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Article

Exploring the Impact of Alginite Mineral on Lactic Acid Bacteria

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Abstract: Studying the uses of different organic-mineral rocks is an expanding area of research. Although these materials have primarily been used in forestry and agriculture, other potential applications include cosmetics and nutrition. Alginite is a volcanic substance that resembles loam and is composed of clay minerals and extinct unicellular algae. Hungary's unique and environmentally friendly agricultural utilisation of alginite has sparked international interest and prompted further exploration of its potential applications. In recent years, studies have proved that alginite can be beneficial in agriculture and as a nutritional supplement, but only if it was further supplemented with lactic acid-producing bacteria (LAB). In contrary, our study investigates the application of alginite already during the LAB fermentation expecting higher probiotic cell number and enhanced positive probiotic effect. Our experiments, conducted using small-scale impedimetric high throughput equipment, revealed that alginite positively influenced the dry matter yield of all four tested probiotic species confirming the enhancing hypothesis. We also thoroughly investigated the fermentations in a lab-scale bioreactor to validate these results. The boosting potential of alginite was verified since depending on the applied strain 30–160% increase in probiotic biomass resulted.

Keywords: Alginite mineral; Lactic acid bacteria; lactose-free and non-dairy probiotic

1. Introduction

The geological team of the Hungarian State Geological Institute discovered the country's first alginite deposit in November 1973 [1]. The formation is similar to conventional oil shale. Alginite colonies are defined more specifically as forming in volcanic craters and are primarily made of algae [1]. We currently know of four volcanic craters in Hungary that contain alginite based on research by the Hungarian State Geological Institute. All locations are in Transdanubia: Pula in Bakony, Gérce in Kemeneshát, in Várkesz villages, and the southwest edge of Egyházaskesz village [1].

Alginite mineral rock formed through the fossilisation of accumulated organic (algae) and inorganic materials such as quartz, carbonates, clay and modified amorphous silicic acid in the watery environment. Humic acids, as a component of alginite's are organic molecules which naturally formed during long-term decomposition and transformation of biomass residues [2,3].

Thanks to its unique composition, alginite has many benefits and uses in agriculture because of its water adsorption capability and pH stabilising effect. Numerous studies on this mineral have reported that, depending on the dose, alginite can significantly boost agricultural productivity [1–3]. Recent studies have explored innovative approaches to using alginite. For instance, turned out that alginite is efficient in demulsification. The mineral contributed significantly to the demulsification of W/O emulsion, which was stable over two months - the emulsion was prepared from 50 wt.% crude oil (Brent type) and 50 wt.% of brine. Furthermore, chemical analysis of the separated oil showed compliance with the industrial standards [4,5].

Other researchers have found alginite beneficial with Lactobacilli (LABs) gastroenterally in animal models [6–8]. In 2017 Hlubeňová K. and colleagues demonstrated the immunomodulatory

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potential of lactobacilli and humic substances present in alginite. The administration of *L. reuteri* and alginite in the group infected with *S. Typhimurium* significantly stimulated the cellular immune response, particularly in the mesenteric lymph nodes of mice. This was evidenced by the activation of CD4+CD8+ lymphocytes, natural killer and natural killer T cells, as well as the activation of phagocytosis within the innate immune system component. [6]. In the same year in a separate study, alginite was introduced into canine diet at a dose of 1% for 14 days, along with probiotic supplementation. The combination of alginite and LAB decreased coliform and Clostridium-like bacteria while increased the lactic acid bacteria of the gastrointestinal system, as well as haemoglobin concentration in blood. At the same time, the treatment stimulated cellular immunity parameters and an improvement in serum mineral levels [7]. Another research study reported comprehensive and comparable positive results as before regarding the alginite combination with probiotic strain. Their research concentrated on the impact of a probiotic strain combined with alginite on the intestinal milieu of SPF mice infected with *Salmonella Typhimurium*. In the investigation, 66 female BALB/c mice were allocated into four experimental groups: control, alginite control, alginite + *Salmonella Typhimurium* CCM 7205NAL, and *Lactobacillus reuteri* CCM 8617 + alginite + *Salmonella Typhimurium* CCM 7205NAL. The group supplemented with *Lactobacillus reuteri* CCM 8617 and alginite exhibited a significant decrease in the growth of *Salmonella Typhimurium* in mouse faeces at 24 and 72 hours ($P < 0.001$) post-infection. The addition of additives significantly influenced nitrogen, enzymatic, hepatic, and energy metabolism in mice. *Typhimurium* infection on the morphology examined in the jejunum and ileum of the LAB group of mice. Mice livers subjected to treatment with both alginite and *Lactobacillus reuteri* CCM 8617 exhibited a significant decrease in overall inflammation, hepatocyte necrosis, and the size of typhoid nodules.These promising findings make us one step closer to making better nondairy probiotic products. A review by Chaudhary et al. (2024) emphasizes the individual roles of site-specific microbiomes in maintaining health and preventing diseases furthermore the complex interactions between different microbiomes across the body are also highlighted [9]. These interactions originate with the mouth and lungs, extending to the vagina, skin, and the central hub of the digestive system. Comprehensive research has demonstrated that the gut microbiota significantly influences the health of other microbiome sites and vice versa. Previously, the health benefits of probiotics were provided by milk/ other dairy products. However, the growth of dairy probiotics is limited by lactose intolerance, and health concerns related to cholesterol, allergic milk proteins and fat content in dairy products [10–12]. Silanikove N. et al. (2015) state that 75% of the world's population suffers from lactose intolerance [13]. The investigation of non-dairy probiotic products, which utilise food matrices derived from fruit, vegetables and cereals, has received significant attention and widely studied due to a growing number of persons with lactose intolerance [14]. The following LABs, such as *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus*, and *B. lactis* are the most employed in the development of novel probiotic products [15–18]. Hence, we have chosen *Bifidobacter adolescentis*, *Lactococcus lactis*, *Lactobacillus rhamnosus*, and *Lactobacillus acidophilus* for our research.

Given the positive influence of alginite and lactic acid bacteria (LABs) on gastrointestinal function in animal models, as well as the growing need for lactose-free probiotic food products, we aimed to conduct an experimental study to examine the impact of alginite on the fermentation of probiotic strains. The assessment involved quantifying the fermentation process using multiple methodologies, like applying two cultivation systems (Bactrac® 4100 and fermentor), monitoring impedance levels, determining the specific colony-forming units (CFU/ml) and employing an online living cell sensor to monitor the fermentation progress.

2. Materials and Methods

2.1. Used Strains and Media

The bacteria were ordered from the National Collection of Agricultural and Industrial Microorganisms (NCAIM). The following microorganisms and media were used in this study: *L.*

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rhamnosus (NCAIM B.02274; ATCC 7469; DSMZ 20021) in MRS medium, *L. acidophilus* (NCAIM B.02085; ATCC:4356; DSM:20079) in MRS medium, *L. lactis* (NCAIM B.0212; DSM 20661) in M17 medium, *B. adolescentis* (NCAIM B.01822, ATCC 15703; DSM 20083) in Bifidobacterium medium.

MRS medium: Glucose 20 g/L, Peptone 10g/L, Meat extract 10 g/L, yeast extract 5,0g/L, Sodium acetate 2,0 g/L; K2HPO4 2,0 g/L, Ammonium citrate 2,0 g/L, MgSO4 · 7· H2O 0,2 g/L; MnSO4 · H2O 0,05 g/L, Tween 80 1,08 g/L.

M17 medium: Lactose 5g/L, Disodium glycerol-β-phosphate 10 g/L, Tryptone 5,0 g/L, Soy peptone 5,0 g/L, Beef extract 5,0 g/L, Yeast extract 2,5 g/L, L-Ascorbic acid 0,5 g/L, MgSO4 0,25 g/L.

Bifidobacterium medium: Glucose 10 g/L, Casein pepton 10 g/L, Yeast extract 5,0 g/L, Meat extract 5,0 g/L, Soy peptone 5,0 g/L, K2HPO4 2,0 g/L, MgSO4 · 7 H2O 0,2 g/L, MnSO4 H2O 0,05 g/L, Tween 80 1,0 mL, NaCl 5,0 g/L, Cystein-HCl · H2O 0,5 g/L, Resazurin (25mg / 100 mL) 4,0 mL/L, Trace elements solution 40 mL/L.

Trace elements solution: 1000 mL distilled water: $NaHCO_3$ 10,00 g, NaCl 2,00g, K2HPO4 1,00 g, KH2PO4 1,00 g, MgSO4 · 7H2O 0,50 g, CaCl2 ·2H2O 0,25 g.

2.2. Fermentations

The small-scale fermentations were conducted in 10ml reusable aerobic glass vials of BacTrac® 4100 (Sy-Lab, Austria) instrument at a temperature of 37°C, with a total liquid volume of 10 mL. The fermentations were monitored using relative impedance changes on the surface (E%) of the electrodes and in the medium (M%) as we had previously reported [17]. Uninoculated vials served as a reference since the relative impedance of the media may also vary. BacMonitor Y 1.39Er program presented the results. Sigma Plot 7.0 was used for curve fitting of the Weibull equation $\left(\boldsymbol{f = if(x \leq x_0 - b * \ln 2^{\left(\frac{1}{c}\right)}; y_0; y_0 + a * (1 - \exp(-(\left|x - x_0 + b * \ln 2^{\frac{1/c^c}{b}}\right|)}\right)$.Weibull was the best fitting model among Log-logistic, Gamma, Log-normal, and exponential in survival analysis using the Akaike information and Bayesian information criterion [20].

The fermentations were conducted in a 1 L benchtop bioreactor with a working volume of 0.8 L (Biostat Q fermenter, B. Braun Biotech International, Melsungen, Germany) with 5% v/v inoculum. The temperature for production was set to 37 °C with an agitation speed of 300 rpm. The pH was regulated using 25% H3PO4 and 25% NaOH. [21].

2.3. Analytic

The BacTrac fermentations were monitored online due to the relative impedance changes on the surface (E%) of the electrodes and in the medium (M%), we used the latter in this article. During the 1L fermentations, the living cells were monitored with a Hamilton InCyte viable cell density sensor. A Waters Breeze HPLC system determined glucose and lactic acid content from samples. The instrument consisted of a Waters 717 Plus Autosampler, Waters 1515 Isocratic Pump, Biorad Aminex HPX 87H (300 × 7.8 mm, 9 µm) column (65 °C) and Waters 2414 RI detector (40 °C). After appropriate dilution steps, the samples were filtered with a 0.2-micron mixed ester syringe filter (ViaLab Ltd). The mobile phase was 5 mM H2SO4 in ion-exchanged water (Simplicity, Millipore, USA), and the elution rate was 0.5 mL/min.

The colony-forming unit (CFU) determination was carried out as follows: Samples (1 ml/sample) were diluted tenfold in a sterile Falcon tube with 9 ml of sterile saline (9,0g/L NaCl). The ten-fold dilution was repeated six and seven times based on the expected cell count, and then 100microL of the diluted samples were spread on MRS agar Petri dishes. The petri dishes were then incubated at 37°C for 24 hours.

The dry matter was quantified by transferring 10 ml of fermentation broth from the BacTrac tubes into Falcon Conical Centrifuge Tubes, which were then centrifuged at 6000 rpm for 10 minutes using a Hermle Z200A centrifuge. The supernatant was decanted, after which the cells were resuspended in distilled water and subjected to centrifugation again. The supernatant was decanted

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once again, and the biomass was placed into the crystallisation cup with 2-3 ml of distilled water and dried expeditiously using the Sartorius MA35 moisture analyser. [19].

To determine what percentage of alginite mineral total mass was lost via solubilisation during fermentation and via dry matter measurement where the water content and also volatile compounds could vaporise, a calibration curve was made using 100g/L lactic acid solution with 2.5, 5, 10, 20 and 40g/L alginite. Solutions were placed into BacTrac, where they could also be checked whether the alginit samples contained contaminants. After three days, the solutions were poured into Falcon tubes, then centrifuged, and the sediments were washed with distilled water as described above. Finally, the dry matter content of the sediment was measured and plotted versus alginite concentrations (Figure 1a). Furthermore, another solubilisation test was prepared with lactic acid solutions of different concentrations, to which 10g/L of alginite was added uniformly, modelling aliginite solubilisation during the lactobacillus fermentation. The concentration of lactic acid solutions were 12.5; 25; 50; 100, and 200 g/L; the sample processing method was the same as the previous one. Finally, the dry matter content of the samples was measured and plotted versus lactic acid concentration (Figure 1b)

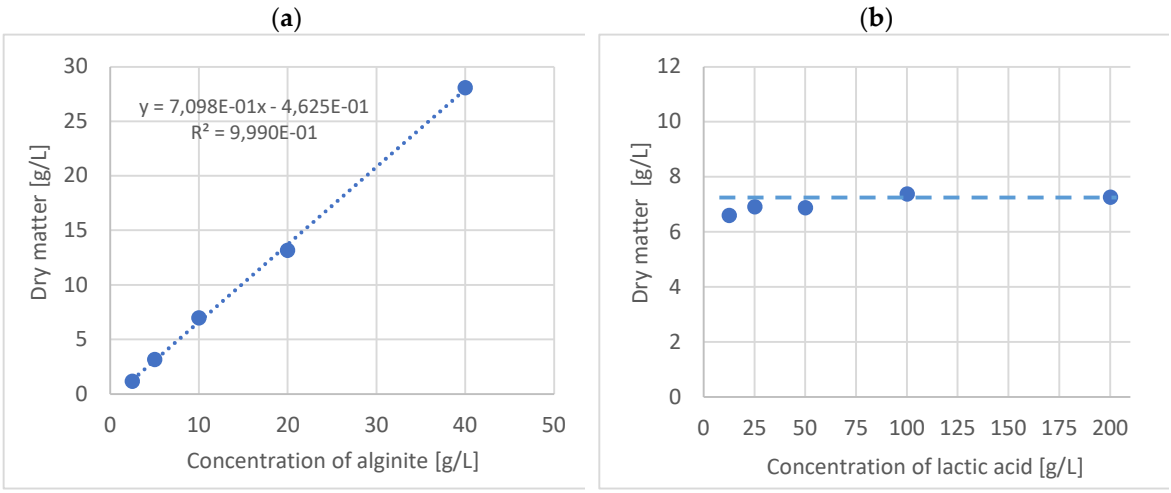

Figure 1. a. - Dry matter of alginite in 100g/L lactic acid solution; b. - Dry matter of alginite in different concentrations of lactic acid.

3. Results

Alginite, characterised by its notable humin and humic acid content, exhibits the potential to enhance the availability of trace elements to plants [22]. Furthermore, its buffering capacity contributes to the soil's pH shift from acidic to neutral. These factors are crucial for promoting optimal plant growth and maximising crop yield [3]. Our expectations were similar in the fermentation experiments. Firstly, we examined the processes in static cultures using BacTrac. We observed a considerable difference between the fermentations supplemented with alginite (with A.) and without it (Figure 2). It suggests that the alginite positively affected these lactic acid-producing bacteria's cell growth (LABs) because they have 2-3 times higher impedance levels than the alginite-free ones.

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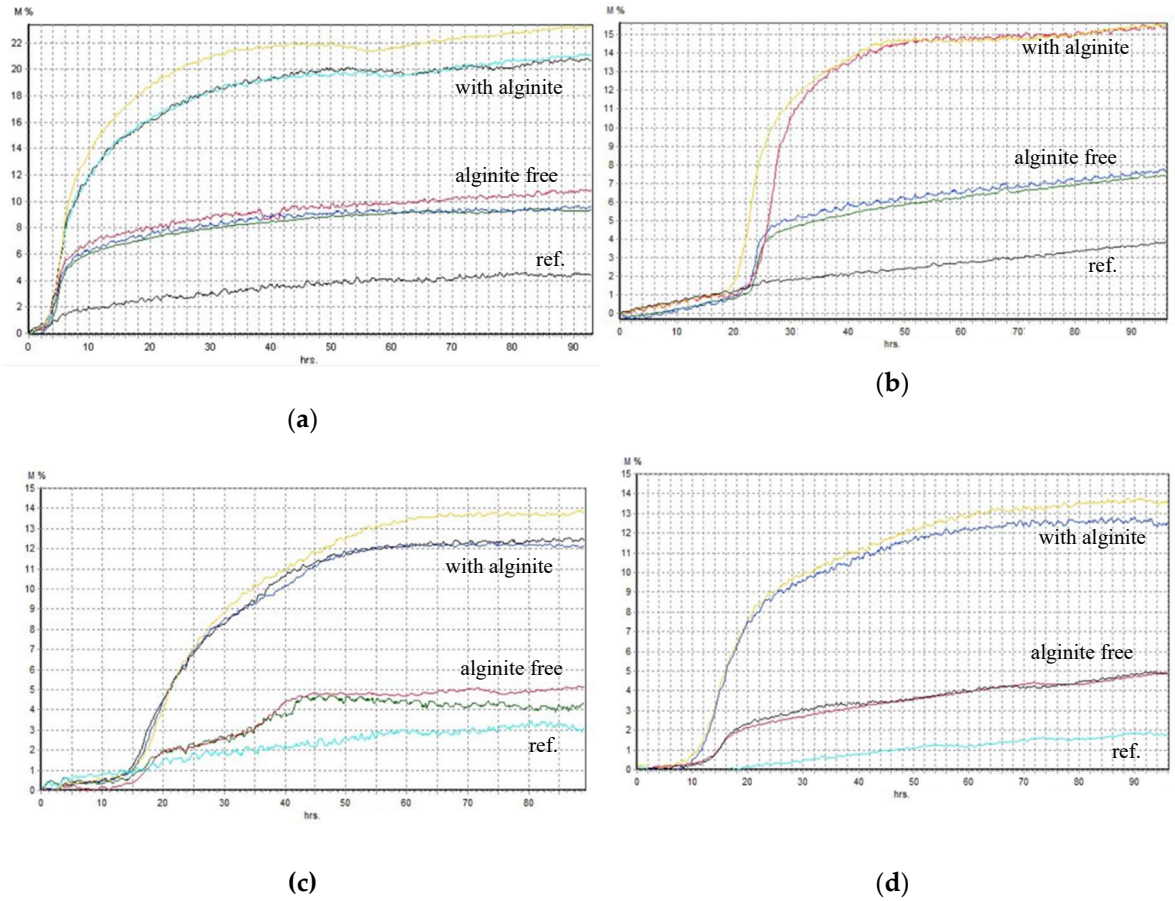

Figure 2. Relative impedance diagram of fermentations (**a** - Bifidobacter adolescentis; **b** - Lactococcus lactis; **c** - Lactobacillus rhamnosus; **d** - Lactobacillus acidophilus.

Maximum specific growth rates were determined through curve fitting and are shown in Figure 3. The results were similar in the case of particular growth rates to maximal impedance values: the μmax is significantly higher in all cases where alginite was used in the fermentation medium compared to the alginite-free cases.

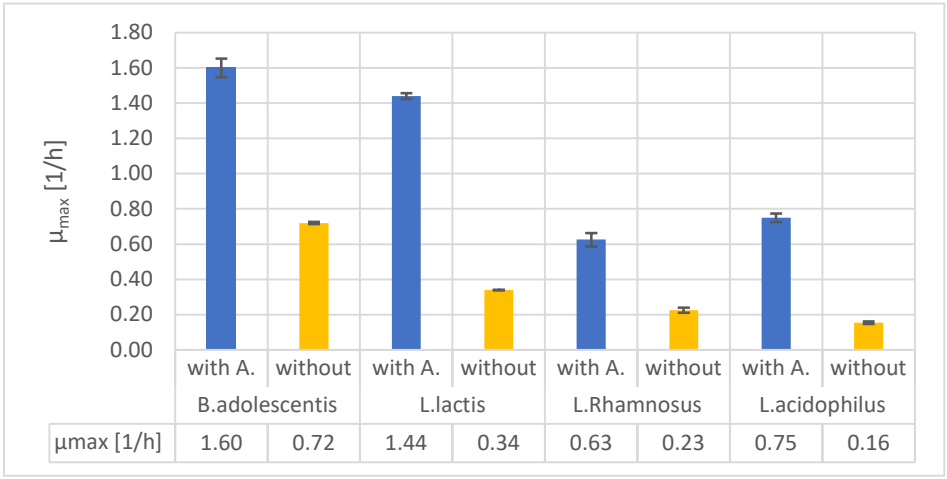

Figure 3. μ_{max} values of fermentations in BacTrac.

To validate the observations above, we conducted measurements of biomass dry matter. In order to accurately determine the dry weight of biomass without interference originating from solid alginite, we used solubilisation curves (see Figure 1). These curves revealed that 70% of the alginate remained as carbonates after reacting with lactic acid. Additionally, soluble and volatile compounds

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were released regardless of the lactic acid concentration at each alginite concentration tested. So, according to the solubilization curves, the remainder of the alginite was subtracted, and the final data is represented in Figure 4.

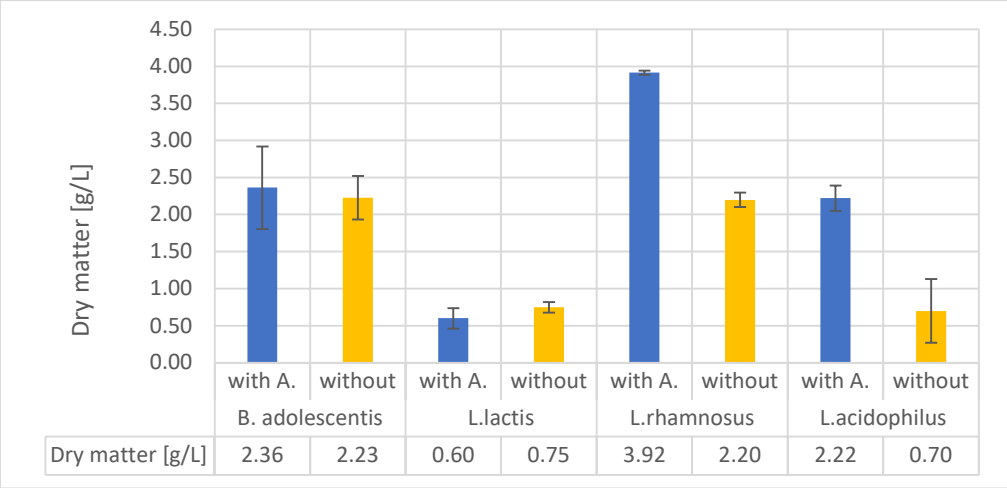

Figure 4. Dry matters of fermentations in BacTrac.

The dry mass results were significantly not different in the case of alginite-containing and alginite-free runs of B. adolescentis and L. lactis. In all other cases, alginite-containing fermentations resulted in significantly higher biomass dry weight, especially in the case of L. acidophilus, reaching three times higher biomass dry weight than in the normal alginite-free fermentations, which was considered a significant enhancement since p-values were lower than 0.05.

The effect of alginite addition to LAB fermentations was also compared in 1L laboratory fermenters since probiotics are rarely produced in static cultures like BacTrac. While offline HPLC measurements monitored substrate and product concentrations, the biomass was followed by CFU determinations since solid alginite disturbed the commonly used optical density and cell dry weight measurements. Comparing time courses of alginite-containing and alginite-free fermentations by B. adolescentis resulted in no significant difference regarding sugar consumption, product formation, and biomass development (Figure 5a,b), which is in accordance with the static culture's (BacTrac) result.

In the case of Lactococcus lactis fermentation, the sugar consumption can be almost the same, but in the case of alginite-free fermentation, ca. 10% more lactic acid was produced (Figure 5c,d). Nevertheless, the CFU in alginite fermentation is ca.1.5 orders higher (on a logarithmic scale) than in alginite-free fermentation.

During the fermentation of Lactobacillus rhamnosus with alginite, the CFU reached a higher value by one order of magnitude, even though, for some reason, the starting sugar amount was less than in the alginite-free fermentation (Figure 5e,f). However, the product reached a higher concentration of 20% in the alginite-free fermentation, following the higher substrate amount consumed, and there was no more production after the 10th hour of fermentation.

We may infer that the production of lactic acid was slower from the flat curve, showing product formation in the Lactobacillus acidophilus alginite supplemented fermentation (Figure 5g,h) and the CFUs were similar.

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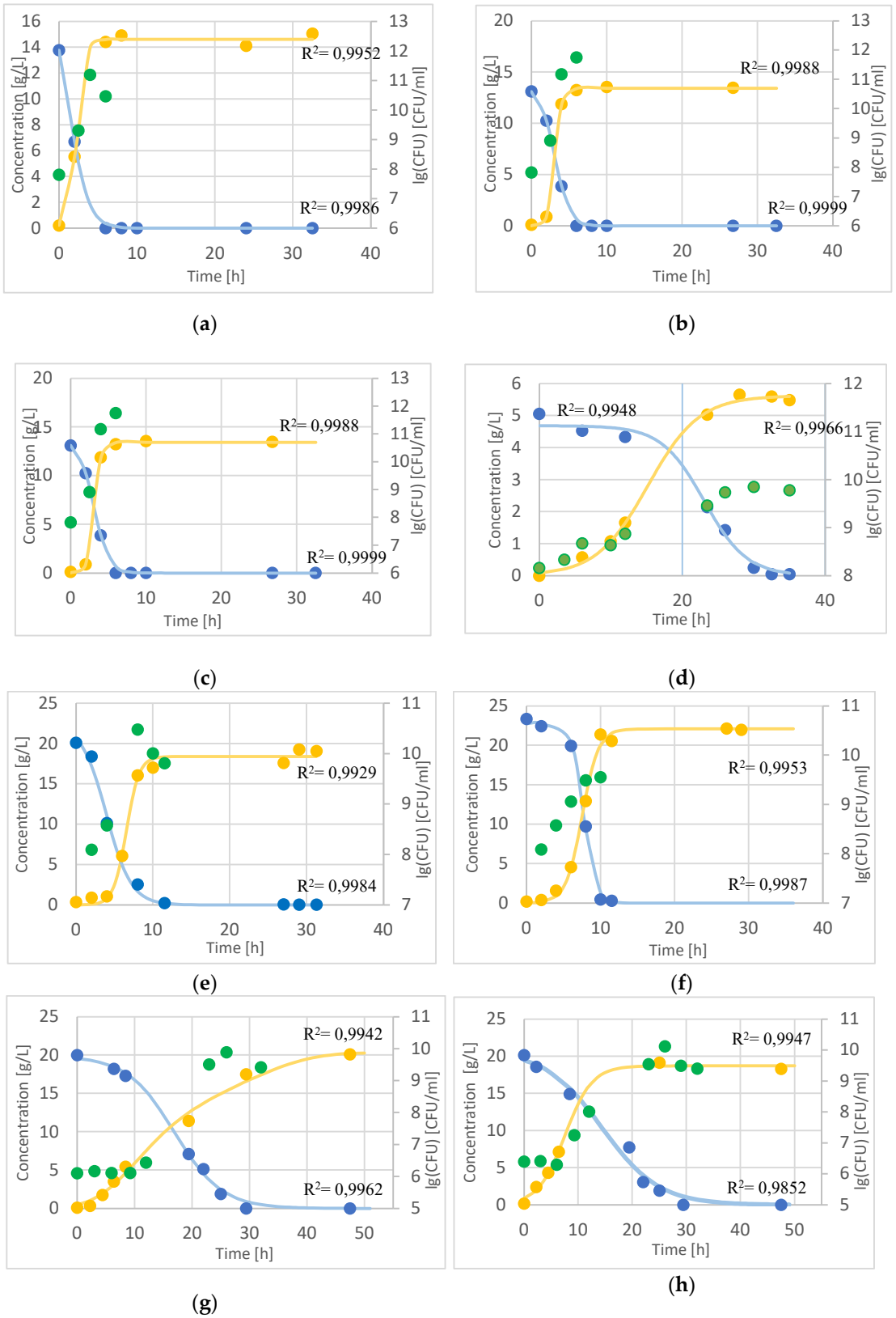

Figure 5. Time course of probiotics fermentation with and without alginite – ● glucose (at *L.lactis* lactose), ● lactic acid, ● CFU (*B. adolescentis* **a.** (with alginite), **b.**; *L. lactis* **c.** (with alginite), **d.**; *L. rhamnosus* ***e.*** (with alginite), **f.**; *L. acidophilus* **g.** (with alginite), **h.**).

Since BacTrac static cultures' CDW (cell dry weight) and bioreactor-based dynamic cultures' CFU results are not in full accordance, a third method was used to clarify results: online capacitance-based living cell sensor was also used and evaluated in bioreactor experiments (permittivity was

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shown which correlates with cell density) Figure 6. In case of all tested lactic acid bacteria, 1,5-2 fold higher cell density could be reached with alginite supplementation compared to alginite free cultures.

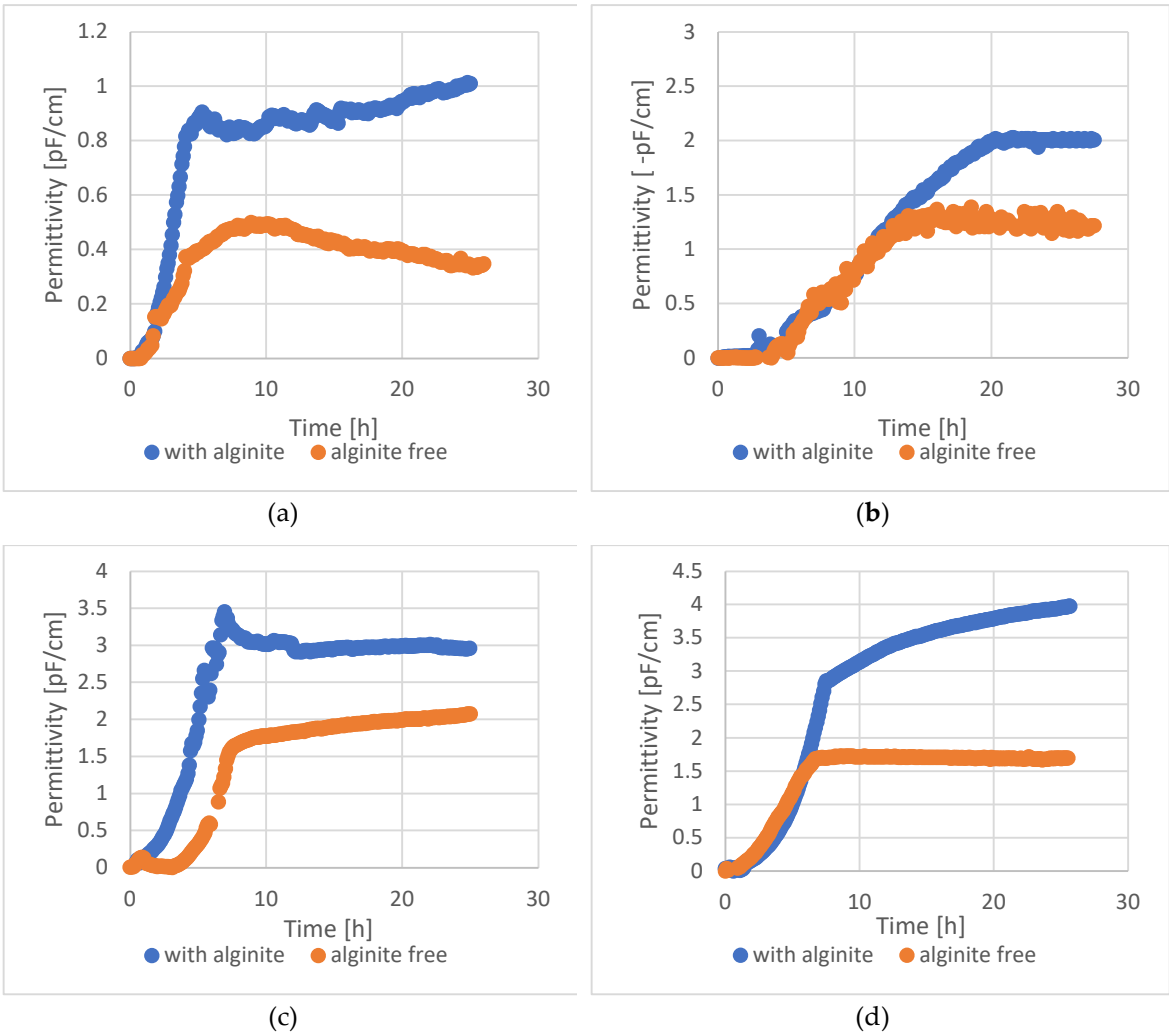

Figure 6. Online cell density results. **a**. - *B. adolescentis*; **b**. - *L. lactis*; **c**. - *L. rhamnosus*; **d**. - *L. acidophilus.*

We also measured dry matter during the fermentation processes to prevent the potential measurement-disturbing effect of alginite-released ions. These measurements consistently confirmed that fermentations involving alginite resulted in higher cell biomass at the end of the process (see Figure 7).

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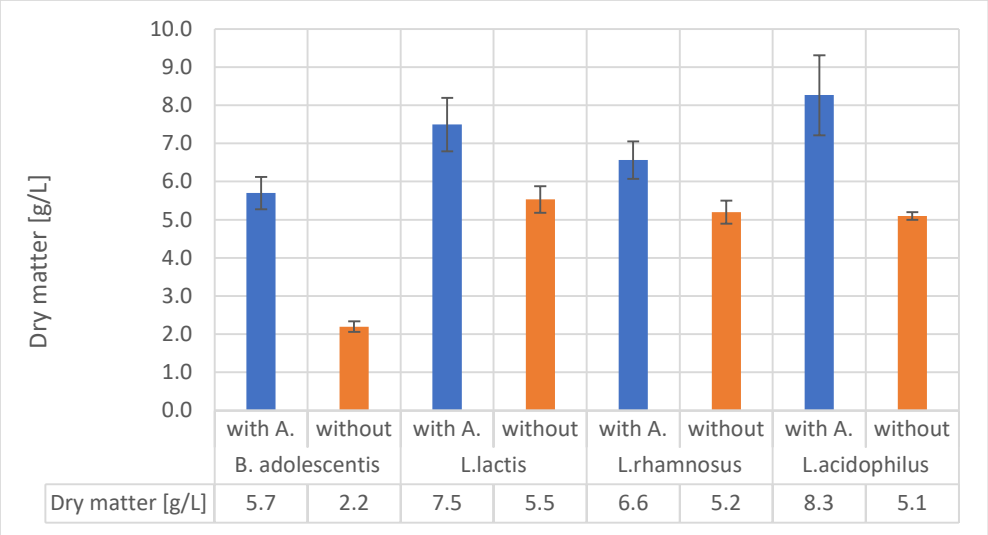

Figure 7. Dry matter of the studied bacteria with and without alginite.

The results were corrected with the previously determined alginite residual amounts (Figure 1). So, in all cases of tested strains's fermentations, the alginite-supplemented ones significantly reached higher biomass dry matter. These observations are based on Figure 7. and contribute to declaring that alginite is beneficial for probiotic biomass production, but based on Figure 5. alginite does not affect the fermentation products.

4. Discussion

Facilitating microbial growth, alginite has been demonstrated to improve the overall fermentation environment by buffering capacity of alginite helps stabilise pH levels during fermentation, hence facilitating ideal conditions for microbial activity [8]. This stabilisation can enhance fermentation rates and product yields, as pH changes can negatively impact microbial metabolism and fermentation efficiency [8]. Following the completion of our initial BacTrac investigations, we got to the same result as well, given that the experiments lacked pH control. For this exact reason, the trials were carried out in a fermenter with a capacity of one litre, with constant mixing and pH that was controlled. Because of this, the buffer capacity that alginite offers cannot be taken into consideration in these experiments. The results were very remarkable in terms of the number of living cells as well as the amount of biomass that was produced.

We postulate that the presence of humic substance (HS) substances in alginite plays a crucial role in enhancing LAB growth. Among the several types of soils, alginite has the lowest amount of aromatic carbon and the highest quantity of aliphatic carbon. The compared soils include lignite, lignohumate, acadian, compost, and alginite [23]. The source of alginite humic acids (HA) are marine organic materials with some contribution of algae and not higher plants as in soil or lignite. Lubica P. et al. (2015) found that alginite-derived humic acid, as well as humic acids obtained from compost, lignohumates, and acadian sources, are newly formed humic acids with a lower level of aromaticity, estimated to be around 40% [23]. Conversely, the humic acids obtained from soils and lignite demonstrate a notable degree of aromaticity, causing enhanced stability and reduced vulnerability to oxidation. The information on alginite is significant in comprehending our findings as previous studies have demonstrated that humic compounds, regardless of their source, can be utilized by 70-80% of bacterial strains [24]. Initially, when Visser [25] introduced this area of study, it was proposed that bacteria might not have easy access to humic compounds. However, further studies conducted by Tranvik and Sieburth [26], Moran and Hodson [27], and Donderski and Wodkowska [28] highlighted the significant role of humic chemicals as nourishment for bacteria. The findings have resulted in diverse study on the interactions between microorganisms and humic chemicals. The

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investigation of a strain of Lactococcus lactis subsp. lactis, Propionibacterium freudenreichii, and Enterococcus cecorum with humic compounds were investigated and they found that these microorganisms were able to reduce humic substances and produce more oxidized products [29]. Notwithstanding, we haven't experienced over oxidized products. Nevertheless, laboratory experiments resembling the ones detailed in our study have not yet been investigated, and according to our information, no one investigated the effect of alginite mineral on the living cell number and biomass.

Humic substances are complex organic materials that arise from the decomposition of plant and animal matter, and they exhibit a variety of properties that are crucial for their roles in soil health, plant growth, and microbial activity. These substances can be broadly categorized into three main fractions: humic acid, fulvic acid, and humin, each with distinct characteristics and functions [30,31]. The numerous positive research results are not surprising since humic substances have many beneficial properties such as redox activity, surface activity and nutrient source, thanks to which they can participate in many microbiological processes [32]. Humic substances also exhibit strong antioxidant properties, which can mitigate oxidative stress in biological systems. They contain polyphenolic components that contribute to their ability to scavenge free radicals, thus protecting cells from oxidative damage [33]. In addition to their effects on soil and plants, humic substances play a crucial role in microbial ecology. They serve as electron donors and acceptors in various redox reactions, facilitating anaerobic respiration in microorganisms [34,35]. This electron transfer capability is vital for the metabolic processes of many soil bacteria and can enhance the degradation of organic matter, thus contributing to nutrient cycling within ecosystems [34,35]. Furthermore, humic substances can stimulate microbial growth and activity (in cow's rumen), leading to increased fermentation efficiency and the production of beneficial metabolites such as short-chain fatty acids [36]. Moreover the humic substance was an increase in the number of LABs found in chicks that were ten days old and had an inclusion level of 0.45% HS. According to this finding, a humic compound that is derived from worm compost has the potential to be utilised as a growth enhancer component in broiler feeds.

In summary, based on our results and other reports, it is assumed that the presence of humic and fulvic acid compounds in alginite leads to a higher yield of LAB biomass. In addition to humic substances, alginite also contains other useful components for microbes, such as amino acids, lipids, polyphenols, and proteins, which the microbes are able to release from the macromolecules of humic- and fulvic acid in alginite [22]. To conclude, it is understandable that the strains we are studying may be able to utilize certain components found in alginite.

Since studies have shown furthermore, that alginite and lactic acid bacteria (LABs) together have positive and synergyc effects on gastrointestinal function in animal models [6–8], our findings on alginit enhancing effect may result more effective probiotic goods with higher living cell number. However, in order to utilise this new useful result, it first needs to be clarified that alginite maintains its positive synergic effects with LABs even if alginite took part in the LAB fermentation, not only added post fermentative as in the above cited earlier reports (Figure 8).

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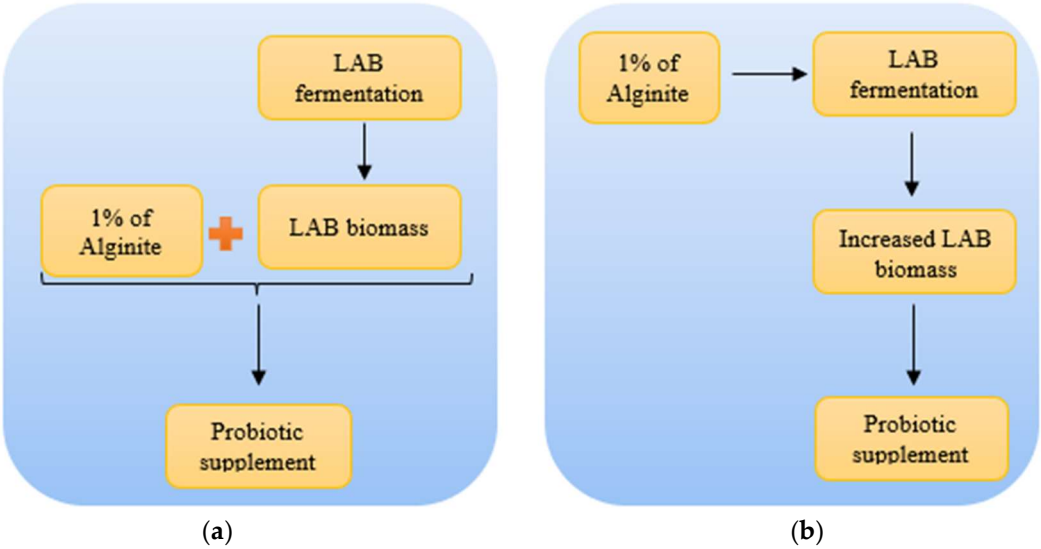

(**a**) (**b**)

Figure 8. Different manufacturing processes of alginite-based probiotic supplement. **a**) Post-fermentation administration of alginite to LAB preparatum; **b**) Pre-fermentation administration of alginite to LAB strains.

The potential for fermented alginite to maintain its beneficial effects on the gastrointestinal system has not been investigated. If this is later confirmed, it could lead to the development of a groundbreaking probiotic product. Prior to that time, we can only emphasize that the use of alginite in LAB fermentation has the potential to enhance biomass production yields without drastically altering lactic acid yield. This, in turn, may contribute to meeting the growing demand for lactose-free probiotics [37].

These findings support the future development and production of probiotic non-dairy-based superfoods or dietary supplements. They also add to our research on LAB-based cosmetics combined with alginite minerals.

Declarations*:* Author contributions: P.T. methodology, investigation, formal analysis, visualisation, writing original draft; Á.N conceptualisation, writing – review &editing, supervision, validation, project administration.

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Data Availability: Authors confirm that all data are involved in the manuscript; further data are available upon request from the corresponding author.

Ethics Approval: No animals and human experiments are involved; therefore, N/A.

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Conflicts of Interest: The authors declare no competing interests.

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