Protoplasma (2010) 241:83–89
DOI 10.1007/s00709-010-0110-3

ORIGINAL ARTICLE

Surface characteristics of isopod digestive gland epithelium studied by SEM

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Received: 12 November 2009 / Accepted: 13 January 2010 / Published online: 14 February 2010

Abstract The structure of the digestive gland epithelium of a terrestrial isopod *Porcellio scaber* has been investigated by conventional scanning electron microscopy (SEM), focused ion beam–scanning electron microscopy (FIB/SEM), and light microscopy in order to provide evidence on morphology of the gland epithelial surface in animals from a stock culture. We investigated the shape of cells, extrusion of lipid droplets, shape and distribution of microvilli, and the presence of bacteria on the cell surface. A total of 22 animals were investigated and we found some variability in the appearance of the gland epithelial surface. Seventeen of the animals had dome-shaped digestive gland "normal" epithelial cells, which were densely and homogeneously covered by microvilli and varying proportions of which extruded lipid droplets. On the surface of microvilli we routinely observed sparsely distributed bacteria of different shapes. Five of the 22 animals had "abnormal" epithelial cells with a significantly altered shape. In three of these animals, the cells were much smaller, partly or completely flat or sometimes pyramid-like. A thick layer of bacteria was detected on the microvillous border, and in places, the shape and size of microvilli were altered. In two animals, hypertrophic cells containing large vacuoles were observed indicating a characteristic intracellular infection. The potential of SEM in morphological investigations of epithelial surfaces is discussed.

Keywords Scanning electron microscopy · Focused ion beam · Digestive gland epithelium · Hepatopancreas · Isopoda · Crustacea

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Introduction

The digestive gland epithelium of the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea) has been thoroughly investigated by many authors (Štrus 1987; Hames and Hopkin 1989; Lešer et al. 2008). Significant alterations in their morphological characteristics were observed to be related to physiological condition (Hames and Hopkin 1989, 1991; Lešer et al. 2008). Additionally, there are also reports of differences in abundance and morphology of bacteria present on the surface of gland epithelium (Wood and Griffith 1988). However, to date no systematic investigation of gland surface characteristics using conventional scanning electron microscopy (SEM) has been conducted.

In terrestrial isopods, the hepatopancreas is composed of four blind-ending tubes, which lie freely in the body cavity. The hepatopancreatic epithelium contains two cell types, the large B cells that project into the lumen of the hepatopancreas and the wedge-shaped S cells that lie between the B cells. The B cells are secretory and absorptive; they usually contain many lipid droplets and glycogen, and they store some metal ions in granules (Hopkin and Martin 1982; Wägele et al. 1992). The S cells accumulate large amounts of metals such as calcium, and uric acid salts (Wägele et al. 1992). Morphological

changes of B cells were attributed to the 24-h digestive cycle (Hames and Hopkin 1991) and recently evidence was presented that B cells have a constant shape and size under normal physiological conditions (Lešer et al. 2008). However, when the animals are subjected to stress, the response is reflected in changes of shape, size, and lipid droplet content of B cells (Köhler et al. 1996; Odendaal and Reinecke 2003; Žnidaršič et al. 2003; Lapanje et al. 2008; Lešer et al. 2008). So far, however, no systematic approach was used to reveal the morphological variability of this epithelium of non-stressed animals.

It is interesting that SEM is rarely selected as a principal method for structural investigation of epithelial morphological characteristics in general. There are many reasons for the paucity of SEM investigations in biology. The development of both transmission electron microscopy (TEM) and SEM began in the 1930s, but TEM reached its full potential for biological imaging almost 30 years earlier than SEM (Pawley 1997). An important reason for this may be that the early SEMs required users to operate with a much higher beam voltage than was necessary to produce low-resolution images of biological samples, and this stimulated the search for alternative microscopy techniques. These were in many cases less suitable than optimized SEM for study of biological samples.

Currently, the focused ion beam–scanning electron microscope (FIB/SEM) system, which has an electron column and an ion column embedded in the same specimen chamber, has opened new and attractive possibilities in biological sample research. A major strength of the application of the FIB/SEM system to investigations of biological samples is its ability to conduct *in situ* site-specific manipulation of a specimen and imaging in a wide range of magnifications (Drobne et al. 2007, 2008).

One aim of this study was to use the potential of SEM for morphological investigation of the digestive gland epithelium surface. Another was to describe morphological characteristics of gland epithelium surface in a population of normal, unstressed animals and so to provide basic morphological characteristics of non-stressed animals, a benchmark for future physiological or (eco)toxicological research. Our objective was a systematic study of the epithelial surface by conventional scanning electron microscopy combined with FIB/SEM and light microscopy.

Materials and methods

Experimental animals and laboratory culture

Terrestrial isopods, *P. scaber* Latreille, 1804 (Crustacea: Isopoda), were collected in gardens under concrete blocks, pieces of decaying wood, or other organic waste. The stock cultures were kept in the laboratory for some weeks to allow animals to acclimatize and they were then analyzed by means of scanning electron microscopy or light microscopy.

The surface morphology of the hepatopancreatic epithelium was analyzed in 22 animals. In 11 of these, the digestive glands were investigated by both, light microscopy and SEM. In some selected animals, FIB/SEM was also used.

Scanning electron microscopy and FIB/SEM

The isolated digestive gland tubes were transferred into 1.0% glutaraldehyde and 0.4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2.5 h at room temperature. The chemically fixed samples were either directly dehydrated in a graded series of ethanol solutions or processed by the OTOTO method with OsO4/thiocarbohydrazide/OsO4/thiocarbohydrazide/OsO4 (Lešer et al. 2009) and then dehydrated in a graded series of ethanol solutions. The samples were then dried at the critical point (Balzers Critical Point Dryer 030, Liechtenstein) and fastened onto mounts with silver paint (SPI) and gold sputtered (Sputter coater SCD 050, BAL-TEC, Germany). Before SEM or FIB/SEM investigation, the tubes were mechanically opened in two or three regions in order to expose the surface of gland epithelial cells.

Digestive gland tubes were investigated with a field emission scanning electron microscope (Jeol JSM-6500F, at the Institute of Metals and Technology, Ljubljana, Slovenia) or by a focused ion beam/scanning electron microscope (FEI Strata DB 235 M, at the University of Modena, Modena, Italy).

The FIB system was used to expose the subsurface structures by ion milling. After rough milling, the sample cross-section was polished. Rough milling conditions to open a trench employed beam currents of 5 to 7 nA, at 30 kV. Lower beam currents of 100 to 300 pA were used to polish the cross-section. The spot size produced by rough milling was approximately 100–150 nm in diameter, and for polishing it ranged from 20 to 35 nm in diameter. The dwell time for milling was 1 μs and the overlap was 50%. In some samples, a 1–2 μm thick protective platinum strip was deposited on the sample prior to milling.

Light microscopy

For light microscopy, the isolated digestive gland tubes were transferred to the Carnoy-B fixative for 2.5 h at room temperature. This is a standard fixative in our histological and histopathological research and provides good results. After fixation samples were dehydrated in absolute alcohol,

transferred to xylene, and embedded in Paraplast Plus wax (Sigma). Cross-sections (8 μm thick; Reichert-Jung 2040 rotatory microtome, Austria) of the entire tube were cut, stained with eosin or hematoxylin and eosin and inspected with a light microscope (Axioskop 2 MOT, Carl Zeiss, Germany at Department of Biology, Biotechnical Faculty, University of Ljubljana).

Results

In digestive gland cells of 22 terrestrial isopods from the stock culture, we investigated the shape of B cells, extrusion of lipid droplets, shape and distribution of microvilli, presence of bacteria on the cell surface, or intracellular infection.

On the basis of these morphological characteristics, the digestive glands were divided into two groups. In the first group, B cells were regularly dome-shaped along the entire gland tube reaching up to 80 μm in height (Fig. 1a–e). In the second group, cells had significantly altered shape and were either much smaller or dome- or pyramid-shaped, or partly, even completely flat (Fig. 2a–e). Sometimes they were significantly hypertrophic (Fig. 3a–e). We refer to those in the first group as *animals with regularly shaped digestive gland epithelium cells*, while members of the

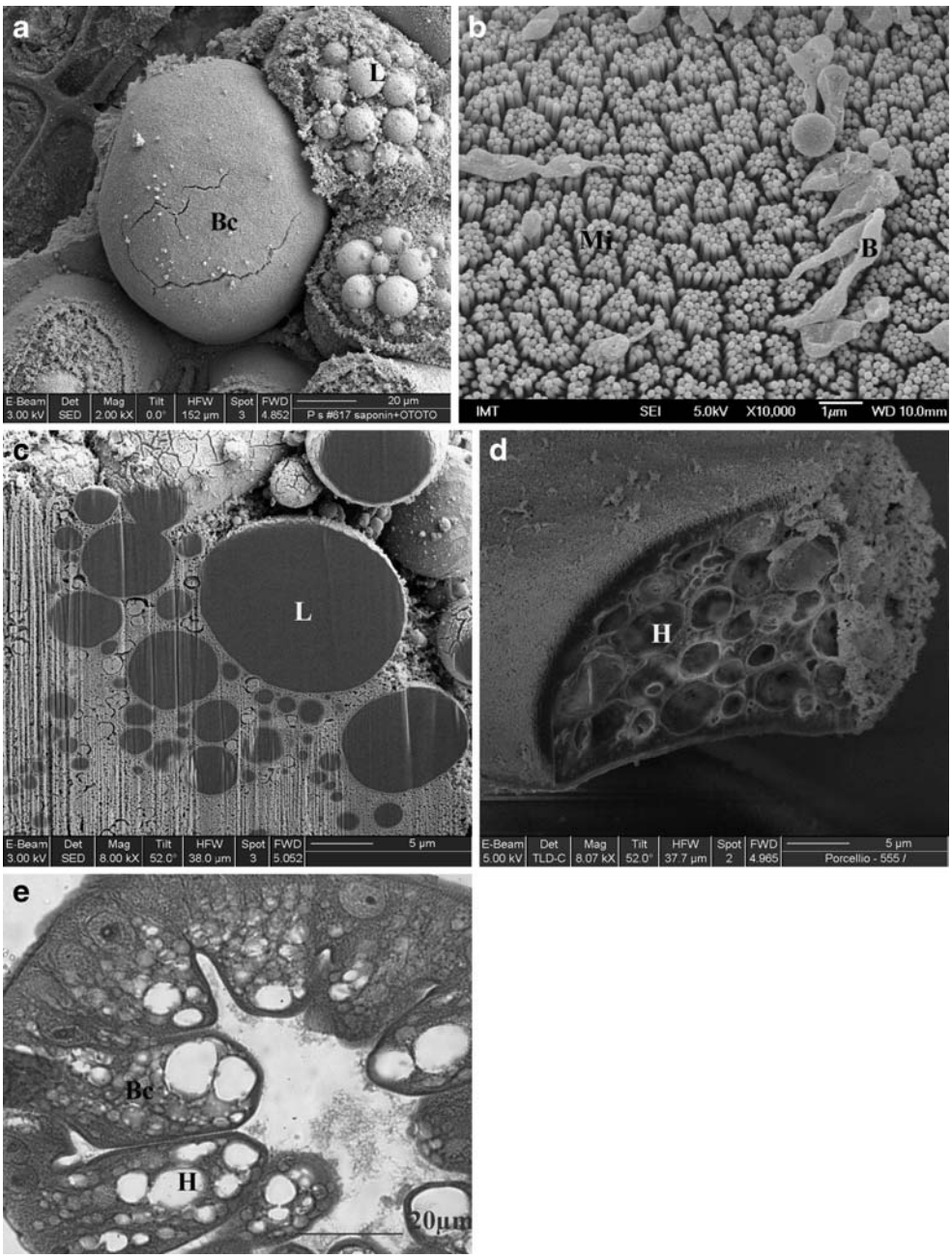

Fig. 1 **a–e** Normal appearance of digestive gland epithelium. **a** Some cells extrude lipid droplets, others do not. **b** Cells are covered by microvilli where bacteria are found. **c** FIB milling revealed that lipid droplets appear as vesicles filled with homogenous material. **d** If lipids are washed out during the preparation procedure. FIB milling revealed the empty voids where lipids were originally deposited. **e** If lipids are washed out during the preparation procedure for light microscopy, empty regions are seen where lipids were originally deposited. *Bc* B cells, *B* bacteria, *H* holes, *L* lipid droplets, *Mi* microvilli

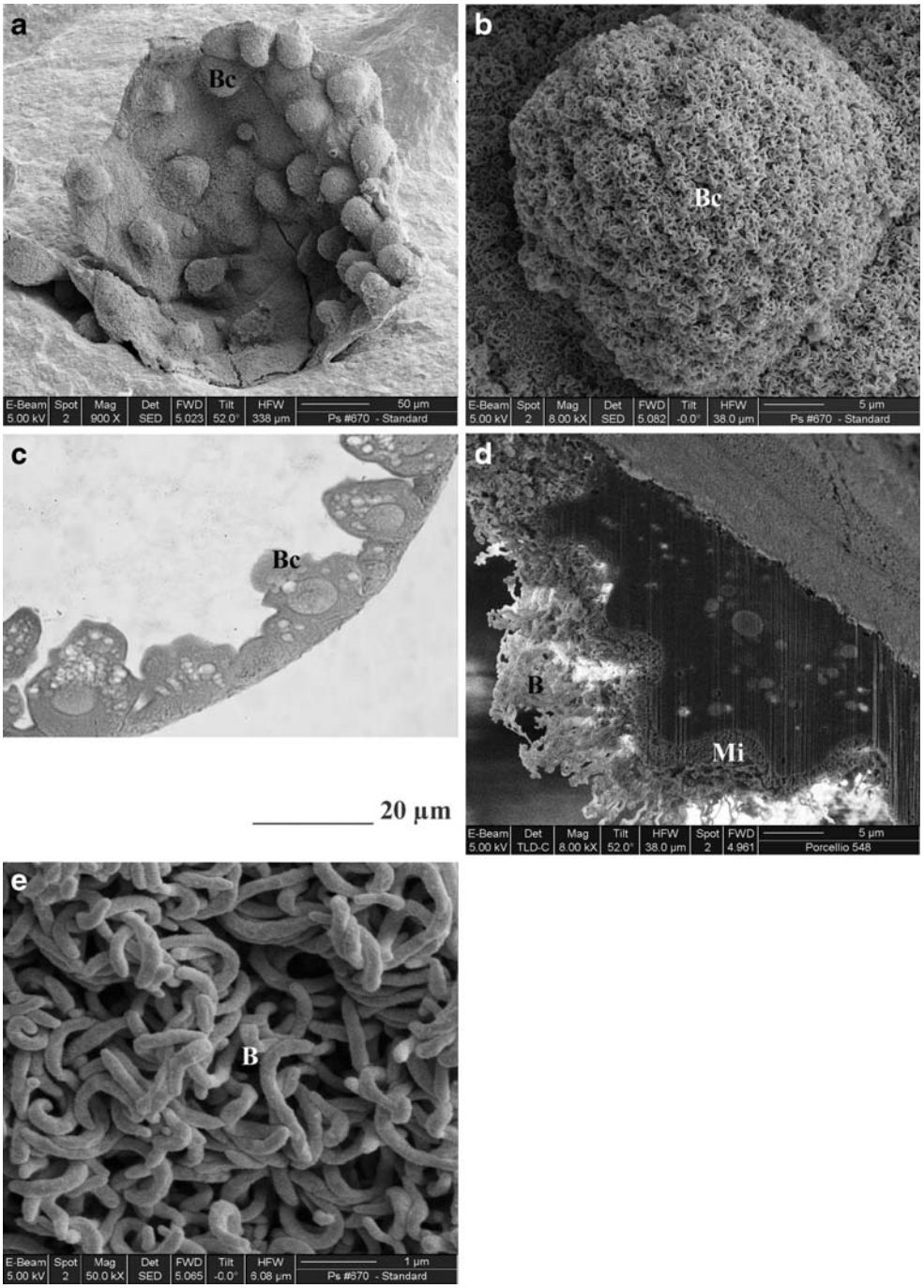

Fig. 2 **a–e** Abnormal appearance of digestive gland epithelium. **a**, **c**, **d** Cells are flattened or pyramid-shaped. **c** The cell surface is sometimes folded. **b**, **d**, **c** Cell surfaces are covered by a thick layer of bacteria. *Bc* B cells, *B* bacteria, *Mi* microvilli

second group are designated as *animals with irregularly shaped digestive gland epithelium cells*.

a. Regularly shaped digestive gland epithelial cells

In the 17 or the 22 animals investigated, the cells were dome-shaped and some of them extruded lipid droplets into the lumen of the gland tube (Fig. 1a, c). These epithelia varied in the proportion of epithelial cells which were extruding lipid droplets. In some glands, a majority of cells were extruding lipid droplets while in others, none of the cells did so. The apical parts of cells which are extruding lipid droplets appeared to be decayed. Other cells were evenly covered by microvilli (Fig. 1b). Here, we regularly observed single bacterial cells lying on the surface of microvillous border. They were sparsely distributed along the tube and had different shapes. They were spherical, quasi-cubic, or shaped like curved or spiral rods. Usually bacteria of similar shapes were detected in a single animal.

We indirectly confirmed the lipid content of extruded material by using a tissue preparation method which dissolves lipids. For this purpose, one tube of the same animal was prepared with the OTOTO preparation proce-

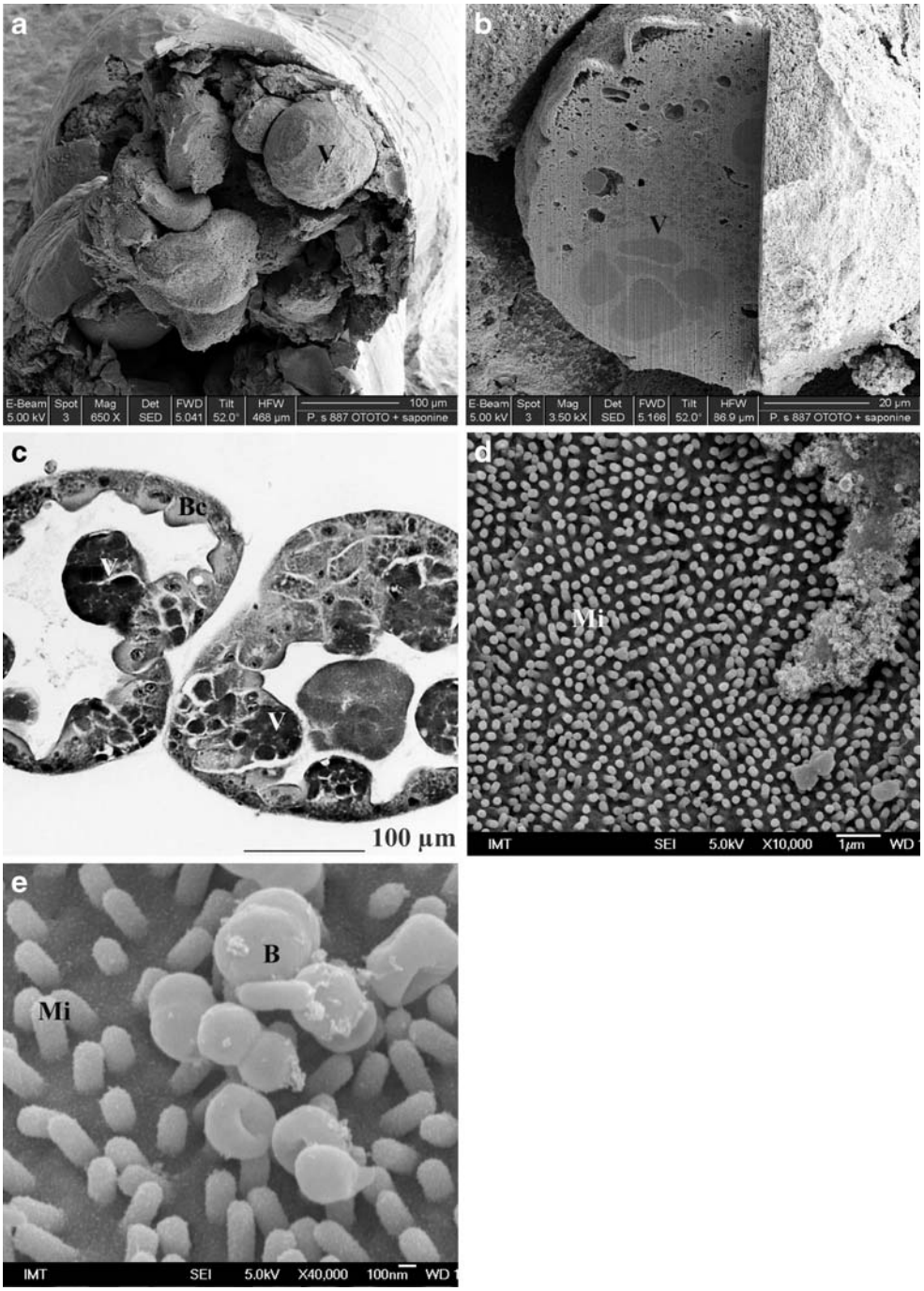

Fig. 3 **a–e** Abnormal appearance of digestive gland epithelium. **a**, **b**, **c** Some cells are hypertrophic. **b** FIB milling revealed that the vacuoles are not filled with homogenous material. **c** Light micrographs confirm that cells are not filled with lipids. **d** Cell surfaces are in spots covered with microvilli of altered shape or **e** spherical-shaped bacteria. *Bc* B cells, *B* bacteria, *Mi* microvilli, *V* vacuoles

dure, which preserves lipids (Fig. 1a, c). The other tube was neither postfixed nor conductively stained therefore lipids were washed out (Fig. 1d). The third tube was prepared for light microscopy following a procedure which also washes out lipids (Fig. 1e).

In addition, FIB milling revealed that OTOTO prepared cells are filled with homogenous material (Fig. 1c), while in those from which lipids were deliberately washed out, holes remained where originally lipids had been deposited (Fig. 1d). The same was confirmed by light microscopy which showed empty regions remaining after lipids were washed out during the preparation (Fig. 1e).

b. Irregularly shaped digestive gland epithelial cells

In three of the 22 animals, the apical and lateral cell surfaces were densely colonized by bacteria (Fig. 2a–e), and a thick layer of bacteria covers the entire epithelial surface. However, cells in distal parts of a gland tube were less densely colonized. In regions of epithelium with a high density of bacteria, alteration in the shape of the cells was

detected, the epithelium becoming flat with pyramidal shaped cells (Fig. 2a, c, d). Cell surfaces were often found to have an irregularly folded appearance (Fig. 2c, d). Where the bacterial coverage was less dense, we also observed changes in shape, size, and density of microvilli. In some regions, microvilli were absent.

In two of 22 animals, cells were hypertrophic, sometimes even more than 150 μm in diameter (Fig. 3a–c). They were filled with large vacuoles, which were not washed out during sample preparation as the lipids were. In cells opened by FIB milling it appeared that the vacuoles were filled with heterogeneous material (Fig. 3b). Similar observations resulted from light microscopy (Fig. 3c). In some cells, microvilli still retained their usual appearance, while in other cells they were short, sparsely distributed or even absent (Fig. 3d). Here we observed single bacterial cells lying on the surface of microvilli (Fig. 3e).

Discussion

This is the first systematic report on an investigation by SEM of the morphological characteristics of digestive gland epithelium of non-stressed terrestrial isopod *P. scaber*.

Our results are in agreement with findings of Lešer et al. (2008) who report that animals in good physiological condition have digestive glands with thick epithelia and B cells filled with lipid droplets. Animals in poor condition, for example starved or chemically stressed animals, have partly or entirely flat digestive gland epithelia with less lipid droplets. The SEM micrographs also reveal that the thinner digestive gland epithelium is related to dense bacterial colonization of cell surfaces. If thinner digestive gland epithelium is linked to stress and poor physiological condition of *P. scaber*, the dense bacterial coverage indicate that these animals are in suboptimal physiological condition. This is not in agreement with some other reports where the authors describe dense bacterial population as symbionts, which as such, play a crucial role in animals' digestion (Wang et al. 2004a, 2004b).

Furthermore, our results are in agreement with reported morphological characteristics of intracellular bacterial infection of hepatopancreas of *P. scaber* (Drobne et al. 1999). The most prominent sign of this infection are white spots between 100 and 200 μm in diameter along the entire gland visible by a naked eye. Previous TEM studies confirmed that these spots are aggregations of vacuoles in the cells that are densely filled with bacteria (Drobne et al. 1999).

Many authors provided scanning electron images of digestive glands of *P. scaber* where rod-shaped bacteria, slightly curved rods, or comma-shaped and spiral-shaped bacteria were observed (Hames and Hopkin 1989, 1991; Storch 1984; Wood and Griffith 1988). Some authors report the variation in their occurrence between sampling sites, collection dates, and substrate they were feeding upon (Wood and Griffith 1988; Lapanje et al. 2008). These reports are consistent with our findings.

In the study presented here, we have confirmed the potential of SEM in morphological investigation of epithelia to provide evidence which could not be obtained by other microscopies. SEM allows navigation through a large area of the sample and subsequently zooming into the area of interest.

Systematic SEM investigation of morphological characteristics of the digestive gland epithelium of the terrestrial isopod *P. scaber* provides a variety of information related to metabolism, nutritional status, and bacterial colonization of gland tubes. The results presented here show that SEM will provide biological structural evidence that contributes to complete morphological information at the tissue level.

Acknowledgments We thank FEI Italy for the access to Strata DB235 M at the University of Modena e Reggio Emilia, Italy. This work was supported by the Slovenian Ministry of Higher Education, Science, and Technology (J1-9475). We thank Bill Milne for English editing.

Conflicts of interest The authors declare that they have no conflict of interest.

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