Hylotrupes bajulus (L.) (Col., Cerambycidae): nutrition and attacked material

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DOI: 10.5073/jka.2010.425.241

Abstract

Hylotrupes bajulus, attacks softwood utilising the cellulose contained in wood walls as food. The fibre is digested in variable percentages, depending on the type of analysis, 20 to 48% and, according to some authors, without the assistance of intestinal symbiotic microorganisms. Furthermore, there is published work referring to *Hylotrupes*, concluding that "starch" … "plays no role in the nutrition of the larvae". Nevertheless, considering that attacks of this species decrease with wood seasoning increasing and having been demonstrated, and that "lignin degradation products of spruce wood do not influence larvae development", it is possible to suppose that cell walls alone are not sufficient to feed this wood boring species. Furthermore, *Hylotrupes* larvae have chisel shaped mandibles, similar to those of powder post beetle larvae that feed on starch and need to pulverise the wood to access the cellular content. Preliminary research suggests an utilization of wood fibre as well as of starch by larvae of *H. bajulus*.

Therefore, the purpose of this research is to test the degree of digestion of wood fibre from different sources (sapwood or heartwood) and the possible role of symbiotic microorganisms. Larvae of *H. bajulus* were grown on synthetic diets made of purified wood fibre and/or starch as main components supplied with mineral and vitamin. Substrates and frass were analysed for fibre fractions, starch and acid insoluble ash, the latter used as an indigestible marker. Larvae purified DNA was analysed by means of metagenomics approaches carried out by direct retrieval and analysis of 16S rRNA gene sequences free of cultural bias in order to discover the bacterial diversity from larva alimentary channel alone. Larvae of *H. bajulus* seem be able to digest either fibre or starch, and a role for symbiotic bacteria is supposed.

Keywords: Cellulose, Starch, Frass, Mouth apparatus, Mandible

1. Introduction

The house longhorn beetle *Hylotrupes bajulus* L. (Coleoptera: Cerambycidae) is an important pest of structural timber. It mainly occurs in roof spaces, where summer temperatures become high enough to permit flight and egg laying. *Hylotrupes bajulus* larvae usually feed in the sapwood of the coniferous genera Pinus, Picea and Abies, but, in the late stages of infestation, can also penetrate in heartwood (Eaton and Hale, 1993). It is an insect of considerable size, reaching, as an adult, 20-30 mm in length. The larva, cream coloured, has the typical form of the Cerambicidae larvae and, fully grown, measures about 22-25 mm.

The female oviposits in cracks within the wood, choosing almost exclusively conifer wood, as it is attracted by α-pinene and β-pinene, volatile substances present in these plants. However, attacks on hardwood trees (e.g. poplar and willow) have also been recorded though it is suspected that these are probably due to misidentification of the larvae, very similar to those of *Trichoferus holosericeus* Rossi: the lignin of hardwoods, in fact, contains a toxic substance that does not allow the development of *Hylotrupes larvae* (Cymorek, 1981). Development lasts from 1 to 7-8 years, with peaks of up to 17 years, depending on the temperatures.

Damage can be detected even in very old structures, but, in this case, involves parts of the structure replaced in more recent years (Cymorek, 1973), as attacks of this Cerambicidae occur predominantly during the first 80 years of the use of the timber, subsequently decreasing markedly, and disappearing after 100 years.

Few insects can digest wood with their own enzymes and they are not very effective in their action. Precisely for this reason it seems that most wood-boring insects have evolved complex symbiosis with micro-organisms specialized in the degradation of wood components (Battisti, 2001).

Hylotrupes seems not to exploit the action of endo-symbiotic microorganisms for the digestion of cellulose: in fact, Falck (1930) states that it secretes an enzyme, cellulase, and can digest about 20% of the cellulose and hemicelluloses of attacked softwood, the remaining indigestible 80% being expelled with the faeces. Its long development cycle, like that of many wood-borers, clearly attests the difficulty in obtaining energy from cellulose and in using the few proteins present in the woody tissues in which it usually lives (Battisti, 2001). In fact, if proteins are added to the diet of the larvae, there is an acceleration of growth (Schuch, 1937) showing that lack of proteins in the diet can be a limiting factor. In the thirties and forties, research carried out on *H. bajulus* nutrition showed that, notwithstanding that this species digests cellulose (Falck, 1930), there are no micro-organisms in the gut and therefore the cellulase is endogenous (Muller 1934; Mansour and Mansour-Bek, 1934). Parkin (1940) concluded that Cerambycidae are able to use cell contents and carbohydrates of the cell wall including cellulose. Cazemier et al. (1997) demonstrated β-glucosidase and carboxy methylcellulasis (CMC-ase) activity in the whole *Hylotrupes* gut (but very high in the foregut), while only a low number of bacteria were present only in the midgut, suggesting the endogenous nature of the cellulolytic enzymes. Höll et al. (2002) found about the same amount of alpha-glucans (considered mainly starch) in faeces and in the alimentary substrate while a significantly greater amount of beta-glucans (result of partial break-down of cellulose during digestion of a wood diet) was present in the faeces. They concluded that starch might "play no role in the nutrition of the larvae" and that the presence of a high amount of beta-glucans in faeces indicates the digestion of cellulose and hemicelluloses and, at the same time, "a surplus uptake of wood into the digestive tract in order to acquire compounds such as vitamins and reduced nitrogen".

Concerning the assessment of *Hylotrupes* larvae microbiota biodiversity, it is well known that several micro-organisms cannot be grown readily in pure culture, and that culturing does not capture the full spectrum of microbial diversity of a given environment (Handelsman, 2004). For this reason, several culture-independent methods were designed to describe the phylogenetic diversity in several environments. Among the methods developed to gain access to the genetics and physiology of uncultured micro-organisms, metagenomics, the isolation of bacterial genomic DNA from an environment followed by its direct analysis, has emerged as a powerful identification technique (Handelsman, 2004).

In this study we applied a metagenomic approach to the analysis of the woodworm's gut microbiota. The aim of the present study was to investigate the alimentary habits of this insect, focusing on starch as a possible key nutrient for larvae and the presence of bacteria in the gut.

2. Materials and methods

2.1. Diet experiment protocol

In order to determine the use of starch, cellulose and hemicelluloses by the *Hylotrupes* larvae, four diets were prepared with different percentages of starch (0, 3, 14) and different fibre sources (heartwood and sapwood): sapwood-starch 0%, sapwood-starch 3%, heartwood-starch 3%, sapwood-starch 14%.

The artificial diet was prepared scraping off the wood in a fine powder, washing it to remove starch, drying it in an oven and mixing it with starch in the specified proportion, minerals and vitamins (1.5%), albumin (6%) and water and arranging it in a square container. After desiccation in a microwave oven, artificial diet cakes were sawn into blocks measuring cm 5 x 5 x 1. Each one was prepared with a hole of the same diameter as that of the larvae and weighed.

The larvae used in this experiment were about 6 month old and were obtained from laboratory-breeding blocks maintained in a temperature-controlled room at 27 ± 2°C and 75 ± 5% r.h. The larvae were extracted from breeding blocks, left in Petri dishes without food for 24 h, weighed, and transferred to the different substrates (1 larva/ block). Each block was isolated in a ventilated container and placed in a temperature-controlled room at 27 ± 2°C and 75 ± 5% r.h. Eight larvae were used for each diet.

After 30 d, the larvae were taken off the blocks, checked for vitality and weighed. The frass produced by each larva during this period of time was weighed, too. Both the frass and the substrate were analysed to assess starch and fibre content. Fibre fractions (neutral detergent fibre= NDF; acid detergent fibre= ADF; acid detergent lignin = ADL) were analyzed according to Goering and Van Soest (1970), and starch by an enzymatic method (AOAC Method 996.11). Hemicelluloses content was calculated as NDF minus ADF, and cellulose as ADF – ADL. All the data were expressed on a dry matter basis.

2.2. Statistical analyses

The statistical processing of the weight of frass product and the weight difference between beginning and end of the larvae feeding using the four different diets was performed using the ANOVAs one-way (univariate) method with the aid of the SPSS 15.0 program: analysis with the diets as the dependent variable was performed for the larvae percentage of weight increase and frass weight (independent variables).

2.3. DNA analyses

Fully grown larvae of H. bajulus taken from laboratory-breeding, were dissected, the separated guts were then isolated in Eppendorf tubes and frozen at -20°C. Prokaryotic and Eukaryotic DNAs were extracted from single guts and the purified DNA was used as target DNA for amplification of 16S rRNA gene.

The selective amplification of bacterial ribosomal RNA gene was achieved by using two universal primers for V2-V3 region of 16SrRNA gene: HDA1-GC (CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-ACT CCT ACG GGA GGC AGC AGT)/HDA2 (GTA TTA CCG CGG CTG CTG GCA C) (Walter et al., 2000) used in the reaction mix (Megamix, Labogen s.r.l) at 0,5 µM final concentration.

A 35-cycle PCR will run in a GeneAmp 9700 (Applied Biosystems, Foster City, CA) using the following profile: 94°C for 1 min, 56°C for 1 min, and 68°C for 60 sec.

The resulting amplification product was a mixture of different bacterial ribosomal gene sequences.

DGGE (Denaturing Gradient Gel Electrophoresis) analysis was performed with a DGene System (Bio-Rad Hercules, California) using 0.8 mm 8% polyacrylamide gel ratio of acrylamide to bisacrylamide, (37.5:1), containing a 35 to 55% gradient of urea and formamide for amplicons obtained with primers Hda1-Hda2. In order to identify at the species level bacteria producing the DNA pattern in DGGE analysis, bands were excised from the stained gels with a sterile scalpel and eluted in 20 µL of sterile water overnight at 4°C. One mL of the eluted DNA of each DGGE band was used as target DNA in PCR reaction using the conditions described above. The resulting PCR products were purified using Wizard® SV Gel and PCR clean-up system (Promega Madison, WI, USA) and sequenced.

The amplified 16S rRNA gene were ligated into the pGEM-T easy vector (Promega) and then transformed by heat shock into chemically competent Escherichia coli cells strain JM109 (Promega). The colonies were screened for α-complementation of β-galactosidase by using X-gal and IPTG. Positive colonies, containing the plasmid with the ribosomal RNA gene, were selected (Fig. 1). Plasmid DNAs were extracted and the inserts were sequenced using the BigDye v3.1 sequencing kit (Applied biosystems). The sequences were loaded and ran on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) (Fig. 2). Finally sequence data were used in search for the closest known relatives to the partial 16S rRNA sequences obtained, using the BLAST and RDP programs.

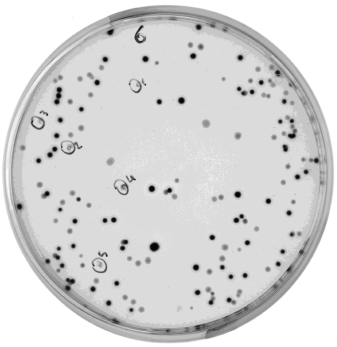

Figure. 1 A Petri dish showing blue and white colonies. The white colonies (positive) contain the plasmid with the desired fragment while the blue colonies (negative) contain the self-ligated plasmid.

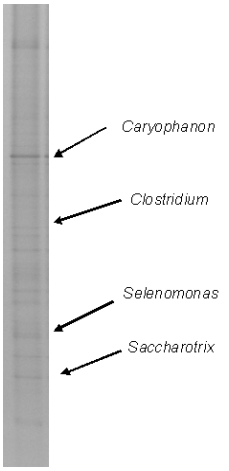

Figure 2 DGGE profile obtained from GI *Hylotrupes* larvae: band 1 corresponds to Caryophanon, band 2 to Clostridium, band 3 Selenomonas, band 4 to Saccharotrix.

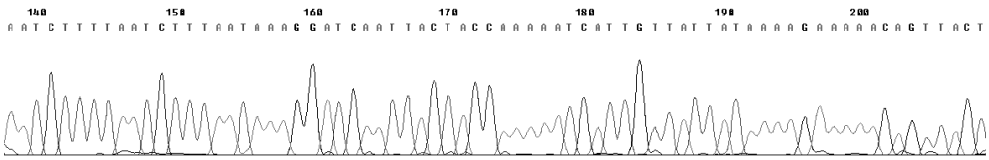

Figure. 3 Example of sequence obtained using the Genetic Analyzer ABIPrism 3100.

3. Results

3.1. Diet experiment

The statistical results of the diet experiment are summarized in Table 1. Larvae fed on diets poor in starch (0 and 3%) showed a weight variation (reduction of about 20%) statistically different (significant at P=0.01) from that (increase of about 0.6%) recorded in those fed on the diet rich in starch (14%).

Table 1 Summary table of analysis of variance on the average values of key variables examined in the various theses. F = F of Fischer; ns = not significant; * = significant per p< 0.05; ** = significant for p< 0.01. Mean values followed by different letters are different from the SNK (Student Neunman Keuls) for P<0.05. The values expressed as percentages were transformed for the compilation of statistical differences, in their angular values.

Diets	initial weight larvae (g)	weight increase (%)		frass weight (g)	
Sapwood 0%	0.2856	-17.73	b	2.839	a;b
Sapwood 3%	0.2865	-22.44	b	4.387	b
Heartwood 3%	0.3331	-17.78	b	3.516	a;b
Sapwood 14%	0.3265	0.632	a	2.052	a
F (significance)	0.288 ns	8.948 **		3.054 *	

The results of chemical analyses are reported in Table 2; all values are expressed as a percentage. NDF values show a similar quantity of fibres in the substrate and frass for all types of diet. The NDF value is obviously slightly higher in the frass because the insect used the soluble components present in the plant cells and therefore the frass-fibre is more concentrated. ADL values were used as indigestible markers in the digestibility estimate, as the amounts of acid-insoluble ash were negligible. The values of starch digestibility in the diets with 0% and 3% of starch were omitted because the amounts were too low to perform a correct analysis. In the diets with 3% of starch the digestibility of all fibre constituents is about the same. The fibre digestibility decreases with increasing amounts of starch in the diet.

Table 2 Digestibility of different fibre fractions and starch in the different diets. The values of starch digestibility in the diets at 0% and 3% of starch were omitted because the amounts were too low to perform a correct analysis; neutral detergent fibre= NDF; acid detergent fibre= ADF.

Fibre fractions	Sapwood 0%	Sapwood 3%	Heartwood 3%	Sapwood 14%
NDF	17.0	9,4	8,6	1,8
ADF	15.3	7,6	5,8	-0,1
Hemicellulose	28.8	16,6	18,9	8,7
Cellulose	23.0	11,5	8,6	-0,2
Starch	-	-	-	11,1

3.2. DNA analyses

DGGE profiles allowed us to identify bands related to the genera Caryophanon, Clostridium, Selenomonas, Saccharotrix. While, considering a partial sequence of about 450 bp of the ribosomal fragment and excluding the uncultured sample record presented in the database, we found sequences showing good homologies (usually with high coverage and max identity greater than 98%) with database records related to several genera: Streptococcus, Staphylococcus, Lactobacillus, Propionibacterium, Pseudomonas, Pelomonas, and Actinobacterium bacteria. Some sequences showed good homologies, but with 96% of max identity, with Gemella bacteria. Finally, with 90% of max identity, we also found some relations with Clostridium and Anaerosporobacter bacteria.

4. Discussion

Hylotrupes larvae, like those of *T. holosericeus*, the other Cerambycid that attacks timber, have chisel shaped mandibles (Schmidt & Parameswaran, 1977), similar to those of powder post beetle larvae that feed on starch and need to pulverise the wood to access the cellular content. The frass they produce is composed of extremely fine wooden fragments derived from the insect tunnelling mixed with faeces (elements cylindrical in shape). The fact that the frass is not made up of by faeces alone, as it is in the case of Anobiidae, proves that the larva does not ingest all the material it tunnels. This could suggest the idea that the larva operates a choice during feeding activity, separating what is necessary from what is not. In fact it seems that the wood powder/faeces proportion is much higher in larvae fed with starch-deprived diets. This is also the reason why we have analysed the whole frass and not only the faeces.

Contrary to what was suggested by Höll (2002), our results indicate that starch is utilized by Hylotrupes larvae as a nutrient source. In the 14% starch diet the larvae gained weight while with the other diets they lost weight. In addition, the total frass produced was less than in the other diets, even if not always significantly. This could demonstrate a lower tunnelling activity of the larvae in the case of higher starch content because of the adequate nutrients in the alimentary substrate.

A depressive effect of higher starch content on fibre digestibility is evident (from 17% of NDF digestibility in sapwood diet with 0% of starch to 1.8% in sapwood diet with 14% of starch) and is probably due to the presence of starch as a more readily available food source than cellulose and hemi-celluloses. This agrees with findings in higher animals with symbiotic micro-organisms (ruminants). However, two mechanisms can be postulated: a depressive effect of starch on symbiotic microorganisms with fibrolytic activity or reduced secretion of fibrolytic animal enzymes in the presence of significant quantities of starch in the diet. In addition, it cannot be excluded that starch availability lowers ingestion of fibrous fragments and then fibre digestion. Nevertheless, a certain digestibility of hemi-celluloses is maintained also in starch-rich diets and this could represent a relevant source of energy for the larvae, besides the starch.

DNA analysis highlights the presence of bacterial DNA in the Hylotrupes larval gut, confirming the results of Cazemier et al. (1997). Metagenomics involves the direct analysis of bacterial DNA extracted from a certain sample bypassing the need for culturing. Considering this, the bacterial 16S rRNA genes were selectively amplified from the extracted DNA by means of some universal primers, producing a mixture of fragments with different sequences that represents a picture, even though partial, of the real bacterial complexity in the starting samples. This mixture of fragments was analysed using two different methodologies: 1) DGGE followed by sequencing of some excised bands, and 2) cloning the PCR

products into a suitable vector, transforming them into a E. coli host strain and finally sequencing the resulting transformant clones.

The combined use of these two approaches shows that the bacterial diversity from Hylotrupes's gut is surprisingly high: indeed we determined the presence of bacteria related to several genera.

It is not surprising that we obtained different results with the different approaches considering that these analyses are still at the beginning, and up to now only a few DGGE bands and single clones (globally 32) have been sequenced.

Concerning the ribosomal DNA analysis, only a fragment of the 16S ribosomal RNA gene, was analysed, obtaining preliminary data. We think that more information about the genera and eventually the species present in Hylotrupes gut will be obtained by a complete sequencing of the different clones analysed.

Relating to the genera Gemella, Clostridium and Anaerosporobacter, it must be noted that the max identity is not very high, ranging from 90 to 96%. This could be due to the fact that the 16S sequence we found belongs to species that are not present in the available sequence databases, yet. Therefore, for these, we will not probably be able to find the true genera, but only the most similar to the obtained sequences, even completing the sequencing.

Finally with a preliminary analysis carried out using primers specific for cellulase encoding genes we also found some indirect evidences of the presence of the cellulosolytic species Bacteroides cellulosolyticus (unpublished data).

The presence of cellulosolytic bacteria and of bacterial cellulases ensures that the larva can digest cellulose at least by means of bacterial activity, in contrast to what has been suggested by the studies conducted so far.

Further studies will be needed to confirm the preliminary data obtained (separated faeces and wood powder analysis, cloning and sequencing etc).

Acknowledgments

We wish to thank Dr. Alberto Vercesi (Istituto di Frutti-Viticoltura,UCSC, Piacenza) for suggestions regarding the statistical analysis.

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