Studies on organogenesis during regeneration in the earthworm, *Eudrilus eugeniae*, in support of symbiotic association with *Bacillus endophyticus*

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Abstract: Organogenesis and animal adaptation upon loss of body parts are crucial events in regeneration of all traits, and both are uncovered areas in biology. The organogenesis of the mouth and anus during regeneration was studied in *Eudrilus eugeniae*. It was found that the earthworm regenerates a functional mouth and anus in six days. During the course of organogenesis, the earthworm adopts unique starvation behavior for 5 days, and the starvation triggers the gut microflora of *E. eugeniae* to produce riboflavin. One of the efficient riboflavin-producing bacteria, *Bacillus endophyticus*, confirmed through 16S rRNA sequencing, has been isolated from its gut. The symbiotic association provides shelter and nutrition to the bacterium and the earthworm in return gets riboflavin to maintain homeostasis during the starvation period of early regeneration.

Key words: Organogenesis, *Eudrilus eugeniae*, riboflavin, starvation, *Bacillus endophyticus*

1. Introduction

Regeneration is an astonishing phenomenon entailing regrowth of a detached body part. It is a genetic characteristic that is more prevalent in certain animal groups, particularly the invertebrates (Bely and Nyberg, 2010). Embryonic development and regeneration are governed by similar principles of cellular development in animals. Organogenesis occurs invariably in embryonic stages of development, since the regeneration capability of certain cells engenders the process of organogenesis for regenerating the lost organs in the adult body. It has been reported that the neoblast cells of planarian flatworm and fragmenting oligochaete *Enchytraeus japonensis* help to regenerate the entire animal (Yoshida Noro and Tochinai, 2010; Wagner et al., 2011). A wide range of invertebrates such as the hydras (Hobmayer et al., 2000; Broun et al., 2005), planarians (Gurley et al., 2008), sea cucumbers (Quiñones et al., 2002; Sun et al., 2011), starfish (Kozlova et al., 2005), and earthworms possess the capacity to regenerate lost body parts. Regeneration of the anterior and posterior body parts was investigated in three earthworm species, namely *Perionyx excavatus* (Gates, 1927; Cho et al., 2009), *Eisenia fetida* (Gates, 1949), and *Eudrilus eugeniae* (Johnson Retnaraj Samuel et al., 2011; Kalidas et al., 2015). The earthworm is a segmented animal whose body is remarkably organized in three regions: preclitellar (1–12 segments), clitellar (13–18 segments), and postclitellar (19–anus) (Daisy et al., 2016). The anterior segmental region starts with a prostomium in the first segment, followed by a long digestive canal connected to several organs, and it ends with a dorsally located periproct. Any injury to either the anterior or the posterior segments of the earthworm leads to a functional loss of the mouth and/or the anus until these organs are restored, thereby disrupting proper ingestion of food and excretion of waste, which are essential biological processes for homeostasis. Interestingly, during regeneration the earthworm *E. eugeniae* would usher in starvation until the regeneration of the lost mouth and anus. However, the survival of the worm without intake or excretion and the sequential events of mouth and anus regeneration were not yet studied in invertebrates.

Nutritional deficiency crucially impairs pre- and postnatal development of animals. It has been reported that a nutritional deficit in pregnant rabbits leads to high abortion rates and weight loss in both the mother and the
fetus (Cappon et al., 2005). Maternal malnourishment alters the body weight and ovarian and adrenal development of water vole progeny (Yakovleva et al., 1997). In addition, micronutrients are also involved in embryonic development and regeneration (McArdle and Ashworth, 1999). Deprivation of vitamin A in animals influences embryonic development and regeneration (Maden et al., 1996, 1998) and the insufficiency of riboflavin retards embryonic growth and cardiac development in mice (Maden et al., 1998). In addition, it was reported that the regeneration process and blastema formation in the earthworm, *E. eugeniae*, is supported by riboflavin (Johnson Retnaraj Samuel et al., 2011). Food deprivation and invertebrate animal survival without essential organs remain unknown.

The present study was undertaken to elucidate the phenomenon of the regeneration of the mouth and anus in the earthworm, *E. eugeniae*, highlighting the role of the underlying factors hitherto unreported. Uniquely, the earthworm regenerated its functional mouth and anus by the 6th day and during the process of organogenesis the earthworm resorted to starvation behavior for the first 5 days. This adaptive behavior of starvation triggered the gut microflora to produce riboflavin, which is the key factor in inducing regeneration.

2. Materials and methods

2.1. Earthworm maintenance

The earthworm *E. eugeniae* was cultured as per the protocol of Johnson Retnaraj Samuel et al. (2011). *E. eugeniae* stock was reared in Department of Biotechnology, Manonmanium Sundaranar University. The earthworms were fed on leaf litter and cow dung maintained in a plastic container in wet conditions with a 12-h photoperiod. Matured *E. eugeniae* worms were identified and separated from the stock by the presence of a clitellum at segments 13–18.

2.2. Wound healing and regeneration analysis

In order to study the wound healing and regeneration processes, 30 worms were selected and equally divided into two groups. The first group of earthworms was amputated at the 10th anterior segment and the second group at the 30th posterior segment. The amputated earthworms were transferred to a Vermi Bed and allowed to regenerate. To analyze the wound healing process on the first day five worms were selected from each group and visualized under a stereo zoom microscope. On the 3rd day after amputation five worms from each group were used for blastema formation analysis. Similarly, the rest of the ten worms from each group were used for differentiation stage analysis.

2.3. Stereo zoom microscope analysis

Live earthworms were anesthetized with 10% ethanol and the morphological changes during organogenesis were observed between days 1 and 6, using a Nikon SMZ800 stereo zoom microscope (Tokyo, Japan).

2.4. Histology

Changes at the cellular level engendering organogenesis were analyzed histologically (Johnson Retnaraj Samuel et al., 2011). The desired tissues were fixed in 10% formaldehyde (HiMedia, Mumbai, India) for 24 h followed by dehydration using gradient isopropanol (HiMedia) at 70%, 80%, 90%, and 100% for 1 h each. The dehydrated tissue was cleansed with xylene (HiMedia) for 45 min followed by wax impregnation and sectioning (6 µm thick) with the help of a Weswox microtome (Haryana, India). The dehydrated and cleaned tissues were stained with hemotoxylin and eosin (HiMedia) and finally mounted under an Olympus BX53 microscope (Singapore).

2.5. Functional analysis of the mouth and anus during regeneration

To study the functional status of the mouth and anus during anterior regeneration, 25 sexually matured earthworms were sampled and fed on tissue paper for 3 days. They were subjected to anterior amputation (10th segment) and organized into two groups, Groups A and B, that included 20 and 5 worms, respectively. The Group A worms were transferred to Vermi media for intake analysis for 4 days (days 3 to 6) to observe body color as well as gut dissection. Each day five worms were sacrificed and documented under a stereo zoom microscope. The Group B worms were individually maintained on fresh tissue paper medium for excretion analysis, confirmed by the presence of brown fecal pellets.

To analyze the functional status of the anus and mouth during posterior regeneration (anus), 25 sexually matured worms were chosen from the Vermi media. These earthworms were amputated at the posterior 30th segment. Of these, 5 worms were individually maintained on fresh tissue paper medium for excretion analysis. The rest of the 20 worms were maintained on fresh tissue paper medium for intake analysis following the procedure described above.

2.6. Estimation of riboflavin

Thin-layer chromatography (TLC) was performed to separate and estimate the riboflavin from the earthworm’s intestinal region (5 µL) and integument tissues (15 µL) using butanol-chloroform-acetic acid-ammonia-water as the mobile phase in a ratio of 7:4:5:1:1. The concentration of riboflavin was estimated by spectrophotometric analysis by generating the standard graph exhibiting the photoluminescent value (PL) on the Y-axis against
concentration of riboflavin on the X-axis using a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, Paris, France). The riboflavin concentration in different organs was estimated using a standard graph (Johnson Retnaraj Samuel et al., 2011).

2.7. Isolation of microbes
First the earthworm sample was washed in water at 48 °C for surface sterilization (Parle, 1963). Subsequently, the earthworm was dissected and the gut material was isolated. It was then plated on nutrient agar medium (HiMedia) by spread plate technique and incubated overnight at 37 °C. Finally, plates with colonies of bacteria were viewed under UV light, and the fluorescent colonies were isolated and cultured for further experiments.

2.8. Staining of isolated bacterium
In order to characterize the isolated bacterium, Gram staining was performed using the HiMedia kit Cat. No. K 001-1KT and the spore staining was performed using the HiMedia kit Cat. No. K006-1KT. The experiments followed the manufacturer’s protocol.

2.9. 16S rRNA sequencing
Single colony isolates were inoculated onto LB broth and incubated overnight. From this, the genomic DNA of the fluorescent bacteria was isolated using the standard protocol. The 16S rRNA sequences of isolates were amplified by PCR primers (forward primer: CGTATGAACATCGGCCAGGT; reverse primer: TCCATTTCGCCGAAGCGCTG) and the PCR products were sequenced accordingly. The DNA sequence was blasted and the 16S rRNA gene sequences of the evolutionarily related bacteria were systematically placed by multiple alignment method using align X software.

2.10. Analysis of riboflavin synthesis by Bacillus endophyticus
The yellowish material of the bacterial colony was scraped and mixed well with sterile water. The mixture was then centrifuged at 5000 rpm for 10 min. Following centrifugation the supernatant was collected and subjected to spectrofluorometric analysis.

2.11. Mass production of riboflavin by Bacillus endophyticus
The Bacillus endophyticus MSU072011 strain was used for production of riboflavin. It was cultured in minimal medium at 38 °C. The overnight culture was used as an inoculum in fermentation. The fermentation process was carried out with a KLF 2000 fermenter (BiOENGiNEERiNG AG, Wald, Switzerland). A sample was taken every 4 h to estimate the riboflavin concentration. The production of riboflavin was estimated by spectrofluorometric analysis using a fluorescence multiwall plate reader (BioTek, Winooski, VT, USA).

2.12. Statistical analysis
Statistical analysis such as standard errors and P-values for the riboflavin concentration of control and regenerated earthworm tissues was performed using Microsoft Office XL 2007 and SigmaPlot 12.0. P < 0.05 was considered as a significant value.

3. Result
E. eugeniae is a segmented annelid (Figure 1A). The anterior segmental region starts with a ventrally situated mouth (Figure 1B), followed by a long digestive canal connected to several organs and ending with a dorsally located anus (Figure 1C). In E. eugeniae, the complex process of regeneration starts with the wound healing process, which incorporates blastema formation, followed by successful organogenesis of lost organs.

3.1. Process of wound healing in E. eugeniae
In order to study the wound healing process followed by mouth and anus development in the earthworm E. eugeniae, ten healthy earthworms were selected and amputated as described in Section 2. Initially, coagulation
was noticed within 1 min at the wounded sites of all amputated earthworms (Supplementary Figures 1A and 1B). Finally, the wound closures of every anterior and posterior amputated earthworm were observed 24 h after amputation (Supplementary Figures 1C and 1D).

3.2. Blastema formation
In our previous report, blastema formation during anterior regeneration of *E. eugeniae* was achieved by the proliferation of the longitudinal cell layer (LCL) (Johnson Retnaraj Samuel et al., 2011; Kalidas et al., 2015). To verify the early events of the mouth and anus at the time of regeneration, ten matured earthworms were selected for the study. On the 3rd day the regenerated blastema was observed under a stereo zoom microscope (Figures 2A and 2B). It was found that both the anterior and posterior blastemas appeared as a colorless mass of freshly formed tissue (Figures 2C and 2D). Consequently, many angiogenic vessels were observed in the blastema (Figures 2C and 2D). To understand the structural development, day 3 blastemas of the anterior and posterior regions of regenerating earthworms were subjected to histological analysis (Figures 2E and 2F). The blastemal cells were newly formed and the epithelial cell layer (ECL) and circular muscle layer (CML) were not found in the regeneration blastema. Unlike the anterior regeneration blastema, the posterior regeneration blastema had a unique appearance; it appeared in a dorsal opening (Figure 2F), which is apparently similar to the intact anus.

3.3. Differentiation and organogenesis state
The mouth and anus reconstitution processes were noted on day 6 using stereo zoom microscopy and histology. Ten earthworms were selected for the anterior and posterior amputation as stated in Section 2. It was observed that in all the earthworms, the regenerating mouth opened ventrally and the regenerating anus opened dorsally (Figures 3A and 3B). Segment formation also occurred in the anterior regenerated part (Figure 3A). The intestinal tract was found in the posterior regenerated part (Figure 3B). Similar to the day 3 blastema, the day 6 regenerated part also contained more vascular networks and the newly formed cells remained unpigmented (Figures 3A and 3B). The dorsal blood vessels were consistently noted in the anterior and posterior regenerated parts (Figures 3C and 3D). Vascular sprouting was also noted in the lateral side of each segment (Figures 3C and 3D). To investigate the cellular changes, the day 6 regenerated earthworm was subjected to histological analysis (cf. Figures 3A and 3B). In contrast to the day 3 blastema, the day 6 anterior and posterior regenerated parts showed more differentiated cells. The newly formed parts of the digestive system, such as the mouth and pharynx, were found at the anterior part (Figure 3E). The ECL, CML, and LCL were noted in both anterior and posterior parts (Figures 3E and 3F). In addition, intestinal lumen was noted at the posterior part (Figure 3F). Figures 3F and 3G reveal that there was a drastic increase in the occurrence of cell differentiation, such as the intestinal epithelial cells (IECs), LCL, CML, and ECL in the distal region as compared to the proximal extremity of the day 6 posterior part (Figures 3F and 3G). The day 6 anterior and posterior regenerated parts (Figures 3E and 3F) resembled the normal mouth and anus (Figures 3H and 3I) of *E. eugeniae*. The intestinal epithelial cell development of day 6 posterior regenerated parts (Figure 3G) were also similar to the intact anus (Figure 3J).

3.4. Functional analysis of regenerated mouth and anus
The digestive system of the earthworm starts at the mouth, followed by a slender tube called the intestinal lumen, from which the process of digestion takes place and ends in the anus. In this context, any physical damage occurring to the mouth or other intestinal parts could lead to the stoppage of food uptake and ejection of excreta until the regeneration of the mouth and anus occurs. As the ventral side of *E. eugeniae* is transparent, the content in the digestive tract is easy to observe.

3.5. Recovery of intake and excretion during regeneration of mouth
The body color of earthworms was used as an indicator to study the functional recovery of the mouth. Under normal feeding (Vermi media) the earthworm appears dark brown in color (Figures 4A and 4B), whereas it seems beige-colored when fed on white tissue paper (Figures 4C and 4D). In order to study the functional recovery of the mouth, 25 earthworms were selected and fed with tissue paper for 3 days. On Day 3, the body color of the earthworms changed to beige due to consumption of white tissue paper. All the earthworms were amputated at the anterior 10th segment and divided into two groups (Group A – 20 earthworms, Group B – 5 earthworms). Group A earthworms were transferred to normal Vermi media for intake analysis whereas Group B earthworms were maintained on fresh white tissue paper media to analyze the excretion process. The functional recovery of the mouth was tested continuously from days 3 to 6 in Group A earthworms. The body color was analyzed to identify the nature of gut materials under a stereo zoom microscope. It was noted that the body color of earthworms took on a beige tinge on days 3 to 5 (Figure 4E). In addition, each day, five earthworms were sacrificed for gut extraction and estimation of enrichment with the tissue paper up to day 5 (Figure 4F), whereby it became clear that the mouth of the regenerating earthworm was not yet developed. Contrastingly, the earthworms were dark brown in color on day 6 (Figure 4G). The color change by day 6 was due to the intake of dark-colored Vermi media (Figure 4H). The presence of Vermi media in the gut on day 6 (Figure 4H) confirms the reconstituted and functional state of the mouth.
To study the functional status of the anus, the Group B earthworms were taken into account. In order to show the morphological appearance of the anterior regenerated part of Group B earthworms (days 3, 5, and 6), they were visualized directly under a stereo zoom microscope (Figures 4I, 4J, and 4K). The presence of fecal pellets confirmed the normal functioning of the anus. In general, the fecal pellet of *E. eugeniae* appears brown in color. It was noted that the fecal pellet was present from days 1 to 3 (Figure 4L) but was not observed on days 4 to 5 (Figure 4M). In addition, the fecal pellet and cast out material in the anal region was again noted from day 6 onwards (Figure 4Q), implying that the earthworm resumes the excretion process subsequently only after complete functional recovery of mouth.

3.6. Recovery of excretion and intake during regeneration of the anus

The histological and stereo zoom microscopical results implied that the initial anus-like structure developed at day 3 blastema. In order to check the functional recovery of the regenerating anus, five earthworms were selected for the experiment. They were amputated at the posterior
30th segment and transferred individually to tissue paper media. The gut material was not observed from days 3 to 5 (Figures 5A and 5B), but gut material was consistently observed on day 6 (Figure 5C). Similarly, on tissue paper media there were no fecal pellets observed from day 3 to day 5 (Figures 5D and 5E), in contrast to fecal pellets observed on day 6 (Figure 5F). The experimental results suggested that the excretion process had actually started on day 6. To investigate the impact of the intake process on anus reconstitution, 30 earthworms were maintained on tissue paper media. Following posterior amputation as described above, the earthworms were transferred to Vermi media individually. Intake analysis was tested by body color and gut. Appearance of beige-colored earthworms and the absence of Vermi media in the gut of the regenerating earthworms on the 5th day indicates that the function of the mouth was not yet restored on days 1 to 5 (Figures 5G and 5H). The body color change and the presence of Vermi media in the gut region was noted on day 6 only (Figures 5I and 5J), which confirmed the day of intake (Figures 5I and 5J).

### 3.7. Concentration of Vitamin B2 during regeneration

The concentration of riboflavin was estimated in different parts of the control earthworms and 3rd and 6th day anteriorly regenerating earthworms by spectrofluorometer. The experimental results showed that the earthworms had stored different concentrations of riboflavin in various parts (Table 1). An elevation in riboflavin amount was notably observed in the skin (Table 1). Thus, accumulation of riboflavin in the skin presumably confirms riboflavin playing a key role in blastema formation in *E. eugeniae* adult organogenesis. The intestine of the intact earthworm

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**Figure 3.** Structure of the regenerated mouth and anus. A & B) Stereo zoom microscopical images (1×) on the 6th day showed a development of the mouth and the anus after amputation at the anterior and posterior parts, respectively. C & D) Magnified (2×) stereo zoom microscopical images of blood vessels of a newly formed mouth and anus, respectively. The dorsal and lateral blood vessels are indicated by arrows. E) Histological image of regenerated mouth on the 6th day shows the formation of pharynx and esophagus in worm amputated at anterior part. F) Histological image (4×) of the anus on the 6th day of a worm amputated at the posterior part. G) Magnified (20×) region shown in panel F shows the formation of intestinal epithelial cells and a septum. H & I) Histological image (4×) of an intact mouth and anus of *E. eugeniae*. J) Magnified (20×) anus region shown in panel I shows the intestinal epithelial cells. rm- regenerated mouth, ra- regenerated anus, m- mouth, a- anus, ph- pharynx, es- esophagus, pm- prostomium, iec- intestinal epithelial cells, il- intestinal lumen, ds- dorsal side, vs- ventral side.
Figure 4. Recovery of intake and excretion during regeneration of mouth. A & B) Stereo zoom microscopical images of *E. eugeniae* body segments (normal and dissected gut) under normal feeding (Vermi media) conditions. C & D) Stereo zoom microscopical images of *E. eugeniae* body segments (normal and dissected gut) with tissue paper feeding. E & F) Images of body segments and dissected gut region of 5th day worm regenerating the mouth. The body and intestine are seen as beige in color because of tissue paper consumption. G & H) Images of body segments and dissected gut region of 6th day worm regenerating the mouth. The body and intestine are dark brown in color because of the presence of normal feed in the gut region. I) Image of 3rd day anterior regenerated blastema. J) Image of 5th day anterior regenerating part. K) Image of 6th day regenerated mouth. L) Image of 3rd day anteriorly amputated worm; the presence of fecal matter confirms anal function. M) Image of 5th day anteriorly amputated worm; no fecal matter was noted. N) Image of 6th day worm regenerating the mouth; the presence of fecal matter confirms anal function. O) Stereo zoom microscopical image of 3rd day anteriorly amputated worm anus region; the presence of cast out material confirms a functional anus. P) Stereo zoom microscopical image of 5th day anteriorly regenerated worm's anal region; the absence of cast out material confirms that the anus did not function. Q) Stereo zoom microscopical image of the anal region of 6th day worm regenerating the mouth; the presence of cast out material confirms anal function. il- intestinal lumen, rb- regeneration blastema, rm- regenerated mouth, ra- regenerated anus.
was estimated to have accumulated 304.79 ± 2.73 µg/g riboflavin. The 3rd day anteriorly regenerating earthworm had the maximum amount of riboflavin, 965.55 ± 0.67 µg/g, in the intestinal region (Table 1).

The riboflavin level was found reduced in the intestine, skin, and other organs on day 6 of regeneration. Though the coelomic fluid had retained a riboflavin level similar to that of the 3rd day (Table 1), the riboflavin concentration was found increased from 386.90 ± 0.72 µg/g on day 3 to 414.5 ± 0.24 µg/g on day 6. The enrichment of riboflavin in coelomic fluid reveals that the fluid carries riboflavin to all body cells, which helps them to withstand adaptive starvation. Our previous report showed that the administration of antibiotics resulted in sixfold reduction
The discovery of riboflavin enrichment in the intestinal region suggests that the gut microbes catalyze a supply of the riboflavin that is used to maintain the homeostasis of *E. eugeniae*.

### 3.8. Production of riboflavin by symbiotic bacteria during adaptive starvation

It is known that the riboflavin is synthesized only by microbes (Prabhakar et al., 1993; Stahmann et al., 1994) and plants (Fischer and Bacher, 2006; Sandoval et al., 2008), not by animals. The autofluorescence property of riboflavin led to the assumption that riboflavin-producing bacterial colonies should also possess the property of autofluorescence. Based on that, agar plates containing the isolates of gut microbial flora of earthworms were visualized under UV light (Figures 6A and 6B). A few bright fluorescent microbial colonies were observed (Figure 6B). The autofluorescent bacteria were selected (Figures 6C and 6D) and the production of riboflavin by the microbes was confirmed by spectrofluorometric analysis (Supplementary Figure 2A). On day 1, the colony was seen to be white in color (Figure 7A). On the second day, it became yellowish. Obvious color change was noted on day 3 of incubation (Figure 7B). The water-soluble material from the colony was extracted and subjected to spectrofluorometric analysis (Supplementary Figure 2B). Interestingly, the yellowish material was found to be riboflavin-rich, synthesized by bacterial isolates. To characterize the bacterial species, Gram staining was performed and it was confirmed that the bacterium was gram-positive (Figure 7C). The spore staining study showed that bacteria formed terminal endospores (Figure 7D). Further, to characterize the bacteria, molecular study of 16S rRNA sequencing was performed. The PCR product was sequenced and the sequence was aligned with the 16S rRNA sequence of other bacterial species retrieved from the NCBI. The sequence of the isolated bacterial isolate was 100% similar to the 16S rRNA of *B. endophyticus* (Supplementary Figure 3). Furthermore, a phylogenetic tree was constructed using align X software.

### Table 1. Riboflavin concentration in different organs of normal and regenerated worms. Higher accumulation of riboflavin was noted in the intestinal region and tissue layer of 3rd day regenerating animals. The reduction of riboflavin was documented in the intestinal region and tissue layer in 6th day regenerating animals.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Different organs</th>
<th>Normal worm (concentration of riboflavin, µg/g)</th>
<th>3rd day regenerated worm (concentration of riboflavin, µg/g)</th>
<th>6th day regenerated worm (concentration of riboflavin, µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Intestine</td>
<td>304.79 ± 2.73</td>
<td>965.55 ± 0.67</td>
<td>320.70 ± 0.54</td>
</tr>
<tr>
<td>2.</td>
<td>Prostate gland</td>
<td>394.67 ± 0.51</td>
<td>279.07 ± 0.29</td>
<td>215.52 ± 0.36</td>
</tr>
<tr>
<td>3.</td>
<td>Setae</td>
<td>284.19 ± 3.02</td>
<td>112.73 ± 0.31</td>
<td>103.23 ± 0.31</td>
</tr>
<tr>
<td>4.</td>
<td>Coelomic fluid</td>
<td>368.19 ± 1.29</td>
<td>386.90 ± 0.72</td>
<td>414.5 ± 0.24</td>
</tr>
<tr>
<td>5.</td>
<td>CML &amp; LCL</td>
<td>202.84 ± 0.66</td>
<td>640.19 ± 1</td>
<td>276.80 ± 1.15</td>
</tr>
</tbody>
</table>

in riboflavin concentration (Johnson Retnaraj Samuel et al., 2011). The discovery of riboflavin enrichment in the intestinal region suggests that the gut microbes catalyze a supply of the riboflavin that is used to maintain the homeostasis of *E. eugeniae*.

4. Discussion

The wound healing process, a complex event, is the first step in regeneration (Gurtner et al., 2008). The commencement of the wound healing process was observed for 24 h (Supplementary Figures 1C and 1D). Observations in different animals suggest that the skin epithelium has a conserved role in wound healing. In humans, new tissue development and epithelialization take 2 to 10 days following the injury (Gurtner et al., 2008). In contrast, the amphibian axolotl can complete the wound healing within 8 h (Kawasumi et al., 2013). Several reports suggested that
the time taken for the wound healing process varies among earthworm species. Generally the earthworm *Eisenia fetida* takes 1 to 3 days, whereas the present investigation of *E. eugeniae* shows that wound healing takes place after 24 h (Supplementary Figures 1C and 1D). A higher degree of microvascularization is required in regenerating the blastema (Figures 2C and 2D). Elevation of vascularization resulting in early regeneration was documented in several animals, e.g., in mice a drastic amount of microvascularization was observed, which triggers the entire regeneration of partial hepatectomic liver (Drixler et al., 2002). Likewise, it plays a significant role in the regeneration of fins in zebrafish (Bayliss et al., 2006) and blastema development of newts (Rageh et al., 2002). Differentiation and pattern formation are the major processes in early development and organogenesis. It was observed in the present investigation that an abundance of differentiated cells such as the ECL, LCL, CML, and IECs was present in the regenerating anterior and posterior regions on day 6 (Figures 3E and 3F). This is similar to the structure of the intact mouth and anus (Figures 3E, 3F, 3H, and 3I).

The impairment of mouth and anal functions should cause food deprivation. Organogenesis is a mechanistic

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**Figure 6.** Isolation of autofluorescent bacteria from earthworm gut. A) Microbial plate shows colonies isolated from the intestinal gut material of earthworm, *E. eugeniae*, as visualized under visible light. B) Microbial plate (panel A) visualized under UV light. The arrow indicates the fluorescent colonies. C) Fluorescent bacteria isolates were visualized under visible light. D) Microbial plate (panel C) visualized under UV light.
process that needs more energy because of huge cell proliferation and turn-over. The impact of food deprivation in embryonic organogenesis was studied in a few animals. For instance, in the development process of *Caenorhabditis elegans*, food scarcity was effectively addressed by undertaking growth and checkpoint adaptations (Kumar et al., 2000; Schindler et al., 2014). Likewise, *E. eugeniae* adapted with unique starvation behavior on regeneration. Naturally, therefore, the liver is the most important organ that stores more riboflavin, and it can maintain the energy balance in early and late stages of starvation due to erratic carbohydrate and lipid metabolism (Potthoff et al., 2009). Riboflavin is the vital substance for carbohydrate and lipid metabolism (Reddi et al., 1979; Depeint et al., 2006). Similar to the liver, tissues of *E. eugeniae* are enriched with riboflavin (Table 1) and both liver and *E. eugeniae* have enormous regeneration ability (Gates, 1949; Goessling et al., 2008; Ding et al., 2010; Johnson Retnaraj Samuel et al., 2011). Thus, we are inclined to hypothesize that the riboflavin is important for cell proliferation and maintains homeostasis during starvation. Intake of riboflavin ahead of starvation helps the animals to sustain life during the period of starvation (Moriya et al., 2013).

In our previous report, we found riboflavin to be an important factor that can alter the regeneration program in *E. eugeniae* and we observed that the stem cells of *E. eugeniae* reside in the CML and ECL (Johnson Retnaraj Samuel et al., 2011). The rich accumulation of riboflavin in the skin layers (Table 1) suggests that the stem cells may require more riboflavin for proliferation and differentiation. Similarly, the report of Zhang and Huang (2012) revealed that there was a higher accumulation of

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**Figure 7.** Characterization of isolated autofluorescent bacterium of *E. eugeniae* gut. White and yellow appearance of day 1 (A) and day 3 (B) incubated bacterial colonies. C) Gram staining image of the bacteria confirms that it is gram-positive. D) Image of spore staining, performed by incubating the bacteria at 37 °C for 48 h followed by staining; the fluorescent microbes form terminal endospores. E) The phylogenetic tree clearly indicates that the fluorescent colony isolated from the earthworm gut was *Bacillus endophyticus.*
riboflavin in peripheral blood mononuclear cells, which are known as multiple progenitor cells (Zhang and Huang, 2012). Ample reports showed that the mitochondrial metabolism plays a pivotal role in stem cell proliferation and differentiation (Funes et al., 2007; Mandal et al., 2011; Xu et al., 2013). It is known that riboflavin and its cofactors are important components for mitochondrial metabolism (Hoppel and Tandler, 1975; Barile et al., 1997; Nelson and Cox, 2013). The collective evidence suggests that riboflavin is an important factor for organ development and homeostasis maintenance in E. eugeniae regeneration.

In the later phase of organogenesis, on day 6, the riboflavin level was reduced in the intestine and skin (Table 1).

Several studies have revealed that host–microbe interaction is an important association for developmental and homeostasis maintenance of animal traits (Nicholson et al., 2005; Zhang et al., 2010). Various reports in mouse and zebrafish revealed that the microbiota played a major role in cell proliferation (Uribe et al., 1997; Rawls et al., 2004). Based on the function, the human body has a unique symbiotic microbial niche (Hooper et al., 2002; Nicholson et al., 2005; Zhang et al., 2010; Aagaard et al., 2014). Some

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Table 2. Spectrofluorometric reading of riboflavin-producing bacteria isolated from earthworm Eudrilus eugeniae. M9 = Minimal medium.

![Figure 8. Production of riboflavin by symbiotic bacteria during adaptive starvation. A) The graph shows the production of riboflavin by the bacteria. B & C) TLC image shows rate of storage of riboflavin in earthworm tissues and the intestinal region, analyzed for normal and regenerating worms (days 1, 3, 5, and 6). cl- control, r- riboflavin. D) Bar diagram of riboflavin concentration in tissues and the intestinal region of control and regenerating worms (days 1, 3, 5, and 6) with standard error. *: P < 0.0012.](image-url)
might have a symbiotic association with the cotton plant. That report speculated that \textit{B. endophyticus} who discovered the isolate from the inner part of a healthy cotton plant. The bacterium \textit{B. endophyticus} adaptive starvation from day 1 to day 5. The bacterium \textit{B. endophyticus} has the same pattern; the \textit{E. eugeniae} \textit{B. endophyticus} has the same pattern; the \textit{E. eugeniae} LeBlanc et al., 2013). The gut of \textit{E. eugeniae} was first reported by Reva et al. (2002), who discovered the isolate from the inner part of a healthy cotton plant. That report speculated that \textit{B. endophyticus} might have a symbiotic association with the cotton plant.

The gut of \textit{E. eugeniae} may contain innumerable riboflavin-producing symbiotic bacteria. A symbiotic microbe, \textit{B. endophyticus} is among those that supply rich amounts of riboflavin during the complex organogenesis process under extreme starvation conditions.

Acknowledgment
We sincerely thank Jason Nefalar, Viral Hepatitis and Gene/Cell Therapy Laboratory, University of California at Los Angeles, USA, for editing the manuscript.

References


Supplementary Figure 1. Process of wound healing in *E. eugeniae*. A & B) Magnified (1×) stereo zoom microscopical image of coagulation at the site of amputation in the anterior and posterior regions, respectively. C & D) Wound healing of worms amputated at anterior and posterior regions, respectively. Arrow shows coagulation and wound healing at the amputation sites (A–D). wc- wound coagulation, wh- wound healing.
Supplementary Figure 2. Analysis of riboflavin from water-soluble material and broth of isolated bacterial colony. A & B) Excitation and emission spectrum of water-soluble yellowish material from the nutrient broth and the bacterial colony in the nutrient agar plate, respectively. The dotted lines indicate the spectra of excitation and the solid lines indicate the spectra of emission. Asterisk indicates unknown fluorophore. PL- photoluminescence.
**Supplementary Figure 3.** Sequence analysis of 16S rRNA gene using align X. The alignment of the 16S rRNA sequence of *Bacillus* sp. retrieved from the NCBI with the 16S rRNA sequence of the isolated bacterium using align X software. The identified sequence shows 100% homology with *Bacillus endophyticus*.  

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---

The identified 16S rRNA sequence of *Bacillus endophyticus* (isolated) shows 100% homology with the NCBI retrieved sequence using align X software.
Supplementary Figure 3. (Continued).
SUBRAMANIAN et al. / Turk J Biol

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B.carboniphilus (241) CGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
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561                                                                          640

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<td>GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG</td>
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<td>B. niabensis (719)</td>
<td>GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG</td>
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<td>B. idriensis (719)</td>
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<td>B. koreensis (719)</td>
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<td>B. aquimarisis (721)</td>
<td>GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG</td>
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<tr>
<td>B. vietnamensis (721)</td>
<td>GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG</td>
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<tr>
<td>B. seohaeanensis (720)</td>
<td>GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG</td>
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<tr>
<td>B. carboniphilus (721)</td>
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<tr>
<td>B. isabeliae (721)</td>
<td>GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG</td>
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<tr>
<td>B. sonorensis (721)</td>
<td>GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG</td>
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</tbody>
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Consensus (721) GCGGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG

Supplementary Figure 3. (Continued).