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Screening of *Weissella* bacterial strains for Production of Bioactive Metabolites on Different Substrates

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POPULAR SCIENCE SUMMARY

What if the bacteria used to ferment the foods we commonly eat, could also help us to better digest plant-based products and create healthier foods? In this project, *Weissella* strains were evaluated on different substrates (the raw material as a food source) to investigate their ability to digest plant proteins and produce bioactive compounds. Bioactive compounds are naturally occurring compounds with significant effect on biological processes that may be beneficial, and support health and reduce the risk of disease.

Fermentation has been used since time immemorial to preserve food and add a wide variety of flavors, but bacteria have shown they can do much more than that. Certain microorganisms can produce certain bioactive compounds that have been shown to improve food quality, optimize industrial processes and even support health.

This degree project focuses on *Weissella*, a group of lactic acid bacteria commonly found in fermented foods. Different *Weissella* strains were screened on various substrates, such as pea protein and potato, to investigate how growth conditions influence their metabolism and the production of useful compounds.

One of the most interesting findings was the production of acetic acid under aerobic conditions. Acetic acid is a well-known compound that gives vinegar its characteristic flavor, but it also plays some important biological and industrial roles. Increased acetate production can enhance bacterial growth and inhibit unwanted microorganisms during fermentation. It is also a valuable intermediate that can be used to produce other compounds relevant for future biotechnological and industrial applications. Understanding how bacteria regulate acetate production could, therefore, help optimize fermentation processes and improve production of valuable metabolites. Lactic acid was also produced, which is of importance due to its ability to improve food safety and shelf life, while also contributing to flavour and texture of fermented foods. In addition, lactic acid has industrial significance as a sustainable raw material for biodegradable plastics and other biobased products.

Another interesting finding was the production of exopolysaccharides (EPS) when potato waste was used as a substrate. EPS are natural polymers produced by bacteria that can improve the texture and stability of foods by acting as natural emulsifiers and thickeners. This makes them very interesting for applications in fermented and plant-based foods. Beyond their food properties, Some EPS have been shown to provide health benefits, including anti-inflammatory and probiotic properties. Lastly, potato waste represents an abundant by-product, and its valorisation as fermentation substrates decreases dependence on raw materials and contributes to sustainability by reducing food waste and promoting circular economy.

Overall, these results highlight the importance of understanding the metabolic behaviour of bacteria and how it can be tailored to produce desired compounds. Bacteria such as

Weissella could contribute in the future to the development of foods and valuable compounds that are not only more sustainable, but also healthier and more functional.

ABSTRACT

Lactic acid bacteria (LAB) have been recognized for their potential to hydrolyse different types of substrates, including plant-based proteins, releasing products of nutritional and biotechnological interest. This thesis investigated three *Weissella* strains, including AYR-17, AYR-1, and AYO-14, for their ability to grow on different substrates, break down proteins and produce bioactive metabolites. Bacterial growth was assessed on different substrates, including a protein-rich medium supplemented with glucose, a starch-rich potato medium, and MRS broth, which served as control. HPLC analysis revealed different organic acid profiles that varied with substrate and oxygen availability, consistent with the heterofermentative metabolism of these strains. Lactate was the dominant organic acid on MRS, reaching ~7.50-8.50 g/L under both conditions, whereas on the protein-glucose medium lactate was lower (approximately 2.60-5.0 g/L), and on potato it remained stable at ~0.5 g/L throughout cultivation. Aerobic cultivation favoured acetate production on both MRS (~6.0-8.0 g/L) and protein-glucose (~4.0-6.0 g/L), while anaerobic cultivation on MRS resulted in the higher concentration of ethanol (3.0-3.6 g/L). On potato, acetate remained at a constant level at approximately 4.0-4.3 g/L, suggesting carryover from the inoculum rather than production occurring. HPAEC-PAD analysis confirmed the strain's ability to break down starch and synthesize glucose-rich heteropolysaccharides containing minor amounts of arabinose and galactose. Strain AYO-14 (anaerobic) exhibited growth-dependent EPS production, yielding 90.23 µg/mL, while strain AYR-17 (aerobic) exhibited growth-independent EPS production, with a yield of 157.04 µg/mL. Although o-phthaldialdehyde (OPA) analysis indicated a net decrease in free amino groups during cultivation, further analyses are needed to determine the strains' ability to digest yellow pea protein. Overall, these findings suggest that when the strains utilise monosaccharide substrates, carbon is directed primarily toward acid fermentation, whereas on polysaccharide substrates such as starch, carbon is directed toward exopolysaccharide synthesis.

Keywords: *Weissella*; yellow pea protein; lactic acid bacteria (LAB); heterofermentative metabolism; exopolysaccharides (EPS); proteolytic activity

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1. INTRODUCTION

1.1. Background

Nowadays there is a growing demand for foods that not only have a higher nutritional value but also provide functional benefits. Food proteins are currently being studied for their potential positive impact on human health. These proteins encode specific sequences that are inactive in the native protein but are released when hydrolysed through gastrointestinal digestion, proteolytic enzymes or microbial cultivation (Korhonen and Pihlanto, 2006). These sequences are called bioactive peptides (BAPs) and present biological functions of great interest (Raveschot et al., 2018).

Lactic acid bacteria (LAB) are a group of Gram-positive, catalase-negative bacteria that produce lactic acid as the main product when cultivated on carbohydrate substrates (Felis and Dellaglio, 2007). In addition to contributing to organoleptic properties, many studies have demonstrated the presence of BAPs in these foods as a result of hydrolysis by these bacteria (Pihlanto, 2013).

The genus *Weissella* was initially classified within the genus *Leuconostoc* and later recognized as a separate genus in 1993 based on phylogenetic analyses. *Weissella* species are characterised as being Gram-positive, catalase-negative, lactic acid-producing bacteria that are typically rod-shaped, although cell morphology can vary from cocci to short rods. Cells usually occur in pairs or short chains, and their colonies are small, circular and grayish white in appearance. They are facultative anaerobes, chemo-organotrophs, and asporogenous. To grow, *Weissella* species use glucose, sucrose, and maltose as primary carbon sources. They thrive under mildly acidic conditions, at pH values ranging from 5.0 to 6.5 and temperatures between 30 and 37°C. *W. hellenica*, *W. paramesenteroides*, *W. confusa* and *W. cibaria* are among the best-known species. (Björkroth et al., 2014; Ma et al., 2025).

These bacteria have recently attracted special interest due to their presence in many fermented foods (Mun and Chang, 2020). In these foods, *Weissella* species exhibited, in addition to probiotic properties and lactic acid production, the ability to produce functional exopolysaccharides (EPS) and bioactive metabolites, including bacteriocins, hydrogen peroxide enzymes and biogenic amines, among others (Kavita et al., 2020).

1.2. Proteolytic Activity

Lactic Acid Bacteria (LAB) are well known for their amino acid auxotrophy (Lin et al., 2022), meaning they rely on external protein resources to meet their specific nitrogen requirements. To compensate for this metabolic limitation, LAB have developed proteolytic systems that enable the hydrolysis of proteins in their environment, resulting in the release of peptides and free amino acids (Raveschot et al., 2018c). The proteolytic activity of LAB not only supports bacterial growth but also leads to the generation of bioactive peptides (BAPs), which offer many health-promoting effects, including angiotensin-converting enzyme (ACE) inhibition, immunomodulatory, antioxidant, and antimicrobial activities. Microbial cultivation has therefore been recognized as an efficient strategy to produce novel BAPs (Daliri et al., 2017).

The LAB proteolytic system is composed of three main components: cell envelope proteinases (CEPs), peptide transporters, and intracellular peptidases (Raveschot et al., 2018c). Protein hydrolysis is initiated by CEPs, which cleave proteins into peptides of varying lengths (Savijoki et al., 2006). These peptides are subsequently transported into

the cell via specific peptide transport systems (e.g., Opp and Dpp) and further degraded into free amino acids by intracellular peptidases (Sadat-Mekmene et al., 2011a; Griffiths and Tellez, 2013). Proteolytic capacity and degradation systems are highly strain-dependent across different bacteria (Jensen & Ardö, 2009). Additionally, they are influenced by the nature of the protein substrate used, as this determines the variety of peptides released (Ayyash et al., 2012).

The LAB strains focused on in this study belong to the genus *Weissella*, which require specific nutritional components for growth, including amino acids, peptides, fatty acids, nucleic acids, carbohydrates, and vitamins (Teixeira et al., 2021). Like other LAB genera, *Weissella* spp. are dependent on proteolytic systems for nutrient acquisition, as described above. According to Lynch et al. (2015), the exoproteome of *Weissella* species involves the secretion of both cell wall-associated and extracellular proteins. Interestingly, *Weissella confusa* and *Weissella cibaria* show similarities to *Lactobacillus rhamnosus* in terms of the number of secreted proteins, including those with potential proteolytic activity. Furthermore, protein breakdown and the generation of bioactive peptides by *Weissella* have been associated with an overall increase in antioxidant activity (Arpitha et al., 2025). One study used Brazilian cheese as a source of LAB and identified three strains with proteolytic potential. One of these strains was *Weissella viridescens* VB11, which caused partial hydrolysis of α S1- and α S2-caseins (Biscola et al., 2018). These caseins are among the most allergen-inducing milk proteins, and microbial hydrolysis may therefore represent a potential strategy to reduce their allergenicity.

1.3. Cultivation substrates

1.3.1. Yellow pea protein

It is important to know the protein substrate, as peptide release and functional properties depend on its interaction with *Weissella*. The most used method for producing pea protein isolate (PPI) is alkaline extraction followed by isoelectric precipitation (AE–IEP), which fractionates pea protein into isolates and concentrates, increasing its value (Cui et al., 2020a). Yellow pea is getting more recognized for human and animal nutrition and its potential in functional foods (Asen et al., 2023). It is also considered as a suitable alternative to soybeans (Barac et al., 2010; Olatunde et al., 2025).

Pea is high in protein, starch, and micronutrients, but low in fats, with a well-balanced amino acid profile compared to other plant proteins. Peas generally contain 20–35% protein, and the chemical composition is influenced by genotype, growth conditions, and extraction method (Olatunde & Bandara, 2025; Al-Karaki & Ereifej, 1999; Vidal-Valverde et al., 2003; Nikolopoulou et al., 2006). Pea proteins are mainly globulins and albumins, with globulins representing 55–80% and albumins 18–25% (Nikolopoulou et al., 2006). Like in other legumes, globulins are further divided into 7S vicilin-convicilin and 11S legumin fractions, important for digestibility and functionality (Tzitzikas et al., 2005). For this study, pea protein isolates were used due to their higher protein content (68–85%). (Lam et al., 2016; Asen et al., 2023).

Pea protein has a relatively balanced amino acid profile, though it is limited in leucine, methionine, and cysteine, while being higher in lysine and phenylalanine compared to other legumes (Asen et al., 2023). However, residual antinutritional components, such as protease inhibitors, may temporarily slow *Weissella*'s protein breakdown and peptide release during cultivation (Röhe et al., 2017).

Protein solubility is pH-dependent and influenced by structure, aggregation, and extraction conditions (Karaca et al., 2011). PPI solubility is lowest at pH 4–5 and highest at pH 8 (Bainto-Ancheta et al., 2025). Due to their solubility and soft-gel formation ability, pea proteins are suitable substrates for cultivation and plant-based beverages (Ehsani et al., 2024; Shanthakumar et al., 2022; Asen et al., 2023). For example, 11 h cultivation of pea protein concentrates with *Leuconostoc plantarum* reduced protease inhibitor activity (Çabuk et al., 2018), and other LAB strains such as *Pediococcus pentosaceus*, *Leuconostoc kimchi*, *W. cibaria*, and *W. confusa* have been isolated from legumes, with *L. plantarum* being the most studied for fermenting faba beans and peas (Auer et al., 2023).

1.3.2. Potato

Potatoes are a cornerstone of Swedish cuisine and agriculture, with around 547,000 tons produced in 2025, while potato waste, is a significant, nutrient-rich substrate which is used for microbial cultivation to produce high-value products like biogas, single-cell protein (SCP), lactic acid, and bioethanol. In the present project the potato waste was provided by Orkla and was evaluated as an alternative cultivation substrate for isolated *Weissella* spp. The substrate was collected as whole raw potato, which was later cut into smaller pieces and homogenized prior to cultivation.

The inclusion of potato waste was motivated by its practical valorization potential, as there is a huge accumulation of potato waste in the food industry every year. One study had shown that potato residues contain approximately 46% dry mass starch, which makes them a suitable starch-rich cultivation substrate (Smerilli et al., 2016). Although some *Weissella* strains have been reported as non-amylolytic (López-Hernández et al., 2018; Sharma et al., 2018), genomic analyses have shown that *Weissella confusa* possesses genes encoding enzymes capable of metabolizing sucrose, hemicellulose, cellulose, and starch residues (Hernández-Oaxaca et al., 2021). This suggests potential for the utilization of the starch-rich substrate provided by homogenized whole potato.

1.4. Antimicrobial activity

Under certain conditions, bacteria can secrete antimicrobial peptides, also known as bacteriocins, which are cationic membrane-permeabilizing peptides with less than 50 amino acid residues. These residues can be modified or not, resulting in two classes of bacteriocins, belonging modified amino acids to class I and non-modified amino acids to class II (Papagianni & Papamichael, 2011). Specifically, the class IIa group, known as pediocin-like antimicrobial peptides (AMPs), are being studied as they have shown to display a strong antilisterial activity. These peptides are used by AMP-producer as a defense tool, where they kill nutrient competing bacteria. These peptides destroy target organisms by permeabilizing the cell membrane and disrupting the proton motive force. However, the structure of the peptides plays an important role in the way they act where subtle variations can affect target-cell specificity. Also, differences in target cells might affect bacteriocin efficiency (Fimland et al., 2005).

A class IIa bacteriocin example is weissellin A, produced by *W. paramesenteroides* isolated from a traditional Greek sausage, which acts against a range of Gram-positive bacteria (Papagianni and Papamichael, 2011). Another example is the bacteriocin weissellicin D produced by *W. hellenica* isolated from a traditional Chinese fermented meat product. This bacteriocin exhibited antimicrobial activity against pathogens such as *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus*, as well as against some molds and yeast (Chen et al., 2014).

1.5. Project motivation and aim

Positive change in society is turning to plant-based proteins and overall, people are becoming more environmentally aware, but this transition still brings some challenges. Unlike animal-based proteins, plant proteins can contain antinutritional factors such as tannins, phytates, and trypsin inhibitors that interfere with digestion and reduce bioavailability (Röhe et al., 2017). Individuals with sensitive gut or gastrointestinal problems do not easily digest such plant-based proteins, making this a relevant concern. Cultivation with *Weissella* could be a possible solution, as proteolytic activity during cultivation can break down proteins into smaller peptides, therefore making them easier to consume and digest. Nevertheless, the potential of specific *Weissella* strains remains insufficiently explored.

The overall aim of this thesis is to evaluate the potential of selected *Weissella* strains for beneficial applications in food biotechnology by assessing their functional properties, specifically proteolytic activity. Specific objectives of the project are:

1. *To evaluate production of bioactive metabolites of the selected Weissella strains during cultivation with different substrates*
2. *To evaluate bacterial proteolytic capacity in selected strains*
3. *To investigate the production exopolysaccharides (EPS) as bioactive metabolites and their functional properties*
4. *To assess the antimicrobial properties of selected Weissella strains and their potential to inhibit pathogenic microorganisms*
5. *To investigate if glucose supplementation influences the cultivation profile and organic acid production on high protein substrate*

2. MATERIALS AND METHODS

2.1. Selection of *Weissella* strains

In this study, cultivation was performed with three *Weissella* strains. These strains were selected based on the best growth curve from previous results (Sivakumar, 2020) and two strains from fermented rice (AYR-17 and AYR-1) and one from fermented oats (AYO-14) were included.

2.2. Cultivation

2.2.1. Cultivation media

2.2.1.1. MRS broth and agar

De Man, Rogosa, and Sharpe (MRS) broth and agar from Merck were prepared according to indications and sterilized by autoclavation. MRS broth and agar composition are found in **Table 1** and **Table 2**.

Table 1. MRS broth composition.

Component	Concentration (g/L)
Glucose	20
Peptone	10
Meat extract	8
Sodium acetate (CH ₃ COONa)	5
Yeast extract	4
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	2
Triammonium citrate ((NH ₄) ₃ C ₆ H ₅ O ₇)	2
Magnesium sulfate monohydrate (MgSO ₄ ·H ₂ O)	0.2
Manganous sulfate tetrahydrate (MnSO ₄ ·4H ₂ O)	0.05

Table 2. MRS agar composition.

Component	Concentration (g/L)
D (+)-glucose	20.0
Agar	15.0
Proteose peptone (vegetable)	10.0
Sodium acetate (CH ₃ COONa)	5.0
Yeast extract	5.0
2-phenylethyl alcohol (C ₈ H ₁₀ O)	3.0
Triammonium citrate ((NH ₄) ₃ C ₆ H ₅ O ₇)	2.0
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	2.0
Magnesium sulfate heptahydrate (MgSO ₄ ·7H ₂ O)	0.1
Manganese sulfate (MnSO ₄ ·H ₂ O)	0.05
Bromocresol green	0.04
Captan	0.004

2.2.1.2. Yellow pea protein media

Yellow pea protein (Bulk, London, UK) was used as the high-protein substrate for bacterial growth. Two types of cultivation were performed: one containing only yellow pea protein, and another to which a glucose solution (20 g/L) was added as a sugar source. The medium was prepared by weighing the corresponding amount of protein (20 g/L) into each baffled shake flask and serum bottle and mixing it with basal medium. The basal medium composition can be found in **Table 3**. The medium was autoclaved, and the filter-sterilized glucose solution was added prior to cultivation.

Table 3. Basal medium composition.

Component	Concentration (g/L)
Sodium acetate trihydrate (CH ₃ COONa·3H ₂ O)	8.29
Ammonium citrate dibasic ((NH ₄) ₂ C ₆ H ₆ O ₇)	4.65
Dipotassium hydrogen phosphate (K ₂ HPO ₄)	2
Magnesium sulfate heptahydrate (MgSO ₄ ·7H ₂ O)	0.356
Manganese sulfate (MnSO ₄ ·H ₂ O)	0.0379

2.2.1.3. Potato media

For this media, potato waste provided by Orkla was used. Potato was thawed and grinded. The whole potato was used, which contained 20 g/L starch. To reach this concentration, 41.63 g of potato were added to 1 L of basal medium containing the components described in **Table 3**. Correspondent volume of medium was added to baffled shake flasks for aerobic cultivation, and serum bottles for anaerobic cultivation, and autoclaved.

2.2.2. Growth and culture conditions

Since *Weissella* species are facultative anaerobes, both aerobic and anaerobic conditions were used. AYR-1 strain was cultivated aerobically, AYO-14 anaerobically, and AYR-17 in both conditions.

In the case of anaerobic conditions, cultivation in serum bottles was used. The serum bottles were prepared by adding the medium, heating them in the microwave until the medium was boiled, and then using a nitrogen flux to remove oxygen from headspace, after which they were immediately capped to seal them.

Inoculum preparation was done in two steps. For 1st inoculum preparation, *Weissella* strains from a frozen stock in glycerol were transferred into flasks containing MRS broth. The flasks were then closed by sterile breathable closure for aerobic conditions. In the case of anaerobic conditions, the stock was injected into serum bottles. Both inoculums were incubated at 37°C, with agitation at 200 rpm in the case of aerobic conditions. The 2nd inoculum was prepared under aerobic conditions by inoculating MRS broth with the 1st inoculum at 5% (v/v), i.e., the inoculum represented 5% of the final culture volume. The flask was sealed with a sterile breathable closure and incubated with agitation at 37 °C for 24 h.

Cultivation in MRS broth was used as a positive control. *Weissella* growth was followed by measuring the optical density at 600 nm (OD₆₀₀) with a spectrophotometer every two hours until 12 hours and at 24 hours. pH measurements were also carried out at every sampling time. The 2nd inoculum was prepared once OD₆₀₀ of 1st inoculum exceeded 1.5. The experiment was performed in duplicate to ensure repeatability.

2.2.3. Assessment of colony forming units (CFUs)

Colony isolation was performed by using the spread plate method on MRS agar-plates. The cultivation broth was thoroughly mixed, and 1 mL was transferred into 9 mL of Ringer's solution. Serial dilutions were prepared as needed, and 0.1 mL aliquots were plated in duplicates onto MRS agar plates using a sterile T-shaped spreader. The plates were incubated aerobically upside down for 24-48 hours at 37°C. All procedures were performed under aseptic conditions. A positive control was included to compare growth of customized substrates to MRS medium.

2.3. Biomass determination

For cell dry weight (CDW) determination, 2 mL of culture broth were centrifugated at 10000 rpm for 5 minutes using an Eppendorf Centrifuge 5425R (Eppendorf SE, Hamburg, Germany), supernatant was removed and kept for further analysis. Pellet was washed with 0.5 mL of distilled water using a vortex and centrifuged again. Supernatant was removed, and pellet washed again. The suspended pellet by vortexing was transferred to pre-weighed aluminium weighing pans and dried in an oven at 105 °C until constant weight. This was checked by weighing the sample and returned it to the oven; after 24 h, it was checked every hour until no further weight loss was observed.

2.4. Proteolytic activity measurement

After cultivation, *Weissella* strains cultivated in de Man, Rogosa and Sharpe (MRS) medium supplemented with yellow pea protein as a protein source were evaluated for proteolytic activity. Proteolytic activity was assessed by monitoring protein degradation during the cultivation process. The release of peptides and free amino groups generated

during protein hydrolysis served as an indirect indicator of protein utilization (Thiele et al., 2019).

2.4.1. Bradford Protein Assay

Residual protein concentration in cultivation samples was determined using the Thermo Scientific™ Coomassie Plus™ Protein Assay Kit based on the Bradford method. The assay is a colorimetric method in which Coomassie dye binds to proteins under acidic conditions, resulting in a shift in absorbance and a color change from brown to blue.

The assay was performed in microplate format according to the manufacturer's instructions. Briefly, 150 μ L of each standard or sample was pipetted into microplate wells, followed by the addition of 150 μ L of Coomassie Plus reagent. The plate was carefully mixed and incubated for 10 min at room temperature. Absorbance was measured at 595 nm using a microplate reader. Protein concentrations were determined using a standard calibration curve prepared with protein standards ranged from 0-2000mg/ml. Changes in residual protein concentration during cultivation were used as an indicator of proteolytic activity.

2.4.2. OPA Assay

As the Bradford assay did not allow reliable protein quantification, proteolytic activity was further evaluated using the o-phthaldialdehyde (OPA) assay. The OPA assay was performed based on the protocols of Church et al. (1983) and Nielsen et al. (2001), with modifications adopted from the laboratory protocol. This method is based on the reaction between o-phthaldialdehyde and primary amino groups in the presence of a thiol compound under alkaline conditions, resulting in the formation of a chromophore measurable at 340 nm.

A freshly prepared OPA reagent was used for the assay. The reagent consisted of sodium borate buffer (50–100 mM, pH ~9.5), sodium dodecyl sulfate (SDS; 0.5–1% w/v), o-phthaldialdehyde dissolved in methanol, and a thiol reagent (β -mercaptoethanol or dithiothreitol, DTT). The reagent was prepared immediately prior to use, protected from light, and used within the same day to ensure stability. Samples were prepared by centrifugation of cultivation supernatants to remove insoluble material, followed by dilution (1:10) prior to analysis. In Eppendorf tubes, 50 μ L of diluted sample was mixed with 2 mL of OPA reagent. After incubation for 2 min at room temperature, absorbance was measured spectrophotometrically at 340 nm. A calibration curve was prepared using amino acid standards, and results were expressed as glycine equivalents of free amino groups.

2.5. Glucose consumption monitoring

Cultivation samples were analysed for assessing glucose consumption. After centrifugation and cell separation, the cell-free supernatant was diluted 1:200 with ultrapure water (Milli-Q grade). Glucose concentration was determined using calibration standards of 5, 10, 20 and 30 mg/L. Both samples and standards were filtered through 0.2 μ m Filtropur S filters and transferred into plastic vials with blue caps. Analysis was performed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Thermo Fisher Scientific, Waltham, USA), equipped with a Dionex CarboPac PA20 guard column coupled to a Dionex CarboPac PA20 analytical column (Weitzhandler et al., 2004). Milli Q water, 2 mM NaOH, and 200 mM NaOH were used as eluents. Standard retention times were determined, and the glucose concentration in the samples was calculated using calibration curves. Glucose

consumption was obtained by subtracting the glucose concentration at the end of cultivation from the initial glucose concentration.

2.6. Screening of short-chain fatty acids and organic acids

Samples collected from cultivations using different media (MRS, protein-only, protein supplemented with glucose, and whole potato) were analysed for the production of organic acids and short-chain fatty acids by high-performance liquid chromatography (HPLC). Prior to analysis, supernatant samples were defrosted and diluted 1:2 by mixing 500 μ L of sample supernatant with 500 μ L of MilliQ water. The pH of each diluted sample was measured using pH indicator strips and adjusted to pH 2–3 by addition of approximately 20 μ L of 20% H₂SO₄ (v/v) per 1 mL of sample, to ensure protein precipitation. Samples were then centrifuged at 10,000 rpm for 5 minutes by a microcentrifuge, and after which the supernatant was filtered through a 0.2 μ m filter using a syringe and transferred to glass HPLC vials.

For quantification, a standard mixture containing succinic, malic, lactic, acetic, formic, propionic, and butyric acid was prepared at five concentration levels: 0.5, 0.75, 1, 2, and 3 g/L. Samples and standards were analysed using a Vanquish HPLC system (Thermo Fisher Scientific) equipped with an Aminex HPX-87H precolumn and analytical column. The mobile phase was 0.5 mM sulfuric acid, at a flow rate of 0.5 mL/min and an oven temperature of 40 °C. Detection was performed using both UV and refractive index (RID) detectors.

2.7. Exopolysaccharide production

Samples from cultivation of potato were analysed for exopolysaccharide (EPS) production. The cell-free supernatant was mixed with ethanol (99.5%) in the ratio of 1:4, mixed thoroughly and left overnight in the fridge at 4 °C. The mixture was centrifuged at 4500 rpm for 10 min at 10 °C using a Sigma 3-16PK refrigerated centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany), and the supernatant was discarded. The pellet (crude EPS) was left to dry in the fume hood overnight for ethanol evaporation. The pellet was mixed with MilliQ water, transferred to glass vials and put in freezer at -80 °C overnight and then pellet samples were freeze-dried for 24-48 hours. After this, 175 μ L of 72% (w/w) sulfuric acid was added to lyophilized samples and incubated at 30 °C for an hour. After this, 4.9 mL of MilliQ water was added to the sample to reach the final concentration of 4% (w/w) sulfuric acid, vortexed and put in a heating block for 3 hours at 100 °C. After the samples were cooled, 1 mL of sample was neutralized with barium hydroxide (0.1 M) until it reached pH 5-7 and filtered into plastic vials using 0.2 μ m filters after proper dilution. (Sardari et al., 2017)

Determination of monosaccharide composition resulting from exopolysaccharide hydrolysis, was performed as described in **Section 2.5**. Standards containing D-glucose, D-xylose, D-fructose, D-mannose, D-galactose, and L-arabinose at concentrations of 5, 10, 20 and 30 μ g/mL were used.

2.8. Antimicrobial activity

The antimicrobial activity of the strains was determined using the agar diffusion method. Indicator bacteria *Escherichia coli* (strain BL21(DE3)) was used to test for this antimicrobial activity. Bacterial inhibition of the bacteriocin was assessed following the methodology described by Chen et al. (2014). In this method a LB agar layer was mixed with 100 μ L of *E. coli* (approximately 10⁶-10⁷ CFU/mL) and spread onto a plate. Small sterile cups (oxford cups) were placed on the agar surface and filled with with 50 μ L

neutralized cell-free supernatant (CFS) containing the bacteriocin. Plates were incubated for 24 hours at 37 °C. Antibacterial activity was assessed by measuring the inhibition zone millimetres around the well.

2.9. Statistical Analysis

All experiments were performed in duplicate, and the values are expressed as the mean $\pm$ standard deviation (SD) of the data obtained.

3. RESULTS

3.1. Cultivation in MRS broth

3.1.1. Growth of *Weissella* strains and glucose consumption

Strain AYR-1 was cultivated under aerobic conditions, strain AYO-14 under anaerobic conditions, and strain AYR-17 under both conditions.

The strains were evaluated in one cultivation based on colony-forming units (CFU) and pH (Fig. 1). In a separate cultivation, optical density, cell dry weight and glucose consumption were assessed (Fig. 2).

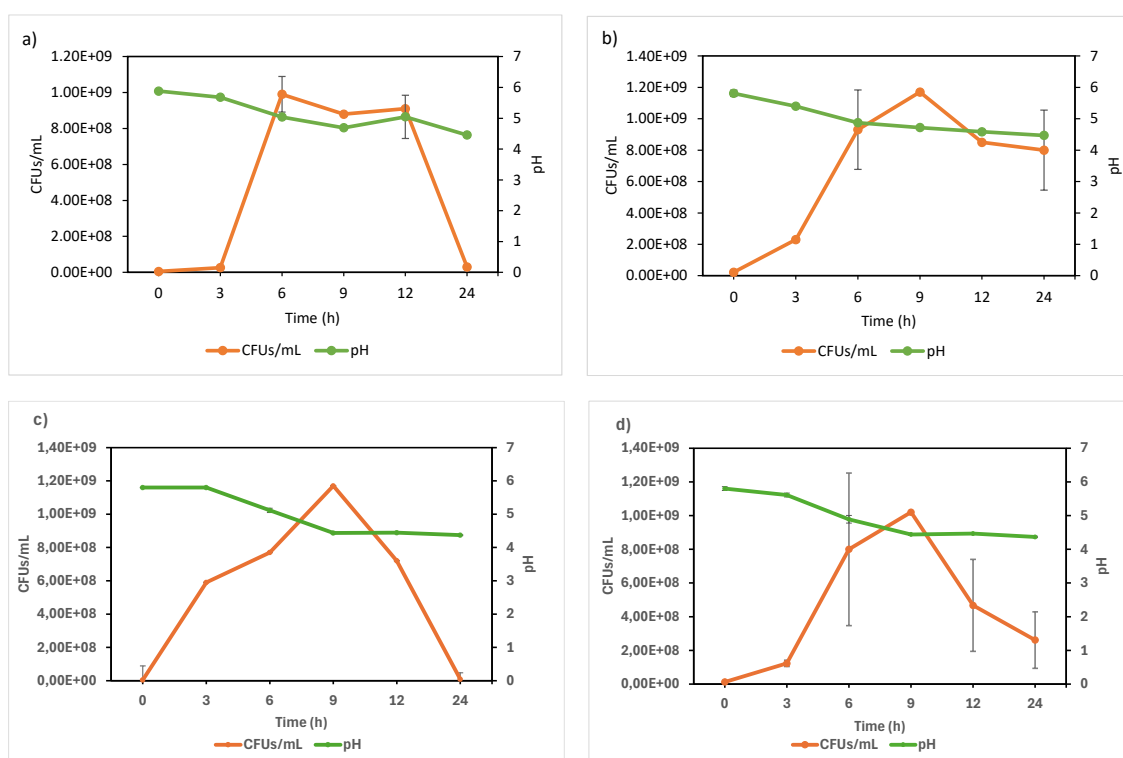


Fig. 1. Colony forming units and pH of *Weissella* in MRS broth cultivation both in aerobic and anaerobic conditions. (a) AYR-17 strain cultivated under aerobic conditions, (b) AYR-17 strain cultivated under anaerobic conditions, (c) AYR-1 strain cultivated under aerobic conditions, (d) AYO-14 strain cultivated under anaerobic conditions.

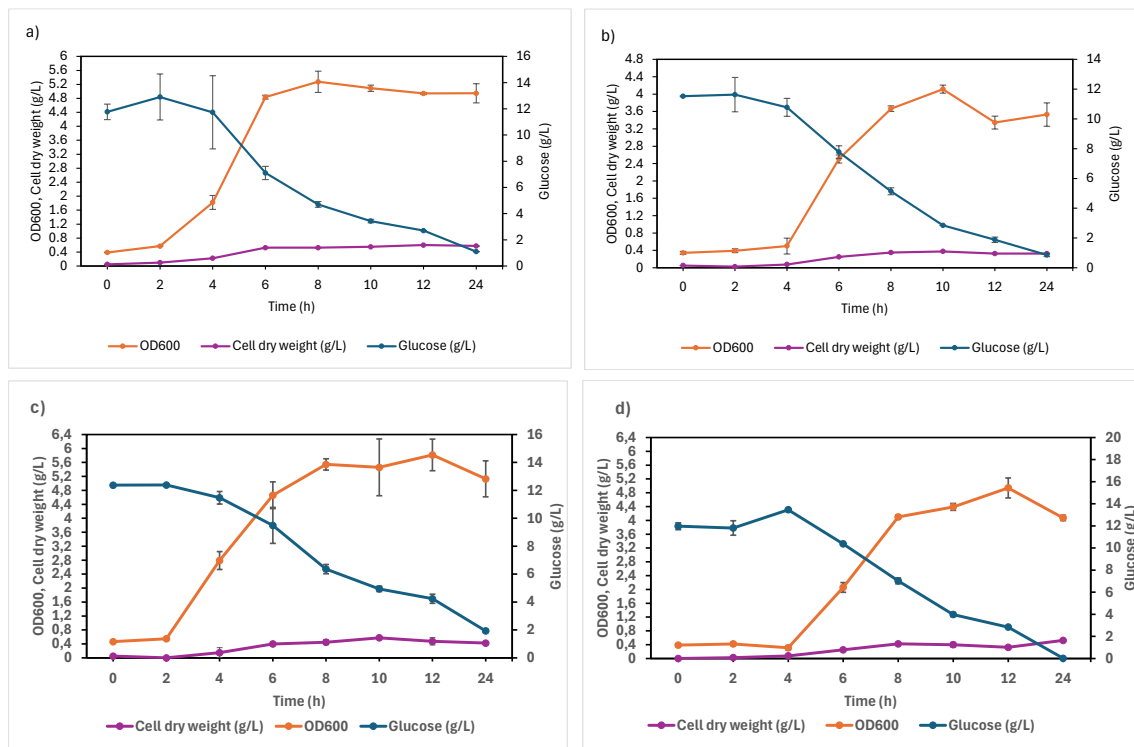


Fig. 2. Glucose consumption, optical density and cell dry weight of *Weissella* in MRS broth cultivation both in aerobic and anaerobic conditions. (a) AYR-17 strain cultivated under aerobic conditions, (b) AYR-17 strain cultivated under anaerobic conditions, (c) AYR-1 strain cultivated under aerobic conditions, (d) AYO-14 strain cultivated under anaerobic conditions.

The CFU and pH measurements (**Fig. 1**) and the OD600, glucose consumption, and cell dry weight measurements (**Fig. 2**) were obtained from two separate cultivations performed under identical conditions on different days.

Based on OD curve, strain AYR-17 exhibited rapid growth under aerobic conditions (**Fig. 2a**). It showed a short lag phase, entered the exponential phase after 3 hours, and reached maximum growth after 6 hours of cultivation. **Fig. 1a**, also shows a growth peak at 6 hours. On the other hand, its anaerobic counterpart exhibited a longer lag phase, beginning its exponential phase after 4 hours and reaching maximum growth at 9-10 hours (**Fig. 2b**). After 10 hours, slight cell death is observed. Strain AYR-17 also showed a similar pH decrease and a nearly complete glucose utilization under both conditions.

Strain AYR-1, cultivated under aerobic conditions, demonstrated significant growth, with OD₆₀₀ rising steeply from 0.40 at inoculation to a maximum of approximately 5.20 after 8 hours of cultivation, followed by a slight decline to 4.80 at 24 hours (**Fig. 2c**). Glucose was almost completely consumed, decreasing from an initial concentration of approximately 12 g/L to near 2 g/L over the cultivation period, representing a net consumption of ~10 g/L (**Fig. 2c**). Cell dry weight increased in parallel with OD600, rising from approximately 0.05 g/L at inoculation to a peak of ~0.58 g/L at 10 hours, before declining slightly to ~0.43 g/L by 24 hours (**Fig. 2c**). CFU counts increased from non-countable to a peak of approximately 1.2×10^9 CFU/mL at 9 hours, followed by a sharp decrease in viable cell numbers, declining to near 0 by 24 hours (**Fig. 1c**). During growth, pH fell from ~6 to ~4.80 and did not change afterward.

Strain AYO-14, grown under anaerobic conditions, exhibited a more gradual growth profile, with OD₆₀₀ rising from 0.40 to a peak of ~4.50 at 12 hours before declining to ~3.50 at 24 hours (**Fig. 2d**). Glucose concentration decreased from ~12 g/L to near 0 g/L

by the end of cultivation, indicating almost complete substrate utilization (**Fig. 2d**). Cell dry weight increased gradually during cultivation, rising from approximately 0.03 g/L at inoculation to ~0.5 g/L by 12 hours and remaining at a similar level until 24 hours (**Fig. 2d**). CFU/mL reached a maximum of $\sim 1.0 \times 10^9$ at 9 hours, followed by a moderate decline to $\sim 2 \times 10^8$ CFU/mL at 24 hours (**Fig. 1d**), suggesting that some cells remained viable even at 24 hours. pH decreased from ~6 at inoculation to approximately 4.30-4.50, where it remained stable through the end of the cultivation (**Fig. 1d**).

3.1.2. Production of metabolites

Cultivated strains in MRS broth with glucose as carbon source were assessed for the production and quantification of organic, short chain fatty acids (SCFAs), and ethanol. The production of organic acids and SCFAs by strains AYR-17, AYR-1, and AYO-14 during cultivation in MRS broth is shown in **Fig. 3**.

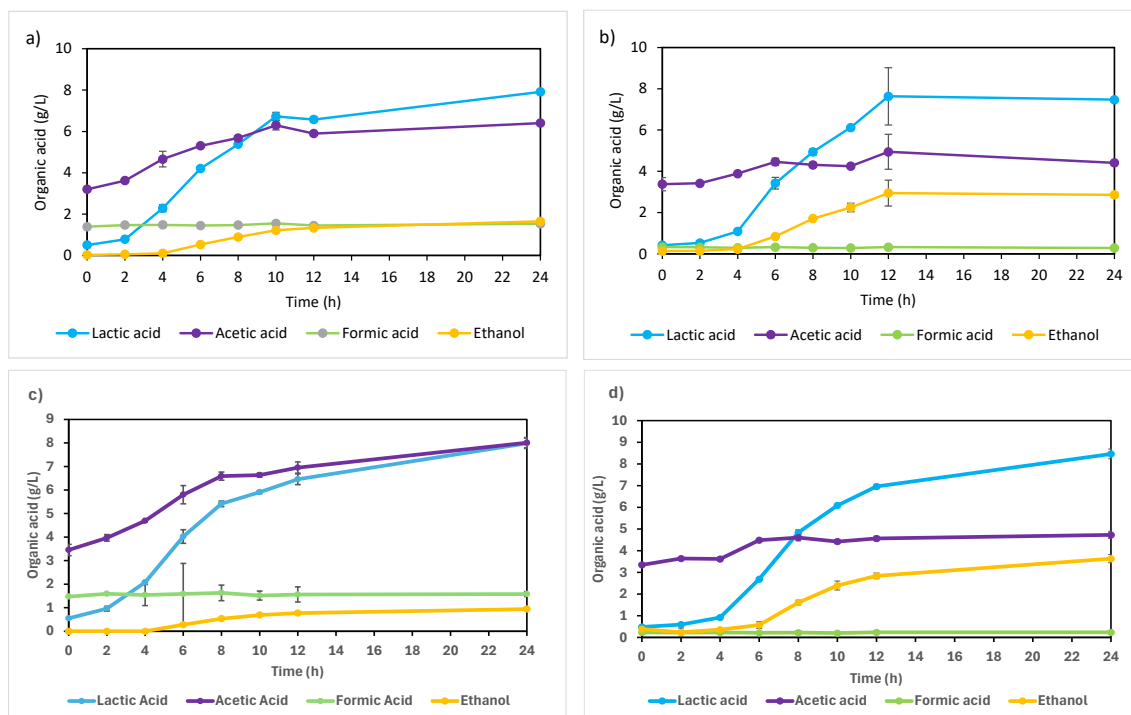


Fig. 3. Organic and short chain fatty acid production by strains AYR-17, AYR-1, and AYO-14 over 24 hours of cultivation in MRS broth. (a) AYR-17 under aerobic conditions, (b) AYR-17 under anaerobic conditions, (c) AYR-1 under aerobic conditions, (d) AYO-14 under anaerobic conditions.

The strains were assessed for the production of lactic, formic, acetic, butyric, propionic and succinic acid. As can be seen in **Fig. 3** all strains demonstrated the ability to produce lactic acid, and two types of SCFAs, acetic acid and formic acid, indicating a heterofermentative metabolism.

Lactic acid was the most abundant acid produced and reached the same concentration under both conditions, nearly 8 g/L. As shown in (**Fig. 3a**), under aerobic conditions lactic acid production by strain AYR-17, reached 6.70 g/L from the early stages of cultivation, and plateaued after 10 hours, with a slight increase to 7.90 g/L at 24 hours. Under anaerobic conditions (**Fig. 3b**), lactic acid concentration increased rapidly after 4 hours of cultivation, reaching 7.60 g/L and remaining constant after 12 hours. In the case of acetic acid, it had already a notable concentration of nearly 4 g/L at the start of cultivation under both conditions. **Fig. 3a** shows higher production, up to 6.30 g/L, which then remained constant with a slight increase towards the end of cultivation. On the other hand,

Fig. 3b shows constant production with slight increases up to 12 hours, reaching a concentration of 4.90 g/L, and then displaying a slight downward trend from that point onward. Ethanol production increased after 4 hours until 12 hours of cultivation, reaching 1.33 g/L aerobically (**Fig. 3a**), and nearly 3 g/L anaerobically (**Fig. 3b**). Formic acid was the least produced acid, showing a very low or almost zero yield throughout the entire fermentative process. As can be seen, it reached a maximum concentration of 1.50 g/L under aerobic conditions (**Fig. 3a**), and 0.28 g/L under anaerobic conditions (**Fig. 3b**).

Furthermore, as shown in **Fig. 3** (c and d), strains AYR-1 and AYO-14 also demonstrated the ability to produce lactic acid, acetic acid, and ethanol, consistent with a heterofermentative metabolism. For strain AYR-1 under aerobic conditions (**Fig. 3c**), lactic acid was the most prevalent acid produced, increasing consistently from ~0.50 g/L at inoculation to approximately 8 g/L at 24 hours, with production rising steeply after 6 hours of cultivation. Acetic acid was already present at a substantial concentration of ~2.50 g/L at the start of cultivation (**Fig. 3c**), rising steadily to approximately 8 g/L by 24 hours, following a similar trend to lactic acid. Formic acid was already present at the start of cultivation at approximately 1.50 g/L and remained at this level throughout, showing no significant production during cultivation. Ethanol remained at low concentration throughout the experiment, becoming detectable from approximately 6 h onwards and reaching only ~0.90 g/L by 24 h (**Fig. 3c**).

For strain AYO-14 under anaerobic conditions (**Fig. 3d**), lactic acid showed a more notable rise, starting from near 0 g/L to approximately 8.50 g/L by 24 hours, with the highest production occurring between 4 and 12 hours of cultivation. Acetic acid was present from the beginning at approximately 3 g/L, increasing moderately to ~4.50 g/L by 24 hours (**Fig. 3d**), where it plateaued. Formic acid was not detected at meaningful levels at any point during cultivation, indicating no production under anaerobic conditions. In contrast to AYR-1, ethanol in AYO-14 was already detectable at low levels in the early hours of cultivation and increased substantially thereafter, reaching approximately 3.60 g/L by 24 h (**Fig. 3d**).

3.2. Cultivation on yellow pea protein

3.2.1. Selection of the best cultivation medium

Two types of cultivation were first performed: one containing only yellow pea protein, and another to which a glucose solution (20 g/L) was added as carbon source. Growth and metabolite production were evaluated throughout 24 hours of cultivation.

Regarding growth, AYR-17 showed opposite behaviours depending on the cultivation substrate. When protein was used alone as the substrate, the strain exhibited a similar trend under both aerobic and anaerobic conditions, beginning with a high CFU count at the start of cultivation followed by a significant decline after 24 hours. In contrast, when both protein and glucose were used as substrates, both conditions showed a good initial CFU count that further increased after 24 hours of cultivation. A different pattern was observed for AYR-1 and AYO-14 under the protein-only condition. AYR-1, cultivated aerobically, started with an initial CFU count of 4.70×10^8 CFU/mL and increased to a density that was too high to be quantified by plate counting after 24 hours. AYO-14, cultivated anaerobically, remained relatively stable throughout the cultivation, with a starting density of 6.95×10^8 CFU/mL and 5.25×10^8 CFU/mL after 24 hours.

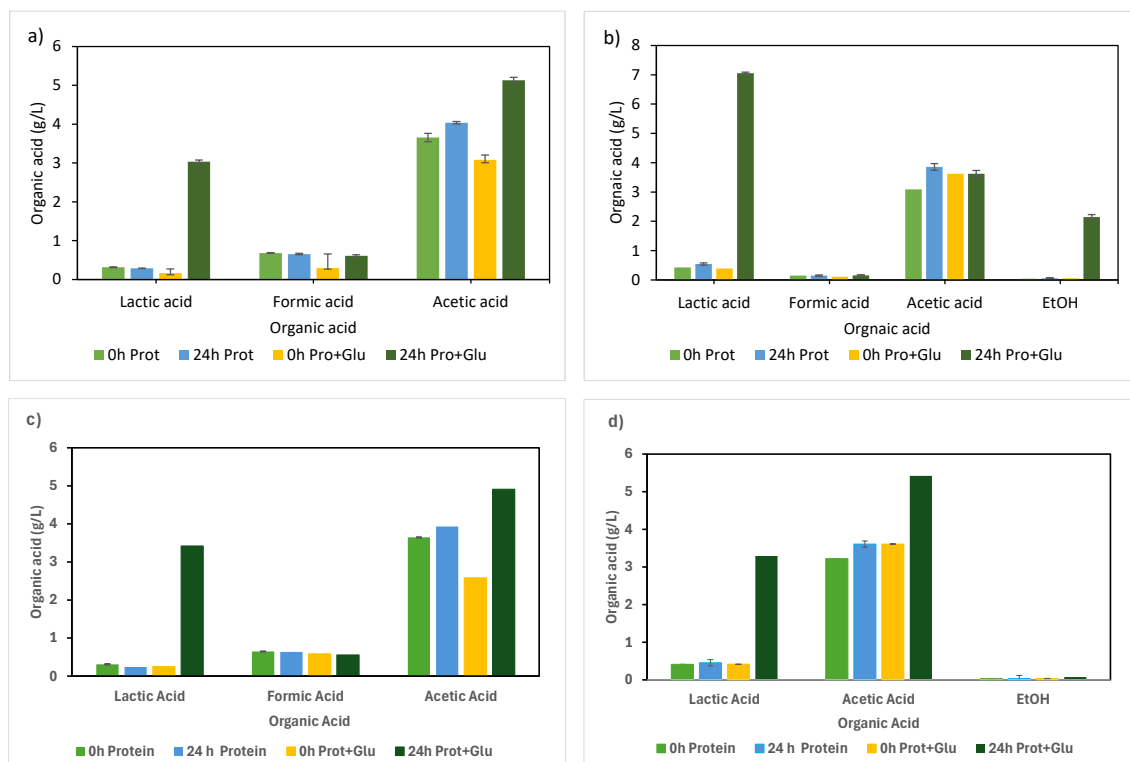


Fig. 4. Comparison of organic acid production by strains AYR-17, AYR-1, and AYO-14 in cultivation with yellow pea protein alone versus yellow pea protein supplemented with glucose. (a) AYR-17 under aerobic conditions, (b) AYR-17 under anaerobic conditions, (c) AYR-1 under aerobic conditions, (d) AYO-14 under anaerobic conditions.

As can be seen in the figure 4, cultivation of the strain AYR-17 on protein only, did not result in production of organic acids, except for a slight production of acetic acid. In the case of cultivation of AYR-17 protein cultivation supplemented with glucose, a higher production of organic acids was observed. Lactic acid was the most produced acid, reaching 3 g/L aerobically (**Fig. 4a**) and 7 g/L anaerobically (**Fig. 4b**). A slight increase in formic acid was observed under both conditions, while acetic acid showed an apparent increase under aerobic conditions, reaching 5.10 g/L (**Fig. 4a**), but remaining constant under anaerobic conditions. Lastly, it should be noted that ethanol was detected only under anaerobic conditions. No ethanol was produced when protein was used as the sole substrate, but when glucose was added, an increase was observed after 24 hours, reaching 2.15 g/L (**Fig. 4b**).

Regarding AYR-1 and AYO-14 strains, cultivation on only protein resulted in very limited acid production over 24 hours in both strains. Lactic acid remained consistently low and unaffected throughout cultivation in both AYR-1 (**Fig. 4c**) and AYO-14 (**Fig. 4d**), at 0.24 g/L and 0.45 g/L, respectively. Notably, acetic acid was already present at high levels at the start of cultivation in both strains - approximately 3.64 g/L (**Fig. 4c**) in AYR-1 and 3.21 g/L (**Fig. 4d**) in AYO-14, with only very small changes observed after 24 hours, reaching 3.9 g/L in AYR-1 and 3.60 g/L in AYO-14. An additional finding was the presence of formic acid in AYR-1 (~0.65 g/L) at the start of cultivation (**Fig. 4c**), which remained unchanged throughout the protein-only cultivation and was not detected in AYO-14 at any point (**Fig. 4d**). When glucose was added as a carbon source, both strains demonstrated a significant increase in lactic acid production after 24 hours. In AYR-1, lactic acid increased from 0.25 to 3.42 g/L (**Fig. 4c**), while in AYO-14 it rose from 0.41 to 3.30 g/L (**Fig. 4d**). Acetic acid similarly increased in both strains, from 2.60 to 4.90 g/L (**Fig. 4c**) in AYR-1 and from 3.60 to 5.40 g/L (**Fig. 4d**) in AYO-14. In AYR-1, with

glucose supplementation, formic acid showed a slight decline from ~ 0.60 to ~ 0.55 g/L (**Fig. 4c**), suggesting it is not a major metabolic product under any of the given conditions. Overall, these results demonstrate that glucose supplementation is necessary to drive significant lactic and acetic acid production in both strains, AYR-1 and AYO-14, while ethanol remained undetectable in AYR-1 (**Fig. 4c**), and at very low levels in AYO-14 (**Fig. 4d**) regardless of substrate composition.

3.2.2. Protein with glucose supplement cultivation

3.2.2.1. Strain growth and glucose consumption

Colony forming unit (CFU) was used to follow bacterial growth during cultivation. Results of colony forming units, pH and glucose consumption from protein cultivation with glucose are shown in **Fig. 5**.

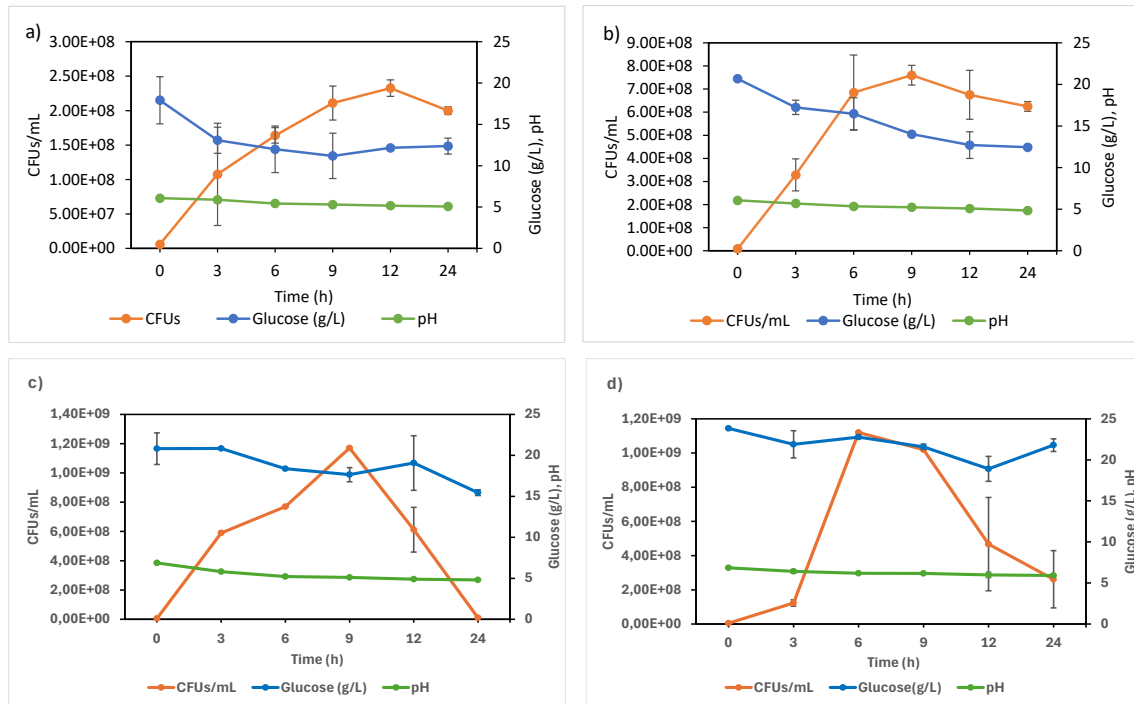


Fig. 5. Colony forming units, pH and glucose consumption of *Weissella* in cultivation media using yellow pea protein and glucose as substrates. (a) AYR-17 under aerobic conditions, (b) AYR-17 under anaerobic conditions, (c) AYR-1 under aerobic conditions, (d) AYO-14 under anaerobic conditions.

Strain AYR-17 exhibited rapid growth under both aerobic and anaerobic conditions using protein and glucose as substrates. In the first case, strain AYR-17 exhibits an exponential phase from the start of cultivation, reaching its maximum growth after 12 hours at $2.33 \times 10^8 \pm 1.21 \times 10^7$ CFU/mL and then declining sharply after 24 hours until nearly complete death at 48 hours (**Fig. 5a**). On the other hand, the strain cultured anaerobically shows greater growth than its aerobic counterpart (**Fig. 5b**), with $7.60 \times 10^8 \pm 4.24 \times 10^7$ CFU/mL at 9 hours. After that time, it enters a death phase, showing a similar result after 24 hours. Regarding pH, it undergoes a slight decrease under both conditions, dropping from 6 to around 5.

Strain AYR-17 also showed some glucose utilization. Under aerobic conditions, glucose is consumed during the first 9 hours of cultivation, reducing the concentration from an initial value of 17.92 g/L to 11.10 g/L (**Fig. 5a**), with no further consumption occurring. In contrast, under anaerobic conditions, strain AYR-17 shows continuous glucose uptake

throughout the entire cultivation, starting from 20.62 g/L and reaching a final concentration of 12.20 g/L (**Fig. 5b**).

Strain AYR-1, cultivated aerobically, showed steady growth throughout the early stages of cultivation, with CFU counts rising from near 0 to approximately 6×10^8 CFU/mL at 3 hours, continuing to increase to $\sim 8 \times 10^8$ CFU/mL at 6 hours, and reaching a peak of $\sim 1.20 \times 10^9$ CFU/mL at 9 hours. After this point, a decline in viable cell numbers was observed, dropping to near 0 by 24 hours (**Fig. 5c**). Glucose consumption was partial, decreasing from an initial concentration of ~ 20 g/L to approximately 15 g/L over the course of cultivation (**Fig. 5c**). pH decreased from ~ 6.90 to ~ 4.80 during the first 6 hours of cultivation, after which it remained stable (**Fig. 5c**).

In contrast, strain AYO-14, cultivated anaerobically, exhibited a different growth pattern, with CFU counts rising from near 0 to a peak of $\sim 1.10 \times 10^9$ CFU/mL at 6 hours, followed by a gradual decline at 9 hours and a sharp decrease by 12 hours. Despite this, viable cells were still detected at 24 hours, indicating that a portion of the population remained cultivable through the end of cultivation (**Fig. 5d**). Glucose consumption was limited throughout cultivation, with the concentration decreasing from an initial ~ 24 g/L to approximately 18.90 g/L by 12 hours (**Fig. 5d**). Towards the end of cultivation, glucose concentration appeared to increase to ~ 21.80 g/L. pH showed a slight decrease from 6.80 at inoculation to 5.90 by the end of cultivation (**Fig. 5d**).

3.2.2.2. Production of metabolites

Strains were assessed for the production of organic and short chain fatty acids (SCFAs). Results from cultivation with protein and glucose as substrates are shown in **Fig. 6**.

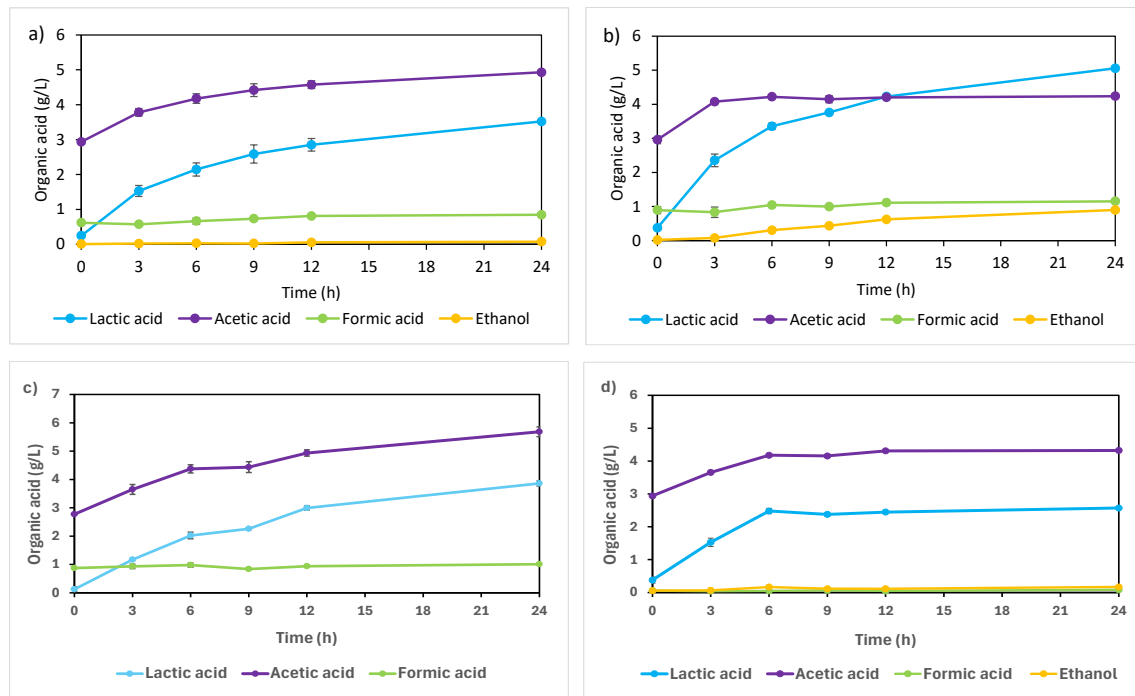


Fig. 6. Organic and short chain fatty acid production over 24 hours of cultivation using yellow pea protein and glucose as substrates. (a) AYR-17 under aerobic conditions, (b) AYR-17 under anaerobic conditions, (c) AYR-1 under aerobic conditions, (d) AYO-14 under anaerobic conditions.

Lactic acid was the most abundant acid produced, showing a rapid increase under both conditions up to 24 hours. For the strain AYR-17 cultivated under aerobic conditions (**Fig. 6a**), lactic acid underwent a moderate increase, reaching 3.50 g/L, while under anaerobic

conditions (**Fig. 6b**), it presented a more pronounced increase, reaching 5 g/L after 24 hours. Acetic acid started at an initial concentration of 3 g/L under both conditions. Under aerobic conditions, strain AYR-17 showed constant production up to 24 hours, reaching nearly 5 g/L (**Fig. 6a**), while under anaerobic conditions, acetic acid production occurred only during the first 3 hours, reaching 4.20 g/L and remaining constant until the end of cultivation (**Fig. 6b**). Formic acid was again the least produced compound, showing very low and constant production. Strain AYR-17 reached barely 0.80 g/L aerobically (**Fig. 6a**) and 1 g/L anaerobically (**Fig. 6b**). Ethanol was the least produced acid, displaying trace amounts under aerobic conditions (**Fig. 6a**), but reaching nearly 1 g/L under anaerobic conditions (**Fig. 6b**).

Fig. 6 (c and d) represents two other strains, AYR-1 and AYO-14, where lactic acid was the most produced acid under both conditions, showing an increase throughout cultivation. Under aerobic conditions (**Fig. 6c**), lactic acid rose steadily from near 0 to ~4 g/L in 24 hours. Under anaerobic conditions (**Fig. 6d**), production was faster, reaching ~2.60 g/L by 6 hours and remaining stable until the end of the cultivation. Acetic acid was already present at the start of cultivation at ~2.70 g/L (**Fig. 6c**) under aerobic conditions and ~3 g/L (**Fig. 6d**) under anaerobic conditions. Under aerobic conditions (**Fig. 6c**), acetic acid increased gradually to ~5.70 g/L by 24 hours. Under anaerobic conditions (**Fig. 6d**), it increased to ~4.30 g/L by 6 hours and then plateaued for the remaining period of cultivation. Under aerobic conditions formic acid was present from the start at ~1 g/L (**Fig. 6c**) and showed no significant change throughout cultivation. Under anaerobic conditions (**Fig. 6d**), formic acid was not detected at meaningful levels at any point during cultivation. Ethanol was not detected in AYR-1 under aerobic conditions (**Fig. 6c**), while in AYO-14 it was present at very low levels throughout cultivation, with only minor accumulation observed by 24 hours (**Fig. 6d**).

3.2.2.3. Antimicrobial activity

Agar diffusion method with using *E. coli* as indicator bacterium was used to test the antimicrobial activity of the strains. After incubation, plates were examined for inhibition zones. No inhibition zones were observed, indicating the absence of peptides with antibacterial activity.

3.2.2.4. Bradford Assay

Although the Bradford assay was performed according to the protocol, no visible color change was observed in the analysed samples after addition of the Coomassie Plus reagent and incubation. Consequently, reliable protein quantification could not be achieved.

3.2.2.5. Peptide production (OPA)

Primary amine content was determined by the o-phthaldialdehyde (OPA) method, with results expressed as glycine equivalents as shown in **Fig. 7**.

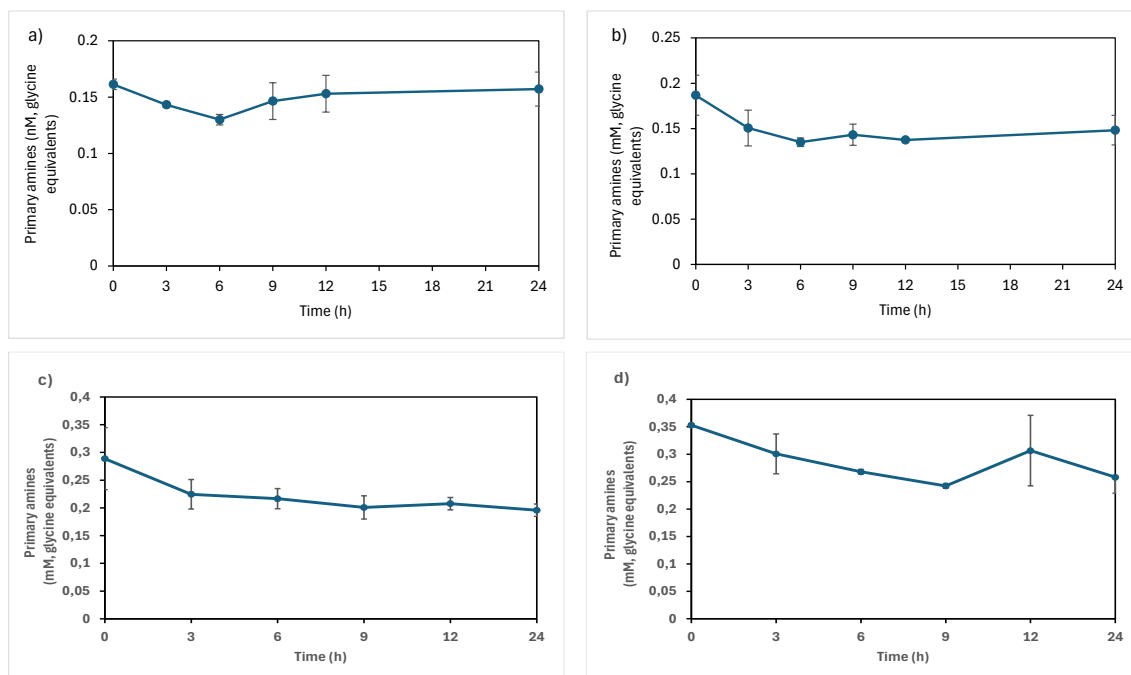


Fig. 7. Primary amine production by strains AYR-17, AYR-1, and AYO-14 over 24 hours of cultivation with yellow pea protein. (a) AYR-17 under aerobic conditions, (b) AYR-17 under anaerobic conditions, (c) AYR-1 under aerobic conditions, (d) AYO-14 under anaerobic conditions.

Primary amine content reflects free amino acids, peptide, proteins, and an overall proteolysis versus a consumption balance (Frister et al., 1988). An increase in OPA signal implies a higher protein/peptide breakdown (proteolysis), therefore a higher content of free amino ($-NH_2$) groups.

AYR-17 strain cultivated under aerobic conditions (**Fig. 7a**) showed a slight drop early in cultivation, reaching 0.13 mM, which suggests a protein initial uptake. After 6 hours, an increase of primary amine content up to 0.15 mM can be observed, indicating a higher proteolytic activity. After 12 hours, a plateau phase is reached, where there is a balance between proteolysis and consumption. AYR-17 strain cultivated under anaerobic conditions (**Fig. 7b**) shows a similar behaviour in the beginning of cultivation, with a clear drop from start, reaching 0.13 mM and indicating rapid amino acid consumption. After 6 hours of cultivation, an overall plateau phase with small fluctuations can be observed, indicating a limiting proteolysis.

The other two strains also showed a decline in primary amine content during cultivation. AYR-1, cultivated under aerobic conditions, exhibited a reduction from ~0.29 mM at 0 h to ~0.22 mM by 3 hours, after which levels remained relatively stable at around 0.20 mM until 24 hours (**Fig. 7c**). AYO-14, cultivated under anaerobic conditions, showed a decrease from ~0.35 mM at 0 h to ~0.25 mM by 6–9 hours, with a short-term increase at 12 hours before returning to ~0.25 mM at 24 hours (**Fig. 7d**).

3.3. Cultivation on potato

AYR-17 cultivated under aerobic conditions and AYO-14 cultivated under anaerobic conditions were used to ferment potato substrate.

3.3.1. Strain growth

Colony forming unit (CFU) was used to follow bacteria growth during cultivation. Results of colony forming units and glucose consumption are shown in **Fig. 8**.

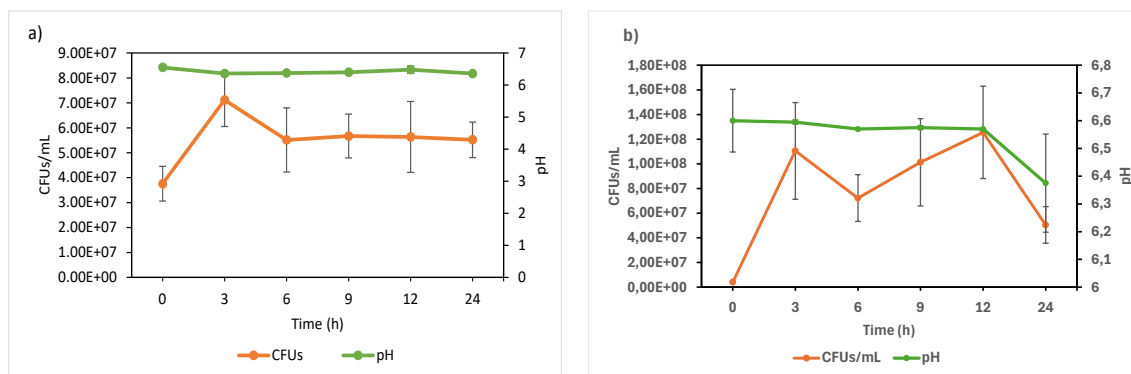


Fig. 8. Colony forming units and pH of *Weissella* in cultivation media using starch from potato as substrate. (a) AYR-17 strain under aerobic conditions and (b) AYO-14 strain under anaerobic conditions.

AYR-17 strain underwent rapid growth during the first 3 hours of cultivation, after which growth declined and entered a stationary phase at 6 hours, remaining at that phase until the end of cultivation. However, as can be seen in **Fig. 8a**, there were no significant differences in terms of variations in CFU and pH.

AYO-14 strain demonstrated fast growth during the first 3 hours of cultivation, reaching $\sim 1.10 \times 10^8$ CFU/mL, followed by a slight decrease at hour 6 to $\sim 7 \times 10^7$ CFU/mL. Maximum growth was observed between hours 6 and 12, with CFU counts recovering and peaking at $\sim 1.20 \times 10^8$ CFU/mL, followed by decrease to $\sim 4.80 \times 10^7$ CFU/mL by 24 hours (**Fig. 8b**). No significant changes in pH were observed throughout the cultivation, remaining stable at around 6.55–6.60 (**Fig. 8b**).

3.3.2. Production of metabolites

Organic acids and SCFAs produced are shown in **Fig. 9**.

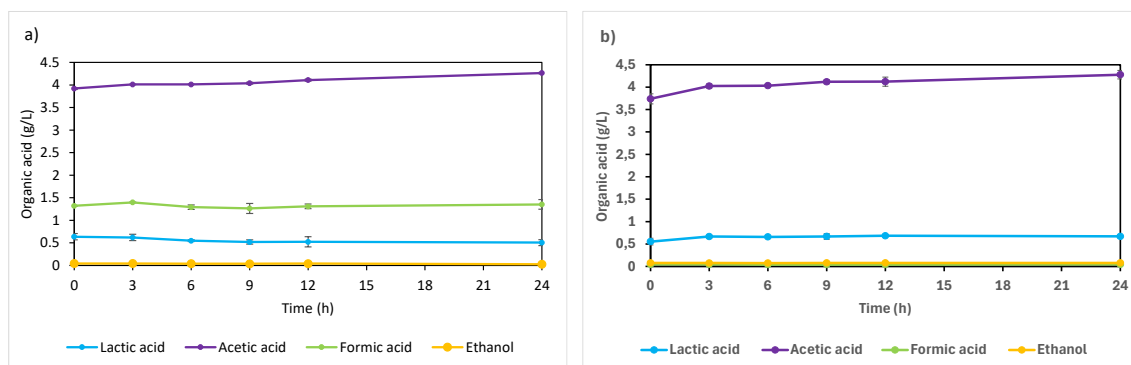


Fig. 9. Organic and short chain fatty acid production by strains AYR-17 and AYO-14 over 24 hours of cultivation with potato substrate. (a) AYR-17 under aerobic conditions and (b) AYO-14 under anaerobic conditions.

As shown in **Fig. 9a**, acetic acid was the most abundant acid in the cultivation medium, with an initial concentration of nearly 4 g/L, and it showed a slight upward trend over time. Ethanol, formic acid and lactic acid maintained constant production levels with minor fluctuations throughout the cultivation process. Formic acid maintained a concentration of 1.30 g/L, lactic acid around 0.50 g/L, and ethanol was present in trace amounts.

Fig. 9b also indicates acetic acid as the predominant organic acid produced during potato cultivation by AYO-14 under anaerobic conditions, increasing gradually from ~ 3.70 g/L at 0h to ~ 4.37 g/L by 24 hours. Lactic acid was consistently detected at low concentrations

throughout cultivation, remaining stable at around 0.50–0.60 g/L. Formic acid and ethanol could not be detected in considerable amounts at any point during cultivation.

3.3.3. Peptide production (OPA)

Primary amine content of strains AYR-17 and AYO-14 during cultivation with potato substrate is shown in **Fig. 10**.

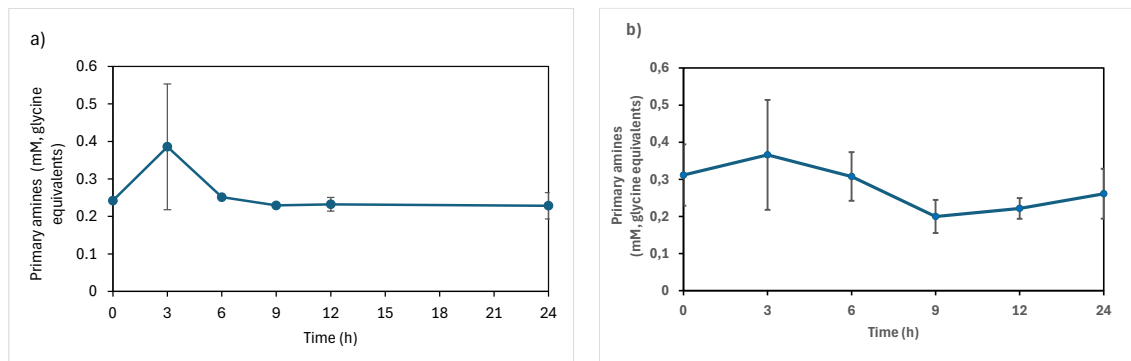


Fig. 10. Primary amine production by strains AYR-17 and AYO-14 over 24 hours of cultivation with potato substrate. (a) AYR-17 under aerobic conditions and (b) AYO-14 under anaerobic conditions.

As can be seen in **Fig. 10a**, there was a rapid increase in primary amine content during the first 3 hours of cultivation, reaching nearly 0.40 mM, followed by a decline and a plateau phase after 6 hours. This indicates an initial proteolysis, followed by protein uptake and a final stable metabolic state with a constant primary amine concentration of 0.20 mM. However, a significant deviation is observed at 3 hours (± 0.17 mM), suggesting possible errors made during the analysis. Therefore, metabolism would be dominated by a plateau phase, in which there would be a balance between proteolysis and consumption.

Primary amine content in AYO-14 during potato cultivation under anaerobic conditions started at 0.31 mM at 0h, with a perceived slight increase observed at 3 hours (0.37 mM). Furthermore, the notably large standard deviation at this time point (± 0.15 mM) indicates considerable variability between replicates and should be carefully interpreted. By 6 hours, primary amine content returned to the initial level (0.31 mM), followed by a more visible decline to 0.20 mM at 9 hours. From 12 hours onwards, levels stabilized at around 0.22–0.26 mM until the end of cultivation at 24 hours (**Fig. 10b**).

3.3.4. Exopolysaccharide production

After hydrolysis, the monosaccharide composition of the crude exopolysaccharides (EPS) was analysed using HPAEC-PAD, and the analysis revealed that all EPS were heteropolysaccharides. The main components of the crude EPS were arabinose, glucose, and galactose. **Fig. 11** showed total EPS produced during cultivation with whole potato as substrate. EPS yield was shown in **Error! Reference source not found.**

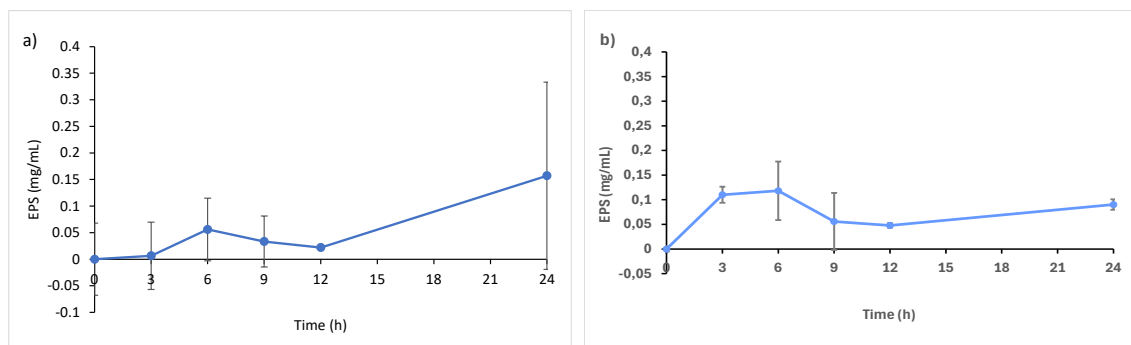


Fig. 11. Exopolysaccharide production by strains AYR-17 and AYO-14 over 24 hours of cultivation with potato substrate. (a) AYR-17 under aerobic conditions and (b) AYO-14 under anaerobic conditions.

Table 4. EPS yield of cultivation with potato substrate.

Strain	EPS yield ($\mu\text{g/mL}$)
AYR-17 (Aerobe)	157.04 ± 188.77
AYO-14 (Anaerobe)	90.23 ± 11.91

As shown in **Fig. 11a**, EPS production by AYR-17 under aerobic conditions started after 3 hours, presenting a notable increase after 12 hours. Among the component monomers, glucose was the main contributor to EPS production, reaching a final EPS yield of $157.04 \pm 188.77 \mu\text{g/mL}$ at the end of cultivation. In contrast, galactose and arabinose remained at consistently low concentrations throughout the entire cultivation, showing only a slight increase over time.

Fig. 11b shows that EPS production by AYO-14 under anaerobic conditions started within the first 3 hours of cultivation and reached its peak at 6 hours. After this point, a decline in EPS concentration was observed until 12 hours, followed by a partial increase in EPS concentration by 24 hours. The monomer profile exhibited a similar trend to that observed for strain AYR-17.

3.4. Comparison of growth rates across cultivations

To quantitatively compare growth rates across strains and substrates, μ_{max} values were calculated from the exponential phase of the $\ln$ (CFU/mL) curves.

Table 5. Maximum specific growth rates (μ_{max}) of strains AYR-17, AYR-1 and AYO-14 across different cultivation conditions.

Substrate	Maximum specific growth rate (h^{-1})			
	AYR-17 (Aerobic)	AYR-17 (Anaerobic)	AYR-1 (Aerobic)	AYO-14 (Anaerobic)
MRS broth	1.21	0.47	1.63	0.69
Yellow pea protein + glucose	0.96	0.73	1.58	0.91
Potato	0.21	n.d.	n.d.	1.11

4. DISCUSSION

In MRS broth cultivation, it is worth mentioning that although CFU counts in MRS medium declined to near zero by 24 hours, OD_{600} could still be noted (**Fig. 1**; **Fig. 2**). This can be explained by the fact that optical density captures total biomass (including dead, intact cells), whereas CFU counts represent only living, cultivable cells (Xiong et al., 2008). Regarding glucose utilization, it is important to note that its concentration started at around 10 g/L (**Fig. 2**), even though the initial glucose concentration in MRS broth is 20 g/L (**Table 1**). This could be explained by possible Maillard reactions, in which reducing sugars, such as glucose, would react with amino compounds to produce a variety of compounds. This reaction is influenced by pH and temperature, among other factors. The Maillard reaction would have occurred during prolonged exposure of MRS broth to high temperatures during autoclave sterilization, which would have reduced the amount of available glucose, possibly decreasing strain growth rate. This was also observed by Marounek & Brezina (1993), who investigated the effect of heat-induced formation of

soluble Maillard reaction products on glucose utilization by rumen bacteria. However, this was not observed during protein cultivations (**Fig. 5**), in which glucose source was added after autoclave sterilization. Lastly, differences between CFU count and OD measurements can be observed. For example, strain AYR-17 cultivated anaerobically displayed a lag phase (**Fig. 2b**) and reached peak growth at 9 hours, while in **Fig. 1** no lag phase was observed and peaked at 10 hours. It should be noted that these results were obtained from separate cultivations, and the inoculum may therefore have exhibited different growth behaviors at the beginning of the fermentation.

To evaluate the proteolytic activity of the *Weissella* strains on yellow pea protein, two cultivation conditions were initially tested: yellow pea protein alone, and yellow pea protein supplemented with glucose. When comparing both conditions, an improved growth and an increase in organic acid production was observed in the glucose supplemented medium. This can be explained by the fact that bacterial growth generally requires both a carbon and a nitrogen source. Yellow pea protein can supply nitrogen through its breakdown into peptides and amino acids, but it does not provide a fermentable carbon source. This is consistent with the metabolic characteristics of LAB such as *Weissella* spp., which efficiently ferment glucose to sustain their metabolism (Fessard & Remize, 2017; Fusco et al., 2023). In addition, the selection of glucose over other carbohydrates was based on previous evidence showing that glucose supports the highest production yields of the bacteriocin Weissellin A (Papagianni & Papamichael, 2012). Glucose supplementation was therefore included to provide a consistent energy source supporting bacterial metabolism. It should be noted, however, that proteolysis by *Weissella* was relatively slowly under these conditions, resulting in only limited peptide accumulation, which is discussed later in this study.

Strain AYR-1 showed the highest maximum growth rate following by AYR-17 among the tested strains when cultivated in MRS broth and protein medium supplemented with glucose under aerobic conditions (**Table 5**). Under anaerobic conditions, strain AYO-14 showed highest maximum growth rate followed by AYR-17 in both MRS broth and protein medium supplemented with glucose.

Strain AYO-14 showed the best growth when potato was used and outperformed aerobically cultivated AYR-17, which exhibited the overall lowest $\mu_{\max}$, at only 0.21 h^{-1} .

All strains grew accompanied by a slight and progressive decrease in pH in all cultivations. This is consistent with organic acid production via the heterofermentative metabolism of *Weissella*, which generates organic acids, among other products (Fessard & Remize, 2017).

Regarding glucose utilization, a more progressive and consistent consumption was observed for MRS broth (**Fig. 2**). In the protein-glucose supplemented media, strain AYR-17 and AYR-1 displayed a similar trend (**Fig. 5**), while strain AYO-14 showed less glucose uptake. Notably, more than half of the initial glucose concentration remained at the end of cultivation, revealing it was not a limiting factor for their growth. Despite the accumulation of both lactic acid and acetic acid in the protein-glucose medium (**Fig. 6**), the pH change shown in **Fig. 5** did not decrease as expected. This may be related to the buffering capacity of the high-protein substrate, as it is possible that amino acids and peptides present in the medium could act as natural buffering agents, and therefore neutralize accumulated acids. The cause of the observed decline in viable cells therefore remains unclear, as neither lack of substrate nor significant acid stress were a cause of it.

Another factor, such as production of inhibitory metabolites may have contributed to the loss of viability.

Under both aerobic and anaerobic conditions, strains AYR-17, AYR-1, and AYO-14 produced notable amounts of lactic acid, highlighting their function as LAB. Lactic acid is a metabolite of interest due its wide range of industrial applications, including the food, pharmaceutical, cosmetic, and chemical industries, where it is used as a preservative, acidulant, and antimicrobial agent. It is also an important sustainable precursor for the production of biodegradable plastics, such as polylactic acid (PLA) (Ojo & De Smidt, 2023). Acetic acid was notably produced by the strain AYR-17 under aerobic conditions, whereas under anaerobic conditions, no production was observed. The same condition-dependent pattern was found for the other strains: in MRS broth, AYR-1 (aerobic) accumulated acetic acid by 24 h (**Fig. 3c**), while AYO-14 (anaerobic) showed only a moderate increase (**Fig. 3d**). This is due to oxygen presence, where it shifts heterofermentative LAB metabolism toward different end products (Zotta et al., 2018). Under aerobic conditions, when oxygen is present, carbon flux shifts toward acetate-producing routes. Pyruvate aerobic metabolic route is shown in **Fig. 12**. As can be observed in **Fig. 3**, **Fig. 4**, **Fig. 6** and **Fig. 9**, when grown anaerobically, AYR-17 and AYO-14 produced mainly lactic acid and ethanol, with small amounts of acetic acid. However, when grown aerobically, lactic acid and ethanol production was reduced with acetic acid experimenting a significant increase. The same outcome was observed by Guo et al. (2017), where oxygen repressed the expression of alcohol dehydrogenase (ADH) of *Lactobacillus brevis* and thus shifted ethanol production to acetate, involving pyruvate oxidase (POX) enzyme.

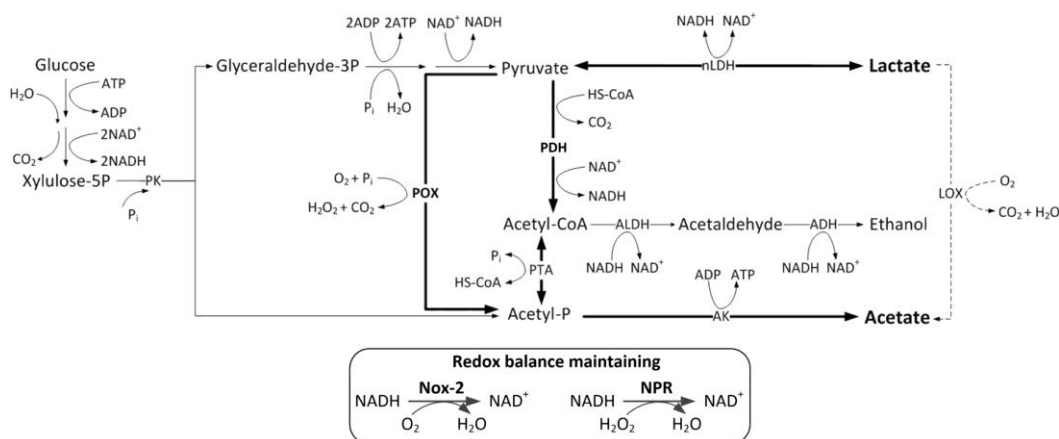


Fig. 12. Pyruvate metabolism. POX, pyruvate oxidase; ADH, alcohol dehydrogenase. (Guo et al., 2017)

Furthermore, Zotta et al. (2018) demonstrated the presence of *pox* gene in *Weissella* and reported improved growth of several *Weissella* strains under aerobic conditions, including *W. cibaria*, *W. confusa* and *W. viridescens*. However, this trend was not observed in the growth curve of **Fig. 5**, where strains AYR-17 and AYO-14 displayed a better growth under anaerobic conditions. Growth efficiency supported by *pox* gene would be counteracted by acid stress. However, slightly improved growth was observed in MRS medium (**Fig. 1**). One possible explanation is that the protein medium supplemented with glucose provided less protection against cell stress. As shown in **Fig. 12**, the POX pathway leads to acetate formation, producing hydrogen peroxide (H₂O₂) as byproduct. MRS broth contains several components, including peptones, yeast extract, meat extract and vitamins (**Table 1**), which may support stress tolerance in LAB. In contrast, the protein medium supplemented with glucose lacks these components, leading to H₂O₂

accumulation, which, together with acetate, might have negatively affected bacterial growth. Furthermore, although the protein substrate theoretically serves as a nitrogen source, it is possible that there was not enough available nitrogen, which is vital for the cells and their growth.

Acetic acid was present at the start of all cultivations with an initial concentration ranging from 3 to 4 g/L. This may be explained by the presence of sodium acetate in both MRS broth (5 g/L) (**Table 1**) and basal medium (8.29 g/L) (**Table 3**). Acetic acid production by LAB under aerobic conditions is of interest due to its role in energy metabolism, oxidative stress adaptation, antimicrobial activity, and flavour development in fermented foods. Increased acetate formation may enhance ATP production and biomass yield, while also contributing to microbial inhibition and product quality. Beyond its role in food cultivation, acetic acid has gained growing interest as a versatile metabolic intermediate and as industrial platform chemical. As highlighted by Kim et al. (2021), acetate can serve as a sustainable feedstock to produce value-added compounds, including tyrosine and fatty acids, and has significant applications in biotechnology and metabolic engineering. This underscores the biotechnological importance of understanding acetate metabolic pathways to optimize cultivation processes and metabolite production. Regarding formic acid production, it is usually associated with anaerobic conditions, where pyruvate formate-lyase (PFL), enzyme responsible for formate generation, is inhibited by oxygen (Andorfer et al., 2021). However, there is no strong evidence of active formate accumulation during cultivation, since concentrations stayed stable over time. This suggests that the PFL pathway may not be fully activated under the tested conditions. Formic acid is widely used as an antimicrobial preservative in food, feed, and silage production, as well as in the leather, textile, and chemical industries. In addition, it is gaining importance in green chemistry, as it is a promising new fuel for hydrogen and fuel cells and enables the generation and storage of electrical energy (Okoye-Chine et al., 2022; Ricke et al., 2020).

To investigate peptide release during cultivation, the OPA assay was used to indirectly assess proteolytic activity by measuring total primary amine content, reflecting the balance between the release of free amino groups through hydrolysis and their consumption by the cells (Frister et al., 1988; Nielsen et al., 2001). The changes in primary amine content observed in this experiment suggest that generally the consumption of free amino acids and peptides by the bacterial cells exceeded the release of free amino groups generated by proteolytic activity (both protein hydrolysis and peptide cleavage) on the yellow pea protein substrate (**Fig. 7**). Only strain AYR-17 cultivated under aerobic conditions showed a more dynamic balance, with an increased proteolytic activity after 6 hours (**Fig. 7a**).

A major limitation of this method is that the OPA assay provides only the net balance between two simultaneous processes - the release of free amino groups through protein and peptide hydrolysis on one hand, and their consumption by the cells on the other. As a result, it cannot be confirmed that proteolysis is occurring at all, only that consumption is faster than any release of free amino groups. This interpretation is supported by the growth and glucose data shown in **Fig. 5**, where a notable glucose fraction was still available at the end of cultivation. Bacteria growth could have also been supported by free amino acids and peptides released during cultivation or autoclave sterilization. These peptides would be taken up by LAB through the oligopeptide permease (Opp) system, which is essential for their nitrogen nutrition (Savijoki et al., 2006).

The progressive decrease in primary amine content observed in **Fig. 7** is therefore consistent with bacterial cells growing and consuming nitrogen sources. After the growth peak **Fig. 5** (b, c and d), the gradual decline in primary amine content throughout cultivation reflects the continuous consumption of nitrogen sources by the growing cells. The apparent increase in primary amine content observed in AYO-14 at 12 hours (**Fig. 7d**) correlates with a sharp drop in viable cell counts between 9 and 12 hours (**Fig. 5d**), suggesting that cell lysis during the death phase may have released intracellular compounds back into the medium. However, the large standard deviation at this time point indicates that this value should be interpreted with caution. The increased amino uptake by AYR-17 under aerobic conditions the first 6 hours of cultivation (**Fig. 5a**), would support its initial growth. From the perspective of peptide production, these findings indicate that, under these conditions, the released peptides were consumed by the bacterial cells rather than accumulating in the cultivation broth. Nonetheless, further analyses, such as amino acid profiling by HPAEC-PAD, would be required to confirm the actual contribution of these nitrogen sources.

Weissella spp. have been reported to display a substrate dependent metabolic shift: on monosaccharide-rich substrates, carbon is primarily directed toward short-chain fatty acid (SCFA) and organic acid production, whereas on polysaccharide-rich substrates the same strains prefer to synthesise exopolysaccharides (EPS), with little or no acid production. Importantly, *Weissella* can utilise polysaccharides directly as a carbon source without the hydrolysis to monomers (Kavitake et al., 2020). For this reason, the strains were also assessed for their ability to synthesise exopolysaccharides. EPS production resulting from cultivation on potato is shown in **Table 4**. Strain selection for this cultivation was based on the superior growth of strain AYR-17, while AYO-14 was included to assess production under anaerobic conditions as well. AYR-17 and AYO-14 showed EPS yields of 157.04 ± 188.77 $\mu\text{g/mL}$ and 90.23 ± 11.91 $\mu\text{g/mL}$, respectively. These results indicate the bacterial ability to use starch for the synthesis of EPS. EPS producing ability of *Weissella* spp. has been previously reported by several authors (Wolter et al., 2014; Yu et al., 2018). For instance, Aregbe et al. (2020) reported a high EPS yield of 824.44 ± 4.19 mg/L for the strain *Weissella* sp. IMAU50209, strains from raw potatoes steamed and subjected to very high pressure.

Potato is a starchy tuber whose granules consist of two types of polysaccharides composed exclusively of glucose monomers (Bertoft & Blennow, 2016). In both strains, glucose was the predominant monosaccharide detected, which aligns with the carbohydrate composition of potato. The elevated levels could also be due to free glucose released during the autoclave sterilization process. For strain AYR-17, glucose was the main contributor to EPS increase after 12 hours of cultivation (**Fig. 11**). Exopolysaccharide production by strains AYR-17 and AYO-14 over 24 hours of cultivation with potato substrate. (a) AYR-17 under aerobic conditions and (b) AYO-14 under anaerobic conditions. **Fig. 11**). It should be noted that production occurred during the stationary phase (**Fig. 8a**), indicating that EPS production is not dependent on growth. In contrast, strain AYO-14 showed a different pattern (**Fig. 11d**). EPS concentration rose during the early hours of cultivation, peaking at 6 hours, when the bacterial population had reached a stable cell density (**Fig. 8b**). After this point, EPS concentration declined from 6 to 12 hours. Compared to AYR-17's increase after 12 hours, the earlier EPS peak in AYO-14 suggests that this anaerobic strain transitions into EPS biosynthesis immediately after the rapid initial growth phase. In parallel with EPS production, primary amine content was monitored using the OPA assay (**Fig. 10**). Throughout the 24-hour cultivation, primary amine levels in both strains fluctuated within a narrow range (~ 0.20 – 0.40 mM). An apparent rise was observed at 3 hours in both

strains, but the high variability between replicates at this time point makes it difficult to distinguish proteolysis from analytical noise. This suggests that proteolytic activity, if present, was limited under these conditions. During cultivation, microorganisms metabolize available carbohydrates and channel them through central metabolic pathways, generating precursors for EPS synthesis. Under favourable environmental and growth conditions, EPS-producing bacteria can thus convert the substrate into extracellular polysaccharides, often resulting in glucose-rich EPS structures (Patrimath et al., 2025). In addition, small amounts of arabinose and galactose were detected in both strains, both of which are components of potato peel polysaccharides (Scharf et al., 2020). Since potato peel was included in the cultivation substrate, it is very likely that bacteria used components from the whole potato as carbon sources for EPS production, supporting the bacteria's ability to produce heteropolysaccharides (HePS). However, the final EPS yield in AYR-17 showed a notable deviation ($392.83 \pm 175.80 \mu\text{g/mL}$) (**Fig. 11a**), so EPS production could not be fully confirmed for that strain. Additional analyses, such as strain identification, EPS purification, molecular weight determination, and structural characterization of the produced EPS, would be necessary to better clarify the relationship between cultivation conditions and EPS biosynthesis.

EPS are of great interest because they play an important role in textural and structural properties of food, act as natural emulsifiers, and offer health benefits, including gut health support, immune-boosting effects, and antimicrobial properties. Aregbe et al. (2020) reported the potential use of EPS produced by LAB, including the species *Weissella*, in potato-based fermented products. Specifically, EPS produced by *Weissella cibaria* have recently attracted growing interest due to their multifunctional and biological properties. Zammouri et al. (2024) reported EPS produced by *W. cibaria* and their potential as bio-hydrocolloid agent in emulsion stability due to their interfacial properties. Furthermore, certain EPS have been described as presenting antioxidant and probiotic properties, promoting SCFA production by gut microbes, and possessing oxidative stress alleviating, anti-inflammatory and anti-cancer potential. (Patrimath et al., 2025; Kavitate et al., 2020; Kavitate et al., 2022)

Future experiments should investigate cultivation parameters to optimize aspects such as growth and cultivation products. For instance, Tuccillo et al. (2022) investigated how cultivation conditions affected the synthesis of certain products by *W. confusa* A16 in faba bean protein concentrate, demonstrating that modifying parameters such as cultivation time, temperature, and inoculum ratio affected the synthesis of volatile compounds, EPS (such as dextran), and organic acids.

5. Conclusion

Lactic acid bacteria (LAB) are recognized for their ability to hydrolyse different types of substrates, including plant-based proteins, releasing products of nutritional and biotechnological interest. In this thesis, three *Weissella* strains, AYR-17, AYR-1, and AYO-14, were investigated for their ability to grow on different substrates, proteolytic activity, and production of organic acids, including short chain fatty acids (SCFAs), and exopolysaccharides (EPS).

The results confirmed heterofermentative metabolism of the bacteria, where aerobic cultivation favoured acetate production, while anaerobic conditions promoted lactate and ethanol production formation. Furthermore, the strains were able to utilize starch substrates and synthesize glucose-rich heteropolysaccharides (HePS). The potential ability to digest plant-based proteins and starch-based substrates makes *Weissella* bacteria

interesting for further application in sustainable fermentation and biotechnological processes. Further studies should focus on optimizing fermentation conditions to enhance bioactive metabolite productions and on investigating the production of functional amino acids, such as γ -aminobutyric acid (GABA).

6. References

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