THE SUSTAINABILITY CHALLENGE: NEW PERSPECTIVES ON THE USE OF MICROBIAL APPROACHES AND THEIR IMPACT ON FOOD AND FEED

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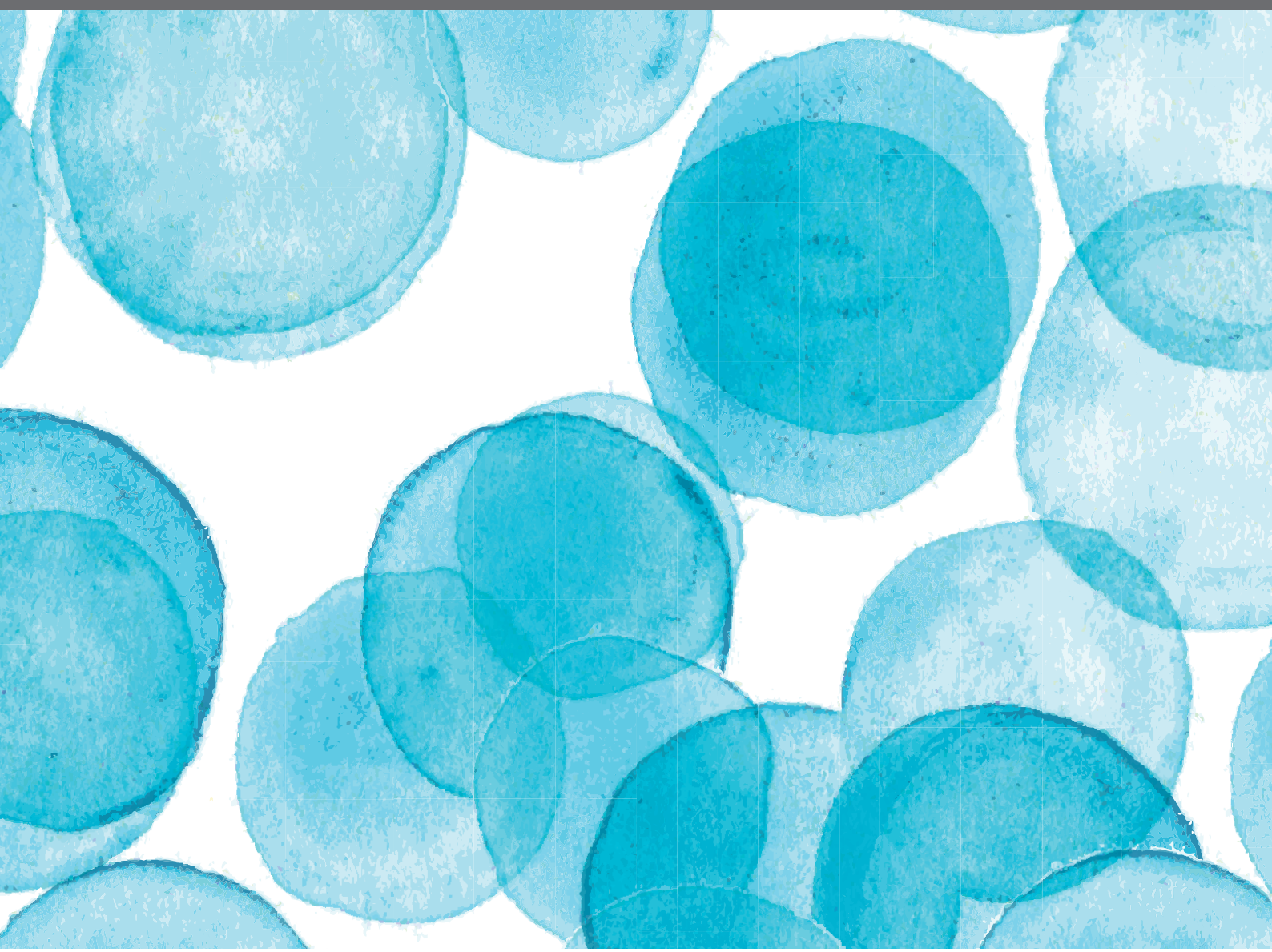

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THE SUSTAINABILITY CHALLENGE: NEW PERSPECTIVES ON THE USE OF MICROBIAL APPROACHES AND THEIR IMPACT ON FOOD AND FEED

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EDITORIAL
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Editorial: The Sustainability Challenge: New Perspectives on the Use of Microbial Approaches and Their Impact on Food and Feed

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Editorial on the Research Topic

The Sustainability Challenge: New Perspectives on the Use of Microbial Approaches and Their Impact on Food and Feed

Building a more resilient food chain, reducing food loss and waste, improving food production practices and increasing plant-based food consumption are some of the fundamental actions suggested in The Sustainable Development Goals adopted by the United Nations Member States in 2015[1]. The objective of this special issue was to explore how the use of microorganisms as direct or indirect sources of transformation could contribute to these sustainability practices. In this context, the following strategies have been presented: (i) valorization of side-streams and underutilized food resources via fermentation, (ii) improvement of the efficiency of bioprocesses for the food and feed industry, and (iii) understanding and applying the microbiome as a resource to improve the agro-food system.

Fermentation was used to upgrade the nutritional and functional value of cereal industry by-products and to promote less utilized but very valuable grains, such as pseudocereals. With a production above 2 billion tons per year, cereals provide a major food resource worldwide but also generate a great amount and variety of side-streams. Verni et al. provide an overview on lactic acid bacteria, yeasts, and fungi fermentation as biotechnological tools for processing cereal side-streams to improve their nutritional properties. Among the positive effects of microbial fermentation, beyond the advantage of reducing the disposal of and reutilizing material still fit for human consumption, an increase in potential health benefits has been observed *in vitro*. More *in vivo* studies are suggested to validate these findings. An example of nutritional benefit was offered in the study of Xie et al. in which wheat bran was fermented by the association of *Propionibacterium freudenreichii* and *Lactobacillus brevis,* obtaining significant amounts of vitamin B12 while maintaining the safety of the food substrate. These results encourage further exploration of microbial biosynthesis of bioactive compounds to fortify food ingredients of plant origin.

Fermentation of maize bran and germ with selected lactic acid bacteria starters previously isolated from different grains enhanced the quality of the raw material by decreasing the phytate content and the lipase activity. At the same time, free amino acids, peptide concentration and the antioxidant activity of fermented maize bran and germ were enhanced. The use of the pre-fermented by-products as ingredients for breadmaking extended the positive attributes to the

[1]https://eatforum.org/eat-lancet-commission/

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bread (Pontonio et al.). Aside from common cereal grains, the increase of the nutritional and functional qualities of pseudocereals via lactic acid bacteria fermentation was reviewed by Rollán et al.. The importance of these crops lies in their resilience and adaptability to harsh climatic conditions, providing an important resource for crop diversification and sustainable agriculture. Among the advantages of fermentation, the decrease of antinutritional factors like phytic acid and the increase in phenolic compounds and B vitamins have been observed.

The reduction of food losses requires an integrated approach to be implemented at different steps of the food chain. Sarwar et al. studied the antagonistic ability of *Streptomyces violaceousniger* against the pathogens responsible for the potato common scab. The selected strain was effective in controlling the disease thanks to the biosynthesis of the antimicrobial compound azalomycin, while simultaneously enhancing the crop yield.

Algae and microalgae are emerging as valuable biomass for different applications, including food and feed. To increase their sustainable use, algal biorefinery processes and purification of bioactive compounds should become more efficient, reducing the environmental impact and side-stream formation. In the study of Chen et al., the extraction of oligosaccharides from the alga *Gracilaria lemaneiformis* was implemented by utilizing the agarolytic activity of *Flammeovirga* sp., a bacterial species inhabiting deep sea water. The resulting environmentally friendly fermentation process allowed the retention of to retain the algal beneficial compounds, reducing by-product formation. Patnaik et al. proposed a novel biorefinery approach to valorize the whole biomass of the microalga *Scenedesmus obliquus,* achieving the conversion of 70% of the microalgal biomass into an industrially relevant product. Furthermore, the positive impact of microalgal protein as feed supplement for fresh-water fish was assessed.

In the feed industry, finding new solutions to enhance the nutrient uptake from plant-based material could contribute to achieving more sustainable, completely plant-based feed, valorizing side-streams, and local resources. Dittoe et al. focused on the search for pectinases to degrade the plant cell wall. Several pectin-related enzymes were found in the genome of *Dickeya dadantii* together with a significant number of pectin degradation-related pathways. This strain could be used as a commercially viable enzyme producer.

Research on microbiome and its applications in the agricultural system has intensified in recent years due to the continuous advancement of genomic information. In the study of Dong et al., the effects of freeze–thaw events on microbial community dynamics and the fermentation quality of red clover silage were investigated. The freeze–thaw events increased the abundance of specific microbial groups at different stages of ensiling, suggesting how to control the quality of silages when forages are subjected to freezing damages. In the study of Wang et al., the capacity of a consortium of different bacterial genera isolated from wheat field in detoxifying deoxynivalenol was defined. The findings highlighted the potential of microbial biotransformation in improving feed and food quality and safety, contributing to a more resilient food chain. Finally, the microbiome along the soil-plant-animal continuum within the pastoral production system was reviewed by Attwood et al.. Understanding the microbial diversity within these environments can be used to improve agricultural processes, helping us to face the upcoming climate change and food security challenges.

The above studies represent an overview on the role of microorganisms as means to design novel industrial applications encompassing the whole food production chain. Microbial biotechnologies focusing on the reduction of food waste and loss and the improvement of process efficiency have the potential to deliver important innovations in the agro-food industry. In the future, we envision that better understanding of the microbiome will lead to a more holistic interpretation of ecosystem functionality, creating a more sustainable agricultural system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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A Potential Biocontrol Agent *Streptomyces violaceusniger* AC12AB for Managing Potato Common Scab

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Potato common scab (PCS) is an economically important disease worldwide. In this study we demonstrated the possible role of *Streptomyces violaceusniger* AC12AB in controlling PCS. Isolates of *Streptomyces scabies* were obtained from CS infected tubers collected from Maine United States, which were confirmed by morphological and molecular analysis including 16S rRNA sequencing and RFLP analysis of amplified 16S-23S ITS. Pathogenicity assays related genes including *txtAB*, *nec1*, and *tomA* were also identified in all *S. scabies* strains through PCR reaction. An antagonistic bacterial strain was isolated from soil in Punjab and identified as *S. violaceusniger* AC12AB based on 16S rRNA sequencing analysis. Methanolic extract of *S. violaceusniger* AC12AB contained azalomycin RS-22A which was confirmed by ^{1}H and ^{13}C-NMR, ^{1}H/^{1}H-COSY, HMBC and HMQC techniques. *S. violaceusniger* AC12AB exhibited plant growth promotion attributes including Indole-3-acetic acid production with 17 μgmL^{-1} titers, siderophores production, nitrogen fixation and phosphates solubilization potential. When tubers were inoculated with *S. violaceusniger* AC12AB, significant ($P < 0.05$) PCS disease reduction up to 90% was observed in greenhouse and field trials, respectively. Likewise, *S. violaceusniger* AC12AB significantly ($P < 0.05$) increased potato crop up to 26.8% in field trial. Therefore, plant growth promoting *S. violaceusniger* AC12AB could provide a dual benefit by decreasing PCS disease severity and increasing potato yield as an effective and inexpensive alternative strategy to manage this disease.

Keywords: *Streptomyces scabies*, biological control, plant growth promoting *Streptomyces*, antagonistic *Streptomyces*, potato common scab

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INTRODUCTION

Potato common scab (PCS) is considered among top five diseases by potato farmers in United States (Slack, 1991). PCS is caused by Gram positive, filamentous bacteria in the genus *Streptomyces*. *Streptomyces* are soilborne saprophytic bacteria, mostly famous for the production of antibiotics (Kemung et al., 2018). However, only few of them are plant pathogens. Although, several species

of *Streptomyces* can cause common scab (CS), *Streptomyces scabies* was considered as predominant plant pathogen (Lambert and Loria, 1989). PCS infection is characterized by superficial, raised or pitted scab lesions on the surface of the tubers. The occurrence of PCS infection is, generally not hazardous to human health. However, they may deteriorate the quality of tubers and effects the market value. For instance, potato industry in Canada reported to experience $1.2 million loss every year due to this disease (Al-Mughrabi et al., 2016).

Scab lesions on the surface of the tubers develop due to a phytotoxin called thaxtomin. In 1898, thaxtomin was firstly described (King et al., 1989; Lawrence et al., 1990) as toxin responsible to produce CS on immature tubers. All PCS causing *Streptomyces* spp. produce thaxtomin A or another member of thaxtomin family (Loria et al., 2008). Biosynthesis of this phytotoxin encompasses non-ribosomal peptide synthetases encoded by *txtA* and *txtB* genes (Loria et al., 2008). The genes responsible for pathogenicity like *txtAB*, *nec1* and *tomA* are clustered together and termed as pathogenicity island (PAI) (Kers et al., 2005). PAI consists of thaxtomin genes including *txtAB* (Healy et al., 2000), *txtH*, *txtC* (Healy et al., 2002), *txtR*, *txtE* (Joshi et al., 2007), *nos/txtD* (Kers et al., 2004), and genes for pathogenicity factors like Tomatinase (*tomA*) and Necrotic protein (*nec1*) (Kers et al., 2005; Barry et al., 2012). Secreted Nec1 protein helps to enhance the virulence by weakening the plant defense mechanism (Bukhalid et al., 1998). *TomA* gene encodes for a virulent protein having high similarity with phytopathogenic fungi tomatinase (Kers et al., 2005).

Although thaxtomin A, which is encoded by *txtAB* gene, is considered as a major player toward plant pathogenicity, other genes including *nec1* and *tomA* are also somehow required for the virulence (Loria et al., 2006). Many unknown factors can play a supportive role in pathogenicity. However, the prevalence of CS pathogens without *txtAB* genes are either very rare or confined to geographic locations (Park et al., 2003; Wanner, 2004).

Over the decades, PCS management remained a serious problem among potato growers. The control strategies are challenging due to limited understanding of genetic diversity of *S. scabies* and genetic differences in various potato cultivars (Dees and Wanner, 2012). Several physiochemical approaches like reducing soil pH, crop rotation, and soil fumigation agents like chloropicrin (trichloronitromethane) have conventionally been used with harmful effects to the environment (Larkin et al., 2011; Xue et al., 2018). In contrast, research in biological control as an alternative strategy is emerging. Several antagonistic bacteria including *Bacillus* spp. (Meng et al., 2013), *Pseudomonas* spp. (Arseneault et al., 2015) and *Streptomyces* spp. (Sarwar et al., 2018) have been used as biocontrol agent against PCS.

The present study was designed to evaluate the pathogens responsible for PCS incidences and to assess the antagonistic ability of *S. violaceusniger*. It was hypothesized that *S. violaceusniger* AC12AB could be used as effective biological control agent due to its ability to promote plant growth and suppress PCS.

MATERIALS AND METHODS

Sample Collection, Bacterial Isolation, and Identification

Potatoes having visible CS symptoms were collected from Presque Isle, ME, United States. All collected samples were carefully transferred to the laboratory at the University of Maine, United States. Samples were stored at 4°C prior to use.

Tubers with CS symptoms were washed and surface sterilized with 5% sodium hypochlorite (NaOCl) for 1 min. Surface sterilized tubers were rinsed with sterile distilled water and air dried. The infected portion from CS tubers were carefully excised with sterile scalpel and triturated to form a homogenized paste by adding 1 mL Tris-HCl. The homogenized suspension was poured into 2 mL Eppendorf tubes, separately. The Eppendorf tubers were placed at 55°C for 2 h to remove unwanted microorganisms. This suspension was ten-fold diluted with sterile distilled water before pouring onto yeast malt extract (YME) agar plates (Shirling and Gottlieb, 1966). An aliquot of 100 μL from diluted suspension was used to spread on YME agar plates and placed in an incubator for 5–7 days at 28°C. After incubation, YME agar plates were checked for the white cottony *Streptomyces* like colonies.

Antagonistic bacterial isolates were collected from agriculture field located at Lahore, Pakistan having no visible CS symptoms over the period of past 5 years. Suppressive soil samples were used to isolate Actinomycetes by serial dilution method (Wang et al., 2015). Colonies were further purified on YME agar plates (Shirling and Gottlieb, 1966). Microorganisms particularly antibiotic producing actinomycetes were targeted as promising candidate as PCS antagonistic bacteria (Kharel et al., 2010).

DNA Extraction and PCR Amplification

Selected bacterial spores were inoculated into YME broth and incubated for 3 days in shaking incubator with 180 rpm at 28°C temperature. After incubation, supernatant was separated from cell pellet by centrifugation. The cell pellet was used for genomic DNA extraction by using the FastDNA® kit (MP Biomedicals, Santa Ana, CA, United States). PCR amplification of DNA samples were performed with 16S rRNA primers (Edwards et al., 1989). PCR reaction was performed in PCR tubes with 25 μL reaction volume which included 1 μL (50ng) DNA (A_{260}/A_{280} ratio was 1.9) template, 5 μL 5X PCR buffer, 0.50 μL 10 mM dNTPs, 0.50 μL 10 μM forward and reverse primers each, 0.10 5 u/μL Taq polymerase and 17.90 μL H_2O. PCR reaction was programmed as, initial denaturation for 5 min at 95°C followed by 30 cycles of denaturation for 30 s at 95°C, annealing for 40 s at 60°C, extension for 40 s at 72°C and final extension was performed for 5 min at 72°C.

Identification of PCS Pathogens

Streptomyces species specific primers were used for the identification of PCS causing pathogens. Species specific primers for *Streptomyces* pathogens including *S. scabies* (Lehtonen et al., 2004), *S. europaeiscabiei*, *S. bottropensis*, *S. stelliscabiei* (Wanner, 2006), *S. acidiscabiei,* and *S. turgidiscabiei*

(Tagawa et al., 2008) were used for the identification by PCR amplification. Amplification of 16S-23S internal transcribed spacer (ITS) sequence was performed with ITS forward and reverse primers (Song et al., 2004). PCR amplified product was digested with *Hpy99I* restriction enzyme which expurgated the amplicon at 1629–1633 nucleotide position.

DNA fragments were visualized under gel electrophoresis. PCR amplified product was sent to DNA sequencing facility, University of Maine for sequencing. 16S rRNA sequences were submitted to NCBI to obtain accession numbers.

Polymerase chain reaction was used to amplify PAI related genes including *txtAB*, *nec1*, and *tomA* (Bukhalid et al., 1998; Wanner, 2006). PCR reaction conditions were same as above except the annealing temperature was adjusted at 60°C, 55°C and 48°C for *nec1*, *tomA*, and *txtAB* genes, respectively.

Disk Diffusion Assay

Antagonistic *Streptomyces* spp. were checked against PCS pathogens by disk diffusion assay (Clinical and Laboratory Standards Institute, 2015). Pure cultures of twelve antagonistic *Streptomyces* spp. were prepared by inoculating few spores into 100 mL YME broth and incubated at 28°C in shaking incubator at 180 rpm for 5–7 days. The broth culture was centrifuged at 9,000 × *g* and supernatant were used for preparation of methanol extract. Meanwhile, YME broth cultures of PCS pathogenic *Streptomyces* strains were spread on YME agar plates with Rattler^{TM} plating beads (Zymo Research Cooperation, United States), separately. 25 μL methanolic extract of antagonistic *Streptomyces* were poured on filter paper disks and placed on YME- agar plates previously spread with pathogenic PCS suspension. The plates were incubated for 48–72 h at 28°C. After incubation, clear zone around filter paper disks were checked and results were recorded in mm.

Plant Growth Promotion

Twelve antagonistic *Streptomyces* spp. were evaluated for plant growth attributes including indole-3-acetic acid (IAA) production, phosphate solubilization, siderophores production and *in vitro* nitrogen fixation. IAA production was estimated by colorimetric method (Gordon and Weber, 1951; Amin and Latif, 2017) and confirmed by HPLC-DAD-MS as mentioned by Sarwar et al. (2018). The IAA production titer from antagonistic *Streptomyces* spp. was performed by observing optical density ($O.D_{530nm}$) against standard curve of IAA and recorded in μg mL^{-1} (Bric et al., 1991). Phosphate solubilization was assessed by the method previously described by Sylvester-Bradley et al. (1982). Glucose yeast medium (GY) along with two solutions; one containing 10% 50 mL K_2HPO_4 and second solution containing 10% 100 mL $CaCl_2$ were prepared and added in 1 L GY medium (Ambrosini et al., 2012). The medium was autoclaved and poured into petri plates after cooling. The addition of two solutions made an opaque insoluble layer of $CaCl_2$. The plates were inoculated with antagonistic *Streptomyces* isolates, separately and incubated for 7 days at 28°C. After incubation, inhibition zone was observed and recorded.

Siderophores production was checked by inoculating bacterial spores on chrome azurol S (CAS) agar plates as mentioned by Schwyn and Neilands (1987). After the incubation of 5–7 days at 28°C, development of yellow to orange color was observed.

Nitrogen fixation potential of antagonistic *Streptomyces* spp. was examined by acetylene reduction assay (ARA) as described by Rice and Paul (1971). Nitrogen free mannitol (NFM) medium (Doty et al., 2009) slants were prepared in glass tubes and inoculated with antagonistic *Streptomyces* spp., separately. The tubes were sealed with a stopper and head space was filled with 2% oxygen. About 10% head space was exchanged with equal amount of acetylene. The tubes were placed in an incubator at 28°C for 2 weeks. Reduction of acetylene to ethylene was measured by gas chromatography (Agilent technologies 7890A GC system), which was equipped with flame ionizing detector and Agilent CP7348 column (25m × 0.25mm). As a positive control, two bacterial strains belonged *Bacillus amyloliquefaciens* (ZM2; accession number JX185642) and *Pseudomonas aerouginosa* (ZS24; accession number JQ990311) were used (kindly provided as positive control by Dr. ZL, University of the Punjab, Pakistan).

Extraction and Analysis of Bioactive Compounds by HPLC-DAD-MS

Antagonistic *Streptomyces* strains were inoculated in 150 mL YME broth in a 500 mL shaking flask. The flasks were incubated for 3 days at 28°C in an incubator shaker with 180 rpm. After incubation, the culture was centrifuged, pellet was discarded, and supernatant was undergone twice extraction with equal amount of ethyl-acetate. The extract was concentrated *in-vacuo* and re-suspended in methanol. For the HPLC-DAD-MS analysis, Agilent 1100 system was used equipped with a XBridge C-18 (3.5 mm, 100 mm × 4.6 mm) reverse phase column, a diode array detector and a quadrupole mass detector. An aliquot of 20 μL diluted crude extract was injected into the HPLC system and eluted isocratically with 95:5 methanol/water at a 0.5 mL min^{-1} flow rate.

Purification and Structural Elucidation of Azalomycin

Culture of *S. violaceusniger* strain AC12AB (100 mL) was used to inoculate in 10 L YME broth at 28°C for 5 days in an incubator shaker with 150 rpm. After incubation, the culture was sonicated for half an hour. The culture was then centrifuged at 11,200 × *g*, supernatant was used for extraction with equal amount of ethyl acetate. The ethyl acetate extract was concentrated *in-vacuo* and powdered extract was re-suspended in methanol. The methanolic extract was used for thin layer chromatography (TLC) and silica gel column chromatography with 5:1 dichloromethane and methanol buffer system. All the fractions were analyzed for their biological activity against *S. scabies* and most active fraction was further purified by Sephadex LH-20 column chromatography system with methanol as mobile phase. The fraction was analyzed by HPLC-DAD-MS system and further purification was performed by SPE Oasis® HLB20 35 cc cartridge (6 g). Fractions were eluted in SPE column with step gradient (20–100%) of methanol. Purified fraction was obtained after final purification with semi-preparative HPLC.

Final purification was achieved with help of semi-preparative HPLC system (Agilent 1100 Series). In HPLC system, as a stationary phase Zorbax B-C C18 (9.4 mm × 20 mm) main column and Zorbax B-C18 (9.4 mm × 150 mm) pre-column was used. The compound was eluted with buffer A (acetonitrile/acetic acid 0.5%) and buffer B (water/acetic acid 0.5%) with 2 mL min^{-1}. Methanolic extract purified from SPE Oasis® HLB20 35 cc cartridge was spiked on the column. 6mg purified azalomycin obtained from semi-preparative HPLC system, was dissolved in CD_3OD and analyzed for one dimensional NMR including ^1H (400 MHZ) and ^{13}C-NMR (100 MHZ) and 2-D NMR including HMQC, $^1H/^1H$-COSY and HMBC on a Bruker DRX-500 NMR spectroscopy (Bruker, Karlsruhe, Germany).

Plant Growth Promotion and Pathogenicity Assay on Potato Tubers

Greenhouse Assay

Pathogenicity assay on tubers was performed in greenhouse assay (Wanner, 2006). From eighteen isolates of pathogenic *Streptomyces* spp., two isolates namely *S. scabies* strain AJ-7 (Accession number MG725948.1) and AJ-10 were selected whereas *S. violaceusniger* strain AC12AB (Accession number MH388022.1) was used as antagonistic and plant growth promoter strain. As a positive control PCS pathogenic *S. scabies* strain AC-46 (Accession number KU560917.1) was also used. To observe normal growth pattern, tubers were also inoculated without any bacterial inoculation. The greenhouse assay was performed twice at greenhouse facility, University of Maine, Orono, United States during 2016-2017. Pathogenic *S. scabies* AJ-7, AJ-10, AC-46, and antagonistic *S. violaceusniger* AC12AB were cultivated separately in YME broth for 3–5 days at 28°C in incubator with shaking at 150 rpm, until they attained 10^6 CFU mL^{-1} conc. After incubation, cultures were separately centrifuged at 9,000 × *g*. Supernatant was discarded, and bacterial cell mass was re-suspended in sterile distilled water to prepare inocula with 10^6 CFU mL^{-1} conc. Pots were filled with compo Sana Universal® (Munster, Germany). Tubers were surface sterilized with 5% NaOCl for 5–10 min and washed with sterilized water. Washed tubers were sown into respective five pots as replicates. After 2–3 weeks of sowing, pots were inoculated by drenching with 100 mL bacterial suspension. The average temperature was maintained between 25 and 28°C. The plants were kept hydrated and continuously monitored for the increase in shoot, root length, tuber weight and decrease in PCS symptoms for 3 months. After harvesting, potato tubers were evaluated against growth and disease parameters. The results were recorded and pathogenic *Streptomyces* spp. was re-isolated from CS infected tubers to confirm the source of CS infection.

Field Trial

Field trial was conducted to determine disease suppression and plant growth promotion of antagonistic *S. violaceusniger* AC12AB. Field trial was conducted in a field available at University of the Punjab, Lahore Pakistan. Indigenous CS pathogen *S. scabies* AC-46 and antagonistic *S. violaceusniger* AC12AB were used. Inoculum of pathogenic and antagonistic strains were prepared as mentioned above. Disease free tubers (cv. Berna; Purchased from Punjab Seed Corporation, Pakistan) were disinfected with 5% NaOCl and washed with sterile water. Tubers were sown in a randomized complete block design in duplicates.

Each block (62 square feet) contained four rows; the length of each row was 1.5 m with 2.5 m distance between each block. Six potato seeds were implanted into each row. After 2–3 weeks of plantation, bacterial spore suspension (prepared as described above) was drenched 20–30 cm deep into the plant's roots. Plants were watered as required under natural light and temperature. Plants were monitored for growth in shoots and roots length. After harvesting, tubers were evaluated for decrease in CS symptoms, increase in tuber weight and yield $acre^{-1}$ were recorded.

Statistical Analysis

All the experiments were performed in triplicates and $P < 0.05$ was considered as statistically significant. The results were subject to one-way analysis of variance (ANOVA) and compared means were separated by Tukey's test. Statistical analysis was performed by using SPSS software (IBM SPSS Statistics, version 21).

RESULTS

Identification and Molecular Characterization of *Streptomyces* Causing PCS

Eighteen bacterial isolates were analyzed by PCR amplification of 16S rRNA gene, out of which, all isolates were confirmed

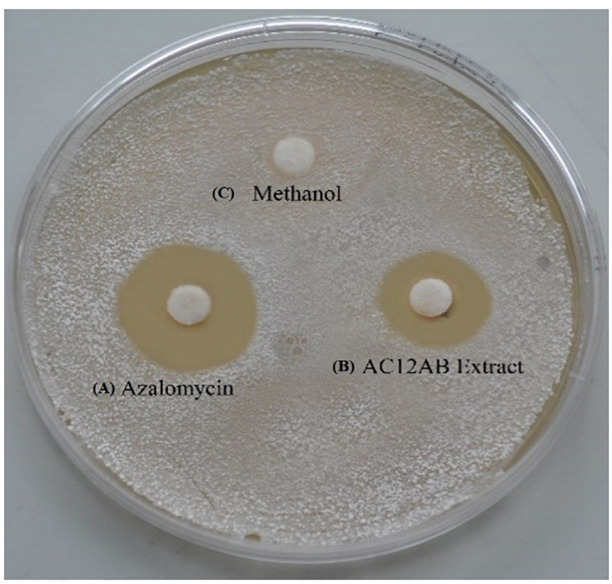

FIGURE 1 | Inhibition of *S. scabies* (AJ-7) by disk diffusion assay. *Streptomyces scabies* (AJ-7) was grown on YME agar plates with filter paper disks containing **(A)** purified azalomycin dissolved in methanol; **(B)** *Streptomyces violaceusniger* AC12AB crude extract; **(C)** methanol only.

as *Streptomyces*. *Streptomyces* isolates were further identified by PCR amplification with using species specific primers, PCR amplification of 16-23S ITS region with ITS primers and digesting the amplicon with *Hpy99I* enzyme. After RFLP pattern analysis, all eighteen *Streptomyces* isolates were found to belong *S. scabies* (Flores-González et al., 2008).

PCR was also performed to identify the pathogenicity-related genes in *Streptomyces* isolates, which were subject to PCR amplification of *txtAB*, *nec1* and *tomA* genes. All *Streptomyces* isolates were found to contain *txtAB*, *nec1*, and *tomA* genes. Antagonistic bacterial isolates were also screened for *txtAB*, *nec1* and *tomA* genes; but, did not test positive for those genes

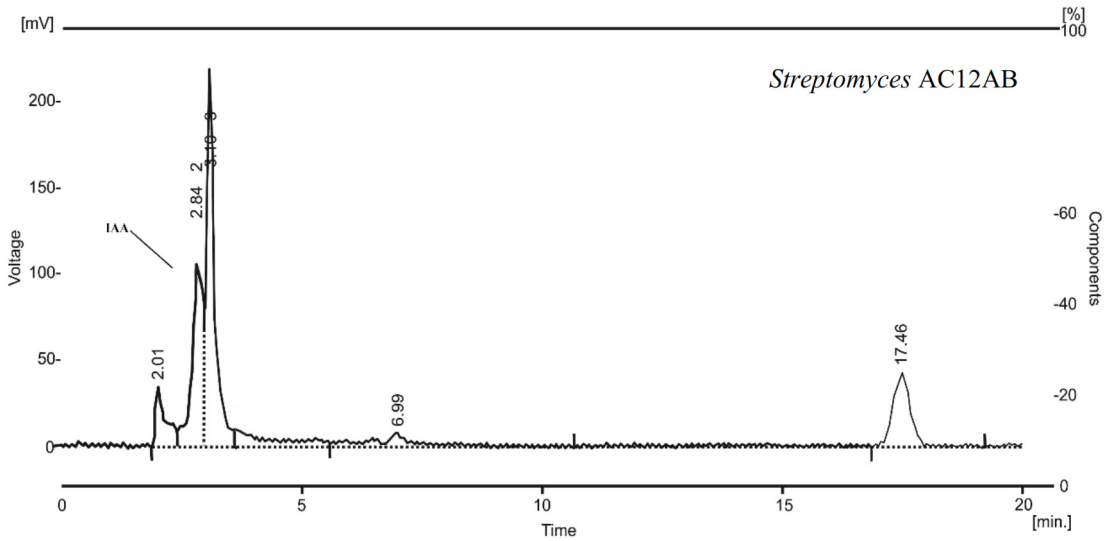

FIGURE 2 | HPLC chromatogram of *S. violaceusniger* AC12AB showing indole-3-acetic acid (IAA) peak at 2.84 min retention time.

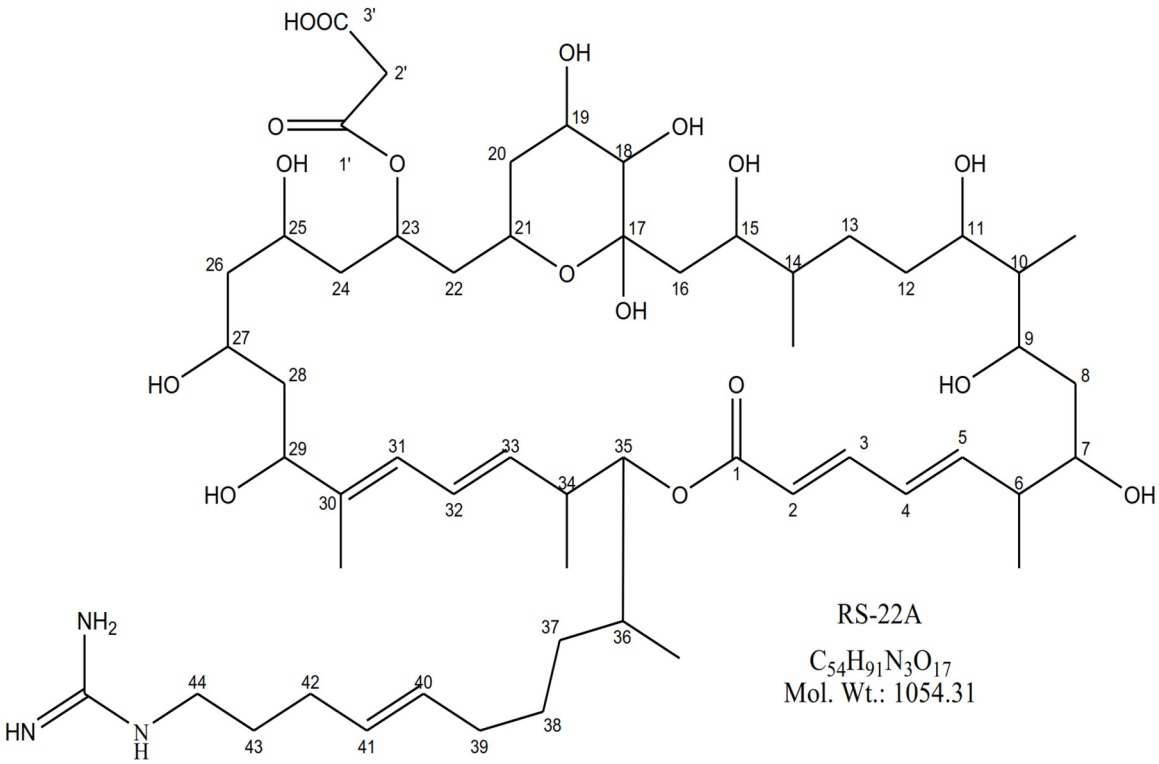

FIGURE 3 | Predicted structure of azalomycin RS-22A purified and isolated from *Streptomyces* AC12AB.

which showed that antagonistic bacterial isolates do not produce thaxtomin A and are non-pathogenic. Among antagonistic bacterial isolates, one bacterial strain AC12AB was identified as *S. violaceusniger* after 16S rRNA analysis with MH388022.1 accession number.

Antagonistic Potential and Isolation of Azalomycin From *Streptomyces violaceusniger* AC12AB

Disk diffusion assay was performed to determine the antibacterial potential of antagonistic *Streptomyces* isolates. The bacterial extract from *S. violaceusniger* AC12AB had high inhibitory activity (18 mm) against *S. scabies* (AJ-7) (**Figure 1** and **Supplementary Table S1**).

Plant Growth Promoting Potential of *Streptomyces violaceusniger* AC12AB

Streptomyces violaceusniger AC12AB was analyzed to produce plant growth promoting attributes including IAA, phosphate solubilization, siderophores production and *in vitro* nitrogen fixation. With colorimetric method, the highest potential of IAA production was estimated in case of *S. violaceusniger* AC12AB as 17 μgmL^{-1} after 4 days of incubation at 28°C. Production of IAA was also confirmed by HPLC analysis (**Figure 2**). From twelve antagonistic bacterial strains, three antagonistic *Streptomyces* isolates (*Streptomyces* A1RT, *S. violaceusniger* AC12AB and *Streptomyces* A-1; data not shown except *S. violaceusniger* AC12AB) were positive for siderophores production by producing blue color around bacterial cultures in NFM medium (**Supplementary Figure S1**). Phosphate solubilization test was performed with antagonistic bacterial strains and only *S. violaceusniger* AC12AB and *Streptomyces* A1RT exhibited clear zone around bacterial colonies (**Supplementary Figure S2**). ARA was performed against antagonistic bacterial isolates. The maximum value for ARA 4351.0 nMole/24h was recorded from *S. violaceusniger* AC12AB. However, 2278 and 1549 nMole/24h ethylene production were estimated from *Streptomyces* A1RT and *Streptomyces* A-1 strains, respectively (**Supplementary Table S2**).

Purification and Structural Elucidation of Bioactive Compound

Through a targeted mass fractionation, a white colored amorphous powder was purified having strong antibacterial activity against *S. scabies*. The molecular mass of the compound was predicted to be $C_{54}H_{91}N_3O_{17}$ based on observed molecular ion $[M\text{-}H]^-$; *m/z* 1054.5 (**Supplementary Figure S3**). Analysis of one-dimensional NMR (^1H and ^{13}C NMR spectra) indicated ten olefinic carbons, twelve oxy-methine and one quaternary hemiacetal carbon (**Supplementary Figures S4, S5**). Analysis of 2D-NMR revealed the characteristic guanidine carbon (**Supplementary Figures S6–S8** and **Supplementary Table S3**). The absorbance spectrum of compound (**Supplementary Figure S9**) exhibited distinct maxima 250–300 nm closely related to azalomycin (**Figure 3**) analog RS-22A (Ubukata et al., 1995).

Plant Growth Promotion and PCS Disease Suppression Under Greenhouse and Field Conditions

In greenhouse assay, pathogenic *S. scabies* isolates (AJ-7, AJ-10, and AC46) caused CS lesions on potato tubers (**Figures 4A,B** and **Table 1**). The inoculation with antagonistic *S. violaceusniger* AC12AB significantly reduced DS index ($P < 0.05$) (**Table 1**). There were 47, 24.6, and 41% increases in shoot length, root length and tuber weight, respectively, when *S. scabies* AJ10 was used in combination with *S. violaceusniger* AC12AB ($P < 0.05$) (**Figures 5**, **6**). Field trial using *S. violaceusniger* AC12AB revealed 83% disease reduction (**Figures 4C,D** and **Table 1**), 26.8% yield increase (**Table 1**), and significant ($P < 0.05$) increase in

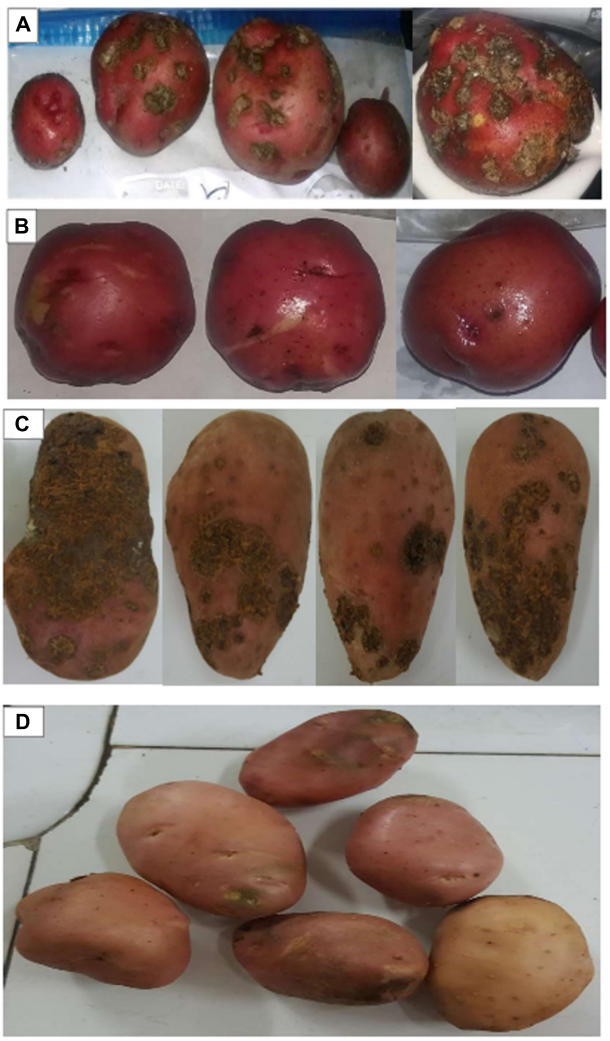

FIGURE 4 | Tubers harvested from greenhouse assay and field trial. **(A)** Tubers harvested from after inoculation with *S. scabies* AJ-7 in greenhouse assay. **(B)** Tubers harvested after inoculation with *S. scabies* AJ-7 + *S. violaceusniger* AC12AB in greenhouse assay. **(C)** Tubers harvested after inoculation with *S. scabies* AC-46 in field trial. **(D)** Tubers harvested after inoculation with *S. scabies* AC-46 + *S. violaceusniger* AC12AB in field trial.

TABLE 1 | Effect of growth promoting *Streptomyces* on potato (*Solanum tuberosum*) grown under greenhouse at the University of Maine, Maine, United States and field conditions in University of the Punjab, Lahore, Pakistan.

Treatment	Greenhouse		Field trial		Field trial	
	DS Index	% decrease	DS index	% decrease	Yield (Kg/h)	% increase
Streptomyces scabies (AJ-7)	153 ± 1f	–	N/A	N/A	N/A	N/A
Streptomyces scabies (AJ-10)	181 ± 1g	–	N/A	N/A	N/A	N/A
Streptomyces scabies (AC-46)	96 ± 1e	–	78 ± 0.5 c	–	7,650 ± 1 b	3.37 a
Streptomyces violaceusniger (AC12AB)	0.2 ± 0.01a	–	0 ± 0.01 a	–	9,100 ± 1 c	18.8 b
AJ7+AC12AB	12.6 ± 0.01c	91.70 b	N/A	N/A	N/A	N/A
AJ10+AC12AB	7.9 ± 0.01b	91.77 b	N/A	N/A	N/A	N/A
AC46+ AC12AB	17.4 ± 0.1d	90.30 a	13.2 ± 0.4 b	83.07	9,701 ± 1 d	26.8 c
Control	0.3 ± 0.01a	–	1 ± 0.01 a	–	7,400 ± 1 a	–

The results are presented as mean of three independent experiments ± SE. N/A, not applicable. Means followed by different letters are significantly different detected by Tukey's test at a significance level P < 0.05.

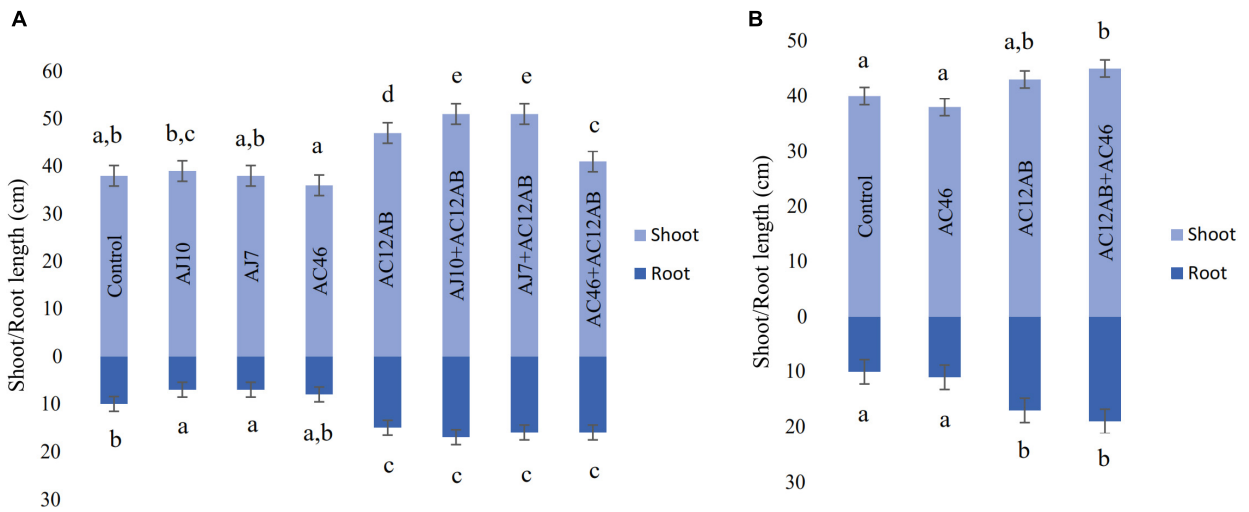

FIGURE 5 | Effect of *S. violaceusniger* AC12AB on potato growth. **(A)** Root and shoot growth in greenhouse trial. **(B)** Root and shoot growth in field trial. Error bars representing ± SE. Measurement was represented by mean ± SE of triplicates. Means followed by different letters show significant differences detected by Tukey's test at a significance level $P < 0.05$.

plant growth attributes (including increase in shoot/root length, number of tubers and tuber weight) (**Figures 5**, **6**).

DISCUSSION

In this research, *S. violaceusniger* AC12AB was assessed for its efficacy in suppressing CS disease and plant growth promotion in potato crop. The results indicated that although *S. violaceusniger* AC12AB application reduced the PCS disease up to 83%, their efficacy of plant growth promotion in field trial varied as compared to greenhouse assay.

Field soil is a complex environment that contains multiple factors which are difficult to control. CS development is not only dependent upon bacterial inoculum, but also by other physical and biological factors, including soil condition, irrigation strategy, plant variety, and weather conditions (Lazarovits et al., 2007). In the current study, field trial was conducted in the soil, and the average disease severity index was recorded as 1.1 (in the control). Moreover, dry and hot weather conditions of Pakistan may favor the development of CS infections in the tubers. All these factors could affect the disease outcome and may hinder the disease management under natural conditions. Previously, disease management remained dependent upon the use of chemical pesticides (Hvězdová et al. , 2018), maintaining high soil moisture level (Powelson and Rowe, 2008), use of resistant cultivars (Dees and Wanner, 2012) and crop rotation (Larkin et al., 2011). Biological control agents have been extensively

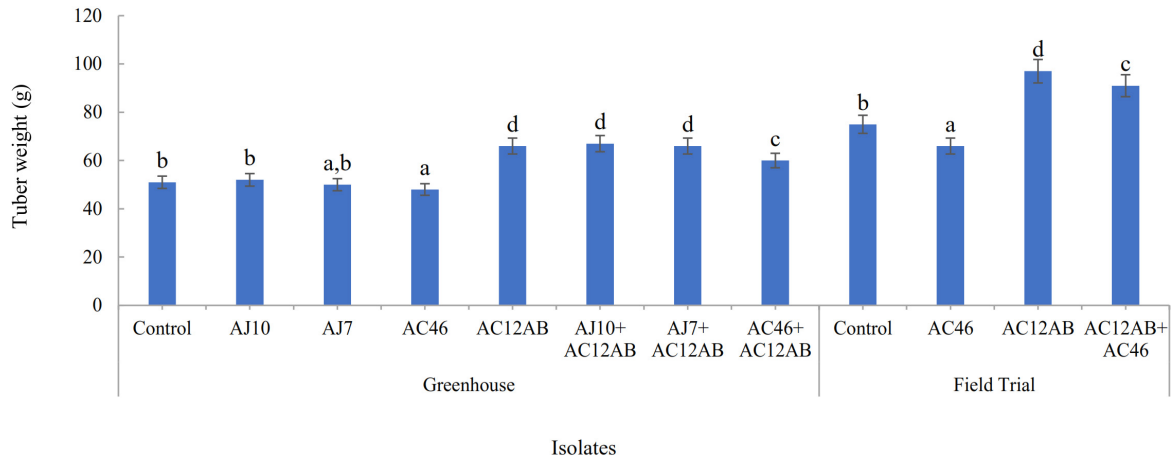

FIGURE 6 | Effect of *S. violaceusniger* AC12AB on potato tuber, which were inoculated with one of *S. scabies* isolates (AJ10, AJ7, and AC46) in greenhouse and field trials. Statistical analysis for greenhouse and field trial was calculated separately. Tuber weight was represented by mean ± SE of triplicates. Means followed by different letters show significant differences detected by Tukey's test at a significance level $P < 0.05$.

studied to control plant pathogens and simultaneously reducing environmental pollution and ecological distribution due to the irrational use of pesticides in fumigation.

Eckwall and Schottel (1997) used *Streptomyces diastatochromogenes* PonSSII as biocontrol agent against PCS by demonstrating antibiosis and competition mechanism. Similarly, Han et al. (2005) and Singhai et al. (2011) used *Bacillus* sp. sunhua and *Pseudomonas* spp. to control CS infections, respectively. Moreover, antimicrobial agents from bacterial spp. such as macrolactin A, iturin A, surfactin, bacillaene, fengycin, isatropolone C and difficidin (Schneider et al., 2007; Chowdhury et al., 2015; Lin et al., 2018; Sarwar et al., 2018) have been used against plant pathogens. In this study, we have identified a novel plant growth promoting *S. violaceusniger* AC12AB, which was confirmed to be an effective and inexpensive method to control PCS and simultaneously enhance the crop yield. PCS management remains unsolved as there is lack of chemical products. In this study, *S. violaceusniger* AC12AB exhibited strong antibacterial activity against *S. scabies*. Further analysis by NMR revealed that the main bioactive compound produced by *S. violaceusniger* AC12AB was azalomycin RS-22A, which has been previously used as a broad-spectrum antibiotic, antifungal and also as a moderate antitumor agent (Cheng et al., 2010; Yuan et al., 2013). To our knowledge, this is the first report of using *S. violaceusniger* AC12AB producing azalomycin as biological control agent in an agriculture system.

For a successful biocontrol agent, it is important to acquire root colonization ability so that the secondary metabolites produced by microorganisms would be available to the plant roots system (Johnston-Monje and Raizada, 2011; Larkin et al., 2011). Plant growth promoting *Streptomyces* (PGPS) are important microorganisms to develop a successful beneficial interaction between plants and microbes in a rhizoplane. PGPS are preferred over other plant growth promoting bacteria due to their enhanced colonization ability, their effect as biofertilization, biostimulation, and bioprotection (Saharan and Nehra, 2011; Rajput et al., 2013; Jog et al., 2014; Qiao et al., 2014). In current study, we demonstrated that *S. violaceusniger* AC12AB had the ability to produce plant growth hormone IAA along with its ability to produce siderophores and solubilize phosphates. Therefore, potato tubers treated with *S. violaceusniger* AC12AB developed in terms of root and shoot growth, tuber weight and better yield.

Results of greenhouse and field trials showed up to 90 and 80%, respectively, decrease in CS disease severity was observed when potato tubers inoculated with *S. violaceusniger* AC12AB. These results coupled with agar plate assay may explain the role of azalomycin as an antagonistic agent against PCS pathogens. Moreover, more than 25% increased yield was observed which could be attributed to the enhanced colonization ability of *Streptomyces*, production of plant growth hormones, siderophores, nitrogen fixation, and phosphate solubilization potential. Application of this type of bacteria will greatly enhance the production of potato and profit, which is especially important in developing countries.

AUTHOR CONTRIBUTIONS

AS conducted all experimental work. ZL assisted with project development and data analysis. SZ and AB assisted in performing NMR analysis. JH assisted in manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00202/full#supplementary-material

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Maize Milling By-Products: From Food Wastes to Functional Ingredients Through Lactic Acid Bacteria Fermentation

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Although recognized as important sources of functional compounds, milling by-products are often removed from the cereal kernel prior milling process. Indeed, the high presence of fiber in bran and the co-presence of lipids and lipase in germ are often considered as downsides for breadmaking. In this work, *Lactobacillus plantarum* T6B10 and *Weissella confusa* BAN8 were used as selected starters to ferment maize milling by-products mixtures made with heat-treated or raw germ and bran. The effects on the biochemical and nutritional features as well as the stability of the milling by-products were investigated. Lactic acid bacteria metabolisms improved the free amino acids and peptides concentrations and the antioxidant activity and caused a relevant phytic acid degradation. Moreover, fermentation allowed a marked decrease of the lipase activity, stabilizing the matrix by preventing oxidative processes. The use of fermented by-products as ingredients improved the nutritional, textural and sensory properties of wheat bread. Fortified breads (containing 25% of fermented by-products) were characterized by a concentration in dietary fiber and proteins of *ca*. 11 and 13% of dry matter, respectively. Compared to the use of the unfermented ones, the addition of pre-fermented by-products to bread caused a significant increase in protein digestibility (up to 60%), and a relevant decrease of the starch hydrolysis index (*ca*. 13%). According to the results, this study demonstrates the potential of fermentation to convert maize bran and germ, commonly considered food wastes, into nutritive improvers, meeting nutritional and sensory requests of modern consumers.

Keywords: maize, milling by-products, lactic acid fermentation, high-fiber, nutritional profile

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INTRODUCTION

The World Health Organization (WHO) stated, in 2016, that more than 1.9 billion adults were overweight and more than 600 million obese (WHO, 2018), probably due to the radical changes of the dietary habits recorded through the past decades (Fernstrand et al., 2017). The average daily intake of fiber in many populations is still lower than those recommended (Stephen et al., 2017), although the consumers are already aware of the advantages of a healthy diet rich in dietary fiber. Indeed, numerous physiological effects have been highlighted, i.e., the prevention of coronary heart

disease, type 2 diabetes, colorectal and other types of cancers (Kaczmarczyk et al., 2012) as well as it seems to be inversely associated with body weight due to the suppressing effect on the energy intake through increasing satiation (Howarth et al., 2001). Fortification of a staple food, i.e., bread, represents a promising way to increase fiber intake. However, it is needed to be able to produce healthy, fiber-rich bakery products with an appealing texture and taste. Optimal diet rich in fiber refers at the same time to the right amount and a suitable balance between them. The production of multi-grain products makes it possible, providing more variety in breads and increasing the diversity in fermentable soluble fiber (Lopez et al., 2001). The need for food companies to produce dietary fibers-enriched as well as low-calorific foods to meet the consumers requirements, is leading to investments in allocating resources in innovative foods developments. In this scenario, several scientific researches have been carried out aiming at substituting wheat flour in bread formulations (Sivam et al., 2010; Preedy et al., 2011; Rosell et al., 2016) and toward sustainable solutions, the fortification with milling by-products has largely been proposed (Katina et al., 2012; Rizzello et al., 2012; Singh et al., 2012; Pontonio et al., 2017). Bran, the outer layers of cereal grains, is rich in dietary fiber as well as other bioactive compounds and germ is usually characterized by a high nutritional value (Katina et al., 2012). However, despite to the positive effects on health, the presence of high content of fiber in bran and lipase and lipoxygenase activities in germ may negatively affect the baked goods quality. Indeed, use of native bran in wheat baking is a technological challenge because of the detrimental effect of bran on the gluten network and subsequent textural attributes of bread (Noort et al., 2010) other than negative influence on the taste of the products. While, the lipase and lipoxygenase activities determine the poor and unstable sensory properties of baked goods made of wheat flour containing the germ (Paradiso et al., 2008). However, lipases are sometimes used in bakery industry as emulsifiers to increase the bread volume, soften the crumb as well as retarding bread staling (Frauenlob et al., 2018). Thermal treatments can be used to inactivate the enzymes, however, collateral negative effects on the bioactive compounds, i.e., destruction of essential fatty acids and vitamins are often highlighted (Sjovall et al., 2000). Therefore, a careful selection of process to pre-treat milling by-products need to be done. Lactic acid bacteria (LAB) have already been proposed as a promising tool to overcome the sensory, structural, functional and nutritional drawbacks related to their use as ingredients in bread-making (Katina et al., 2012; Rizzello et al., 2012; Pontonio et al., 2017).

Maize (*Zea mays*) is a domesticated grass that originated in what is now Mexico. Worldwide, 60–70% of maize production is used domestically as livestock feed, and the remaining 30–40% is used for production of items for human consumption (Pranjal et al., 2017). Nevertheless, due to the processing wastes and the preparation of non-food products, the use of these cereals is lower than what estimated (Ranum et al., 2014). Milling by-products (bran and germ), which contain most of the bioactive compounds, are often removed from the kernel prior processing thus causing a loss of nutritional quality (Ranum et al., 2014).

In this study, selected LAB were used to ferment raw- and heat-treated milling by-products from maize, investigating the effects of the fermentation on their stabilization biochemical and nutritional properties. Based on the above considerations and aiming at increasing the fiber content of wheat bread valorizing food processing wastes, fermented and unfermented mixtures of maize germ and bran were used as ingredients to fortify wheat breads, evaluating biochemical and nutritional characteristics, structural properties and sensory profiles.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions and Starter Selection

Aiming at investigating a wide microbial diversity, starters were selected among 100 LAB strains (**Supplementary Table S1**) previously isolated from matrices with different chemical composition and sharing either most of the functional compounds and anti-nutritional factors with maize milling by-products. In detail, LAB strains belonging to the Culture Collection of the Department of Soil, Plant and Food Science (University of Bari, Italy) were previously isolated from raw or spontaneously fermented wheat, quinoa, hemp, hop, and wheat germ (Rizzello et al., 2010, 2016; Nionelli et al., 2014, 2018a,b; Pontonio et al., 2015; Mamhoud et al., 2016) (**Supplementary Table S1**). Strains were routinely cultivated on modified de Man, Rogosa and Sharpe medium (mMRS, maltose and fresh yeast extract were added to MRS at 1 and 5%, respectively, and the final pH was 5.6) until the late exponential phase of growth was reached (*ca*. 8 h), as previously determined by the analysis of the kinetics of growth (Rizzello et al., 2010, 2016; Nionelli et al., 2014, 2018a,b; Pontonio et al., 2015; Mamhoud et al., 2016).

Aiming at selecting strains to be used as mixed starter for maize milling by-products fermentation, the pro-technological and functional features of LAB were evaluated when singly inoculated in their own isolation matrix (wheat, quinoa, hemp and hop flours and wheat germ). Cells were harvested by centrifugation (10,000 × *g*, 10 min, 4°C), washed twice in 50 mM phosphate buffer, pH 7.0, and re-suspended in tap water. The DY (dough yield, dough weight × 100/flour weight) was 200 and the initial cell density of each LAB was *ca*. 7.0 log10 cfu/g. Fermentation was carried out in triplicate at 30°C for 24 h. After fermentation, samples were stored at 4°C and analyzed within 2 h. Non-inoculated doughs were used as controls. Proteolytic by means of total free amino acids (TFAA), phytase and radical scavenging (in the methanolic extract) activities were considered as functional features, however, kinetics of growth and acidification were considered as pro-technological traits.

Kinetics of growth and acidification were determined and modeled in agreement with the Gompertz equation, as modified by Zwietering et al. (1990): $y = k + A \exp\{-\exp[(\mu\text{max or Vmax } e/A)(\lambda\text{-}t) + 1]\}$; where y is the growth expressed as log10 cfu/g/h or the acidification rate expressed as dpH/dt (units of pH/h) at the time t; k is the initial level of the dependent variable to be modeled (log10 cfu/g or pH units); A is the cell density or pH (units) variation (between inoculation and the

stationary phase); μmax or Vmax is the maximum growth rate expressed as Δ log10 cfu/g/h or the maximum acidification rate expressed as dpH/h, respectively; λ is the length of the lag phase measured in hours. The experimental data were modeled by the non-linear regression procedure of the Statistica 12.0 software (Statsoft, Tulsa, OK, United States). The values of pH of doughs were determined by a M.507 pHmeter (Crimson, Milan, Italy) equipped with a food penetration probe.

Water/salt-soluble extracts (WSE) from doughs were prepared according to the method originally described by Osborne (1907) and modified by Weiss et al. (1993). Briefly, 5g of sample were suspended in 10 ml of 50 mM Tris–HCl (pH 8.8), incubated at 4°C for 1 h under stirring conditions (150 rpm) and centrifuged at 12,000 × *g* for 20 min. The supernatant was used for the determination of TFAA concentration and phytase activity. TFAA were analyzed by a Biochrom 30 series Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, United Kingdom) with a Na-cation-exchange column (20 by 0.46 cm internal diameter), as described by Rizzello et al. (2010). Phytase activity was determined by monitoring the rate of hydrolysis of *p*-nitrophenyl phosphate (p-NPP) (Sigma, 104-0). The assay mixture contained 200 μL of 1.5 mM *p*-NPP (final concentration) in 0.2 M Na-acetate, pH 5.2, and 400 μL of WSE. The mixture was incubated at 45°C and the reaction was stopped by adding 600 μL of 0.1 M NaOH. The *p*-nitrophenol released was determined by measuring the absorbance at 405 nm (Rizzello et al., 2010). One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μmol/min of *p*-nitrophenol under the assay conditions. The radical scavenging activity was determined on the ME methanolic extract (ME) of doughs. Three grams of each sample were mixed with 30 ml of 80% (vol/vol) methanol to get ME. The mixture was purged with nitrogen stream for 30 min, under stirring condition, and centrifuged at 4,600 × *g* for 20 min. The supernatants (MEs) were transferred into test tubes, purged with nitrogen stream and stored at *ca*. 4°C before analysis. The radical $DPPH^{\cdot}$ was used for determining the free radical scavenging activity (Rizzello et al., 2010). The synthetic antioxidant butylated hydroxytoluene (BHT) was included in the analysis as the reference (75 ppm). The reaction was monitored by reading the absorbance at 517 nm.

Based on the results collected under the above conditions, the two best performing strains, *Lactobacillus plantarum* T6B10 and *Weissella confusa* BAN8, were selected and used as a mixed starter for sourdough fermentation of maize milling by-products.

Microbiological and Chemical Analysis of Milling By-Products

Commercial samples of maize milling by-products, certified for mycotoxins levels (aflatoxins, zearalenone, deoxynivalenol, ochratoxin A, and fumonisin) under the thresholds defined by Reg. UE 1881/2006, Reg. UE 1126/2007, and Reg. UE 165/2010, were supplied by Molino Favero (Padova, Italy). Raw germ (RG), germ stabilized by heat-treatment (TG) and bran (B) were used in this study. The heat-treatment was carried out at *ca*. 200°C until the product temperature of 110°C was achieved. Temperature was monitored during treatment trough an RTD temperature probe (Jumo Food temp insertion RTD 902350, Sesto San Giovanni, Italy). Proximal analysis of the milling by-products prior doughs preparation was carried out.

Protein (total nitrogen × 5.7), lipids, moisture, total dietary fiber and ash of RG, TG and B were determined according to Approved Methods 46-11A, 30-10.01, 44-15A, 32-05.01, and 08-01.01 of the American Association of Cereal Chemists (American Association of Cereal Chemists [AACC], 2010). Available carbohydrates were calculated as the difference [100 − (proteins + lipids + ash + total dietary fiber)]. Proteins, lipids, carbohydrates, total dietary fiber and ash were expressed as % of dry matter (d.m.).

Microbiological analyses were carried out on milling by-products as specified below. In details, 10 g of RG, TG and B were suspended in 90 ml of sterile sodium chloride (0.9%, wt/vol) solution and homogenized in a Stomacher lab blender for 2 min at room temperature. Mesophilic presumptive LAB were determined on mMRS at 30°C for 48–72 h, under anaerobiosis. Yeasts were plated on Sabouraud Dextrose Agar (SDA, Oxoid, Basingstoke, Hampshire, United Kingdom), supplemented with chloramphenicol (0.1 g/l) at 25°C for 48h. Molds were enumerated on Potato Dextrose Agar (PDA, Oxoid) at 25°C for 48 h. Total *Enterobacteria* were determined on Violet Red Bile Glucose Agar (VRBGA, Oxoid) at 37°C for 24 h and total mesophilic bacteria were determined on Plate Count Agar (PCA, Oxoid) at 30°C for 48 h.

Milling By-Products Fermentation

Doughs (100 g) consisting of milling by-products mixtures and water (1:1) were obtained by an IM 58 high-speed mixer (Mecnosud, Flumeri, Italy). DY was 200. Mixtures of RG and B (ratio 2:1, $FMBP_{RG}$) or TG and B (ratio 2:1, $FMBP_{TG}$) were used. Doughs were inoculated with *L. plantarum* T6B10 and *W. confusa* BAN8 each at the cell density of *ca*. 7 log10 cfu/g of dough. Fermentations were carried out in triplicate at 30°C for 24 h. After fermentation, samples were stored at 4°C and analyzed within 2 h. Non-fermented doughs (MBP_{RG} and MBP_{TG}) were used as controls.

Microbiological, Biochemical and Nutritional Characterization of Fermented Milling By-Products

Lactic acid bacteria and pH values of MBP_{RG}, MBP_{TG}, $FMBP_{RG}$, and FMB_{TG} were determined as reported above. Ten grams of MBP_{RG}, MBP_{TG}, $FMBP_{RG}$, and FMB_{TG} were homogenized with 90 ml of distilled water for the determination of total titratable acidity (TTA). TTA is expressed as the amount (ml) of 0.1 M NaOH to reach pH of 8.3. WSE from fermented and un-fermented doughs were used for the determination of organic acids, peptides, TFAA concentrations and radical scavenging activity. Organic acids were determined by High Performance Liquid Chromatography (HPLC), using an ÄKTA Purifier system (GE Healthcare, Buckinghamshire, United Kingdom) equipped with an Aminex HPX-87H column (ion exclusion, Bio-Rad, Richmond, CA, United States), and an UV detector operating at 210 nm. Elution was at 60°C, with a flow rate of 0.6 ml/min,

using H_2SO_4 10 mM as mobile phase (Rizzello et al., 2010). The quotient of fermentation (QF) was determined as the molar ratio between lactic and acetic acids. TFAA were analyzed as reported above. For the peptides analysis, WSE were treated with trifluoroacetic acid (0.05% wt/vol) and subject to dialysis (cut-off 500 Da) to remove proteins and FAA, respectively. Then, peptides concentration was determined by the *o*-phtaldialdehyde (OPA) method as described by Church et al. (1983). All analyses were carried out in triplicate.

Tributyrin was used as the substrate to determine the lipase activity of the MBP_{RG}, MBP_{TG}, $FMBP_{RG}$, and $FMBP_{TG}$ extract by agar diffusion assay (Lawrence et al., 1967). Agar plates contained 1% (wt/vol) of triglyceride, 0.02% (wt/vol) sodium azide, and 50 mM phosphate buffer, pH 8.0. As reported by Lin et al. (1983), this value of pH was the optimum for maize germ endogenous lipase activity. Activity was expressed as the minimum dilution of the enzyme preparation that failed to give a detectable zone of hydrolysis after 24 h of incubation at 30°C.

Phytic acid concentration was measured using K-PHYT 05/07 kit assay (Megazyme Intl., Ireland), following the manufacturer's instructions. Total phenols and radical scavenging activity were determined on the ME of MBP_{RG}, MBP_{TG}, $FMBP_{RG}$, and $FMBP_{TG}$. The concentration was determined as described by Slinkard and Singleton (1977) and expressed as gallic acid equivalent. The radical scavenging activity was determined as reported above.

Breadmaking

Experimental breads (DY, 180) were manufactured at the pilot plant of the Department of Soil, Plant and Food Science of the University of Bari (Italy), according to the two-stage protocol commonly used for sourdough breadmaking (Rizzello et al., 2016). MBP_{RG} and MBP_{TG} were fermented at 30°C for 24 h with the mixed starters as described before (step I); then, $FMBP_{RG}$ and $FMBP_{TG}$ were mixed with wheat flour, water, and baker's yeast at 60 × *g* for 5 min with an IM 58 high-speed mixer (Mecnosud, Flumeri, Italy) and incubated for 1.5 h at 30°C (step II). The characteristics of the flour (*Triticum aestivum*, cv Appulo) used were the following: moisture, 14.2%; protein (N × 5.70), 11.5% of d.m.; fat, 1.6% of d.m.; ash, 0.6% of d.m. and total soluble carbohydrates, 1.5% of d.m. In detail, MBP_{RG} and MBP_{TG} and $FMBP_{RG}$ and $FMBP_{TG}$ were used at the percentage of 12.5 and 25% (wt/wt), respectively, of the total dough weight (Nionelli et al., 2014). At the end of step II, doughs (300 g) were baked at 220°C for 50 min (Combo 3, Zucchelli, Verona, Italy), obtaining breads fortified with raw and fermented milling by-products (MBP_{RG}-B/MBP_{TG}-B and $FMBP_{RG}$-B/$FMBP_{TG}$-B, respectively). A baker's yeast wheat bread (WB) was manufactured without the addition of milling by-products (DY, 180) and used as the control. Baker's yeast was added at the percentage of 1.5% (wt/wt), corresponding to a final cell density of *ca*. 9 log10 cfu/g in all the doughs only for the step II. Salt was not used. All breads were cooled for a period of 2 h on cooling racks at room temperature prior analysis.

The Texture Profile Analysis (TPA) of bread was carried out by means of a Universal Testing machine (model 3344, Instron, Norwood, MA, United States), equipped with 3.6 cm diameter cylindrical probe, 1000 N load cell. The chromaticity co-ordinates of the bread crust (obtained by a Minolta CR-10 camera) were also reported in the form of a color difference, dE*ab, as follows:

$$dE^*ab = \sqrt{(\mathrm{d}L)^2 + (\mathrm{d}a)^2 + (\mathrm{d}b)^2}$$

where dL, da, and db are the differences for *L*, *a*, and *b* values between sample and reference a white ceramic plate having $L = 67.04$, $a = 2.44$, and $b = 18.28$.

The values of pH and TTA, concentration of organic acids, TFAA, total phenols and phytic acid and radical scavenging activity were determined as reported above. The specific volume and moisture content of breads were measured determined according to the approved methods AACC 10-05.01 and 44-15.02, respectively (American Association of Cereal Chemists [AACC], 2010). Water activity (a_w) was determined at 25°C by the Aqualab Dew Point 4TE water activity meter (Decagon Devices Inc., United States). Fermentations were carried out in triplicate and each bread was analyzed twice.

Nutritional Characterization of Breads

The *in vitro* protein digestibility (IVPD) of breads was determined by the method proposed by Akeson and Stahmann (1964) with some modifications (Rizzello et al., 2014). Samples were subjected to a sequential enzyme treatment mimicking the *in vivo* digestion in the gastro intestinal tract and IVPD was expressed as the percentage of the total protein which was solubilized after enzyme hydrolysis. The concentration of protein of digested and non-digested fractions was determined by the Bradford method (Bradford, 1976). The analysis of starch hydrolysis was carried out on breads. The procedure mimicked the *in vivo* digestion of starch (De Angelis et al., 2009). Aliquots of breads, containing 1 g of starch, were undergo to enzymatic process and the released glucose content was measured with D-Fructose/D-Glucose Assay Kit (Megazyme). The degree of starch digestion was expressed as the percentage of potentially available starch hydrolyzed after 180 min. Wheat flour bread (WB) leavened with baker's yeast was used as the control to estimate the hydrolysis index (HI = 100). The predicted GI was calculated using the equation: $GI = 0.549 \times HI + 39.71$ (Capriles and Areas, 2013).

Sensory Analysis

Sensory analysis of breads was carried out by 10 trained panelists (5 male and 5 females, mean age: 35 years, range: 18–54 years), according to the method described by Haglund et al. (1998). After a roundtable discussion about the attributes, 13 were selected as the most frequently recognized by all the members of the panel. These were included in a score sheet for the quantitative evaluation with a scale from 0 to 10, with 10 the highest score. Elasticity of crumb, softness of crumb, crust and crumb color were considered visual/kinesthetic attributes Taste was evaluated as nuts, sourness, bitterness and toasted, while for aroma was evaluated the intensity, the rancid and aromatic characteristics of mixed nuts (Georgsson, 2015). Besides, the typical aroma of a fermented

dough was evaluated. According to the IFST Guidelines for Ethical and Professional Practices for the Sensory Analysis of Foods, assessors gave informed consent to tests and could withdraw from the panel at any time, without penalty or having to give a reason.

Statistical Analysis

Data were subjected to one-way ANOVA; pair-comparison of treatment means was achieved by Tukey's procedure at $P < 0.05$, using the statistical software, Statistica 12.5 (TIBCO Software Inc., Palo Alto, CA, United States) for Windows. Principal Components analysis was performed through Xlstat 2014 (Addinsoft, New York, NY, United States).

RESULTS

Starters Selection for Lactic Acid Fermentation

Lactic acid bacteria strains were singly used to ferment wheat, quinoa, hemp and hop flours and wheat germ at 30°C for 24 h. To allow the comparison between results from different food matrices, the increase (%) of TFAA concentration and phytase and radical scavenging activities, as compared to the corresponding non-inoculated doughs, were considered (**Figure 1A**). Increases of TFAA were in the range 13–87%, being the highest for *L. plantarum* T6B10 and the lowest for *Lactobacillus farciminis* S3N2. Similarly, wide increase of the phytase activity was found among the LAB strains, with highest and lowest values reached when *L. plantarum* T6B10 (81.7%) and *Lactococcus lactis* LVS 26 (3.8%) were used, respectively. Highest value of radical scavenging activity (44.3%) were found when *L. plantarum* LIN 2 was used to ferment wheat flour (**Figure 1A**). According to the pro-technological features, *W. confusa* BAN8 showed highest cell density increase (A_G, 2.4 log10 cfu/g). Moreover, both *L. plantarum* T6B10 and *W. confusa* BAN8 fell into the 75 and 25% percentile of the A_A and λ_A and λ_G, respectively (**Figure 1B**).

Based on the above results, *L. plantarum* T6B10 and *W. confusa* BAN were selected and used as mixed starter to ferment maize milling by-products.

Milling By-Products Characterization

The proximal composition and microbiological characterization of RG, TG and B used in this study are reported in **Table 1**. The heat-treatment led to a TG having moisture four times lower than RG. Although B was also subjected to heat-treatment, its moisture was 10.6 ± 0.9%. As expected, RG and TG contained high level of fat (up to *ca.* 33% of d.m.) and B was characterized by the highest concentration of carbohydrates and especially total dietary fiber (up to *ca.* 50%). Probably due to the heat treatment, any of the microbial groups investigated were detectable in 1 g TG, however, total mesophilic bacteria and molds were detected in B at cell density ≤ 2 log10 cfu/g (**Table 1**).

Fermented Milling By-Products Characterization

Either RG or TG and B (MBP_{RG} and MBP_{TG}, respectively) were mixed before use in a ratio 2 (RG or TG) to 1 (B). MBP_{RG} and MBP_{TG} had similar values of pH and TTA, being *ca.* 6.3 and 9 ml NaOH 0.1 M, respectively (**Table 2**). However, the concentration of lactic acid was significantly higher in MBP_{RG}. Acetic acid was not detectable in any of the sample prior the fermentation. Significant differences were also found for TFAA and peptides concentrations, being higher in MBP_{RG} (**Table 2**). As expected, after 24 h of fermentation with *L. plantarum* T6B10 and *W. confusa* BAN8, the value of pH of $FMBP_{RG}$ and $FMBP_{TG}$ was lower compared to the corresponding unfermented doughs (MBP_{RG} and MBP_{TG}, respectively), with lower value in $FMBP_{TG}$. On the contrary, values of TTA increased during fermentation, being significantly higher in $FMBP_{TG}$ compared to $FMBP_{RG}$. Lactic acid concentration in $FMBP_{RG}$ and $FMBP_{TG}$ was *ca.* 10–100 times higher than MBP_{RG} and MBP_{TG}, respectively (**Table 2**). Similar trend was found for acetic acid. Moreover, $FMBP_{RG}$ and $FMBP_{TG}$ contained different concentrations of both lactic and acetic acids, being higher in $FMBP_{TG}$ and $FMBP_{RG}$, respectively. QF was determined only in the fermented samples, being *ca.* 4.7. Fermented samples ($FMBP_{RG}$ and $FMBP_{TG}$) had significantly higher concentrations of TFAA (up to 80%) and peptides (up to 21%) compared to MBP_{RG} and MBP_{TG}. Moreover, the presence of raw germ in the mixture (MBP_{RG} and $FMBP_{RG}$) led to higher values compared to heat-treated germ containing samples (MBP_{TG} and $FMBP_{TG}$). As regard to the nutritional properties, MBP_{RG} contained higher contents of phytic acids as compared to MBP_{TG}, however, any significant differences were found in term of total phenols. Moreover, the radical scavenging activity in the WSE, was lower in MBP_{RG}. The fermentation led to a decrease and increase of the phytic acid concentration (up to 50%) and radical scavenging activity in the WSE (up to 30 times), respectively (**Table 2**). A slight increase of the concentration of total phenols was also found (**Table 2**). The minimum concentration of the crude enzyme extract that failed to give a detectable zone of hydrolysis was 35 ± 2.7 μg/ml for MBP_{RG}.

Biochemical and Nutritional Characterization of Breads

Biochemical and nutritional characteristics of breads are summarized in **Table 3**. Similar values of moisture and a_w were found between breads (MBP_{RG}-B, MBP_{TG}-B, $FMBP_{RG}$-B, and $FMBP_{TG}$-B). No significant differences were found between enriched breads and WB (**Table 3**). As expected, the values of pH, TTA and concentrations of lactic and acetic acids (up to 6 times) were lower and higher, respectively, in breads enriched with fermented milling by-products ($FMBP_{RG}$-B and $FMBP_{TG}$-B) as compared to MBP_{RG}-B, MBP_{TG}-B, and WB. Moreover, MBP_{RG}-B was characterized by higher value of pH and concentrations of lactic and acetic acids as compared to MBP_{TG}-B, however, the TTA did not differ significantly. Any significant differences were found in term of acetic acids between in $FMBP_{RG}$-B and $FMBP_{TG}$-B. According to these results, the

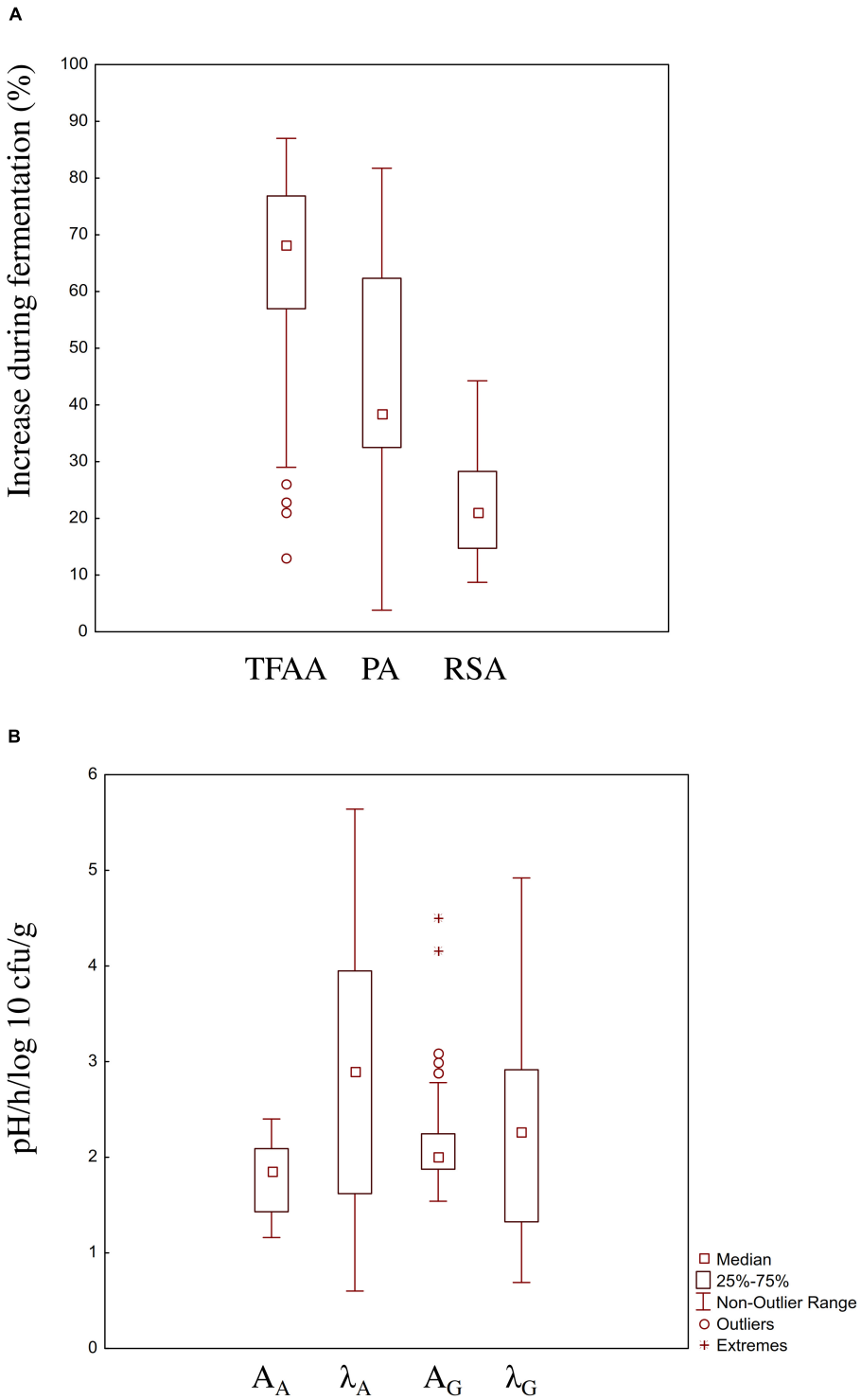

FIGURE 1 | Boxplot showing the functional **(A)** and pro-technological **(B)** characterization of 100 strains of lactic acid bacteria belonging to the species *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus helveticus*, *Lactobacillus farciminis*, *Lactobacillus nantensis*, *Lactobacillus plantarum*, *Lactobacillus rossiae*, *Lactococcus lactis*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Weissella cibaria*, *Weissella confusa*, *Leuconostoc citreum*, and *Leuconostoc mesenteroides* of the Culture Collection of the Department of Soil, Plant and Food Science of the University of Bari, Italy and isolated from raw or spontaneously fermented wheat, hemp, hop, quinoa, wheat germ and bran. The increase (%) of TFAA concentration, phytase (PA) and radical scavenging (RSA) activities in wheat, hemp, hop, quinoa, wheat germ and bran (DY 200) singly inoculated with the strains and fermented for 24 h at 30°C, compared to a not inoculated dough incubated in the same conditions were considered as functional features. Panel B displays the boxplot of the acidification (A_A, pH; λ_A, h) and growth (A_G, log 10 cfu/g; λ_G, h) kinetics parameters of the strains in the above-mentioned conditions, respectively. The top and the bottom of the box represent the 75th and 25th percentile of the data, respectively. The top and the bottom of the bars represent the 5th and the 95th percentile of the data, respectively.

TABLE 1 | Proximal composition and microbiological characterization of the maize raw (RG) and heat-treated (TG) germ and bran (B).

	RG	TG	B
Proximal composition*			
Moisture (%)	7.9 ± 0.3^{b}	2.3 ± 0.2^{a}	10.6 ± 0.9^{c}
Protein (%)	21.9 ± 0.2^{b}	19.8 ± 0.2^{a}	18.4 ± 0.6^{a}
Fat (%)	32.3 ± 0.3^{b}	33.5 ± 0.8^{c}	3.2 ± 0.1^{a}
Available carbohydrates (%)	9.5 ± 0.9^{b}	5.7 ± 0.9^{a}	23.3 ± 0.9^{c}
Total dietary fibers (%)	32.7 ± 0.8^{a}	32.7 ± 0.4^{a}	50.0 ± 0.8^{b}
Ash (%)	8.2 ± 0.4^{b}	8.3 ± 0.6^{b}	5.0 ± 0.2^{a}
Microbiological characterization			
Mesophilic aerobic bacteria (log10 cfu/g)	5.3 ± 0.7^{c}	<10 cfu/1g^{a}	2.0 ± 0.3^{b}
Yeast (log10 cfu/g)	<10 cfu/1g^{a}	<10 cfu/1g^{a}	< 10 cfu/1g^{a}
Molds (log10 cfu/g)	4.3 ± 0.6^{c}	<10 cfu/1g^{a}	1.5 ± 0.2^{b}
LAB (log10 cfu/g)	3.5 ± 0.5^{b}	<10 cfu/1g^{a}	<10 cfu/1g^{a}
Enterobacteriaceae (log10 cfu/g)	2.0 ± 0.3^{b}	<10 cfu/1g^{a}	<10 cfu/1g^{a}

*The data are the means of three independent experiments ± standard deviations (n = 3); *Data of protein, fat, carbohydrates, total fiber and ash are expressed on dry weight; LAB, Lactic acid bacteria; $^{a-c}$Values in the same row with different superscript letters differ significantly ($p < 0.05$).*

TABLE 2 | Biochemical and nutritional properties of maize milling by-products doughs: MBP_{RG}, unfermented mixture of raw germ and bran; MBP_{TG}, unfermented mixture of heat-treated germ and bran; $FMBP_{RG}$ fermented mixture of raw germ and bran; $FMBP_{TG}$, fermented mixture of heat-treated germ and bran.

	MBP_{RG}	MBP_{TG}	$FMBP_{RG}$	$FMBP_{TG}$
Biochemical characteristics				
pH	6.41 ± 0.4^{c}	6.23 ± 0.5^{c}	4.21 ± 0.02^{b}	4.04 ± 0.01^{a}
TTA (ml NaOH 0.1 M)	8.4 ± 0.6^{a}	9.6 ± 0.5^{b}	31.2 ± 0.5^{c}	35.5 ± 0.3^{d}
Lactic acid (mmol/Kg)	5.22 ± 0.2^{b}	0.70 ± 0.4^{a}	60.47 ± 0.6^{c}	66.48 ± 0.4^{d}
Acetic acid (mmol/Kg)	n.d.	n.d.	7.0 ± 0.6^{b}	4.67 ± 0.4^{a}
QF	n.d.	n.d.	4.0 ± 0.2^{b}	5.4 ± 0.3^{a}
TFAA (mg/Kg)	1431 ± 12^{b}	816 ± 15^{a}	1905 ± 14^{d}	1470 ± 20^{c}
Peptide concentration (mg/g)	38.4 ± 0.4^{b}	35.3 ± 0.4^{a}	46.5 ± 0.6^{d}	42.1 ± 0.4^{c}
Nutritional characteristics				
Phytic acid (g/100 g)	1.10 ± 0.03^{c}	0.81 ± 0.02^{b}	0.53 ± 0.02^{a}	0.55 ± 0.04^{a}
Total phenols (mmol/Kg)	1.6 ± 0.4^{a}	1.7 ± 0.2^{a}	1.8 ± 0.4^{a}	2.0 ± 0.3^{a}
Radical scavenging activity (%) on ME	$52.3 \pm 0.3^{b,c}$	51.3 ± 0.7^{b}	52.8 ± 0.4^{c}	50.9 ± 0.3^{a}
Radical scavenging activity (%) on WSE	1.4 ± 0.04^{a}	5.8 ± 0.03^{b}	42.5 ± 0.3^{d}	41.4 ± 0.6^{c}

The data are the means of three independent experiments ± standard deviations (n = 3). $^{a-d}$Values in the same row with different superscript letters differ significantly ($p < 0.05$). WSE, Water/Salt extract; ME, Methanolic extract. Fermented milling by-products doughs ($FMBP_{RG}$ and $FMBP_{TG}$) (DY 200) were fermented with Lactobacillus plantarum T6B10 and Weissella confusa BAN8 at 30°C for 24 h.

QF was higher in fermented milling by-products containing breads. Significant higher (up to *ca*. 3 times) concentrations of TFAA were found in enriched breads as compared to WB. Among experimental breads, $FMBP_{RG}$-B and $FMBP_{TG}$-B contained higher (58–91%, respectively) concentration of TFAA as compared to the corresponding MBP_{RG}-B and MBP_{TG}-B, however, MBP_{RG}-B had higher contents than MBP_{TG}-B. Similar trend was found for peptides contents. Lowest content was found for WB. Higher values were found in enriched breads (24–70%) as compared to WB, however, the use of fermented milling by-products led to a higher content of peptides (up to *ca*. 30%) as compared to the corresponding un-fermented milling by-products breads. Overall, the use of RG corresponded to higher values, as compared to TG (**Table 3**).

The higher concentrations of TFAA and peptides in breads containing the fermented milling by-products reflected on the IVPD which was up to 70% with respect to MBP_{TG} and MBP_{RG} (*ca*. 40%). WB had the lowest IVPD, 13–23% and 79–84% higher values were found for MBP_{RG}-B and MBP_{TG}-B and $FMBP_{RG}$-B and $FMBP_{TG}$-B, respectively. Compared to WB, MBP_{RG}-B and MBP_{TG}-B and $FMBP_{RG}$-B and $FMBP_{TG}$-B were characterized by significant lower values of the HI. Moreover, up to 13% lower values of the HI were observed in $FMBP_{RG}$-B and $FMBP_{TG}$-B as compared to the corresponding un-fermented milling by-products enriched breads. The lowest value was found for $FMBP_{TG}$-B (*ca*. 77%). The use of milling by-products as ingredient in breadmaking led to high fiber (up to *ca*. 11% of d.m) and protein of *ca*. (13% of d.m) contents in all breads as compared to WB, regardless the fermentation process and the germ heat-treatment.

According to the results of MBP, phytic acid content was lower in MBP_{TG}-B compared to MBP_{RG}-B. Lower values were

TABLE 3 | Biochemical and nutritional properties of breads: MBP_{RG}-B, containing unfermented mixture of raw germ and bran (MBP_{RG},12.5%, wt/wt); MBP_{TG}-B, containing unfermented mixture of heat-treated germ and bran (MBP_{TG}, 12.5%, wt/wt); $FMBP_{RG}$-B, containing fermented mixture of raw germ and bran ($FMBP_{RG}$, 25% wt/wt); $FMBP_{TG}$-B, containing fermented mixture of heat-treated germ and bran ($FMBP_{TG}$, 25% wt/wt); WB, wheat flour bread.

	MBP_{RG}-B	MBP_{TG}-B	$FMBP_{RG}$-B	$FMBP_{TG}$-B	WB
Biochemical characteristics					
Moisture (%)	32.9 ± 0.3^{b}	31.9 ± 0.6^{a}	32.5 ± 0.8^{b}	$31.6 \pm 0.7^{a,b}$	31.0 ± 0.2^{a}
a_w	0.93 ± 0.05^{a}	0.94 ± 0.06^{a}	0.94 ± 0.04^{a}	0.94 ± 0.06^{a}	0.92 ± 0.02^{a}
pH	5.59 ± 0.05^{c}	5.46 ± 0.06^{b}	4.22 ± 0.04^{a}	4.17 ± 0.01^{a}	5.61 ± 0.3^{c}
TTA (ml NaOH 0.1 M)	8.2 ± 0.6^{a}	8.2 ± 0.4^{a}	15.4 ± 0.4^{b}	16.6 ± 0.3^{c}	9.1 ± 0.3^{a}
Lactic acid (mmol/Kg)	1.53 ± 0.02^{b}	0.76 ± 0.04^{a}	25.1 ± 0.6^{c}	29.2 ± 0.4^{d}	3.3 ± 0.5^{a}
Acetic acid (mmol/Kg)	3.93 ± 0.05^{b}	0.83 ± 0.03^{a}	5.7 ± 0.5^{c}	5.3 ± 0.6^{c}	1.27 ± 0.3^{a}
QF	0.4 ± 0.1^{a}	0.9 ± 0.2^{b}	4.4 ± 0.2^{c}	5.5 ± 0.3^{d}	2.6^{a}
TFAA (mg/Kg)	214 ± 10^{b}	142 ± 11^{a}	338 ± 12^{d}	272 ± 15^{c}	134 ± 10^{a}
Peptide concentration (mg/g)	33.1 ± 0.5^{b}	30.2 ± 0.4^{a}	41.2 ± 0.5^{d}	38.4 ± 0.4^{c}	24.2 ± 0.1
Nutritional properties					
Protein (%)	13.0 ± 0.2^{b}	13.1 ± 0.4^{b}	12.9 ± 0.6^{b}	13.1 ± 0.3^{b}	6.3 ± 0.1^{a}
Fat (%)	5.9 ± 0.5^{b}	6.5 ± 0.3^{b}	5.9 ± 0.3^{b}	6.5 ± 0.5^{b}	0.61 ± 0.04^{a}
Available carbohydrates (%)	70.2 ± 1.7^{b}	69.4 ± 1.3^{b}	69.4 ± 1.3^{b}	69.5 ± 0.4^{b}	76.5 ± 0.9^{a}
Total dietary fibers (%)	10.9 ± 0.6^{b}	10.8 ± 0.5^{b}	10.8 ± 0.7^{b}	10.8 ± 0.3^{b}	1.87 ± 0.02^{a}
IVPD (%)	48 ± 3^{b}	44 ± 1^{b}	72 ± 1^{c}	70 ± 2^{c}	39 ± 1^{a}
HI (%)	93 ± 3^{c}	87 ± 1^{c}	82 ± 2^{b}	77 ± 1^{a}	100 ± 1
Phytic acid (mg/100 g)	340 ± 4^{d}	270 ± 5^{c}	70 ± 4^{a}	140 ± 2^{b}	234 ± 6^{e}
Total phenols (mmol/Kg)	0.82 ± 0.04^{a}	0.99 ± 0.03^{b}	1.07 ± 0.04^{c}	1.20 ± 0.02^{d}	2.39 ± 0.03^{e}
Radical scavenging (%) on ME	39.4 ± 0.5^{b}	53.1 ± 0.4^{d}	49.8 ± 0.4^{c}	58.0 ± 0.4^{e}	20.3 ± 0.3^{a}
Radical scavenging (%) on WSE	16.6 ± 0.3^{a}	22.4 ± 0.5^{c}	31.5 ± 0.5^{d}	33.7 ± 0.4^{e}	18.2 ± 0.3^{b}

The data are the means of three independent experiments ± standard deviations (n = 3). Data of protein, fat, carbohydrates and total fiber are expressed on dry weight. $^{a-e}$Values in the same row with different superscript letters differ significantly ($p < 0.05$). WSE, Water/Salt extract; ME, Methanolic extract. Fermented milling by-products doughs ($FMBP_{RG}$ and $FMBP_{TG}$) (DY 200) were fermented with Lactobacillus plantarum T6B10 and Weissella confusa BAN8 at 30°C for 24 h. Doughs for breadmaking had DY 180.

found when $FMBP_{RG}$ and $FMBP_{TG}$ were included in bread formula (**Table 3**). Nevertheless, higher values of phytic acids were found in MBP_{TG}-B and MBP_{RG}-B compared to WB (**Table 3**). The presence of milling by-products in bread led to higher concentration of total phenols than WB. Values 10–34% and 45–62% higher were found in MBP_{RG}-B and MBP_{TG}-B and $FMBP_{RG}$-B and $FMBP_{TG}$-B, respectively, as compared WB. MBP_{TG}-B was characterized by higher concentration of total phenols as well as higher radical scavenging in both ME and WSE as compared to MBP_{RG}-B. When $FMBP_{TG}$ and $FMBP_{RG}$ were used to fortify the bread, higher values of radical scavenging activities (ME and WSE) and total phenols concentration were found as compared to the corresponding un-fermented samples (**Table 3**).

Structural Properties and Sensory Profile of the Breads

After baking, structural and sensory analysis were carried out on MBP_{RG}-B, MBP_{TG}-B, $FMBP_{RG}$-B, $FMBP_{TG}$-B, and WB. The specific volume of the breads was influenced by the addition of milling by-products and fermentation process indeed the inclusion of milling by-products caused a decrease of the specific volume up to *ca.* 6% (MBP_{RG}-B, MBP_{TG}-B) as compared to WB. However, $FMBP_{RG}$-B, $FMBP_{TG}$-B showed higher specific volume than the corresponding MBP_{RG}-B and MBP_{TG}-B, respectively. Nevertheless, WB was characterized by the highest value. On the contrary, the hardness seemed to be influenced by both heat-treatment (increase) and fermentation (decrease) (**Table 4**). Compared to WB, the inclusion of milling by-products led to an increase of the hardness, however, it was lower in fermented products compared to un-fermented. Moreover, the presence of RG led to an increase of the value as compared to TG.

Aiming at highlighting the effect of the fermentation on the sensory profile of fiber-rich breads, MBP_{RG}-B, MBP_{TG}-B, $FMBP_{RG}$-B, and $FMBP_{TG}$-B were subjected to sensory analysis. The results are shown in **Figure 2**. PCA, representing 88% of the total variance of the data, showed that $FMBP_{TG}$-B and $FMBP_{RG}$-B were both scattered on the right zone of the plane because sharing the fermented, sourness, softness and bitterness aroma and taste. On the left part of the plane, the MBP_{TG}-B, MBP_{RG}-B were distributed. The perception of rancidity, although with very low scores, was found in MBP_{RG}-B and $FMBP_{RG}$-B. The analysis also pointed up perfect separation between breads containing RG and TG. Attributes such as toasted, nuts and nutty were perceived, as demonstrated by PCA, only in MBP_{TG}-B and $FMBP_{TG}$-B.

DISCUSSION

The growing interest of consumers in balanced nutrition, due to the increase in the number of overweight people in Western society, has refocused the food industry on the merits of

TABLE 4 | Structural properties of breads: MBP_{RG}-B, containing unfermented mixture of raw germ and bran (MBP_{RG},12.5%, wt/wt); MBP_{TG}-B, containing unfermented mixture of heat-treated germ and bran (MBP_{TG}, 12.5%, wt/wt); $FMBP_{RG}$-B, containing fermented mixture of raw germ and bran ($FMBP_{RG}$, 25% wt/wt); $FMBP_{TG}$-B, containing fermented mixture of heat-treated germ and bran ($FMBP_{TG}$, 25% wt/wt); WB, wheat flour bread.

	MBP_{RG}-B	MBP_{TG}-B	$FMBP_{RG}$-B	$FMBP_{TG}$-B	WB
Specific volume (cm^3/g)	2.27 ± 0.4^{c}	2.29 ± 0.3^{c}	2.80 ± 0.2^{b}	2.70 ± 0.2^{b}	2.91 ± 0.02^{a}
Resilience	0.79 ± 0.02^{a}	0.89 ± 0.04^{b}	0.82 ± 0.03^{a}	$0.86 \pm 0.03^{a,b}$	$0.85 \pm 0.04^{a,b}$
Cohesiveness	0.47 ± 0.05^{a}	0.48 ± 0.02^{a}	0.49 ± 0.04^{a}	0.49 ± 0.02^{a}	0.70 ± 0.07^{b}
Gumminess (N)	24.5 ± 0.6^{c}	17.1 ± 0.4^{b}	38.2 ± 0.7^{d}	25.1 ± 0.8^{c}	7.3 ± 0.2^{a}
Chewiness (g)	1930 ± 21^{c}	1530 ± 13^{b}	3140 ± 26^{e}	2150 ± 17^{d}	825 ± 13^{a}
Hardness (g)	7720 ± 47^{d}	5070 ± 35^{c}	5150 ± 49^{c}	3590 ± 56^{b}	2890 ± 22^{a}
Color crust					
L	46.3 ± 1.9^{b}	42.3 ± 1.2^{a}	47.3 ± 2.7^{b}	42.6 ± 1.8^{a}	68.1 ± 0.7^{c}
a	3.2 ± 0.3^{b}	5.01 ± 1.2^{c}	3.6 ± 0.4^{b}	5.9 ± 0.4^{c}	2.5 ± 0.1^{a}
b	17.4 ± 0.6^{a}	17.3 ± 0.4^{a}	18.7 ± 0.7^{b}	$18.2 \pm 0.5^{a,b}$	23.4 ± 0.3^{b}
ΔE	48.5 ± 1.2^{b}	52.8 ± 0.7^{c}	47.9 ± 0.6^{b}	52.8 ± 0.6^{c}	33.1 ± 0.5^{a}

The data are the means of three independent experiments ± standard deviations (n = 3) $^{a-e}$*Values in the same row with different superscript letters differ significantly ($p < 0.05$). Fermented milling by-products doughs ($FMBP_{RG}$ and $FMBP_{TG}$) (DY 200) were fermented with Lactobacillus plantarum T6B10 and Weissella confusa BAN8 at 30°C for 24 h. Doughs for breadmaking had DY 180.*

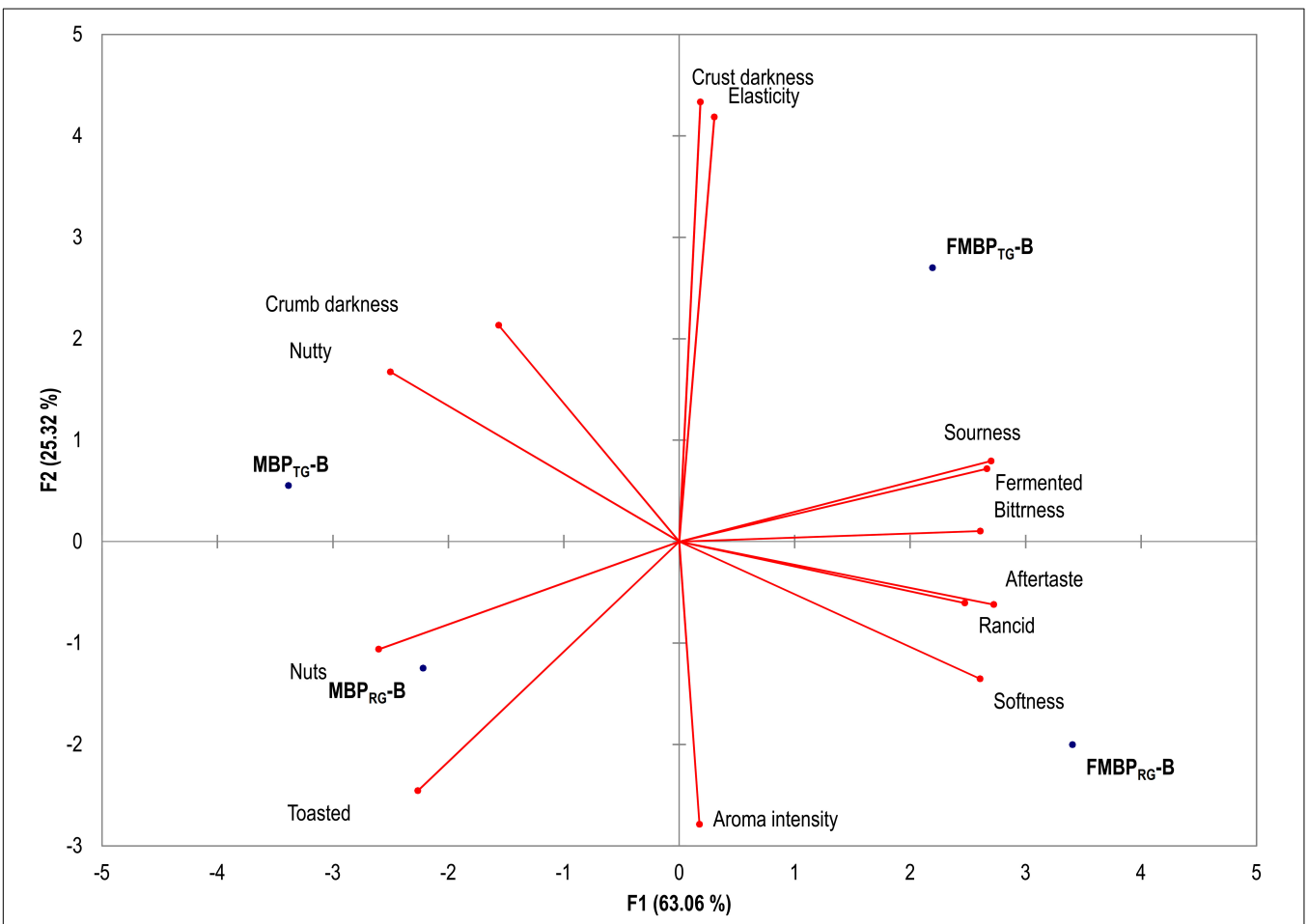

FIGURE 2 | Principal Components Analysis (PCA) based on the sensory attributes of breads: MBP_{RG}-B, containing unfermented mixture of raw germ and bran (MBP_{RG},12.5%, wt/wt); MBP_{TG}-B, containing unfermented mixture of heat-treated germ and bran (MBP_{TG}, 12.5%, wt/wt); $FMBP_{RG}$-B, containing fermented mixture of raw germ and bran ($FMBP_{RG}$, 25% wt/wt); $FMBP_{TG}$-B, containing fermented mixture of heat-treated germ and bran ($FMBP_{TG}$, 25% wt/wt). Fermented milling by-products doughs ($FMBP_{RG}$ and $FMBP_{TG}$) (DY 200) were fermented with *Lactobacillus plantarum* T6B10 and *Weissella confusa* BAN8 at 30°C for 24 h. Doughs for breadmaking had DY 180.

including some form of dietary fiber in food products (Redgwell and Fischer, 2005). Besides the well-known functional effects related to the nutrient absorption modification and the prebiotic capability, the importance of the dietary fiber is increasing due to its beneficial effects on the reduction of cholesterol levels and the risk of colon cancer (Lattimer and Haub, 2010). Health authorities, world-wide, recommend an increase of cereal derived foods in diet, since recognized as potential source of dietary fiber, especially when employed as whole grains (Dahl et al., 2015). The widespread consumption of cereals all over the world and the tradition of breadmaking give bread an important position in international nutrition being a popular staple food for ages. Nevertheless, white bread, obtained by refined flour, is the most consumed type of bread. Therefore, aiming at meeting the recommendations regarding the dietary fiber intake, the development of high fiber bread could be a promising strategy. Producing fortified/enriched products without compromising their sensory appeal, seems to be a real challenge for the food industry. Indeed, the consumer wants variety, good taste but no constraints and sometime placed nutrition second in importance to taste in factors for food selection (Sandvik et al., 2018). Among ingredients, milling by-products (bran and germ) are considered suitable fiber sources to be used as bread ingredients (Hemdane et al., 2016). Nevertheless, the increase in fiber content causes several issues, such as the decrease of loaf volume and softness, and the decrease in consumer acceptability (Wang et al., 2002). Moreover, in the case of germ, a stabilization prior the inclusion in flour is strictly required to avoid oxidative rancidity (Leenhardt et al., 2006), due to the concomitant presence of lipids and lipases (Tovey and Hobsley, 2004). Maize and its milling by-products are often contaminated with mycotoxins produced by fungi of the genera *Aspergillus*, *Fusarium*, and *Penicillium* (Fountain et al., 2014; Munkvold et al., 2019). Management of mycotoxin problems requires a multifaceted approach including preharvest and postharvest strategies (Okeke et al., 2015; Burns et al., 2018) for preventing mycotoxin accumulation, and a strict control of the matrix intended for food and feed uses (Munkvold et al., 2019). From the nutritional point of view, besides the positive contribution of tocopherols, fiber, high quality protein, iron, zinc and vitamins (Naves et al., 2009), the abundance of anti-nutritional factors (e.g., phytic acid) characterizing milling by-products may lower the overall quality of fortified breads (Rizzello et al., 2012; Hemdane et al., 2016).

The application of the sourdough-type fermentation, involving LAB as natural or selected starters, has largely been proposed as valuable biotechnology able to solve the above mentioned technological, stabilization and nutritional problems related to the employment of cereal milling by-products as bread ingredient (Arte et al., 2015; Pontonio et al., 2017), allowing the exploitation of by-products generally treated as wastes. The production of value-added products from food processing wastes have gained worldwide attention (Wang and Chen, 2010; Burns et al., 2018) due to the economical but also environmental implications (Galanakis, 2012).

Among cereals, maize (Pranjal et al., 2017) is grown throughout the world and although the good nutritional profile its use as food ingredient is second to the fuel production (Ranum et al., 2014). Similarly, to wheat, maize milling methods produce a variety of economically and nutritionally valuable co-products, which can be used as food ingredient (Naves et al., 2009).

This study aimed at improving the nutritional profile of wheat bread using raw- and heat-treated wastes of maize milling process fermented by selected LAB.

Apart from the moisture, RG and TG had similar proximate composition. As expected, cell density of the different microbial groups considered was in TG very low, as the consequence of the thermal process, while in RG and B samples microbial contaminations were observed, at different level. However, *Enterobacteriaceae* were not found (<10 cfu/1 g) showing the good hygienic status of all the samples (Cordier, 2006) meeting food quality and safety standards. Either RG or TG were combined, with B in a ratio of 2:1 according to their presence in the maize kernel (*ca*. 11 and 5%, respectively) (Gwirtz and Garcia-Casal, 2013), before use and characterization. Doughs were prepared (MBP_{RG} and MBP_{TG}) mixing milling by-products and water in a ratio of 1:1 and fermented ($FMBP_{RG}$ and $FMBP_{TG}$) with two LAB strains, *L. plantarum* T6B10 and *W. confusa* BAN8, previously selected according to their pro-technological, biochemical and nutritional features (kinetics of growth and acidification, proteolytic, phytase and antioxidant activity) (Pontonio et al., 2015; Rizzello et al., 2016). Overall, MBP_{TG} was characterized by lower concentration of lactic acid, TFFA, peptide and phytic acid as compared to MBP_{RG}. The partial denaturation of the endogenous proteases and the significant decrease of the resident microbiota due to the heat-treatment might explain the lower values of TFFA and peptides concentrations. Similarly, the dejection of LAB, and more in general, microbial density led to a reduction of lactic acid in the TG.

Lactic acid bacteria fermentation led, as expected, to a relevant decrease of the pH due to the synthesis of lactic and acetic acids (Gobbetti et al., 2005). Moreover, $FMBP_{RG}$ and $FMBP_{TG}$ were characterized by higher concentration of TFAA compared to MBP_{RG} and MBP_{TG}. Sourdough fermentation resulted in an increase of amino acid concentrations due to the proteolytic activity of sourdough LAB and endogenous proteases which have been activated under the acidic conditions of sourdough fermentation (Thiele et al., 2002). TFAA and peptides concentrations were higher in $FMBP_{RG}$ than in $FMBP_{TG}$, thus hypothesizing a contribution of the activity of endogenous proteases of the raw germ to the microbial proteolysis. The lower lactic acid concentration detected in $FMBP_{RG}$ may be explained by the reduced acidification efficiency of the inoculated LAB, affected by the competition of the endogenous microbiota.

The digestibility of protein, bioavailability of amino acids and protein quality of foods can be weakened by the presence of dietary anti-nutritional factors (Soetan and Oyewole, 2009). Phytic acid is an anti-nutritional factor because works as an excellent chelator of minerals, complexes the basic amino acid group of proteins, thus decreasing their dietary bioavailability (Febles et al., 2002). Fermentation with LAB contributed to create the optimal environment for phytase (myo-inositol-hexakisphosphate phosphohydrolase, EC 3.1.3.8)

(Poutanen et al., 2009) which decreased the concentration of phytic acid, down to trace in $FMBP_{RG}$. The optimal pH of a purified phytase from maize seedlings was 4.8 (Laboure et al., 1993). The proteolysis operated by endogenous proteases and microbial peptidase during the fermentation may have led to the release of peptides with antioxidant activity, thus explaining the increase of the radical scavenging activity in the WSE in $FMBP_{RG}$ and $FMBP_{TG}$. Although the LAB fermentation often leads to an increase of the solubilization of phenolic compounds having antioxidant activity, due to the biological acidification and the microbial enzymatic activity (e.g., feruloyl-esterase and β-glucosidase activities) radical scavenging activity as well as in the ME and total phenols concentration $FMBP_{RG}$ and $FMBP_{TG}$ were not significantly different than MBP_{RG} and MBP_{TG}.

Data of the biochemical and nutritional characterization of MBP_{RG}, MBP_{TG}, $FMBP_{RG}$, and $FMBP_{TG}$ were subjected to Principal Components Analysis (PCA) (**Figure 3**). The factors explained the 98.7% of the total variance and clearly differentiated the MBP_{RG} and MBP_{TG} form the corresponding fermented $FMBP_{RG}$ and $FMBP_{TG}$. Indeed, the former and the latter are scattered on the left and right part of the plane, respectively. Overall, $FMBP_{RG}$ and $FMBP_{TG}$ seem to be characterized by a more complex profile than the corresponding MBP_{TG} and MBP_{RG}, with improved nutritional features. According to the literature, maize germ contains lipases which are responsible for the fatty acid oxidation leading to unstable product with poor quality (Paradiso et al., 2008; Rizzello et al., 2010). Under this study conditions, the lactic acid fermentation was proposed as valuable alternative to heat treatment aiming at decreasing the lipase activity of maize germ. Indeed, MBP_{RG} was the only sample showing activity, however, a strong inhibition was achieved through the fermentation ($FMBP_{RG}$). As expected, similar results were observed when the heat treatment (MBP_{TG} and $FMBP_{TG}$) was used; however, it may have decrease of the nutritional value of maize germ (Paradiso et al., 2008; Rizzello et al., 2010).

$FMBP_{RG}$ and $FMBP_{TG}$ were used as ingredient for the manufacture of wheat bread, whose characteristics were compared to those of breads manufactured with MBP_{RG} and MBP_{TG}. Overall, the nutritional properties of white bread have been improved through the inclusion of milling by-products in the formula. However, the best improvements were observed when $FMBP_{RG}$ and $FMBP_{TG}$ were used. Indeed, according to the high content of organic acids in fermented samples, $FMBP_{RG}$-B and $FMBP_{TG}$-B were characterized by optimal QF for sensory profile (Minervini et al., 2012) and acetic acid concentration, associated to the extended microbiological shelf-life

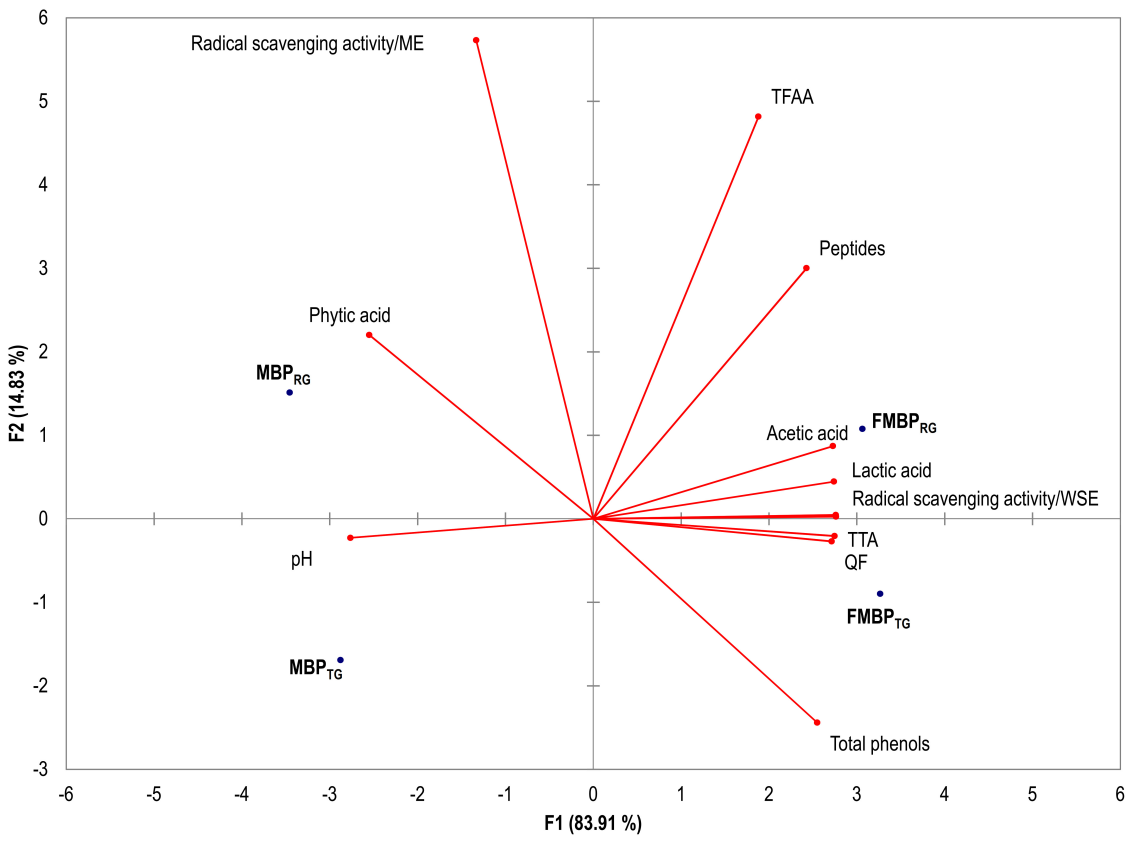

FIGURE 3 | Principal Components Analysis (PCA) based on the biochemical and nutritional properties of the milling by-products doughs: MBP_{RG}, unfermented mixture of raw germ and bran; MBP_{TG}, unfermented mixture of heat-treated germ and bran; $FMBP_{RG}$ fermented mixture of raw germ and bran; $FMBP_{TG}$, fermented mixture of heat-treated germ and bran. Fermented milling by-products doughs ($FMBP_{RG}$ and $FMBP_{TG}$) (DY 200) were fermented with *Lactobacillus plantarum* T6B10 and *Weissella confusa* BAN8 at 30°C for 24 h.

(Katina, 2005). Improvements in TFAA and peptides concentrations, as well as the phytic acid degradation and the enhancement of the radical scavenging activity (WSE) in bread containing fermented milling by-products, were also found. According to the literature, when dietary fiber is used in breadmaking, the loaf volume and shelf-life are often compromised. Bran is the main responsible for the weak structure and poor baking quality (low bread volume and elasticity of crumb) in fiber enriched bread (Katina, 2005). These effects on the dough structure are due to the dilution of the gluten network, which in turn impairs gas retention rather than gas production. Thus, the supplementation of dietary fiber requires changes in processing techniques to produce baked goods with good consumer quality. The sourdough was proposed as strategy to overcome the downsides in breadmaking due to the high fiber contents (Katina, 2005; Pontonio et al., 2017). Under these study conditions, the specific volume and the hardness of $FMBP_{TG}$-B and $FMBP_{RG}$-B were higher and lower, respectively, than the corresponding MBP_{TG}-B and MBP_{RG}-B corroborating the previous findings (Katina, 2005; Katina et al., 2006b; Rizzello et al., 2012).

High fiber content (Regulation EC No. 1924/2006, 2006) was achieved including milling by-products in bread, however, the fermentation with LAB positively affected other nutritional features, i.e., IVPD and HI, leading to a bread with high nutritional profile. High protein content and digestibility was achieved when fermented milling by-products were used to fortify wheat bread. A high content of protein (*ca*. 13% of d.m.) as well as values of IVPD up to *ca*. 60% were observed. The latter were mainly due to the intense proteolysis operated by endogenous and microbial enzymes (Pontonio et al., 2017). The predicted GI was also investigated, through the study of the starch hydrolysis kinetic *in vitro* conditions. Due to the biological acidification, lactic acid bacteria fermentation can be used to decrease the starch hydrolysis during digestion (De Angelis et al., 2009). Indeed, $FMBP_{RG}$-B and $FMBP_{TG}$-B showed lower values as compared to the corresponding (MBP_{RG}-B and MBP_{TG}-B).

The sensory acceptability of the breads was assessed by a panel test, and although all breads were appreciated, those containing fermented milling by-products showed a more balanced profile. Moreover, breads fortified with $FMBP_{RG}$ and $FMBP_{TG}$ were characterized by acidic aroma and taste typical of sourdough breads (Katina et al., 2006a).

The results of this study demonstrate that fermentation with LAB led the improvement of nutritional properties of maize milling by-products through the increase of free amino acids, peptides concentrations and antioxidant activity, and the decrease of the antinutritional phytic acid. Fermentation also caused the chemical stabilization of the by-products through the inhibition of the lipase activity, thus suggesting an alternative use to heat-treatment which often impair the nutritional profile of germ.

CONCLUSION

This study demonstrates the potential of fermentation to convert maize bran and germ, commonly considered food wastes, into nutritive improvers, meeting nutritional and sensory requests of modern consumer. The fortification of wheat bread with maize milling by-products allows the increase in dietary fiber and proteins compared to a conventional wheat bread. When fermented, milling by-products also conferred to fortified bread the advantages commonly related to the sourdough fermentation, such as the increase of the protein digestibility, the decrease of the starch hydrolysis, the degradation of phytic acid. Moreover, LAB fermentation as pre-processing treatment positively affects the textural properties and the sensory profile of the breads.

AUTHOR CONTRIBUTIONS

EP conceived the study, elaborated the results, and wrote the draft of the manuscript. CD carried out the experiments. MG was the scientific advisor and responsible for financial contribution. CR supervised the work and critically revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00561/full#supplementary-material

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REVIEW
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Fermentation Biotechnology Applied to Cereal Industry By-Products: Nutritional and Functional Insights

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Cereals are one of the major food sources in human diet and a large quantity of by-products is generated throughout their processing chain. These by-products mostly consist of the germ and outer layers (bran), deriving from dry and wet milling of grains, brewers' spent grain originating from brewing industry, or others originating during bread-making and starch production. Cereal industry by-products are rich in nutrients, but still they end up as feed, fuel, substrates for biorefinery, or waste. The above uses, however, only provide a partial recycle. Although cereal processing industry side streams can potentially provide essential compounds for the diet, their use in food production is limited by their challenging technological properties. For this reason, the development of innovative biotechnologies is essential to upgrade these by-products, potentially leading to the design of novel and commercially competitive functional foods. Fermentation has been proven as a very feasible option to enhance the technological, sensory, and especially nutritional and functional features of the cereal industry by-products. Through the increase of minerals, phenolics and vitamins bioavailability, proteins digestibility, and the degradation of antinutritional compounds as phytic acid, fermentation can lead to improved nutritional quality of the matrix. In some cases, more compelling benefits have been discovered, such as the synthesis of bioactive compounds acting as antimicrobial, antitumoral, antioxidant agents. When used for baked-goods manufacturing, fermented cereal by-products have enhanced their nutritional profile. The key factor of a successful use of cereal by-products in food applications is the use of a proper bioprocessing technology, including fermentation with selected starters. In the journey toward a more efficient food chain, biotechnological approaches for the valorization of agricultural side streams can be considered a very valuable help.

Keywords: cereal by-product, fermentation, yeast, lactic acid bacteria, bioactive compounds, antioxidant activity, anticancer

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INTRODUCTION: OVERVIEW OF THE FATE OF CEREAL INDUSTRY BY-PRODUCTS

Cereals are the edible seeds of the grass family of *Poaceae,* also known as *Gramineae,* and their cultivation dates to thousands of years ago. Wheat, maize, rice, barley, sorghum, millet, oat, and rye are the cereals most important on a global scale (1). Among them, wheat and rice represent the dominant crops, in Western and Asian countries, respectively (2, 3). Cereals are one of

the most important food sources for human consumption, with a production of more than 2 billion tons/year. However, unfortunately, roughly 30% of this amount is wasted or lost due to several reasons (2, 4). Overall, food losses include all the edible parts discarded during the supply chain, while food wastes are residues of high organic load, removed during raw materials processing to foodstuff (5). In developing countries, substantial food losses occur during agricultural production, whereas in industrialized countries losses also include processed products during the distribution and consumption stages (5). Considering the unused food matrix as waste does not enforce the possibility of re-utilizing it in the food chain. For this reason, the use of the term "by-product" is increasing and identifies those wastes that become substrates for the recapture of functional compounds and the development of new products with a market value (6).

In cereals processing, the two major by-products obtained during the traditional milling procedures are bran and germ. The initial purpose of milling was mostly to grind the grain, successively, the separation of the starchy endosperm from the outer layers (dry milling) became more important. The main reason for germ and bran removal is that, despite being rich in vitamins, minerals and dietary fiber, they adversely affect the processing properties of flours and this is one of the main reasons why the majority of cereal foods consumed are made of refined flour (7, 8). However, refined flour is then lacking many compounds important for nutrition (8). Wet milling, on the other hand, is mainly used to produce starch and gluten, separating them from germ, bran and a precipitate solid fraction (9).

The employment cycle of these by-products can follow different paths (**Figure 1**). The most common way of disposal is to use them as feed or for compost. However, to alleviate the environmental and economic burden of such losses, different approaches have been explored (10). One of them is biorefinery to produce biofuels such as ethanol (11). Ethanol is often produced from the cellulosic fractions of cereal bran, especially from maize. It is estimated that 4% of the global grains are used for ethanol production (12). Besides ethanol the production of chemical compounds such as lactic acid is also pursued (13). Lactic acid is traditionally applied in food industry as well as in pharmaceutical, textile and chemical industry. It can be obtained from an extensive range of carbon sources, of which cereal industry by-products such as maize cob, wheat bran, brewer's spent grains are very rich (14). Whereas, maize, rice and wheat bran are often used to produce phytic acid. After phytic acid extraction, the resulting by-product is deprived of an antinutritional factor, making it a more valuable ingredient in animal feed (12). Insoluble dietary fibers, fructans, antioxidants, and many other bioactive compounds are extracted from cereal by-products and used in food manufacturing (10). The oil obtained from wheat germ finds wide application in vitamin production, and cosmetic industry as well as in food, feed and as insect biological control agent, while the defatted wheat germ and wheat germ proteins are used as ingredients for several food products (15).

A more recent approach involves the generation, from rice and wheat bran, husk and straw, of nanoparticles exhibiting antibacterial activity or the production, through microbial fermentation, of biodegradable plastics (10). Cereal by-products are considered an ideal candidate to produce commercially important enzymes, due to their richness in nutrients but also to their low costs and wide availability as cultivation substrates. The use of cereal by-products is considered one of the most effective means in producing high value compounds (10).

Besides enzymes production or bioplastics synthesis, microbial fermentation is a very efficient way to improve the nutritional and functional properties of cereal by-products to implement their use in food production. Fermentation, alone ore coupled with technological or biotechnological processing techniques, offers a variety of tools to modify cereal matrices. During fermentation, both endogenous and bacterial enzymes are able to modify the grain constituents affecting the structure, bioactivity, and nutrient bioavailability (16). Since fermentation with either lactic acid bacteria, yeasts or fungi is extensively employed to produce cereal-based foods with enhanced health properties (17, 18), its application to cereal by-products could, to a wider extent, improve the overall eco-sustainability of the food system, providing a suitable alternative to reduce malnutrition and hunger (19). In light of the above considerations, this review aims at providing a comprehensive overview of all the nutritional and functional positive outcomes deriving from fermentation of cereal industry waste and by-products.

FERMENTATION OF THE MILLING BY-PRODUCTS

Wheat Bran

The multiple outer layers of wheat (outer and inner pericarp, seed coat, and nucellar epidermis) are commonly referred to as bran (20). During conventional wheat roller milling, most of the endosperm is separated and further ground to wheat flour. Therefore, bran, together with the aleurone layer and remnants of endosperm, becomes a milling by-product. Different types of bran (i.e., coarse bran or regular bran, coarse weatings or fine bran, fine weatings or middlings or shorts, and low-grade flour or "red dog") can be distinguished depending on the particle size and the endosperm content (21). The most abundant polysaccharides of the bran layers, arabinoxylans and β-glucans, have a role in lowering the risk of type II diabetes and colorectal cancer as well as cardiovascular and diverticular diseases (8). However, bioactive compounds such as dietary fibers and phenolic acids are trapped in the cell wall structures resisting conventional milling and thus having low bioaccessibility (22). Thereby, new milling techniques, enzymatic treatments and fermentation processes targeting the structure of bran, have been studied with the aim of enhancing its nutritional potential (23).

Over the last years, the interest of the scientific community toward wheat bran fermentation, alone or combined with other approaches, markedly increased. Fermentation with two selected

Abbreviations: ABTS, 2,20-azino-di-[3-ethylbenzthiazoline sulphonate]; BSG, Brewers' Spent Grain; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GABA, γ-aminobutyrric amino acid; SHIME, Simulator of the Human Intestinal Microbial Ecosystem.

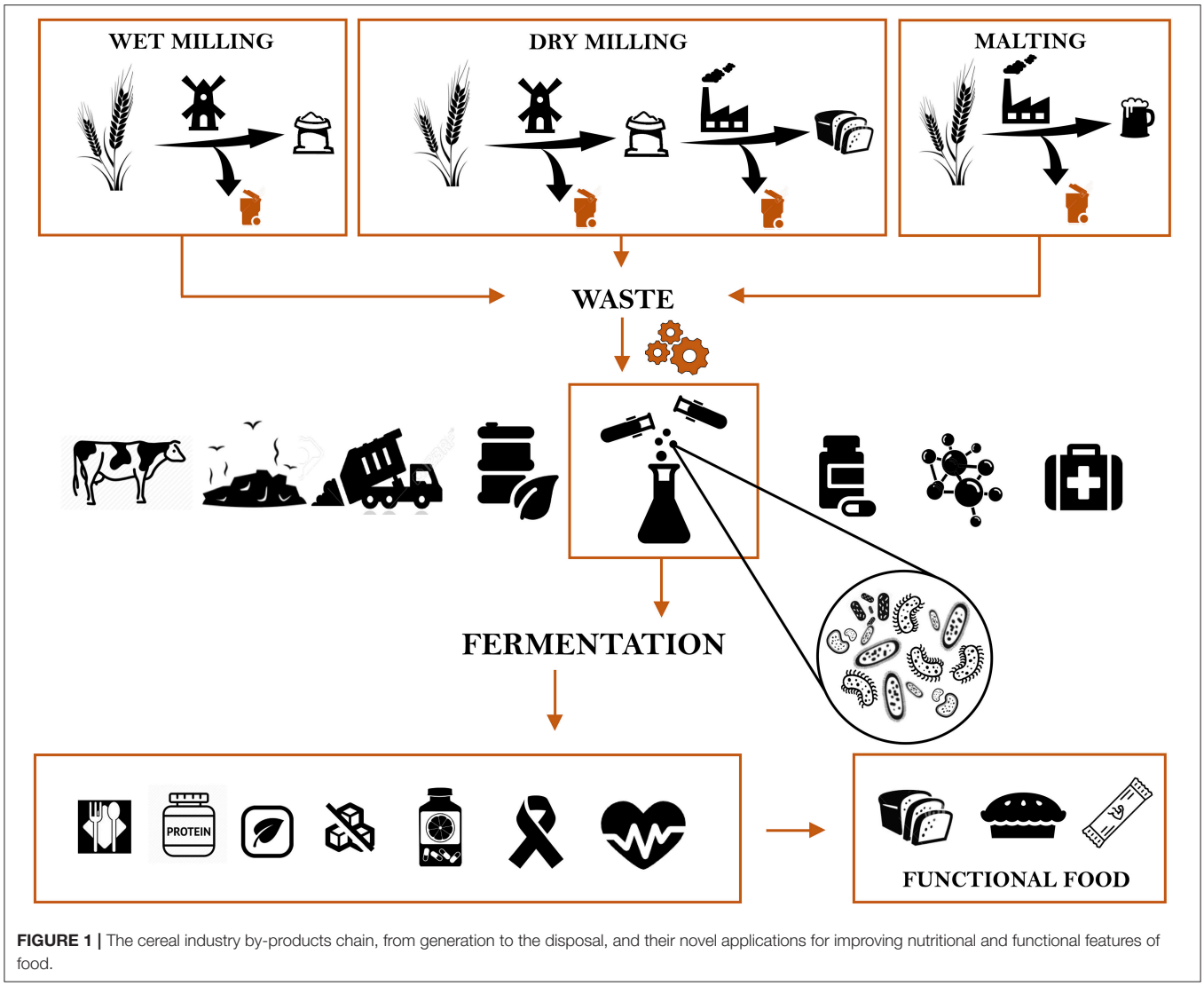

FIGURE 1 | The cereal industry by-products chain, from generation to the disposal, and their novel applications for improving nutritional and functional features of food.

microbial strains (*Lactobacillus brevis* E95612 and *Kazachstania exigua* C81116) combined with hydrolytic enzymes, mainly xylanase, endoglucanase, and β-glucanase was employed to obtain bran with higher nutritional quality than the native one (24). Bioprocessing influenced the microstructure of the bran, causing an extensive breakdown of the cell wall structures entailing an increase of the solubility of arabinoxylans, more than 11-fold compared to the native bran (24). These results confirmed those obtained in a previously study (6) in which yeast fermentation of wheat bran led to a 66% increase in soluble arabinoxylans. Wheat bran was also fermented following a traditional fermentation process consisting of a back-slopping procedure of daily refreshments until a stable microbiota of lactic acid bacteria and yeasts was established. After fermentation, an increase up to 30% of soluble dietary fiber was obtained (25). Similar results were observed in a recent study fermenting bran with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* combined with a commercial baker's yeast (26).

Wheat bran is also a rich source of proteins and amino acids, which, although different from those found in the endosperm, still have high biological and nutritional value (27). However, the bioavailability of proteins in bran is limited by multiple factors, (i) the structure of the layers (composed by insoluble and complexed carbohydrates and lignin) and (ii) the high content of antinutritional factors such as phytate which forms insoluble phytate-protein complexes (28). Because of lactic acid bacteria proteolytic activity and endogenous proteases activated by the low pH, increases of peptides and free amino acids concentration, including the functional non-protein γ-aminobutyrric amino acid (GABA), have been reported during fermentation (24, 25, 29). Consequentially, the *in vitro* digestibility of proteins, which gives information on their stability and on how they withstand to digestive processes, increased. The digestible protein fraction can also give information about the quality of the protein itself. It was indeed proven that the biological value, the ratio of essential amino acids, and the nutritional index were higher in

fermented bran compared to the native one, and further increase of the above indexes was obtained when enzymes were used in combination with microbial fermentation (24). The use of the starter cultures *L. brevis* E-95612 and *Candida humilis* E-96250, especially when cell-wall-degrading enzymes were added, affected not only the release and the composition of free amino acids, improving protein digestibility, but also enabled the release of phenols (29). Hydroxycinnamic acids are the most common phenolic acids found in wheat bran. In particular, ferulic acid, a structural component of the cell walls of aleurone and pericarp is mainly esterified with arabinoxylans, and therefore has a very low bioaccessibility. The potential health effect of ferulic acid is ascribed to its antioxidant properties. In particular, its ability to inhibit the lipid peroxidation and the oxidation of low-density lipoprotein (the main cholesterol carrier in blood), is greater than other hydroxycinnamic acids (30). Ferulic acid also has anti-inflammatory effects (30). Bioprocessing of wheat bran with baker's yeast and an enzymes mixture containing ferulic acid esterase allowed the increase of free phenolic acids content (31). Spontaneous fermentation carried out by lactic acid bacteria (mainly belonging to the genera *Lactobacillus*, *Leuconostoc* and *Pediococcus*) and yeasts was also found to be effective on the release of ferulic acid, which increased of 82% (25). The release of ferulic acid occurred also during fungal fermentation. Among the edible mushrooms employed in a previous study, *Hericium erinaceus* was the one that allowed the highest release of ferulic acid (44% higher than the unfermented bran) due to the combined action of cellulase and ferulic acid esterase which were able to decompose wheat bran cell walls (32). An improvement in total phenol content and antioxidant activity toward free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,20-azino-di-[3-ethylbenzthiazoline sulphonate]) occurred when wheat bran was fermented for 6 days at 30°C with *Aspergillus oryzae* MTCC 3107 (33). Maximum antioxidant activity was noticed for fermented bran, in both methanol and ethanol extracts, compared to fermented wheat intact kernels and flour (33). The role of oxygen during the fermentation of a liquid wheat bran sourdough was also investigated. The amount of oxygen influenced the microbial community, as well as the metabolite profile of fermented bran. It was indeed observed that anaerobic conditions, in which lactic acid bacteria and endogenous heterotrophic bacteria grew better, induced the conversion of ferulic and caffeic acids into their corresponding derivatives, and increased the amount of sinapic acid. On the contrary, wheat bran fermented in aerobic conditions, which favored yeasts growth, was characterized by the presence of the phenolic compounds dihydroxyphenyl ethanol and hydroxyphenylacetaldehyde. Moreover, a higher amino acids content was found after anaerobic fermentation compared to aerobic one (34).

Minerals and vitamins contained in wheat grains are mostly located in the bran fraction, especially in the aleurone layer. The bioavailability of minerals strongly depends on the content of phytic acid, which is generally very abundant in wheat bran and is considered an antinutritional factor. An improvement in phytase activity was observed when both fermentation and enzymes were used to bioprocess bran (24, 29) and a reduction of phytic acid was reported by several authors (25, 26, 35).

Several B-vitamins, mainly niacin, pantothenic acid, biotin, thiamin and small amount of riboflavin are present in wheat bran (30). Yeast fermentation successfully increased the folate content of wheat bran over 40% (36). In the study conditions, folate synthesis, which varies extensively between *Saccharomyces cerevisiae* strains, was also partially ascribed to the presence of indigenous lactic acid bacteria. The fortification in limiting vitamins was also proposed. For instance, the intake of vitamin B12, also known as cobalamin, is mainly possible through consumption of food from animal origin (37), therefore there is a risk of its deficiency for people consuming limited amount of animal food products. Thus, the fortification of plant-based food with this vitamin through fermentation represents a good strategy. *Propionibacterium freudenreichii* DSM 20271, one of the few microorganisms recognized as vitamin B12 producers, was used to ferment wheat bran. The content of the active form of vitamin B12 in fermented bran increased of about 5 times, and a higher content in riboflavin was also detected, proving that bran can be a potential substrate for vitamins synthesis (38).

The use of milling by-products in food processing entails technological drawbacks, which make their application more challenging. For instance, in wheat bread baking, gluten has a key role in the structure formation and bran addition weakens the gluten network structure, therefore affecting the gas-holding capacity of doughs. As a result, volume and elasticity of baked goods decrease (23).

An increase in the phenolic content and therefore in the antioxidant activity of bread containing wheat bran fermented with either yeasts or lactic acid bacteria was reported by few authors (36, 39, 40). Anson et al. (31), fermented wheat bran with baker's yeast and used it to produce a fortified bread having almost 3-fold the amount of free ferulic acid and 8-fold when combined with hydrolytic enzymes treatment. *p*-coumaric and sinapic acids also increased with the processing. When subjected to gastro-intestinal digestion *in vitro*, despite the substantial increase in the bioavailability of phenolic compounds, mostly recovered from the jejunal compartment, only a small part of them was further metabolized in the colon section, especially those that were already partially degraded by the bran fermentation and enzymatic treatments. Colonic metabolites have been found to have anti-inflammatory properties suggesting that bread enriched in fermented wheat bran could show the same properties (31).

Fermented wheat bran was used to prepare a composite wheat-rye bread containing a β-glucan hydrogel. Although the flavonoid content was significantly higher in the experimental bread compared to the control, the concentration of phenolic acids decreased during sourdough fermentation. However, higher radical scavenging activity against both ABTS and DPPH radical were found. The same bread was found to lower the glucose response 120 min after the consumption in a small group of volunteers. Insulin response did not change compared to the control bread (wheat-rye bread), but the authors indicated that an increased amount of fermented wheat bran and β-glucan

could further improve the nutritional impact of the wheat bran-rye bread (41).

Wheat Germ

Wheat germ is a high nutritional value by-product separated during the milling process. It is the primary source of vitamin E in wheat kernel and a rich source of vitamins of the group B, proteins, dietary fiber and minerals (30, 42). Most of the essential amino acids are present in wheat germ proteins at concentrations higher than in the reference egg protein pattern (43, 44). Wheat germ is also rich in unsaturated fatty acids, mainly oleic, linoleic and α-linoleic acids and functional phytochemicals especially flavonoids, sterols, octacosanols, and glutathione (45). However, its consumption is limited by some anti-nutritional factors (raffinose, phytic acid, and wheat germ agglutinin) and by the high lipase and lipoxygenase activity that favor lipid oxidation, negatively affecting the stability of wheat germ (15).

To solve this issue, the effects of sourdough fermentation on wheat germ stabilization were studied. Two lactic acid bacteria (*Lactobacillus plantarum* LB1 and *Lactobacillus rossiae* LB5) isolated from wheat germ were used as starters for sourdough fermentation (46). After 40 days of storage, compared to the raw germ, the fermented one had very low percentage of the aldehydes usually responsible for the rancidity perception, as well as of alcohols, ketones, furanones, and lactones, other volatile compounds occurring in lipid oxidation. The low pH achieved with fermentation was indeed responsible of the lower lipase activity. Fermentation also increased of ca. 50% the concentration of total free amino acids, more specifically Lys, the major limiting amino acid of wheat flour, and GABA were present in fermented wheat germ at the concentration of almost 2 g/kg (46). During sourdough fermentation of wheat germ, the phytase activity increased and an enhanced the bioaccesibility of Ca^{++}, Fe^{++}, K^{+}, Mn^{++}, Na^{+}, and Zn^{++}. Concomitantly, raffinose concentration decreased by 45% and a 33% increase in phenol content occurred, which resulted in higher scavenging activity toward free radical DPPH and ABTS (46). Antioxidant activity in food matrixes is often due to the presence of phenolic compounds; nonetheless, this functional property can also be ascribed to bioactive peptides. Biologically active peptides, often encrypted in the native sequence, can be produced from their protein precursor by digestive enzymes or during food processing (47). The interest toward bioactive peptides from vegetable sources has increased thanks to the recent evidence of their wide potential functional effects (antihypertensive, antioxidant, antitumoral, antiproliferative, hypocholesterolemic, antinflammatory activities) (48). After 48 h of fermentation of a medium composed by 5% of defatted wheat germ, the maximum yield of peptides was obtained. The protein hydrolysate showed high antioxidant activity, determined as scavenging activity on DPPH, hydroxyl, and superoxide radicals (49).

One of the most promising features of fermented wheat germ is represented by the cytotoxic activity toward cancer cell lines. A commercially available wheat germ extract known as Avemar® is obtained by fermentation of wheat germ water-soluble extract with *Saccharomyces cerevisiae*, followed by concentration and drying (50, 51). The anticancer properties of this extract have been shown *in vitro* on various human cancers cell lines (including leukemia, melanoma, breast, colon testicular, head and neck, cervical, ovarian, gastric, thyroid, and brain carcinomas), as well as on the prevention of chemical carcinogenesis, and some autoimmune conditions (50, 52). These features are mainly attributed to two quinones, 2-methoxy benzoquinone and 2,6-dimethoxybenzoquinone which are naturally present in wheat germ as glycosylated and non-physiologically active form. For this purpose, selected strains of lactic acid bacteria possessing high β-glucosidase activity, therefore potentially able to release the two quinones, were used for wheat germ fermentation (53). During 24 h of incubation, the release of the non-glycosylated and physiologically active forms was almost complete. Compared to the control, the concentration of the above bioactive compounds increased up to 6-folds. While no effect was found for the raw wheat germ, the preparation fermented by *Lb. plantarum* LB1 and *Lb. rossiae* LB5 exerted anti-proliferative effect on human tumor cell lines (colon carcinoma and ovarian carcinoma), as showed by *ex vivo* assays (53).

Germ is rarely used for food processing mainly because of the short shelf-life, due to the presence of large amounts of unsaturated fat acids and of hydrolytic and oxidative enzymes (54), as well as to the negative effects on the technological properties of the flour. Fermented wheat germ, having lipase activity lower than raw wheat germ, was incorporated in bread effectively reducing the technological obstacles that prevent its use in baking (55). The concentration of total free amino acids, especially that of lysine, which is limiting in cereals, increased if compared to wheat bread. GABA content increased as well reaching 223 mg/kg. Among the nutritional benefits deriving from wheat germ fermentation and supplementation in bread manufacturing there were improved *in vitro* protein digestibility and decreased phytase activity, all without compromising baking properties (55). In a recent study, a sourdough composed by both wheat germ and bran was used to fortify wheat flour bread (39). In addition to the release of free amino acids and phenolic compounds, which reflected on the antioxidant activity, the fortified bread containing 15% of the above fermented milling by-products had 6.53% of dietary fiber, almost 5% higher than that of wheat flour bread. The *in vitro* and, especially, the *in vivo* glycaemic index were markedly lower in the fortified bread, reaching 36.9%, a value way below the threshold needed for a food product to be considered "low glycaemic index" (39).

Rye Bran

Rye, one of the most important sources of dietary fiber in European Nordic countries, is often used as whole grain flour in the making of cereal based products. Nonetheless, rye bran is also a by-product of conventional milling and can be used as ingredient to increase food nutritional value (56). Besides fibers, the bran fraction is rich in many other bioactive compounds (phenolic acids, phytosterols, tocopherols), among which alkylresorcinols and steryl ferulates have been studied for their cancer preventive and antioxidant potential (7, 57, 58). The influence of fermentation conditions and type of bran (native or peeled) on the levels of bioactive compounds was studied (59). Bran fractions, deriving from native or peeled grains were

fermented with baker's yeast for 6-20 h at temperatures ranging from 20 to 35°C. Fermentation of both peeled and native bran increased free ferulic acid and free total phenolics, indicating increased liberation of bound phenolic compounds from the polymeric rye bran structure. The increase in free phenolics after fermentation was reported to be 90% for native rye bran, and 30% for peeled bran (59). The level of folates increased over 100% with fermentation and the highest level was obtained when higher growth of indigenous lactic acid bacteria occurred, which was strongly dependent on fermentation conditions (higher temperature and longer time). However, the strong acidity was found to be deleterious for cinnamoyl esterases, the enzyme responsible for the release of ferulic acid esterified to the arabinofuranosyl residues (60). For this reason, the highest level of free ferulic acid was obtained in fermentation conditions with a pH value of 6–6.5 (59).

Several authors studied the influence of rye bran bioprocessing on bread nutritional quality. When combining the action of hydrolytic enzymes and baker's yeast fermentation the main change was represented by the degradation of the cell wall structure which consequentially led to an increase in the solubility of dietary fibers, arabinoxylans, proteins, and free phenolics (61). As shown by the *in vitro* colon model, bioprocessing made carbohydrates more fermentable by the microbiota and allowed a higher release of ferulic acid. In a follow up study, the phenolic profile of bread fortified with bioprocessed rye bran was evaluated (62). *p*-cumaric, sinapic, and caffeic acids were detected at concentration 20–30-fold higher than the bread containing native rye bran. Additionally, although a slight alkylresorcinol degradation was observed, benzoxazinoid aglycones increased after the enzymatic bioprocessing. Despite the extensive phenolic acid release caused by the bioprocessing, when breads were subjected to *in vitro* colon model, only subtle differences were observed in the microbial metabolites (63).

Rye bran proved to be an excellent substrate for the synthesis of polyunsaturated fatty acids and β-carotene by fungal solid-state fermentation (64). Four *Mucor* spp. strains selected based on the ability to synthesize γ-linolenic acid (GLA) and β-carotene were used. Compared to oat flakes, barley groats and wheat bran, rye bran fermented with *Mucor circinelloides* CCF-2617 contained the highest content of both compounds, which was further increased by the addition of brewers' spent grain (64).

Rye and wheat bran were used as substrates for the synthesis of exopolysaccharides through lactic acid bacteria fermentation. Fermentation with *Lactobacillus reuteri* in the presence of sucrose enabled high glucan formation within 8 h of incubation of rye bran (65). In a recent study, two strains of *Weissella confusa*, known for the ability to produce significant amounts of dextran, were employed to ferment both wheat and rye bran. Rye bran proved to be an optimal substrate for *in situ* dextran production, reaching concentrations of 2–3% on dry matter (66). The *in situ* synthesis of EPS and oligosaccharides represents a way to obtain a food substrate with increased functional properties. In fact, they may act as prebiotic and have been shown to possess antitumor and immunomodulating activities *in vitro* (67). Additionally, EPS also act as hydrocolloids and can improve the technological properties of material like cereal bran, otherwise characterized by poor structure-forming capacity.

Rice Germ and Bran

Asian Countries are the major producers of rice, representing 50% of the daily energy supply of the diet of the local population (68). From the commercial white rice, germ and bran are removed because the oils they contain are quickly subjected to rancidity, reducing its shelf-life (7). It is estimated that every year 120,000 tons of rice husks alone are wasted worldwide (10). Rice milling by-products are currently underutilized, since their further exploitation is possible. Rice bran oils and proteins have demonstrated antioxidant properties and chronic disease preventing activity, particularly toward cardiovascular disease and certain cancers (69–71). However, the content of these bioactive compounds is not equally distributed among rice varieties (72). Microbial fermentation of rice by-products is an emerging area of scientific and industrial research. Rice bran fermented with *S. cerevisiae* was shown to exert anti-stress and anti-fatigue effects on rats (73). Moreover, water-soluble extracts of fermented rice bran had an anti-photoaging effect on human skin fibroblasts cultures (74). During the last decade, solid-state fungal fermentation of rice bran was extensively studied. The main results achieved concerned the increase of protein content and antioxidant activity (75–77), particularly efficient when the substrate had small particle size (0.18 mm) (78). When defatted rice bran was fermented with *Rhizopus sp.* and *Aspergillus oryzae*, a high amino acids release and consequently a substantial (from 37.5 to 54.3%) chemical score increase (79) were obtained. Apart from proteins, fibers and minerals, rice bran is a good source of oil, which can reach up to 20% of its weight (70). Fermentation with either *Rhizopus oryzae* or *A. oryzae* significantly increased palmitic and linoleic acids content, causing a decrease in saturated fatty acids and an increase in the unsaturated ones (80, 81), thus improving the overall nutritional quality; additionally, when *R. oryzae* was used as starter for rice bran fermentation, a 10% reduction of total lipid content was also observed (80).

Several studies investigated rice bran anticancer properties. Rice bran fermentation with *Lentinus edodes* allowed the production of an exo-biopolymer, consisting of sugars (mainly arabinose, galactose, glucose, mannose, and xylose), uronic acid and a small amount of proteins. Oral administration of the polysaccharide extract induced the activation of natural killer cells and prolonged the life spans of mice transplanted with Sarcoma-180 cells, while inhibiting cancerous cells growth in the intraperitoneum (82). Metabolite formation from extracts of rice bran fermented with *Saccharomyces boulardii* were studied by Ryan et al. (71). It was indeed observed that fermentation altered bioactive compounds, reducing the growth of human B lymphomas *in vitro*. Another study showed that the extract obtained by co-fermentation of rice bran with *Lactobacillus rhamnosus* and *S. cerevisiae*, was able to reduce the cytotoxicity and inhibit melanogenesis in B16F1 melanoma through downregulation of microphthalmia-associated transcription factor (83). Anticancer properties were also found in brown rice bran fermented with *Aspergillus oryzae*.

It was demonstrated that fermented rice bran acts as preventive agent against colon carcinogenesis in rats (84).

The antioxidant potential of fermented rice bran was also studied. Phenolic compounds are present in rice bran at high concentrations, however, 70% of them are esterified to the arabinoxylans present in the bran cell wall. Rice bran fermented by *Issatchenkia orientalis,* a yeast isolated from rice bran, showed higher free phenolic content compared to the native one. The extracts strongly inhibited reactive oxygen species generation and ameliorated oxidative stress-induced insulin resistance by neutralizing free radicals and upregulating adiponectin in adipocytes (85). Phenolic extracts obtained by solid-state fermentation of rice bran with *Rhizopus oryzae*, were evaluated for their ability to reduce free radical DPPH and inhibit the enzymes peroxidase and polyphenol oxidase (86). Compared to the native bran, the phenolic content doubled with fermentation and changed in composition, in fact, ferulic acid increased over 20-fold reaching 765 mg/g in fermented bran. Although no inhibition of the polyphenol oxidase enzyme was found, the phenolic extracts DPPH scavenging and peroxidase inhibitory activities (86). The release of phenolic compounds from the bran was also obtained by fermenting heat-stabilized defatted bran with *Bacillus subtilis* subsp. *subtilis*. Compared to the control, which only had low levels of *p*-cumaric and ferulic acids, fermentation allowed the release of gentistic, caffeic, syringic, p-coumaric, ferulic, sinapic, and benzoic acids (87). The potential of fungal solid-state fermentation on the release of bioactive compound having antioxidant activity was investigated (88). Changes in the phenolic profile by *Rhizopus oligosporus* and *Monascus purpureus* fermentation used as single or mixed starters were observed. Although total polyphenols content and ferric reducing ability of plasma increased upon fermentation, DPPH radical-scavenging activity decreased in some cases, due to a different composition in phenolic acids. Ferulic acid was the only phenolic acid present in all samples, before and after fermentation, whereas vanillic, caffeic, and 4-hydroxybenzoic acids were found only after fermentation. The use of the two fungi combined was the condition that allowed the highest release of ferulic acid (almost 8-fold higher than the unfermented bran) (88).

The other by-product of rice milling is the germ, commonly separated by sieving and vibrating rice bran (68). The literature on rice germ is very limited, and studies on fermentation of rice germ initiated only few years ago. Extracts from rice germ fermented by the GABA-producing *Lactobacillus sakei* B2-16 accumulated 15% (of dry weight) of GABA and were found to have a positive impact on sleep disturbance in mice (89).

Regarding their use as food ingredients, the literature is very meager. Fermented rice bran was used in bread-making with the aim of balancing the lacking essential amino acids and enriching the protein content of wheat-based products. The authors evaluated the effect of different substitution levels of wheat with protein concentrates from natural and yeast fermented rice bran. At 10% substitution level, the composite bread had higher total amino acid content than control wheat bread. An increase in the radical scavenging activity and ferric reducing ability power were also observed (90).

Milling By-Products From Other Cereals

Barley and oat significantly differ in their chemical composition from other cereals; their cell walls are rich in the non-starchy polysaccharide β-glucan, which is the major component of the soluble dietary fiber, and has been associated with the reduction of plasma cholesterol and glycemic index, and a decreased risk of colon cancer (91). Despite the beneficial advantages deriving from the consumption of barley and oat dietary fibers, very little information in the literature deals with the fermentation of their by-products. Catechin and proanthocyanidins are among the polyphenol compounds contained in barley bran. Hordeumin, an anthocyanidin-tannin purple pigment produced from barley bran fermented using *Salmonella typhimurium*, was found to have antimutagen properties (92). Barley bran hydrolysates were used to obtain xylitol through bioconversion of xylose-containing solutions by the yeast *Debaryomyces hansenii* under microaerophilic conditions (93). Xylitol is employed in the food industry to manufacture sugar-free products because of its high sweetening power, anticaries properties, and its tolerance by diabetics (94).

Fermentation was used as means to enrich oat bran with folate. Folate is a generic name for several derivatives of pteroylglutamic acid (folic acid) and is necessary for methylation reactions in cell metabolism and for neural development of fetus during pregnancy (95). Oat bran was fermented with yeasts isolated from barley kernels and selected for the ability to synthesize folate, alone or together with lactic acid bacteria isolated from oat bran. The best folate producers were *S. cerevisiae*, followed by *Pseudozyma* sp., *Rhodotorula glutinis,* and *Kluyveromyces marxianus*. Many yeasts, beyond the considerable amount of folate produced, caused a decrease in the viscosity, suggesting a possible generation of soluble fibers, with positive repercussion on the nutritional effect. When inoculated together with *Streptococcus thermophilus* or *L. rhamnosus*, *S. cerevisiae* and *Candida milleri* produced significant amount of folates reaching 120 ng/g, suggesting that the consumption of 100 g of fermented oat bran could represents 15% of the recommended folates daily intake (95). Fermentation of oat bran with rye sourdough, previously obtained with a commercial starter culture containing lactobacilli and *Candida milleri*, allowed to double protein and β-glucan solubility (96). Since oat β-glucan can stimulate the growth of *L. rhamnosus*, a fermented oat bran suspension was used as a carrier for the probiotic strain. A simulator of the human intestinal microbial ecosystem (SHIME) was used to evaluate the effect on gut microbiota, concluding that *Lb. rhamnosus* colonized the SHIME and oat bran favored the growth of bifidobacteria (97).

Lactobacillus plantarum T6B10 and *Weissella confusa* BAN8 were used as selected starters to ferment maize milling by-products mixtures made with raw or heat-treated germ and bran (98). Lactic acid bacteria metabolisms improved the free amino acids and peptides concentrations as well as the antioxidant activity and induced phytic acid degradation. As previously reported for wheat germ (46), fermentation allowed the decrease of the endogenous lipase activity, stabilizing the

matrix by preventing oxidative processes. When fermented maize by-products were used as ingredient for bread making (25% on total weight) dietary fiber and proteins content were of ca. 11% and 13% of dry matter, respectively. Compared to the use of the same amount of unfermented ones, the addition of pre-fermented maize by-products to bread caused a significant increase in protein digestibility (up to 60%), and a relevant decrease of the starch hydrolysis index (ca. 13%) (98).

FERMENTATION OF THE CEREAL INDUSTRY WASTE

Brewers' Spent Grain

Brewers' spent grain (BSG) is recovered from mashing, one of the initial steps of brewing. During the boiling process, all soluble matter is extracted into the mash from the barley malt which, after lautering (or mash filtration), is separated into wort (liquid) and spent grain (solid) components. BSG represents 85% of the total residues from the brewing process and it is estimated that 30,000 tons of BSG are wasted yearly worldwide. The main current destination of BSG is cattle feed or discarded (10). BSG is rich in cellulose (17%) and non-cellulosic polysaccharides, especially arabinoxylans from the barley grain hull (39%) (99). BSG contains up to 20% of proteins, particularly rich in lysine and approximately 30% of the total protein content is made of essential amino acids (100). Apart from uses in animal nutrition or recovery of valuable compounds such as carbohydrates, proteins and phenolic compounds (101), thanks to the health benefits associated with BSG ingestion, some attempts to exploit its use in food industry have also been made. The research so far has shown that due to BSG challenging technological properties, pre-treatments aiming at reducing its detrimental impact on food quality are necessary (101). Overall, milling and bioprocessing technologies, including fermentation, are potential means to use more of the BSG in food applications, conferring important health benefits. In one study, BSG was used as substrate to immobilize *Lactobacillus casei* suggesting that BSG can act as prebiotic, stimulating lactic acid bacteria growth (102).

A fermented liquid product from BSG having nutraceuticals properties was also developed. After a first substrate optimization phase aiming at improving bacterial growth and polyphenolic compounds release, *L. plantarum* ATCC 8014 was used to produce a fermented beverage displaying high antioxidant potential, due to the high content of total phenolic compounds and flavonoids released during the fermentation, as consequence of the acidification and microbial metabolism (103). Bread containing BSG fermented with a strain of *L. plantarum*, was positively judged for the main structural and sensory properties. Thanks to the high content of proteins, fibers and lysine, a 10% replacement of wheat flour with either BSG or fermented BSG, improved the nutritional properties of the resulting bread (104). Spontaneous fermentation of BSG also showed positive impact on bread properties, characterized by lower levels of phytic acid (more than 30% lower compared to the native counterpart) and higher antioxidant activity (up to 36%) compared to the bread containing unfermented BSG (105).

Other Cereal-Derived Waste: Bread and Starch

On a global scale, one of the major food waste is bread, reaching thousands of tons daily. Industrial bread waste is generated at different stages: during the manufacturing process, because of substandard products or other processing factors such as crusts removal for sandwich bread production, or as unsold bread from retail (106). If not discarded or used as feed, over the last decades, wasted bread was used to produce chemicals, aroma compounds, enzymes and biofuels (106). The processes to produce a seasoning sauce from the hydrolysis of wheat bread or to produce a syrup from bioprocessed bread were even patented (107, 108). The first attempt involving lactic acid bacteria fermentation was proposed a couple of decades ago. In this study, more than one hundred starters were screened based on the ability to acidify a medium containing bread crumbs. Three strains of *L. plantarum*, *Staphylococcus carnosus*, *Pediococcus acidilactici* and *Micrococcus* spp. were selected as able to resist for long fermentation periods (48–96 h) without producing off-flavors (109). A sourdough containing bread waste was produced with the aim of enhancing bread aroma and flavor. Fermentation with a commercial *L. plantarum* strain as starter (35°C for 48 h) of a dough made of 50% whole wheat bread crumb, favored the highest organic acids production (109). Although no specific nutritional features have been highlighted by the authors for the fermented bread, the role of the organic acids, the main responsible of reduced glycaemia and insulinemic responses, should be considered. Indeed, lactic acid lowers the rate of starch digestion in bread whereas acetic and propionic acids appear instead to prolong the gastric emptying rate (110). Therefore, it is assumable that fermented bread can have similar features of sourdough.

Starch production industry mostly interests cereals such as maize, rice, and wheat, and crops like potato, and like any other industry, has its own by-products. Broken rice, which is an inevitable by-product of rice milling, can be used to extract powder and crystal starch generating another by-product rich in proteins (111). The protein residue of starch extraction was treated with a combination of enzyme hydrolysis and microbial fermentation. The hydrolysate resulting from the action of proteolytic enzyme and a cell suspension of *Bacillus pumilus* AG1 displayed antioxidant activity toward ABTS radical. Almost all the peptides contained in the hydrolysate showed one or more features typical of well-known antioxidant peptides, most probably conferring a synergic antioxidant effect to the mixture with the potential to be used as functional ingredient for novel food formulations (112).

Trends and Perspectives

The future bio-economy concept, based on a more sustainable use of agricultural by-products, will require a more efficient utilization of side streams and waste from food processing industry to reduce the environmental burden of their generation and disposal. The exploitation of cereal by-products for the extraction of their functional compounds, whether for food, cosmetics, or pharmaceutical industry, offers promising

TABLE 1 | Main nutritional and functional effects of the use of fermentation in cereal industry by-products.

Cereal by-product	Bioprocessing employed	Effect	References
Wheat bran	*Lb. brevis* E95612 and *K. exigua* C81116 with enzymes; baker's yeast; spontaneous fermentation; *Lb. bulgaricus* and *St. thermophilus* combined with baker's yeast	Higher fiber solubility	(24–26, 59)
	Lb. brevis E-95612 and *Candida humilis* E-96250 with cell wall-degrading enzymes; *Lb. brevis* E95612 and *K. exigua* C81116 with enzymes; spontaneous fermentation	Increased peptides and free amino acids content and *in vitro* protein digestibility	(24, 25, 29)
	Spontaneous fermentation; baker's yeast; *Lb. bulgaricus* and *St. thermophilus* combined with baker's yeast	Decreased phytic acid content	(25, 26, 35)
	Baker's yeast; *A. oryzae* MTCC 3107; *Hericium erinaceus;* spontaneous fermentation; lactic acid bacteria and yeasts with enzymes	Higher phenols content and antioxidant activity	(25, 31–34)
	Propionibacterium freudenreichii DSM 20271	Fortification in vitamins	(38)
	Mucor spp.	Increase of gamma-linolenic acid and β-carotene content	(64)
Wheat germ	*Lb. plantarum* LB1 and *Lb. rossiae* LB5	Increased free amino acids content, protein and minerals bioavailability, decreased anti nutritional factors	(46)
	B. subtilis B1; *Lb. plantarum* LB1 and *Lb. rossiae* LB5	Increased antioxidant activity due to phenolics or bioactive peptides	(46, 49)
	S. cerevisiae; *Lb. plantarum* LB1 and *Lb. rossiae* LB5	*In vitro* and *ex vivo* anticancer and antiproliferative properties	(50–53)
Rye bran	Baker's yeasts	Release of phenolic compounds, increase of folates content	(59)
	Lactobacillus reuteri; *Weissella confusa*	Exopolysaccarides synthesis	(65, 66)
	Mucor spp.	Increase of gamma-linolenic acid and β-carotene content	(64)
Rice bran	*S. cerevisiae*	Anti-stress, anti-fatigue and anti-photoaging effect	(73, 74)
	Aspergillus oryzae; Rhizopus oryzae; *Rhizopus oligosporus and Monascus purpureus; Lb. rhamnosus* and *S. cerevisiae*;	Higher protein and phenols content and antioxidant activity	(75–78, 85–88, 113)
	Aspergillus oryzae; Rhizopus oryzae	Decrease of saturated fatty acids and total lipids and increase of unsaturated fatty acids	(80, 81)
	Lentinus edodes; *A. oryzae*; *S. boulardii*; *Issatchenkia orientalis*;	*in vivo* and *ex vivo* anticancer and antiproliferative properties	(71, 82–84)
Rice germ	Spontaneous fermentation	Increased in GABA content and improvement of sleep disturbances in mice	(89)
Barley bran	*Salmonella typhimurium*; *Debaryomyces hansenii*	Production of phenolics and xylitol	(92, 93)
Oat bran	*Streptococcus thermophilus, Lb. rhamnosus*, *S. cerevisiae* and *C. milleri*	Folic acid fortification	(95)
	C. milleri	Higher fiber solubility	(96)
BSG	*Lb. plantarum* ATCC 8014	Higher phenols content and antioxidant activity	(103)
Bread waste	*Lb. plantarum*	Production of a sourdough with high content in organic acids	(109)
Protein from rice starch extraction	*Bacillus pumilus* AG1	Bioactive peptides with antioxidant activity	(112)

alternative to synthetic compounds and it is an increasing trend. Nevertheless, this approach implies that more by-products will be generated once the specific compound is extracted. Furthermore, if the generation of by-products from food industry is unavoidable, the best possible valorization of these by-products should be sought which, in the case of by-products still fit for human consumption, as described in this review, implies their re-utilization within the food chain.

As recently pointed out by the EAT Lancet report[1], a diet rich in plant-derived food and less relying on animal derived foods is the most beneficial for human health and environment[1]. In this context, the use of the fiber and protein rich part of cereal by-products in food formulations represents a very good opportunity to enrich our diet with beneficial compounds. To contribute to the above objective, the development of technologies allowing the use of the whole by-product, without the undesirable features and with improved nutritional quality is a crucial step.

[1]https://eatforum.org/eat-lancet-commission/

By exerting an impact on the nutritional properties and potential health effects of bran, germ and all the by-products of the cereal industry, fermentation technology well-responds to the challenge of turning poorly utilized waste into products of interest (**Table 1**). Increased minerals and vitamins bioavailability, protein content and digestibility, fiber and phenolic compounds solubility are the most common aspects fermentation acts on. However, in some cases other effects have been found, such as the production of bioactive compounds with anti-cancer properties *in vitro*. Some of the nutritional and functional properties of these by-products are well-known, others less, and despite the need and the increased awareness of the impact of diet on health, sensory and organoleptic properties remain the main drivers of consumers' choice. Unfortunately, health benefits and good taste or even appearance, do not always go together; thus, the modulation of processing parameters is required to reach a balance between desired and undesired features. Therefore, more research on the supplementation of these by-products, fermented or as such is needed not only to fill the current technological gaps but also to validate with *in vivo* studies the benefits found *in vitro*: the potential outcome of this approach is worth to be explored further.

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One-Step Process for Environment-Friendly Preparation of Agar Oligosaccharides From *Gracilaria lemaneiformis* by the Action of *Flammeovirga* sp. OC4

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Oligosaccharides extracted from agar *Gracilaria lemaneiformis* (*G. lemaneiformis*) have stronger physiological activities and a higher value than agar itself, but the pollution caused by the extraction process greatly restricts the sustainable use of agar. In this study, four bacterial strains with a high ability to degrade *G. lemaneiformis* were isolated from seawater by *in situ* enrichment in the deep sea. Among them, *Flammeovirga* sp. OC4, identified by morphological observation and its 16S rRNA sequencing (98.07% similarity to type strain JL-4 of *Flammeovirga aprica*), was selected. The optimum temperature and pH of crude enzyme produced by *Flammeovirga* sp. OC4 were 50°C and 8, respectively. More than 60% of the maximum enzyme activity remained after storage at pH 5.0–10.0 for 60 min. Both Mn^{2+} and Ba^{2+} could enhance the enzyme activity. A "one-step process" for preparation of oligosaccharides from *G. lemaneiformis* was established using *Flammeovirga* sp. OC4. After optimization of the Plackett–Burman (PB) design and response surface methodology (RSM), the yield of oligosaccharides was increased by 36.1% from 2.71 to 3.09 g L^{-1} in a 250-mL fermenter with optimized parameters: 30 g L^{-1} *G. lemaneiformis* powder, 4.84 g L^{-1} $(NH4)_2SO_4$, 44.8-mL working medium volume at 36.7°C, and a shaking speed of 200 × *g* for 42 h. The extracted oligosaccharides were identified by thin layer chromatography (TLC) and ion chromatography, which consisted of neoagarobiose, agarotriose, neoagarotetraose, agaropentaose, and neoagarohexaose. These results provided an alternative approach for environment-friendly and sustainable utilization of algae.

Keywords: ***Gracilaria lemaneiformis*, oligosaccharides, *Flammeovirga* sp. OC4, one-step process, response surface methodology, optimization**

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INTRODUCTION

Oligosaccharides are carbohydrates that contain 2–20 sugar units joined by glycoside bonds. Some oligosaccharides are biologically active substances that possess antimicrobial activity, immunoenhancing ability, antitumor activity (Fang et al., 2012; Zou et al., 2016; Chen et al., 2017), and antioxidant activity (Chen et al., 2015; Malunga and Beta, 2015; Hamley-Bennett et al., 2016)

and have great potential in nutrition (Kashtanova et al., 2016; Marcotuli et al., 2016), health care (Mensink et al., 2015; Zhang et al., 2016; Di et al., 2017), disease diagnosis, and control (Kazłowski et al., 2012; Zhang et al., 2016). To date, oligosaccharides derived from plants and chitins have been used widely (Wojtkowiak et al., 2013; Hamley-Bennett et al., 2016; Yin et al., 2016). On the other hand, algae, an inexpensive and abundant biomass, is attracting increasing attention because it can be used for the production of algae oligosaccharides that have better physiological activity and a higher value than algae polysaccharides (such as agar, carrageenan, and alginate). However, current studies are mostly focused on agarase (Kim et al., 2017, 2018; Qu et al., 2018; Schultz-Johansen et al., 2018), and little has been done in the direct and efficient preparation process of algae oligosaccharides from algae. Currently, algae oligosaccharides are prepared by a two-step process: step 1 is the extraction of algae polysaccharides from algae, which consumes a lot of strong acids and alkalis, diatomite and perlite, and produces useless algal dregs; step 2 is the degradation of polysaccharides by expensive agarases or strong acids, which can result in great pollution to the environment. However, with growing ecological awareness, the preparation process with high pollution has raised concerns in the food industry and is becoming a major obstacle limiting the sustainable use of algae oligosaccharides. Thus, there is a need to develop a more efficient and environment-friendly approach of direct enzymatic degradation of algae for oligosaccharide production, which is also named "one-step process."

To establish a "one-step process," suitable agarolytic bacterial strains that can produce enzymes should be screened first. The deep sea environment harbors a massive pool of biodiversity, which is a good source for agarolytic bacterial strains (Kamjam et al., 2017; Huo et al., 2018). Because of extreme ecological environment such as high pressure (Case et al., 2017), high or low temperature (Mino et al., 2013), and oligotrophic environment (Picard and Ferdelman, 2011; Sohlberg et al., 2015), deep sea microorganisms can form unique physiological structures and metabolic mechanisms, which are excellent sources for acquiring the strain with an inventory of enzymes that can be used for producing oligosaccharides directly from algae.

Fermentation conditions of the deep sea strains are important parameters for biotransformation to oligosaccharides, which should be optimized to achieve higher productivity of oligosaccharides. Several techniques have widely been used to increase the production of oligosaccharides, including the experimental–statistical method (Dotsenko et al., 2016; Sen et al., 2016), the two-level Plackett–Burman (PB) design (Purama and Goyal, 2008; Zhou et al., 2014), and the response surface methodology (RSM) (Yoshida et al., 2010; Vega and Zúniga-Hansen, 2011).

In this study, some agarolytic bacterial strains were isolated from the deep sea through *in situ* enrichment. *Gracilaria lemaneiformis* (*G. lemaneiformis*) was used as the sole carbon source. One of these isolated agarolytic strains was selected to produce oligosaccharides by degrading *G. lemaneiformis*. PB design was used to investigate the factors affecting the production of oligosaccharides, and the RSM method was used to further optimize the parameters of biotransformation to oligosaccharides from *G. lemaneiformis*. The objectives of this study were to provide a theoretical basis for producing algae oligosaccharides from *G. lemaneiformis* with less pollution to the environment and to provide algae oligosaccharides for potential use in agro-food systems and in the nutraceutical industries.

MATERIALS AND METHODS

In situ Enrichment in the Deep Sea

Gracilaria lemaneiformis was washed by tap water and spin dried, which was repeated three times, and then air dried. The sample was then cut 2 or 3 cm in length and ground to powder for further use. The deep sea strains were enriched using a special *in situ* sampling equipment, which was loaded with *G. lemaneiformis* powders, and placed at the sampling site in the sea water with

TABLE 1 | Experimental variables at different levels for oligosaccharide production by *Flammeovirga* sp. OC4 using the PB design.

Symbol code	Variables	Units	Level	
			−1	+1
A	Peptone	g L^{-1}	2	5
B	KCl	g L^{-1}	10	15
C	Dummy	–	–	–
D	$(NH_4)_2SO_4$	g L^{-1}	2	6
E	Initial pH	–	7	8
F	Dummy	–	–	–
G	Inocula	% (*v/v*)	3	5
H	Medium volume	mL	40	60
J	Dummy	–	–	–
K	Temperature	°C	33	37
L	Dummy	–	–	–

PB, Plackett–Burman.

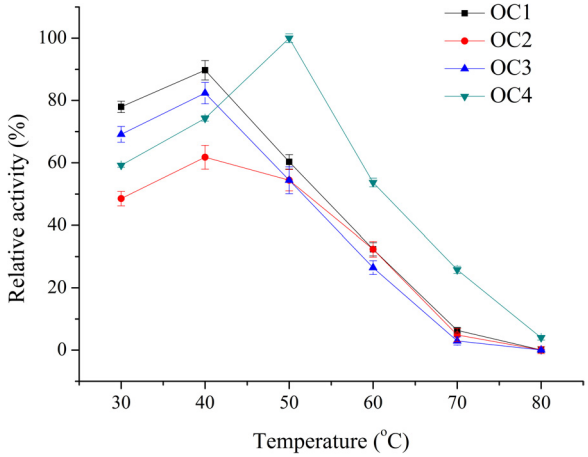

FIGURE 1 | Temperature effect on the activity of the crude enzyme solution.

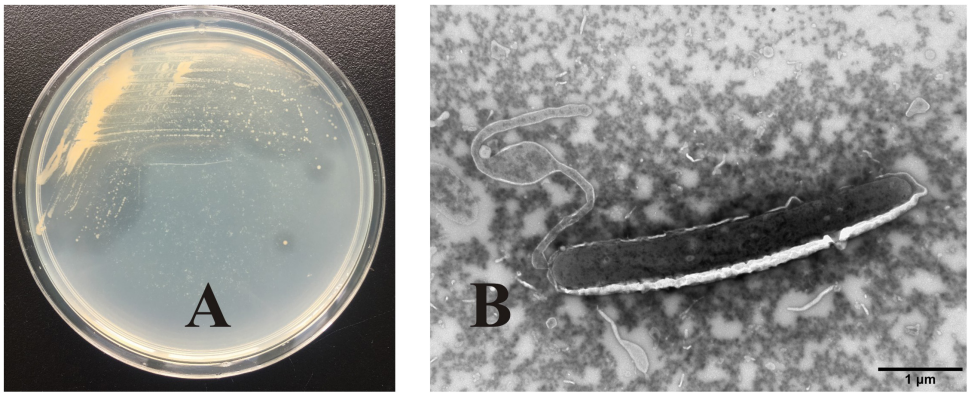

FIGURE 2 | Colony morphology and cell morphology identification of strain OC4. **(A)** Colony morphology. **(B)** Cell morphology.

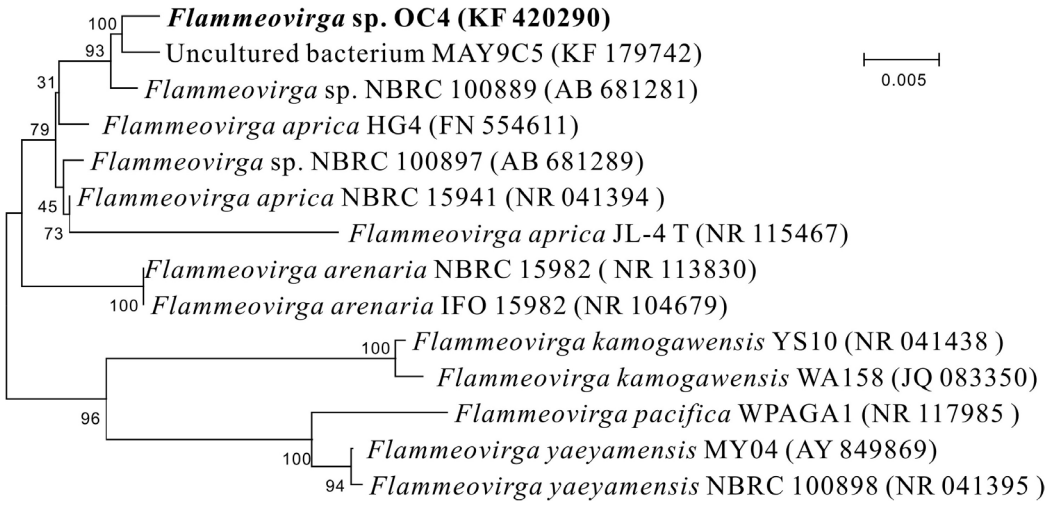

FIGURE 3 | Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of *Flammeovirga* sp. OC4 and representatives of related taxa. Bootstrap values based on 100 replications are shown at branch nodes. The scale bar indicates the average number of substitutions per site.

a depth of 2,000 m, 21°03′N, 118°23′E of the South China Sea for 1 year (**Supplementary Figure S1**).

Screening and Identification of Agarolytic Strains

The enriched samples were transferred onto the plate of the basic culture medium (BCM) that contained the following components (g L^{-1}): *G. lemaneiformis* powder, 30; peptone, 5; $(NH_4)_2SO_4$, 4; KCl, 10. Strains with a transparent ring were acquired and purified. The optimal temperature and relative activity of the crude enzyme of the strains were investigated by shake flask experiments. The single colony with a transparent circle was selected and transferred to another BCM plate and cultured at 28°C. The newly grown strains were observed and photographed under a transmission electron microscope (TEM). A bacterial genomic DNA extraction kit (Sangon Biotech. Co., Ltd., Shanghai, China) was used to extract the genomic DNA of the strain as a template for PCR amplification of 16S rRNA with an upstream primer (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′) and a reverse primer (1492R: 5′-GGTTACCTTGTTACGACTT-3′) (Delong, 1992) with the following program: 94°C for 4 min (94°C for 30 s, 55°C for 30 s, 72°C for 60 s, repeated 30 times); 72°C for 5 min. Phylogenetic analysis was performed using MEGA version 5.1 after multiple alignment of data using DNAMAN version 7.0.

Characterization of Crude Agarase Enzyme

The strain mentioned above was cultured in BCM by shaking at 28°C for 36 h and then centrifuged at 13,000 × *g* for 10 min to acquire the supernatant as crude enzyme solution, whose activity was tested by detecting the release of oligosaccharides according to the 3,5-dinitrosalicylic acid (DNS) method (Hou et al., 2015; Chen et al., 2016).

The effect of temperature was studied by measuring the enzymatic activity at several temperatures ranging from 30

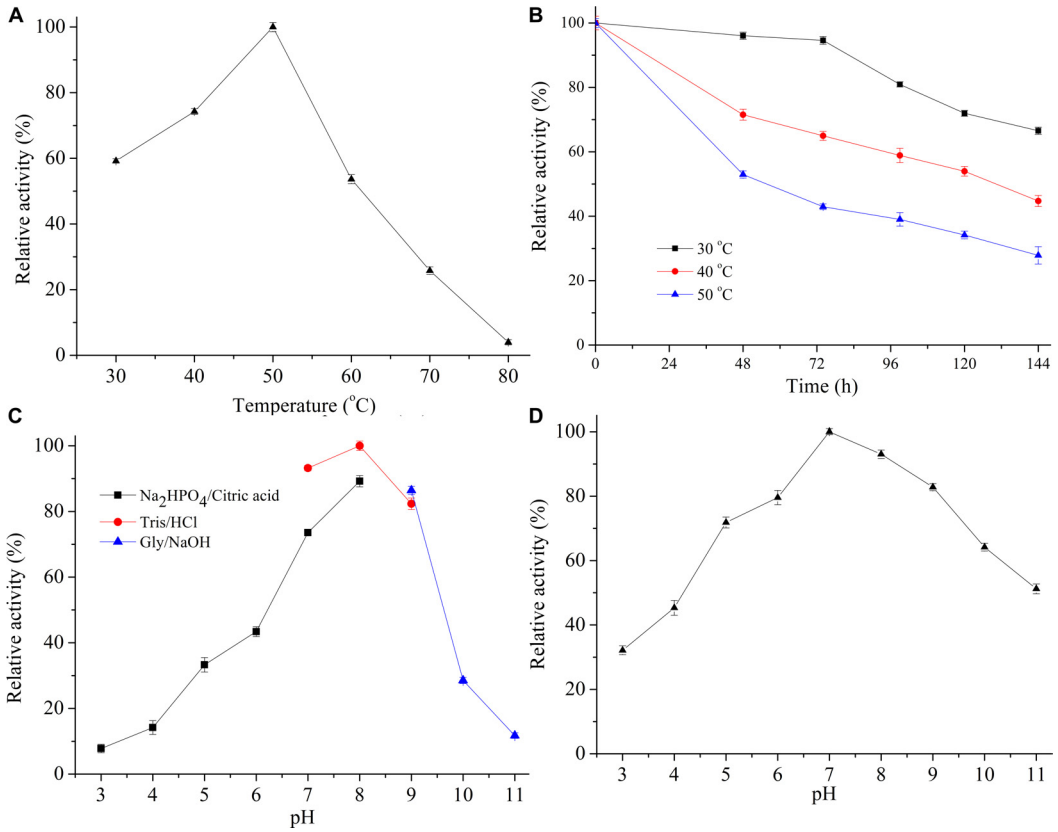

FIGURE 4 | Effect of temperature and pH on enzyme activity of *Flammeovirga* sp. OC4. **(A)** The effect of temperature on enzyme activity. The activity was detected at the temperature ranges from 30 to 80°C at pH 7.4. **(B)** The effect of temperature on the stability of the enzyme. The remaining activity was measured after incubating crude enzyme in the absence of substrate at 30°C, 40°C, and 50°C for various durations. **(C)** pH effects on the activity of crude enzyme. pH profiles were determined by incubating crude enzyme at 50°C in the following buffers: Na2HPO4/citric acid solution (pH 3.0–8.0), Tris-HCl buffer (pH 7.0–9.0), and Gly/NaOH buffer (pH 9.0–11.0). **(D)** pH effects on the stability of crude enzyme. Prior to determination of residual activity, the crude enzyme was first incubated in buffers of desired pH (pH 3.0–11.0) at 50°C for 60 min. For all of the above plots, values are presented as percentages of the maximum activity of crude enzyme (taken as 100%) and are expressed as mean of three parallel trials with standard deviation.

TABLE 2 | Effects of metal ions and chemical agents on the enzyme activity.

Metal ion (10 mM)	Relative activity (%)	Metal ion (10 mM)	Relative activity (%)
K^+	58.49 ± 2.73	Fe^{2+}	92.02 ± 4.03
Na^+	55.03 ± 3.05	Zn^{2+}	48.38 ± 1.89
Sr^{2+}	94.91 ± 1.95	Ba^{2+}	107.83 ± 2.33
Mg^{2+}	74.56 ± 4.16	Ca^{2+}	60.23 ± 3.21
Cu^{2+}	40.57 ± 3.17	Co^{2+}	96.35 ± 2.45
Mn^{2+}	114.56 ± 2.56	EDTA	51.56 ± 1.68
Control	100		

to 80°C at pH 7.4 of phosphate-buffered saline (PBS) buffer. Meanwhile, crude enzyme in pH 7.4 of PBS buffer without substrate was incubated for a period of time (0, 48, 74, 99, 120, and 144 h) at several temperatures (30, 40, and 50°C) to investigate the effect of thermostability by measuring the remaining enzyme activity.

The effect of pH was investigated by measuring the crude enzyme activity at 50°C in several pH values ranging from pH 3.0 to 11.0 in the buffers with the concentration of 50 mM: Na_2HPO_4/citric acid solution (pH 3.0–8.0), Tris-HCl buffer (pH 7.0–9.0), and Gly/NaOH buffer (pH 9.0–11.0). Meanwhile, the effect of pH stability was evaluated by measuring the remaining activity of the crude enzyme preincubated at 50°C for 60 min in several solutions from pH 3.0 to 11.0.

The effects of the chelators and metal ions were studied by detecting the enzyme activity at 10 mM of ethylene diamine tetraacetic acid (EDTA) or different metal ions, which were listed below: chelator (EDTA) and metal ions (Co^{2+}, Mg^{2+}, Cu^{2+}, Na^+, Ca^{2+}, Mn^{2+}, K^+, Fe^{2+}, Ba^{2+}, Sr^{2+}, and Zn^{2+}). All the above-mentioned experiments were conducted in triplicate.

Single Factor Optimization Experiments

Seven experimental groups were set with concentration of *G. lemaneiformis* from 10 to 40 g L^{-1}, five values per interval. Other factors were exactly the same as the BCM condition.

Nitrogen sources, which were mainly used in the synthesis of metabolites and cell substances, could be divided into

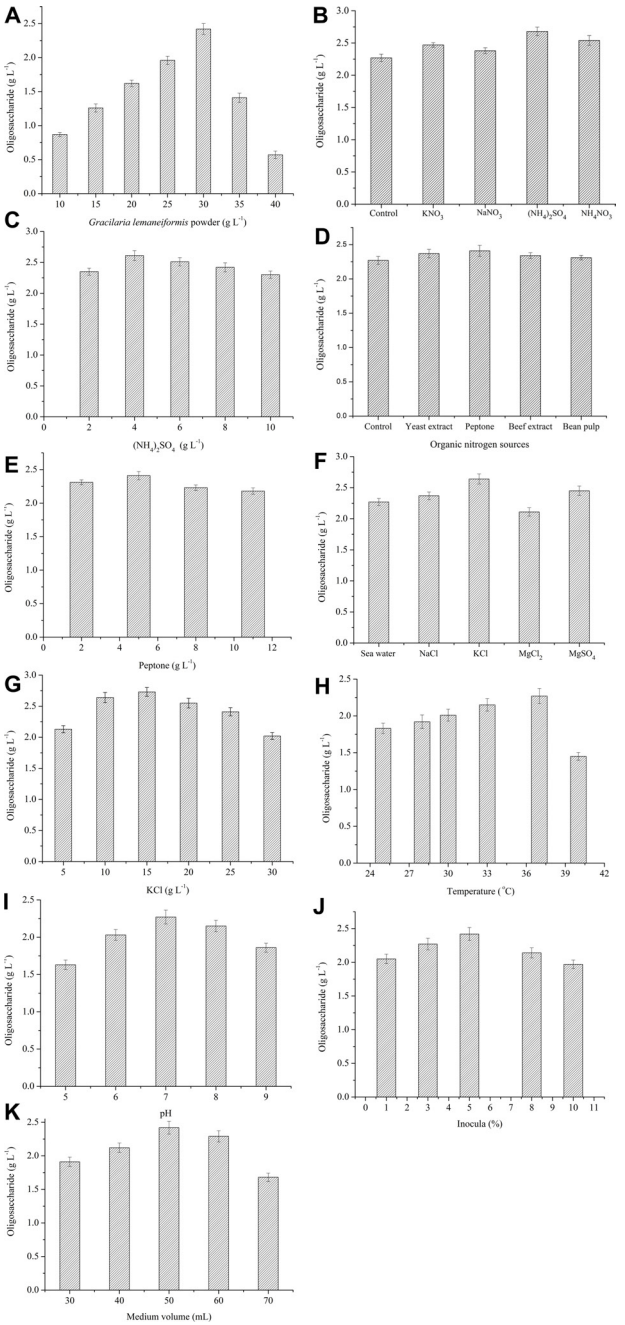

FIGURE 5 | Effects of each single variable on the production of oligosaccharides. **(A)** Effect of *G. lemaneiformis*. The concentration was from 10 to 40 g L^{-1}. **(B)** Effect of inorganic nitrogen sources. 5 g L^{-1} of KNO_3, $NaNO_3$, $(NH_4)_2SO_4$, $NH4NO_3$ were tested. **(C)** Effect of $(NH_3)_2SO_4$. The concentration of $(NH_4)_2SO_4$ was from 2 to 10 g L^{-1}. **(D)** Effect of organic nitrogen sources. 4 g L^{-1} of yeast extract, peptone, beef extract, bean pulp were tested. **(E)** Effect of peptone. The concentration of peptone was from 2 to 11 g L^{-1}. **(F)** Effect of inorganic salts. 10 g L^{-1} of NaCl, KCl, $MgCl_2$, $MgSO_4$ and sea water were tested. **(G)** Effect of KCl. The concentration of KCl was from 5 to 30 g L^{-1}. **(H)** Effect of temperature. Different temperatures (25, 28, 30, 33, 37, and 40°C) were tested. **(I)** Effect of initial pH. Different initial pH (5, 6, 7, 8, 9) were tested. **(J)** Effect of inocula. Different inocula (1%, 3%, 5%, 8%, 10%) were tested. **(K)** Effect of medium volume. Different medium volume (30, 40, 50, 60, and 70 mL) were tested.

inorganic nitrogen sources or nitrogen sources. KNO_3, $NaNO_3$, $(NH_4)_2SO_4$, NH_4NO_3 (5 g L^{-1}; inorganic nitrogen sources) and yeast extract, peptone, beef extract, bean pulp (4 g L^{-1}; organic nitrogen sources) were evaluated to determine the best one. Each group was a BCM containing this type of nitrogen source instead of the original one. After that, different concentrations of the best nitrogen sources were compared to determine the optimal concentration.

A solution of different inorganic salts (NaCl, KCl, $MgCl_2$, and $MgSO_4$) was prepared with distilled water to a final concentration of 10 g L^{-1}. The oligosaccharide products in these solution and seawater were compared to select the best one, following concentration screening from 5 to 30 g L^{-1}.

Based on the BCM condition, different temperatures (25, 28, 30, 33, 37, and 40°C), initial pH (5, 6, 7, 8, 9), inocula (1%, 3%, 5%, 8%, and 10%), and medium volume (30, 40, 50, 60, and 70 mL) were carried out separately.

Experimental Design for Oligosaccharide Production

Since every single variable mentioned above was obtained under certain conditions, the PB design was used to screen significant variables that influence the oligosaccharide production among all the medium components. N variables could be evaluated in N+1 experiment by PB design and examined at two levels: +1 for a high level, and −1 for a low level. In this study, these variables were selected: peptone, KCl, $(NH_4)_2SO_4$, initial pH, temperature, 250-mL Erlenmeyer flask with a working medium volume, and inocula. The factors and the levels of each factor are illustrated in **Table 1**.

Response surface methodology was used for optimizing the screened variables to increase the yield of oligosaccharides from *G. lemaneiformis* powders by the action of *Flammeovirga* sp. OC4. Developed by Design Expert 8.0.6, central composite design (CCD) under RSM was used for optimizing the concentrations of the significant factors and determining a total of 20 experimental runs with six replicated center points. Each factor was investigated in five levels (+1.682, +1, 0, −1, and −1.682). Each of the factors was calculated according to Eq. (1).

$$x_i = (X_i - X_0)/\Delta X_i, i = 1, 2, 3, \ldots, k \quad (1)$$

where x_i and X_i are the codified and actual values, respectively. X_0 is the value of X_i at the center point, and ΔX_i is the step change value. The relationship between the dependent and independent variables was explicated by the second-order polynomial Eq. (2) below.

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j, i = 1, 2, 3, \ldots, k \quad (2)$$

where Y is the predicted response, x_i and x_j are the coded independent factors that influence the response variable Y, β_0 is the intercept, β_i represents the linear effect of x_i, β_{ij} represents the interaction between x_i and x_j, and β_{ii} represents the quadratic effect of x_i.

The analysis of variance (ANOVA) was assessed by analyzing the model, whose quality could be further confirmed by the

TABLE 3 | PB experimental design for evaluation of 12 components with coded values for oligosaccharide production by *Flammeovirga* sp. OC4.

Run	A	B	D	E	G	H	I	K	Oligosaccharides (g L^{-1})
1	1	1	1	1	−1	−1	−1	−1	2.42 ± 0.09*
2	−1	1	−1	1	1	−1	−1	1	2.31 ± 0.07*
3	1	−1	1	−1	1	1	−1	−1	1.92 ± 0.06*
4	−1	1	1	1	1	1	1	−1	1.99 ± 0.08*
5	−1	−1	−1	1	−1	1	1	−1	2.17 ± 0.08*
6	−1	−1	1	−1	1	−1	1	1	2.45 ± 0.10*
7	1	−1	−1	1	1	1	−1	1	2.30 ± 0.01*
8	1	1	−1	−1	−1	1	1	1	2.06 ± 0.13*
9	1	1	−1	−1	1	−1	1	−1	2.71 ± 0.12*
10	−1	1	1	−1	−1	1	−1	1	2.18 ± 0.67*
11	1	−1	1	1	−1	−1	1	1	2.25 ± 0.09*
12	−1	−1	−1	−1	−1	−1	−1	−1	2.36 ± 0.11*

*All experiments were conducted in triplicates. *Statistical analysis by t-test, $P < 0.05$.*

coefficient of determination (R^2). The statistical significance was tested by the *F*-test, and the significance of regression coefficients was confirmed by the *t*-test.

Validation of the Experimental Model

This model was confirmed with regard to all of the three variables. The maximum productions of oligosaccharides predicted by Design Expert 8.0.6 in six combinations of medium components were conducted in triplicates and then compared with the predicted values.

Identification of Oligosaccharides Derived From *G. lemaneiformis*

Samples were withdrawn from the fermenter (Erlenmeyer flask) for analysis at regular intervals, and 50 mL of suspensions was collected by centrifugation (10,000 × *g*, 5 min), which was carried out in triplicate. The kind of oligosaccharides in the supernatant was identified by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC; Dionex, U-3000, United States) using a PA-100 anion exchange chromatography column (250 mm × 4 mm, CarboPac, Dionex, United States). The mobile phase was 100 mM NaOH and 150 mM NaAc. The mobile phase was filtered with a 0.22-μm microfiltration membrane before use. The sample injection volume was set to be 25 μL, and the column was eluted at 25°C with a flow rate of 0.25 mL/min using a pulse ampere detector (PAD).

Pure neoagarobiose, agarotriose, neoagarotetraose, agaropentaose, neoagarohexaose, agaroheptaose, neoagarooctaose, agarononaose, neoagarodecaose, agaroundecaose, and neoagarododecaose (Qingdao BZ Oligo Biotech Co., Ltd., China) were used as reference standards in the TLC experiment. Pure neoagarobiose, agarotriose, neoagarotetraose, agaropentaose, and neoagarohexaose (Qingdao BZ Oligo Biotech Co., Ltd., China) were used as reference standards in HPLC.

Statistical Analyses

All experiments were conducted in triplicates. Each value in the tables and figures was presented as the mean ± standard error (n = 3). Statistical analyses of the tested data were conducted using the *t*-test.

RESULTS

Screening and Identification of Agarolytic Strains

Four bacterial strains with transparent circle in the BCM plate were selected and named as OC1, OC2, OC3, and OC4. Crude enzyme of these strains was detected in the shaking flasks. The optimum temperature of the crude enzyme solution of OC4 was 50°C, while it was 40°C for OC1, OC2, and OC3. The relative enzyme activity at the optimal temperature of the agarolytic strains was OC4>OC1>OC3>OC2 (**Figure 1**). Thus, OC4 was selected for further research.

OC4 formed small yellowish round colonies, which were lustrous, regular, and easy to be picked up. There was a clear transparent circle around the colony, indicating that the secreted agarase degraded the agar in the medium (**Figure 2A**). TEM images showed that the strain cells with flagellum at the end of the tail were straight rod like, about 4 μm long and 0.6 μm wide (**Figure 2B**).

The 16S rRNA gene sequence of strain OC4 was acquired and then submitted to GenBank with the accession number KF 420290. Sequences of related taxa were obtained from GenBank using the program BLAST, and strains with the top hits are listed in **Supplementary Table S1**. Phylogenetic analysis showed that the closest relative for the new isolate was *Flammeovirga aprica* strain JL-4 with 98.07% 16S rRNA gene similarity. According to the position in the phylogenetic tree (**Figure 3**), this strain was named as *Flammeovirga* sp. strain OC4 and deposited in the Marine Culture Collection of China (MCCC) with accession number MCCC 1A07090.

Characterization of Crude Agarase Enzyme

The effect of temperature on the crude enzyme activity of *Flammeovirga* sp. OC4 was conducted at a wide temperature range (30–80°C) (**Figure 4A**). The optimum temperature was determined at 50°C, at which the enzyme had maximum activity and was set as 100%. The crude enzyme of *Flammeovirga* sp. OC4 retained 45% and 28% after incubation at 40°C and 50°C for 144 h, respectively (**Figure 4B**). The pH profile showed that the crude enzyme of *Flammeovirga* sp. OC4 had its optimum pH at 8.0, with more than 40% of its maximum activity in the spectrum from pH 6.0 to 9.0 (**Figure 4C**), and had more than 60% of the maximum activity after incubating for 60 min at pH 5.0–10.0 (**Figure 4D**).

The effects of EDTA and several metal ions on the activity of *Flammeovirga* sp. OC4 were investigated and listed in **Table 2**. Among these metal ions, Mn^{2+} and Ba^{2+} promoted the activity of *Flammeovirga* sp. OC4 especially. Mn^{2+} obviously enhanced the activity of *Flammeovirga* sp. OC4 by more than 14%. However, other metal ions (K^{+}, Na^{+}, Mg^{2+}, Sr^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, and Cu^{2+}) exerted inhibitory effects on *Flammeovirga* sp. OC4 activity. Moreover, EDTA distinctly restrained the activity of the crude enzyme, deducing the important roles of certain divalent metal ions in the *Flammeovirga* sp. OC4 activity.

Effects of Each Single Factor on the Production of Oligosaccharides

The production of oligosaccharides by *Flammeovirga* sp. OC4 in different conditions was measured, respectively. To evaluate the effect of the concentration of *G. lemaneiformis* on the production of oligosaccharides, the fermentation experiments

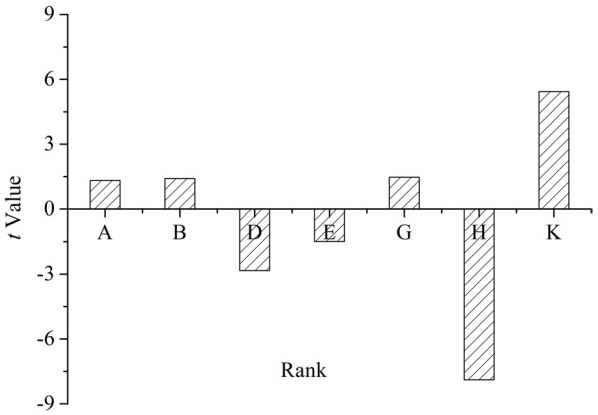

FIGURE 6 | *T*-value of the medium constituents for oligosaccharide production by *Flammeovirga* sp. OC4 based on the Plackett–Burman (PB) experimental results. (A) Peptone; (B) KCl; (D) $(NH_4)_2SO_4$; (E) initial pH; (G) inocula; (H) working medium volume; and (K) temperature.

TABLE 4 | Experimental design using CCD of three independent variables with their actual and coded values and six center points showing the experimental and predicted responses.

Run	Factors			Oligosaccharides (g L^{-1})
	Medium volume (mL)	Temperature (°C)	$(NH_4)_2SO_4$ (g L^{-1})	Experimental
1	40 (− 1)	33 (− 1)	2 (− 1)	2.99 ± 0.14*
2	60 (1)	33 (− 1)	2 (− 1)	2.75 ± 0.09*
3	40 (− 1)	37 (1)	2 (−)	3.10 ± 0.11*
4	60 (1)	37 (1)	2 (− 1)	2.92 ± 0.15*
5	40 (− 1)	33 (− 1)	6 (1)	2.94 ± 0.08*
6	60 (1)	33 (− 1)	6 (1)	2.78 ± 0.09*
7	40 (− 1)	37 (1)	6 (1)	3.14 ± 0.11*
8	60 (1)	37 (1)	6 (1)	2.97 ± 0.12*
9	26.4 (− 1.682)	35 (0)	4 (0)	3.03 ± 0.09*
10	73.6 (1.682)	35 (0)	4 (0)	2.75 ± 0.08*
11	50 (0)	31.6 (− 1.682)	4 (0)	2.82 ± 0.08*
12	50 (0)	38.4 (1.682)	4 (0)	3.11 ± 0.12*
13	50 (0)	35 (0)	0.636 (− 1.682)	3.03 ± 0.16*
14	50 (0)	35 (0)	7.364 (1.682)	3.07 ± 0.10*
15	50 (0)	35 (0)	4 (0)	3.09 ± 0.10*
16	50 (0)	35 (0)	4 (0)	3.11 ± 0.11*
17	50 (0)	35 (0)	4 (0)	3.08 ± 0.11*
18	50 (0)	35 (0)	4 (0)	3.11 ± 0.10*
19	50 (0)	35 (0)	4 (0)	3.13 ± 0.10*
20	50 (0)	35 (0)	4 (0)	3.09 ± 0.09*

*All experiments were conducted in triplicates. *Statistical analysis by t-test, $P < 0.05$. CCD, central composite design.*

were carried out at 37°C for 42 h using various amounts of *G. lemaneiformis*. The results show that the production of oligosaccharides increased from 0.86 to 2.27 g L^{-1} with increase in the concentration of *G. lemaneiformis* from 10 to 30 g L^{-1} (**Figure 5A**). However, when the concentration of *G. lemaneiformis* was higher than 30 g L^{-1}, the production of oligosaccharides decreased (**Figure 5A**), which illustrated that a suitable concentration of *G. lemaneiformis* was beneficial for producing oligosaccharides. A high concentration of *G. lemaneiformis* in the fermentation could remarkably increase the viscosity of the culture, which might limit the oxygen and mass transfer and, thus, result in greatly reducing the production of oligosaccharides. Based on the above results, the concentration of *G. lemaneiformis* (30 g L^{-1}) was used as the carbon source concentration in the optimization of the fermentation process.

Both organic and inorganic nitrogen sources have influence on the production of oligosaccharides from *G. lemaneiformis* by the action of *Flammeovirga* sp. OC4. As shown in **Figures 5B,D**, $(NH_4)_2SO_4$ and peptone were the most suitable inorganic and organic nitrogen source. The maximum concentration of oligosaccharide was observed at 4 g L^{-1} $(NH_4)_2SO_4$ and 5 g L^{-1} peptone (**Figures 5C,E**). It means that suitable nitrogen sources can accelerate the growth of *Flammeovirga* sp. OC4, which increased the production of oligosaccharides, whereas the high concentration of nitrogen sources made *Flammeovirga* sp. OC4 grow too fast, thus inhibiting its degrading activity on *G. lemaneiformis*.

Since seawater was difficult to get inland on a large scale, the impact of inorganic salt water solution on the production of oligosaccharides was studied. As shown in **Figure 5F**, the highest production of oligosaccharides was observed in KCl solution compared to seawater, NaCl, $MgSO_4$, and $MgCl_2$ solution. The optimal concentration of KCl was found to be 15 g L^{-1} (**Figure 5G**).

The effects of different temperatures, initial pH, inocula, and working medium volume on the production of oligosaccharides from the biotransformation of *G. lemaneiformis* by the action of *Flammeovirga* sp. OC4 are shown in **Figures 5H,I,K**. The highest production of oligosaccharides among different temperatures, initial pH, inocula, and working medium volume were observed at 37°C (**Figure 5H**), pH 7 (**Figure 5I**), 5% inocula (**Figure 5J**), and 50-mL working medium volume (**Figure 5K**), respectively.

Screening of Significant Factors by the PB Design

To increase the yield of oligosaccharides, seven medium components were screened by the PB design, which contained 12 experimental runs and two levels for each component. The effects of each medium component on the yield of oligosaccharides are summarized in **Table 3**.

The *t*-value on the effect of each medium component is shown in **Figure 6**, which reflected the contributions of each component to the yield of oligosaccharides.

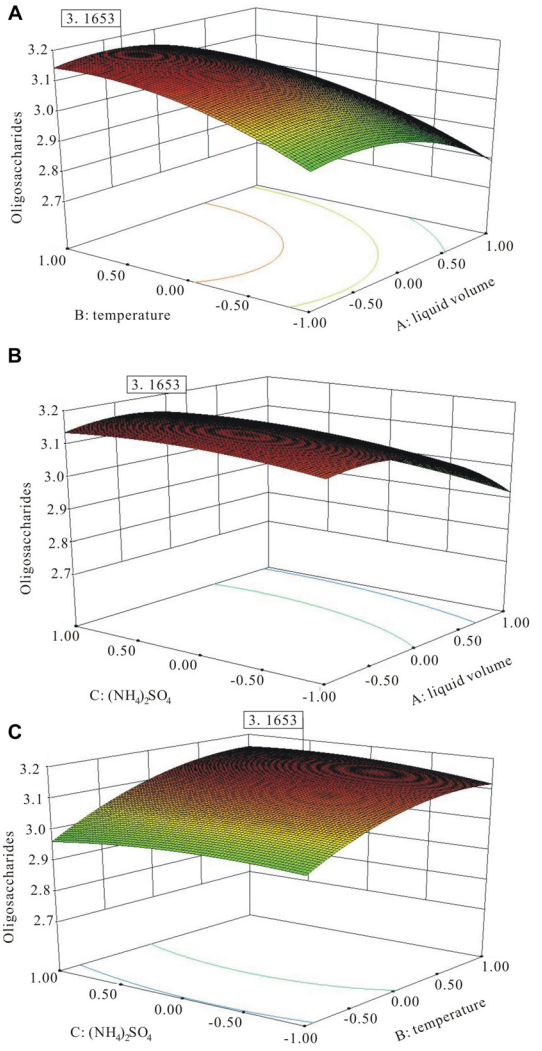

FIGURE 7 | The three-dimensional (3D) response surface curves of the combined effects of medium volume, temperature, and $(NH_4)_2SO_4$ on oligosaccharide production. **(A)** Medium volume and temperature at the fixed level of $(NH_4)_2SO_4$. **(B)** Medium volume and $(NH_4)_2SO_4$ at the fixed level of temperature. **(C)** $(NH_4)_2SO_4$ and temperature at the fixed level of medium volume.

The medium components influencing the yield of oligosaccharides were ranked as follows: working medium volume > temperature > $(NH_4)_2SO_4$ > inocula > initial pH > KCl > peptone. It was clear that working medium volume, initial pH, and $(NH_4)_2SO_4$ affected oligosaccharide production in a negative way, and temperature, inocula, KCl, and peptone affected the yield in a positive way (**Figure 6**). According to the PB design, the highest oligosaccharide production (2.71 g L^{-1}) was found in run 9, while the lowest yield (1.92 g L^{-1}) was in run 3 (**Table 3**).

From these experimental runs, the standard ANOVA results were reckoned (**Supplementary Table S2**). The *F*-value (14.76) and *P*-value (0.0103) of the model indicate that the model and its terms were significant. Based on the ANOVA results,

TABLE 5 | Validation of the experimental model.

Experiment	Medium volume (mL)	Temperature (°C)	$(NH_4)_2SO_4$ (g L^{-1})	Oligosaccharides (g L^{-1})	
				Experimental	Predicted
1	46.6	34.7	2.08	3.02 ± 0.075*	3.00
2	35.7	33.2	1.56	2.98 ± 0.082*	3.13
3	39.6	32.6	3.20	3.01 ± 0.102*	3.08
4	44.8	36.7	4.84	3.09 ± 0.087*	3.16
5	32.1	32.9	3.70	3.03 ± 0.093*	3.11

*All experiments were conducted in triplicates. *Statistical analysis by t-test, $P < 0.05$.*

working medium volume, temperature, and $(NH_4)_2SO_4$ were selected as significant model terms. Moreover, the value of R^2 for the model was 0.9627. The predicted R^2 values corresponded with the adjusted R^2 values for all equations. The adequate precision that measures the signal-to-noise ratio was 12.768 for oligosaccharide production. A desirable ratio of >4 was recorded in this study. According to the results, the three most significant variables [working medium volume, temperature, and $(NH_4)_2SO_4$] were selected for further study.

Optimization Using RSM

As the highest oligosaccharide production was found in run 9, their level was determined as the center points. The optimal condition for the three significant factors was determined using CCD under RSM. One of the 20 predicted combinations from the regression equation was set in each run, and the experimental oligosaccharide production in each run was obtained (**Table 4**). It was found that the maximal yield of oligosaccharides (3.14 g L^{-1}) by *Flammeovirga* sp. OC4 was in run 7, while run 10 showed the minimum production (2.75 g L^{-1}).

The regression equation below displayed the dependence of the yield of oligosaccharides on constituents of the medium.

$$\begin{aligned} Y(\text{oligosaccharides, g}L^{-1}) &= 3.10 - 0.089A + 0.084B \\ &+ 9.319E^{-003}C + 5.000E^{-003}AB + 0.01AC \\ &+ 0.015BC - 0.077A^2 - 0.05B^2 - 0.02C^2 \end{aligned}$$

The response (Y) represented the yield of oligosaccharides, and A, B, and C, respectively, represented the working medium volume, temperature, and $(NH_4)_2SO_4$.

Supplementary Table S3 presented the ANOVA results for the quadratic model. The *P*-value of the model was less than 0.05, which implied that the model was significant. In this case, A, B, C, AC, BC, A^2, B^2, and C^2 were significant model terms. The lack of fit of $P = 0.047$ shows that the lack of fit was significant. These results reveal that the second-order equation could be used to predict the effects of the variables [working medium volume, temperature, and $(NH_4)_2SO_4$] on the yield of oligosaccharides by *Flammeovirga* sp. OC4 in shaking Erlenmeyer flask cultures.

The coefficient values of the regression equation are also listed in **Supplementary Table S3**. R^2 close to 1 meant that the correlation between the predicted values and experiment results was high.

The three-dimensional (3D) response surface plots (**Figure 7**) were the graphical representation of the regression equation, which indicated the following predicted optimal fermentation condition: $A = -0.52$, $B = 0.87$, and $C = 0.42$, corresponding to the optimal levels of 44.8 mL working medium volume, 36.7°C temperature, and 4.84 g L^{-1} $(NH_4)_2SO_4$ for the maximal oligosaccharide production of 3.16 g L^{-1}.

Validation of the Model

To test the accuracy of the predicted results, several experiments were replicated three times, and the medium components are presented in **Table 5**. Some of the experimental results are slightly lower or higher than the predicted results, but all of the deviations are smaller than 5%. This might be due to unavoidable experimental errors.

After optimization, a 36.1% increase in oligosaccharide production to 3.09 g L^{-1} was observed with fermentation performed using CCD, which was compared with the oligosaccharide production (2.27 g L^{-1}) obtained using the original culture medium.

Identification of Oligosaccharides Derived From *G. lemaneiformis*

The TLC chromatogram of the reference standards of oligosaccharides and oligosaccharides derived from *G. lemaneiformis* is displayed in **Figure 8A**. It could be deduced that the oligosaccharides derived from *G. lemaneiformis* were agarotriose, agaropentaose, neoagarobiose, neoagarotetraose, and neoagarohexaose. The HPLC chromatograms, which are displayed in **Figures 8B,C**, respectively, further confirmed this deduction. Peaks 1, 2, 3, 4, and 5 in **Figure 8B** represented the peak of reference substance of agarotriose, agaropentaose, neoagarobiose, neoagarotetraose, and neoagarohexaose, respectively. A comparison to the HPLC chromatogram of the reference standards of oligosaccharides showed that there were the same peaks 1, 2, 3, 4, and 5 in the HPLC chromatogram of the oligosaccharides derived from *G. lemaneiformis* (**Figure 8C**), indicating that the oligosaccharides derived from *G. lemaneiformis* in this work contained agarotriose, agaropentaose, neoagarobiose, neoagarotetraose, and neoagarohexaose.

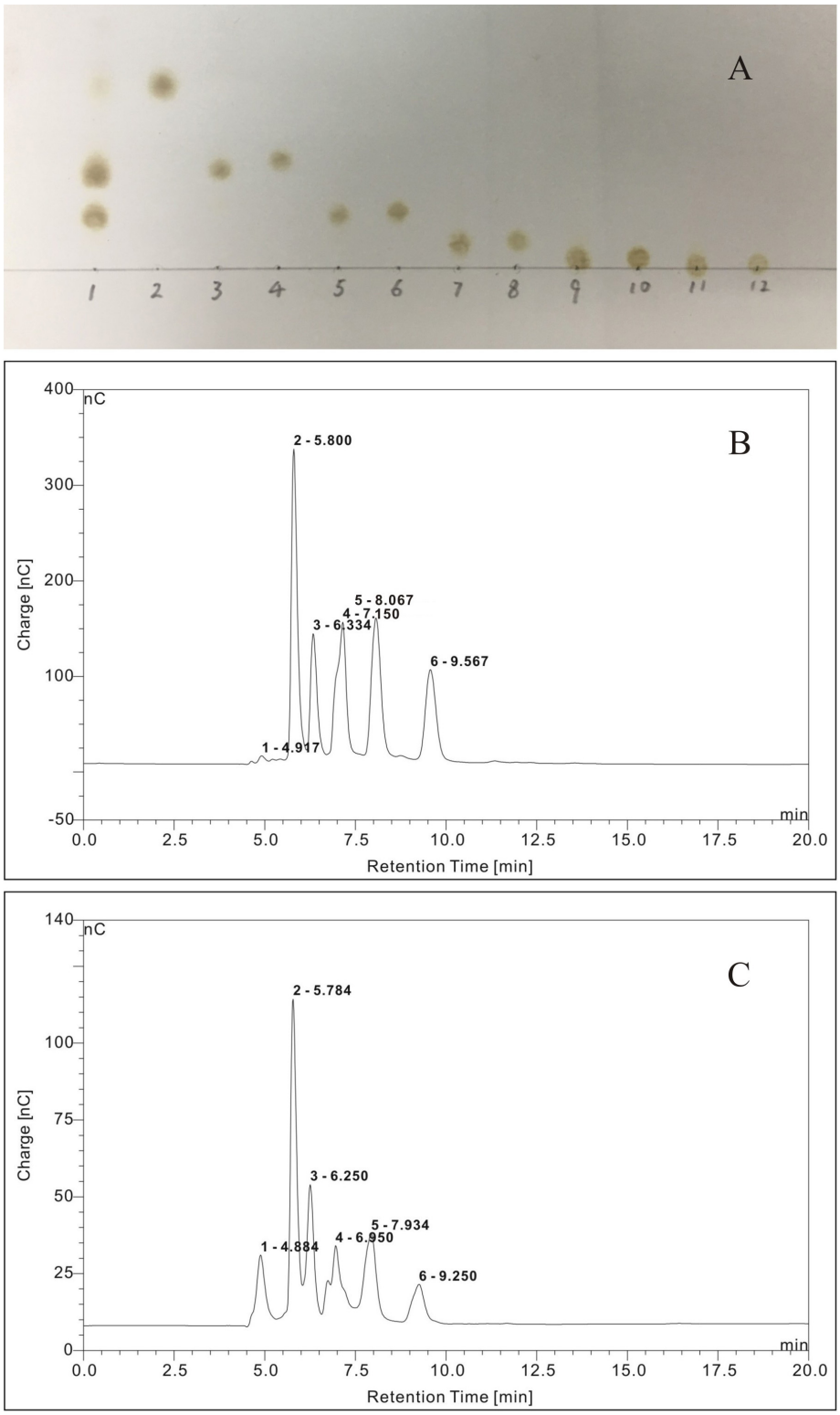

FIGURE 8 | Thin layer chromatography (TLC) analysis **(A)** and high-performance liquid chromatography (HPLC) chromatograms **(B,C)** of purified fermentation products of *Flammeovirga* sp. OC4. **(A)** Lane 1, purified fermentation products; lane 2, neoagarobiose; lane 3, agarotriose; lane 4, neoagarotetraose; lane 5, agaropentaose; lane 6, neoagarohexaose; lane 7, agaroheptaose; lane 8, neoagarooctaose; lane 9, agarononaose; lane 10, neoagarodecaose; lane 11, agaroundecaose; and lane 12, neoagarododecaose.

DISCUSSION

In situ enrichment was beneficial for screening target strains. According to the traditional method, samples collected in the deep sea were treated in the laboratory; thus, screening of target strains was challenged by both the randomness of sampling and the change of habitats. In the separation process, if the target strain did not have absolute competitive edge, it was likely to submerge in a large number of other strains. However, *in situ* enrichment created a favorable habitat for target strains, which still circulated in the environment of the deep sea. After a period of time, the target strain will occupy the advantage to facilitate our subsequent screening. In this study, four strains with agar-degrading activity were screened without much interruption from other irrelevant strains.

Compared with traditional methods of producing algae oligosaccharides from algae polysaccharides, the "one-step process" established in this study had at least two advantages. The first one is the reduction in the pollutants (i.e., by-products) of algal dregs in the extraction process of agar from *G. lemaneiformis*. Algal dregs contain diatomite and perlite, which were added in the extraction process to facilitate filtering of agar, and a high concentration of salt produced by acid–base neutralization. The dregs would obstruct the growth of plants, crops, fish, and shrimps, causing dead corners of environmental sanitation if they were accumulated in large quantities. The second one is the retaining of the beneficial ingredients of algae. It is mentioned above that algae oligosaccharides are the degradation products of the cell walls of algae. In addition to cell walls, algae also have many beneficial components, such as algae proteins, polyterpenoids, and steroids, which have the potential of promoting growth of plants and crops and would be destroyed by strong acid and alkali. In this study, oligosaccharides with different degrees of polymerization were produced by "one-step process," thus retaining beneficial ingredients without producing dregs.

Response surface methodology effectively combined mathematical methods with statistical analysis. Through mathematical modeling and analysis of several response process variables, the optimal conditions of a multifactor system could be determined efficiently. Compared with the orthogonal experiment method, RSM could make a comprehensive study of the experiment with less experiment quantity and time and scientifically provide the relationship between the whole and the partial, which could obtain more definite and effective conclusions. In this study, three factors that had the greatest influence on the number of oligosaccharides produced by the "one-step process" were determined by the PB test. After optimization of RSM, the yield of oligosaccharides was increased by 36.1%, which was from 2.71 to 3.09 g L^{-1}. The results demonstrated that the "one-step process" had potential for large-scale industrial applications.

CONCLUSION

A "one-step process" for the preparation of oligosaccharides from *G. lemaneiformis* was established through acquisition of *Flammeovirga* sp. OC4 by *in situ* enrichment in the deep sea and the following optimization methods. This study provided an alternative approach for environment-friendly and sustainable utilization of algae on a large scale. In addition to creating more opportunities for the application of *G. lemaneiformis* in the industry, the "one-step process" described in this study could be extended to other kinds of algae in the future.

AUTHOR CONTRIBUTIONS

HL and RZ designed the research program. XC, LL, and ZC performed the experiments. XC analyzed the experimental data and wrote the manuscript. HL revised the manuscript and ML edited the English language of the manuscript. All authors have approved the submission and publication of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00724/full#supplementary-material

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REVIEW
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Lactic Fermentation as a Strategy to Improve the Nutritional and Functional Values of Pseudocereals

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One of the greatest challenges is to reduce malnutrition worldwide while promoting sustainable agricultural and food systems. This is a daunting task due to the constant growth of the population and the increasing demands by consumers for functional foods with higher nutritional values. Cereal grains are the most important dietary energy source globally; wheat, rice, and maize currently provide about half of the dietary energy source of humankind. In addition, the increase of celiac patients worldwide has motivated the development of gluten-free foods using alternative flour types to wheat such as rice, corn, cassava, soybean, and pseudocereals (amaranth, quinoa, and buckwheat). Amaranth and quinoa have been cultivated since ancient times and were two of the major crops of the Pre-Colombian cultures in Latin- America. In recent years and due to their well-known high nutritional value and potential health benefits, these pseudocereals have received much attention as ideal candidates for gluten-free products. The importance of exploiting these grains for the elaboration of healthy and nutritious foods has forced food producers to develop novel adequate strategies for their processing. Fermentation is one of the most antique and economical methods of producing and preserving foods and can be easily employed for cereal processing. The nutritional and functional quality of pseudocereals can be improved by fermentation using Lactic Acid Bacteria (LAB). This review provides an overview on pseudocereal fermentation by LAB emphasizing the capacity of these bacteria to decrease antinutritional factors such as phytic acid, increase the functional value of phytochemicals such as phenolic compounds, and produce nutritional ingredients such as B-group vitamins. The numerous beneficial effects of lactic fermentation of pseudocereals can be exploited to design novel and healthier foods or grain ingredients destined to general population and especially to patients with coeliac disease.

Keywords: lactic acid bacteria, pseudocereals, vitamins, phytate, phytochemicals

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INTRODUCTION

According to the Food and Agriculture Organization (FAO), global hunger is in ascent again after constantly decreasing for over a decade (1). The number of chronically undernourished people in the world is estimated to have increased to 815 million (11% of the global population) in 2016, up from 777 million in 2015, as reported in the edition of the Annual United Nations on World Food Security and Nutrition published in September 2017, based on reports by five organizations

[ONU, FAO (Food and Agriculture Organization), WHO (World Health Organization), IFAD (International Fund for Agricultural Development (IFAD), and World Food Program (WFP)] (1). At the same time, different forms of malnutrition are threatening the health of millions of people worldwide. Nearly 795 million people have eating disorders and do not carry out healthy and active lifestyles with an estimated 41 million children that are now overweight according to the World Food Program. Added to this serious situation, the world population is expected to reach nine billion persons in the coming decades, imposing the need for urgent solutions to increase food supplies (2). In addition, climate change is rapidly degrading the conditions of crop production, affecting the availability of water and arable land, increasing salinization and aridity, generating a serious problem in the yield of food. It is estimated that approximately one billion hectares or crop land will be affected worldwide due to these problems, especially those in the hottest and most arid regions of the world (3–7). In addition to climate change, global staple crop production is also threatened by restrictions such as accelerated erosion of soil and natural resources (8). Frison et al. (9) also reported that modern agriculture generates serious problems in the environment causing soil degradation and erosion, water pollution and biodiversity decline. Therefore, it is essential to increase food production for a growing population that uses low input regimes. The FAO urges to expand the response to climate change in agriculture. According to their 2017 document "A systemic approach that involves the relevant agricultural and food sectors and those interested in the adoption of agroecology, has the potential to greatly accelerate the transition to sustainable and resilient food systems, in line with the various international commitments assumed by the member countries." Agroecology, in an integral manner, can support the execution of different social, economic, environmental, nutritional, and health objectives.

Diets throughout the world are based on two dozen crops with a dominant proportion of the "big three" cereals: wheat (*Triticum aestivum*), maize *(Zea mays*), and rice (*Oryza sativa*), which contribute to approximately 60% of the total caloric intake (10). However, these crops may not intrinsically be the best-suited species to face up to extreme weather events that are becoming more frequent due to climate change; thus, world grain production per capita is expected to decline by at least 14% between 2008 and 2030 (11). The rapid growth of the world population and per capita food consumption worldwide puts great pressure on the food industry to produce more food (12). The food supply must double by 2050 to counterbalance the effects of climate change and population pressure on global food systems and thus novel food sources must be found (6). Less than 0.6% of plant species that are suitable for human consumption have reached the world markets (13). The diversification of main crops and the systems in which they grow is essential for agriculture to be sustainable, resilient, and suitable for local environments and soils in the future. One critical measure to ensure future food availability for all is to provide more diverse food sources and develop agricultural systems that are resistant to climate change. Furthermore, the new challenge for the food industries and scientific areas such as chemistry, biology, medicine, pharmacology, and food technology is to obtain foods with a higher nutritional value that also possess functional properties which go beyond traditional health requirements. In response to this issue, one of the leading strategies is unlocking the potential of underutilized crops. Most of these crops have high nutritional value, resilience traits, with the ability to withstand drought, flooding, extreme temperatures, and pests and diseases better than current major staples and thus they should be investigated, developed, and now more than ever used (14). Current emphasis is now placed on the use of ancient cereals and pseudocereals that include amaranth, buckwheat, quinoa, teff, millets amongst others.

The aim of this review is to highlight certain nutritional and functional properties of pseudocereals and how lactic acid fermentation can be used as an advantageous biotechnological strategy to improve the natural potential of these grains. This review provides an overview on pseudocereal fermentation by lactic acid bacteria (LAB) emphasizing the capacity of these bacteria to decrease antinutritional factors such as phytic acid, increase the functional value of phytochemicals such as phenolic compounds, and produce nutritional ingredients such as B-group vitamins.

CROP DIVERSIFICATION: PSEUDOCEREALS

Crop diversification is an important strategy to protect global food supplies and to fight against malnutrition. Sustainable diets should provide nutritious food at affordable costs, while having a low impact on the environment (15). Effective analysis of sustainable plant resources is an important assignment for ensuring global food security in the future (16).

The need for the diversification of grains for human consumption and the consumer's demands for gluten-free and more nutritious products caused the resurgence and valoration of underutilized crops, so-called minor grains such as sorghum, millets, and pseudocereals through the world during the last several decades (17). In the "International AACC list of recognized grains" pseudocereals are also mentioned (18) where the most important species are quinoa (*Chenopodium quinoa* Willd), amaranth (*Amaranthus* sp.), and buckwheat (*Fagopyrum esculentum*). Pseudocereals are dicotyledonous species unlike true cereals (Poaceae family), that are monocotyledonous species. Pseudocereals are known as such since they are similar to cereals in their physical appearance and their seeds are edible with high starch content that can be milled into flour (19). Their high nutritional value is mostly due to their elevated content and quality of proteins (20, 21).

Celiac disease (CD) is one of the most common lifelong disorders worldwide with as estimated mean prevalence of 1% of the general population (22). The increase of celiac patients throughout the world has led to intensify the search for alternative flours to wheat (19). The development of gluten-free (GF) products is therefore essential and poses novel challenges for food producers (23). In the last decade, due to the pseudocereals characteristics, GF and good nutritional

advantages, the use of these grains has increased for their addition in healthy diets especially for people allergic to cereals. Thus, the integration of these grains into GF diets could be a valuable contribution for improving the quality of the existing GF products, which have been mainly based on rice and maize flour (24). Despite the fact that the interest in pseudocereals due to its high nutritional value has increased, only a few products including these grains are available on the market [**Figure 1**; (25)].

PSEUDOCEREALS HISTORICAL BACKGROUND

Pseudocereals nourished the Native Americans populations and allowed them to increase their endurance and mental development; because of their properties, Mayans and Incas considered these grains sacred. The conquest of America meant not only a political and social domination of indigenous civilizations, but also a change in their feeding habits. Jacobsen (26) reported that quinoa is one of the oldest crops in the Andean region, having been grown for approximately 7000 years; it is considered the principal crop of the pre-Columbian cultures in Latin America (27, 28). The Incas called quinoa "the mother grain" for many reasons: (i) it is one of the few crops able to grow in high salt soils in Southern Bolivia and Northern Chile, (ii) it has high tolerance to abiotic stresses, (iii) it grows in soils with water scarcity, and (iv) it is resistant to extreme temperatures (−4 to 38°C) and harsh climate conditions (15, 27, 29, 30). The FAO (United Nations) declared, "quinoa has the balance of proteins and nutrients closest to the ideal food for humans."

Amaranth (*Amaranthus* sp.) is an ancient crop consumed during the Mayan and Aztec periods. It was called "the Inca wheat" by the Spanish conquerors. Amaranth grain species are annual herbaceous plants native of America but they are also now distributed in Asia and Africa (31, 32). *Amaranthus caudatus* was discovered in the north of Argentina (Salta) 2000 years ago (33). When the Spaniards arrived, they decided to exterminate pseudocereals because of their religious implications. Ironically, it is now Europeans who teach us how to consume the grains that were used by the Native American civilizations. The interest in these Andean ancestral crops in the world has led to an increase in their cultivation and production in recent years.

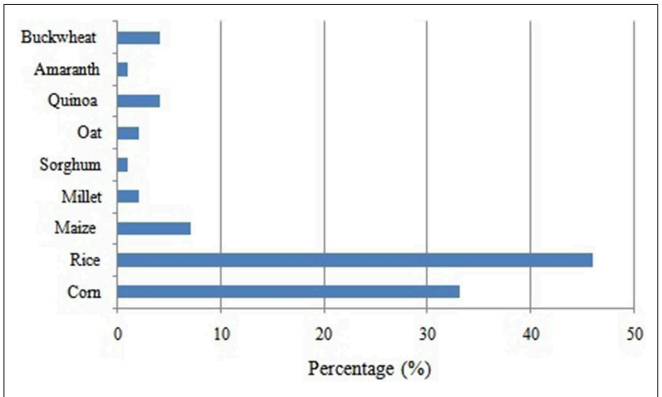

FIGURE 1 | Principal grains used for gluten-free products on the market [adapted from (25)].

Buckwheat (*Fagopyrum esculentum* Moench) has its origin in Asia and it is believed to have been cultivated in China during the fifth and sixth centuries. It came to Europe after some 800–900 years and to North America in seventeenth century (34).

Underutilized species by means of sustainable intensification, adaptation and mitigation can accelerate the process to obtain climate-smart agriculture [**Figure 2**; (35)].

NUTRITIONAL VALUE

Charalampopoulos et al. (36) reported that 73% of the total world harvested area corresponds to cereal crops and contributes to more than 60% of the world food production, furnishing proteins, minerals, dietary fiber, and vitamins necessary for human health. Cereals contribute around 50% of the mean daily energy intake in most populations, and 70% in some developing countries, converting them into one of the most important sources of energy in the world (37). However, most grains are, to a greater or lesser extent, deficient in a number of elemental nutrients such as the essential amino acids threonine, lysine, and tryptophan. Their protein digestibility is also lower than that of animal origin, due partially to the presence of phytic acid, tannins, and polyphenols which bind to protein thus making them indigestible (38). Pseudocereals in turn have been described as "the grains of the twenty-first century" (39, 40). The FAO classified quinoa as one of humanity's promising crops destined to contribute to food security in the twenty-first century by its high nutritive potential and genetic diversity [Food and Agriculture Organization Regional Office for Latin America and PROINPA, (27, 41)]. Quinoa and amaranth have tender leaves that are used in food preparation; however, it is their grains that attract the most interest due to their high nutritional value. They are rich in proteins of excellent quality with a balanced essential amino acid composition that include abundant amounts of sulfur- rich amino acids (42). They are also a good source of minerals (calcium, iron and zinc), vitamins, and natural antioxidants (43). They are a significant source of compounds such as flavonoids, polyphenols and phytosterols with potential nutraceutical benefits. From a food provision perspective, pseudocereals are potentially important crops due to their properties (exceptional nutritional value, ability to grow in dry conditions, and their resilience to climatic conditions). Several reviews have reported the nutritional value of Andean grains (19, 43–49).

The General Assembly of the United Nations declared 2013 as the International Year of Quinoa (IYQ), with the goal of focalizing global attention on the role it can perform in contributing to food security, nutrition, and poverty eradication (50, 51). The rapid expansion of the harvested area, with a doubling of countries from 2013, is rapidly changing the perception and representation of quinoa from a minor to a potential major crop. The excellent properties of quinoa led to

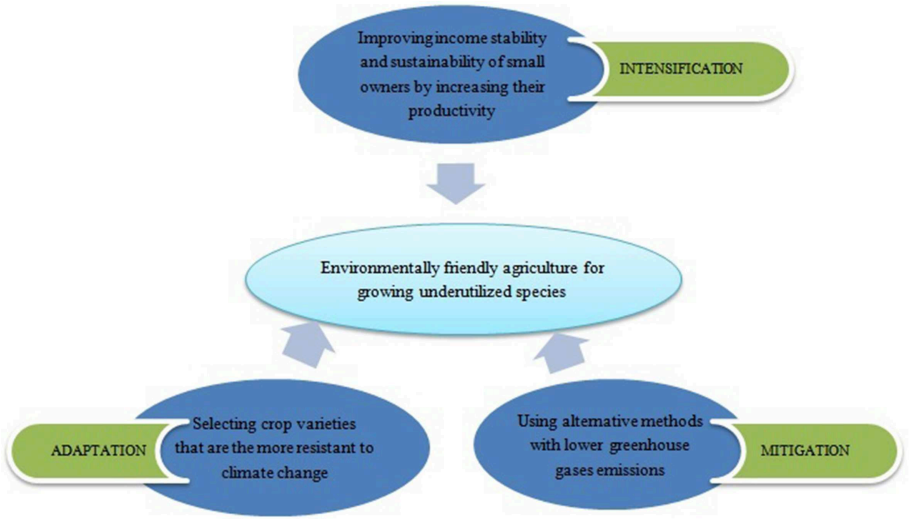

FIGURE 2 | Strategies to use underutilized species and increase the crops biodiversification for a sustainable agriculture [adapted from (35)].

this ancestral grain to be considered a possible crop in NASA's Controlled Ecological Life Support System for long-duration manned space flights (39).

Vitamin in Pseudocereals

Vitamins are essential micronutrients since only small quantities are required for adequate growth and function of numerous metabolic reactions. Vitamins are divided based on their solubility in fat (A, D, E, and K), or in water (C and the B-group vitamins). Since there are no foods that contain all vitamins, there is a worldwide increase in their deficiencies due to unbalanced diets. Other causes of vitamin deficiencies are malabsorption that can be due to certain drug treatments or diseases, by the presence of antinutritional factors found in certain foods. Although most vitamins are present in cereals and pseudocereals, a large portion of water-soluble vitamins are lost during processing and cooking, especially when water is used for grain soaking. In this sense, many countries have adopted mandatory fortification programs with specific vitamins and minerals. Folic acid is frequently added in foods of mass consumption (such as different flours) in order to prevent deficiencies in the general population. However, the chemical form of the vitamin used in these programs is controversial. Folic acid, a chemical derivative of folates, is not found in nature and can cause many side effects, especially masking vitamin B_{12} deficiency and affecting the activity of certain liver enzymes, but also has been associated with increased risks of colon and prostate cancers (52). Natural folate, present in numerous different chemical forms in vegetables or produced by certain microorganisms does not cause these undesirable side effects. For this reason, more and more researchers are now searching for more natural methods to increase water soluble vitamins such as folate and riboflavin in foods to not only prevent deficiencies, but also to reduce the use of chemical additives in the food chain.

It was demonstrated that staple foods produced from amaranth contained total folate contents of 35.5 μg/100 g in bread, 36.3 μg/100 g in cookies, and 38.9 μg/100 g in noodles, whereas when wheat was used, breads contained only 12.0 μg/100 g (53). The riboflavin content in amaranth flour is in the range of 0.29–0.32 mg/100 g, which is about 10-fold higher than that of wheat (54). In general, significant reductions of all vitamins take place during processing, which affect the nutritional value of the products (55). The production of fermented food products with high levels of B-group vitamins increases their commercial and nutritional value and eliminates the need for fortification (56).

Phytochemical Profile of Pseudocereals

Pseudocereals are important phytochemical sources in the diet. Like cereal, these grains contain a great amount of functional phytochemicals including the phenolic compounds (PC) (57–60). The PC constitute a group of secondary metabolites with important functions in cereals and pseudocereals. The chemical structures of these compounds include an aromatic ring with one or more hydroxyl substituents, and vary from simple phenolic molecules to highly polymerised compounds (61). PC are broadly divided in four classes; phenolic acids (benzoic or hydroxycinnamic acid derivatives), flavonoids (flavonols, flavones, isoflavones, flavanones, and anthocyanidins), stilbenes and lignans. In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids (60). In general, ferulic, *p*-coumaric, caffeic, isoferulic, vanillic, sinapic, *p*-hydroxybenzoic, syringic, and protocatechuic acids are present in all grains; with ferulic acid the most abundant phenolic acid (62, 63). Gorinstein et al. (64, 65) reported a high content of polyphenols, anthocyanins and flavonoids in pseudocereals such as buckwheat, quinoa and amaranth. Likewise, the highest amount of PC was reported in quinoa (490.2 mg/kg DW), slightly

lower in amaranth v. Aztek (464 mg/kg DW), and the lowest in amaranth v. Rawa (424.6 mg/kg DW) (66).

PC may provide health benefits to humans since they are associated with a reduced risk of chronic diseases such as anti-allergenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective, and stimulates insulin secretion in diabetes mellitus type 2 (57, 67–71). The dietary PC contribute to the maintenance of a healthy gut by modulating the gut microbial balance (beneficial bacteria/pathogen bacteria). Metagenomic and metabolomic studies providing more insight into the health effects of PC in humans are needed to understand the dietary PC/gut microbiota relationship and their mechanisms of action. The PC effect on the modulation of the gut ecology and the two-way relationship "polyphenols ↔ microbiota" is currently being studied (72).

The biological effects of PC depend principally on their bioaccessibility (release of the food matrix in an absorbable form during digestion) and bioavailability (absorption and transference to the bloodstream), and both depend on their chemical structure, matrix interactions, antioxidant activity, and food processing (73–79). Natural PC usually occur as glycosides, esters or polymers that have no biological activity (80). Of the total PC intake, only 5–10% is absorbed in the small intestine and the remaining PC (90–95%) accumulate in the large intestinal lumen where they are subjected to the enzymatic activities of the gut microbial community (81). Food technologists need to find the operating conditions to increase bioaccessibility and bioavailability of PC from the matrix. The addition of purified enzymes such as feruloyl and *p*-coumaryl esterases, xylanase, β-glucanase, and α-amylase from natural sources has been proposed to increase the active PC content in cereals [i.e., wheat and rye; (82–84)]. However, these studies in pseudocereals are still missing.

ANTINUTRITIVE FACTORS IN PSEUDOCEREALS

Grains of cereals, pseudocereals, and legumes are of global importance in the feeding of monogastric animals (humans and domestic animals) since they are a good source of proteins, bioactive compounds and trace elements (85). However, they contain certain antinutrients compounds, such as phytic acid, saponins, tannins, polyphenols, and protease inhibitors (86). In this sense the bioavailability of minerals in whole grain foods is negatively affected by the presence of phytate (87). Since phytate is an antinutritional factor that is found in the highest quantities in pseudocereals and due to its important negative effect on malnutrition, this review will focus on this antinutritive factor.

Phytates

Phytic acid (PA) (myo-inositol 1,2,3,4,5,6-hexakisphosphate) is an abundant plant constituent, comprising 1–5% (w/w) of legumes, cereals, pseudocereals, oil seeds, pollen and nuts and represents the largest form of phosphorus storage (88, 89). Besides phytate, myo-inositol 1,2,3,4,5 pentaphosphate and myo-inositol 1,2,3,4 tetraphosphates are also present in seeds, but to a much lower extent (<15%) (90).

Phytic acid is negatively charged at physiological pH, which gives it an extraordinary chelating power with affinity for various components present in foods that are positively charged such as minerals and trace elements. The formed complexes are stable, insoluble and difficult to digest at physiological pH, thus decreasing their bioavailability in the human digestive tract (91).

In certain world populations where staples like wheat, maize and rice are the major or the only source of nutrition, PA as antinutritional factor attracts higher attention because the reduced bioavailability of minerals complexed by it can lead to significant deficiencies in humans (92). Also, Arendt et al. (93) reported that gluten free flours/ingredients have variable concentrations of phytate, i.e., rice, 0.12%; pearl millet, 0.25%; amaranth, 0.47%; teff, 0.70%; lupin, 0.77%; corn, 0.92%; oats,1.13%; quinoa, 1.18%; and soybean, 1.33%. Micronutrient deficiencies affect more than half of the world population, especially in developing countries where plants are the major source of food. Thus, improving the nutritional value of such type of food will improve the nutritional status of entire population (94). High content of phytates in the diet, especially of infants, children, elderly, and people in clinical situations, can significantly decrease the retention of calcium, iron and zinc (95). Reddy et al. (96) reported that PA also is present in the diets of non-ruminant animals, representing 50–80% of total phosphorus content in cereal grains and legumes frequently used in livestock animal feeds. However, phytate phosphorus present in food and feed has low bioavailability and is underutilized due to the lack or low levels of gastrointestinal phytases in monogastric animals (swine, poultry, and fishes) (97, 98). In order to meet the phosphorus requirements in these animals, inorganic phosphorus has to be added to the animal feedstuff as an additional nutrient, which in turn increases the feed cost and phosphorus pollution (99, 100). Undigested phytate and unabsorbed inorganic phosphate are excreted to a large extent (70%) and remains in manure and can lead to its accumulation in the soil and waters. This fact can generate the eutrophication of water, a serious phosphorus pollution problem in areas of intensive livestock production. The eutrophication of water surfaces can then generate cyanobacterial blooms, hypoxia and death of aquatic animals and nitrous oxide production, a potential green- house gas producing a severe environmental problem (101).

PA forms a strong complex with some proteins (the free portion of the basic amino acids such as Lys, Arg, His) and resists their proteolysis. PA negatively affects the absorption of proteins present in foods because inhibits enzymes that are necessary to their digestion such as pepsine. In general, the interaction of phytate with protein is dependent on pH (102).

Lee et al. (103) reported that dietary phytate forms complexes with carbohydrates, reducing their solubility and negatively affecting glucose absorption, leading to a decrease in the glycemic index (blood glucose response). In addition, it was postulated that phytate, by complexing with Ca^{2+} ion, inhibits amylase activity (104).

FUNCTIONAL FOODS AND BIOACTIVE COMPOUNDS

The demand of consumers for healthier foods has led the food industry to formulate new products within the area of so-called functional foods. Functional foods were defined by Bech-Larsen and Grunert (105) as "Foods that may provide health benefits beyond basic nutrition" and "Food similar in appearance to conventional food that is intended to be consumed as part of a normal diet, but has been modified to subserve physiological roles beyond the provision of simple nutrient requirements." According to these definitions, certain fruits and vegetables, rich in fiber and bioactive phytochemicals, can be considered functional products. Bioactive compounds are phytochemicals present in plants that can promote health but are not essential for life (106). In the last years, cereals have also been explored due to their potential utilization in developing functional foods (107–109). The key bioactive components of whole grain cereals provide health benefits, principally due to their content of flavonoid and dietary fiber. The covalent interactions between these two components increase their individual anti-inflammatory effects and their positive impact on the gut microbiome (67, 69, 110). In addition to their exceptional nutritional value, pseudocereals are characterized for being rich in many "health-promoting" phytochemicals, such as polyphenols and dietary fiber which exhibit anti-oxidant and free-radical scavenging activity (28, 64, 66, 111–115). Amaranth oil has high levels of tocotrienols and squalene, which are involved in the cholesterol metabolism and could play a significant role in lowering the low-density lipoprotein (LDL)—cholesterol in blood (116). Also, dietary fiber and polyphenols intake has been associated with reduced risk for a number of cardiovascular diseases including stroke, hypertension, and heart disease (117, 118). Pasko et al. (119) reported that the supplementation of a fructose-containing diet with quinoa in male Wistar rats reduced serum total cholesterol, triglycerides, glucose, LDL and plasma total protein and suggests the potential ability of this pseudocereal to prevent cardiovascular disease.

Furthermore, Berti et al. (120) reported that good glycemic control is especially important in CD, as there appears to be a higher incidence of type I diabetes among CD patients. Certain studies *in vivo* demonstrated that pseudocereals have hypoglycemic effects, for this reason they have been suggested as an alternative to habitual ingredients in the production of cereal-based GF products with low GI (120–123). Hence, the utilization of pseudocereals has increased not only in special diets for people allergic to cereals, but also as part of healthy diets (65).

FERMENTATION

Fermentation is a metabolic process in which carbohydrates are oxidized to liberate energy in the absence of external electron acceptor. This process is one of the oldest and most economical techniques applied in food preservation and processing (124). Fermented foods, produced and consumed since the development of human civilizations, form part of normal human diet (125). The original purpose of fermentation was the preservation effect. Subsequently, with the development of numerous available preservation technologies, plenty of fermented foods were therefore manufactured because of their unique flavors, aromas, and textures that are much appreciated by consumers. The fermentation of cereals plays a vital role in the production of compounds of great influence on the organoleptic characteristics (such as aroma, taste, and texture) and on the improvement of nutritional properties with a final positive impact on human health (126). Microorganisms are found in almost all ecological niches; cereals and pseudocereals are, in general, a good medium for microbial fermentations. They are rich in polysaccharides, which can be used as a source of carbon and energy by microorganisms during fermentation. Besides carbohydrates, they also contain minerals, vitamins, sterols, and other growth factors (127). Fermented products prepared from more common cereals (such as rice, wheat, corn, or sorghum) and pseudocereals are widespread around the world (128–130). In certain developing countries such as Asia and Africa, high consumption of cereals was reported where these grains are mixed with legumes to improve overall protein quality of the final fermented products (131). Cereal and pseudocereal grains normally have an indigenous microbiota composed by molds, LAB, enterobacteria, aerobic spore formers, etc., which compete for nutrients. The type of microbiota present in each fermented food depends on the pH value, water activity, salt concentration, temperature and composition of the food matrix (132).

Lactic Acid Fermentation

Lactic acid bacteria are Gram positive, non-sporulating, cytochrome deficient, catalase negative, aerotolerant, fastidious, acid-tolerant, and strictly fermentative microorganisms, produce lactic acid as the major metabolic end product of carbohydrate fermentation (133–135). LAB is a heterogeneous group of microorganisms with GRAS (Generally Recognized as Safe) status that have traditionally been associated with food fermentation (136). The effective carbohydrate fermentation coupled to substrate-level phosphorylation is essential characteristic of LAB; the ATP produced is then employed for biosynthetic functions. LAB are generally related with habitats rich in nutrients, for example different foods (milk, beverages, vegetables, meat, cereals); however, some LAB are members of normal flora of the intestine, mouth, and vagina of mammals (137, 138).

Hammes and Ganzle (139) reported that "Sourdough is a leavening agent traditionally obtained through a backslopping procedure, without the addition of starter microorganisms, whose use in bread making has a long history." Likewise, according to Hammes et al. (140) "The concerted hydrolytic activities of the grain and microorganisms (LAB and yeasts) are the origin of all cereal fermentations and are best represented by the traditional sourdough fermentation." The application of selected autochthonous LAB to ferment sourdough constitutes an adequate biotechnology to exploit the potential of cereals, non- wheat cereals and pseudocereals in breadmaking (141, 142). This criterion is of great importance when considering the different biochemical, technological, nutritional,

and functional characteristics of different flours. The activity of LAB during cereal fermentation is well-documented. A wide variety of metabolites and compounds, such as organic acids, exopolysaccharides (EPS), antimicrobial compounds, and useful enzymes, among others, are produced by LAB (143–151). There are several different ways how the nutritional and functional quality of cereals and pseudocereals could be improved by their fermentation such as: production of bioactive peptides that may stimulate immune system (152); elimination of cereal gluten (153–158); production of gamma-aminobutyric acid (141); increasing total phenolic content and antioxidant capacity (159–162); improving antiproliferative activity (162); decreasing of antinutritional factors, such as phytic acid, tannins and enzyme inhibitors (163–167).

Traditional cereal- and pseudocereal-fermented products are made all over the world, mainly widespread in Asia and Africa (168). Innovative functional fermented foods were formulated using cereal matrices and LAB (169–172). Currently, there are many products derived from cereals fermented by LAB, however only a few are derived from pseudocereals. Fermented quinoa-based beverages were developed by Ludena Urquizo et al. (173) and Jeske et al. (174). Jeske et al. (175), reported the beneficial effect of fermentation by mannitol-producing LAB in combination with various exogenous enzymes in the reduction of sugar in a quinoa-based milk substitute.

Improving the Functional Phytochemical Value by Lactic Fermentation

Numerous commercial microbial enzymes have been used to increase the functional value of phytochemicals present in plant sources however, lactic acid fermentation is preferred to improve the nutraceutical value of these foods because it is relatively inexpensive and improve overall organoleptic and nutritional characteristics (84). Contradictorily, PCs are able to exert an inhibitory effect on LAB (176). In addition, the incidence of certain chemical and physical parameters, such as the lack of fermentable carbohydrates, osmotic stress, and the acidic environment, are adverse conditions for bacterial growth. However, several LAB can adapt and grow in these substrates, being *L. plantarum* the most isolated species (166, 167, 177–179). The adaptation and survival strategies of LAB during cereal fermentation by activation of specific metabolic pathways have been investigated through a panel of various interacting omics approaches (metabolomic, phenomic, and transcriptomic profile) (180–183). The study of these adaptation responses would allow the optimal design of fermentation strategies for cereals and others plant matrices; however, these "omics" studies were not reported in pseudocereals fermentation.

The effects of LAB on the release of PC and modification of phenolic profiles in both cereals and pseudocereals have been reported. They depend mainly on the grains types, species of microorganisms, fermentation conditions, particularly time, temperature, and pH values (159, 183–185). Some studies have highlighted the capacity of lactic fermentation of pseudocereals to enhance the PC in bread (159, 161, 186), beverages (173, 187, 188), tarhana soup (189) and pasta (190). The PC metabolism in LAB has two important physiological functions, it is an efficient mechanism to detoxify such compounds (191), and can have a role in the cellular energy balance because LAB employ hydroxycinnamic acids as external acceptors of electrons (192). The metabolism of PC by LAB was described principally in *L. plantarum* strains, and only few studies were reported in *Weissella* spp., *Leuconostoc mesenteroides*, *L. paracollinoides*, *L. hilgardii,* and *Oenococcus oeni* (192–194). The enzymes involved in the PC metabolism by LAB such as decarboxylases (PAD), reductases (PAR), esterases and/or glycosidases were reported [**Figure 3**; (176, 179, 196–198)]. The production of vinyl-phenol, vinyl-guaiacol and vinyl catechol from the *p*-coumaric, ferulic and caffeic acids, respectively, by PAD activities, are the most relevant (176, 177). Subsequently, these hydroxycinnamates by action of reductase are transformed to their corresponding phenylpropionic acids (199).

The pseudocereals (i.e., buckwheat and quinoa) have a higher content of flavonoids, mainly rutine, kaempferol, and quercetin, with respect to cereals such as rye and wheat (112, 200–204). The flavonoid aglycones are more potent in their functional action (i.e., antioxidant activity) than their corresponding glycosides. Shin et al. (205) showed that a strain of *Enterococcus avium* was able to metabolize rutine, a flavonol glycoside, in quercetin, a flavonol with many beneficial effects on health. Recently, Zielinski et al. (206) observed a decrease in rutin content in buckwheat flours fermented by different species of *Lactobacillus*. According to Yang et al. (207), quercetin has numerous biological

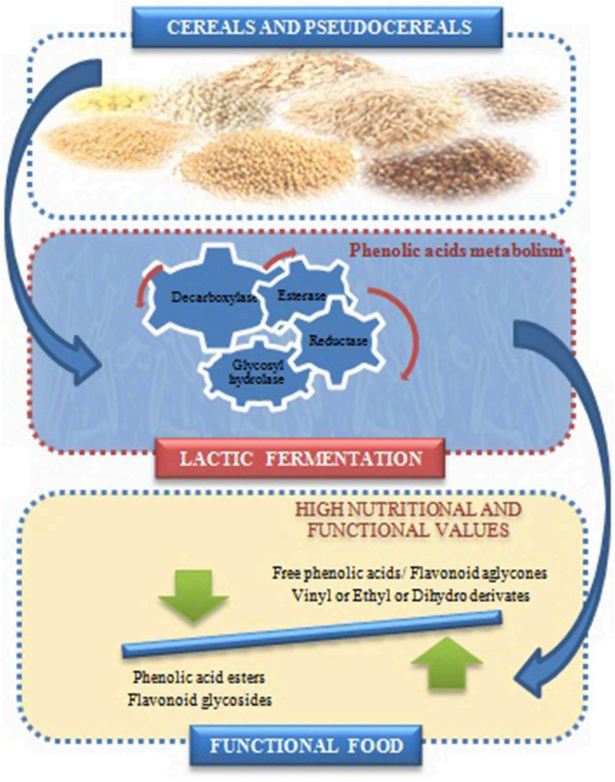

FIGURE 3 | Effect of lactic acid fermentation on phenolic compounds profile of cereal and pseudocereals [adapted from (195)].

and pharmacological effects, such as anticancer, antioxidative, antiviral, anti-inflammatory, and antiatherogenic activities. Fan et al. (208) reported the inhibition mechanism of quercetin on tyrosinase (rate-limiting enzyme in the melanogenesis pathway) and its potential use in the treatment of pigmentation disorders. In addition, Xiao (209) reviewed different biological benefits and pharmacokinetic behaviors between flavonoid glycosides and their aglycones. The elucidation of the metabolic pathways of these compounds will lead to obtain strains resistant to PC or adequate enzymes for cereals/pseudocereals processing and products with higher functional values, such as antioxidants.

Vitamins Produced by LAB

LAB and other vitamin-producing microorganisms can be used as an alternative to mandatory fortification in many countries to reduce deficiencies. Some LAB strains can produce elevated concentrations of the natural form of vitamins, which reduces the side-effects of chemically synthesized vitamins (masking of vitamin B_{12} deficiency, reduced enzyme activities in the liver, etc.) that are normally used (210, 211). Besides being a more natural alternative, vitamins producing LAB can also lower production costs by eliminating the need to add synthetic vitamins. The search for natural LAB strains from different ecological niches that can produce vitamins, such as folate, is essential in order to produce novel fermented foods that have high concentrations of this vitamin (212, 213). Vitamin producing strains have been able to revert and prevent vitamin deficiencies in animal models (52, 214).

LAB diversity is interesting not only at a species level, but also at the strain level, since most technological and nutritional properties are strain dependent. Raw cereals/pseudocereals constitute an interesting ecological niche to isolate new LAB strains with important characteristics to be used as a starter culture in the preparation of fermented cereal food. It was shown that folate producing LAB were isolated from wheat, sorghum and triticale (215). Previously, a few strains have been studied for this capacity in oat brans and rye sourdoughs (216, 217). In terms of vitamin-producing strains in pseudocereals, it has been shown that certain strains of LAB isolated from quinoa and amaranth sourdough have the capacity to produce elevated concentrations of riboflavin and folate in vitamin-free media (166, 167). These strains were used to obtain a B_9 and B_2 bio enriched pasta, which was able to prevent and revert vitamin deficiency in different rodent models (218).

In Africa, folate deficiency is related to the low dietary diversity and nutrient concentrations in complementary foods for infants (219, 220). In several African countries, cereal-based porridges are consumed as an alternative or in complement to breast feeding (221) but this product does not contain sufficient nutrients to prevent folate deficiencies (222). It has been suggested that porridges can also be consumed after fermentation with LAB, which can improve their overall nutritional quality, especially by increasing vitamin B_9 concentrations (131, 223).

Several vitamin B_2–producing LAB were isolated from durum wheat flour (224). Two *L. plantarum* over producer strains used as starter cultures were able to increase between 2 and 3 times the initial concentration of vitamin B_2 in both, bread and pasta fermentations.

Russo et al. (225) reported that *L. fermentum* PBCC11 isolated from sourdough was able to produce riboflavin. Bread produced using the co-inoculum yeast and *L. fermentum* PBCC11.5 led to an approximately 2-fold increase of final vitamin B_2 content compared to the wild-type strain (*L. fermentum* PBCC11). It was also reported that some LAB strains that are able to produce pseudo-vitamin B_{12} could also be used to increase the concentrations in cereal-based foods (226). These authors stated

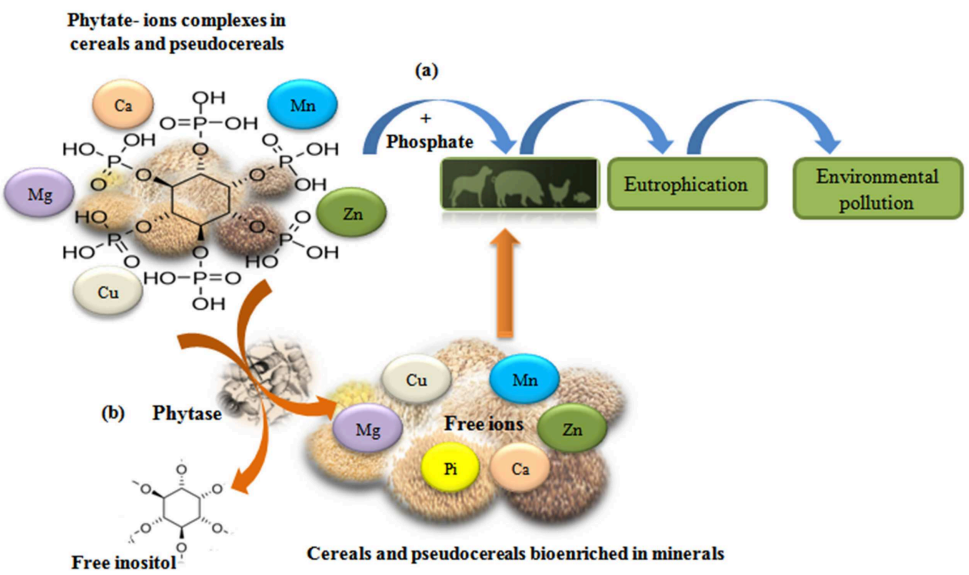

FIGURE 4 | Phytate-ions complexes reduce bioavailability of minerals in cereal and pseudocereals. **(a)** Phosphorus supplementation in animal feedstuff cause pollution problems. **(b)** Phytase activity increase mineral biodisponibility and improve nutrition value of foods [adapted from (240)].

that the first pseudo-cobalamin producing strain of LAB was *L. reuteri* CRL 1098 that was isolated from sourdough (227). It has been suggested that the pseudo-cobalamin produced by LAB would not be biologically available since the intrinsic factor has a low affinity for this compound (228). However, it has been shown that soy fermented with this strain was able to prevent vitamin B_{12} deficiency in mice (229), demonstrating that pseudo cobalamins are bioavailable. The analysis of sequenced genomes of LAB will provide more insights and new potential candidates that could be used to ferment cereal-based foods.

Strategies to Decrease Phytates in Pseudocereals: Phytases

Different methods have been applied to reduce the PA content in grains and food to improve their nutritional value (230). Phytases, [myo-inositol (1–6) hexakisphosphate phosphohydrolase] constitute a particular subgroup of phosphatases capable of initiating the gradual dephosphorylation of phytate [myo-inositol (1–6) hexakisphosphate] forming myo-inositol phosphate intermediates decreasing or eliminating its antinutritional effect (231, 232). Phytases can be derived from different sources including plants, animals and microorganisms such as yeast and LAB; however, their structures are different (233).

One strategy to reduce phytate in cereals/pseudocereals includes treatments, such as soaking and malting (94) or germination (234, 235), that activate phytases present in plants (236). However, this activity is considered insufficient to eliminate the phytate present in these substrates (237). Recent research has shown that microbial sources are more promising for the production of phytases on a commercial level and in cereal based foods (238, 239).

Nowadays, phytase is one of the most important enzymes for non-ruminant animal production. The application of phytases is broad, can be used to eliminate phytates in the food and feed industries, protect the environment by reducing phosphorus contamination and the eutrophication of water surfaces [**Figure 4**; (100, 239, 241, 242)]. Phytases have been successfully used in monogastric feeds for about to 20 years. In the beginning, marketable phytases were of fungal origin, mostly from *Aspergillus* species. Different studies have shown that the bioavailability of phosphorus increases, and the amount of phosphorus excreted is reduced (30–50%) by supplementing animal feeds with phytases (243–245). Recently, Theodoropoulos et al. (246) reported that treatment with commercial phytase decreased the content of myo-inositol phosphates and improved the nutritional value of soy drink, by improving the solubility of Ca^{2+}, Fe^{2+}, and Zn^{2+}. Currently, the significance of bacterial phytases as potential tools in biotechnology is increasing (247).

LAB phytase activity

The prevalence of LAB in cereal ecosystems and their contribution to the improvement of this particular fermentation processes could be due to their biochemical and metabolic characteristics (248). Studies of enzymes, like phytases involved in nutritional aspects in determined ecosystems, are important for the understanding of particular traits of LAB that are relevant for their right exploitation as starters (249). The PA contained in gluten free flours can be reduced by lactic acid fermentation, directly by LAB phytase activity or indirectly providing the optimal conditions to the endogenous phytase activity (250). Several studies were carried out on different aspects of LAB phytases in cereals fermentation (129, 130, 163, 238, 251–253), nevertheless, there are only few reports of these enzymes in autochthonous LAB isolated from pseudocereals. Carrizo et al. (166, 167) reported high phytase activities in LAB strains isolated from quinoa and amaranth (grains and sourdough), such as *E. durans* CRL 2122 (1,041 ± 48 U/mL), *E. mundtii* CRL 2007 (957 ± 25 U/ml) and *L. plantarum* CRL 2106 (730 ± 25 U/mL), among other. Afterwards, the minerals bioavailability present in pasta made with quinoa flour as a dietary matrix and fermented by selected LAB producing phytase was evaluated in an animal model. The animal group fed with the bio-enriched pasta fermented by LAB (*L. plantarum* CRL 2107 + CRL 1964) showed higher concentrations of minerals (P, Ca^{+2}, Fe^{+2}, and Mg^{+2}) with respect to control animal group (218). Also, Rizzello et al. (186) reported that the use of quinoa sourdough with autochthonous LAB (*L. plantarum* T6B10 and *L. rossiae* T0A16) increased phytase activity during the fermentation respect to non-fermented flour. The results confirmed that quinoa fermented with selected starters had a phytase activity ca. 2.75- times higher than raw quinoa flour.

Recent studies showed that phytate degradation by recombinant probiotic LAB could provide a solution for phosphate utilization in humans (254, 255). Regarding this topic, Vasudevan et al. (247) reviewed the contributions of recombinant technology to phytase research during the last decade with specific emphasis on new generation phytases. These results are relevant in the design of new functional foods with improved nutritional quality by using food-grade strains expressing microbial phytases.

CONCLUSIONS

Throughout the world and especially in developing countries, the interest in pseudocereals has increased for both consumers and small businesses. Recent studies strongly suggest that non-essential nutrients like phytochemicals of pseudocereals can also have potential health beneficial effects. This fact has promoted different processing techniques that may enhance the biological value of pseudocereals. Despite the important nutritional and functional value of these grains, their commercialization is still quite limited. Lactic acid fermentation is an ancestral process of food preservation but with renewed interest over time. It has become an important strategy to exploit the bioactive potential of pseudocereals by hydrolysing anti-nutrients factors and increasing the level of health beneficial compounds. The multiple beneficial effects of pseudocereals fermented by selected LAB can be exploited in different ways leading to the design of novel plant-based foods that can target specific populations.

This review summarized recent research reporting some different beneficial effects of pseudocereals and contributes to increase the knowledge on LAB capacity to produce B-group vitamins, metabolize phytochemicals, and decrease phytates present in Andean grains. In this way, lactic acid fermentation can contribute to improve the nutritional and functional

potential of fermented foods based on these grains for wide use throughout the world.

AUTHOR CONTRIBUTIONS

GR: writing—original draft, conceptualization, funding acquisition, and project administration. CG and JL: writing—original draft.

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Co-fermentation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* in Wheat Bran for *in situ* Production of Vitamin B12

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The present study investigated the effect of co-fermentation on vitamin B12 content and microbiological composition of wheat bran. *Propionibacterium freudenreichii* DSM 20271 was used as the producer of vitamin while *Lactobacillus brevis* ATCC 14869 was selected to ensure the microbial safety of the bran dough. Fermentation trials were conducted in bioreactors to monitor and adjust the pH of the ferments. Vitamin B12 level reached 357 ± 8 ng/g dry weight (dw) after 1 day of pH-controlled fermentation with *P. freudenreichii* monoculture and remained stable thereafter. In co-fermentation with *L. brevis*, slightly less vitamin B12 (255 ± 31 ng/g dw) was produced in 1 day and an effective inhibition of the growth of total *Enterobacteriaceae* and *Bacillus cereus* was obtained. On day 3, vitamin B12 content in pH-controlled co-fermentation increased to 332 ± 44 ng/g dw. On the other hand, without a pH control, co-fermentation resulted in a stronger inhibition of *Enterobacteriaceae* and *B. cereus* but a lower level of vitamin B12 (183 ± 5 ng/g dw on day 3). These results demonstrated that wheat bran fermented by *P. freudenreichii* and *L. brevis* can be a promising way to produce vitamin B12 fortified plant-origin food ingredients, which could reduce cereal waste streams and contribute to a more resilient food chain.

Keywords: *Propionibacterium freudenreichii*, *Lactobacillus brevis*, bioreactor, vitamin B12, wheat bran, co-fermentation

INTRODUCTION

Vitamin B12 plays an important role in human body and its deficiency may result in megaloblastic anemia, peripheral arterial diseases and various neurological disorders (Nielsen et al., 2012; Zsori et al., 2013). Previously, deficiency of this vitamin was considered as rare, but recent studies found that varying degrees of suboptimal vitamin B12 status, ranging from insufficiency to outright deficiency, have wide prevalence and affect people of all ages (Green et al., 2017; Smith et al., 2018). Considering animal products are the main dietary source of vitamin B12, developing plant-origin food products fortified with vitamin B12 is a promising way to increase dietary vitamin B12 intake of people consuming limited amounts of animal products (Watanabe et al., 2014). Among

the plant-based food matrices, cereal and cereal bran are the most abundant in the world and are an excellent material for innovative food applications. As the by-product of wheat (*Triticum aestivum*) milling process, a huge quantity of wheat bran is produced every year and yet most of it is discarded or used for feed due to its poor technological performance (Coda et al., 2015). *Propionibacterium freudenreichii* is a generally recognized as safe (GRAS) bacterium with the ability to produce active vitamin B12 in different plant-based matrices (Chamlagain et al., 2017; Signorini et al., 2018; Wolkers-Rooijackers et al., 2018). In our previous work, we demonstrated the possibility of producing physiologically significant amount of active vitamin B12 in non-sterile wheat bran using fermentation with *P. freudenreichii* (Xie et al., 2018). However, growth of potential pathogens, such as enterobacteria, from endogenous microbiota during wheat bran fermentation may result in safety concerns of the fermented dough to be used in food applications (Xie et al., 2018).

Co-fermentation with lactic acid bacteria (LAB) could be a feasible solution to improve the microbiological safety of the fermented bran matrix. LAB are a group of bacteria widely used in cereal fermentation to improve the flavor, nutrient contents and texture of products (De Vuyst and Neysens, 2005). Moreover, LAB can also produce various natural antimicrobials, contributing to the safety of fermented food products (Leroy and De Vuyst, 2004; Axel et al., 2017; Leyva Salas et al., 2017). Cultivation of propionibacteria (PAB) with LAB in cheese production is a typical example of commensalism because lactic acid produced by LAB is the preferential carbon source for PAB (Smid and Lacroix, 2013). Co-cultivation of PAB and LAB is also an appropriate choice for industrial biopreservation due to their production of various antimicrobial compounds (Smid and Lacroix, 2013). In addition, a co-fermentation process of LAB–PAB has been shown to produce vitamin B12 and folate in sterilized whey permeate medium (Hugenschmidt et al., 2011). However, producing vitamin B12 during the co-fermentation of LAB–PAB in non-sterile wheat bran matrices has not yet been reported.

There is only a limited number of studies on co-fermentation of LAB-PAB in cereal-based products, and most of them are focused on the preservative effect of these cultures. For instance, a mixed culture pre-fermentation of LAB and PAB can improve the shelf-life of wheat or rye sourdough breads as a result of the acid production (Javanainen and Linko, 1993a,b). Tinzl-Malang et al. (2015) also reported the antifungal, texture-building and anti-staling ability of LAB-PAB co-fermentation in wheat bread due to the synergistic effects of exopolysaccharide and acid productions. Notably, pH control was used in all above mentioned studies, most probably to avoid inhibition of PAB growth and metabolism by rapid pH drop caused by acid producing LAB (Chaia et al., 1994).

The aim of this study was to investigate the production of vitamin B12 by *P. freudenreichii* DSM 20271 in wheat bran during its co-fermentation with *Lactobacillus brevis* ATCC 14869 with or without a pH control. The strain of *L. brevis* was chosen based on a pre-screening study to improve the microbial safety of the fermented bran. The acidification properties, microbial growth, sugar metabolism and the change in riboflavin (a precursor for synthesis of vitamin B12) content were also monitored to follow the microbial metabolism during the co-fermentation.

MATERIALS AND METHODS

Pre-screening of Culture Combinations

To select a suitable culture in co-fermentation with *P. freudenreichii* for improving the microbial safety of fermented bran, *Saccharomyces cerevisiae* H10 and 7 strains of LAB belonging to the species: *Lactobacillus reuteri*, *Leuconostoc pseudomesenteroides*, *Lactobacillus delbrueckii*, *Weissella confusa*, *Leuconostoc mesenteroides*, and *L. brevis* (strain codes and origins in **Supplementary Table S1**), previously used for cereal or bran fermentation, were separately used for co-fermentation with *P. freudenreichii* DSM 20271. Wheat bran doughs (400 g) were prepared by mixing 80 g bran with 320 g water. After transferring into 500 ml bottles, doughs were inoculated at the initial cell density of 9.0 log colony forming units (CFU)/g of *P. freudenreichii* and 6.0 log CFU/g of LAB or yeast. The initial inoculum level of *P. freudenreichii* was performed according to our previous study to produce sufficient content of vitamin B12 (Xie et al., 2018). The inoculum levels of LAB or yeast were determined by preliminary experiments to achieve a significant inhibition on *Enterobacteriaceae* growth with a minor inhibition on production of vitamin B12. Doughs were fermented in shaking conditions (200 rpm) for 3 days at 25°C and during fermentation, pH value was measured every 12 h. When the value dropped below 5.5, pH was adjusted to 6.0 with 3M NaOH (no adjustment at 60 h). The fermentations were carried out in biological duplicate. After day 0, 1, and 3, samples of 20 g were taken out for cell count measurement of PAB and total *Enterobacteriaceae*. Based on the acidification properties (**Supplementary Table S2**) and the inhibitory effect on the propagation of *Enterobacteriaceae* (**Supplementary Table S3**), *L. brevis* ATCC 14869 was selected for further co-fermentation experiments.

Raw Material, Microbial Strains, and Culture Preparation

The milled wheat bran was obtained from Fazer Mills (Lahti, Finland). More than 99% of bran particles are smaller than 790 μm and about 80% of them were larger than 224 μm. The composition of the bran was 16.0% protein, 20.0% available carbohydrate, 43.0% fiber, 4.8% lipids, 12.5% moisture, and 3.9% ash, as provided by the manufacturer.

Both *P. freudenreichii* and *L. brevis* cultures were cryopreserved at −60°C in glycerol. *P. freudenreichii* was propagated in the yeast extract lactate (YEL) medium (Malik et al., 1968) at 30°C for 3 days and *L. brevis* was propagated in de Man, Rogosa and Sharpe (MRS) medium (Lab M, Lancashire, United Kingdom) at 37°C for 1 day. After incubation, the cultures were recovered by centrifugation (3,200 × g, 10 min) and resuspended in MillQ water before inoculation.

TABLE 1 | PH and NaOH consumption (ml) during controlled fermentation.

Sample code	Starter*	Initial pH	Final pH	Time (h)**	NaOH consumption (5 M) after 1 day	NaOH consumption (5 M) after 3 day
Control	–	6.5	5.0	19	11 ± 1^{a}	32 ± 2^{a}
PF_C	*P. f*	6.5	5.0	20	14 ± 1^{b}	36 ± 2^{a}
CO_C	*P. f* + *L. b*	6.5	5.0	11	24 ± 4^{c}	43 ± 1^{b}
CO_U	*P. f* + *L. b*	6.5	3.7	11	–	–

*Control, spontaneously fermented bran with pH control; PF_C, bran fermented with P. freudenreichii and pH control; CO_C, bran fermented with P. freudenreichii and L. brevis with pH control; CO_U, bran fermented with P. freudenreichii and L. brevis without pH control. *P. f, P. freudenreichii; L. b, L. brevis. **The time required for pH decreasing to 5.0. Values bearing different superscripts (a–c) in the same column are significantly different ($p < 0.05$).*

Fermentation

Four different wheat bran doughs were fermented as outlined in **Table 1**: spontaneously fermented bran dough with pH control (Control); bran dough fermented with *P. freudenreichii* monoculture with pH control (PF_C); bran dough fermented with *P. freudenreichii*/*L. brevis* co-culture with pH control (CO_C); bran dough fermented with *P. freudenreichii*/*L. brevis* co-culture without pH control (CO_U). In each fermentation, 1 kg of wheat bran dough was prepared by mixing bran and water in a 15:85 ratio. Wheat bran doughs were transferred in three bioreactors (Sartorius, Goettingen, Germany) and successively inoculated with the microbial starters. Fermentation was carried out for 72 h at 25°C, with stirring set at 600 rpm. In doughs fermented with pH control, 5 M NaOH solution was used to maintain the pH value at 5.0.

Propionibacterium freudenreichii was inoculated at the initial cell density of 9.0 log colony forming units (CFU)/g and *L. brevis* at the level of 6.0 log CFU/g. At time 0, 24, and 72 h, samples of 80 g were taken. An aliquot of 10 g was used for the cell count determinations and the rest of the samples were stored (−20°C) for other analyses. Three biological replicate fermentations were carried out for each dough type.

Microbial Counts

To estimate the number of viable cells, bran doughs (10 g) were serially diluted in sterile saline solution (8.5 g/L of NaCl) and appropriate dilutions were plated on the agar plates. YEL plates were incubated anaerobically for 4 days in anaerobic jars with Anaerogen (Oxoid, Basingstoke, United Kingdom) followed by 1 day incubation under aerobic conditions at 30°C. In these conditions, the colonies of *P. freudenreichii* turn brownish to be distinguishable from colonies of other bacteria. MRS agar (Lab M) for the cell counts of LAB was supplemented with 0.01% of cycloheximide (Sigma Chemical Co., United States) and microaerobically incubated at 30°C for 48 h. Plate count agar (PCA) plates (Lab M) were used for the cell counts of total aerobic bacteria. Yeast and mould (YM) agar plates (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L dextrose, and 0.01% chloramphenicol) were used for the cell counts of yeast. Total *Enterobacteriaceae* were enumerated on the violet red bile glucose agar (VRBGA) plates (Lab M). Polymyxin egg yolk mannitol bromothymol blue agar (PEMBA) plates (Lab M) were used for the cell counts of *Bacillus cereus*. PCA, YM, and PEMBA plates were incubated aerobically at 30°C for 48 h, VRBGA plates were incubated aerobically at 37°C for 48 h.

Determination of Acids

Lactic acid, acetic acid and propionic acid were determined using a high-performance liquid chromatography (HPLC) method. Dough samples (1 g) after dilution (1:10, w/v) in MilliQ water were centrifuged (3,200 × g, 10 min) and supernatants were filtered (0.45 μm, Pall, United States) before injection. HPLC analysis was performed with the same instrument and the method as reported in the earlier study (Xie et al., 2018).

Determination of Monosaccharides

Arabinose, galactose, xylose, glucose, and fructose were analyzed by high performance anion exchange chromatography equipped with a pulse amperometric detection system (HPAEC-PAD). Before analysis, dough samples diluted in water (1:10, w/v) were filtered by an Amicon Ultra-0.5 centrifugal filter unit (Millipore, Billerica, MA, United States) at 12,000 × g for 10 min to get rid of polymeric molecules. Monosaccharides were separated on a CarboPac PA1 column (250 × 4 mm i.d., Dionex, Sunnyvale, CA, United States) and detected using a Waters 2465 pulsed amperometric detector (Waters, United States). The solvents used were 200 mM NaOH and MilliQ water. A gradient elution was maintained at a constant flow rate of 1 ml/min: 0–31 min, 2 mM NaOH; 31–33 min, 200 mM NaOH; and 33–50 min, 2 mM NaOH, with an additional 10 min washing and regeneration steps. The injection volume was 10 μl. Glucose (Merck, Germany), fructose (Merck), xylose (Merck), arabinose (Merck), and galactose (Merck) were used as external standards and 2-deoxy-D-galactose (Sigma-Aldrich, Germany) was used as the internal standard for quantification.

Determination of Vitamin B12

Vitamin B12 in the bran dough was extracted in cyano form and determined by an Ultra-HPLC (UHPLC) method as described by Xie et al. (2018). During determination, the presence of other corrinoids, especially pseudovitamin B12, was followed in the chromatograms based on their retention times and absorption spectra according to our previous studies (Chamlagain et al., 2015, 2017; Deptula et al., 2015).

Briefly, dough samples (3 g) were mixed with 15 ml of extraction buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid, pH 4.5) and 100 μl of sodium cyanide (1% w/v in water).

After extraction in boiling water (30 min), cooled mixtures were incubated in a water bath (30 min, 37°C) with addition of 300 μl α-amylase (50 mg/ml; St Louis, MO, United States) to allow the breakdown of starch before centrifugation (6,900 × *g*, 10 min). Residues after centrifugation were suspended in 5 ml of extraction buffer and centrifuged again. Both supernatants were combined and adjusted to the same volume (25 ml) with the extraction buffer. Finally, 10 ml of the extracts were purified using an immunoaffinity column (Easi-Extract; R-Biopharm; Glasgow, Scotland) and analyzed with a Waters UPLC system (Milford, MA, United States) as explained by Chamlagain et al. (2015).

Determination of Riboflavin

Content of riboflavin in doughs was determined with a UHPLC method according to Chamlagain et al. (2016) with minor modification. Samples (2 g) were mixed with 15 ml of 0.1 M hydrochloric acid and extracted in a boiling water bath (60 min). After cooling on ice, the pH of the mixture was adjusted to 4.5 with 2.5 M sodium acetate and incubated at 37°C with Taka-Diastase (50 mg; Pfaltz and Bauer, CT, United States) and β-amylase (5 mg; Sigma-Aldrich) for 24 h. The extract was filtered (0.2 μm, Pall, United States) and analyzed on a Waters UPLC system with an Acquity BEH C18 column (2.1 × 100 mm, 1.7 μm) and a Waters fluorescence detector using aqueous methanol (30% v/v) containing 20 mM ammonium acetate as an eluent (0.2 ml/min).

Statistical Analysis

Statistical analysis was performed using SPSS 24.0 for Windows (IBM Corporation, NY, United States). One-way analysis of variance (ANOVA) and Tukey's *post hoc* test were used to determine significant differences at a p-value < 0.05 among the samples.

RESULTS

Pre-screening of Co-fermentation Cultures

Supplementary Tables S2–S4 show the change in pH values and the cell counts of total *Enterobacteriaceae* and PAB during bran dough fermentation with *P. freudenreichii* monoculture and in co-fermentation of *P. freudenreichii* with LAB/yeast strains. In general, dough pH dropped more rapidly and lower cell densities of *Enterobacteriaceae* were counted in doughs co-fermented with LAB compared to doughs co-fermented with yeast or fermented with *P. freudenreichii* monoculture. Fastest drop in pH and the lowest cell counts of *Enterobacteriaceae* on both day 1 (2.4 ± 0.2 log CFU/g) and day 3 (3.2 ± 0.2 log CFU/g) were observed in fermentations including *L. brevis* as a starter. The cell density of *P. freudenreichii* in all combinations varied from 8.9 to 9.4 log CFU/g during fermentation.

Microbial Counts of Bran Doughs

In the control dough, no PAB were detected throughout the fermentation (**Table 2**). The initial cell density of *P. freudenreichii* was ca. 8.7 log CFU/g on day 0 due to the inoculum. In the PF_C and CO_C doughs, cell density of *P. freudenreichii* increased from ca. 8.7 log CFU/g to ca. 9.2 log CFU/g during the first day and remained stable thereafter. In the CO_U dough, the cell density of *P. freudenreichii* remained constant from day 0 to day 3.

In doughs without *L. brevis* inoculation (control and PF_C), the initial cell density of LAB was ca. 3.0 log CFU/g and increased to ca. 9.8 log CFU/g on day 1. In the CO_C and CO_U doughs, the initial cell densities of LAB were ca. 6.3 log CFU/g and increased to ca. 10.2 log CFU/g and ca. 9.6 log CFU/g on day 1, respectively. From day 1 to day 3, cell density of LAB remained stable in the CO_C dough but decreased of 0.5 log units in the CO_U dough. The initial cell densities of the total aerobic

TABLE 2 | Cell counts (log CFU/g) of *Propionibacteria* (PAB), lactic acid bacteria (LAB), total aerobic bacteria, yeasts, total *Enterobacteriaceae*, and *Bacillus cereus* during bran dough fermentation.

Time (days)	0	1	3
PAB			
Control	nd*	nd	nd
PF_C	$8.8 \pm 0.1^{a,x}$	$9.2 \pm 0.1^{b,y}$	$9.0 \pm 0.1^{b,y}$
CO_C	$8.7 \pm 0.1^{a,x}$	$9.1 \pm 0.1^{b,y}$	$8.9 \pm 0.2^{b,xy}$
CO_U	$8.6 \pm 0.1^{a,x}$	$8.6 \pm 0.2^{a,x}$	$8.5 \pm 0.1^{a,x}$
LAB			
Control	$2.7 \pm 0.3^{a,x}$	$9.8 \pm 0.2^{a,y}$	$9.8 \pm 0.2^{a,y}$
PF_C	$3.0 \pm 0.2^{a,x}$	$9.7 \pm 0.2^{a,y}$	$9.6 \pm 0.1^{a,y}$
CO_C	$6.3 \pm 0.2^{b,x}$	$10.2 \pm 0.0^{b,y}$	$10.3 \pm 0.2^{b,y}$
CO_U	$6.4 \pm 0.2^{b,x}$	$9.6 \pm 0.2^{a,z}$	$9.1 \pm 0.1^{a,y}$
Total aerobic bacteria			
Control	$5.2 \pm 0.2^{a,x}$	$9.8 \pm 0.4^{a,y}$	$9.8 \pm 0.1^{b,y}$
PF_C	$5.2 \pm 0.0^{a,x}$	$9.6 \pm 0.2^{a,y}$	$9.6 \pm 0.2^{b,y}$
CO_C	$6.4 \pm 0.3^{b,x}$	$9.9 \pm 0.3^{a,y}$	$10.2 \pm 0.2^{c,y}$
CO_U	$6.5 \pm 0.2^{b,x}$	$9.7 \pm 0.2^{a,z}$	$9.2 \pm 0.2^{a,y}$
Yeasts			
Control	$3.7 \pm 0.3^{a,x}$	$5.1 \pm 0.0^{b,y}$	$5.4 \pm 0.1^{b,y}$
PF_C	$3.7 \pm 0.1^{a,x}$	$5.1 \pm 0.2^{b,y}$	$5.3 \pm 0.1^{b,y}$
CO_C	$3.6 \pm 0.2^{a,x}$	$3.6 \pm 0.2^{a,x}$	$5.1 \pm 0.3^{b,y}$
CO_U	$3.6 \pm 0.1^{a,x}$	$3.4 \pm 0.2^{a,x}$	$3.6 \pm 0.2^{a,x}$
Total *Enterobacteriaceae*			
Control	$4.8 \pm 0.0^{a,y}$	$6.1 \pm 0.1^{b,z}$	$3.7 \pm 0.3^{b,x}$
PF_C	$4.7 \pm 0.1^{a,y}$	$6.0 \pm 0.1^{b,z}$	$3.7 \pm 0.1^{b,x}$
CO_C	$4.7 \pm 0.1^{a,y}$	$3.3 \pm 0.3^{a,x}$	$3.4 \pm 0.4^{b,x}$
CO_U	$4.8 \pm 0.1^{a,z}$	$3.4 \pm 0.3^{a,y}$	$2.8 \pm 0.1^{a,x}$
Bacillus cereus			
Control	$3.2 \pm 0.1^{a,x}$	$3.1 \pm 0.0^{b,x}$	nd
PF_C	$3.3 \pm 0.0^{a,x}$	$3.4 \pm 0.1^{b,x}$	nd
CO_C	$3.2 \pm 0.2^{a,y}$	$2.4 \pm 0.1^{a,x}$	nd
CO_U	3.1 ± 0.1^{a}	nd	nd

**nd, not detected. Control, spontaneously fermented bran with pH control; PF_C, bran fermented with P. freudenreichii and pH control; CO_C, bran fermented with P. freudenreichii and L. brevis with pH control; CO_U, bran fermented with P. freudenreichii and L. brevis without pH control. The results are expressed as the mean ± standard deviation (n = 3). Values from the same day and microbial group bearing different superscripts (a–c) are significantly different ($p < 0.05$). Values from the same dough type and microbial group bearing different superscripts (x–z) are significantly different ($p < 0.05$).*

bacteria ranged from ca. 5.2 log CFU/g to 6.4 log CFU/g. During fermentation, their cell densities increased in the range of ca. 9.2 log CFU/g to 10.2 log CFU/g.

The initial cell density of yeasts was approximately 3.7 log CFU/g on day 0. The cell density of yeasts increased to ca. 5.1 log CFU/g on day 1 in doughs without *L. brevis* inoculation but remained unaltered afterward in co-fermented doughs. On day 3, yeast cell number in the CO_U dough was significantly ($p < 0.05$) lower than in the other doughs.

Before fermentation, ca. 4.8 log CFU/g of total *Enterobacteriaceae* and ca. 3.2 log CFU/g of *B. cereus* were found in wheat bran dough. In the control and PF_C doughs, cell densities of *Enterobacteriaceae* increased to ca. 6.0 log CFU/g on day 1 and decreased to ca 3.7 log CFU/g on day 3. In the CO_C dough, cell density of total *Enterobacteriaceae* was ca. 3.3 log CFU/g on day 3. In the CO_U dough, the final cell density of *Enterobacteriaceae* (2.8 ± 0.1 log CFU/g) was significantly ($p < 0.05$) lower than in the other doughs. *B. cereus* was not detected in the CO_U dough but a cell density of 2.4 to 3.0 log CFU/g of *B. cereus* was still detected in the other 3 doughs after 1 day of fermentation. However, on day 3, *B. cereus* was not found in any of the four doughs.

Acidification of the Doughs

On day 0, the pH value of all the doughs were ca. 6.5 (**Table 1**). In the doughs inoculated with *P. freudenreichii*/*L. brevis* co-culture pH dropped most rapidly and reached pH 5.0 already after 11 h. In the dough inoculated with *P. freudenreichii* monoculture and in the control dough, pH dropped similarly and reached pH 5.0 after 20 and 19 h, respectively. At the end of fermentation, pH 3.7 was reached in the CO_U dough while in other doughs pH remained at 5.0.

Among the doughs with pH control, the highest consumption of NaOH solution (5 M) was found in the CO_C dough both on day 1 (24 ± 4 ml) and day 3 (43 ± 1 ml). The consumption of NaOH in the PF_C dough (14 ± 1 ml) was significantly ($p < 0.05$) higher than in the control dough (11 ± 1 ml) on day 1 but there was no significant ($p > 0.05$) difference in NaOH consumption between these two doughs on day 3.

Before fermentation, lactic, acetic and propionic acid were not detected in any of the doughs (**Figure 1**). On day 1, the concentration of lactic acid in the CO_C dough was 204 ± 14 mg/g dry weight (dw), which was significantly ($p < 0.05$) higher than in the other doughs (ranging from ca. 79 to 96 mg/g dw). On day 3, lactic acid content in the doughs with a pH control had no significant ($p > 0.05$) difference and varied from 212 to 250 mg/g dw. In the CO_U dough, the concentration of lactic acid was 98 ± 2 mg/g dw at day 3.

On day 1, the highest amount of acetic acid was found in the PF_C dough (20.3 ± 1.0 mg/g dw). Concentration of acetic acid in the control dough (12.8 ± 3.5 mg/g dw) was significantly ($p < 0.05$) higher than in the CO_C (3.6 ± 0.6 mg/g dw) and the CO_U dough (1.8 ± 0.1 mg/g dw). On day 3, the highest concentration of acetic acid was found in the PF_C dough (40.3 ± 1.6 mg/g dw) and the lowest concentration was found in the CO_U dough (3.1 ± 0.6 mg/g dw).

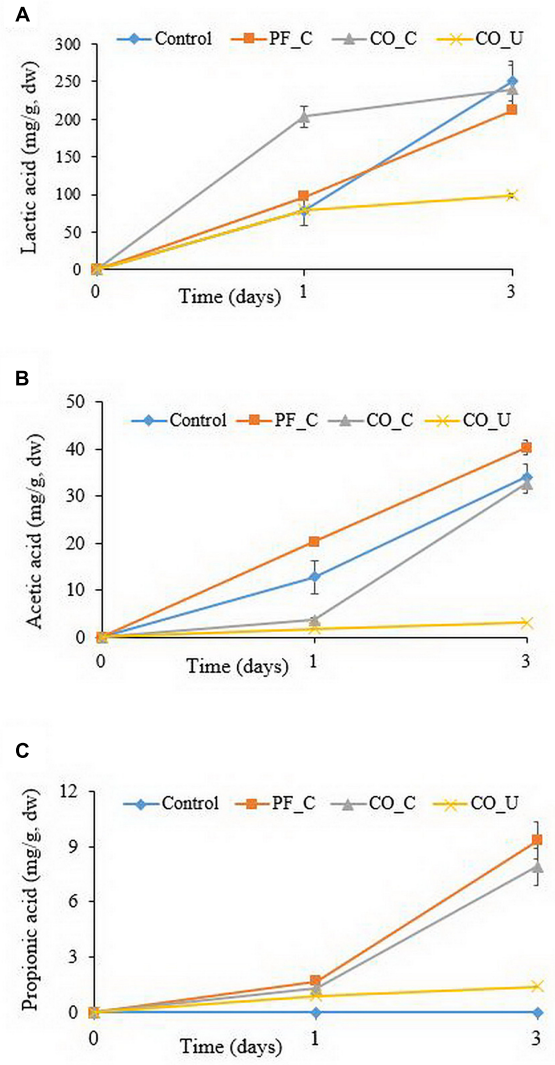

FIGURE 1 | Concentration (mg/g, dry weight) of lactic acid **(A)**, acetic acid **(B)**, and propionic acid **(C)** during fermentation. Values are means and standard deviations of three replicates. Control, spontaneously fermented bran with pH control; PF_C, bran fermented with *P. freudenreichii* monoculture and pH control; CO_C, bran fermented with *P. freudenreichii*/*L. brevis* co-culture with pH control; CO_U, bran fermented with *P. freudenreichii*/*L. brevis* co-culture without pH control.

Propionic acid was not detected in the control dough throughout the fermentation. In other doughs, the level of propionic acid ranged from 0.9 to 1.7 mg/g dw on day 1. On day 3, the CO_U dough had the lowest concentration of propionic acid (1.4 ± 0.1 mg/g dw) and the level of propionic acid in the other two doughs had no significant ($p > 0.05$) difference and ranged from 7.9 to 9.3 mg/g dw.

Monosaccharides in Bran Doughs

On day 0, the main monosaccharides in wheat bran doughs were glucose (3.4 mg/g dw) and fructose (2.3 mg/g dw) while

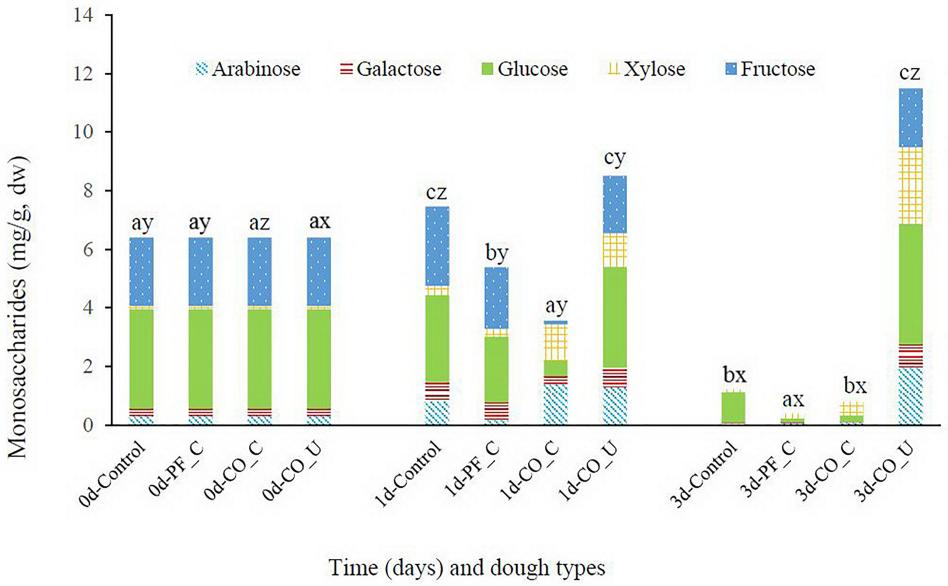

FIGURE 2 | Concentration (mg/g, dry weight) of arabinose, galactose, glucose, xylose, and fructose during fermentation. Values are means and standard deviations of three replicates. Control, spontaneously fermented bran with pH control; PF_C, bran fermented with *P. freudenreichii* monoculture and pH control; CO_C, bran fermented with *P. freudenreichii/L. brevis* co-culture with pH control; CO_U, bran fermented with *P. freudenreichii/L. brevis* co-culture without pH control. Values of total monosaccharide concentration from the same day bearing different superscripts (a–c) are significantly different ($p < 0.05$). Values of total monosaccharide concentration from the same dough type bearing different superscripts (x–z) are significantly different ($p < 0.05$).

xylose, galactose and arabinose were present at levels ranging from 0.1 to 0.3 mg/g dw (**Figure 2**). In the control dough, concentration of galactose, xylose and arabinose increased during the first day and the sum of monosaccharides increased from ca. 6.4 mg/g dw to ca. 7.5 mg/g dw. However, only ca. 1.2 mg/g dw of monosaccharides was detected in the control dough on day 3. Content of monosaccharides in the PF_C dough was ca. 5.4 mg/g dw and was mainly composed by glucose and fructose on day 1, while in the CO_C dough there was ca. 3.6 mg/g dw of monosaccharides on day 1, mostly composed of xylose and arabinose. On day 3, there were ca. 0.4 mg/g dw and ca. 0.8 mg/g dw of monosaccharides in the PF_C dough and the CO_C dough, respectively. In the CO_U dough, the concentration of all monosaccharides, except fructose, increased from day 0 to day 3 and reached a sum of ca. 11.5 mg/g dw at the end of fermentation.

Vitamin B12 in Bran Doughs

In the control dough, no vitamin B12 was detected during fermentation (**Figure 3**). In other doughs, ca. 40 ng/g dw of vitamin B12 were found on day 0 from the *P. freudenreichii* inoculum. On day 1, the highest content of vitamin B12 was found in the PF_C dough (357 ± 9 ng/g dw). In the CO_C dough and the CO_U dough, vitamin B12 concentration were 255 ± 31 and 214 ± 35 ng/g dw on day 1, respectively. From day 1 to day 3, there was no significant ($p > 0.05$) increase of vitamin B12 concentration in the PF_C dough and the CO_U dough. In the CO_C dough, concentration of vitamin B12 increased to 332 ± 34 ng/g dw on day 3, which was on the same level as in the PF_C dough.

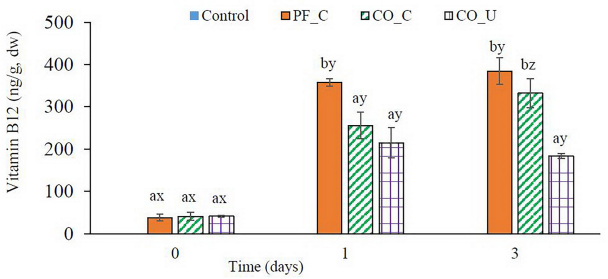

FIGURE 3 | Concentration (ng/g, dry weight) of vitamin B12 during fermentation. Values are means and standard deviation from three replicates. Control, spontaneously fermented bran with pH control; PF_C, bran fermented with *P. freudenreichii* monoculture and pH control; CO_C, bran fermented with *P. freudenreichii/L. brevis* co-culture with pH control; CO_U, bran fermented with *P. freudenreichii/L. brevis* co-culture without pH control. Values from the same day bearing different superscripts (a,b) are significantly different ($p < 0.05$). Values from the same dough type bearing different superscripts (x–z) are significantly different ($p < 0.05$).

Riboflavin in Bran Doughs

Before fermentation, wheat bran doughs contained ca. 4.0 μg/g dw of riboflavin (**Figure 4**). In the control dough, concentration of riboflavin had no significant ($p > 0.05$) change during fermentation. In other doughs, riboflavin concentrations were significantly ($p < 0.05$) lower on day 1 varying from ca. 3.2 μg/g dw to ca. 3.5 μg/g dw. From day 1 to day 3, concentration of

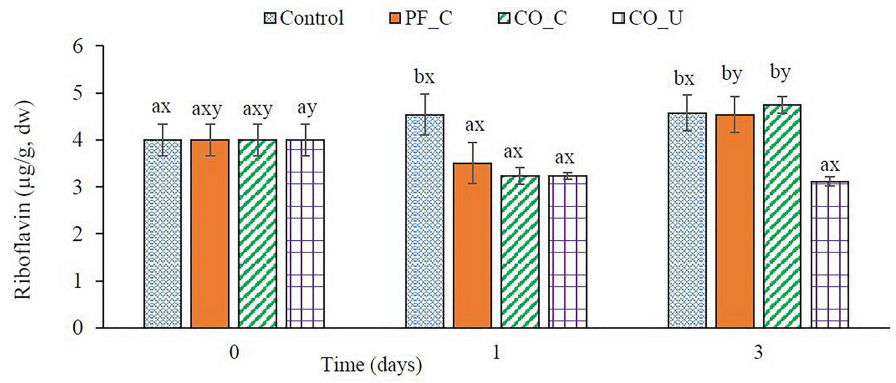

FIGURE 4 | Concentration (μg/g, dry weight) of riboflavin during fermentation. Values are means and standard deviation from three replicates. Control, spontaneously fermented bran with pH control; PF_C, bran fermented with *P. freudenreichii* monoculture and pH control; CO_C, bran fermented with *P. freudenreichii/L. brevis* co-culture with pH control; CO_U, bran fermented with *P. freudenreichii/L. brevis* co-culture without pH control. Values from the same day bearing different superscripts (a,b) are significantly different ($p < 0.05$). Values from the same dough type bearing different superscripts (x,y) are significantly different ($p < 0.05$).

riboflavin increased to ca. 4.6 μg/g dw in the PF_C dough and the CO_C dough while it remained stable in the CO_U dough.

DISCUSSION

In the present study, non-sterile wheat bran was used for *in situ* fortification of vitamin B12 by co-fermentation of *P. freudenreichii* DSM 20271 and *L. brevis* ATCC 14869 in controlled conditions. This strain of *P. freudenreichii* was found to be a promising vitamin B12 producer in our previous study (Xie et al., 2018). A pre-screening phase was conducted to select the best performing starter for co-fermentation with *P. freudenreichii* in wheat bran to reduce the growth of *Enterobacteriaceae*, and improve the microbial safety of fermented wheat bran. *L. brevis* ATCC 14869 was selected as it exhibited the strongest antagonistic activity. The adaptability and performance of different strains of *L. brevis* in bran has been shown previously, including the positive effect on the quality of bread containing fermented wheat bran (Coda et al., 2014; Valerio et al., 2014; Messia et al., 2016).

Microbiological Characteristics of Fermentation

The microbiota of the wheat bran doughs was composed of endogenous microorganisms and the added microbial inocula. In the doughs with starters, the cell density of *P. freudenreichii* was ca. 8.7 log CFU/g before fermentation while the cell density of LAB was ca. 6.3 log CFU/g in the doughs with addition of *L. brevis* and ca. 3.0 log CFU/g in doughs without *L. brevis* inoculation. However, either endogenous or inoculated LAB outnumbered the cell density of *P. freudenreichii* and dominated fermentation already after 1 day of fermentation. Yeasts are a group of microorganisms commonly found in sourdough, co-existing with LAB and the LAB:yeast ratio has been shown to be generally 100:1 during traditional wheat sourdough fermentation (De Vuyst and Neysens, 2005). Similarly to our previous results (Xie et al., 2018), in the present study, LAB:yeast ratio in the control dough was about 10000:1 during fermentation. Meanwhile, addition of *L. brevis* inhibited the growth of yeasts on day 1. However, the cell density of yeasts increased to ca. 5.3 log CFU/g on day 3 in the CO_C dough but remained stable in the CO_U dough suggesting that inhibition of *L. brevis* on yeasts may be due to the decrease of pH.

Native wheat bran also contained some undesirable potential pathogens, such as *Enterobacteriaceae* and *B. cereus*. *Enterobacteriaceae* is a large family of Gram negative bacteria and some members among this group are able to cause infections of the human gastrointestinal tract or might produce various endotoxins (Singh et al., 2015). *B. cereus* is a pathogenic foodborne species commonly existing in plant-origin products such as bread, rice and vegetables (Rosenquist et al., 2005). By producing heat-stable toxins in food, *B. cereus* can cause mild to severe nausea, vomiting and diarrheal illness in humans (Bottone, 2010). Therefore, controlling the growth of these bacteria during bran fermentation is very important for the safety of bran derived food.

Lactic acid bacteria can inhibit the growth of pathogens by production of acids and antimicrobial compounds as well as by competitive exclusion (Kostrzynska and Bachand, 2006). However, outgrowth of *Enterobacteriaceae* (ca. 6.0 log CFU/g on day 1) in the control dough showed that spontaneous fermentation with endogenous LAB was not effective in controlling the cell propagation of *Enterobacteriaceae*. This may be due to the low initial level of endogenous LAB (ca. 3.0 log CFU/g) and pH control condition during fermentation which may diminish the inhibitory effect of produced acids on potential pathogens. Inoculum with *P. freudenreichii* monoculture did not show inhibitory effect on *Enterobacteriaceae* growth either, and under the pH control conditions used here, only the additional starter culture provided a promising inhibition on *Enterobacteriaceae*.

The pre-screening revealed that all the LAB cultures tested could, to a varying extent, inhibit the growth of

Enterobacteriaceae while *L. brevis* showed the strongest inhibition among them. During co-fermentation with *L. brevis*, the cell density of *Enterobacteriaceae* started to decrease on day 1 irrespective of whether pH was controlled or not. This inhibitory effect of *L. brevis* in the early stage can reduce the microbial risk e.g., potential production of endotoxins during fermentation and increase the overall quality of wheat bran. Moreover, the lower cell density of *Enterobacteriaceae* in CO_U dough (2.8 ± 0.1 log CFU/g) than in CO_C dough (3.4 ± 0.4 log CFU/g) on day 3 showed that the lower pH (3.7 vs. 5.0) can enhance the inhibition of *L. brevis* on *Enterobacteriaceae*. Although in this study the dominance of *L brevis* ATCC 14869 was not confirmed, the high level of inoculum (ca. 6.0 log CFU/g) compared to the endogenous LAB of wheat bran (ca. 3.0 log CFU/g), and the significant ($p < 0.05$) difference of lactic acid (97 vs. 204 ug/g dw) and acetic acid (20 vs. 4 ug/g dw) content between the PF_C dough and CO_C dough suggest that the starter culture was able to steer the fermentation process.

Utilization of Carbohydrates and Production of Acids

Wheat bran contains various endogenous and microbial enzymes which can result in the release of various monosaccharides from complex carbohydrates during fermentation (Apprich et al., 2014; Immerzeel et al., 2014). Additionally, both inoculated and endogenous microorganisms in bran doughs also consumed monosaccharides to produce acids and other metabolites. In the control dough, endogenous LAB brought intensive acidification. However, from day 0 to day 1, the level of monosaccharides in the control dough increased as a result of liberation of xylose and arabinose by hydrolysis of arabinoxylan, which comprises 10.9 to 26.0% of wheat bran (Onipe et al., 2015).

Propionibacterium freudenreichii prefers lactic acid as the carbon source during fermentation and produces propionic acid and acetic acid as the main metabolites (Lee et al., 1974). Addition of *P. freudenreichii* had no effect on the cell density of endogenous LAB but resulted in a faster utilization of monosaccharides and higher production of acids. When *L. brevis* ATCC 14869 was added (CO_C), higher level of lactic acid and lower level of acetic acid were found compared to the dough containing only *P. freudenreichii* (PF_C) and the spontaneously fermented dough on day 1. It was previously observed that co-fermentation of glucose and other carbon sources is a typical feature of *L. brevis* ATCC 14869, in which a less rigorous hierarchical consumption of carbohydrates occurs. Additionally, simultaneous fermentation of glucose and fructose resulted in lactic acid and ethanol as the main products (Kim et al., 2009). After day 1, the level of acetic acid in the CO_C dough increased drastically, likely because of *L. brevis* ATCC 14869 started to use xylose and arabinose after fructose and glucose were almost depleted. In fact, the addition of *L. brevis* largely increased the hydrolysis of arabinoxylan during the first day of fermentation. From day 1 to day 3, microorganisms in doughs with pH control still utilized monosaccharides and continued acid production. However, in the dough without pH control, no further acid production was observed after day 1 suggesting that the low pH reached might have inhibited the metabolic activity of the microorganisms. For example, PAB cannot produce acids when pH is lower than 4.5 (Piwowarek et al., 2018). On the other hand, contents of monosaccharides increased throughout the fermentation because some monosaccharide-releasing enzymes, such as xylanolytic enzymes, may still be active in this pH condition (Bajpai, 2014).

Production of Vitamin B12

The fact that vitamin B12 was not found in the control dough confirmed that vitamin B12 was only synthesized by the inoculated *P. freudenreichii*. In previous studies, the possibility to fortify plant-based substrates with vitamin B12 by *P. freudenreichii* fermentation was shown. For instance, it was found that 9 ng/g to 37 ng/g fresh weight (41 ng/g to 200 ng/g dry weight) of vitamin B12 were produced by fermentation of *P. freudenreichii* in autoclaved aqueous barley and wheat aleurone matrices (Chamlagain et al., 2017). Moreover, an increase of vitamin B12 content (up to 9.7 ng/g fresh weight) in lupin tempeh by co-fermentation of *Rhizopus oryzae* and *P. freudenreichii* was also reported (Wolkers-Rooijackers et al., 2018). In our former study, ca. 155 ng/g dw of vitamin B12 was produced in wheat bran dough after a 7-day fermentation by *P. freudenreichii* with a final cell density at ca. 9.2 log CFU/g (Xie et al., 2018). In the present study, ca. 200 ng/g dw of vitamin B12 was produced in the CO_U dough after only 1 day of fermentation by *P. freudenreichii* with a cell density at ca. 8.5 log CFU/g. Considering that cobalt is one of the limiting factors for vitamin B12 production by *P. freudenreichii* during fermentation (Hugenschmidt et al., 2011; Deptula et al., 2017; Xie et al., 2018), the higher production of vitamin B12 in the present study was probably due to the higher cobalt content in the wheat bran used here than in the one used in the previous study (0.27 vs. 0.1 μg/g dw; data not shown). In addition, according to Quesada-Chanto et al. (1994), the production of vitamin B12 by *P. freudenreichii* was strongly depending also on pH level. The optimal pH for the production was around 6.5. The higher concentration of vitamin B12 in the PF_C dough than in the CO_C dough on day 1 could be a result of slower acidification in PF_C dough. Moreover, from day 1 to day 3, the concentration of vitamin B12 increased continuously in the CO_C dough, which was not observed in the CO_U dough, implicating that *P. freudenreichii* was still able to produce vitamin B12 at pH 5.0. However, no further increase of vitamin B12 content was observed in the PF_C dough from day 1 to day 3, possibly due to the depletion of available cobalt in the dough.

Synthesizing Lower Ligand of Vitamin B12 From Riboflavin

Since *P. freudenreichii* mainly produces the active form of vitamin B12 with a 5, 6-dimethylbenzimidazole (DMBI) as the lower ligand (Deptula et al., 2015) and no DMBI were added during fermentation, all the DMBI in the synthesized vitamin B12

was from *de novo* biosynthesis. Riboflavin has been found to be the precursor for the *de novo* biosynthesis of DMBI in *P. freudenreichii* in the presence of oxygen (Hollriegl et al., 1982). After day 1, the content of riboflavin in doughs with vitamin B12 production was significantly ($p < 0.05$) lower than in the control dough confirming that *P. freudenreichii* synthesized DMBI from riboflavin. The content of riboflavin in the PF_C dough and the CO_C dough increased to the same level as in the control dough on day 3 because riboflavin can also be synthesized by *P. freudenreichii* and various LAB species commonly existing in wheat based sourdough microflora (Burgess et al., 2006; Capozzi et al., 2011; Russo et al., 2014).

CONCLUSION

This work demonstrated that *P. freudenreichii* can produce nutritionally relevant amount of vitamin B12 in wheat bran during co-fermentation with *L. brevis* and that the vitamin B12 production can be markedly enhanced by maintaining the medium pH above 5. Meanwhile, addition of *L. brevis* with *P. freudenreichii* can effectively inhibit the growth of total *Enterobacteriaceae* and *B. cereus* to ensure the safety of fermentation when pH was controlled around 5. Therefore, wheat bran fermented with *P. freudenreichii* and *L. brevis* can be a promising alternative to produce vitamin B12 enriched ingredient for various food products. These applications could increase the use of wheat bran, thus reducing cereal waste streams and contributing to a more resilient food chain.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

CX performed the experiments and drafted the manuscript. RC, BC, PV, VP, and KK conceived the experiments and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01541/full#supplementary-material

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Effects of Freeze–Thaw Event on Microbial Community Dynamics During Red Clover Ensiling

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Freezing damages in forages represents a major economic loss to agriculture. This study was conducted to investigate the effects of freeze–thaw (FT) event on microbial community dynamics of red clover silage. Results showed that the FT-treated material displayed higher proportions of *Weissella* and aerobic bacteria, while lower *Pantoea* and *Enterobacter* compared with the control material. The FT event promoted the development of *Lactobacillus* in silage microflora, inducing more intense lactic fermentation after an initial short lag. The aerobic bacteria were suppressed immediately after the onset of ensiling. Microbiomes of the two silages tended to be almost similar after 2 days of ensiling. However, a small number of aerobic bacteria tended to revitalize in the FT silage with prolonged ensiling time, indicated by apparent abundances of *Acinetobacter* and *Pseudomonas* at the end of ensiling. The results obtained here suggest that the FT event could promote the development of *Lactobacillus* during ensiling and the control of aerobe revitalization need to be concerned with silages made from the freeze-damaged forages.

Keywords: freeze–thaw event, red clover, microbial community, fermentation quality, next-generation sequencing

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INTRODUCTION

In boreal and temperate regions, forage crops are frequently exposed to freezing temperature during autumn, spring, and mild winter. This leads to reduction in field productivity and, particularly, problem in forage utilization due to altered physical properties after thawing (Koponen et al., 2006). Direct ensiling could be an advisable choice as it avoids the waste resulting from the aerobic spoilage in field. Ensiling is a conservation method based on spontaneous lactic acid fermentation under anaerobic condition. It could be expected that the freeze–thaw (FT) stress would cause physical damages to plant, promote nutrient release from plant cell (Phalakornkule et al., 2017) and consequently benefit the development of lactic acid bacteria (LAB) during ensiling (Charmley et al., 1997). However, on the other hand, the damaged forages may also serve as an open culture medium allowing vigorous growth of various bacteria. Although it is known that a great majority of these bacteria are aerobes and will be suppressed after anaerobiosis is achieved during ensiling, some undesirable facultative aerobes, such as enterobacteria, might remain active during ensiling (McDonald et al., 1991). This brings the uncertainties of silage fermentation quality made from the FT-damaged forages. Evaluation of potential of the FT-damaged forages in silage making would be important as to supporting the current shifts toward sustainable and low-cost agricultural systems.

Red clover (*Trifolium pratense* L.) is a leading legume forage widely cultivated in boreal and temperate regions. Compared to alfalfa (*Medicago sativa* L.), red clover is characterized by rapid spring establishment and superior performance on acid and wet soils (Bertrand et al., 2016).

However, as a promising forage crop, characterization of microbial communities in red clover silage is far behind from those in other forage species, such as alfalfa (Zheng et al., 2017; Yang et al., 2019) and whole crop corn (Keshri et al., 2018; Ni et al., 2018). Understanding of the microbial communities involved in the ensiling process would provide insight into approaches to improve the conservation of red clover.

Many techniques have been developed to describe microbial communities in silage. These include culture-based techniques (Ercolini, 2004), real-time PCR (Stevenson et al., 2006), and denaturing gradient gel electrophoresis (Ni et al., 2017a). In general, none of these techniques are powerful enough to identify species present in low abundance, some of which may nevertheless be critical for optimal fermentation (Gharechahi et al., 2017). The current next-generation sequencing (NGS) technique allows a much greater level of resolution than has been available in the past and therefore widely used in various micro-ecological environments (Liu et al., 2019). The objective of this study was to characterize the effects of freeze–thaw (FT) event on microbial community dynamics and fermentation quality of red clover silage with the application of NGS high-throughput sequencing.

MATERIALS AND METHODS

Silage Preparation and Experiment Design

Red clover was cultivated in experimental field of Nanjing Agricultural University, Nanjing, Jiangsu, China (N31°14″, E118°22″), and harvested on November 12, 2017. The growth stage was at late bud to early bloom. The stubble was about 10 cm above ground level. After harvest, red clover was immediately transferred to laboratory and chopped to a theoretical length of 1∼2 cm with a forage chopper. The chopped red clover was equally divided into two groups after thoroughly mixing. The first group was untreated control. The second group was frozen in a commercial refrigerator (−20°C) for 2 h and then thawed for 1 h at room temperature (10∼16°C). This FT process was repeated three times. After that, these forages (approximately 500 g for each bag) were picked into thick polyethylene bags (200 μm thick), vacuumed, and stored in a light-blocking box at room temperature. CK is abbreviation of untreated control silage and FT is FT treated silage. These silos were opened after 1, 2, 4, 8, and 30 days of ensiling, respectively. There were three replicates for each treatment per day. After opening, fermentation quality, microbial population, and microbial community were determined.

Chemical Analyses

Approximately 35 g silage sample was extracted in 70 ml of deionized water at 4°C for 24 h to obtain the extract. The pH of the extract was measured with an electrode pH meter (HANNA pH 211, Hanna Instruments, Italy). The extract was centrifuged for 10 min at 10,000 × *g*, and the supernatant was reserved for organic acid (including lactic, acetic, propionic, butyric acid, and ethanol) analysis. The quantification of organic acid was conducted using an Agilent 1260 HPLC system equipped with a refractive index detector (Carbomix H-NP5® column, 2.5 mM H_2SO_4, 0.5 mL/min). Nitrogen compounds including free amino acid (FAA-N), ammonia (NH_3-N) and non-protein nitrogen (NPN) were measured in the extracts using the method presented by Li et al. (2018) and the results were all expressed as g/kg of total nitrogen (TN).

The plant tissue damage degree (TTD) was measured in the ensilage materials according to the method of Lee et al. (2009). Ensilage material and silage samples were freeze-dried for 48 h to determine dry matter (DM) content. TN was determined with the method of Association of Official Analytical Chemists [AOAC] (1990). The water-soluble carbohydrates (WSC) were quantified as the method of Dong et al. (2017).

Microbial Analysis by Culture-Based Method

Ten grams of sample was blended with 90 mL of sterilized water and serially diluted from 10^{-1} to 10^{-9} in sterilized water. The LAB was counted on de Man, Rogosa and Sharpe agar medium, incubated in an anaerobic incubator at 37°C for 3 days. Yeasts were enumerated on potato dextrose agar after aerobic incubation at 28°C for 2 days. The yeasts were distinguished from moulds and other bacteria by colony appearance and cell morphology. The aerobic bacteria were estimated by using nutrient agar after aerobic incubation at 30°C for 24 h.

Microbial Diversity Analysis

Microbial DNA Isolation

For the molecular analysis of the microbial communities, genomic DNA was extracted through the following steps: 10 g of silage sample was mixed with 90 mL of sterile 0.85% NaCl solution, and treated with a table concentrator at 120 r/m for 2 h. After filtering with carbasus, and the liquor was centrifuged at 10,000 r/m for 10 min at 4°C. The supernatant was discarded, and the deposit was suspended in 1 mL of sterile 0.85% NaCl solution. The liquor was centrifuged at 12,000 r/m for 10 min at 4°C, and the supernatant was discarded. The pellet was used for DNA extraction. Total DNA were extracted with TIANamp Bacteria DNA isolation kit. The agarose gel electrophoresis and Nano Drop 2000 118 UV-vis spectrophotometer (Thermo Fisher Scientific, Wilmington, United States) were used to check the quality of DNA after extraction.

PCR Amplification

Approximately 10 ng of DNA isolated from each sample was used for amplification. The primers were 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT- 3′) targeting the V3-V4 regions of 16S rRNA genes.

The PCR reaction system consisted of 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mmol/L dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. These reactions were performed by thermocycler PCR system (GeneAmp 9700, ABI, United States) under the following condition: a prior denaturation at 95°C for 30 min, followed by 27 cycles of denaturation 30s at 95°C, annealing at 55°C

for 30 s, elongation at 75°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were extracted by 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, 129 CA, United States). To reduce PCR deviation, PCR reaction for one treatment was performed in triplicate.

MiSeq Processing and Data Analysis

The DNA samples were sequenced with an Illumina MiSeq 133 PE300 platform (Shanghai Majorbio Bio-pharm Technology Co., Ltd., China). To control sequencing quality, sequences with scores lower than 20 were discarded based on the QIIME quality control process (version 1.7.0). The operational taxonomic units (OTUs) at 97% similarity level were clustered using Usearch (vsesion 7.0[1]). The RDP Classifier (version 2.2[2]) in the Silva (Release128[3]) database was applied to perform sequence-level taxonomic assignment using confidence threshold of 70%. The non-metric multi-dimensional scaling (NMDS) was conducted using the Vegan software based on the unweighted uniFrac distance of genus.

Statistical Analysis

Fermentation parameters and microbial counts data were analyzed by two-way ANOVA for a 2 × 5 (treatment × storage periods) factorial arrangement of treatments by using the SPSS 19.0. Duncan's multiple comparison was used for the means separation. Significant differences were declared when $P < 0.05$.

RESULTS

Forage Characteristics

The characteristics of red clover forages upon ensiling are shown in **Table 1**. The DM, WSC, TN, FAA-N, and NPN of red clover were 21.0%, 109 g/kg DM, 29.3 g/kg DM, 65.2 g/kg TN, and 145 g/kg TN, respectively. The LAB, yeast and aerobic bacteria numbers were 6.21, 4.33, and 6.16 lg cfu/g FM, respectively. The FT event caused increases ($P < 0.05$) in FAA-N and NPN concentrations and TDD in the fresh material. The numbers of all enumerated microbes, particularly aerobic bacteria, increased ($P < 0.05$) in FT material compared with those in CK material.

TABLE 1 | The characteristics of red clover forages upon ensiling.

Items[1]	CK[2]	FT	SEM	*P*-value
DM (% FM)	21.0	20.7	0.22	0.536
WSC (g/kg DM)	109	97.1	6.62	0.884
TN (g/kg DM)	29.3	28.7	0.42	0.471
NPN (g/kg TN)	145	246	5.71	0.012
FAA-N (g/kg TN)	65.2	102	4.69	0.009
TTD (%)	14.4	70.0	3.47	0.002
LAB (lg cfu/g FM)	6.21	7.12	0.12	0.017
Yeast (lg cfu/g FM)	4.33	4.87	0.07	0.024
Aerobic bacteria (lg cfu/g FM)	6.16	7.68	0.26	0.002

[1]FM, fresh matter; DM, dry matter; WSC, water-soluble carbohydrates; TN, total nitrogen; NPN, non-protein nitrogen; FAA-N, free amino acids N, TTD, tissue damage degree; LAB, lactic acid bacteria. [2]CK, untreated control; FT, freeze-thawed red clover. SEM, standard error of means (n = 3, significant at P < 0.05).

Fermentation Parameters

As presented in **Table 2**, the pH, WSC concentrations, yeast and aerobic bacteria numbers declined, and fermentation products, such as lactic acid and acetic acid, and LAB counts increased with prolonged ensiling time. The propionic and butyric acids was below the detection limit. Despite higher ($P < 0.05$) LAB number was observed in FT material compared with CK material, the FT event seemed to result in a transitory lag in the fermentation process during the initial 1 day of ensiling, as indicated by higher ($P < 0.05$) pH and lower ($P < 0.05$) lactic acid concentrations in FT silage compared with those in CK silage at day 1. After 1 day of ensiling, more intense lactic fermentation was observed in FT silage, as indicated by lower ($P < 0.05$) pH and higher ($P < 0.05$) lactic acid concentration. At the end of ensiling, the lower ($P < 0.05$) pH and higher ($P < 0.05$) lactic acid concentrations indicated better fermentation quality of FT silage compared with CK silage.

Bacterial Diversity

Approximately 1,837,106 valid sequences were obtained after removing unqualified sequences, with an average length of 449 bp per sequence for bacteria. These reads were clustered into 361 OTUs at 97% sequence identity. The rarefaction curve (**Figure 1**) and Good's coverage (>99%, **Table 3**) indicated that sequencing depth had adequately captured most of the bacterial communities in all samples. Based on alpha diversity, the richness, and diversity of bacterial communities were evaluated in the two silages (**Table 3**). According to OTUs and Chao 1 index, the richness of the bacterial community dropped sharply after the onset of ensiling, especially in FT silage. Shannon index, a measure of the diversity based on the number and evenness of species, showed that among all material and silage samples the highest bacterial diversity was observed in FT material whereas lowest in CK material.

The dynamic variance of bacterial community of the two silages during ensiling was observed by NMDS analysis. As shown in **Figure 2**, it can be seen that a clear separation and difference of bacterial communities in fresh materials from that in ensiled samples. Furthermore, the bacterial community of FT material was distinctive from that of CK material.

Bacterial Community Dynamics

Three phyla (*Firmicutes*, *Proteobacteria*, *and Cyanobacteria*) were detected at high abundance in fresh red clover (**Figure 3**). Among them, *Proteobacteria* was the highest abundance phylum, accounting for 82.8% of the bacterial community. The FT event increased the relative abundances of *Firmicutes* from 16.2 to 28.2% and *Cyanobacteria* from 0.76 to 6.31%, and decreased the relative abundance of *Proteobacteria* to 60.5% in the ensilage

[1]http://drive5.com/uparse/

[2]http://sourceforge.net/projects/rdp-classifier/

[3]https://www.arb-silva.de/

TABLE 2 | Fermentation parameters and microbial counts during ensiling.

Treatment[1]	Day	pH	DM[2](% FM)	WSC (g/kg DM)	NH_3-N (g/kg TN)	Fermentation parameters (g/kg DM)				Microbial counts (lg cfu/g FM)		
						LA	AA	LA/AA	EOL	LAB	Yeast	Aerobic bacteria
CK silage	1	4.74e	20.3bc	92.9c	39.4ab	17.9b	11.2a	1.57b	30.2b	6.21a	4.20cd	4.12d
	2	4.61d	18.8a	63.0b	54.4e	34.5c	12.4ab	2.55c	40.0d	8.12cd	4.10cd	4.02d
	4	4.46c	19.8abc	36.8ab	52.8cde	50.5d	16.1bcde	3.14d	38.0cd	7.95cd	3.71b	3.48b
	8	4.39c	19.2ab	18.2a	57.9ef	55.9e	17.9de	3.10d	37.2cd	8.30cd	3.54ab	3.14a
	30	4.39c	20.3bc	20.7a	64.8f	67.5f	23.2f	2.91d	35.0c	7.42b	3.44a	3.10a
FT silage	1	4.84f	20.8c	57.5b	36.9a	4.88a	13.9abc	0.35a	25.4a	7.22b	4.45e	3.98cd
	2	4.44c	19.4ab	58.8b	39.0ab	44.4d	15.2bcd	2.93d	27.9ab	8.81e	4.41de	3.87c
	4	4.29ab	19.0ab	53.1b	45.9bcd	60ef	17.1cde	3.51e	30.2b	8.04cd	3.92c	3.40b
	8	4.31b	19.5abc	38.3ab	45.5bc	67.9f	18.9e	3.59e	29.2ab	8.45d	3.41a	3.50bc
	30	4.22a	18.7a	21.6a	53.6de	78.2g	22.3f	3.53e	26.1ab	7.48b	3.54ab	3.67c
SEM		0.008	0.130	3.338	0.799	1.046	0.351	0.027	0.403	0. 210	0.173	0.073
P-value												
Treatment (T)		<0.001	0.450	0.595	<0.001	0.011	0.135	0.026	<0.001	<0.001	<0.001	<0.001
Day (D)		<0.001	0.019	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
T × D		<0.001	0.073	0.454	0.136	0.005	0.583	<0.001	0.151	<0.001	<0.001	<0.001

[1]CK, untreated control; FT, freeze-thawed red clover. [2]FM, fresh matter; DM, dry matter; WSC, water-soluble carbohydrates; TN, total nitrogen; LA, lactic acid; AA, acetic acid; EOL, ethanol; LAB, lactic acid bacteria; cfu, coloning forming unit. SEM, standard error of means. Values in the same column with different letters differ significantly (n = 3, significant at P < 0.05).

material. In addition, two extra phyla (*Actinobacteria* and *Bacteroidetes*) were detected abundant in the FT material.

After the beginning of ensiling, *Firmicutes* quickly superseded *Proteobacteria in* CK silage to be the dominant phylum, and kept its dominant role (>87.2% of the bacterial communities) until the end of ensiling; the relative abundance of *Proteobacteria* phylum decreased sharply to <12.7%. Relative to CK silage, the FT event increased the relative abundance of *Firmicutes* and decreased the abundance of *Proteobacteria* at day 1 of ensiling, while the bacterial community structures of the two silages became similar after 2 days of ensiling. Phyla *Actinobacteria* and *Bacteroidetes* in the FT silage decreased to undetectable level after the onset of ensiling. However, their relative abundances became apparent again at the end of ensiling.

To further understand the effects of FT event on microbial community dynamics during ensiling, bacterial community structures of the two silages were examined at genus level (**Figure 4**). The most prevalent genus in CK material was *Pantoea* (63.8%), followed by *Enterobacter* (11.3%), *Weissella* (9.72%) and *Pseudomonas* (7.04%), and *Lactococcus* (3.08%) and *Pediococcus* (2.25%). Compared with CK material, the abundant presence of various aerobic bacteria, such as *Pseudomonas* (14.1%), *Cyanobacteria* (6.40%), *Rhizobium* (2.86%), *Acinetobacter* (2.85%), *Comamonas* (1.93%), *Sphingomonas* (1.67%), *Methylobacterium* (1.24%) as well as some unclassified genera (11.9%), was found in FT material. Furthermore, it was observed that the relative proportions of genera *Pantoea* (26.6% vs. 63.8%) and *Enterobacter* (4.71% vs. 11.3%) deceased in the material microflora, whereas genera *Lactobacillus* (1.14% vs. undetectable level) and *Weissella* (20.3% vs. 9.72%) increased.

After the onset of ensiling, *Weissella* proliferated quickly, reaching to 65.7% of the bacterial community at day 1. However, its abundance decreased thereafter with prolonged ensiling time. The relative abundance of *Pediococcus* increased after the onset of ensiling and peaked (26.4%) at day 4 of ensiling. Along with the changes in *Weissella* and *Pediococcus* abundances, the relative abundance of *Lactobacillus* increased and became dominant after 4 days of ensiling. After the onset of ensiling, the all aerobic bacteria quickly decreased to undetectable levels. With respect to the *Pantoea*, they also dropped to marginal levels in the bacterial community. However, members of *Enterobacter* were still readily apparent throughout ensiling. Compared with CK silage, the FT event reduced *Enterobacter* (3.29% vs. 9.41%) at the day 1 of ensiling. In addition, it was observed that the relative proportions of *Lactobacillus* were always higher in FT silage compared with CK silage before 4 days of ensiling.

With prolonged ensiling time, microbiomes of the two silages were mostly constituted by LAB species and tended to be almost similar after 2 days of ensiling. However, genera *Acinetobacter* (2.74%) and *Pseudomonas* (1.65%) became apparent again in FT silage at the end of ensiling.

DISCUSSION

In practice, the FT event frequently occur before harvest or during silage making, leading to physical damages and vigorous

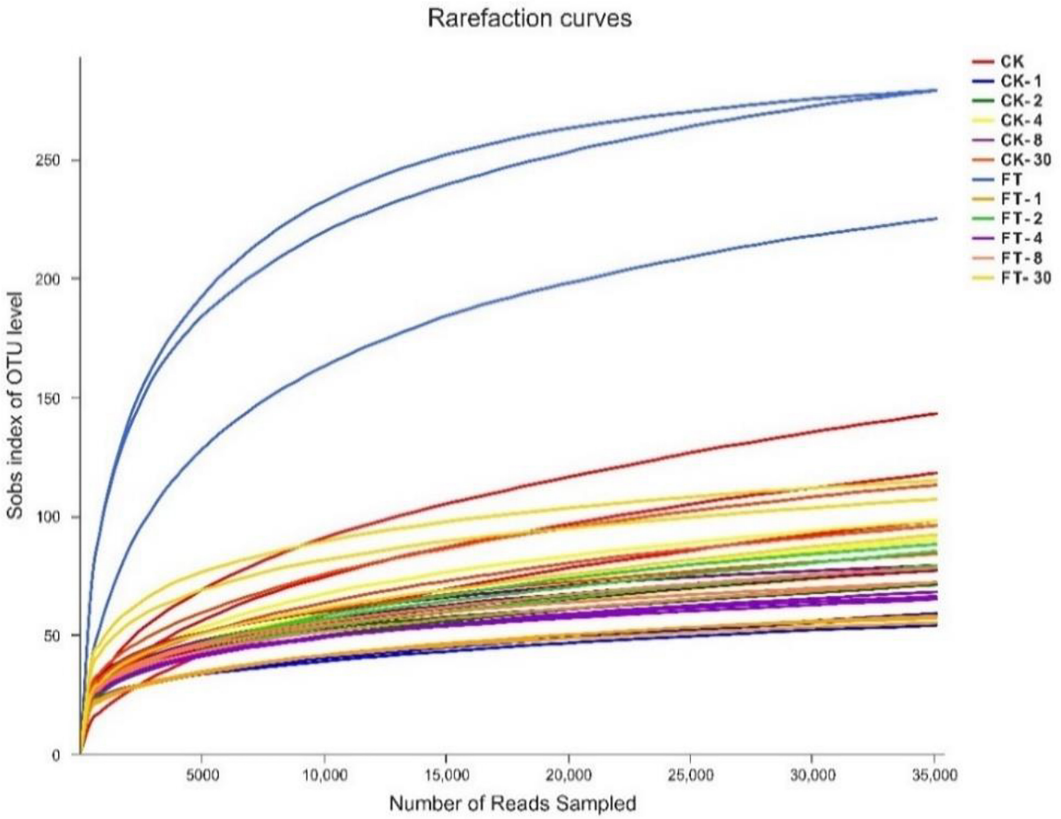

FIGURE 1 | Rarefaction curves for all samples. CK, untreated control; FT, freeze-thawed samples. Arabic numbers following the treatment abbreviation indicate the ensiling day.

growth of various bacteria in forage crops. It is known that microbial communities originally present on ensilage material play an important role in fermentation quality (Gharechahi et al., 2017). This means uncertainties of silage quality made from the damaged forages. The NGS technique has far superior resolution and is more adept at describing bacterial diversity than conventional methods (McAllister et al., 2018). Herein we compared the microbial community dynamics of silages made from FT-treated and untreated materials using NGS approach. As far as we know, this is the first report of the bacterial community in red clover silage.

Changes in Forage Characteristics and Fermentation Parameters After FT Event

As indicators of protein hydrolysis (Li et al., 2018), the increased FAA-N and NPN concentrations suggested larger extent of protein hydrolysis in the FT material upon ensiling. In living plant, extensive degradation of plant protein is prevented by cellular compartmentation (Carrión et al., 2014). The FT event disrupted the cellular compartmentation, thereby increasing the exposure of forage proteins to plant proteases (Grabber and Coblentz, 2009). The greater proteolysis extent confirmed the significant physical changes in plant cell after the FT event, consistent with the observation of increased TDD of the FT material.

Despite the FT procedures were conducted under conditions where as possible as to avoid soil and water contamination, the numbers of all enumerated microbes, particularly aerobic bacteria, and increased significantly in the FT material compared with the CK material upon ensiling. The increased microbial counts were assumed to link the increased nutrients release that favored proliferation of indigenous microbes and likely some airborne bacteria (Leifert et al., 1994).

Legume forages are generally regarded as difficult to ensile because of high buffering capacity and low sugar content (McDonald et al., 1991). Clostridial fermentation is easy to occur particularly when legume forages are ensiled at high moisture (Dong et al., 2017). In this study, butyric acid, the product of clostridial fermentation, were absent in all silages. These observations, coupled with the low terminal pH and dominance of lactic acid, indicated satisfactory fermentation quality of the red clover silages. The quality fermentation might be attributed to the high WSC content (109 g/kg DM) that encouraged lactic fermentation. This was in accordance with numerous studies where fermentable sugar contents were observed to be low in other legume forages, such as alfalfa, whereas usually high in red clover (Raguse and Smith, 1966; Owens et al., 1999).

TABLE 3 | Alpha diversity of bacterial diversity during ensiling.

Treatment[1]	Day	Sequence number	OTUs[2]	Shannon	Chao 1	Coverage
CK silage	0	56091	153	1.52	142	1.00
	1	48263	63.7	2.03	115	1.00
	2	58790	90.1	2.34	119	1.00
	4	56091	111	2.19	97.6	1.00
	8	43168	95.3	2.43	96.6	1.00
	30	41347	125	2.39	165	1.00
FT silage	0	58790	302	2.89	257	1.00
	1	51562	69.9	1.90	76.6	1.00
	2	44872	102	2.36	117	1.00
	4	58790	81.9	2.26	84.4	1.00
	8	41347	87.3	2.45	89.5	1.00
	30	48263	129	2.52	148	1.00

[1]CK, untreated control; FT, freeze-thawed red clover. [2]OTUs, number of operational taxonomic units; coverage, good's coverage.

Under anaerobic conditions, LAB ferment soluble carbohydrates to organic acids, mainly lactic acid, results in decline in silage pH (Pahlow et al., 2003). The fast dominance of LAB in silage microflora is crucial for the resultant fermentation. Yang et al. (2019) previously showed that inoculation of LAB to sterilized material than to non-sterilized material resulted in a faster predominance of LAB. This leads to the deduction that complex microbial composition of FT material may increase the difficulty of LAB to outgrow in the microflora at the onset of ensiling and explained the initial short lag in lactic fermentation of FT silage compared with CK silage. At the end of ensiling, better fermentation quality was observed in FT silage. The plausible explanation could be that the FT event increased the leakage of plant cellular contents, making plant nutrients more readily available for LAB growth (Charmley et al., 1997). However, information obtained by these conventional fermentation parameters was very limited, increasing the need for deeper insights into microbial community dynamics during the ensiling.

Changes in Bacterial Diversity and Composition After FT Event

Bacteria present in fresh forage crop and silage can be simply classified into three groups based on their contributions to silage quality and abilities to thrive in anerobic environment: LAB, which are desirable bacteria contributing to the production of lactic acid and pH decline; undesirable facultative aerobes, which compete with LAB for nutrients during ensiling and contribute little to the pH decline; strict aerobes, which are also undesirable bacteria whereas remain active only before oxygen is depleted in silo (McDonald et al., 1991).

Our results showed that *Proteobacteria* and *Firmicute* were two most abundant phyla in fresh red clover, comprising 99.2% of the microflora. After the FT event, the relative abundance of *Firmicute* increased from 16.2 to 28.2%. The increased *Firmicute* was attributed to increased abundance of *Weissella*, which belong to *Firmicute*. *Weissella*, an obligatory heterofermentative LAB species, are major components of the microflora in various types of forage crops (Cai et al., 1998; Yang et al., 2019). They are initial LAB population that play a key role in the fast acidification at the onset of ensiling. The enrichment of *Weissella* in FT material indicated the beneficial effects of FT event on the silage fermentation and was consistent with higher numbers of LAB in FT material. The accurate reason for the increased *Weissella* abundance is unclear, probably associated with the liberation of chemical signals from the damaged plant, such as amino acids, that play as a specific

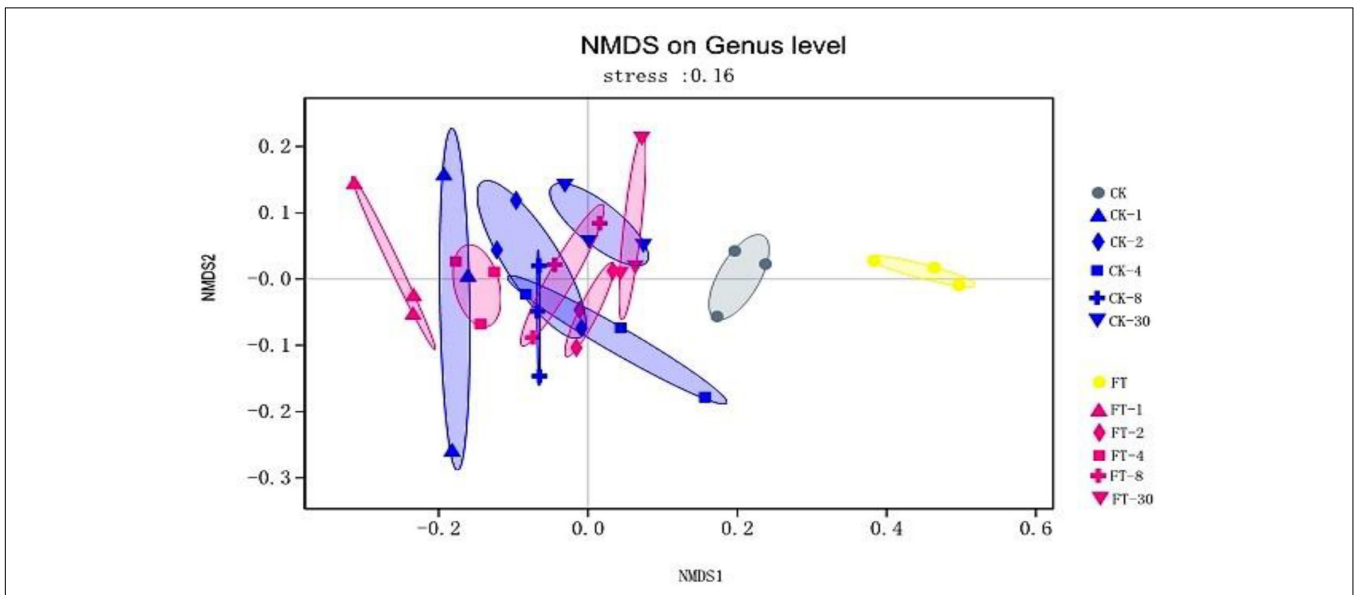

FIGURE 2 | The dynamic variance of bacterial community at genus level shown by NMDS analysis. CK, untreated control; FT, freeze-thawed samples. Arabic numbers following the treatment abbreviation indicate the ensiling day.

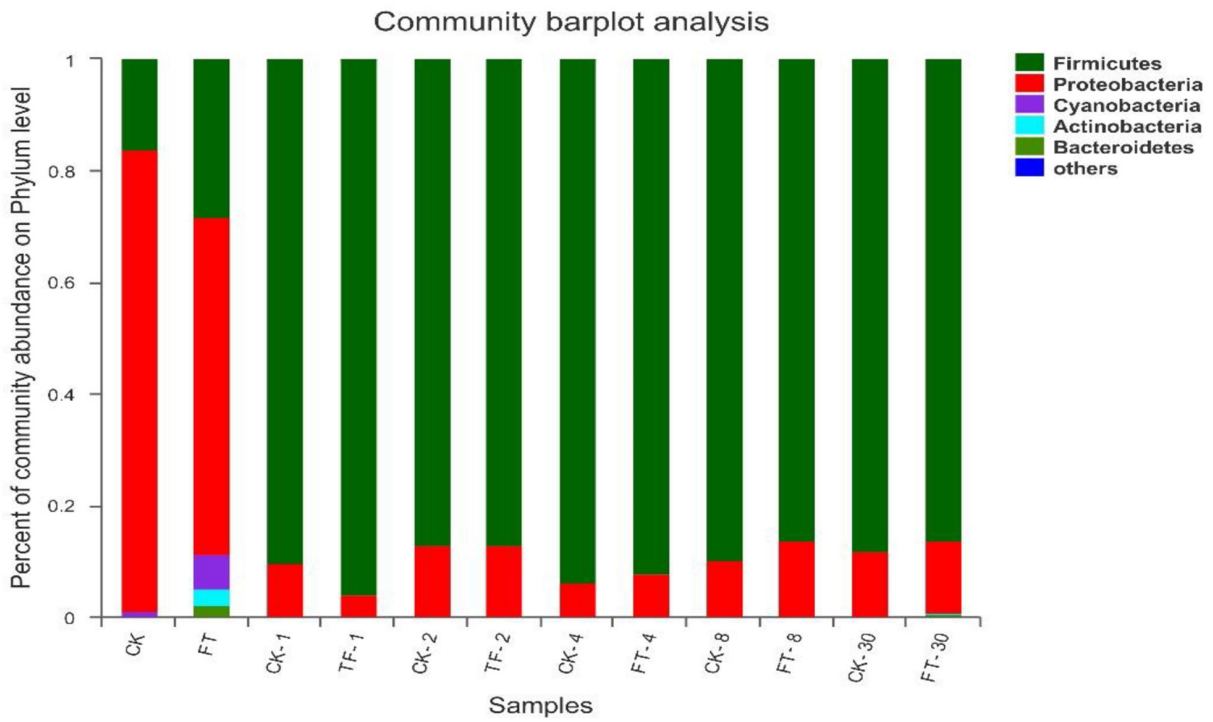

FIGURE 3 | Dynamics of microbial community structure during ensiling at phylum level. CK, untreated control; FT, freeze-thawed samples. Arabic numbers following the treatment abbreviation indicate the ensiling day.

stimulus to growth of LAB (Barnett, 1952). In addition, it could be expected that the release of plant juice from the damaged plant may also help LAB spread during handling (Stirling and Whittenbury, 1962).

Lactobacillus, *Pedicoccus*, and *Weissella* are considered as the 3 most predominant LAB genera responsible for driving lactic fermentation during ensiling (Pang et al., 2011; Liu et al., 2019). After the onset of ensiling, significant shift in bacterial community from *Proteobacteria* to *Firmicute* could be attributed to the increased abundances of genera *Lactobacillus*, *Weissella* and *Pediococcus*, which flourished in the environmental conditions developed during ensiling (Keshri et al., 2018). Furthermore, in this study the intensive sampling points revealed a dominance succession from *Weissella* and *Pediococcus* to *Lactobacillus* in lactic fermentation. It is known that *Weissella* is an early colonizer (Graf et al., 2016), and *Pediococcus* contributes to initial decline in silage pH, creating an environment suitable for the development of *Lactobacillus* (Yang et al., 2019). These two LAB genera are not as tolerant as *Lactobacillus* to acidifying environment and thus active only during the early stages of ensiling. The follow-up lactic acid production mainly depends on *Lactobacillus*, which become active and grow vigorously as pH decreases (Cai et al., 1998). Compared with CK silage, it was observed that the relative proportions of *Lactobacillus* were always higher in FT silage before 4 days of ensiling. This probably explained the more intense lactic fermentation. The higher proportions of *Lactobacillus* were assumed to be related with the enrichment of *Weissella* in the FT material that created an initial acid environment favorable for the following *Lactobacillus* development (Cai et al., 1998).

This study showed that *Pantoea* and *Enterobacter* were two major facultative aerobe genera present in fresh red clover microflora. *Pantoea* and *Enterobacter* have been reported to be present in various forages, such as alfalfa (Ogunade et al., 2018) and soybean (Ni et al., 2017b). Their populations are affected by environmental factors, such as climate, geographical location, and type of fertilizer used (Yang et al., 2019). The FT event did not favor their growth; in contrast, it deceased their relative proportions in the fresh material. The deceased *Pantoea* and *Enterobacter* might have two reasons. First, increased *Weissella* abundance exhibited an inhibitory effect on other bacteria by producing bacteriocin (Papagianni and Papamichael, 2011); Second, vigorous multiplication of other bacteria, such as aerobic bacteria, reduced the relative proportions of *Pantoea* and *Enterobacter* in the microbial community.

After the beginning of ensiling, drops in *Pantoea* abundances reflected their high sensitivity to pH decline (Ogunade et al., 2018). However, the members of *Enterobacter* were apparent throughout the ensiling, likely because of the presence of some acid-tolerant *Enterobacter* species (McGarvey et al., 2013). Compared with CK silage, the FT silage exhibited reduced relative abundance of *Enterobacter* at day 1 of ensiling, probably owing to its relative lower abundance in FT material. From this aspect, the facultative aerobes might not be the threat for the silage quality made from the FT-damaged forages. However, facultative

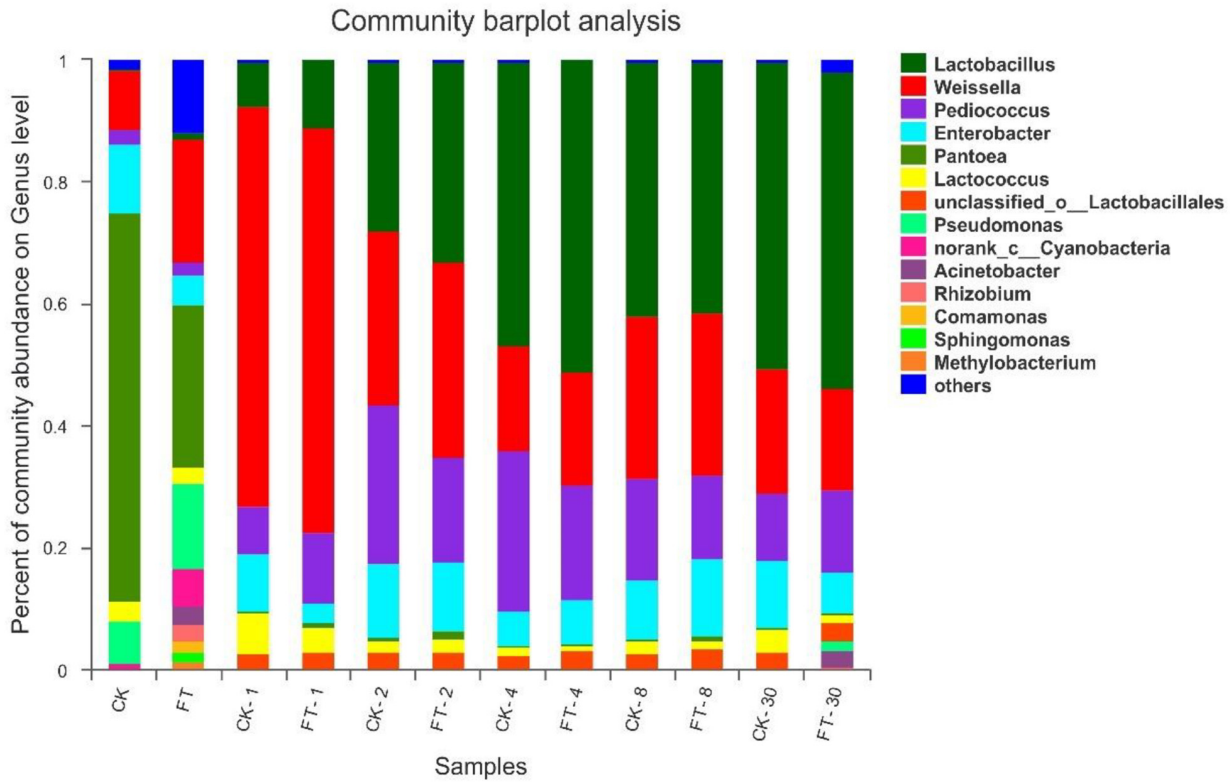

FIGURE 4 | Dynamics of microbial community structure during ensiling at genus level. CK, untreated control; FT, freeze-thawed samples. Arabic numbers following the treatment abbreviation indicate the ensiling day.

aerobes are abundantly present in various environmental sources, such as soil and animal manure (Ogunade et al., 2018). Under field conditions, the altered physical features, induced by the FT event, may increase the risk of the damaged forages being contaminated with the facultative aerobes from the environment, which also highlights the importance of hygienic quality during harvesting and processing.

In the current experiment, aerobic bacteria flourished after the FT event, contributing to the high bacterial richness, and diversity in the FT material. These aerobic bacteria were supposed to compete with the LAB for nutrients during the initial aerobic phase of ensiling (Dong et al., 2017), explaining the initial short lag in lactic fermentation of the FT silage. As expected, due to the achievement of acidic and anaerobic environment in silage, the aerobic bacteria all fell to undetectable levels after the onset of ensiling. The decreases in abundance of aerobic bacteria could be responsible for the drops in bacterial richness and diversity in FT silage after the onset of ensiling. It is worth noting that genera *Acinetobacter* (2.74%) and *Pseudomonas* (1.65%) became apparent again in FT silage at the end of ensiling. *Acinetobacter* and *Pseudomona* are aerobic, non-fermenting bacteria, which can be found in different environments (Palleroni, 2010; Kampfer and Glaeser, 2012). The roles of these bacteria in silage have not been extensively studied. Although they are supposedly absent in silage (Li and Nishino, 2011), some species are able to survive in anaerobic environment in the presence of acetate as a substrate (Fuhs and Chen, 1975). When silages are exposed to air, these survived aerobic bacteria may proliferate firstly if silage pH is not low enough to suppress them, leading to aerobic deterioration of the silage (Liu et al., 2013). Liu et al. (2019) previously found that *Acinetobacter* was the dominated spoilage organisms in aerobically deteriorating barley silage. The survival of these bacteria also increases the evidence that the microbes responsible for aerobic deterioration after silo opening are indigenous to the silage rather than aerial-borne invaders.

CONCLUSION

Weissella played an important role in the initiation of lactic fermentation in red clover silage. The FT event promoted the development of *Lactobacillus* during ensiling, and reduced the relative abundances of *Pantoea* and *Enterobacter* at the onset of ensiling, indicating the beneficial effects on fermentation quality. However, due to vigorous growth of aerobic bacteria in FT material, *Acinetobacter*, and *Pseudomonas* became apparent again in FT silage at the end of ensiling. This reveals that the control of aerobe revitalization could be a challenge for the silage quality made from the freeze-damaged forages. In fact, knowledges obtained in this study might be applicable not only to those circumstances where forages are subjected to freezing

damages and also to circumstances where forages are imposed by mechanical damages (for e.g., rolling, crushing, and maceration).

AUTHOR CONTRIBUTIONS

TS designed the experiments. JL, LC, SW, and ZD conducted the experiments. ZD analyzed the data and wrote the manuscript.

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REVIEW
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Applications of the Soil, Plant and Rumen Microbiomes in Pastoral Agriculture

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The production of dairy, meat, and fiber by ruminant animals relies on the biological processes occurring in soils, forage plants, and the animals' rumens. Each of these components has an associated microbiome, and these have traditionally been viewed as distinct ecosystems. However, these microbiomes operate under similar ecological principles and are connected via water, energy flows, and the carbon and nitrogen nutrient cycles. Here, we summarize the microbiome research that has been done in each of these three environments (soils, forage plants, animals' rumen) and investigate what additional benefits may be possible through understanding the interactions between the various microbiomes. The challenge for future research is to enhance microbiome function by appropriate matching of plant and animal genotypes with the environment to improve the output and environmental sustainability of pastoral agriculture.

Keywords: genomics, metagenomics, pasture, ecosystems, food, soil

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INTRODUCTION

The arrival of the next generation sequencing (NGS) era has opened up new opportunities for understanding biological processes and implementing new strategies for improving these processes and monitoring the environmental impacts of agriculture (1–7). This has enabled the development of tools to implement genomic selection (GS) particularly in the livestock industry, and provide genome wide association studies (GWAS) to further elucidate genomic regions of importance in production traits. Genome assemblies, together with re-sequencing, have helped to establish SNP arrays for assessing genetic variation within and between genomes of individuals. This has become well-established for diverse breeds and species from around the globe in animal [e.g., (8–13)], and plant species [e.g., (14–18)]. In addition, genomic selection methods (19–29) used in conjunction with imputation strategies (30–33) that utilize various SNP densities in a cost effective manner, encourage the uptake of GEBVs by the breeding industry with the view to increase the rate of genetic gain in both animals (22) and forage plants (34). Furthermore, utilization of genotypes that are imputed to a whole genome sequence equivalent level for use in GWAS and GS are now a reality (35–39). Continued reductions in DNA sequencing costs together with an improvement in longer read technology has generated more refined genome assemblies that are being annotated at the functional level via assays designed to establish chromatin architecture, accessibility, modification and subsequent transcription and translation profiles (40–45). The human and mouse ENCODE projects (46–49) have paved the way for the international

consortium FAANG [Functional Annotation of the Animal Genome; (50, 51)], which aims to identify all the functional elements in animal genomes. A significant challenge in the post-genomic era is connecting genotype to quantitative phenotype in basic and applied biology, represented as the genome to phenome challenge. Understanding the genotype to phenotype link is not only important from a genomic selection perspective but also assists in improving the fundamental understanding of the biology of the system.

The use of genomic tools in livestock research has had a substantial effect on genetic gain for the industry (22). Similar technologies are now being developed and implemented in forage species (34, 52). However, the animal and plant genomes constitute only two components of the "pasture to plate ecosystem." They exist and function in association with their own microbiomes, and the microbiome of the soil on which pastures are grown and animals graze. The Human Microbiome Project has lead the way in characterizing the contributions that microbiomes make to host phenotypes, with an ever-increasing list of human attributes which are influenced by microbial activities [e.g., (53–69)]. Subsequently, NGS technology has advanced research in the characterization and understanding of the microbiome of the rumen of grazing animals (70), as well as the microbiome of forage plants and soils. Microbiome characterization initially involved sequencing of marker genes within microbial communities mainly targeting rRNA gene sequences but has grown to include deep metagenomic and metatranscriptomic sequencing. This has allowed global characterization of both culturable and unculturable microbial species within an environment coupled with quantification of their gene expression which enables functional profiling. In addition to understanding the contribution microbiomes make to production in grazing animals, genomic techniques can also be extended to monitoring sources of food or water contamination, thereby having important potential impacts for human health.

The integration of genomic information from host organisms with their microbiomes and with other environmental parameters of the target ecosystem has become an important challenge to research projects seeking to enhance agricultural output while reducing its environmental footprint. These host × microbiome × environment relationships in agricultural production systems involve extremely broad and complex interactions along the soil-plant and animal continuum, and its investigation needs to be divided into more specific research questions to enable detailed dissection and analysis. In this context, an "ecological genomics" approach is appropriate, whereby the microbiomes associated with soil, plants and animals are recognized as an integral part of an interconnected system that influence the functions of their hosts and thereby contribute significantly to productive processes in the pastoral sector (71). The key features of, and interactions between the soil-plant-animal microbiomes need to be identified so that their contributions to these agricultural processes and their impacts on the environment can be quantified.

In this paper, we consider the recent developments in genomics that provide new tools to understand the microbiome along the soil-plant-animal continuum within the pastoral production system. We summarize how these tools provide more precision in the identification and quantification of the structure of the microbial communities and how the emerging tools in metagenomics can be applied. Within the soil-plant-animal continuum we look at the animal and farm management opportunities arising from advanced understanding of microbial diversity and ecosystem function and how that can be used to improve soil processes, forage growth and pasture utilization and help withstand the challenges of diseases and climate change. These opportunities are summarized via three case studies involving: the microbiomes of the soil, the pasture, and the rumen of grazing animals. The potential for interdependencies, interplay and interactions between the microbiomes of the ecosystems along this continuum are considered along with other downstream impacts on ecosystems associated with water runoff. We finally propose how an "ecological genomics" approach can contribute to improved understanding of these microbiomes to improve the performance of the pastoral sector.

CASE STUDY 1: SOIL MICROBIOME

The biology of soils has long been recognized as being central to the productive capacity of natural and managed ecosystems (72, 73). While we cannot directly observe much of the soil microbiome, its function shapes the world around us. Soils microbiomes are highly diverse ecosystems, comprising complex assemblages of bacteria, archaea, and eukaryotic taxa, and are considered the most genetically diverse ecosystems on earth (74). Estimates of the total of life in soil vary widely; bacterial species alone, are present in the order of thousands to tens-of-thousands of species (inferred from 16S rRNA gene phylotypes) per gram of agricultural soil (74–76). Soils provide a reservoir of microbial species that may either support or inhibit the growth of plants and animals directly; as beneficial symbionts or as pathogens, respectively, or indirectly via actions which affect the biological availability of nutrients and toxins (77). Furthermore, functions supported by the soil microbiome provide a range of enabling and provisioning ecosystem services that support the natural environment, including interaction between above- and below-ground terrestrial biomes, aquatic ecosystems (rivers, lakes, groundwater), and the earth's atmosphere [e.g., (78)].

New soil management approaches are aimed at opportunities based on the understanding of soil microbiomes for improved processes and lowered environmental impact (79, 80). These management strategies increasingly use ecological genomics approaches (81) where soil is treated as an ecosystem hosting a rich diversity of species which harbor diverse "functional" genetic elements (e.g., genes conferring antibiotic resistance or nitrogen fixation). Assessing these at an ecosystem level is technologically challenging and requires the development of new bioinformatic and statistical tools for ecological analysis [e.g., (82, 83)]. Most importantly, an ecological genomics approach necessitates a shift in conceptual thinking from the organism or gene using ecosystem property/function interactions, to embracing the complexity of interactions among organisms, their genetic elements, and the biotic and abiotic factors that are

expressed collectively to deliver ecosystem processes (84, 85). Ecological genomics offers a key opportunity to further advance the understanding of soil ecology and function and thereby help unravel the complexity of their ecosystems across spatial and temporal scales (86–88).

The application of molecular-based tools has become essential to characterize and understand soil ecosystems, because the soil is hyper-diverse and therefore genetically complex. A single gram of soil is estimated to contain up to 1,000 Gbp of metagenome DNA (80, 89) and current NGS platforms can only provide partial coverage of the metagenomic DNA in a soil sample. To date, most soil metagenome research has relied on the characterization of specific elements within the metagenome. Examples include the use of meta-barcoded primers to assess community composition [e.g., (90)], application of NGS or high-density environmental microarrays to determine functional status/composition of the community [e.g., (91, 92)], or functional screening of libraries of cloned DNA fragments for novel enzymes and bioactive compounds (93).

A good example illustrating metagenomics applications to study the soil microbiome involves soils suppressive to soil-borne plant diseases. These are defined as those in which the activity of the resident soil microbiota reduces the occurrence or severity of plant disease caused by soil-borne pathogens (94). Examples of such disease reduction in soils include suppression in wheat of take-all (*Gaeumannomyces graminis* var. *tritici*) and *Rhizoctonia* bare patch (*Rhizoctonia solani* AG-8) diseases, and the role *Streptomyces* spp. in the plant rhizosphere and endosphere play in promoting plant growth and the induction of resistance via antibiotic production and competitive exclusion (95). Given the high cost of soil-borne disease on agricultural production [e.g., estimated costs of 28–50% of pasture production in New Zealand; (96, 97)] and the lack of practicable and economic control options, the development of disease suppression in soil microbial communities represents an important soil service that serves to maintain agricultural activity and the food and fiber it produces (86). Disease suppression has been observed in a number of soils, with different disease-host interactions, and can develop naturally over time (94, 98). In instances where disease suppression develops, it is underpinned by alteration in the soil microbial community structure toward a greater number of disease suppressive taxa, or expression of potential (latent) disease suppressive activity (86, 99). Not surprisingly, the development of disease suppression in soils is highly desirable, and there have been considerable efforts to understand how this can be facilitated through changes in system management [e.g., via fertilizer use and plant residue management; or via reduced tillage and crop rotation; (94, 100)]. However, the characterization of the community and functions associated with general disease suppression has been very difficult, particularly as they potentially represent a small fraction of the total microbial diversity in soils (101). Furthermore, in the case of "general" disease suppression (102) underpinned by phylogenetically diverse consortia of microbiota, functions such as lytic enzyme production, antibiotic secretion, and elicitation of plant defense mechanisms, may be collectively responsible (94).

Advances in understanding changes within the soil community during development of disease suppression are being supported through application of ecosystem genomic tools [see Dignam et al. (86) review]. These include the application of high density oligonucleotide microarrays (88), tag-based NGS (103), and shotgun metagenomics (104, 105). In each case, phylogenetically diverse microbial consortia were associated with disease suppression. Resolving these taxa against the rich background of soil microbial diversity would not have been possible without an ecological genomics approach.

More recently, the focus on assessment of soil-borne disease suppression has been extended from approaches focused on identifying the taxa responsible, toward assessing soil ecosystems based on functional genes. This has followed recognition that, in many instances, a phylogenetic description of a microorganism can be a poor reflection of the metabolic (functional) ability outside of its base metabolism. This is particularly important where functions, such as antibiotic production, antibiotic resistance, host compatibility, and virulence, are borne on mobile/transferrable genetic elements such as plasmids (106). The acquisition or loss of a plasmid can change the biology and wider ecology of individuals of the same species in the soil. In these cases, the identification of a species only indicates the presence of a "potential host" that may or may not harbor the functional genes of interest [e.g., (107)]. As such, the detection of multiple functional genes associated with disease suppression is likely to provide a richer understanding of the ecosystem potential for this important ecosystem function (108). To achieve this, technology platforms such as functional environmental microarrays (91) are being constantly updated to include information on genes either directly or putatively associated with disease suppression. These include many antibiotic production genes, such as *phz*F and *phz*A (phenazine), *bac*A (bacilysin), *pab*A (chloramphenicol), *phl*D (DAPG), *lgr*D (gramicidin), *lmb*A, (lincomycin), *prn*D (pyrolnitrin), *str*R (streptomycin), *spa*R (subtilin), and *pcb*C (β-lactam) genes, alongside sub-sets of existing gene probes for detection of lytic enzyme production, e.g., *hcn*B (cyanide formation) (109). By assessing the abundance and distribution of these genes in soil environmental DNA (eDNA) samples, the functional ecology of disease suppressive communities may be determined. Impacts of farm management practices on disease suppression can then be interpreted through the lens of functional changes in the soil biology. Over time, this knowledge is expected to provide novel opportunities for on-farm management of soil biological resources toward enhanced disease suppression. Furthermore, molecular-based tools may enable the rapid identification of soils suppressive to specific diseases. These soils will represent important natural resources enabling the transmission of disease suppression from one soil to another by deliberate soil inoculation (94).

Functional properties of the soil microbial ecosystem are being inferred based on the taxa present. Using phylogenetic marker gene information (e.g., 16S rRNA or ITS gene sequences), bioinformatic tools such as PICRUSt [Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; (110)] and FUNGuild (111) can predict the metagenome level functional content (e.g., C and N cycling genes) or functional

guilds (pathogens, saprotrophs, symbionts, etc.) to provide deeper ecological insights into the functional ecology of the data sets. The expansion of these and similar tools, alongside better reference data (annotated genomes) which these tools reference, will provide further cost-effective approaches to describe the ecology and functioning of complex ecosystems such as soils.

The transfer of microbial species from one soil to another can confer new ecosystem phenotypes. This has been well-established for various microbial species such as mycorrhizal fungi, plant pathogens, and rhizobia. The movement of these taxa among soils has direct impact on the productive capacity and success of various plant species in the receiving environment (112, 113). This demonstrates the potential to manage soil biology for specific production-based and/or environmental outcomes.

Soil biology is the "engine room" that recycles plant material, either from direct inputs (leaf fall, root senescence), or secondary deposition (animal manure, urine) (114). The nutrients in these materials are either recycled within the biosphere, or mineralized into the geochemical matrix of the soil (114). In terrestrial systems, the soil microbiology provides an interface between the biological and abiotic worlds, affecting movement of essential major and minor elements between the geologic reserves and the biosphere. As such, there are a broad range of opportunities to harness the potential of soil ecosystems to optimize nutrient cycling. These include increasing the supply of many major and minor essential elements for plant use, stimulating the long-term storage of carbon in soils and promoting "closed" nutrient cycling within specific environments such that N within NO_3 and N_2O, for example, stay on-farm. Given our current lack of understanding of soil biology, we still have only a rudimentary knowledge of the extent of species interactions that may potentially affect critical rate regulating biogeochemical transformations in the mineralization, immobilization, and cycling of nutrients and the coupling of nutrient cycles. Indeed, it is highly likely that cryptic species and/or functional processes will have hitherto unrecognized importance in many aspects of soil nutrient cycling.

CASE STUDY 2: PASTURE MICROBIOME

Grassland composition and forage production is finely balanced under the influence of interactions among many factors (115). These include the physical environment of soil, water, nutrient availability, temperature, extreme climatic events [e.g., (116)], pasture management of the grazing process (117), plant genetics (118), and the soil and plant microbiomes (see case study 1, above). Pasture-based livestock industries are primarily based on relatively simple mixtures of temperate grass and legume species as the main feed source for ruminant animals. Yet even these "simple" vegetation communities vary greatly in space and time (119), often for reasons that are not obvious using traditional scientific monitoring or analytical methods.

Well-studied components of the microbiome in grass-legume pastures involve the symbiotic association of *Epichloë* endophytes in grasses and *Rhizobium* nodules on legume roots. We illustrate ecological interactions involving these critically important microbiome components with an example based upon pasture dynamics under dairy cattle grazing in a warm-temperate region of New Zealand.

New knowledge of the ecology of pasture communities in northern New Zealand has revealed a clear instance where the microbiome drives change in community structure, with consequent feedback loops that engage other microbial communities. Sustained high densities of the root-feeding insect pest black beetle (*Heteronychus arator*) since 2007/08 in the Waikato and Bay of Plenty regions of New Zealand (120), combined with other stress factors, particularly increasing summer-autumn soil moisture deficit (121), has led to widespread but spatially disaggregated instances of near-complete failure of pastures based on perennial ryegrass (*Lolium perenne*). For example, when a ryegrass population contains a strain of the endophyte *Epichloë festucae* var. *lolii* [formerly *Neotyphodium lolii* (122)] that offers minimal protection against the insect pest black beetle, pasture collapse is observed within 2 years after sowing (123). In contrast, when the ryegrass population contains an *Epichloë* endophyte strain effective against black beetle, ryegrass populations are maintained (124).

A signature of ryegrass failure is the content of white clover (*Trifolium repens*) in the pasture. When white clover is sown together with ryegrass cultivars containing the endophyte strain AR1 (which does not protect against black beetle), the white clover content of the pasture increases rapidly. In contrast, in pastures sown with perennial ryegrass containing endophytes that are effective against black beetle such as wildtype endophyte or strain AR37 (125) the ryegrass/clover balance is stable. The plant genome does not explain this different survival pattern, although there can be subtle host genotype x endophyte strain interactions that mediate the speed and scale of change (126). The outcome of clover dominance, resulting from reduced competitive pressure from the grass (127) leads to *Rhizobium* symbiosis becoming a dominant process in the community. This illustrates the connectivity between the plant and soil microbiomes—mediated through ecological processes, in this case competition. Furthermore, clover dominance changes the nutritional composition of the feed eaten by livestock, reducing total fiber content and increasing soluble protein (128), which in turn creates a change in the rumen microbiome where the microbial composition changes in response to the altered substrate. This change results in a further interaction involving the microbiome impacting on the host animal. A direct outcome of this interaction is greater ammonia release in the rumen, flowing through to increased excretion of surplus nitrogen via higher urinary nitrogen concentrations (129) and heightened risk of nitrate leaching from beneath the urine patches returned to the grazed pasture (130).

This example illustrates the influence of, and in some case control by, the microbiome on the productivity and sustainability of a pastoral ecosystem. It highlights feedback loops, precipitated by a mis-match between the plant microbiome and the environment, led to the transformation of a grass-dominant and relatively nitrogen-efficient pasture to a legume dominant pasture with a leaky nitrogen cycle. The new pasture state with strong clover content may increase total herbage

accumulation (compared with a ryegrass-dominant sward) in the short term. However, if clover contribution remains in the range of 15–30% of herbage mass (131), the consequences for the long-term yield potential of the system cannot be easily predicted. For instance, the new pasture composition may accentuate patch selection if grazing animals express partial preference for clover (132, 133). The resulting bimodal frequency of pasture mass observed in cattle grazed systems [e.g., (134)] will likely reduce the total production of the system (135). Questions that then arise include: Where and how should we intervene to manipulate the microbiome in an ecological system such as this? With what purpose and consequences? What benefit can we expect, relative to the manipulation of the plant or animal genome itself, from going down this pathway?

CASE STUDY 3: RUMEN MICROBIOME

By virtue of converting human-indigestible plant polymers (cellulose and hemicellulose) into edible animal protein, ruminants enable high value food production from pasture plant resources. In the New Zealand context, ruminant animals are therefore an important part of the pastoral sector and produce a wide range of food and fiber products of considerable value to the economy. The digestion of plant material is achieved via the ruminant's specialized digestive systems, consisting of a multi-chambered stomach which supports the growth and fermentation activities of a diverse array of anaerobic microbes. The main digestive processes are carried out in the first two stomach compartments, called the reticulo-rumen, where microbes colonize and degrade forage plant material. The microbes ferment the released sugars into volatile fatty acids (VFA), which are absorbed from the reticulo-rumen and used by the animal to drive growth and formation of food and fiber products. The process is regulated so that only a partial fermentation occurs, allowing the ruminant host to absorb and utilize the intermediate fermentation products for its own metabolism and growth. The ruminant also benefits from the provision of vitamins and from the microbial cells flowing further down the digestive tract.

There has been a continual drive by livestock breeders and farmers to improve the efficiency of digestion in the rumen. Studies of the rumen microbiome have focused on understanding the contribution that the microbes make to the digestion and metabolism of particular feeds, or that are involved in production traits that are selected during animal breeding. However, microbiome analyses are increasingly being used to identify new ways to manipulate microbial metabolism, to enhance digestive capacity and drive greater output of food and fiber products by the host animal, while reducing waste or detrimental end products of the fermentation that have negative impacts on digestive efficiency, rumen function or the environment. There are many previous examples of microbial manipulations in ruminants to influence digestion, including additives such as buffers, antibiotics [ionophores and non-ionophores; (136–139)], methane inhibitors (140–148), vitamins, minerals, iso-acids, enzymes, and exogenous bacteria and/or yeast (149). These additives target different processes in the rumen and have varying degrees of effectiveness, depending on the ruminant species targeted and the diet fed to the animals. Many of these additives are non-selective or have unknown modes of action, and there is a need to have a better understanding of rumen microbiome responses so that these manipulations can be more precisely tailored for delivery of the desired improvements while removing, or minimizing, any unintended consequences.

Rapid advances in DNA and RNA sequencing, and new high throughput screening technologies for proteins and metabolites, are now making a complete description of the rumen microbiome an achievable goal (150). Combined with the ability to interpret the "omics" information using new bioinformatics approaches, this is transforming our understanding of the rumen microbial ecosystem (70, 151–154) and will inevitably lead to new ways of manipulating ruminal fermentation processes. Although these technologies are relatively new, they are being used to address recurring questions about the contribution the rumen microbiome makes to the nutritional functions of the ruminant. This will allow assessment of the types of microbes that are present, how many organisms are there, their relative quantities, and their functional role. Furthermore, as a better appreciation is gained of the importance of gastrointestinal microbes to their host, new questions around their protective, immunological, and developmental benefits to the host are being posed (155–157).

An example which illustrates the interactions between the host animal and its rumen microbiome involves methane yield differences in sheep that are related to expression of genes encoding the hydrogenotrophic methane formation pathway (158). Methane is produced in the rumen by the methanogenic archaea and is released from animals via eructation, or belching, and is also respired via the breath (159). Methane is an important agricultural greenhouse gas and has a global warming potential (GWP) of 28, meaning it is 28x the GWP of CO_2. Agricultural methane emissions contribute ∼14% of all anthropogenic emissions and therefore reducing emissions from ruminant animals is an important goal globally. While the main rumen methanogens are known, the process of methane formation is not clearly linked to either the number (160–162) or a particular community structure of methanogens (163, 164). However, it is known that the concentration of methanogenic substrates (mainly hydrogen and methyl compounds such as methanol and methylamines) and the interactions between methanogens and microbes producing and consuming hydrogen in the rumen (165, 166) are important factors contributing to methane emissions. To better understand methane formation, there has been a concerted effort to accurately measure methane emissions from ruminant animals, to examine the variation in methane yield (g methane/kg dry matter intake) between animals, and to assess the effects of different diets or dietary additives on methane output. Measurements made in sheep have shown methane yields vary considerably between individual animals within flocks (167–169), by as much as 34% between the low and high methane emission phenotypes. These variations in methane yield have been linked to differences in particle retention time in the rumen (167, 170, 171) and rumen volume (172). Furthermore, the variations were found to persist under different grazing

conditions and to be a heritable trait in sheep (169). The genetic basis for the methane phenotype in sheep is indicative of a key interaction between the host animal and the rumen microbiome. Because methane is produced solely by the action of methanogenic archaea, rumen methanogens must make some contribution to the methane phenotype in sheep, either directly or via changes to the microbial community in the rumen.

To examine the contribution that the microbiome makes to methane yield, sheep with high or low emission status were rumen sampled and DNA and RNA were extracted to enable both metagenome and metatranscriptome analysis of their rumen microbiomes (158).

Surprisingly, these analyses showed no differences in the relative abundance of bacteria, archaea or eukaryotes between the low and high methane yield sheep (158). Even detailed genus-level analysis of methanogens showed only slightly elevated levels of *Methanosphaera* spp. in the low methane yield sheep and slightly higher *Methanobrevibacter gottschalkii* in the high methane yield sheep, however these were not sufficient to explain the differences in animal methane yield. An analysis of abundance of genes encoding the methanogenesis pathway also showed no significant differences, which confirmed the rRNA gene analyses. However, when the metatranscriptomic data were examined, there were clear increases in transcripts of genes encoding the methane metabolism pathway in high methane yield sheep. In particular, the genes encoding the hydrogenotrophic methanogenesis pathway (in which methane is formed from hydrogen and carbon dioxide) were significantly up-regulated compared to the methylotrophic methanogenesis pathway (where methane is formed from methyl compounds). Specifically, high methane yield sheep had high transcript levels of the methyl coenzyme M reductase enzyme (*mcr*, EC: 2.8.4.1) which catalyzes the final step in the methane formation pathway. A detailed comparison of these *mcr* genes found that they clustered into three distinct groups, called sheep rumen MCR groups 1, 2, and 3. The SRMR1 group of *mcr* genes were derived from a new group of rumen methanogens which belong to the order Methanomassiliicoccales. The SRMR2 group was identified as encoding an isozyme of methyl coenzyme M reductase (MCRII encoded by the *mrt* gene) and was found in both *Methanobrevibacter* spp. and *Methanosphaera* spp., while the SRMR3 was derived from *Methanobrevibacter* spp. only. The vast majority of methyl coenzyme M reductase transcripts were from the SRMR1 and SRMR3 groups and were 2.84- and 2.85-fold more abundant in high methane yield sheep, respectively, while SRMR2 transcripts were very low (158). These results showed that transcriptional up-regulation of the hydrogenotrophic methanogenesis pathway was an important microbial mechanism contributing to higher methane yield in sheep.

It makes biological sense that an up-regulation of methanogenesis genes in rumen methanogens results in more methane emissions from animals, but why does this happen in some sheep and not in other grazing animals? A possible mechanism has been proposed which incorporates differences in rumen size and feed particle retention time, leading to altered microbial growth kinetics and fermentation thermodynamics which affects ruminal dissolved hydrogen levels (165). It is proposed that low methane yield sheep have a smaller sized rumen, which causes increased particle passage rate that leads to higher rumen hydrogen concentrations (**Figure 1**). The higher hydrogen concentration causes a negative feedback that results in less hydrogen formation by fermentative microbes, leading to less methane formation. Conversely, high methane yield animals are predicted to have a larger rumen with slower particle passage, which results in lower hydrogen concentrations, enhanced hydrogen formation during fermentation, and more methane. Under ruminal conditions of slower particle passage rate and lower hydrogen concentrations, it is predicted that there is a higher turnover rate of a smaller hydrogen pool through the methanogenesis pathway to account for the elevated methane formed. The lower ruminal hydrogen concentration means that methanogens have to increase expression of methanogenesis genes to produce more enzymes to scavenge the hydrogen and maintain its turnover rate. This is because enzyme concentrations as well as substrate concentrations can limit the flux through a pathway, and increasing enzyme expression partially overcomes the limitation of lower substrate concentrations. Conversely, a high particle passage rate and high hydrogen conditions would require a lower level of expression of methanogenesis pathway genes to permit the same flux. More recent studies have shown that the dissolved hydrogen concentrations in the rumen are higher than predicted from calculations that assume equilibrium with the gas phase (173). The concentrations of hydrogen measured *in vivo* show that hydrogen is super-saturated in the rumen, and significantly affects the calculated ΔG of hydrogen-forming and hydrogen consuming reactions in the liquid phase of rumen (174).

The strong relationship between expression levels of the hydrogenotrophic methanogenesis pathways in rumen methanogens and methane yield in sheep, is the first example of rumen microbial gene expression being directly linked to an animal phenotype of relevance to environmental sustainability and production.

DISCUSSION

The case studies described above illustrate the complex nature of interactions within the microbiomes of the soil, plants and the rumen of grazing animals. The potential for further higher-level interdependencies and interactions between the microbiomes along the soil, plant and animal continuum dramatically increases the overall complexity in a wider ecological context. Associations between the microbiomes of soil, plants, above and below-ground animals, and the environment are massively complex. These involve soil "genotype" x plant genotype x animal genotype x rumen "genotype" $\times$ environment interactions. Thus, while there are many examples of the importance of management of individual and simple microbiomes, most ecosystem outcomes are supported by the activities of multiple consortia of microorganisms. These outcomes are the result of many microbial species and strains, with a collective functional capacity resulting in an altered ecosystem phenotype. The

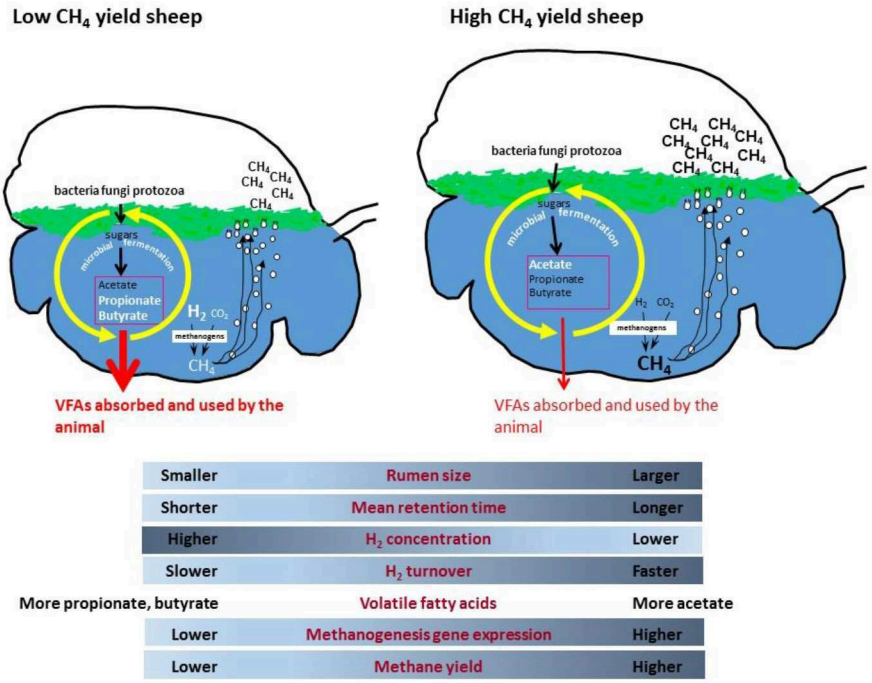

FIGURE 1 | Proposed rumen model for methane yield phenotypes in sheep.

opportunities to harness these interactions are immense, and offer great potential if they can be understood, directed and actively managed.

SOIL-PLANT MICROBIOME INTERACTIONS AND OPPORTUNITIES

Critically, the soil microbiome has a number of direct influences on plant performance. With the exception of seed-borne (vertically transmitted) endophytes, the soil biology provides the primary reservoir of microorganisms that colonize the root rhizoplane, rhizosphere, and ultimately the wider endophytic microbiome within the plant (175–179). The discovery of plant endophytes remains in its infancy, and estimates of 1 million endophytic species of higher plants may be reasonable (180). The consequences of the endophytic colonization of plants are profound, as the plant microbiome has wide ranging impacts on expression of plant phenotypes. Plant-associated microbiomes have been shown to confer drought tolerance (181), alter flowering phenology and timing (182, 183), influence plant shoot dry matter production (179), and induce systemic resistance to diseases (184). The interaction between the microbiome and plant genetics also affects aspects of plant quality via altering changes in the production of plant metabolites, or providing additional metabolic capacity via ancillary metabolic pathways encoded in the microbiome (185). An interesting example of interactions within the soil-plant microbiomes involves assessment of transgenic potato plants expressing an antimicrobial protein that is secreted into the apoplastic space between cells (186). While minor differences in the microbiomes were found in the rhizosphere of transgenic vs. non-transgenic plants, these changes were negligible compared to differences between non-transgenic plants of different potato cultivars. Another example is in flavor development in strawberries where the quantity and profile of their flavor is influenced by microbiome regulation of fuaranol synthesis (187, 188). Furthermore, the soil microbiome influences the microbial community on the grape berry and subsequent wine properties (189). It is also likely that microbiomes affect the quality of resins, fruit, honey, and essential oils (185). While the manipulation of plant traits through the microbiome have been vastly understudied, this represents major opportunities for production of novel products or additional value of current products (190). Indeed, the microbiome background in which plants are grown can be seen to contribute to the wider *terroir* of the plant product, and may be used to add value to the provenance of products grown in different soils.

There are many direct and indirect interactions between below and above ground ecosystems, and these converge into terrestrial ecosystem function (191, 192). Collectively these express as an "ecotype," or "functional status" to the soil. Across a multitude of functions, a "normal operating range" of soil ecosystems can be defined. These can be assessed by sampling across a range of sites to give a generalized understanding of the performance of a soil, allowing an assessment of measured vs. expected system function (81). This framework can be expanded to investigation of factors such as expression of plant-genotype effects, impacts on soil ecosystems due to disturbance (e.g., by humans or climate), and assessing ecosystem recovery. Future decisions about plant

or cultivar selection for different farming systems (e.g., pastoral, arable, horticulture, and forestry) will include an understanding of the underlying soil biology. Furthermore, these decisions are likely to extend to precision use of fertilizers, agri-chemicals, and seed dressings (including biological ingredients), that consider the wider ecosystem parameters. These opportunities will need to find a balance between optimal output of products and sustainable environmental outcomes, which are not obtainable with the current laissez-faire approach.

WHAT CONNECTIONS EXIST BETWEEN SOIL, PLANT, AND ANIMAL MICROBIOMES?

Research is now striving to understand the interactions between the soil, plant, and animal microbiomes within different environmental situations (**Figure 2**). Such holistic, community-level approaches to assess complex, multi-trophic linkages and communication among microorganisms, plants, and animals, within a wide environmental spatiotemporal heterogeneity, will require application of a range of emerging tools and approaches such as those based on ecological genomics (71). These will need to deliberately embrace the inherent complexity of microbiomes as "meta-ecosystem" containing an assortment of biological elements (species, mobile genetic elements), with different functional potentials resulting in an overall ecosystem phenotype. An integrative ecological genomics approach, that explores interactions among and across these meta-ecosystems and their collective ecological control, will be required to translate the biology to useful applications in a control-analysis approach (81).

As indicated in the dairy pasture dynamics case study, animal production systems in New Zealand are based on year-round grazing of ruminants on pastures, which are dominated by perennial ryegrass-clover mixtures (125). There are clear connections between the plant and animal microbiomes via the ingestion and fermentation of plant material in the rumen. However, there is very little information on how the above ground plant microbiomes (i.e., endophytic and ectophytic microbes colonizing the internal and external parts of plant stems and leaves) affect digestive processes in the rumen. It is known that as soon as plant material enters the rumen, it is colonized by a succession of different rumen microbes which initiate digestion (193–195). There is also evidence that ingested plant material continues to metabolize and undergoes a cell death response which leads to DNA fragmentation and protein breakdown, independent of rumen bacterial activities (196). This autolytic plant protein breakdown contributes to the inefficient use of plant protein which can result in much of the ingested nitrogen being lost from the animal in the form of ammonia and urea, which can cause environmental problems when excreted from the animal. The types of endo- and ecto-phytic plant microbes entering the rumen, and their activities during the plant digestion process, are poorly understood. Knowing what type of microbes are carried into the rumen in, or on, plant forages may reveal opportunities for microbial manipulation of the plant autolytic processes, allowing for the enhancement of rumen microbial colonization of the plant material or improvement of the ruminal digestion process itself.

Investigation of plant microbiomes entering the rumen may also offer some new insights and perspectives into facial eczema (FE), a significant disease in ruminants caused by saprophytic fungi growing on the dead litter at the base of pastures. The fungus, *Pithomyces chartarum,* proliferates under the warm moist conditions typically found in late summer and early autumn, and produces large numbers of spores which are subsequently ingested by the animal. In the rumen, the spores release a mycotoxin, sporidesmin, which is absorbed and causes damage to the liver and bile ducts. The damaged liver is unable to breakdown chlorophyll normally and a toxin, phylloerythrin, builds up in the blood causing sensitivity to sunlight and skin inflammation, leading to the FE symptoms of skin irritation and peeling, lowered production and sometimes death of the animal. Current management practices for FE include treatment of stock with zinc sulfate (supplied via their drinking water, drenched as a liquid product or delivered via an intraruminal slow release capsule), or applying fungicide to pastures before the spore counts become too high. There are also animal breeding programmes underway to select for FE-resistant sheep and cattle. Non-toxigenic isolates of *P. chartarum* have also been investigated as potential competitive exclusion biocontrol agents under New Zealand conditions (197). While sporidesmin levels were reduced by the application of the non-toxigenic isolates to pastures, the percentage of such isolates declined from 90 to 54% of all *P. chartarum* isolates retrieved during the 19-week trial, and after 15 months represented only 4% of the isolates from treated plots. This indicates that non-sporidesmin-producing isolates did not persist in the environment, at least under the field conditions examined. These observations suggest that better understanding of the microbiomes associated with decaying plant material in pastures, and of the ingestion and subsequent degradation of spores in the animal gut, may lead to easier and more effective means of controlling this serious animal disease.

While the interactions between plant and ruminant microbiomes are obvious, it is less appreciated that ruminants also ingest significant quantities of soil. The amounts ingested by ruminants depends on the amount of soil that becomes attached to the portions of the plant grazed by the animal, which is influenced by the weather conditions and soil type. Soil ingestion is lowest in the summer months on soils with strong structure and is highest in the winter months on soils with poor structure, where there is greater opportunity for rainfall to mediate soil transfer to the above ground parts of the forage plants (198, 199). Soil ingestion was originally studied in relation to teeth wear in sheep, as particularly abrasive soil types contribute significantly to teeth wear, limiting the productive lifetime of ewes. These studies showed that ewes can ingest up to 400 g of soil per day (198), while similar studies carried out in dairy cows found they could ingest more than 2 kg of soil per day under certain conditions (199). Soil ingestion is also a major route of uptake of trace elements for ruminants (198). The best known example of this is the uptake of cobalt (Co) to alleviate the symptoms of "bush sickness" or "wasting disease." Co is required in ruminant diets for the bacterial synthesis in the rumen of cobalamin,

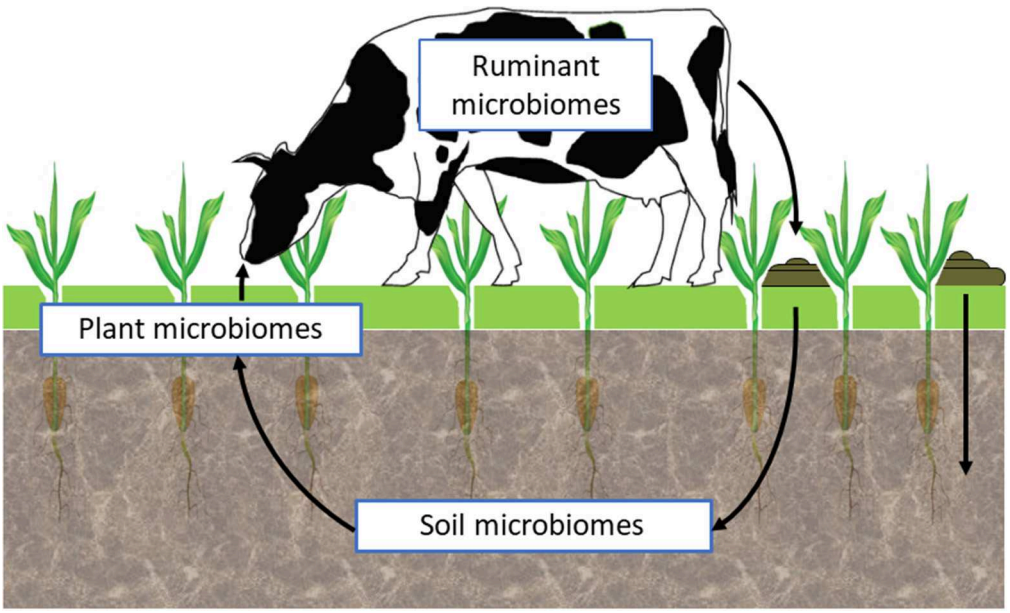

FIGURE 2 | Harnessing microbiome function.

which is also known as vitamin B_{12}. Cobalamin is an essential cofactor for the enzyme methylmalonyl-CoA mutase, involved in an important metabolic pathway of ruminants, converting propionic acid (one of the main VFA produced by the rumen fermentation) to glucose (200, 201).

Given that soil can contain 10^9-10^{10} organisms per g, the amount of soil ingested by ruminants reported above (198, 199) represents up to 4×10^{11} to 4×10^{12} soil microorganisms ingested by sheep per day, or 2×10^{12}-2×10^{13} microorganisms per day by cattle. The potential impact of this ingested soil microbiome is estimated to be from ∼8 to ∼2.6% of the rumen microbiome of sheep and cattle, respectively. These estimates of soil-borne microbes entering the rumen are consistent with recent findings of a global rumen census, in which exogenous microbes (likely to be derived from ingested plant material, water or soil) on average made up ∼3% of the rumen microbiome sequences (151). The changes in dairy pasture dominance by clover over ryegrass in Case Study 2 described above, has shown the indirect effects of soil microbes on ruminant production via altered forage species mix and elevated N fixation. However, there has been no examination of the direct influence that ingested soil microbes themselves might have in the rumen. The rumen and the soil environments have very different physico-chemical conditions which select for dissimilar microbiomes, therefore one would not expect soil organisms to survive, or be metabolically active, for long periods in the rumen. However, the effects of enhanced trace element supply to ruminants via ingestion of soils and the subsequent co-factor biosynthesis by bacteria in the rumen, suggest that some interactions of relevance to ruminant growth and health do occur at this interface, and are worthy of further characterization.

After digestion in the rumen, the remaining material is further fermented in the hind gut before being passed from the animal as dung. In the New Zealand dairy grazing system, dung is mainly deposited as "cow pats" onto the pasture, and eventually is broken down by a combination of microbial, insect and earthworm activity and incorporated into the soil or is volatized as ammonia. The amounts of manure produced by cattle varies considerably; beef cattle consuming 12 kg dry matter intake (DMI) per day produce about 5–6% of their body weight as manure each day (average ∼27 kg wet weight), while dairy cattle with a 22 kg DMI produce closer to 70 kg per day. The microbial density in manure is roughly equivalent to the density in the rumen, but the phylogenetic distribution of microbes within the manure differs. While the rumen microbiome is usually dominated by Firmicutes and Bacteroidetes, manure can have altered Firmicutes to Bacteroidetes ratios (202) or elevated levels of Proteobacteria (203). The type of diet consumed by the animal also influences the composition of the fecal microbiome (204). Studies on the effects of manure deposited onto pastures of upland soils, indicate that dung deposition provides additional substrate for microbial growth and metabolism, and alters nutrient availability (205–207). The contribution of ruminant gut microbes in the manure, to these soil processes remains unknown, and represents a potential point of intervention to affect beneficial changes to the availability of nutrients from the soil.

DOWNSTREAM IMPLICATIONS FOR ENVIRONMENTAL AND FOOD MONITORING

While focusing on increasing the benefits of enhancing agricultural production through the microbiomes along the soil-plant-animal continuum, there is also a need to consider the

potential effects of animal pathogens on human health. We know that changes in animal diets and/or farm systems can affect the zoonoses carried by farm animals (208–210). These zoonoses can impact on human health via multiple pathways. The first is direct animal contact which impacts predominantly on the people who work in the industry, as well as non-occupational contact (211). The second pathway is via contamination of the food products consumed (212, 213). The third major pathway is via water contamination (214) which in itself can exhibit three separate pathways such as drinking water (215), contact recreation (216) and irrigation of food crops (217). These outbreaks of zoonotic disease events can have considerable economic cost to the agricultural industries (218–220).

Genomic technologies present a major opportunity to have a transformational impact on environmental monitoring and food monitoring. This will result in increased use and adoption of genomics tools for diagnostic purposes associated with the monitoring of "risk microbes" involved with environmental health, food safety and well-being of people. Genomic techniques have already shown great potential in linking and understanding sources of food or water contamination (221–223). Highly sensitive targeted amplicon sequencing can readily detect specific pathogens and environmental metagenomics will generate huge data sets in which risk microbes can be identified.

Genomic technologies are extremely sensitive, therefore interpreting a positive signal for the presence of a DNA sequence in a sample becomes absolutely critical. There is an urgent need to establish "genomic thresholds" for water quality or food contamination to allow appropriate interpretation of genomic diagnostic data by environmental and food regulatory authorities. Similar issues are also posed for the implementation of genomic diagnostics in biosecurity decisions at border controls or clinician/veterinarian interpretations in human/animal health. A critical requirement will be the up-skilling of end-users in genomics to ensure that genomic-based diagnostic data can be effectively interpreted and appropriate actions implemented by stakeholders when enforcing policy decisions.

FUTURE OPPORTUNITIES FOR UNDERSTANDING THE MICROBIOME INTERACTIONS

The principal advantage of using genomic tools to improve the understanding of microbiome interactions is the greater precision in identification and quantification of the structure of the microbial communities. Enhanced detection and characterization of the microbial members of each microbiome (the number of different species, the number of individuals within the species, and the detection of unculturable microbes), along with predictions of their metabolic capabilities from retrieved genomic information, will greatly enhance our understanding of microbiome community structure and function.

Assessment of species rank-abundance curves (RAC's) show that the soil microbiome contains many rare species (224, 225). This has particularly been brought to light with NGS-based community sequencing analysis; with increasing depth of sequencing, generally using SSU rRNA phylogeny, more species are discovered. That is, the tails of the RAC's generated for soil microbial ecosystems are very long. However, do these rare species matter in relation to soil-provided ecosystem function? In many cases, the rare biosphere is the reservoir of many novel lineages, colloquially referred to as "microbial dark matter" (226). Our understanding of these taxa is slight, particularly as many of these novel, and sometimes candidate phyla remain to be isolated in pure culture (227). As such, the ecological importance of the rare biosphere is unclear. Genomic analysis has shown that these taxa harbor unexpected metabolic features (226), and are therefore a potential source of novel enzymes and "stored ecosystem potential" (228). Furthermore, the recruitment of taxa, with unique ecophysiological adaptations, has been shown to be essential in recovery of soil ecosystem function after disturbance events, such as ammonia oxidation (229). Thus, the rare biosphere has wider impacts on ecosystem function than the total size of the community. It represents an important "seed bank" of organisms with which we may begin to have a functional role as opportunities arise, for example recruitment by a host plant or animal, or edaphic or environmental changes.

Numerous microbiome studies have been performed using rRNA gene targeted approaches. While this marker gene has worked well for many examples it is dependent on organisms within the sample having matches to the primer sequences used. New primer-independent, metagenomic shotgun sequencing approaches are rapidly increasing the volume of sequence data of microbiome samples across numerous environments along the soil-plant-grazing animal continuum. This is producing large databases of sequence information which is providing the science community with a significant resource for data mining to better understand these microbiomes, and will also act as a reference for characterization of future microbiomes. The continual increase in sequencing capacity at lower cost and access to constantly improving computational resources (e.g., more powerful data processing hardware and purpose-written, open source software) will allow new science questions to be asked about microbial functions in systems that were previously not possible. These advances will substantially improve the degree of replication and depth of sequencing required to cover the variables present in a given microbiome, or compensate for variation within samples that was not previously feasible (230).

The metagenomic shotgun sequencing of significant components of entire microbial communities is now becoming achievable at a reasonable cost. Coupled with improved computational power and bioinformatic analyses, this will dramatically improve investigation of microbiomes. Having the technologies to understand how the organisms within a microbiome interact to support ecosystem functions, such as nutrient cycling, is an exciting prospect and will undoubtedly lead to opportunities for discovery of novel microbiome features to improve ecosystem production and environmental outcomes. However, there are still significant hurdles to overcome (231, 232), and achieving a more comprehensive description of the soil-plant-grazing animal microbiome continuum would

represent a remarkable advance in our ability to characterize and understand complex ecosystems.

FUTURE QUESTIONS FOR UNDERSTANDING THE SOIL-PLANT-GRAZING ANIMAL MICROBIOME CONTINUUM

Understanding of the various microbiomes that make up the soil-plant-grazing animal microbiome continuum involves a major effort. Integrating metagenomic data from multiple microbiomes to get a more holistic view of ecosystems is rare, but is beginning to be addressed. Trying to understand the soil-plant-grazing animal microbiome continuum requires a clear framework, informed by the answers to some key questions. These include: how do the different microbes within a microbiome contribute to the overall phenotype? how do the different microbiomes interact with each other? and how do we move to an ecosystem-wide approach to understand the (role of the) microbiomes across the ecosystem? While these questions are answered, the main metabolic pathways in each microbiome need to be characterized along with how the composition of the microbiome can predict the phenotype. Genomics has already allowed for a vast amount of data to be generated, but the knowledge on how to translate this genomic knowledge into a phenotype requires further attention.

With the ever increasing volume of data available for each microbiome sample, microorganisms present in minute quantities will be increasingly detected with greater accuracy. This will provide a greater understanding of the relationship between the quantity of microorganisms in a sample and the contribution ("quality") or ecological "phenotype" of that organism. For example, do microorganisms present in high frequency (quantity) contribute more to the overall phenotype than microorganisms which are rare (quality)?

Improved understanding of the microbiome composition with respect to quantity and quality will raise potential options to manipulate the microbiome to our advantage. Examples include the potential of keeping nutrients in the soil through using diverse plant genotypes/ plant species to manipulate the microbiome. The challenge ahead is to use the expanding genomics knowledge not only to increase the resilience of pastoral systems (and pasture persistence) by manipulating the microbiome, but to achieve this with less environmental impact while maintaining or improving agricultural outputs.

AUTHOR CONTRIBUTIONS

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Utilization of *Scenedesmus obliquus* Protein as a Replacement of the Commercially Available Fish Meal Under an Algal Refinery Approach

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The approach of algal refinery as a method to reduce the cost of algal biodiesel by co-production of various value-added chemicals is the most up-coming strategy suggested for the economic viability of microalgal biodiesel. This concept being relatively new and novel, abundant literature on the subject is not available although fragmented data on some feedstocks are present. The main objective of this research paper is to propose an algal refinery design through utilization of *Scenedesmus obliquus* biomass for production of various industrially important products. For this purpose, first a protocol was standardized for maximum extraction of protein from *S. obliquus* biomass. Then, different experiments were conducted for 90 days each to find out the optimum concentration of microalgal protein that can be substituted in the diets of freshwater fishes for their maximum growth. During these experiments eight different growth parameters and seven water quality parameters were tested. Results showed that the standard + whole microalgal biomass + extracted microalgal protein diet (25:25:50) was the best diet for maximum growth of the freshwater fishes. After conducting these experiments, a detailed sequential extraction process for maximum valorization of the *S. obliquus* biomass or in other words an algal refinery was designed. The detailed sequential process developed, yielded 0.06 g of β-carotene, 10 g of protein, 38 g (43 mL) of biodiesel, 2 g of omega-3 fatty acid, 3 g (2.4 mL) of glycerol and 18 g (23 mL) of bioethanol from 1 Kg wet (≈100 g dry) *S. obliquus* biomass thus converting 70% of the test microalgal biomass into biodiesel and other value-added products by using an algal refinery approach.

Keywords: algal refinery, biodiesel, bioethanol, omega-3-fatty acids, protein-rich algae meal

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INTRODUCTION

The use of microalgal lipids for biodiesel production is a widely practiced strategy these days for countering the problem of over-exploitation of non-renewable sources of energy. But the production process for microalgae-derived biodiesel is extremely cost-intensive (Delrue et al., 2012; Borowitzka, 2013; Banerjee et al., 2016). Hence, application of algal refinery approach is

being explored and looked upon as a possible strategy for improving the economics of microalgal biodiesel. This approach suggests the valorization of the whole microalgal biomass through production of industrially important co-products along with biodiesel for countering the costs incurred with more earnings from high-value products.

In the paper published by Patnaik and Mallick (2015), the green microalga *Scenedesmus obliquus* was used as a model organism to produce biodiesel and other industrially important co-products such as β-carotene, omega-3 fatty acids, glycerol and bioethanol in a sequential process in the designed algal refinery, but the use of protein present in the same microalgal biomass was not shown. Hence in this research study, for improved valorization of the *S. obliquus* biomass, the utilization of the leftover microalgal protein has been demonstrated by using the protein-rich algae meal as a replacement of the commercially available fish meal in the diets of the freshwater fishes.

Fishmeal is a widely used source of protein in the fish feeds. But the continued consumption of this commercially available fish meal in large quantities has reduced the availability of this protein-rich resource (Grammes et al., 2013; Jones et al., 2014; Camacho-Rodríguez et al., 2017). Hence, it is essential to find a sustainable substitute for the commercially available fish meal without affecting the quality of the feeds. Although conventional terrestrial crops such as oilseeds and grains have been successfully used as alternatives to fishmeal their effect on the nutritional quality of the fishes due to lack of certain essential amino acids in the terrestrial crops (Valente et al., 2006; Dawczynski et al., 2007; Neori, 2011) have shifted the focus toward algae which are reservoirs of protein, lipid, carbohydrate, vitamins and pigments and which form the base of the aquatic food chain (Cyrus et al., 2014; Haas et al., 2016; Kiron et al., 2016; Shah et al., 2017; Vadstein et al., 2018). Additionally, although the protein content in the different microalgal species vary, they generally contain all the essential amino acids required for the better growth and survival of the aquatic organisms (Abdulrahman, 2014; Egerton et al., 2018; Vestrum et al., 2018).

In consideration of all these facts, this research study has focused on selecting a protocol for maximum protein extraction from *S. obliquus* (Trup.) Kutz. (SAG 276-3a) biomass after which, the extracted protein has been used to formulate a protein-rich algae meal for the freshwater fishes. After completion of the fish feeding experiments, the algal refinery design shown by Patnaik and Mallick (2015) has been further enriched by addition of the protein component of the test microalga in the valorization process, eventually utilizing the major components present in *S. obliquus* under the optimized condition for production of industrially important products through the designed refinery.

MATERIALS AND METHODS

Microalgal Growth Conditions for Fish Feeding Experiments

Photoautotrophic culture condition of the green microalga, *S. obliquus* (Trup.) Kutz. (SAG 276-3a), was maintained by cultivating the algae in 100 ml of N 11 medium contained in 250 ml Erlenmeyer flasks (Soeder and Bolze, 1981) without aeration. The cultures were maintained under sterile conditions. Light at an intensity of 75 μmol photon $m^{-2}s^{-1}$ PAR was continuously supplied for a photoperiod of 14:10 h with pH adjusted at 6.8 and temperature at 25 ± 2°C. Continuous shaking of the culture flasks was done, two to three times a day, to prevent sticking of the culture to the bottom of the flask. To produce enough biomass for the fish feeding experiments, the green microalga, *S. obliquus* was grown in 5 L tubular glass photobioreactors (38 cm height and 15 cm diameter) with working volume of 4 L capacity (courtesy: ICG, Forchungszentrum, Juelich, Germany) as per the protocol detailed by Bagchi and Mallick (2016).

Selection of Protocol for Protein Extraction From *S. obliquus* Biomass

Five different protocols were used for protein extraction from wet *S. obliquus* biomass.

Protocol 1

Extraction of protein was carried out as per the methodology suggested by Barbarino and Lourenco (2005) in which alkali hydrolysis method and ultrapure millipore water were used for extraction of proteins followed by precipitation using trichloroacetic acid. 200 mg of wet microalgal biomass was suspended in 4 mL of ultrapure millipore water and incubated for 12 h at 4°C. After the incubation period, the microalgal suspension was centrifuged at 5000 rpm for 15 min. The supernatant obtained, was collected in another tube and the pellet was re-extracted with 4 mL of 0.1N NaOH containing 0.5% β-mercaptoethanol (v/v), after incubation at room temperature for 1 h with occasional manual shaking. The mixture was then centrifuged again at 5000 rpm for 15 min. The supernatant was collected and mixed with the supernatant obtained in the first phase of the extraction procedure.

The sample was then processed for protein precipitation. Twenty five percent TCA was mixed with the protein containing supernatant in a proportion of (2.5:1, v/v) and kept in an ice bath for 30 min. The mixture was then centrifuged at 5000 rpm for 15 min. The supernatant was discarded and the pellet formed was washed with 10 mL of cold 10% TCA and centrifuged again. The pellet after the second centrifugation was again washed with 5 mL of 5% TCA and centrifuged under the same conditions as mentioned above for separating the protein precipitated at the bottom of the centrifuge tube.

The protein pellet was then processed for quantification using Bradford method (Bradford, 1976). The protein pellets obtained following the above mentioned extraction protocol were suspended in 500 μL of distilled water, and out of this suspension, 100 μL was pipetted into 15 × 1.5 cm test tubes. Five mL of Bradford reagent was added to the test tube and the contents were mixed by vortexing. The absorbance at 595 nm was taken within 1 h in 3 ml cuvettes against a reagent blank prepared from 100 μL of distilled water and 5 mL of Bradford reagent. The concentration of the protein was estimated after comparison with a standard curve.

Protocol 2

Protein extraction using Tris-EDTA saturated phenol and precipitation using 0.1 M ammonium acetate prepared in 80% methanol was done as per the protocol suggested by Wang et al. (2006). Microalgal biomass (wet) was homogenized in a mortar and pestle using liquid nitrogen and out of that 200 mg was transferred to a 2 mL tube. The tube was filled with 2 mL of 10% acetone and mixed with the microalgal biomass using a cyclomixture. After 30 min of incubation at room temperature, the mixture was centrifuged at 6000 rpm for 10 min. After centrifugation, the supernatant was discarded and the tube was filled with 2 mL of 0.1 M ammonium acetate prepared in 80% methanol. After proper mixing by vortexing and incubation at room temperature for 15 min, the mixture was centrifuged at 6000 rpm for 10 min. The supernatant was again discarded and the pellet was mixed with 2 mL of 80% acetone. After keeping the mixture undisturbed for 15 min, the acetone was removed after centrifugation under the above mentioned conditions. The pellet was then air dried to remove the residual acetone. To this microalgal pellet, 1 mL of phenol was added, mixed thoroughly and incubated for 5 min at room temperature. The mixture was then centrifuged at 6000 rpm for 5 min and the supernatant containing the proteins dissolved in phenol, was transferred to a fresh 2 mL tube. To this separated supernatant, 1 mL of 0.1 M ammonium acetate prepared in 80% methanol was added, mixed properly and incubated overnight at −20°C for proper protein precipitation. The other day, the mixture was centrifuged at 6000 rpm for 5 min, the supernatant was carefully discarded and the pellet obtained was washed once with 80% methanol and once with 80% acetone. The protein pellet was then processed for quantification using Bradford method (Bradford, 1976).

Protocol 3

The protocol recommended by Cilia et al. (2009) was followed for extraction and precipitation of protein from wet *S. obliquus* biomass using 10% TCA prepared in 80% acetone containing 2% β-mercaptoethanol. The wet microalgal biomass (200 mg) was dissolved in 10 mL 10% TCA prepared in 80% acetone containing 2% β-mercaptoethanol. The mixture was mixed thoroughly by vortexing. It was then incubated at −20°C for at least 12 h for protein precipitation. The precipitated protein was separated from the supernatant by centrifugation at 5000 rpm for 15 min and then washed three times in 5 mL of cold acetone. The protein pellet was then processed for quantification using Bradford method (Bradford, 1976).

Protocol 4

The protocol suggested by Bhardwaj and Yadav (2013) was followed for extraction of proteins using Tris-HCl saturated phenol and precipitation using 0.1 M ammonium acetate prepared in 80% cold methanol. After homogenization of the wet microalgal biomass using liquid nitrogen in a mortar pestle, 200 mg of homogenized microalgal biomass was suspended in 3 mL of extraction buffer in a 15 mL centrifuge tube, vortexed and incubated for 10 min on ice. Afterward, equal volume, i.e., 3 mL of Tris-HCl saturated phenol was added and the solution was incubated with intermittent shaking at room temperature for 10 min for phase separation to occur. Following this, the solution was centrifuged for 10 min at 5000 rpm. The phenolic phase on the top of the tube, was collected carefully to avoid contact with the interphase, and was transferred to a fresh tube. This phenol phase was then re-extracted with 3 mL of extraction buffer. The sample was shaken and incubated at room temperature for 5 min, after which, it was centrifuged for 10 min at 5000 rpm. The phenol phase, still on the top of the tube, was recovered carefully and transferred to a new tube. To this 10 mL of precipitation solution was added. The contents were mixed by a cyclomixture and then incubated overnight at −20°C. The other day, the protein was finally pelleted after centrifugation at 5000 rpm for 10 min. The pellet was washed once with 80% cold methanol and once with 80% cold acetone. The protein pellet was then processed for quantification using Bradford method (Bradford, 1976).

Protocol 5

As proposed by Gerde et al. (2013), protein extraction was carried out by alkali hydrolysis method and ultrapure millipore water followed by precipitation using 2M HCl. 200 mg of wet microalgal biomass was dissolved in 20 mL of 2 M NaOH prepared in ultrapure millipore water containing 0.05% (v/v) of β-mercaptoethanol thus raising the pH of the biomass suspension to pH 11. The mixture was incubated for 5 h at 60°C. The biomass suspension was then centrifuged at 5000 rpm for 15 min and the supernatant collected was acidified using 2M HCl (added drop wise) till the pH was brought down to pH 3.2. After incubation of the acidified mixture at room temperature for 30 min, it was centrifuged at 5000 rpm for 15 min. The supernatant was discarded and the protein pellet was then processed for quantification using Bradford method (Bradford, 1976).

Ethics Statement

This study was carried out in accordance with the principles of the Basel Declaration and recommendations of Institutional Animal Ethics Committee (IAEC), IIT Kharagpur. The protocol was approved by the IAEC, IIT Kharagpur.

Experimental Set-Up for Fish Feeding Experiments

Freshwater fish, namely, rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*) and catla (*Catla catla*), were obtained from natural ponds at Agricultural and Food Engineering Department, IIT Kharagpur. Before distributing the fish in different tanks, fish fingerlings (no = 90) were treated with potassium permanganate solution (1 mgL^{-1}) for 1 h to remove any external parasites. Then they were randomly divided over six 50 L glass tanks fitted with lights on top of the tanks (15 fish fingerlings of mixed species in each tank). The fingerlings were then allowed to acclimatize to the new growth conditions for 2 weeks. The weight of the fish fingerlings ranged from 3.7 to 4.0 g at the beginning of the acclimatization period. During this period of acclimatization, the fish were fed with their standard diet at 2% of their body weight, only once during the daylight period between 9:00 – 10:30 am. The fecal matter and uneaten feed were removed once every 7 days from each tank by draining approximately 80% of the tank water volume using a siphon pipe and replacing it with

an equal volume of clean ground water. The rectangular glass tanks/aquariums were properly aerated with the use of air stones.

Experimental Set-Up 1

90 days feeding experiment was conducted with fish fed with three different test diets in three different tanks. The three test diets were standard diet (control), whole *S. obliquus* biomass and standard (control) + whole microalgal biomass (50:50) diets. The control diet consisted of a standard fish meal-based pelleted feed containing fishmeal, groundnut-oil cake, rice bran, wheat flour, and vitamins and minerals mixture in appropriate proportions. Biochemically the control feed had 30% crude protein, 3.5% lipid, and 40% carbohydrate, with the rest of the composition being crude fiber, minerals, ash, and moisture. Contrarily, the whole *S. obliquus* biomass was the harvested microalgal cells without any pre-treatment consisting of 53.2% protein, 12.5% lipid, and 22% carbohydrate. These diets were fed to the fish fingerlings at 2% of their body weight per day. At the start of the experimental set-up 1, the weight of the fish fingerlings ranged from 4.5 to 4.9 g. The amount of feed was calculated and readjusted every 15 days according to change in the body weight. The treatments were executed in duplicate. During this experiment, seven water quality parameters (analyzed as explained below in Section "Analysis of Water Quality Parameters," daily for pH, temperature and DO, and once every 3 days for the rest of the parameters) and eight growth parameters (analyzed as explained below in Section "Analysis of Fish Growth Performance and Nutrient Utilization Parameters," once every 15 days) were tested for ensuring proper fish health and management through the progress of the experiment.

Experimental Set-Up 2

Another 90 days feeding experiment was conducted with fish fed with three different test diets, standard diet (control), standard (control) + whole microalgal biomass (50:50) diets and standard (control) + extracted microalgal protein (50:50) diets at 2% body weight per day. The treatments were done in duplicate and all the experimental analyses were done in a similar manner as explained above.

Experimental Set-Up 3

The third feeding experiment for 90 days was conducted with fish fed with three different test diets, standard diet (control), standard (control) + whole microalgal biomass (50:50) diets and standard (control) + whole microalgal + extracted microalgal protein (25:25:50) diets at 2% body weight per day. The treatments were done in duplicate and all the experimental analyses were done in a similar manner as explained above.

Analysis of Water Quality Parameters

Water quality parameters such as temperature, pH, dissolved oxygen (DO) and turbidity were measured using standard instruments. Nitrate, nitrite and total ammonia nitrogen (TAN), contents in the water samples were measured according to the protocols suggested by Nicholas and Nason (1957), Lowe and Evans (1964), and Herbert et al. (1971), respectively.

Analysis of Fish Growth Performance and Nutrient Utilization Parameters

Body weight gain

The body weight gain (BWG) was calculated as per the formula suggested by Badwy et al. (2008) and was expressed as g fish^{-1}. The weight of the fish was measured using a weighing balance (Pioneer Scale Industries, Kolkata, India). Fish were caught using a fish net and were then placed on a weighing balance. Weight of the fish was measured after the fish became stable on the balance. An average value of the wet weight was taken after noting down three independent readings. The fish fingerlings were not anesthetized during their weight measurement.

Body weight gain (g fish^{-1}) = Weight (g) at the end of the experimental period – weight (g) at the beginning of the experimental period.

Specific growth rate

The specific growth rate (SGR) of the fish was calculated as per the formula suggested by Badwy et al. (2008).

$$\text{Specific growth rate (SGR)} = (\ln W_1 - \ln W_0)/(t_2 - t_1).$$

where W_1 was the final body weight of the fish after time t_2 and, W_0 was the initial body weight of the fish at time t_1.

Feed conversion ratio (FCR)

The FCR was calculated as per the formula suggested by Siddhuraju and Becker (2003). Total feed fed to the fishes for every 15 days period was calculated and expressed as (g fish^{-1}). Total weight gained by the fishes (in each tank) after every 15 days of feeding was measured as per the method detailed for measurement of BWG.

$$\text{Feed conversion ratio (FCR)} = \text{Total feed fed (g fish}^{-1}\text{)} / \text{Total wet weight gain (g)}.$$

Protein efficiency ratio (PER)

The PER was calculated as per the method suggested by Siddhuraju and Becker (2003). Fish were caught in a fish net and euthanized by rapid chilling. Fish flesh were then collected and grounded using liquid nitrogen. Protein extraction using ultrapure water, NaOH and TCA was done following Barbarino and Lourenco (2005). Protein estimation was done following Bradford (1976).

$$\text{Protein efficiency ratio (PER)} = \text{Wet weight gain (g fish}^{-1}\text{)} / \text{Amount of protein fed (g fish}^{-1}\text{)}.$$

Apparent net lipid utilization (ANLU)

The ANLU was calculated as per the formula suggested by Becker et al. (1999). Fish were first caught in a fish net and euthanized by rapid chilling. Fish flesh was then collected and grounded using liquid nitrogen. Lipid extraction using methanol and chloroform was done following Bligh and Dyer (1959). The amount of lipid obtained/lipid yield was calculated by subtracting W_1 (weight of the empty vial) from W_2 (weight of the vial containing the

extracted lipid) and was expressed as gL^{-1}.

$$\text{ANLU} = [\text{final fish body lipid (g)} - \text{initial fish body lipid (g)} / \text{crude lipid fed (g)}] \times 100.$$

Protein productive value (PPV)

The PPV was calculated using the formula suggested by Siddhuraju and Becker (2003).

$$\text{Protein productive value (PPV)} = [\text{gain in fish body protein (g)}/\text{crude protein fed (g)}] \times 100.$$

Metabolic growth rate (MGR)

The MGR was calculated as per the formula suggested by Becker et al. (1999).

$$\text{Metabolic growth rate (MGR)} = \text{live body weight gain (g)}/[(\text{initial body weight (g)}/1000)^{0.8} + (\text{final body weight (g)}/1000)^{0.8}/2]\text{day}^{-1}.$$

Omega-3 Fatty acid analysis

Fish were first caught in a fish net and euthanised by rapid chilling. Fish flesh was collected and grounded using liquid nitrogen. Lipid extraction was done following Bligh and Dyer (1959) following which the amount of lipid obtained was expressed as gL^{-1}. Transesterification of the extracted lipids was done following Mandal et al. (2013) and analyzed using GC-MS as per the method detailed in Patnaik and Mallick (2015).

Proposition for an Algal Refinery

This manuscript being a sequel to the previously published paper by Patnaik and Mallick (2015), the test microalga for this part of the study has been grown under the optimized condition for designing the algal refinery. The optimized condition comprised of *S. obliquus* grown in N 11 medium supplemented with 0.17% acetate, 0.17% citrate and 0.4 g L^{-1} nitrate and incubated for a period of 9 days in a temperature controlled culture room. The sequential extraction of all the components from the test microalga other than protein was done as per the methods detailed in Patnaik and Mallick (2015) whereas the protein part was extracted following the method standardized in this manuscript.

To find out the correct sequence of extraction of different components (so as to avoid any negative impact on the extraction yields of different components) four different sequences were tried as shown in **Table 1**.

Statistical Analysis

Microsoft Excel (Microsoft Corporation, United States) was used for graphical representations and determination of the standard error values (obtained from experiments carried out by using three independent cultures to confirm their reproducibility) for all experiments related to standardization of protein extraction protocols and microalgal refinery design. Fish feeding experiments were done in duplicate. Statistical analysis and graphical representations of the fish growth and nutrient utilization studies were also done using Microsoft Excel (Microsoft Corporation, United States).

RESULTS AND DISCUSSION

Selection of Protocol for Protein Extraction From *S. obliquus* Biomass

For extraction of protein from *S. obliquus* biomass, five different protocols were tested by following the methods described in Section "Selection of Protocol for Protein Extraction From *S. obliquus* Biomass." The extracted proteins were then analyzed using Bradford method (Bradford, 1976). The protein yield from the whole *S. obliquus* biomass grown under control condition for a period of 21 days was found to be 0.65 gL^{-1} (53.2% dcw), as measured before the initiation of the extracting protocols. Of all the protocols tested, Protocol 1 proved to be the most efficient. The selected protocol produced maximum extraction of protein up to 0.58 gL^{-1} (47.5% dcw) after 21 days of incubation. So, this protocol was able to extract 89% of the protein present in the whole *S. obliquus* biomass under control condition. As compared to the other protocols, Protocol 1 was found to extract maximum amount of proteins, probably due to the presence of more water-soluble proteins in the test microalgal biomass (Algae PARC, 2016), the denaturation of the disulfide bonds in the protein molecules by β-mercaptoethanol (Nelson and Cox, 2000), and the disruption of the solvation layers of the proteins and their partial denaturation, thus exposing even more hydrophobic surface to the solvent and enhancing the hydrophobic aggregation of the protein molecules by using TCA (trichloroacetic acid) (Barbarino and Lourenco, 2005; Cilia et al., 2009).

Hence, this experiment suggested the significance of a thorough knowledge of the type of proteins present inside the test organism along with their solubility and structure for selecting/designing an efficient protein extraction protocol. Additionally, the selected method should be compliable for a broad range of microalgal species, in both, their wet and lyophilized forms. Furthermore, the extraction protocol should not be extremely harsh so as to jeopardize the complete protein functionality ultimately compromising with quantification of the extracted protein using dye-based reactions. Another important point worth keeping in mind is that, the extraction protocol

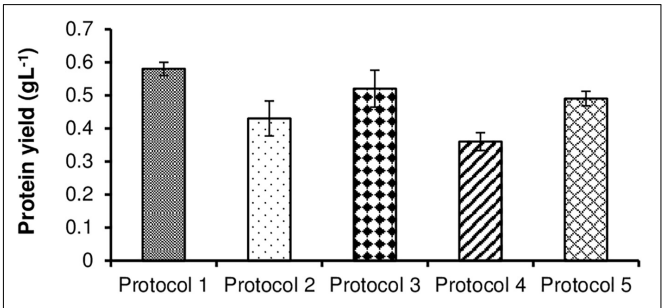

FIGURE 1 | Comparative representation of the amount of protein extracted from *S. obliquus* biomass using different extraction protocols.

alone, ultimately decides the use to which the extracted protein can be put to in a refinery design. The results of different protein extraction protocols used have been given in **Figure 1**.

Fish Feeding Experiment

Trial Experiment

After selection of the protein extraction protocol, experiments to formulate a protein-rich algae meal for substitution in appropriate quantities in the standard fish diet, was carried out. Trial experiment for investigating the ability of the freshwater fish species, rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), and catla (*Catla catla*), to feed on *S. obliquus* whole biomass as their only feed resource was performed for a period of 30 days. Additionally, the ability of the dried microalgal biomass pellet to sink to the bottom and remain undispersed till eaten by the fishes, was also checked. It was observed that the microalgal biomass was being consumed by the fishes with significant improvement in growth without much wastage of the supplied feed. The water quality parameters measured, were found to be well maintained within the tolerable limits of the fishes during the trial period.

Experimental Set-Up 1

After confirmation of the acceptability of *S. obliquus* biomass as a feed supplement by the freshwater fishes, the first experimental set-up for 90 days was conducted with fish fed with three different test diets at 2% body weight per day. The body weight of the fish in the test tanks on the 0 day (on the day of the start of the experiment) was 4.5 ± 0.72, 4.9 ± 0.65, and 4.7 ± 0.69 g in the tank fed with the standard diet (control), whole *S. obliquus* biomass diet, and standard + whole microalgal biomass (50:50) diet, respectively. The control diet consisted of a standard fish meal-based pelleted feed with 30% crude protein, 3.5% lipid, and 40% carbohydrate, with the rest of the composition being crude fiber, minerals, ash, and moisture. The *S. obliquus* biomass consisted of 53.2% protein, 12.5% lipid, and 22% carbohydrate.

Fish growth performance and nutrient utilization parameters

The growth parameters for assessing the effect of different diets on the freshwater fishes was studied as per the methods described in Section "Analysis of Fish Growth Performance and Nutrient Utilization Parameters." Eight different growth performance and nutrient utilization parameters i.e., BWG, SGR, FCR, PER, ANLU, omega-3 fatty acid content, PPV and MGR were observed. The results of the growth study have been shown in **Figures 2A–H**. It was observed that, all the above parameters showed maximum improvement in the fishes fed with 50:50 ratio of standard + whole *S. obliquus* biomass diet. Under this diet condition, maximum BWG of 6.95 g was observed in the third fortnight, i.e., within 30–45 days of feeding. The maximum SGR when fed with this diet was 0.026 μ. Out of the three test diets, this diet was observed to have the lowest FCR value, indicating the cost-effectiveness of the formulated diet. Hence it was implied that the maximum portion of 50:50 ratio of standard + whole *S. obliquus* biomass diet was able to be assimilated in the body of the fishes. Similarly, the PER value of this diet was found to be the highest among all three diet conditions indicating higher contribution of the protein in the standard + whole *S. obliquus* biomass feed toward raising the weight of the fish. It was also observed that the proportion of the lipid assimilated into the body of the fish out of the total crude lipid fed, i.e., ANLU was maximum (45.7%) in the third fortnight of feeding the 50:50 ratio of standard + whole *S. obliquus* biomass diet. Hence the omega-3 fatty acid content of 11.2% was also found to be maximum during that period. Similar to these observations, the PPV, i.e., the amount of protein gained by the fish after being fed with the test diet was found to be maximum (30.6%) within 30–45 days. With all the growth performance and nutrient utilization parameters showing maximum value in the third fortnight of feeding with 50:50 ratio of standard + whole *S. obliquus* biomass diet, the MGR was consequently found to be maximum during the same time period. This finding was found to be well in agreement with Radhakrishnan et al. (2015) in which 50% inclusion of *C. vulgaris* in the control diet, increased the growth parameters in *Macrobrachium rosenbergii*. Higher feeding rate and better assimilation of the protein fraction of the microalgal diet in the prawn were reasons cited by the authors for such an observation. The other two diets, i.e., standard (control) diet and whole *S. obliquus* biomass diet showed comparatively poorer performance as assessed through the above mentioned parameters. The 100% inclusion level of whole *S. obliquus* biomass diet in this research study must have reduced the palatability of the feed thus showing comparatively lower growth (**Figure 2**). Contrary to this observation, 100% inclusion percentage of *Spirulina platensis* was found to improve the specific growth rate and PER of the freshwater fish, *Labeo rohita* as observed in the report of Nandeesha et al. (2001). Algal inclusion percentages of <50% have also been recommended by few researchers (Teimouri et al., 2013; Ghosh and Mitra, 2015; Basri et al., 2015) for improving growth and/or functional component accumulation in some fish species. The examples cited, thus justify the species-specific dependence of both, the microalga (to be included in the fish diet) and the fishes (to be fed with the microalga included diet) on each other for improvement in the fish growth and nutrient utilization parameters. Moreover, standardization of the inclusion percentage of protein-rich algal meal in the diets of the freshwater fishes is also suggested to be of utmost importance.

Water quality parameters

The water quality parameters of the fish tanks were analyzed as per the protocols described in Section "Analysis of Water Quality Parameters." As the experiment was carried out from September to November, the temperature gradually saw a declining trend due to gradual decline in the atmospheric temperature in this geographical locale. The three parameters, i.e., temperature, pH and DO were not found to have any significant difference in the fish tanks administered with the three test diets. However, in case of turbidity, TAN, nitrate and nitrite content, the fish tanks provided with standard diet, showed higher values of the said parameters (data not shown) which could have been due to the accumulation of the residual feed and inadequate oxidation of the fecal matter (Ghosh and Mitra, 2015).

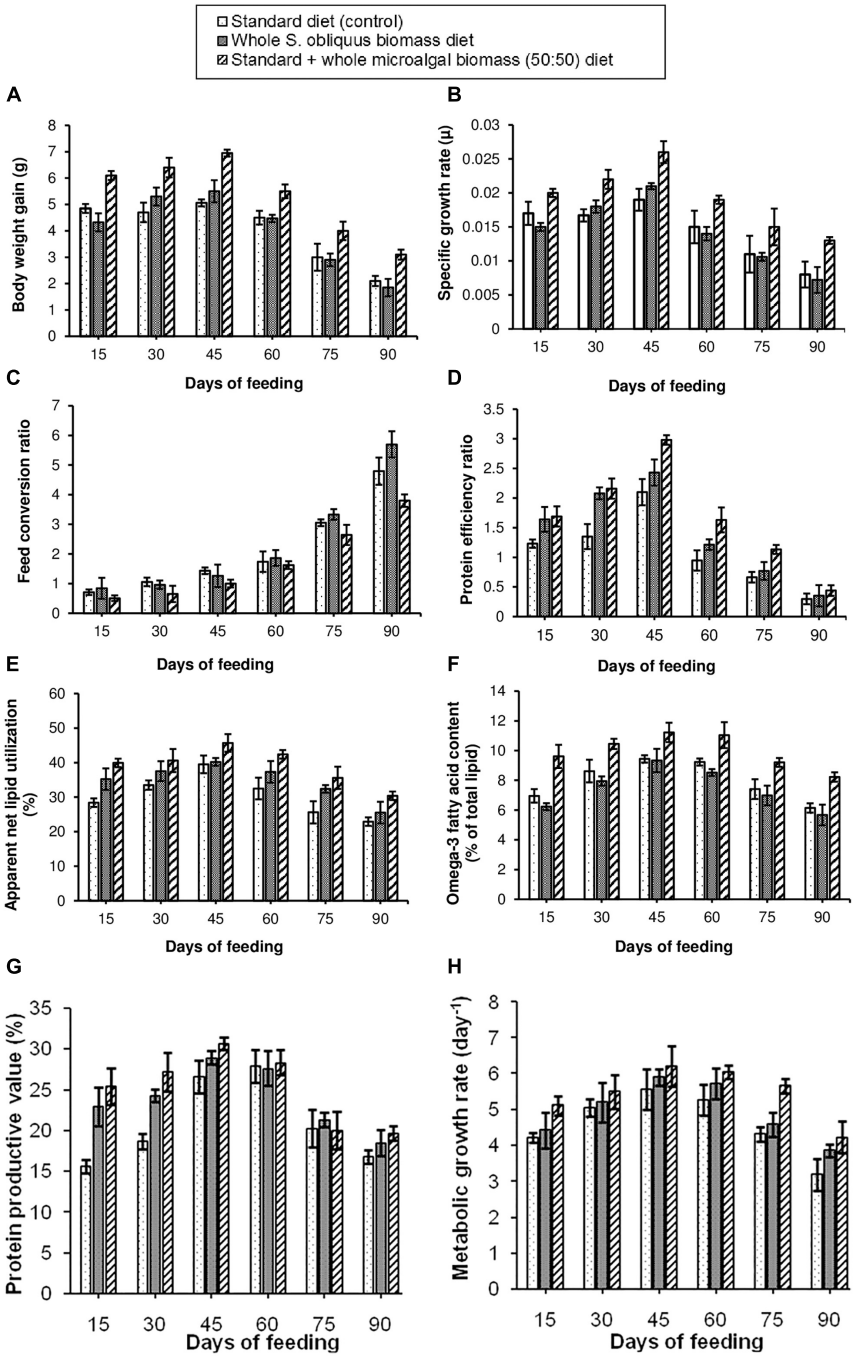

FIGURE 2 | Comparative representation of the **(A)** body weight gain, **(B)** specific growth rate, **(C)** feed conversion ratio, **(D)** protein efficiency ratio, **(E)** apparent net lipid utilization, **(F)** omega-3 fatty acid content, **(G)** protein productive value, and **(H)** metabolic growth rate in three different diet conditions.

Experimental Set-Up 2

As 50% inclusion of whole *S. obliquus* biomass showed the best results in the first set-up (**Figure 2**), the second set-up was designed to reconfirm the results of the first set-up, and in addition explore the effect of 50% inclusion of extracted microalgal protein in the standard diet. Another 90 days feeding experiment was conducted with fish fed with three different test diets, standard diet (control), standard + whole microalgal biomass (50:50) diet, and standard + extracted microalgal protein (50:50) diet at 2% body weight per day. The body weight for the fish in the test tanks on the 0 day (on the day of the start of the experiment) was 4.6 ± 0.83, 5.3 ± 0.79, and 5.8 ± 0.72 g,

respectively. The experimental set-up was similar to that of the first experiment.

Fish growth performance and nutrient utilization parameters

The results of the experiment have been shown in **Figures 3A–H**. Out of the three test diets, the standard + whole *S. obliquus* biomass (50:50) diet in this experimental set-up also, showed the best growth performance. It was observed that, BWG of the fishes in these test tanks increased gradually with maximum BWG of 5.65 g in the third fortnight of feeding. The SGR of the fish fed on 50:50 ratio of control and whole *S. obliquus* biomass diet was also found to be maximum (0.02 μ), with a gradual decline in the subsequent time periods. An observation of the FCR, PER and PPV values further attested the best growth performance in the fish fed with this test diet. The percentage of ANLU and omega-3 fatty acid content, were also found to be maximum between 30 to 45 days of feeding with their respective values being, 35.7 and 10.5%. The diet formulation with standard + extracted microalgal protein (50:50) diet was however, not found to be very significant as compared to the other two test diets which could be due to the lower palatability of this diet which was clearly demonstrated in the poorer growth performance in the fishes administered with this diet, as compared to the control and 50% whole *S. obliquus* biomass included diet. Such an experiment with inclusion of only extracted plant or animal protein has not been carried out by any researcher, to the extent of our knowledge and hence is not available for reference.

Water quality parameters

The temperature, as recorded daily was found to drift toward higher degrees gradually due to the change in season during the months of December–February, in this geographical locale. The three parameters, i.e., temperature, pH and DO were not found to have any significant difference in the fish tanks administered with the three test diets. However, in case of turbidity, TAN, nitrate and nitrite content, the fish tanks provided with standard + extracted microalgal protein (50:50) diet, showed a higher value of the said parameters due to higher accumulation of the uneaten feed. Comparatively, the fish tanks with the standard diet showed lower wastage of the administered feed, hence lower values of turbidity, TAN, nitrite and nitrate but the lowest values of the three measured parameters was observed in the tanks with the standard + whole *S. obliquus* biomass (50:50) diet. These four parameters were found to increase gradually in the test tanks with increased days of feeding (data not shown).

Experimental Set-Up 3

As 50% inclusion of the extracted microalgal protein along with 50% standard diet, did not show any improvement in the growth parameters of the freshwater fish varieties in the second experimental set-up, the proportion of the standard diet was reduced to 25 and 25% of whole *S. obliquus* biomass was included along with 50% extracted algal protein in the third set-up, to increase the palatability of the test diet for consumption by the fishes. The third experimental set-up for 90 days was carried out in a similar way as was done in the other two experimental set-ups. The weight of the fishes before the start of the experiment were 5.6 ± 0.72, 5.9 ± 0.66, and 5.5 ± 0.71 g in the fish tanks administered with standard diet (control), standard + whole microalgal biomass (50:50) diet, and standard + whole microalgal biomass + extracted microalgal protein diet (25:25:50) diet, respectively.

Fish growth performance and nutrient utilization parameters

The results of the experiment have been shown in **Figures 4A–H**. Out of the three test diets, the standard + whole microalgal biomass + extracted microalgal protein (25:25:50) diet showed the best growth performance. It was observed that, BWG of the fishes in this test tank increased gradually with maximum BWG (7.3 g) in the third fortnight of feeding. The SGR of the fish fed on the same diet was also found to be 0.028 μ. Higher incorporation of protein due to higher nutrient digestibility (Nandeesha et al., 1994; Mustafa and Nakagawa, 1995) could have led to this increased body weight gain and SGR in the selected test diet. Although the microalgal protein during extraction and precipitation could have been denatured, the amino acids to which it was broken down to were successfully incorporated in the body of the fishes (Sward, 2014). While this diet condition showed maximum gain in body weight, the BWG in case of standard + whole microalgal biomass (50:50) diet was found to be only marginally different. An observation of the FCR and PER values further confirmed the best growth performance in the fish fed with this test diet. The percentage of ANLU and omega-3 fatty acid content, were however, found to be maximum within 30–45 days of feeding with the standard + whole microalgal biomass (50:50) diet, their respective values being, 46.8 and 15.5%. But in case of PPV and MGR, maximum improvement was seen in the third fortnight of feeding the fishes with standard + whole microalgal biomass + extracted microalgal protein (25:25:50) diet. The maximum PPV was recorded to be 36.3% and maximum MGR was found to be 6.21 day^{-1}, thus, attesting the use of the microalgal protein as an aquafeed, during the formation of an algal refinery.

Water quality parameters

The water quality parameters in the fish tanks were analyzed as per the protocols described in Section "Analysis of Water Quality Parameters." Temperature, as recorded daily was found to drift toward higher degrees gradually due to the change in change in atmospheric temperature during March – May. As observed in the other two experimental set-ups, these three parameters, i.e., temperature, pH and DO, were not found to have any significant difference in the fish tanks administered with the three different test diets. However, in case of turbidity, TAN, nitrate and nitrite content, the fish tanks provided with standard diet, again showed higher values of the said parameters. Comparatively, the fish tanks with the standard + whole microalgal + extracted microalgal protein (25:25:50) and standard + whole microalgal biomass (50:50) diets showed lower wastage of the administered feed, hence lower values of turbidity, TAN, nitrite and nitrate, varying marginally from each other (data not shown).

Fish feeds account for a significant portion of the costs in an aquaculture enterprise. Hence, to minimize the feed costs while simultaneously looking into environmental sustainability,

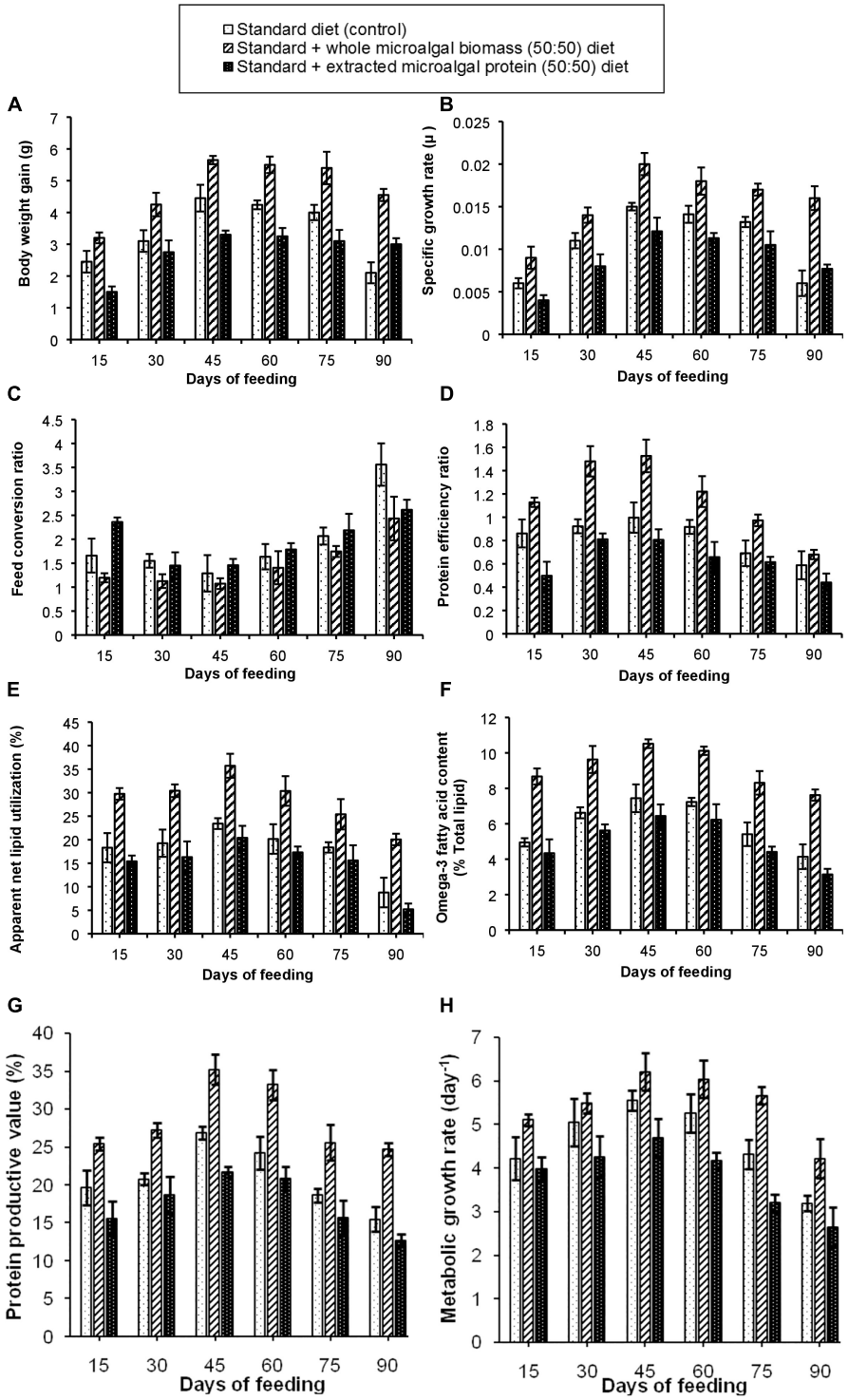

FIGURE 3 | Comparative representation of the **(A)** body weight gain, **(B)** specific growth rate, **(C)** feed conversion ratio, **(D)** protein efficiency ratio, **(E)** apparent net lipid utilization, **(F)** omega-3 fatty acid content, **(G)** protein productive value, and **(H)** metabolic growth rate in three different diet conditions.

accurate information about the nutritional requirement of the fishes is imperative so as to adopt balanced diet formulations and feeding practices for their optimal growth and development. Fishes mostly require diets composed of proteins, lipids and carbohydrates for good growth and maintenance, however, amongst them, proteins are required in maximum proportions, comprising about 65–70% of the fish muscle. Proteins consumed by the fishes not just provide the essential and non-essential

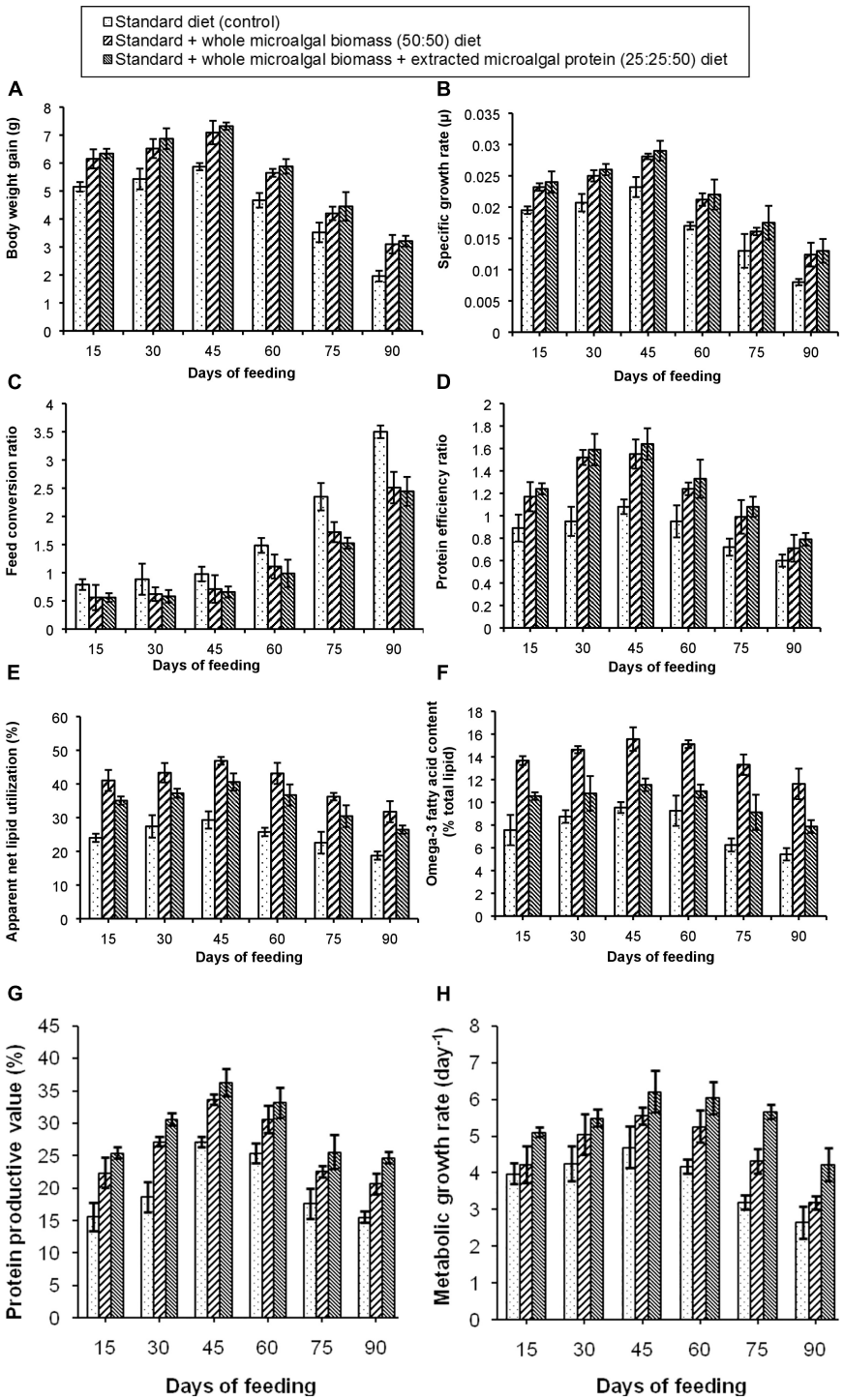

FIGURE 4 | Comparative representation of the **(A)** body weight gain, **(B)** specific growth rate, **(C)** feed conversion ratio, **(D)** protein efficiency ratio, **(E)** apparent net lipid utilization, **(F)** omega-3 fatty acid content, **(G)** protein productive value, and **(H)** metabolic growth rate in three different diet conditions.

amino acids for their muscle formation and enzymatic function, but also provide energy for their maintenance. Insufficient amounts of protein in the fish diets lead to a decline or diminution of their growth parameters probably due to restricted supply of proteins to more vital organs and tissues. While, excessive amounts of protein in the fish diets result in accelerated energy requirements, higher costs, more nitrogenous waste excretions and decreased fish growth. Moreover, as proteins

are the most expensive item in the commercially available fish feeds, it is indeed wise to include optimum dietary protein levels in their diets, thus avoiding biological and economical loss without compromising with their growth and maintenance. Furthermore, as fish growth also depends on the effectiveness of the withholding time of the dietary protein, optimum protein proportions for each species including their cultivation phase needs should also be determined for maximum profitability.

Proposition of an Integrated Sequential Processing Approach for an Algal Refinery

Sequential Production of Biodiesel and Various Co-products

Selection of the sequence for maximum extraction of the microalgal components

The extraction of the components from *S. obliquus* biomass in this part of the study was done under the optimized culture condition as detailed in Section "Proposition for an Algal Refinery." During a sequential extraction process, the biggest problem posed is by the interference of the solvents and the methodologies used for extraction of one component on the yield of the other microalgal components. Hence proper placement of the lipids and other components one after the other, without much effect on their extracted yields is essential. For this purpose four different sequences were tried. A display of the variation in the yields of the different components when arranged in different sequential positions has been given in **Table 1**.

Before elaborating on the data given in **Table 1**, a mention of the yields of the different components (each component extracted individually from the whole *S. obliquus* biomass without any sequential processing) obtained under the optimized condition from 100 g of dry *S. obliquus* biomass is important. The β-carotene, lipid, carbohydrate and protein yield, extracted individually was 0.06, 56.2, 30.2, and 10.2 g, respectively.

From the Table above, it was observed that when sequence 1 was followed, the yields of β-carotene, lipid and carbohydrate were insignificantly affected, but the protein yield was reduced drastically to 5.4 g from 1 Kg wet (≈100 g dry) *S. obliquus* biomass. This observation indicated that neither did the use of acetone for β-carotene extraction affect the lipid yield from the microalgal biomass as the amount of lipid obtained after β-carotene extraction was nearly the same as that obtained directly from the test microalga, nor did the solvents acetone, chloroform and methanol affect the carbohydrate yield, extracted sequentially after β-carotene and lipid, but the positioning of protein extraction at the end of the sequential process affected the protein yield severely. The reduced yield of protein could possibly have been due to leakage during acid hydrolysis of the biomass for carbohydrate extraction. The loss in biomass during the sequential extraction procedure was however, found to be negligible. In case of sequence 2, a reduction in both, the lipid and protein yield to 32.2 and 2.4 g, respectively was observed when the lipid and protein extractions were preceded by carbohydrate extraction from the dry microalgal biomass due to loss of lipids and proteins during acid hydrolysis of the dry microalgal biomass. β-carotene extraction on the top of the sequence was not found to be responsible for this observation which can also be confirmed from the results of the first sequential extraction process.

The results of the third sequential extraction process showed that if β-carotene and lipid extraction are followed by protein extraction instead of carbohydrate extraction then the yield of the extracted carbohydrate was reduced marginally to 26.7 g but the yield of the protein obtained was still significantly lower than that obtained directly from the whole *S. obliquus* biomass under the optimized condition which was due to the interference of the solvents used during lipid extraction in the extraction of proteins using ultra-pure water (Wessel and Flugge, 1984), although after being placed in the third position of the sequential extraction process the protein yield showed an increase to 6.4 g.

However, implementation of the fourth sequential extraction process resulted in maximum yields of the microalgal components which were unaffected by any form of loss due to use of solvents or methods of cell hydrolysis. The chosen sequence extracted β-carotene followed by protein, lipid and carbohydrate. β-carotene and protein extraction were done from wet *S. obliquus* biomass, but the data presented, has been given in terms of dry cell weight for a better representation of the microalgal biomass composition.

TABLE 1 | Variation in the yields of the different microalgal components when arranged in different sequential positions.

Sequence 1	Sequence 2	Sequence 3	Sequence 4
β-carotene yield (0.061 ± 0.07 g)	β-carotene yield (0.054 ± 0.03 g)	β-carotene yield (0.063 ± 0.09 g)	β-carotene yield (0.057 ± 0.05 g)
↓	↓	↓	↓
Lipid yield (54.78 ± 1.97 g)	Carbohydrate yield (28.77 ± 1.32 g)	Lipid yield (56.16 ± 1.85 g)	Protein yield (9.79 ± 0.72 g)
↓	↓	↓	↓
Carbohydrate yield (30.05 ± 1.63 g)	Lipid yield (32.21 ± 1.95 g)	Protein yield (6.43 ± 0.46 g)	Lipid yield (55.83 ± 1.94 g)
↓	↓	↓	↓
Protein yield (5.41 ± 0.53 g)	Protein yield (2.42 ± 0.22 g)	Carbohydrate yield (26.71 ± 0.78 g)	Carbohydrate yield (29.78 ± 1.28 g)

Values represent the mean ± SE of data based on three independent determinations.

Microalgal refinery

The design of the proposed algal refinery has been shown in **Figure 5**. The harvested wet biomass of *S. obliquus* was treated with acetone for β-carotene extraction followed by protein extraction using ultrapure water and alkali hydrolysis of the microalgal cells. The *S. obliquus* biomass was then dried at 60°C followed by lipid extraction using a binary solvent system (chloroform-methanol). The de-fatted biomass was then subjected to acid hydrolysis for the breakdown of the complex polysaccharides to simple monomers for bioethanol production through fermentation by *Saccharomyces cerevisae*. 1 Kg wet (≈100 g dry) *S. obliquus* biomass under the optimized condition yielded 0.06 g of β-carotene, 10 g of protein, 38 g (43 mL) of biodiesel, 2 g of omega-3 fatty acid, 3 g (2.4 mL)

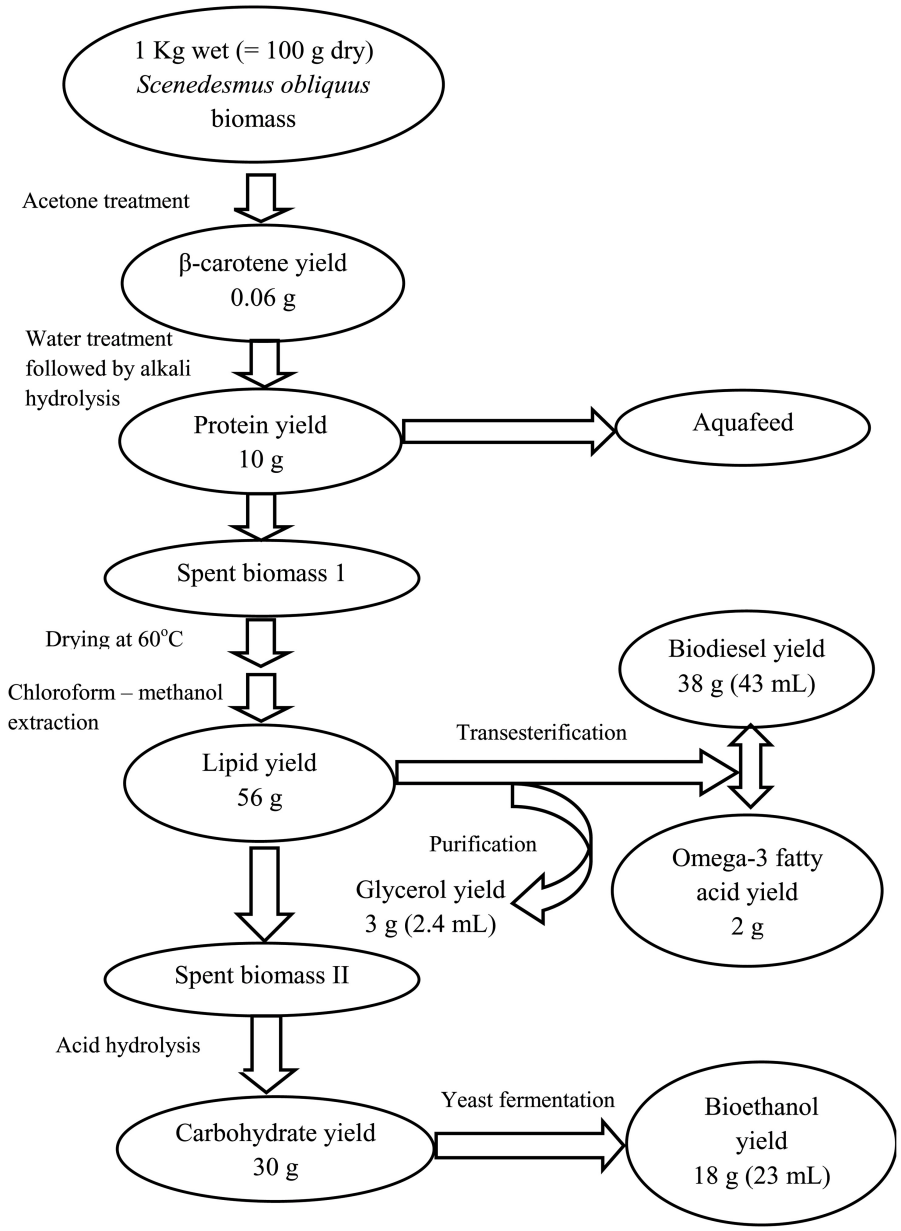

FIGURE 5 | Modified schematic representation of the detailed process for sequential production of biodiesel and other industrially valuable co-products from *S. obliquus* biomass.

of glycerol and 18 g (23 mL) of bioethanol. As shown in **Figure 5**, during conversion of carbohydrates to ethanol, it was observed that ∼60% of the total carbohydrate contained in the microalgal biomass under the optimized condition was converted to bioethanol. Although theoretically, 50% conversion of the carbohydrates in the microalgal cells is possible, conversions of carbohydrates to bioethanol by >50% may reflect fermentation of additional carbohydrates beside glucose and mannose, which was not accounted for in the theoretical calculations (Laurens et al., 2015). Hence 70% of the test microalgal biomass was successfully used for production of biodiesel and other value-added products by using an algal refinery approach.

The approach of algal refinery as a method to reduce the cost of the algal biodiesel by co-production of various value-added chemicals, antioxidants, fertilizers etc. is the most up-coming strategy suggested for the economic viability of the microalgal biodiesel. This concept is a relatively new and novel idea where abundant literature is not available although fragmented data on some feedstocks are present. A report by Dong et al. (2016) on the valorization of *Scenedesmus acutus* by simultaneous

production of bioethanol and biodiesel in a combined algal processing method has shown to utilize nearly 66% of the microalgal biomass for energy generation thus bringing down the cost of the total energy produced by 9% from $10.86/GGE (Gallons of Gasoline equivalent) to $9.91/GGE. In another report by Marinho et al. (2016), the macroalga *Saccharina latissima* in a biorefinery approach has been shown to produce succinic acid after which the leftover solid residue has been tested for their total phenolic compounds and macronutrients (Ca, K, Na, Mg, P, N, and Fe) content. These phenolic compounds and macronutrients contained in the leftover residue have been proposed to be used as antioxidants and fertilizers, respectively, thus, demonstrating the potential of ∼70% of the *S. latissima* biomass to be converted to value-added products. In the present study, a successful conversion of 70% of the *Scenedesmus obliquus* biomass into industrially important products such as β-carotene, protein for aqua-feed, biodiesel, omega-3 fatty acid, glycerol and bioethanol through defined sequential processing steps has been demonstrated unlike the report by Marinho et al. (2016) in which conversion of the phenolic compounds and macronutrients to antioxidants and fertilizers has not been carried out. Additionally, the production of multiple high-value products as shown in the refinery design further confirms the comparative richness and relevance of the present research study in providing an economically sustainable model in future during application of the refinery design in a pilot scale.

Cost-intensive production process of microalgal biodiesel is a major obstacle in its commercialization. Hence efforts should be made to reduce the costs associated with microalgal production and conversion of the algal intermediates to biodiesel. But merely improving the biomass productivity or augmenting the quantity of lipids in microalgae is not expected to bring enough cost reductions. For further progress, complete utilization of all algal components is essential. Previous research reports have shown substantial reductions in microalgal biofuel production costs by applying combined algal processing techniques through simultaneous production of proteins, carbohydrates and other valuable cellular compounds. But, the production of higher value co-products differs depending on the algal biomass composition. Therefore for making maximum profit from algal biodiesel production, decisions should be made judiciously regarding the algal strains possessing maximum potential for producing different high-value products; methodologies used for sequential extraction of different cellular components so as to incur minimal losses in terms of loss of compounds; the potential for nutrients and solvents recycling; and degradation or denaturation of the cellular compounds during the extraction process. Furthermore, colocation of the refinery units with algal farms can reduce the transportation and pre-processing costs. Algal refineries are expected to lead to high quality job creation and energy independence by bridging the gap between high-value small-market products and low-value large-market products. Hence extensive research on these refinery designs is expected to be of immense benefit.

CONCLUSION

An algal refinery is a concept that is being followed nowadays as a strategy to reduce the high costs incurred during microalgal biodiesel production. Hence, a study with a sequential processing design for production of biodiesel and other industrially important products such as β-carotene, protein, biodiesel, omega-3 fatty acid, glycerol and bioethanol, might appear conceptually incomplete without an assessment of the economics of the entire process to ascertain its viability and sustainability. But the experiments having been carried out in the laboratory under controlled culture condition, calculation of the costs incurred in the entire process will not be realistic. Therefore, scaling up of the present study to pilot scale under outdoor condition should be carried out in future to valuate and validate the economic sustainability of the designed algal refinery. Furthermore, as the present study demonstrates conversion of 70% of the *S. obliquus* biomass into industrially important products, efforts to convert the remaining 30% of the microalgal biomass into value-added products through improved methodologies to valorize the whole algal biomass with minimal loss would further enrich the proposed strategy in future.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/the supplementary files.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Ethics Committee, IIT Kharagpur.

AUTHOR CONTRIBUTIONS

RP wrote the article, conducted all the three fish feeding experiments including the experiments for the final algal refinery design, analyzed and interpreted the results of all the experiments. NS performed the first two set-ups of the experiments during which he analyzed four growth parameters and seven water quality parameters. SB also contributed to this work through his help during data analysis. NM and PR supervised the design of the experiments. NM checked the manuscript. All authors have read and approved the final manuscript.

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Biodegradation of Deoxynivalenol by a Novel Microbial Consortium

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Deoxynivalenol (DON), a common mycotoxin of type B trichothecene, is produced mainly by several *Fusarium* species. DON causes great losses in farming and poses severe safety risks to human and animal health. Thus, DON contamination in cereals and DON toxicity are of worldwide concern. In this study, we screened the bacterial consortium C20, which efficiently degraded almost 70 μg ml^{-1} DON within 5 days. The bacterial consortium also had the ability to degrade 15-acetyl-DON, 3-acetyl-DON, and T-2 toxin. The bacterial consortium C20 was able to degrade DON under a wide range of pH and temperature conditions. The optimal temperature and pH for DON degradation were 30°C and pH 8.0, respectively. The bacterial consortium C20 comprised of different bacterial genera, and several strains were found to significantly increase when cultured in Mineral Medium with 100 μg ml^{-1} DON based on the analysis of the sequences of the hypervariable V3-V4 region of the 16S rRNA gene. 3-keto-DON was confirmed as a degradation product of DON by liquid chromatography/time-of-flight/mass spectrometry (LC-TOF-MS) and nuclear magnetic resonance (NMR) analyses. The results indicated that the bacterial consortium C20 is a potential candidate for the biodegradation of DON in a safe and environmentally friendly manner.

Keywords: deoxynivalenol, bacterial consortium, biodegradation, 3-keto-DON, mycotoxin

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INTRODUCTION

Mycotoxins have adverse effects on grain quality, resulting in economic losses, besides the threat to human and animal health. The main groups of mycotoxins in world-wide concern are aflatoxin, ochratoxin, patulin, fumonisin, trichothecene, deoxynivalenol, and zearalenone due to their toxicity and their contamination of cereals (James and Zikankuba, 2018). Among these mycotoxins, trichothecenes are mainly produced by a range of fungi, including *Fusarium, Myrothecium*, *Trichoderma*, and *Stachybotrys* (Pessu et al., 2011). Trichothecenes not only cause growth impairment and immune dysfunction at low doses but also contribute to diarrhea, emesis, leukocytosis, hemorrhage, endotoxemia, and death at higher doses (Ueno, 1983). Deoxynivalenol (DON), also known as vomitoxin, produced by *Fusarium graminearum* and

Fusarium culmorum, is one of the most important type B-trichothecene mycotoxins (Sobrova et al., 2010). It causes acute toxicity, vomiting, anorexia, growth retardation, reproductive and teratogenic effects, skin disorders, diarrhea, carcinogenesis, and even immune dysregulation (Pestka, 2010; Sobrova et al., 2010). DON is a frequent contaminant of grain cereals, such as wheat, maize, and barley, which can be also detected in the foods of many countries (Thuvander et al., 2001; Rasmussen et al., 2003; Leblanc et al., 2005; Schollenberger et al., 2005; Castillo et al., 2008; Sugiyama et al., 2009; Alkadri et al., 2014; Stanciu et al., 2016; Shi et al., 2018). Since DON is commonly detected in grains and is chemically stable, it represents a permanent health risk to humans and domestic animals. Therefore, the development of efficient decontamination strategies is essential to DON in grains and agricultural products.

Several strategies have been developed to control DON contamination. Firstly, cultural practices may help to combat plant diseases and fungal growth, such as the multi-field rotation of crops, the selection of seeds that are resistant to *Fusarium* head blight, and the early harvest of crops (Awad et al., 2010). The suitable storage of grains after harvest can also help to avoid increased DON contamination (Shapira et al., 2004). Physical methods, such as damaged grain removal, sieving, dehulling, radiation, and adsorbent use, were successful in detoxifying DON in cereal crops (Leibetseder, 2006). A wide variety of chemical methods have also been found to be effective against DON, including heating, alkaline hydrolysis, and oxidation (Young et al., 1986; He et al., 2010).

Compared with physical and chemical detoxification, microbial biotransformation attracted considerable interests due to high degradation efficiency, the metrics of mild reaction condition, sustainable and eco-friendly process (Awad et al., 2010). Several studies have demonstrated that DON could be metabolized to lower toxicity compounds performed by several microorganisms. For instance, DON was found to be converted into 3-keto-DON under aerobic conditions by the *Agrobacterium-Rhizobium* strain E3–39, isolated from soil samples (Shima et al., 1997). Zhou et al. revealed that two metabolites of DON during degradation by *Barpee* were identified as stereoisomers of DON and 3-keto-DON (Zhou et al., 2008). In addition, microbial consortia have shown the potential to degrade DON. One mixed culture was able to convert DON into two chromatographically separable metabolites (Andrea et al., 2004). Chicken intestinal microbes were also capable of degrading DON by de-epoxidation (Young et al., 2007).

Compared with single bacterium and anaerobic microbes, bacterial consortia with ability of DON degradation under aerobic conditions are rarely reported. Moreover, the DON degradation conditions of these anaerobic bacteria were still limited. In the present study, we isolated a novel bacterial consortium C20 from the wheat field in Jiangsu Province, China, which can stably and efficiently degrade DON into 3-keto-DON under a wide range of pH and temperature with aerobic condition. Further, the performance of biodegradation of other trichothecene mycotoxins was also evaluated.

MATERIALS AND METHODS

Samples

A total of 174 samples from different locations, including soil, rice, corn, wheat, rice panicles and fresh corn leaves, were collected during 2016–2017 in Jiangsu Province, China (**Table 1**). Notably, soil samples were randomly collected from the upper layers (0–30 cm) of different crop fields, such as rice, soybean, corn, and winter wheat fields.

Culture Media and Mycotoxins

Minimal medium (MM) was used as a culture medium for the enrichment and isolation of DON-degrading strains. The composition of MM (pH 7.0) was described by Wang et al. (2019b). Ingredients were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), Guanghua Sci-Tech Co., Led. (Shantou, Guangdong, China), XILONG SCIENTIFIC (Shantou, Guangdong, China), and Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Toxin standards for DON, HT-2, T-2, 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON) were purchased from Romer Labs (Erber Campus, Getzersdorf, Austria).

Selection of Deoxynivaleno-Degrading Microbes

Five-gram aliquots of each sample from the 174 field samples were smashed by a pulverizer (JYL-c020E, Joyoung, China), dispersed in 50 ml distilled water, and cultured with shaking at 180 rpm for 30 min. About 500 μl of the suspension was immediately transferred to 4.5 ml MM broth media with DON (10 μg ml^{-1}) as the sole carbon source. Tebuconazole and Carbendazim (1 or 10 μg ml^{-1}) were added in the cultures to inhibit fungal growth, respectively. The cultures were grown at 30°C, 180 rpm. To exclude physical adsorption, samples autoclaved at 121°C for 20 min were set as negative controls. The extent of DON biotransformation was determined by high-performance liquid chromatography (HPLC). Then, the cultures with the ability of DON degradation were further inoculated on the MM broth containing 10 μg ml^{-1} DON at intervals of

TABLE 1 | Information for samples collected during 2016–2017 in Jiangsu province.

Number	Sample	Location
A1–A3	Panicles of rice	Suqian
A4–A7	Panicles of rice	Wuxi
A8–A10	Panicles of rice	Nanjing
B1–B4	Rice	Xuzhou
B5–B11	Rice	Nantong
C1–C10	Soil	Nanjing
C11–C16	Soil	Xuzhou
C17–C25	Soil	Suqian
D1–D8	Fresh leaves of corn	Xuzhou
E9–E17	Corn	Taizhou
F1–F24	Wheat	Nanjing
F25–F48	Wheat	Taizhou
F49–F73	Wheat	Yangzhou
F74–F91	Wheat	Xuzhou
F92–F111	Wheat	Yancheng

7 days. To investigate DON degradation activity, the cultures were used to degrade 100 μg ml^{-1} DON. MM with DON (100 μg ml^{-1}) was set as negative control. The bacterial growth was measured by optical density. The changes in DON concentration and OD_{600} values were monitored every day.

Sample Preparation and High-Performance Liquid Chromatography Analyses

DON was extracted from fermentation broth with an equal volume of ethyl acetate for three times. After dried under an N_2 stream, it was re-dissolved in methanol and quantified by HPLC (Waters 2695, Milford, USA) equipped with a PDA (photo-diode array) detector at a wavelength of 218 nm and an Atlantis T3 column (octadecylsilyl, ODS; 4.6 mm × 150 mm, 3 μm; Waters), as described previously (Wang et al., 2019b). The mobile phase was methanol/water (20/40, v/v) with a flow rate of 0.6 ml min^{-1} at 35°C. The identification of mycotoxin was achieved by comparing the retention times while using DON as a standard. DON and degradation products were quantified by measuring peak areas and comparing with MS calibration curves. All data are presented as mean ± standard deviation of three replicates. Degradation percentage of DON was calculated as follows:

$$D_r = \left(1 - C_t / C_{\mathrm{ck}}\right) \times 100\%$$

where D_r represents the degradation percentage, and C_t and C_{ck} are the DON concentrations in the experimental treatment and the control groups, respectively (Wang et al., 2019b).

Analysis of Bacterial Population Diversity

To analyze the bacterial population diversity and its change at high concentrations of DON, the enriched microbial consortium C20 was isolated from environmental samples and was added to MM to degrade DON, with DON concentrations of 10 and 100 μg ml^{-1}. The bacteria were incubated in media with different concentrations of DON at 30°C for 72 h. Genomic DNA of the enriched microbial cultures was extracted and purified with Gentra Puregene Yeast/Bacteria Kit (Qiagen Inc., Mississauga, Ontario, Canada). The hypervariable V3-V4 region of the 16S rRNA gene was amplified by PCR with primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) (Ahad et al., 2017) and sequenced by Sangon Biotech Co. (Shanghai, China). Further, the 16S rRNA (V3-V4 region) gene sequences were compared with the ribosomal database project (RDP)[1]. All treatments were conducted in triplicates.

Factors Influencing Deoxynivaleno Degradation

To screen for optimal degrading conditions of DON by C20, the degradation rates of DON were determined at the different conditions including temperature, pH, and bacterial concentrations. An aliquot (200 μl) of each microbial culture was added to 1.8 ml of MM supplemented with 10 μg ml^{-1} DON. The effect of incubation temperature (20, 25, 30, 35, and 40°C), medium pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), and inoculation size (1, 3, 5, 10, 15, and 20%) on biodegradation of DON were investigated. All treatments were performed by three independent experiments with three replicates. The optimal degradation conditions of temperature, pH, and inoculation amount were determined by the degradation rates of DON.

Type A- and B-Trichothecene Mycotoxins Degradation

To examine the ability of C20 to transform type A- and B-trichothecene mycotoxins, which included two type A-trichothecene mycotoxins (HT-2 toxin and T-2 toxin) and three type B-trichothecene mycotoxins (DON, 3-ADON, and 15-ADON), 10 μg ml^{-1} of each mycotoxin was added to MM in 2 ml microcentrifuge tubes. Each tube was inoculated with 200 μl of C20, which was cultured in MM with 10 μg ml^{-1} DON for 72 h. Three replicate cultures were evaluated for each trichothecene mycotoxin, and the negative controls lacked C20. All cultures were incubated at 30°C and 180 rpm in a shaking incubator for 72 h, extracted three times with ethyl acetate, as described, re-dissolved in methanol and analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS).

The LC-MS/MS system was equipped with a Shimadzu 20 AD XR (Kyoto, Japan) coupled with an AB Sciex QTRAP 3500 mass spectrometer (Foster City, CA, USA) and an XDB-C_{18} analytical column (2.1 mm × 150 mm, 3.5-μm bead diameter; Agilent) maintained at 30°C. Nitrogen was used as the drying gas. The capillary voltage was 4 kV, the nebulizer pressure was 30 psi, and the drying gas temperature was 300°C. The gradient elution conditions were methanol/water (5/95, v/v) to methanol/water (90/10, v/v) in 10 min and were held at 90% methanol for 3 min, the flow rate was set at 0.2 ml min^{-1} (Wang et al., 2019b). Analyst 1.6.1 software was used for data acquisition and processing.

Identification of Deoxynivaleno Degradation Products

To analyze the transformation of DON, C20 was added to broth containing 10 μg ml^{-1} DON and incubated at 30°C on a rotary shaker (180 rpm) for 72 h. Culture broth containing C20 alone was used as a control. Samples were prepared as described above and detected by HPLC. The mobile phase was methanol/water (50:50, v/v) with the flow rate of 0.8 ml min^{-1}.

To investigate the structure of DON degradation products, C20 was inoculated into MM medium with 100 μg ml^{-1} DON and cultured for 72 h at 30°C with shaking at 180 rpm. The DON degradation products were extracted three times with ethyl acetate. The organic phase was pooled, concentrated and applied to a Waters 1525 Prep-HPLC system (Milford, MA, USA) equipped with a UV detector. Each 20 μl extract was injected onto an XBridge™ Prep C_{18} column (19 mm × 100 mm, 5 μm, Waters) with methanol/water (50:50, v/v) at a flow rate of 2 ml min^{-1}. DON and its degradation products were detected at a wavelength of 218 nm at 35°C. The resulting degradation products were dried under an N_2 stream and re-dissolved in $CDCl_3$ for nuclear magnetic resonance (NMR) analysis.

[1]http://rdp.cme.msu.edu/misc/resources.jsp

The ^{1}H NMR spectra of the biodegradation products were measured by a Bruker DRX-600 spectrometer operated at 600 MHz. The chemical shift (δ) was recorded in parts per million (ppm) relative to the solvent signals [δ(H) 7.26], and the coupling constant (*J*) was measured in Hz.

Data Analysis

Data were organized using Microsoft Excel 2010. All values are expressed as the mean of three replicates ± standard deviation. The mean differences among treatments were considered significantly at $p < 0.05$ and were evaluated with Tukey's-honestly significant difference (HSD) test (Ahad et al., 2017).

RESULTS

Isolation of Deoxynivaleno-Degrading Bacteria

To screen for the microbes that degraded DON, DON was used as the sole carbon source for the enrichment and isolation of the mixed culture. The mixed microbial culture C20, isolated from a soil sample from Suqian, degraded 74.29 μg ml^{-1} DON after incubation at 30°C for 10 days. DON was degraded rapidly within 5 days of incubation and was continuously degraded after incubation for 10 days. The biomass increased continuously with the amount of DON decreased (**Figure 1**). Furthermore, no difference was found between the samples with and without fungal inhibitors. And the DON concentration in the autoclaved sample was not reduced (**Supplementary Figure S5**). Importantly, the enriched bacterial consortium C20 could efficiently degrade DON, even after more than 100 subcultures in broth (**Supplementary Figure S3**).

Bacterial Diversity Analysis of the Microbial Consortium C20

To examine mainly bacteria involved in DON degradation, the microbial diversity dynamics of C20 with different concentrations of DON was defined through analyzing the sequences of the 16S rRNA genes, as described in methods. The results showed that the enriched bacterial consortium C20 mainly consisted of the genera *Hyphomicrobium* (69.54%), followed by *Acidovorax* (12.09%), unknown microbes (7.04%), *Prosthecomicrobium* (2.71%), *Pseudomonas* (2.68%), and *Methylophilus* (2.31%) in MM with 10 μg ml^{-1} DON (**Figure 2A**). In contrast, the culture in MM with 100 μg ml^{-1} DON contained a heterogeneous community composed of *Methylophilus* (35.48%), followed by *Hyphomicrobium* (27.71%), *Ancylobacter* (14.73%), *Pseudomonas* (4.87%), *Prosthecomicrobium* (4.6%), unknown microbes (2.39%), *Taonella* (2.33%), *Bosea* (2.33%), and others (**Figure 2B**). Noticeably, as the DON concentration increased to 100 μg ml^{-1}, there was a marked increase in *Methylophilus*, *Ancylobacter*, *Pseudomonas*, *Prosthecomicrobium*, *Taonella*, *Bosea*, *Terrimonasin*, and *Devosia* in C20.

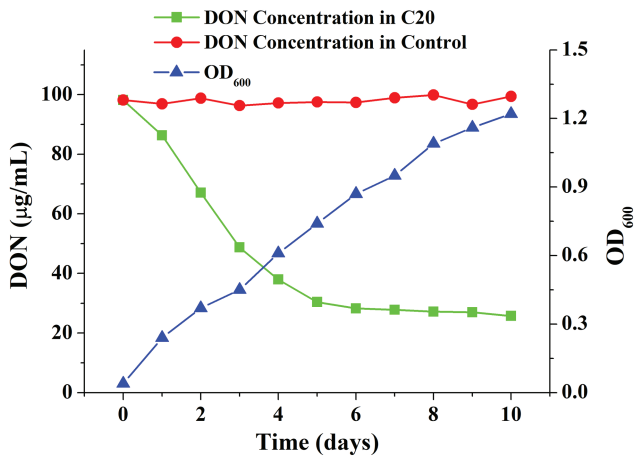

FIGURE 1 | Growth and DON-degrading efficiency of the enriched culture C20.

Factors Influencing Deoxynivaleno Degradation

The effects of the temperature, pH, and inoculum on DON degradation by C20 were investigated (**Figure 3**). DON degradation activity was examined at various temperatures from 20 to 40°C in the enriched cultures (**Figure 3A**). The highest degradation rate occurred at 30°C and decreased only slightly at 25 and 35°C. Importantly, approximately 50% of DON degradation was observed even at 40°C, indicating that the consortium C20 can effectively degrade DON over a wide range of temperatures (25–35°C). Moreover, the effects of pH on DON degradation was detected under six different pH levels conditions. The degradation rate of DON increased markedly from pH 4.0 to 8.0. The maximum DON degradation rate was observed at pH 8.0, and the degradation for 10 μg ml^{-1} DON at pH 9.0 was reduced to 70.08% (**Figure 3B**). The inoculum size significantly affected the degradation of DON. The degradation rates of 10 μg ml^{-1} DON after 72 h of incubation increased from 30 to 85% when the inoculum size was raised from 1 to 10% (**Figure 3C**).

Degradation of Type A- and B-Trichothecene Mycotoxins by Consortium C20

To investigate the degradation of other trichothecene mycotoxins by C20, the consortium C20 was incubated with T-2, HT-2, 3-ADON, and 15-ADON for 72 h. The results demonstrated that the consortium C20 degraded 58% of 15-ADON, 28% of 3-ADON, and 21% of T-2. However, the degradation of HT-2 was not observed (**Figure 4**). 15-ADON and 3-ADON are the main acetylated derivatives of DON, and the degradation of 15-ADON and 3-ADON indicates that C20 possesses the ability to degrade acetylated DON derivatives (Payros et al., 2016).

Identification of Deoxynivaleno Degradation Products

The metabolic products of DON were identified by using HPLC, LC-TOF-MS, and NMR. HPLC analysis showed that DON

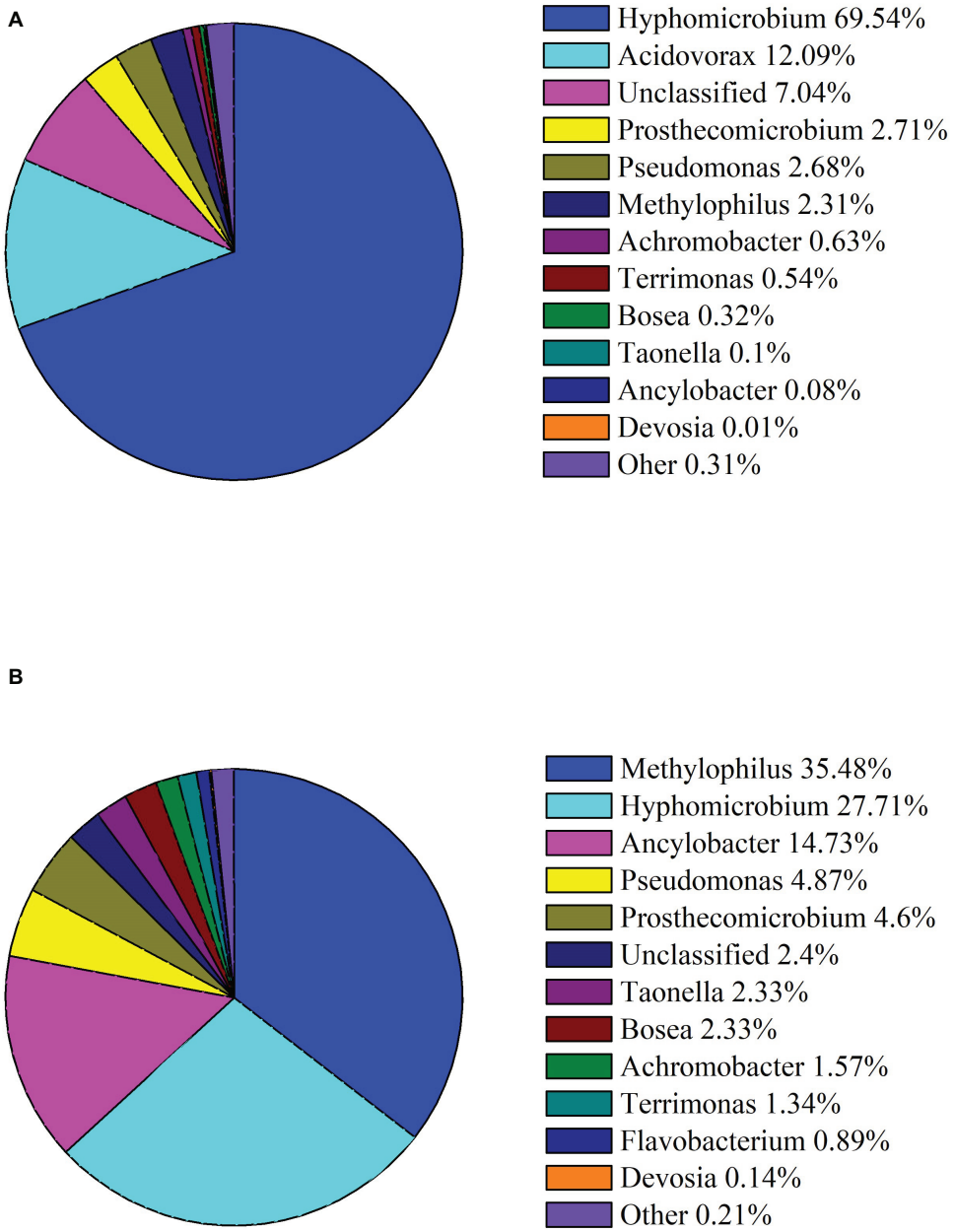

FIGURE 2 | Abundances of bacterial genera in the enriched culture C20. **(A)** Relative abundance of bacterial genera in C20 in MM with 10 μg ml^{-1} DON. **(B)** Relative abundance of bacterial genera in C20 in MM with 100 μg ml^{-1} DON. The different colors and areas represent different bacterial genera and their relative abundance present in C20.

was transformed to another metabolite during degradation by the enriched bacterial consortium C20 (Compound P, **Figure 5**). LC-TOF-MS results (**Supplementary Figure S1**) showed that the molecular ion of the metabolite was at *m/z* 295.1151 ([M + H]$^+$, $C_{15}H_{19}O_6^+$; calcd. 295.1182), which indicated a di-dehydrogenation of the DON molecule. The ^{1}H-NMR spectrum for compound P (**Supplementary Figure S2**) was 6.55 (dd, J = 6.0, 1.5), 4.91 (d, J = 1.3), 4.57 (d, J = 6.0), 3.91 (d, J = 11.6), 3.81 (d, J = 1.5), 3.75 (d, J = 11.6), 3.52 (s), 3.36 (d, J = 4.2), 3.23 (d, J = 4.2), 3.13 (d, J = 19.5), 2.29 (d, J = 19.5), 1.90 (3H, s), and 1.34 (3H, s). The chemical formula and the ^{1}H-NMR signals of compound P were identified as 3-keto-DON (Hassan et al., 2017; Wang et al., 2019b).

DISCUSSION

DON is one of the most commonly detected contaminants of crops in worldwide, such as maize, wheat, and rice (Lee and Ryu, 2015). The widespread occurrence of this mycotoxin,

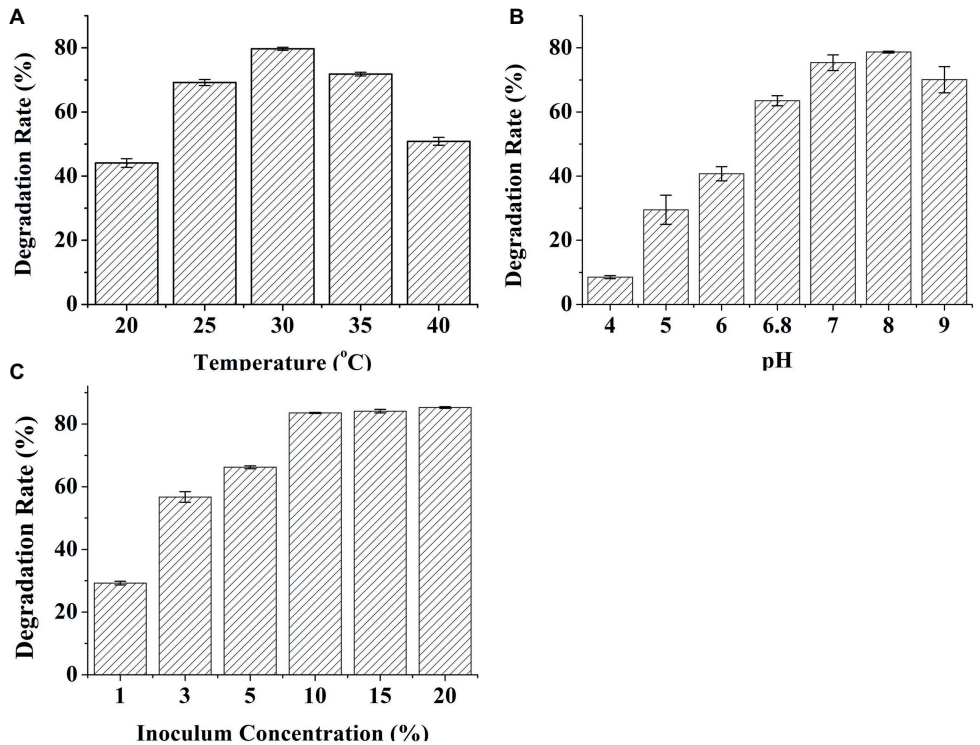

FIGURE 3 | Effects of temperature, pH, and inoculum concentrations on the degradation of DON by C20. **(A)** The effect of incubation temperature on the degradation of DON. **(B)** The effect of pH on the degradation rate of DON. **(C)** The effect of inoculum concentrations on the degradation of DON. Results are the mean of five replicate observations, and bars shown are ± standard errors of the means.

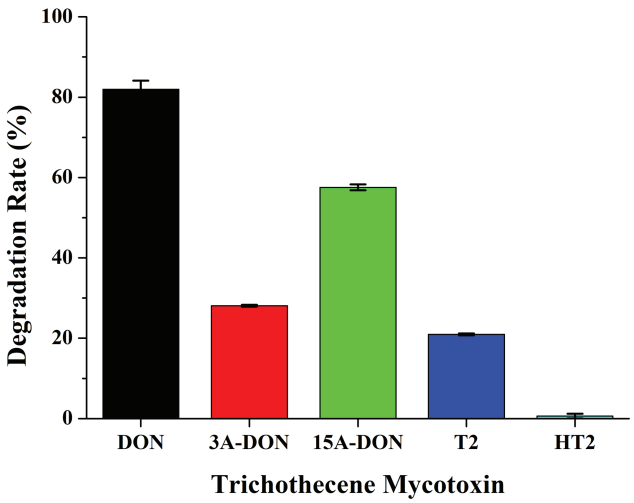

FIGURE 4 | Degradation rate of DON, 3-ADON, 15-ADON, T-2, and HT-2 by the enriched culture C20.

coupled with the detriments to human health and animal productivity resulting from its consumption, has promoted intensive efforts to identify detoxification measures, including microbial biotransformation (Pestka, 2010; Sobrova et al., 2010; Kris et al., 2014). Although several single microbial strains for the degradation of DON were isolated, there are still many limitations to application for agricultural fields because of the diversity and complexity of crop cultivation and storage environments (Brenner et al., 2008). Fortunately, microbial consortia provide a promising application for mycotoxin decontamination owing to their special advantages in the degradation of complex compounds. Microbial consortia combine multiple metabolic capacities of different species, improving the ability and efficiency of complex substrates biotransformation process, as well as the tolerance of complex environments (Shong et al., 2012). To date, microbial consortia has been widely used for a variety of important processes, such as the treatment of wastewater (Daims et al., 2006), the biosynthesis of compounds for medicine and industry (Shong et al., 2012), the biodegradation of bispyribac-sodium and phenanthrene (Janbandhu and Fulekar, 2011; Ahmad et al., 2018), and the production of hydrogen (Zhang et al., 2017), due to their high adaptability and broad substrate spectra (Kleerebezem and Van Loosdrecht, 2007). However, few attempts have been made to degrade DON by a microbial consortium. In this study, the highly enriched, stable and effective microbial consortium C20 was found to be able to degrade DON, resulting in a significant reduction in DON (**Figure 1**). In addition, tebuconazole and

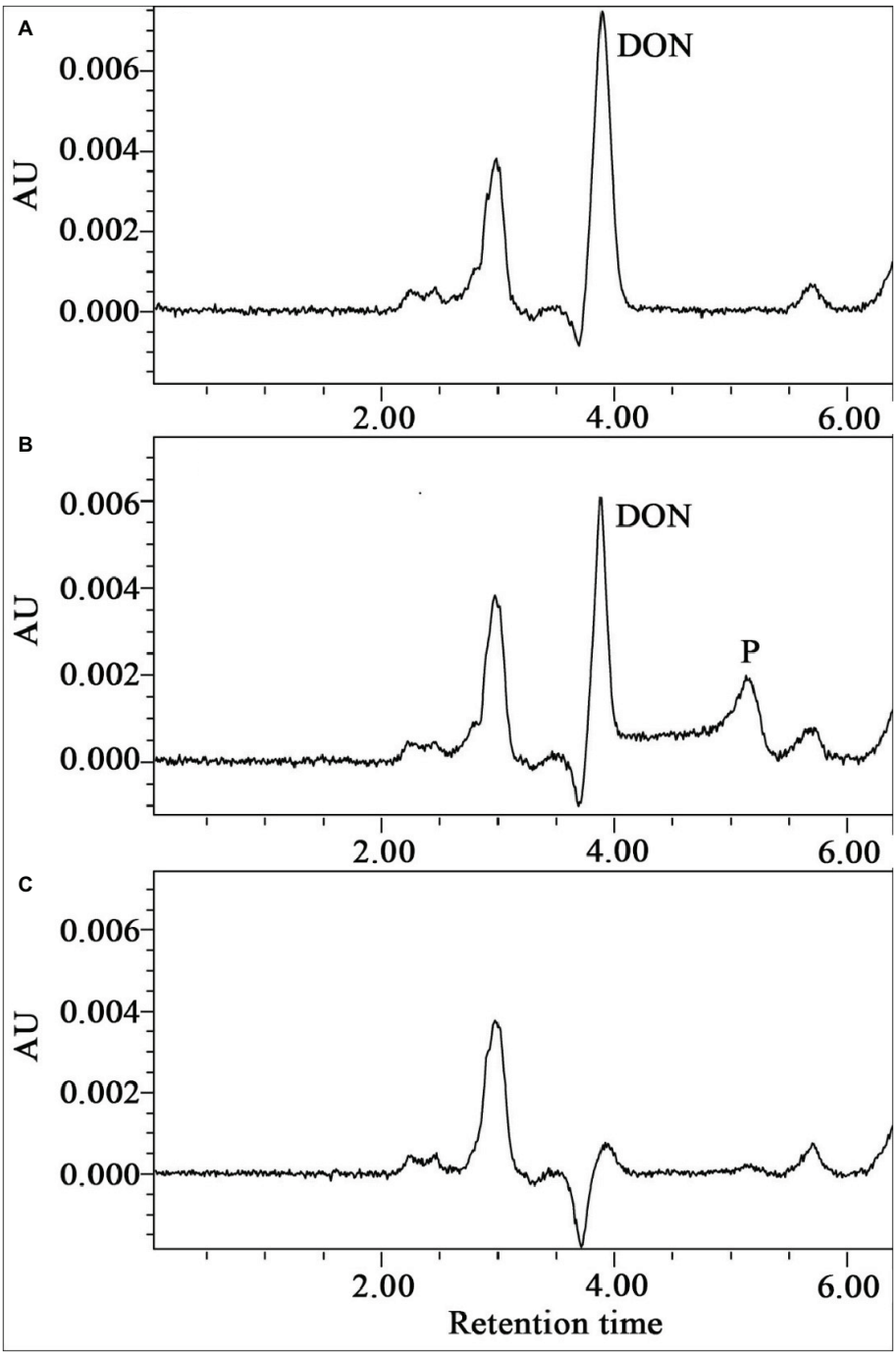

FIGURE 5 | The HPLC spectra of the metabolism of DON by the enriched culture C20. **(A)** Substrate control broth containing the substrate DON alone. **(B)** Broth containing DON and C20. **(C)** Culture control broth containing C20 alone.

carbendazim have no effects on the degradation of DON by the enriched culture, indicating that the degradation of DON is owing to bacteria. Furthermore, there was no change in DON concentration in the autoclaved sample, suggesting that the soil sample cannot detoxify DON by physical adsorption (**Supplementary Figure S5**).

The characterization of DON degradation in this study showed the activity of the microbial culture at a wide range of pH (6.0–9.0) and temperatures (25–35°C) compared with previously studied microbial cultures (Guan et al., 2009; Wang et al., 2019b). The microbial consortium C20 might degrade DON in complex and diverse environments. The optimal pH

(8.0) and temperature (30°C) of the microbial consortium C20 for DON degradation are similar to that of most arable plants. It has been suggested that microbial consortia have potential applications for the detoxification of DON in field crops (Karlovsky, 1999; Islam et al., 2012).

The functional and stability of microbial consortium are usually modulated through interactions among microorganisms. These interactions can be indirect, such as exchanging different metabolites and information signals (Wintermute and Silver, 2010). For example, *Bacillus cereus* A1 and *Brevundimonas naejangsanensis* B1 mutually cooperate to enhance hydrogen production and starch utilization. In this consortium, strain A1 produced lactate as carbon source for strain B1 to cell growth and hydrogen production. In return, formate produced by strain B1 as an electron shuttle for strain A1 to generate hydrogen (Wang et al., 2019a). In direct interactions, microorganisms exchange electrons or deliver macromolecules such as DNA and proteins by direct interspecies contact. Rotaru et al. reported that besides H_2 or formate as the electron donor for interspecies electron transfer, direct electrical connections was also found in the production of methane between *Geobacter metallireducens* and *Methanosaeta harundinacea* (Rotaru et al., 2014).

As the sole carbon, DON provided more substrate to the microbial consortium C20 in MM with 100 μg ml^{-1} DON than in the original culture. The increasement of DON might enhance the proportion of DON-degraders during the consecutive enrichment, resulting in a change in the community composition (Ahad et al., 2017). Strains belonging to *Methylophilus*, *Ancylobacter*, *Pseudomonas, Prosthecomicrobium, Bosea*, *Taonella* and *Devosia* were significantly increased ($p < 0.05$, one-way ANOVA) in C20 after enrichment with 100 μg ml^{-1} DON. These results suggest that the changes in these bacterial populations might be related to the microbial degradation of DON. It was demonstrated that many bacteria isolated for the degradation of DON were identified as *Devosia* strains (Sato et al., 2012; Onyango et al., 2014; Yin et al., 2016; Zhao et al., 2016; Hassan et al., 2017). Moreover, DON was transformed by these bacteria to 3-epi-DON, 3-keto-DON, or other products. Therefore, the *Devosia* strains in C20 have great potential for DON degradation.

The 3-OH group in DON, as well as the epoxide group on the trichothecene backbone, is responsible for its toxicity. When DON was epimerized or degraded, its interaction with ribosome will be changed, leading to an absence of MAP kinase activation and toxicity reduction. It was reported that the intestinal toxicity of DON would be decreased when it was converted into depoxy-DON or 3-epi-DON by microbial transformation (Pierron et al., 2016). In this study, 3-keto-DON was found to the main degradation product of DON analyzed by HPLC, LC-TOF-MS and NMR. It is all known that the oxidation of DON to 3-keto-DON plays a vital role in the detoxification of DON-contaminated grains (Shima et al., 1997). In addition, the 3-keto-DON showed a reduction of more than 90% in immunosuppressive toxicity compared with that of DON (He et al., 2015).

A DON degrading bacterial strain was isolated from the consortium C20, but the strain did not maintain its DON degradation activity after a few generations of culture in broth medium (**Supplementary Figure S4**). It is still challenging to isolated DON-degrader through selective enrichment procedures because the degradation reaction involves the removal of an oxygen molecule (Islam et al., 2012; Yoshizawa et al., 2014), which does not supply any nutrients for bacteria (Horvath, 1972; Fedorak and Grbić Galić, 1991) and prevents the utilization of DON as the sole carbon or nitrogen source for the selective isolation of degrader from the consortium C20 (Ahad et al., 2017).

The enriched culture C20 had the ability to degrade 15-ADON, 3-ADON and T-2, as well as DON in this study. Previous studies demonstrated that 15-ADON is higher toxicity than DON or 3-ADON (Pinton et al., 2012; Kadota et al., 2013). Moreover, both DON and 15-ADON are frequently found in wheat and other cereals (Han et al., 2014; Dong et al., 2016). Thus, the microbial consortium C20 has great potential to degrade DON and 15-ADON in agricultural products.

In conclusion, the enriched bacterial consortium C20, which can efficiently degrade DON and its derivatives 15-ADON and 3-ADON through the combined strengths of individual organisms, was isolated from environmental samples in this study. C20 is comprised of different bacterial genera, with *Methylophilus*, *Ancylobacter,* and *Devosia* significantly increasing in cultures with high concentrations of DON. Importantly, about 70 μg ml^{-1} DON could be degraded by consortium C20 within 5 days under a wide range of pH and temperature conditions. And the final degradation product was identified as 3-keto-DON with less toxicity. These results suggest that the C20 microbial consortium provides a new potential method for DON degradation. Extending DON degradation capabilities from single bacteria to microbial consortia represents a new frontier in DON microbial biotransformation. Although stable community behavior remains a great challenge in this area, it still has great potential to be used to degrade DON in food/feedstuffs and to improve food/feed quality and safety.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YaW performed experiments and wrote the paper. GW, YuW, and Y-WL designed and performed experiments. JX, JS, and YD designed experiments, supervised the students, and wrote the paper. All authors have approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02964/full#supplementary-material

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Assessment of a Potential Role of *Dickeya dadantii* DSM 18020 as a Pectinase Producer for Utilization in Poultry Diets Based on *in silico* Analyses

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Currently, the poultry industry has been faced with consumer pressure to utilize only vegetable feedstuffs in poultry diets, eliminate antibiotics from poultry production, and rear poultry in free range systems. To maintain current production standards, the industry must determine ways to enhance nutrient uptake and utilization further. One possible solution is the supplementation of pectinase, an enzyme that degrades pectin within the cell walls of plants, in poultry diets. Therefore, the objective of the current study was to determine the potential role of a pectinase producer, *Dickeya dadantii* DSM 18020, as a commercially utilized pectinase producer in poultry diets against other known pectinase producers, *in silico*. In the current study, whole genomes of *Dickeya dadantii* DSM 18020 (Dd18020), *D. dadantii* 3937 (Dd3937), *D. solani* IPO 2222 (Ds2222), *Bacillus halodurans* C-125 (BhC125), and *B. subtilis* subsp. *subtilis* str. 168 (Bs168) were compared using bioinformatic approaches to compare the chromosomal genome size, GC content, protein coding genes (CDS), total genes, average protein length (a.a.) and determine the predicted metabolic pathways, predicted pectin degrading enzymes, and pectin-degradation pathways across pectinase producers. Due to insufficient information surrounding the genome of Dd18020 (lack of annotation), the genome of Dd3937, a 99% identical genome to Dd18020, was utilized to compare pectinase-associated enzymes and pathways. The results from the current study demonstrated that Dd3937 possessed the most significant proportion of pathways presented and the highest number of pathways related to degradation, assimilation, and utilization of pectin. Also, Dd18020 exhibited a high number of pectinase-related enzymes. Both Dd3937 and Dd2222 shared the pectin degradation I pathway via the EC 3.1.1.11, EC 3.2.1.82, and EC 4.2.2.- enzymes, but did not share this pathway with either *Bacillus* species. In conclusion, Dd18020 demonstrated the genetic potential to produce multiple pectinase enzymes that could be beneficial to the degradation of pectin in poultry diets. However, for Dd18020 to become a commercially viable enzyme producer for the poultry industry, further research quantifying the pectinase production *in vitro* and determining the stability of the produced pectinases during feed manufacturing are necessary.

Keywords: free range, pectinase, *Dickeya dadantii*, all-vegetable diets, *in silico*

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INTRODUCTION

Dickeya dadantii, previously classified as *Erwinia chrysanthemi* by Burkholder (1957), is one the many bacteria responsible for bacterial soft rot disease (*Dickeya* spp., *Erwinia* spp., *Pectobacterium* spp., etc.) that occurs in a wide range of crops (Samson et al., 2005; Toth et al., 2011). The facultative anaerobic, Gram-negative bacilli typically grow at an optimal temperature of 39°C, but can grow between the ranges of 25–40°C (Samson et al., 2005). The typical transmission of *D. dadantii* to plants is through direct contact with contaminated soil (Grenier et al., 2006). Contamination of soil can occur through several vectors such as water (irrigation, runoff, etc.), various insects, and specific agricultural techniques such as plowing (Grenier et al., 2006). Due to the pectinolytic nature of *D. dadantii*, it causes an infection in crops that induces soft rot that can be characterized by the rapid disorganization of the parenchymatous tissues (Hugouvieux-Cotte-Pattat et al., 1996). Although the colonization of plants by soft rot *Erwinia* is primarily driven by the production of pectic enzymes, the process is multifactorial requiring cellulases, iron assimilation, an Hrp type III secretion system, exopolysaccharides, motility, and proteins involved in resistance against plant defense mechanisms (Barabote et al., 2003; Toth et al., 2003; Ravirala et al., 2007). Due to the capability of *D. dadantii* to produce pectinases, it has the potential to be advantageous to multiple industries.

Pectinases consist of a group of enzymes that hydrolyze pectin present in plant cell walls and exist in higher order plants and microorganisms (Whitaker, 1991). In plants, pectinases are found to enhance cell wall extension (Jayani et al., 2005) and promote softening of specific plant tissues during maturation and later storage (Aguilar and Huirton, 1990; Sakai, 1992). Therefore, pectinases are used to degrade plant materials in multiple food processing techniques such as reducing the time it takes to extract fruit juice from fruit puree (Junwei et al., 2000) and enhancing the clarity in wine (Kobayashi et al., 2001). Currently, pectinase is applied in several processes such as textile processing and bio-scouring of cotton fibers, the degumming and retting of plant bast fibers, wastewater treatment, coffee and tea fermentation, paper and pulp industry, purification of plant viruses, and in animal feed (Sharma et al., 2013). Across industries, pectinases are mass manufactured by utilizing pectinase producing bacteria and fungi. Of such bacteria and fungi, *Bacillus and Aspergillus* are the most characterized pectinase producers utilized across manufacturing of agricultural and food products (Garg et al., 2016).

Of the many uses of pectinases, the poultry industry would greatly benefit from the addition of pectinases in poultry diets. Recently, consumer pressure has motivated the poultry industry to introduce no antibiotic ever (NAE), all -vegetable fed broilers (Weil, 2017). In addition, there has also been an increased demand for free range poultry and forage feeding. In efforts to utilize locally available feed sources, sectors of the poultry industry have initiated forage feeding, pasture farming, or supplementary feeding regimens (Buchanan et al., 2007). With the move to all -vegetable diets and forage feeding, broiler diets may contain an increased concentration of cereals containing non-starch polysaccharides (NSPs) in their endosperm (Broz and Beardsworth, 2002). Poultry are unable to produce many of the enzymes necessary to degrade NSP's and broiler performance is decreased due to the impairment of digestive enzymes, decreased nutrient absorption, and increase in viscous excreta (Broz and Beardsworth, 2002).

The supplementation of diets with pectin has specifically resulted in the decrease of growth rate, decrease in feed efficiency, increase in feed intake, increase in sticky droppings, and overgrowth of *Clostridium* species (Wagner and Thomas, 1977, 1978; Ricke et al., 1982). More recently, research has demonstrated that pectinases have the ability to degrade pectin more readily in poultry diets alone and in combination with other NSP-hydrolyzing enzymes. In addition, the supplementation of pectinase, cellulase, or hemicellulase alone in poultry diets has proven to be less effective than their combination on improving poultry performance (Tahir et al., 2005, 2006).

Currently, the major sources of NSP-containing plant material within poultry diets is corn and soybean meal (Pierson et al., 1980; Chesson, 2001). However, due to the increasing demand for corn and soybean meal from the swine industry and other sectors, the poultry industry has explored alternative sources of carbohydrates. Such sources are wheat, barley, and lupins (Australia) which contain high amounts of NSPs compared to those contained in corn and soybean meal, specifically pectin (Bailoni et al., 2005; Leeson, 2008; Kim et al., 2011).

Therefore, there is a need for a wide variety of enzymes, especially those that break down plant material that poultry are unable to utilize on their own due to the vast differences in plant composition. For example, of the pectins contained within the cell wall of plants vary in amount and in the physiochemical properties such as structural differences among the types of plants and fruits (Kamnev et al., 1998). Pectin structure consists of an α(1-4)-linked polygalacturonans with varying chain lengths, intermittent rhamnosyl residues, and other potential structural variations (Selvendran, 1985; Piknik, 1990; King, 1993). Further, there are currently no products on the market that solely target the complex structures of pectins, especially those contained in lupins (Kim et al., 2011). Pectin contain galactose residues of the polygalacturonans that are methyl-esterfied at C-6, which protects the substrate from the access of pectinases (Ali et al., 2005). Further, the *in vitro* supplementation of pectin methyl esterase and polygalacturonase in combination with ground dehulled lupins (*Lupinus angustifolius* in 70 mL acid buffer) reduced the length of pectin chains by 65% and molecular weight of pectin by 56% (Ali et al., 2005). As such, it is imperative to identify bacterial enzyme sources capable of producing a wide variety of pectinases for potential commercial development.

Pectinases have been provided in poultry diets in addition to other enzymes to enhance nutrient digestibility of broilers and other poultry species. Previous research has demonstrated that the use of pectinases in an enzyme cocktail has the capability to reduce the viscosity of the feed, release nutrients through the hydrolysis of non-biodegradable fibers or those blocked by these fibers and reduce the total amount of excreted

feces (Jayani et al., 2005). Therefore, the overall objectives of the current study were to determine if the genome of *Dickeya dadantii* DSM 18020 (NZ_CP023467.1) encodes pectinases, if, in turn, those pectinases are comparable to those currently utilized in the poultry industry, and if *D. dadantii* would make a suitable alternative source of novel pectinases to those bacteria currently used commercially to produce pectinases.

MATERIALS AND METHODS

Genomic Data Retrieval

In the current study, genomic data was accessed and collected from the National Center for Biotechnology Information (NCBI) on December 7, 2018 (O'Leary et al., 2016). The genomic data for *Dickeya dadantii* DSM 18020 (NZ_CP023467.1), *D. dadantii* 3937 (NC_014500.1; Glasner et al., 2011), *D. solani* IPO 2222 (NZ_CP015137.1; Pédron et al., 2014), *Bacillus subtilis* subsp. *subtilis* str. 168 (NC_000964.3; Kunst et al., 1997; Barbe et al., 2009; Belda et al., 2013; Borriss et al., 2018) and *B. halodurans* C-125 (NC_002570.2; Takami et al., 2000), was utilized in the current study to compare novel pectinase homologs (*Dickeya* spp.) to known industrialized pectinase producers (*Bacillus* spp.) and their associated metabolic pathways.

Genomic Relation Across Pectinase Producers

Genomic data that was retrieved from NCBI was utilized to compare the genomic characteristics of *Dickeya dadantii* DSM 18020 (Dd18020), *D. dadantii* 3937 (Dd3937), *D. solani* IPO 2222 (Ds2222), *Bacillus subtilis* subsp. *subtilis* str. 168 (Bs168), and *B. halodurans* C-125 (BhC125). Genomic characteristics that were compared among bacterium were chromosomal genome size (Mb), Guanidine to Cytosine content (GC Content; %), protein-coding genes (CDS), total genes, and average protein length (a.a).

Dd3937, a wild-type strain originally isolated from *Saintpaulia ionantha*, is most commonly used as a model organism for soft rot pathogenesis since the 1980's (Glasner et al., 2011). As Dd3937 is a known model organism, the authors wanted to explore the candidacy of a more recently discovered strain, DSM 18020. Dd18020 was recently isolated from *Pelargonium capitum* in Comoros and deposited to the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell

TABLE 1 | Comparative genomic information of five pectinase producing bacteria.

Bacterium	NCBI accession number	Chromosomal genome size (Mb)	GC content	Protein coding genes (CDS)	Total genes	Average protein length (a.a.)	Isolated from	References
Dickeya dadantii DSM 18020	NZ_CP023467.1	5.00	56.40%	4,271	4,531	326	*Pelargonium capitatum*	Cheng et al., 2017
Dickeya dadantii 3937	NC_014500.1	4.92	56.30%	4,244	4,485	323	*Saintpaulia ionantha*	Glasner et al., 2011
Dickeya solani IPO 2222	NZ_CP015137.1	4.92	56.20%	4,132	4,329	339	*Solanum tuberosum*	Khayi et al., 2016
Bacillus subtilis subsp. *subtilis* str. 168	NC_000964.3	4.22	43.50%	4,237	4,871	290	Mutant strain of *B. subtilis Marburg*[1]	Kunst et al., 1997; Belda et al., 2013; Borriss et al., 2018; Barbe et al., 2009
Bacillus halodurans C-125	NC_002570.2	4.20	43.70%	3,950	4,134	298	Soil	Nakasome et al., 2000; Takami and Horikoshi, 2000; Takami et al., 2000

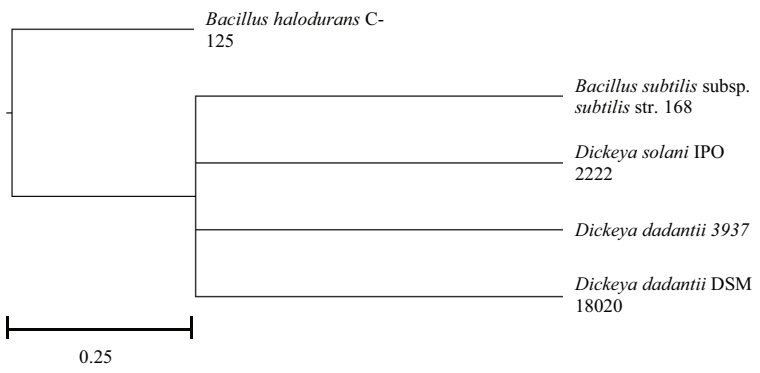

FIGURE 1 | Rooted phylogenic tree of five pectinase-producing bacteria after multiple alignment in MAUVE.

Culture (Braunschweig, Germany). The authors were particularly motivated to choose Dd18020 as the candidate pectinase producer in the current study as there are no current Nagoya Protocol restrictions to its use, it is obtainable through multiple collections of plant-associated bacteria, and has a complete genome assembly available on NCBI. Therefore, Dd18020 can be annotated (genome), purchased, and applied in research or industrial use consistently and without restrictions.

However, due to the absence of Dd18020 in several databases, Dd18020 and Dd3937 were compared against one another by comparing syntany and homogeneity. Syntany was explored using the progressive MAUVE whole genome alignment (Darling et al., 2004). Homogeneity was investigated using Web Blast on NCBI (Altschul et al., 1990). As other research has utilized reference genomes to identify close proximity to the target bacteria or organism (Tyson et al., 2004; Iverson et al., 2012; Wrighton et al., 2012; Albertsen et al., 2013; Sharon et al., 2013), the comparison of Dd18020 and Dd3937 was utilized to validate if Dd3937 was an appropriate reference genome for Dd18020. Therefore, further genomic comparisons were done in the absence of Dd18020, with Dd3937 in its stead. In addition, a rooted phylogenetic tree was constructed from the output obtained from progressiveMAUVE whole genome alignment of Dd18020, Dd3937, Ds2222, Bs168, and BhC125 (Darling et al., 2010).

Predicted Pathways Across Pectinase Producers

In the current study, genomes of Dd3937, Ds2222, Bs168, and BhC125 were compared in MetaCyc (MetaCyc 22.5; Caspi et al., 2014) to evaluate the protein and pathway similarities between genomes of the four pectinase producers. Numerical comparisons were performed in Microsoft Excel for Office 365 MSO (16.0.11601.20174; Microsoft, Redmond, WA, United States) to highlight the numerical difference in pathways between the four pectinase producers utilized in the current study.

Predicted Pectin-Related Enzymes Across Pectinase Producers

The list of proteins and enzymes encoded in the genomes of Dd18020, Dd3937, Ds2222, Bs168, and BhC125 was retrieved from NCBI (O'Leary et al., 2016) and utilized to determine the pectin-related proteins and enzymes encoded. Proteins encoded in the genome of the pectinase producers were aligned in MegaX (Kumar et al., 2018) using ClustalW (Thompson et al., 1994) where Maximum Likelihood was utilized to produce a rooted phylogenetic tree. In addition, Metacyc was subsequently utilized to compare pectinase related enzymes and genes across the four pectinase producers: Dd3937, Ds2222, Bs168, and BhC125. The top eleven related proteins were chosen for comparison in the current study.

Pectinase Pathway

As Dd18020 is less studied, Dd3937 was chosen to elucidate the pectin-related pathways. MetaCyc 22.5 was utilized to determine the pathways of Dd3937. Dd3937 was subsequently compared to the other pectinase producers utilized in the current study: Ds2222, Bs168, and BhC125.

TABLE 2 | Direct comparison of *Dickeya dadantii* DSM 18020 and 3937 complete genomes.

Description	Max score	Total score	Query cover	*E*-value	Ident	Accession
Dickeya dadantii 3937, complete genome	1.661e + 05	9.161e + 06	91%	0.0	99%	NC_014500.1

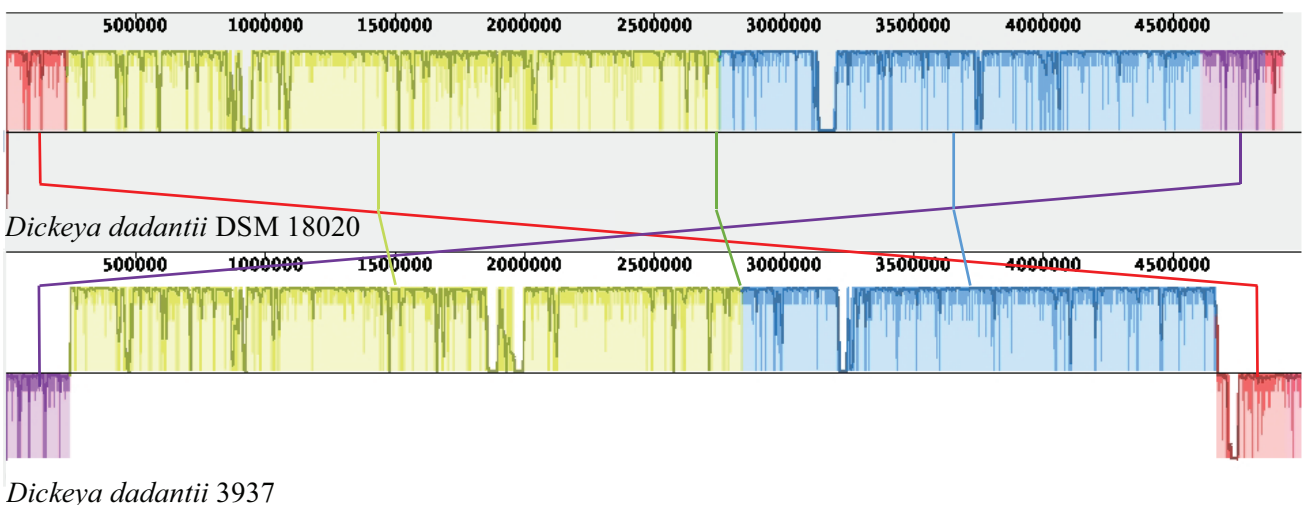

FIGURE 2 | Synteny analysis of *Dickeya dadantii* DSM 18020 (NZ_CP023467.1) and 3937 (NC_014500.1). Synteny analysis was performed using MAUVE whole genome alignment. Arrows indicate the regions that are located in different positions in both genomes.

TABLE 3 | Analysis of metabolic pathways in the genomes of four pectinase producing bacteria.

Pathway class[a]	*Dickeya dadantii* 3937 NC_014500.1	*Dickeya solani* IPO 2222 NZ_CP015137.1	*Bacillus subtilis* subsp. *subtilis* str. 168 NC_000964.3	*Bacillus halodurans* C-125 NC_002570.2
Biosynthesis	178	164	155	151
Amine and polyamine biosynthesis	2	4	3	4
Amino acid biosynthesis	22	24	28	32
Aminoacyl-tRNA charging	1	1	2	3
Aromatic compound biosynthesis	4	4	4	3
Carbohydrate biosynthesis	14	12	8	6
Cell Structure biosynthesis	6	5	2	2
Cofactor, prosthetic group, electron carrier, and vitamin biosynthesis	51	41	44	43
Fatty acid and lipid biosynthesis	13	15	11	10
Hormone biosynthesis	3	1	0	0
Metabolic regulator biosynthesis	2	2	2	1
Nucleoside and nucleotide biosynthesis	21	18	19	18
Other biosynthesis	0	0	0	0
Secondary metabolite biosynthesis	4	4	8	3
Storage compound biosynthesis	0	0	0	0
Degradation/utilization/assimilation	106	98	97	94
Alcohol degradation	3	3	3	2
Aldehyde degradation	2	3	2	0
Amine and polyamine degradation	3	6	4	7
Amino acid degradation	14	13	21	19
Aromatic compound degradation	2	3	1	3
C1 compound utilization and assimilation	3	2	2	3
Carbohydrate degradation	17	20	20	18
Carboxylate degradation	12	14	13	11
Chlorinated compound degradation	1	0	0	0
Cofactor, prosthetic group, electron carrier degradation	0	0	0	0
Degradation/utilization/assimilation – other	2	1	0	0
Fatty acid and lipid degradation	4	4	3	3
Hormone degradation	0	0	0	0
Inorganic nutrient metabolism	13	7	5	5
Nucleoside and nucleotide degradation	15	7	9	10
Polymeric compound degradation	5	6	4	3
Protein degradation	0	0	0	0
Secondary metabolite degradation	14	16	10	8
Generation of precursor metabolite and energy	16	18	18	18
Signal transduction pathways	0	0	17	0
Total	252	234	252	226

[a]*Green shading represents the highest incidence of pathways between the four pectinase producing bacteria, with white representing the lowest incidence of pathways between the four pectinase producing bacteria.*

RESULTS

In relation to the genomic make-up of the pectinase producers, all *D. dadantii* species, Dd18020 and Dd3937, and Ds2222 were more similar in their genome size (5.00, 4.92, and 4.92 Mb, respectively), GC content (56.40, 56.30, and 56.20%, respectively) and average protein-coding length (326, 323, 339 a.a., respectively) than the size, GC content, and average protein-coding length of the two *Bacillus* species (4.22 and 4.20 Mb, 43.50 and 43.70%, and 290 and 298 a.a., respectively, **Table 1**). All bacteria in the current study possessed similar levels of proteins and genes, with BhC125 having the fewest number of proteins and genes (3,950 CDS and 4,134 total genes). In addition, both bacilli possessed smaller average protein lengths than the *Dickeya* species. The differences in size, GC content, average protein-coding length, proteins, and genes may be attributed to *Dickeya* and *Bacillus* species belonging to different phyla

TABLE 4 | Pectin degradation related enzymes produced by *Dickeya dadantii* DSM 18020.

NCBI accession number	Protein length	Protein description
WP_038911228.1	576	Pectate lyase
WP_038912610.1	551	Pectin acetylesterase
WP_038901807.1	366	Pectinesterase A
WP_013318064.1	110	MULTISPECIES: pectin degradation protein kdgF
WP_013316284.1	315	Pectate lyase
WP_038911625.1	344	Pectate lyase
WP_013319746.1	374	Pectate lyase
WP_013319745.1	375	Pectate lyase
WP_038911695.1	392	Pectate lyase
WP_038901806.1	392	Pectate lyase
WP_038901804.1	404	Pectate lyase
WP_038911484.1	425	Pectate lyase
WP_038911249.1	543	Pectate lyase

(Proteobacteria and Firmicutes, respectively) (**Figure 1**, Galperin, 2015; Lockwood et al., 2019).

Due to the paucity of literature and available genomic research concerning Dd18020, comparison of Dd18020 and Dd3937 genomic information was performed in Web BLAST (**Table 2**). The results of the global alignment in Web BLAST revealed that the two genomes were a 99% match and therefore the genome of Dd3937 was sufficient to compare to other pectinase producers in lieu of Dd18020. The *E*-value, maximum score, and maximum identity of Dd18020 and Dd3937 genomes were 0.0, 1.661e + 05, and 99%, respectively, indicating that the local coverage was high. The total metrics were also relatively high, with a total score and query coverage of 9.161e + 06 and 91%, respectively. In addition, synteny was explored between Dd18020 and Dd3937 (**Figure 2**). The Dd18020 genome is highly syntenic with the Dd3937 genome, exhibiting only two disruptions. One region of about 250 kb is present at different locations in both chromosomes (location 4.6–4.85 Gb in Dd18020 and 0–242 kb in Dd3937). A second region in Dd18020 chromosome is split into two regions. The first region of Dd18020 is at position 0 to 228 kb and the second region at position 4.85–4.92 Gb.

The metabolic pathways of the four pectinase producers were compared in MetaCyc 22.5, with Dd3937 representing Dd18020. The pathways did differentiate in several ways (**Table 3**). In all, Dd3937 and Bs168 exhibited the highest representation of pathways, however, Dd3937 had the highest proportion of total pathways presented. In relation to degradation, assimilation, and utilization, Dd3937 possessed the highest number of related pathways. Carbohydrate biosynthesis was present in the highest number (14) in Dd3937 when compared to the three other pectinase producers. Ds2222 had the second highest number of carbohydrate biosynthesis pathways (12) than both *Bacillus* species (8 and 6); however, when carbohydrate degradation pathways were examined, Dd3937 had the lowest representation of pathways (17), with Ds2222 and Bs168 possessing more carbohydrate degradation pathways (20 and 20, respectively).

Dd18020 was screened for pectinase-related genes by exploring the genome in NCBI (**Table 4**). The genome of Dd18020 contained 13 identified extracellular pectinase-related proteins: pectin acetylesterase, pectinesterase A, MULTISPECIES: pectin degradation protein KdgF, and several pectate lyases. The pectin degrading enzymes and proteins

FIGURE 3 | Rooted phylogenic tree of pectin degrading enzymes encoded in the genomes of *Dickeya dadantii* 18020, *D. dadantii* 3937, *D. solani* IPO 2222, *Bacillus subtilis* subsp. *subtilis* str. 168, and *B. halodurans* C-125. Proteins encoded in the genome of the pectinase producers were aligned in MegaX using ClustalW where Maximum Likelihood was utilized to produce a Newick tree.

encoded in the genome of the five pectinase producers in the current study demonstrated no distinct pattern in genetic divergence (**Figure 3**). For example, Dd18020, Dd3937, Ds2222, and Bs168 possess the genetic potential to produce pectin lyase which evolutionarily is the most recent with Dd18020, Dd3937, and Ds2222 being a polytomy. Overall, the evolutionary

TABLE 5 | Comparison of key pectinase enzymes between four pectinase producing bacteria.

Protein homolog		*Dickeya dadantii* 3937	*Dickeya solani* IPO 2222	*Bacillus subtilis* subsp. *subtilis* str. 168	*Bacillus halodurans* C-125
1	Product	DDA3937_RS15625	None	YesY	AYT26_RS19885
	Gene	Pectin acetylesterase	None	Rhamnogalacturonan acetylesterase	Hypothetical protein
2	Product	DDA3937_RS11325	None	None	AYT26_RS02680
	Gene	Pectin degradation protein kdgF	None	None	Cupin
3	Product	DDA3937_RS15615	None	Pel	AYT26_RS03755
	Gene	Pectate lyase	None	Pectate lyase	Hypothetical protein
4	Product	DDA3937_RS16955	None	yesO	AYT26_RS05885
	Gene	Sugar ABC transporter substrate-binding protein	None	Pectin degradation byproducts-binding lipoprotein	Hypothetical protein
5	Product	DDA3937_RS19500	None	None	AYT26_RS19165
	Gene	Pectate lyase	None	None	pectate lyase
6	Product	DDA3937_RS11370	None	ytmA	AYT26_RS16630
	Gene	Pectin acetylesterase	None	Putative hydrolase	Peptidase
7	Product	DDA3937_RS02860	None	pelB	None
	Gene	Pectate lyase	None	Pectin lyase	None
8	Product	DDA3937_RS11370	None	ytmA	AYT26_RS16630
	Gene	Pectin acetylesterase	None	Putative hydrolase	Peptidase
9	Product	DDA3937_RS09860	None	ywoF	None
	Gene	Pectate lyase	None	Putative pectate lyase	None
10	Product	DDA3937_RS11105	None	pelC	None
	Gene	Type III secreted protein	None	Secreted pectate lyase	None
11	Product	DDA3937_RS15625	None	yesY	AYT26_RS19885
	Gene	Pectin acetylesterase	None	Rhamnogalacturonan acetylesterase	Hypothetical protein

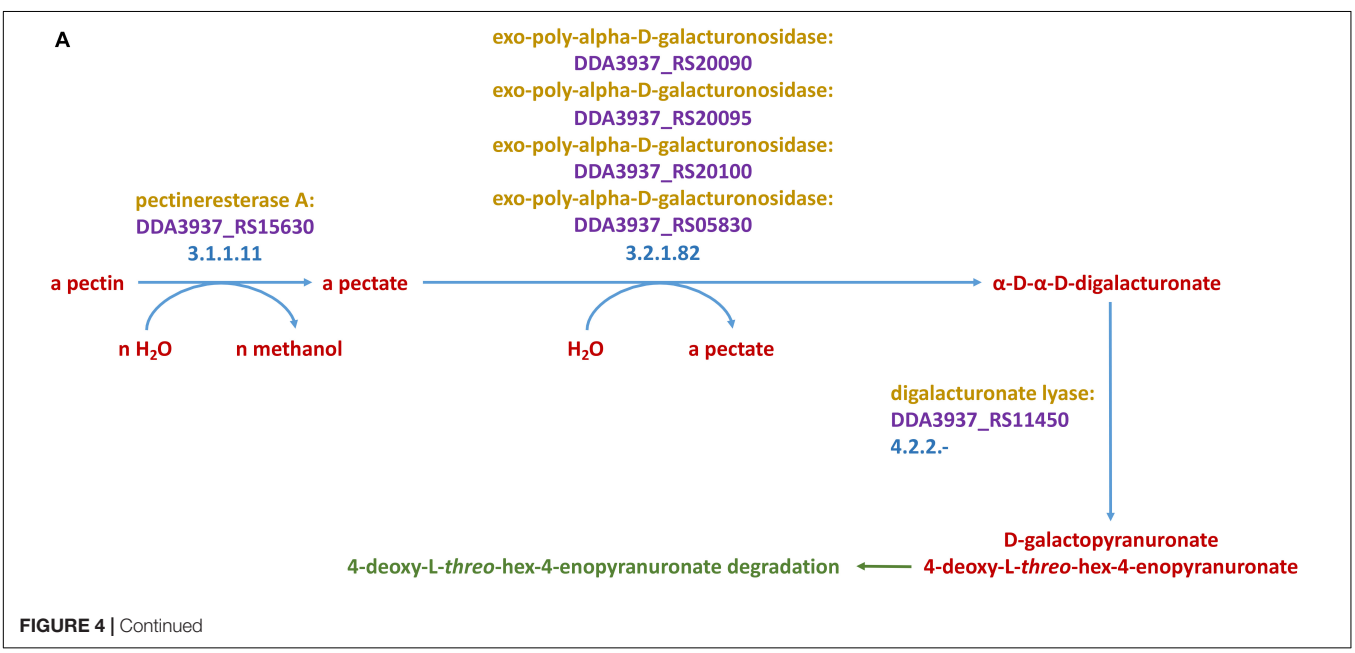

FIGURE 4 | Continued

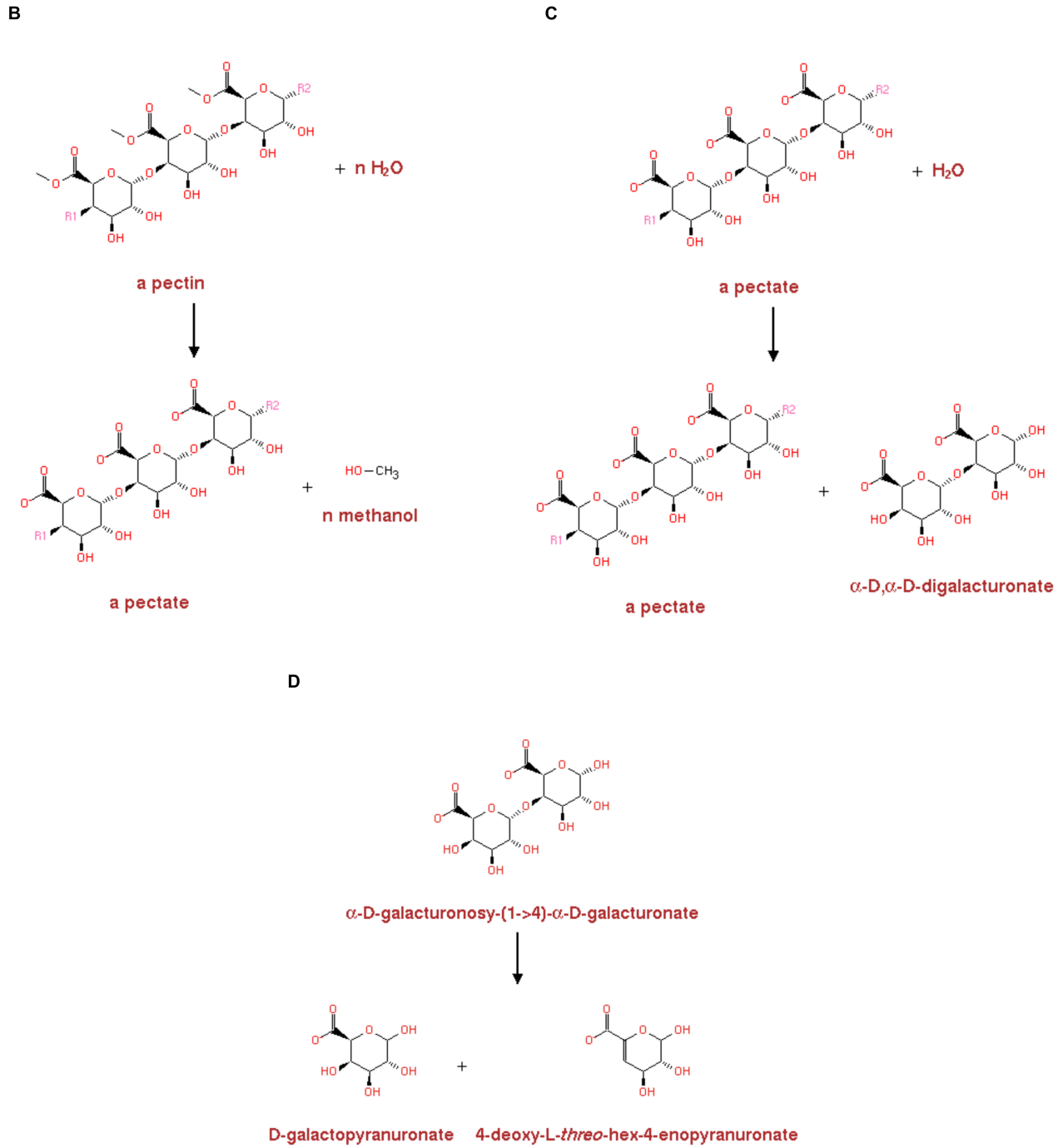

FIGURE 4 | Enzymes, genes, and products for pectin degradation in *Dickeya dadantii* 3937 **(A)**. Pectin is first acted upon by pectinesterase A (3.1.1.11) which yields a pectate and n-methanol **(B)**. Pectate is further degraded to α-D-α-D-digalacturonate by an exo-poly-α-D-galacturonosidase (3.2.1.82) with a pectate as a byproduct **(C)**. The α-D-α-D-digalacturonate is then degraded to D-galactopyranuronate 4-deoxy-L-threo-hex-4-enopyranuronate by the enzyme catalyst digalacturonate lyase (4.2.2.-) **(D)**.

divergence of pectin degrading enzymes is primarily due to the emergence of new proteins and enzymes, rather than the differences between pectinase producing bacteria. Pectin degrading enzymes encoded by *Dadantii* species were less divergent between one another than those encoded by the Bacilli.

Due to the paucity of genomic research related to Dd18020, Dd3937 was compared to the three other pectinase-producing bacteria in MetaCyc 22.5 (**Table 5**). The top eleven pectinase-related enzymes that were shared among pectinase producers are also present in Dd18020 (**Tables 4**, **5**), however, Ds2222

did not appear to share similar enzymes to either Dd3937 or both *Bacillus* species. It appears that *D. dadantii* possesses more proteins dedicated to degradation, utilization, and assimilation (105) than Ds2222, Bs168, and BhC125 (97, 95, and 94 proteins, respectively). However, *in vitro* modeling should be done to quantify the pectinase production of *D. dadantii* compared to other pectinase-producing bacteria.

In addition to comparing the pectinase-related proteins produced by Dd18020 and the four other bacteria utilized in the current study, the metabolic pathways related to pectin degradation were compared simultaneously for shared pectinase related pathways in MetaCyc 22.5. Of the shared pathways, only the two *Dickeya* species shared similar pathways, EC 3.1.1.11, EC 3.2.1.82, and EC 4.2.2.- (**Figure 4A**). The presence of shared pathways between the two *Dickeya* species was relatively surprising, as the genome-encoded pectinase-related enzyme comparison did not reveal similarities in the proteins encoded by the two *Dickeya* species. In fact, Ds2222 did not possess similarly coded proteins to that of the other three bacteria (**Table 5**). However, the metabolic pathway related to pectin degradation I was shared between Dd3937and Ds2222.

Pectinases have been divided into three separate groups dependent on the cleavage site. These three groups of pectinases are comprised of (1) hydrolases consisting of polygalacturonase, PG (EC 3.2.1.15); (2) lyase/trans-eliminases comprising pectin lyase, PNL (EC 4.2.2.10), and pectate lyase, PL (EC 4.2.2.2); and (3) pectinesterase, PE (EC 3.1.1.11) (Yadav et al., 2009; Visser et al., 2004). In the current study, similar pathways (**Figures 4A–D**) of all categories were observed in Dd18020, Dd3937, and Ds2222.

In the pectin degradation pathway, both Dd3937and Ds2222 possessed the same enzyme, pectinesterase A (EC 3.1.1.11), encoded for by the DDA3937_RS15630 and DSOIPO2222_RS15080 genes (data not shown). In the same pathway, the enzymes and genes of similar relation from Dd3937and Ds2222 were several exo-poly-α-D-galacturonosidases (EC 3.2.1.82) encoded for by the DDA3937_RS20090, DDA3937_RS20095, DDA3937_RS20100, and DDA3937_RS05830 genes of Dd3937and the DSOIPO2222_RS19410, DSOIPO2222_RS19420, and DSOIPO2222_RS19425 genes of Ds2222. Lastly, in the metabolic pathway EC 4.2.2.-, the enzymes, and genes of similar relation from Dd3937and Ds2222 were oligogalacturonate lyase encoded in the genes DDA3937_RS11450 and DSOIPO2222_RS11150, respectively. Neither *Bacillus* species utilized in the current study shared any similar enzymes or genes in the pectin degradation I pathway to the *Dickeya* species.

The pectin degradation pathway I shared by the *Dickeya* species degrades pectin to D galactopyranuronate + 4-deoxy-L-threo-hex-4-enopyranuronate (**Figure 4A**). Pectin is degraded to pectate via pectinesterase A in the metabolic reaction catalyzed by EC 3.1.1.11, with methanol byproduct (**Figure 4B**). Pectate is degraded to α-D, α-D-digalacturonate via several exo-poly-α-D-galacturonosidases in the metabolic reaction catalyzed by EC 3.2.1.82 with a pectate byproduct (**Figure 4C**). The α-D, α-D-digalacturonate is degraded to D galactopyranuronate + 4-deoxy-L-threo-hex-4-enopyranuronate in the metabolic pathway catalyzed by EC 4.2.2.- in Dd3937 via oligogalacturonate lyase (**Figure 4D**).

DISCUSSION

As pectinase is a vital enzyme utilized in the degradation of different fruit and vegetable products, it has value to the food industry (Sharma et al., 2013). Likewise, based on consumer preference, poultry industry diets are increasingly becoming vegetable-based void of animal protein (Weil, 2017). To make all-vegetable diets commercially viable, the poultry industry will need additional sources of feed grade pectinases with different hydrolytic capabilities to break down vegetable products with widely different compositional levels and subsequently improve broiler performance. Therefore, the utilization of a more flexible set of supplementary enzymes will become increasingly crucial to poultry nutritionists in enhancing the nutrient uptake in all-vegetable diets.

However, as *D. dadantii* has been reported to be a pathogen to plants and insects, another potential strategy for use of *D. dadantii* DSM 18020 pectinase genes would be to incorporate them into a current commercial poultry probiotic. Therefore, the genes of pectin degrading enzymes of Dd18020 could be inserted into a *Bacillus*, a known probiotic currently utilized in poultry diets. A *Bacillus* spp. would be a suitable candidate as they have the potential to withstand feed manufacturing conditions due to spore forming capabilities and can be genetically modified for industrial use (Ricke and Saengkerdsub, 2015). Though it is feasible, the insertion of Gram-negative genes (*D. dadantii*) into a Gram-positive organisms (*Bacillus*) is not easily accomplished as it requires proper plasmid origin recognition, promoter recognition, and codon usage between the organisms. Another limitation to consider is that certain *Bacillus* species such as *B. subtilis* produce proteases that have been known to hinder the production of heterologous proteins (Puohiniemi et al., 1992). As such, the meticulous insertion of Gram-negative genes into *Bacillus* spp. has been possible through plasmid insertion (Luchansky et al., 1988; Ohta et al., 2005) and more recently through CRISPR-Cas9 systems (Toymentseva and Altenbuchner, 2019).

In the current study, data demonstrated that *D. dadantii* DSM 18020 possessed several pectinase-related enzymes such as polygalacturonases (PG), pectin lyase (PL), polygalacturonate lyase (PGL), and pectinesterase or pectin methylesterase (PE) (Sharma et al., 2013). The optimal pH for most endo PG, PL, PGL, and PEs are 2.5–6.0, 4.0–7.0, 6.0–11.0, and 4.0–7.0, respectively (Sharma et al., 2013). The optimal temperatures range from 30 to 50°C (PGs) and 40–60°C (PEs) (Sharma et al., 2013). The internal body temperature of poultry is 41.5°C (Dawson and Whittow, 2000) and the pH of the avian gastrointestinal tract (GIT) varies from highly acidic (below 4) in the proventriculus and gizzard and neutral in the lower intestines (above 7; Sturkie, 1999).

Therefore, these enzymes should function as the avian GIT is within the optimal parameters for enzyme utilization.

In fact, the *in vitro* evaluation of PG and pectin methylesterase (PME) demonstrated an improvement in the digestion of pectin and a reduction in the water holding capacity of lupins in comparison to PG or PME alone (Ali et al., 2005). Therefore, Ali et al. (2005) concluded that the inclusion of legumes such as lupin could be increased by utilizing the combination of the two pectinases in poultry diets. Furthermore, the supplementation of pectinase with either cellulase or hemicellulase in corn-soybean meal broiler diets from days 15 to 27 has demonstrated the improvement in the ileal digestibility of crude protein (CP) and organic matter and increased apparent metabolizable energy content of the diet (Tahir et al., 2006). Other researchers have also found the supplementation of a multi-enzyme preparation with pectinase improved CP digestibility (Marsman et al., 1997; Kocher et al., 2002; Saleh et al., 2005). The improvement in CP digestibility may in part be due to the degradation and subsequent digestion of pectic polysaccharides (Slominski and Campbell, 1990; Simbaya et al., 1996). Due to 10% of the protein being entrapped in the cell wall matrix of soybeans, the degradation of the cell wall via the utilization of pectinase-related enzymes may release the contained proteins (Chesson, 2001). Also, half of the NSP's contained within the cell matrix of the soybean meal are pectic polysaccharides (Chesson, 2001). Their subsequent depolymerization may improve CP digestibility, which has been noted previously (Tahir et al., 2006).

CONCLUSION

The current study validates the genetic potential of *D. dadantii* DSM 18020 as an enzymatic pectinase producer to utilize commercially within the poultry feed industry. The results demonstrated that several pectin-related enzymes are encoded in the genome of *D. dadantii,* and *D. dadantii* possesses the most significant number of pectin degradation-related pathways. Also, the study demonstrates the genomic capability for metabolic breakdown of pectin via the pectin degradation pathway belonging to *D. dadantii*; however, further studies are necessary to evaluate the capabilities of *D. dadantii* DSM 18020 as a source of commercially viable pectinase for large scale production. For example, more in-depth knowledge of pectin degradation by *D. dadantii* is necessary to quantify the pectinase production of *D. dadantii* to design scale up production. Based on the current *in silico* research study, *D. dadantii* does appear to produce pectinases that can further enhance the utilization of poultry diets and the continual supplementation via production by poultry probiotics fed to poultry may enhance nutrient absorption and subsequent performance. In relation to poultry, further analyses are necessary to determine if the supplementation of *D. dadantii* pectin-related enzymes in poultry diets is feasible. Some caution should be exercised as diet manufacturing involves steam and high temperature; this could potentially denature these key enzymes, thus reducing the effectiveness of pectin-related enzymes. Therefore, further research evaluating the survival of pectinases in poultry diets is necessary.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI, Dickeya dadantii DSM 18020 (NZ_CP023467.1), *D. dadantii* 3937 (NC_014500.1), *D. solani* IPO 2222 (NZ_CP015137.1), *Bacillus subtilis* subsp. *subtilis* str. 168 (NC_000964.3) and *B. halodurans* C-125 (NC_002570.2).

AUTHOR CONTRIBUTIONS

DD wrote the manuscript with the assistance from SR, RB, and MR. All authors significantly contributed to the work of the current manuscript.

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