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Food quality and microbial succession in ageing earthworm casts: standard microbial indices and metabolic fingerprinting

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Summary

The complex and dynamic nature of soil makes the assessment of soil biodiversity difficult. Findings presented here form part of a wider scheme to develop 'bio-sampling' methods based on earthworm cast analysis. Our overall aim is to achieve high throughput, metabolic procedures to monitor soil biological activity and, in particular, to investigate the potential for gene transfer from plants to soil micro-organisms and its consequences.
Here experiments were carried out to assess several potential foods (oat grain, fresh and aged tobacco) for *Lumbricus terrestris* and *Lumbricus rubellus*, species being considered for later experimentation. These experiments provided temporal microbial composition and activity data in ageing casts, an environment where gene transfer might occur. For both earthworm species, there was a predictable microbial succession (from bacteria to fungi) as casts aged. Species also differed in their cast microbiology in response to food type, but less so with more processed food. Analysis of ageing casts by FT-IR spectroscopy coupled with cluster analysis indicated greater chemical changes in casts of *L. terrestris* than for *L. rubellus*, regardless of food type.

Introduction

Earthworm casts provide a sample representative of soil receiving inputs of fresh plant residues. Enhanced gene transfer between bacteria has been found in earthworm casts (Daane et al. 1996). Therefore, casts were considered suitable material for evaluating metabolomic procedures for monitoring soil biological activity, particularly the potential for gene transfer from plants to soil micro-organisms.

Fine comminution of litter by earthworms (Schulmann & Tiunov 1999) modifies its microbial community and accelerates nutrient mineralization (Brown et al. 1998; Tiunov & Scheu 1999). Parle (1963) described a microbial succession in ageing casts, although Tiunov & Scheu (2000) found that trends in microbial activity and composition with time varied depending on litter type ingested.

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Experiments were carried out to assess several food substrates for *Lumbricus terrestris* and *Lumbricus rubellus*. These experiments provided data describing microbial composition and activity in ageing casts, the environment in which gene transfer might occur.

Materials and Methods

Earthworms were incubated in 2.2L pots (8 replicates) containing a mix of sand, moss peat and kaolin (OECD 1984). Similar live weights, though different numbers (8 or 20 respectively), of adults of the two species were used. An excess of food (processed oat grain, fresh tobacco (*Nicotiana tobaccum*) or aged tobacco) was added to the surface of these pots at weekly intervals. Tobacco was 'aged' by storing fresh leaves on foil at 28 °C for 7 days. These substrates differed in their composition (Table 1), with the oat grain markedly lower in lignin and cellulose; the most notable effect of ageing was the large increase in soluble carbohydrates.

Table 1. Constituents (as % of dry weight) of processed oat grain, fresh tobacco or aged tobacco as percentage of total dry weight

Food type	N	P	WSC*	Lignin	Cellulose
Fresh tobacco	4.77	0.48	3.75	1.23	9.54
Aged tobacco	2.53	0.47	23.51	1.58	9.43
Oat grain	2.40	0.51	4.02	0.87	0.75

*WSC – water-soluble carbohydrates

After 2 weeks, pot surfaces were cleared of casts, fresh food was added and all casts were collected on each of the next three consecutive days. Cast materials were sub-divided, with some analysed immediately whilst the remainder were aged for up to 16 days (22 days for respiration) and sampled at intervals. Cast incubations were carried out in the dark at 15 °C in a humid atmosphere; *L. rubellus* produced insufficient material for some of the final (16 day) analyses.

A range of microbial indices was measured, including viable bacteria counts (based on Weinbauer et al. 1998), hyphal length using calcofluor staining (based on Bardgett 1991) and CO_2 production (Sparling 1981). Species and food type differences were assessed by two-way analysis of variance

FT-IR analysis

Cast samples were frozen at −80 °C until analysed. The final concentration was 125 mg cast/ml physiological saline. Aliquots (10 µl) of cast suspensions were added to wells on an aluminum plate and dried at 50 °C for 30 min. Each sample was triplicated. FT-IR data were collected using Opus software (version 2.1) (Goodacre et al. 1998). The scan range of spectra was 4,000 to 600 cm^{-1} and resolution was 4 cm^{-1}; each spectrum was represented by 882 points.

To minimize baseline shift problems, spectra were normalised for absorbance from 0 to +1 and the smoothed first derivatives of these spectra calculated using the Savitzky-Golay algorithm. Absorbance data were then analysed by principal components analysis (PCA) to reduce their dimensionality from 882 to 20 PCs. Next discriminant function analysis (DFA) differentiated between groups based on these retained PCs and the *a priori* knowledge of which spectra were replicates, a process that does not bias the analysis. Only DFA analyses are presented here. Statistical analysis was performed using Matlab version 5 (MathWork Inc., Natick, MA, USA) as detailed in Goodacre et al. (1998).

Results

Bacterial counts (Fig. 1) showed a large initial increase followed by a rapid decline by day 4. Counts were highly variable and with the only significant difference ($P < 0.05$) between food types on day 4, aged tobacco having lower values. For most treatments, hyphal length (Fig. 2) increased to day 4, then declined by day 8 but increased again to day 16. Oat grain gave consistently higher ($P < 0.001$) hyphal lengths than tobacco. When averaged across the three food types hyphal lengths tended to be greater for *L. rubellus*, but these differences were significant ($P < 0.01$) only on days 4 and 8. Respiration (Fig. 3) generally peaked on day 2 with a secondary peak occurring on day 10. On most days, respiration across the three food types was significantly higher ($P < 0.05$) for *L. rubellus*, mainly due to the very high respiration rates for this species when fed fresh tobacco; respiration was higher for oat grain on day 1 but lower on day 22 ($P < 0.01$). There were several significant earthworm species × food type interaction effects ($P < 0.01$). Respiration for *L. rubellus* casts was markedly higher than corresponding *L. terrestris* casts only for fresh tobacco. Also, hyphal lengths were higher in casts from *L. terrestris* fed on fresh tobacco than for *L. rubellus*, but the reverse was the case when aged tobacco was the food type. Overall, variations between earthworm species in microbial indices tended to be less with processed oat grain than was the case for tobacco.

FT-IR data showed separation by earthworm species and age of cast for all food types. However, this sepa-

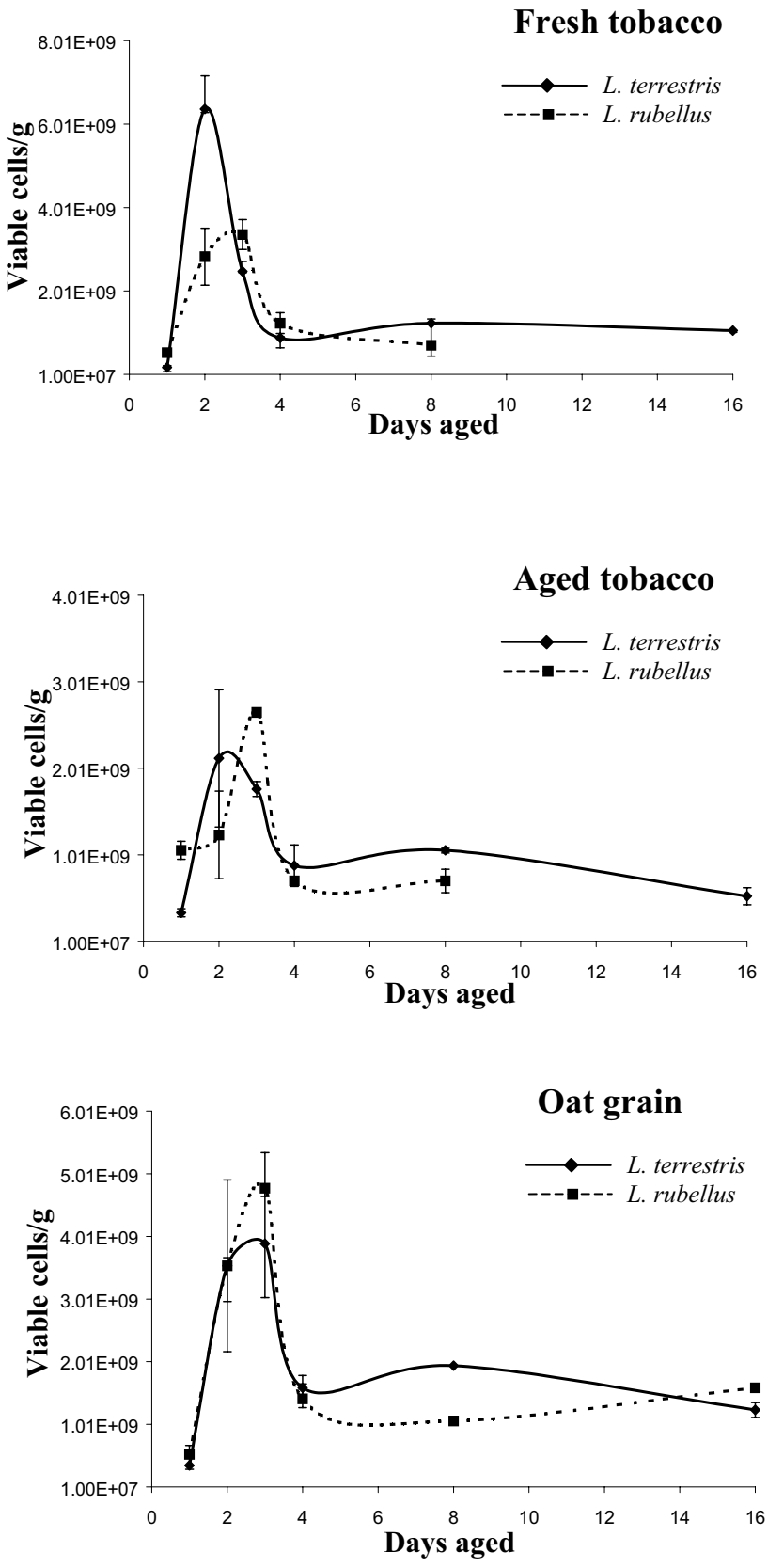

Fig. 1. Viable bacterial counts in ageing casts for different earthworm species and food types

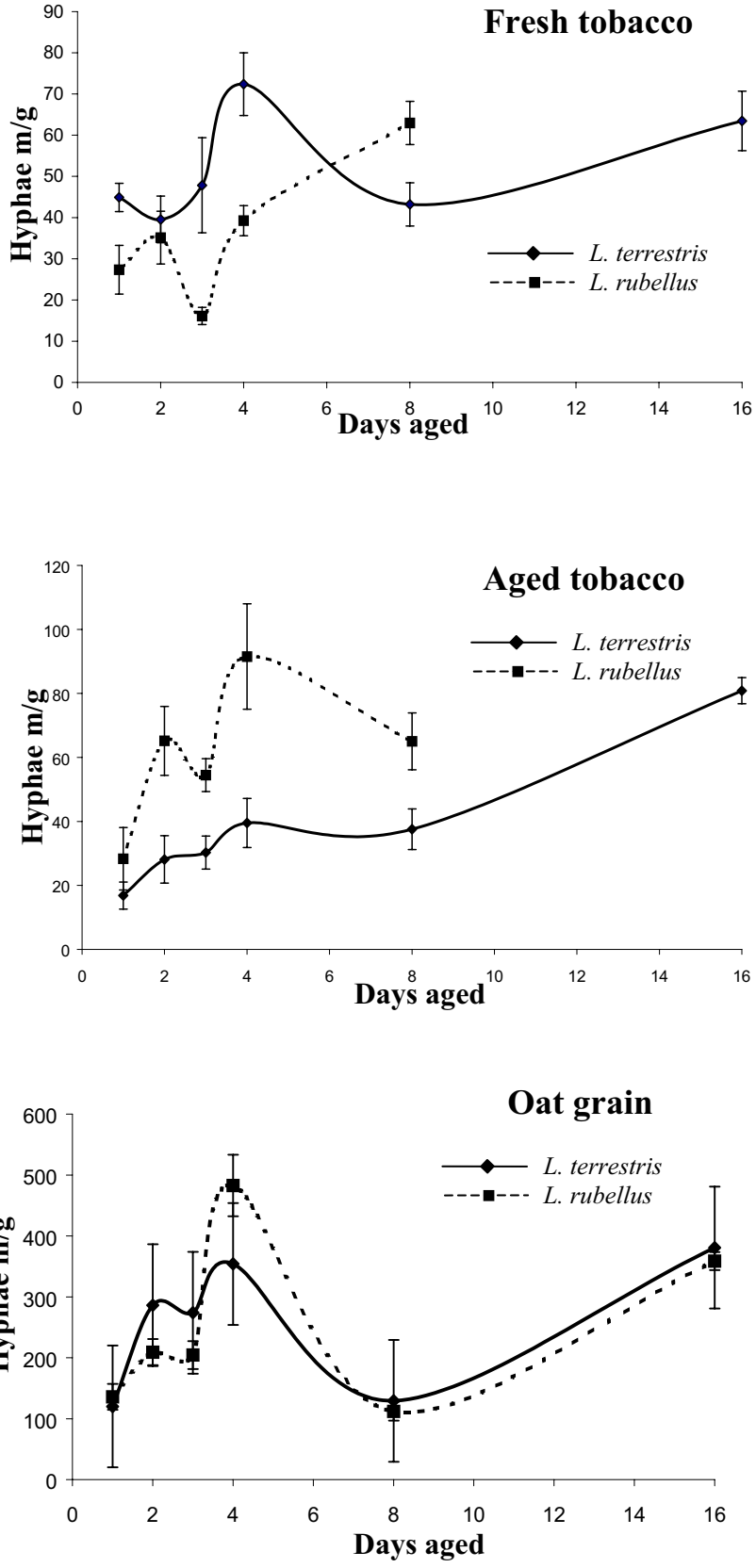

Fig. 2. Hyphal length in ageing casts for different earthworm species and food types

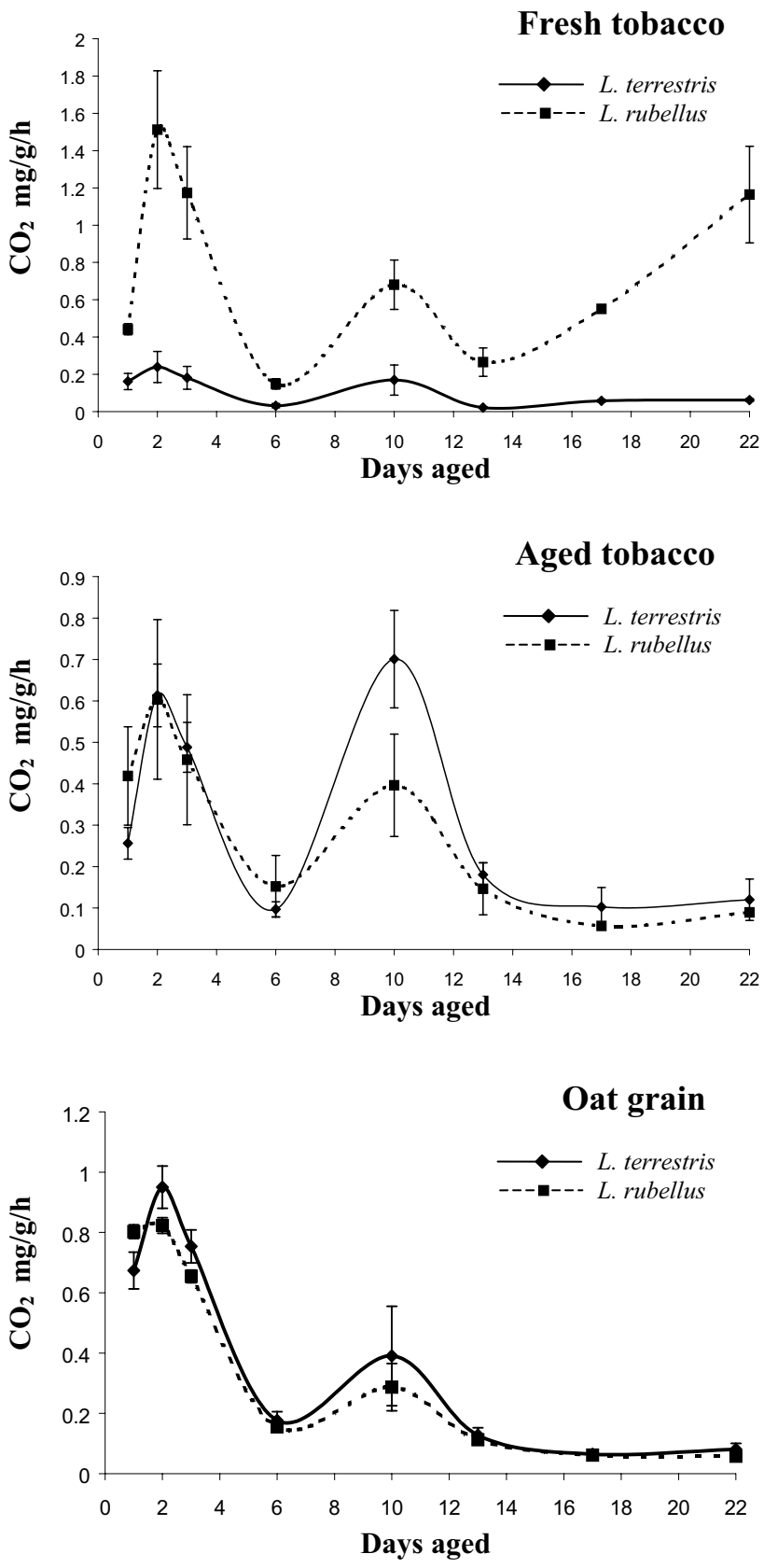

Fig. 3 CO_2 production in ageing casts for different earthworm species and food types

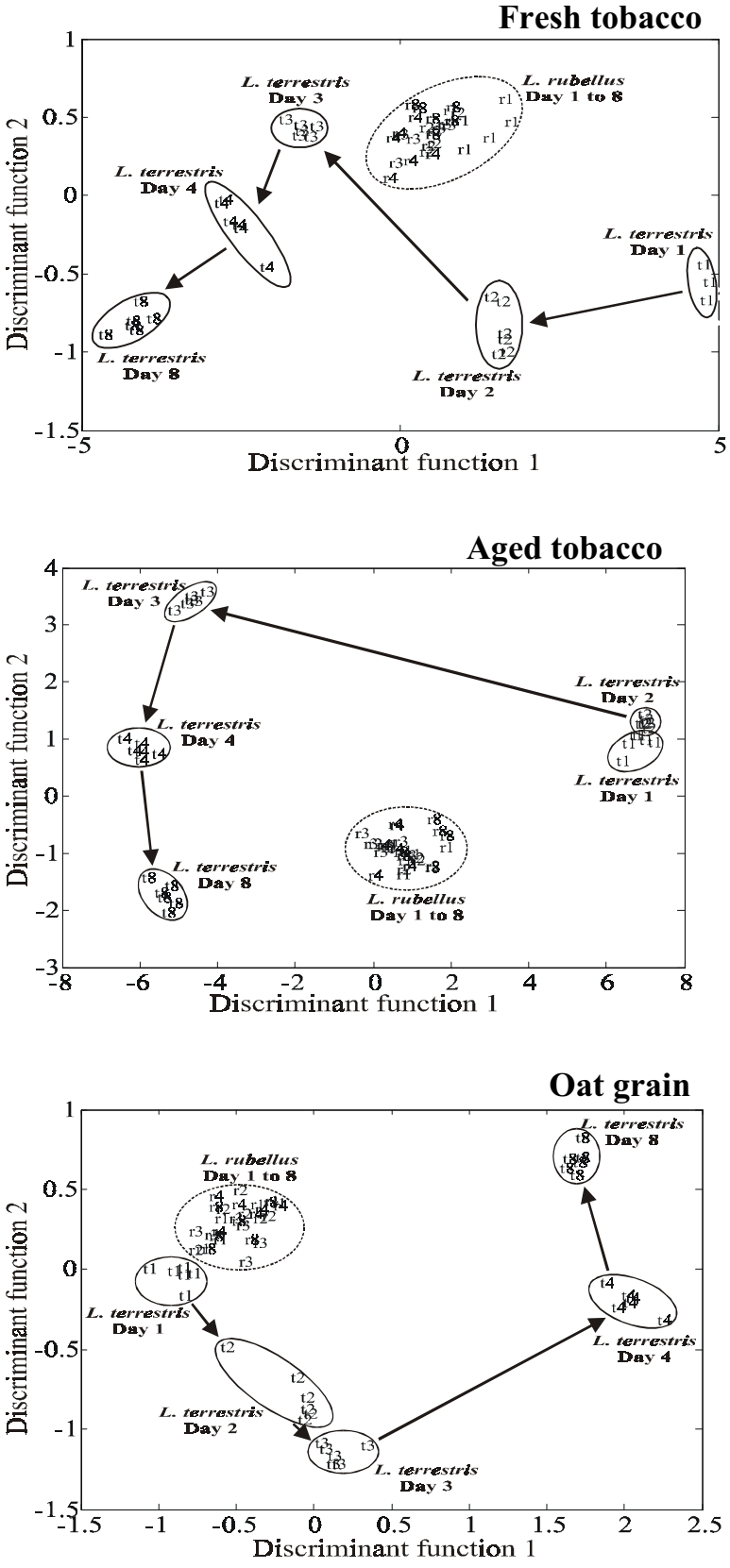

Fig. 4. Joint cluster analysis (DFA) of FT-IR data from ageing casts for different earthworm species and food types

ration was more pronounced (Fig. 4) for *L. terrestris* than for *L. rubellus*, and for tobacco compared with processed oat grain. When *L. rubellus* data were analysed separately (data not shown), DFA clearly separated casts by age.

Discussion

There were predictable microbial successions within casts of both earthworm species as they aged, broadly similar to those found in previous studies (Parle 1963; Tiunov & Scheu 2000). With ageing, activity decreased and there was a shift from bacterial to fungal dominance within the population. Where differences occurred, microbial indices tended to be higher for *L. rubellus*. The particularly high hyphal lengths for the oat grain could be explained, in part, by observed extensive fungal colonisation prior to ingestion by earthworms.

Casts of the two earthworm species differed in the microbiological response to the fresh or aged tobacco leaves, but less when fed with more processed food. Oat grain processing physically disrupted plant structures, a 'pre-treatment' not affecting tobacco. One explanation for the limited species differences for grain fed earthworm casts may be that variations in the extent to which earthworms comminute their food could not be expressed in material that had already been 'comminuted' during the manufacturing process and thus could not have affected cast microbiology. Variations in their gut flora and activity and/or variations in residence time may also have an effect.

FT-IR spectroscopy, coupled with DFA clearly tracked changes in cast material. There were larger changes in casts of *L. terrestris* than in those of *L. rubellus*, regardless of food type. This finding was not apparent in the microbial data measured and contrasted with the generally higher, more variable microbial indices for *L. rubellus*; work is continuing to elucidate relationships between FT-IR data and cast microbiology.

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