Indigofera Tinctoria Leaf Powder As A Promising Additive To Improve Indigo Fermentation Prepared With Sukumo (Composted Polygonum Tinctorium Leaves)

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Research Article

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Abstract

Being insoluble in the oxidize form, indigo dye must be solubilized by reduction for it to penetrate textile. One of the procedures is the reduction by natural bacterial fermentation. *Sukumo*, composted leaves of *Polygonum tinctorium*, is a natural source of indigo in Japan. Although *sukumo* has an intrinsic bacterial seed, the onset of indigo reduction with this material may vary greatly. Certain additives improve indigo fermentation. Here, we studied the effects of *Indigofera tinctoria* leaf powder (LP) on the initiation of indigo reduction, bacterial community, redox potential (ORP), and dyeing intensity in the initial stages and in aged fermentation fluids prepared with *sukumo*. *I. tinctoria* LP markedly decreased ORP at day 1 and stabilised it during early fermentation. These effects could be explained by the phytochemicals present in *I. tinctoria* LP that act as oxygen scavengers and electron mediators. Using next generation sequencing results, we observed differences in the bacterial community in *sukumo* fermentation treated with *I. tinctoria* LP, which was not influenced by the bacterial community in *I. tinctoria* LP *per se*. The concomitant decrease in *Bacillaceae* and increase in *Proteinivoraceae* at the onset of fermentation and the ratio of facultative to obligate anaerobes are vital to the for initiation and maintenance of indigo reduction. Hence, *I. tinctoria* LP improved early indigo reduction by decreasing the ORP and hasten the appropriate transitions in the bacterial community in *sukumo* fermentation.

Introduction

Indigo is one of the oldest dyes in human history. It was used in many ancient civilisations around the world (Chavan, 2015). Natural indigo is extracted from various plants. While *Indigofera tinctoria* was the main source in India, *Polygonum tinctorium* was found in China and Japan, and woad (*Isatis tinctoria*) was the major source of indigo in Europe (Cardon 2007). The requirement for natural indigo declined after the first commercially available synthetic indigo was developed and commercialised in 1890. Synthetic indigo has almost entirely replaced natural indigo (Chavan 2015; Głowacki et al. 2012). Today, the global indigo production is in the thousands of tonnes. More indigo is manufactured than any other dye worldwide and it is used mainly to dye denim for blue jean fabrication (Głowacki et al. 2012). However, synthetic indigo production generates environmentally harmful chemicals (Hus et al. 2018).

Oxidized indigo dye is insoluble in water and must be solubilised by reduced in alkali solution. The reduced product is known as leuco-indigo and readily penetrates fabrics. On contact with atmospheric oxygen, it oxidises to a blue form (Clark et al. 1993; Głowacki et al. 2012). Fermentation by bacterial, chemical and electrochemical reduction, and catalytic and electrocatalytic hydrogenation can be used to reduce indigo (Chavan 2015; Yi et al. 2020; Nakagawa et al. 2021). Industrial dyeing commonly involves the reducing agent sodium dithionate (Głowacki et al. 2012). Whilst it is fast and inexpensive chemical reduction requires the continual addition of the reductant and generates toxic and corrosive waste products (Chavan 2015; Hus et al. 2018).

Sukumo is a traditional Japanese dyeing material prepared by composting *P. tinctorium* leaves This method concentrates the indigo and facilitates its storage, transportation and preservation. *Sukumo* also

harbours microorganisms that can serve as an inoculum for indigo-reducing fermentation. Moreover, the plant materials derived from the composting may be used as nutrients for the microorganisms (Aino et al. 2018). Hence, *sukumo* is a fermented raw material that contains nutrients for microorganisms and microbial seed.

The indigo in *sukumo* must be solubilised by reducing it in a liquid alkaline medium. To this end hot wood ash extract (60–80°C; pH 11) is combined with *sukumo* at 1/3 fermentation volume. After onset of indigo reduction, the wood ash extract is topped up to 2/3 volume. After indigo reduction starts, wood ash extract is the added to make up the final volume. The fluid is maintained at pH ≈ 10.3 by adding lime hydrate ($Ca(OH)_2$) and stirring once daily. Japanese rice wine is initially added the preparation and wheat bran (substrate) is periodically introduced after the onset of indigo reduction. The period between the initiation of the process and dyeing varies depending on the *sukumo* quality and preparation. The process may take from 3 days to several weeks (Aino et al. 2018).

The microbiota in *sukumo* preparations have been studied via clone library analysis (Aino et al., 2010; Okamoto et al. 2017) and next generation sequencing (NGS) (Tu et al. 2019ab). Clone library analysis focused on aged fluids and identified *Proteinivoraceae, Tissirellaceae, Anaerobacillus, Amphibacillus, Alkalibacterium* and *Polygonibacillus* as the dominant taxa (Okamoto et al. 2017). It also revealed that changes in microbiota occurred more slowly in the later than the earlier fermentation phases. NGS investigated the changes that occur in early fermentation and the entire fermentation process. In the initial stages, *Bacillaceae* predominated and consumed oxygen in the fluid. *Bacillaceae* were then succeeded by the obligate anaerobes *Anaerobranca* (*Proteinivoraceae*) (Tu et al. 2019a). A comparative study on fermentation batches in different phases of indigo reduction indicated the early predominance of obligate anaerobes such as *Anaerobranca* (*Proteinivoraceae*) followed by gradual successive changes into stable microbiota. The study suggested that these transformations are necessary for the establishment of stable indigo fermentation fluid (Tu et al. 2019b).

Traditional fermentation may be difficult to initiate and maintain for long periods. It depends on numerous factors such as the correct abundance and diversity of natural microorganisms and appropriate pH, stirring, and feeding. Thus, ample experience in traditional fermentation preparation and management is required for the successful execution of this process (Aino et al. 2018). In Medieval times, madder (*Rubia tinctorum*) was added to woad vats to improve fermentation. Madder has numerous anthraquinones. In fermentation experiments using indigo-reducing *Clostridium isatidis*, the addition of anthraquinones and madder stimulated indigo reduction (Nicholson and John 2004). In the case of extracted indigo, since it lacks sufficient microorganisms to initiate the fermentation, Senna tora (originally named Cassia tora by Linnaeus), tamarind, and ripe fruits may be added to accelerate microbial indigo reduction (Samanta and Agarwal 2009; Chavan 2015, Zhang et al. 2019). These plant materials also contribute with phytochemicals and microbial substrates. In the present study, we evaluated *I. tinctoria* as a candidate for acceleration of indigo reduction. This plant has numerous carbohydrates, flavonoids, saponins, proteins, tannins, phenols, steroids, anthraquinone and triterpenoids (Sharma et al. 2016). *I. tinctorum* leaf powder (LP) has been used as a hair dye. It is readily accessible

and has been used by certain artesian in fermentation fluid for cloth dyeing. However, its mechanism for the acceleration of indigo reduction is still unknown.

The present study aimed to assess the effect of *I. tinctoria* in terms of accelerating the initiation of indigo reduction in *sukumo* fermentation. For this purpose, we examined the bacterial community profiles and characteristics of multiple *sukumo* fermentation batches over different fermentation periods with and without the presence of *I. tinctorum* LP. The result of this study provided valuable knowledge regarding the initiation and maintenance of indigo reduction in *sukumo* fermentation fluid.

Materials And Methods

Sukumo fermentation fluid preparation

A small-scale batch fermenter (0.5 L) was prepared to simulate the large-scale craft centre *sukumo* fermentation. *Sukumo* occurs in an alkaline solution made of wood ash extract. The extract was produced by boiling 380 g wood ash (*Quercus phillyracoides* A. Gray, Nagomi Co., Gobo, Wakayama, Japan) in 5 L tap water for 10 min. The supernatant was carefully decanted and boiled again and distributed into 500-mL Erlenmeyer flasks. After the supernatant cooled to 60°C, 38 g *sukumo* was added to it. When the fluid mixture cooled to 30°C, 0.5 g *I. tinctoria* LP (NCC Agro Industries, Tamil Nadu, India) was added to one of two flasks. Two batches of *I. tinctoria* LP from the same company were used, with LP1 referencing to the oldest batch and LP2 to the recently open package. The flask containing no LP was named as control. The flasks were maintained at 26°C. For Experiment 1, the *sukumo* source was by TT (Tokushima Prefecture, Shikoku, Japan). For Experiment 2 and 3, the *sukumo* source was KS (Date City, Hokkaido, Japan) (Table 1). To test indigo reduction, small cotton cloth squares were dipped in the fluid for 1 min, exposed to air for 10 min to oxidise the indigo, washed under running water to remove impurities, air-dried, and stored. The staining intensity of each batch was periodically evaluated by visual observation and comparing the colour of the dyed cloth against a colour scale graded from 0–3.0. Dyeing efficiency of the *I. tinctoria* LP treatment was assessed by comparing it against the control. The pH and redox potential (ORP) of the fermentation fluids were measured with a D-71 pH meter (Horiba, Kyoto, Japan) and a D-75 pH/ORP/DO meter (Horiba), respectively. The pH was maintained between 10.5 and 11 with $Ca(OH_2)$. Before sampling and after pH adjustments, the fluids were stirred on a magnetic stirrer for 1 min. Aliquots of 500 µL were collected, and glycerol was added to them at 25%. They were then and stored − 80°C until DNA extraction.

Table 1
Preparation of indigo fermentation fluids used in this study

Batch	*Sukumo*	Leaf powder	Microbiota analysis period	Substrate added	Increase in redox potential (ORP) in control at early fermentation stages	Fermentation volume (L)
Experiment 1	Date (by KS)	LP1	Days 1–72	No	Yes (Days 5–17)	0.5
Experiment 2	Date (by KS)	LP1	Days 36–94	No	No	0.5
Experiment 3	Tokushima (by TT)	LP2	Days 115–226	No	Yes (Days 10–21)	0.5

I. tinctoria LP and LP viable cell counts

Sukumo or *I. tinctoria* LP (0.5 g) was suspended in either 5 mL physiological saline (0.9% NaCl) or wood ash extract. The *sukumo* suspended in wood ash extract was maintained at 55°C for 30 min. The LP suspended in wood ash extract was maintained at 26°C for 30 min. Each suspension was serially diluted and inoculated onto Plate Count Agar (PCA) agar (5 g Bacto-Peptone (Becton Dickinson, Franklin Lakes, NJ, USA), 2.5 g yeast extract (Becton Dickinson), 1g dextrose and 10 g agar in 1 L distilled water; pH 7.0) and aerobically incubated at 26°C for 48 h. Colony counts (CFU) were then determined for incubated samples.

DNA extraction and PCR

Sukumo fermentation fluids with and without *I. tinctoria* LP were centrifuged at 15,000 × *g* for 10 min at 25°C to obtain a pellet used for DNA extraction. The latter was performed with an ISOIL extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. For the final step, however 50 µL TE was added instead of 100 µL. The extracted DNA was stored at -30°C. The bacterial 16S rRNA sequence of the V3–V4 region was amplified with the primer pairs: V3–V4f_MIX (ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-CCTACGGGNGGCWGCAG) and V3-V4r_MIX (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GACTACHVGGGTATCTAATCC) provided by Bioengineering Lab. Co. Ltd. (Sagamihara, Kanagawa, Japan). The primers consisted of an adaptor sequence followed by insertions with 0–5 bases with random sequences and lengths to improve quality (represented by N). The final sequence was homologous to 16S rDNA (341F and 805R). The PCR solution (40 µL) consisted of 4 µL of 10× Ex buffer (Takara Bio, Otsu, Shiga, Japan), 3.2 µL dNTPs (Takara Bio), 2µL forward primer, 2 µL reverse primer, 2 ng DNA template (extracted sample), and 0.4 Ex Taq polymerase (Takara Bio). The amplification reaction was performed as follows: 94°C for 2 min; 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. The final step was 72°C for 5 min. Product identity and quality were confirmed by agarose gel electrophoresis. The *I. tinctoria* LP source material was suspended in physiological solution. LP1 was processed as previously described. For LP2, the extracted DNA was

sent to the Bioengineering Lab. Co. Ltd., where was treated with Peptide Nucleic Acid (PNA) to block chloroplast and mitochondrial DNA sequences.

PCR and NSG

The second PCR was performed by Bioengineering Lab. Co. Ltd. The samples were amplified with a tailed primer set and purified. The NGS was conducted on an Illumina MiSeq platform (Illumina, San Diego, CA. USA). The raw reads were pre-processed by Bioengineering Lab. Co. Ltd. and fastq.gz output files were processed with QIIME2 ver. 2020.2 (Bolyen et al. 2019). Cutadapt version 1.18 removed the primers and adapters. The sequences were filtered, and merged and had chimeras and singletons were removed with the Demux command (Callahan, 2016). DADA2 was used to cluster the sequences with 99% similarities into amplicon sequence variants (ASVs). Taxonomy classification were made by his research group and the SILVA database (Pruesse et al. 2007; Quast et al. 2013). For the I. tinctoria LP source material sequences classified as chloroplasts were filtered. ASV nucleotide sequence identities with > 1.5% participation were compared against bacterial 16S rRNA reference sequences in the BLAST database. Rarefaction curves were plotted for the sequences and OTUs at 99% similarity level with respect to the total number of reads per sample. Rarefaction curves of the observed species were plotted with the QIIME2 alpha diversity analysis script. A Bray-Curtis dissimilarity matrix was constructed by principal coordinate analysis (PCoA). The corresponding plots were prepared with the beta diversity analysis script in QIIME 2. EMPEROR software visualised the PCoA plots (Vázquez-Baeza et al. 2013).

The NGS data from this study are publicly available in the DNA Data Bank of Japan (DDBJ) under accession no. SAMD00289726 to SAMD00289754, and SAMD00289756.

Results

Effects of *I. tinctoria* LP on early fermentation phase

Samples of the early fermentation phase of Experiment 1 were observed to determine the effect of *I. tinctoria* LP on the bacterial community. Results of dyeing and changes in pH and ORP are shown in Figure 1 and Supplementally Figure 1. There were evident differences in terms of fluid dyeing between *I. tinctoria* LP treatment and the control after day 5. The dyeing intensity of *I. tinctoria* LP slightly decreased after the pH increased at day 10. The effects of this sudden change especially in the control were reflected in the ORP curves. These changes persisted from days 10–21. No increase in ORP was observed in *I. tinctoria* LP treatment during the foregoing time period. Nevertheless, its dyeing intensity transiently decreased (Fig. 1, Supplementally Figure 1).

Firmicutes was the dominant phylum in all samples (including facultative, obligate and aerotolerant anaerobes) followed by *Proteobacteria* and *Actinobacteria* (Fig. 1). The microbiota detected in both *I. tinctoria* LP (LP1 and LP2) in natural state are shown in Supplementary Fig. 2. The estimated CFU for *I. tinctoria* LP and *sukumo* are shown in Supplementary Table 1. The bacterial community profiles in both *I. tinctoria* LP in raw state dose not correlates with those of the fluid with *sukumo* at day 1. Hence, the *I.*

tinctoria LP bacterial flora did not markedly influence the bacterial flora profile in the broth. This finding corroborated with the results of the estimated bacterial counts for *I. tinctoria* LP compared against those for *sukumo* (Supplementary Table 1). The ORP-lowering effect of *I. tinctoria* LP might be explained by endogenous aerobic bacterial oxygen consumption. Thus, the aerobic bacterial colony count was estimated and maximized using the neutral pH medium. The expected neutralophilic bacterial count for *I. tinctoria* LP in prepared fermentation fluid was ~0.9% that for the *sukumo*. Wood ash extract treatment at 26 °C for 30 min lowered the *I. tinctoria* LP bacterial count. In contrast, wood ash extract treatment even at 55 °C for 30 min had no effect on the *sukumo* bacterial count. There were more *Bacillaceae* in fluid where *I. tinctoria* LP was added than in the control at day 1. Samples from day 1 showed that the bacterial community had four major operational taxonomic units (OTUs) related to *Bacillus cohnii*, *Bacillus taeanensis*, *Alkaliphilus oremlandii*, and *Mogibacterium neglectum*. All other taxa except *Mogibacterium neglectum* were more abundant in *I. tinctoria* LP treatment than the control. All five OTUs decreased in both *I. tinctoria* LP treatment and the control over next 5 d. However, the sum of the decrease in *I. tinctoria* LP (50%) was greater than the control (36.2%). The control treatment exhibited relatively higher bacterial diversity on day 1. The proportion of taxa grouped as “others” (□ 1.5%) was 21.8 % higher than *I. tinctoria* LP. The control contained more *Proteinivoraceae* than *I. tinctoria* LP at day 5. In *I. tinctoria* LP treated batch, an OTU related to *Alkalihalobacillus alkalinitrilicus* was highly active by day 5. In Experiment 1, there were comparatively more microorganisms such as *Tissierellaceae* in *I. tinctoria* LP treated batch after day 10. In addition, an OTU related to *Polygonibacillus indicireducens* appeared in *I. tinctoria* LP after 58 d.

At fermentation onset, there was a decrease in numbers of facultative anaerobes. However, a subsequent increase in the ratio of facultative anaerobes to absolute anaerobes modulated staining intensity. The ratio of facultative anaerobes (F) to obligate anaerobes (O) (designated F/O), the total abundance of facultative anaerobes (F) and obligate anaerobes (O) (designated F + O), and their correlation with dyeing intensity are plotted in the Fig. 2. Nevertheless, the strength of this relationship fluctuated because of the differences in the constituent microorganisms and time lag until the effects reflected in the dyeing intensity. In most case, F/O increased with dyeing intensity.

Diversity decreased on days 5 and 25 according to the observed changes in the OTUs at the sample depth of 18512 (Figure 3). Relatively fewer OTUs on day 1 of the *I. tinctoria* LP treatment indicated that *I. tinctoria* LP addition affected the microbiota. The diversity decreased on day 5 coinciding with changes in redox potential. The diversity decreased on day 25 was explained by depletion of the readily assimilated substrates which coincided with the increase in redox potential and decrease in dyeing intensity in *I. tinctoria* LP (Fig. 2 and Supplementary Fig. 1).

The relative difference between *I. tinctoria* LP and the control in terms of their microbiota were estimated by principal coordinate analysis (PCoA) using a Bray-Curtis dissimilarity matrix (Figure 4). The microbiota of *I. tinctoria* LP rapidly changed until day 10. However, the rate of change declined after day 10. In contrast, 58 d were required to reach the final stage in the control. There were three stages in both *I. tinctoria* LP and the control. However, the duration of each phase varied between treatments. Thus, *I.*

tinctoria LP addition may affect both the bacterial community composition and the rate of change in the microbiota.

Effects of *I. tinctoria*LP on the middle and late fermentation phases

Samples from Experiments 2 and 3 were used to determine the effects of *I. tinctoria* LP no the microbiota in the middle and later fermentation phases. The effects of *I. tinctoria* LP on the *sukumo* fermentation in terms of fabric dyeing and pH and ORP changes are shown in Fig 5 and Supplementary Figs. 3 and 4. *I. tinctoria* LP addition was correlated with an early, steep decline in ORP (near -600 mV) compared with the control. This finding was true for Experiments 1–3 inclusive (Supplementary Figs. 1, 3, and 4). *I. tinctoria* LP addition during initial *sukumo* fluid preparation hastened the onset of indigo reduction compared with the control (Experiment 3) (Supplementary Fig. 4). In contrast, despite of *I. tinctoria* LP on the ORP at the start of fermentation, there was no marked difference in dyeing intensity for Experiment 2. A possible reason is that unlike the other experiments in Experiment 2, neither *I. tinctoria* LP nor the control reached pH ≥ 10.7 in the initial phase. The ORP for both *I. tinctoria* LP and the control) in Experiment 2 were lower than those in Experiment 1 and 3 for most of the fermentation period.

Both the control and *I. tinctoria* LP in Experiment 2 had similar amounts of OTUs related to *Erysipleothrix inopinata* (93.2–93.4% similarity). This taxon predominated but its abundance decreased with increasing fluid age. On days 50 and 94, *I. tinctoria* LP and the control had similar quantities of two OTUs identified with *Pseudomonas* spp. In Experiment 2, OTUs related to *Proteinivorax tanatarenses* (93.1–93.8% similarity) predominated in the control but not *I. tinctoria* LP samples. The latter had relatively higher numbers of an OTU related to *Tissierella creatinini* (94.5% similarity) compared with the control. An OTU related to indigo reducing *P. indicireducens* was also detected.

Experiment 3 showed that decrease in the amounts of obligate anaerobes and increase in the numbers of facultative anaerobes were similar for both *I. tinctoria* LP and the control by day 142. However, there were abundant OTUs related to *P. indicireducens* in *I. tinctoria* LP (97.7–98.6%) and OTUs related to *Alkalihalobacillus hemicellulosilyticus* (98.1–99.3%) in the control. *Tissierellacea* usually predominates compared to *Proteinivoraceae* in *I. tinctoria* LP. Nevertheless, this difference was not observed in Experiment 3.

In Experiment 2, the ratio of facultative anaerobes (F) to absolute anaerobes (O) (F/O) was nearly constant for the control. In contrast, F/O varied with fermentation time for *I. tinctoria* LP. Changes in F/O were not correlated dyeing intensity for *I. tinctoria* LP in Experiment 2. However, both *I. tinctoria* LP and the control presented with the highest dyeing intensities (Figs. 6A–6B). In contrast, changes in F/O varied with dyeing intensity in Experiment 3 (Figs. 6C–6D).

The changes in OTU number at the 18512-sampling depth (Supplementary Fig. 5) in Experiment 2 indicated a decrease in diversity with fermentation time for *I. tinctoria* LP added fluid but not the control. In Experiment 3, the observed OTU more rapidly increased with fermentation time for *I. tinctoria* LP than the control. PCoA by the Bray-Curtis dissimilarity matrix showed a distinct separation between *I. tinctoria*

LP and the control for in Experiments 2 and 3 (Supplementary Figs. 6A–6B). The microbiota changed faster in *I. tinctoria* LP than the control in Experiment 2, but the opposite was true for Experiment 3.

Discussion

Traditional indigo reduction methods are becoming more popular as they are comparatively more sustainable than modern techniques. *Sukumo* is a traditional Japanese material used in indigo fermentation. It contains both seed culture and bacterial substrates. However, the onset of indigo reduction greatly varies greatly among batches depending on the fermentation's preparation procedures and *sukumo* quality. In the present study, we used dried *I. tinctoria* LP to increase the homogeneity and synchronicity of *sukumo* fermentations. The addition of *I. tinctoria* LP to the indigo fermentation fluid containing *sukumo* improved the dyeing results and caused an early decrease in ORP. Adjustment to the target pH is challenging because the alkalizer, $Ca(OH_2)$, does not immediately dissolve in the fluid. Abrupt increases in pH during early fermentation might alter the bacterial ecosystem that sustains the indigo reduction. The addition of *I. tinctoria* LP affected the ORP values at the onset of fermentation impacting the bacterial flora in the early fermentation and aged fluid.

The early decrease in the ORP was caused by the addition of *I. tinctoria* LP during the initial fermentation stages and had an important influence on early indigo reduction. The formation of a low ORP fermentation condition is vital to indigo reduction. This naturally occurs during *sukumo* fermentation because of bacterial oxygen metabolism (Tu et al. 2019a). On day 1 of Experiment 1, there were substantially more oxygen-consuming facultative anaerobes in the *I. tinctoria* LP treated group than in the control. Hence, the *I. tinctoria* LP treatment affected the decrease in the ORP. This effect may even be enhanced by *I. tinctoria* LP treatment via oxygen-scavenging activity, such as the transformation of indigo precursors in *I. tinctoria* LP into indigo. Moreover, *I. tinctoria* has flavonoids, saponins, and terpenoids with strong antioxidant activity and reducing power that help lower the dissolved oxygen content (Srinivasan et al. 2016). Ethanolic and hydroethanolic extracts of *I. tinctoria* LP contain anthraquinone (Sharma et al. 2016) which is an electron mediator. Considering these findings, it can be determined that *I. tinctoria* LP may contain certain oxygen scavengers and electron mediators that reduce and stabilise ORP.

On day 1 of Experiment 1, *I. tinctoria* LP exhibited lower microbial community diversity than the control. Therefore, *I. tinctoria* LP may have exerted a selection pressure on the bacteria present in the *sukumo* fermentation. Both the control and the fluid treated with *I. tinctoria* LP, displayed a decrease in the bacterial diversity from day 1 to day 5. Bacterial diversity in *sukumo* preparations usually decrease in the initial phases (from *sukumo* to day 2), possibly because a hot alkaline solution is used in its preparation. This decrease of bacterial diversity is related to the dominance of obligately anaerobic bacteria (Tu et al. 2019a). Therefore, heat treatment and the decrease of ORP influenced the bacterial diversity. In Experiment 3, bacterial diversity increased in the aged fluids for both the *I. tinctoria* LP treated fluid and the control but especially in the former treatment. Similar patterns were reported for the *sukumo* fermentation fluid in previous studies. These late changes in the microbial flora are associated with the

environmental transformation that occurs during fluid aging. This process occurs despite the addition of *I. tinctoria* LP to the *sukumo* fermentation.

In an earlier study, *Bacillaceae* dominated the community on days 2 and 5 of *sukumo* fermentation and was considered responsible for the ORP reduction via the consumption of dissolved oxygen. After the predominance of *Bacillaceae*, the OTU exhibited sequence similarity of 93.1% to obligate anaerobic *Anaerobranca gottschalkii* predominated (Tu et al. 2019a). In Experiment 1, the predominance of obligate anaerobes in the early days happened in both the *I. tinctoria* LP treated fluid and control. However, in the *I. tinctoria* LP treated batch, the dominance of the obligate anaerobic *Proteinivoraceae* was not as strong as seen in the control on day 5. The *I. tinctoria* LP treated fluid contained relatively larger amounts of *Bacillaceae* on the first day, and the ORP declined comparatively faster than the control.

These alterations in microbial community composition (i.e., *Bacillaceae* → *Proteinivoraceae*) may be necessary to initiate indigo reduction. Before the initiation of indigo reduction in *sukumo* fermentation, obligate anaerobic bacterial OTUs predominate, including *Proteinivoraceae* (93.3–93.8% similarity with *P. tanatarense*). These OTUs might be equivalent to *Anaerobranca*, which was found in previous studies on *sukumo* fermentation fluids. *Anaerobranca* decreases before indigo-reducing bacteria such as *P. indicireducens* increase (Tu et al. 2019ab). A similar pattern was observed in the present study for *Proteinivoraceae*. Increases in *Anaerobranca* were accompanied by an increase of dyeing intensity suggesting that this taxon participates in indigo reduction (Tu et al. 2019ab). However, this association was not observed for the control samples in Experiment 1. Therefore, *Proteinivoraceae* predominance is not directly associated to indigo reduction. The relationship between the initiation of indigo reduction and this change in the microbial community remain unknown as this species is difficult to isolate from indigo fluids. The OTUs that make up *Proteinivoraceae* were most closely related to the saprophyte *P. tanatarenses* (Boltyanskaya and Kevbrin 2016). *P. tanatarenses* related OTUs considerably increased soon after the decline in microbial diversity and the decrease of *Bacillaceae*. Hence, *P. tanatarenses* related OTUs might have been consuming the dead *Bacillaceae*. We hypothesize that the importance of this event for indigo reduction relies on the fact that anthraquinones and flavins are biosynthesised for electron transport by some bacteria and can be released after bacterial lysis, helping to reduce indigo (Nicholson and John 2005; Light et al. 2018; You et al. 2018).

The abundances of obligately anaerobic *Proteinivoraceae* (formerly *Anaerobranca*) and *Tissierella* increased with culture age (Tu et al. 2019b). This pattern was also observed in the current study. OTUs distantly related to *Erysipelothrix* increased on day 58 in Experiment 1 and reached even higher levels in Experiment 2. *Erysipelothrix inopinata*, was originally isolated from vegetable broth (Verbarg et al. 2004), supporting the fact that the material for the fermentation fluid are plant-based. A strain belonging to *Erysipelotrichaceae* was isolated from other sukumo preparations and showed reducing ability to indigo carmine (unpublished data).

The presence of *Pseudomonas* in the medium has been linked to fluid acidification and the formation of neutralophilic niches by acid producing bacteria (Tu et al. 2019b). The occurrence of *Pseudomonas* spp.

in Experiment 1 may be associated with the accumulation of acid, which is generated by obligate anaerobic bacteria. Nevertheless, *Pseudomonas* sp. can produce metabolites that enable gram-positive bacteria to perform extracellular electron transfer (Pham et al. 2008) and a *Pseudomonas* strain with reduction activity has been isolated from dyeing vat (Park et al. 2012). In previous studies with *sukumo* fermentation, it was detected in short-lived fluid as well as in decaying long-lived batches (Tu et al. 2019b). *Pseudomonas peli* was closely associated with the observed OTUs. This species was isolated from textile wastewater and can decolorise other dyes (Dellai et al. 2013). In Experiment 2, the amount of *Pseudomonas* spp. did not seem to impede indigo reduction. Therefore, it is more likely that it has a positive or neutral affect on the dyeing intensity.

Hirota et al. (2016), reported indigo-reducing bacteria such as *P. indicireducens* playing a major role in indigo-reducing bacterial communities. *P. indicireducens* might be the key contributor to indigo reduction in both the *I. tinctoria* LP treated fluid and control during the latter stages of Experiment 1 (~ day 58) and in the *I. tinctoria* LP treated batches in Experiments 2 and 3. In earlier studies, the presence of *P. indicireducens* was connected to aging batches (Tu et al. 2019ab). This explains why *P. indicireducens* was absent or low in the *I. tinctoria* LP treated fluid and control in Experiment 1 on earlier days. However, it was not present in the aged control in Experiments 2 and 3. In both case, no other known indigo-reducing bacteria were detected. OTUs related to *Alkalihalobacillus hemicellulosilyticus* (95.6–98.1% similarity) are the presumed indigo reducers as they strongly correlated with dyeing intensity in Experiment 3. In addition, this taxon is also closely related to *P. indicideducens* using 16S rRNA gene sequence similarity. OTUs from the same genus, related to *Alkalihalobacilus alkalinitrilicus* (95.6–98.1% similarity), also predominated in the *I. tinctoria* LP treatment of Experiment 1, which presented the strong staining intensity. *Proteinivoracea*e and *Tissierellaceae* occurred in large quantities but their role in fermentation remains to be clarified. Nevertheless, in the case of *Proteinivoraceae* (formerly *Anaerobranca*), the shift of its dominance to *P. indiciresucens* was linked to the onset of a stable reducing state in *sukumo* fermentation fluids (Tu et al. 2019b). Other known indigo-reducing bacteria had a very sporadic presence in the samples. Indigo-reducing bacteria *Alkalibacterium* spp. was not detected in any sample while *Amphibacillus* spp. occurred in trace amounts. This observation was not reported in earlier studies (Tu et al. 2019ab). The fermentation fluid contains microorganisms with overlapping functions and this property might account for the resilience of this system.

Conclusion

The present study examined the effects of the *I. tinctoria* LP addition on the initial steps of *sukumo*-based indigo fermentation. Experiment 1 demonstrated that a decrease in ORP was essential for accelerating the onset of indigo reduction and that *I. tinctoria* LP could hasten this reaction. Despite the use of different *sukumo* and packaged *I. tinctoria* LPs, the addition of *I. tinctoria* LP rapidly lowered ORP by day1 and stabilised it in the early fermentation phase. Moreover, the microbial taxa profiles differed between the control and the *I. tinctoria* LP treatment and the community structure changed in early-stage and late-stage fermentation batches. Initiation of indigo reduction depended on a rapid decline in ORP simultaneous with oxygen consumption by facultative anaerobes followed by a decrease in facultative

anaerobes from days 1–7. An overall decrease in bacterial community diversity was also required to initiate indigo reduction. *I. tinctoria* LP accelerated the establishment of the biological and physicochemical conditions required for indigo reduction. The present study showed that *I. tinctoria* LP compensate for the inhibitory effect of incorrect pH adjustment on indigo reduction. However, the effect of *I. tinctoria* LP on indigo reduction must be validated on a larger batch fermenter scale. The complex community, limited identity of certain taxa with known BLAST sequences, and lack of recognized indigo-reducing bacteria indicate that *sukumo* harbour numerous bacteria that remain to be characterised.

Declarations

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Author contributions

HFSL, HS, and IY. conceived and designed the experiments. HFSL and ZT performed the experiments. HFSL, ZT and IY analysed the data. HFSL and IY wrote the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare that there is no conflict of interest.

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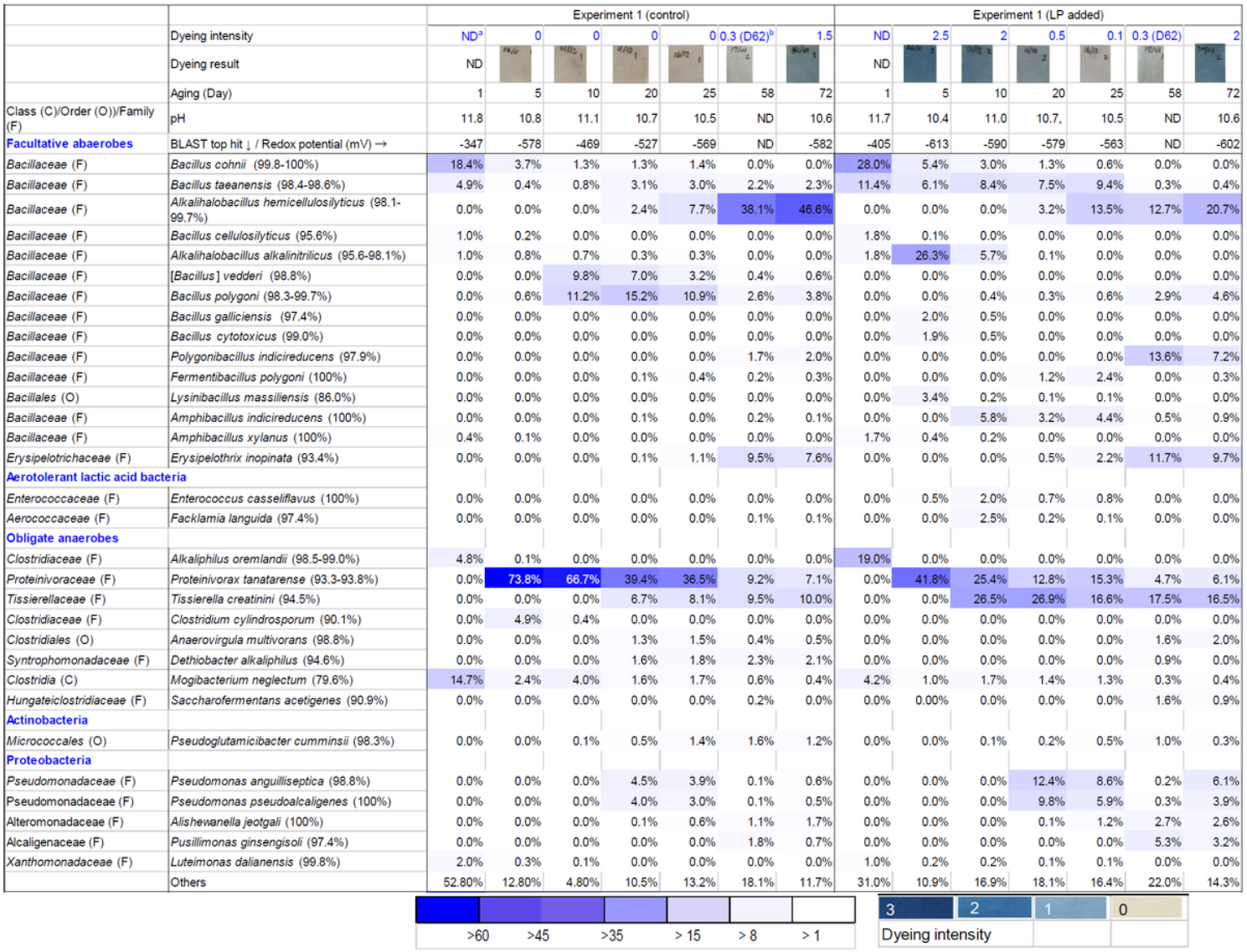
Figures



		Experiment 1 (control)							Experiment 1 (LP added)						
	Dyeing intensity	ND[a]	0	0	0	0	0.3 (D62)[b]	1.5	ND	2.5	2	0.5	0.1	0.3 (D62)	2
	Dyeing result	ND							ND						
	Aging (Day)	1	5	10	20	25	58	72	1	5	10	20	25	58	72
Class (C)/Order (O))/Family (F)	pH	11.8	10.8	11.1	10.7	10.5	ND	10.6	11.7	10.4	11.0	10.7,	10.5	ND	10.6
Facultative abaerobes	BLAST top hit ↓ / Redox potential (mV) →	-347	-578	-469	-527	-569	ND	-582	-405	-613	-590	-579	-563	ND	-602
Bacillaceae (F)	*Bacillus cohnii* (99.8-100%)	18.4%	3.7%	1.3%	1.3%	1.4%	0.0%	0.0%	28.0%	5.4%	3.0%	1.3%	0.6%	0.0%	0.0%
Bacillaceae (F)	*Bacillus taeanensis* (98.4-98.6%)	4.9%	0.4%	0.8%	3.1%	3.0%	2.2%	2.3%	11.4%	6.1%	8.4%	7.5%	9.4%	0.3%	0.4%
Bacillaceae (F)	*Alkalihalobacillus hemicellulosilyticus* (98.1-99.7%)	0.0%	0.0%	0.0%	2.4%	7.7%	38.1%	46.6%	0.0%	0.0%	0.0%	3.2%	13.5%	12.7%	20.7%
Bacillaceae (F)	*Bacillus cellulosilyticus* (95.6%)	1.0%	0.2%	0.0%	0.0%	0.0%	0.0%	0.0%	1.8%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacillaceae (F)	*Alkalihalobacillus alkalinitrilicus* (95.6-98.1%)	1.0%	0.8%	0.7%	0.3%	0.3%	0.0%	0.0%	1.8%	26.3%	5.7%	0.1%	0.0%	0.0%	0.0%
Bacillaceae (F)	*[Bacillus] vedderi* (98.8%)	0.0%	0.0%	9.8%	7.0%	3.2%	0.4%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Bacillaceae (F)	*Bacillus polygoni* (98.3-99.7%)	0.0%	0.6%	11.2%	15.2%	10.9%	2.6%	3.8%	0.0%	0.0%	0.4%	0.3%	0.6%	2.9%	4.6%
Bacillaceae (F)	*Bacillus galliciensis* (97.4%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.0%	0.5%	0.0%	0.0%	0.0%	0.0%
Bacillaceae (F)	*Bacillus cytotoxicus* (99.0%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.9%	0.5%	0.0%	0.0%	0.0%	0.0%
Bacillaceae (F)	*Polygonibacillus indicireducens* (97.9%)	0.0%	0.0%	0.0%	0.0%	0.0%	1.7%	2.0%	0.0%	0.0%	0.0%	0.0%	0.0%	13.6%	7.2%
Bacillaceae (F)	*Fermentibacillus polygoni* (100%)	0.0%	0.0%	0.0%	0.1%	0.4%	0.2%	0.3%	0.0%	0.0%	0.0%	1.2%	2.4%	0.0%	0.3%
Bacillales (O)	*Lysinibacillus massiliensis* (86.0%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.4%	0.2%	0.1%	0.1%	0.0%	0.0%
Bacillaceae (F)	*Amphibacillus indicireducens* (100%)	0.0%	0.0%	0.0%	0.1%	0.0%	0.2%	0.1%	0.0%	0.0%	5.8%	3.2%	4.4%	0.5%	0.9%
Bacillaceae (F)	*Amphibacillus xylanus* (100%)	0.4%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	1.7%	0.4%	0.2%	0.0%	0.0%	0.0%	0.0%
Erysipelotrichaceae (F)	*Erysipelothrix inopinata* (93.4%)	0.0%	0.0%	0.0%	0.1%	1.1%	9.5%	7.6%	0.0%	0.0%	0.0%	0.5%	2.2%	11.7%	9.7%
Aerotolerant lactic acid bacteria															
Enterococcaceae (F)	*Enterococcus casseliflavus* (100%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%	2.0%	0.7%	0.8%	0.0%	0.0%
Aerococcaceae (F)	*Facklamia languida* (97.4%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.0%	0.0%	2.5%	0.2%	0.1%	0.0%	0.0%
Obligate anaerobes															
Clostridiaceae (F)	*Alkaliphilus oremlandii* (98.5-99.0%)	4.8%	0.1%	0.0%	0.0%	0.0%	0.0%	0.0%	19.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Proteinivoraceae (F)	*Proteinivorax tanatarense* (93.3-93.8%)	0.0%	73.8%	66.7%	39.4%	36.5%	9.2%	7.1%	0.0%	41.8%	25.4%	12.8%	15.3%	4.7%	6.1%
Tissierellaceae (F)	*Tissierella creatinini* (94.5%)	0.0%	0.0%	0.0%	6.7%	8.1%	9.5%	10.0%	0.0%	0.0%	26.5%	26.9%	16.6%	17.5%	16.5%
Clostridiaceae (F)	*Clostridium cylindrosporum* (90.1%)	0.0%	4.9%	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Clostridiales (O)	*Anaerovirgula multivorans* (98.8%)	0.0%	0.0%	0.0%	1.3%	1.5%	0.4%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%	1.6%	2.0%
Syntrophomonadaceae (F)	*Dethiobacter alkaliphilus* (94.6%)	0.0%	0.0%	0.0%	1.6%	1.8%	2.3%	2.1%	0.0%	0.0%	0.0%	0.0%	0.0%	0.9%	0.0%
Clostridia (C)	*Mogibacterium neglectum* (79.6%)	14.7%	2.4%	4.0%	1.6%	1.7%	0.6%	0.4%	4.2%	1.0%	1.7%	1.4%	1.3%	0.3%	0.4%
Hungateiclostridiaceae (F)	*Saccharofermentans acetigenes* (90.9%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%	0.00%	0.0%	0.0%	0.0%	1.6%	0.9%
Actinobacteria															
Micrococcales (O)	*Pseudoglutamicibacter cumminsii* (98.3%)	0.0%	0.0%	0.1%	0.5%	1.4%	1.6%	1.2%	0.0%	0.0%	0.1%	0.2%	0.5%	1.0%	0.3%
Proteobacteria															
Pseudomonadaceae (F)	*Pseudomonas anguilliseptica* (98.8%)	0.0%	0.0%	0.0%	4.5%	3.9%	0.1%	0.6%	0.0%	0.0%	0.0%	12.4%	8.6%	0.2%	6.1%
Pseudomonadaceae (F)	*Pseudomonas pseudoalcaligenes* (100%)	0.0%	0.0%	0.0%	4.0%	3.0%	0.1%	0.5%	0.0%	0.0%	0.0%	9.8%	5.9%	0.3%	3.9%
Alteromonadaceae (F)	*Alishewanella jeotgali* (100%)	0.0%	0.0%	0.0%	0.1%	0.6%	1.1%	1.7%	0.0%	0.0%	0.0%	0.1%	1.2%	2.7%	2.6%
Alcaligenaceae (F)	*Pusillimonas ginsengisoli* (97.4%)	0.0%	0.0%	0.0%	0.0%	0.0%	1.8%	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	5.3%	3.2%
Xanthomonadaceae (F)	*Luteimonas dalianensis* (99.8%)	2.0%	0.3%	0.1%	0.0%	0.0%	0.0%	0.0%	1.0%	0.2%	0.2%	0.1%	0.1%	0.0%	0.0%
	Others	52.80%	12.80%	4.80%	10.5%	13.2%	18.1%	11.7%	31.0%	10.9%	16.9%	18.1%	16.4%	22.0%	14.3%

Figure 1

Relative abundance (%) of bacterial constituents based on 16S rRNA analysis (≥ 1.5% in sample), dyeing intensity, pH, and redox potential (ORP) of Experiment 1 sukumo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder. Percentages in brackets in BLAST top hit column indicate the ranges of similarities to known species in the database. Classification hierarchy (columns A and B) are described as follows: G, genus; F, family; O, order; C, class. aND, no data; b(D62), result of Day 62 sample.

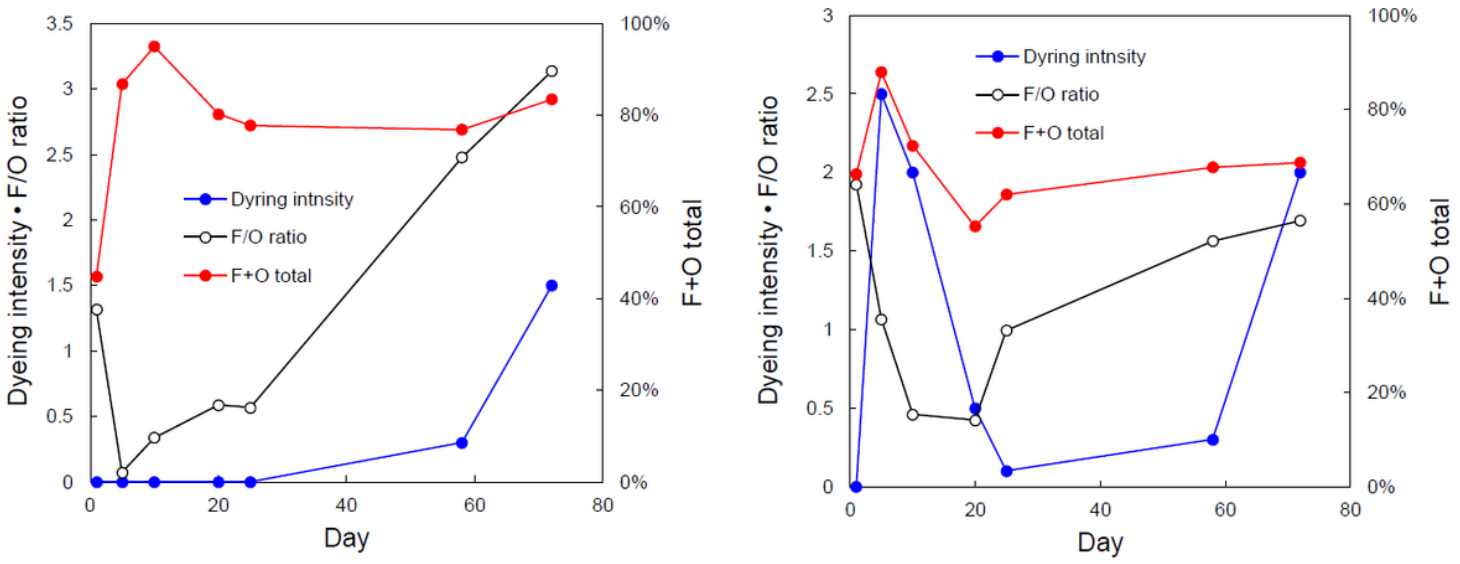


Figure 2

Changes in dyeing intensity, abundance ratios of facultative anaerobes and absolute anaerobes (F/O), and total abundance ratios of facultative anaerobes and absolute anaerobes (F + O) in sukumo determined for Experiment 1 indigo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder.

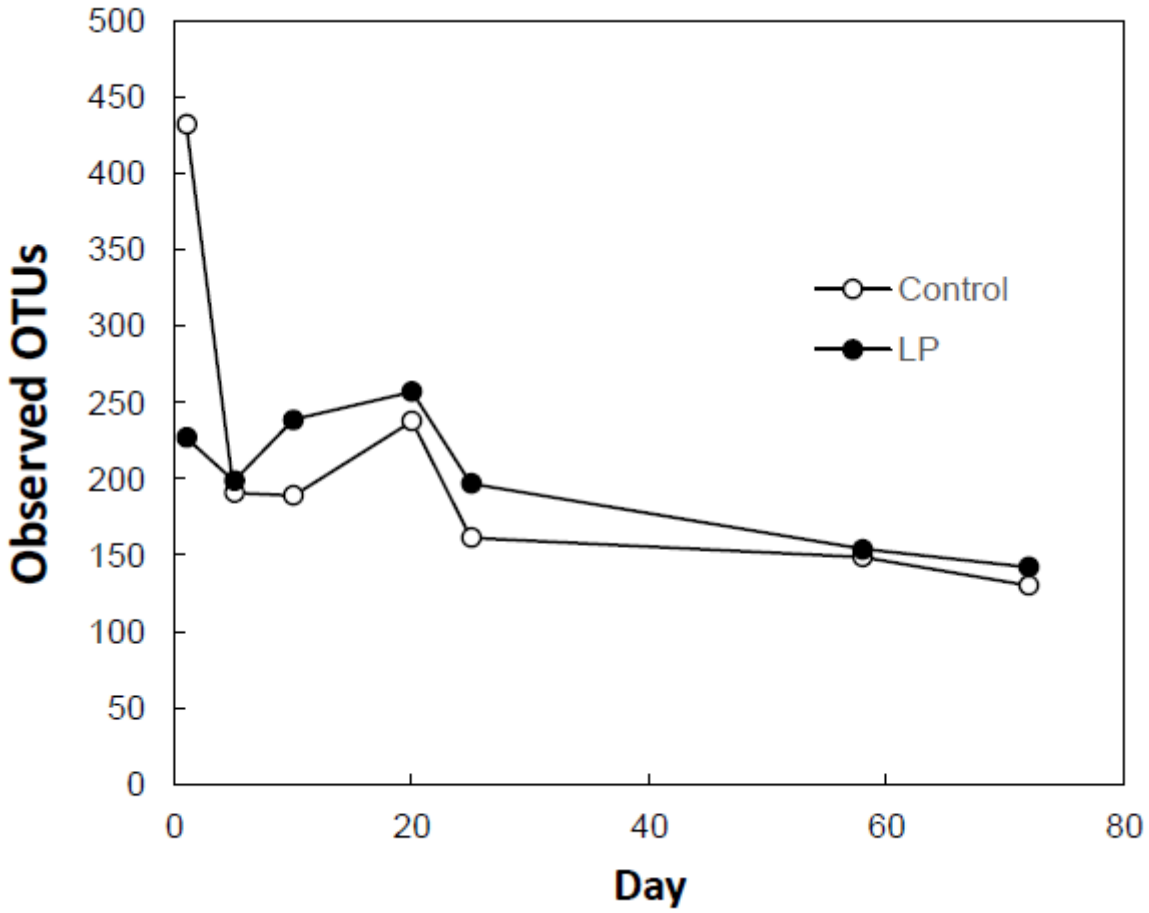


Figure 3

Changes in observed OTUs at sample depth of 18512 varying with sukumo fermentation period using Experiment 1 indigo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder. Analysis was performed with the Divisive Amplicon Algorithm (DADA2) and depended on the fermentation period.

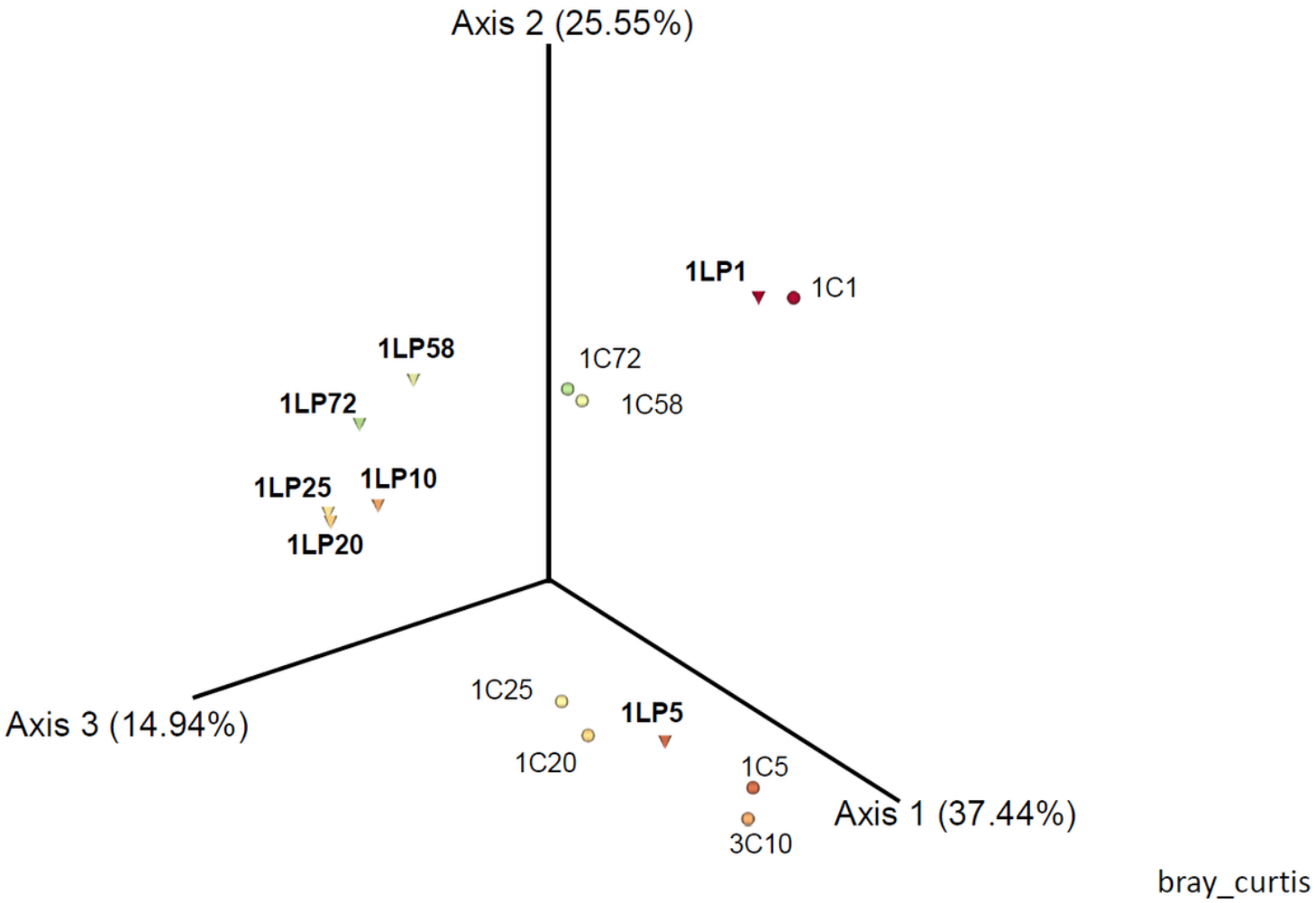


Figure 4

Principal coordinate analysis (PCoA) using Bray-Curtis dissimilarity matrix in Experiment 1. Triangles and circles indicate samples with (LP) and without (control) Indigofera tinctoria leaf powder, respectively.

		Experiment 2 (control)			Experiment 2 (LP added)			Experiement 3 (control)				Experiment 3 (LP added)			
	Dyeing intensity	2.5	2.5	2.5 (D98)[a]	2.5	2.5	2.5 (D98)	1.5	0.5 (D147)[c]	1.0	1.0	3	1.5 (D147)	2.5	1.0
	Dyeing result														
	Aging (Day)	36	50	94	36	50	94	115	142	176	226	115	142	176	226
Order (O)/Family (F) /Genus (G)	pH	10.7	10.8	ND[b]	10.8	10.7	ND	10.8	ND	10.7	10.9	10.2,	ND	11.0,	10.8
Facultative anaerobes	BLAST top hit ↓ / Redox potential (mV) →	-647	-641	ND	-656	-644	ND	-629	ND	-579	-619	-652	ND	-630	-613
Bacillaceae (F)	*Bacillus cohnii* (98.3-100%)	1.5%	5.7%	7.8%	2.7%	9.8%	4.0%	0.6%	1.0%	0.5%	0.4%	0.2%	0.2%	0.2%	0.2%
Bacillaceae (F)	*Alkalihalobacillus hemicellulosilyticus* (98.1-99.3%)	0.1%	0.0%	0.0%	0.4%	0.0%	0.0%	22.2%	10.4%	42.7%	41.3%	4.1%	4.4%	4.4%	2.1%
Bacillaceae (F)	*Bacillus polygoni* (99.7%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.2%	0.5%	2.4%	2.6%	1.4%	2.0%	0.7%	2.4%
Bacillaceae (F)	*Bacillus kokeshiiformis* (99.8%)	0.7%	0.6%	0.5%	0.7%	0.3%	0.3%	0.6%	0.8%	0.4%	0.3%	0.4%	0.4%	0.2%	0.5%
Bacillaceae (F)	*Bacillus licheniformis* (99.8-100%)	0.2%	0.3%	0.2%	0.2%	0.1%	0.0%	2.6%	1.8%	0.9%	1.1%	1.1%	0.4%	0.6%	1.1%
Bacillaceae (F)	[*Bacillus*] vedderi (98.8%)	0.0%	0.0%	0.0%	0.6%	0.7%	0.1%	0.4%	2.2%	1.3%	1.2%	0.2%	0.0%	0.0%	0.0%
Bacillaceae (F)	*Polygonibacillus indicireducens* (97.7-98.6%)	0.0%	0.0%	0.0%	4.4%	9.0%	13.2%	0.4%	0.1%	0.0%	0.0%	33.8%	38.3%	46.7%	23.5%
Erysipelotrichaceae (F)	*Erysipelothrix inopinata* (93.2-93.4%)	30.1%	15.6%	11.3%	36.7%	17.5%	4.4%	24.2%	9.4%	3.4%	4.8%	12.1%	3.4%	6.0%	5.8%
Aerotolerant lactic acid bacteria															
Bacillaceae (F)	*Amphibacillus xylanus* (99.3%)	0.9%	0.2%	0.1%	0.0%	0.0%	0.2%	0.3%	0.3%	0.4%	0.6%	0.4%	0.2%	0.1%	0.5%
Obligate anaerobes															
Clostridiaceae (F)	*Alkaliphilus metalliredigens* (79.8%)	1.2%	1.7%	2.0%	1.0%	0.8%	2.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Proteinivoraceae (F)	*Proteinivorax tanatarense* (93.1-93.8%)	28.2%	24.1%	19.8%	6.5%	3.5%	9.4%	9.0%	31.3%	9.2%	1.9%	13.0%	17.6%	5.2%	1.4%
Tissierellaceae (F)	*Tissierella creatinini* (94.5%)	9.3%	3.2%	2.7%	12.5%	9.1%	19.9%	11.0%	6.0%	5.0%	9.3%	5.4%	2.9%	3.9%	10.7%
Clostridiaceae (F)	*Clostridium septicum* (89.0%)	1.5%	0.4%	0.2%	0.4%	0.8%	0.4%	1.0%	1.2%	1.1%	0.7%	0.7%	1.6%	1.1%	1.3%
Bacillaceae (F)	*Anaerobacillus macyae* (98.8%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%	1.3%	1.1%	1.5%
Syntrophomonadaceae (F)	*Dethiobacter alkaliphilus* (94.6%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.5%	0.3%	0.1%	1.4%	0.0%	0.0%	0.0%	0.0%
Hungateiclostridiaceae (F)	*Saccharofermentans acetigenes* (90.9%)	0.0%	0.0%	0.0%	0.5%	0.1%	0.2%	1.9%	0.2%	0.0%	0.1%	0.7%	0.2%	0.3%	0.3%
Actinobacteria															
Micrococcales (O)	*Pseudoglutamicibacter cumminsii* (98.3%)	0.7%	0.8%	1.0%	1.2%	0.8%	1.6%	1.1%	4.2%	2.3%	2.1%	0.6%	0.7%	0.5%	1.0%
Corynebacteriales (O)	*Corynebacterium pollutisoli* (100%)	0.0%	0.1%	0.1%	0.0%	0.0%	0.0%	1.5%	1.0%	0.6%	0.2%	0.0%	0.0%	0.0%	0.0%
Micrococcales (O)	*Arthrobacter endophyticus* (98.5%)	0.0%	0.0%	0.0%	1.8%	0.5%	0.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Proteobacteria															
Pseudomonadaceae (F)	*Pseudomonas anguilliseptica* (98.8%)	1.1%	9.7%	9.0%	0.9%	11.2%	10.6%	0.0%	0.0%	3.1%	0.2%	0.9%	0.7%	0.0%	0.7%
Pseudomonadaceae (F)	*Pseudomonas pseudoalcaligenes* (99.8-100%)	0.9%	9.9%	10.5%	0.9%	10.4%	12.0%	0.0%	0.1%	2.5%	0.2%	0.9%	1.3%	0.1%	0.5%
Pseudomonadaceae (F)	*Pseudomonas peli* (98.4%)	0.1%	0.4%	7.3%	0.3%	0.5%	4.8%	0.0%	0.0%	0.0%	0.0%	0.5%	2.0%	0.1%	0.2%
Pseudomonadaceae (F)	*Pseudomonas nitrititolerans* (97.7%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	3.7%	4.9%	2.9%	1.7%
Alteromonadaceae (F)	*Alishewanella jeotgali* (100%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.1%	0.2%	1.1%	1.4%	0.4%	1.0%	0.7%	1.6%
Rhodobacterales (O)	*Paracoccus cavernae* (98.8-99.0%)	0.4%	2.4%	1.8%	0.1%	0.9%	0.6%	0.1%	0.4%	0.3%	0.6%	0.6%	1.1%	1.3%	2.2%
Alcaligenaceae (F)	*Pusillimonas ginsengisoli* (97.4%)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.6%	0.2%	2.9%	5.5%	0.7%	0.6%	1.4%	1.5%
	Others	23.2%	24.9%	25.7%	28.3%	24.1%	15.3%	20.8%	28.4%	20.0%	24.2%	17.6%	14.8%	22.5%	39.1%

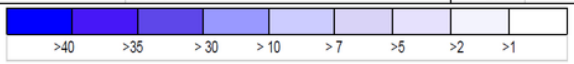


Figure 5

Relative abundance (%) of bacterial constituents based on 16S rRNA analysis (≥ 1.5% in sample) dyeing intensity, pH, and redox potential (ORP) in sukumo using indigo fermentation batches from Experiment 2 and 3 with (LP) and without (control) Indigofera tinctoria leaf powder. Percentages in brackets BLAST top hit column indicate range of similarities to known species in the database. Classification hierarchy (columns A and B) described as follows: G, genus; F, family; O, order; C, class. a(D98), result of Day 98 sample; bNA, no data; c(D147), result of D147 sample.

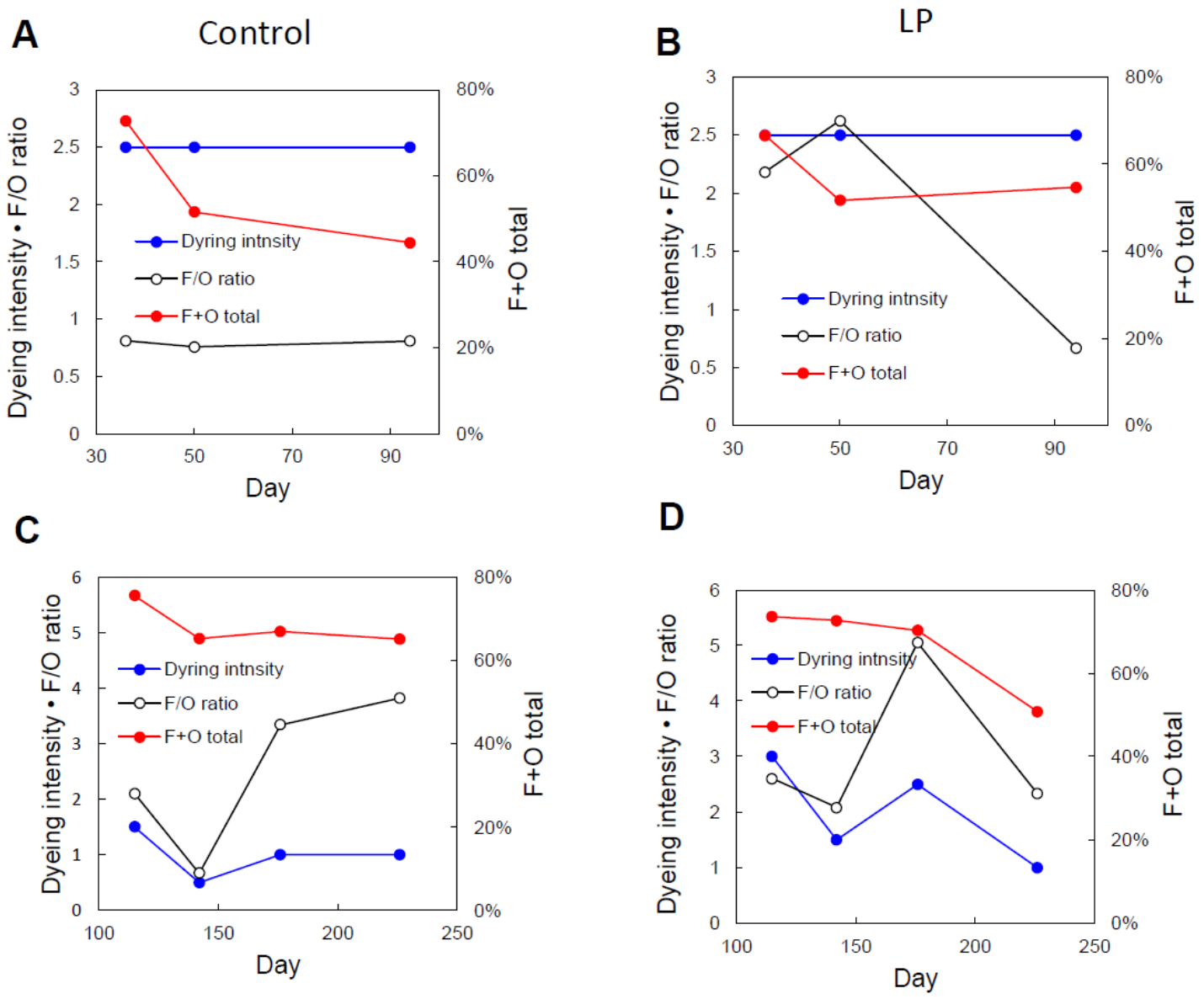


Figure 6

In contrast, changes in F/O varied with dyeing intensity in Experiment 3 (Figs. 6C–6D). The changes in OTU number at the 18512-sampling depth (Supplementary Fig. 5) in Experiment 2 indicated a decrease in diversity with fermentation time for I. tinctoria LP added fluid but not the control. In Experiment 3, the observed OTU more rapidly increased with fermentation time for I. tinctoria LP than the control. PCoA by the Bray-Curtis dissimilarity matrix showed a distinct separation between I. tinctoria LP and the control for in Experiments 2 and 3 (Supplementary Figs. 6A–6B).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- WJBMSupplementaryMaterial.docx