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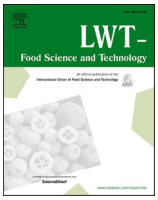

Spatial-temporal distribution of oxygen and its effect on microbial dynamics and vitamin B_{12} content in lupin tempeh

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ABSTRACT

Vitamin B_{12} is an essential vitamin for humans that can only be produced by some bacteria and archaea and is usually only found in animal (derived) products. In order to move from animal-based towards plant-based diets, supplementation of this vitamin is essential, especially for vegetarians and vegans. Tempeh is a popular meat replacer which has shown to be suitable for *in situ* vitamin B_{12} fortification. In this study, we analyzed the presence of fungal biomass, *Propionibacterium freudenreichii*, oxygen and vitamin B_{12} in lupin tempeh, produced by co-culturing *Rhizopus microsporus* var. *oligosporus (Saito)* CBS 338.62 and *P. freudenreichii* subsp. *freudenreichii* DSM 20271. We demonstrated that for all parameters mentioned above, spatial and temporal gradients exist within the solid state fermented matrix. These gradients occur inside the tempeh due to the interplay between the fungus, the bacterium and the lupin substrate during the course of fermentation. As fungal biomass increases, less oxygen is available due to the fast metabolism of the mold. When fungal metabolism slows down, enough oxygen can diffuse back into the tempeh which allows for *P. freudenreichii* to remain active and subsequently produce up to 20 μg vitamin B_{12} per 100 g of fresh lupin tempeh.

1. Introduction

Plant based foods are becoming increasingly popular, especially in countries with a Western diet (Singh et al., 2021). For the implementation of plant-based foods in current diets, enrichment or supplementation with vitamin B_{12} is an important aspect. Vitamin B_{12} is among others nutrients, essential for a healthy nervous system and cell division, especially for red blood cells. It can only be found in animal (derived) products and is exclusively produced by some bacteria and archaea (Schneider & Stroiński, 1987; Watanabe & Bito, 2018) which makes it even more important to supplement it in a vegetarian (with increasing focus on plant-based foods), but especially vegan diet (Niklewicz et al., 2023). In order to enrich a plant-based food product *in situ*, one can incorporate a vitamin B_{12} producing bacterium, like *Propionibacterium freudenreichii*. It is a food safe bacterium, known for its vitamin B_{12} production in Swiss-type cheese (Gomes Soares, Bevilaqua, Marcondes Tassi, & Reolon Schmidt, 2023; Martens, Barg, Warren, & Jahn, 2002; Piwowarek, Lipińska, Hać-Szymańczuk, Kieliszek, & Ścibisz, 2018). Studies have shown that incorporation of *P. freudenreichii* into a food product (for example cereals, sunflower seed milk, soy tempeh) resulted in elevated levels of vitamin B_{12} (Chamlagain et al., 2018; He & Howell, 2022; Tangyu et al., 2022). A previous study demonstrated that *in situ* vitamin B_{12} fortification of lupin tempeh using *P. freudenreichii* is promising (Wolkers – Rooijackers, Endika, & Smid, 2018). Lupin is a suitable alternative for soy as raw material because of its nutritional quality (digestibility, protein quality and dietary fiber content) (Prusinski, 2017). Lupin can be grown in moderate climate zones and qualifies as a native European legume (Huyghe, 1997) making it more sustainable than soy.

In order to be able to steer the production of vitamin B_{12}, it is necessary to know the environmental conditions inside the tempeh and the interplay between the fungus, the bacterium and the lupine substrate. We anticipate a delicate balance between the two microorganisms in the tempeh process. Tempeh is a solid state fermented product in which oxygen concentration decreases due to its consumption by the mold and oxygen diffusion to the center of the bed is limited (Nout & Kiers, 2005). At the same time heat is generated by the metabolism of the fungus. This increase in temperature and change in oxygen availability during the actual fermentation process leads to circumstances in which *P. freudenreichii* can grow well and is able to produce active vitamin B_{12} (Orla-Jensen, 1921). Only with 5,6-dimethylbenzimidazole (DMBI) as lower ligand, vitamin B_{12} is able to play an active role in the

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human body and the synthesis of DMBI requires oxygen (Deptula et al., 2015; Martens et al., 2002). There are conflicting data with respect to the presence of oxygen and its effect on vitamin B_{12} production. (Quesada-Chanto et al., 1998) found decreased levels of vitamin B_{12} produced in an oxygen rich environment. High concentrations of oxygen inhibit the synthesis of cytochrome and thereby these conditions also inhibit growth of *P. freudenreichii* (Thierry et al., 2011) which ultimately leads to less vitamin B_{12} being produced. (Ye, Shijo, Jin, & Shimizu, 1996) however showed that increased yields of vitamin B_{12} were found when *P. freudenreichii* was grown in alternate presence/absence of oxygen. A recent study by (Dank, Biel, Abee, & Smid, 2022) also showed that a low oxygen regime had a positive effect on both biomass formation and vitamin B_{12} production, supporting that oxygen is one of the key-factors for high vitamin B_{12} production by *P. freudenreichii* (Thierry et al., 2011). The aforementioned studies have been executed in cultures or bioreactors where the presence of dissolved oxygen could be manipulated but not in a solid state fermented food product. The interplay between the aerobic fungus and its substrate over time defines the low oxygen surrounding in which the aerotolerant bacterium is able to grow and produce vitamin B_{12}.

The aim of this research was to confirm the role of oxygen in vitamin B_{12} production by *P. freudenreichii* in a mixed-culture fermentation with *Rhizopus microsporus* var. *oligosporus (Saito)* on lupin beans. Fungal biomass, temperature, presence of *P. freudenreichii* and oxygen were measured and used to determine whether spatial-temporal gradients of the aforementioned factors exist and whether these influence the amount of vitamin B_{12} formed.

2. Materials and methods

2.1. Lupin beans

Chopped dehulled sweet white lupin beans (*Lupinus albus*) from France (Inveja Food) were used as substrate for lupin tempeh. The lupin bits (which had a particle size of 2–4 mm) were provided by De Hobbit (Maldegem, Belgium).

2.2. Fungal spore inoculum

Rhizopus microsporus var. *oligosporus* (Saito) CBS 338.62 was obtained from the public culture collection of the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands and was originally isolated from soy tempeh in the Netherlands. The strain, kept in 20% (v/v) glycerol at −80 °C, was cultured onto Malt Extract Agar (MEA from Oxoid, Basingstoke, UK) and incubated at 30 °C for 48 h. Fungal spore suspension was prepared according to (Wolkers – Rooijackers et al., 2018) with a final spore count of approximately 10^7 spores per ml.

2.3. Bacterial inoculum

The food grade vitamin B_{12} producing bacterium, *Propionibacterium freudenreichii* subsp. *freudenreichii* DSM 20271, was stored at −80 °C as a stock culture in 20% (v/v) glycerol. For preparing a bacterial suspension, the bacterium was cultured from the frozen stock on Potassium Lactate (PL) agar (Burgess, Smid, Rutten, & Van Sinderen, 2006). Plates were incubated for 5 days at 30 °C anaerobically. After incubation, a single colony was transferred aseptically into 10 ml PL broth. An aliquot of 1 ml of grown culture in broth medium was centrifuged at 17000×*g* for 5 min and the supernatant was discarded. The bacterial pellet was then re-suspended in 1 ml phophate buffered saline (PBS, pH 7.2) and again centrifuged at 17000×*g* for 5 min. Supernatant was discarded and the washed bacterial pellet was suspended in PBS for use in tempeh production.

2.4. Lupin tempeh production

Approximately 200 g of lupin bits was weighed into a 2L plastic jar (Nalgene) containing 600 ml of tap water for soaking for a period of 15 h at 20 °C. After soaking, the lupin bits were drained, rinsed with running tap water and then boiled for 20 min in water (weight of ratio beans to water 1:3). After the boiling stage, lupin bits were drained and spread out on a metal mesh (cooling tray) with approximately 1 cm layer thickness and then dried in the open air for 2 h. After drying, 200 g of lupin bits were weighed into a stomacher bag.

An aliquot of 4.5 ml of *Rhizopus microsporus* var. *oligosporus (Saito)* spore suspension (containing approximately 10^7 spores per ml) was transferred into a sterile 15 ml Falcon tube and 0.5 ml of a concentrated bacterial suspension ($2*10^{11}$ CFU/ml) was added. The inoculum suspension was mixed and added to the stomacher bag containing 200 g lupin bits. The lupin bits and tempeh inoculum inside stomacher bag were mixed by shaking vigorously and poured from the stomacher bag into the a food grade perforated polyamide foil casing (Viscofan SA, Spain) which was provided by De Hobbit (Maldegem, Belgium). This resulted in a cylinder shape lupin tempeh with a total length of 12 cm and 6 cm diameter (with 1.5 mm holes every 8 mm × 10 mm). In the end, the concentration of fungal spores and *P. freudenreichii* cells were approximately 10^5 and $5*10^8$ per gram of lupin bits respectively. The inoculated lupin was incubated at 28 °C for up to 44–46 h.

2.5. Temperature measurements

The temperature during fermentation inside the lupin tempeh was measured by using Maxim's iButtons® type DS1922L. iButtons were inserted at the core (3 cm) of each sampling location (edge, middle, center, see Fig. 1). Data were collected every 10 min with a measuring precision of 0.06 °C. After fermentation, data were retrieved using OneWireReader software by MaximIntegrated™.

2.6. Oxygen measurements

Oxygen was measured with a NTH Microsensor (PreSens, Precision sensing). The oxygen probe was inserted at different locations (Fig. 1) in the lupin tempeh (edge, middle and center), at different depths, from the core of the tempeh to the outside (3 cm–0.6 cm deep) at 4 mm intervals and the percentage of oxygen was recorded. The analysis was done after 18, 24 and 44 h of tempeh incubation.

2.7. Fungal biomass quantification

2.7.1. Solvent extraction

The method for extraction of ergosterol from lupin substrate containing samples and from dried fungal biomass was adapted from the miniaturized bead-beating method described by (Sae-Tun et al., 2020). The latter method is a variation of the method described by (Gong, Guan, & Witter, 2001). Approximately 0.16 g of homogenized samples were weighed into 2 ml lysing matrix C tubes (MP Biomedicals). To measure the extraction method's recovery, a lupin blank homogenate was spiked with 50 µl per gram of a 200 µg/ml ergosterol solution in methanol which was then subjected in triplicate to the same treatment as other homogenized samples. Analytical grade methanol ≥99.9% (Emsure) was added at volume of 0.8 ml. The tubes were then vortexed for 10 s, and subsequently placed in the FastPrep-24 bead-beater (MPBiomedicals) which was operated at 6.5 m/s for 60 s. Tubes were then centrifuged in a Pico 21 microcentrifuge (Thermo Scientific) at 10.8×*g* for 10 min at 20 °C. Supernatant was then transferred to a clean 2 ml Eppendorf tube and centrifuged again with the same settings. Finally, supernatant was syringe filtered through a 0.2 µm filter into an HPLC vial.

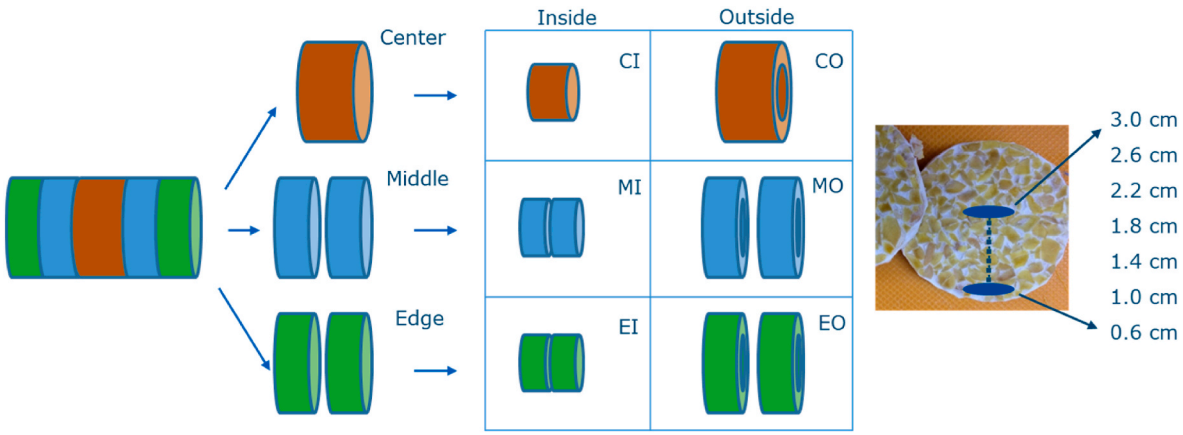

Fig. 1. Sampling locations within the lupin tempeh Edge, Middle and Center at different depths (0.6–3.0 cm).

2.7.2. Ergosterol quantification

Ergosterol was determined by chromatographic separation via a reversed-phase chromatography, using an Ultimate 3000 UHPLC with UV detector set at 282 nm (Thermo Scientific), fitted with UPLC BEH C18 1.7 μm 2.1 × 100 mm column (Acquity). The column oven temperature was set at 40 °C, and a mixture of 95% MeOH and 5% MilliQ water was used as the mobile phase for isocratic elution at a flow rate of 0.5 ml/min. Sample injection volume was 20 μl. The total retention time for ergosterol was 3.5 min. Standard solutions were prepared containing ergosterol in MeOH ranging from 0.1 to 200 μg/ml. Ergosterol was calculated in mg/g tempeh unit based on the extraction ratio of sample to volume as well as the dilution factor of homogenization. A correction factor based on the mean recovery of triplicate recovery samples was also applied. Analysis was performed within 3 days after extraction to avoid loss of ergosterol by degradation. A standard curve was constructed using Chromeleon software (Thermo Scientific) and used to quantify samples. The standard curve is available in Appendix A.

2.8. qPCR quantification of Propionibacterium freudenreichii

Quantification of *P. freudenreichii* gDNA copies was conducted with technical duplicates for each of the biological triplicates. Genomic DNA was extracted using FastDNA Spin Kit for Soil (MPBiomedicals) according to the manufacturer's instructions. The bead-beating step was done using the FastPrep-24 (MPBiomedicals).

The following primer pair was used (Forward: CAAGGACAAGTTCGTGCTGC; Reverse: CTTCACAGAGGGCTTCTCGG). This primer pair targeted the genomic sequence of *P. freudenreichii* DSM 20271 at nucleotide range: 670133-670292 of sequence accession number CP010341.1 of the NCBI database. Target amplicon length was 160 bp. Each reaction well was composed of 14 μl of the specific primer pair reaction mix and 6 μl of either template gDNA dilution or autoclaved MilliQ as NTC, giving a 20 μl total reaction volume per well. Run parameters for the qPCR were: 5 min at 95 °C; 30x cycles of 15 s at 95 °C and 45 s at 60 °C; melt curve from 55°C to 95 °C with 0.5 °C increment at 5 s/step.

2.9. Vitamin B_{12} analysis

2.9.1. Sample extraction

Samples were extraction using the extraction protocol provided by Vitafast's immunoaffinity columns (IAC), with modifications.

Samples were snap frozen using liquid nitrogen and immediately grinded using a coffee grinder for 25 s. After grinding, the powder was transferred in amber Greiner tubes of 50 ml and stored at −20 °C. Upon analysis, approximately 10 g of frozen powder was transferred into 100 ml glass bottles containing 35 ml 50 mM acetate buffer (pH 4.5). Lysozyme (0.2 ml; 0.15 g/ml) and taka diastase (0.5 g) were added and bottles were put in a shaking water bath (160 rpm) at 37 °C for 30 min. Then, pepsin (0.5 ml, 4% w/v solution) and KCN (0.25 ml; 4% w/v solution) were added and incubated for another 45 min. KCN is added to replace the upper ligand to cyanide (resulting in cyanocobalamin). Enzymes were deactivated by transferring the bottles to a water bath of 99.9 °C for 30 min followed by cooling the samples on ice down to ~25 °C. After cooling, samples were centrifuged (8382 g, 20 min at 4 °C) and filtered (2V folded filters, diameter 240 mm, Whatman®, Darmstadt, Germany) to obtain a clear filtrate. This filtrate was concentrated using immunoaffinity columns (R-Biopharm, Darmstadt, Germany). After a washing step with MilliQ, samples were eluted using 100% methanol (4 ml) followed by evaporation of the methanol using a heating block (50 °C) and nitrogen gas to speed up the drying process and prevent oxidation. The dried extracts were redissolved in 1 ml of mobile phase (10 mM ammonium formate + 0.1% formic acid in water) and filtered through Nalgene Syringe Filters with PES membrane of 0.2 μm (Thermo Scientific, Rochester, NY) to remove any remaining solid particles and transferred into 1.5 ml HPLC vials.

2.9.2. LC-MS analysis of vitamin B_{12}

Vitamin B_{12} was analyzed using an Agilent LC-MS, with C18 column (HSS, 100 mm × 3.0 mm, 3.5 μm) and column temperature of 30 °C. Mass detection was set at 678.4 SIM (to target cyanocobalamin with DMBI as lower ligand). The injection volume was 15 μl. The two mobile phases used for the analysis were constituted of 10 mM ammonium formate in 0.1% formic acid in water (mobile phase A) and 10 mM ammonium formate in 0.1% formic acid in methanol (mobile phase B) and had a flow rate of 0.4 ml/min with gradient elution (Table 1).

A calibration curve was made with a range of 0–2 μg/ml.

The amount of vitamin B_{12} was calculated using the following equation:

$$Concentration_{\ product}\left(\frac{\mu g}{100g}\right) = concentration_{\ vial}\left(\frac{\mu g}{ml}\right) \times \left(\frac{total\ mass_{extract}}{mass_{column}}\right) \times \left(\frac{100}{sample\ amount\ (g)}\right)$$

Table 1
Gradient elution for vitamin B_{12} analysis.

Time	0 min.	30.0 min.	30.1 min.	35.1 min.
Mobile phase A	99	2	99	99
Mobile phase B	1	98	1	1
0 -> 30 min.	analysis time			
15 -> *30.*1 min.	purge time			
30.1 -> 35 min.	equilibration time			

2.10. Microbiome profiling

Microbial communities present in the final lupin tempeh (three samples with and three samples without added *P. freudenreichii*) were analyzed using INVIEW Microbiome 3.0 sequencing (Eurofins). These samples were subjected to paired-end sequencing of the V3–V5 hypervariable region of the bacterial 16S-rRNA gene amplicon. This was performed on the MiSeq Illumina platform with 300 bp paired end read module by Eurofins Genomics (Ebersberg, Germany). Reads were processed by demultiplexing, primer clipping, merging, quality filtering, chimera filtering, picking of Operational Taxonomic Units (OTUs), taxonomical assignment and read abundance estimation down to species level if possible. Further processing was performed with the QIIME pipeline.

2.11. Data analysis

Data were analyzed statistically using SPSS. One or two way ANOVA was performed, followed by post-hoc Tukey honestly significant difference (HSD). Significance level (p-value) was set at 0.05.

3. Result and discussion

Fungal growth is an important characteristic of tempeh. The presence of dense, white mycelium defines a successful solid state fermentation process. In order to be able to quantify the biomass of the fungus, the concentration of ergosterol can be measured. Ergosterol is an important cell membrane sterol that is found in yeast and fungi, but is absent in bacteria, plants and animals. The unique association of this molecule with fungi makes it a valuable biomarker for the estimation of fungal biomass in mold fermented food products like tempeh (Gessner, 2005). Changes in ergosterol concentration during the course of the fermentation were measured compared to the corresponding amount of ergosterol at time zero hours (Fig. 2).

For each part of the lupin tempeh (center, middle and edge) all timepoints are significantly different ($P < 0.05$) and it is clearly shown that fungal biomass increases as a function of time which was confirmed by visual observations. Rapid growth of fungal mycelium is known to produce heat which increases the actual temperature of the tempeh (Ikasari, Mitchell, & Stuart, 1999; Nout & Kiers, 2005). This was confirmed in our experiment (Fig. 3) where a maximum internal temperature of 35 °C was reached after approximately 18 h of fermentation. After 18 h, fungal metabolism starts to slow down which leads to a gradual decrease in temperature which stabilizes at 30 °C during the remaining fermentation time.

In the core of the edge, the maximum reached temperature is higher than in the core of the center part and this corresponds to more

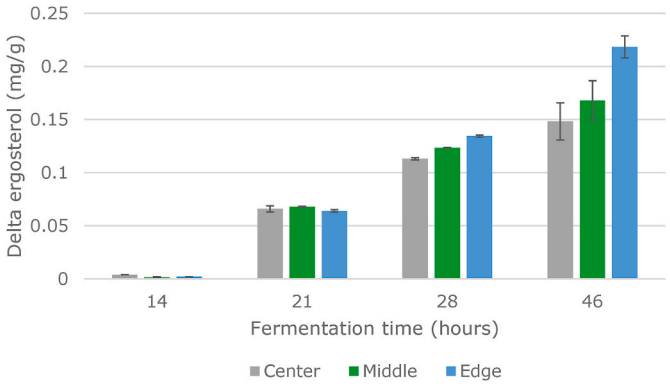

Fig. 2. Average change (and standard deviation) in total ergosterol concentration (mg/g) across fermentation time (14, 21, 28 and 46 h) compared to 0 h for different parts of the lupin tempeh (center, middle and edge).

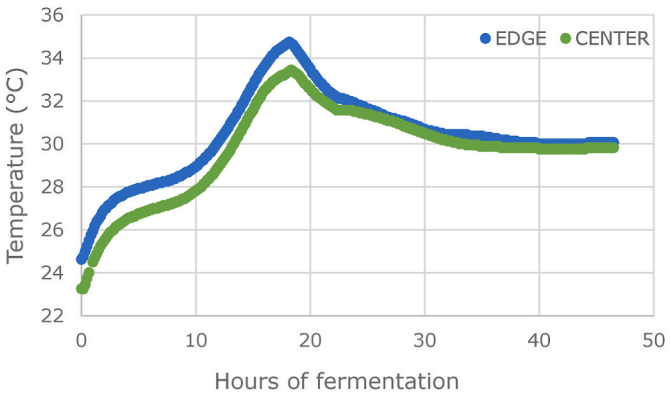

Fig. 3. Temperature profile during 46 h of fermentation inside lupin tempeh. The temperature was measured with ibuttons inserted at core of both edge and center part of the tempeh.

mycelium being produced at the edge which corresponds to faster fungal metabolism (Fig. 2). Since the mold is aerobic, higher levels of ergosterol (and densities of mycelium) are seen at the middle and edge, especially towards the outside of the tempeh, consistent with previous literature (Feng, Olsson, Swanberg, Schnürer, & Rönnow, 2007). Although it seems that the mold completely claims the substrate niche, we know from previous studies that also bacteria are able to grow alongside the mold in tempeh (Mulyowidarso, Fleet, & Buckle, 1990; Suwanto, 2021). Since we co-inoculated our lupin tempeh with *P. freudenreichii* we had a closer look at the diversity of the microbiome present at the end of fermentation. The V3–V5 hypervariable region of the bacterial 16S-rRNA gene amplicon was analyzed for 6 lupin tempeh samples (three without and three with added *P. freudenreichii*). Results clearly show that when *P. freudenreichii* is added (+PF), less bacterial diversity can be seen compared to the control samples (Fig. 4).

Lupin tempeh is not a sterile product and different species of bacteria are able to grow during the course of fermentation (Mulyowidarso et al., 1990). Although not all obtained amplicons of the V3–V5 hypervariable region of the bacterial 16S-rRNA encoding gene could be identified to genus level, the main message from Fig. 4 is that in samples where *P. freudenreichii* was added, it dominated the bacterial community. In the control samples, the majority of the bacteria present belonged to the *Enterobacter* genus. *P. freudenreichii* was inoculated at a level of 10^8 CFU/g at the onset of tempeh production and increased approximately 10 fold in abundance during the course of the fermentation (Wolkers – Rooijackers et al., 2018). The presence of *P. freudenreichii* was found to suppress the proliferation of other microbes which is in line with several other studies. (Gwiazdowska & Trojanowska, 2006) reported bacteriostatic activity of bacteriocins produced by *P. freudenreichii* against Gram-negative bacteria (*Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Yersinia enterocolitica*). (Hajfarajollah, Mokhtarani, & Noghabi, 2014) reported production of a lipopeptide biosurfactant that was effective against several bacteria (*P. aeruginosa* and *Escherichia coli* amongst others). Also the production of organic acids like acetate and propionate can have an inhibitory effect on other bacteria (Taniguchi et al., 1998).

Once we had confirmed the presence of fungal biomass and established the presence of *P. freudenreichii* along with bacterial biodiversity in the lupin tempeh samples we analyzed the spatial and temporal distribution of fungal biomass in lupin tempeh. At different time points during the fermentation process, six different sections of the cylinder shaped tempeh were sampled and ergosterol was determined for each section and displayed as ergosterol increase compared to time zero hours (Fig. 5).

As can be seen from Fig. 5, spatial differences across the different sections exist with increased fermentation time. To facilitate contextualization of the spatial variation of ergosterol concentration at 46 h

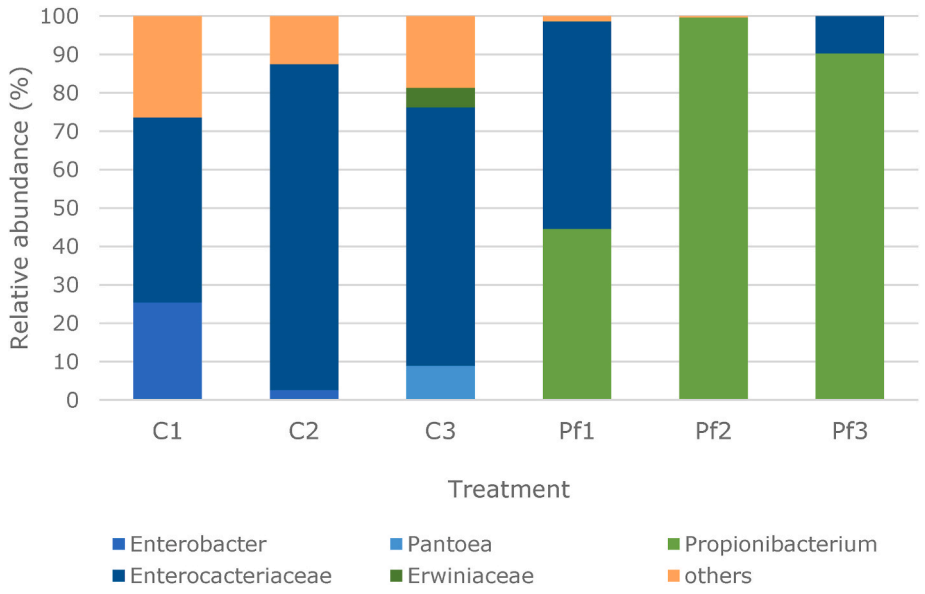

Fig. 4. *Relative abundance of lupin tempeh microbial communities (based on OUT assignment) on genus and family level per sample (with others comprising undefined genera and genera with a relative abundance < 1% (*Klebsiella, Pseudomonas, Acinetobacter, Kocuria, Pseudoescherichia and Micrococcaceae*)). Samples were made in triplicate per treatment, treatments being control lupin tempeh and lupin tempeh with added* P. freudenreichii.

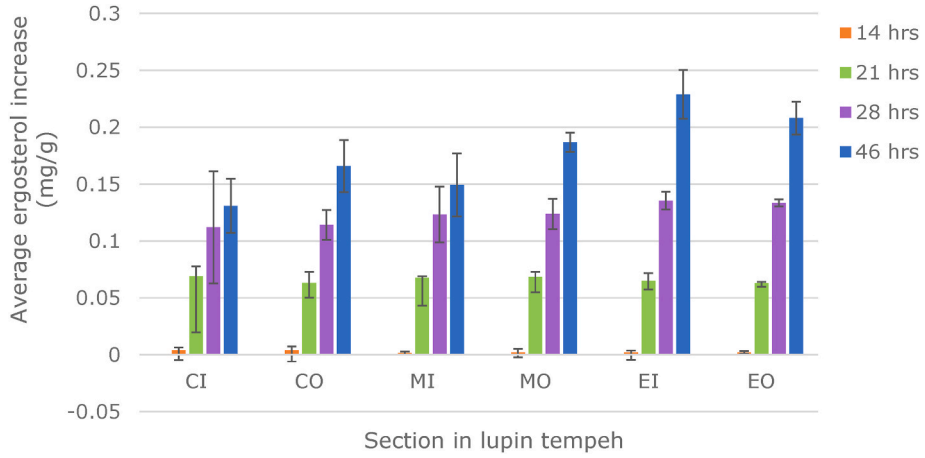

Fig. 5. Mean increases in ergosterol concentration (and standard deviation) from time 0 h for each incubation time (14, 21, 28 and 46 h) within each spatial positioning group (CI, center inside; CO, center outside; MI, middle inside; MO, middle outside; EI, edge inside; EO, edge outside).

incubation, a heat map was constructed, where the changes in ergosterol concentration are mapped onto their corresponding areas of a cross-section of lupin tempeh, as shown in Fig. 6.

A significant effect at 46 h of fermentation within the different sections was measured. As expected, the increase in ergosterol concentration was lowest in the CI area, with larger increases observed at positions CO and MI, and largest at the EO and EI positions, which represent areas at smaller radial and lengthwise depths, respectively. The packaging consisted of a flexible plastic sleeve, consistently perforated but with two open ends in which the inoculated substrate was inserted. These open ends were closed using tape, therefore allowing a greater flux of air (which boosts growth of the fungus) than the relatively restricting perforation holes in the rest of the foil. The fact that the fungus is unevenly distributed in the solid state fermented product raises the question whether this also applies for the distribution of *P. freudenreichii*. For quantification of *P. freudenreichii* with qPCR (log DNA copy numbers), sections at different timepoints were taken where the focus was core and edge (in and out, 0.6 cm and 3.0 cm deep respectively) of the lupin tempeh (Fig. 7).

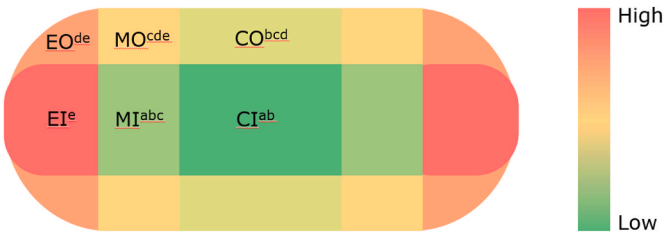

Fig. 6. Heat map showing relative differences in mean change in ergosterol concentration between spatial samples of 46 h incubated lupin tempeh (CI, center inside; CO, center outside; MI, middle inside; MO, middle outside; EI, edge inside; EO, edge outside). Sections with different superscripts are significantly different ($P < 0.05$).

As expected, none of the control samples revealed the presence of *P. freundenreichii* (Cq value > 30) and this corresponds to the results obtained from microbiome amplicon sequencing. For all samples where *P. freudenreichii* was added, more than 8.7 log DNA copy numbers per

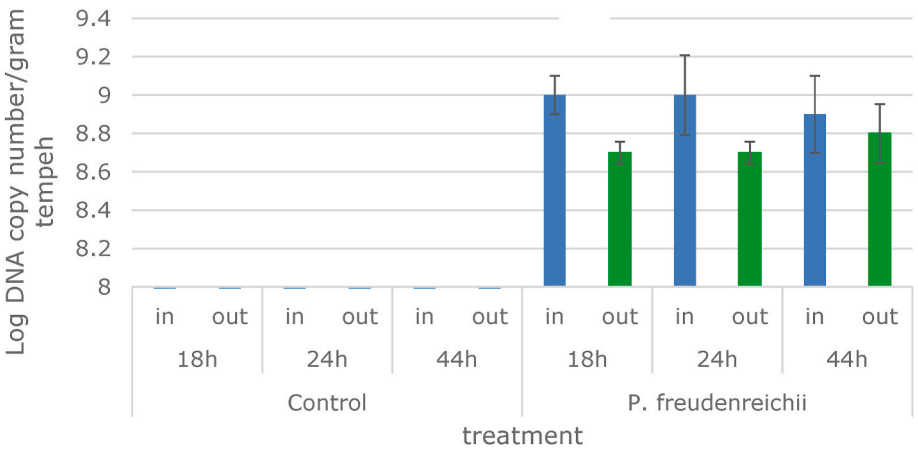

Fig. 7. *Average and standard deviation of* log *DNA copy numbers of* P. freudenreichii *in control lupin tempeh samples and with added* P. freudenreichii *at different sample locations (in: core of the center; out: core of the edge (see Fig. 1). Samples were analyzed with technical replicates for each biological triplicate.*

gram of lupin tempeh were found. A significant difference in log DNA copy number was observed between the inside and outside of the lupin tempeh with more *P. freudenreichii* present inside; interestingly, an opposite spatial difference was found for fungal biomass. This suggested a relation between presence of the fungus and the bacterium with oxygen being a possible factor; less oxygen available inside of the tempeh compared to outside. In order to prove this reasoning, oxygen was measured in lupin tempeh's as a function of fermentation time (18, 24 and 44 h of incubation) and at different depths (according to Fig. 1) within the lupin tempeh (Fig. 8).

We demonstrated an oxygen gradient in the lupin tempeh ranging from relatively high to low while going deeper into the developing tempeh (see Fig. 8). The lowest oxygen concentrations were measured after 18 h of fermentation with less than 1.5% at 3 cm depth and 5.2% at 0.6 cm depth. Compared to the situation at 18 h, we observed an increase in the oxygen concentration inside the tempeh after 24 and 44 h of fermentation. This increase coincides with a decreased metabolic activity of the fungus evidenced by a decreased heat production which peaked at 18 h (see Fig. 3). The same trends in oxygen gradients were observed in the middle part and the edge of the tempeh (see Appendix B). Most likely, less oxygen (<2 %) is available in the center of the tempeh due to dense packing of the lupin bits in the plastic foil and the metabolism of the mold. Oxygen is consumed as the mold grows and diffusion of oxygen to the core of the tempeh is too slow to compensate for this oxygen consumption, making the inside of the tempeh a low oxygen surrounding. (Mitchell, Doelle, & Greenfield, 1988; Nout & Rombouts, 1990) showed the influence of particle size (an thus packing of the bits) and diffusion on the availability of oxygen. These results are consistent with literature on soy tempeh (Nout & Kiers, 2005). In addition, the amount of available oxygen changes in time, as the mold is highly active (and forms aerial hyphae which extend into the gaseous phase where they can restrict airflow through the tempeh) within the first 18 h of fermentation (as shown in Fig. 8) and slows down after this time period allowing oxygen to diffuse back inside the tempeh (Ghildyal, Ramakrishna, Lonsane, & Karanth, 1992; Nout & Rombouts, 1990) also showed this trend in reduced oxygen present at greater depths in fermentation beds (18 and 27 cm) during fermentation time followed by increased oxygen concentration after the metabolism of the fungus slowed down. In our experiments, the total diameter of the fermentation bed was only 6 cm in which the perforated plastic foil allowed oxygen to enter the outside of the tempeh shape but this perforation was not sufficient to allow sufficient oxygen influx towards the inner part to sustain substantial fungal growth there. For both 18 and 24 h the amount of available oxygen seems smaller in samples with the

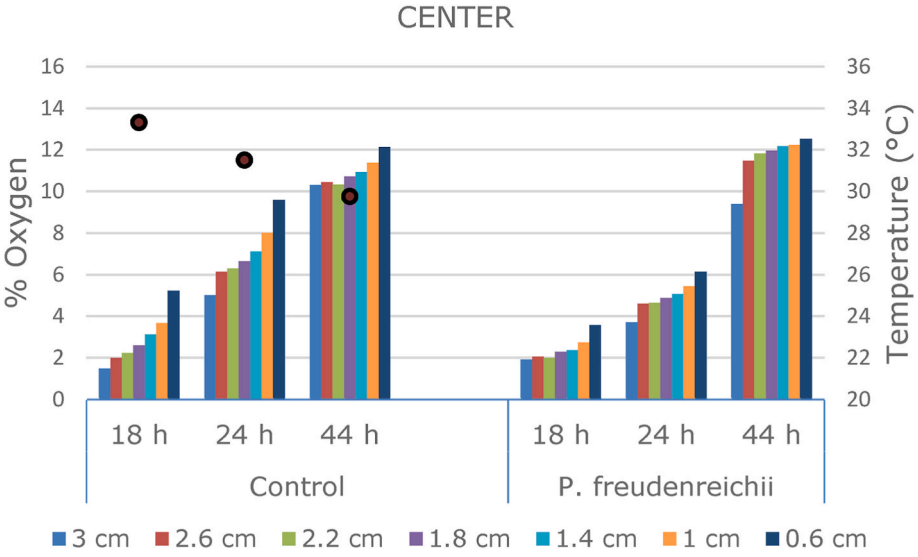

Fig. 8. *Oxygen (left Y-axis - bars) measurements at different timepoints of fermentation and at different depths (according to Fig. 1) in the center of control and* P. freudenreichii *enriched lupin tempeh samples. Temperature (right Y-axis – closed black dots) measurements at different time points at* 3 cm *depth in the center of control tempeh.*

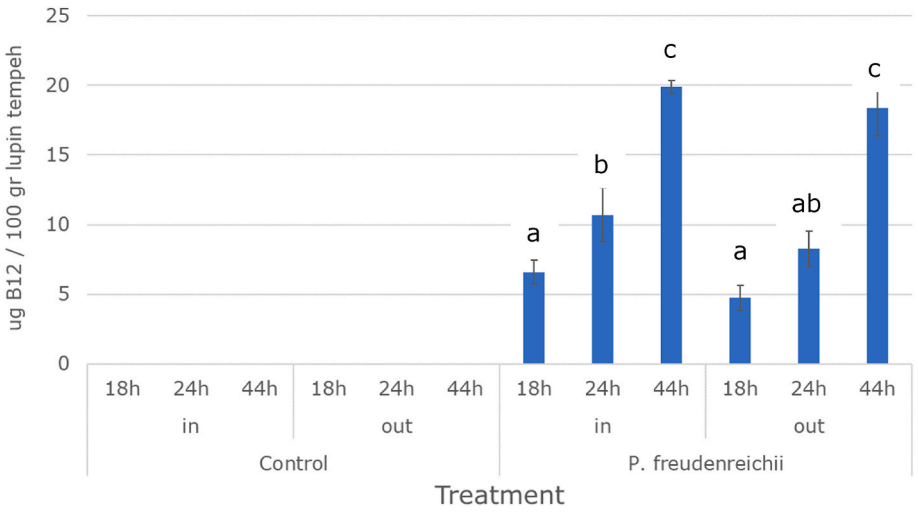

Fig. 9. *Vitamin B_{12} (μg/100 gr lupin tempeh) for control and added* P. freudenreichii *lupin tempeh at different timepoints of fermentation and at different sample locations (in: core of the center; out: core of the edge (see Fig. 1). Averages of 3 biological replicates are shown with standard deviation. Significant differences ($P < 0.05$) within each sample location (in/out) are marked with different letters.*

bacterium added. *P. freudenreichii* benefits from low oxygen concentrations and uses it for growth and metabolism. (Dank et al., 2021; Dank et al., 2022). Our observation of higher log DNA copy numbers of *P. freudenreichii* inside of the lupin tempeh (Fig. 7) confirms the preference of this organism for a niche characterized by a low oxygen concentration. Oxygen is also needed for the final metabolic conversion of cobamide into an active form of vitamin B_{12} by production of the lower ligand (DMBI) and coupling it to cobamide (Martens et al., 2002). Because tempeh production is a static solid state fermentation process, the amount of oxygen available cannot be manipulated by active mixing. Passive diffusion of oxygen back into the tempeh takes place after fungal metabolism slows down (Fig. 8, after approximately 18 h). Since the availability of oxygen, along with the concentration of *P. freudenreichii* cells present in the matrix could influence the concentration of vitamin B_{12} produced we measured vitamin B_{12} at the same positions in the tempeh where oxygen was measured (Fig. 9).

Fig. 9 displays clearly that when no *P. freudenreichii* was added, no vitamin B_{12} was produced. All data for control lupin tempeh were below detection limit (N.D.) of the LCMS. The concentration of vitamin B_{12} increased in time to up to 20 μg/100 gr lupin tempeh (after 44 h). This is a very promising amount, even after 24 h of fermentation, since the adequate intake (AI) for adults varies from 2.4 to 4 μg/day (Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference, its Panel on Folate, & Choline, 1998; EFSA Panel on Dietetic Products, Nutrition, and Allergies NDA, 2015), meaning that consuming

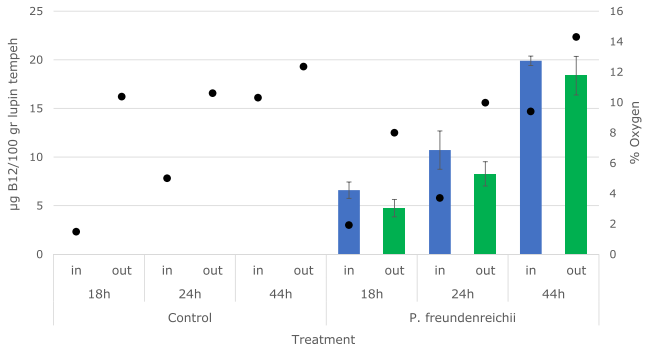

Fig. 10. *Vitamin B12 amounts with standard deviation (left Y-axis - bars) and % oxygen (right Y-axis – closed black dots) for control and added* P. freudenreichii *lupin tempeh at different timepoints of fermentation for each of the 3 biological replicates.*

only 8.5–20 g of 44 h fermented tempeh would supply this amount on a daily basis. When looking at possible spatial differences of vitamin B_{12} produced in the lupin tempeh, it can be seen that inside the tempeh more vitamin B_{12} is produced that at the outside, which correlates to the log DNA copy number of *P. freudenreichii* measured in similar sections (Fig. 7) and to a smaller amount of oxygen present inside the tempeh (Fig. 10). This difference is however not significant.

With respect to the presence of oxygen there is a fine balance between anaerobic and aerobic as also shown by (Tangyu et al., 2022; Ye et al., 1996). They demonstrated that alternation of aerobic and anaerobic incubation (thereby influencing the amount of dissolved oxygen present in the culture) greatly improved vitamin B_{12} production. The size and diameter of the lupin tempeh could also play a role in the oxygen influx, with a higher surface to volume ratio allowing for a better influx. Though the supply of oxygen is not controlled during solid state fermentation of tempeh we can see an effect of available oxygen on the amount of vitamin B_{12} being produced.

4. Conclusions

The results of this work show that variance exists in spatial and temporal distribution of fungal biomass (determined by ergosterol quantification) and *P. freudenreichii* (determined by qPCR) during the production of lupin tempeh. This combination in turn leads to spatial and temporal differences in available oxygen which influences the amount of vitamin B_{12} produced during fermentation. A significant increase in vitamin B_{12} content (up to 20 μg/100 g fresh lupin tempeh) was achieved by fermenting lupin bits using a mixed starter consisting of *Rhizopus microsporus* var. *oligosporus (Saito)* spores and *Propionibacterium freudenreichii*. The achieved vitamin B_{12} concentrations are enough to sustain the daily AI of 2.4–4 μg vitamin B_{12} in only 8.5–20 g of fresh lupin tempeh.

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CRediT authorship contribution statement

Judith C.M. Wolkers–Rooijackers: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis,

Data curation, Conceptualization. **Owen Turner:** Writing – review & editing, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Eva Almekinders:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Eddy J. Smid:** Writing – review & editing, Visualization, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116275.

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