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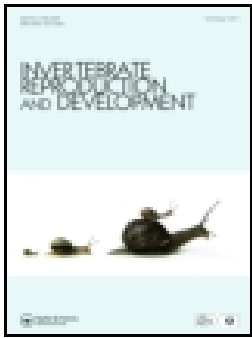
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# Effect of symbiont acquisition on growth, survival and fertility of the terrestrial isopod *Porcellionides pruinosus* (Crustacea, Oniscidea)

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## ABSTRACT

Growth rate, survival, mean size of reproductive females and fertility were assessed in the terrestrial isopod *Porcellionides pruinosus* fed an artificial diet supplemented with potential sources of symbionts. The acquisition of symbionts was experimentally manipulated through feeding experiments to investigate the effect of the acquisition mechanism on isopod performances over 4 months. Five groups of isolated newborn were fed differently (basal diet or control; basal diet with leaf litter, faeces, gut; and basal diet mixed with antibiotic), weighed and counted at 0, 2 and 4 months. Growth rate differed significantly between diet groups at 2 and 4 months. No significant difference of survival was detected, but groups fed on a diet with supplements (leaf litter, faeces and gut) had a higher rate of survival. Size and fertility of reproductive females differed significantly across diet groups. Our findings showed the importance of the acquisition mode of symbionts on the life history of Oniscidea and the role of diet intake on the performance of *P. pruinosus*.

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Feeding; leaf litter; life history; terrestrial woodlouse; symbiont transmission

## Introduction

Among crustacean taxa, terrestrial isopods constitute the most adapted group to terrestrial life benefiting from a set of morphological and physiological adaptations. The acquisition of a bacterial partner appears to have been a key step for isopod terrestrialization (Zimmer et al. 2001, 2002). Indeed, the main challenge in terrestrial life is the low quality and the recalcitrant nature of plant material. The association with bacterial symbionts coincided with the appearance of new digestive capabilities which permitted these macroinvertebrates to digest phenolic compounds characteristic of a terrestrial diet. Thus, the digestion of lignin, cellulose and other polymers is enhanced by bacterial and fungal enzymes in the gut of herbivorous mammals and arthropods (Douglas 1994; Zimmer et al. 2001, 2002). Bacterial symbionts, therefore, are involved in host fitness and their nutritional ecology by providing metabolic advantages (Douglas 2009, 2011), protection of the host by enhancing thermal tolerance and defense against enemies (Russell and Moran 2006; Tsuchida et al. 2010).

Terrestrial isopods benefit from their microbial community qualitatively rather than quantitatively especially for lipids, vitamins (Carefoot 1984) and other essential components (Douglas 2009; Horváthová et al. 2015). Digestive tract symbionts such as bacteria

associated with the hepatopancreas of Oniscidea are mainly involved in cellulose digestion and phenol oxidation. Such symbionts probably have a role in terrestrial colonization (Zimmer and Topp 1998; Zimmer et al. 2002) and appear to improve survival under conditions of poor diet (Fraune and Zimmer 2008).

The suborder Oniscidea plays an important role in nutrient recycling during the decomposition processes. With around 3710 species belonging to 527 genera and 37 families (Sfenthourakis and Taiti 2015) of terrestrial isopod, a symbiotic association between oniscidean representatives and microbes has been confirmed only recently for seven species (Zimmer 2002a). Terrestrial isopods need cellulolytic enzymes for the digestion of cellulose and recalcitrant compounds of terrestrial leaf litter (Zimmer 2002b), which is why they need host microorganisms from their environment. In contrast to vertical transmission where the symbiont genotype in offspring depends mainly on the symbiont genotype within the parent (Baumann et al. 1995), the symbiont genotype horizontally transmitted to the host is determined by the host (Troll et al. 2010; Nyholm and Graf 2012; Garcia et al. 2014) and environmental factors (Finney et al. 2010; Porter and Rice 2012). Wang et al. (2007) suggested that the environmental transmission

appears to be the mode of bacterial acquisition in oniscidean isopods. We can discriminate between three kinds of symbiont transmission in arthropods: (1) a vertical pathway of symbiont transmission from mother to juveniles as for reproductive manipulator symbionts like *Wolbachia*; (2) horizontal transmission of transient bacteria which occurs via ingested food, cannibalism, predation or by coprophagy (Rigaud and Juchault 1995; Zimmer 2002a; Brandstädter and Zimmer 2008; Le Clec'h et al. 2013); and (3) transmission from environment to host as for the hepatopancreatic symbiont bacteria *Candidatus Hepatoplasma crinocheatorum* and *Candidatus Hepatincola porcellionum* (Wang et al. 2004a, 2004b, 2007).

Terrestrial isopods provide an excellent model to study the acquisition mode of bacterial symbionts since they constitute the only crustacean group that is strictly independent of aquatic life. This study has two main objectives: (1) to determine the effect of diet type on growth rate, survival, and fertility of *P. pruinus* and (2) to test the mechanism of symbiont acquisition and its effect on life history traits of juveniles.

## Materials and methods

### Sampling

Adult specimens of *P. pruinus* were hand collected from the region of Tunis (Tunisia) in March 2016. After identification of the specimens using a Leica MS 5 stereomicroscope, 10 gravid females of similar size and weight were kept alive in individual plastic boxes with sterile wet soil under laboratory conditions (a temperature between 20°C and 25°C and a daily photoperiod of 12 h). The females were fed with a small amount (1–2 g) of artificial sterile diet that is a mixture of chemical compounds used as a control diet for feeding juveniles (see Appendix).

### Preparation of diets

The basal diet was obtained after mixing the chemical constituents (see Appendix) with hot distilled water and agar. After cooling, the basal diet was poured into sterile Petri dishes and stored at 4°C.

### Experimental diets and feeding

After hatching, juveniles were immediately removed to avoid contact with their mother, weighed to the nearest 0.01 mg, and placed in groups of four to five into plastic boxes containing sterile wet soil with a sterile clay pot to serve as an artificial refuge.

A total of 133 juveniles from seven females hatched over 48 h were mixed and placed haphazardly into five groups with different diets. The first diet was an artificial basal diet (Carefoot 1984; Zimmer and Topp 1998; Horváthová et al. 2015) that was considered a control diet without supplements (**B**) ( $n = 27$ ). The second group of isopods were fed on the basal diet supplemented with 1% of streptomycin antibiotic (**A**) ( $n = 27$ ) (Zimmer and Topp 1998) to affect the microbial flora. For the remaining diet types, in addition to the control diet, we added a small supplementary diet that served as a potential source of bacterial symbionts. The third group had a diet supplemented with a small piece of leaf litter (**L**) (0.5 mg) ( $n = 26$ ), for the fourth group the basal diet was complemented with a single faecal pellet (**F**) (0.4–0.7 mg) ( $n = 27$ ), and in the fifth group the basal diet was supplemented with fresh gut (**G**) (0.4–0.6 mg) ( $n = 26$ ) from adult conspecifics. The clay pot, the basal diet, and the supplements were changed twice per week. Uneaten food and faecal pellets were removed from the boxes to avoid the contamination of fresh diets.

### Growth rate, survival and fertility

Growth rate was expressed as the difference between the initial juvenile body mass at hatching and juvenile weight after 2 and 4 months. Survival, determined as the number of surviving isopods compared to the initial number, was provided by continuous monitoring of the surviving juvenile isopods until the end of experiments which was indicated by embryo release by the gravid females (a few days after 5 months). Fertility was measured as the number of living embryos hatched per brood. The size of gravid females was noted for all diet types.

### Statistical analysis

To assess the effect of diet type on growth rate, juvenile mass at hatching, 2 and 4 months were compared by ANOVA. The same test was used to compare reproductive female size and fertility in each diet group. In the case of a significant difference, a *post hoc* Tukey test was used to compare all pairs of group means. Comparison of survival across different diet groups was performed using a  $\chi^2$  test. Statistical analyses were carried out with the free version MINITAB 17.

## Results

### Growth rate

No significant difference of mean initial body mass of juveniles was found among different diet groups

( $F = 1.85$ ,  $p = 0.124$ ) at hatching. Growth rate differed significantly among 2-month-old juveniles fed different diets ( $F = 5.51$ ,  $p = 0.001$ ) (Figure 1(a)). A *post hoc* test showed that mass increase was significantly different between the Basal diet (**B**) and supplemented diets **F** and **G** (Figure 1(a), Table 1). Juvenile mass after 4 months also differed significantly between diet groups ( $F = 2.71$ ,  $p = 0.049$ ). The mass increase of juveniles at 4 months was greater within groups fed on supplemented diets (**L**, **F**, **G** and **A**) (Figure 1(b), Table 1).

### Survival

Our results showed that no effect of diet type was detected on survival of *P. pruinosus* embryos, but juveniles fed on diets supplemented with a potential source of symbiont (**L**, **F** and **G**) exhibited higher survival in comparison with those fed only on basal diet (**B**) or on a basal diet mixed with antibiotic (**A**) (Figure 2(a)). After 4 months, juveniles fed a diet supplemented with faeces (**F**) exhibited the highest number surviving ( $n = 11$ ) followed by in descending order diets (**G**) ( $n = 10$ ); (**L**) ( $n = 7$ ); (**A**) and (**B**), ( $n = 3$ ) (Figure 2(b)).

### Life history trade-offs

The mean length of females attaining the reproduction stage differed significantly among diets ( $F = 3.77$ ,  $p = 0.02$ ) but not between (**B**), (**A**), (**L**) and (**G**) (Figure 3(a) and Table 2). Fertility (the number of juveniles released per brood) differed significantly among diets ( $F = 2.99$ ,  $p = 0.045$ ), whereas the number of embryos did not differ between females fed on supplemented diets **L**, **F** and **G** and the antibiotic diet (**A**) ( $F = 3.06$ ,  $p = 0.055$ ) (Figure 3(b) and Table 2).

### Discussion

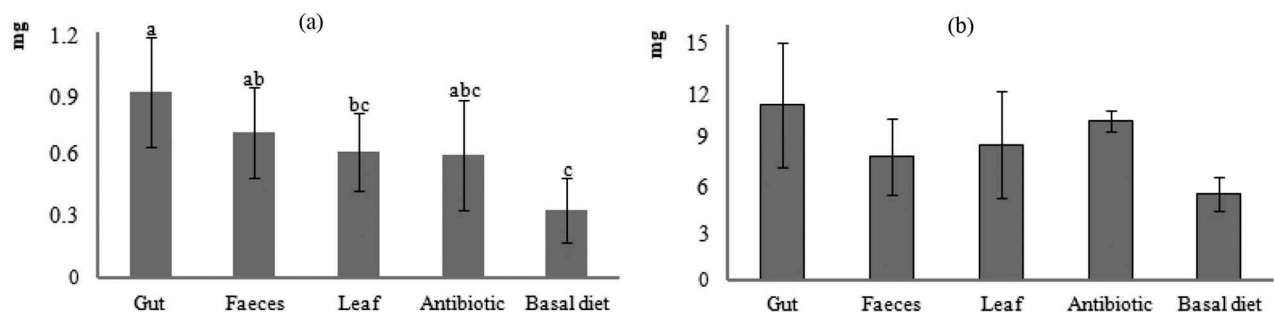
The growth rate of juveniles fed on a basal diet (**B**) was clearly lower than those fed a basal diet supplemented with an antibiotic (**A**) and diets inoculated with symbiont

**Table 1.** New born mean weight (mg) ( $\pm$ SD) under different diet types after hatching, 2 and 4 months: basal diet (**B**) supplemented with antibiotic (**A**), leaf litter (**L**), faeces (**F**) and gut (**G**).

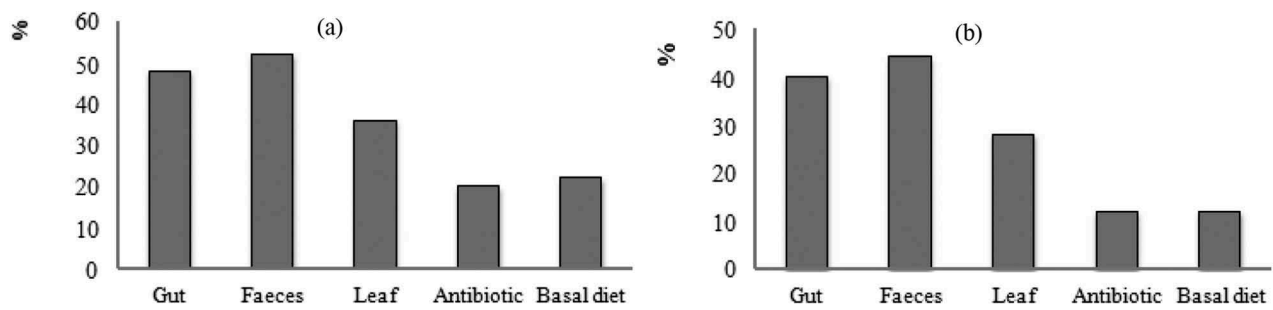
Diet type	Hatching	2 months	4 months
B ( $N = 27$ )	$0.48 \pm 0.04$	$0.82 \pm 0.16$	$5.86 \pm 1.07$
A ( $N = 27$ )	$0.45 \pm 0.05$	$1.06 \pm 0.27$	$10.43 \pm 0.65$
L ( $N = 26$ )	$0.46 \pm 0.07$	$1.07 \pm 0.19$	$8.94 \pm 3.36$
F ( $N = 27$ )	$0.43 \pm 0.05$	$1.14 \pm 0.22$	$8.16 \pm 2.42$
G ( $N = 26$ )	$0.45 \pm 0.07$	$1.36 \pm 0.27$	$11.42 \pm 3.87$

sources; (**L**), (**F**) and (**G**). This difference can be ascribed to the probable role of symbionts in enhancing growth as previous studies have shown that fitness and population dynamics of terrestrial isopods are positively influenced by food sources densely colonized by bacteria (Zimmer and Topp 1997; Kautz et al. 2000; Horváthová et al. 2016). It has been proposed that in *Porcellio scaber*, microbial colonization of leaf litter and artificial diets act positively on growth, survival and reproductive female success (Kautz et al. 2000; Zimmer et al. 2002; Horváthová et al. 2015). Zimmer and Topp (1997) also mentioned the negative effect on reproduction in *P. scaber* fed on leaf litter with little microbial activity. According to literature data, when given the choice, woodlice prefer to consume old densely colonized litter than fresh litter. Indeed, the high density of microorganisms in dead plant material makes it a palatable source of food with a high quality (Rushton and Hassall 1983b; Zimmer et al. 2003). In this context, Horváthová et al. (2016) demonstrated the importance of plant biofilm as a high quality nutritional source for the terrestrial isopod *P. scaber*.

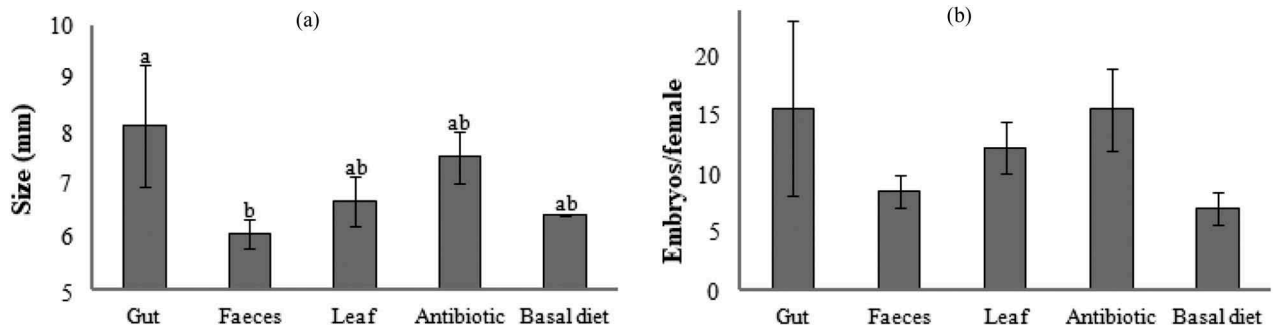
Macroarthropod decomposers benefit quantitatively and qualitatively from food sources inoculated with microorganisms (Kautz et al. 2000; Zimmer and Topp 2000). This occurs by a reduction in phenolic compounds and the C/N ratio (Zimmer 1999), and the production of digestive enzymes necessary for cellulose breakdown (Zimmer et al. 2002). Apart from digestive capabilities, microorganisms colonizing the digestive tract via nutrients like leaf litter are mainly involved in



**Figure 1.** Mean increase in mass (mg) after 2 (a) and 4 months (b) (different letters indicate that the values differ at  $p < 0.01$ ).



**Figure 2.** Survival (%) of juveniles after 2 months (a) and 4 months (b).



**Figure 3.** Size of reproductive females (mm) (a) and fertility (b) according to different types of diet (different letters indicate that the values differ at  $p < 0.05$ ).

**Table 2.** Reproductive female size (mm) and fertility according to type of diet: basal diet (B) supplemented with antibiotic (A), leaf litter (L), faeces (F) and gut (G).

Diet type	B	A	L	F	G
Mean female size ( $\pm$ SD)	6.4 $\pm$ 0	7.55 $\pm$ 0.49	6.67 $\pm$ 0.47	6.06 $\pm$ 0.29	8.1 $\pm$ 1.15
Mean fertility ( $\pm$ SD)	7 $\pm$ 1.41	15.5 $\pm$ 3.53	12.25 $\pm$ 2.21	8.5 $\pm$ 1.41	15.6 $\pm$ 7.53

the equilibrium of physiological conditions including pH homeostasis (Zimmer and Topp 1997), oxygen concentration and redox potential (Bignell 1984; Zimmer and Brune 2005).

Depriving terrestrial isopods of their faeces reduces their growth rate (Hassall and Rushton 1985). Among advantages provided by recycling of faeces is the maintenance of an adequate level of copper and nitrogen (Hassall and Rushton 1985; Zimmer 2002a). Total nitrogen content is considered one of the most important parameters in the dynamics of a saprophagous population (Lardies et al. 2004). The highest rates of growth and survival in *Amadillidium vulgare* have been recorded in feeding experiments with increasing nitrogen content (Rushton and Hassall 1983a). Lardies et al. (2004) established the importance of dietary nitrogen in the life history of *P. laevis* juveniles, being beneficial for growth and survival.

Studies of feeding behaviour in terrestrial isopods showed that *P. scaber* prefers feeding on their own faeces,

especially aged ones rather than newly deposited (Hassall and Rushton 1985; Hassall et al. 1987; Ullrich et al. 1991). Coprophagy seems to be an efficient path to gain essential nutrients and to acquire the capability to breakdown phenolic compounds (Carefoot 1984; Ullrich et al. 1991; Zimmer 2002). In addition, several studies note the significant value of faeces as a bacterial and nutrient source (Wieser 1966, 1978; Horv athov a 2015).

Wang et al. (2007) found that the midgut of juveniles at an age of 1 week, and kept in non-sterile conditions, were colonized by bacteria that were different to those of conspecifics under sterile conditions. This finding supports the probability of environmental transmission of symbionts from the supplements of diets **L**, **F** and **G** to the isopod embryos in the present study. Hence, the higher rate of growth and survival within juveniles fed on basal diets supplemented with faeces (**F**) and gut (**G**) of conspecifics may be due to the colonization of the isopod gut by hepatopancreatic bacteria via these supplements. Hepatopancreatic bacteria colonize

the digestive tissues of terrestrial isopods through food sources, cannibalism or coprophagy (Brandstädter and Zimmer 2008). *Candidatus* Hepatoplasma crinocheatorum (*Mollicutes*) (Wang et al. 2004a) and *Candidatus* Hepatincola porcellionum (*Rickettsiales*) (Wang et al. 2004b) are two bacterial symbionts detected in the hepatopancreas of some terrestrial isopod species. These two bacteria may enable the digestive process by facilitating the breakdown of leaf litter (Zimmer and Topp 1998). Using faeces and gut as dietary supplements may pave the way for hepatopancreatic bacteria to reach the digestive tract of terrestrial isopods given that *Hepatincola* bacteria were detected in the gut of *A. vulgare* (Dittmer et al. 2016) and *P. pruinus* (Delhoumi et al. unpublished data). The hepatopancreatic symbiont *Hepatincola* and *Hepatoplasma* enhance host survival when fed a low quality diet (Fraune and Zimmer 2008).

In our study, maximal rate of growth and fertility were noted within juveniles fed on a diet supplemented with guts of conspecifics in comparison with those fed a control diet. This clear difference can be ascribed to the nutritional advantages provided by feeding on gut and especially hepatopancreatic symbionts. According to Leclercq et al. (2014), the genome of *Hepatoplasma* contains a set of genes presumably involved in the digestive process. Moreover, isolating bacteria from the gut of *P. pruinus* showed the presence of cellulolytic bacteria potentially involved in the breakdown of cellulose and then terrestrial leaf litter (Delhoumi et al. unpublished data).

An extensive literature exists on the subject of antibiotic interactions with arthropod microbiota, growth and fitness. Information is controversial according to antibiotic treatment and animal taxa. Indeed, despite that they showed the lowest survival, juveniles fed on a diet supplemented with streptomycin exhibited a higher rate of growth, ovigerous female size and fertility in comparison with those fed on the control diet. Antibiotics have been used to improve the weight gain and the development acceleration within arthropods (Ruan et al. 2006; Kopecky et al. 2014; Thakur et al. 2016). Equally, these biological compounds enhance the immune process of animals by eliminating the pathogenic strains and selecting the beneficial bacteria (Gibson and Roberfroid 1995; Sritunyalucksana et al. 2005; Anuta et al. 2011). So, apart from the effectiveness of streptomycin against a broad spectrum of bacteria (Greewood and Whitley 2003), a possible reason for the low survival of juveniles fed on a basal diet enriched with streptomycin is the antibiotic toxicity.

Juveniles fed on a mixture of foods grew faster than those with a basal diet alone (Rushton and Hassall

1983a). It has been shown that a single food source is generally not sufficient to cover the nutritional needs of isopods when compared to a mixed diet (Rushton and Hassall 1983b; Hassall and Rushton 1985). In this context, several reports investigating the life history of terrestrial isopods have shown the importance of food source type and quality on Oniscidea such as *Porcellio olivieri* (Kheirallah and El-Sharkawy 1981), *A. vulgare* (Rushton and Hassall 1983a), *P. scaber* (Zimmer and Topp 1997; Horváthová et al. 2015, 2016), and *P. laevis* (Lardies et al. 2004).

The transmission and acquisition mechanisms of bacterial symbionts are still poorly understood. In isopods, it has been proposed that horizontal and environmental transmission constitute pathways of acquiring bacteria especially given the different bacterial content in the midgut of terrestrial and freshwater isopods (Wang et al. 2007). So, in accordance with Horváthová et al. (2015) who assured the role of horizontal and environmental transmission in isopod performance, juveniles fed only on basal diet (where only the vertical transmission of symbionts is possible) exhibited the lowest rate of growth and survival.

The clear difference of growth rate between juveniles aged 2 and 4 months may be owing to accelerated development during first stages of life in comparison with the adult stage as mentioned by Achouri and Charfi-Cheikhrouha (2001) within this species. Horváthová et al. (2015) found a significant difference in growth between *P. scaber* juveniles weighed at 1 and 2 months excepting those fed on diets without supplements. Carefoot (1990) documented that the increase of age and size coincided with the decrease of growth efficiency and the increase of growth costs within the supralittoral isopod *Ligia pallasii*. Nevertheless, we can ascribe the difference in the number of embryos per brood between females to the reproductive strategy adopted. Thus, according to the environmental conditions such as food resources, reproductive females have the capability to choose an appropriate strategy which serves their specific performances and consequently that of progeny. Herein, females fed on a rich diet tend to maximize the number of embryos even though they are small. However, those reared with a poor diet quality produce a limited number of larger embryos able to surpass predation risk and food shortage hindrances (Lardies et al. 2004).

In conclusion, the stimulation of symbiont acquisition in *P. pruinus* provided evidence of the importance of horizontal and especially environmental transmission of symbionts. Symbiont acquisition allows isopods to meet their feeding requirements given the

low nutritive quality of terrestrial leaf litter. Individuals fed on a basal diet solely showed the lowest rate of growth, survival and fertility. Thus, apart from their palatability, supplements with a potential source of symbionts improve the nutritive status of a diet that acts positively on oniscidean life history. In the field, terrestrial isopods resort to nutritional alternatives like coprophagy, cannibalism and feeding on biofilm to surpass the nutritional boundaries like the low quality and the recalcitrant nature of phenolic compounds in leaf litter. Antibiotics are defined as growth promoters but also used to alter the intestinal microbiota. Herein they had a controversial effect; the low survival coincided with higher mass increase, reproductive female size and fertility in comparison with the control diet. Further antibiotic feeding experiments can shed the light on their accurate effect in the life history of Oniscidea.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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## Appendix The composition of artificial diet

Basal diet (%): casein 15%, cellulose 30%, starch 25%, sucrose 10%, maltose 5%, glucose 5%, lactose 5%, dipotassium hydrogen phosphate 1.15%, magnesium sulphate anhydrous 0.65%, copper chloride dihydrate 0.2%, sodium dihydrogenphosphatemonhydrate 0.45%, sodium chloride 0.2%, calcium hydrogenphosphate 0.65%, calcium lactate pentahydrate 1.55%, iron citrate 0.15%.