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Fast acquisition of a polysaccharide fermenting gut microbiome by juvenile green turtles Chelonia mydas after settlement in coastal habitats

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Abstract

Background: Tetrapods do not express hydrolases for cellulose and hemicellulose assimilation, and hence, the independent acquisition of herbivory required the establishment of new endosymbiotic relationships between tetrapods and microbes. Green turtles (*Chelonia mydas*) are one of the three groups of marine tetrapods with an herbivorous diet and which acquire it after several years consuming pelagic animals. We characterized the microbiota present in the feces and rectum of 24 young wild and captive green turtles from the coastal waters of Brazil, with curved carapace length ranging from 31.1 to 64.7 cm, to test the hypotheses that (1) the ontogenetic dietary shift after settlement is followed by a gradual change in the composition and diversity of the gut microbiome, (2) differences exist between the composition and diversity of the gut microbiome of green turtles from tropical and subtropical regions, and (3) the consumption of omnivorous diets modifies the gut microbiota of green turtles.

Results: A genomic library of 2,186,596 valid bacterial 16S rRNA reads was obtained and these sequences were grouped into 6321 different operational taxonomic units (at 97% sequence homology cutoff). The results indicated that most of the juvenile green turtles less than 45 cm of curved carapace length exhibited a fecal microbiota co-dominated by representatives of the phyla *Bacteroidetes* and *Firmicutes* and high levels of *Clostridiaceae*, *Prophyromonas*, *Ruminococcaceae*, and *Lachnospiraceae* within the latter phylum. Furthermore, this was the only microbiota profile found in wild green turtles > 45 cm CCL and in most of the captive green turtles of any size feeding on a macroalgae/fish mixed diet. Nevertheless, microbial diversity increased with turtle size and was higher in turtles from tropical than from subtropical regions.

Conclusions: These results indicate that juvenile green turtles from the coastal waters of Brazil had the same general microbiota, regardless of body size and origin, and suggest a fast acquisition of a polysaccharide fermenting gut microbiota by juvenile green turtles after settlement into coastal habitats.

Keywords: Tetrapods, Herbivorous, Microbial communities, *Chelonia mydas*, 16S rRNA, Fermentation

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Background

Herbivory has evolved independently in several groups of tetrapods belonging to diverse evolutionary lineages [1]. Unlike some invertebrates, tetrapods do not express hydrolases for cellulose and hemicellulose [2], and hence, the independent acquisition of herbivory required the establishment of new endosymbiotic relationships between tetrapods and microbes [1, 3–5]. As a consequence, the composition, abundance, and diversity of the gut microbiota of herbivorous tetrapods vary widely across groups, reflecting not only their evolutionary relationships but also their foraging habits and the location of the cavity of fermentation into the gut–hindgut vs. foregut fermenters [6–8].

Several groups of tetrapods have recolonised the marine environment after independent evolution in land, but only three of them are herbivores: sirenians (manatees and the dugong), the marine iguana (*Amblyrhynchus cristatus*), and the green turtle (*Chelonia mydas*). Sirenian diet is dominated by seagrasses [9–12] which are vascular plants rich in cellulose [13, 14]. Consequently, sirenians host microorganisms producing the enzymes needed for the fermentative digestion of cellulose [15, 16]. On the other hand, marine iguanas feed only on macroalgae [17]. The cell wall of macroalgae differs from that of seagrasses and other vascular plants in the abundance of sulfated polysaccharides and alginic acid and low levels of cellulose [18]. As a consequence, the microbiota of marine iguanas is characterized by the presence of some specific groups of methanogens and differs largely from that of terrestrial iguanas, despite a close evolutionary relationship [3]. Green turtles exhibit a much larger dietary flexibility than sirenians and marine iguanas, as they undergo a major ontogenetic dietary shift from animal-based to plant-based diets following settlement in coastal areas [19–25]. Nevertheless, they also exhibit a high level of regional variability in the degree of omnivory after settlement and the relative importance of seagrasses and seaweeds in their diets [20, 21, 23, 26–34].

The acquisition of a specialized microbiota is facilitated by lactation and intimate calve/mother relationships in mammals [35] and the consumption of conspecific excrements in marine iguanas [17]. On the contrary, the solitary lives of green turtles may delay the acquisition of a specialized gut microbiota, which in combination with the higher body temperature of larger turtles in winter may explain the improved digestibility and assimilation of plant material as green turtles grow [13, 20]. This is because green turtles are ectothermic, and the body temperature of inactive adult green turtles can be 2 °C above water temperature thanks to gigantothermy [36], whereas that of juveniles matches that of the environment [37]. It has also been suggested that mixed seagrass/macroalgae diets are uncommon in green turtles because the entirely different structure of polysaccharides in their cell walls would require different compositions of the gut microbiota [38]. In such case, frequent and short-term shifts in diet may reduce the efficiency of plant digestion [39].

Unfortunately, very little is known about the gut microbiota of green turtles, how it changes after settlement in coastal areas in association to the increase in the consumption of plant material, and the influence of turtle diet on microbiota composition. The only information available to our knowledge is about the microbiota present in the cloaca of pelagic and recently settled green turtles, which reveals a high prevalence of *Proteobacteria* and a low occurrence of bacteria associated to the fermentation of structural polysaccharides [40]. In this study, we characterize the microbiota present in the feces and rectum of young wild and captive green turtles from Brazil to test the hypotheses that (1) the ontogenetic dietary shift after settlement is followed by a gradual change in the composition and diversity of the gut microbiome, (2) differences exist in the composition and diversity of the gut microbiome of green turtles from tropical and subtropical regions, and (3) the consumption of omnivorous diets modifies the gut microbiota of green turtles.

Methods

Study area

Two different areas of Brazil were sampled in February to March 2016. Most samples ($n = 20$) were collected from subtropical Ubatuba (23° 26′ S, 45° 05′ W), in the northern coast of the state of Sao Paulo. Rocky reefs and sandy beaches dominate the coastline of Ubatuba [41]. A few additional samples ($n = 5$) were collected from tropical Praia do Forte (12° 38′ S 38° 05′ W), located 70 km from Salvador do Bahia. The coastline is characterized by the presence of shallow coral reefs with substantial air exposition during low tide [42].

Sampling

Fecal samples were collected from 8 turtles held in captive at the facilities of Projeto Tamar at Ubatuba and 11 wild turtles from Ubatuba. Some wild green turtles were captured alive in weirs ("*Cercos flutuantes*") used by local fishermen and consisting on fixed nets attached to the seafloor [43], and others were captured alive through free diving by members of Projeto Tamar (www.tamar.org.br), as part of the long-term study on the abundance and habitat use of green turtles along the Brazilian coast. After capture, curved carapace length (CCL) was measured with a flexible tape (CCL, notch to tip) and turtles were moved to the facilities of Projeto Tamar in Ubatuba. These turtles were confined in individual PVC tanks until the moment they defecated, between 24 and 36 h after capture, and then released back to the sea at the same place of capture.

Tanks had been previously disinfected with regular bleach. The core of each fecal pellet was accessed using sterilized forceps and sampled with a swab, to reduce as much as possible contamination from water. Additionally, rectal samples ($n = 5$) were collected with a swab during the necropsy of recently dead turtles at Praia do Forte.

Fecal and rectal samples were stored at 4 °C immediately after collection and then at − 20 °C until DNA extraction. No buffers were used. All procedures were non-invasive and conducted in accordance with guidelines from the Projeto TAMAR and ICMBio.

DNA extraction and next-generation sequencing

DNA was extracted from a subsample of 0.25 g from each fecal or rectal sample using the PowerSoil DNA kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. All DNA extracts were kept frozen at − 20 °C until further analysis. Massive barcoded 16S rRNA gene-based libraries in the *Eubacteria* domain were sequenced by using the MiSeq Illumina platform (Molecular Research DNA LP, Shallowater, USA). These gene libraries were constructed by targeting the V1–V3 hypervariable regions with the primer set 27F (5′-AGRGTTTGATCMTGGCTCAG-3′)/519R (5′-GTN TTACNGCGGCKGCTG-3′) as previously described in [44]. The obtained DNA reads were compiled in FASTq files for further bioinformatic processing. Trimming of the 16S rRNA barcoded sequences into libraries was carried out using QIIME software version 1.8.0 [45]. Quality filtering of the reads was performed at Q25, the default set in QIIME, prior to the grouping into operational taxonomic units (OTU) at a 97% sequence homology cutoff. The following steps were performed using QIIME: Denoising of sequence data using Denoiser [46], picking up of OTU reference sequences via the first method of the UCLUST algorithm [47] and, for sequence alignment and chimera detection, processing by PyNAST [48] and ChimeraSlayer [49]. OTUs were then taxonomically classified using BLASTn against GreenGenes and RDP (Bayesian Classifier) databases and compiled into each taxonomic level [50].

Biostatistical methods

A general lineal model (GLM) using locality (Ubatuba vs. Praia do Forte) as a fixed factor and turtle curved carapace length as a covariable was used to test the hypothesis that the microbial diversity of wild green turtles increases with turtle size and varies across localities. A general lineal model using origin (captive vs. wild) as a fixed factor and turtles curved carapace length as a covariable was used to test the hypothesis that the microbial diversity of green turtles increases with turtle size and differs between captive and wild green turtles from subtropical Ubatuba. GLMs were run in IBM SPSS Statistics

23. Multivariate principal coordinate analysis (PCoA) based on Bray-Curtis similarity distances was carried out on the OTUs incidence matrix using the CANOCO software package, version 5 (Microcomputer Power, Ithaca, NY, USA), to identify clusters of green turtles differing in the community structure of their microbiomes.

Results

The gut microbiome of 24 green turtles ranging in curved carapace length (CCL) from 31.1 to 64.7 cm was studied. A genomic library of 2,187,066 valid eubacterial 16S rRNA reads was obtained from their feces (Additional file 1). These sequences were grouped into 6321 different OTUs (at 97% sequence homology cutoff), ranging from 473 to 1952 in individual turtles (Table 1). The Good's coverage estimator on the percentage of the total species (as OTUs) represented in any given sample was above 98%, indicating that the observed species encompassed a very significant proportion of the entire sample populations. With this respect, the number of expected OTUs (Chao 1) ranged from 959 to 2818 and the Shannon index from 2.17 to 5.38 (Table 1). The number of recovered and expected OTUs in wild turtles form Praia del Forte was larger than those in wild turtles from Ubatuba and increased significantly with curved carapace length in both areas according to GLM (Table 2). However, the indices of microbial diversity did not differ between wild and captive turtles from Ubatuba (GLM; OTUs: $F_{2,18} = 1.750$, $p = 0.205$; Chao1: $F_{2,18} = 1.922$, $p = 0.179$; Shannon: $F_{2,18} = 2.445$, $p = 0.118$).

The dominant phyla in the majority of wild and captive turtles were *Bacteroidetes*, ranging 20–70% of relative abundance (RA), and *Firmicutes* with a 24–56% of RA (Fig. 1). In most of the studied turtles (Fig. 2), the predominant families within *Bacteroidetes* phylum were *Bacteroidaceae* and *Porphyromonadaceae*, while within *Firmicutes* phylum the predominant families were *Clostridiaceae*, *Lachnospiraceae*, and *Ruminococaceae*, with the exception of two wild individuals and one captive individual from Ubatuba. The bacterial community structure of these two anomalous wild turtles (UB7 and UB10) was characterized by a high RA of representatives from the phyla *Proteobacteria* (approximately 60% RA) and *Actinobacteria*, which in this latter phylum belonged to the *Mycobacterium* genus (1.2 and 4.7% RA in UB7 and UB10, respectively). The main OTUs of the former *Proteobacteria* phylum were related to *Burkholderia* spp. (*Betaproteobacteria*), *Sphingopyxis* spp. (*Alphaproteobacteria*), and *Pseudomonas* spp. (*Gammaproteobacteria*), which combined represented 49.5 and 38.3% RA for UB7 and UB10, respectively. Except for *Sphingopyxis*, these genera have been associated to the presence of *Staphylococcus* spp., in the phylum *Firmicutes* (5.0% and 3.5% of RA in UB7 and UB10, respectively). Regarding the bacterial community of the anomalous captive turtle

Table 1 Descriptors of bacterial diversity in fecal and rectal samples of juvenile green turtles *Chelonia mydas* from Brazil

Study area	Origin	Turtle	CCL (cm)	Total reads	OTUs	Coverage (%)	Shannon (ave ± SD)[a]	Chao1 (ave ± SD)[a]
Praia do Forte–BA	Wild	PF1	31.1	70,792	1589	99	4.69 ± 0.006	2015 ± 78
Praia do Forte–BA	Wild	PF2	35.0	111,405	1794	99	4.17 ± 0.008	1790 ± 88
Praia do Forte–BA	Wild	PF3	38.8	70,850	1997	98	5.14 ± 0.006	2523 ± 85
Praia do Forte–BA	Wild	PF4	40.0	90,045	1911	99	4.22 ± 0.008	2148 ± 87
Praia do Forte–BA	Wild	PF5	44.0	89,351	2211	98	5.05 ± 0.007	2466 ± 90
Ubatuba–SP	Wild	UB6	37.0	127,862	1217	99	2.16 ± 0.009	1071 ± 69
Ubatuba–SP	Wild	UB7	39.7	68,389	601	99	2.59 ± 0.006	956 ± 80
Ubatuba–SP	Wild	UB8	40.0	98,513	1954	99	4.70 ± 0.007	2053 ± 77
Ubatuba–SP	Wild	UB9	41.3	76,055	1947	99	4.82 ± 0.006	2389 ± 90
Ubatuba–SP	Wild	UB10	44.7	61,852	598	99	3.08 ± 0.005	953 ± 67
Ubatuba–SP	Wild	UB11	45.0	119,273	2150	99	4.37 ± 0.008	2036 ± 93
Ubatuba–SP	Wild	UB12	47.0	119,764	2206	99	4.47 ± 0.008	2050 ± 81
Ubatuba–SP	Wild	UB13	53.3	84,889	2006	99	4.60 ± 0.008	2264 ± 79
Ubatuba–SP	Wild	UB14	54.2	107,097	1670	99	3.24 ± 0.009	1657 ± 71
Ubatuba–SP	Wild	UB15	58.3	90,582	1951	99	4.53 ± 0.007	2187 ± 90
Ubatuba–SP	Wild	UB16	61.4	79,361	2179	98	5.15 ± 0.006	2540 ± 83
Ubatuba–SP	Captivity	UB17	32.5	103,168	2284	99	4.55 ± 0.008	2355 ± 88
Ubatuba–SP	Captivity	UB18	34.9	121,100	1481	99	2.88 ± 0.009	1374 ± 75
Ubatuba–SP	Captivity	UB19	38.6	56,987	1723	99	4.85 ± 0.005	2447 ± 77
Ubatuba–SP	Captivity	UB20	40.0	123,937	1442	99	2.79 ± 0.009	1302 ± 71
Ubatuba–SP	Captivity	UB21	41.3	101,478	2436	98	4.68 ± 0.008	2549 ± 93
Ubatuba–SP	Captivity	UB22	53.5	99,346	2079	99	4.17 ± 0.009	2118 ± 75
Ubatuba–SP	Captivity	UB23	58.6	70,520	2330	98	5.38 ± 0.006	2802 ± 77
Ubatuba–SP	Captivity	UB24	64.7	43,980	1036	99	4.70 ± 0.001	1875 ± 34
Range			31.1–64.7	70,792–127,862	1589–2436	98–99	2.16–5.38	953–2549

Fecal samples were collected at Ubatuba and rectal samples at Praia do Forte
CCL curved carapace length, *BA* State of Bahia, *SP* State of Sao Paulo, *ave* average
[a]Calculated upon sample rarefaction at 43000 reads

Table 2 Summary statistics of general lineal models describing the relationship between indices of microbial diversity in fecal and rectal samples of wild juvenile green turtles *Chelonia mydas*, sampling area (subtropical Ubatuba and tropical Praia do Forte) and curved carapace length (CCL)

Microbial diversity		*F*	df	*p*	*r²*
OTUs	Model	4.155	2.15	*0.040*	0.296
	CCL	6.016	2.16	*0.023*	
	Area	6.205	2.16	*0.028*	
Chao 1	Model	4.517	2.16	*0.032*	0.319
	CCL	6.177	2.15	*0.027*	
	Area	7.671	2.15	*0.016*	
Shannon	Model	3.180	2.16	0.075	NA
	CCL	3.939	2.15	0.069	
	Area	2.708	2.15	0.033	

Microbial diversity is higher in tropical Praia do Forte and increases with turtles size. Italics denote statistical significance
NA not applicable

(UB18), it was characterized by a high abundance of the phylum *Fusobacteria* (27% RA). Such dominance was primarily caused by OTU7, affiliated with the microaerotolerant fermentative *Cetobacterium* sp. (96% of similarity to *Cetobacterium ceti*), also found in whale, dolphin, and porpoise gut flora (Bik et al. 2016).

When those three anomalous turtles (UB7, UB10, and UB18) were removed from the analysis, the abundance of *Proteobacteria* was consistently higher in captive (range 0.7–7.7% RA) than in wild (range 0.2–1.9% RA) turtles from Ubatuba (Mann-Whitney test; $U = 57.00$, $p = 0.046$). On the other hand, *Akkermansia* spp., belonging to the phylum *Verrumicrobia*, was found with a RA of 8–15% in captive turtles UB18, UB20, and UB21. It is noteworthy that in one of the wild individuals (UB14), *Akkermansia* was enriched up to a 30% of RA and, curiously, the microbiome of this individual was rather different from that of other wild turtles.
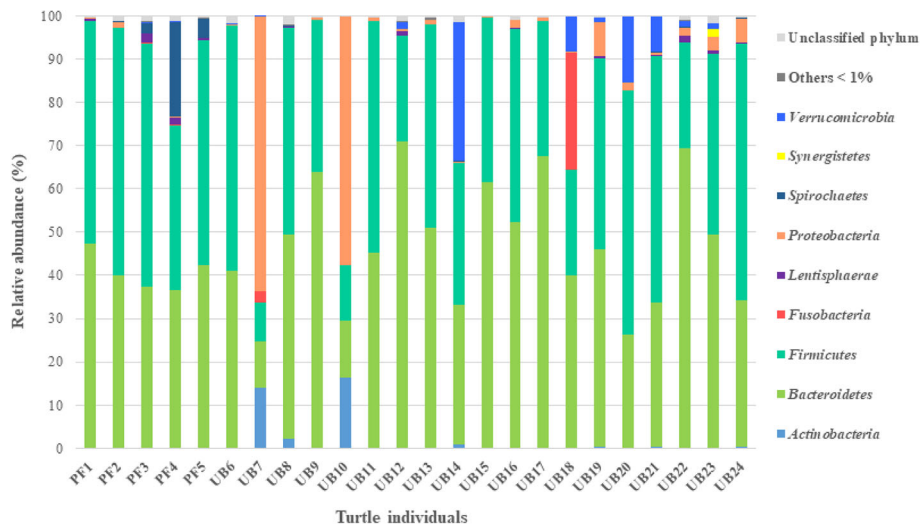
Fig. 1 Percentages of sequences from each individual turtle, fecal or rectal sample assigned at the phylogenetic level of phylum, according to the RDP Bayesian Classifier database with a bootstrap confidence above 80%. PF1 to PF5 = wild turtles from Praia do Forte; UB6 to UB16 = wild turtles from Ubatuba; UB17 to UB24 = captive turtles from Ubatuba. Taxa with a RA lower than 1% is grouped as "others"

The family *Clostridiaceae* comprised a ribotype (OTU1) that was predominant in almost all samples (from 1 to 8% RA). OTU1 belongs to the unclassified *Clostridiaceae* 1 subfamily. Interestingly, the RA of OTU1 in the wild turtles with the most dissimilar microbiome (UB7 and UB10) was < 0.1% RA (Figs. 1 and 2). Moreover, predominant

OTUs of *Lachnospiraceae* and *Bacteriaceae* in those anomalous turtles were present at a comparatively low RA. On the other hand, representatives of the genus *Spirochaetes* were detected in all samples, but only in turtles from Praia do Forte this phylum appeared in significant amounts, especially in PF3, PF4, and PF5, where
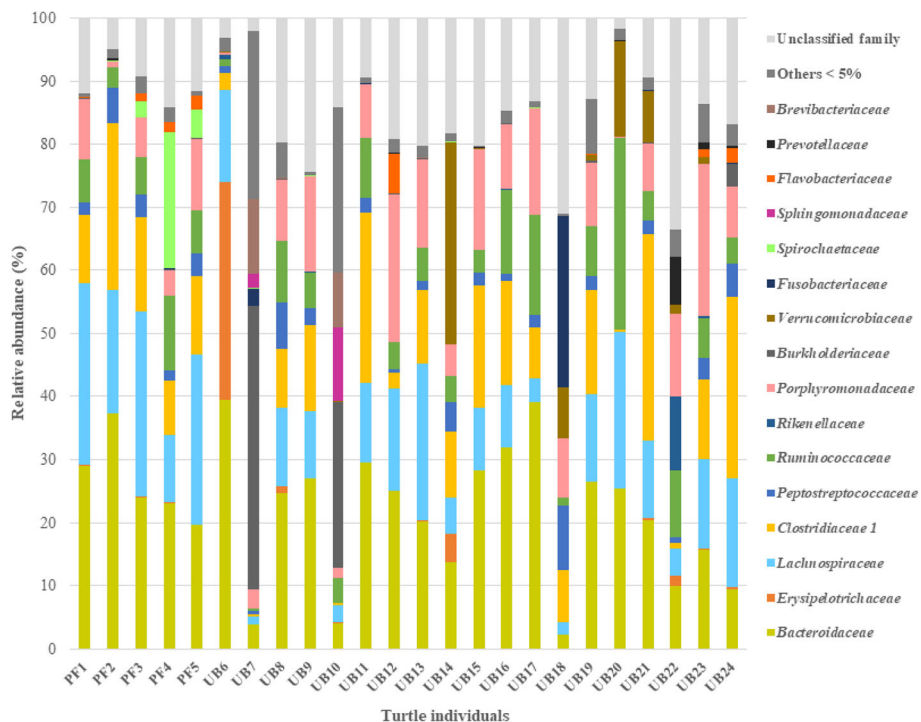

Fig. 2 Percentages of sequences from each individual turtle, fecal or rectal sample assigned at the phylogenetic level of family, according to the RDP Bayesian Classifier database with a bootstrap confidence above 80%. PF1 to PF5 = wild turtles from Praia do Forte; UB6 to UB16 = wild turtles from Ubatuba; UB17 to UB24 = captive turtles from Ubatuba. Taxa with a RA lower than 5% is grouped as "others"

OTU6 was predominant. This OTU was distantly related (88% in sequence homology) to *Treponema brennaborense* and might therefore correspond to an undescribed species. Furthermore, samples from Praia do Forte had a lower abundance of representatives in the *Actinobacteria* and *Verrumicrobia*, when compared to the Ubatuba individuals.

Multivariate analysis (PCoA of samples' Bray-Curtis distances based on OTUs incidence) (Fig. 3) showed three major clusters in relation to the microbial community structure of the gut microbiome from the studied turtles (Fig. 3). The smallest and more specific group confirmed the uniqueness of the bacterial community in the two anomalous wild turtles described above, UB7 and UB10. No significant segregation was observed between wild and captive turtles, but individuals from Ubatuba displayed a significant variability, and two major groups were apparent. The minor cluster encompassed the previously described individuals that were characterized by a relatively high abundance of *Akkermansia* spp., while a second larger one also contained the samples from Praia do Forte forming a very compact subcluster.

Discussion

Green turtles settle in the coastal habitats of the southwestern South Atlantic when they are 30–45 cm in CCL [34, 41, 51]. The results reported here indicated that most

Fig. 3 PCoA biplot of the gut microbiome in Brazilian green turtles based on the Bray-Curtis distance matrix. Wild turtles came from Praia do Forte (PF1 to PF5) and Ubatuba (UB6 to UB16). Captive turtle came only from Ubatuba (UB17 to UB24). The percentage of explained variation encompassed by the two main axes has been indicated. The main sample score clusters (dashed contours) and the more specific subcluster from Praia do Forte (dotted contour) have been highlighted

of the green turtles less than 45 cm CCL from Brazil exhibited a fecal microbiota co-dominated by phyla *Bacteroidetes* and *Firmicutes* and high levels of *Clostridiaceae*, *Porphyromonas*, *Ruminococcaceae*, and *Lachnospiraceae* within the latter phylum. Furthermore, this was the only microbiota profile found in wild green turtles > 45 cm CCL and in most of the captive green turtles of any size feeding on a macroalgae/fish mixed diet. These results suggest a fast acquisition of a polysaccharide fermenting gut microbiota by juvenile green turtles after settlement into coastal habitats.

A high abundance of *Proteobacteria* had been previously reported from the cloaca of pelagic (range 17.1–21.7 cm CCL) and recently settled (29.4–34.6 cm CCL) juvenile green turtles from Florida and from the gut of omnivorous marine fishes, but not from other groups of herbivorous vertebrates (Table 3). A high abundance of *Proteobacteria* has been observed also in two wild and one captive green turtles from Brazil less than 45 cm CCL (this study), but this is probably because they were immunodepressed and not because of recent settlement. We hypothesize that the prevalence of the *Proteobacteria* phylum in those three individuals was because of lesions from anthropogenic impacts [52]. The same is true for *Mycobacterium,* from the *Actinobacteria* phylum, a genus very uncommon in turtles but which includes several well-known pathogens for reptiles and amphibians [53, 54]. Furthermore, three captive and one wild turtle shared OTUs affiliated to the *Akkermansia* genus (*Verrumicrobiaceae* family). *Akkermansia* is a mucin-degrading bacterium commonly found in the human gut and recently isolated in reptiles [55, 56]. Several studies showed that the enrichment of *Akkermansia* induces gut inflammation and is associated with colonic diseases in mammals, but nothing is known about its pathogenicity in reptiles. It is also worth noting a small captive turtle (34.9 cm CCL) with a microbiota dominated by *Bacteroidetes* and *Firmicutes* but with a high relative abundance of *Fusobacteria*, a group occurring sporadically in carnivorous marine mammals [4].

High levels of *Firmicutes* are characteristic of the gut and fecal microbiota of herbivorous vertebrates (Table 3), as this phylum plays a critical role in the fermentation of complex polysaccharides [3, 57]. The families *Ruminococcaceae* and *Lachnospiracea* are particularly relevant, as both are obligate anaerobes with capacity to degrade structural polysaccharides into short-chain volatile fatty acids [3, 58–62] and occur in large numbers only in the gut and feces of herbivorous tetrapodes [3, 8, 62, 63]. Short-chain volatile fatty acids are indeed the main product of fermentation of plant material in the large intestine of green turtles [39, 64], and the analysis of the green turtle microbiota reported here revealed that *Ruminococcaceae* and *Lachnospiraceae* represented 3–30% of the OTUs recovered from the rectal and fecal samples of most
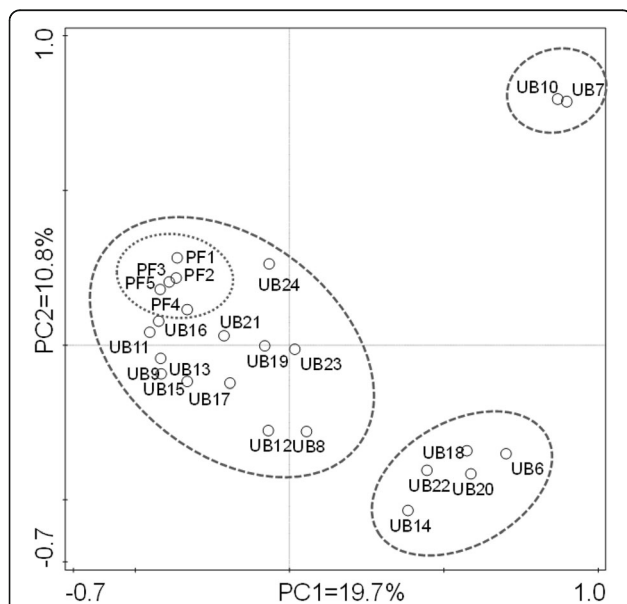
Table 3 Relative abundance of bacterial phyla to the gut microbiota of omnivorous and herbivorous vertebrates

Species	Diet	Firmicutes	Bacteroidetes	Verrucomicrobia	Spirochaetes	Proteobacteria	Actinobacteria	Other	Source
Teleosteans									
Acanthurus gahhm[1]	Omn/Alg	**29.5**	0.6	0.0	1.3	**49.4**	7.7	9.1	Miyake et al. (2015)
Naso elegans[1]	Herb/Alg	**97.4**	0.0	0.0	0.0	0.0	0.0	2.6	Miyake et al. (2015)
Naso unicornis[1]	Herb/Alg	**83.3**	9.0	**2.6**	0.0	2.6	1.3	1.2	Miyake et al. (2015)
Siganus stellatus[1]	Omn/Alg	42.3	11.5	0.0	2.6	**37.2**	0.0	6.4	Miyake et al. (2015)
Turtles									
Chelonia mydas[a,2]	Omn/Alg	6.5	27.1	0.6	0.0	**60.5**	0.1	5.2	Price et al. (2017)
Chelonia mydas[b, 2]	Herb/Seg	8.3	15.4	0.2	0.2	**66.6**	1.7	7.6	Price et al. (2017)
Chelonia mydas[c,e,3]	Herb/Alg	10.8	11.8	0.1	0.1	**60.7**	15.2	1.3	This study
Chelonia mydas[d,3]	Herb/Alg	**44.8**	**46.6**	**3.8**	1.3	1.1	0.3	2.1	This study
Geochelone nigra[3]	Herb/Ter	**81.1**	4.4	0.1	0.0	2.0	0.8	11.6	Hong et al. (2011)
Gopherus polyphemus[3]	Herb/Ter	38.4	36.9	**3.0**	4.4	<3.0	<3.0	7.4	Yuan et al. (2015)
Lizards									
Amblyrynchus cristatus[3]	Herb/Alg	**75.1**	8.2	1.0	0.0	0.6	0.6	14.5	Hong et al. (2011)
Conolophus spp.[3]	Herb/Ter	**63.9**	4.2	0.2	0.0	1.4	1.3	29.0	Hong et al. (2011)
Iguana iguana[3]	Herb/Ter	**74.0**	10.1	1.0	0.6	3.1	0.1	11.1	Hong et al. (2011)
Mammals									
Antidorcas marsupialis[3]	Herb/Ter	**75.6**	24.4	0.0	0.0	0.0	0.0	0.0	Ley et al. (2008)
Dugong dugong[3]	Herb/Seg	57.5	**42.5**	0.0	0.0	0.0	0.0	0.0	Eigeland et al. (2012)
Gorilla gorilla[3]	Herb/Ter	**67.4**	3.5	**10.5**	2.3	0.0	11.6	4.7	Ley et al. (2008)
Loxodonta africana[3]	Herb/Ter	**80.5**	2.5	**1.8**	0.2	10.1	4.7	0.2	Ley et al. (2008)
Ovis canadensis[3]	Herb/Ter	**64.0**	3.0	**2.7**	0.0	2.1	25.8	2.4	Ley et al. (2008)
Trichechus manatus[3]	Herb/Seg	**77.3**	19.5	0.0	0.1	0.3	2.0	0.8	Merson et al. (2014)

Bold type denote accumulated RA higher that 60%. Superscript numbers denote sample source as follows: [1] whole intestinal tract, [2] cloaca; [3] rectum or feces. Diet: omnivores (Omn) or herbivores (Herb). Major group of plants in diet: algae (Alg), seagrasses (Seg) and terrestrial plants (Ter). Length of green turtles Chelonia mydas: [a] 17.1–21.7 cm CCL, [b] = 29.4–34.6 cm CCL, [c] 39.7–44.7, [d] 31.1–64.7. [e] potentially immunodepressed individuals

juvenile green turtles, thus confirming their capacity to ferment structural polysaccharides. This suggests that juvenile green turtles with a *Firmicutes-Bacteroidetes* dominated fecal microbiota were plant-based omnivores or herbivores, which agrees with available dietary information [31, 33, 34, 65–68].

Interestingly, *Ruminococcaceae* prevail over *Lachnospiraceae* in terrestrial herbivorous reptiles [3] but the opposite appears to be true in marine iguanas [3] and in green turtles. Macroalgae are the staple food of both groups and differ from seagrasses and terrestrial plants in high levels of sulfated polysaccharides and alginic acid and low levels of cellulose [18]. This suggests that the prevalence of *Lachnospiraceae* over *Ruminococceea* in marine iguanas and green turtles is related to the similar composition of the polysaccharides in their diets. Nothing is known about the microbiota of green turtles feeding on seagrasses, but the profiles of the short-chain volatile fatty acids produced in the large intestine of green turtles feeding on seagrasses and those feeding on macroalgae differ [39, 64], thus suggesting potential differences in their microbiota worth exploring in further research.

Another major difference between the rectal and fecal microbiota of green turtles and those of other herbivorous vertebrates is the high abundance of *Bacteroidetes* in the former, a pattern reported previously only from dugongs (*Dugong dugong*) and gopher tortoises (*Gopherus polyphemus*) (Table 3). *Bacteroidetes* may contribute significantly to the initial attack on both simple and complex carbohydrates [69], and Yuan et al. (2015) speculated that the high prevalence of *Bacteroidetes* in gopher tortoises might be related to the seasonally low temperatures experienced in subtropical environments. However, *Bacteroidetes* had a similar prevalence in green turtles from tropical Praia do Forte and from subtropical Ubatuba (this study), thus suggesting that seasonal differences in temperature are unlikely to not induce major changes in the relative abundance of *Bacteoidetes* and *Firmicutes*, although samples were collected in summer in both areas. A high abundance of *Bacteroidetes* is neither characteristic of the gut microbiota of herbivorous chelonians, as they represent only 4% of the relative abundance of bacteria in the microbiota of Galapagos giant tortoises (*Geochelone nigra*) [3]. It is suggested that the high presence of this phylum in all the samples of green turtles from Brazil, except those of the three anomalous individuals, could be related to the presence of high levels of organic matter in coastal waters, which allow copiotrophs (such as *Bacteroidetes*) to thrive and dominate the microbial community structure [70]. Moreover, a recent study of gut microbiota of the loggerhead sea turtle *Caretta caretta* [71] found that *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were the most predominant microbial population in turtle feces.

Spirochaetes is another group of non-cellulolytic bacteria associate with specific plant substrates during digestion [72], facilitating the breakdown of cellulose by co-occurring bacteria [73]. Within this phylum, the *Spirochaetes* members exhibit enormous diversity in a free-living or host-associated life, being pathogenic or non-pathogenic, and aerobic or anaerobic [74]. This phylum has also been reported to be a major component of the microbiota of gopher tortoises, omnivorous fishes and gorilla, but not in other herbivorous reptiles (Table 3). OTU 6, an unidentified *Spirochaetes*, was detected in all the samples, but only in the rectal samples of three individuals from Praia do Forte (PF3, PF4, and PF5) did it represented more than 2% of the relative abundance.

The fact that *Bacteroidetes* and *Firmicutes* were the dominant bacteria in the feces and the rectal samples of most juvenile green turtles less than 45 cm CCL, including four specimens ranging 31.1–35.0 cm CCL, indicates that they acquired a microbiota adapted to digest polysaccharides shortly after settlement. How this specialized bacterial flora is acquired by settlers remains unknown, but land and marine iguanas have been observed consuming conspecific excrements [17, 75], which certainly facilitate acquiring a plant degrading microbiota. Juvenile green turtles are not gregarious, but may form dense aggregations [31, 76], which might facilitate feces consumption and hence the quick acquisition of a bacterial flora adapted to digest polysaccharides. Alternatively, fermenters might be transferred through the diet, as they can be associated with algal surfaces [77].

Algae and seaweeds are typically rich in sulfated polysaccharides that are absent in terrestrial plants. Hence, microbiota from the phylosphere of seaweeds are characterized by high copy numbers of sulfatases in their genomes [78]. A recent study suggested that traditional sushi food, which is largely composed of seaweeds, significantly affected the gut microbiome of the Japanese population [79, 80]. It was then observed that carbohydrate-active enzymes (CAZymes) in the gut microbiome, which are absent in the human genome, were acquired by horizontal gene transfer (HGT) from the marine bacteria associated with seaweeds. Moreover, [81], reviewed several studies on the HGT phenomena between environmental and gut bacteria within the phyla of *Bacteroidetes* and *Firmicutes* in different organisms, including the grazer surgeonfish. Hence, it is well possible that the seaweed-based diet of turtles could similarly affect their gut microbiota by gene acquisition, considering that CAZymes and sulfatases are required for efficient seaweed degradation [82]. This topic merits further research taking advantage of the existing programs on captive breeding of green turtles by performing gut metagenomics analysis.

In any case, the fast acquisition after settlement in coastal areas of a microbiota adapted to ferment polysaccharides should enable green turtles to adopt an herbivorous diet soon after recruitment. This is the pattern reported from tropical areas [25, 83], but in warm temperate and subtropical regions, juvenile green turtles are best described as plant-based omnivore and only adults are primarily herbivores [19–21, 23, 33, 34, 37, 84, 85]. The results presented here indicate an increase in the taxonomic richness of the gut microbiome as turtles grow, but this is an unlikely explanation by the progressive ontogenetic dietary shift, because even small turtles had a high abundance of *Ruminococcaceae* and *Lachnospiraceae*. Consumption of animal material results into a slight and statistically significant increase in the relative abundance of *Proteobacteria*, as revealed by the differences between captive and wild healthy turtles, but the abundance of *Ruminococcaceae* and *Lachnospiraceae* remains high anyway. This suggests that omnivore is unlikely to reduce the capacity of green turtles to digest plant material.

Digestibility of plant material in green turtles increases with temperature [13] and the body temperature of juvenile green turtles inhabiting subtropical regions is close to that of water during winter months [86]. Conversely, the body temperatures of inactive adult green turtles can be 2 °C above water temperature thanks to gigantothermy [36], which explains why the digestibility of plant material by green turtles increases with body size even in tropical settings [13]. Interestingly, the apparent digestibility of plant material does not increase with body size in marine iguanas [86], because even very small individuals can rise significantly their body temperature through basking in black lava [17]. Green turtles bask regularly in the beaches of Hawaii and Galapagos [87, 88] and this behavior has been suggested to improve digestion, but beach basking has never been reported in other areas to our knowledge. If green turtles inhabiting subtropical and warm temperate regions do not bask in winter, the digestibility of plant material by small individuals can be compromised during winter, even if they support a specialized microbiota rich in *Ruminococcaceae* and *Lachnospiraceae*, which may explain the progressive dietary shift as they grow.

Conclusions

This study revealed that juvenile green turtles from the coastal waters of Brazil had the same general microbiota profile, regardless of size and origin (wild vs. captive; subtropical Ubatuba vs. tropical Praia do Forte). This indicates a fast acquisition of a microbiota with capacity to ferment structural polysaccharides soon after settlement in the coastal waters of Brazil and that the regular consumption of animal prey does not significantly reduce the presence of *Ruminococcaceae* and *Lachnospiraceae* and, hence, does not impair the capacity to ferment structural polysaccharides. However, subtropical specimens displayed a larger variability in the gut microbial community structure, which in the most extreme cases was clearly related to poor physical condition. In summary, there is no reason for a delayed ontogenetic dietary shift after settlement, unless low winter temperature reduces their capacity to digest plant material.

Additional file

Additional file 1: Phylogeny and incidence of gut eubacteria. (XLSX 1339 kb)

Authors' contributions
LC directed the overall research project. LC and FPB designed the experiments and drafted the manuscript. PC carried out the field work and was the first author. MG performed the DNA sequencing and bioinformatics processing. All authors participated in the analysis and interpretation of the data, and contributed significantly in writing the final manuscript. All authors read and approved the final manuscript.

Ethics approval
Field work in natural reserves and handling of wild animals was carried out under the authority of the Instituto Chico Mendes de Conservação da Biodiversidade–ICMBio (license reference ICMBio/SISBIO 52128-1), and of the Convention on International Trade in Endangered Species of Wild Fauna and Flora–CITES (license reference CITES 16BR020234/DF).

Competing interests
The authors declare that they have no competing interests.

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