Modulation of neuronal activity by symbiotic bacteria in the early-branching metazoan *Hydra*

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Abstract

Accumulating evidence indicates that symbiotic microbiota are able to communicate with the host nervous system, thereby modulating the host neuronal activity. This modulation is reflected in, among others, changes in behaviour and physiological processes that are under neuronal control. This communication has been mainly studied in vertebrate models, whose complexity hamper the complete understanding of this interaction. In particular, contribution from endocrine, immune, nervous and metabolic signalling are hard to distinguish from one another. In addition, the restricted experimental manipulability of the highly diverse vertebrate microbiota, makes difficult to address causality. In consequence, the basic molecular and cellular mechanisms underlying this communication are not completely understood yet. The fresh-water polyp *Hydra* is a suitable alternative model to study this communication. Its simple body plan, the presence of an anatomically simple but molecularly complex nerve net that coordinates several behaviours and physiological functions, and the presence of a relatively simple and stable associated microbiota, make hydra an attractive model to study the causal effect of symbiotic bacteria and the host neuronal activity. Here we show that the feeding response and the spontaneous contractions of hydra's body column, both actions that are under the control of the nerve net, are modulated by hydra symbiotic bacteria. Compared to animals harbouring undisturbed microbiota, germ-free animals display a shorter feeding response duration. Similarly, germ-free polyps show strongly reduced and less regular spontaneous contraction frequencies. The effects on contraction frequency were partially restored by reconstituting the natural microbiota. Moreover, soluble molecule(s) produced by symbiotic bacteria may be involved in contraction frequency modulation. As the absence of bacteria does not impair the contractile ability itself, a microbial effect on the pacemakers responsible for timing the spontaneous contractions, seems plausible. Our findings suggest that the influence of bacteria on neuronal activity is the outcome of an evolutionary ancient interaction between bacteria and metazoans, opening a window into investigating the basic mechanisms of for example, dysmotility disorders in vertebrates.

Zusammenfassung

Es gibt zunehmende Beweise, dass die symbiotische Mikrobiota mit dem Nervensystems des Wirtsorganismus kommuniziert und dadurch die neuronale Aktivität des Wirtes modulieren kann. Als Ergebnis einer solchen Aktivitätsänderung kann es unter anderem zu Veränderungen im Verhalten oder von physiologischen Vorgängen des Wirtes kommen, sofern diese unter neuronaler Kontrolle stehen. Diese Verbindung wurde bisher primär in Wirbeltieren studiert, deren Komplexität das vollständige Verständnis dieser Interaktion allerdings erschwert. Insbesondere die Einflüsse des Immunsystems und endokriner Faktoren sowie neuronaler und metabolischer Signalweiterleitung sind schwer voneinander zu unterscheiden. Außerdem erschwert die begrenzte experimentelle Manipulierbarkeit der extrem vielfältigen Wirbeltiermikrobiota die Aufklärung der Zusammenhänge der Wirts-Bakterien-Interaktion in solch komplexen Organismen. Dies hat zur Folge, dass die molekularen und zellularen Grundlagen der Kommunikation bis heute nicht vollends verstanden sind. Der Süßwasserpolyp *Hydra* eignet sich sehr gut als Modell, um die Interaktion zwischen Wirt und Bakterien im Detail zu untersuchen. Der simple Körperbau, die übersichtliche mikrobielle Diversität auf den Polypen, sowie das anatomisch einfache, jedoch molekular komplexe Nervensystem, das unterschiedliche physiologisch Prozesse und Verhaltensweisen koordiniert, machen *Hydra* zu einem attraktiven Modell, um die kausalen Effekte von symbiotischen Bakterien und neuronaler Aktivität des Wirts zu untersuchen. Diese Arbeit zeigt, dass sowohl das Fressverhalten als auch die spontanen Kontraktionen der Körpersäule von *Hydra,* die unter der Kontrolle des Nervensystems stehen, von symbiotischen Bakterien reguliert werden. Im Vergleich zu Kontrolltieren mit ungestörter Mikrobiota, weisem keimfreie Polypen im Durchschnitt ein zeitlich verkürztes Fressverhalten auf. Darüberhinaus zeigen keimfreie Polypen eine stark reduzierte Häufigkeit der spontanen Kontraktionen sowie ein weniger regelmäßiges Kontraktionsmuster. Die Häufigkeit der spontanen Kontraktionen konnte durch Hinzugabe der natürlichen Mikrobiota teilweise wiederhergestellt werden. Daher ist es wahrscheinlich, dass sezernierte Moleküle der symbiotischen Bakterien für die Regulation der Kontraktionen verantwortlich sind. Zudem scheinen die bakteriellen Moleküle auf die Schrittmacherneuronen zu wirken, die für die Kontraktionsfrequenz verantwortlich sind, da die Abwesenheit der Bakterien keinen Einfluss auf die Kontraktionsfähigkeit des Epithels hat. Die hier präsentierten Ergebnisse lassen darauf schließen, dass der Einfluss von Bakterien auf die neuronale Aktivität das Ergebnis eines evolutionär uralten Zusammenspiels zwischen Bakterien und vielzelligen Tieren ist und könnte den ersten Schritt zur Aufklärung der Ursachen von z. B. gastrointestinalen Motilitätsstörungen darstellen.

1. Introduction

1.1 Animals as metaorganisms

Interactions between animals and microorganisms have taken place along the whole metazoan evolutionary history, developing symbiotic relationships that have shaped both host and microbiota evolutionary trajectories (McFall-Ngai et al., 2013). From this point of view, a host and its associated microbes can be seen as a metaorganism, a dynamic assembly of organisms facing and responding to environmental pressures as individuals and as a community (Douglas and Werren, 2016; Theis et al., 2016). Under this view, it is not surprising that microorganisms would play a major role in the animal host's physiological functions and adaptation. For instance many marine invertebrates require environmental bacterial clues for the settlement and further development of the larvae (Shikuma et al., 2016). In the squid *Euprymna scolopes* for example, the development of the light organ, which is involved in camouflage and anti-predator defence, is triggered by the presence of lipopolysaccharide and peptidoglycan fragments of the symbiont *Vibro fisheri* (Koropatnick et al., 2004). In insects, the gut microbiota is crucial for immune and epithelial cell homeostasis of the gut, as well as food supplier (Engel and Moran, 2013). Similarly in vertebrates, the gut bacteria provide the host with metabolic products such as short-chain fatty acids, which promote intestinal gluconeogenesis and in turn improve glucose metabolism (De Vadder et al., 2014). In humans and mice the gut microbiota affect fat uptake, leading to weigh loss or gain depending on the bacterial composition harboured by the host (Ley et al., 2006; Ridaura et al., 2013). On the other hand, in mice the presence of specific bacteria is necessary for tuning up the host immune system, promoting lymphoid organogenesis, activating the T regulatory cells and balancing pro- and anti-inflammatory cytokines, preventing inflammatory diseases (Mazmanian et al., 2005; Round and Mazmanian, 2009). In addition, in zebrafish the gut microbiota is required for the development of the digestive tract and the differentiation of gut epithelia (Bates et al., 2006). Similar results have been reported in mice, where leaky gut and abnormal mucosa is observed in the absence of gut bacteria (Smith et al., 2007). In summary, these studies show that associated microbiota in particular bacteria, are involved in the host's normal metabolism, development and immune system maturation.

1.2 The gut-brain-axis

Since in vertebrates the major concentration of microorganisms is located in the gut, the research on the effect of associated microbiota on host physiology has mainly focused on the digestive tract function and related digestive pathologies. These studies have found that the gut bacteria is required for the normal development of the enteric nervous system, including enteric neurogenesis and gliogenesis (Collins et al., 2014; Kabouridis and Pachnis, 2015; Kabouridis et al., 2015). Gut motility problems have also been observed in the absence of gut microbiota or when the microbiota composition is disturbed (Abrams and Bishop, 1967; Husebye et al., 2001; Quigley, 2011). In particular the ever increasing prevalence of conditions such as irritable bowel syndrome that are associated with both gut dysmotility and dysbiosis (Kostic et al., 2014), has raised the interest to understand the role of commensal microbiota in the host's health and disease. This increasing interest has also revealed that the influence of commensal gut bacteria on animal physiology extends beyond the gut to other systems. One of the most surprising findings was the recognition that in vertebrates, the gut microbiota is involved in the normal development of the central nervous system. It has been demonstrated, that the presence of gut microbiota is necessary for the establishment of the blood-brain barrier, adult hippocampal neurogenesis and proper microglia function (Braniste et al., 2014; Erny et al., 2015; Möhle et al., 2016). Moreover, cognitive, emotional and behavioural processes are also dependent on the gut microbiota (Cryan and Dinan, 2012; Forsythe and Kunze, 2013; Sharon et al., 2016). For instance, compared with animals harbouring undisturbed gut microbiota, germ-free mice show anxiolytic behaviour, exaggerated stress response, impaired memory, learning and sociability, and altered brain function. These effects are associated with changes in brain chemistry and differential gene expression such as abnormal neurotransmitter signalling and higher levels of stress hormones as corticosterone and lower levels of brain-derived neurotrophic factor (BDNF), glutamate, dopamine and serotonin receptors (Diaz Heijtz et al., 2011; Gareau et al., 2011; Neufeld et al., 2011; Sudo et al., 2004). Similar results are obtained when the gut microbiota is disrupted for example, by antibiotic treatment (Möhle et al., 2016; O'Mahony et al., 2014). In addition, sociability in rodents, as shown by reduced time spent with conspecifics and increased engagement in repetitive behaviours like self-grooming, is also affected in the absence of gut microbiota (Desbonnet et al., 2014; Hsiao et al., 2013). Notably, restoration of native gut microbiota or administration of probiotics, ameliorates and might even reverse the behavioural abnormalities observed in these animals (Bravo et al., 2011; Hsiao et al., 2013; Liu et al., 2015; Sudo et al., 2004)

This interaction between the gut microbiota and the nervous system is known as the "microbiota-gut-brain axis" (Grenham et al., 2011). This concept is gaining acceptance as it opens news ways to interpret not only gastrointestinal pathologies but also neurological disorders. In fact, emerging evidence suggests that diseases such as Alzheimer, Parkinson and multiple sclerosis are associated with gut microbiota dysbiosis (Cekanaviciute et al., 2017; Harach et al., 2017; Jangi et al., 2016; Sampson et al., 2016). Therefore it is of clinical importance to understand how the commensal microbiota affects the host brain function. Although endocrine, immune, nervous and metabolic signalling as well as epigenetic regulation are involved in establishing this communication (Cryan and Dinan, 2012; Stilling et al., 2014), the basic molecular and cellular mechanisms have not been discovered yet. In part, this might be due to the fact that mainly vertebrate models such as rodents, have been employed so far to study the microbiota-gut-brain axis. The complexity of the candidate pathways involved and the still limited knowledge about vertebrate microbiota function and composition (Kowarsky et al., 2017) has proven to be an obstacle to achieve a complete understanding of this communication. For this reason, disentangling the roots of the microbiota-gut-brain axis requires the use of alternative models, a relatively simpler organism that would allow manipulations of both the host and the microbiota (Chen et al., 2013).

1.3 *Hydra*: Emerging model for metaorganism research

In addition to vertebrate, invertebrates have contributed as alternative models to study metaorganisms dynamics. In fact, the concept of "holobiont", the starting point of the current metaorganisms research, was postulated using corals as models (Zilber-Rosenberg and Rosenberg, 2008). However, corals do not allow for the extensive manipulation necessary for deep molecular and cellular analysis, for example, there is no transgenesis or RNA interference available in corals. Therefore, other cnidarians have entered the scene: host-microbe interactions, framed in the community view of the metaorganim perspective, have already been explored in *Nematostella, Aurelia* and *Hydra* (Grasis et al., 2014; Mortzfeld et al., 2016; Weiland-Bräuer et al., 2015).

In particular, the fresh-water polyp *Hydra* would suit the role as a model organism to study the microbiota-gut-brain axis. First, *Hydra* belongs to the phylum Cnidaria, the sister group of Bilateria (Fig. 1a), which is considered one the first groups where the nervous system first appeared (Jákely et al., 2015; Pisani et al., 2015). Its body plan is very simple: two epithelial layers, the ecto- and the endoderm, form the body column, which is a tube with an apical

head and a basal foot (Fig. 1b). The body column lumen is known as the gastric cavity because it has digestive functions and therefore can be considered as an evolutionary ancient intestine (Schröder and Bosch, 2016). Second, as other cnidarians, *Hydra* possesses a relatively simple nerve net, composed of only sensory and ganglion neurons, which spread throughout both epithelial layers (Fig. 1c-d) (Koizumi, 2002). Although the hydra nerve net possesses no centralized structures, nerve cells are concentrated in the head and foot region. The anatomical simplicity of the nerve net contrasts its molecular complexity, since cnidarians possess almost the complete set of homolog genes involved in vertebrate neurogenesis, neuronal specification, migration and synapsis formation (Watanabe et al., 2009). This, together with the increasing molecular and imaging tool availability, has renewed the interest in cnidarian neuroscience and the use of cnidarians as model to study the origin and function of the nervous system (Bosch et al., 2017).

Third, *Hydra* harbours species-specific microbiota (Franzenburg et al., 2013), mainly found in the glycocalyx, a mucus layer covering the outside surface of the ectoderm (Fig. 1b) (Fraune et al., 2015; Schröder and Bosch, 2016). Compared to vertebrates, *Hydra* microbiota is composed by relatively few species most of which can be cultured (Fig. 1e) and thus amenable for experimental manipulation. In fact, it is possible for example to create germ-free hydras, devoid of symbiotic bacteria, and to conventionalize them animals by recolonizing them with native microbiota (Fraune and Bosch, 2007; Fraune et al., 2015). As a result of this manipulability, it was revealed that the presence of symbiotic bacteria is critical for tissue homeostasis and health of the polyp. In the absence of symbiotic bacteria, hydra polyps are more susceptible to fungal infection with lethal consequences (Fraune et al., 2015). Similarly, recently it has been shown that the abundance of hydra's symbiotic bacteria can be altered by neuropeptides secreted by hydra (Augustin et al., 2017). This evidenced that the interplay between neurons and microbiota also takes place outside Bilateria, and is an on-going process in *Hydra*. These results have promoted the current intensive research on the dynamics governing hydra microbiota community (Deines and Bosch, 2016; Li et al., 2015; Pietschke et al., 2017).

Forth, *Hydra* is a convenient model to study the microbiota-gut-brain axis due to the set of simple of movements and reactions it displays. The feeding response, spontaneous and mechanically-stimulated body column contractions, somersaulting displacement, photo- and gravitaxis are among them (Wagner, 1905).

In summary, a simple body plan, the presence of an anatomically simple but still complex enough nerve net that coordinates several behaviours and physiological functions, together with the presence of stable associates microbiota, supports hydra as an attractive model to study the casual effect of symbiotic bacteria and the host neuronal activity.

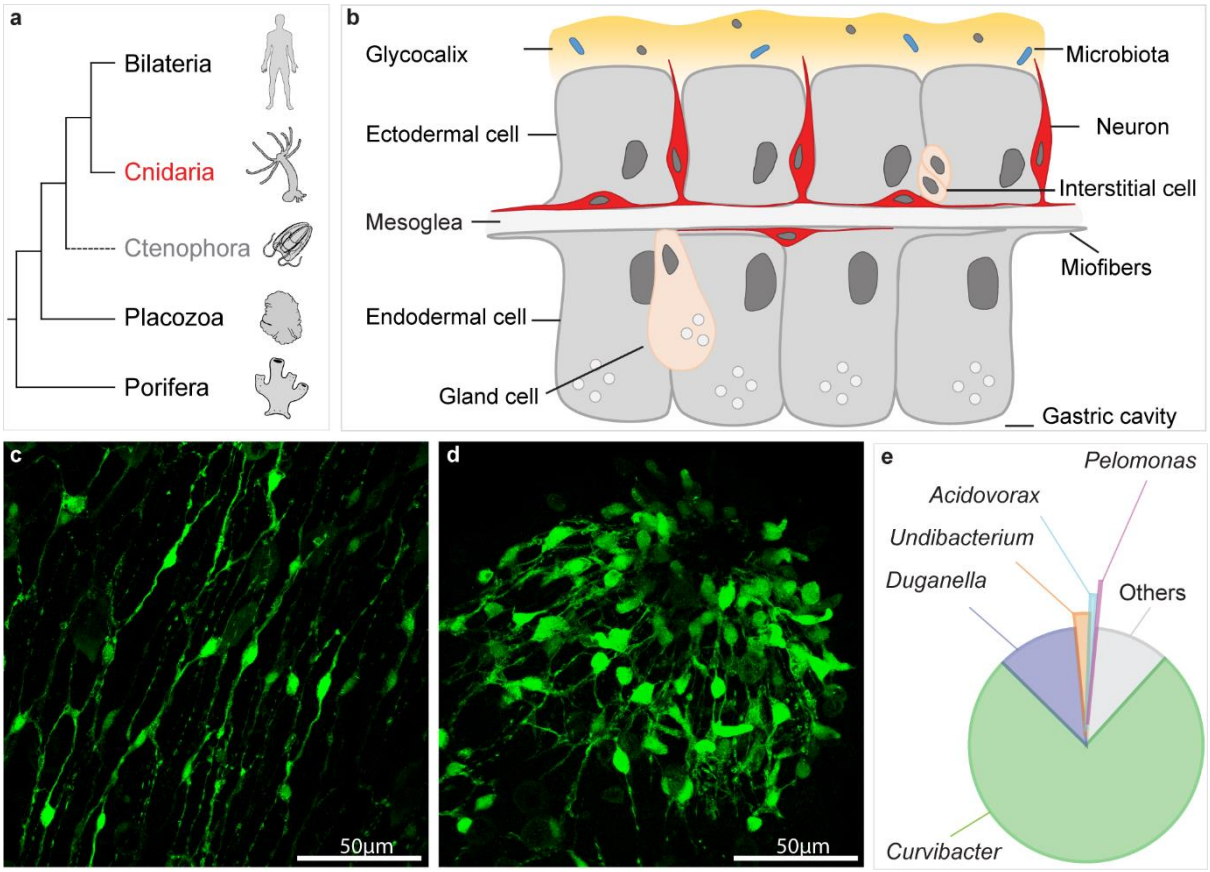


Figure 1. *Hydra* as a model to study host-symbiont interactions. (**a**) *Hydra* belongs to the phylum Cnidaria, the sister group of Bilateria. (**b**) The body wall of *Hydra* is composed of three cell lineages: the ectodermal and the endodermal epithelia that are separated by an extracellular matrix (mesoglea), and the lineage of interstitial cells that differentiate into neurons, gland cells, cnidocites and gametes. The outer surface of the ectoderm is covered by a glycocalyx, the habitat of symbiotic bacteria. The endoderm lining the gastric cavity, is free of glycocalyx and stable microbiota. (**c-d**) *Hydra* has a simple nerve net. Here ganglion neurons (**c**) in the body column and sensory neurons in the hypostome (**d**) are revealed by expression of RFP (green pseudo-color) under the actin promoter. (**e**) 90% of *H. vulgaris* AEP microbiota is composed by five bacterial strains, with less abundant strains making up the remainder. Picture credit for (c-d) Alexander Klimovich.

1.4 *Hydra* behavioural repertoire

The feeding response and the spontaneous body contractions are of particular interest, as they have been subject to extensive behavioural and physiological studies (for review see

Kass-Simon and Pierobon, 2007 and Pierobon, 2012). Moreover, these studies provided evidence that both the feeding response and the spontaneous contractions are dependent on neural activity, since neither is observed in nerve-free hydra (Campbell, 1976a; Campbell et al., 1976) or animals treated with anaesthetics (Loomis, 1955; Passano and McCullough, 1964). Therefore, the feeding response and the spontaneous contractions can be used as read-outs to assess the influences of microbiota on hydra's neuronal activity, on a behavioural and a physiological process respectively.

1.4.1 Feeding response in *Hydra*

The feeding response in *Hydra* (Fig. 2a) is triggered when a tentacle captures a prey by discharging nematocytes. The tentacles bend towards the mouth carrying the prey, then the mouth opens and the prey is ingested (Lenhoff, 1961). Afterwards, the mouth closes and the prey is pushed down to the gastric cavity. The mouth opening and tentacle bending can be activated by γ-glutamil-cysteinyl-glycine or reduced glutathione (GSH) in micromolar concentrations (Fig. 2b) (Loomis, 1955). This makes possible to control the initiation of the response and register the time and sequence of the events occurring during it. Although the response to GSH is robust and well described, the receptor and the downstream signalling cascade activated by GSH are not yet reported. However, pharmacological studies suggest that classical neurotransmitter signalling may be controlling the feeding response: in particular glutamate, GABA and glycine acting through their ionotropic receptors have shown to modulate the duration of the response (Pierobon, 2012; Pierobon, 2015).

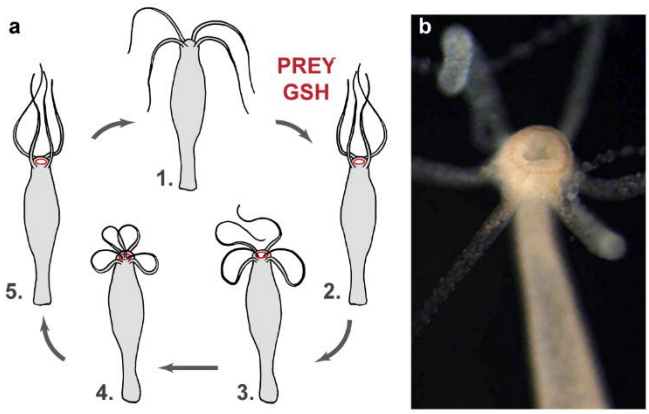


Figure 2. Feeding response in *Hydra*. (**a**) Schematic representation of the feeding response triggered upon stimulation (*i.e.* GSH or a prey). (**b**) Mouth opening in a polyp exposed to GSH 1µM.

As every animal, *Hydra* has particular way to feed and therefore it is difficult to compare its feeding behaviour with a feeding-related activity found in Bilateria. However, regardless the peculiarities of a feeding strategy, feeding is a classic example of behaviour, where an effector

system produces a motor output as a response to external and/or internal stimuli, output that is under neuronal control in cnidarians as well as in bilaterians (Jekely et al., 2015). Thus, assessing the impact of the microbiota on the feeding response would provide evidence of the microbiota influence on hydra neuronal activity.

There are no standard methods to register and quantify hydra behaviour. The classical approach to quantify the feeding response is to measure the time the mouth stays open upon stimulation, or as the percentage of hydras displaying a feeding reaction in a population (Koizumi et al., 1983; Lenhoff, 1961). It has been also proposed to measure the "tentacle spread" during the initial moments of the response (Kulkarni and Galande, 2014). Here we intend to describe the response in terms of events occurrence upon GHS exposure, their timing and sequence.

1.4.2 The spontaneous contractions

The spontaneous body contractions are another type of activity exhibited by *Hydra*. As in other hydrozoan polyps, hydra's spontaneous contractions are shrinkages of the body column that occur periodically in the absence of any exogenous stimuli (Stokes and Rushforth, 1979). A full body column contraction is also called a contraction burst because it reduces the polyp to a tight ball in a series of step-wise contractions (Fig. 3). Although the cell population(s) responsible for generating the spontaneous contractions have not been characterized yet, the evidence suggests that they are neurons, as the spontaneous contractions are abolished in nerve-free hydras (Campbell et al., 1976) and drastically reduced when gap junction communication among neurons is inhibited (Takaku et al., 2014). Moreover, contraction bursts are always preceded by periodic endogenous electrical activity generated in the head and the foot region, suggesting the existence of underlying pacemaker activity (Kass-Simon, 1972; Passano and McCullough, 1963; Passano and McCullough, 1964; Passano and McCullough, 1965). In addition, environmental factors such as light and chemical signals (*e.g.* glutathione) modulate contraction frequency (Passano and McCullough, 1964; Rushforth et al., 1964). Therefore the spontaneous contractions are both a behaviour as they respond to external stimuli, and a physiological process which is internally coordinated by neuronal activity (Jekely et al., 2015).

The spontaneous contractions have been assessed by counting the number of contraction per unit of time by direct observation or from videos recording groups of animals under a light microscope (Benos and Prusch, 1973; Takaku et al., 2014). In the present work we will base

our assessments on video recordings, to estimate contraction frequency, contraction regularity and contractile patterns among individuals.

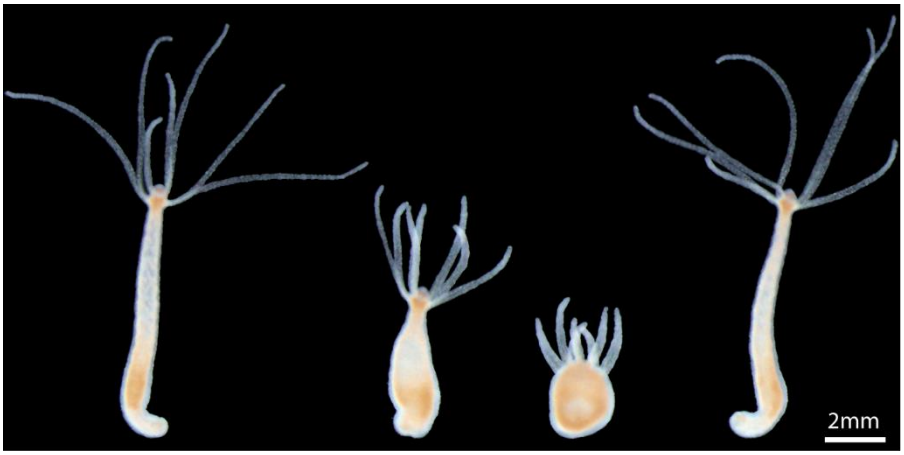


Figure 3. Sequence of pictures demonstrating the spontaneous contraction of the body column, hydra's most common behaviour.

Contrary to the feeding response, the spontaneous contractile activity displayed by *Hydra* has physiological equivalents in other animals, including humans. Gastrointestinal peristalsis and heart beat are examples of spontaneous contractile activity found across the animal kingdom. This kind of activity is driven by intrinsic rhythmic electrical pulses generated by pacemaker cells. Pacemakers are able to periodically depolarize themselves without the need of external stimuli and to transmit their depolarizing waves to effector cells. For example in vertebrates, the pacemakers underlying the movements of the digestive tract are the interstitial cells of Cajal (ICC) (Hulzinga et al., 1995). These cells spread depolarizing currents, known as slow waves, via gap junctions to smooth muscular cells that subsequently contract. Although the generation of the pacemaker activity *per se* does not depend on neuronal input, its modulation, for instance the speed and amplitude of the bursts, is under neuronal control (Sanders et al., 2012). In invertebrates, the pacemaker cells regulating rhythmic contractions are neurons embedded in neuronal networks known as central pattern generators (CPG) (Katz, 2016; Marder and Caiabrese, 1996). In insects and molluscs, CPGs are responsible for the motility of the gut (Dickinson, 2006; Okamoto and Kurokawa, 2010; Rand and Ayali, 2010; Robertson et al., 2012). In cnidarians CPGs control the swimming beat of cubozoans and hydrozoans medusa (Garm and Bielecki, 2008; Satterlie, 2002).

In vertebrates, there is a strong correlation between contractile activity disruptions (*e.g.* intestinal dysmotility) and dysbiosis (Hadizadeh et al., 2017b; Scott and Cahall, 1982; Vandeputte et al., 2016b) and this association is observed in conditions such as small intestine

bacterial overgrowth and irritable bowel syndrome (Kostic et al., 2014; Toskes, 1993; Vantrappen et al., 1977). Studies in mice have demonstrated excitatory effects of the gut microbiota on the enteric nervous system (Mao et al., 2013; McVey Neufeld et al., 2013) speaking for the causal effect of microorganisms on motility. In addition, there is also evidence showing a correlation between disrupted electrical activity which leads to dysmotility, and disrupted microbiota (Caenepeel et al., 1989; Husebye et al., 1994; Husebye et al., 2001; Scott and Cahall, 1982). However whether dysbiosis is the cause or the consequence of motility disruption is not completely understood. For instance, the whole range of bacterial molecules provoking the excitatory effect or what are the targets of those molecules, for example whether the gut microbiota has a direct impact on pacemaker activity, is not known.

1.5 Summary

Associated microbiota in particular bacteria, are involved in diverse aspects of the host's physiology, such as normal metabolism, development and immune system maturation. The development and function of the central nervous system has also emerged as another system under the influence of the gut microbiota. This interaction is known as the "microbiota-gut-brain axis" and has been mainly studied in mouse. However the complexity of the vertebrate nervous system and the still poorly understood vertebrate microbiota composition impose difficulties for the deep understanding of this interaction.

Contrary to the mouse, the fresh-water cnidarian polyp *Hydra* possesses an anatomically simple nerve net consisting of two types: sensory and ganglion neurons. The nerve net is nevertheless complex enough to coordinate several behaviours such as the feeding response and the spontaneous contraction of the polyp's body column. Moreover, *Hydra* houses a stable microbiota which in 90% is made up by less than ten bacterial strains that are culturable.

This, together with the experimental accessibility of *Hydra*, allows combining the experimental and molecular approaches necessary to tackle complex questions in a simple background: behavioural and physiological manipulation of the host, microbiota modification, up- and down-regulation of nerve cells function and depletion of the entire nerve net are possible in *Hydra*. Finally, its basal phylogenetic position will allow studying the influence of the microbiota on the host neuronal activity from an evolutionary perspective. As a cnidarian, *Hydra* belongs to one of the groups where the metazoan nervous system emerged and consequently where the signalling between the microbiota and nervous system first took

place. Therefore the ancestral mechanisms and the fundamental implications of this communication for the host's fitness could be better understood in a system that is evolutionarily close to the point of this cross-talk emergence.

2. Objectives

The main goal of this project is to assess whether the microbiota-gut-brain axis is an ancient evolutionary trait. In other words, whether associated bacteria affect neuronal activity in early branching metazoans, using *Hydra* as a model, as is observed in vertebrates. To address this goal, the following specific questions have to be addressed:

1. Does hydra commensal bacteria affect the feeding response?
2. Does hydra commensal bacteria affect the spontaneous body contractions?
3. Does the microbiota composition, in terms of strains richness and abundance, affect the neuronal activity controlling the spontaneous contractions?
4. What are candidate molecular mechanisms behind the bacterial modulation of hydra's neuronal activity?
5. As there are no standard protocols to assess hydra behaviour, we intent to establish methods to quantitatively describe the feeding response and the spontaneous contractions, which allow the assessment of the impact of symbiotic bacteria on hydra behaviour.

3. Results

3.1 Establishment of the method to quantify the feeding response

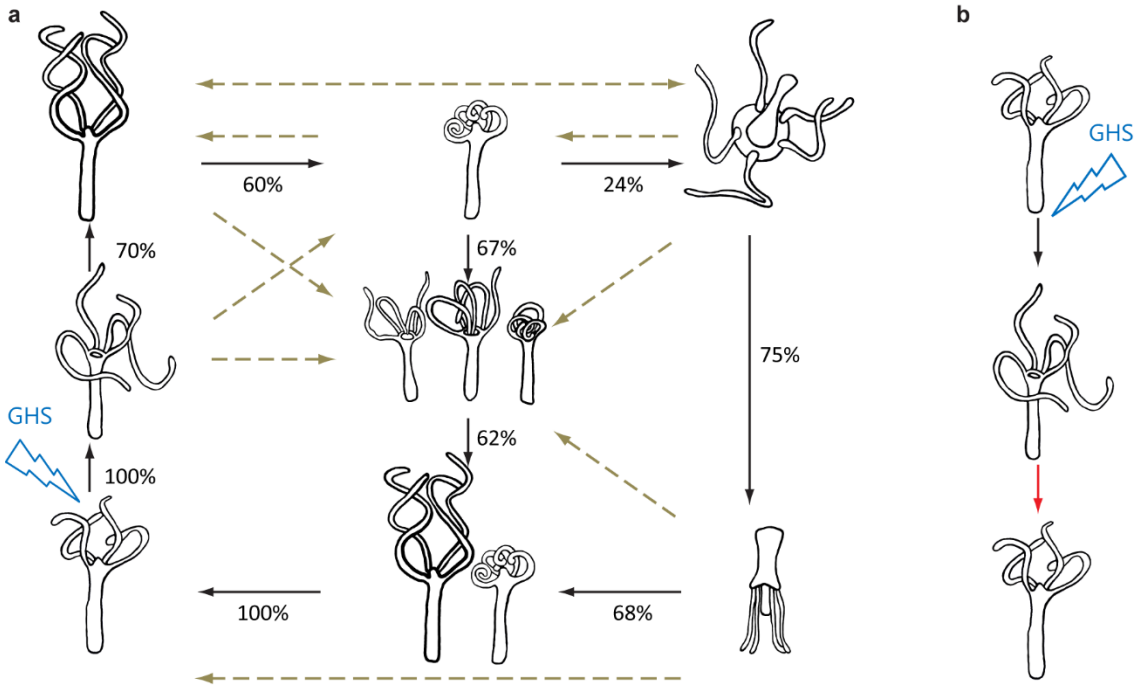
The first step in the analysis of the feeding behaviour was to describe the feeding response elicited by reduced glutathione (GHS) in *Hydra vulgaris* AEP polys, in terms of events taking place, their timing and whether they the follow any occurrence sequence.

We were able to elicit the feeding response in control polyps using 1µM GSH, (see Methods), and observed the following stereotypical events occurring upon GSH exposure:

1. Mouth opens: appearance of a visible opening on the tip of the hypostome.
2. Tentacle writhing: convulsive tentacle movements with tentacles stretched out over the hypostome.

3. Tentacle curling: curling up of tentacles towards the hypostome.
4. Touching the mouth: tentacles bend towards the mouth opening and touch it with tentacle tips.
5. Ball: tentacles contract and fold themselves over the hypostome forming a ball.
6. Tentacles inside the mouth: tentacles are inserted in the body cavity through the mouth opening.
7. Mouth spreading and partial inversion of the body: the mouth attaches to the bottom of the container the polyp is in and expands in an attempt to swallow the container. In doing so, the body of the polyp flattens into an expanding disc, sometimes until such an extent that the expanded mouth tissue falls off, folds backwards along the body column, partially inverting the polyp.
8. Mouth closes: no visible opening is seen on the hypostome.

Among these events, mouth opening and tentacle writhing, have been described as the most characteristic ones (Lenhoff, 1961; Loomis, 1955), and in our experiment were observed in all polyps during the GHS-elicited response. Mouth opening eventually followed by mouth closure, were the only events occurring in a consistent sequence, occurring in 100% of the observed polyps. All other events occurred without following any particular sequence, as measured by the percentage of polyps making a transition from a given event to another (Fig. 4a). Therefore the time to mouth opening and the duration of the response, which is the interval between mouth opening and mouth closure (Fig. 4b), were chosen for further analysis.



<- Figure 4. Events taking place during the GSH-induced feeding response do not follow a strict pattern. (**a**) Graphic representation of events happening upon exposure of GSH 1µM. Solid lines indicate the course of events undertaken by more than 20% of the tested polyps (n=40). Broken lines correspond to transitions undertaken by less than 20% of the animals and exemplify the absence of a robust pattern in the occurrence of the events. (**b**) In the absence of a strict pattern, time to mouth opening (black arrow) and the duration of the response, the time between mouth opening and closure(red arrow), were selected for further analyses.

In agreement with previous studies on the feeding response (Lenhoff, 1961; Pierobon, 2015), mouth opening occurred within the first minute (mean±SE = 0.67±0.06 min, n=40; Fig. 5a) in wild-type polyps, after the exposure to GSH. The mouth stayed opened in average 35.05±1.68 minutes (Fig. 5b). These results indicate that GSH-activated feeding response can be described in quantitative terms (i.e. frequency and timing) and therefore comparisons between different treatments are possible (e.g. animals with disturbed microbiota).

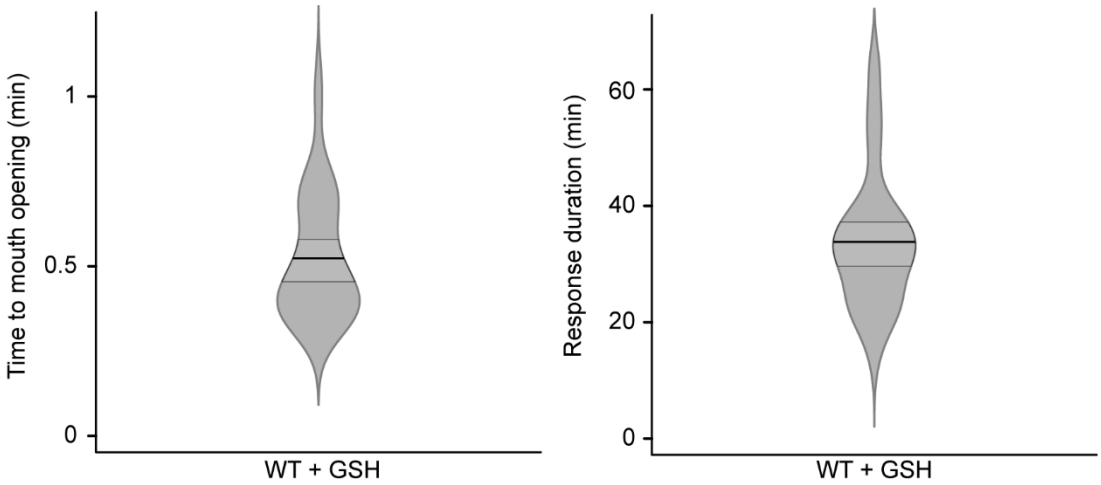


Figure 5. Feeding response induced by GSH in wild-type animals. (**a**) Time to mouth opening and (**b**) duration of the response in 40 wild-type (WT) polyps, starved for 2-3 days. Here and further in similar diagrams, boxes in beans represent the Highest Density Intervals (HDI) with 95% confidence for the mean (middle line) and the shape of the beans shows the distribution of the raw data.

3.2 Duration of the feeding response is altered in germ-free polyps

After describing the wild-type feeding response, we assessed this behaviour in germ-free (GF) polyps, obtained by adjusting the procedure initially described in Franzenburg et al. (2013). Briefly, the antibiotic treatment was extended to two weeks as opposed to the one-week period originally employed (Fig. 3). After the antibiotic treatment the polyps were transferred to sterile hydra medium, which was replaced every two days, for two weeks. Once

in sterile hydra medium, the germ-free state of the polyps was checked at least once per week by plating tissue macerates of treated polyps on R2A agar plates. At the end of the experiment, GF polyp macerates were analysed by 16S rDNA amplification (See Methods for details). Absence of bacteria growth on the plates and absence of amplification product confirmed the GF status of the polyps. Only verified GF-polyps were used for further experiments.

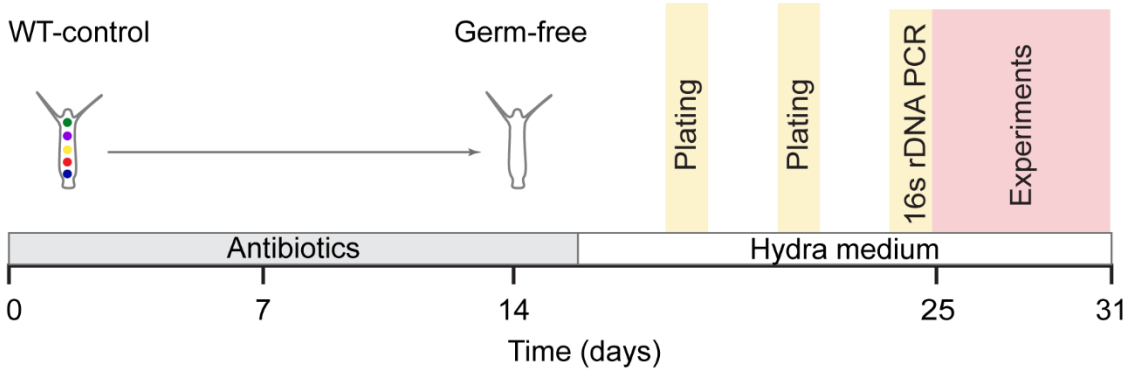


Figure 6. Schematic representation exemplifying the handling of GF animals. Wild-type polyps were treated for 15 days with an antibiotic cocktail. Afterwards the animals were transferred to sterile hydra culture medium. Regular assessment for germ-free state of the treated animals was performed after the end of the antibiotic treatment (*e.g.* plating, 16S rDNA PCR). Detailed description is found in the Methods section.

To evaluate the effect of microbiota on feeding behaviour, we compared time to mouth opening and duration of the response in control and GF animals, upon exposure to 1µM GSH (Fig. 7a). There was no difference in time to mouth opening between control and GF animals (0.53±0.04min and 0.69±0.07min respectively, Fig. 7b). On the contrary, the duration of the response was significantly shorter in GF than in control animals (18.59±0.99 min and 32.54±1.16 min respectively, Fig. 7c). This suggest that the absence of associated bacteria does not affect the pathway(s) leading to mouth opening, but instead modulates the downstream signalling that keeps the mouth open or that leads to mouth closure. To test whether this difference is due to the absence of bacteria, it would be necessary to recolonize GF animals with native hydra microbiota (*i.e.* conventionalized polyps) and to compare their feeding response to that of control and GF animals. Given that this test is extremely laborious, we were not able to study this behaviour in depth.

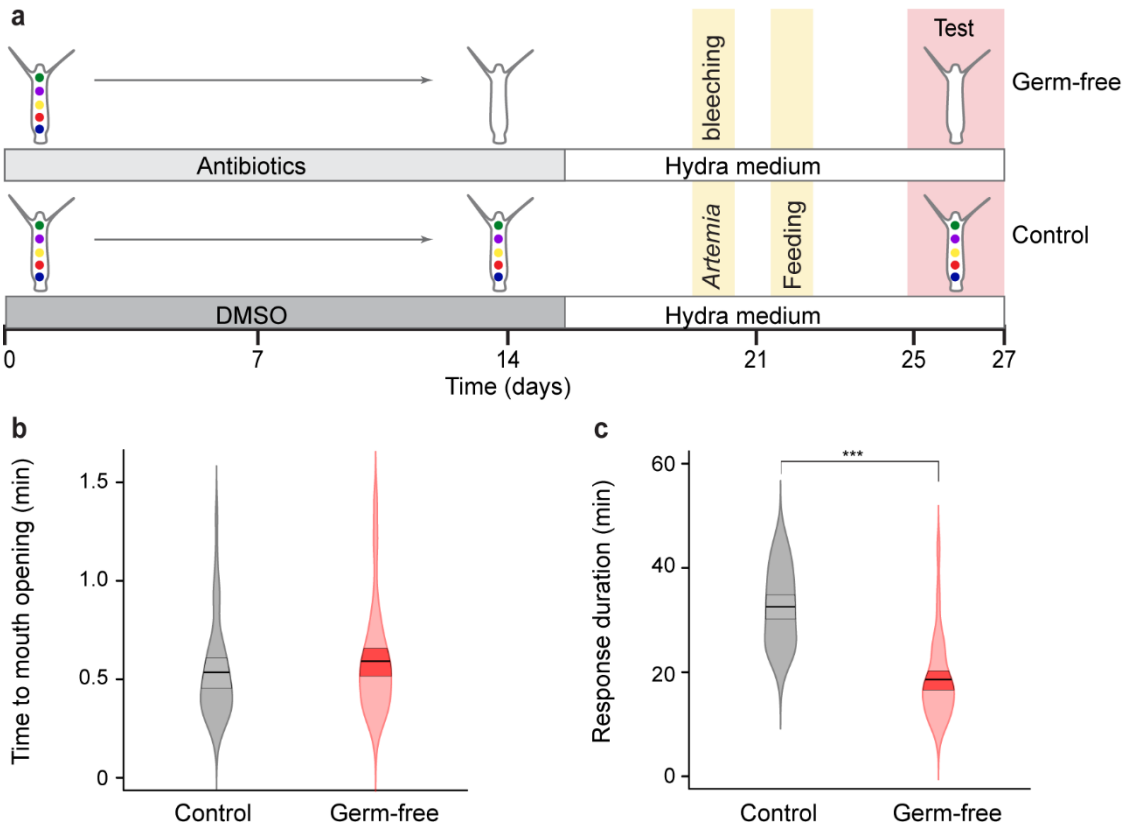


Figure 7. Feeding response is altered in GF animals. (**a**) Experimental design. GF animals were obtained by treating WT (control) polyps with an antibiotic cocktail for two weeks. Control polyps were treated with the antibiotic solvent (DMSO) for the same amount of time. GF and control polyps were feed 3-5 five days before the experiment with sterile-hatched *Artemia* naupilli (See methods for details). (**b**) Control and GF polyps have similar mouth opening times after exposure to GSH. Wilcoxon rank sum test, W=1011, p=0.26. (b) The duration of the response is shorter in GF polyps compared to control animals. Wilcoxon rank sum test, W=2228, *** p≤ 0.001. Sample size in (a) and (b): GF=60, control= 41.

3.3 Establishment of the method to assess the spontaneous contractions of *Hydra*

In order to quantitatively describe the contractile behaviour of hydra, we recorded time-lapse videos of individual undisturbed light-adapted control polyps. We measured the contraction frequency of each animal by counting the amount of full body contractions occurring in one hour (Fig. 8a, Suppl. Video 2). Polyps starved for three days contracted on average 10.86±0.53 times per hour (mean±SE, n=22; Fig. 8b). These contractions appeared in a regular temporal pattern with an average interval between two consecutive contractions (Δt, Fig. 8a) of 5.35±0.26 min (mean±SE, median=4.5min, n=217) and 91% of consecutive contractions occurring within an interval of 10 min (mode= 3.25 min), 9% with intervals between 10-15min and, only 2.7% taking longer than 15 min (Fig. 8c).

The contraction frequency as well as the time in between two consecutive contractions obtained with the method presented here are in agreement with the values reported by other studies using different techniques such as direct counting or electrophysiological measurements (Dupre et al., 2017; Gonzales et al., 2017; Takaku et al., 2014).

Moreover, besides contraction frequency and time between contractions, our setup also allows for assessing differences in stretching and contraction capacities of the polyps (S_{max} and ΔS**,** Fig. 8a), providing additional tools to quantify the behaviour. The method quantifies the polyp shape at a given time point relative to all the shapes the polyp takes during the hour of recording. In this way, contracted states are expected to get standardized stretching scores (z-scores) below zero and elongated states scores above zero, with zero representing average metric. Scoring the shape in this way allows us to compare relative changes among polyps independently of their size. For more details see Methods section.

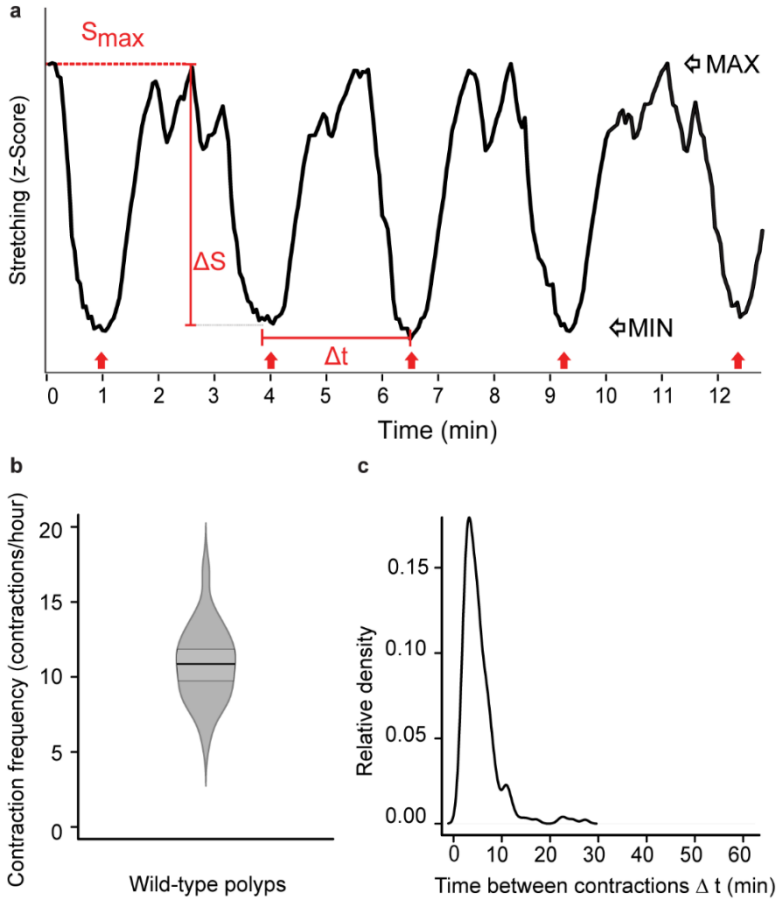


Fig. 8. Spontaneous contractions assessment in wild-type polyps. (**a**) Exemplary profile of body contractions obtained from a wild-type polyp. The contractile behaviour was described in terms of contraction frequency, defined as the number of full body contractions (red arrows) occurred in one hour, the time interval between two consecutive contractions (Δt), the stretching capacity (S_{max}), which is the maximum body elongation achieved after a contraction and the contractile capacity (ΔS), the

difference in a polyp's shape between MAX and MIN states. (**b**) Contraction frequency of undisturbed wild-type polyps as the number of contractions per hour. (**c**) Time between two consecutive contractions displayed by control polyps. In (**b-c**) n=22, polyps were starved for three days.

To test the ability of our method to capture relative differences in a polyp shape, *i.e.* elongation and contraction capacities, we tested animals with known differential contractile abilities. It has been reported that decapitated polyps do not contract or contract rarely compared to intact animals (Passano and McCullough, 1964), therefore our method should register them in a "permanent" elongated state.

When compared with intact control polyps (Fig. 9a), decapitated polyps (Fig. 9b) indeed displayed dysregulated spontaneous contractions shown by decreased contraction frequency and radically different distribution of time intervals between consecutive contractions (Δt, Fig. 9c-d). When decapitated polyps contracted more than once in one hour, most of the consecutive contractions occurred within intervals around 6 min, twice longer than in control animals. In addition, in decapitated polyps 33% of the intervals were longer than 20 min, while there were no intervals longer than 20 min in control animals (Fig. 9d). The presence of longer intervals in decapitated animals compared to controls is also evidenced by the difference in the median values of both distributions, with the median interval distribution of decapitated animals being three times larger (Fig. 9d).

The contractile capacity ΔS is also different in decapitated polyps (Fig. 9e). As described before, the relative differences in stretching (*i.e* polyp's shape) that a polyp acquire during an hour, are quantified using z-scores in such a way that relative mean stretching (*i.e* mean shape) values will oscillate around zero. Contractions, which relative to the average correspond to stretched states, will be scored with values below zero.

The shape of control polyps is constantly changing, oscillating between z-scores of 2 and -2 (Fig. 6a). These are average extreme values that correspond to maximum elongated and maximum contracted states. In decapitated polyps however, the scores oscillate around zero with maximum values around 1 (Fig. 6b). This means that the maximum values displayed by these polyps correspond to average shape values, evidencing the "permanent" elongated state of decapitated polyps. For the same reason, relative to the average, a contraction will represent a greater change in the polyp shape compared to controls, and therefore the contractile capacity ΔS (*i.e.* the difference between maximum and minimum elongations, Fig. 8a) of decapitated polyps will be larger (Fig. 6e).

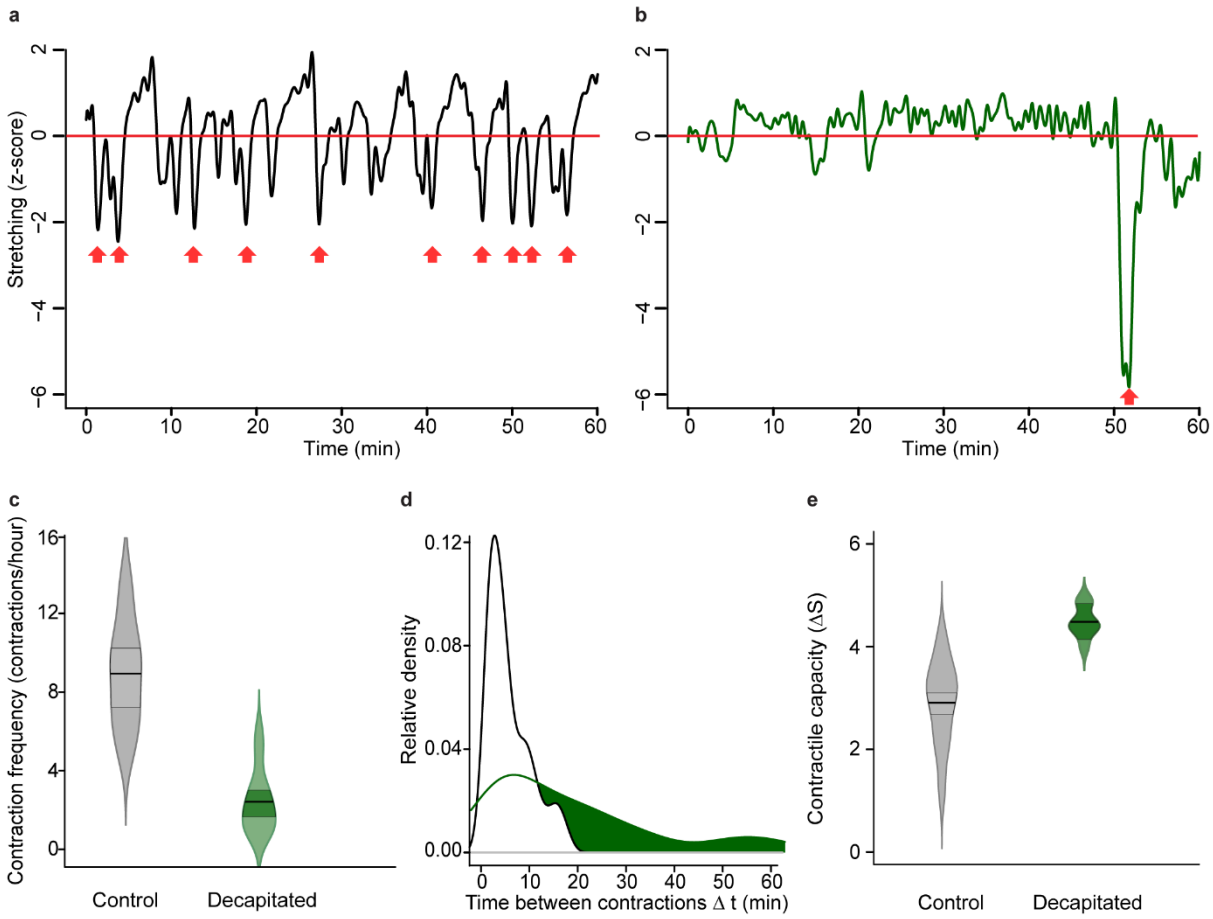


Figure 9. Validation of the method to assess the spontaneous contractions using decapitated hydras. Example of the variation of the shape in a control (**a**) and a decapitated (**b**) polyp over an hour. The red line indicates the average shape value and the red arrows mark full contractions: 10 contractions in control polyps and 1 in decapitated polyps. (**c**) Contraction frequency is reduced in decapitated polyps (n=25, mean±se = 2.42±032 contractions/hour) compared with control polyps (n=18, mean±SE = 8.94±0.68 contractions/hour. Wilcoxon test = 409, p <0.001). (**d**) The time between contractions is longer in decapitated (mode=6.34 min) than in controls animals (mode = 2.82 min), with longer intervals occurring in decapitated than in control polyps (median= 12.65 min and 4.35min respectively, green-shaded area). (**e**) Contractile capacity ΔS is larger in decapitated polyps (mean z-score: wt = 2.90, decapitated = 4.48), since full contractions represent greater deviations from the mean shape values, given that average shape scores in decapitated animals correspond to maximum elongated states too.

In summary, the method presented here has the ability to distinguish differential contraction dynamics, as long as there is internal variation in the shape of a polyp. Therefore it allows us to assess whether there are differences in the spontaneous contractions displayed by wild-type and GF animals, and in turn to evaluate the impact of microbiota on hydra neuronal activity controlling this aspect of the polyp physiology.

3.4 Spontaneous contraction behaviour is altered in germ-free polyps

To study the impact of the microbiota on spontaneous contractile behaviour, we measured contraction frequency in control and GF polyps. GF animals were obtained by treating *H. vulgaris* polyps with an antibiotic cocktail, as previously describe (Franzenburg et al., 2013). Because of technical reasons, both GF and control polyps were starved for entire duration of the experiment (Fig. 10a). Control polyps, which harboured undisturbed microbiota, contracted on average 7.8±0.1 times per hour (mean±SE, n=271) (Fig. 10b). The contraction frequency in starved controls is a bit lower compared to non-starved controls (Fig. 8b). As a consequence, the average time interval between contractions in starved controls increased in about two minutes (mean±SE=7.32±0.14 min; n=1658), keeping the regularity in the contraction pattern. The observed decrees in contraction frequency due to starvation agrees with what has been already reported (Passano and McCullough, 1964; Rushforth and Hofman, 1972).

Most strikingly, the contraction frequency of GF animals decreased to an average of 4.6±0.1 contractions/hour (mean±SE, n=236), corresponding to about 60% of the corresponding control contraction frequency (Fig. 10b). This suggests symbiotic bacteria are involved in modulating the spontaneous contraction frequency.

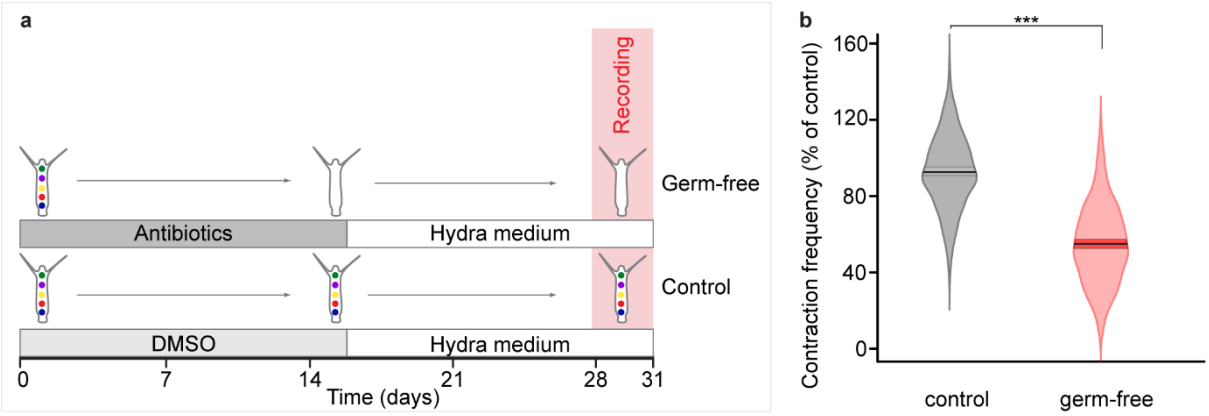


Figure 10. Absence of bacterial microbiota affects *Hydra* spontaneous contraction behaviour. (**a**) Experimental design: GF animals were generated by antibiotic treatment and then either kept in GF conditions, monocolonized with single bacterial isolates (Monocol.), with a mixture of the five main bacteria in equal proportions (5 bact.), or with natural hydra microbiota (Convent.). Control polyps were treated with the antibiotic solvent (DMSO) for as long as the time of antibiotic treatment lasted. Animals were not fed during the course of the experiment. (**b**) Contraction frequency is reduced in GF (batch=23, *n* = 236) compared to control (batch=23, *n* = 271) animals (X^2 = 426, df=1, ****P*≤0.001; linear mixed effects model with batch as random effect). Batch= number of independent groups of polyps treated to obtain the reported sample size n. Data are means±s.e.m.

To exclude that this reduction in contraction frequency between controls and GF animals was due to disrupted epithelium contractile ability, we compared the stretching (Smax) and contractile (ΔS) capacity of the body column in control and GF polyps (Fig. 8a). There was no difference in stretching capacity between control and GF animals, as evidenced by similar Smax values (Fig. 11a). Likewise, there was no difference in the contractile capacity of GF and control polyps, since the changes in the polyps' shape between a maximum stretched and the next full contraction states were comparable (Fig. 11b). These findings indicate that the removal of the associated bacteria does not impair the contractile ability of the epithelio-muscular cells in GF animals but instead affects the number of contractions per unit of time.

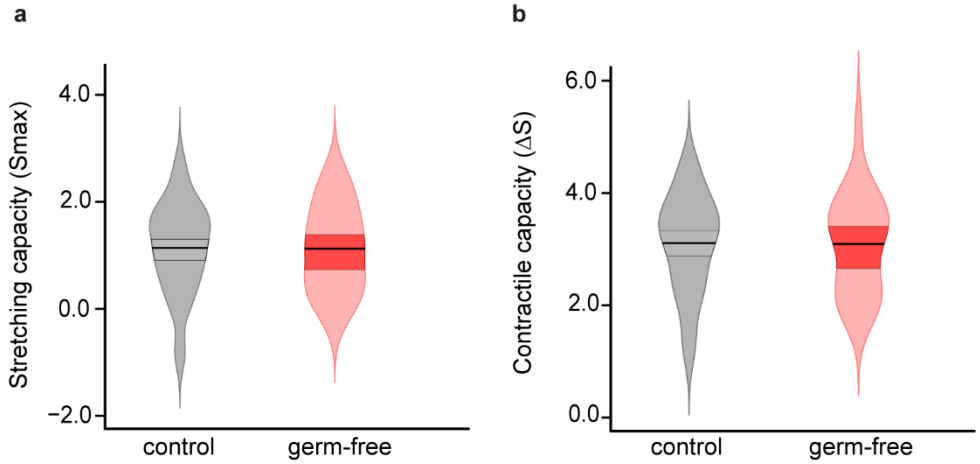


Figure 11. Epithelium ability to elongate and contract is not impaired in GF animals. (**a**) Stretching capacity Smax and (**b**) contractile capacity ΔS, is not disturbed in GF animlas, evidenced by the lack of statistical difference in Smax and ΔS between GF and control animals (**a**, $t = 0.07$, df=47.63, $P = 0.94$; maximum elongated states in GF $n = 22$, 0.96±0.10; in control $n = 63$; 0.97±0.12 and **b**, $t = 0.60$, df=40.00, $P = 0.60$; number of delta MAX-MIN in GF $n = 26$, 2.80±0.16; in control $n = 66$; 2.90±0.10). Data are means±s.e.m.

The observed reduction in contraction frequency in GF animals may reflect two possible scenarios, taking into account the two-fold difference in average contraction frequency between GF and control polyps. First, that the reduction is due to extended but still regular intervals between contractions, in other words, that the intervals between contractions increase proportionally to the reduced number of contractions per hour (Fig. 12a). If this is the case, one would expect a shift in the distribution of Δt (*i.e.* intervals between contractions, Fig. 8) towards longer intervals, reflected in an increment in the distribution mode. Second, that intervals between contractions do not increase proportionally with the reduction in contraction number, but longer breaks appear between them. If this is the case, one would expect no major shifts in the interval distribution mode. However, the appearance of longer

intervals would create a tail in the Δt distribution towards greater interval values. This scenario would imply that contractions are triggered less regularly.

To test which of the two possible scenarios was the case for GF polyps, we next analysed the distribution of time intervals between two consecutive contractions in these animals. We found that the majority of the contractions of GF polyps took place in a similar time frame as in control animals. This is shown by similar modes in both distributions, with most contractions separated by intervals of around 3-4 min (Fig. 12c). Moreover, the interval distributions of control and GF polyps did not differ for intervals lasting up to 15 min, with 75% of all intervals being shorter than 15 min in both control and GF animals. However, a significantly larger fraction of intervals in GF polyps lasted longer than 15 min with 4% of all intervals spanning longer than 30 min, creating a tail in the GF Δt distribution (red-shaded area in Fig. 12c). Such long breaks were not observed under any circumstance in control polyps. Together, these observations indicate that although most consecutive contractions in GF animals occur within normal 3-4 min intervals, these are intermitted by intervals lasting longer than 15 min. This situation corresponds to the second scenario (Fig. 12a, below). Thus, in the absence of bacteria the contractions seem to occur in a less regular pattern. This suggests that symbiotic bacteria are potentially involved in supporting pacemaker activity and/or its transmission to the effector epithelio-muscular cells.

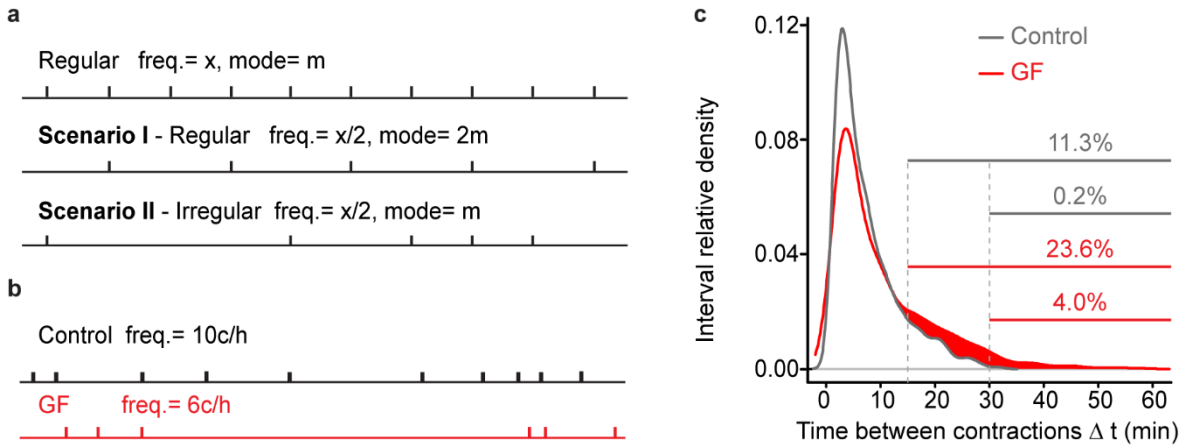


Figure 12. Contraction pattern is disturbed in GF animals. (**a**) Possible scenarios that would explain the contraction frequency reduction in GF polyps. A normal polyp contracting x times per hour (freq.= x) with most of the contractions separated by similar time intervals (mode= m), thus the contractions appear evenly spread over time (Regular). Scenario I: A polyp displaying reduced contraction frequency (freq.= x/2) and a proportional increase in the interval between contractions (mode= 2m), thus the contractions are still evenly spread over time (Regular). Scenario II: A polyp displaying reduced

contraction frequency (freq.= x/2) but not a proportional increase in time interval between contractions (mode= m). Therefore the contractions appear irregularly over time. (**b**) Contraction pattern of a representative control and GF polyp. The GF polyp (red) contracts six times per hour (c/h), however this reduction is not due to longer regular intervals, since most of the intervals have similar length as those of control polyps. Instead several contractions are "skipped" leading to extended pauses interrupting the regular pattern. Therefore contrary to control animals, contractions in GF polyps do not appear uniformly spread over time. (**c**) Distribution of time intervals between two consecutive contractions (Δt) in control and GF animals. Most contractions in both GF and control polyps occur within an interval of 3-4 minutes (mode = 3.55 min, n = 787 and mode = 2.93 min, n = 1658, respectively; n = number of intervals analysed per treatment). The distributions do not differ for intervals up to 15 minutes. Red-shaded area shows the time interval distribution for which GF and control significantly differ (Fisher exact test, $P \leq 0.001$). Percent values represent the fraction of intervals taking longer than 15 min (upper line) and 30 min (lower line) in control and GF polyps (grey and red, respectively).

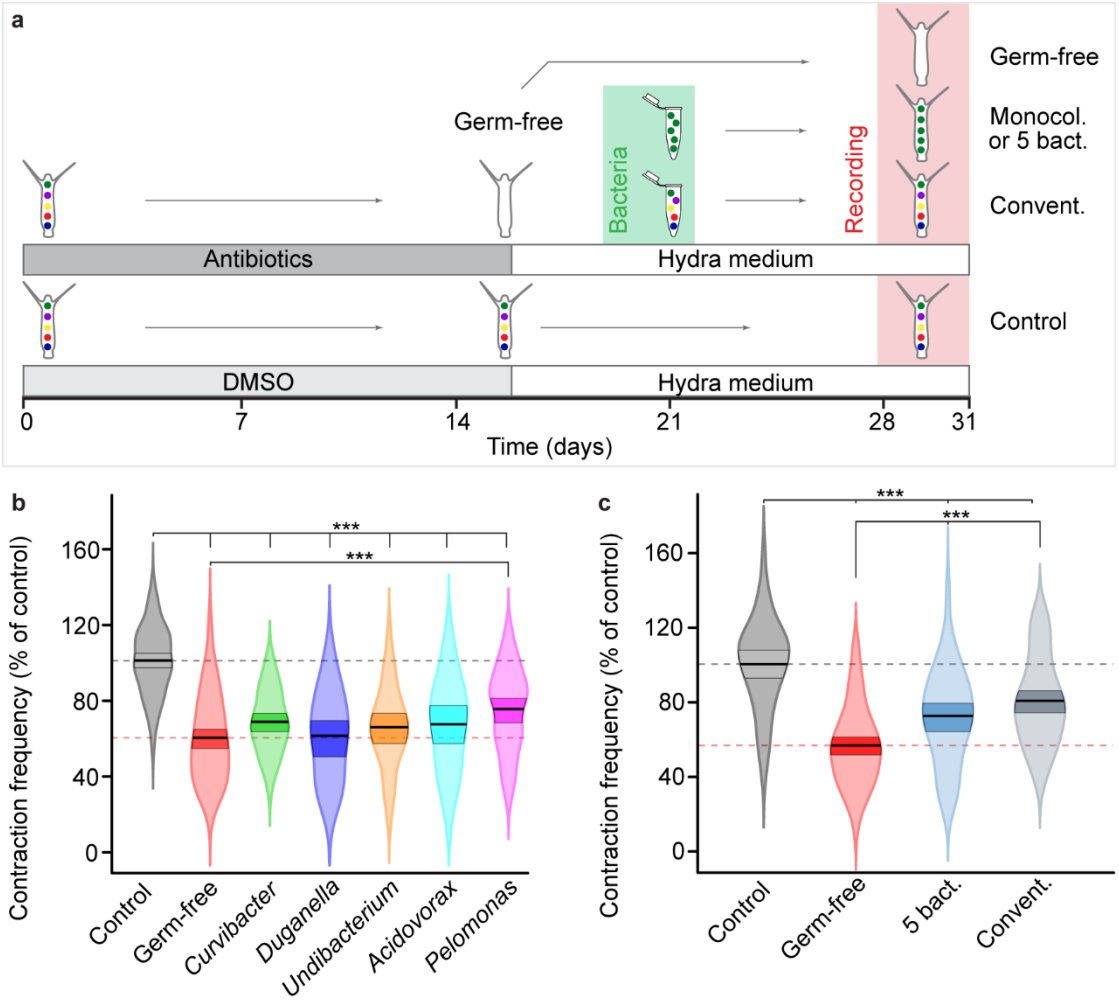
3.5 Reconstitution of microbiota improves contraction frequency

To assess causality between the presence of microbiota and contraction frequency, GF polyps were recolonized with hydra commensal bacteria, and their contraction frequency was measured. The experimental approach is summarized in Fig. 13a. Since hydra's symbiotic microbiota is relatively simple in comparison to that of vertebrates (Fig. 1e) (Fraune and Bosch, 2007), it allows analysing the role of each member individually. Therefore, we first tested the role of the main bacterial symbionts individually, by mono-colonizing GF polyps with isolates of the five strains that make up the majority of the microbiome: strains of *Curvibacter*, *Duganella*, *Undibacterium*, *Acidovorax* and *Pelomonas* genera (Fig. 1e) (Fraune et al., 2015). See Methods for details. Colonizing GF animals with single bacteria strains did not restore the contraction frequencies to control levels (Fig. 13b). In fact, frequencies of most monocolonized animals did not differ from GF values (Fig. 13b). Only recolonization with *Pelomonas* increased the contraction frequency in comparison to GF (Fig. 13b). These marginal effects of monocolonizations on contraction frequency contrast the striking behavioural difference between control and GF animals, and suggests that the presence of a complex bacterial ensemble may be necessary to restore the normal contractile behaviour.

We next investigated the role of the microbiota composition in modulating the contraction frequency. Two different microbial assemblages were assessed. First, GF animals were recolonized with the five main hydra bacteria strains combined in equal proportions. This composition does not mirror the *in vivo* situation, since the most abundant member (*Curvibacter*) is under-represented, while the other four (*Duganella*, *Pelomonas*,

Undibacterium, *Acidovorax*; Fig. 1e) are over-represented. However, the rare bacterial strains ("*Others*" on Fig. 1e) are missing in this assemblage, allowing us to assess the relative contribution of rare and dominant bacteria. Animals recolonized with this 5-strain mixture showed significantly higher contraction frequency than GF polyps, although it was not completely restored to control levels (Fig. 13c).

The second assemblage used reflects more accurately the natural microbiota community in terms of strain composition and abundance (Fig. 1e) since it was directly prepared from tissue homogenates of control polyps (Fig. 13a). GF polyps recolonized with this microbiota (referred to as conventionalized) showed significantly higher contraction frequency compared to GF polyps (Fig. 13c). These animals also had a tendency to display a higher frequency than polyps recolonized with the five main bacteria (P=0.067). Taken together, these results may indicate that not just the presence, but the relative abundances of the main colonizers play an important role in modulating the frequency of spontaneous contractions. Moreover, the presence of low-abundance bacterial strains may be essential for normal contractile activity. Finally, potential interactions among the members of the community may contribute to normal contraction dynamics.



←**Figure 13. Bacterial microbiota composition affects *Hydra* spontaneous contraction behaviour**. (**a**) Experimental design: GF animals were monocolonized with single bacterial isolates (Monocol.), with a mixture of the five main bacteria in equal proportions (5 bact.), or with natural hydra microbiota (Convent.). Control polyps were treated with the antibiotic solvent (DMSO) for as long as the time of antibiotic treatment lasted. Animals were not fed during the course of the experiment. (**b**) Contraction frequencies of control (batch = 8, *n* = 84), GF (batch = 8, *n* = 79) and monocolonized polyps. There is no difference in contraction frequency between GF and monocolonized animals (LME, GF compared to *Curvibacter* *P* = 0.09, batch = 4, *n* = 47; *Duganella P* = 0.94, batch = 3, *n* = 29; *Acidovorax* *P* = 0.22, batch = 3, *n* = 25; *Undibacterium* *P* = 0.37, batch = 3, *n* = 31), except for GF and *Pelomonas* (*P* = 0.001, batch = 4, *n* = 47). All frequencies are significantly lower than those of controls (GF 58.3±3.3%, *Curvibacter* 66.5±3.9%, *Duganella* 58.9±4.7%, *Undibacterium* 63.2±4.6%, *Acidovorax* 66.1±4.9% and Pelomonas 72.5±3.9%,; $X^2 = 199$, df = 6, ****P*≤0.001). (**c**) Contraction frequency of polyps recolonized with the five main bacteria (5 bact.) and conventionalized polyps (Convent.) is significantly higher than that of GF animals (bact. 72.8±4.9%, batch = 5, *n* = 48; convent. 80.9±4.6%, batch = 6, *n* = 62; GF 57.0±4.4%, batch = 6, *n* = 76; anova $F_{(3,228)} = 33.8$, ****P*≤0.001). Control: batch = 6, *n* = 46. Batch= number of batches produced to obtain the reported sample size n.

3.6 Microbiota supernatant extract improves contraction frequency in GF animals

To gain first insights into the mechanisms by which bacteria affect hydra spontaneous contractions, we prepared a microbiota supernatant extract (M.ext.), which is essentially a concentrated purified culture medium conditioned by bacteria for 24 or 48 hours (Fig. 14b). GF polyps were incubated overnight in these extracts before recording (Fig. 14a). GF polyps treated with the extracts displayed significantly higher contraction frequency than GF animals incubated only in hydra medium, and these frequencies were only about 12% lower than the control contraction frequency (Fig. 14c). There was no difference between the contraction frequencies of polyps incubated in the 24h and the 48h extracts (*P*=0.80). Since bacterial cells were removed from the supernatant by centrifugation and filtration before the extraction, the supernatant was assumed to contain only molecules secreted by bacteria. Moreover, as methanol was the solvent employed for the extraction followed by a heating step at 40°C to remove the solvent (see methods), it is likely that the bacteria-secreted products present in the final extracts are small rather than big molecular complexes, polar and thermoresistant molecules. Taken together, our findings suggest that some water-soluble and small product(s) secreted by hydra-associated bacteria is involved in modulating the spontaneous contraction frequency. This is in accordance with previous views (Kass-Simon et al., 2003; Ruggieri et al., 2004) that molecules such as aminoacids may influence the pacemaker activity in hydra.

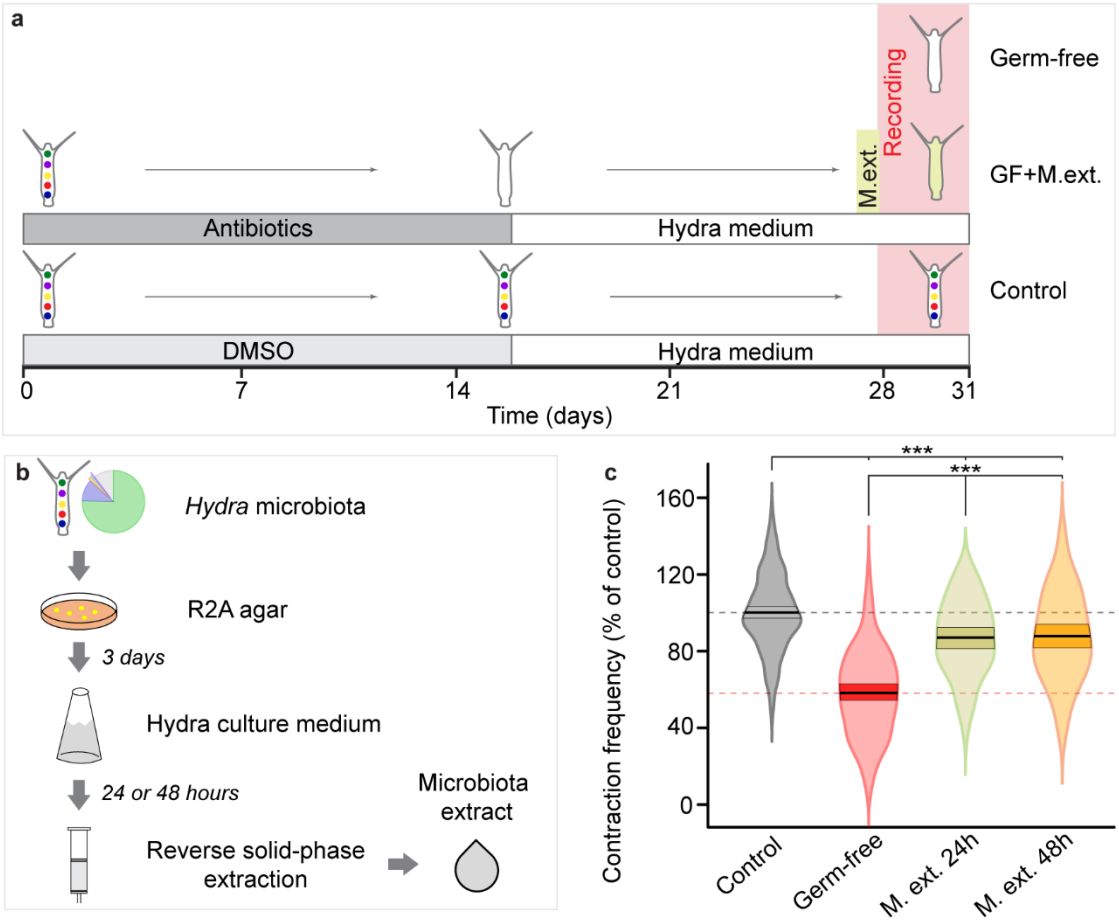


Figure 14. Microbiota supernatant extract improves contraction frequency in GF animals. (**a**) Experimental design: GF animals were incubated overnight in 24 or 48 microbiota extracts (M.ext.) before recording. GF and control animals were incubated in hydra culture medium. Animals were not fed during the course of the experiment. (**b**) Workflow used to obtain the microbiota supernatant extracts. Tissue homogenates from control polyps were plated on R2A agar and grown for three days. The resulting colonies were rinsed from the plate into sterile hydra culture medium and kept in agitation for 24 or 48 hours. The filtered supernatant was purified and concentrated using reverse solid phase extraction. (**c**) Contraction frequency of GF polyps incubated in both microbiota extracts is higher than that of GF animals incubated in hydra medium alone (M.ext.24h 86.6±3.4%, batch = 5, $n = 49$; M.ext.48h 87.4±3.4%, batch = 6, $n = 54$; GF 58.6±2.6%, batch = 12, $n = 110$; anova $F_{(3,351)} = 73.56$, ***$P \leq 0.001$). Control: batch = 12, $n = 153$. Batch= number of batches produced to obtain the reported sample size n.

3.7 Preliminary search for the active bacterial molecule

Among small, water-soluble and polar molecules produced by bacteria, short-chain fatty acids (SCFA) are well known for their role in microbiota-host communication as signalling molecules in vertebrates. They are produced naturally as products of dietary fibre

fermentation by anaerobic gut bacteria in vertebrates (Macfarlane and MacFarlane, 2003), and their effects may reach the host nervous system (Kimura et al., 2011; Selkrig et al., 2014).

To test the hypothesis whether SCFAs may be involved in the modulation of hydra spontaneous contraction, we tested the ability of hydra symbiotic bacteria to ferment simple carbohydrates, since fermentation of different carbon sources is the only known mechanism so far, for the production of SCFAs (Louis and Flint, 2017). For that we used the Oxidation/ Fermentation (OF) test. This test determines whether an organisms is able to metabolize carbohydrates by oxidation or by fermentation. In other words, whether they produce acid from breaking down carbohydrates only in aerobic (oxidation) or in both aerobic and anaerobic conditions (fermentation). Briefly, bacteria are inoculated in OF medium, which is supplemented with different sugars. Two tubes are prepared per sugar: one for aerobic and another for anaerobic growth conditions. Due to a pH indicator added to the medium, pH reduction by the production of acid will turn the originally blue medium into yellow, while a pH rise will turn it green/blue. Upon bacterial growth, if both tubes change to yellow, it means the bacterium is fermentative. If only the aerobic tube turns yellow, it is an oxidative bacterium. When the colour turns to green/blue, it means that the bacterium in nonsaccharolitic but instead is metabolizing aminoacids, which leads to amine production and the alkalinisation of the medium. *Escherichia coli* growing on glucose was used as positive control (C+) for fermentation.

After three weeks of growth, the results suggest that *Curvibacter* would mainly use aminoacids as source of energy, as most of the tubes turned green/blue. *Curvibacter* is also able to oxidase sucrose (Suc) and mannose (Mann), since only the aerobic tubes (*i.e.* O_2 +) turned yellow (Fig. 15, black arrows). There was no evidence for fermentative abilities in *Curvibacter* (no anaerobic tube changed to yellow, Fig. 15), the main colonizer of *H. vulgaris* AEP (Fig. 1e). *Duganella*, *Undibacterium* and *Pelomonas* (not shown), the second, third and fifth most abundant bacteria on hydra (Fig. 1e), showed no capacity for fermentation either. The results suggest that *Duganella* may be able to oxidase sucrose, mannose and galactose. *Pelomonas* and *Undibacterium* may have weak oxidative potential as the colour change was slight. Therefore it might be necessary to extend the essay period for another week or two, to assure that the uncomplete colour change was not due to insufficient bacterial growth. The only bacterium that showed evidence of fermentation of glucose (Glu) and mannose (Mann) was *Acidovorax*, since both aerobic and anaerobic tubes in these sugars changed to yellow (Fig. 15, red arrows).

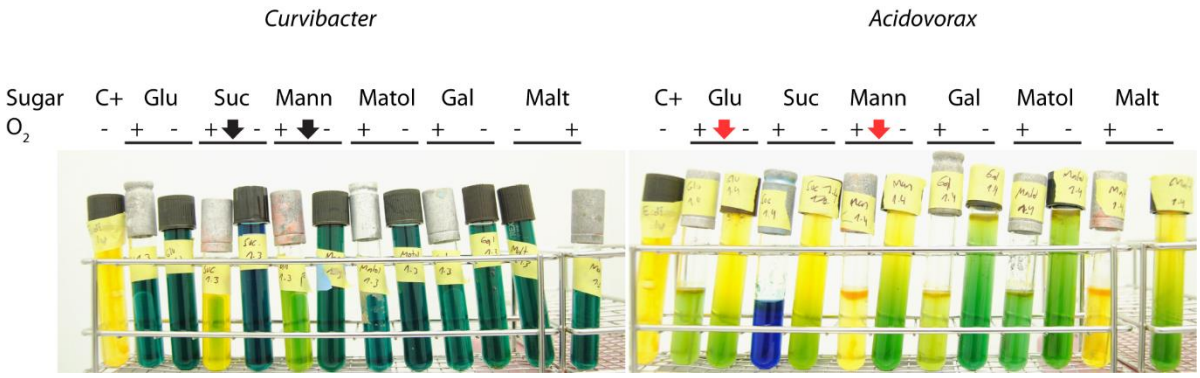


Figure 15. Most of hydra main colonizers are not able to ferment simple sugars. The results of the Oxidation/Fermentation test showed that the main colonizer *Curvibacter* mainly metabolizes aminoacids resulting in pH increase (green/blue medium). If it uses sugars, it oxidases them (black arrows). *Acidovorax* was the only one from the five main colonizers that showed evidence of fermentative capabilities, being able to produce acid in aerobic and anaerobic conditions (red arrows). Sugars: glucose (Glu), sucrose (Suc), mannose (Mann), mannitol (Matol), galactose (Gal), maltose (Malt). Aerobic (O_2 +) and anaerobic (O_2 -): conditions. Positive control (C+).

Since the OF evaluates in general the ability to ferment but not the type of fermentation, in addition, we searched for the presence of enzymes necessary for butyrate catabolism in the genomes of *H. vulgaris* AEP five main colonizers. Butyrate is one of the three major SCFA produced by gut bacteria, together with acetate and propionate. Among others beneficial effects of butyrate, it was shown to be involved in the maintenance of the blood brain barrier in vertebrates (Braniste et al., 2014). There are only few pathways known so far that may lead to butyrate synthesis, however all of them converged in pyruvate or in crotonyl-CoA as the main substrates to finalize the synthesis (Fig. 16) (Louis and Flint, 2017; Vital et al., 2014). The final step in the production of butyrate is the conversion of butyryl-CoA into butyrate by the action of either two enzymes: the butyryl-CoA:acetate CoA transferase (But) and the butyrate kinase (Buk) (Fig. 16), therefore But and Buk are usually used as biomarkers for the identification of butyrate-producing bacteria (Vital et al., 2014). Besides But and Buk, the 4-hydroxybutyrate transferase (4Hbt) may also produce butyrate from 4-hydrxybutyrate and butyryl-CoA. Thus we looked for the presence of But, Buk and 4Hbt in the genomes of hydra five main colonizers, as markers for butyrate production. We found no evidence for the presence of But and Buk in the genomes of any of the genomes assessed (Table 1). On the contrary 4Hbt may be present in *Curvibacter*, *Pelomonas* and *Undibacterium*. Care must be taken however, as CoA transferases are enzymes with broad substrate spectrum that exhibit high levels of sequence similarity (Vital et al., 2014). Therefore functional analyses at the

protein level would be necessary to confirm the presence of 4Hbt in the mentioned hydra bacteria.

In summary, these preliminary results suggest that in general hydra bacteria are not able to ferment. According to the tests, fermentation may be carried out by *Acidovorax*, however this pathway may be undertaken only if oxygen concentrations are very low, which are not the normal conditions were hydra and its bacteria live. In addition, the genome search showed weak to no support for the presence of terminal enzymes of butyrate synthesis in *H. vulgaris* AEP five main colonizers. Therefore it seems unlikely that SCFA are produced by hydra bacteria and in turn modulate the contraction frequency.

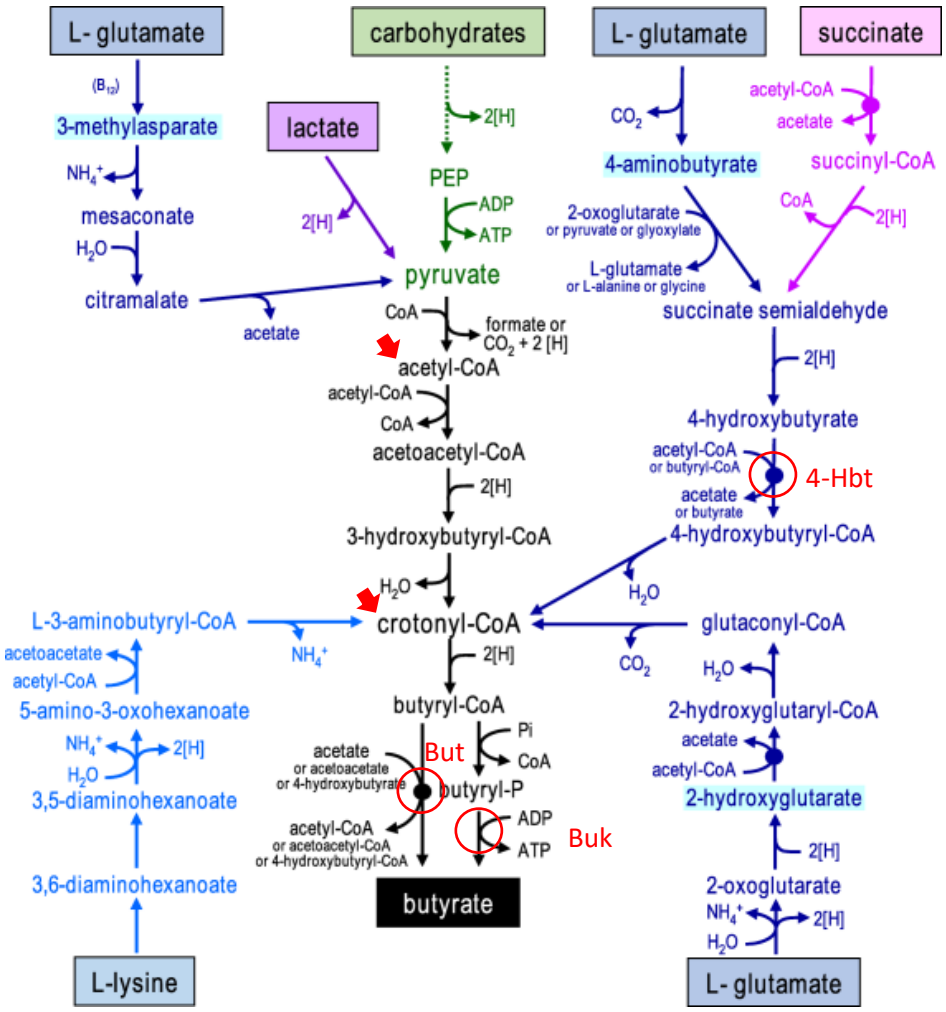


Figure 16. Known pathways leading to the production of butyrate. Carbohydrate fermentation (*i.e.* pyruvate pathway), aminoacid fermentation (e.g. glutamate and lysine) and organic acid fermentation (e.g. lactate and succinate), all pathways converge in the formation of acetyl-CoA or crotonyl-CoA (red arrows). The terminal enzymes But, Buk and 4-Hbt are highlighted in red. Taken and modified from *Louis and Flint (2017) Formation of propionate and butyrate by the human colonic microbiota.*

Table 1. Terminal enzymes for butyrate synthesis present in hydra symbiotic bacteria genomes.

		Enzyme		
		Buk	**But**	**4Hbt**
	Pathway	Acetyl-CoA	Acetyl-CoA	Succinate / 4-aminobutyrate
	Source	*Clostridium acetobutylicum*	*Roseburia intestinalis*	*Clostridium kluyveri*
Hydra symbiotic bacteria	*Curvibacter sp.*	-	-	+
	Duganella sp.	-	-	-
	Undibacterium sp.	-	-	+
	Acidovorax sp.	-	-	-
	Pelomonas sp.	-	-	+

An enzyme was considered present (+), if after blasting the source sequence against a bacterium genome a related predicted protein was obtained and if after blasting back this predicted protein the same enzyme was retrieved, even if it was not from the source organisms. Otherwise the enzyme was considered absent (-). But, butyryl-CoA:acetate CoA transferase; Buk, butyrate kinase; 4Hbt, butyryl-CoA:4-hydroxybutyrate CoA transferase.

4. Discussion

The nervous system plays three major roles in animals: control of behaviour, physiology and development (Jekely et al., 2015). These implies sensing inputs from the environment as well as coordinating internal activities of the organism. Microorganisms are not only part of the environment but also may be in tight association with the host, therefore microbial inputs are also to be sensed and coordinated by the nervous system. The aim of this work was to assess whether the communication between symbiotic microbiota and the nervous system, is an ancient evolutionary trait. We used the early-branching metazoan *Hydra* as the model to study this interaction. In particular, we evaluated whether hydra microbiota would affect the polyp feeding response as well as the spontaneous contractions of the body column, both activities that are under the control of *Hydra*'s nerve net.

We found that associated microbiota indeed affects neuronal activity in *Hydra*, since in the absence of the bacterial community the feeding response duration and the frequency of the spontaneous contractions, decreased. This suggests that the ability of associated microbiota

to modulate neuronal activity controlling behaviour and physiology of the host, appeared early in animal evolution, before appearance of an anatomically defined gut and a centralized nervous system. Later in the evolution of bilaterians, this interaction developed into what in vertebrates is known as the microbiota-gut-brain axis.

4.1 *Hydra* microbiota and the feeding response

The feeding behaviour, as it is the way to obtain energy, is critical for the survival of heterotroph organisms. It is well known that the gut microbiota is essential for energy metabolism as it provides nutrients that otherwise will not be accessible to the host (Flint et al., 2012). However, another equally important role of the gut microbiota is emerging: its role in host appetite control (Fetissov, 2016). This implies that the gut microbiome could modulate host feeding-related activities such as food preferences or over-eating. In fact, the role of the gut microbiome in the feeding behaviour of the host is increasingly recognized, since particular microbiota compositions are associated with obesity and metabolic diseases (Joyce et al., 2014; Turnbaugh et al., 2009; van de Wouw et al., 2017). Emerging data show that in mammals, the gut microbiota is able to stimulate hunger/satiation pathways of the host via bacterial metabolites, such as short-chain fatty acids (De Vadder et al., 2014), and small peptides with high similarity to appetite control hormones such as leptin, ghrelin, peptide YY, neuropeptide Y (Fetissov et al., 2008). In addition, the gut microbiota could also alter the host food taste sensing, generating craving for food types that would support the growth of certain microorganism (Duca et al., 2012; Swartz et al., 2012).

Modulation of feeding behaviour by the gut microbiota is also seen in invertebrates. In *Drosophila* for instance, the presence of *Acetobacter* and *Lactobacilli*, both regular members of the fly gut microbiota, is responsible for supressing protein appetite, while in the absence of microbiota, flies show an increased appetite for both protein and sugar (Leitão-Gonçalves et al., 2017). Feeding behaviour is also affected by microbial cues coming from the food. As diet helps shaping the gut microbiota, choosing what to eat is relevant to keep the “right” microbiota composition, leaving out pathogens. In *Drosophila*, bacterial metabolites in the food induce behavioural changes that lead to the choice of food where gut microbiota members are present (Fischer et al., 2017). Interestingly, these behavioural changes towards choosing food with the “right” microbes is mediated by gut microbiota in *Drosophila* (Wong et al., 2017). Similarly in *C. elegans*, recognition of pathogenic bacteria secondary metabolites by amphid neurons triggers avoidance behaviour which reduces the ingestion of pathogens (Meisel et al., 2014). In sum, modulation of feeding behaviour by environmental and symbiotic

microbiota is common in vertebrates and invertebrates, and is fundamental for the physiology of both host and its microbiota.

Here we show that symbiotic microbiota also modulates feeding behaviour in *Hydra.* We found that hydra's feeding behaviour may also be sensitive to bacterial cues, since the absence of its commensal bacteria affects the duration of the feeding response. An interesting question would be what signalling pathways and molecular mechanisms are involved in this interaction. Our experimental setup did not allow us dissecting these aspects in details. However, previous studies on hydra's feeding response showed that some aminoacids and fatty acids affect its duration (Koizumi et al., 1983; Pierobon et al., 1997). Therefore they might be targets for the observed microbial modulation. On the other hand, reduced glutathione (GSH) is a known powerful elicitor of the feeding response in *Hydra*. During the normal feeding, GSH leaks out from the wounded prey that has been pierced by *Hydra*'s nematocysts, activating the feeding response (Loomis, 1955). Furthermore, GSH stimulates production of nitric oxide (NO), via endogenous activity of NO synthase (NOS), and as a consequence increases cGMP production (Colasanti et al., 1997). It has been shown that inhibition of NOS activity delays mouth closure (*i.e.* increases response duration), while exogenous cGMP shortens the duration of the response (Colasanti et al., 1995). This indicates that NO and cGMP promote the mouth closing process in *Hydra*. Here we found that in the absence of bacteria, the response duration is shorter (*i.e.* the mouth closes faster). Therefore we can speculate that bacteria may modulate the duration of the response by decreasing the NO concentration, thereby keeping the mouth open for longer time. This is plausible given that exogenous NO, in this case NO produced by *Hydra*, is known to be toxic for microorganisms (Bogdan, 2001; Bogdan, 2015). Bacteria might keep NO levels low by altering *Hydra* NOS activity, for instance through depletion of host arginine, the substrate for NOS to produce NO. Interestingly, arginine is the only aminoacid reported to elicit mouth opening (Koizumi et al., 1983), therefore its depletion could be indeed associated with a shorter duration of the response. Bacteria could also reduce NO levels by directly scavenging NO. Since free NO would easily react with molecular oxygen or superoxide (O_2^-) to produce dinitrotrioxide (N_2O_3) and peroxynitrite ($ONOO^-$) respectively, both cytotoxic, bacteria would deplete NO before it reacts with these free radicals, for example by oxidizing it to non-toxic, less-reactive nitrate (NO_3^-) and nitrite (NO_2^-). The hypothetical scenario how bacteria would modulate the feeding behaviour by interfering with NO/cGMP pathway is presented in Figure 17. An important question is what parts of this scenario would take place in neurons. While the feeding response is neuron-dependent (Campbell, 1976b), the location of GSH and cGMP receptors and NO production remains unknown.

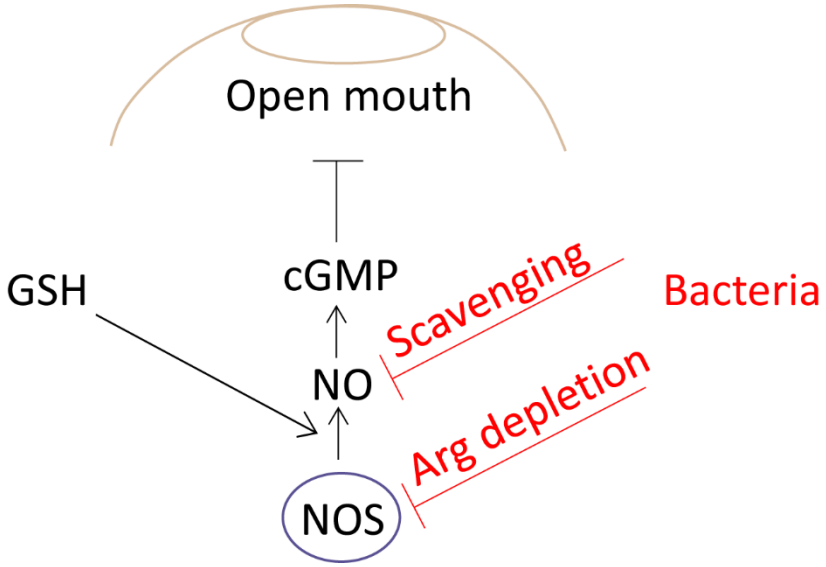


Figure 17. Hypothetical scenario for bacterial modulation of the feeding behaviour. NO/cGMP pathway is involved in the mouth closure process during *Hydra* feeding response. When NO levels are reduced, the time the mouth stays open (*i.e.* the response duration) is extended. As in the absence of bacteria the response duration is shortened (*i.e.* mouth closes faster), we hypothesise that under normal conditions, symbiotic bacteria would keep NO level low, either by interfering with hydra NOS activity (e.g. Arg depletion) or by scavenging released NO (*e.g.* driving the oxidation to NO_2^-), thereby modulating the response duration.

To address causal connection between bacteria and duration of the response, it would be first necessary to assess whether conventionalizing GF polyps with native *Hydra* symbiotic bacteria would restore the response duration. An approach similar to the one used to address the role of bacteria in spontaneous contractions (Fig 13), would be necessary.

To further test whether hydra bacteria modulate the feeding response by interfering with NOS activity, comparing NOS activity and NO production in wild-type, GF and conventionalized polyps would be necessary. To tests for bacterial NO scavenging capabilities, screening genomes of symbiotic bacteria for genes coding for NO sensors and scavenging enzymes should be performed. If any of these molecules are present, it would be interesting to produce bacteria deficient in these gene products or to specifically inhibit them, and then to assess the duration of the feeding response in either GF polyps recolonized with enzyme-deficient bacteria or in polyps treated with the specific inhibitors.

In summary, elucidating the mechanisms underlying bacterial modulation of the feeding response, would be informative to understand the evolution of this interaction and establishment of the microbiota-gut-brain-axis.

4.2 *Hydra* microbiota and the spontaneous contractions

To evaluate the influence of the associated microbiota on *Hydra* neuronal activity, we analysed a second action displayed by *Hydra* polyps, which is also under neuronal control: the spontaneous contractions of body column. These contractions are the outcome of endogenous rhythmic electrical oscillations, also known as pacemaker activity (Passano and McCullough, 1964) and therefore, the spontaneous contractions do not require sensory input to be triggered. Being autonomous, they however respond to endogenous inputs, such as starvation or internal hydrostatic pressure, that modulate the contraction frequency (Benos and Prusch, 1973; Passano and McCullough, 1964; Rushforth et al., 1964). In addition, environmental inputs (*e.g.* light and medium osmolarity) can also affect the frequency of the contractions (Benos and Prusch, 1973; Rushforth et al., 1964). Therefore, the spontaneous contractions are the outcome of internally-coordinated processes in contrast to the feeding behaviour which is a response to sensory inputs (Jekely et al., 2015).

All cnidarians display certain contractile activity, however, the physiological role of the contractions is not completely understood. In corals, jellyfish and colonial hydrozoans, this type of motility is involved in multiple functions including feeding, morphogenesis, regeneration, respiration and propulsion (Abrams et al., 2015; Beloussov and Dorfam, 1974; Jantzen et al., 2010; Kremien et al., 2013; Satterlie, 2002). In hydra, the contractions are suggested to be involved in regulating the hydrostatic pressure of the polyp. As water passively diffuses through hydra epithelium following an osmotic gradient, but does not go out in the same way, it accumulates in the gastric cavity, increasing the pressure there. If the fluid is not removed, the polyp would burst. The spontaneous contraction of the body column would be the mean to periodically expel the accumulated fluid from the gastric cavity (Benos and Prusch, 1973; Macklin et al., 1973). The contractions seem also to be involved in budding and body patterning during polyp regeneration (Wanek et al., 1980).

Here we analysed the interaction between symbiotic microbiota and the spontaneous contractions in *Hydra*. We found that the bacterial microbiome directly affects the spontaneous body contraction pattern of the polyps, since the contraction frequency is reduced in GF polyps (Fig. 10b). Moreover, we found that most monocolonization experiments did not improve the contraction frequency in contrast to conventionalized animals (Fig. 13b). In fact, when GF polyps were recolonized with natural hydra microbiota (*i.e.* conventionalization), the contraction frequency significantly increased compared to GF animals, although it did not reach control levels. This suggests that the bacterial community

and interactions among its members, rather than single bacterial species, are essential for normal spontaneous contraction dynamics. Interestingly, the least abundant of the main members of *H. vulgaris* AEP microbiota, *Pelomonas* sp., showed a positive effect on the contraction frequency similar to conventionalized animals (Fig. 13b). This points to the important role of less abundant members of the community supporting these interactions. We note however, that the effects of monoassociations do not reflect the natural situation encountered by the host or the bacteria, and that hydra bacteria behave differently when growing *in vitro* and *in vivo* (Li et al., 2015). Therefore, the effect of *Pelomonas* has to be further investigated before more conclusions can be drawn.

Since the absence of microbiota affects the contraction frequency and contraction regularity (Fig. 10b, Fig. 12) but not the amplitude of the contractions (Fig. 11), we propose that bacteria influence the pacemaker activity that is responsible for timing of the contractions. Early behavioural and electrophysiological studies on hydra showed that pacemaker activity is located in the hypostome and foot region, and suggested that the pacemakers were neurons (Kass-Simon, 1972; Passano and McCullough, 1963; Passano and McCullough, 1964; Passano and McCullough, 1965). Recently this idea was confirmed by functional alteration of neurons in the foot (Takaku et al., 2014) as well as by calcium imaging of neuronal activity (Dupre et al., 2017). Using genetically engineered hydra, the later study identified separate non-overlapping neuronal networks, which are associated with specific behaviours, including the spontaneous contractions (Dupre et al., 2017). The molecular signature of the pacemaker neurons, including the receptors that would be responsive to the microbiota-derived signals remain to be characterized.

Hydra spontaneous contractile activity resembles one kind of peristaltic movement of the vertebrate gastrointestinal tract, the fasting intestinal motor activity, also called the migrating motor complex (MMC). It rises from endogenous electrical pacemaker activity and is abolished by food ingestion (Deloose et al., 2012). Interestingly, there is a strong correlation between the MMC and the microbial assemblage in the gut of humans and rodents. However, it is still unclear whether dysmotility is cause or consequence of an altered microbiota composition (Hadizadeh et al., 2017a; Vandeputte et al., 2016a; Vantrappen et al., 1977). Given the experimental accessibility of hydra, we were able to test the effect of the five main bacterial colonizers on hydra motility disruptions. We were able to evaluate the relative effect of less abundant bacteria as well as the role of potential bacterial interactions in the spontaneous contraction pattern. Our results provide evidence for the causal role of bacteria in modulating spontaneous contractile behaviour, which is the basis for gut motility and the according to

these results, the outcome of an ancient interaction between bacteria and early emerging animals.

4.3 Molecular mechanisms behind bacterial modulation of *hydra*'s neuronal activity

Further, we sought to get insights into the potential molecular mechanisms involved in the interaction between symbiotic microbiota and the spontaneous contractions in *Hydra*. We found that the in the absence of bacteria, the spontaneous body contraction pattern of the polyps is disturbed, and that products secreted by the bacteria are likely responsible for this effect. A cell-free supernatant extract from the natural microbiota culture greatly improved the contraction frequency (Fig. 14b). Before the extraction, the bacterial cells were removed from the supernatant by centrifugation and filtration, therefore the supernatant was assumed to contain only molecules secreted by bacteria. This indicates that the presence of bacteria *per se* is not necessary for but that a secreted product is sufficient to normalize the contraction frequency.

The extracts were prepared using bacteria cultures from tissue homogenates of control polyps (Fig. 14b), therefore they likely retained the natural microbiota composition and abundance, as well as the potential bacterial interactions normally occurring among undisturbed microbiota. Thus we assume that the supernatant extract fairly recapitulates the processes occurring *in vivo* on hydra. Interestingly, the presence of hydra is not necessary to induce the production of the candidate molecule.

So far we have not isolated and identified the active compound from the extract, however the way the extract was produced may provide a hint for the nature of the molecule. It is likely that a small, water-soluble, secreted bacterial product is involved in modulating hydra body contractions. As short-chain fatty acids (SCFAs) comply with these characteristics, we tested whether hydra bacteria are capable of fermentation, one of the prerequisite for SCFAs production. In vertebrates, SCFA are involved in regulating host metabolisms, gut immunity and are also able to affect nervous system functions (Koh et al., 2016). Our results showed that under relevant biological conditions, neither of the main colonizers is able to ferment (Fig. 15). Only *Acidovorax* may ferment if oxygen concentration is low, which is not the case in hydra's natural aerobic environment. Since the bacteria used to prepare the supernatant grew in aerobic conditions as well (Fig. 14a), it is unlikely that the extract contains products of fermentation. In addition, a search in the genomes of hydra main colonizers for terminal enzymes in the known butyrate synthesis pathways, one of major SCFA produced by gut

bacteria, showed no evidence for the presence of Buk and But enzymes, the classical biomarkers for butyrate-producing bacteria. Taken together, our preliminary results though point to a small molecule, showed weak support for SCFAs as the active compounds modulating the contraction frequency. This indicates that although the communication between host associated microbiota and the nervous system is a universal phenomenon, the molecular language might differ across evolutionary linages.

Taking together the outcome of the recolonization experiments and extracts treatment, we propose the following model of interaction between bacteria, neurons and contractions (Fig. 18). The contractions are driven by pacemaker cells, these cells are autonomous in generating electrical activity, however they receive inputs for the environment via sensory neurons that may change the pacemaker output. For instance light and GSH activate sensory neurons and inhibit pacemaker activity, thereby reducing contraction frequency. Hydra symbiotic bacteria produce small secreted molecule(s) that would support the pacemaker activity. Whether they directly affect the generation of periodic impulses or act indirectly by inhibiting sensory neurons, is not known.

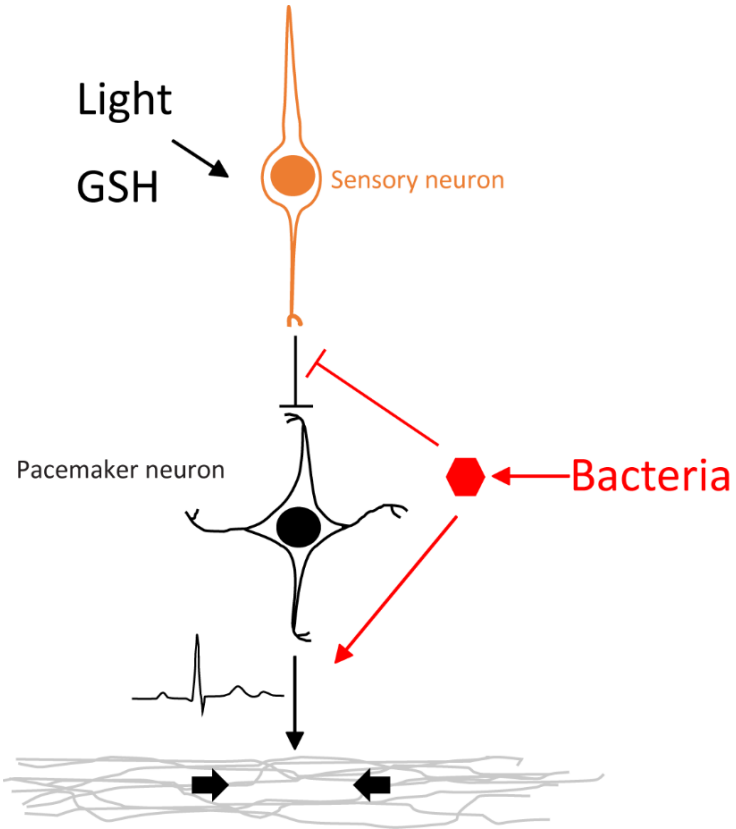


Figure 18. Hypothetical scenario for bacterial modulation of Hydra spontaneous contractions. For detailed explanation see text.

The approach employed here to track hydra behaviour was useful for finding out that indeed there are differences between GF and conventional animals. Yet, the hunt for the active compound involved in the behavioural modulation, would require methods that allow to scale-up the behavioural tracking. Here we used the feeding response and the spontaneous contractions as proxies for hydra neuronal activity, however an alternative to achieve high throughput screening is to measure directly the in neuronal activity. Calcium imaging (Dupre et al., 2017) and high throughput electrophysiology (Gonzales et al., 2017) are now available for cnidarians, and have been successfully applied to hydra in the context of behavioural tracking.

In summary, this work assessed the microbiota-nervous system communication from an evolutionary perspective. Since cnidarians are among the first animals where the nervous system emerged (Bosch et al., 2017; Kelava et al., 2015), the ability of the microbiota to modulate the host neuronal activity, which in hydra is reflected in the modulation of behaviour and internal coordination, appears to be an evolutionary ancient feature. It also provides evidence for the bidirectionality of the interaction between microbiota and neurons. A previous study has shown that *Hydra* neurons secrete peptides that can shape the microbiota composition (Augustin et al., 2017). Here we show that *Hydra* microbiota in turn, modulates host spontaneous contraction dynamics. In agreement with this, studies in mice have demonstrated excitatory effects of the gut microbiota on the enteric nervous system (Kunze et al., 2009; Mao et al., 2013; McVey Neufeld et al., 2013; Wang et al., 2010) while in zebrafish it has been shown that the enteric nervous system, acting through its control over gut motility, may influence the composition of the gut microbiota (Rolig et al., 2017). Our observations highlight the value of cnidarians as model organisms to study complex questions, such as nervous system-microbiota interactions, in a simple background. Their relative simplicity contrasts the variety of nervous systems and behavioural repertoires (Bosch et al., 2017). This, together with the increasing availability of imaging and gene manipulation techniques as well as genomic and transcriptomic tools, makes cnidarians attractive models to further study the interactions among the kingdoms of life.

5. Conclusions

Based on our observations, we are able to draw the following conclusions:

1. Hydra neuronal activity is modulated by its symbiotic bacteria. The feeding response and the spontaneous contractions, both outputs of neuronal activity, are disturbed in germ-free animals: in the absence of associated bacteria, the duration of the response and the contraction frequency are reduced. Therefore the ability of the microbiota to modulate the neuronal activity of the host, appears to be an evolutionary ancient feature.

2. In the absence of associated bacteria, the contraction frequency and contraction regularity are affected, but not the stretching and contractile ability of the animals. Therefore associated bacteria may modulate the contraction frequency by supporting regular pacemaker activity.

3. The bacterial community composition plays an important role in modulating the frequency of spontaneous contractions. This is because 1) the relative abundances of the main colonizers, 2) the presence of low-abundance bacterial strains and 3) the potential interactions among the members of the community, appeared to contribute to normal contraction dynamics.

4. A cell-free supernatant extract from the natural microbiota culture improved the contraction frequency of germ-free animals. This strongly suggests that a small, water-soluble, secreted bacterial product is involved in modulating hydra body contractions.

5. The methods we established here enable us to discover the phenomenon of bacteria-neuron interaction and to approach the possible mechanisms behind. However, to further understand the molecular basis of this cross-talk, methods would be necessary for high throughput screening of candidate molecules.

6. Materials

6.1 Chemicals

Antibiotics

Ampicillin	Sigma-Aldrich GmbH
Neomycin	Roth
Rifampicin	Sigma-Aldrich GmbH
Spectinomycin	AppliChem

Streptomycin	Roth
Sugars	
Galactose	Roth
Glucose	Roth
Maltose	Roth
Mannose	Roth
Manitol	Roth
Sucrose	Roth
General chemicals	
Agar	Roth
Agarose	Roth
Bromothymol blue	Sigma-Aldrich GmbH
Dipotassium Phosphate	Roth
Ethanol	Roth
Methanol	Roth
PeqGREEN	Peqlab
Peptone from casein/Tryptone	Roth
Reduced gluthatione (GSH)	Roth
Sodium Chloride	Roth
Solutions and Buffers	
TAE buffer (50 x)	2 M Tris acetate, 1 mM EDTA, pH 8
DNA loading dye	10 mM EDTA pH 8.0, 0.1% SDS, 0.025%, bromophenol blue, 0.025% xylene cyanol
Enzymes, DNA ladders, primers	
Polymerase-chain reaction (PCR)	GoTaq® Reaction Buffer (1x), dNTP-Mix (0,2mM), up- and downstream primers (1 µM each), GoTaq® DNA polymerase (0,25 U), template DNA (~100 ng), Millipore H_2O. Promega
GeneRuler™ DNA Ladder Mix (100 10.000 bp)	Thermo Scientific
Eubacteria (5'-3')	Eub_27F AGRGTTTGATCMTGGCTCAG T_m=57,3°C Eub_1492R GGHTACCTTGTTACGACTT T_m=53,1°C

6.2 Media

Hydra medium	0.29 mM $CaCl_2$ x 2 H_2O, 0.33 mM $MgSO_4$ x 7 H_2O, 0.5 mM $NaHCO_3$, 0.08 mM K_2CO_3
Sterile hydra medium (100-fold diluted solution was autoclaved. Filtered-sterilized 1000-fold diluted solution 2 was added.)	Stock solution 1 (1000x): 42.18 g CaCl2 x 2 H2O / 1L Millipore H_2O Stock solution 2 (100x): 8.116 g $MgSO_4$ x 7 H_2O, 4.238 g $NaHCO_3$, 1.0958 g K_2CO_3 / 1L Millipore H_2O
Artificial sea water	31.8g sea salt/1L Millipore H_2O
Bleaching solution	1:1 5M NaOH and NaClO 12%
R2A liquid medium	3 g R2A Broth / 1 l Millipore H_2O
R2A solid medium	18 g R2A-Agar / 1 l Millipore H_2O
Oxidation/Fermentation medium	NaCl (5g/L), Pancreatic Digest of Casein (2g/L), K_2HPO_4 (0.3g/L), agar (2g/L), bromothymol blue (0.08/L), 1% filter- sterilized sugar solution.

6.3 Devices

Centrifuges	
Centrifuge 5417 R (refrigerated centrifuge)	Eppendorf
Mini Spin	Eppendorf
Centrifuge 5424	Eppendorf
Centrifuge 5415 D	Eppendorf
Gel Electrophoresis	
Electrophoresis Power Supply Consort EV 231	peqLab
SE250 PAGE gel chamber	Hoefer Scientific Instr.
Incubators/shakers	
Thermo-Inkubator	Heraeus Instruments
REAx 2000 (vortex)	Heidolph
VF2 (vortex)	Jankel and Kunkel
PCR thermal cyclers	
MWG-Biotech	MWG-Biotech
Primus 96 *plus*	MWG-Biotech
Primus 96 *advanced*	peqLab
Miscelaneous	
1205 MP balance	Sartorius
BioPhotometer	Eppendorf
C-mount 5-megapixel digital cameras	Breukhoven Microscopes Sytems
Freezer -20°C	Liebherr
Freezer -80°C	Forma Scientific
HI9321 (pH-Meter)	Hanna Instruments
Ice machine Scotsman AF 10	Mathner Kalte-Klima
Julabo 5B (water bath)	Eydam
LaminAir HB 2448 (clean bench)	Heraeus Instruments
M3C binoculars	Wild Heerbrugg
Mili-Q Academic System	Milipore
Molecular Imager® Gel Doc™ XR+	Bio-Rad
Refrigerator	Liebherr
Rotavapor R-114 (rotary evaporator)	Büchi
Vakuum-Controller CVC 24	Vacuubrand

6.4 Software

Image editing and processing	Image J
	Adobe Photoshop CS4
Illustration	Adobe Illustrator CS4
	Microsoft Powerpoint 2010
Microscopy	Chronolapse
	BMS_pix2 3.6
	CELL*
Statistics	R

6.5 Others

Solid phase extraction columns	Supelco Superclean^{TM} LC-18 (60ml, 10g)

7. Methods

7.1 Cultivation of Organisms

7.1.1 *Hydra* polyps

Hydra vulgaris strain AEP was used in all experiments presented in this work. The animals were maintained according to standard procedures (Lenhoff and Brown, 1970) and fed three times per week on newly hatched *Artemia salina* nauplii. Only polyps without buds or gonads were used in the experiments. The animals were fed for the last time one day before starting the antibiotic treatment (see below). All animals used in the experiments were clones coming from the same culture dish.

7.1.2 Conventional *Artemia salina* nauplii

Hydra polyps were fed newly hatched *Artemia salina* nauplii. *A. salina cysts* were incubated for 24 hours in artermia medium at 30°C with constant aeration. After hatching, larvae were kept at 18°C. For feeding, nauplii were collected and rinsed with hydra culture medium.

7.1.3 Germ-free *Artemia salina* nauplii

Artemia salina cysts (~0,2g) were rehydrate in 4ml artificial sea water for 1 hour at room temperature (RT). Afterwards, 4ml of bleaching solution was added to the hydrated cysts and kept in agitation for 10 min, or until the cysts start turning to yellow colour. The bleached cysts were rinsed using a sterile 125µm sieve with abundant autoclaved water. Bleaching solution should be completely removed. The washed cysts were transferred into a sterile 500ml-flask with vented cap in ~50-100ml filtered artificial sea water. The cysts were kept in slow agitation at RT, protected from light. At 20-25°C it takes around 24h for the cysts to hatch. Around 18°C, hatchlings will appear after 1 day. To check the germ-free state of the nauplii obtained after cyst bleaching, 200-500 µl hatchlings were macerated and subsequently plated into R2A agar plates. Lack of bacterial growth after two days of incubation was taken as proof of germ-free state. Before feeding hydras with these nauplii, they were rinsed with

hydra culture medium. All GF hydras fed with naupilli produced in this way, were check for germ-free state after feeding and before using them in experiments.

7.1.4 Germ-free and recolonized *H. vulgaris* AEP

Germ-free (GF) polyps were obtained by treating control animals for two weeks with an antibiotic cocktail containing rifampicin, ampicillin, streptomycin and neomycin in final concentrations of 50µg/ml each and spectinomycin at 60µg/ml, as previously described (Franzenburg et al., 2013). Since rifampicin stock is dissolved in DMSO, control polyps were incubated in the corresponding final DMSO concentration (0.1%) for the same period of time. Antibiotic solution and control polyp medium was replaced every 48 hours. After 17 days in the antibiotic cocktail, the animals were transferred to sterile hydra medium that was further replaced every 48 hours until the behavioural tests were performed (days 29-31). The GF status of the polyps was tested twice per week, starting at day 19-20, by plating an antibiotic-treated macerated polyp from every batch produced on R2A agar, which supports growth of hydra microbiota (Franzenburg et al., 2013). Absence of colonies following three days of incubation at 18°C showed the GF status. GF polyp macerates were further analysed by 16S rDNA amplification, using universal 16S rRNA primers Eub-27F and Eub-1492R (Weisburg et al., 1991). PCR conditions were: 3' 95°C, 40x (30'' 95°C, 30'' 53°C, 1'30'' 72°C), 5' 72°C. Absence of amplification product confirmed the GF status.

GF animals were monocolonized by incubating the polyps with a pure culture of each of the main colonizers *Curvibacter* AEP 1.3*, Duganella* C 1.2*, Undibacterium* C 1.1*, Acidovorax* AEP 1.4 and *Pelomonas* AEP 2.2. These bacteria were cultured from existing isolate stocks (Fraune et al., 2015) in R2A medium at 18°C for three days. Approximately $5x10^5$ cells (based on OD_{600} quantification as previously described (Fraune et al., 2015)) of each bacterial strain were added separately to 50ml sterile hydra medium containing 20-30 GF polyps on day 20. Likewise, GF polyps were recolonized with a mixture of the five main colonizers in equal proportions ($5x10^5$ cells /strain). After three days of incubation, the polyps were washed with and transferred to sterile hydra medium. Conventionalized animals were obtained by incubating GF polyps with tissue homogenates of control animals (one control polyp per one GF polyp) in 50ml sterile hydra medium. The conventionalized polyps were washed and transferred to sterile hydra medium 24 hr after colonization. This medium was replaced every 48 hr until the behavioural tests were performed (days 29-31). Recolonizations were verified by plating tissue homogenates on R2A agar and by 16S rDNA sequencing as described above.

7.1.5 Cultivation of *H. vulgaris* AEP symbiotic bacteria for oxidation/fermentation assay

H. vulgaris AEP main bacteria colonizers *Curvibacter* AEP 1.3*, Duganella* C 1.2*, Undibacterium* C 1.1*, Acidovorax* AEP 1.4 and *Pelomonas* AEP 2.2 from existing isolate stocks (Fraune et al., 2015), were cultured in R2A broth for three days at 18°C. 1:1000 dilutions were performed and 1.5ml were plated into R2A agar. After three days of growth at 18°C, bacteria were inoculated by stabbing the medium, 0.5 cm from the bottom of the tube. The medium was supplemented with 1% of either of the following sugars: glucose, sucrose, mannose, mannitol, galactose and maltose. Two tubes per sugar were inoculated per bacteria: one was kept with constant oxygen supply (aerobic conditions) while the other was filed up completely with medium and sealed to reduce oxygen availability (anaerobic conditions). After three weeks of growth at 18°C, bacterial growth was verified and medium colour changes were resisted.

7.2 Production of microbiota supernatant extract

Tissue homogenates from three control polyps, previously rinsed with sterile hydra medium, were plated on R2A agar and grown at 18°C for three days (Fig. 14b). The resulting colonies were rinsed from the agar into 500ml sterile hydra medium and kept agitated at 18°C for either 24 hours or 48 hours. The suspensions were centrifuged (1400g, 10min) and the supernatant collected, filtered (0.2 µm pores) and stored at -80°C. The supernatants were loaded onto solid phase extraction (SPE) columns (Supelco Superclean^{TM} LC-18, 60ml, 10g) previously activated and washed with 100% methanol followed by 10% (vol/vol) methanol, at a flow rate of 3ml/min. The columns were directly eluted with 100% methanol without a wash step. If necessary, the elution products were store at -80°C prior evaporation. The eluates were dried using a rotary evaporator and a vacuum pump (35-40°C, 50-100 mbar) and resuspended in 5 ml of hydra culture medium. This produced 100x-concentrated extracts that were subsequently filter-sterilized and stored at -80°C. GF polyps were incubated in 20x diluted microbiota extracts overnight (~16 hours) before behavioural tests were performed.

7.3 Behavioural tracking

7.3.1 *Hydra* feeding response

Wild-type (control) polyps were placed individually into wells from a 12-well plate, containing reduced glutathione (GSH) 1µM. Under a dissecting microscope the main events occurring during the normal feeding response as well as their timing, were registered for each polyp.

Since upon exposure to GSH the polyps increased their movements, the 12-well plate was placed onto a small mirror in order to facilitate the observation of the mouth. For the analysis of the feeding response in GF animals, the same setup was employed, and only time to mouth opening (t_o) and time to mouth closure (t_f) were registered in GF and control polyps. The duration of the response was calculated as $t_o - t_f$.

The sample size (n) reported is the total amount of animals used in each treatment (*e.g.* control, GF). Each animal was recorded only once, therefore each animal is considered an independent sample. Time registration was not blinded to treatment. Control and GF polyps were fed germ-free *Artemia* nauplii 3-5 days before the experiment (Fig. 7a). The experiments were carried out at 18-20°C.

7.3.2 *Hydra* Spontaneous contractions

Individual polyps were placed in 200-500µl hydra medium (Lenhoff and Brown, 1970) on microscope concave glass slides and recorded using C-mount 5-megapixel digital cameras (Breukhoven Microscopes Sytems) fitted onto M3C Wild Heerbrugg binocular microscopes. The animals were light-adapted overnight before recording and recorded in an insulated climate chamber at 18°C, to avoid any external stimuli that could induce other than spontaneous contractions. A time series was recorded for 90 min, taking one frame every 3 seconds, with dim non-localized white light. After trimming the first 30-min acclimation period, the remaining 60-minute time series were used to quantify the contractions. Using ImageJ (Schneider et al., 2012) to visualize the time lapses, contractions were manually identified and registered with the number of the frame where they occurred. As the natural variability in contraction frequency is high and it is only possible to treat and record a reduced amount of animals at once, several independent batches were produced in order to reach adequate sample sizes. The contraction frequency expressed as number of contractions per hour was converted into percentages of the mean values of control polyps in each batch. The sample size (n) reported is the total amount of animals used in each treatment (*e.g.* control, GF), unless otherwise is stated. Each animal was assigned to only one treatment and was recorded only once, therefore each animal is considered an independent sample. Contraction counting was blinded to treatment.

The time interval between two consecutive contractions Δt (Fig. 8a), was calculated in minutes. Variation in the polyps' shape was used to quantify the stretching (Smax) and contractile (ΔS) capacity of the animals. Smax was defined as the average of the maximum elongations achieved by the polyps between two contractions (MAX, Fig. 8a), and ΔS as the

average difference in the polyp's shape between a maximum elongated state and the next contraction (MAX-MIN, Fig. 8a).

We used the radius of gyration (RoG) to assess the shape of the polyps and thus to calculate Smax and ΔS in control and GF animals. The RoG describes the shape of an object by assessing how compact it is, therefore, the more stretched an object is, the larger its RoG will be. For each image in a given time series, after converting it to black and white, the edges (*i.e.* the silhouette) of the polyp and its geometric center (or center of mass, gd(x,y)) were determined. An image of a polyp has as many edges as black pixels adjacent to white pixels. Using these two variables, the RoG corresponding to a given image was calculated as the average distance from the edges of the polyp to the geometric centre. To account for size difference among the polyps, we used z-scores to standardize the RoG values corresponding to maximum elongations and full contractions (MAX and MIN respectively, Fig. 8a). The z-scores were calculated by subtracting the average and dividing by the standard deviation of all the RoG values in a given time lapse (*i.e*. for a given polyp). A z-score may be positive or negative, depending on whether the value is greater or smaller than the mean. A z-score equal to zero means that the value has the same value as the mean. The magnitude of the Z-score also tells how many standard deviations the score is above or below the mean. The images were processed in ImageJ and the RoG values calculated using custom-made functions in R (R Core Team, 2014).

7.4 Statistical analysis

7.4.1 Feeding response

The percentage of polyps undertaking a transition between two events was calculated using the TraMineR package in R (Gabadinho, et al., 2011). This package was designed to analyse state or event sequences that describe life courses in the field of social sciences. Differences in time to mouth opening and duration of the response were testes using Wilcoxon rank sum test. Normality in the distribution of the variables was tested graphically by means of quantile-quantile (Q-Q) plots. All analyses were done in R (R Core Team, 2014).

7.4.2 Spontaneous contractions

Differences in contraction frequency among the treatments (i.e. control, GF, recolonizations, GF incubated in the microbiota extract) were analysed using ANOVA, with "treatment" and "batch" as explanatory variables and "contraction frequency" (expressed as percentage of the

control) as response variable. If “batch” had no effect, this variable was removed from the analysis. In cases where the variance in contraction frequency due to the batch was greater than zero, linear mixed effects models (LME) were employed using “batch” as random effect. As the batches for the experiment were spread over more than six months, LME allows accounting for batch variability before calculating the variation in contraction frequency due to the treatment (*i.e.* the fixed effect). Differences in stretching (Smax) and contractile (ΔS) capacities were tested using unpaired *t*-test. Contraction interval distributions (Δt) were analysed using χ^2 test.

All analyses were done in R (R Core Team, 2014). LME analysis was performed using the lme4 package (Bates et al., 2015). The p-values reported for LME are taken from ANOVA type II tests on the final models. For post-hoc analysis we used the multicomp package (Hothorn et al., 2016), with false discovery rate (fdr) for p-values adjustments. Normality in the distribution of the variables was tested graphically by means of quantile-quantile (Q-Q) plots.

8. Contributions to the Thesis

The results obtained from the analysis of the spontaneous contraction behaviour in hydra reported in this thesis were published in the journal Scientific Reports with title "**Spontaneous body contractions are modulated by the microbiome of *Hydra***" doi:10.1038/s41598-017-16191-x

I designed the experiment, collected the data, performed the analyses, and wrote the first version of the manuscript. Alexander Klimovich contributed with writing the final version of the manuscript and finalizing the figures. Tim Lachnit assisted with the bacteria supernatant extraction. Jan Traubenheim contributed with the R script to analyse hydra polyp's shape. Benedickt Mortzfeld provided the microbiota abundance data pre- and post-recolonization. Eileen Pemöller contributed with the establishment of the method.

9. Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation nach den Regeln guter wissenschaftlicher Praxis eigenständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen benutzt habe. Dabei habe ich keine Hilfe, außer der wissenschaftlichen Beratung durch meinen Doktorvater Prof. Dr. Dr. h. c. Thomas C. G. Bosch in Anspruch genommen. Des Weiteren erkläre ich, dass ich noch keinen Promotionsversuch unternommen habe. Teil dieser Arbeit wurde veröffentlicht.

Kiel, den 29. Januar 2018

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Andrea Patricia Murillo Rincón

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2010-2012	Master of Science, Evolutionary Biology Erasmus Mundus Master (MEME) LMU, Germany/ Uni Groningen, The Netherlands
2014-2017	Doctoral studies IMPRS for Evolutionary Biology Zoological Institute, Christian Albrecht University Kiel, Germany

Stipends / Awards

2014-2016	Scholarship for doctoral studies International Max Planck Research School (IMPRS) for Evolutionary Biology, Germany
2010-2012	Scholarship for master studies Talent Grant Groningen University, the Netherlands
2006-2007	Research scholarship “Jorge Ignacio Hernandez Camacho” Conservación Internacional Colombia, Fondo para la Acción Ambiental, Unidad Administrativa Especial del Sistema de Parques Nacionales Naturales y Fundación Omacha.