applied sciences

MDPI

Article

Oncom from Surplus Bread Enriched in Vitamin B12 via In Situ Production by *Propionibacterium freudenreichii*

Bożena Stodolak *, Anna Starzyńska-Janiszewska and Dagmara Poniewska

Department of Biotechnology and General Technology of Food, Faculty of Food Technology, University of Agriculture in Krakow, al. Mickiewicza 21, 31-120 Kraków, Poland; anna.starzynska@urk.edu.pl (A.S.-J.); dagmara.poniewska@urk.edu.pl (D.P.)
* Correspondence: bozena.stodolak@urk.edu.pl; Tel.: +48-126-624-789

Abstract: Bread is a frequently wasted food product. Surplus or stale bread can be successfully processed by solid-state fermentation and used as the only fermentation substrate. Oncom, which originated in Indonesia, is made with moulds of the *Neurospora* genus. This experiment aimed to obtain oncome from stale bread enriched in vitamin B12. Co-fermentation with *N. sitophila* and *Propionibacterium freudenreichii* was carried out on two types of bread differing in chemical composition and initial pH value. Oncom obtained after 5 days of fermentation, depending on the substrate used and the fermentation variant (fungal, fungal-bacterial), contained from 35 to 40% dry mass, from 17.5 to about 23% protein, about 2 to max 5% fat, and from 65 to 74% carbohydrates by weight in dry mass. Vitamin B12 content depended largely on the bacterial strain, the colony-forming unit dose in the inoculum, and also the initial pH of the substrate. The oncom product obtained after co-fermentation with *P. freudenreichii* DSM 20271 contained a maximum of 1.3 µg/100 g, which corresponds to the vitamin B12 level in a chicken egg.

Keywords: stale bread; *Neurospora sitophila*; solid-state fermentation; co-fermentation

Citation: Stodolak, B.; Starzyńska-Janiszewska, A.; Poniewska, D. Oncom from Surplus Bread Enriched in Vitamin B12 via In Situ Production by *Propionibacterium freudenreichii*. *Appl. Sci.* **2024**, *14*, 4879. https://doi.org/10.3390/app14114879

Academic Editors: Francesca Melini and Valentina Melini

Received: 26 March 2024
Revised: 27 May 2024
Accepted: 28 May 2024
Published: 4 June 2024

1. Introduction

Bread plays a significant role in the daily diet of the vast majority of Poles. In terms of the quantitative structure of grain product consumption, bread accounts for the largest share (over 50%) within the entire group [1]. It is an important source of energy, building components, B group vitamins, minerals, and dietary fibre. On the other hand, 52% of surveyed Poles admit to wasting bread [2]. Such waste at the consumer level as well as in the producer–seller chain is a well-known phenomenon. The extent of the losses can vary greatly. In Western countries, households generate more than half of total food wastage, and losses in bread processing range from approximately 1 to 14% [3]. The main reasons are the short shelf life associated with bread staleness as well as consumer demands for freshness, the ability to choose different types of bread, and its specific quality characteristics.

Wasted bread has numerous potential applications. Research is underway to obtain active enzymes of microbial origin for application in the food industry [4]. Bread can be used to create feed products enriched in protein, lipids, and carotenoids by fermentation with moulds of the genus *Neurospora* [5]. Bread also has the potential to be utilized for producing fungal biomass abundant in protein and fatty acids, simultaneously serving as a source of microfibre [6].

Stale or leftover bread can find application in the creation of food. Research is being conducted on its use as a substitute for flour in baking new batches of bread [7] and as an ingredient in fried wheat chips [8]. Additionally, surplus or stale bread can be used to produce fungi burgers by applying solid-substrate fermentation such as oncom, treating bread as the sole substrate for fungal growth.

Oncom is a fermented food from West Java traditionally made from by-products, such as peanut press cake or residue after tofu or cassava starch production [9]. During the

fermentation process, moulds from the *Neurospora* genus, considered a Generally Recognized as Safe (GRAS) organism, are utilized [10]. This traditional method of converting food waste into products for consumption appears suitable for bread fermentation as an alternative substrate.

To date, several articles related to the possibility of using this type of fermentation in the management of stale and surplus bread have been published [11–14]. Fungi burgers obtained with *N. intermedia* were quite well received by consumers [11]. They can be considered meat substitutes, and similarly to tempe, which is more recognizable in Western countries, they can serve as a valuable source of protein in vegetarian diets. Following a vegetarian and vegan diet for a long period can be associated with deficiencies in some essential nutrients, including iron, long-chain omega-3 fatty acids, and vitamin B12 [15,16].

Vitamin B12 can only be synthesized by bacteria and archaebacteria. In the human diet, the best sources of this vitamin are animal products, namely milk, cheese, eggs, and meat [16]. The recommended cobalamin intake depends on age and physiological status; for healthy adults, it is 2.4 μg/day [17]. Cobalamin in the appropriate form, namely methylcobalamin, plays a role in methionine biosynthesis as a coenzyme of methionine synthase. On the other hand, 5′-deoxyadenosylcobalamin has a role in branched-chain amino acid and odd-chain fatty acid catabolism as a coenzyme of methylmalonyl-CoA mutase [15]. The effects of vitamin B12 deficiency can manifest as pernicious anaemia through the alteration of erythropoiesis and as correct nerve transmission disorders [16]. In light of this, there is a necessity for vitamin B12 supplementation among individuals following a vegetarian and vegan diet. This can be achieved by consuming supplements containing vitamin B12 or by utilizing fortified foods. Typical fortified products available on the Polish market include breakfast cereals, juices, nectars, and plant-based beverages [18].

Over the past few years, the enrichment of food products with vitamin B12 through in situ fermentation has increased [19]. *Propionibacterium freudenreichii*, used in Swiss cheese production, has GRAS status [20] and qualified presumption of safety (QPS) status [21]. It is the only GRAS source to produce an active form of vitamin B12 suitable for human consumption [19].

From Indonesia, similar to oncom, comes the more well-known tempe, a product derived from the solid-state fermentation of soybeans (a traditional substrate) involving moulds from the *Rhizopus* genus. Reports on the presence of vitamin B12 in tempe go as far back as the early 80s [22]. The bacteria *Klebsiella pneumoniae* and *Citrobacter freundii* have been shown to be producers of this vitamin. These species can accompany the main mould in traditional tempe starters, ragi tempe [23]. However, to date, few studies have been published on this issue. In addition to those mentioned, the production of tempe by co-fermentation with *P. freudenreichii* was undertaken by the teams of Krusong et al. [24], Wolkers-Rooijackers et al. [25], and Signorini et al. [26]. The need to produce tempe enriched in vitamin B12, which could become an alternative source of this vitamin in the diets of vegans/vegetarians, was also recognised by Kustyawati et al. [27].

To the best of our knowledge, no one has attempted to enrich oncom with vitamin B12 by means of solid-state fermentation.

The main aim of this study was to obtain red oncom from stale bread enriched in vitamin B12 by *Propionibacterium freudenreichii* activity. The secondary goal was to determine the influence of the chemical composition and pH of the bread used, and the effect of *P. freudenreichii* strain, on the levels of vitamin B12 in the oncom.

2. Materials and Methods

2.1. Fermentation Substrates

As fermentation substrate, the following breads were used: stale sourdough wheat bread and stale light rye sourdough bread, dried at 70 °C (from the artisan bakery Handelek, Kraków, Poland), and dried returned wheat yeast Okin bread, specifically the Big Gourmet (from the industrial bakery Artadi Alimentación S.L, Zumaia, Spain), kindly provided by Rebread Upcycled Food sp. z o.o. (Kraków, Poland).

All breads were ground in a meat mincer (sieve ø = 2.7 mm; type 887, ZELMER, Warsaw, Poland). Next, the sourdough wheat and rye bread were mixed in a 1:1 ratio (mixed bread). Mixed bread and Okin bread were roasted at 250 °C for 2 min in a layer approximately 0.5 cm thick (PolEko SLW115 STD (POL-EKO, Wodzisław Śląski, Poland) dryer was used for roasting). This method of preparing dry bread ensured the microbiological purity of the fermentation substrate.

2.2. Neurospora Sitophila Inoculum for the Production of Red Oncom—Preparation of Rice Starter

The *N. sitophila* strain was sourced from an oncom starter produced by the company Agrotekno.lab in Bandung, Indonesia (Ragi Oncom; *Neurospora sitophila* fungi), purchased through Raprima, Indonesia. To isolate a pure mould culture, a portion of the starter suspension in sterile water was spread onto Petri dishes containing sterile PDA (potato dextrose agar) medium. After cultivation at 30 °C in darkness for 48 h, visible *N. sitophila* spores were collected using a sterile loop and transferred to a fresh sterile medium. Multiple passages were performed.

As a substrate for mould growth, 50 g of rice flour sterilized in an autoclave (20 min, 121 °C) and hydrated to 40% with sterile water was used. The flour mixture was inoculated with three loops of *N. sitophila* mycelium and spores from the PDA medium, thoroughly mixed, and spread onto a larger Petri dish, compacted, and closed. After two days of incubation (upon the appearance of white mycelium), the lid was removed. On the fifth day, the humidity in the chamber was reduced, and the temperature increased to 40 °C for two days to dry the starter under mild conditions. The prepared starter was stored in a refrigerator at around 4 °C.

2.3. Inoculum of Propionic Bacteria

Two strains of *Propionibacterium freudenreichii* were used in the experiment. One was obtained from an easily accessible, cheap dairy starter culture, the other from a microbiological collection.

2.3.1. *Propionibacterium freudenreichii* ssp. *shermanii* Dairy Starter Culture

'PP-starter culture' (Biochem s.r.l), containing *P. freudenreichii* ssp. *shermanii*, was purchased through the online store Serowar.

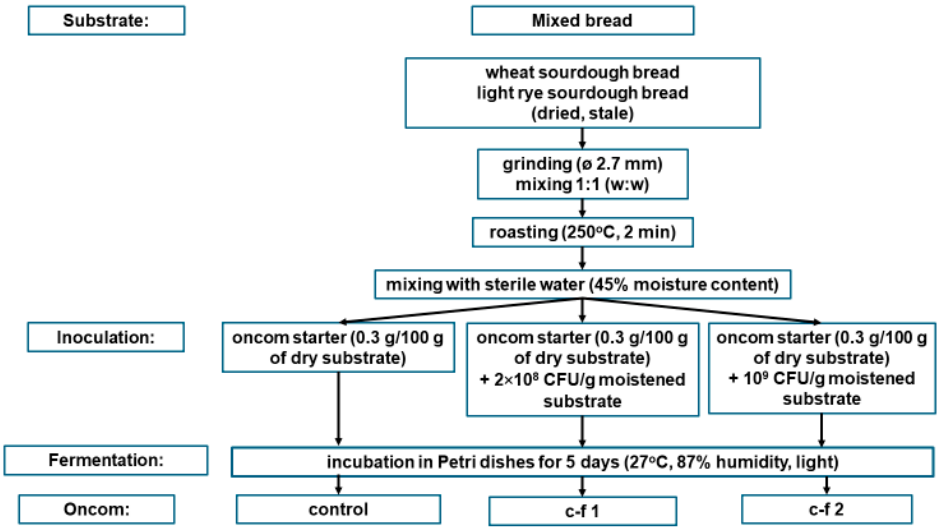
A total of 300 mg of starter culture was weighed into a sterile Falcon tube, and 5 mL of saline with Tween 80 was added. A total of 1 mL of the suspension was transferred to a flask with 50 mL of medium (containing 0.1% casein peptone, 0.05% yeast extract, and 0.1% sodium lactate, pH 7.0–7.2) and incubated at 30 °C. After 48 h, the bacteria were centrifuged in sterile Falcons. The precipitate was resuspended in sterile distilled water and the density of the bacterial cell suspension was determined from absorbance measurements at λ = 600 nm, and a standard curve in the McFarland method.

2.3.2. *Propionibacterium freudenreichii* DSM 20271 Strain

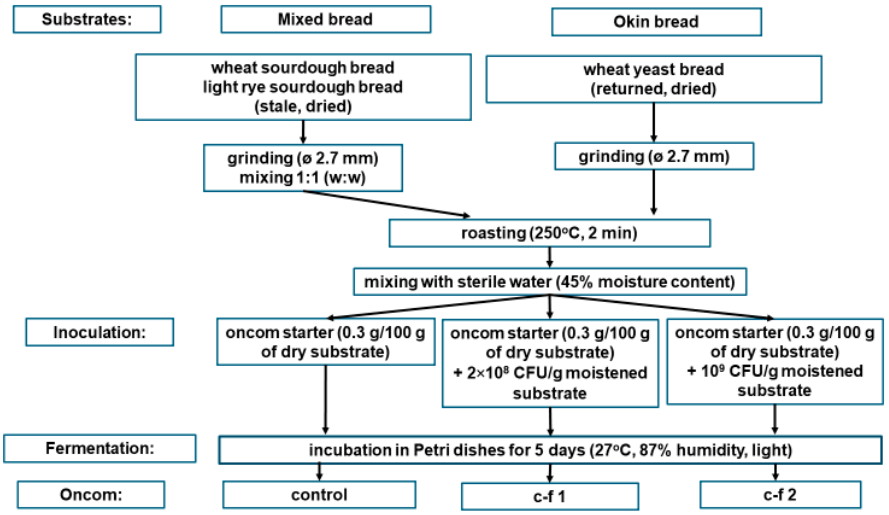
P. freudenreichii DSM 20271 (Leibniz Institut-DSMZ German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was previously applied in bacterial-fungal co-fermentation [25]. The strain was activated according to the manufacturer's recommendations, and then the bacteria were cultured in a liquid medium, and the procedure described above was followed thereafter.

2.4. Fermentation Experiment

Two separate fermentation experiments, I and II, were performed, as presented in Schemes 1 and 2, respectively. In Experiment I, mixed bread as a fermentation substrate and *P. freudenreichii* strain from dairy starter culture were used. In Experiment II, mixed bread and Okin bread as fermentation substrates and *P. freudenreichii* DSM 20271 strain were used.



Scheme 1. Schematic representation of Experiment I with *P. freudenreichii* strain from dairy starter culture.



Scheme 2. Schematic representation of Experiment II with *P. freudenreichii* DSM 20271 strain.

To 250 g of roasted bread, cooled to room temperature, 0.75 g of powdered oncom starter (0.3 g/100 g of dry substrate) was added, along with a sufficient amount of sterile water to achieve a final substrate moisture content of 45% (control oncom). Additionally, for two portions of the substrate, besides the oncom starter, a suspension of *P. freudenreichii* cells containing either 2×10^8 CFU/g FW or 10^9 CFU/g FW was introduced (oncom after co-fermentation—c-f 1 and c-f 2, respectively). The material was thoroughly mixed and spread, firmly compacted, onto sterile Petri dishes (ø = 9 cm). Each portion of the prepared substrate was spread onto 6 Petri dishes. Cultivation was carried out in a climatic chamber (Pol_Eko Aparatura, Wodzisław Śląski, Poland, Type KK 115 Top+/Fit P) under the following conditions: temperature 27 °C, humidity 87%, light (70%), fan (25%) for 5 days.

2.5. Determination of Dry Mass, pH and Tritatable Acidity in Fresh Oncom

In fresh oncom, dry mass was determined using the weighing method (drying at 105 °C for 3 h, followed by additional drying for 0.5 h). pH value and titratable acidity were measured as well.

For pH determination, 5 g of product (two replicates), taken with a sterile scalpel from two dishes of a given oncom variant, was crushed in 20 mL of redistilled water and stirred using a rotator (kamush® LP360AMP, Kamush, Zblewo, Poland) for 30 min. After this time,

pH was measured using a pH meter (CP-401, Elmetron, Zabrze, Poland). The material was then transferred quantitatively to beakers using 20 mL of redistilled water and acidity was determined by potentiometric titration. Samples were titrated with 0.1 M NaOH until a pH of 8.2 was reached. Titratable acidity was expressed in degrees, i.e., the amount of mL of 1 M NaOH needed to neutralize the acids present in 100 g of material.

2.6. Determination of the Amount of P. freudenreichii (CFU) after Oncom Fermentation

A total of 10 g (in duplicate) of product taken from two Petri dishes of each oncom variant obtained after co-fermentation in Experiments I and II were weighed into a sterile plastic bag, and 90 mL of sterile saline was added and homogenized for 2 min (Stomacher 80, Seward Medical London SE1 1PP, UK). Subsequently, the pour plate method (in triplicate) of the supernatants, suitably diluted in saline, was performed in a medium containing 0.1% casein peptone, 0.05% yeast extract, 0.1% sodium lactate, and 1.5% agar with 100 IU/mL nystatin. The Petri dishes were incubated in a climatic chamber at 27 °C for 48 h. The CFU reading was taken on a manual bacterial colony counter (LBB 2002, Pol-Eko Aparatura, Wodzisław Śląski, Poland).

2.7. Determination of Chemical Composition of Oncom

The remaining oncom product was dried under mild conditions (55 °C) for about 20 h. It was ground in a planetary ball mill (BKBM-V2, Biobase, Jinan, China) and stored at about 4 °C until analysis.

In the dried material, dry mass was determined by weight method, protein by the Kjeldahl method, using a conversion factor of the amount of amino nitrogen to protein of 6.25, total fat by weight method after extraction in a Soxhlet apparatus [28], and ash by weight method after combustion in a muffle furnace (type SM 2002, Czylok, Jastrzębie-Zdrój, Poland) at 550 °C [29]. All analyses were performed in triplicate. Total carbohydrate content was calculated from the difference [100-(protein content + fat content + ash content)]. In addition, reducing sugars in aqueous extracts (1 g in 20 mL) was determined using the DNS method [30]. The determinations were made in 4 replicates.

2.8. Determination of Vitamin B12

In each variant of the oncom product, vitamin B12 was determined using the microbiological method (VitaFast®Vitamin B12, Institut für Produktqualität GmbH, Berlin, Germany, AOAC-RI-Certificate No. 101002).

Preparation of the extract: 1 g of ground oncom was weighed into a Falcon tube (50 mL), to which 20 mL of acetate buffer (0.1 M, pH 4.5) and 250 µL of 1% NaCN solution were added. The mixture was mixed thoroughly. Taka-Diastase (Taka-Diastase from *Aspergillus oryzae* 100 U/mg, Sigma-Aldrich, Saint Louis, MO, USA) was added (5 mg) and stirred. All samples were then incubated at 37 °C in the dark for one hour. Next, the extract was made up to 40 mL with redistilled water. Falcon tubes were heated in a 96 °C water bath for 30 min. Then, they were immediately cooled in cold water and then centrifuged (9000 rpm, 10 min). The supernatant from each sample was transferred to separate sterile Eppendorf tubes. Extracts were made in duplicate.

Further procedures were carried out strictly according to the manufacturer's recommendations for the vitamin B12 assay kit. Different dilutions of extracts were prepared and each was introduced into 3 wells on a microplate. A microplate reader (TECAN, Männedorf, Switzerland) was used to read absorbance at λ = 540 nm.

2.9. Statistical Analysis

Data were statistically processed in Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) using a one-way analysis of variance and Fisher's test to determine statistically significant differences at $p \leq 0.05$.

3. Results

3.1. Characteristics of Fermentation Substrates

In this experiment, dried bread from two different sources was used. Table 1 presents the basic chemical composition on a dry weight basis and the initial pH of the substrate prepared for inoculation.

Table 1. Basic chemical composition of dried bread—fermentation substrate.

Bread Type	Protein (g/100 g DM)	Lipids (g/100 g DM)	Carbohydrates (g/100 g DM)	Sugars (g/100 g DM)	pH
Mixed bread	10.65	0.63	82.62	1.08	4.27
Okin bread	11.7 [1]	1.25 [1]	79.04 [1]	1.76 [1]	5.69

[1] Data according to Okin bread manufacturer's declaration.

The fermentation substrates differed in composition. Okin bread had two times more lipids and 63% more sugars than bread from the Handelek bakery. In addition, Okin bread had a significantly higher pH than mixed bread.

3.2. Experiment I

Experiment I was performed on mixed bread with the oncom stater and *P. freudenreichii* isolated from the 'PP-starter culture' (Biochem s.r.l, Rome, Italy) as presented in Scheme 1.

3.2.1. Changes in Dry Mass, pH, Titratable Acidity and *P. freudenreichii* Amounts after Oncom Fermentation

All the obtained oncom products had similar dry mass after cultivation, around 36% (Table 2). It is worth noting that fermentation substrates inoculated with starters had a dry mass of 55%. Hence the recorded approx. 50% decrease in the dry weight of the material taking place during cultivation. A similar pH value was determined in all oncom variants, at an average of 5.7. Therefore, it can be concluded that an increase in pH of about 1.4 units took place during the fermentation process. The titratable acidity, expressed in degrees, in the fresh oncom product, regardless of the fermentation variant, averaged 7.4.

Table 2. Dry mass, pH, and titratable acidity after mixed bread fermentation.

Oncom [1]	Dry Mass (%)	Dry Mass Loss (%)	pH	Titratable Acidity (Degrees) [2]
control	36.12 ± 0.53 a [3]	50.00 ± 0.27 a	5.68 ± 0.05 a	8.20 ± 1.19 a
c-f 1	34.15 ± 2.29 a	54.02 ± 5.55 a	5.82 ± 0.05 a	6.88 ± 0.15 a
c-f 2	38.43 ± 0.29 b	46.13 ± 1.85 a	5.72 ± 0.03 a	7.26 ± 0.81 a

[1] control—oncom fermented with *N. sitophila*; c-f 1—oncom after fermentation by *N. sitophila* and 2×10^8 CFU/g FW of *P. freudenreichii*; c-f 2—oncom after fermentation by *N. sitophila* and 10^9 CFU/g FW of *P. freudenreichii*. [2] mL of 1 M NaOH needed to neutralize acids in 100 g of oncom. [3] Data are shown as the mean ± SD. Mean values within a column followed by different letters differ significantly ($p \leq 0.05$).

After cultivation using the plate method, it was determined that in oncom c-f 1, there were 1.2×10^5 CFU/g FW, while in oncom c-f 2, there were 1.4×10^6 CFU/g FW. This indicates that the number of living bacterial cells during the cultivation period decreased compared to the amount introduced in the inoculum.

3.2.2. Chemical Composition of Oncom and Vitamin B12 Level

The resulting oncom products from mixed bread, regardless of the fermentation variant, were characterized by an average of 22% protein content in dry mass, two times higher in relation to the fermentation substrate (Tables 1 and 3). The fermentation process also increased lipid levels, 8-fold for the control product and 5-fold for the co-fermentation variants. In the fermentation products, a lower amount of total carbohydrates per dry

weight was determined than in the substrate. However, a more than 10 times higher level of reducing sugars was measured (Tables 1 and 3).

Table 3. Chemical composition of oncom obtained from mixed bread.

Oncom [1]	Protein (g/100 g DM)	Lipids (g/100 g DM)	Ash (g/100 g DM)	Carbohydrates (g/100 g DM)	Reducing Sugars (g/100 g DM)	Vitamin B12 (µg/100 g DM)
control	22.74 ± 0.20 a [2]	5.51 ± 0.10 b	7.08 ± 0.16 b	65.01	12.99 ± 0.68 a	0.41 ± 0.00
c-f 1	22.38 ± 1.71 a	3.61 ± 0.15 a	7.76 ± 0.10 c	66.48	11.98 ± 1.10 a	0.72 ± 0.0
c-f 2	21.06 ± 2.89 a	3.49 ± 0.13 a	6.79 ± 0.05 a	68.88	13.26 ± 1.44 a	0.56 ± 0.0

[1] control—oncom fermented with *N. sitophila*; c-f 1—oncom after fermentation by *N. sitophila* and 2×10^8 CFU/g FW of *P. freudenreichii*; c-f 2—oncom after fermentation by *N. sitophila* and 10^9 CFU/g FW of *P. freudenreichii*. [2] Data are shown as the mean ± SD. Mean values within a column followed by different letters differ significantly ($p \leq 0.05$).

The introduction of *P. freudenreichii* isolated from the 'PP starter culture' into the inoculum allowed the oncom to be enriched in vitamin B12. In this part of the experiment, no proportional relationship between the amount of CFU introduced into the substrate and the determined level of the vitamin was observed. The oncom obtained with a lower dose of bacteria in the inoculum proved to be a better option. The level of vitamin B12 in this product was 75% higher compared to the initial level, determined in the fermentation substrate (Table 3).

3.3. Experiment II

Experiment II was conducted on mixed bread and Okin bread, with oncom starter and *P. freudenreichii* DSM 20271, as presented in Scheme 2.

3.3.1. Changes in Dry Mass, pH, Titratable Acidity, and *P. freudenreichii* Amounts after Oncom Fermentation

The dry mass content in the obtained oncom products was similar, averaging 40%. After fermentation, large dry mass losses were again determined, although slightly smaller than in Experiment I, reaching a maximum of 43% (Tables 2 and 4). The pH values determined in the oncom ranged from 5.35 to 5.7 (Table 4) and again were higher by over 1 unit in the case of mixed bread compared to the initial value, while in the case of Okin bread, they were at the level indicated in the substrate (Table 1). The titratable acidity in the products was at a similar level of about 10–11 degrees. The highest value, significantly higher than the others, was determined in the oncom obtained with mixed bread after the co-fermentation process with a lower dose of bacteria. It is worth noting at this point that in Experiment II, slightly higher titratable acidity levels were determined for oncom from mixed bread than in Experiment I, with similar indications of pH values. This may be indicative of the higher buffering capacity of the resulting products.

Table 4. Dry mass, pH, and titratable acidity after mixed bread and Okin bread fermentation.

Oncom [1]		Dry Mass (%)	Dry Mass Loss (%)	pH	Titratable Acidity (Degrees) [2]
Mixed bread	control	40.17 ± 0.97 a [3]	41.69 ± 2.07 c	5.57 ± 0.03 cd	10.19 ± 0.48 ab
	c-f 1	42.15 ± 0.74 a	38.69 ± 2.23 ab	5.50 ± 0.10 bc	13.06 ± 1.52 c
	c-f 2	38.12 ± 1.36 a	43.27 ± 1.64 c	5.67 ± 0.01 d	9.55 ± 0.60 a
Okin bread	control	42.62 ± 1.03 a	37.33 ± 2.01 a	5.41 ± 0.09 ab	10.10 ± 0.13 ab
	c-f 1	40.50 ± 2.37 a	41.33 ± 0.94 bc	5.35 ± 0.06 a	11.68 ± 0.93 bc
	c-f 2	38.96 ± 1.36 a	43.34 ± 1.37 c	5.70 ± 0.00 d	10.19 ± 0.31 ab

[1] control—oncom fermented with *N. sitophila*; c-f 1—oncom after fermentation by *N. sitophila* and 2×10^8 CFU/g FW of *P. freudenreichii*; c-f 2—oncom after fermentation by *N. sitophila* and 10^9 CFU/g FW of *P. freudenreichii*. [2] mL of 1 M NaOH needed to neutralize acids in 100 g of oncom. [3] Data are shown as the mean ± SD. Mean values within a column followed by different letters differ significantly ($p \leq 0.05$).

After fermentation, in oncom products obtained from mixed bread, 2.6×10^8 CFU/g FW was determined in the c-f 1 variant, and 3×10^8 CFU/g FW in the c-f 2 variant. Similar amounts of CFU were determined in fresh 5-day oncom made from Okin bread, in the c-f 1 variant it was 2.2×10^8 CFU/g FW, and in c-f 2 2.6×10^8 CFU/g FW. This means that the amount of viable bacterial cells of strain DSM 20271 in the course of the culture did not decrease or decreased slightly compared to the amount introduced into the inoculum.

3.3.2. Chemical Composition of Oncom and Vitamin B12 Level

The protein content of oncom obtained from mixed bread in Experiment II was slightly lower than in Experiment I (Tables 3 and 5), averaging at just over 18%. Slightly higher levels of protein (22%) were determined in products obtained from Okin bread. Again, it can be concluded that the fermentation process resulted in the enrichment of the product in protein compared to the initial substrate (in this case from 65 to 95%). The obtained oncom was characterized by a higher level of lipids per dry weight than the stale bread prepared for fermentation. The change in lipid levels ranged from 40% (Okin bread c-f 2) to 550% (mixed bread c-f 2). In addition, the fermentation process again resulted in a significant accumulation of reducing sugars in the product. There were 11-fold changes in the oncom made with mixed bread and a maximum of 7-fold changes in the oncom made with Okin bread (c-f 2).

Table 5. Chemical composition of oncom.

Oncom [1]		Protein (g/100 g DM)	Lipids (g/100 g DM)	Ash (g/100 g DM)	Carbohydrates (g/100 g DM)	Reducing Sugars (g/100 g DM)	Vitamin B12 (µg/100 g DM)
Mixed bread	control	18.51 ± 0.46 b [2]	1.72 ± 0.06 a	5.96 ± 0.05 e	73.79	11.81 ± 0.72 b	0.25 ± 0.00 a
	c-f 1	17.55 ± 0.38 a	2.75 ± 0.27 c	5.70 ± 0.09 d	73.98	11.73 ± 1.48 b	0.37 ± 0.0 b
	c-f 2	18.34 ± 0.39 b	4.14 ± 0.12 d	5.42 ± 0.01 c	72.24	11.61 ± 0.22 b	2.22 ± 0.0 d
Okin bread	control	21.08 ± 0.35 c	2.32 ± 0.05 b	4.62 ± 0.05 a	71.95	6.20 ± 0.016 a	0.25 ± 0.0 a
	c-f 1	22.46 ± 0.27 d	2.75 ± 0.12 c	5.04 ± 0.03 b	69.74	6.15 ± 0.60 a	0.78 ± 0.0 c
	c-f 2	22.87 ± 0.40 d	1.76 ± 0.10 a	6.08 ± 0.03 f	69.28	12.52 ± 0.15 b	3.31 ± 0.0 e

[1] control—oncom fermented with *N. sitophila*; c-f 1—oncom after fermentation by *N. sitophila* and 2×10^8 CFU/g FW of *P. freudenreichii*; c-f 2—oncom after fermentation by *N. sitophila* and 10^9 CFU/g FW of *P. freudenreichii*. [2] Data are shown as the mean ± SD. Mean values within a column followed by different letters differ significantly ($p \leq 0.05$).

In the control oncom, obtained only with *N. sitophila*, 0.25 µg/100 g DM vitamin B12 was recorded, regardless of the type of bread. The introduction of *P. freudenreichii* DSM 20271 into the inoculum resulted in the enrichment of the oncom in the vitamin. Changes in vitamin levels were dependent on the CFU/g FW dose and the type of fermentation substrate (Table 5). The highest amount of vitamin B12 was found in oncom made with Okin bread inoculated with a higher dose of bacterial cells. The level of vitamin in this product was 13 times higher compared to the control oncom and 50% higher compared to the analogous product after mixed bread fermentation (Table 5).

4. Discussion

In this experiment, oncom was obtained from stale bread using a cultivation method previously developed. Co-fermentation products were prepared using a standard procedure, except that at the inoculation stage, *P. freudenreichii* cells were additionally introduced into the substrate. It should be noted that the oncom obtained by co-fermentation did not differ in appearance, smell, or taste from the control oncom. While a sensory test was not conducted, the above observations were made by individuals conducting this experiment and familiar with the typical characteristics of the bread-based oncom produced in the unit's laboratory. Figure 1 shows the appearance of a typical oncom from mixed bread after fermentation on a Petri dish.

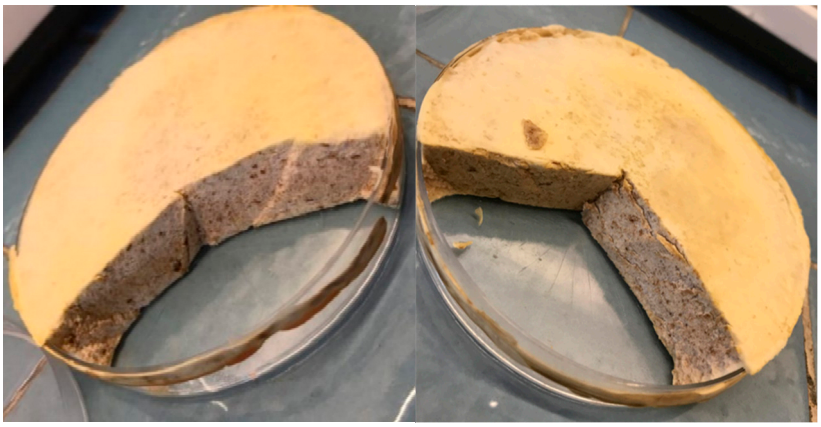


Figure 1. Examples of mixed bread oncom products from Experiment I: control on the left, c-f 1 on the right (photo: P. Majka).

The level of protein in products after 5-day fermentation was around 20%, regardless of the experiment and the kind of bread. This was in line with the observations of Gmoser et al. [31], who determined 21% (total amino acid residues) after a 6-day fermentation of stale bread with *N. intermedia*, and with those of Hellwig et al. [32], who measured 19.6 to 21.9% (laboratory Petri dish culture, in household conditions). Mycelial activity contributes to the enrichment of the final product in protein. Similar changes in protein levels in the grain products were determined by Starzyńska-Janiszewska et al. [33], from 13 to 21%, after fermentation of red quinoa with *N. intermedia*.

In this experiment, an increase in the level of fat was noted. Gmoser et al. [31] also observed such a tendency, an increase from 2.4% in stale bread to as much as 10.5% in 6-day oncom. Hellwig et al. [32] noted an increase, from roughly 1% in the substrate to 4–5% depending on the culture type. The increase in lipid content in high-carbohydrate substrates is not surprising, as moulds produce lipids, which they incorporate into cellular structures, utilizing other carbon and energy sources—primarily carbohydrates in this case. The rise in lipid content observed after the fermentation might also result from the utilization of non-lipid components, altering the percentage contribution of basic components in the dry mass of the product [34]. Although moulds of the *Neurospora* genus exhibit lipolytic properties, the utilization of lipid substrates depends on their level in the material. In contrast to the bread experiment, flaxseed oil-cake fermentation with *N. intermedia* resulted in a reduction in the fat content of the product, from 9.1 to 7.7 g/100 g DM [35]. A similar trend was observed in tempe fermentations with *Rhizopus oligosporus* [36], where the growth of the strain in the lower-fat substrate (white quinoa) resulted in an enrichment of fat in the product, while the opposite relationship was noted for the more fat-rich coloured quinoa.

Hellwig et al. [32] reported a decrease in starch content after fermentation of leftover bread with *N. intermedia*. In the case of bread fermentation in our experiments, an increase in the level of reducing sugars was observed, from seven- to eleven-fold. Amylolytic enzyme activity was detected by Kumbhare after 48 h of *N. intermedia* fermentation on a typical oncom substrate of peanut press cake [37], as well as by Shahryari et al. [38] in the conditions of submerged fermentation.

From the results obtained in both experiments, it is clear that the amount of vitamin B12 determined in oncom products depended significantly on the strain of *P. freudenreichii*, on the CFU dose used as inoculum, but also significantly on the type of culture medium—in this case, stale bread.

The number of bacteria determined after 5 days of culture in both experiments was lower than the amount introduced into the fermentation substrate. This either means that *P. freudenreichiii* had unfavourable conditions for growth or that after such a long time (5 days) the culture went into the die-off phase. A significantly higher amount of CFU was determined in the second experiment regardless of the type of substrate used. It is

known, however, that these bacteria poorly proliferate when growing in a solid medium. Additionally, their growth depends on ensuring anaerobic conditions and optimal pH, which typically oscillates around 7.0 [19]. The higher levels of vitamin B12 determined when Okin bread was used as a fermentation substrate compared to mixed bread, may have been related to a more favourable pH value at the start of the culture (5.7 vs. 4.3), but also a higher content of sugars (1.76/100 g DM vs. 1.08/100 g DM). Bacteria from the species *P. freudenreichii* primarily utilize carbohydrates as a carbon source, including glucose, lactose, fructose, ribose, and galactose, as well as organic acids such as lactic acid [19]. Krusong et al. [24] pointed out the significant influence of substrate pH during fungal-bacterial co-fermentation aimed at enriching tempe with vitamin B12. Attempts at simultaneous co-fermentation of *Rhizopus oligosporus* and *P. shermanii* with the addition of *Lactobacillus casei* resulted in less success, leading to the maintenance of acidic pH over the period of time.

In the cited work, vitamin B12 was determined in tempe at a level of 0.18 µg/100 g DM [24]. Significantly higher levels of the vitamin in tempe from lupine were obtained by Signorini et al. [26], at 1.2 µg of the vitamin in 1 g DM. The authors ensured that the initial pH of the fermentation substrate exceeded 5 (5.3–5.5), and after cultivation, the pH increased to a level of approximately 7.0. This increase in pH may have promoted the proliferation of *P. freudenreichii* and the production of vitamin B12. Comparing, in turn, the density of the bacterial inoculum, it must be said that Signorini et al. [26] introduced significantly more CFU into the substrate than in our experiments.

Values of vitamin B12 in the produced tempe from lupin at levels similar to oncom from Okin bread (dose of 10^9 CFU/g substrate) were obtained by Wolkers-Rooijackers et al. [25] using the same *P. freudenreichii* strain DSM 20271 (0.97 vs. 1.3 g/100 g fresh weight). Despite initially using a CFU dose two orders of magnitude smaller than in our experiment, these authors likely observed bacterial proliferation due to the more favorable pH for bacterial growth (6–7) or a shorter fermentation time. This could have resulted in obtaining a comparable amount of vitamin B12 as in the case of using a higher inoculum dose. The same authors noted a correlation between the CFU dose in the inoculum and the level of vitamin B12 produced, which is confirmed in our study of this particular strain of propionic bacteria. Xie et al. [39], on the other hand, found that the limiting factor in the synthesis of vitamin B12, even under conditions favourable to the growth of *P. freudenreichii* (access to substrates, optimal pH), may be the depletion of Co resources in the fermentation substrate, necessary for the synthesis of new cobalamin molecules.

In fresh oncom, the maximum content of vitamin B12—in the variant made with Okin bread after co-fermentation with a higher dose of *P. freudenreichii* DSM 20271—was about 1.30 µg/100 g, which corresponds to the typical level in a chicken egg [15]. The oncom obtained after culture conducted in a Petri dish, which could be directly consumed as a fungi burger, weighed an average of 40 g. Thus, consuming such a serving of oncom could cover 20% of the daily requirement for vitamin B12.

Author Contributions: Conceptualization, B.S. and A.S.-J.; validation, B.S. and D.P; formal analysis, B.S. and D.P.; investigation, B.S., A.S.-J. and D.P.; resources, D.P.; writing—original draft preparation, B.S.; writing—review and editing, A.S.-J.; visualization, B.S.; project administration, D.P.; funding acquisition, B.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Rebread Upcycled Food sp. z o.o. (Kraków, Poland), grant number P/406/2022/WTŻ.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: The authors thank Patrycja Majka for technical support.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of this study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Available online: https://bdl.stat.gov.pl/bdl/dane/podgrup/wymiary/3/10/2456 (accessed on 24 February 2024).
2. Raport "Nie Marnuj Jedzenia 2023" 16 Października 2023. Available online: https://bankizywnosci.pl/swiatowy-dzien-zywnosci-2023/ (accessed on 24 February 2024).
3. Goryńska-Goldman, E.; Gazdecki, M.; Rejman, K.; Kobus-Cisowska, J.; Łaba, S.; Łaba, R. How to prevent bread losses in the baking and confectionery industry?—Mesurement, causes, management and prevention. *Agriculture* **2021**, *11*, 19. [CrossRef]
4. Melikoglu, M.; Lin, C.S.K.; Webb, C. Stepwise optimisation of enzyme production in solid-state fermentation of waste bread pieces. *Food Bioprod. Process.* **2013**, *91*, 638–646. [CrossRef]
5. Gmoser, R.; Sintca, C.; Taherzadeh, M.J.; Lennartsson, P.R. Combining submerging and solid-state fermentation to convert waste bread into protein and pigment using the edible filamentous fungus *N. intermedia*. *Waste Manag.* **2019**, *97*, 63–70. [CrossRef] [PubMed]
6. Svensson, S.E.; Bfova, L.; Ferreira, J.A.; Souza Filho, P.F.; Taherzadeh, M.J.; Zamani, A. Valorization of bread waste to a fiber and protein-rich fungal biomass. *Fermentation* **2021**, *7*, 91. [CrossRef]
7. Meral, H.; Karaoğlu, M.M. The effect of the stale bread flour addition on flour and bread quality. *Int. J. Food Eng.* **2020**, *16*, 20190100. [CrossRef]
8. Yuksel, F.; Kayacier, A. Effects of addition of stale bread flour on the acrylamide, fatty acid composition, resistant starch content, and in vitro glycemic index in wheat chips production using response surface methodology. *LWT* **2022**, *161*, 113354. [CrossRef]
9. Surono, I.S. Ethnic fermented foods, and beverages of Indonesia. In *Ethnic Fermented Foods and Alcoholic Beverages of Asia*; Tamang, J.P., Ed.; Springer: New Delhi, India, 2016; pp. 341–382.
10. Perkins, D.D.; Davis, R.H. Evidence of the safety of *Neurospora* species for academic and commercial uses. *Appl. Environ. Microbiol.* **2000**, *66*, 5107–5109. [CrossRef]
11. Hellwig, C.; Gmoser, R.; Lundin, M.; Taherzadeh, M.J.; Rousta, K. Fungi burger from stale bread? A case study on the perception of a novel protein-rich food product made from an edible fungus. *Foods* **2020**, *9*, 1112. [CrossRef] [PubMed]
12. Wang, R.; Gmoser, R.; Taherzadeh, M.J.; Lennartsson, P.R. Solid-state fermentation of stale bread by an edible fungus in a semi-continuous plug-flow bioreactor. *Biochem. Eng. J.* **2021**, *168*, 107959. [CrossRef]
13. Brancoli, P.; Gmoser, R.; Taherzadeh, M.J.; Bolton, K. The use of life cycle assessment in the support of the development of fungal food products from surplus bread. *Fermentation* **2021**, *7*, 173. [CrossRef]
14. Gmoser, R.; Lennartsson, P.R.; Taherzadeh, M.J. From surplus bread to burger using filamentous fungi at bakeries: Techno-economical evaluation. *Clean. Environ. Syst.* **2021**, *2*, 100020. [CrossRef]
15. Watanabe, F.; Yabuta, Y.; Bito, T.; Teng, F. Vitamin B_{12}-containing plant food sources for vegetarians. *Nutrients* **2014**, *6*, 1861–1873. [CrossRef] [PubMed]
16. Rizzo, G.; Laganà, A.S.; Rapisarda, A.M.C.; La Ferrera, G.M.G.; Buscema, M.; Rossetti, P.; Nigro, A.; Muscia, V.; Valenti, G.; Sapia, F.; et al. Vitamin B12 among vegetarians: Status, assessment and supplementation. *Nutrients* **2016**, *8*, 767. [CrossRef] [PubMed]
17. Human Vitamin and Minerals Requirements. Report of a Joint FAO/WHO Expert Consultation, 2001, Bangkok, Thailand. *Chapter 5. Vitamin B12.* Available online: https://www.fao.org/3/Y2809E/y2809e0b.htm#bm11. (accessed on 15 April 2024).
18. Available online: https://ncez.pzh.gov.pl/ciaza-i-macierzynstwo/plodnosc-i-ciaza/witamina-b12-a-dieta-wegetarianska-i-ciaza (accessed on 24 February 2024).
19. Piwowarek, K.; Lipińska, E.; Hać-Szymańczuk, E.; Kieliszek, M. *Propionibacterium* spp.—Source of propionic acid, vitamin B_{12}, and other metabolites important for the industry. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 515–538. [CrossRef]
20. Meile, L.; Le Blay, G.; Thierry, A. Safety assessment of dairy microorganisms: *Propionibacterium* and *Bifidobacterium*. *Int. J. Food Microbiol.* **2008**, *126*, 316–320. [CrossRef] [PubMed]
21. EFSA Panel on Biological Hazards. The maintenance of the list of QPS microorganisms intentionally added as food or feed. *EFSA J.* **2008**, *923*, 1–48.
22. Okada, N.; Hadioetomo, R.S.; Nikkuni, S.; Katoh, K.; Ohta, T. Vitamin B12 contents of fermented foods in the tropics. *Rep. Natl. Food Res. Inst.* **1983**, *43*, 126–129.
23. Keuth, S.; Bisping, B. Formation of vitamins by pure cultures of tempe moulds and bacteria during the tempe solid substrate fermentation. *J. Appl. Bacteriol.* **1993**, *75*, 427–434. [CrossRef]
24. Krusong, W.; Yongsmith, B.; Sanchez, P.C. Influence of *Lactobacillus casei* in production of high vitamin B_{12}-tempeh. *Agric. Natur. Res.* **1991**, *25*, 458–462.
25. Wolkers-Rooijackers, J.C.M.; Endika, M.F.; Smid, E.J. Enhancing vitamin B_{12} in lupin tempeh by in situ fortification. *LWT* **2018**, *96*, 513–518. [CrossRef]
26. Signorini, C.; Carpen, A.; Coletto, L.; Borgonovo, G.; Galanti, E.; Capraro, J.; Magni, C.; Abate, A.; Johnson, S.T.; Duranti, M.; et al. Enhanced vitamin B12 production in innovative lupine tempeh is due to synergic effects of *Rhizopus* and *Propionibacterium* in cofermentation. *Int. J. Food Sci. Nutr.* **2018**, *69*, 451–457. [CrossRef] [PubMed]
27. Kustyawati, M.E.; Subeki; Murhadi; Rizal, S.; Astuti, P. Vitamin B12 production in soybean fermentation for tempeh. *AIMS Agric. Food* **2020**, *5*, 262–271. [CrossRef]
28. *PN-EN ISO 11085*; Cereal Grains, Products Based on Cereal Grains and Animal Feed-Determination of Crude Fat and Total Fat by Randall Extraction Method. The Polish Committee for Standardization (PKN): Warsaw, Poland, 2010.

29. *ICC-STANDARD, No. 104/1*; Determination of Ash in Cereals and Cereal Products. International Association for Cereal Science and Technology: Stubenring, Austria, 1990.
30. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
31. Gmoser, R.; Fristedt, R.; Larsson, K.; Undeland, I.; Taherzadeh, M.J.; Lennartsson, P.R. From stale bread and brewers spent grain to a new food source using edible filamentous fungi. *Bioengineered* **2020**, *11*, 582–598. [CrossRef] [PubMed]
32. Hellwig, C.; Rousta, N.; Wikandri, R.; Taherzadeh, M.J.; Häggblom-Kronlöf, G.; Blton, K.; Rousta, K. Household fermentation of leftover bread to nutritious food. *Waste Manag.* **2022**, *150*, 39–47. [CrossRef] [PubMed]
33. Starzyńska-Janiszewska, A.; Stodolak, B.; Duliński, R.; Mickowska, B.; Sabat, R. Fermentation of colored quinoa seeds with *Neurospora intermedia* to obtain oncom-type products of favorable nutritional and bioactive characteristics. *Cereal Chem.* **2017**, *94*, 619–624. [CrossRef]
34. Beuchat, L.R.; Worthington, L.E. Changes in the lipid content of fermented peanuts. *J. Agr. Food Chem.* **1974**, *22*, 509–512. [CrossRef]
35. Stodolak, B.; Starzyńska-Janiszewska, A.; Bączkowicz, M. *Aspergillus oryzae* (Koji Mold) and *Neurospora intermedia* (Oncom Mold) application for flaxseed oil cake processing. *LWT* **2020**, *131*, 109651. [CrossRef]
36. Starzyńska-Janiszewska, A.; Duliński, R.; Stodolak, S.; Mickowska, B.; Wikiera, A. Prolonged tempe-type fermentation in order to improve bioactive potential and nutritional parameters of quinoa seeds. *J. Cereal Sci.* **2016**, *71*, 116–121. [CrossRef]
37. Kumbhare, P.H. Activity assay of the enzymes amylase, protease, and lipase produced by *N. intermedia* MTCC 1230 and *R. oligosporus* MTCC 556 during peanut press cake fermentation. *Int. J. Res. Biosci. Agric. Technol.* **2015**, *2*, 183–187.
38. Shahryari, Z.; Fazaelipoor, M.M.; Ghasemi, Y.; Lennartson, P.R.; Taherzadeh, M.J. Amylase and xylanase from edible fungus *Neurospora intermedia*: Production and characterisation. *Molecules* **2019**, *24*, 721. [CrossRef] [PubMed]
39. Xie, C.; Coda, R.; Chamlagain, B.; Varmanen, P.; Piironen, V.; Katina, K. Co-fermentation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* in wheat bran for in situ production of vitamin B12. *Front. Microbiol.* **2019**, *10*, 1541. [CrossRef] [PubMed]