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Gut bacteria associated with different diets in reared *Nephrops norvegicus*

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ABSTRACT

The impact of different diets on the gut microbiota of reared *Nephrops norvegicus* was investigated based on bacterial 16S rRNA gene diversity. Specimens were collected from Pagasitikos Gulf (Greece) and kept in experimental rearing tanks, under *in situ* conditions, for 6 months. Treatments included three diets: frozen natural (mussel) food (M), dry formulated pellet (P) and starvation (S). Gut samples were collected at the initiation of the experiment, and after 3 and 6 months. Tank water and diet samples were also analyzed for bacterial 16S rRNA gene diversity. Statistical analysis separated the two groups fed or starved (M and P *vs.* S samples). Most gut bacteria were not related to the water or diet bacteria, while bacterial diversity was higher in the starvation samples. M and P samples were dominated by *Gammaproteobacteria*, *Epsilonproteobacteria* and *Tenericutes*. Phylotypes clustering in *Photobacterium leiognathi*, *Shewanella* sp. and *Entomoplasmatales* had high frequencies in the M and P samples but low sequence frequencies in S samples. The study showed that feeding resulted in the selection of specific species, which also occurs in the natural population, and might be associated with the animal's nutrition.

Introduction

The Norway lobster *Nephrops norvegicus* is a decapod crustacean living at depths of 20–800 m in the Mediterranean, the North Sea and the North East Atlantic Ocean. It is a commercially important species [4], but overexploitation and inappropriate management strategies have led to possible depletion of the existing stocks. Several studies of *N. norvegicus* have been performed in the past on their biology [4], population abundance and structure [1], molting and growth [17], feeding ecology and behavior [9] and reproductive biology [35,46,47]. However, the lack of knowledge concerning their nutritional requirements and rearing under laboratory conditions [45] is the constraint limiting the successful culture of the Norway lobster, either for restocking or for commercial size production. Recent studies [34] have provided valuable data on the survival, growth and feeding behavior of *N. norvegicus* kept in controlled laboratory conditions, and they have shown, among others, the need for high-quality dry food.

N. norvegicus mainly feeds on fish, mollusks, crustaceans, polychaetes, echinoderms and foraminifers [9]. However, the synthetic feed provided to *N. norvegicus* in past studies [34] was mostly pellets used in fish aquaculture that consisted mainly of fishmeal and soy. The use of probiotics, although very important in the aquaculture of crustaceans [15], has never been attempted in the rearing of *N. norvegicus*. The probiotics used in crustacean aquaculture have been shown to increase the growth and survival of the species ([15] and references therein) without the use of antibiotics. The probiotics used for each species are determined by several factors, such as the non-pathogenicity and non-toxicity of the strain used, as well as its ability to survive in and adhere to the gut [15]. Additionally, probiotics should benefit their host in certain ways, such as promoting growth or protecting against pathogens [2,25].

Recent studies have shown that the gut microbial communities of several animals are influenced by the nutritional habits of their hosts [29] and, at the same time, they metabolize part of the ingested food and provide the host with important nutrients (*e.g.* cellulose digestion). Resident gut microbes that are able to metabolize complex compounds are very good candidates as probiotics since they fulfill all the above-mentioned criteria. In the case of the gut microorganisms of *N. norvegicus*, the only study that has been performed in wild specimens showed that a seasonal variation of mid-gut bacterial communities was mostly related to differences in food supply from the overlying water column [36].

During the last 20 years, *N. norvegicus* catches per unit of effort have steadily declined within the Mediterranean Sea and, thus, support the need for measures to conserve this crustacean species. Aquaculture has been a very useful tool for restocking and stock enhancement programs for a number of fish and shellfish species. Although commercial cultivation of Norway lobsters might be hindered by the slow growth rate exhibited by juveniles of this species in nature [38] and in captivity [10], there is a lot of interest in the intensive cultivation of these animals due to their high nutritional and commercial value. Although there are studies on husbandry and rearing conditions for the Norway lobster [34,45], knowledge

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Table 1
Codes of samples collected and analyzed. Samples from the 2nd batch (April 2009) are indicated in boldface.

Samples	t0 (start)	t1 (3 months)	t2 (6 months)
Natural population	Nat1/**Nat2**		
Starvation		S3m1	S6m1/S6m2
Mussels		M3m1	M6m1/M6m2
Pellets			**P6m1/P6m2**

on the nutritional physiology of the species remains limited. Therefore, the present study contributes to our knowledge of the possible role of its gut microbiota in nutrition. Such information can provide the basis for diet formulation in commercial scale culturing of the Norway lobster. In particular, this study aimed at investigating the factors that affected the dominant gut bacterial communities of *N. norvegicus* grown under stable laboratory conditions. For this purpose, *N. norvegicus* individuals were reared in three different groups where mussels (natural feed) and pellets were provided, respectively, while the third group was starved. Mid-gut samples were collected at the beginning of the experiment and 3 and 6 months afterwards, and they were investigated by 16S rRNA gene diversity analysis.

Materials and methods

Collection of N. norvegicus individuals

Individuals were collected from Pagasitikos Gulf (Greece) in March (1st batch) and April 2009 (2nd batch). Sampling depth varied from 60 to 88 m and only male individuals were kept for further analysis, since it has been shown from previous studies that males have better survival rates than females under rearing conditions [41], and that sex is not a significant factor for gut bacterial diversity [36]. After collection, *N. norvegicus* individuals were immediately transferred to the laboratory in aerated seawater, and animal weight and carapace length were measured. One individual from each batch (Nat1–Nat2) was immediately sacrificed, while the rest were kept for rearing (Table 1).

Rearing procedure

Individuals for rearing were placed in 100 L glass tanks in the laboratory. Water from Pagasitikos Gulf was transported in the tanks before *N. norvegicus* transport. In order to establish the bacterial nitrifying communities on the filters of the tanks, bacteria from commercial solutions (Stability, Seachem Inc., USA) were added to the tanks. Water was constantly recycled through carbon and biological filters. Water temperature, salinity and photoperiod were maintained at $11.9 \pm 0.8\,^{\circ}C$, 374 ± 0.2 ppm and 24 h darkness, respectively, reflecting *in situ* conditions.

After a 15-day acclimatization period, the animals were divided into three groups (M, P, S), and each animal was placed in a separate compartment in tanks made from Plexiglas and plastic netting. Groups M and P were supplied with 1–4 g frozen mussels (natural feed) and approximately 1 g fish pellets (synthetic feed), respectively, three times per week, while group S was starved. The diet's chemical composition was 69% protein, 7.5% lipid and 23.5% carbohydrate for dry mussels and 42% protein, 11.1% lipid and 46.9% carbohydrate for pellets (Rotllant, unpublished data).

Collection of samples

Three and 6 months after the initiation of the experiment, animals from each group (Table 1) were sacrificed and their morphometric characteristics were measured. All animals were healthy when sacrificed. During the rearing period, molting had been observed only in one individual from group P (P6m2) 2 days before the animal was sacrificed (post-molt stage).

Water samples were collected from the tanks 2–3 days before the sampling of the animals. Water samples were collected in sterile 1 L bottles and 1 L, 500 mL and 800 mL were filtered from samples wt2 (t1), wt3 (t2, 1st batch) and wt4 (t2, 2nd batch), respectively. Filtering was performed under vacuum using 0.2 μm filters (GTTP, Millipore, USA) and filters were kept at $-20\,^{\circ}C$ until further processing. Samples from mussels and pellets from the batches provided for feeding were also kept.

Mid-gut isolation

The animals were dissected using sterile lancets and the intestine was extracted using sterile forceps, as described in Meziti et al. [36]. Since the bacterial communities established on or in the gut tissue were required, the intestine was emptied by applying mechanical force and by rinsing three times in autoclaved particle-free seawater in order to remove all gut content. The posterior part of the mid-gut was used for further analysis. All dissecting tools were alcohol-flame sterilized between each individual sample.

DNA extraction

DNA extraction was performed on 10 *N. norvegicus* gut tissues using the QIAamp DNA Mini Kit (Qiagen Inc., USA) and following the manufacturer's standard protocol. At the final step, DNA was diluted in 100 μL of the elution buffer provided with the kit and it was stored at $-20\,^{\circ}C$.

DNA extraction from the water samples was performed using the UltraClean Soil DNA Kit (MoBio Laboratories Inc. USA), following the manufacturer's protocol. DNA was finally eluted in the 50 μL elution buffer provided by the manufacturer.

DNA was extracted from pooled foot and mantle tissues of three mussels from the batch used for feeding (frozen *Mytilus edulis*) following the same procedure as for the mid-gut samples. For the pellets, DNA was extracted from 10 pooled pellets using the UltraClean Soil DNA kit (MoBio Laboratories Inc.), following the manufacturer's standard protocol.

Cloning and sequencing of 16S rRNA genes

Part of the bacterial 16S rRNA gene was amplified from all mid-gut, water, mussel and pellet samples using the primers 27f BAC (5′-AGAGTTTGATCMTGGCTCAG-3′) [27] and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) [37]. PCR conditions were 5 min at $94\,^{\circ}C$ followed by 22–27 cycles of 1 min at $94\,^{\circ}C$, 1 min at $52.5\,^{\circ}C$ and 1 min at $72\,^{\circ}C$ and a final step of 7 min at $72\,^{\circ}C$. The number of PCR cycles was adjusted when needed in order to decrease non-specific products. The total number of cycles for all samples varied from 23 (sample S6m1) to 27 cycles (sample P6m1). PCR products were purified with the Montage Purification Kit (Millipore, USA) and were cloned directly using the TOPO TA Kit for sequencing (Invitrogen Inc., USA) with electrocompetent cells. The insert size was checked using PCR with M13f–M13R vector-binding primers. Positive clones were grown overnight in 1.5 mL of Luria–Bertani medium containing kanamycin (50 $\mu g\,mL^{-1}$), and plasmids were purified from the pelleted cells using the Nucleospin Plasmid QuickPure Kit (Macherey-Nagel GmbH and Co., KG, Germany). Plasmids were partially sequenced with primer M13f (5′-GTAAAACGACGGCCAG-3′). After alignment with ClustalW [28], manual correction, elimination of chimeras using the Pintail software [3] and visual examination of the alignments, clones were grouped based on a 16S rRNA similarity cut-off level of 98% and representatives from each group were sequenced using primer M13R