Bacteria associated with the intestinal tract of three predators of *Dactylopius opuntiae* (Hemiptera: Dactylopiidae)

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Abstract

Insects depend on gut bacteria for many metabolic functions including detoxification. *Dactylopius* (Hemiptera: Dactylopiidae) species (e.g. *D. opuntiae* and *D. coccus*) produce carminic acid possibly acting as part of their immune response to predation; thus predators of *Dactylopius* species may require intestinal symbionts to metabolize carminic acid acquired from their prey. The average content of carminic acid in *D. opuntiae* and *D. coccus* is 3–5% and 19–25% respectively; the predators *Leucopina bellula* and *Hyperaspis trifurcata* are specialists on *D. opuntiae* while *Laetilia coccidivora* feeds on both *D. opuntiae* and *D. coccus*. We determined the diversity of bacteria associated with the gut of these predators to test the hypothesis that it would vary significantly depending on the level of prey specialism. Bacteria were isolated from the intestine of *Le. bellula*, *L. coccidivora* and *H. trifurcata* larvae and also adults of *H. trifurcata*. Bacteria were identified morphologically and by partial sequencing of the 16S rRNA gene. The greatest bacterial diversity was found in *L. coccidivora* and *H. trifurcata* adults, followed by *H. trifurcata* larvae. The lowest diversity was found in *Le. bellula*. Sequencing revealed the presence of *Bacillus cereus*, *Enterococcus gallinarum* and *E. casseliflavus* in *L. coccidivora* larvae; *Enterobacter* sp. in larvae of *H. trifurcata*; *Lactococcus lactis* in adults of *H. trifurcate*; and *Staphylococcus* sp. in larvae and adults of *H. trifurcate*. *Bacillus pumilus* was only found in *Le. bellula*. The possible role of these bacteria in the ability of predators to feed on *D. opuntiae* and *D. coccus* is discussed.

Introduction

Insects have complex symbiotic inter and intracellular associations with various microorganisms [1, 2]. These microorganisms can be free in the intestinal lumen or in the body [1]. The microbial community is diverse, but bacteria are the most abundant microorganisms involved in these associations [3]. The intestine of insects harbours bacteria with specific physiological functions [2, 4]; for example, some are responsible for supplementing dietary deficiencies in amino acids and vitamins [5, 6]. Many of the bacteria that have a symbiotic association with insects belong to the families Enterobacteriaceae, Bacillacea, Pseudomonacea, Staphylococcaceae and Enterococcaceae [7], and produce enzymes that break down natural and synthetic organic molecules into forms that can be assimilated by the host [8]. For, example *Lactobacillus* and *Enterobacteria* in the digestive tract of coleopterans, are important for digestion of celluloses and lignocellulosic compounds [9]. Perhaps one of the most explored bacterial populations is the one associated with the midgut of termites where a great diversity of bacteria are associated with the breakdown of lignin or aromatic compounds, and the fixation of nitrogen [4, 10]. Despite this, interactions between insects and symbionts still remains largely unstudied [11].

Predator-prey biological models that include carminic acid production by prey insects in the genus *Dactylopius* have not been studied previously. It is known that the concentration of carminic acid varies between species and stages of development. For example, carminic acid content is 3–5% of the dry weight of *D. opuntiae* but 19–25% of the dry weight of *D. coccus* [12, 13]. It has been suggested that carminic acid is produced as a defence compound within the immune system of *D. coccus* [14], most likely targeted at limiting predation [15, 16]. This might account for the limited number of known predators of species in the genus *Dactylopius*. Currently, *D. opuntiae* is considered one of the most important global pests of *Opuntia ficus-indica* (L.) (Caryophyllales: Cactaceae) [17]. *Dactylopius coccus* is also a pest of *O. ficus-indica* although damage and losses are not as important as those caused by *D. opuntiae* [18]. Only in Ethiopia has *D. coccus* been reported as a highly significant pest [19]. In fact, the economic importance of *D. coccus* in some parts of Mexico is not as a pest but as a source of carminic acid which is used as a natural colourant [20].

Although the anti-predatory effect of carminic acid on natural enemies of *Dactylopius* species has long been suggested, only two specialist predators have been reported, the coccinellid *Hyperaspis trifurcata* Schaeffer (Coleoptera: Coccinellidae), and the dipteran *Leucopina bellula* Williston (Diptera: Chamaemyiidae); these species use the carminic acid acquired from predation of *Dactylopius* species, to repel predatory ants [15, 16]. The generalist predator, *Laetilia coccidivora* Comstock (Lepidoptera: Pyralidae), has also been reported preying on *Dactylopius* species; they excrete the carminic acid when threatened by other predators [15]. The ability to metabolize carminic acid has a fitness cost for predators; carminic acid from the consumption of *D. coccus* has negative effects on the development and reproductive capacity of *L. coccidivora* [13]. Larvae of the specialist predator, *Le. bellula*, have received attention as predators of *D. opuntiae* [21] because they feed on all development stages [17]. Both larvae and adults of the specialist *H. trifurcata* [18] have been associated with *D. opuntiae* in the field and occasionally feed on commercially established colonies of *D. coccus* [20]. This apparent preference for *D. opuntiae* over *D. coccus* could be due to the difference in carminic acid concentrations present in the two species of prey. However, larvae of *L. coccidivora* are one of the most voracious generalist predators of both *D. opuntiae* and *D. coccus*, suggesting that they are not affected by the difference in carminic acid concentration [17]; as a result they can cause considerable losses in commercial production of *D. coccus* [20].

Various studies have demonstrated the ability of bacteria to metabolize dyes, and that the efficiency of this varies amongst species [22]. Some *Bacillus* species have the ability to metabolize dyes such as recalcitrant anthraquinone, triphenylmethane and azo dyes [23]. *Enterobacter* species are known for their capacity for biodegradation of the anthraquinone group including reagent blue 19 [22]. *Enterococcus* species have the ability to metabolize anthraquinones using carbon sources such as glucose, sucrose and fructose, as well nitrogen sources such as yeast extracts [24]. *Staphylococcus* species have the ability to metabolize anthraquinones [25], and *Lactococcus* species can metabolize dyes such as reagent black 5 [26]. Carminic acid is an anthraquinone and all these bacterial genera have the ability to metabolize anthraquinones. Bacterial

species within these genera can grow in nutrient-rich culture media such as Luria Bertani [22, 27], and R2A, the latter of which is a medium low in nutrients that allows growth of bacteria that are inhibited by high nutrient concentrations [27]. Both Luria Bertani and R2A are frequently used for isolation of intestinal bacteria from insects [28]. The diversity of bacteria in the intestines of predators is an indicator of the complexity of the assimilation process which can require several steps to metabolize carminic acid.

The preference of *Le. bellula* and *H. trifurcata* for *D. opuntiae* over *D, coccus* in response to lower concentrations of carminic acid (in *D. opuntiae*), and the capacity of *L. coccidivora* to feed on both *D. opuntiae* and *D. coccus* in addition to other insect species, suggests variation in the bacterial community of these contrasting predatory species as a mechanism for differences in prey preference. Based on this, the objective of this study was to compare the diversity of bacterial species associated with the intestinal tract of larvae of *L. coccidivora* and *Le. bellula*, and the larvae and adults of *H. trifurcata*, all predators of *D. opuntiae* and *D. coccus*.

Materials And Methods

Predators

The three species of predators were obtained from laboratory colonies maintained at the Colegio de Postgraduados, Texcoco, Mexico State, Mexico. They were fed on different development stages of their natural prey (*D. opuntiae*) *ad libitum*, according to the methodologies described by Barreto-García et al., [13]. To maintain the natural bacterial diversity of the insects in the colonies, field-collected predators were introduced into the colonies every 10 weeks. Prior to introduction into colonies, field-collected predators were maintained under observation for 15 days, to avoid the introduction of pathogens. Groups of each predator species and developmental stage were established (n = 10 individuals per group): third instar larvae (L3) of *Le. bellula*, fifth instar larvae (L5) of *L. coccidivora*, fourth instar larvae (L4) and adults of *H. trifurcata*. Each group of individuals were then placed in Ziploc® bags and exposed to CO_2 for 20 minutes; all individuals were then transferred to an ethyl acetate chamber for 15 minutes. Subsequently, individuals were surface-sterilized with 1% sodium hypochlorite for 5 minutes, rinsed six times with sterile distilled water, and placed individually onto sterile absorbent paper [29]. Finally, the intestine of each individual was extracted using entomological forceps (No. 5) through a longitudinal cut made with a sterile scalpel. The ten intestines from each group of insects were placed in 100 mL of Luria-Bertani Broth (Dibico, USA) which was incubated at 550 rpm and room temperature for 48 h for *Le. bellula* and *L. coccidivora*, and 72 h for *H. trifurcata*. Differences in incubation time were necessary to achieve sufficient bacterial growth for further analysis.

Isolation of bacterial colonies

Cultures from the Luria-Bertani (LB) broth were logarithmically diluted from 10^{-1} to 10^{-6}. From each dilution, 0.1 mL was inoculated in triplicate into 9 cm Petri dishes of Luria Bertani agar (Dibico, USA) or R2A (Difco, USA) culture media, and distributed with a Digralsky bacterial loop. The plates were incubated at 30°C until bacterial growth was observed. Bacteria were purified using the cross-streak technique [30] and preserved in 80% glycerol at -20 ° C prior to evaluation.

Morphological characterization of bacteria

After 24, 48 and 72 h incubation at 30°C bacterial colonies were characterized morphologically using a stereoscopic microscope (Leica Model EZ4, Germany). The form of the colonies was classified as circular, irregular or filamentous, and the margin as undulate, filamentous or entire. The surface or elevation of colonies was considered as flat, convex or umbonate. Pigmentation was categorized as yellow, white, cream or greyish; and the texture as membranous or butyrose, according to the criteria described by Seeley et al. [30].

Microscopic characterization was done using Gram staining according to the protocol described by Seeley et al. [30]. The preparations of each bacterial smear were observed under oil immersion in an optical microscope (Leica Model DM500, Germany) at 100 X objective magnification. All bacterial colonies were classified according to the Gram staining results, and their morphology as cocci, bacilli, coccobacilli or spirals based on the criteria described by Seeley et al. [30].

Taxonomic placement of bacteria

Bacterial identification was achieved by amplifying a region of the 16S gene of the rDNA with the universal primers 8F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´- GGTTACCTTGTTACGACTT-3´) [31]. DNA was extraction from pure bacterial colonies using the DNeasy Plant Mini kit (Qiagen®, USA), following the manufacturer's instructions. The quality and quantity of DNA in each sample was verified by spectrophotometry in a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The reaction mix for PCR was prepared in a final volume of 25 µL: 17.37 µL of nuclease-free water (Promega®, USA), 5 µL of 5X Buffer, 0.2 mM of dNTP (Promega®, USA), 0.4 µM of each primer, 1.25 U of DNA polymerase (Promega®, USA) and 1 µL of DNA (15–25 ng μL^{-1}). Amplifications were made in a Thermal Cycler (BioRad) with an initial denaturation cycle at 95 ° C for 15 min; 30 cycles of 95 ° C for 1 min, 54 ° C for 1 min, an extension at 72 ° C for 2 min; and a final extension at 72 ° C for 10 min [32]. To identify the PCR products, an agarose gel electrophoresis (Promega®, USA) was run and visualized on a transilluminator. The PCR products were sent to Macrogen Inc. (Korea) for direct sequencing.

Sequences were edited using Bioedit v.7.2.5 software [33], and compared with the National Center for Biotechnology Information (NCBI) database using the basic local alignment tool BLAST implemented in GenBank, as well as with sequences from the European Nucleotide Archive. Based on BLAST analyses, sequences with the highest similarity (e-score closer to zero) to the sequences obtained in this study were retrieved and used as references for identification. Sequences were analysed using Maximum Likelihood analysis based on the Tamura-Nei model with MEGA ver. 5. software [34]. The robustness of branches was estimated by bootstrap analysis with 1000 repetitions [35].

Results

A total of 45 bacterial colonies (morphotypes) were obtained from the intestinal tract of the three predators. Of these, 26 were isolated using the LB medium and 21 using the R2A medium (Table 1). There was a large diversity of margin, colour, form and elevation amongst the morphotypes (Table 1). Phylogenetic analysis, all with 100% probability, grouped the 45 morphotypes into eight species distributed amongst five genera, specifically the: genus *Enterobacter* (n = 21); species *Staphylococcus sciuri* (n = 1); species *S. capitis* (n = 2); species *Bacillus cereus* (n = 3); species *B. pumilus* (n = 7); species *Enterococcus gallinarum* (n = 1); species *E. casseliflavus* (n = 1); species *Lactococcus lactis* (n = 9) (Fig. 1).

Table 1

Bacterial isolates obtained in this study from three predators of *Dactylopius coccus* and *Dactylopius opuntiae*. Species were determined after phylogenetic analysis of partial 16S sequences. LB = Luria-Bertani broth, R2A = medium with low nutrient concentrations.

Predator	Isolate	Genus/Species	Medium	Margin	Colour	Form	Elevation	Texture	Gram	GenBank
Leucopina bellula	B1Leu	*Bacillus pumilus*	LB	undulate	cream	circular	rugose	membranous	+	
	B2Leu	*Bacillus pumilus*	LB	undulate	cream	circular	rugose	membranous	+	
	B3Leu	*Bacillus pumilus*	LB	undulate	cream	circular	rugose	membranous	+	
	B4Leu	*Bacillus pumilus*	LB	undulate	cream	circular	rugose	membranous	+	
	B5Leu	*Bacillus pumilus*	LB	undulate	cream	circular	rugose	membranous	+	
	B6Leu	*Bacillus pumilus*	R2A	undulate	white	circular	umbonate	membranous	+	
	B7Leu	*Bacillus pumilus*	R2A	undulate	white	circular	umbonate	membranous	+	
Laetilia coccidivora	B8Lc	*Enterococcus gallinarum*	LB	entire	yellow	circular	convex	smooth	+	
	B9Lc	*Enterococcus casseliflavus*	BL	entire	yellow	circular	convex	smooth	+	
	B10Lc	*Bacillus cereus*	BL	entire	yellow	circular	convex	smooth	+	
	B11Lc	*Bacillus cereus*	R2A	filamentous	white	rhizoid	flat	smooth	+	
	B12Lc	*Bacillus cereus*	R2A	filamentous	white	rhizoid	flat	smooth	+	
Hyperaspis trifurcata	B13Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
Larvae	B14Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B15Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B16Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B17Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B18Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B19Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B20Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B21Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B22Htl	*Enterobacter* sp.	LB	entire	white	circular	convex	smooth	-	
	B23Htl	*Staphylococcus sciuri*	LB	entire	yellow	circular	convex	smooth	+	
	B24Htl	*Enterobacter* sp.	LB	entire	cream	circular	convex	smooth	-	
	B25Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B26Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	

Predator	Isolate	Genus/Species	Medium	Margin	Colour	Form	Elevation	Texture	Gram	GenBank
	B27Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B28Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B29Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B30Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B32Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B33Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B34Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
	B36Htl	*Enterobacter* sp.	R2A	entire	cream	circular	convex	smooth	-	
Hyperaspis trifurcata	B37Hta	*Lactococcus lactis*	LB	entire	cream	circular	flat	smooth	+	
Adult	B38Hta	*Lactococcus lactis*	LB	entire	cream	circular	umbonate	smooth	+	
	B39Hta	*Lactococcus lactis*	LB	entire	cream	circular	umbonate	smooth	+	
	B40Hta	*Lactococcus lactis*	LB	entire	cream	circular	umbonate	smooth	+	
	B41Hta	*Lactococcus lactis*	LB	entire	cream	circular	umbonate	smooth	+	
	B42Hta	*Lactococcus lactis*	LB	entire	cream	circular	umbonate	smooth	+	
	B44Hta	*Lactococcus lactis*	R2A	entire	cream	circular	raised	smooth	+	
	B45Hta	*Staphylococcus capitis*	R2A	entire	cream	circular	raised	smooth	+	
	B46Hta	*Staphylococcus capitis*	R2A	entire	white	circular	raised	smooth	+	
	B47Hta	*Lactococcus lactis*	R2A	entire	white	circular	umbonate	smooth	+	
	B48Hta	*Lactococcus lactis*	R2A	entire	white	circular	flat	smooth	+	

The greatest bacterial diversity was found in the intestine of *L. coccidivora*, with three bacterial species (*B. cereus*, *E. gallinarum* and *E. casseliflavus*), followed by *H. trifurcata* adults with two species (*La. lactis* and *S. capitis*); *H. trifurcata* larvae with *Enterobacter* spp. and *S. sciuri*, and finally, *Le. bellula* with only *B. pumilus* (Table 1).

Discussion

This study is the first description of culturable bacteria from the intestines of *Le. bellula*, *L. coccidivora* and *H. trifurcate*, the most important predators of *D. opuntiae* and *D. coccus*. The diversity of the bacteria present corresponded with groups known to be capable of metabolizing dyes, pigments and high molecular weight compounds [25]. Therefore, it is likely that the bacteria we found have a functional role in these predators. The diversity of intestinal bacteria found in the generalist predator *L. coccidivora* was greater than the two specialist predators, *Le. bellula* and *H. trifurcate*; this could be related to their feeding habits and the ability of *L. coccidivora* to feed on both species of *Dactylopius* regardless of carminic acid content. The effect of diet on the composition of gut microbial communities in insects has been widely accepted [36] because they are involved in the metabolism of ingested compounds and in other beneficial metabolic processes [37].

Gut bacteria contribute to the nutrition of insects in different ways; these include the production of compounds and enzymes that break down complex molecules into simpler forms that are easier to assimilate, thereby improving digestion [38]. We found *E. gallinarum, E. casseliflavus* in *L. coccidivora.* These two *Enterococcus* species have been reported before as part of the microbiota of *Musca domestica* (Diptera: Mussisidae) and *Blattella germanica* (Blattodea: Blattelidae); both were associated with resistance to various groups of antibiotics including aminoglycosides, streptomycin and kanamycin (*E. casseliflavus*); and streptomycin, neomycin and tetracycline (*E. gallinarum*) [41]. *Enterococcus casseliflavus* is one of the most widely distributed bacteria in Lepidoptera [7]. Many of the known *Enterococcus* species are common symbionts in the intestinal tract of insects and have important functions [42]. Some *Enterococcus* species produce bactericidal substances that selectively target invasive bacteria, but have no effect on ubiquitous members of gut fauna [7]. They can also increase intestinal pH providing the conditions necessary to metabolize complex molecular substances [43]. Similarly, some *Enterococcus* species can metabolize methyl red dye [40] and anthraquinone dye (acid blue 25) [24]; carminic acid is an anthraquinone dye.

We also found *B. cereus* in *L. coccidivora*. This genus is frequently found in the intestines of insects and, in some predators may play an important role in the degradation of carminic acid acquired during feeding on *D. opuntiae*. Some researchers have shown that *Bacillus* species have the ability to metabolize dyes, for example, one *Bacillus* sp. isolate could metabolize more than 98% of the toxic azo dye methyl red it was provided with [39]. Furthermore, *B. cereus*, *B. pumilus, B. subtilis* and *B. megaterium* could metabolize 50–60% of the Congo red, Bordeaux and blue dyes they were provided with [40]. We suggest that this is why *B. pumilus*, the only species found in *Le. bellula* larvae, could be related to metabolism of carminic acid.

We found *Enterobacter* sp. and *S. sciuri* in *H. trifurcata* larvae. *Enterobacter* species are known to assimilate phenolic compounds and metabolize anthraquinones, such as reagent blue 19 [22, 44]. We only found *S. sciuri* in the larval stages of *H. trifurcata*, although it has also been found in larvae of the beetle *Anoplophora chinensis* (Coleoptera: Cerambicidae) [2]. It has been reported that *S. sciuri* can metabolize remasol bright blue dye via secretion of enzymes such as laccase, manganese and lignin peroxidase [25].

We found *La. lactis*, and *S. capitis* in *H. trifurcata* adults. Previous reports showed that members of *Lactococcus* produce lactic acid, hydrogen peroxide, diacetyl, carbon dioxide and bacteriocins with antimicrobial activity [36, 45]. *Lactococcus* species also metabolize dyes such as reagent black 5 (> 99% in 5.5 h) [26]. The genus *Staphylococcus* has been isolated from other insects such as *Spodoptera frugiperda* (Lepidoptera: Noctuidae), and has been shown to break down insecticides [8] and metabolize dyes [25]. *Staphylococcus* species have been found in larvae and adults of *Anoplophora chinensis* Thomson (Coleoptera: Cerambycidae) [2], which is in line to our findings. As they are present throughout the entire life cycle, this suggests that these microorganisms have a beneficial function for the insect. We suggest that, finding bacteria from the genus *Staphylococcus* in larvae and adults of *H. trifurcate*, is strongly correlated with their role as a top predator; both developmental stages evaluated feed on more than one developmental stages of *D. opuntiae*, including partial consumption of gravid females.

In conclusion, bacterial diversity found in the generalist predator *L. coccidivora* (larvae) was greater than in the two specialist predators, *Le. bellula* (larvae) and *H. trifurcata* (larvae and adults). Bacterial species isolated from the intestinal tract of the three predators of *Dactylopius*, are known to be capable of metabolizing anthraquinone dyes, to which carminic acid belongs, suggesting a relationship between the capacity to metabolize carminic acid and the diversity of bacteria in the gut of specialist and generalist predators. Our methods enabled identification of culturable bacterial species; this is important because it will allow us to determine the ability of these bacteria to metabolize carminic acid experimentally. Additional studies should also focus on identifying bacterial species that are not culturable as these could also have an important role in the metabolism of carminic acid.

Declarations

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Conflicts of interest/Competing interests

No conflict of interest is declared by the authors.

Availability of data and material

All data generated or analysed during this study are included in this published article

Code availability

Not applicable

Authors' contributions

Conceptualization: [SMM, ERL, SAO, MTSG, AHL and AWGF]; Methodology: [SMM, ERL, SAO and AWGF], Formal analysis and investigation: [SMM, ERL, AWGF]; Writing - original draft preparation: [SMM, ERL, SAO, MTSG, AHL and AWGF]; Writing - review and editing: [SMM, ERL and AWGF]; Funding acquisition: [ERL, MTSG and AWGF]; Resources: [ERL, SAO, MTSG and AWGF]; Supervision: [ERL and AWGF].

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

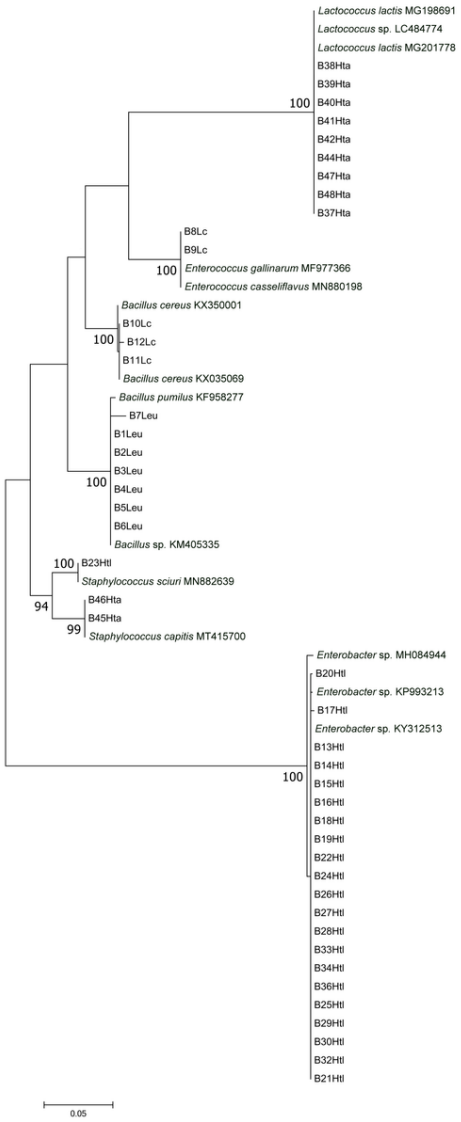

Figure 1

Phylogeny of bacterial species and genera inferred from Maximum Likelihood (ML) analysis of the partial 16S sequences data. Sequences used as a reference are labelled according to their GenBank accession numbers. Only supporting bootstrap values above 80% are shown. The scale bar corresponds to 0.05 nucleotide substitutions per site.