

THE ROLE OF *Chlorella vulgaris* SUPPLEMENTATION IN *Lactiplantibacillus plantarum* FERMENTATION ACTIVITY

Diajeng Sriyana Saraswati^{1*}, Bustari Hasan¹, Sumarto¹,
Tjipto Leksono¹, Dian Iriani¹, Yusmarini²

¹Department of Fisheries Product Technology, Faculty of Fisheries and Marine,
Universitas Riau, Pekanbaru, 28293 Indonesia

²Department of Agriculture Technology, Faculty of Agriculture,
Universitas Riau, Pekanbaru, 28293 Indonesia

* diajeng.sriyana7863@grad.unri.ac.id

ABSTRACT

Chlorella vulgaris, a unicellular green microalga, is widely recognized for its rich nutritional profile and bioactive compounds that support microbial growth, making it a promising ingredient in functional food development. Supplementation in lactic acid bacteria (LAB) fermentation may enhance bacterial activity and improve product quality. This study aimed (1) to evaluate the nutritional composition of *C. vulgaris* and (2) to assess the effect of its supplementation in LAB growth substrates on the fermentation activity of two probiotic strains: *Lactiplantibacillus plantarum* 1 R 11.1.2 and *L. plantarum* 1 R 1.3.2. Four concentrations of *C. vulgaris* (0.25%, 0.5%, 0.75%, 1%) were applied, and proximate composition (moisture, ash, protein, fat, fiber, carbohydrate by difference) and fermentation parameters (pH value, total LAB count, total titratable acidity) were observed. Proximate analysis showed *C. vulgaris* contained 4.35% moisture, 6.72% ash, 30.01% protein, 1.24% fat, 15.10% fiber, and 42.58% carbohydrate (by difference), with a pH of 6.59. Fermentation results indicated that supplementation increased LAB growth (>9.49 log CFU/ml), accelerated pH reduction (4.67–4.34), and enhanced titratable acidity (>1.85%). *L. plantarum* 1 R 11.1.2 showed the strongest effect, with higher growth and acidification than *L. plantarum* 1 R 1.3.2. In conclusion, *C. vulgaris* supplementation, particularly at higher concentrations, significantly improved the fermentation performance of *L. plantarum* 1 R 11.1.2, supporting its potential use in functional and synbiotic food formulations.

Keywords: *Chlorella vulgaris*, *Lactiplantibacillus plantarum*, Nutritional value.

1. INTRODUCTION

The scope of food microbiology has significantly expanded from fundamental safety and quality assurance to the development of dietary innovations that promote human health, most notably through functional foods¹. Within this rapidly growing sector of food biotechnology, products fortified with probiotics and prebiotics have garnered substantial attention. Probiotics, defined as live microorganisms that confer physiological health benefits when

administered in adequate amounts, have become central to this shift, with lactic acid bacteria (LAB) being the most extensively investigated and applied microbial group². The strategic combination of these probiotics with prebiotics (indigestible compounds that selectively stimulate beneficial gut microbiota) has led to the development of synbiotics, representing a highly promising frontier in functional nutrition³.

To optimize these symbiotic systems, researchers are increasingly integrating

sustainable resources such as microalgae into food biotechnology to enhance both the nutritional profile and functional efficacy of food products. *Chlorella vulgaris*, a unicellular green microalga, stands out as a promising candidate because its dense biochemical matrix includes proteins, lipids, essential vitamins, minerals, and potent bioactive compounds such as chlorophyll and carotenoids. These intrinsic properties make *C. vulgaris* an ideal substrate for enhancing probiotic fermentation. Previous research indicates that its rich nutrient composition exerts both protective and growth-stimulatory effects on LAB, thereby bolstering bacterial viability and metabolic activity even under environmental stress⁴.

Recent investigations further corroborate that incorporating *C. vulgaris* into LAB-based fermentation systems significantly amplifies overall fermentation performance by increasing microbial proliferation, lactic acid yield, and antioxidant capacity⁵. Moreover, the lactic acid fermentation of *C. vulgaris* biomass by specific bacteria has been proven to enhance nutrient bioavailability and facilitate the synthesis of bioactive metabolites, reinforcing its viability for functional food applications⁶⁻⁷.

Among probiotic species, *Lactiplantibacillus plantarum* is exceptionally suited for these applications because of its robust metabolic flexibility and strong capacity to adapt to diverse fermentation substrates⁸. This bacterium synthesizes key beneficial metabolites, including organic acids and antimicrobial agents, which serve both food preservation and physiological health enhancement⁹. Co-utilization of *L. plantarum* with a nutrient-dense substrate like *C. vulgaris* is expected to induce a synergistic interaction that maximizes fermentation efficiency and the functional quality of the final product. Therefore, investigating the specific impact of *C. vulgaris* supplementation on the fermentative dynamics of particular *L. plantarum* isolates, such as strains 1 R 11.1.2 and 1 R 1.3.2, is essential for advancing the

design of optimized, probiotic-driven functional foods.

The objectives of this research are as follows 1). to evaluate the nutritional composition of *C. vulgaris* using proximate analysis, and 2). to assess the impact of *C. vulgaris* supplementation at concentrations of 0.25%, 0.5%, 0.75%, and 1% on the fermentation activity of *L. plantarum* 1 R 11.1.2 and 1 R 1.3.2, measured through pH, total LAB count, and total titratable acidity..

2. RESEARCH METHOD

Time and Place

This research was conducted in February 2025. *C. vulgaris* cultivation was performed at the Laboratory of Microbiology and Biotechnology of Fishery Products, Department of Fishery Products Technology, Faculty of Fisheries and Marine Sciences, Universitas Riau. Proximate characteristics and fermentation activity were analyzed at the Laboratory of Agricultural Technology, Department of Agricultural Technology, Faculty of Fisheries and Marine Sciences, Universitas Riau.

Method

This study employed an experimental method. The experiment consisted of eight treatments with three replications. Data from proximate analysis and fermentation activity were analyzed using analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) in Statistical Package for the Social Sciences (SPSS) version 26 (SPSS, 2022). A significance level of 1% was applied, where H_0 was accepted if $p > 0.01$ and rejected if $p < 0.01$, corresponding to a 99% confidence level. Proximate and microbiological characteristics were analyzed using the AOAC method; proximate characteristics consisted of moisture, ash, protein, fat, fiber and carbohydrate by difference, while fermentation activity of *L. plantarum* 1 R 11.1.2 and 1 R 1.3.2 consisted of pH, total LAB, and total titratable acidity (lactic acid).

Procedures

Cultivation and processing of *C. vulgaris* powder

500 mL of *C. vulgaris* inoculum was introduced into 4.5 L of nutrient-enriched mineral water containing 7.5 g KNO₃, 6.25 g KH₂PO₄, 5 g MgSO₄·7H₂O, and 0.249 g FeSO₄·7H₂O. The culture system was assembled using tubing, an aeration stone, and an aerator to ensure continuous oxygen supply. The setup was arranged vertically on a glass rack and exposed to sufficient natural sunlight. Cultivation was carried out for 14 days, with daily morning monitoring. After the cultivation period, *C. vulgaris* biomass was harvested and centrifuged to separate the wet biomass from the culture medium. The centrifugation process was conducted at 4°C and 4500 rpm for 3 minutes. The resulting biomass was then transferred onto trays and dried using a cabinet dryer at 65°C for 8 hours. The dried biomass was subsequently stored in an airtight container under refrigerated conditions until further use.

Starter preparation

Starter preparation followed the method described by Yusmarini et al.¹⁰, with slight modifications. Initially, de Man Rogosa Sharpe (MRS) broth was prepared and sterilized at 121°C under 1 atm pressure for 15 minutes. After sterilization, the medium was cooled to approximately 43–45°C before inoculation. We prepared eight treatment groups by combining different concentrations of *C. vulgaris* with two strains of *L. plantarum*. The treatments consisted of: 0.25% *C. vulgaris* + *L. plantarum* 1 R.11.1.2 (C1L1), 0.5% *C. vulgaris* + *L. plantarum* 1 R.11.1.2 (C2L1), 0.75% *C. vulgaris* + *L. plantarum* 1 R.11.1.2 (C3L1), 1% *C. vulgaris* + *L. plantarum* 1 R.11.1.2 (C4L1), 0.25% *C. vulgaris* + *L. plantarum* 1 R.1.3.2 (C1L2), 0.5% *C. vulgaris* + *L. plantarum* 1 R.1.3.2 (C2L2), 0.75% *C. vulgaris* + *L. plantarum* 1 R.1.3.2 (C3L2), and 1% *C. vulgaris* + *L. plantarum* 1 R.1.3.2 (C4L2). Each bacterial culture (*L. plantarum* 1 R.1.3.2 and 1 R.11.1.2) was

inoculated at 2% (v/v) into the MRS broth and incubated at 37°C for 24 hours.

Next, we performed a stepwise adaptation by preparing a second medium consisting of 75% MRS broth and 25% *C. vulgaris*. The procedure was identical to the initial step, except that the inoculum was derived from the previously cultured medium. This gradual adaptation continued with successive media compositions of 50% MRS broth and 50% *C. vulgaris*, followed by 25% MRS broth and 75% *C. vulgaris* and finally 100% *C. vulgaris* as the growth medium. The resulting liquid starter culture was then considered ready for further application.

Data Analysis

Moisture Content

Moisture content was determined according to the AOAC¹¹ standard method. The percentage of moisture content was calculated using the following equation:

$$\% \text{ Moisture} = \frac{B - C}{B - A} \times 100\%$$

where:

- A = weight of the empty porcelain dish (g)
- B = weight of the dish containing the sample before drying (g)
- C = weight of the dish containing the dried sample (g)

Ash Content

Ash content was determined according to the AOAC¹¹ standard method. The percentage of ash content was calculated using the following equation:

$$\% \text{ Ash} = \frac{C - A}{B - A} \times 100\%$$

where:

- A = weight of the empty crucible (g)
- B = weight of the crucible containing the sample before the furnace (g)
- C = weight of the crucible containing the ash after incineration (g)

Protein Content

Protein content was determined according to the AOAC¹¹ standard method.

The percentage of protein content was calculated using the following equation:

$$\% \text{ Protein} = \frac{(V_1 - V_2) \times N \times 14 \times \text{fp} \times \text{fk}}{W} \times 100\%$$

where:

- W = sample weight (g)
 V_1 = volume of 0.01 N HCl used for sample titration (mL)
 V_2 = volume of HCl used for blank titration (mL)
 N = normality of HCl
 Fp = dilution factor
 fk = protein conversion factor (generally 6.25)

Fat Content

Fat content was determined according to the AOAC¹¹ standard method. The percentage of fat content was calculated using the following equation:

$$\% \text{ Fat} = \frac{(W_3 - W_2)}{W_1} \times 100\%$$

where:

- W_1 = sample weight (g)
 W_2 = weight of the empty fat extraction flask (g)
 W_3 = weight of the flask after fat extraction (g)

Fiber Content

Fiber content was determined according to the AOAC¹¹ standard method. The percentage of fiber content was calculated using the following equation:

$$\% \text{ Crude fiber} = \frac{(W_a - W_k)}{W_s} \times 100\%$$

where:

- W_a = final weight after treatment (g)
 W_k = weight of the filter paper (g)
 W_s = weight of the sample (g)

Carbohydrate Content

Carbohydrate content was determined using the by-difference method according to AOAC¹¹. In this method, total carbohydrate is calculated by subtracting the sum of other proximate components (moisture, ash, protein, and fat) from 100%. This approach provides the total carbohydrate content, including dietary fiber. The calculation was performed using the following equation:

$$\% \text{ Carbohydrate} = 100\% - (\%M + \%A + \%P + \%F)$$

where:

- 100% = total sample composition
 $\%M$ = moisture content (oven-drying method)
 $\%A$ = ash content (furnace/incineration method)
 $\%P$ = crude protein content (typically Kjeldahl method)
 $\%F$ = crude fat content (Soxhlet or solvent extraction method)

Total Lactic Acid Bacteria (LAB)

The total lactic acid bacteria (LAB) count was determined according to the method described by Fardiaz¹². Microbiological analysis was performed using the spread plate technique on appropriate agar media (MRSA). Colony counts were conducted after incubation. Incubation was carried out using an incubator under aerobic conditions at 37°C for 24 hours. Only plates containing countable colonies were considered. The total LAB count was calculated using the following equation:

$$\text{Total LAB (CFU/mL)} = \frac{\text{Number of colonies}}{\text{dilution factor}} \times 100\%$$

Total Titratable Acidity (TTA)

Total titratable acidity (lactic acid) content was determined according to the AOAC¹¹ standard method using an alkalimetric titration with 0.1 N NaOH. Titrate the sample to the endpoint, then calculate the lactic acid concentration based on the volume of NaOH consumed. The total lactic acid content was calculated using the following equation:

$$\% \text{ Lactic acid} = \frac{V_{\text{NaOH}} \times N_{\text{NaOH}} \times 90}{V_{\text{sample}} (\text{mL}) \times 1000} \times 100\%$$

where:

- V_{sample} = sample volume (mL)
 V_{NaOH} = volume of NaOH used for titration (mL)
 N_{NaOH} = normality of NaOH solution
 90 = equivalent weight of lactic acid
 $\% \text{ Fat}$ = crude fat content (Soxhlet or solvent extraction method)

pH Value

The pH value was determined following the method described by Muhtadi et al.¹³. Measurements were carried out using a calibrated pH meter.

3. RESULT AND DISCUSSION

Water Quality

Proximate Analyses

The nutritional profile of *C. vulgaris* was determined via proximate analysis to evaluate its suitability as a functional food ingredient; Figure 1 illustrates the moisture, ash, protein, fat, crude fiber, and carbohydrate levels.

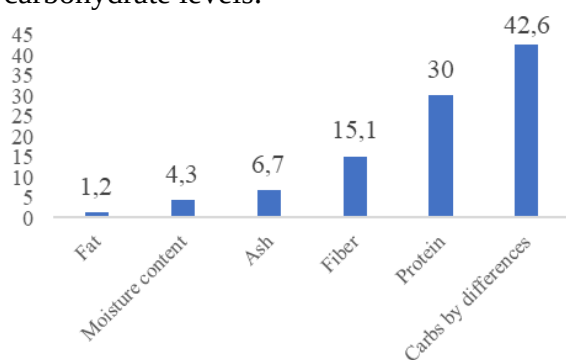


Figure 1. Nutritional value of *C. vulgaris*

Figure 1 illustrates the proximate composition of *C. vulgaris*, dominated by carbohydrates (42.6%) and protein (30.0%), followed by crude fiber (15.1%), ash (6.7%), moisture (4.3%), and fat (1.2%). This distribution indicates that the biomass is primarily composed of energy-providing macronutrients with moderate structural and mineral components. The relatively high carbohydrate content suggests the accumulation of storage polysaccharides, which is commonly observed in microalgae under nutrient-balanced or stress-modulated cultivation conditions.

Similar trends have been reported by Safi et al.¹⁴, who found carbohydrate levels in *C. vulgaris* ranging from 30–50% depending on growth conditions. Likewise, Becker¹⁵; Yaakob et al.¹⁶ confirmed that environmental factors such as light intensity and nitrogen availability influence carbohydrate accumulation, which may

explain the relatively elevated carbohydrate fraction observed in this study.

Protein content is the second major component, although it falls slightly below the typical range of 40–60% reported in several studies¹⁵⁻¹⁷. Despite this lower yield, *C. vulgaris* remains a high-quality functional ingredient due to its balanced amino acid profile and superior digestibility, making it a viable sustainable alternative to conventional plant-based proteins^{15,17-18}.

Structural components, specifically crude fiber and ash, reflect rigid cell wall polysaccharides (cellulose and hemicellulose) and essential minerals. Crude fiber exhibits prebiotic potential for gut microbiome modulation and gastrointestinal health^{16,18-19}, while ash content aligns with the 5–10% range, retaining vital cofactors such as iron, magnesium, and phosphorus for metabolic functions²⁰. The high fiber concentration necessitates downstream processing, such as enzymatic hydrolysis or fermentation, to enhance nutrient bioavailability.

Fat content is substantially lower than the typical 7–20% reported in microalgal literature, indicating cultivation conditions unfavorable to lipid storage, which is typically induced by stressors like severe nitrogen deprivation, thereby prioritizing carbohydrate and protein biosynthesis. Despite the low yield, these lipids remain nutritionally valuable because they contain essential omega-3 and omega-6 polyunsaturated fatty acids. The low moisture content (4.3%) indicates effective post-harvest drying, which is crucial for preventing microbial spoilage and extending product stability^{3,17}.

The nutritional value of *C. vulgaris* in this study reflects a carbohydrate- and protein-rich biomass with moderate fiber and mineral content and low lipid levels. Compared with previous studies, the observed variations likely reflect differences in cultivation systems, nutrient availability, and processing methods. Overall, these results reaffirm the suitability of *C. vulgaris* as a sustainable and versatile ingredient for

nutraceutical and food applications, especially as a fermentation starter^{14,18}.

Total Lactic Acid Bacteria

The impact of *C. vulgaris* supplementation on the growth of lactic acid bacteria (LAB) was assessed using two distinct *L. plantarum* strains. Total LAB counts at different *C. vulgaris* concentrations are shown in Figure 2.

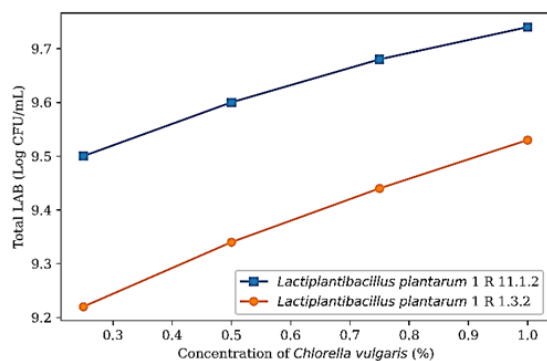


Figure 2. Total LAB counts at different *C. vulgaris* concentrations

Figure 2 demonstrates that increasing concentrations of *C. vulgaris* (0.25 – 1.0%) consistently enhanced the growth of LAB for both *L. plantarum* 1 R 11.1.2 and 1 R 1.3.2, as indicated by the progressive increase in total LAB counts (log CFU/mL). Strain 1 R 11.1.2 showed higher cell density than strain 1 R 1.3.2 across all treatments, suggesting strain-dependent responses to *C. vulgaris* supplementation. This trend is consistent with previous findings showing that adding *C. vulgaris* accelerates bacterial growth and shortens the lag phase due to its rich nutrient composition, including proteins, vitamins, and bioactive compounds⁴. Furthermore, *C. vulgaris* has been reported to act as a prebiotic-like substrate, promoting LAB proliferation and enhancing metabolic activity. Similar studies also confirmed that microalgae supplementation significantly improves LAB growth performance, highlighting its role as a functional fermentation enhancer²¹.

From a comparative perspective, the higher growth performance observed in strain 1 R 11.1.2 indicates a stronger adaptability to *C. vulgaris*-enriched media.

This phenomenon may be attributed to differences in metabolic pathways, enzyme activity, or nutrient utilization efficiency between strains. Previous studies have shown that LAB strains respond differently to microalgal substrates, with some strains exhibiting enhanced enzymatic activity and faster proliferation rates when exposed to *C. vulgaris*⁴. Additionally, fermentation studies have demonstrated that combining *L. plantarum* with *C. vulgaris* can significantly increase microbial biomass and lactic acid production, further supporting the synergistic interaction between the microalga and LAB²¹. The observed increase in LAB counts with rising *C. vulgaris* concentration is therefore likely driven by improved nutrient availability and enhanced metabolic efficiency.

The increasing trend in LAB counts suggests a positive dose-response relationship between *C. vulgaris* concentration and bacterial growth. Similar studies have reported significant differences ($p < 0.05$) in LAB growth when supplemented with *C. vulgaris* compared to control conditions. The gradual increase in log CFU/mL observed in this study aligns with previous reports indicating that microalgal supplementation enhances LAB viability under both optimal and stress conditions²¹. Compared with literature values, the LAB counts obtained in this study (~9.2–9.7 log CFU/mL) fall within the typical range for probiotic viability ($>10^7$ CFU/mL), indicating that the fermentation system successfully supports probiotic growth. Overall, these findings confirm that *C. vulgaris* acts as an effective growth-promoting supplement for LAB, with strain-specific differences influencing the magnitude of the response.

pH Value

We evaluated the effect of *C. vulgaris* supplementation on pH changes during fermentation by two *L. plantarum* strains to assess acidification performance. Figure 3 presents the variation in pH values at different concentrations of *C. vulgaris*.

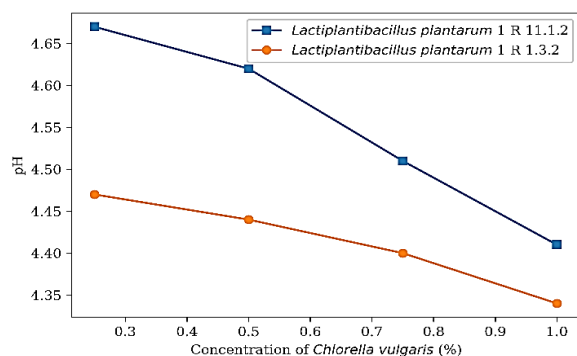


Figure 3. pH value at different *C. vulgaris* concentrations

The data shown in Figure 3 demonstrate that elevating *C. vulgaris* supplementation from 0.25% to 1.0% resulted in a consistent decline in pH for both *L. plantarum* 1 R 11.1.2 and 1 R 1.3.2. This downward trend indicates intensified metabolic activity, as the LAB effectively converted available substrates into organic acids, predominantly lactic acid⁴. This accelerated acidification aligns with existing research showing that *C. vulgaris* provides the fermentable compounds and essential nutrients needed to stimulate LAB metabolic output. Furthermore, previous investigations have confirmed that fermenting microalgal biomass can lower pH, underscoring the strong impact of *C. vulgaris* on acid production and the efficiency of LAB in metabolizing microalgal substrates into organic acids²²⁻²³.

A comparison between the two strains shows that *L. plantarum* 1 R 11.1.2 consistently achieved a greater pH reduction than strain 1 R 1.3.2, indicating stronger acidification capacity. This suggests strain-dependent metabolic differences, particularly in carbohydrate utilization and lactic acid production pathways. Previous research has demonstrated that LAB strains exhibit distinct acidification kinetics depending on their enzymatic systems and substrate adaptability⁴. Moreover, studies involving *L. plantarum* fermentation of *C. vulgaris* have shown enhanced lactic acid production and improved metabolic activity due to increased availability of fermentable sugars released from microalgal biomass²².

The observed strain variability in this study aligns with earlier reports indicating that microalgae supplementation can differentially affect LAB strains, with some strains exhibiting higher acid production efficiency²⁴⁻²⁵.

Furthermore, the pH values obtained in this study (4.67–4.34) fall within the typical range for LAB fermentation systems, confirming effective acid production and microbial activity. Compared to other studies, where pH values may drop below 4.0 under optimized fermentation conditions²², the relatively moderate decrease observed here suggests that further optimization of substrate concentration or fermentation conditions could enhance acidification efficiency.

Total Titratable Acidity

The effect of *C. vulgaris* supplementation on total titratable acidity (TTA) during fermentation by two *L. plantarum* strains was evaluated to assess acid production performance. The variation in TTA values at different concentrations of *C. vulgaris* is presented in Figure 4.

Figure 4 demonstrates that increasing concentrations of *C. vulgaris* (0.25–1.0%) led to a progressive increase in total titratable acidity (TTA) for both *L. plantarum* strains. This indicates enhanced production of organic acids, primarily lactic acid, during fermentation. The increase in TTA is consistent with previous studies reporting that supplementation with *C. vulgaris* significantly enhances the acidifying activity of lactic acid bacteria due to its high content of fermentable substrates and bioactive nutrients. For example, adding *C. vulgaris* has been shown to accelerate LAB growth and increase lactic acid production, leading to higher titratable acidity values⁴. Furthermore, microalgal biomass has been demonstrated to serve as an efficient carbon source for lactic acid fermentation, enabling increased acid production through improved sugar availability²⁶. Scieszka & Klewicka⁴ have also confirmed that increasing microalgae

concentration enhances acidification efficiency and metabolic activity of LAB

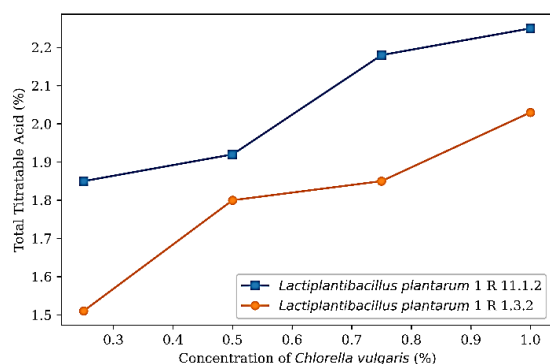


Figure 4. TTA value at different *C. vulgaris* concentrations

Lactiplantibacillus plantarum 1 R 11.1.2 consistently produced higher TTA values than strain 1 R 1.3.2 across all treatment levels, indicating superior acid-producing capability. This strain-dependent variation likely reflects differences in metabolic pathways, enzyme activity, and substrate utilization efficiency. Previous research has demonstrated that LAB strains exhibit distinct fermentation kinetics when exposed to microalgal substrates, with some strains producing significantly higher levels

of lactic acid than others. In addition, *C. vulgaris* has been reported to enhance LAB enzymatic activity, including carbohydrate-degrading enzymes that improve substrate utilization and acid production⁴. Other studies also highlight *L. plantarum* as one of the most efficient species for fermenting microalgal biomass and producing organic acids. Therefore, the higher TTA observed in strain 1 R 11.1.2 suggests greater metabolic adaptability and efficiency in utilizing *C. vulgaris* components. Compared to previous studies, where optimized fermentation conditions resulted in even higher acid production, the moderate increase observed here suggests that further optimization of substrate concentration, fermentation time, or pretreatment methods could enhance acidification efficiency²⁶.

4. CONCLUSION

Chlorella vulgaris supplementation, particularly at higher concentrations, significantly improved the fermentation performance of *L. plantarum* 1 R 11.1.2, supporting its potential use in functional and synbiotic food formulations.

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