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Screening and bioreactor cultivation of wild-type lactic acid bacteria for high purity D-lactic acid production

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ABSTRACT

Biodegradable polymers are the green alternative to conventional oil-based plastics and have a key role in the achievement of the Sustainable Development Goals of Agenda 2030. Nowadays, polylactic acid (PLA) is one of the most common bioplastics present in the global market. Optically pure D-lactic acid (D-LA) is a valuable monomer for the production of high-performance PLA materials. This study aims to select wild-type lactic acid bacteria capable of producing D-LA with high optical purity and to define an optimized fermentation process in bioreactors to maximize production. A total of 150 LAB strains from the University of Parma Culture Collection (UPCCO) were screened using enzymatic assays and HPLC analysis. Among them, *Lactobacillus delbrueckii* UPCCO 2214 and *Leuconostoc citreum* UPCCO 4516 were selected for fed-batch cultivations for their high D-LA production and purity (>97 %). After optimization of fermentation parameters, *L. delbrueckii* UPCCO 2214 was found to be the most efficient strain for D-LA production, with a yield of 0.74 g/g and a volumetric productivity of 0.96 g/L/h, outperforming *L. citreum* UPCCO 4516. Bioreactors cultivation has helped to understand microbial production and boost their potentialities. This work supports further investigations and improvements in D-LA production processes to advance the field of biomaterials with benefits for both industry and the environment.

1. Introduction

In recent years, the European Union has reinforced its commitment to sustainable plastic use through initiatives like the Circular Economy Action Plan [1] and the EU Policy Framework for Bioplastics [2]. These policies promote innovation in recyclable and biodegradable materials, aiming for all plastic packaging to be recoverable by 2030. As a result, bioplastics—particularly biodegradable polymers—have gained traction as eco-friendly alternatives to conventional plastics, driven by growing environmental awareness and market demand [3].

Among biodegradable bioplastics, polylactic acid (PLA) stands out due to its biodegradability, biocompatibility, and mechanical properties comparable to conventional plastics like polystyrene and PET [4]. PLA accounts for a significant share of global bioplastic production, driven by its versatility and expanding applications across packaging, textiles, and biomedical sectors [5–7].

The total lactic acid (LA) produced annually worldwide is not only destined for polymerization (30 % market share in 2022) but it is addressed to different economic sectors, where it finds several applications [8]. In 2024, the global market of LA was valued at 4.07 billion US dollars, however, it is estimated to grow up to 8.70 % by 2034 due to its use as a building block of polylactic acids [9–12]. Corbion (Amsterdam, The Netherlands), Galactic (Celles, Belgium), and NatureWorks LLC (Minnetonka, MN, USA) are the global leader companies for lactic acid production [13].

Lactic acid ($C_3H_6O_3$) exists in two enantiomeric isoforms L(+)-LA

Abbreviations: LA, Lactic Acid; PLA, Polylactic acid; PLLA, Poly-L+ -lactic acid; PDLA, Poly-D–lactic acid; PDLLA, Poly-D/L-lactic acid; LAB, Lactic Acid Bacteria; UPCCO, University of Parma Culture Collection; D-LDH, D+ -Lactate Dehydrogenase; L-LDH, L–Lactate Dehydrogenase; $Y_{p/s}$, Yield of LA; P, Volumetric Productivity; *r*, Glucose Consumption Rate.

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and D(-)-LA, or in a racemic mixture of them (D/L-LA) based on the production process [14]. LA can be produced both via chemical synthesis and microbial fermentation [15,16]. The chemical synthesis mostly gives LA as a racemic mixture, whereas microbial fermentative processes guarantee optically pure enantiomers [17,18].

PLA can be obtained from the racemic mixture (PDLLA), or either from L-LA (PLLA) or D-LA (PDLA) [19]. The polymerization process and enantiomeric ratio affect both the chemical and physical properties of the final polymer [20]. PDLLA is thermally and mechanically unstable due to its amorphous form, while PLLA and PDLA have better properties, but are still unstable to heat. However, it was studied that the stereo complex obtained from the combination of PLLA and PDLA has enhanced mechanical performance, hydrolysis resistance, and thermal stability [21]. This is due to the chemical stabilization via hydrogen and van der Waals bonding which increases the melting point of the polymer from 60°C (PLLA) to 230°C [22].

L-LA is more commonly produced and diffused than D-LA for its major use in the food and pharmaceutical industry [23]. D-LA, instead, stayed behind the scenes because of its toxicity [24], but it has now gained attention for the more significant stability of PDLA.

The production of pure enantiomers and the possibility of recovering waste substrates, thus implementing the circular economy, are some of the advantages of fermentation over chemical synthesis. This has led the fermentative processes to drive the LA production market, estimating that about 90 % of the total LA is obtained by microorganisms [22]. LA is the main end-product of the lactic acid bacteria (LAB) metabolism, therefore they are doubtlessly involved in the LA market [25].

Among them, *Leuconostoc* spp. and *Lactobacillus delbrueckii* strains may produce D(-)-lactic acid with high optical purity and yield [23, 26–28]. Compared to L-LA-producing microorganisms, optically pure D-LA producers have lower fermentative performances. Currently, promising results are emerging by the development of less frequent LAB species such as *Sporolactobacillus inulinus* [29], by using innovative bioreactor setup to improve the D-LA production. On the other hand, metabolic and genetic engineering has represented a key method to improve microbial production of D-LA biosynthetic capabilities. This approach has been successfully applied to bacteria, as is the case of *Corynebacterium glutamicum*, that was genetically modified to inhibit L-LA production, reaching 17.92 g/l (optical purity higher than 99.9 %), over 30 % higher than the originating strain [30]. More recent works have focused on yeasts, both conventional and unconventional, for D-LA production. Works performed on *Saccharomyces cerevisiae* [31] and, more recently, on *Yarrowia lipolytica* [32], showcase the possibility offered by these cell factories, that have high yield, and better tolerance to acidic environments. Besides, they are more easily adapted to cheaper and/or complex media. However, genetically modified microorganisms face restricted industrial use because of limitations posed by biosafety regulations and issues related to genetic instability. Therefore, industrial production facilities continue to favour the use of wild strains [33].

In this perspective, culture collections (CCs) and microbial biological resource centers (mBRCs) are essential for preserving microbial biodiversity supporting both research and industrial innovation [34].

In the field of industrial microbiology, wild-type strains from microbial represent a valuable resource. Frequently, this microbial diversity has represented a source of product innovation and diversification in the food sector, but recently this potential is starting to be developed for chemical industries [35].

The Microbial Resource Research Infrastructure – European Research Infrastructure Consortium (MIRRI-ERIC) promotes the systematic investigation and valorisation of microbial resources [36]. The University of Parma hosts the UPCCO collection, comprising over 6000 isolates, mainly composed of Lactic Acid Bacteria (LAB) isolated from different food matrices, and associated with the Joint Research Unit (JRU) of MIRRI-IT. While initially oriented towards food-related traits, UPCCO strains are now being investigated for potential industrial microbiology applications [37,38].

This work aimed to identify D-LA-producing strains within a microbial collection of 150 wild-type LAB strains from the University of Parma Culture Collection (UPCCO).

An enzymatic method was employed to find D-LA producers out of 150 strains. Successively, 20 were selected and cultivated on a small scale for 72 h and LA production was followed by HPLC analysis.

After the selection of the most promising strains, fermentation was optimized in bioreactors to investigate process optimization for the production of high-purity D-LA from glucose.

2. Material and methods

2.1. Primary screening

A set of 150 Lactic Acid Bacteria strains was selected from the University of Parma Culture Collection (UPCCO). The screening involved species belonging to 12 different genera, previously isolated from several food matrices, especially of dairy origin, since 2012. Cultures were stored at −80 ∘C in their optimal growth medium (MRS, TSB or M17) containing 15 % glycerol (v/v). Strains were propagated three times in a fresh medium and incubated overnight. Using glass tubes with cap, 6 ml of MRS, TSB (VWR International bvba, Leuven, Belgium) or M17 (Merck, Darmstadt, Germany) were inoculated with 3 % overnight preculture and incubated at the optimal temperature of strains for 24 h in anaerobiosis (AnaeroGen, ThermoScientific, Landsmeer, The Netherlands). After incubation, 1 ml of culture was centrifuged at 13,400 rpm for 10 min and the supernatant was analysed. L-LA and D-LA were separately detected by enzymatic method using the D-/L-lactic acid rapid assay kit (Megazyme®, Ireland). Samples were diluted to a detectable concentration (0.005–0.3 g/L for each isomer) and two sequential enzymatic reactions were performed by using D-LDH and L-LDH. The absorbance of every reaction was read at 340 nm and the concentration of the two isomers was calculated as follows:

$$c = \frac{V * MW}{\varepsilon * d * v} * \Delta A_{D-LA\ orL-LA} \tag{1}$$

where c = concentration of D-LA or L-LA (g/L)
V = final volume (ml)
MW = molecular weight of lactic acid (g/mol)
ε = extinction coefficient of NADH at 340 nm
d = light path (cm)
v = sample volume (ml)
$\Delta A_{D\text{-}LA}$ = difference between the absorbance of the first enzymatic reaction (after addition of D-LDH) and that of the sample before the reaction
$\Delta A_{D\text{-}LA}$ = difference between the absorbance of the second enzymatic reaction (after addition of L-LDH) and that of the first enzymatic reaction

The supernatants were also analysed by HPLC (Shimadzu, Korneuburg, Austria) to accurately quantify the total lactic acid produced. The HPLC was equipped with an Aminex HPX-87H column (300 × 7.8 mm, Biorad) operated at a temperature of 60 °C, a flow of 0.6 ml/min and by using 4 mM H_2SO4 as mobile phase. Samples and standards for HPLC analysis consisted of 900 μL of sample/standard added with 100 μL 40 mM H_2SO4. Subsequently, samples and standards were filtered. For the detection of metabolites, a refraction index detector (RID-10A, Shimadzu, Korneuburg, Austria) was used.

2.2. Secondary screening

Strains producing 80 % or more of D-LA were selected (n = 20). Cultures were repeated on a small scale on MRS (pH = 6.2) to further reduce the number of strains to scale in bioreactors. MRS medium contained per litre: 10 g casein peptone, 10 g meat extract, 5 g yeast extract, 1 g tween 80, 2 g K_2HPO_4, 5 g sodium acetate, 2.60 g tri-sodium citrate·2 H_2O, 1.17 g $NH_4(HPO_4)_2$, 0.20 g $MgSO_4·7\,H_2O$, 0.05 g $MnSO_4·H_2O$ and 22 g glucose·H_2O. Using glass tubes with cap, 6 ml MRS were inoculated to an $OD_{600} = 0.2$ and subsequently incubated at 30°C for 72 h in anaerobic conditions (AnaeroGen, ThermoScientific, Landsmeer, The Netherlands). The growth was followed every 24 h by $OD_{600\ nm}$ measurement on a spectrophotometer (Hitachi U-1900) and the pH decrease was monitored. The supernatant was centrifuged (13,400 rpm for 10 min), and glucose, lactic acid and ethanol were quantified by HPLC analysis (Shimadzu, Korneuburg, Austria). Based on the highest amount and purity of lactic acid produced, two strains were selected for the scaling up: *Leuconostoc citreum* UPCCO 4516 and *Lactobacillus delbrueckii* UPCCO 2214.

2.3. Scaling-up in bioreactors

2.3.1. Analysis

Pre-cultures were prepared to inoculate a batch with a specific OD_{600}. Fermentations in a DASGIP® parallel bioreactor system (Eppendorf International, Jülich, Germany) were set up as suggested by Russmayer et al. [39]. The batch process was carried out with an initial volume of 700 ml MRS with 22 g/L glucose monohydrate as a carbon source, inoculated to an OD_{600} of 0.2 with an overnight culture. After the total glucose depletion, we started the fed-batch process initially characterised by a constant feeding rate of 2.5 ml/h 50 % (w/v) glucose. The pH was kept constant at 6.2 via the addition of 4 M NaOH, temperature set at 30°C for *L. citreum* UPCCO 4516 and 37°C for *L. delbrueckii* UPCCO 2214, anaerobiosis, agitation was set to 200 rpm. The fermentations were stopped at 74 h. This process was successively optimized: i) by starting the feeding as soon as the glucose was depleted (7 h for both strains); ii) by adjusting the feeding rate to 4 ml/h for *L. delbrueckii* UPCCO 2214 and 6 ml/h for *L. citreum* UPCCO 4516; iii) by increasing the NaOH concentration to 8 M; iv) by adding 10 times concentrated fresh medium without glucose as soon as the growth stopped (24 h) to provide new nutrients. These experiments were conducted in duplicate. Cultures were sampled periodically: the OD_{600} was measured to follow the growth, and glucose consumption, lactic acid and ethanol production were analysed through HPLC.

2.3.2. Calculations

The yield of LA ($Y_{p/s}$), the volumetric productivity (P), and the glucose consumption rate (*r*) were calculated using the following equations:

$$Y_{p/s} = \frac{lactic\ acid\ produced(g)}{glucose\ consumed(g)} \tag{2}$$

$$VP = \frac{total\ lactic\ acid\ concentration(g/L)}{fermentation\ time(h)} \tag{3}$$

$$r = \frac{glucose\ consumed(g)}{fermentation\ time(h)} \tag{4}$$

Productivity in terms of lactic acid and biomass produced was also evaluated by estimating the cell dry mass (CDM) according to the method reported by Pflügl et al. [40]. Results from duplicate experiments are reported as mean ± standard deviation.

3. Results

3.1. Primary screening

A set of 150 LAB strains was selected from the UPCCO, by ensuring a large biodiversity of the sampled strains. As reported in Table 1, the screening involved species belonging to 12 genera, previously isolated across a 10 years temporal range (2012–2022) from several food matrices, especially of dairy origin.

Table 1

Strains selected from UPCCO for the primary screening.

Genera	*Species*	*No. strains*	*Isolation source*
Companilactobacillus	*farciminis*	2	Sourdough
Enterococcus	*avium*	1	Sourdough
	faecalis	10	Cheese, cream, curd, milk
	faecium	6	Cheese, milk, whey starter
Lacticaseibacillus	*casei*	1	Cheese, milk
	paracasei	15	Cheese, milk
	rhamnosus	12	Cheese, curd, milk
	zeae	8	Cheese
Lactiplantibacillus	*plantarum*	6	Cheese
Lactobacillus	*amylolyticus*	1	Curd
	delbrueckii	11	Curd, milk, natural whey starter
	gasseri	1	Milk
	helveticus	9	Curd, milk, natural whey starter
Lactococcus	*lactis*	1	Milk
Latilactobacillus	*curvatus*	10	Cheese
Lentilactobacillus	*diolivorans*	1	Milk
	hilgardii	2	Milk
	otakiensis	2	Milk
Leuconostoc	*citreum*	8	Milk, sourdough
	lactis	1	Milk
	mesenteroides	6	Cheese, milk
	pseudomesenteroides	1	Milk
Levilactobacillus	*brevis*	7	Sourdough
Limosilactobacillus	*fermentum*	8	Cheese, curd, whey starter
Streptococcus	*macedonicus*	2	Milk
	pasteurianus	2	Milk
	porcorum	2	Milk
	thermophilus	9	Milk, cheese
	uberis	5	Milk

Out of 150 strains, setting 80 % as a threshold for isomeric lactic acid, 74 produced L-LA, 20 produced D-LA and the rest (56 strains) produced a racemic mixture of both isomers (Fig. 1). The total lactic acid production was successively quantified by HPLC analysis: results related to the selected D-LA-producing strains are reported in Table 2.

3.2. Secondary screening

The secondary screening was aimed at monitoring the production of primary metabolites by the selected strains over time. As shown in Fig. 2, LAB's growth (OD_{600}), glucose consumption, lactic acid and ethanol production were analysed over time (72 h). Compared to *Leuconostoc* spp. strains, *L. delbrueckii* ones showed a greater production of biomass (Fig. 2A), a slower consumption of glucose (Fig. 2B), and constant production of lactic acid until 72 h (Fig. 2D). *Leuconostoc* spp. strains finished all the sugar by 24 h and, accordingly, stopped producing lactic acid. Only *Leuconostoc* spp. strains produce ethanol because of the heterofermentative metabolism.

Despite all strains of *L. delbrueckii* produced more lactic acid than *Leuconostoc* spp. by using the same amount of glucose, the produced lactic acid is characterised by a lower optical purity (Table 2). However, considering the greatest amount of LA produced and the highest isomeric purity, *L. delbrueckii* UPCCO 2214 and *L. citreum* UPCCO 4516 were selected for the scaling process. At the end of the fermentation, *L. delbrueckii* UPCCO 2214 and *L. citreum* UPCCO 4516 produced 20.40 g/L and 11.69 g/L lactic acid, respectively (Table 3). The yield of LA ($Y_{p/s}$) and the volumetric productivity (P) were calculated as reported in Section 2.3.2, and were respectively $Y_{p/s} = 0{,}90$ g/g and $P = 0{,}28$ g/L/h for *L. delbrueckii* UPCCO 2214, and $Y_{p/s} = 0{,}63$ g/g and $P = 0{,}15$ g/L/h for *L. citreum* UPCCO 4516.

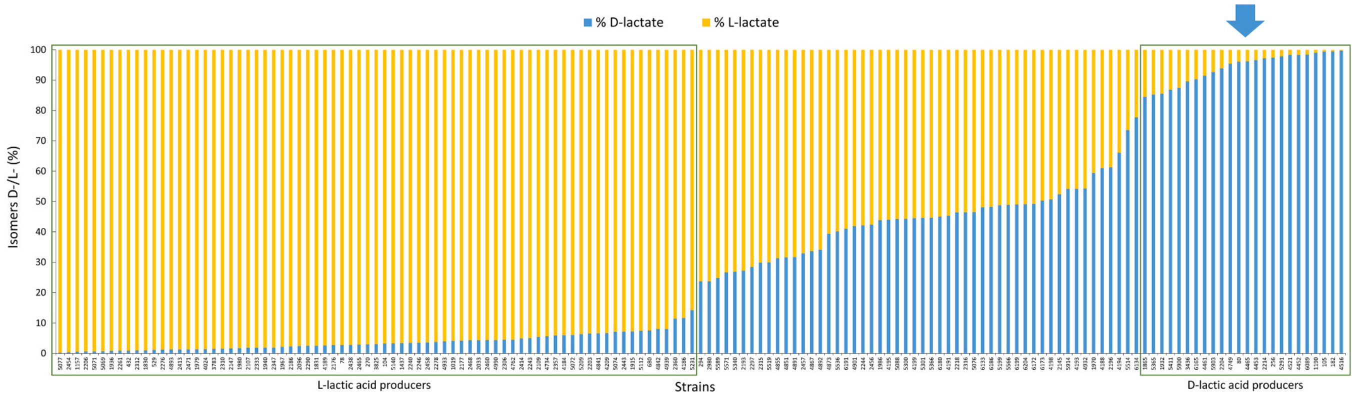

Fig. 1. Percentage of lactic acid isomers produced by screened LAB and quantified by enzymatic method. Strains producing more than 80 % of D-LA were selected (right side panel).

Table 2
Selected strains producers of high purity D-lactic acid (> 80 %). Percentage of the D-LA isomer produced was measured by enzymatic method and lactic acid production (g/L) was detected at 24 h by HPLC analysis.

ID strain	Species	Lactic acid (g/L)	D-LA (%)
4516	*Leuconostoc citreum*	8.77	99.70
182	*Leuconostoc citreum*	8.84	99.46
105	*Leuconostoc mesenteroides*	8.96	99.39
1190	*Leuconostoc lactis*	7.72	99.03
6089	*Leuconostoc mesenteroides*	8.02	98.42
4452	*Leuconostoc citreum*	8.91	98.35
4521	*Leuconostoc citreum*	7.85	98.32
5291	*Leuconostoc pseudomesenteroides*	8.51	97.87
256	*Lactobacillus delbrueckii*	13.55	97.40
2214	*Lactobacillus delbrueckii*	17.05	97.21
4453	*Leuconostoc citreum*	8.76	96.64
4465	*Leuconostoc citreum*	8.24	96.20
80	*Leuconostoc mesenteroides*	8.98	96.08
4749	*Lactobacillus delbrueckii*	8.95	95.43
2204	*Leuconostoc citreum*	8.26	93.87
4461	*Leuconostoc citreum*	8.08	91.44
6165	*Leuconostoc mesenteroides*	7.62	90.30
1932	*Lactobacillus delbrueckii*	17.24	85.56
5365	*Lactobacillus delbrueckii*	13.88	85.26
1865	*Lactobacillus delbrueckii*	11.75	84.46

3.3. *Fermentation in bioreactors*

L. delbrueckii UPCCO 2214 and *L. citreum* UPCCO 4516 were selected for bioreactor cultivations and optimization. An experimental fed-batch process was set, in a parallel cultivation system comprising two 1.2 L vessels filled with 700 ml of MRS media.

The batch process lasted 7 h: *L. delbrueckii* UPCCO 2214 and *L. citreum* UPCCO 4516 consumed 22.05 ± 0.59 g/L and 21.21 ± 0.60 g/L of glucose, respectively, and produced 19.59 ± 0.56 g/L and 10.58 ± 0.33 g/L of lactic acid, respectively. *L. delbrueckii* showed a yield$_{p/s}$ ($Y_{p/s}$) of 0.82 ± 0.003 g/g and a volumetric productivity (P) of 2.80 ± 0.08 g/L/h. *L. citreum*, instead, had $Y_{p/s} = 0.46 \pm 0.0002$ g/g and $P = 1.51 \pm 0.05$ g/L/h (Table 4).

During this phase, considering the biomass produced by strains, *Leuc. citreum* showed higher productivity (1.08 ± 0.16 $g_{LA}/g_{Biomass}$/h) than *L. delbrueckii* (0.78 ± 0.003 $g_{LA}/g_{Biomass}$/h) which produced a great amount of biomass.

The last 2 h of the batch phase allowed us to calculate the theoretical maximum glucose consumption rate (*r*) to subsequently set the optimal feeding rate: $r = 4.55 \pm 0.05$ g/h for *L. delbrueckii* UPCCO 2214 and $r = 4.70 \pm 0.13$ g/h for *L. citreum* UPCCO 4516.

After 7 h, the fed-batch phase was started, and the process continued until 74 h, with supplementation of ten times concentrated MRS media after 30 h from the cultivation start. At the end of the fermentation, *L. citreum* and *L. delbrueckii* produced 63.08 ± 0.73 g and 55.18 ± 0.16 g, respectively). However, *L. citreum* consumed much more glucose (149.64 ± 0.81 g, Table 4) and more constantly until 49 h (Fig. 3B) compared to *L. delbrueckii* (74.26 ± 0.35 g glucose consumed, Table 4) that started accumulating the sugar after 24 h (Fig. 3A).

At 74 h, as reported in Table 4, *L. delbrueckii* reached twice the yield$_{p/s}$ of *L. citreum* (0.74 ± 0.001 g/g and 0.42 ± 0.003 g/g, respectively), although they present a similar final volumetric productivity (0.96 ± 0.009 g/L/h and 0.85 ± 0.01 g/L/h, respectively). *L. delbrueckii* UPCCO 2214 was selected as the most promising strain of the screening for D-lactic acid production as it is the fastest and the least C-consuming strain with the highest yield ($Y_{p/s} = 0.74 \pm 0.001$).

4. Discussions

This study aimed to identify high purity D-LA producers among wild-type lactic acid bacteria strains within the UPCCO, following a top-down approach. First, a large number of isolates was subjected to a primary screening, followed by a narrower secondary screening on the best-performing strain, and finally scaling up and optimizing fermentation on two high-producing strains.

The primary screening, performed with an enzymatic method, elucidated the production capability of D-LA and L-LA of 150 strains from UPCCO collection. Based on the result of this screening, *Lactobacillus delbrueckii* and *Leuconostoc* spp. UPCCO strains were found to produce high purity D-LA, thus confirming what was reported in the literature [33,41–45]. After 24 h of fermentation, it is already possible to distinguish two different behaviours: *L. delbrueckii* strains produced more than 10 g/L of lactic acid, while *Leuconostoc* spp. showed a lower titre. On the other hand, D-lactic acid obtained from *Leuconostoc* spp. has a higher optical purity than from *L. delbrueckii*.

The secondary screening confirmed the two different trends, due to the type of metabolism characterizing the strains. *L. delbrueckii* is a homofermentative bacteria that produces primarily lactic acid from sugars via the Embden-Meyerhof Pathway (EMP), while *Leuconostoc* strains are characterised by a heterofermentative metabolism, which addresses sugars for lactic acid, ethanol/acetic acid and carbon dioxide production via the Phosphoketolase Pathway (PP) [46,47]. In the first 24 h of fermentation, *Leuconostoc* spp. strains finished all the glucose supplied, therefore they stopped producing LA before *L. delbrueckii,* which instead consumed the sugar more slowly and continued to produce LA until the end of fermentation. Both strains also showed a different biomass yield on glucose, which was higher for *L. delbrueckii* strains than *Leuconostoc* ones, suggesting a different carrying capacity of the two species.

Based on the highest amount and purity of D-LA produced, two strains were selected for bioreactor cultivation: *L. delbrueckii* UPCCO 2214 and *L. citreum* UPCCO 4516. It is important to acknowledge that

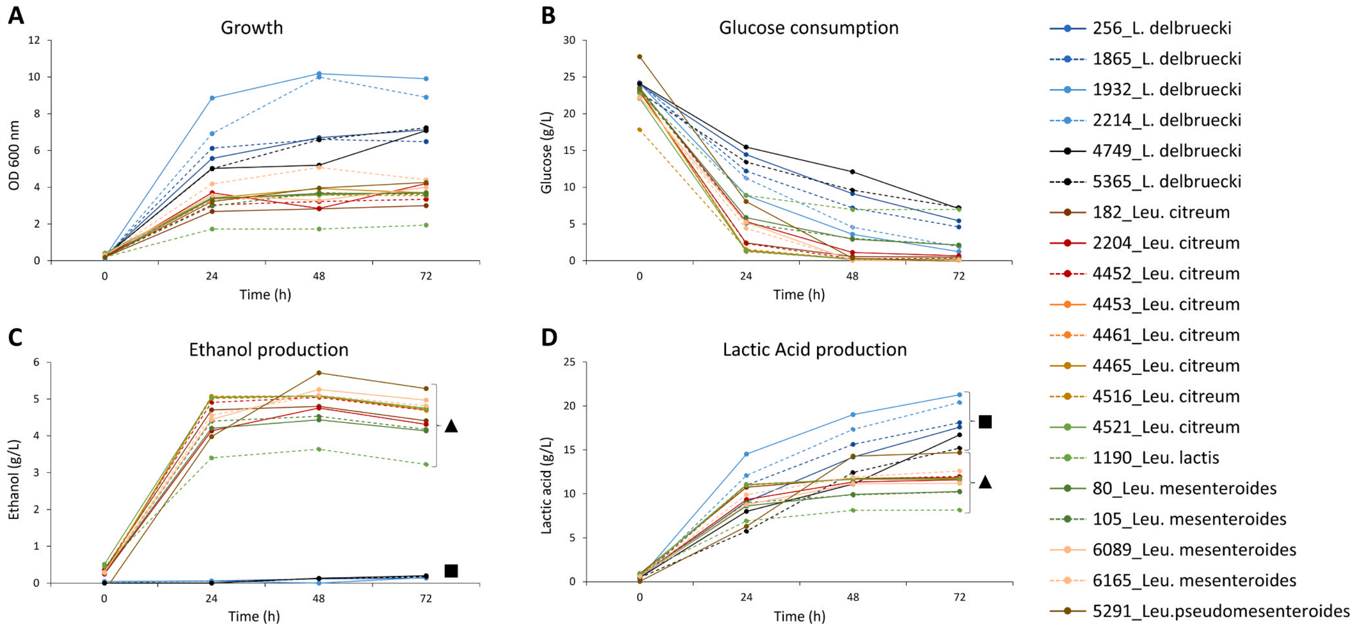

Fig. 2. Small-scale fermentations: A) growth (OD_{600}); B) glucose consumption (g/L); C) ethanol production (g/L); D) lactic acid production (g/L). In panels C and D, the closed black square indicates compounds produced by *L. delbrueckii* strains, while the closed black triangle indicates *Leuconostoc* spp. strains.

Table 3
Selected strains from the secondary screening for the simultaneous high lactic acid titre and high optical purity.

ID strain	Species	Lactic acid 24 h (g/L)	Lacic acid 48 h (g/L)	Lactic acid 72 h (g/L)
2214	*Lactobacillus delbrueckii*	12.09	17.32	20.41
4516	*Leuconostoc citreum*	10.97	11.68	11.69

the scale-up experiments were limited to two selected strains, based on their performance in the secondary screening. While this approach allowed for focused research, it may have overlooked other strains with latent potential under bioreactor conditions.

With the same initial amount of sugar, after 72 h, one produced a double quantity of lactic acid than the other since, as cited above, *Leuconostoc* addresses part of the carbon to produce ethanol (4.73 g/L at 72 h).

Moving on to a bioreactor experiment, results still support the higher efficiency of *L. delbrueckii* UPCCO 2214. It had twice the yield of *L. citreum* UPCCO 4516 that took longer and consumed more glucose to finally produce about the same amount of lactic acid.

The first 7 h of the bioreactor process (batch phase) highlights the natural capabilities of microorganisms, i.e. their productivity without any external action. Indeed, comparing the volumetric productivity calculated both in the secondary screening and in bioreactors, for the two selected strains, this parameter was enhanced by scaling the process. Hence, the results from the batch phase were used to characterise the LA production from the strains. The advantages of batch cultivation are less technical equipment is needed, easy management, high titre and yield, and minor risk of contamination. However, the accumulation of potential by-products is a disadvantage of the batch culture. Excessive concentration of the substrate can prolong the lag phase and cause cell lysis, thus reducing the sugar utilization and fermentation rate. Moreover, the inhibitory effects of the end-product concentration may cause reduced productivity, while a limitation of nutrients results in a low concentration of cells [48]. During the batch phase cultivation, the two strains used the same amount of glucose and, accordingly, at the same rate. However, in this first phase, *L. delbrueckii* already shows a double yield$_{p/s}$ and productivity than *L. citreum.*

Once the fed-batch phase is started, microorganisms are subjected to an artificial manipulation to prolong the exponential phase. The fed-batch mode gives low substrate inhibition by supplying calculated substrate concentration during the fermentation. This allows to maintain cell growth and increase productivity. However, the bottlenecks are represented by the design and optimization of the feeding, and the inhibitory effect of the end-product accumulation [11]. Around 34 h, the lactic acid production capabilities are comparable among the two strains, until *L. citreum* UPCCO 4516 overtakes *L. delbrueckii* UPCCO 2214 by reaching a greater lactic acid concentration. However, this great productivity is reached with a great request of glucose by *L. citreum* UPCCO 4516 (high consumption rate) that kept constantly consuming it

Table 4
Parameters monitored during different stages of cultivation in bioreactors. For reference on how the parameters were calculated please refer to Section 2.3.2. Data are reported as mean ± standard deviation.

Parameter	Batch		Fed-batch		Constant glucose consumption		End-point	
	UPCCO 2214	UPCCO 4516	UPCCO 2214	UPCCO 4516	UPCCO 2214	UPCCO 4516	UPCCO 2214	UPCCO 4516
Time (h)	7	7	67	67	24.25	48.50	74	74
Δ glucose (g)	15.95 ± 0.43	15.88 ± 0.45	58.31 ± 0.78	133.77 ± 0.36	49.65 ± 0.49	133.14 ± 0.06	74.26 ± 0.35	149.64 ± 0.81
Δ lactic acid (g)	13.04 ± 0.40	7.24 ± 0.21	42.15 ± 0.56	55.84 ± 0.52	40.90 ± 1.09	60.54 ± 0.53	55.18 ± 0.16	63.082 ± 0.73
Yield$_{p/s}$ (g/g)	0.82 ± 0.003	0.46 ± 0.0002	0.72 ± 2.60E−05	0.42 ± 0.003	0.82 ± 0.01	0.45 ± 0.004	0.74 ± 0.001	0.42 ± 0.003
Volumetric productivity (g/L/h)	2.80 ± 0.08	1.51 ± 0.05	0.77 ± 0.02	0.78 ± 0.006	2.24 ± 0.04	1.18 ± 0.01	0.96 ± 0.009	0.85 ± 0.01
Glucose consumption rate (g/h)	2.28 ± 0.06	2.27 ± 0.06	0.87 ± 0.01	2.00 ± 0.005	2.05 ± 0.02	2.75 ± 0.001	1.00 ± 0.005	2.02 ± 0.01

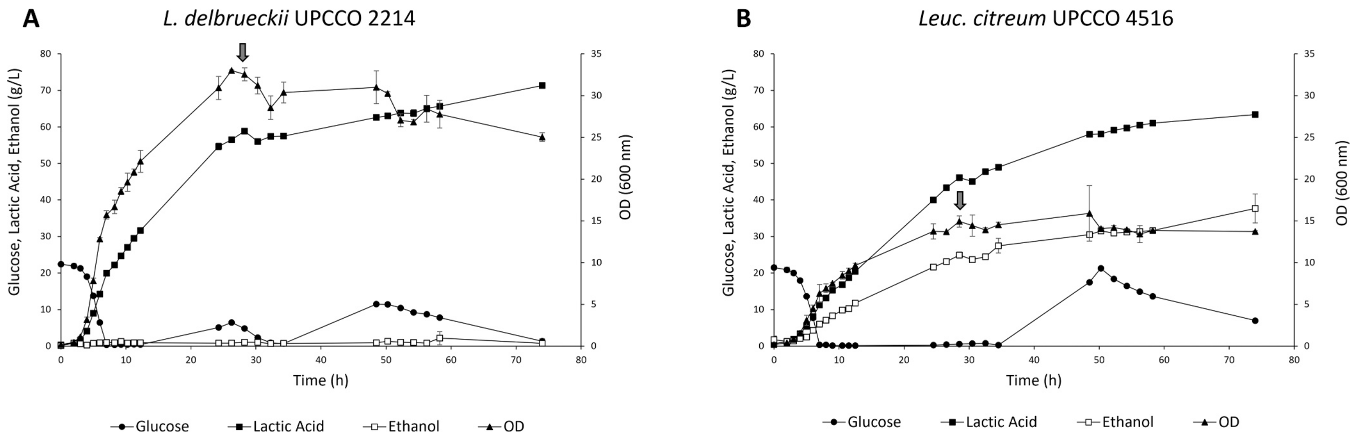

Fig. 3. Comparison between the fermentation in bioreactors of *L. delbrueckii* UPCCO 2214 and *L. citreum* UPCCO 4516. Data represent the average of duplicate experiments with experimental error bars. During 74 h of fermentation, four parameters were monitored: ●) glucose consumption (g); ■) lactic acid production (g); □) ethanol production (g); ▲) cellular growth (OD_{600}). Ten times concentrated fresh medium was added at 30 h (grey arrow).

until 49 h, thus strongly affecting yield$_{p/s}$ and productivity. Furthermore, *L. citreum* UPCCO 4516 produced ethanol (37.70 ± 4 g/L at 74 h) along with LA, maintaining a ratio of 1:1.73. Kim et al. [17] explained that such a characteristic is genetically determined and sugar concentration independent.

As soon as the OD_{600} stopped increasing, indicating the beginning of the stationary phase, a concentrated fresh medium without glucose was added to reintegrate missing nutrients. The lack of nutrients could lead to growth restriction, which also negatively affects lactic acid productivity. Results show that the supplementation of new nutrients did not restart the cellular growth of any of the two bacteria.

Nevertheless, it is noteworthy to analyse the stationary phase of both strains. LA production curve of *L. delbrueckii* UPCCO 2214 is paired with the microbial growth curve, right as the primary metabolite production trend should be [49]: cells and metabolites are produced more or less simultaneously. Furthermore, we can suppose that cells do not react to the addition of new nutrients because they reached their physiological limit of lactic acid tolerance, thereby they start being inhibited by their metabolism product [50,51], or they achieve the carrying capacity of the environment [52].

L. citreum UPCCO 4516, instead, did not stop producing lactic acid when it reached the stationary phase. Within this phase, available carbon is still converted to metabolites, as is usually the case for secondary metabolite biosynthesis. It leads to high glucose consumption, which seems to be entirely destined for lactic acid conversion. In this case, *L. citreum* UPCCO 4516 has probably reached its carrying capacity, whereas it no longer produces biomass, but its metabolism is still active.

Looking at Table 5, the results obtained from this work are promising since, with minimal optimization, we reached values fully in line with the data present in the literature. Indeed, the productivity and yield values of D-LA production obtained by this study can be compared to those observed by other authors, such as [53]. However, by employing the same species *L. delbrueckii* various work have proven the possibility of utilising various complex substrates, reaching interesting results upon the utilisation of substrates such as saccharified rice [54] or, in a more recent perspective oriented towards circular economy, the use of agro-industrial by-products, that are still capable to provide a high productivity and yield [41,42,55,56].

Interestingly, not many examples were found in the literature regarding the use of fed-batch cultivation of *L. delbrueckii*, except for the work of Beitel et al. [57], that proved the possibility of further upscaling D-LA yield by optimizing culture conditions, and using cheaper substrates (molasses and corn steep liquor) that can have an impact on process cost.

Bioreactor tests reflected what was already seen on the small scale. However, in the small-scale screening, the fast pH drop caused a decreased productivity, therefore cultivation in bioreactors was necessary to understand the natural limitations of the studied strains and try in future to boost their potential.

Future outlooks for this study are investigating on lactic acid tolerance of strains and improve production capabilities by designing adaptive laboratory evolution experiments. Moreover, to further optimize the

Table 5
Comparison of D-lactic acid production by *L. delbrueckii* using different C sources and processes in the literature.

Microorganism	Fermentation mode	C source	Productivity$_{LA}$ (g/L/h)	Yield$_{p/s}$ (g/g)	Reference
L. delbrueckii UPCCO 2214	Batch	Glucose	2.80	0.82	This study
L. delbrueckii UPCCO 2214	Batch + Constant feeding rate fed-batch (24 h)	Glucose	2.24	0.82	This study
L. delbrueckii sp. *lactis* QU 41	Batch	Glucose	0.52	n.d.	[53]
L. delbrueckii IFO 3202	Batch	Saccharified rice	0.65	n.d.	[54]
L. delbrueckii sp. *delbrueckii* CECT 286	Batch	Orange peel waste hydrolysate	2.35	0.92	[42]
L. delbrueckii	Batch	Molasses sugar	3.36	0.61	[57]
L. delbrueckii	Constant feeding rate fed-batch	Molasses sugar	3.96	0.61	[57]
L. delbrueckii sp. *delbrueckii* CECT 286	Batch	Orange peel waste	0.63	0.86	[41]
L. delbrueckii sp. *bulgaricus* CECT 5037	Batch	Orange peel waste	0.55	0.84	[41]
L. delbrueckii sp. *bulgaricus* WT	Batch	Glucose	n.d.	0.86	[55]
Lb. delbrueckii	Batch	Broken rice	3.58	0.81	[9]
Lb. delbrueckii NCIM 2025	Batch SSF	Cassava bagasse	1.36	0.94	[56]

fermentative process in bioreactors, it is suggested to prolong the batch phase by supplying a higher concentration of sugar and increasing the inoculum, thus leveraging the natural capabilities of microorganisms. It is very interesting also to implement different process modes, such as repeated batch or continuous fermentation. The first one gives high growth rates and it is time and labour-saving, although it requires specific devices for cell concentration. The second one, instead, guarantees high productivity and controlled growth rates, but it implies carbon leftovers and a high risk of contamination [11].

5. Conclusions

In conclusion, this investigation provides useful insights into the production of D-lactic acid, precursor of PDLA, by *L. delbrueckii* and *L. citreum*. The screening of a large collection of LAB strains and cultivation in bioreactors confirmed the D-LA biosynthetic potential of the selected strains.

Notably, *L. delbrueckii* UPCCO 2214 demonstrated interesting performances in both batch and fed-batch fermentations, using glucose as the carbon source. The productivity and yield reached by this strain both in batch and fed-batch cultivation are competitive with, and in some cases exceed, those reported in the current literature for wild-type LAB strains. The regulation and optimisation of the fermentation parameters (pH, temperature, anaerobic condition, and substrate concentration), are essential to maximize D-LA yield and productivity. Cultivation in bioreactor allowed to obtain reliable and reproducible results for potential industrial applications. Future studies may be addressed to different fermentation approaches or to the improvement of microbial capabilities. This research is an additional piece of the puzzle for further exploration and optimization of D-LA production processes by wild type LAB strains, supporting environmentally friendly PLA manufacturing.

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Declaration of Competing Interest

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

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Data availability

Data will be made available on request.

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