Bacterial profile of different indigo fluids and the effect of the addition of *Indigofera tinctoria* leaf powder on *sukumo* preparations

（原料の異なる藍染め発酵液の微生物叢の解析およびスクモを用いた藍染め発酵液へのインド藍粉の効果）

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General Introduction

Indigo has been used by mankind since ancient times and its oldest indication of used dates to 6000BP. It can be extracted from many plants, and it is present in all the corners of the world. Natural indigo was a valuable trade commodity acquiring status of "blue gold" until synthetic indigo and the chemical process of dyeing were created in the 19th century.

Natural indigo can be extracted from many plants, but only its precursor form is present in them. For the precursor to become indigo it must be hydrolyzed and then oxidized. Because of its vast distribution around the world different processes to extract and preserve indigo were created. Somewere based on the direct extraction of indigo by adding the plant leaves to water and using beating and aeration tanks or lime to decant indigo. The supernatant is discarded, and the decanted material is heated, filtered, and dried (Indian indigo and Ryukyu ai). Others rely on the fermentation of the leaves in special conditions and the resulting material is rich in organic matter and bacteria, characteristics that will be useful in the following fermentation step (coached woad and *sukumo*). Nevertheless, independent of the extraction method this process is necessary for the transformation of the precursor into indigo, its concentration and stabilization for storage.

Indigo has another peculiar characteristic; it is insoluble in water and needs to be transformed into its soluble form (leuco-indigo) to penetrate the textile fibers. The reduction can happen by several ways. In the case of bacterial fermentation, the fluid is prepared using an indigo source and additives (rice wine or wheat bran for example). Bacterial seed can be added if the indigo source did not come from a fermentative extraction. The fermentation process happens in an alkaline environment and depending on the material used it can take days to weeks for the indigo reduction to start and the fluid be ready to dye. When the fabric that has been immerse in the reduced indigo fluid enters in contact with the air's oxygen the leuco-indigo oxidize to indigo and stay impregnated into the fibers. The chemical reduction of indigo, although cheap and fast, it produces a large amount of contaminated wastewater that is damaging to the environment. The study of the traditional techniques has opened the possibility of creating more sustainable processes.

Currently, many different indigo fermentation methods have been the focus of studies. *Sukumo* fermentation studies revealed many characteristics of this process, but still couldn't elucidate all the aspects of it, especially regarding the initial changes and the effect of outside

factors. Furthermore, preparation using different types of indigo sources other than *sukumo* or woad have never been characterized.

While the bacterial community of *sukumo* fluids has already been outlined, the bacterial community of fermentations fluids made with other indigo sources lack investigation. Therefore, the first chapter of this study focus on the characterization of fermentation fluids using other sources of indigo (Ryukyu ai paste and Indian cake) and comparing them with different *sukumo* fermentations. Next generation sequencing (NGS) was used to identify the bacterial community and analyze its diversity.

The deep understanding of the process of complex fermentations can give clues for the creation of more effective fermentation process. The present study aims to show the importance of the bacterial seed in the fermentation, and that the reduction can happen despite the differences in diversity and complexity of the community. Some specific microorganisms can be the key for the indigo reduction onset and stability. In addition, the conditions of the fluid are also important, such as the effect of the presence of debris in the fermentation fluid. The manipulation of the factors in the preparation process can affect the outcome of the fermentation.

Taking in consideration the characteristics of how *sukumo* is prepared (open air fermentation), and that the bacterial community for the fermentation comes from *sukumo,* it is expected that the onset of indigo reduction may vary (from days to weeks) according to the quality of the *sukumo* and preparation procedures for the fermentation. Because of the high diversity of *sukumo* fluids, the presence of an adequate bacterial community is important. Chapter 2 target the effects of the addition of *Indigofera tinctoria* leaf powder (LP) during the preparation of *sukumo* fluid. To understand the effects in all aspects of the fermentation, the pH, oxidation and reduction potential (ORP), dyeing and bacterial community (using NGS) were studied. The LP might have certain effect for the initial microbial community.

Since there is an increasing interest in finding environmentally friendly reductions of indigo, new processes have been emerging. Nevertheless, the answer for the environmental problem of dyeing procedures is still far from be solved due to its complexity. The focus on traditional methods have increased lately in the attempt of finding viable alternatives for the current methodology. Although hard to control and not efficient enough to supply the global indigo dyeing demand, traditional indigo reduction still can promote the discovery of new techniques. Therefore, the present work aims to characterize and compare the bacterial community of different *sukumo* fluids and fluids made with different indigo sources, and the effect of *I. tinctoria* leaf powder addition to the *sukumo* fermentation. Hopefully this research

will provide knowledge for the creation of new techniques and guidelines to improve the efficiency of fermentations.

General overview

1.1. Brief history on Indigo Dye

Indigo is one of the oldest dyes used by mankind worldwide. Recently findings at the Peruvian Preceramic site in Huaca Prieta dating back to 6200BP are the earliest evidence of its use known so far [1]. Additionally, Egyptian Mummies from about 2500BC were covered in indigo dyed hemp fabrics, and woad, native from Southeast Russia, was used in Northern Europe during the bronze age (2500-850 BC). Its presence can also be found in other ancient civilizations from Mesopotamia, Egypt, Greece, Rome, Britain, Iran, and Africa [2].

The origin of the dye's name come from the Greek word *indikón* (ινδικόν), meaning "coming from India". The Phoenician trades and migrating people were responsible for gradually introducing indigo dye from India to the Mediterranean area, and from there it spread to Europe. During the Middle Ages (5th to 15th centuries), France and Germany were renowned woad trade centers, but in the late fifteenth century, Portuguese explores established a sea route to India which resulted in the tropical indigo overtaking European markets. Soon it became the largest export business in the world, acquiring a high value and being referred as "blue gold" [2].

The demand of indigo dramatically increased during the industrial revolution, which was in part due to the popularization of blue denim jeans. The potential for color fading, and the possibility of creating vintage looks by abrasion and other finishing processes, helped the popularization of the blue denim jeans. Natural extraction was expensive and could not provide the quantities required for the industry, therefore it was necessary to find cheap and easier sources of Indigo. In 1883 the chemical structure of indigotin was determined, and in 1897 the synthetic product was launched into the market [3]. As a result, the exports of natural Indian indigo fell from 187000tons in 1895 to 11000tons in 1913. The appearance of synthetic Indigo destroyed almost completely the production of tropical indigo [2].

Since the 18th century, the scientists searched for a process to reduce indigotin that would be quicker and more easily controlled than the traditional fermentation vats. Various cold vat processes were adopted using the reducing potential of substances like arsenic trisulphide, ferrous sulphate, or zinc dust. In 1870, just before the creation of synthetic indigo, the hydrosulphite vat, made with the powerful reducing agent sodium hydrosulphite (sodium

dithionite), was developed. This recently discovered method rapidly became the most used method worldwide for both synthetic and natural indigo [3].

1.2. Indigo proprieties

Indigo is also known as indigo blue and indigotin (C.I. Vat Blue 1, CI7300, CAS number: 482/582-89-3, IUPAC name 3H-indol-3-1, 2-(2,3-dihydro-3oxo-2H-indol-2-ylidene)-1,2-dihydro-, chemical formula $C_{16}H_{10}N_2O_2$) (Figure 1). In ambient temperature and normal pressure, it presents itself as dark blue-violet needles or prisms with a distinctive coppery luster. In the electromagnetic spectrum the color is placed between 420 and 450 nm wavelength. Indigo is insoluble in water and poorly soluble in most common solvents, most likely due to the strong inter- and intramolecular hydrogen bonds that are formed in indigo crystals. This also explaining indigo's relatively high melting point, 300 °C. It is more soluble in polar organic solvents than non-polar ones. It has a low mammalian toxicity and there is no indication of sensibilization in humans after repeated skin applications [2].

1.3. Natural Indigo

Many different species of plants can be used to extract natural indigo. Commonly, *Indigofera* plant species, such as *Indigofera tinctoria*, were used in tropical regions like India. In Europe, dye's woad (*Isatis tinctoria*) was the traditionally cultivated plant for indigo production, and dyer's knotweed (*Polygonum tinctorium*) was used mainly in China and Japan. In the colonial North America, there were three commercially important species: the native *Indigofera caroliniana*, the introduced *I. tinctoria* and *Indigofera suffruticosa*. Worldwide, there are more than 300 species of plants that can produce indigo [2].

However, the indigo molecule is not present in the plant, instead, the substance that will be converted into indigo is present. These indigo precursors are colorless glycosides of indoxyl (ß-hydroxy-indole) (Figure 2). Their attachment to glucose while inside the fresh green leaves gives them stability. However, when leaves are fermented, the precursor is hydrolyzed (cleavage of the sugar residue) by glucosidase enzymes to yield indoxyl, which can be transform into indigo by oxidative dimerization (Figure 3). The enzyme is present in the leaf, but in different compartments in the cell, and when the leaf is damaged it is released causing the transformation of the precursor [2,3].

Indican is one of those glycosides of indoxyl and was the first precursor to be identified and characterized from *Indigofera* plants. Presently it has also been identified in many species of plants belonging to many different families. From woad, different precursors were also isolated in addition to indican: Isatan A and B, also present in Chinese woad (*Isatis indigotica)* [3]. Young leaves of *I. tinctoria* had yet another indigo precursor which was named isatan C, which favors the production of isoindirubin rather than indigo [4]. Whatever is the structure of the percussors present, they will all basically produce indoxyl which will be transformed in the many different constituents of natural indigo [3] (Figure 4).

Indigotin, the main coloring matter formed by the combination of two indoxyl molecules by air oxidation, and it is the blue substance mostly responsible for the dyeing proprieties of Indigo. Isatin, another substance that can be produced by the precursors, is favored by special circumstance related with alkali and acid environment, as well as temperature. Indirubin (red shade of indigo) is made by the condensation of isatin with indoxyl, and it is an indigotin isomeric with its formation still not completely understood, appearing to be related to pH and temperature. Other minor indigoid colorants can also be formed in varying proportions: *cis*-forms of idigotin (also blue) and idirubin (called isoindirubin red) and the brown isoindigo. Natural indigo usually contains varying proportions of these constituents, together with mineral impurities and sometime with colorants of another chemical nature such as flavonoids [3].

A large amount of plant is required to produce a significant quantity of dye. Additionally, the conditions of plant cultivation, such as water availability can also affect the yield of indigo [5]. The purity of plant-derived indigo even with modern extraction methods is somewhat low when compared to synthetic indigo. Natural indigo contains impurities such as indirubin, indigo-brown, indigo gluten and mineral matter. The purity of indigo from woad has been reported to be 20-40%, and *Indigofera* from 50% to 70% [2].

1.4. Methods to extract and preserving natural indigo

1.4.1. Drying

Leaves can be preserved by drying, which is not a traditional procedure, but it is convenient for small scale use. Depending on the drying method, Indican levels can be increased in relation to other precursors, and oxidation of indoxyl may not happen in the drying

product, mainly because of powerful antioxidants present in the dried leaves [6,7]. Therefore, indigo is not completely formed in dried leaves product unless the drying happened after grinding of fresh leaves [6]. To use the dye, the powder is soaked in cold water for several hours or have hot water poured over it. The procedures are similar to the ones used for dyeing with fresh leaves [3].

1.4.2. Crushing and fermenting

To produce indigo dye from woad the leaves are crushed to a pulp which is kneaded into balls, and then allowed to dry for several weeks. The dried ball stage can already be stored for later processing. To obtain the final product, the balls needed to be couched. To do so, the balls are crushed into powder and wetted allowing the material to ferment for several weeks. After couching the woad become a dark clay like material that is dried and tightly packed to storage and use [2].

To produce *sukumo* (composted leaves of *Polygonum tinctorium*), the leaves are harvested and air-dried. Approximately 3tons are mixed with the same amount of water and stacked to a high of 1 m. Once every 2-3 days the leaves are mixed up and down with water for sufficient aeration (oxidative fermentation). The temperature reaches 60°C due to fermentation heat during plant tissue decomposition. This operation takes 100 days to produce *sukumo* [8 9]. During this process the indigo precursors inside the plant are transformed into indoxyl and later react with oxygen to form indigo.

The characteristics of the fermentation (pH, OD, temperature and length of fermentation) can impact the yield of indigo [10]. The advantage of coached woad and Japanese *sukumo* is that they can be kept for a long time and are easily transported. They are also good for the onset of bacterial fermentation vats because they still contain microorganisms and many vegetable matters [3].

1.4.3. Direct extraction of indigo

Although there are several variations to the method, traditionally, cut plants are tied in bundles, packed into the fermenting vat, and covered with clear water. There the plant is allowed to steep for 10 -15h, during which natural fermentation start. The liquor, which varies from a pale straw color to yellow is run into the beating vat where is agitated either manually

or mechanically. This step in important to transform the indigo precursors into indigo. The liquid becomes green, then blue, and finally indigo separates into flakes and precipitates to the bottom of the vat. Indigo is them allowed to thoroughly settle and the supernatant liquid is drawn off. The pulpy mass of indigo is boiled with water for a few hours to remove impurities, filtered and them pressed to remove as much moisture possible, after which it is cut into cubes and finally air dried. [2].

1.5. Dyeing with natural indigo

Indigo differs from other dyes in many aspects. First because it is not present in the in the plants but is rather produced when its precursors present in the plant react with the oxygen in the air. Secondly, because its blue form is insoluble and can't impregnate in the textile fibers, even when a mordant is used. To use indigo as a textile dye, it needs to be transformed into a colorless soluble substance *leuco*-indigo (from the Greek *leukos*, meaning white), an acid form that is transformed into a salt through reaction with alkalis and can be absorbed by the fibers. After the fabric is immersed into the dyebath and removed, its oxidizes to the insoluble blue form again [3] (Figure 5).

The various methods used for dyeing obey this core concept: the utilization of the soluble form of indigo. This can be done by dyeing with idoxyl before it oxidizes to indigo or reducing indigo and making it soluble again. Most of the methods involve the process of indigo reduction in an alkaline medium to produce *leuco*-indigo [3].

1.5.1 Dyeing with fresh indigo plants

The complex chain of reaction necessary to obtain indigo has not been studied in detail in all plants, so the scheme proposed for the dyeing with fresh leaves should be seen as a likely hypothesis. Variation may occur from one plant to another based on the anatomy of the leaves, the bacteria present, enzymes, and precursors presents [3].

The fresh leaves (sometimes previously shredded or pounded) are macerated in hot water. In woad case, the enzyme present in the leaves and produced by bacteria present in the vegetable matter provoke the hydrolysis of the indigo precursors. In Knotweed the enzyme is present in the chloroplasts [3].

After the hydrolysis, an alkali is added (usually lime, wood ash lye or sodium carbonate) to help preserve the vat and neutralize acids from the bacterial fermentation. The bacterial proliferation maintains anoxia of the environment preventing the oxidative dimerization of indoxyl into indigotin. The dye remains soluble and can impregnate into the textile fibers. The fabric is removed from the vat and the indoxyl in the fibers combines with the oxygen becoming insoluble indigo [3].

1.6. *Sukumo* fermentation

One important characteristic of *sukumo* fermentation is that its community is very dynamic. The bacterial community changes according to the material used and fermentation stage. *Sukumo* quality is very important since it is the bacterial source for the fermentation process. Usually the important microorganism are in very low amount in *sukumo* and the preparation procedures (temperature and pH) are responsible for selecting them [11]. Despite always changing, some characteristics are shared among *sukumo* fermentation. In recent studies using NGS, 3 major phyla appear to be prevalent *Proteobacteria, Firmicutes*, and *Actinobacteria* [12]. There is also some trends observed in the microbial community during the aging of the fluids. The community changes from aerobes and facultative aerobes to anaerobes, and then it changes again to the predominance of facultative aerobes (usually indigo reducers) [12]. There is a big difference from *sukumo* community to the community in the day 2 of fermentation, which is expected since it is a big change of environment (from the sukumo environment to a alkaline fluid) .There is an peaking in *Anaerobranca* abundance when the fluid gets closer to 10 days of fermentation which will decrease with time [12] [11]. Another anaerobic group that appears around the same period is *Tissierella* [12] [11]. These bacteria are usually related to a low redox and strong dyeing. Regarding indigo reducing bacteria, the genera *Alkalibacterium* and *Amphibacillus* are usually present in the young fluids while older fluids are usually dominated by *Polygonibacillus* [11]. Others indigo reducing genus such as *Oceanobacillus* can also be sometimes found in the fluid [12].

1.7. Synthetic indigo and chemical reduction vats

With the increasing demand for indigo scientists searched for processes to produce and reduce indigo that would be fast and cheaper. The chemical structure of indigo was discovered

by Adolf von Baeyer, who later obtained the first patent for making synthetic indigo from toluene in 1880. Ten years later, K. Heumann published his synthesis of indigo from benzene, but the yield was not enough. In the same year he published a modification using anthranilic acid derived from naphthalene, a substance that was available in large quantities from coal tar [13]. Despite being the same chemical substance, natural and synthetic indigo are different. Plant indigo had 3 oxidation peaks in electroanalysis while synthetic indigo had only 2. This difference may be due to slightly differences in chemical structures, but also the presence of other substances in the natural indigo that accelerate the transfer of electron on the electrode surface, thus reducing the overpotential [14].

Other than the indigo production, another necessary point was to find a quicker and more easily controlled way to reduce indigo than the traditional fermentation vats. Various cold vat processes were adopted using the reducing potential of substances like arsenic trisulphide, ferrous sulphate, or zinc dust. In 1870, just before synthetic indigo was discovered, the hydrosulphite vat prepared with the powerful reducing agent sodium hydrosulphite (sodium dithionite) was developed and it is the most used worldwide for both synthetic and natural indigo [3].

Although the new chemical vat process was easier, cheaper, and fast it still has drawbacks. The process uses ammonia or sodium carbonate to adjust the alkalinity which can affect the fabric. It is almost impossible to build up darker tones by repeated immersions since the indigo already present inside then fabric can be reduced again, therefore the intensity will depend on the quantity of indigo in the preparation of the vat. The vat needs to be maintained at temperature of 55°C [3].

There are also drawbacks from the environmental point of view, such as the problem of how to deal with the enormous quantity of waste. Dyeing and finishing wastewater are the highest pollution load in textile industry accounting for about 80% of the total wastewater generation having the most environmental impact due to the high COD and color loads [15]. To reduce 3kg of indigo 2kg of sodium dithionite are necessary which break down to polluting Sulphur compounds [3]. This water cannot be directly reused in the processing plant, but after proper treatment some can become reusable [15].

1.8. Indigo reducing bacteria

To understand indigo reduction in fermentation preparations, the study of the reducing bacteria is necessary. In the last few years many new indigo reducing bacteria has been isolated. From the genus *Alkalibacterium*, 3 indigo reducing species were isolated *Alkalibacterium psychrotolerans* [16], *Alkalibacterium iburiensis*[17] and *Alkalibacterium indicireducens* [16]. *Alkalibacterium* is currently the genus with the highest number of indigo reducers. The *Amphibacillus* and *Bacillus* genera both have 2 species each: *Amphibacillus iburiensis* [18] *and Amphibacillus indicireducens* [19]; *Bacillus fermenti* [20] and *Bacillus cohnii*. In addition, *Polygonibacillus indicireducens* [21], *Paralkalibacillus indicireducens* [22], *Fermentibacillus polygoni* [23] and *Oceanobacillus indicireducens* [24] were also isolated . All these species have their origin from fluids prepared with *sukumo*. In the case of preparations using woad only the indigo reducing specie *Clostridium isatidis* was isolated [25]. Indigo reducer *Alkalibacterium* spp. and *Amphibacillu*s spp. are aerotolerant anaerobes while other indigo reducing bacterial are facultative anaerobes. *Alkalibacterium* spp. and *Amphibacillu*s spp. are able to hydrolyze cellulose and xylan, which are components present in wheat bran, a commonly additive to indigo fermentations. *P. indicireducens* is able to decompose starch and cellulose.

1.8.1 Bacterial reducing mechanisms

The reducing mechanisms used by the bacterial community to transform indigo into its leuco form is still under investigation, and despite the efforts, was not yet fully understood. Bacillus sp. AO1, a bacterium isolated from the polygonum alkaline liquor was able to convert indigo carmine. The indigo carmine reduction, which happens even in cell free extract, occur in the presence of NADH. It's believed to also reduce indigo, since NADH was being oxidize, but the presence of *leuco* indigo was not detected in free cell extraction. It is possible that this intracellular enzyme is being released when the cells are lysed and therefore helping the indigo reduction [26]. Model systems showed that acetaldehyde can both donate and accept electrons. In the proposed mechanisms indigo in *sukumo* is reduced by acetaldehyde with an (NAD-independent) acetaldehyde dehydrogenase to generate leuco-indigo and acetate in the bottom of the fermentation vat. Acetaldehyde can be supplied by fermentation of ethanol and alcohol dehydrogenase, making the addition of sake (rice wine) in the fermentation preparation and the fermentation sour aroma of acetic acid very reasonable [8]. Consequently, there isn't a consensus on the mechanisms of indigo reduction by bacteria and further studies are necessary.

1.9. Leaf powder

Indigofera tinctoria is a deciduous shrub or subshrub belonging to the Fabaceae family. It can reach 1-2m in height, can be annual to perennial , and has nitrogen fixing activity [27] [3]. It is commonly used for extraction of indigo dye but has also application in medicine and cosmetics. Analysis of ethanolic and hydroethanolic extract showed the presence a wide range of carbohydrates, flavonoids, anthraquinones, saponins, proteins, tannins, phenols, steroids, and triterpenoids [28]. Bioactive, antioxidant potential and free radical scavenging activity were found in both ethanolic and hydroethanolic extracts of *I. tinctoria*. Plant polyphenols, are a group of important secondary metabolites related to the defense mechanisms of the plant, they possess potent antioxidant properties, are good electron donors and have the ability to terminate free radical chain reactions by converting them into more stable compounds [27].

One of the cosmetic applications of *I. tinctoria* leaf powder is for hair dyeing. The hair product consists of dried and pulverized leaves of *I. tinctoria* and present itself as a finely divided greenish dispersible powder containing a complex chemical mixture of indican, indigo and indirubin and insoluble matter such as cellulose fibers. The Scientific Committee on Consumer Safety (SCCS) of the European commission considered it safe to use (on-head concentrations of 25%), with a weak potential for skin sensitization [29].

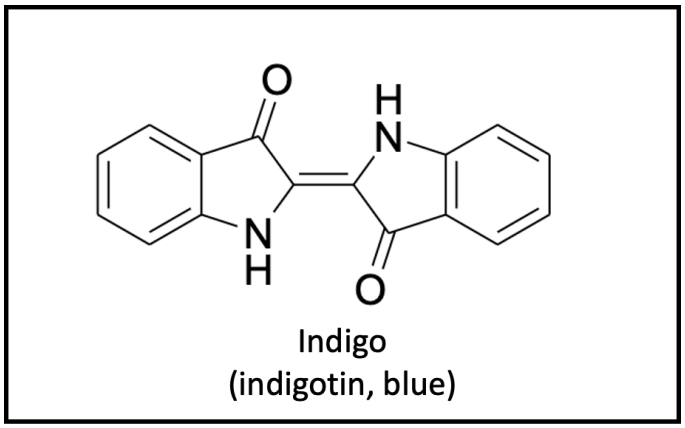

Figure 1 Indigo chemical structure

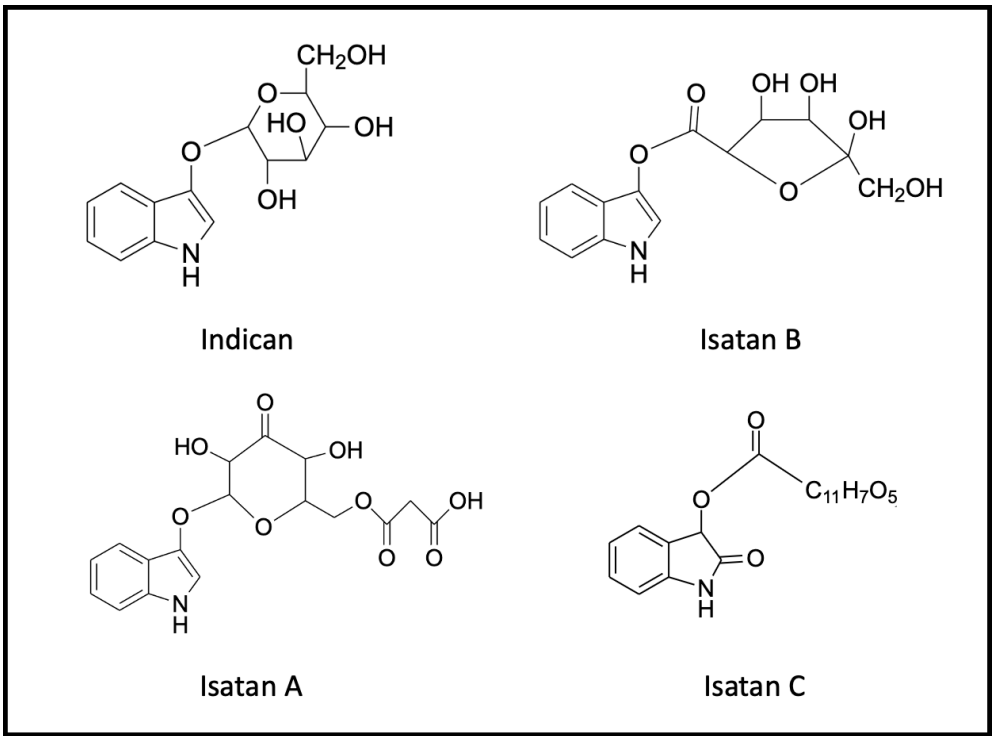

Figure 2 The different indigo precursors present in the indigo bearing plants.

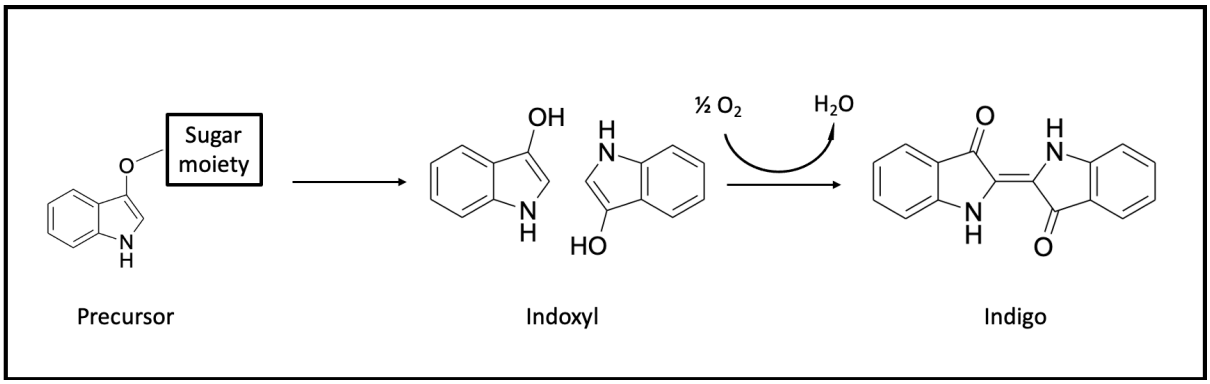

Figure 3 Transformation of indigo precursors into indigo.

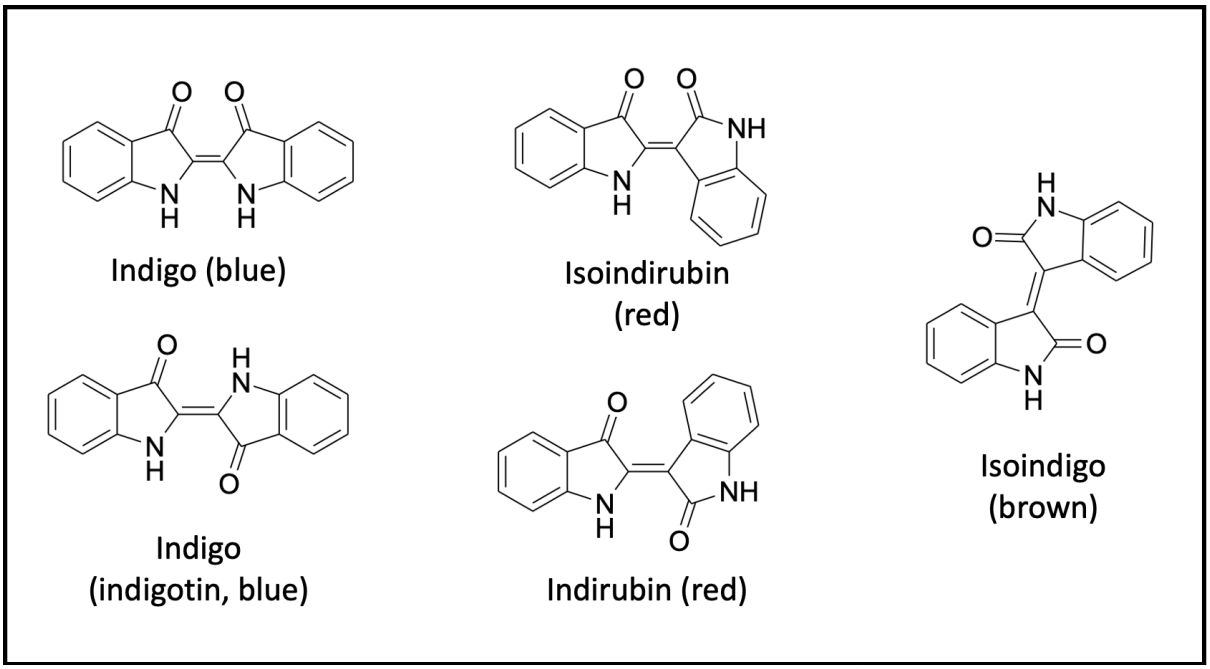

Figure 4 Indigo and other indigoid components that can be produced by the precursors from plants

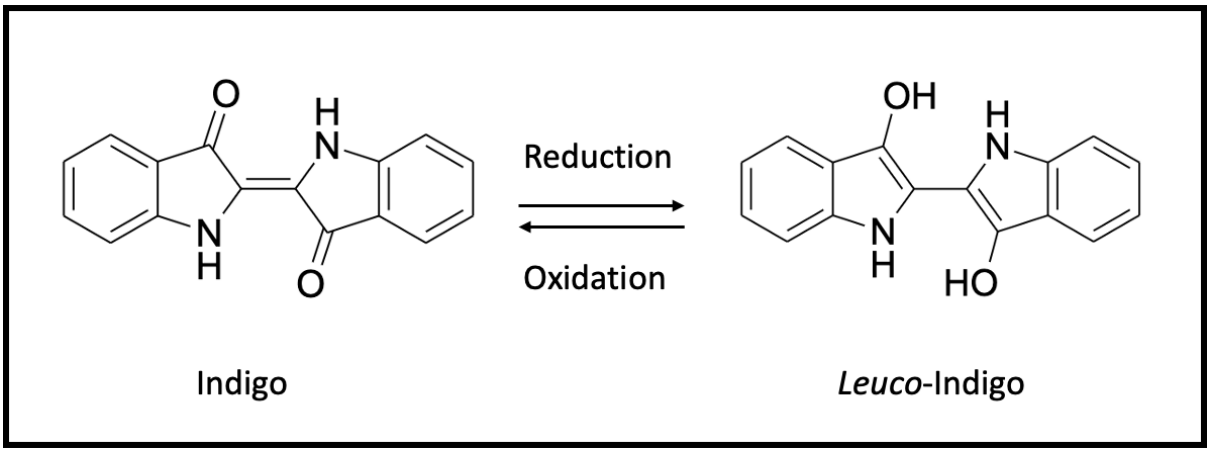

Figure 5 Formula with the reduction of indigo to leuco-indigo and the oxidation of leuco-indigo back to indigo.

Chapter 1

Analysis of bacterial flora of indigo fermentation fluids utilizing composted indigo leaves (*sukumo*) and indigo extracted from plants (Ryukyu-ai and Indian indigo)

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Abstract

Indigo is a fabric dye that requires reduction by microbial activity or chemical reagents to render it soluble in water. Sources of indigo for fermentation are primarily divided into composted indigo-containing plants and indigo extracted from plants. To elucidate the factors responsible for bacterial diversity, and for sustaining the reduced state of indigo in different preparations, this study assessed fermentation-derived fluids using composted plant leaves, *sukumo*, and extracted indigo (Ryukyu-ai paste, and Indian indigo cake) prepared using different procedures. Regardless of the indigo source, obligate anaerobic bacteria, including the families *Proteinivoraceae* and *Tissierellaceae*, predominate (16.9–46.1%), suggesting their high affinity for this fermentation ecosystem (hyperalkaline and low redox potential). Moreover, bacterial communities in *sukumo* fermentations are more diverse than those from indigo extracts with the diversity tending to increase based on the fermentation period. Our results further suggest that the microbiota composition in *sukumo* fermentation is associated with the various bacterial nutrients derived from *sukumo*, including seed microorganisms. In addition, the debris derived from *sukumo* can reduce the pH stress experienced by the microorganisms. Further, regardless of 5.4 years difference in the fermentation age, the bacterial flora in two Ryukyu-ai batches exhibit similar features with low microbial diversities. The uniformity of the nutrient, along with the simple, yet strong, bacterial network in Ryukyu-ai fluids may be responsible for the stable bacterial flora composition. Taken together, these results indicate that the microbiota in indigo fermentation is highly influenced by the seed culture, the nutrient derived from raw materials, and the fermentation conditions.

Introduction

Indigo is an organic dye that has been used in textiles worldwide since ancient civilization. Until the development of artificial indigo in 1897, indigo dye was derived from plants [2]. Although artificial indigo is cheaper than the natural product, it requires $Na_2S_2O_2$ reduction as only its reduced form is soluble in water and capable of penetrating fabrics. However, artificial synthesis and reduction procedures are environmentally damaging [30–32].

The application process of natural indigo from plants can be roughly divided into microbial composting and direct extraction. Both processes are important to transform indican (the indigo precursor in plants) into indoxyl, which is further oxidized to indigo. These processes are also used to concentrate indigo, facilitate transportation, and for storage. A composting procedure developed in medieval Europe involves the use of leaves from the plant *Isatis tinctoria*, which are cut into small pieces, gathered into a ball the size of an orange, and dried. Before preparation of the dye fluid, they are crushed into a powder, arranged into heaps, sprinkled with water, and fermented under appropriate humidity and temperature [3,13,33,34]. In Japan, the most common source of indigo is "*sukumo*," which is composed of composted *Polygonum tinctorium* leaves. The harvested leaves are arranged into a 1-m pile on an earthen floor and subsequently fermented for approximately 100 d, with regulated water supply and temperature [9,35,36]. Composting procedures have the advantage of retaining microorganisms and organic matter, which is important for the next fermentation step, wherein indigo is reduced to its dye form.

In Okinawa Prefecture, Japan, an extraction procedure known as "Ryukyu-ai" was developed, wherein the leaves of the plant *Strobilanthes cusia* are immersed in water for a few days and $Ca(OH)_2$ is used to precipitate indigo into a paste ("indigo paste"). Similar procedures are also popular in the northeastern part of India, southeast Asia, and southern China [37]. The indigo extraction procedure used for "Indian indigo" involves an aeration precipitation technique in place of chemical addition. This procedure uses the plant *Indigofera tinctoria*, which is collected in tanks and allowed to ferment for a few days, after which, it is transferred to a series of aeration and decantation tanks. A paste is collected and air dried into an "indigo cake", that is often heated to reduce its microbial and moisture content for preservation [2,3].

For its utilization as a dye, indigo must be reduced to its soluble form. Composted products from indigo leaves, designated *sukumo*, contain seed bacteria as well as the bacterial nutrient for indigo reduction fermentation. The nutrients present in *sukumo* comprise the fermentation residue of *Polygonum tinctorium* and living and dead bacterial cells from the long composting

process. Aqueous hot wood ash extract (50–80°C) is mixed with the composted product, forming an alkaline environment. Indigo reduction spans days to several weeks for initiation and can continue for 4–6 months with proper maintenance. Compared to composting procedures, indigo prepared by extraction does not possess sufficient microorganisms to initiate the fermentation step; thus, chemicals, such as $Na_2S_2O_2$, are required for indigo reduction. Alternatively, several plants, including *Senna tora* (originally described by Linnaeus as *Cassia tora*), tamarind, or ripe fruits, have been used to accelerate indigo reduction by microorganisms in fermentation using extracted indigo [38,39]. Changes of the bacterial composition in the initial and aged states in *sukumo* fermentation fluid have been reported. The study of bacterial succession in the early stages of *sukumo* fermentation revealed that there are two steps. In the initial stage, the family *Bacillaceae* predominate, surviving the initial heat treatment and contributing to oxygen consumption in the fluid. Thereafter, following redox potential reduction in the fluid, *Bacillaceae* is succeeded by the obligate anaerobe *Anaerobranca* [11,12]. Bacterial flora subsequently approaches a stable state of fermentation, in which changes occur much slower than those in the initial stage [12,36]. Hence, bacterial flora stability may be an important factor for endurance of the fermentation system. Indeed, a comparative study using fermentation batches with different durations in the indigo-reducing state has suggested that early predominance of obligate anaerobes, followed by the successive change into a stable microbiota is important to establish stable indigo fermentation fluid [12].

Several microorganisms contribute to the indigo-reducing fermentation step, with eleven species having been described, to date [9]. In fact, indigo-reducing bacteria considered to be major constituents in indigo-reducing *sukumo* fermentation fluid [9,11,12] Meanwhile, obligate anaerobic *Clostridium isatidis*, a spore-forming indigo-reducing species, has been isolated from a couched woad preparation [40–44]. However, previously reported indigo-reducing bacteria comprise only a small fraction of the microbiota found in a couched woad fermentation [40]. Thus, a more detailed investigation in indigo-reducing obligate anaerobic bacteria present in *sukumo* and couched woad fermentations is required to understand the ecosystem sustaining indigo reduction.

Moreover, although the bacterial community of fluids using *sukumo* in their preparation has been explored, the bacterial composition of fluids made with extracted indigo, such as Ryukyu-ai and Indian indigo, remains to be investigated. Differences between the fermented dyeing material and extracted dye are listed in Table 1. The present study, to the best of our knowledge, is the first to investigate and compare the bacterial community of indigo-reducing fluids produced from composted dye source with extracted dye materials.

Although the dyeing process in *sukumo* fermentation is performed in an open system, the indigo reduction state can be maintained for more than 6 months, even in the high likelihood of bacterial contamination. We aimed to determine the structure of bacterial flora in the reduction state, as well as its formation, sustainability, and resilience. Further, considering that *sukumo* possesses external seed microorganisms necessary for the subsequent indigo-reducing fermentation step, while extracted indigo possesses fewer microorganisms in dye material, requiring an external seed culture to ensure suitable indigo reduction, we also analyzed the bacterial flora of various fermentation fluids using different indigo sources. The present study will provide insights on the diversity of microbiota as well as the features important for the sustainability of indigo fermentation systems.

Materials and methods

Samples from dyers

Seven samples, originating from fermentation fluids containing *sukumo* (composted *Polygonum tinctorium*), Ryukyu-ai [extracted indigo from *Strobilanthes cusia* and precipitated by $Ca(OH)_2$], and Indian indigo (extracted indigo from *Indigofera tinctorial* and precipitated by aeration), were collected (Table 2). Samples, SSHP [3-year fermented *sukumo* (craftsperson: mainly AS) + homemade precipitated indigo; maintained with wheat bran], RWTE (80-d fermented Ryukyu-ai: tamarind was added at the preparation of the fluid and maintained with ethanol and wheat bran), RWB1 (180-d fermented Ryukyu-ai maintained with wheat bran), RWB2 (5.9-year fermented Ryukyu-ai maintained with wheat bran), IWS270 (270-d fermented Indian indigo prepared with woad fermentation as a seed), and IWS470 (470-d fermented Indian indigo prepared with woad fermentation as a seed) were obtained from the North-Indigo Textile Arts Studio in Otaru, Hokkaido (43°20’45.0” N, 140°59’94.5” E). Sample SNFU [*sukumo* (craftsperson: ON) fermentation fluid produced in Fukushima] was obtained from Fukushima Senkou (a dyeing factory) in Fukushima City, Fukushima Prefecture (37°48’04.4” N, 140°30’09.9” E). In *sukumo* preparations, except SSHP, were treated with hot wood ash aqueous extract (50–60°C).

IWS470 (Indian indigo based fermentation fluid) was prepared by adding 130 g of Indian indigo (net indigo content 21.5%) to 20 L of freshly prepared (6 d) couched woad fermentation, prepared using 1.3 kg of couched woad, 20 L of wood ash extract, and 20 g of wheat bran. Prior to its introduction to the fermentation fluid, Indian indigo was drenched in wood ash

extract and kneaded well. IWS270 (Indian indigo based fermentation fluid) was prepared using 500 g of Indian indigo, 50 g of wheat bran, 5 L of IWS470 fermentation fluid (aged 1 year and 2 months), and 90 g Indian indigo (*I. tinctoria*) leaf powder (mixed with 1 L of tap water and kept for one day at room temperature), brought to a final volume of 50 L by the addition of wood ash extract. Both fermentation fluids (IWS270 and IWS470) were maintained at a pH of approximately 9.5–10.2 via addition of limestone. Boiled wheat bran was added (10 g to IWS470 and 25 g to ISW270) once every 1–1.5 months.

RWB1 (Ryukyu-ai based fermentation fluid) was prepared by adding 5 kg Ryukyu-ai indigo paste, 15 L Ryukyu-ai paste immersion liquid, 50 g wheat bran, and 5 L of a previously prepared Ryukyu-ai fermentation fluid, which was brought to a final volume of 100 L by adding wood ash extract. The previously prepared Ryukyu-ai fermentation fluid (total volume of 100 L) used in RWB1 was made by adding 8 kg of Ryukyu-ai indigo paste, 12 L of Ryukyu-ai immersion liquid, 50 L of wood ash extract produced by 5 kg of wood ash, 500 g of limestone, 80 g of malt starch syrup, 200 mL of awamori (Okinawan alcoholic beverage), and 50 g of sugar cane molasses. This fermentation fluid was maintained by adding 80 g of malt starch syrup once every two months.

RWB2 (Ryukyu-ai based fermentation fluid) was prepared by adding 10 kg of Ryukyu-ai indigo paste, 10 L Ryukyu-ai immersion liquid, and 50 g of wheat bran, which was brought to final volume of 100 L by adding wood ash extract. Both Ryukyu-ai-based fermentation fluids (RWB1 and RWB2) were maintained at a pH of approximately 9.5–10.2 by adding limestone, and 10 g of boiled wheat bran was added as a bacterial nutrient once every 1–1.5 months.

RWTE (Ryukyu-ai based fermentation fluid) was prepared by adding 2 kg of Ryukyu-ai indigo paste, 2 L Ryukyu-ai immersion liquid, and 10 g wheat bran, which was brought to final volume (20 L) by adding wood ash extract. The fermentation fluid was maintained in Thailand by a local craft person. Ethanol and tamarind were added to the fermentation, however, the precise volumes/weights, as well as the maintenance conditions, are unknown as the local craft person adjusted it according to the dyeing intensity.

SNFU (*sukumo* based fermentation fluid) was prepared using 6.0 kg of *sukumo* previously drenched in wood ash extract (50 L) and kneaded well. After allowing the treated *sukumo* to rested for 5–6 h, wood ash extract was added to a final volume of 60 L. To this fermentation fluid, 100 g of wheat bran and 360 mL of rice wine was added. On the next day (day 1), the pH was adjusted to 10.5–11 by with limestone and wood ash extract. The final volume (80 L) of the fermentation fluid was achieved by adding wood ash extract.

SSHP (*sukumo* based fermentation fluid) was prepared using 6.0 kg of *sukumo* drenched in wood ash extract and stored overnight. On the next day (day 1), the treated *sukumo* was transferred to a 100 L fermentation vat to which 60 L of wood ash extract and 50 g of wheat bran were added. From the second day, the pH of the fermentation fluid was adjusted to 10.5–11 by adding limestone and wood ash extract. The final volume (100 L) of the fermentation fluid was achieved by adding wood ash extract.

The wheat bran used in all of the above preparations. The tap water was added to wheat bran and boiling for 5 min before being added to the various fermentation fluid. Dyeing conditions were reported by the craft persons, and all samples were obtained from batches that were active (being used for dyeing fabrics) at the time of sampling. While laboratory prepared fermentation fluids were used only for occasional test staining of small cotton cloths, and large-scale fluids using by craftsperson were frequently used for dyeing textiles. The obtained samples were homogenized and a 500 μL aliquot from each sample was subsequently used for DNA extraction.

Laboratory preparation of *sukumo* fermentation batches

For laboratory scale *sukumo* fermentation, wood ash (*Quercus phillyraeoides* A. Grey, Nagomi Company, Gobo, Wakayama, Japan; 380 g) was mixed with 5 L of tap water and boiled for 10 min). Following settling of the contents, the supernatant was transferred to a new receptacle and re-boiled. Thereafter, the supernatant was cooled to 60°C and a 500 mL volume was transferred to Erlenmeyer flasks with the immediate addition of 38 g of *sukumo*, flasks were subsequently incubated at 26°C. The batches were prepared with two types of *sukumo* produced by different crafts persons (Table 2). The first batch was prepared with *sukumo* produced in Tokushima, Japan (*sukumo* produced by TT, STT142 was 142-d old; STT226 was 226-d old). The second batch was prepared with *sukumo* produced in Date, Japan (*sukumo* produced by KS, SSD50 was 50-d old; SSD94 was 94-d old). The reducing state was detected by testing the dying ability of the fermentation fluid after dipping a small piece of cotton cloth into the fluid for 1 min and exposing it to air. After 2–5 min, the cloth was rinsed and dried, and a blue color indicated whether the fluid had reached a reduced state (Supplementary information - Figure 9). Fluid pH was maintained between 10.5 and 11 by adding $Ca(OH)_2$, and measured using the pH meter D-71 (Horiba, Kyoto, Japan). Before sampling, the fluid was stirred on a magnetic stirrer for 1 min, and 500 μL aliquots were collected and stored in 25% glycerol at −80°C until DNA extraction.

DNA extraction and PCR

Samples were centrifuged at 15,000 × *g* for 10 min to obtain a pellet that was subsequently used for DNA extraction. The extraction was performed using the ISOIL extraction kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions with a slight modification (extracted DNA was dissolved in 50 µL of TE, instead of 100 µL). Extracted DNA was stored at −30°C. Prior to sending the samples for next generation sequencing (NGS), the DNA was amplified by PCR using primers provided by Seibutsu Giken Inc. (Sagamihara, Kanagawa, Japan). The bacterial 16S rRNA sequence of V3-V4 region was amplified using primers V3-V4f_MIX (5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-CCTACGGGNGGCWGCAG-3′) and V3-V4r_MIX (5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GACTACHVGGGTATCTAATCC-3′). Primers were composed of an adaptor sequence, followed by the insertion of several bases, varying in sequence and length, to improve quality of amplification (represented by the letter N) and the sequence homologous to the 16S DNA (341F and 805R). The reaction mixture (40 µL) was composed of 4 µL 10× Ex buffer (Takara, Otsu, Shiga, Japan), 3.2 µL 2.5 mM dNTPs (Takara), 2 µL of forward primer, 2 µL of reverse primer, 2 ng of DNA template (extracted sample), and 0.4 µL of 5 units μL^{-1} Ex Taq polymerase (Takara). The amplification reaction was performed under the following conditions: 94°C for 2 min; 25 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; final extension of 72°C for 5 min. PCR products were confirmed by agarose gel electrophoresis.

Next generation sequencing

Samples were sent to Seibutsu Giken Inc. for NGS, where they were reamplified using PCR with a tailed set of primers, and purified. NGS was conducted on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The adapters and primers from the delivered fastq files were removed using Cutadapt version 1.18. QIIME 2 version 2020.2 [45] was used to conduct bioinformatic analysis and output data annotation. The latest database Silva_132_release from SILVA database [46,47] with training feature classifiers was used for 16S rRNA taxonomic annotation based on the above primer pair 341F-805R. A cluster analysis based on operational taxonomic units (OTUs) with a 97% identity threshold and taxonomic classification was performed using QIIME2 version 2020.2 [45]. Demux command was used to examine the quality,

and DADA2 analysis was used to filter sequences, trim and merge sequences, remove chimeras, and construct an amplicon sequence variant (ASV) table. Taxonomic analysis and annotation of output data were also performed using QIIME2, including OTU picking and taxonomic assignment. Nucleotide sequence identity was estimated with bacterial 16S rRNA reference sequences in the BLAST database. Rarefaction curves were computed for sequences and observed OTUs with 97% similarity regarding the total number of reads for each sample were selected using the UCLAST algorithm [48]. Rarefaction curves of observed species were determined using the QIIME2 alpha diversity analysis script. Jaccard and unweighted UniFrac distances were presented as principal coordinate analysis (PCoA) and the corresponding plots were prepared using the beta diversity analysis script in QIIME 2. EMPEROR software was used to visualize PCoA plots [49]. Detrended correspondence analysis (DCA) was performed using taxonomy based BIOM file obtained from QIIME2 transformed into percentages, and Vegan package under R version 4.0.5 (https://www.R-project.org/).

Results

Next generation sequencing

In NGS analysis, 469,005 sequences were obtained from 11 samples. Following quality filtering and exclusion of chimeric sequences, 288,572 reads remained, with an average of 26,234 (maximum: 43,207; minimum: 20,211) reads per sample, which was considered sufficient to characterize the community as reflected by the plateau on the rarefaction curves (Fig. 1). The raw sequenced data were deposited under DDBJ/EMBL/GenBank with accession numbers SAMD00233309 to SAMD00233319.

Community characterization

Sukumo samples were characterized by a high ratio of the genera *Bacillus* or *Alkaliholobacillus* and/ or family *Proteinivoraceae* (formerly *Anaerobrancae*), followed by families *Tissierellaceae* or *Erysipelotrichaceae*, regardless of differences in the *sukumo* maintenance procedure, fermentation quantity, or incubation period (Figure 7). Taxa belonging to family *Bacillaceae* were frequently observed. The most predominant family *Bacillaceae* OUTs in *sukumo* samples (45.6% and 40.4% of the OTUs in STT226 and SUFU, respectively) exhibited

high similarity with the *Alkalihalobacillus hemcellulosilyticus* (98.4% similarity; STT226), followed by the *Bacillus fermenti* (99.8–100% similarity; SNFU), with the latter reported as indigo-reducing bacteria [20]. In SSHP, the most predominant family *Bacillaceae* OTU exhibited high similarity with *Polygonibacillus indicireducens* (98.6% similarity), which is a reported indigo-reducing species [21]. The obligate anaerobes belonging to families *Proteinivoraceae*, *Tissierellaceae* and order *Clostridiales* were frequently observed in all samples except for IWS-470. The SSD sample could be differentiated from the rest of the *sukumo* fermentation fluids as having the genus *Pseudomonas* as one of the major components of its community. The SSD sample contained a low *Bacillaceae* family ratio. This may be attributed to the antagonism between facultative anaerobic and aerotolerant *Bacillaceae*, and gram-negative bacteria. A similar relationship was also observed in Ryukyu-ai and Indian indigo samples. Batches SNFU and SHHP were larger in volume than the other *sukumo* samples and exhibited higher abundance of aerotolerant *Bacillaceae* and *Carnobacteriaceae* and lower abundance of gram-negative bacteria. Differences in microbiota between SNFU and SHHP are likely attributed to fermentation age. The observed predominant taxa in *sukumo* samples have been observed in previous studies using *sukumo* [11,12,35,36].

Ryukyu-ai samples without additives contained the genus *Halomonas* [similarity: *Halomonas johnsoniae* (98.8–100%)] as the dominant taxon (38.3–45.0%), followed by the order *Clostridiales* [similarity: *Proteinivorax tantarense* (88.4%)] (Figure 7). The similarity between the two samples (RWB1 and RWB2) was significant considering the fermentation age difference of approximately 5.4 years. This similarity suggested that bacterial seed in RWB1 (previous fermentation) is equivalent to that in RWB2 (Ryukyu-ai indigo paste and Ryukyu-ai paste immersion liquid). Facultative anaerobic family *Bacillaceae*, frequently observed in *sukumo* samples was rarely observed in Ryukyu-ai samples. In addition, aerotolerant lactic acid bacteria were scarcely observed in Ryukyu-ai samples (0.6–1.7% in total/sample). The dominant microorganism in the RWTE sample contained the genus *Marinospirillum* (13.2%) followed by order *Tissierellales* (11.7%) in comparison with other Ryukyu-ai samples. Notably, the predominance of the genera *Halomonas* or *Marinospirillum* is specific to Ryukyu-ai samples, which the low bacterial diversity in RWB1 and RWB2 samples is also a specific feature. Although the reported indigo reducing genus *Amphibacillus* [18,19] existed in proportions of 13.9% and 4.2% in RWB1 and RWB2 communities, respectively, other reported indigo-reducers were not observed, as most bacteria present have never been isolated. The most predominant OTU belonging to *Amphibacillus* in RWB1 and RWB2 was *Amphibacillus iburiensis* (100% similarity).

In Indian indigo fermentation fluid, bacterial flora varied distinctively according to preparation procedure (i.e., woad fermentation fluid as the seed) or additive (i.e., leaf powder; Figure 7). Comparing microbiota with other fermentation batches, a particular member in Indian indigo samples, lactic acid bacteria *Facklamia* (similarity: *Facklamia languida* 97.4%), was predominantly observed (24.9%) in IWS-470. Like Ryukyu-ai samples, facultative anaerobic family *Bacillaceae*, which was frequently observed in the *sukumo* sample, was scarcely observed in Indian indigo fermentation fluid. The diversity of the obligate anaerobes was higher than that in *sukumo* and Ryukyu-ai samples. The diversity of gram-negative bacteria was higher in Indian indigo fluid than in the *sukumo* sample. This may be attributed to microbial diversity and woad-derived plant components contained in the seed culture. Thus, the specific microbiota in the Indian indigo may be attributed to the woad fermentation-based seed culture.

Rarefaction curve and statistical analysis

The rarefaction curve showed low microbial diversity in two Ryukyu-ai samples, RWB1 and RWB2, compared with other samples (Figure 6). Despite the fermentation period being different among groups, the consistently low level of diversity was significant. The complexity in microbiota diversity was lowest in Ryukyu-ai, highest in *sukumo* and medium in Indian indigo. Diversity in the microbiota of RWTE was higher than that of the other Ryukyu-ai samples. This suggests that additives (e.g., tamarind) not only provide nutrients for enhancing the bacterial diversity but also plant materials contribute their attached microorganisms to the fermentation fluid. Freshly prepared *sukumo* (SNFU) exhibited low diversity while aged *sukumo* samples exhibited relatively high diversity.

The three-dimensional Jaccard distance and unweighted PCoA analyses presented in Fig. 3A and 3B, respectively, reveal that bacterial flora of samples differed depending on the dye materials used. As long as the preparation and maintenance procedures are similar, each dyeing source exhibited similar positionings, which was also confirmed by DCA; Supplementary information - Figure 10. According to the results, type of *sukumo*, fermentation batch size, fermentation age, and/or addition of wheat bran may influence bacterial communities in *sukumo* samples (i.e., SNFU and SSHP are larger in volume and contain wheat bran; STT226 and SSHP are aged fermentation). Comparatively, if the fermentation was managed by the same preparation and maintenance procedures, and the source of *sukumo* was also the same, the fermentation period exhibited little influence on the coordinate positioning of laboratory

prepared *sukumo* samples (i.e., SSD and STT samples in Figure 8). Moreover, regardless of the large difference in fermentation period, the positioning of two Ryukyu-ai samples, RWB1, and RWB2 was similar. That is, the Ryukyu-ai sample containing ethanol and tamarind as additives exhibited a far distant position from the other Ryukyu-ai samples.

Discussion

To understand the fundamental basis of bacterial diversity and sustainability of the indigo-reducing state in indigo fermentation fluid, we analyzed microbiota in fermentations of various ages and indigo sources under different preparation and maintenance procedures. The presented results suggest that the complexity of microbiota in *sukumo* fermentations is attributed to various bacterial nutrients derived from *sukumo,* which contains composting products knotweed leaves and their associated microorganisms. In addition, the debris derived from *sukumo* in the bottom of the fermentation fluid aids in the production of flocs and reduces the pH stress, thereby affecting the microbial composition [50]. Induction of microbial diversity by plant sourced materials was also demonstrated in Ryukyu-ai fermentation using tamarind (difference between RWTE and RWB1 and 2) and Indian indigo fermentation using Indigofera leaf powder and woad fermentation (difference between IWS270 and other fermentation batches). The addition of materials derived from plants (tamarind or *Indigofera tinctoria* leaf powder), not only provided nutrients for the microorganisms but also phytochemicals that act as oxygen scavengers or electron mediators for indigo reduction [28,51]. Conversely, the direct pH stress effects of internal and external microorganisms, the uniformity of the nutrients (i.e., wheat bran and dead bacterial cells during the fermentation), and the simple and strong bacterial network in Ryukyu-ai fluids (RWB1 and RWB2) are responsible for the strong sustainability of bacterial flora. The above results suggest that the fermented liquid of indigo is maintained by a network of microbial functions using plant-derived components such as components of *sukumo*, wheat bran, and plants that were used as additives. Therefore, when added, the *Senna tora* seed (methi; Indian method) [2] and *Ricinus communis* seed (Chinese method) [39] affected the bacterial community in the fluid. In the case of *sukumo*, in addition to the plant-derived nutrients that were already present, microbial-derived components including metabolites and dead cells derived from composting processes for its preparation, are also involved. The present study demonstrates that the complexity of the bacterial community depends on the complexity of the nutrients, seed bacteria and the fermentation environment.

Based on predominant taxa, the bacterial components of indigo fermentation fluids used in this study can be roughly classified into (i) facultative anaerobes containing indigo-reducing bacteria, (ii) aerotolerant bacteria containing indigo-reducing bacteria (including lactic acid bacteria), (iii) obligate anaerobic bacteria with unknown function, and (iv) aerobic bacteria (mostly gram-negative bacteria, containing non-alkaliphiles) with unknown function. Regardless of the indigo source used, obligate anaerobic *Firmicutes* were observed at a high ratio (16.9–46.1%). The major obligate anaerobic constituents in *sukumo* (STT142, SSD50, SSHP, and SNFU) and Ryukyu-ai fermentations are the members of *Proteinivoraceae* (closest taxon: *Proteinivorax tanatarense* 92.3–93.6%) and *Clostridiales* (closest taxon: *Proteinivorax tanatarense* 88.4%), respectively. The family *Tissierellaceae* was observed as the major obligate anaerobic constituent in *sukumo* (STT226 and SNFU) and Indian indigo (IWS-470). The closest taxon for *Tissierellaceae* was *Tissierella creatinine*, and the sequence similarity was 94.5–95.0% in the most predominant OTU. Several reports have shown that genera *Anaerobranca* (belong to *Proteinivoraceae*) and/or *Tissierella* are major constituents of the bacterial flora in the production of short chain fatty acids from sludge under anaerobic alkaline conditions [52–54]. In addition, *Proteinivorax* spp. and *Acetoanaerobium pronyense* (in IWS470) utilize proteinaceous substrates [55–57]. These obligate anaerobes observed in indigo fermentation fluids may also play a role in the degradation or consumption of degraded substances, which can be derived from debris of *sukumo*, wheat bran, and dead bacterial cells. The predominance of these obligate anaerobic *Firmicutes* across samples suggests that they have an important role in the indigo reducing ecosystem of the indigo fermentation fluid. However, to verify this, these microorganisms must be isolated and their various properties, including indigo-reducing ability and substrate utilization, must be characterised.

The hyperalkaline pH and the lack of debris in extracted dye samples allow microorganisms present in the fluid to be directly exposed to hyperalkaline pH [50]. This may be the reason for the observed low microbial diversity in the Ryukyu-ai fermentation fluids, RWB1 and RWB2. However, this minor difference is significant considering the immense difference in their fermentation periods. The proposed function of these major taxa are diverse: *Amphibacillus* spp. reduces indigo and decomposes wheat bran [18,19]; the order *Clostridiales* decomposes dead and/or weakened bacteria while producing organic acids [56,57]; *Halomonas* spp. uses organic acids produced by other major taxa [58]. In addition, *Halomonas* spp. (gram-negative) possess higher adaptability in aqueous alkaline environments than *Firmicutes* (gram-positive) [59], while the predominance of *Halomonas* spp. in bacterial flora may contribute to the stability of this system. Hence, trials with isolated strains are necessary to determine the functionality of this

predicted ecosystem. Additionally, in Ryukyu-ai-based samples, *Halomonas* species were predominantly observed, which is not a feature observed in *sukumo* fluids. The most abundant OTU from the genus *Halomonas* in the three Ryukyu-ai samples was the *Halomonas johnsoniae*. *Halomonas* spp. have often been observed in alkaline environments [35,60]. *Alkalibacterium indicireducens* contained in RWTE involve indigo-reducing bacteria, which produce lactic acid [16]. It has also been reported that *Halomonas* spp. consume lactic acid produced by the *Alkalibacterium* spp. [61]. In addition, *Halomonas* spp. can reportedly consume several organic acids, such as propionate, valerate, and lactate consume lactic acid produced by the *Alkalibacterium* spp. [61,62]. Although localized acid production may allow opportunities for non-alkaliphiles to inhabit indigo fermentation fluid, *Halomonas* may have a role in maintaining the microflora by consuming acids and suppressing the growth of non-alkaliphilic contaminating microorganisms in Ryukyu-ai fermented broths.

Although the high microbial diversity of *sukumo* may represent risk for proliferation of unfavorable microorganisms in the fluid, it could also be responsible for the rapid recovery of appropriate bacterial flora by its buffering effect attributed to functional redundancies [63]. This study demonstrated that the extracted indigo fermentation fluids exhibited extraordinary microbial community stability despite having lower diversity and a different bacterial flora. Comparative studies on recovery of microbial community between *sukumo*- and extracted dye-fermentation after intense use, remain warranted. While the starter culture and additives strongly influence bacterial flora in fermentations using extracted indigo, bacterial flora in *sukumo* fermentations was highly influenced by the selection of intrinsic bacteria present in *sukumo.* Alternatively, these bacteria in *sukumo* originated from *sukumo* production procedures, which may also explain the increased diversity in *sukumo* fermentations.

Cumulatively, the results of study indicated that microbiota in indigo fermentation is highly reflected by seed culture and the materials derived from plants; the latter includes *sukumo*, which contains degraded plant components. Moreover, indeed, *sukumo* contains fermentation residues of *P. tinctorium* and living and dead bacterial cells derived from the long composting process during production of *sukumo*.

Table 1 Characteristics of indigo materials used in this study.

Dye source	Form	The place mainly used.	Seeds microorganisms for fermentation	Contents	Presence of debris in fermentation
Sukumo (composted indigo plant)	Composted plant	Japan	The content is high enough to initiate the fermentation for indigo reduction	Indigo plant fermentation residues Metabolites of microorganisms Live and dead microorganisms	Yes
Ryukyu-ai (extracted dye)	Indigo paste	Okinawa Prefecture Japan	The content is not sufficient to initiate the fermentation for indigo reduction	Mainly indigo dye	No
Indian indigo (extracted dye)	Indigo cake	India	The content is not sufficient to initiate the fermentation for indigo reduction	Mainly indigo dye	No

Table 2 Differences in preparation of indigo fermentation fluids used in this study.

Sample ID	Source of dye	Bacterial seed	Substrate	Other additives	Fermentation period	Size (L)	Temp (°C)	pH
STT142[a]	*Sukumo* (TT[b])	*Sukumo*	No	No	142 d	0.5	26	10.66
STT226	Sukumo (TT)	*Sukumo*	No	No	226 d	0.5	26	10.96
SSD50	*Sukumo* (KS[c])	*Sukumo*	No	No	50 d	0.5	26	10.82
SSD94	*Sukumo* (KS)	*Sukumo*	No	No	94 d	0.5	26	10.66
SNFU	*Sukumo* (ON[b])	*Sukumo*	Wheat bran	No	14 d	80	30	ND[g]
SSHP	*Sukumo* (Major:AS[b]; Minor: ON[b], Harima[d], KS)	*Sukumo* + homemade precipitated indigo	Wheat bran	No	3 years	100	25	11.95
RWTE	Ryukyu-ai	Ryukyu-ai indigo paste + Ryukyu-ai paste immersion liquid	Ethanol + wheat barn	Tamarind	80 d	20	25	ND
RWB1	Ryukyu-ai [e]	Ryukyu-ai indigo paste + Ryukyu-ai paste immersion liquid + Previous Ryukyu-ai fermentation fluid	Wheat bran	No	180 d	100	27	10.81
RWB2	Ryukyu-ai	Ryukyu-ai indigo paste + Ryukyu-ai paste immersion liquid	Wheat bran	No	5.9 years	100	27	ND
IWS270	Indian indigo [f]	IWS470 fermentation fluid	Wheat bran	Indigofera leaf powder	270 d	50	28	ND
IWS470	Indian indigo	Woad fermentation fluid	Wheat bran	No	1.3 years	20	25	10.25

[a] Except STT and SSD, all independent batch.

[b] TT, AS and ON are *Sukumo* producers in Tokushima.

[c] KS is producer in Date, Hokkaido.

[d] Harima is *Sukumo* producer in Nishiwaki, Hyogo.

[e] Ryukyu-ai was obtained from the Higa Ryukyu-ai manufacturing factory.

[f] Indian indigo cake was obtained from NCC AGRO Industries.

[g] ND: no data

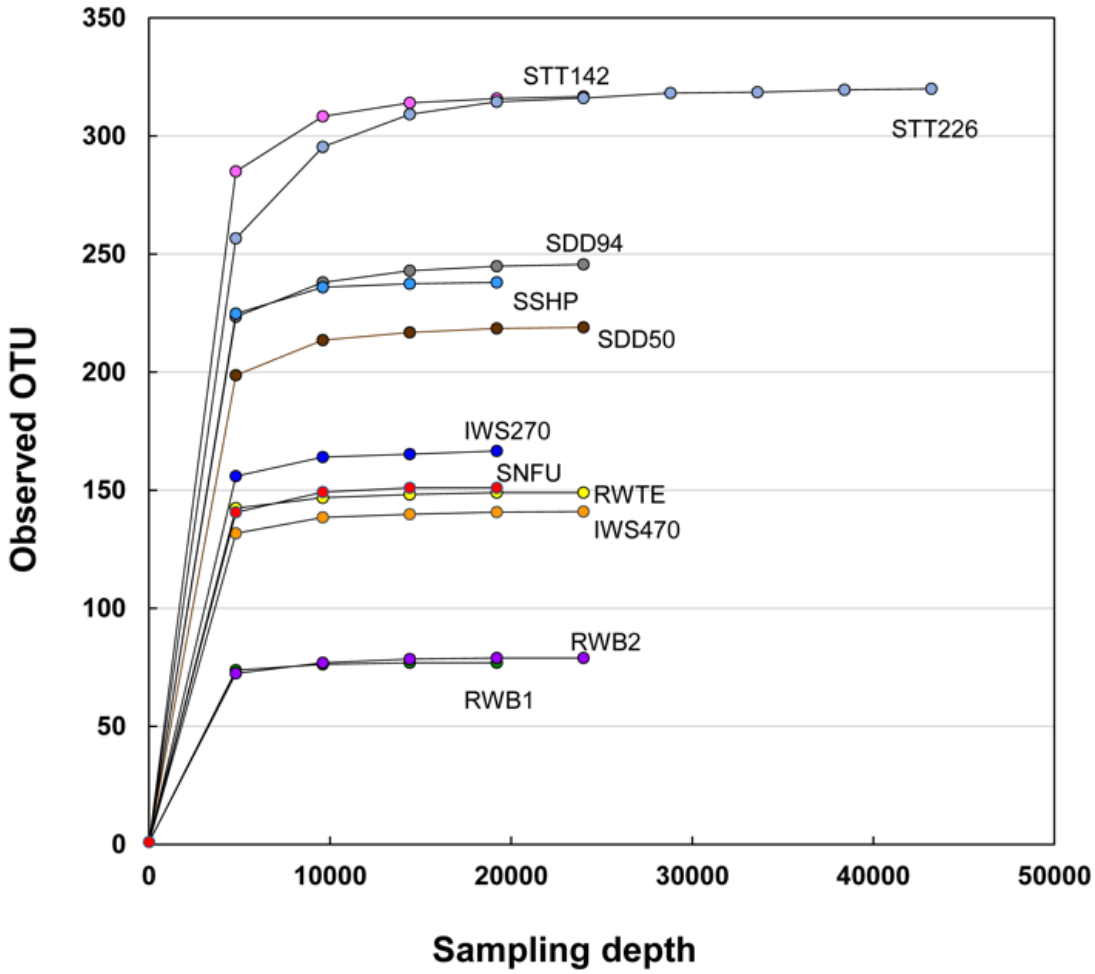

Figure 6 Rarefaction curve of operational taxonomic units (OTUs) defined by 3% sequence variation in 16S rRNA sequencing reads in samples from the indigo fermentation system. STT142, 226: sukumo (produced in Tokushima by TT) fermentation prepared in the laboratory, aged 142 and 226 d; SDD50, 94: sukumo (produced in Date, Hokkaido by KS) fermentation prepared in the laboratory, aged 50 and 94 d; SNFU: sukumo (produced in Tokushima by ON) fermentation; SSHP: sukumo (main constituent was produced in Tokushima by AS) fermentation; RWB 1, 2: Ryukyu-ai based fermentation with wheat bran as the nutrient; RWBE: Ryukyu-ai based fermentation with wheat bran and ethanol as nutrients, and tamarind as the additive; IWS270, 470: Indian indigo-based fermentation with wheat bran as the nutrient.

A	B	C	D									
Phylum (P)/Class (C)/Order (O))/Family (F)	Order (O)/Family (F) /Genus (G)	BLAST top hit	STT142	STT226	SSD50	SSD94	SNFU	SSHP	RWTE	RWB1	RWB2	IWS-270	IWS-470
Facultative anaerobes or aerotolerants			(%)										
Bacillaceae (F)	*Bacillus* (G)	*Bacillus cohnii** (98.36%)	0	0	5.0	7.6	0.4	0	0	0	0	0	0
Bacillaceae (F)	*Bacillus* (G)	*Bacillus fermenti** (99.8-100%)	0	0	0	0	23.2	0	0	0	0	0	0
Bacillaceae (F)	*Bacillus* (G)	*Bacillus dakarensis* (99.8%)	0	0	0	0.1	4.5	0	0	0	0	0.6	0.2
Bacillaceae (F)	*Alkalihalobacillus* (G)	*Alkalihalobacillus hemicellulosilyticus* (98.1-98.4%)	11.0	41.2	0	0	0	1.8	0	0	0	0	0
Bacillaceae (F)	[*Bacillus*] (G)	[*Bacillus*] *vedderi* (98.8-99.1%)	2.8	3.8	0	0	0	0.2	0	0	0	0	0
Bacillaceae (F)	*Polygonibacillus* (G)	*Polygonibacillus indicireducens** (98.6%)	0	0	0	0	0	13.5	0	0	0	0	0
Bacillaceae (F)	*Oceanobacillus* (G)	*Oceanobacillus luteolus* (97.2%)	0	0	0	0	2.2	0.3	0	0	0	0	0
Bacillaceae (F)	*Amphibacillus* (G)	*Amphibacillus indicireducens** (100%)	0	0	0	0	3.5	8.0	0.9	5.5	0.9	1.0	0
Bacillaceae (F)	*Amphibacillus* (G)	*Amphibacillus xylanus* (99.3-100%)	0.3	0.6	0.2	0.1	7.0	2.7	0.1	0.6	0.4	1.2	3.7
Bacillaceae (F)	*Amphibacillus* (G)	*Amphibacillus iburiensis** (100%)	0	0	0	0	0	0.4	0.2	5.8	2.1	0.4	0
Bacillaceae (F)	*Amphibacillus* (G)	*Amphibacillus sediminis* (98.1%)	0	0	0	0	0	0	0	2.0	1.7	0	0
Bacilli (C)	*Bacillales* (O)	*Bacillus pseudofirmus* (85.6%)	0	0	0	0	0	0	8.9	0	0	0	0
Erysipelotrichales (O)	*Erysipelotrichaceae* (F)	*Erysipelothrix inopinata* (93.2-93.4%)	9.9	4.8	15.7	11.8	0	8.0	0	1.4	2.1	6.6	3.5
Aerotolerants lactic acid bacteria					0								
Carnobacteriaceae (F)	*Alkalibacterium* (G)	*Alkalibacterium indicireducens** (100%)	0.1	0.1	0.3	0.3	2.4	1.9	1.5	0	0	0	0.2
Carnobacteriaceae (F)	*Alkalibacterium* (G)	*Alkalibacterium kapii* (98.1%)	0	0	0	0	0	0	0	0	0	0.2	1.5
Carnobacteriaceae (O)	*Atopostipes* (G)	*Atopostipes suicloacalis* (99.1%)	0	0	0	0	4.3	0.4	0	0.6	0.8	0.2	0.2
Aerococcaceae (F)	*Facklamia* (G)	*Facklamia languida* (97.4%)	0	0	0.1	0	0	0.1	0.2	0	0	0.8	24.9
Enterococcaceae (F)	*Enterococcus* (G)	*Enterococcus aquimarinus* (99.8%)	0	0	0	0	0	0	0	0	0	4.3	2.8
Obligate anaerobes													
Clostridiales (O)	*Proteinivoraceae* (F)	*Proteinivorax tanatarense* (92.3-93.6%)	28.1	1.9	24.1	20.4	10.0	12.9	1.7	1.4	1.8	0.2	0
Clostridia (C)	*Clostridiales* (O)	*Proteinivorax tanatarense* (88.4%)	0	0	0	0	0	0	0	18.7	21.6	0	0
Clostridiales (O)	*Acetoanaerobium* (G)	*Acetoanaerobium pronyense* (100%)	0	0.1	0	0	0	0.1	0	0	0	0.2	11.2
Clostridiales (O)	*Hungateiclostridiaceae* (F)	*Ercella succinigenes* (90.4%)	0	0	0	0	0	0	0	0.1	0.1	2.7	5.4
Clostridiales (O)	*Proteiniclasticum* (G)	*Proteiniclasticum ruminis* (99.5%)	0	0	0	0	0	0	0	0	0	0.7	4.2
Clostridiales (O)	*Eubacteriaceae* (F)	*Rhabdanaerobium thermarum* (92.4%)	0.1	0.7	0.1	0	0	1.6	0.1	0.2	0	0.1	0
Clostridia (C)	*Clostridiales* (O)/ *Hungateiclostridiaceae* (F)	*Saccharofermentans acetigenes* (89.8-90.9%)	0.2	0.1	0	0	0	0.2	1.2	0.6	0.5	5	0.7
Clostridia (C)	*Clostridiales* (O)	*Abyssisolibacter fermentans* (86.9%)	0	0	0	0	0	0	2.1	0	0	0	0
Clostridia (C)	*Clostridiales* (O)	*Clostridium septicum* (89.0%)	1.3	0.7	0.4	0.2	0.5	3.8	0.3	0	0	1.6	0
Firmicutes (F)/ *Clostridia* (C)	*Clostridia* (O)	*Alkaliphilus metalliredigens* (79.8%)	0	0	1.7	2	0	0	0	0	0	0	0
Tissierellales (O)	*Tissierellaceae* (F)/*Tissierella* (G)	*Tissierella creatinini* (94.5- 95.0%)	6.4	9.5	4.1	3.9	6.4	14.2	4.8	2.0	2.0	10.8	1.8
Tissierellia (C)	*Tissierellales* (O)	*Sporanaerobacter acetigenes* (88.0%)	0	0	0	0	0	0	11.7	0	0	0	0
Actinobacteria					0								
Micrococcales (O)	*Pseudoglutamicibacter* (G)	*Pseudoglutamicibacter cumminsii* (98.3%)	4.4	2.1	0.7	1.0	0	2.8	0.1	0.1	0.2	0.1	0.2
Corynebacteriales (O)	*Corynebacterium* (G)	*Corynebacterium sputi* (100%)	0	0	0	0	0	0	1.9	0	0	0	0
Corynebacteriales (O)	*Corynebacterium* (G)	*Corynebacterium pollutisoli* (100%)	1.0	0.2	0.1	0.1	0	0	0	0	0	0.2	0.1
Proteobacteria					0								
Rhodobacterales (O)	*Paracoccus* (G)	*Paracoccus cavernae* (98.8-99.0%)	0.5	0.6	2.4	1.9	0	1.9	0	0	0	0	0
Caulobacterales (O)	*Brevundimonas* (G)	*Brevundimonas naejangsanensis* (100%)	0	0	0	0	0.3	0	0	0	0	0.2	2.3
Caulobacterales (O)	*Brevundimonas* (G)	*Brevundimonas terrae* (99.5%)	0	0	0	0	0	0	1.8	0	0	0.1	0
Halomonadaceae (F)	*Halomonas* (G)	*Halomonas johnsoniae* (98.8-100%)	0	0	0	0	0	0	7.7	45.0	38.3	0.2	0.1
Halomonadaceae (F)	*Halomonas* (G)	*Halomonas lactosivorans* (96.0%)	0	0	0	0	0	0	0	0.5	16.2	0	0
Halomonadaceae (F)	*Halomonas* (G)	*Halomonas axialensis* (99.5%)	0	0	0	0	0	0	1.5	0	0	0	0
Oceanospirillaceae (F)	*Marinospirillum* (G)	*Marinospirillum minutulum* (95.6%)	0	0	0	0	0	0	13.2	0	0	0	0
Pseudomonadaceae (F)	*Pseudomonas* (G)	*Pseudomonas pseudoalcaligenes* (100%)	0.1	0.2	9.9	7.5	0	0.3	0	0	0	6.6	1.0
Pseudomonadaceae (F)	*Pseudomonas* (G)	*Pseudomonas peli* (98.4-98.8%)	0	0.2	10.1	16.8	0	0.3	0	0	0	10.0	3.9
Pseudomonadaceae (F)	*Pseudomonas* (G)	*Pseudomonas salegens* (97.9%)	0	0	0	0	0	0	6.4	5.0	1.7	0	0
Moraxellaceae (F)	*Acinetobacter* (G)	*Acinetobacter indicus* (99.8-100%)	0	0	0	0	0	0	0.2	0	0	0.4	4.8
Alteromonadaceae (F)	*Alishewanella* (G)	*Alishewanella jeotgali* (100%)	0.2	1.4	0	0	0	0.2	0	0.1	0.2	3.2	0.2
Alcaligenaceae (F)	*Parapusillimonas* (G)	*Parapusillimonas granuli* (97.4%)	0.2	5.5	0	0	0	0.1	0	0.1	0.1	9.8	5.0
Alcaligenaceae (F)	*Pusillimonas* (G)	*Pusillimonas ginsengisoli* (97.4%)	0	0	0	0	0	0	0	0	0	2.8	0
Tenericutes													
Acholeplasmatales (O)	*Acholeplasmataceae* (F)	*Acholeplasma axanthum* (94.13%)	0	0	0	0	0	0	3.4	0	0	0	0
Acholeplasmatales (O)	*Acholeplasma* (G)	*Acholeplasma laidlawii* (98.1%)	0	0.4	0	0	0	0	1.7	0	0	0.9	0
		Others	33.4	25.9	25.1	26.3	35.3	24.3	28.4	10.3	9.3	28.9	22.1
			>30	>24	>15	> 8	> 3	> 1					

Figure 7 Relative abundance (%) of bacterial constituents based on the 16S rRNA analysis (≥1.5% in sample). The percentages in the brackets (column C) indicate the highest similarities with the known species in the database. Classification hierarchy (column A and B) described in the brackets as follows: G, genus, F, family, O, order; C, class and F, Phylum. Classification hierarchy (column C) depends on database similarity as follows: order level, <90% similarity; family level, ≥ 90, < 95%; genus level, ≥ 95, < 98.6%; and S, species level (≥ 98.6). The known indigo-reducing bacterial species are marked with an asterisk.

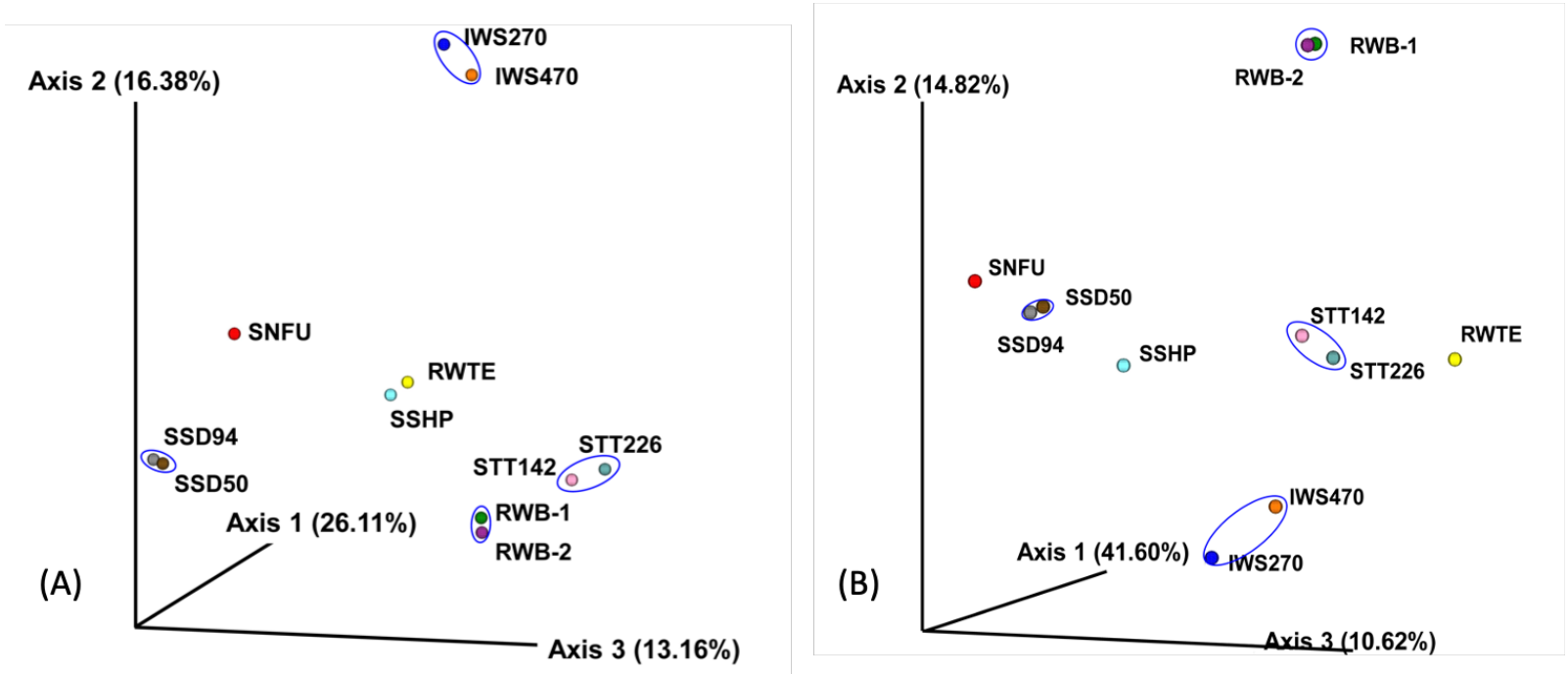

Figure 8 Principal coordinates analysis (PCoA) plot of the bacterial community constructed using Jaccard (A) and unweighted UniFrac (B) distances. Sample identities include: STT142, STT226: sukumo (produced in Tokushima by TT) fermentation prepared in the laboratory, aged 142 and 226 d, respectively; DD50, SDD94: sukumo (produced in Date, Hokkaido by KS) fermentation prepared in the laboratory, aged 50 and 94 d, respectively; SNFU: sukumo (produced in Tokushima by ON) fermentation; SSHP: sukumo (main constituent produced in Tokushima by AS) fermentation; RWB 1, 2: Ryukyu-ai-based fermentation with wheat bran used as the nutrient; RWBE: Ryukyu-ai-based fermentation with wheat bran and ethanol as nutrients and tamarind as the additive. IWS270, 470: Indian indigo-based fermentation with wheat bran as the nutrient. Samples with similar procedures are marked with a circle.

Supplementary Information

Sample ID	STT142	STT226	SSD50	SSD94
ORP (mV)	ND	-619	-641	ND
Dyeing result				

Figure 9 Dyed cotton fabric with associated oxidation reduction potentials (ORPs mV) in sukumo fermentation solutions prepared in laboratory conditions. STT142 and STT226: sukumo produced in Tokushima Prefecture by TT, aged 142 d and 226 d, respectively. SDD50 a and SDD94: sukumo produced in Date City, Hokkaido by KS, aged 50 d and 94 d, respectively. ND: no data

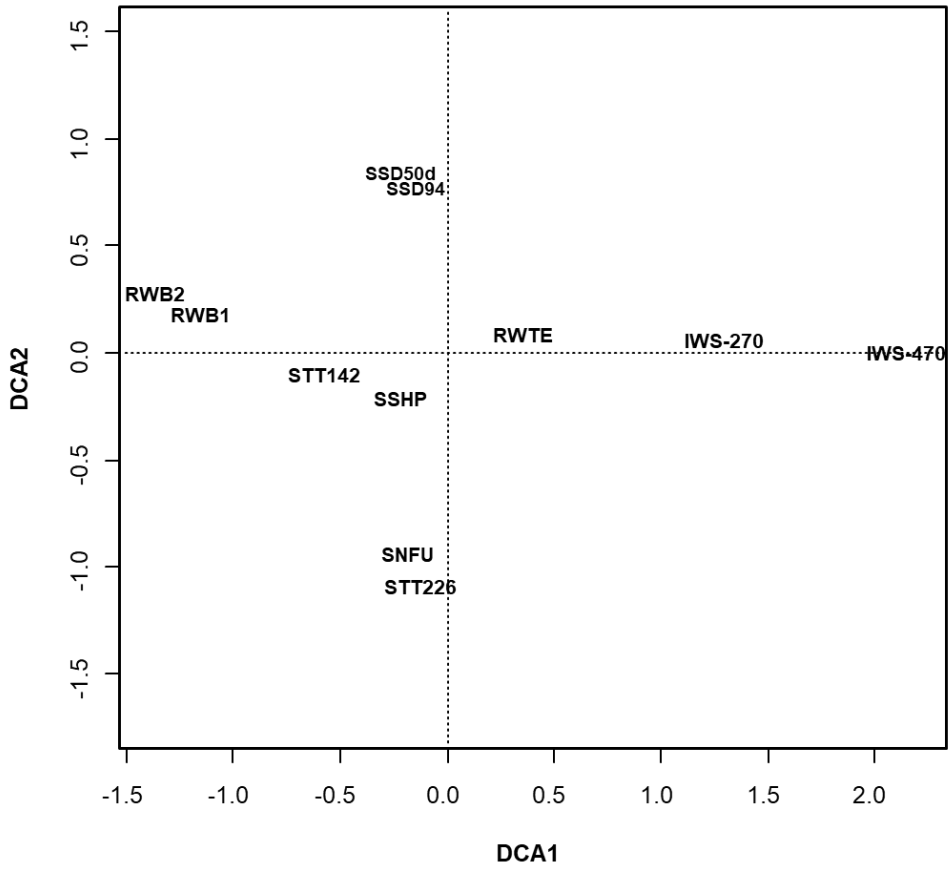

Figure 10 Detrended correspondence analysis (DCA) of the different samples, constructed using taxonomy based BIOM file obtained from QIIME2 transformed into percentages, and R software (https://www.R-project.org/). Sample identities are: STT142, STT226: sukumo (produced in Tokushima by TT) fermentation prepared in the laboratory, aged 142 and 226 d, respectively; SDD50, SDD94: sukumo (produced in Date, Hokkaido by KS) fermentation prepared in the laboratory, aged 50 and 94 d, respectively; SNFU: sukumo (produced in Tokushima by ON) fermentation; SSHP: sukumo (main constituent produced in Tokushima by AS) fermentation; RWB 1, 2: Ryukyu-ai-based fermentation with wheat bran used as the nutrient; RWBE: Ryukyu-ai-based fermentation with wheat bran and ethanol as nutrients and tamarind as the additive. IWS270, 470: Indian indigo based fermentation with wheat bran as the nutrient. DCA analysis showed that the material and procedures used to make the fluids impact the samples.

Chapter 2

Indigofera tinctoria leaf powder as a promising additive to improve indigo fermentation prepared with *sukumo* (composted *Polygonum tinctorium* leaves)

(Submitted to World Journal of Microbiology and Biotechnology)

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 increase in the ratio of facultative to obligate anaerobes (F/O ratio), or the total abundance of facultative anaerobes and obligate anaerobes (O) (deignated F+O) are vital for 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 civilizations around the world [2]. 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 [3]. The requirement for natural indigo declined after the first commercially available synthetic indigo was developed and commercialized in 1890. Synthetic indigo has almost entirely replaced natural indigo [2,64]. Today, the global indigo production is in the thousands of tones. More indigo is manufactured than any other dye worldwide and it is used mainly to dye denim for blue jean fabrication [64]. However, synthetic indigo production generates environmentally harmful chemicals [32].

Oxidized indigo dye is insoluble in water and must be solubilized by reduced in alkali solution. The reduced product is known as leuco-indigo and readily penetrates fabrics. On contact with atmospheric oxygen, it oxidizes to a blue form [13,64]. Fermentation by bacterial, chemical and electrochemical reduction, and catalytic and electrocatalytic hydrogenation can be used to reduce indigo [2,8,65]. Industrial dyeing commonly involves the reducing agent sodium dithionate [64]. Whilst it is fast and inexpensive chemical reduction requires the continual addition of the reductant and generates toxic and corrosive waste products [2,32].

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 harbors 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 [9]. 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 (nutrient) 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 [9].

The microbiota in *sukumo* preparations have been studied via clone library analysis [35,36] and next generation sequencing (NGS) [11,12]. Clone library analysis focused on aged fluids and identified *Proteinivoraceae, Tissirellaceae, Anaerobacillus, Amphibacillus, Alkalibacterium* and *Polygonibacillus* as the dominant taxa [36]. 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*) [11,12]. 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 [11,12].

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 [9]. 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 [66]. 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 [2,38,39]. 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 [28]. *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 Experiments 1 and 2, the *sukumo* source was produced by KS (Date City, Hokkaido, Japan). For Experiment 3, the *sukumo* source was produced by TT (Tokushima Prefecture, Shikoku, Japan) (Table 3). Both *sukumo* sources used in this study were chosen because the start of indigo reduction took more than 6 days, whereas the *sukumo* normally used in our laboratory exhibited indigo reduction within 5 days. 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. Dyeing efficiency of the *I. tinctoria* LP treatment was assessed by comparing it against the control. The dyeing intensity of the samples sent to NGS were measured by scanning the dyed cloth and analyzing the image using Mathematica (version 12.2). The intensity was expressed as the square root of a^2+b^2 in CIE $L^*a^*b^*$ color system. 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.

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.

Next generation sequencing

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 [45]. 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 [67]. 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 [46,47]. 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. Principal coordinates analysis (PCoA) based on the Jaccard distance was performed using beta diversity analysis script in QIIME 2. EMPEROR software was used to visualised the PCoA plots [49]. The predictive functions of the metagenomes of LP treatment and the control at day 5 were estimated using PICRUSt2 [68]. KEGG pathway mapping was used to search the KEGG database according to the output results.

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 11 and Supplementary information - Figure 14. 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 (Figure 11 and Supplementary information - Figure 14).

Firmicutes was the dominant phylum in all samples (including facultative, obligate and aerotolerant anaerobes) followed by *Proteobacteria* and *Actinobacteria* (Figure 11). The microbiota detected in both *I. tinctoria* LP (LP1 and LP2) in natural state are shown in Supplementary information - Figure 17. The estimated CFU for *I. tinctoria* LP and *sukumo* are shown in Supplementary information - Table 4. The bacterial community profiles in both *I. tinctoria* LP in raw state does 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 information - Table 4). 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 four 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 Figure 12. From these results, it seems that the intensity of dyeing is strongly influenced by the initial abundance of F+O in LP added fluids, which is lower than the control. In addition, an imbalance of the F/O ratio with a dominance of obligate anaerobes seems detrimental in both fermentations. 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 13). 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 (Figure 12 and Supplementary information - Figure 16).

The relative difference between *I. tinctoria* LP and the control in terms of their microbiota were estimated by principal coordinate analysis (PCoA) using Jaccard distance (Figure 14). The microbiota of *I. tinctoria* LP rapidly changed from day 10 to day 25 compared with the control. However, both *I. tinctoria* LP and the control reached the final stage around day 58. In addition, 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.

Predicted metabolic functional differences between *I. tinctoria* LP added fluid and the control at day 5 were estimated (Supplementary Figure 18). The results showed that carbohydrate, lipid and amino acids metabolism are superior in LP added fluid than in the control. Specifically, the results point to a predominance in phosphotransferase system, which is known for sugars uptakes, including glucose, mannose, fructose, and cellobiose in bacteria. It is presumed that the regeneration of microbial flora triggered by the fermentation preparation (heat and high pH treatments) and the sugars uptakes derived from *sukumo* as an energy source are more active in the LP-added fermentation broth than in the control.

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 on 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 Figure 15 and Supplementary information - Figure 19 and Figure 20. *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 information - Figure 16, Figure 19, and Figure 20). *I. tinctoria* LP addition during initial *sukumo* fluid preparation hastened the onset of indigo reduction compared with the control (Experiment 3) (Supplementary information - Figure 20). 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 F/O ratio was nearly constant and F+O values was decreased in the first two weeks and then maintained stable in the control. In the *I. tinctoria* LP added fluid, the F/O ratio varied with fermentation time and the F+O values decreased in the first two weeks and then maintained stable until the final date. The dyeing intensity had an opposite behavior in the first two weeks and then almost consistent behavior to the F+O change in both the *I. tinctoria* LP added fluid and the control (supplementary Figure 21 A - B). In Experiment 3, the dyeing intensity followed the same trend as the F+O or F/O in both the LP treated fluid and the control.

The F/O ratio was higher in the LP added fluids and fluctuated more drastically than in the control (supplementary Figure 21 C - D).

The changes in OTU number at the 18512-sampling depth (Supplementary information - Figure 22) 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. Jaccard distance PCoA exhibited a distinct separation between *I. tinctoria* LP and the control for in Experiments 2 and 3 (Supplementary information - Figure 23). 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 [12]. 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 [51]. Ethanolic and hydroethanolic

extracts of *I. tinctoria* LP contain anthraquinone [28] 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 stabilize 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 [11,12]. 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 [11]. 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 [11,12]. 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 [11,12]. 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* [56]. *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 [41,69,70].

Since there are many factors involved, it is not easy to clarify the relationship between staining intensity and microbial flora. The possible factors could be 1) the abundance of the substrate leading up to indigo reduction and the presence or absence of microorganisms that can use it 2) the abundance of microorganisms involved and the abundance of electron mediators in the fermented liquor and an event for the circulation of the mediator in the fluid such as cell disruption as the dye itself may be involved in the redox state in the system; and 3) the amount of oxidized and reduced dyes in the fluid. From these results, it seems that the dyeing intensity is strongly influenced by F+O or F/O depending on what is rate-determining factor in that situation. The relationship between F/O and staining intensity is not always parallel because it takes certain days for an aspect of the microbiol community to reflects in the staining intensity. As there are many factors and a time lag, it is difficult to judge the current status of staining intensity from only one point of the analysis of the microbial community; however, by estimating of the tendency of changes in F+O and F/O over an approximately 5 day period, for example, it may be possible to predict the staining intensity over the upcoming days.

Previous studies have shown that the *sukumo* fermentation can be divided into different phases. Based on the F+O and F/O graphics it can be seen that the changes in these values have different influences in the dyeing intensity. In the initial phase, the onset of an already high F+O ratio seems to have a positive effect on the dyeing intensity, which can be observed by comparing the control and treatment. After some time, the decrease in the F+O ration is most likely caused by the consumption of readily available nutrients and changes in the environment, which in turn impact the dyeing intensity. At this moment the increase of facultative anaerobes as seen in the F/O ratio will play a bigger role on the dyeing intensity. This increase may reflect the availability of new nutrients and mediators made available via metabolism by obligate

anaerobes or the appearance of facultative anaerobes capable of using more complex nutrients. After the stabilization of F+O ratio varying around 60%–70% of the community the changes in F/O ration seems to have a smaller impact.

In a previous report the abundances of the facultative anaerobic *Polygonibacillus* increased with culture age [12]. A similar pattern was observed in the current study. OTUs distantly related to the facultative anaerobic *Erysipelothrix* increased on day 58 in Experiment 1 and reached even higher levels in Experiment 2. *Erysipelothrix inopinata*, was originally isolated from vegetable broth [71], 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 [12]. 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 [72]and a *Pseudomonas* strain with reduction activity has been isolated from dyeing vat [73]. In previous studies with *sukumo* fermentation, it was detected in short-lived fluid as well as in decaying long-lived batches [12]. *Pseudomonas peli* was closely associated with the observed OTUs. This species was isolated from textile wastewater and can decolorise other dyes [74]. 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 effect on the dyeing intensity.

Hirota et al. [21], 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 [11,12]. 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 cases, 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 [12]. 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 [11,12]. The fermentation fluid contains microorganisms with overlapping functions and this property might account for the resilience of this system.

Conclusion

The present study examined effects of the *I. tinctoria* LP addition on the initial steps of *sukumo*-based indigo fermentation. This study demonstrated that an early decrease in ORP was essential for accelerating the onset of the appropriate microbial community for indigo reduction and that *I. tinctoria* LP can assist in the appropriate transitional change of the microbial community. Despite the use of different *sukumo* sources and differently packaged *I. tinctoria* LPs, the addition of *I. tinctoria* LP rapidly lowered and stabilised the ORP in the early fermentation phase. Moreover, *I. tinctoria* LP treatment affected the microbiota in the early stages and these changes were observed even in the late stages. Initiation of indigo reduction is dependent on the rapid decline in ORP attributed to oxygen consumption by facultative anaerobes, followed by a decrease in facultative anaerobes from days 1–7. In addition, an overall decrease in the bacterial community diversity is required to initiate indigo reduction. *I. tinctoria* LP accelerated the establishment of the biological and physicochemical conditions required for indigo reduction by affecting those parameters. Moreover, *I. tinctoria* LP changed the F/O ratio and F+O, which may impact the staining intensity in the initial phase of fermentation.

Despite the optimistic results, the effect of *I. tinctoria* LP on indigo reduction must be validated using a larger scale fermenter. The complex community, limited identity of certain taxa, and absence of known indigo-reducing bacteria indicate that *sukumo* harbours numerous bacteria that need to be characterised. Nevertheless, the present study shows that some environmental characteristics are important for the onset of indigo reduction in bacterial fermentation and the manipulation of these characteristics by the use of additives can be useful for the development of new methodologies.

Table 3 Differences in preparation of indigo fermentation fluids used in this study.

Table 1 Differences in preparation of indigo fermentation fluids used in this study					
Batch	Sukumo	Leaf powder	Period of analysis of microbiota	Addition of nutrient	Fermentation volume (L)
Experiment 1	Date (by KS)	LP2	Days 1–72	No	0.5
Experiment 2	Date (by KS)	LP1	Days 36-94	No	0.5
Experiment 3	Tokushima (by TT)	LP1	Days 115–226	No	0.5

		Experiment 1 (control)							Experiment 1 (LP added)						
	Dyeing intensity ($L^*a^*b^*$ value × 10)	ND[a]	0.34	0.37	0.44	0.29	1.03 (D62)[b]	1.14	ND	1.93	1.63	1.08	0.23	1.02 (D62)	1.69
	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 anaerobes & aerotolerants	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%	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%
[a]ND, no data; [b](D62), result of Day 62 sample															
		>60	>45	>35	> 15	> 8	> 1								

*Figure 11 Relative abundance (%) of bacterial constituents based on 16S rRNA analysis (≥ 1.5% in sample), dyeing intensity (exhibited as L*a*b* value × 10), 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; order; C, class. [a]ND, no data; [b](D62), result of Day 62 sample.*

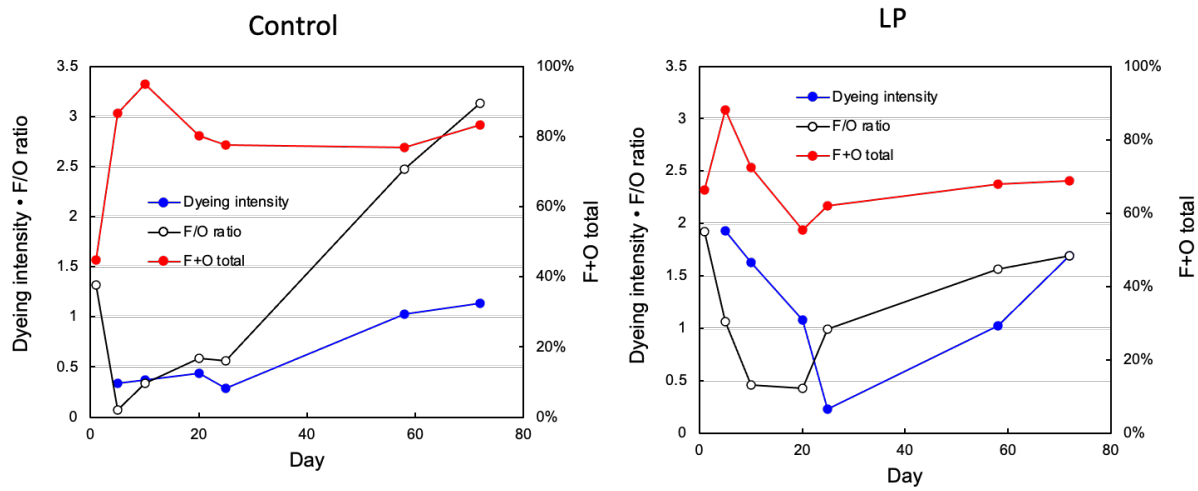

*Figure 12 Changes in dyeing intensity, abundance ratios of facultative anaerobes and absolute anaerobes (F/O), and total abundance ratios of facultative anaerobes and absolute anaerobes (F + O) in sukumo determined for Experiment 1 indigo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder. Dyeing intensity is exhibited as L*a*b* value × 10.*

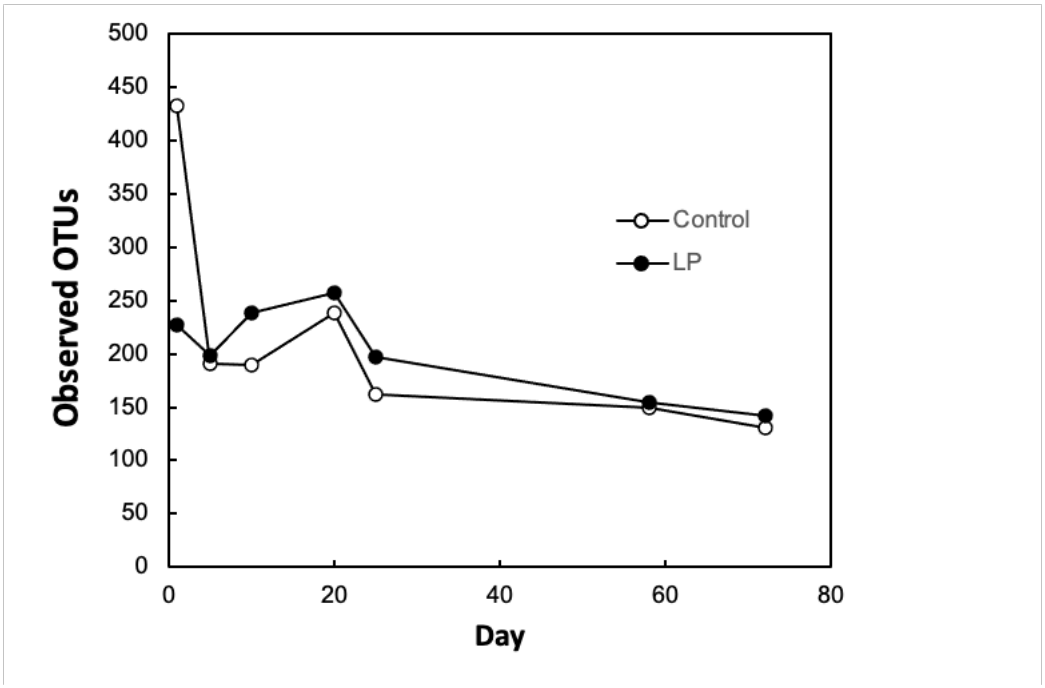

Figure 13. Changes in observed OTUs at sample depth of 18512 varying with sukumo fermentation period using Experiment 1 indigo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder. Analysis was performed with the Divisive Amplicon Algorithm (DADA2) and depended on the fermentation period. The standard error is less than 0.4% for each value

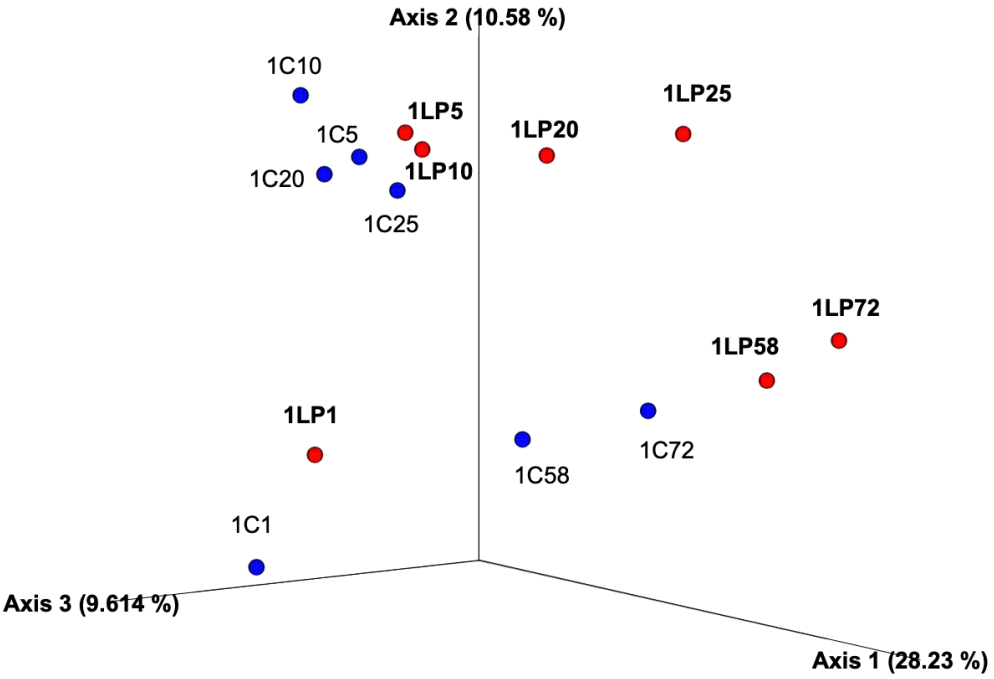

Figure 14 Principal coordinate analysis (PCoA) based on Jaccard distance in Experiment 1. Red and blue circles indicate samples with (LP) and without (control) Indigofera tinctoria leaf powder, respectively.

		Experiment 2 (control)			Experiment 2 (LP added)			Experiement 3 (control)				Experiment 3 (LP added)			
	Dyeing intensity ($L^*a^*b^*$ value × 10)	1.45	2.01	1.92 (D98)[a]	1.56	2.10	2.16 (D98)	1.73	1.24 (D147)[c]	1.56	1.22	2.29	1.84 (D147)[c]	2.23	1.18
	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 & aerotoleants	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%
[a](D98), result of Day 98 sample; [b]NA, no data; [c](D147), result of D147 sample			>40	>35	> 30	> 10	> 7	>5	>2	>1					

*Figure 15 Relative abundance (%) of bacterial constituents based on 16S rRNA analysis (≥ 1.5% in sample) dyeing intensity (exhibited as L*a*b* value × 10), 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; [b]NA, no data; [c](D147), result of D147 sample.*

Supplementary information

Table 4 Changes of bacteria count (CFU) of leaf powder and sukumo

LP	Dilution with physiological saline (cell count in original sample)	Exposure to wood ash extract at 26 °C for 30 min
Bacterial count in 0.5 g/5 mL suspension (CFU/ mL physiological saline or wood ash extract)	5.3×10^5	3.4×10^5
Expected bacterial count in fermentation fluid (CFU/mL)	5.8×10^3	3.7×10^3
Change from original count	100%	64%

Sukumo	Dilution with physiological saline (cell count in original sample)	Exposed to wood ash extract at 55 °C for 30 min
Bacterial count in 0.5 g/5 mL suspension (CFU/ mL physiological saline or wood ash extract)	4.6×10^5	4.9×10^5
Expected bacterial count in fermentation fluid (CFU/ mL)	3.9×10^5	4.1×10^5
Change from original count	100%	105%

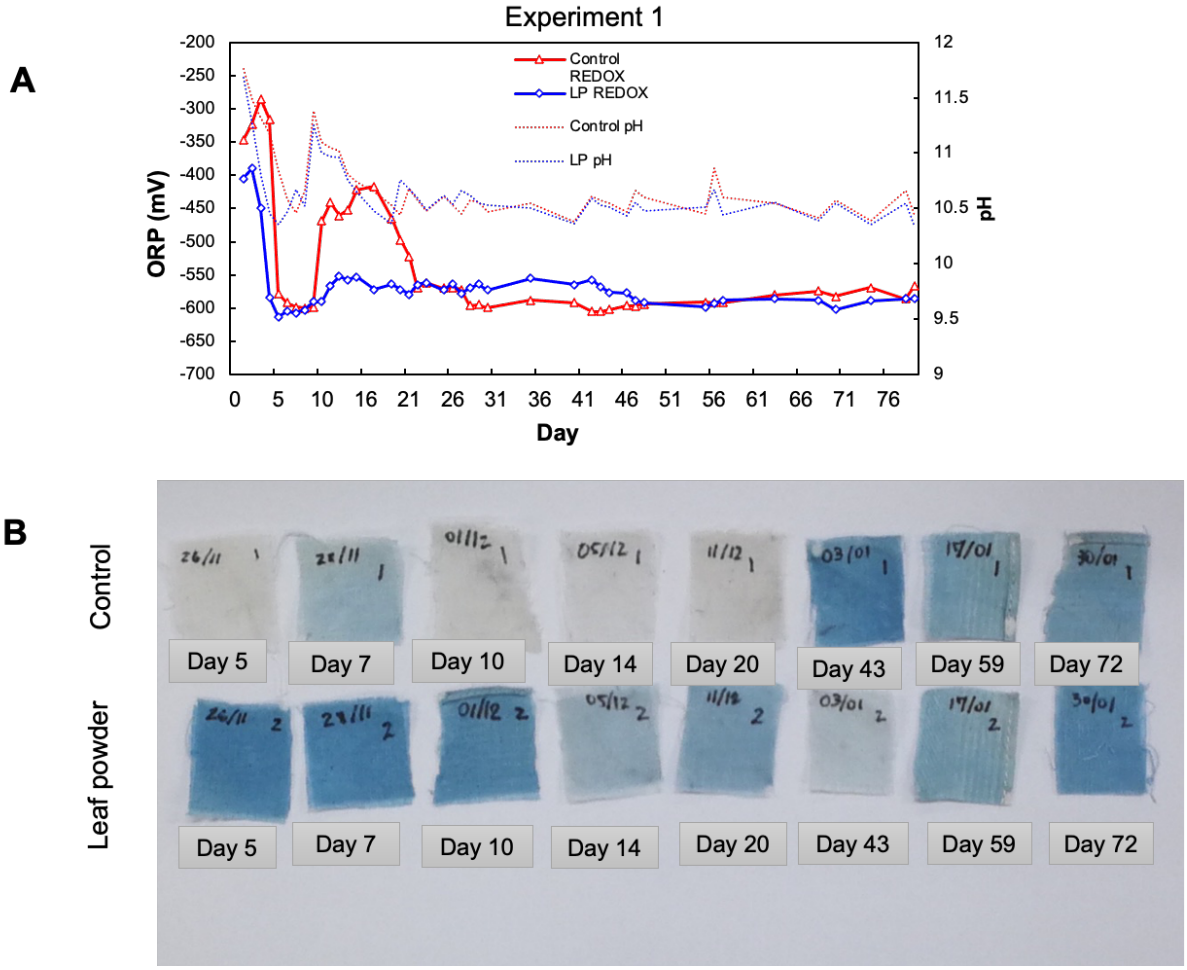

Figure 16 Changes in redox potential (ORP) and pH from day 1 to day 70 (A) and dyeing results from day 5 to day 72 of Experiment 1 with (LP) and without (control) Indigofera tinctoria leaf powder.

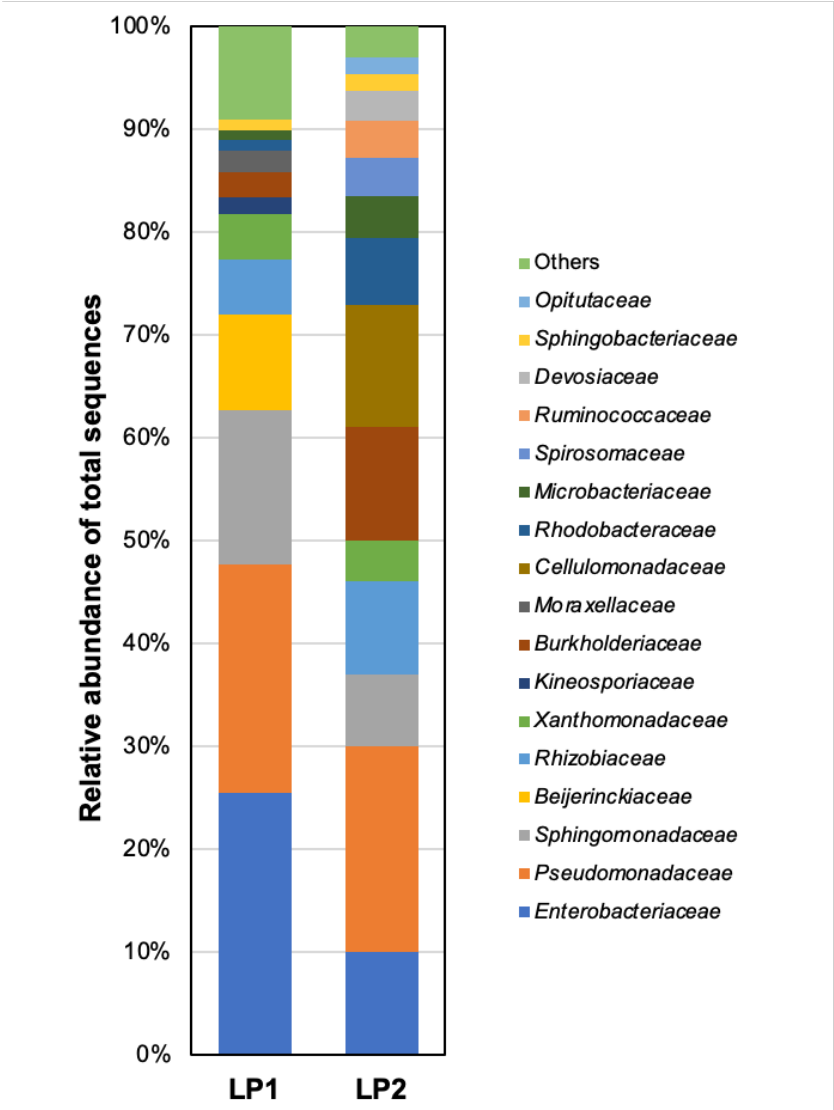

Figure 17 Relative abundance (%) of bacterial constituents based on 16S rRNA analysis (≥2.0% in sample) in leaf powders 1 (LP1) and 2 (LP2).

Level 2	KEGG pathway mapping	Relative abundane in LP added compared with control at day 5
Carbohydrate metabolism	Ascorbate and aldarate metabolism (Ko00053)	62.4%
Metabolism; Lipid metabolism	Secondary bile acid biosynthesis (ko00121)	56.1%
Metabolism; Metabolism of terpenoids and polyketides	Tetracycline biosynthesis (ko00253)	41.0%
Metabolism; Amino acid metabolism	Valine, leucine and isoleucine degradation (ko0028)	54.9%
Metabolism; Amino acid metabolism	Lysine degradation (ko00310)	52.7%
Metabolism; Amino acid metabolism	Tryptophan metabolism (ko00380)	68.2%
Metabolism; Metabolism of other amino acids	Phosphonate and phosphinate metabolism (ko00440)	169.8%
Metabolism; Metabolism of other amino acids	D-Arginine and D-ornithine metabolism (ko00472)	156.7%
Metabolism; Xenobiotics biodegradation and metabolism	Aminobenzoate degradation (ko00627)	117.8%
Metabolism; Metabolism of cofactors and vitamins	Lipoic acid metabolism (ko00785)	22.4%
Environmental Information Processing; Membrane transport	Phosphotransferase system (PTS) (ko02060)	378.6%

Figure 18 Difference in relative abundances between Indigofera tinctoria leaf powder (LP) and the control at day 5 based on KEGG categories predicted by PICRUSt2 (Level 2 and KEGG pathway mapping). The KEGG pathway entry number is shown brackets in the KEGG pathway mapping column

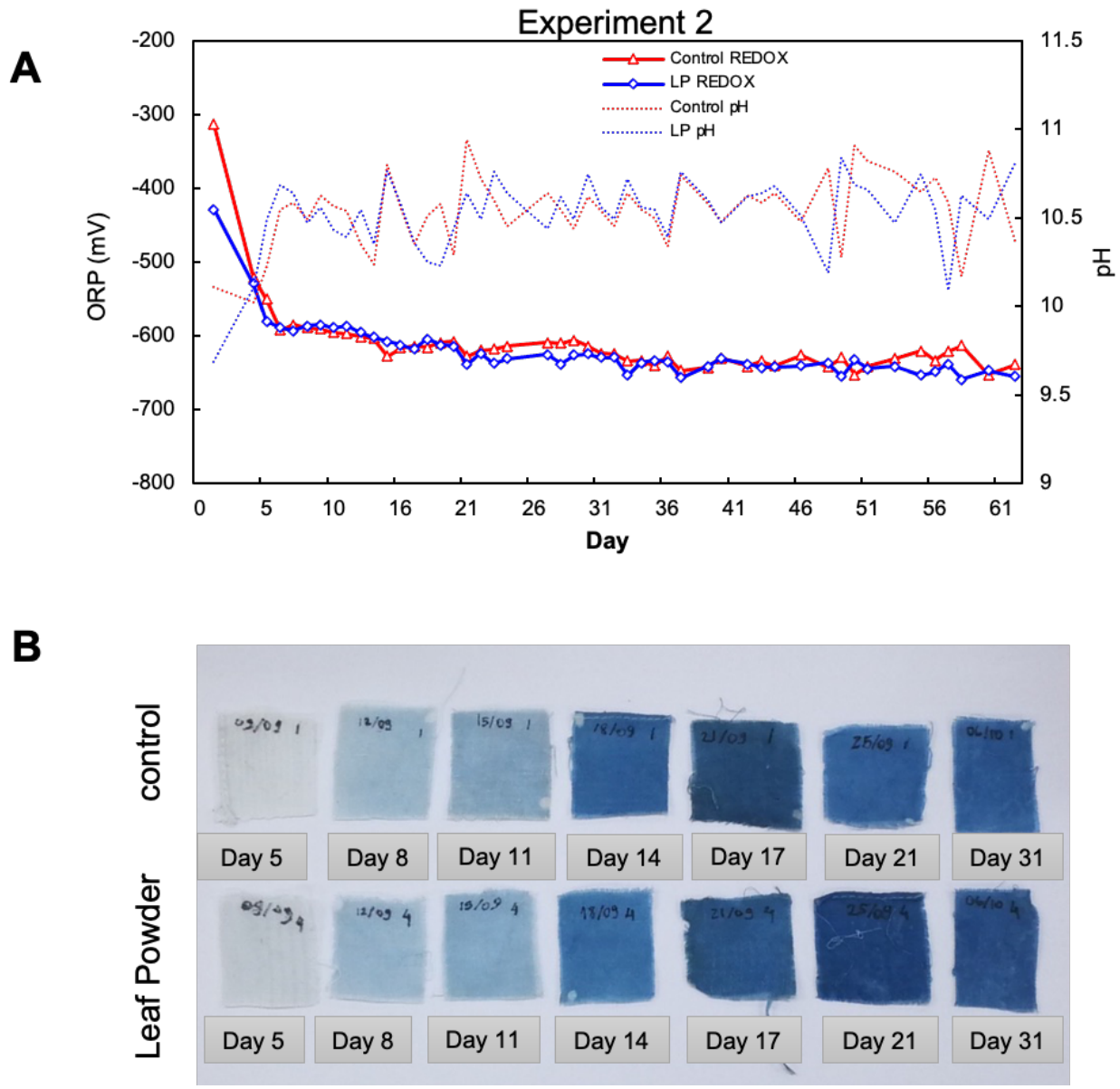

Figure 19 Changes in redox potential (ORP) and pH from day 1 to day 62 (A) and dyeing results from day 5 to day 31 of Experiment 2 with (LP) and without (control) Indigofera tinctoria leaf powder.

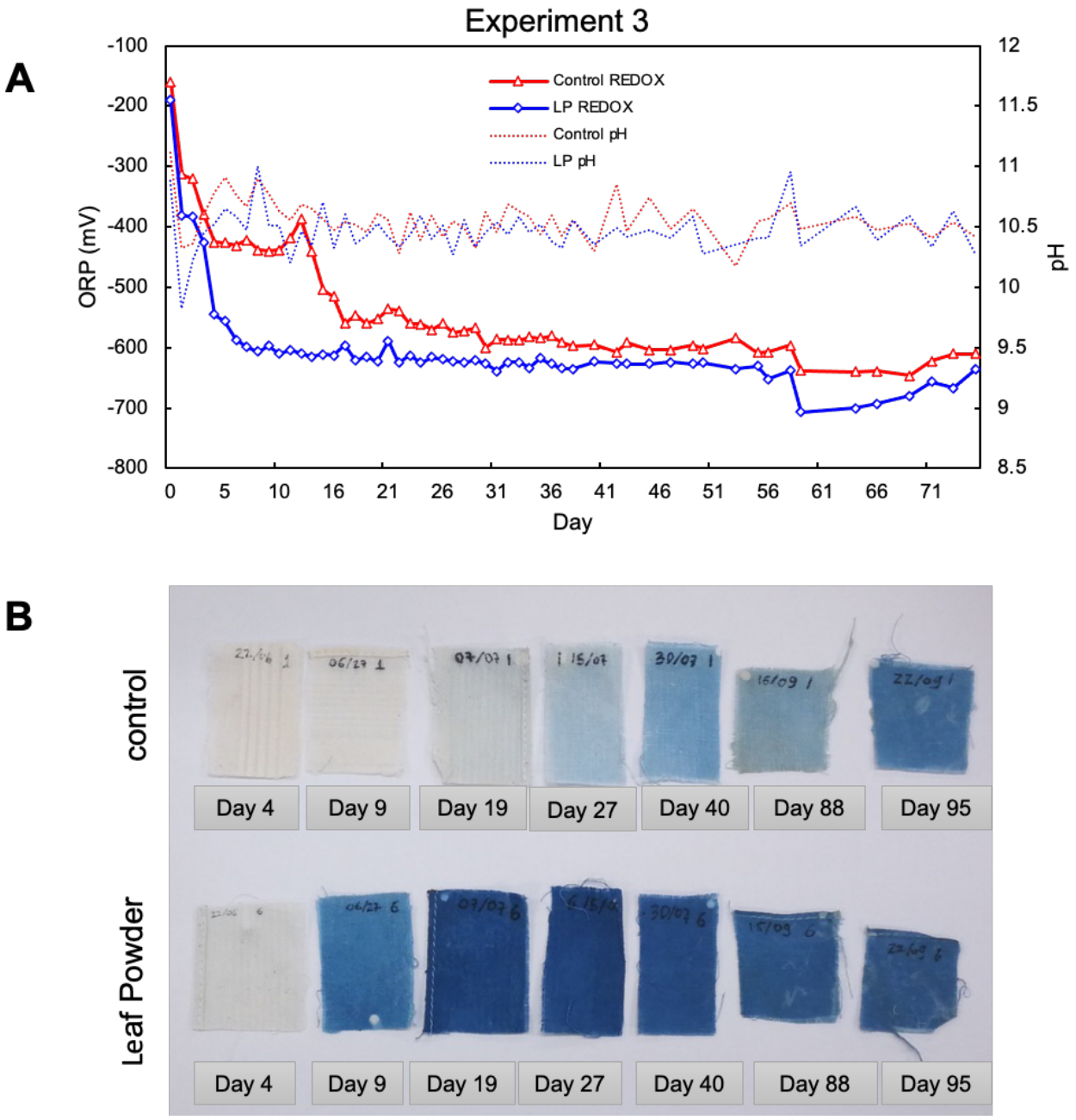

Figure 20 Changes in redox potential (ORP) and pH from day 1 to day 75 (A) and dyeing results from day 4 to day 95 of Experiment 3 with (LP) and without (control) Indigofera tinctoria leaf powder.

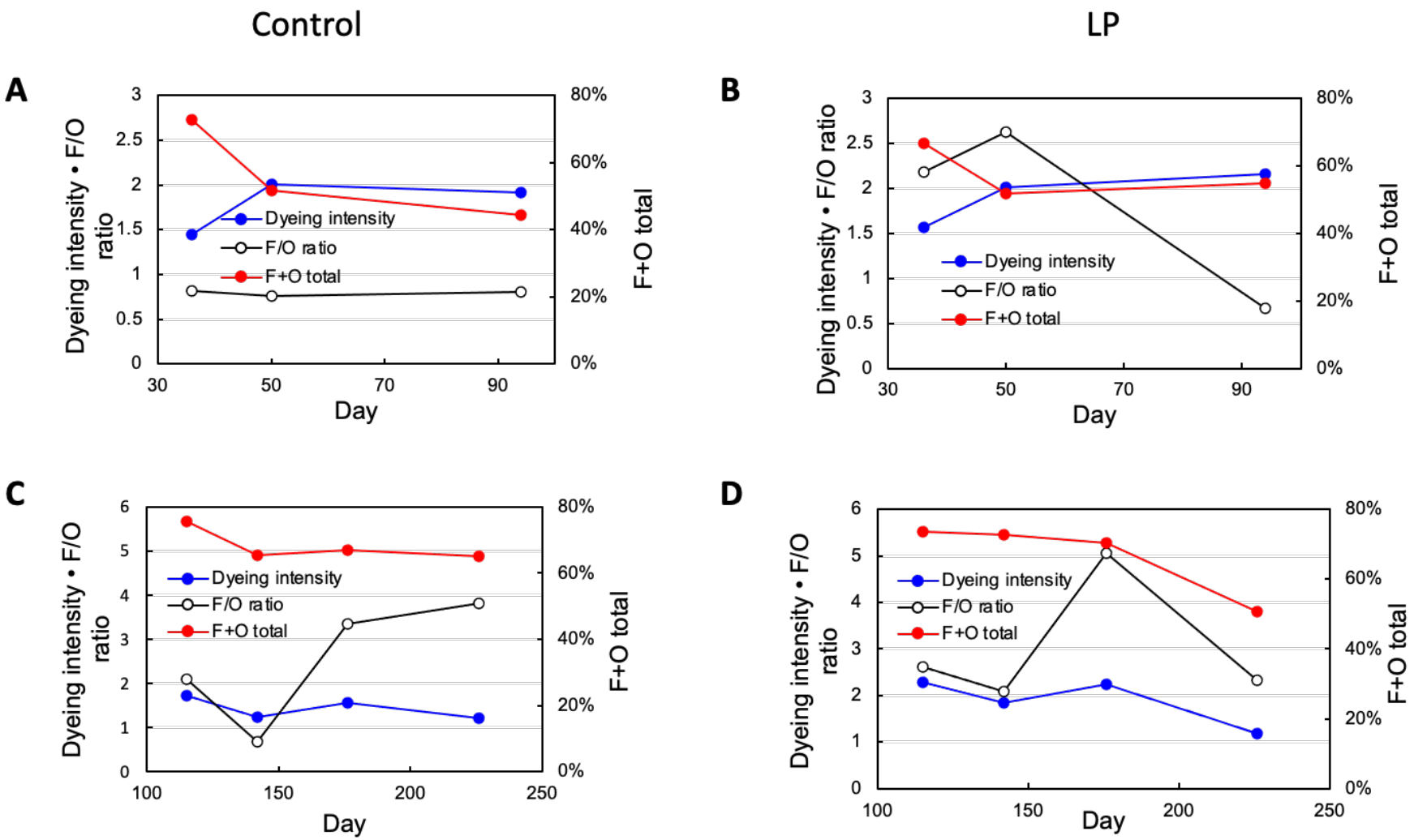

*Figure 21 Changes in dyeing intensity, abundance ratios of facultative anaerobes and absolute anaerobes (F/O), and total abundance ratios of facultative anaerobes and absolute anaerobes (F + O) in sukumo determined for Experiment 2 (AB) and Experiment 3 (CD) indigo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder. Dyeing intensity was exhibited as L*a*b* value × 10.*

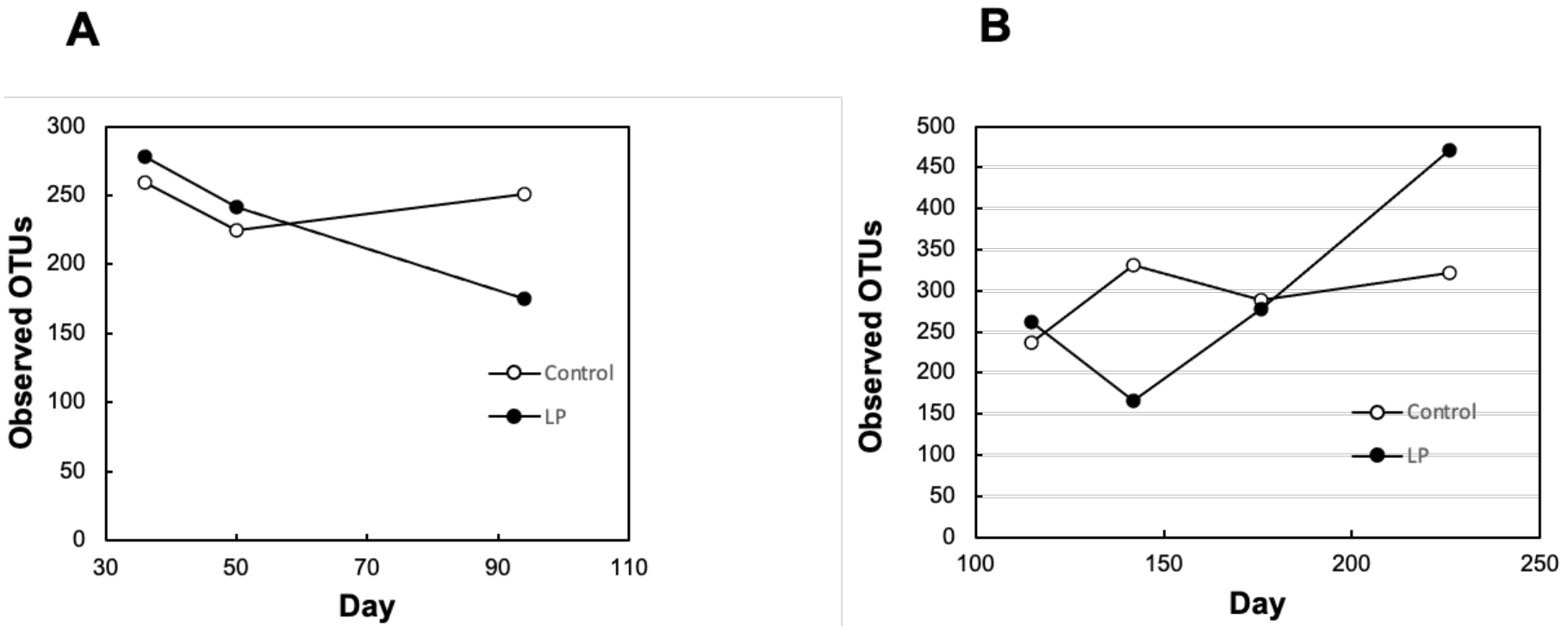

Figure 22 Changes in observed OTUs at 18512 sample depths varying with sukumo fermentation period in Experiment 2 (A) and Experiment 3 (B) indigo fermentation batches with (LP) and without (control) Indigofera tinctoria leaf powder. Analysis was performed using Divisive Amplicon Algorithm (DADA2) and depended on the fermentation period. The standard error is less than 0.4% for each value.

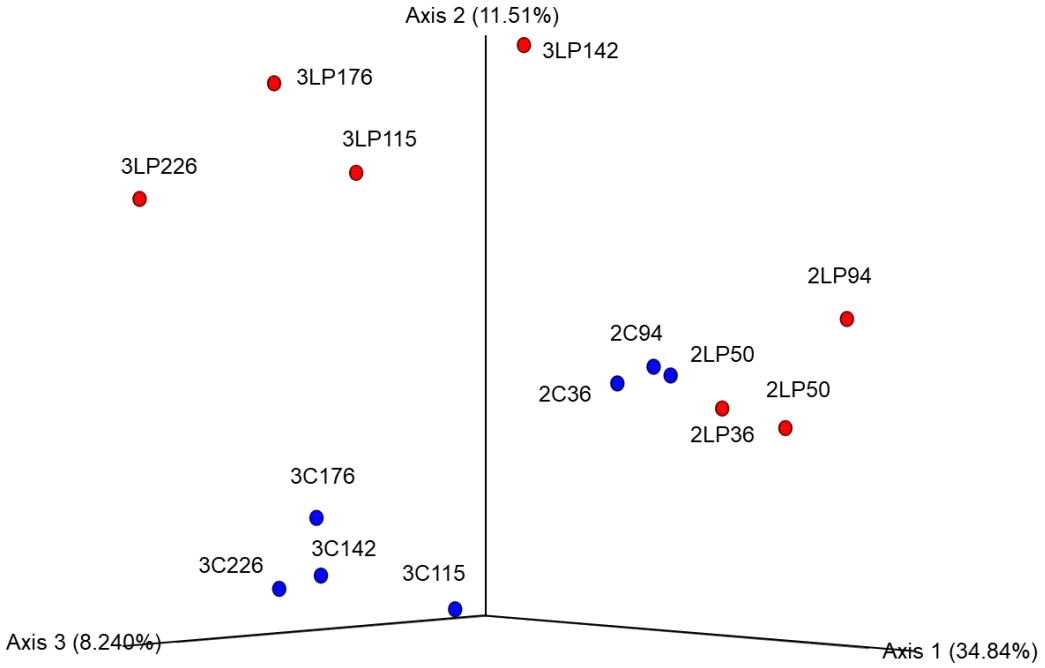

Figure 23 Principal coordinate analysis (PCoA) using Jaccard distance on Experiment 2 and Experiment 3. Red and blue circles indicate samples with (LP) and without (control) Indigofera tinctoria leaf powder, respectively.

General discussion

There are many traditional processes to extract and reduce natural indigo, therefore the present study aims to understand the effect in each preparation and try to improve a traditional method using *Indigofera tinctoria* leaf powder.

The bacterial community of fermentation fluids made with *sukumo* have been the focus of previous studies, while fermentations using extracted indigo remained unknown. Understanding the many possibilities of how a stable bacterial community can present itself and how each preparation can affect it is an important step in the creation and improvement of dyeing methodologies based on fermentation. For this reason, the bacterial community of fluids made with different indigo sources and *sukumo* fluids from different origins were analyzed and compared (Chapter 1). The results showed that the bacterial community was deeply influenced by the materials used. The community of fluids using extracted indigo were influenced by the microbial seed added, while *sukumo* fermentation have a more diverse community influenced by the aging of the fermentation and the complex nature of *sukumo* (seed microorganisms, nutrients, and debris). The bacterial community of extracted indigo achieved a reducing state despite having a different community than *sukumo*, therefore different communities can still sustain indigo reduction.

Sukumo fluids are dependent on the intrinsic bacterial seed present in them. The time from the fermentation preparation to the start of indigo reduction varies depending on the nature of the *sukumo* used and the method used on the preparation of the fermentation. To address this problem, the effect of the addition of *Indigofera tinctoria* leaf powder (LP) in different *sukumo* fermentations was evaluated (Chapter 2). Possibly because of the rich diversity present in the *sukumo* bacterial seed, the addition of *I. tinctoria* LP in the beginning of *sukumo* preparations don't directly influence the identity of the bacteria present in the community. The results suggest that the effect of *I. tinctoria* LP is due to the acceleration of the necessary transitional changes in the bacterial community by the rapid decrease of ORP.

Sukumo is the product of the composting of *P. tinctoria* and recently has been target of several studies, with many of its fermentation characteristics being described. The preparation procedures play a very important role in the characteristics of the indigo fermentation. One important point of *sukumo* fermentations is its characteristic bacterial diversity. This high diversity and constant changing of the microbiota during the fermentation period has been used

to explain the stability of the fermentation. In face of changes in the environment, nutrients availability, and possible contaminations, the diverse bacterial community would shift to a more favorable profile [9,12,75,76]. The results of the analysis of preparations using extracted indigo (which is dependent of an added seed) showed that this diversity is not necessarily mandatory for a stable fermentation. The stability and similarity of the Ryukyu-ai samples, even with a disparity of age, is a good example of how a successful seed and environment can stabilize the fermentation. Both characteristics (high and low diversity) are important, and the applicability of each one will depend on the source material and fermentation method. While a more diverse and dynamic community can be use in the recuperation of dye capacity of dye effluents or in fermentation with complex additives as nutrients, a simple seed fermentation is less complex for the industrial setting.

Regarding the bacterial profile, *Proteinovorax*, *Tissierella* and *Elysipelotrix* were present in almost all samples independent of the preparation procedure and material. The function of these genus is still unclear, especially because of the difficulties of isolating them from the fermentation. Nevertheless, apart from *Tissierela*, which is under investigation (unpublished results), both the other genera have not yet been associated to the indigo reduction. This fact doesn't exclude them of having an important role in the fermentation. *Proteinovorax* for example, has species that can degrade death cell making this bacterium able to explore this nutrient source in the fluid [56,57]. In *sukumo* samples, the *sukumo* used was a strong determinant of the community profile, and age also played an important role on the prevalence of certain characteristics. Ryukyu ai fermentations were characterized by the presence of the genus *Halomonas*, having a variable intensity among the samples. *Halomonas* species are able to use organic acids which can help maintain the alkaline pH [58]. Preparation procedure was also a big factor in the fermentations such as the inclusion of additives. The additives in RWTE sample were probably the responsible for the variability in this sample. In regard of Indian indigo samples, it presented many identities in their community which were not so present in the other samples showing that there are more species in fermentation to be explored.

As discussed previously a stable seed can be a key factor for a stable fermentation. The complexity present in *sukumo* which is seen as an advantage for the fermentation (different nutrients and innate bacterial seed) can also work against it. The impact of different types of *sukumo* is clear in the results (chapter 1). Since the quality of the *sukumo* after the composting process can vary, the initiation of the fermentation can also vary from days to weeks [35].

As also observed previously additives can impact fermentation in the extracted indigo fermentations (chapter 1) and it was also reported in previous studies additive effects in the

indigo reduction[38,39]. The leaf powder of *I. tinctoria* was chosen because it is easily available and safe [29]. Beside it has already been use as additive to make *sukumo* fermentations by a few craftsperson. The hypothesis was that the community present in the dried leaves would compensate the lack of essential bacteria in a low quality *sukumo*. Therefore, samples of 3 different preparations in different stages were analyzed, with each preparation possessing a control and a LP added fluid (chapter 2).

Dyeing results showed that LP had a bigger impact in the beginning of the fermentation rather than in the long run. Taking this information into account, one of the major differences between control and LP is the steep decrease of ORP in the beginning of the fermentation. This early decrease in ORP may have collaborated to the earlier arrival of some groups and their higher abundance. Other impact related to the addition of LP is the difference of diversity in the beginning of the fermentation. The fluid with LP, from day one had lower diversity than control. The diversity decrease in the beginning of the fermentation is a common feature in *sukumo* preparations [12,76], but in LP it was already lower than the control since the start. After some time, the values of diversity between control and LP added fluids became similar and had similar changing pattern. An important point to consider in the beginning of the fermentation is the changes of the environment characteristics. Aerobic bacteria have been considered the sole responsible for the dissolved oxygen depletion in *sukumo* fermentations. Based on the results it is clear that *I. tinctoria* LP possess many components that can also affect the dissolved oxygen, therefore reducing the ORP. One explanation is the presence of indigo precursors in *I. tinctoria* LP that react with the dissolve oxygen to form indigo [29] and, on top of that, *I. tinctoria* LP also possesses antioxidants and oxygen scavengers [27,28,51].

Previously was mentioned that the groups *Proteinovorax*, *Tissierella* and *Elysipelotrix*, were common to most samples regardless of the preparation (chapter 1). These groups were also observed independent of the addition or not of *I. tinctoria* LP. The major difference seen was regarding the time of appearance and/or abundance of these groups. *Tissierella* and *Elysipelotrix* appeared earlier and were more abundant in LP fluids compared to control. *Proteinovorax* appeared at the same time in both fermentations but was more abundant in control. When observing the community present in the *I. tinctoria* LP (raw material), there is no explicit correlation that can explain these small changes in the bacterial profile.

Indigo still one of the most used dyes. With the advent of synthetic indigo and the development of reduction vat using sodium dithionite, traditional methodologies based in natural extraction and reduction almost disappeared. Despite having amazing advantages like the cheap and reduction speed, these methodologies are very polluting being accountable for

80% of wastewater generation [15]. Recently there has been an increase interested in alternative methodologies and a revival of traditional methods. Because of the limitations and complexity of these methods, a lot of investigation is still necessary to develop practices that can substitute the current methodology used in the textile industry.

Fermentation methods have some advantages over other methodologies. Firstly, the indigo reduction inside the fermentation can be done continuously since the indigo oxidized can be reduced again by the bacteria present. The fermentation occurs in an open system despite its community be constituted mainly of anaerobic or facultative anaerobic individuals. It also can occur in "room temperature" which decrease the energy costs.

This study showed some new perspective regarding the assumptions of previous studies. Since the stability of *sukumo* fermentations rely on the syncretism of the present species in a constant interchanging of dominant species it was believed that a diverse community was necessary to keep a stable community. The low diversity present in the extracted indigo fermentation fluids shows that a simpler community can also be stable. *Proteinivoraceae* abundance, which was previously related to high dyeing intensity, did not showed the same behavior. This and the fact that there were samples where the known indigo reducing bacteria had low abundance or were not even present showed that a deeper understand of the community identity needs to be addressed. The fluid environment plays an important role. The early decrease of the ORP is important to quickly select the appropriate community.

These findings are important pieces that can help the improvement and creation of dyeing methodologies. They show the importance of the seed culture and additives to the fermentation and that the reducing communities can have a low diversity and still be stable. The manipulation of the environment in the beginning of the fermentation with regard of the depletion of oxygen and ORP reduction helps achieving an efficient bacterial community for indigo reduction in *sukumo* fluids. In addition, there is a small group of individuals in the community that are practically universal to the indigo fermentation.

Conclusion and perspectives

The reduction of indigo using fermentation procedure is very complex. The bacterial community is deeply influenced by the material used to make the fermentation. While the bacterial community of preparations with extracted indigo will be influenced by the microbial seed added, fermentations using *sukumo* will have more diverse community being influenced by the complex nature of the *sukumo* used (natural occurring microorganisms, nutrients, and debris) and its aging. The use *I. tinctoria* LP as an additive in *sukumo* fermentations showed improvement on the dyeing initiation. Despite not influencing the identities of the community present, possible because of the complexity of *sukumo*, it changed the environment of the fermentation (rapid ORP decrease). This was sufficient to induce the necessary transitional changes for the initiation of indigo reduction. These findings can contribute for the creation of new fermentation procedures, stable artificial communities for reduction, and manipulation of the environmental characteristics to accomplish a more efficient fermentation

Summary

Indigo is the most common dye used by mankind. Because of its worldwide availability and presence in many cultures, there are many methods for preservation and extraction of natural indigo. They can be basically divided into 2 distinct categories: microbial composting and direct extraction. The process of extraction of indigo is not only necessary for its concentration and preservation, but also to the transformation of the indigo precursor in the plants into indigo.

In the composting method, the leaves of the indigo bearing plants undergo a long fermentation period ranging from weeks to months. This methodology is exemplified by the production of woad (*Isatis tinctoria*) in Europe and *sukumo* (*Polygonum tinctorium*) in Japan. In the case of direct extraction, leaves are fermented briefly (up to hours) to release the precursors, and then aerated in a beating vat to react with oxygen. The newly formed indigo is decanted, boiled, filtered, and pressed to form an indigo cake, which is the case of Indian indigo. In the method used in Okinawa, Japan, the leaves are fermented for days and the indigo is decanted by the reaction with $Ca(OH)_2$ to obtain an indigo paste.

To be used as a dye, Indigo, that is insoluble in water, needs to be transformed into leuco-indigo, which is its reduced form. There are many ways to accomplish that. In the microbial method, the indigo source (woad, sukumo, Indian indigo and others) is fermented in an alkaline environment and specific bacteria from the community reduce indigo into its leuco form. Indigo sources originated from composting, such as woad and *sukumo*, already have the necessary microorganisms to start the fermentation, whereas Indian indigo and other extracted indigo may need the addition of a seed culture.

While fermentation fluids made with *sukumo* have already been characterized, the bacterial community of different preparations using different indigo sources lack definition. Therefore, one of the focus of this study is the comparison between *sukumo* fluids from different origins and fluids made from other sources of indigo. Furthermore, the composting process that produce *sukumo* is not always stable and the quality of the final product can fluctuate. This characteristic also affects the onset of indigo reduction which sometimes can take more than 2 weeks to start. To address this problem, we also evaluated the effect of the addition of *Indigofera tinctoria* leaf powder (LP) in different *sukumo* fermentations.

Analysis of bacterial flora of indigo fermentation fluids utilizing composted indigo leaves (*sukumo*) and indigo extracted from plants (Ryukyu-ai and Indian indigo)

To clarify the factors behind the bacterial diversity and the sustainability of indigo fermentations, the bacterial community of preparations using different sources of indigo, composted (*sukumo*) and extracted (Ryukyu-ai paste and Indian indigo cake), were studied. Next Generation Sequencing (NGS) technology was used to access the bacterial community profile. The common feature of all preparations was the strong presence of obligate anaerobic bacteria, the bacterial profile, *Proteinovorax*, *Tissierella* and *Elysipelotrix* were present in almost all samples independent of the preparation procedure and material. Communities from *sukumo* fermentations were more diverse when compared to preparations using extracted indigo. This characteristic is related to the presence of seed microorganisms and various nutrients in *sukumo*. In addition, the debris in *sukumo* fermentations allows the formation of low pH niches which promotes the diversity by reducing pH stress. On the other hand, fermentation using extracted indigo presented a simpler community and was highly influenced by the seed culture added. These results show that the diversity is not necessarily mandatory for a stable fermentation. Nutrients and the strong microbial network inside these fermentations were responsible for their stability. Ryukyu-ai fermentation, for instance, had a very stable community regardless of the fermentation age.

Indigofera tinctoria leaf powder as a promising additive to improve indigo fermentation prepared with sukumo (composted *Polygonum tinctorium* leaves)

The onset of the indigo reduction in *sukumo* fermentations can vary greatly (from 3-4 days to weeks). Since certain additives can improve indigo fermentation, the effects of *Indigofera tinctoria* LP on the reduction initiation and bacterial community were studied. To observe the effects on the reduction, pieces of cloth were dyed using the fermentation fluid. The intensity of the dye was then compared between control and LP treatment. The redox potential (ORP) was also measured and the bacterial community in the initial phase and in older fluids were analyzed using NGS. The addition of LP improved the speed of the dyeing in most experiments, and a markedly decrease of ORP was observed since day one. *Proteinovorax*, *Tissierella* and *Elysipelotrix*, were common to most samples regardless of the preparation. *Tissierella* and *Elysipelotrix* appeared even earlier and were more abundant in LP fluids compared to control. The observed effect on ORP was likely a result of the phytochemicals present in *I. tinctoria* LP acting as oxygen scavengers. Regarding the effects in the microbial community, there were no significant changes in the identity directly related to the bacteria present in LP. The decrease

in *Bacillaceae* and increase in *Proteinivoraceae* at the onset of fermentation and the ratio of facultative to obligate anaerobes are vital to the initiation and maintenance of the indigo reduction. Both characteristics appeared earlier in fluids treated with LP.

Conclusions

The bacterial community is deeply influenced by the materials used to make the fermentation. While the community of fluids originated from extracted indigo will be influenced by the microbial seed added, *sukumo* fermentation will be influenced by the aging of the fermentation and the complex nature of *sukumo* (seed microorganisms, nutrients, and debris) presenting a more diverse community. Possibly because of the complex microbial seed already present in *sukumo*, the addition of *I. tinctoria* LP in the preparation phase is not sufficient to change the identity of the bacteria present in community. Nonetheless, it can change the environment of the fermentation, by the rapid decrease of ORP, and accelerated the necessary transitional changes for indigo reduction. These findings can contribute to the development of new fermentation procedures, the creation of stable artificial communities for reduction, and the manipulation of environmental characteristics to accomplish a more efficient fermentation.

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