, PACAP$^{-/-}$ mice were suffering from more severe chronic DSS-induced colitis, whereas 60% of diseased animals additionally developed colorectal tumors with aggressive phenotype [8]. Interestingly, naive PACAP$^{-/-}$ mice did not display histopathological changes in their intestinal mucosa. Hence, lack of endogenous PACAP resulted in increased susceptibility of the murine host to intestinal inflammation and inflammation-induced colonic cancer development.

There is compelling evidence that the host microbiota constitutes a key factor in health and disease of vertebrates. In fact, it has been shown that the gut microbiota is essentially involved in a multitude of physiological processes including food digestion [9], fat metabolism [10], vitamin synthesis [11], intestinal angiogenesis [12], enteric nerve function [13], and protection from pathogens [14, 15] as well as immune cell development [16]. Conversely, pertubations of this complex intestinal ecosystem termed dysbiosis are associated with increased susceptibility of the host to distinct intestinal (e.g., inflammatory bowel disease, irritable bowel syndrome, coeliac disease) as well as to extra-intestinal immunopathological conditions (e.g., multiple sclerosis, autism, depression, allergy, asthma, cardiovascular morbidities) [17]. Hence, the fine-tuned orchestrated interplay between the intestinal commensal microbiota and the host plays a pivotal role in immune homeostasis, host cell physiology, and resistance to disease [17].

To date, however, no data are available regarding the intestinal microbiota composition of PACAP$^{-/-}$ mice. In the present study, we therefore addressed whether PACAP deficiency was associated with qualitative and/or quantitative differences in the gut microbiota repertoire that in turn may be linked to the phenotypes and increased susceptibility to immunopatholocal conditions observed in PACAP gene-deficient mice. To accomplish this, we performed a comprehensive kinetic survey of the intestinal microbiota composition in PACAP$^{-/-}$ and corresponding WT mice starting 2 weeks postpartum until 18 months of age applying culture-independent molecular techniques.

Methods

Mice

PACAP gene-deficient (PACAP$^{-/-}$) mice and corresponding WT counterparts were raised and maintained in open cages within an experimental semi-barrier (accessible only with laboratory coat, overshoes, caps, and sterile gloves) under standard conditions (22–24 °C room temperature,

55 ± 15% humidity, 12 h light/12 dark cycle) in the animal facilities of the University of Pecs (Pecs, Hungary). Mice had unlimited access to autoclaved tap water and standard chow. Comparable numbers of male and female age-matched PACAP$^{-/-}$ and WT mice were included in the study at defined time points postpartum. Genotypes of mice were confirmed by polymerase chain reaction (PCR) as described elsewhere [18].

Molecular analysis of fecal microbiota

Fresh fecal pellets were collected from corresponding mice at 2 weeks, 1 month, 3 months, 6 months, 12 months, and >15 months (i.e., between 15 and 18 months) of age, immediately snap-frozen in liquid nitrogen and stored at −80 °C until further processing. DNA was extracted from fecal samples as described previously [19, 20]. In brief, DNA was quantified by using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng/μl. Then, the main bacterial groups abundant in the murine intestinal microbiota including enterobacteria, enterococci, lactobacilli, bifidobacteria, *Bacteroides*/*Prevotella* spp., *Mouse Intestinal Bacteroides*, *Clostridium coccoides* group, and *Clostridium leptum* group as well as total eubacterial loads were assessed by quantitative real-time PCR (qRT-PCR) with species-, genera-, or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as described previously [15, 21–23], and numbers of 16S rRNA gene copies per nanogram DNA of each sample were determined.

Statistical analysis

Medians and levels of significance were determined by Mann–Whitney *U* test (GraphPad Prism v5, La Jolla, CA, USA) as indicated. Two-sided probability (*p*) values of ≤0.05 were considered significant.

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments (BA02/2000-15024/2011).

Results

Comprehensive culture-independent analysis of the fecal microbiota in PACAP$^{-/-}$ and WT mice over time

In order to provide a comprehensive kinetic survey of the main bacterial groups abundant in the intestinal tract of PACAP$^{-/-}$ and WT mice, we collected fecal samples of age- and sex-matched mice starting 2 weeks postpartum until the age of 18 months. Notably, due to limited num-

bers in progressively aged and surviving mice lacking the PACAP gene, results derived from mice aged 15 to 18 months of either genotype were pooled (indicated as >15 months age group) and subjected to statistical analyses.

Total eubacterial gene numbers were highest in fecal samples derived from both PACAP$^{-/-}$ and WT mice at the beginning of the observation period as early as 2 weeks postpartum and higher as compared to any time points analyzed thereafter (i.e., >15 months; $p < 0.001$; *Fig. 1*). Furthermore, irrespective of the genotype, total bacterial loads were lower in fecal samples taken from mice ≥6 months as compared to 1-month-old animals ($p < 0.05–0.01$; *Fig. 1*). By the age of ≥15 months, WT but not PACAP$^{-/-}$ mice dis-

played lower eubacterial gene numbers in their intestines as compared to corresponding mice ≤6 months of age ($p < 0.05–0.001$; *Fig. 1A*). It is important to note, however, that the observed differences were rather minor (approximately 0.5 order of magnitude).

When assessing gene numbers of commensal enterobacteria including *Escherichia coli*, 2-week-old and 1-month-old WT mice harbored up to four orders of magnitude more enterobacteria in their intestines as compared to WT mice aged ≥3 months ($p < 0.001$; *Fig. 2A*). In fact, fecal enterobacteria burdens were lower in mice of the oldest cohort as compared to any other time point ($p < 0.05–0.001$; *Fig. 2A*). In 2-week-old PACAP$^{-/-}$ mice, fe-
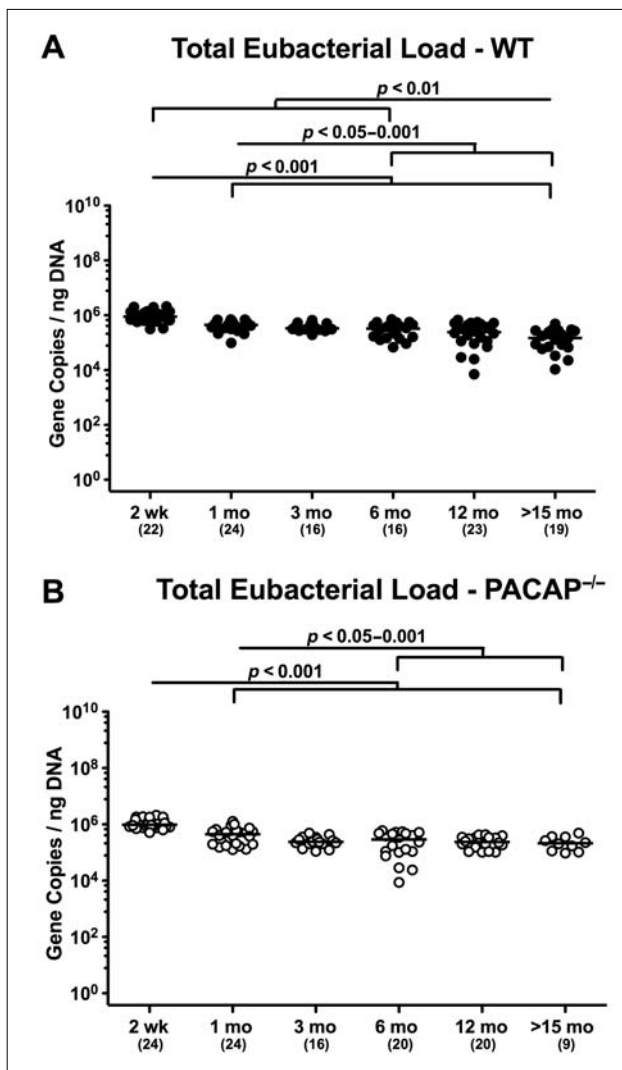
Fig. 1. Total eubacterial gene numbers in fecal samples derived from PACAP-deficient and wildtype mice over time. Individual intestinal total eubacterial loads were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated
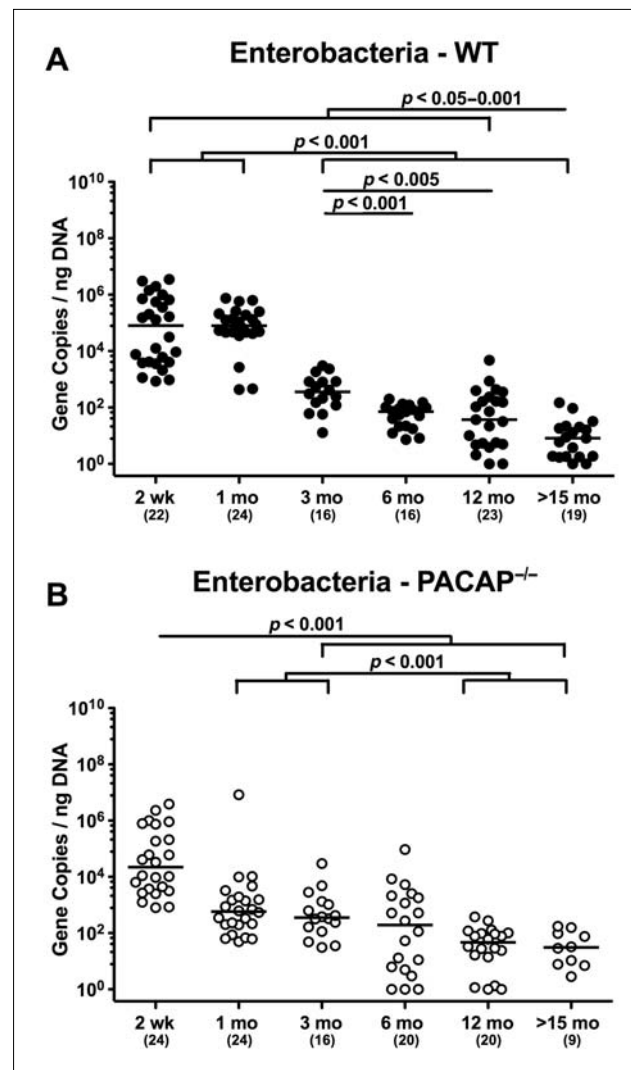
Fig. 2. Fecal gene numbers of enterobacteria in PACAP-deficient and wildtype mice over time. Individual intestinal loads of enterobacteria were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated

cal enterobacteria loads were higher as compared to animals aged ≥3 months ($p < 0.001$; *Fig. 2B*). Furthermore, PACAP$^{-/-}$ mice over 12 months of age exhibited less enterobacteria in their feces than 1-month-old and 3-month-old animals ($p < 0.001$; *Fig. 2B*). The overall decrease in fecal enterobacteria burdens observed during the entire survey period was approximately 2.5 orders of magnitude in PACAP$^{-/-}$ animals *(Fig. 2B)*.

In mice of either genotype, not only fecal enterococci gene numbers were rather low but also kinetic changes were minor. Three-month-old WT mice displayed 1.0–1.5 orders of magnitude lower enterococci burdens as compared to younger counterparts ($p < 0.005$; *Fig. 3A*). In ≤1-month-old PACAP$^{-/-}$ mice, enterococci gene numbers

were higher than those detected in feces of ≥3-month-old animals ($p < 0.005$; *Fig. 3B*). Notably, enterococci loads in gene-deficient mice older than 3 months were close to the detection limit *(Fig. 3B)*.

Irrespective of the genotype, intestinal lactobacilli loads were approximately two orders of magnitude higher in ≤3-month-old mice than in older counterparts ($p < 0.05$–0.001; *Fig. 4*). In addition, WT mice in the youngest cohort displayed slightly higher fecal burdens of lactobacilli as compared to 1-month-old animals ($p < 0.001$; *Fig. 4A*).

Two-week-old WT mice harbored higher bifidobacteria gene numbers in their feces as compared to younger (except for 3-month-old) mice ($p < 0.001$; *Fig. 5A*). Even though 6-month-old WT animals exhibited very low intes-
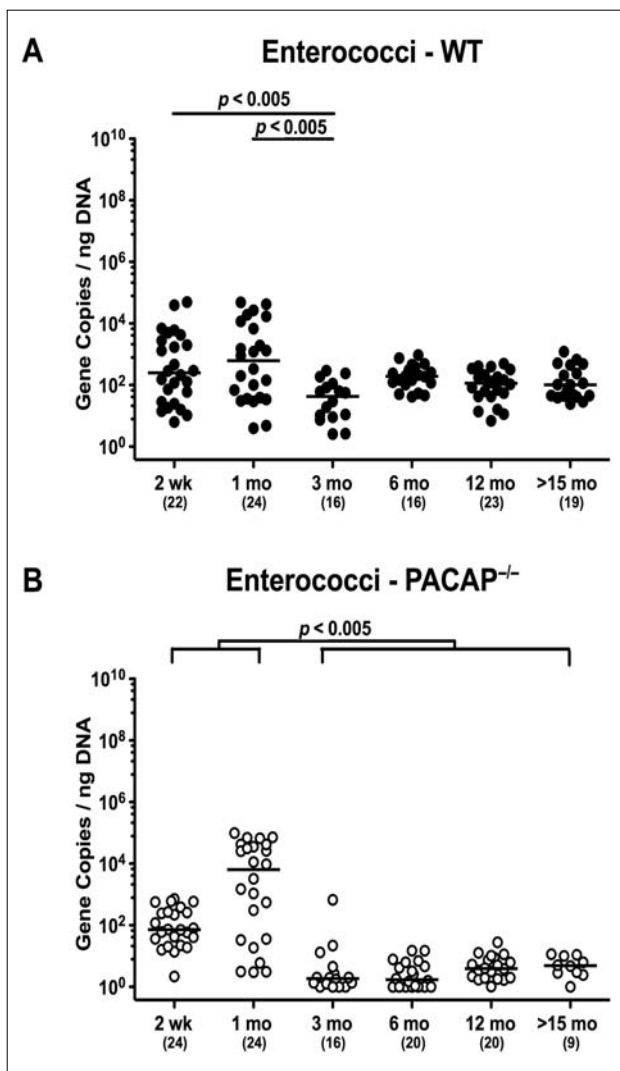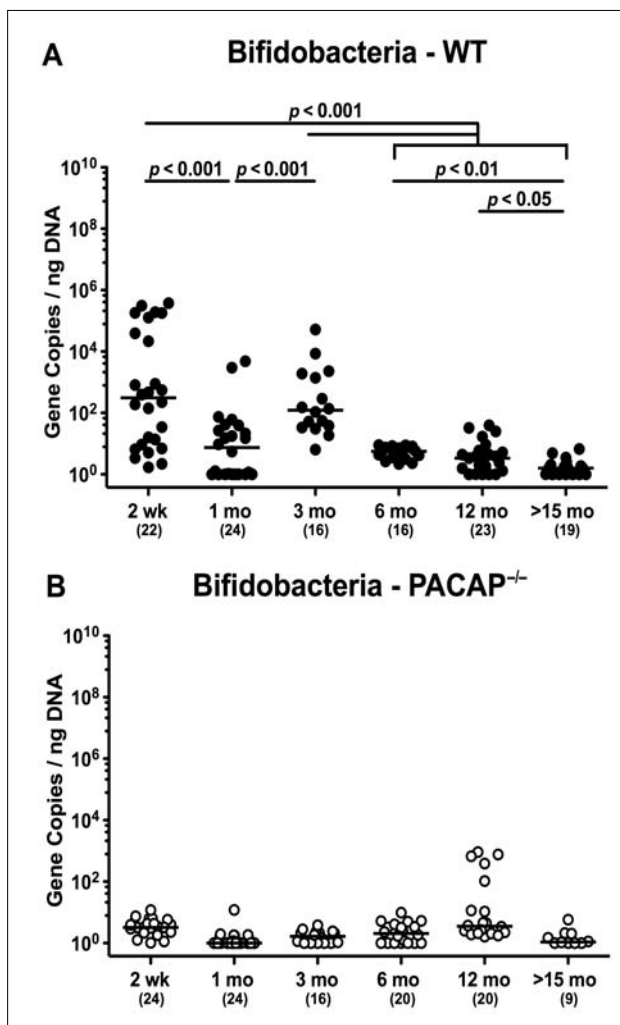

Fig. 3. Fecal gene numbers of enterococci in PACAP-deficient and wildtype mice over time. Individual intestinal loads of enterococci were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated
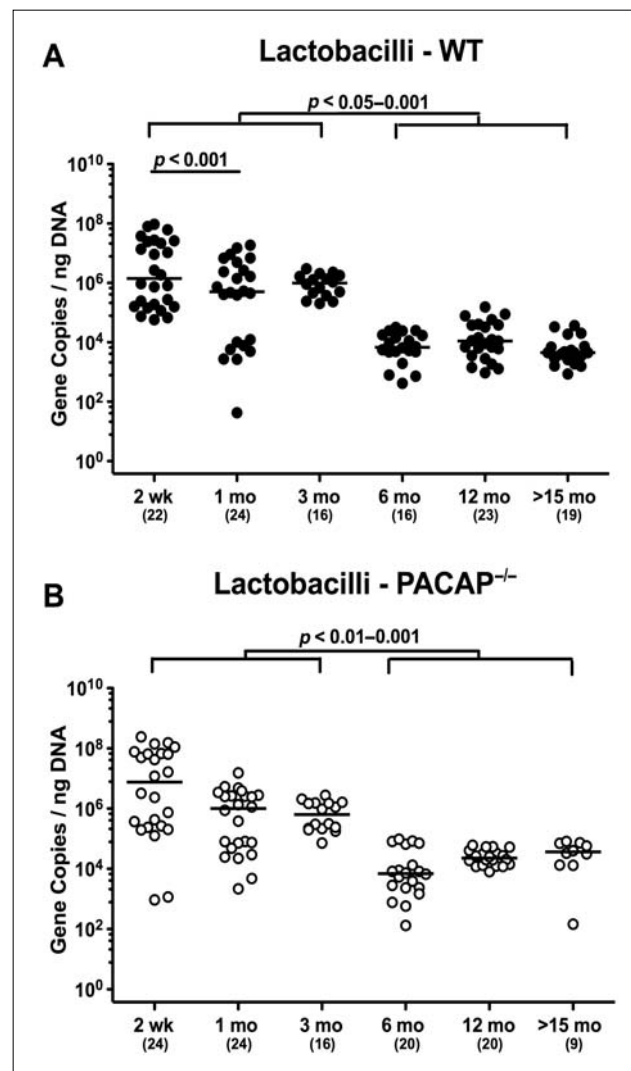
Fig. 4. Fecal gene numbers of lactobacilli in PACAP-deficient and wildtype mice over time. Individual intestinal loads of lactobacilli were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated
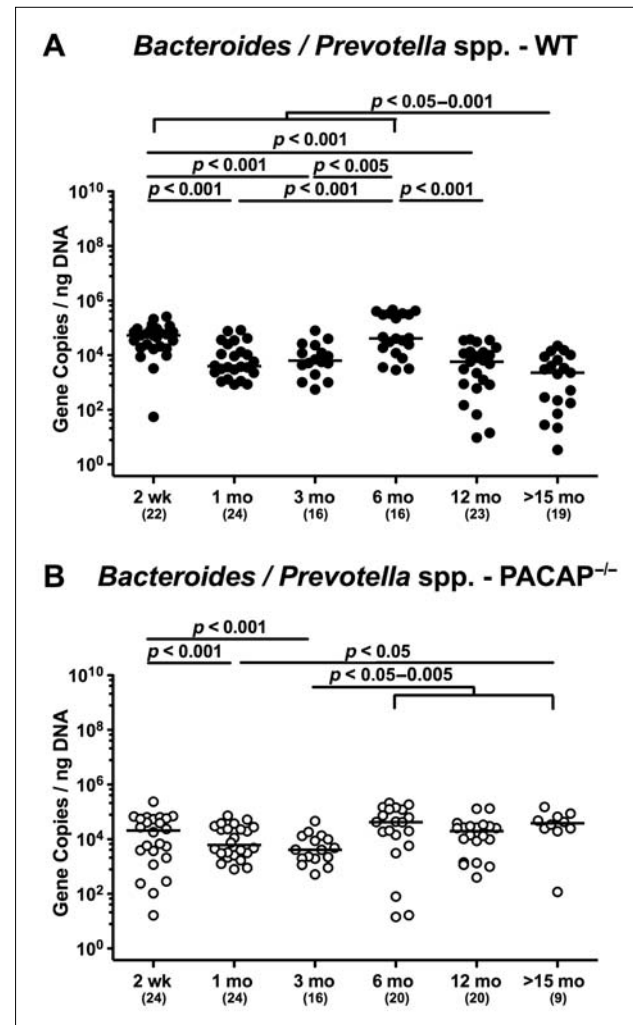
tinal bifidobacteria numbers in their intestines, the fecal loads further declined to the detection limit thereafter ($p < 0.05$–0.01; *Fig. 5A*). Remarkably, even in the youngest cohort, bifidobacteria were (if at all) abundant only in very low numbers (i.e., close to the detection limit) in the intestinal tract of PACAP$^{-/-}$ mice *(Fig. 5B)*.

When analyzing obligate anaerobic gram-negative commensals, fecal loads of *Bacteroides/Prevotella* spp. were highest in the youngest and lowest in the oldest cohort of WT mice ($p < 0.05$–0.001; *Fig. 6A*). In PACAP$^{-/-}$ mice, however, fecal gene numbers of *Bacteroides/Prevotella* spp. slightly decreased until 3 months of age ($p < 0.001$) but were higher thereafter ($p < 0.05$–0.005) and comparable to loads observed in the youngest cohort *(Fig. 6B)*.

In both PACAP$^{-/-}$ and WT mice, mouse intestinal *Bacteroides* were up to 1.5 orders of magnitude higher in the fecal samples derived from animals aged ≥3 months as compared to 2-week-old and 1-month-old counterparts ($p < 0.05$–0.001; *Fig. 7*). Conversely, fecal gene numbers of *Clostridium coccoides* were slightly lower in >15-month-old as compared to ≤3-month-old WT mice ($p < 0.05$–0.005; *Fig. 8A*). In >6-month-old PACAP$^{-/-}$ mice, *C. coccoides* loads were up to 1.5 orders lower in the feces as compared to those detected in younger animals ($p < 0.05$–0.001; *Fig. 8B*). Fecal gene numbers of the *C. leptum* group increased after 2 weeks of age in WT animals by approximately one order of magnitude and remained stable until the end of the observation pe-

Fig. 5. Fecal gene numbers of bifidobacteria in PACAP-deficient and wildtype mice over time. Individual intestinal loads of bifidobacteria were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated
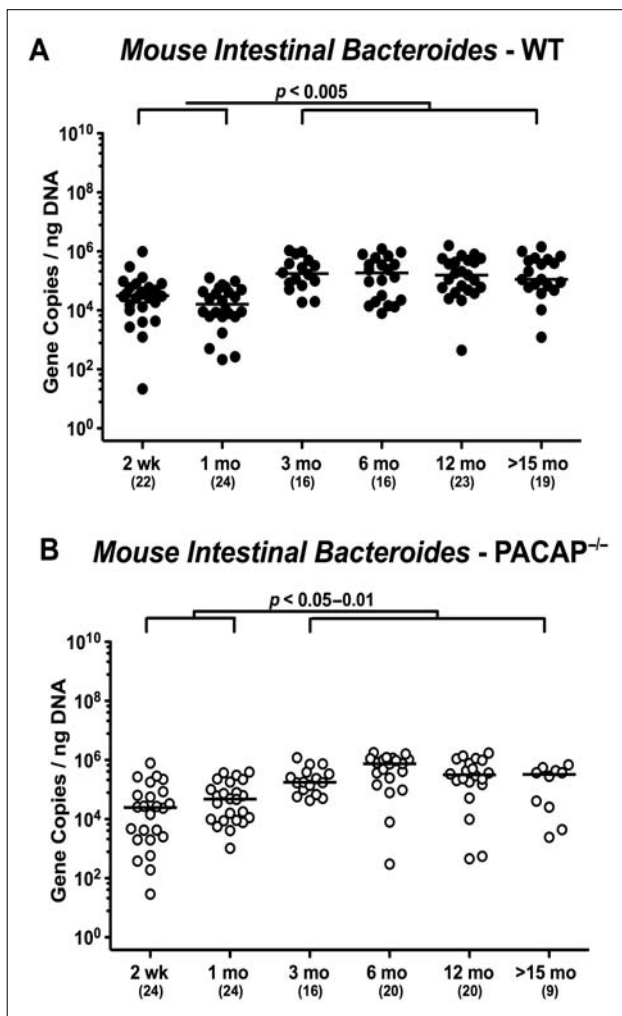
Fig. 6. Fecal gene numbers of *Bacteroides/Prevotella* spp. in PACAP-deficient and wildtype mice over time. Individual intestinal loads of *Bacteroides/Prevotella* spp. were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated

riod ($p < 0.05$–0.001; *Fig. 9A*). Interestingly, intestinal *C. leptum* loads were virtually comparable over time in PACAP$^{-/-}$ mice (n.s.; *Fig. 9B*).

Genotype-dependent differences in fecal microbiota of PACAP$^{-/-}$ and WT mice at defined time points postpartum

When comparing total eubacterial gene numbers in fecal samples derived from WT and PACAP$^{-/-}$ mice at defined time points postpartum, no differences could be observed (n.s.; *Fig. 10*). Fecal enterobacteria loads, however, were two orders of magnitude lower in 1 month old PACAP$^{-/-}$ as compared to age-matched WT mice ($p < 0.001$; *Fig. 11A*),

whereas, conversely, respective loads were slightly higher in the former versus the latter when older than 15 months ($p < 0.05$; *Fig. 11A*). PACAP$^{-/-}$ mice harbored more enterococci in their intestines as compared to WT counterparts, whereas the opposite was true for ≥6-month-old mice ($p < 0.001$; *Fig. 11B*). Whereas lactobacilli were higher in fecal samples derived from PACAP$^{-/-}$ as compared to WT mice of the respective oldest cohorts (i.e., >15 months of age, $p < 0.005$; *Fig. 12A*), intestinal bifidobacteria were virtually absent in PACAP$^{-/-}$ mice and up to two orders of magnitude lower until the age of 6 months ($p < 0.001$; *Fig. 12B*). Obligate anaerobic gram-negative commensals such as *Bacteroides*/*Prevotella* spp. were up to 1.5 order of magnitude higher in ≥12-month-old PACAP$^{-/-}$ as compared to age-matched WT control animals ($p < 0.05$–0.001;

Fig. 7. Fecal gene numbers of *Mouse Intestinal Bacteroides* in PACAP-deficient and wildtype mice over time. Individual intestinal loads of *Mouse Intestinal Bacteroides* were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated
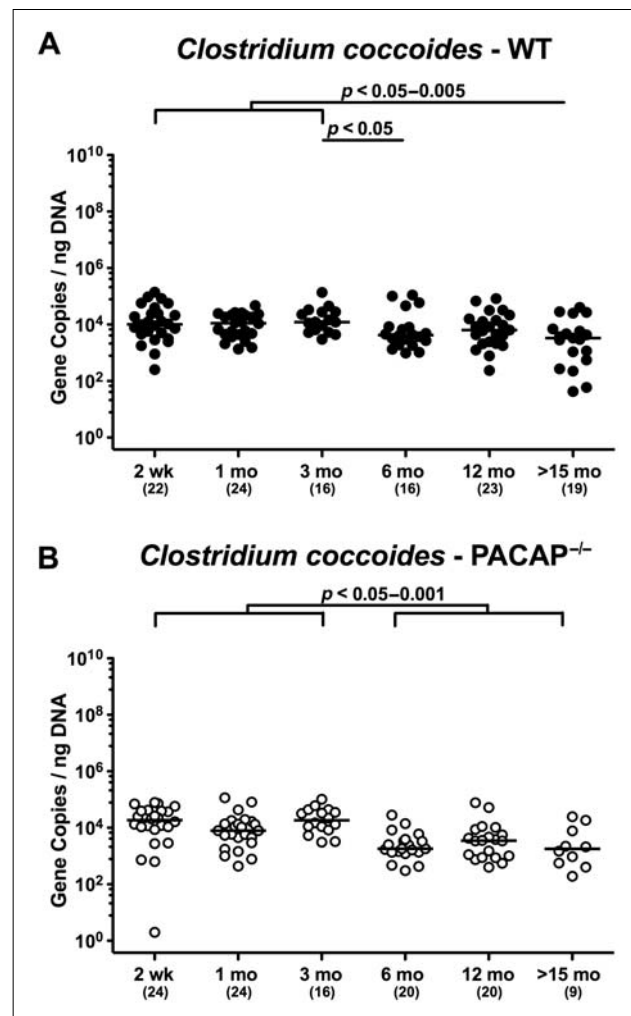
Fig. 8. Fecal gene numbers of *Clostridium coccoides* in PACAP-deficient and wildtype mice over time. Individual intestinal loads of *Clostridium coccoides* were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated
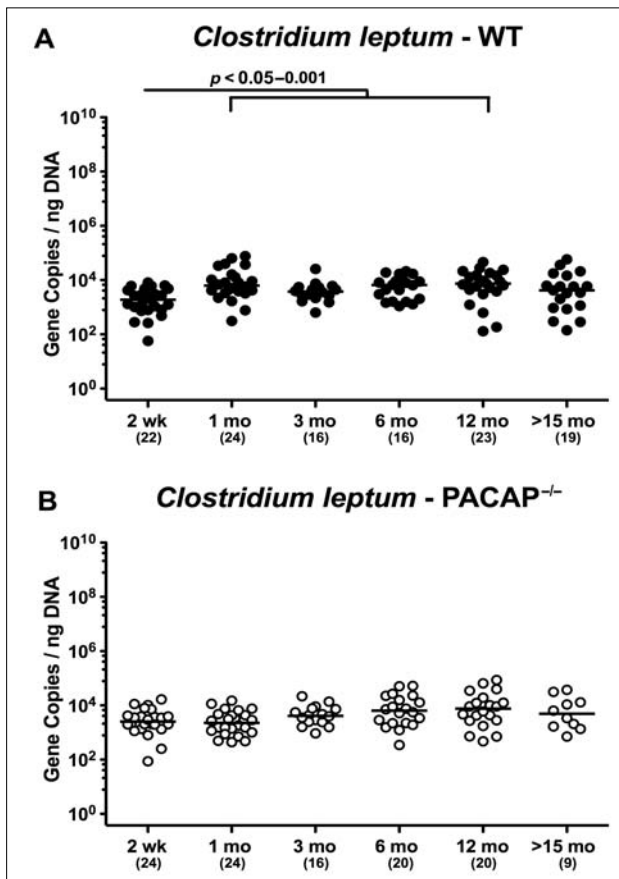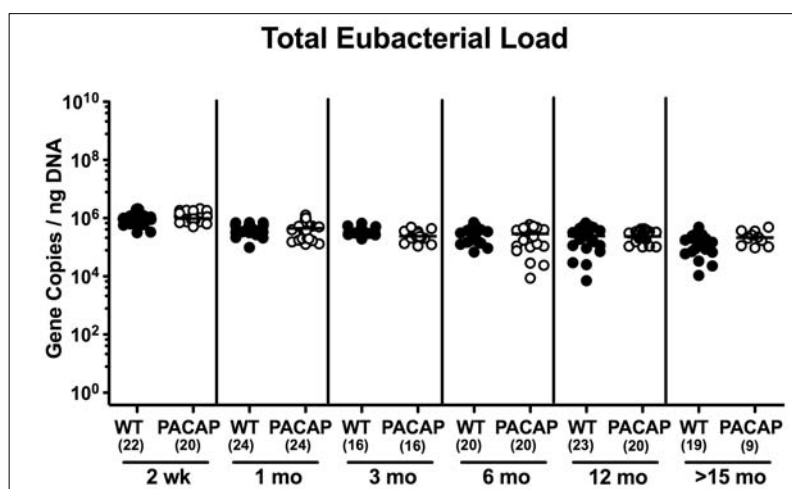
Fig. 9. Fecal gene numbers of *Clostridium leptum* in PACAP-deficient and wildtype mice over time. Individual intestinal loads of *Clostridium leptum* were quantitatively assessed in fecal samples derived from (A) wildtype (WT, closed circles) and (B) pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses) and significance levels (*p* values), determined by Mann–Whitney *U* test are indicated

Fig. 13A), which also held true for *Mouse Intestinal Bacteroides* in respective 1-month-old and 6-month-old mice ($p < 0.05$; *Fig. 13B*). Interestingly, fecal loads of obligate gram-positive species such as *C. coccoides* and *C. leptum* groups did not differ in PACAP$^{-/-}$ and WT mice at either time point postpartum (n.s.; *Fig. 14*).

Discussion

During the past years, the commensal microbiota has gained increasing attention concerning their impact in health and disease [24]. Microbial colonization and host immune development and maturation run in parallel and are essentially involved in regulation of intestinal (patho) physiology in mice and men [25, 26]. Given the pivotal interactions between the intestinal microbiota and host physiology, pertubations of this fine-tuned, orchestrated interplay result in increased vulnerability of the host to external (harmful) stimuli and increased susceptibility to disease not only of the intestinal tract [17, 27], in fact, phenomena that are shared by PACAP gene-deficient mice [6]. This prompted us to present a comprehensive kinetic survey of the intestinal microbiota composition and their changes "during lifetime" of PACAP$^{-/-}$ and corresponding WT mice for the first time.

The microbial colonization of the intestinal tract starts immediately after birth with facultative anaerobic commensal species such as enterobacteria, enterococci, and lactobacilli as the first colonizers [28], as we could confirm with highest respective fecal loads in the youngest murine cohort (i.e., 2 weeks postpartum) in our study. Irrespective of the genotype, fecal gene numbers of respective commensals declined with progressive aging, particularly after 3 months of age. Conversely, obligate anaerobic bacterial species including *Bacteroides* and *Clostridium* species, but also bifidobacteria, usually establish gradually in

Fig. 10. Fecal total eubacterial gene numbers in PACAP-deficient as compared to wildtype mice over time. Individual intestinal total eubacterial loads were quantitatively assessed in fecal samples derived from wildtype (WT, closed circles) and pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians and numbers of analyzed animals (in parentheses) are indicated
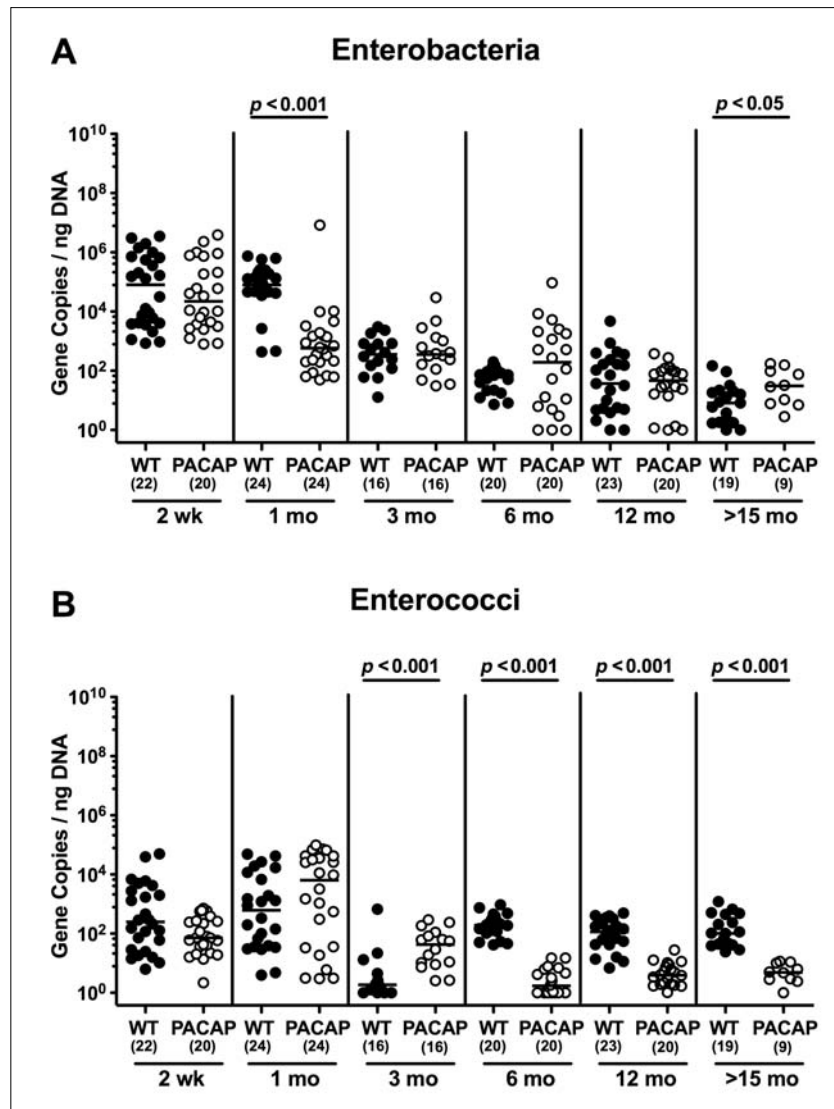
Fig. 11. Fecal gene numbers of enterobacteria and enterococci in PACAP-deficient as compared to wildtype mice over time. Individual loads of (A) enterobacteria and (B) enterococci were quantitatively assessed in fecal samples derived from wildtype (WT, closed circles) and pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the x axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (p values) determined by Mann–Whitney U test are indicated

mice and men [29, 30]. Our results are well in line, given that *Mouse Intestinal Bacteroides* increased in either mice after 1 month of age, for instance, whereas *Bacteroides/Prevotella* spp. were lowest in feces of >15-month-old WT but not PACAP$^{-/-}$ mice. Strikingly, bifidobacteria were virtually absent in the intestinal tract of PACAP$^{-/-}$ mice, even in still breastfed 2-week-old infants. In WT mice, however, bifidobacterial loads were highest in 2-week-old mice and subsequently declined after 3 months of age.

Bifidobacteria belong to the phylum Actinobacteria and constitute the predominant microbial group in healthy breastfed babies. It remains relatively stable during adulthood and tend to decrease upon senescence [28]. Whereas bifidobacteria are considered important for intestinal homeostasis, a delayed bifidobacterial colonization of the intestinal tract during infancy results in increased susceptibility to diseases in childhood and later in life [31]. For instance, dysbiosis with decreased bifidobacterial numbers have been associated with distinct immunopathologies such as inflammatory bowel disease, irritable bowel disease, celiac disease, and atopic disease [28]. In our own studies, conventionally colonized mice gene-deficient for nucleotide-binding oligomerization domain (NOD) 2 or Toll-like receptor (TLR) 9 were virtually deprived from intestinal bifidobacteria but suffered from more distinct *Toxoplasma gondii*-induced inflammatory sequelae in intestinal and extra-intestinal compartments including the brain [20, 32].

Genome-based analyses revealed a number of factors that might explain host-beneficial mechanisms exerted by bifidobacteria as reviewed by Klijn et al. [33]: production of 1) bacteriocins, further of 2) lactic acid and 3) acetic acid, thereby lowering the intraluminal pH, 4) exerting functional oxidative stress response mechanisms, and thus
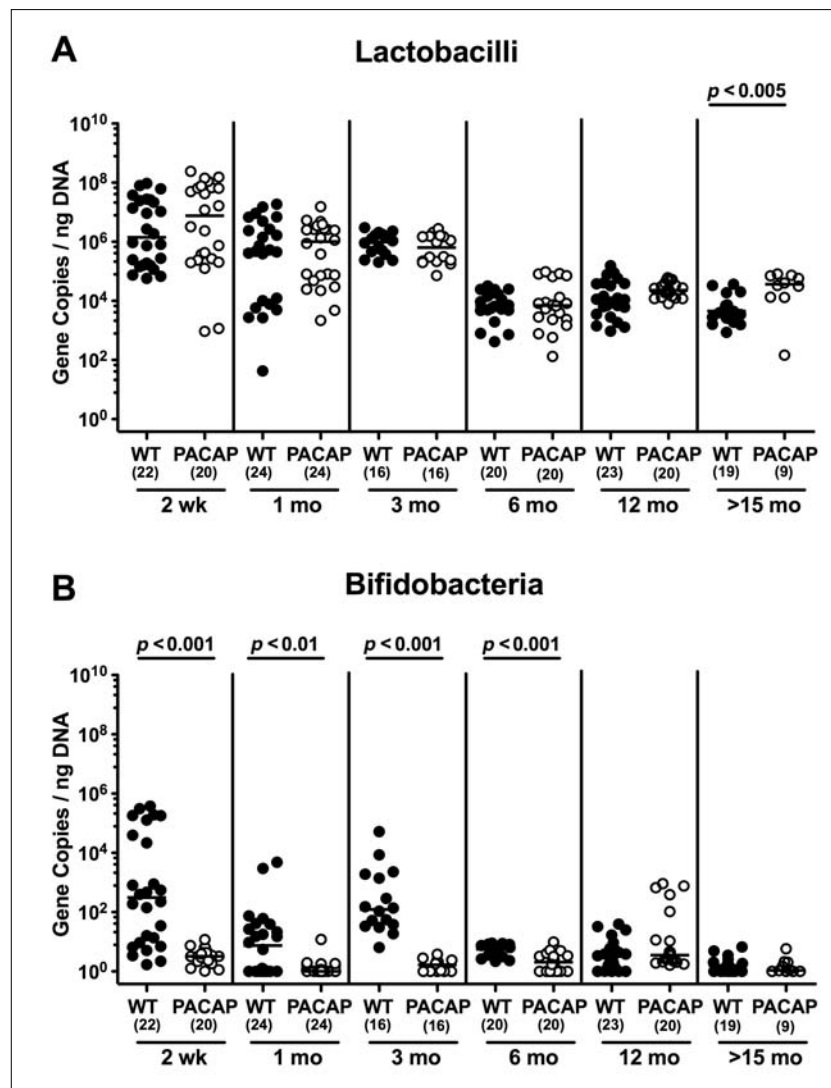
Fig. 12. Fecal gene numbers of lactobacilli and bifidobacteria in PACAP-deficient as compared to wildtype mice over time. Individual loads of (A) lactobacilli and (B) bifidobacteria were quantitatively assessed in fecal samples derived from wildtype (WT, closed circles) and pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated

supporting the host phalanx angainst invading (entero) pathogens as well as providing metabolic features such as 5) production of vitamins, 6) hydrolysis of bile salts, 7) production of conjugated linoleic acid, and 8) degradation of oxalate. 9) Importantly, a very recent report provided a direct link between bifidobacteria and neuromodulatory activities, given that a particular *Bifidobacterium dentium* strain was able to modulate visceral sensitivity in the intestinal tract by production of γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the central nervous system of mammals [34]. This report together with our study further underlines the (patho)physiological importance of the gut–brain axis.

Given their health-promoting features, viable bifidobacterial strains have been introduced into probiotic formulations such as VSL#3 and have been shown efficient in ameliorating symptoms and maintaining remission of patients suffering from inflammatory bowel disease [28].

Very recently, we were able to demonstrate the extensive impact of VSL#3 treatment on mucosal, peripheral, and systemic innate and adaptive immunity exerting beneficial anti-inflammatory effects in intestinal as well as systemic compartments of mice that had been subjected to broad-spectrum antibiotic treatment [27]. In addition, VSL#3 treatment was sufficient to attenuate intestinal, but also extra-intestinal including systemic pro-inflammatory responses upon murine infection with the enteropathogen *Campylobacter jejuni* [35].

It is, hence, highly likely that the lack of intestinal bifidobacteria observed in PACAP$^{-/-}$ mice might be one key factor explaining their vulnerability to cell-toxic stimuli and increased susceptibility to immunopathological conditions in both intestinal and extra-intestinal compartments.

In addition, we were able to show that facultative anaerobic commensals such as enterobacteria (e.g., *E. coli*) were lower in the intestines of 1-month-old PACAP$^{-/-}$ as
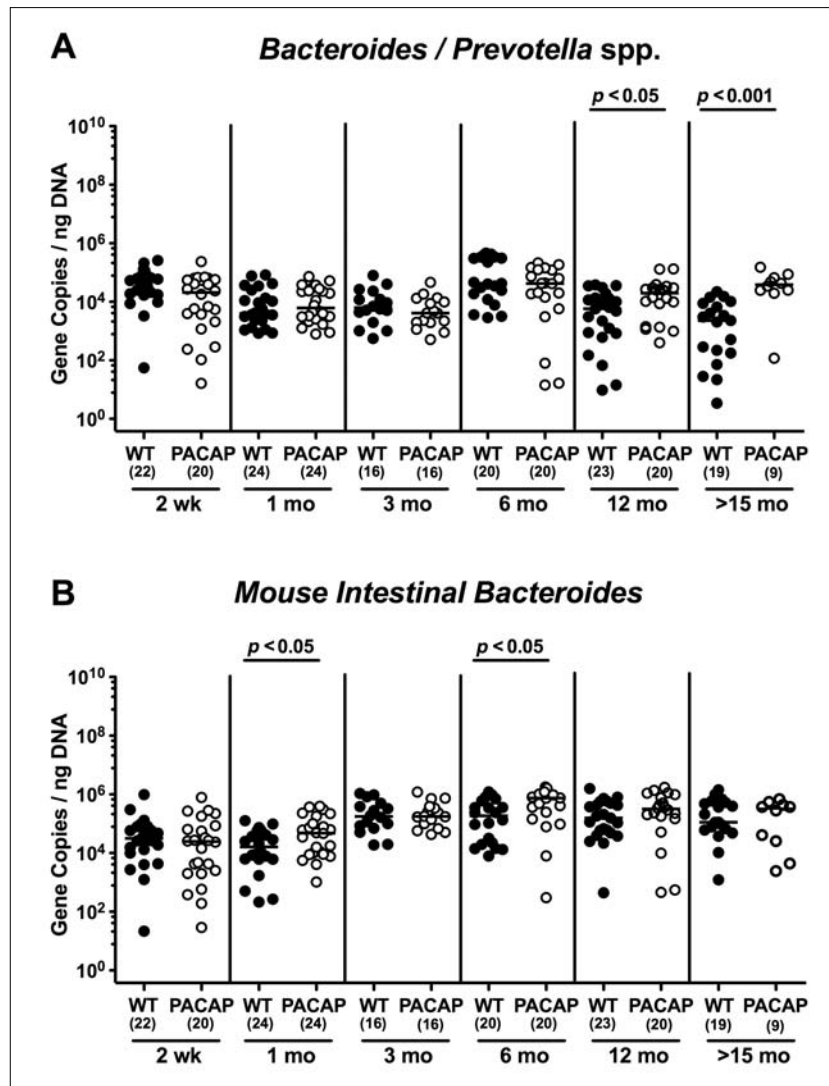
Fig. 13. Fecal gene numbers of *Bacteroides*/*Prevotella* spp. and *Mouse Intestinal Bacteroides* in PACAP-deficient as compared to wildtype mice over time. Individual loads of (A) *Bacteroides*/*Prevotella* spp. and (B) *Mouse Intestinal Bacteroides* were quantitatively assessed in fecal samples derived from wildtype (WT, closed circles) and pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians, numbers of analyzed animals (in parentheses), and significance levels (*p* values) determined by Mann–Whitney *U* test are indicated

compared to WT mice, which also held true for enterococci later in murine life (i.e., ≥6 months of age). Whereas gene numbers of obligate anaerobic gram-negative commensals such as *Mouse Intestinal Bacteroides* were slightly higher in the feces of PACAP$^{-/-}$ versus WT mice aged 1 and 6 months, this held true for *Bacteroides*/*Prevotella* spp. in the intestines later in life (i.e., ≥12 months of age).
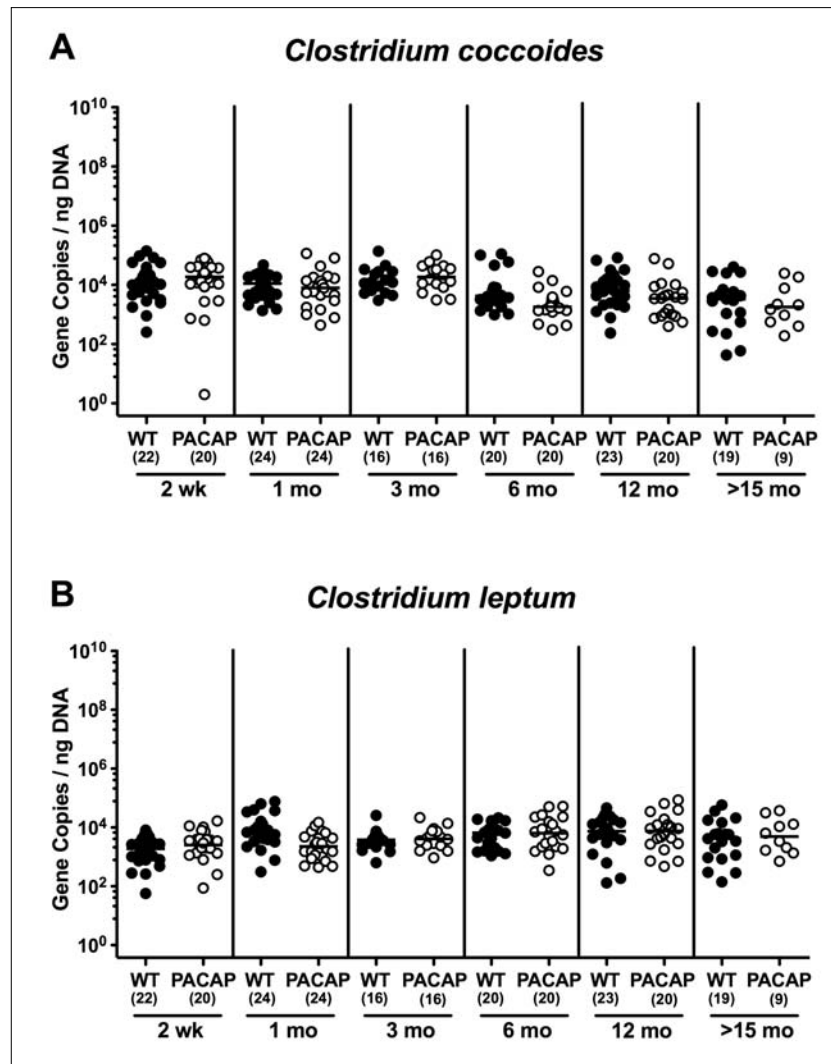
Conclusion

We conclude that the described genotype-dependent differences in gut microbiota composition and their development over time with the virtually absent intestinal bifidobacteria as hallmark might explain the distinct phenotypes observed in PACAP$^{-/-}$ mice upon cell-toxic (i.e., oxidative, traumatic, inflammatory) challenges further supporting the major (patho)physiological impact of the gut–brain axis.

Fig. 14. Fecal gene numbers of *Clostridium coccoides* and *Clostridum leptum* in PACAP-deficient as compared to wildtype mice over time. Individual loads of (A) *Clostridium coccoides* and (B) *Clostridum leptum* were quantitatively assessed in fecal samples derived from wildtype (WT, closed circles) and pituitary adenylate cyclase activating polypeptide-deficient (PACAP$^{-/-}$, open circles) mice at defined time points postpartum (as indicated on the *x* axis; wk: weeks; mo: months) by qRT-PCR and expressed as 16S rRNA gene numbers per nanogram DNA. Medians and numbers of analyzed animals (in parentheses) are indicated

Conflict of interest

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