UNLOCKING THE POTENTIAL OF BASIDIOMYCETES IN VALORISATION OF EMPTY FRUIT BUNCH: A BUZZING ALTERNATIVE FOR RUMINANT'S ROUGHAGE IN THE POST-PANDEMIC ERA

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Abstract. The shortage of feed sources in Malaysia's livestock industry was a major issue well before the COVID-19 outbreak. The prices of imported ingredients have increased substantially over the past few years. In the meantime, the impact of climatic change is exacerbating a food crisis already fuelled by COVID-19**,** which compromised the existing sources of local feed supply. Alternatively, the continuous production of palm oil generates a large amount of lignocellulosic by-product known as empty fruit bunch (EFB). The EFB is a good candidate for roughage replacers, known as a valuable source of hemicellulose and cellulose. However, further treatment is required to render the highly recalcitrant cell wall of the EFB. The changes in the quality of shredded EFB after being subjected to bio-treatment of inoculation with *S. commune, P. pulmonarius*, and *G. lucidum* in flasks for 5 weeks of incubation were observed. A total of 45 flasks containing inoculated EFBs were placed randomly in an incubator, and weekly triplicate samples were harvested for further analysis. The treated EFB is composed of two times higher carbohydrate concentrations in comparison to untreated EFB. Based on the carbohydrates-to-lignin ratio (C/L), *P. pulmonarius* and *G. lucidum* had the highest tendency towards lignin degradation, yielding higher *in vitro* gas production, which was reflected by the improvement in the fermentation of structural carbohydrates in comparison to *S. commune*. The morphological results of treated-EFB demonstrated severe damage with a prominent striation along the fibre length, which increased the total surface area for rumen microbial attachment, except for *S. commune*. Therefore, *P. pulmonarius* and *G. lucidum* are the best candidates to improve the quality of EFB and have the potential to be fed as ruminant roughage replacements.

Keywords: Biological treatment, low-quality roughages, *in vitro* gas production, oil palm by-products, edible mushroom.

Article Info

Received 16th June 2023
Accepted 11th December 2023
Published 12th June 2024

ISSN: 1823-7010, eISSN: 2600-7444

1. INTRODUCTION

Retail meat prices have risen by 7% from 2021 to 2022 as the Coronavirus (COVID-19) pandemic changes the worldwide economic landscape [1]. The global inflation of livestock commodities is mainly impacted by price fluctuation of the raw ingredients. Feed costs represent the single largest expense in most livestock operations. To mitigate higher prices, low-cost feed replacements such as empty fruit bunches (EFB) are necessary to compose animal feed formulations. Rumen microbiota may allow the conversion of low-quality feed and lignocellulosic material into meat and milk. However, direct feeding of EFB is not advisable, and the extensive lignification bound by the fermentable sugar fractions limits the digestibility of these by-products. An effective bio-treatment, such as white-rot fungi (WRF), naturally degrades the lignin aid in liberating the cell wall components, which is readily fermentable when exposed to rumen microbes.

The major enzymes responsible for degrading the lignin structure are laccase, lignin peroxidase, and lignin peroxidase. Promising results in upgrading the quality of EFB were previously compiled by Kume and co-workers [2]. Among the tested WRF species, *Pleurotus* spp. Showed an outstanding performance. A treated EFB based-feed named “Sterifeed” was later developed; however, this product was not well-sustained. Recently, the interest in exploiting the EFB as a potential ruminant feed has been growing. *S. Commune* ENN1 demonstrated a great potential in degrading lignin within a short week of incubation [3]. Still, no findings have been reported on the fungi preferences and the degradability of treated-EFB by rumen microbes. Most recent findings are reported by Nur-Nazratul and her team [4]. The changes in the nutritional content and ruminal kinetics fermentation of EFB treated using *G. lucidum* over 12 weeks have been compiled. The authors observed a considerable loss of silica bodies detached from the fibre surface and major modification of fibre structures at the end of incubation.

Based on the available literature, the treatment using WRF on EFB within 4 to 6 weeks had significantly improved the quality of EFB. This study was, therefore, conducted by exploring the potential of treated EFB as animal feed replacement using *S. commune*, *G. lucidum* and *P. pulmonarius*. The parameters, such as *in vitro* ruminal kinetic fermentation and morphological surface of treated EFB over 5 weeks of incubation, were determined.

2. MATERIALS AND METHODS

2.1 Collection of Substrates and Inoculum

Shredded fibres of EFB were obtained from a local oil palm mill, which is situated at 2°50’43.86” North latitude and 101°28’4.825 East longitude. Three WRF species, *Pleurotus pulmonarius*, *Schizophyllum commune* and *Ganoderma lucidum* were grown and maintained on Potato Dextrose Agar containing chloramphenicol (0.01%) at 28 °C for 7 days.

2.2 Substrate Preparation, Fungal Inoculation and Sampling

The EFB fibres were cut into 3 cm lengths and soaked in tap water overnight at room temperature. Water was drained for 30 minutes, and the moistened EFB was placed in an Erlenmenyer flask with weight of approximately 25 g of each flask. All EFB flasks were autoclaved at 121 °C and allowed to cool overnight at room temperature.

The autoclaved EFB was aseptically inoculated with 5 mycelial discs (1 cm Ø). These flasks were incubated in triplicate at 28 °C for 5 weeks in an incubator oven. A total of 45 flasks (3 species, 5 incubation week x 3 replicates) were placed in a similar incubator. At each week, independent samples were weighed, thoroughly mixed and oven-dried at 65 °C until constant weight was achieved. Samples were ground over for further analysis.

2.3 Fibre Fractions, In Vitro Gas Production Studies and Surface Morphology Observation

Fibre fractions were determined using Van Soest's detergent system [5]. Gas released from the fermentation of substrate within 72 hours of incubation was measured according to Menke and Steingass [6]. The surface morphological study was performed using a high-magnification Philips XL30 ESEM electron microscope. The scanning electron microscopy micrograph was recorded at different magnifications.

2.4 Statistical Analysis

The data analysis was performed in R software v4.2.0 [7]. The interaction effects of WRF species and incubation week on lignin degrading enzymes activities and carbohydrate to lignin ratio were determined using a two-way analysis of variance using agricolae package v1.3-5 [8]. The Post hoc analysis between treatment group was done using least-square means with emmeans package v 1.7.4-1 [9] with a significant level at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Dry Matter Content and Fibre Fractions of EFB

The EFB was mainly composed of structural carbohydrates, which represented 70.78% of the total contents, as reported in Table 1. The composition of lignin indicated that this structure was highly lignified and had a value (20.93%) above the average value of local tropical grasses.

Table 1: Dry matter content and fibre fractions of untreated EFB

Compositions	Concentration	SEM (standard error of means)
Dry matter (g/100 g fresh weight)	45.50	0.32
Fibre fractions (g/100 g DM basis)		
• Cellulose	40.43	0.49
• Hemicellulose	30.35	0.58
• Lignin	20.93	0.32

3.2 Residual Lignin Concentration

There is an interaction of residual lignin concentration between tested species across the incubation weeks (Figure 1). Lignin content was gradually decreased, and significant differences ($P<0.001$) between tested species were observed from week 4 onwards. The degradation of lignin by *P. pulmonarius* and *G. lucidum* were 10 – 20% higher than that of *S. commune* in week 4 and 5. Overall, more than 50% of lignin degraded in all tested species after 4 weeks of incubation.

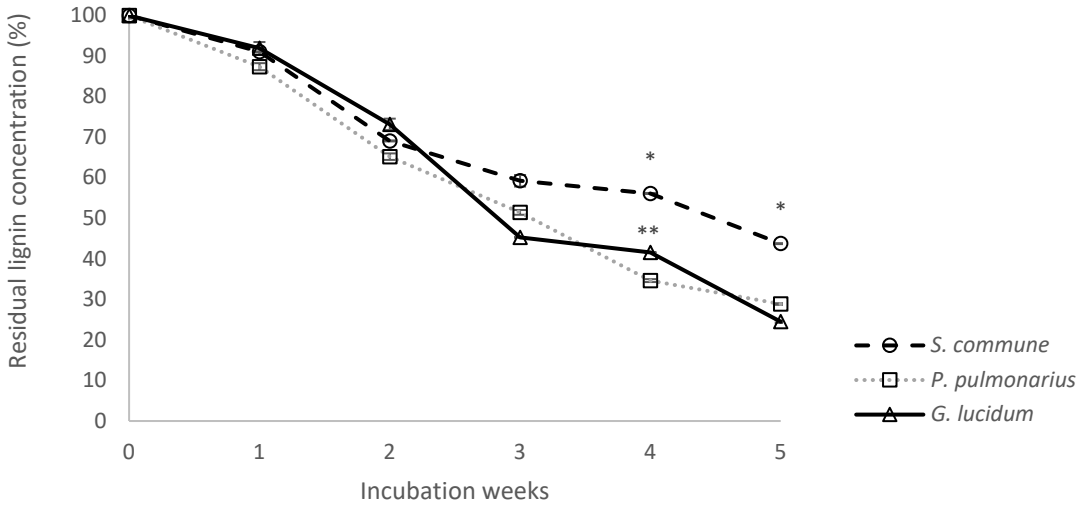

Figure 1: Residual lignin concentration of *S. commune*, *P. pulmonarius*, and *G. lucidum* treated EFB over 5 weeks of incubation. The asterisk indicates a significant (P<0.05) difference between species within a similar colonisation week.

3.3 Carbohydrate to Lignin Ratio

The C/L indicates the preference of WRF as a selective lignin degrader. Based on Table 2, there was no changes in C/L of *S. commune* over 5 weeks of incubations. The C/L differed between WRF species (P<0.001) at week 4 onwards. The carbohydrate fraction of *P. pulmonarius* was 6 times higher than lignin fractions when the incubation weeks were prolonged for 4 to 5 weeks. Similar results of C/L (6.0) were demonstrated in *G. lucidum* when incubated for 5 weeks.

Table 2: Carbohydrate-to-lignin ratio of *S. commune*, *P. pulmonarius* and *G. lucidum*-treated EFB over 5 weeks of incubation. SEM (standard error of means).

Species	Incubation weeks						SEM	Sig. level		
	Untreated	1	2	3	4	5		S	W	S*W
S. commune	3.39^{bx}	3.45^{bx}	4.38^{ax}	3.99^{abx}	3.62^{abz}	3.74^{aby}	0.06	<0.001	<0.001	<0.001
P. pulmonarius	3.39^{cx}	3.76^{bcx}	4.42^{bx}	4.30^{bx}	6.40^{ax}	6.03^{ax}	0.28			
G. lucidum	3.39^{cx}	3.59^{cx}	4.12^{cx}	5.21^{bx}	4.98^{by}	6.40^{ax}	0.23			
SEM	0.05	0.12	0.16	0.25	0.42	0.43				

^{a,b,c} Within a row means without a common superscript differ (P<0.05)
^{x,y,z} Within a column without common superscript differ (P<0.05)

According to the C/L results, the degradation rate of carbohydrates in *S. commune*-treated EFB was consistent with that of lignin during a 5-week incubation period. *P. pulmonarius* and *G. lucidum* were both selective lignin degraders. These species tended to degrade lignin in comparison to carbohydrate fractions, and a distinct improvement of carbohydrate fractions appeared at 4 weeks onwards when compared to untreated EFB. This indicated that treatment using *P. pulmonarius* and *G. lucidum* increased the quantity of carbohydrate (kg^{-1} DM of treated EFB) to be utilised by rumen microbes when fed to ruminants.

3.4 In Vitro Gas Production and Kinetic Fermentation

Figure 2 shows an *in vitro* kinetic fermentation of untreated and treated-EFB of *S. commune*, *P. pulmonarius* and *G. lucidum* at week 5 over 72 hours of incubation in ruminal fluid. The gas produced (GP) from degradation of untreated EFB slowly increased at a rate of 0.017/hour, and reached the highest volume at 72 hours of incubation. The GP from *S. commune*-treated EFB was the highest for the first 12 hours ($P>0.05$), resulted by the highest ($P<0.001$) degradation rate of 0.035/hour among the tested species. However, the GP of *S. commune* slowly increased over the incubation hours, and the volume released was similar ($P>0.05$) to untreated EFB. The GP released from *G. lucidum* and *P. pulmonarius*-treated EFB were similar to each other over 72 hours of incubation, which was significantly higher than untreated and *S. commune* after 24 hours incubations. Both species had similar degradation rates ($P>0.05$) of 0.030/hour and 0.032/hour, respectively. The GP of *G. lucidum* and *P. pulmonarius*-treated EFB increased at a faster rate in comparison to other tested species at 12 hours incubation; reaching plateau at 32 hours incubation, and slowly increasing to the highest volume at the end of incubation. Based on the potential gas production results, extended fermentation of *P. pulmonarius*-treated EFB were able to produce the highest value of 231.8 ml/g OM, followed by untreated EFB (215.9 ml/g OM), *G. lucidum* (204.6 ml/g OM) and *S. commune* (138.6 ml/g OM). When comparing with other by-products, the potential GP of treated EFB using *P. pulmonarius* was comparable to GP produced from oil palm fronds [10] and other tropical grasses such as *Brachiaria. decumbens* (212.68 ml/g OM), *Panicum maximum* (235.58 ml/g OM); however, lower than common roughages such as *Pennisetum purpureum* (268.08 ml/g OM) [11].

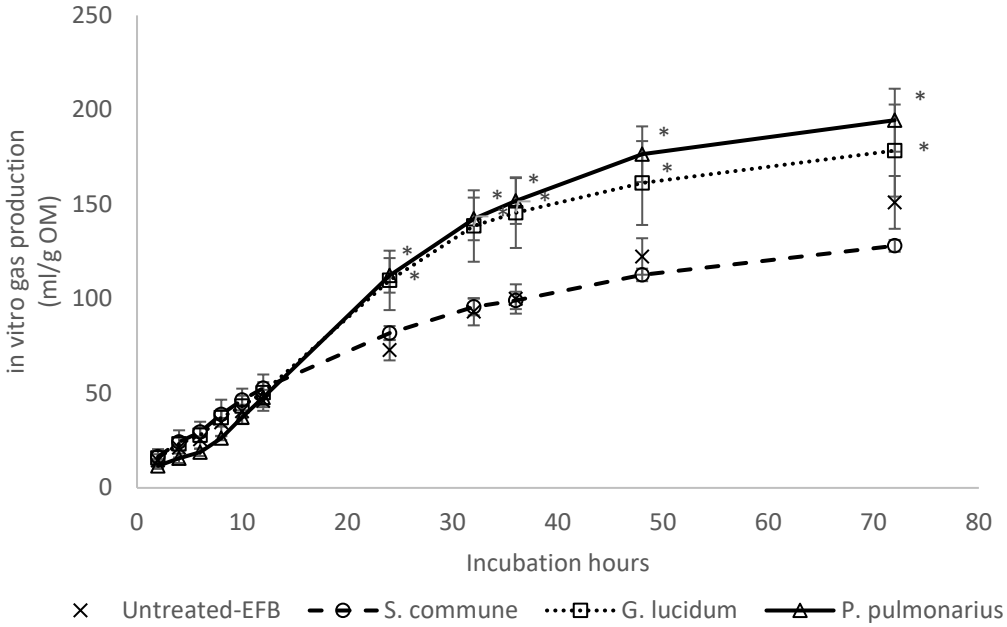

Figure 2: *In vitro* gas production over 72 hours of untreated and treated-EFB using *S. commune*, *G. lucidum* and *P. pulmonarius* at 5 weeks of incubation.

The quality of the EFB was improved by *G. lucidum* and *P. pulmonarius* based on the GP and the kinetic fermentation over 72 hours incubated in ruminal fluid. The GP was reflected by microbial fermentation of accessible substrates available on the EFB. Based on the results, treatment using *S. commune* could improve the accessibility of carbohydrates due

to a rapid degradation rate during early incubation. However, due to the considerable loss of CHO consumed by the fungi, the GP was lower than those produced from untreated EFB. The GP produced by fermentation of *G. lucidum* and *P. pulmonarius*-treated EFB was maximised after 32 hours of incubation. The intersection of GP at 12 hours had showed an increase in degradation rates of fermentable CHO as compared to *S. commune* and untreated EFB. This indicated that both *G. lucidum* and *P. pulmonarius*-treated EFB had improved the structure of the EFB by increasing the amount of accessible CHO to be fermented by rumen microbes.

3.5 Surface Morphology

Scanning electron microscopy was applied to investigate the morphological changes of degraded EFB treated with different species of fungi. Figure 3 depicts the non-degraded cell wall with an intact surface morphology. It could be seen that silica bodies were embedded on the fibre surface (Figure 3(a)). Other impurities were observed covering the surface of untreated EFB (Figure 3(b)).

However, none of the impurities fractions were observed on the surface of FTEFB. The EFB samples colonised by *S. commune* revealed that the plant cell walls remained mostly intact despite the fungal mycelia covering the EFB surface (Figure 4). The filamentous *S. commune* hyphae network showed a typical branching, septation pattern and a smooth surface. An extensive modification of EFB tissues was observed on the surface of *P. pulmonarius* (Figure 5) and *G. lucidum*-treated EFB (Figure 6); the EFB samples were broken, and distortions appeared with the striations along the fibre length becoming prominent. The surface of EFB treated with *P. pulmonarius* was enveloped in a slime sheath (Figure 5(a) and (b)), which increased mycelia contact with the cell wall. Fungal bore holes were observed like those reported by Wahab and co-researchers [12], indicating the features of the cell wall degradation by hyphae of WRF. A dense web-like hyphal network was observed on the surface of *G. lucidum*-treated EFB at 5 weeks of incubation (Figure 6(b)).

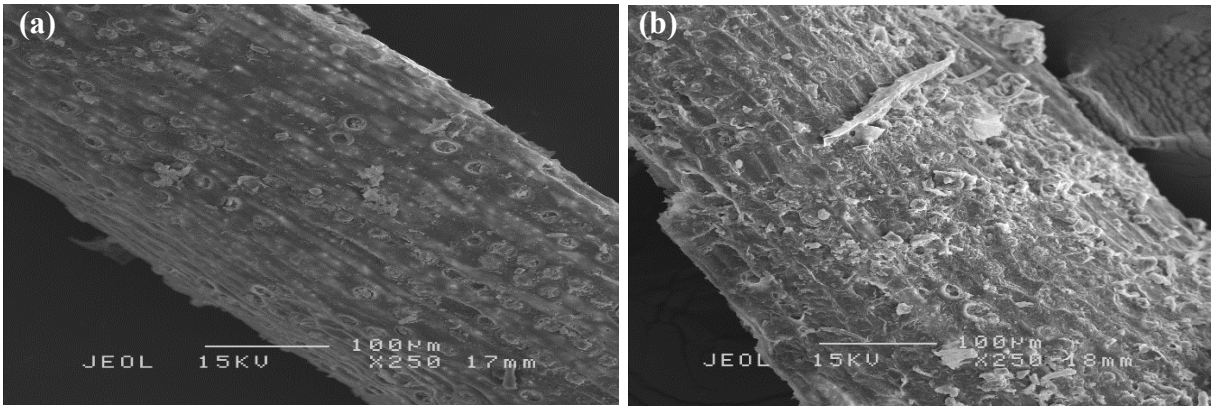

Figure 3: Surface morphology structure of untreated EFB at 250x magnification. (a) Embedded silica bodies on the surface of EFB fibre, and (b) Intact EFB with impurities covering the surface structure.

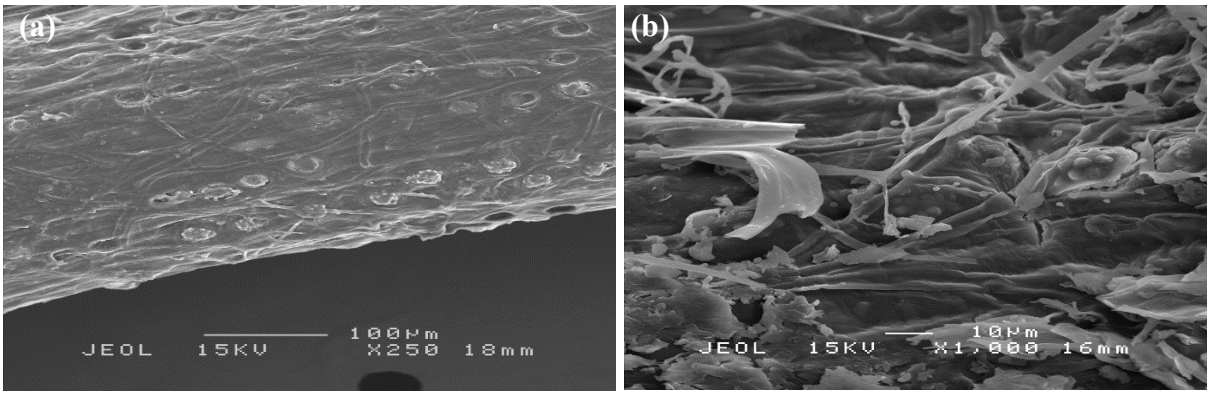

Figure 4: Surface morphology structure of *S. commune*-treated EFB at 5 weeks incubation at (a) 250x and (b) 1000x magnifications.

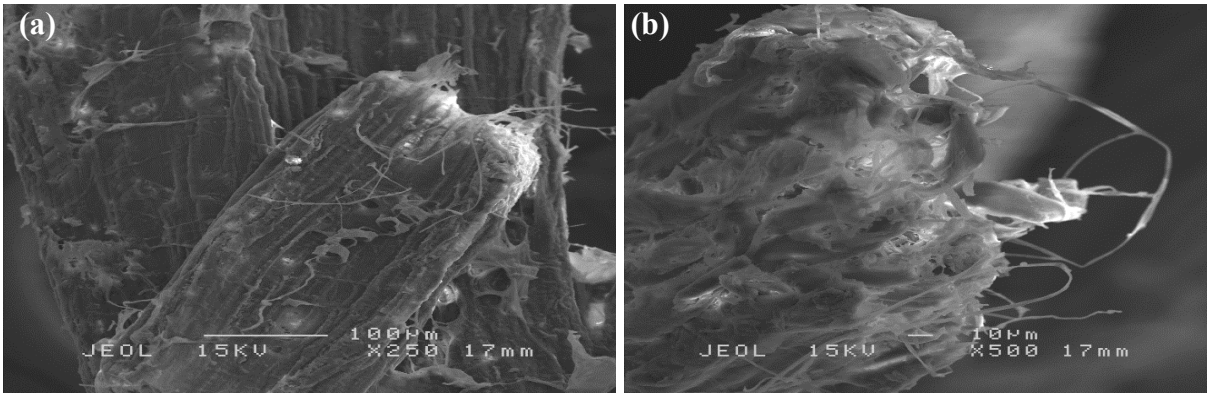

Figure 5: Surface morphology structure of *P. pulmonarius*-treated EFB at 5 weeks of incubation at (a) 250x and (b) 500x magnifications.

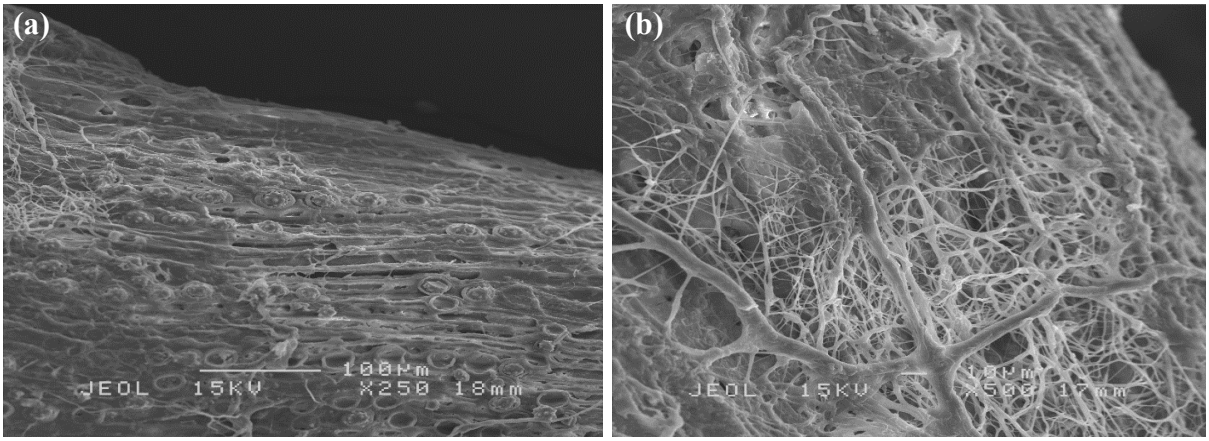

Figure 6: Surface morphology structure of *G. lucidum*-treated EFB at 5 weeks incubation at (a) 250x and (b) 500x magnifications.

The surface morphology of untreated EFB was rough, covered with a thick layer of waxy materials, and embedded with spiky silica bodies. Based on observations, impurities and waxy substances were found on the surface of untreated EFB strands (Figure 3). This was in line with our unpublished observations (Figure 7) and was also reported by Wan Nadhari and co-researchers [13]. A lot of silica bodies were still intact on the surface of EFB strands after 5 weeks of being treated with fungi.

A dense and continuous mycelia network of *G. lucidum* was observed in the present study (Figure 6) and also as illustrated in Figure 7 of our previous findings. A fine mycelia threads spreads perpendicular to the fibre direction (Figure 8(a)) and a small hollow centre (Figure 8(b)) were observed in *G. lucidum*-treated EFB for 6 weeks. The differences in the density of mycelia growth by *G. lucidum* on EFB might be due to the difference in the proportion of mycelia applied on the EFB when compared between the studies. Moreover, another study had found higher densities of mycelium composites in chopped fibre substrates compared to loose substrates [14], thus resulting in different effects on the composition and surface morphology of the substrate.

Our previous findings reported a considerable loss of the silica bodies, leaving the perforated bottom of the silica crater after 12 weeks of incubation with *G. lucidum* [4]. Deeper silica crates, which were expansion of the pit pores, formed, and more protruded silica bodies were found detaching from the surface. The removal of silica bodies exposed the pores connected to the interior surfaces through the siliceous pathway. Considerable destruction of fibre structures created a larger hollow cross-section of the treated-EFB. The silica bodies acted as a barrier to enzymes and delayed the degradation process. The silica bodies were synthesised by polymerisation and deposition of silicic acid through the siliceous pathways [15]. Thus, removing silica bodies would provide an access of enzymes into the internal layer of the EFB, exposing more surface area to degradation. However, the present study showed intact deposited silica bodies when treated using tested WRF over 5 weeks of incubation. The cleavage and severe delamination of fibre strands resulting in a rougher surface, especially when treated with *P. pulmonarius* and *G. lucidum*. This was due to the mercerisation of fibre, which resulted in the elimination of fractions such as chemicellulose, lignin and other waxes from the surface. Both *P. pulmonarius* and *G. lucidum* showed greater effects on the surface morphological changes of EFB, indicating a better removal of structural fractions of EFB than *S. commune*.

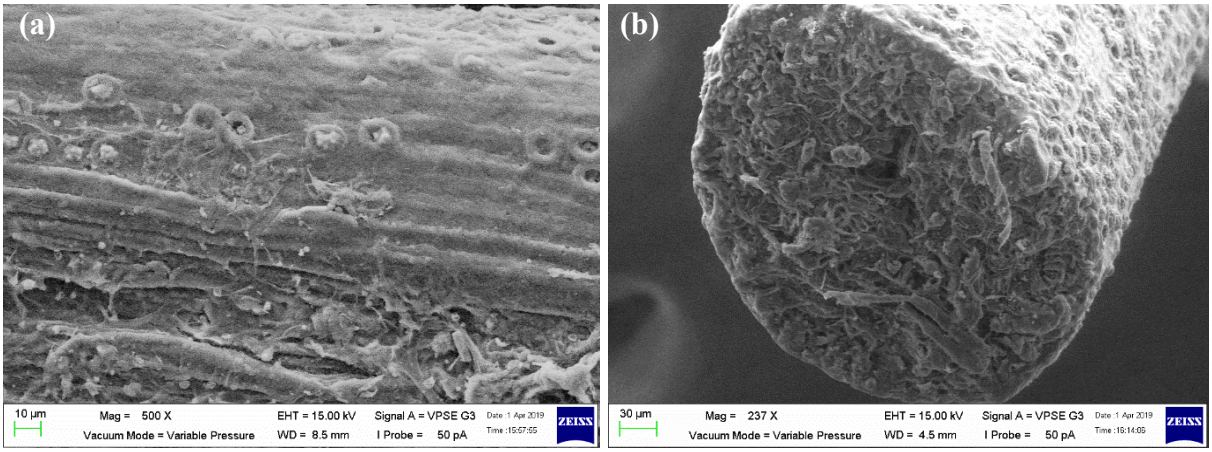

Figure 7: Scanning electron microscopy images of untreated EFB (Unpublished). (a) Surface morphology at 500x magnification, and (b) Cross-sectional view at 237x magnification.

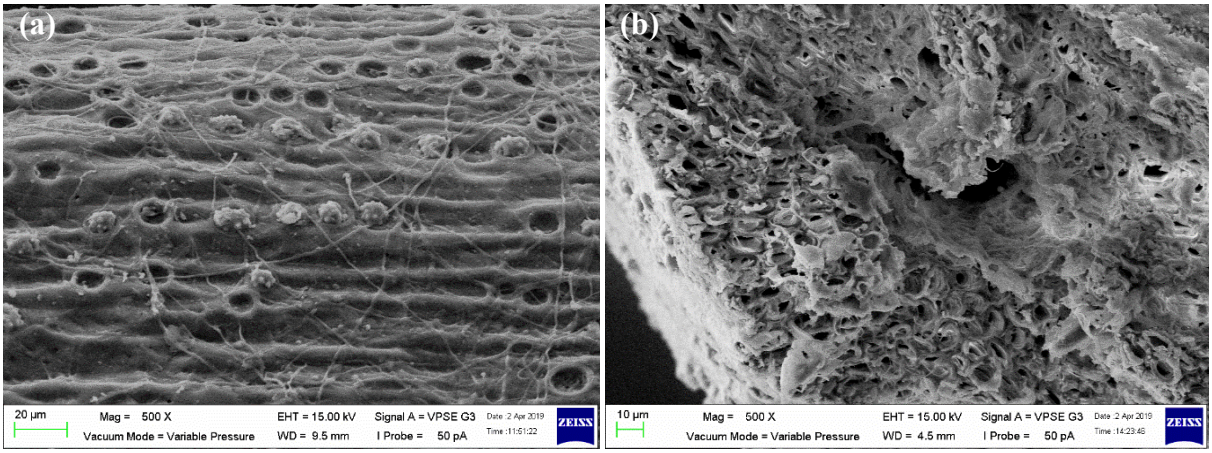

Figure 8: Scanning electron microscopy images of *G. lucidum*-treated EFB at 6 weeks incubation (Unpublished). (a) Surface morphology at 500x magnification, and (b) Cross-sectional view at 500x magnification.

4. CONCLUSIONS

The results clearly demonstrated the variation of species on substrate preferences and *in vitro* ruminal kinetic fermentation. The degradation of EFB by rumen microbes improved when treated with *P. pulmonarius* and *G. lucidum* for 5 weeks. These fungi had increased the C/L as well as the volume of gas produced. The surface morphology of *P. pulmonarius* and *G. lucidum* treated-EFB was rougher, with a deeper striated fibre that improved the total surface area for ruminal microbial attachments. No improvement was observed in the quality of EFB when treated using *S. commune*. The quality of treated EFB using *P. pulmonarius* was on par with several tropical forages; thus, it has great potential as a roughage replacer in ruminant diets.

Acknowledgements

The authors gratefully acknowledge the financial support provided by Universiti Putra Malaysia (GP-IPS/2020/9687100) and the scholarship by the Ministry of Higher Education Malaysia and Universiti Malaysia Sabah.

Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no conflicts of interest to declare relevant to this article's content.

Compliance with Ethical Standards

The work is compliant with ethical standards. All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (Ref: AUP-R063/2020).

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