Exploring encapsulation strategies as a protective mechanism to avoid amensalism in mixed populations of *Pseudomonas taetrolens* and *Lactobacillus casei*

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ABSTRACT

Pseudomonas taetrolens constitutes an efficient platform for the biosynthesis of lactobionic acid, a potentially prebiotic compound. Unfortunately, an amensalistic interaction has been demonstrated between *P. taetrolens* and probiotic lactic acid bacteria (LAB), characterised by the competitive exclusion of *P. taetrolens*, hindering the *in situ* production of fermented dairy products with synbiotic properties.

In the present research, encapsulation was explored as a barrier to the diffusion of the antimicrobial metabolites generated by LAB. Mixed fermentations involving *P. taetrolens* LMG 2336 and *Lactobacillus casei* CECT 475 were cultivated, entrapping

both microorganisms alternately. Alginate, alginate/starch and carboxymethyl cellulose/k-carrageenan were tested as encapsulating agents. The immobilization of *L. casei* in 2% alginate/2% starch beads was found to be the best strategy, improving the production of lactobionic acid by 182% with respect to co-cultures with free cells. This study proves the potential of LAB encapsulation for the protection of sensitive strains in mixed food fermentations.

Keywords: Microbial encapsulation; mixed fermentations; *Pseudomonas taetrolens*; *Lactobacillus casei*, lactobionic acid.

Introduction

Microbial immobilization through encapsulation is seen as a promising technique, especially with probiotic microorganisms, in order to provide them with a protective environment during the manufacturing process and storage of probiotic products, and their passage through the gastrointestinal tract [1-3]. Different hydrogels used in food applications have been tested for encapsulation purposes. Alginate remains the most commonly used, due to its non-toxicity, the simplicity of its use and low cost [4, 5]. It has been reported that alginate produces a hydrogel barrier in solution which retards the permeation of acid fluid [6]. However, alginate is susceptible to damage in harsh environments and has high permeability owing to its porous and hydrophilic nature. The addition of other polymers as fillers, such as starch, allows the formation of matrices with improved structural properties [7]. Carboxymethyl cellulose (CMC) is the most widely used cellulose ether, employed in many food applications as a viscosity modifier or thickener. Blends of CMC and k-carrageenan (CMC/k-carr) have been studied for probiotic encapsulation with k-carrageenan as a coating material [8, 9].

A few studies have employed encapsulation to control the strain ratios and to provide physical and chemical protection to microorganisms in mixed fermentations [10-12]. Microbial associations are present in most food fermentation processes, providing the final product with the desired characteristics. But obtaining stable mixed cultures is a complex task due to the different nutritional requirements, optimal growth conditions and growth rate of each population [13]. In traditional and novel fermented dairy products it is common to find a complex microbiota [14], normally including LAB which produce a wide range of inhibitory compounds, such as organic acids, ethanol, diacetyl, hydrogen peroxide or bacteriocins [15]. In this context, cell immobilization could be employed to exercise some control over mixed cultures containing LAB and sensitive species, making use of the limited diffusion of such inhibitory substances through the wall of the capsules [6, 12, 13, 16].

Lactobionic acid, an aldonic acid derived from the oxidation of lactose, has become a subject of major interest as an additive in dairy products. It possesses valuable technological properties, but also provides health benefits as an agent promoting calcium absorption and it is potentially prebiotic [17, 18]. An efficient and sustainable bioprocess has been optimized to obtain lactobionic acid from dairy substrates, employing the bacterium *Pseudomonas taetrolens* as the producer microorganism [19-21]. The coupling of *P. taetrolens* lactose oxidation to traditional fermentation carried out by probiotic LAB would make it possible to obtain functional synbiotic products, containing the probiotic bacteria and the prebiotic lactobionic acid. But an amensalistic association was found between LAB such as *Lactobacillus casei* and *P. taetrolens,* in which the release of antimicrobial substances by the LAB caused the inhibition of *P. taetrolens* growth and

productive capacity. This antagonistic interaction makes the simultaneous production of lactic and lactobionic acids for commercial purposes unfeasible [22].

In the present study, the effect of encapsulation on the interaction of *L. casei* and the sensitive strain *P. taetrolens* was studied by employing combinations of alginate, starch, CMC and k-carrageenan as encapsulating agents. Mixed fermentations of *P. taetrolens* and *L. casei* were carried out, alternately encapsulating one or other of the two microorganisms in the different hydrogels. Operating conditions were chosen with reference to the optimum conditions for *P. taetrolens* in pure culture, as determined previously by Alonso et al. [19-21]. A dairy substrate based on skimmed milk was employed, in consideration of the interest of this study for the dairy food sector.

Materials and methods

Microorganisms

L. casei CECT 475, obtained from the Spanish Type Culture Collection (Valencia, Spain), was maintained frozen (in 40% v/v glycerol solution at -20 °C) and subsequently incubated on MRS (de Man Rogosa and Sharpe, Biokar Diagnostic, France) agar plates, cultured for 48 h at 37 °C and then stored at 4 °C.

P. taetrolens LMG 2336 was obtained from the Belgian Coordinated Collection of Microorganisms (Ghent, Belgium). The strain was conserved frozen in 40% (v/v) glycerol at -20 °C and subsequently subcultured on NB agar plates (Nutrient Broth, containing 1g L^{-1} meat extract, 2 g L^{-1} yeast extract, 5 g L^{-1} peptone and 5 g L^{-1} NaCl). The agar plates were incubated for 48 h at 30 °C and preserved at 4 °C.

Inocula and substrate preparation

L. casei was reactivated on MRS under microaerophilic conditions. A loopful from an MRS agar plate was used to inoculate a 250 mL storage media bottle containing 250 mL MRS broth. The culture was incubated in an orbital shaker (New Brunswick Scientific Co., model G25, USA) at 37 °C without agitation for 16 h.

In the case of *P. taetrolens* the culture method was adapted to its aerobic metabolism. A 500 mL Erlenmeyer flask containing 100 mL of NB broth was inoculated with a loopful from an NB agar plate. The culture was incubated at 250 rpm and 30 °C for 10 h.

Skimmed cow's milk was heated in a water bath at 90ºC for 10 minutes for sterilization [23] and subsequently used as substrate in fermentations.

Bead-forming procedure

Three different hydrogel formulations were prepared by dissolving the corresponding polymeric mixture in distilled water: 2% [w/v] sodium alginate (Acros Organics); 2% sodium alginate/2% starch (Panreac); and 2% sodium carboxymethyl cellulose (Sigma Aldrich)/1% k-carrageenan (Sigma Aldrich). The choice of these proportions was based on the information obtained from hardening studies carried out with different concentrations of hydrogels and on the results previously reported by other authors [8, 24].

L. casei and *P. taetrolens* were alternately encapsulated, whilst leaving the other species free in the fermentation medium. In each case, 40 mL from the MRS or NB inoculum cultures containing actively growing cells were centrifuged at 12,000 *x g* for 10 min. The resulting pellet was used for immobilization, by the extrusion methodology described by Alonso et al. [1], with modifications. The biomass was re-suspended in 25 mL of the hydrogel solutions. A peristaltic pump was used to transfer the solutions dropwise into

400 mL of $CaCl_2$ 0.54 M as a gelling solution. The resulting beads were collected, washed in phosphate-buffered saline (PBS, pH 7.4 sterile and filtered at 0.22 µm), filtered and subsequently used as inoculum for fermentations.

Culture conditions and fermentation experiments

The biomass from 40 mL of MRS or NB precultures was introduced in free suspension, together with the encapsulated biomass, into 2 L storage media bottles containing 400 mL of skimmed milk (10% v/v inoculum level). Thus, fermentations were carried out employing a working volume to air ratio of 1:4, with agitation at 250 rpm and at 30ºC for 72 hours. These operating conditions, favourable to *P. taetrolens*, were chosen in order to avoid it undergoing environmental stress and maximize lactobionic acid production. Pure cultures of *P. taetrolens* and mixed fermentations with both microorganisms in free suspension, under the same operating conditions, were used as controls. Samples were periodically taken to determine bacterial growth, pH and for the chemical analysis of substrate consumption and the production of organic acids. All fermentations were carried out in duplicate as independent experiments and the reported results correspond to the mean value of at least three measurements. Positive and negative error values are shown as error bars in the figures. The experimental data obtained were fitted to the Gompertz kinetic model.

Quantification of *L. casei* and *P. taetrolens* cells

Growth of free and encapsulated bacteria was determined by means of the spread plate method in MRS and NB agar for *L. casei* and *P. taetrolens*, respectively. In the case of the immobilized biomass, beads were solubilized and encapsulated cells were released by suspending one bead in 1 mL of sodium citrate 1% (v/v). Colony Forming Units (CFU)

were counted after incubating the agar plates for 48 h at 30º C in all cases. Results are expressed as the increase in the number of CFU during fermentations with respect to initial concentration (CFU_0), according to the formula CFU mL^{-1}/CFU_0 for free bacteria and CFU $bead^{-1}/CFU_0$ for encapsulated bacteria.

Hydrogels and bead characterization

The textural properties of the different encapsulating hydrogels were studied using a TA.XTplus Texture Analyzer (Stable Micro Systems). The Bloom test, for the determination of bloom strength of gelatin according to the International Standard ISO 9665, was implemented. The Bloom test measures the weight in grams needed by a specific plunger to depress the surface of the gel by 4 mm without breaking it and the result is expressed as the Bloom number. A higher Bloom number indicates higher gel strength. Measurements were carried out using 100 mL samples of each hydrogel suspension, mixed with the biomass and preserving the same proportion used for the encapsulation. The Bloom test was conducted at room temperature at a speed of 0.5 mm s^{-1}, a penetration distance of 4 mm and a data acquisition rate of 200 pps. Measurements were carried out in triplicate for each material.

A visual characterization of the different types of beads was carried out at time 0, with a LEICA M205FA fluorescence stereo microscope (Leica Microsystems Inc., Heidelberg, Germany), without giving the beads any special treatment. A magnification of 22x was employed and image processing was performed with the Leica Application Suite v4.0 software platform, in order to determine the bead size. The shape of the beads was characterized using the sphericity factor (SF), calculated according to the following equation [25]:

$$SF = \frac{d_{max} - d_{min}}{d_{max} + d_{min}} \quad (1)$$

Where d_{max} is the largest diameter and d_{min} is the smallest diameter perpendicular to d_{max}. The SF varies from 0 for a perfect sphere to 1 for an elongated object.

Encapsulation efficiency (EE) and cell leakage profiles

Entrapment efficiency was calculated for the different encapsulating hydrogels according to Sandoval et al. [26] by the following equation:

$$Efficiency = (A/B)\ x\ 100 \quad (2)$$

Where *A* = CFU of bacteria mL^{-1} of hydrogel solution after encapsulation; and *B* = CFU of bacteria mL^{-1} of hydrogel solution before encapsulation (10^8 CFU mL^{-1} in all cases).

To study the cell leakage phenomenon in entrapped cells, 0.5 g of beads were suspended in 4.5 mL of buffer solution and incubated for 24 h with constant agitation at 250 rpm. Samples were taken from the surrounding medium to quantify the bacterial growth outside the capsule. The counting of viable cells was carried out by the spread plate method as previously described for free bacteria.

Substrate and product analysis

Lactose, lactic acid, and lactobionic acid concentrations were measured by High Performance Liquid Chromatography (HPLC). The liquid chromatography system used for the analysis (Agilent 1200, Agilent Technologies Inc., CA, USA) was equipped with an ICSep ICE-ION-300 column (Transgenomic Inc., CA, USA) coupled to a refractive index detector. The mobile phase was a sulphuric acid solution (0.450 mmol L^{-1}, pH 3.1),

196 employing a 0.3 mL min^{-1} flow rate and a column temperature of 75ºC. Data acquisition
197 and analysis were performed using ChemStation software (Agilent).
198
199 ## Results
200 ### Hydrogel strength and encapsulation efficiency
201 The strength of the encapsulating hydrogels was measured according to the Bloom test.
202 A decrease in degradation and higher encapsulation efficiencies have been reported for
203 gels when the Bloom value increases [27, 28]. As can be observed in Table 1, in the
204 present study the highest Bloom value was obtained for the gelling blend composed of
205 alginate/starch, corresponding to the highest encapsulation efficiency (53.30 and 83.50%,
206 respectively).
207
208 **Table 1** – Bloom values (g) and encapsulation efficiencies (%) for the different
209 encapsulating hydrogels tested

Hydrogel composite	Bloom value (g)	Encapsulation efficiency (%)
Alginate 2%	43.73	63.98
Alginate 2% + starch 2%	53.30	83.50
CMC 2% + k-carr 1%	37.03	76.82

210
211 The lowest encapsulation efficiency (63.98%) was obtained when only alginate was
212 employed as the encapsulating material. Reduction in encapsulation efficiency is mainly
213 attributed to the cell damage caused by detrimental conditions during the encapsulation
214 process itself, in addition to the loss of cells into the hardening solution [29]. The loss of
215 cells during the preparation of the beads, favoured by their high porosity, constitutes the
216 major limitation in alginate solutions [25]. The addition of starch to alginate solutions

leads not only to stronger composites, but also to an increase in the encapsulation efficiency [24, 25, 30] by promoting the stabilization of the alginate matrix [6].

Optical characterization of beads

Photographs and stereo microscope images corresponding to the three types of beads are shown in Fig. 1. The surface of the alginate/starch beads appears smoother (b.2), without the cracks that can be observed in the alginate and CMC/k-carr beads (b.1 and b.3). This smoothing effect is caused by the starch, which acts as a filler, occupying the interstitial space in the alginate matrix [25]. Regarding the size, all beads had an approximate diameter of 2.5-3.5 mm (Table 2). The "tail" in the alginate capsules can be explained by the surface tension which is generated when the droplets are extruded. Hydrogel mixtures containing starch become more viscous and the droplets tend to be retained longer before falling into the gelling solution, generating longer "tails" (a.2 and b.2). For this reason, the sphericity factor (SF) shows an increase from 0.04 in alginate beads to 0.11 in alginate/starch beads (Table 2), indicative of an elongation in the bead shape. Systems with SF < 0.05 can be considered spherical [25]. Because of the absence of elongation in the CMC/k-carr beads, they are larger in terms of volume. This difference in size may be partly due to their greater swelling capacity, caused by the strong electrostatic repulsion between the sulphate groups of the k-carrageenan [8].

Fig. 1 – Photographs of (a.1) alginate, (a.2) alginate/starch and (a.3) CMC/k-carrageenan beads at time 0 of cultivation; stereo microscope images of (b.1) alginate, (b.2) alginate/starch and (b.3) CMC/k-carrageenan beads at time 0 of cultivation. Scale bars = 1 mm

242 **Table 2** – Largest diameter (mm), smallest diameter (mm), sphericity factor and weight
243 (g) for the different encapsulating hydrogels tested

Hydrogel composite	d_{max} (mm)	d_{min} (mm)	SF	Weight (g)
Alginate 2%	3.139	2.906	0.04	0.013
Alginate 2% / starch 2%	3.673	2.965	0.11	0.016
CMC 2% + k-carr 1%	3.380	2.477	0.15	0.016

244

245 **Cell leakage**

246 The same cell loading conditions were used in experiments with the different entrapment
247 materials (10^8 CFU/ml). After encapsulation, bacterial growth in the liquid phase was
248 monitored with the aim of determining the degree of cell leakage from the beads. Fig. 2
249 compares the increase in the number of free cells in the liquid medium for each
250 encapsulating hydrogel, represented as $CFUml^{-1}/CFU_0$ of *L. casei*. As can be observed,
251 the largest increase in free cells occurred in the case of the alginate beads, especially
252 during the first hours of incubation, revealing the low mechanical stability that has been
253 reported by other authors [31]. Similarly, a significant degree of cell leakage was
254 observed in previous studies employing alginate beads at 250 rpm of agitation [1]. In
255 addition to the mechanical factors, alginate presents low stability in the presence of
256 chelating agents, which share affinity for calcium and destabilize the gel. Therefore,
257 problems are encountered during lactic fermentations [32] and these could be exacerbated
258 in the mixed fermentation of *L. casei* and *P. taetrolens*, due to the presence in the medium
259 of the lactobionic acid, another calcium chelating agent [33].

260

261 Mixing with starch produces an improvement in the stability of the beads, resulting in
262 better retention of encapsulated microbial cells [32, 31]. As can be observed in Fig. 2.,

the addition of 2% starch to the alginate matrix led to a reduction in cell leakage. Beads prepared with CMC/k-carr showed an intermediate cell leakage profile, influenced by the swelling capacity of the hydrogel mixture. The swelling phenomenon influences their retention capacity, leading to greater porosity and facilitating the release of the entrapped molecules [34]. A high degree of swelling implies high water uptake and the consequent solubilization of the hydrogel matrix [35]. This disintegration would involve the progressive release of cells observed in Fig. 2 for CMC/k-carr beads.

Fig. 2 – Increase in the cell leakage during the first 24 h of cultivation for the three types of beads tested

Mixed fermentations with immobilized *L. casei* and free *P. taetrolens*

Bearing in mind the efficient productivity achieved by the encapsulation of LAB [36-38] and the low production of lactobionic acid reported in previous studies with encapsulated *P. taetrolens* cells [1], tests with *L. casei* immobilized and *P. taetrolens* free in suspension were carried out first.

L. casei growth

Fig. 3a shows the growth of *L. casei* inside the three types of beads (expressed as $CFUbead^{-1}/CFU_0$). The lowest increase in biomass was registered when only alginate was used as the encapsulating material. The curve corresponding to *L. casei* encapsulated in alginate/starch beads shows a large increase in biomass, reflecting the improved retention of the entrapped cells due to starch addition. In the case of the CMC/k-carr, the growth curve shows that the density of *L. casei* cells did not increase significantly until 32 hours of incubation.

Fig. 3 - Evolution of the *L. casei* growth inside beads (A), lactic acid production (B), *P. taetrolens* growth in the free medium (C), lactobionic acid production (D), lactose consumption (E) and medium pH (F) in mixed fermentations with *L. casei* encapsulated in alginate, alginate/starch and CMC/k-carr beads. Pure cultures of *P. taetrolens* and mixed fermentations with both microorganisms free in the medium are used as controls

Lactic acid production

Regarding the productive capacity, the immobilization of *L. casei* did not imply a reduction in the lactic acid synthesized in the case of alginate and alginate/starch beads (Fig. 3b). In fact, the encapsulation of *L. casei* with alginate/starch led to an increase in the final lactic acid concentration with respect to the mixed fermentations employing free cells (from 8.15 gL^{-1} to 10.68 gL^{-1}), as can be observed in Table 3. In previous studies with mixed free cultures under the same operating conditions (30ºC and highly aerobic environment), very different from the optimum for *L. casei* (37ºC and microaerophilic conditions), it was found that the LAB could survive but their productive capacity was harmed [22]. Immobilization of *L. casei* by encapsulation would improve lactic acid productivity by protecting cells exposed to these harsh environmental conditions [31]. This preservation of the healthy status of *L. casei* is important, given the significance of this study in contributing to the development of a synbiotic product containing probiotic active cells.

In the case of CMC/k-carr beads, according to the registered growth, no lactic acid production was obtained until 32 hours of incubation. Consequently, the final concentration of lactic acid for the CMC/k-carr beads was only 5.77 gL^{-1} (Table 3).

Table 3 - Summary of the final values obtained in the different fermentation systems tested: controls (pure cultures of *P. taetrolens* and mixed fermentations with both microorganisms in free suspension), mixed fermentations with *L. casei* encapsulated (*L. casei* cap) and mixed fermentations with *P. taetrolens* encapsulated (*P. taetrolens* cap)

	Controls		***L. casei* cap**			***P. taetrolens* cap**	
Fermentation system	Pt pure	Free cells	Alginate	Alg/ starch	CMC/ k-carr	Alginate	Alg/ starch
Lactose ($g\ L^{-1}$)	18.14	27.96	18.89	13.76	20.61	18.30	24.60
Lactic acid ($g\ L^{-1}$)	-	8.15	8.26	10.68	5.77	10.30	9.99
Lactobionic acid ($g\ L^{-1}$)	31.32	5.99	13.02	16.93	14.24	6.80	8.10
Lactobionic acid productivity ($g\ L^{-1}\ h^{-1}$)	0.43	0.08	0.18	0.23	0.20	0.09	0.11

P. taetrolens growth

In Fig. 3c, the effect of *L. casei* encapsulation on the growth capacity of *P. taetrolens* when both coexist in mixed fermentations can be observed. In the mixed fermentations with free cells used as control, the CFU count showed a very low increase compared to that obtained for *P. taetrolens* in pure culture, starting from 10^8 $CFUmL^{-1}$ at time 0 in all cases (CFU_0). This limited growth constitutes a clear sign of the inhibition exerted by *L. casei* on *P. taetrolens* growth. The increase in the number of CFUs followed a similar curve in the case of mixed fermentations with *L. casei* entrapped in the alginate and CMC/k-carr beads. Nevertheless, in mixed fermentations with *L. casei* encapsulated in alginate/starch, the growth curve of *P. taetrolens* reached levels comparable to those obtained with *P. taetrolens* in pure culture (Fig. 3c).

330 *Lactobionic acid production*

331 The above results are consistent with the lactobionic acid concentration registered in the
332 cultures (Fig. 3d). A quantity of 5.99 gL^{-1} of lactobionic acid was obtained in mixed
333 fermentations with free cells, compared to the 31.32 gL^{-1} synthesized by *P. taetrolens* in
334 pure culture. The encapsulation of *L. casei* increased the final concentrations of
335 lactobionic acid to 13.02, 14.24 and 16.93 gL^{-1} for alginate, CMC/k-carr and
336 alginate/starch beads, respectively (Table 3). The entrapment of *L. casei* in the
337 alginate/starch beads resulted in the greatest increase in lactobionic acid productivity,
338 from 0.08 g L^{-1} h^{-1} for mixed fermentations with both microorganisms free, to 0.23 g L^{-1}
339 h^{-1}.

340

341 It has been reported that encapsulation allows mass transfer between the bead core and
342 the external environment to be limited by the shell material acting as a physical barrier
343 [2, 29]. Although some previous studies have determined that encapsulation may not
344 affect the diffusion of certain antimicrobial substances towards the external medium, it
345 has been seen that the diffusion capacity is related to the size of the bead. Therefore, in
346 smaller capsules the release of encapsulated compounds is faster due to the greater surface
347 to volume ratios, while in large capsules the diffusion path length increases and most of
348 the release starts when the hydrogel matrix begins to degrade [29, 39]. In the present
349 study, the lowest release profile, coinciding with the greatest concentration of lactobionic
350 acid found in the medium, was achieved by encapsulating *L. casei* in the alginate/starch
351 beads (Figs. 2 and 3). These beads would constitute an impediment to the diffusion of the
352 inhibitory compounds generated by *L. casei* towards the medium in which *P. taetrolens*
353 was free. This result corresponds with those reported by other authors, according to which

354 the blend of alginate and starch slows the release of antimicrobial substances such as the
355 bacteriocin nisin [30].
356
357 *Lactose and pH evolution*
358 The lactose concentration and the pH varied during the cultures in agreement with the
359 production results. The greatest decrease in lactose during the first 24 h was registered in
360 mixed fermentations with *L. casei* encapsulated in alginate/starch (Fig. 3e), which also
361 achieved the lowest final concentration (13.76 gL^{-1}, as can be observed in Table 3). The
362 higher production of both lactic and lactobionic acids also resulted in the lowest final pH
363 in fermentations with *L. casei* encapsulated in alginate/starch (Fig. 3f).
364
365 *Kinetic modelling*
366 The modified Gompertz model was used to describe the fermentative behaviour of *L.*
367 *casei* and *P. taetrolens* in experiments with *L. casei* encapsulated in the three
368 encapsulating materials. The Gompertz kinetic model defines the asymmetrical sigmoid
369 curve of microbial growth composed of the initial lag phase, the exponential growth phase
370 and the stationary period [40]. The kinetics of the bacterial population growth is given by
371 the following equation:

$$Y = A \exp\left\{-\exp\left[\frac{\mu_m e}{A}(\lambda - t) + 1\right]\right\} \qquad (3)$$

372 Where Y is the logarithm of the relative population size [$Y = \log(N/N_0)$], A is the
373 maximum potential growth [$A = \log(N_\infty/N_0)$], μm is the maximum specific growth rate (h^{-1})
374 and λ is the lag time (h).

375 The relationship between biomass, organic acids production and substrate degradation
376 was determined using the following equations:

$$r_{p1} = Y_{\frac{p1}{x1}} \mu_{m1} x_1 \quad (4)$$

$$r_{P2} = Y_{\frac{p2}{x2}} \mu_{m2} x_2 \quad (5)$$

$$r_s = -Y_{\frac{X_1}{S}} \mu_{m1} x_1 - Y_{\frac{X_2}{S}} \mu_{m2} x_2 \quad (6)$$

Where r_s is the substrate consumption rate ($gL^{-1}h^{-1}$), r_p is the product formation rate of lactic and lactobionic acids ($gL^{-1}h^{-1}$), $Y_{p/x}$ is the product yield/biomass (gg^{-1}) and $Y_{x/s}$ is the biomass yield/lactose (gg^{-1}).

Fitting of experimental data to the kinetic model is shown in Fig. 4, corresponding to the lag, exponential growth and stationary phases, until 48 hours of incubation, before the appearance of the cell death phase.

Fig. 4 – Fitting of experimental data (●) to the kinetic model (-) corresponding to the growth curves of *L. casei* (a), *P. taetrolens* (b), the lactic acid (c), lactobionic acid (d) and lactose concentrations (e) for mixed cultures with *L. casei* encapsulated in alginate (1), alginate/starch (2) and CMC/k-carr beads (3)

The presence of growth from time 0 in all the cultivations resulted in λ=0 in all cases.

In accordance with results previously mentioned, the highest maximum specific growth rate and the maximum potential growth of *L. casei* were found when alginate/starch was used as encapsulating material (μm=0.21 h^{-1} and A=1.95, compared to μm=0.07 h^{-1} and A=1.54 for alginate, as can be observed in Fig.4 and Table 4). In the case of CMC/k-carr

beads, the low maximum potential growth obtained (A=0.66) reflects the inactivity of *L. casei* during the first hours of cultivation.

Table 4 – Values of parameters λ (h), A [$\log(N_\infty/N_0)$], μmax (h^{-1}), $Y_{p/x}$ (g product/g biomass) and $Y_{x/s}$ (g biomass/g lactose) resulting from the fitting of experimental data to the kinetic model

	P. taetrolens					***L. casei***				
Hydrogel	**λ**	**A**	**μ_{max}**	**$Y_{p/x}$**	**$Y_{x/s}$**	**λ**	**A**	**μ_{max}**	**$Y_{p/x}$**	**$Y_{x/s}$**
Alginate	0	1.19	0.74	0.12	100.00	0	1.54	0.07	2.02	0.19
Alginate/starch	0	1.62	0.17	0.19	100.00	0	1.95	0.21	0.29	0.95
CMC/k-carr	0	1.43	0.13	0.18	33.33	0	0.66	0.13	0.69	0.23

The lactic acid yield/biomass ($Y_{p/x}$) is greater in the alginate and CMC/k-carr beads than in the alginate/starch beads (Table 4). Thus, lactic acid concentrations registered in fermentations with alginate and CMC/k-carr beads are high in relation to the amount of biomass quantified inside the beads. These results confirm the low contribution of *L. casei* cells encapsulated in these hydrogels to the production of lactic acid, which can be attributed to the cell leakage phenomenon.

Regarding the performance of *P. taetrolens*, the maximum potential growth (A) was obtained for fermentations with alginate/starch beads (Table 4). However, the maximum specific growth rate was not the highest (μm=0.17 h^{-1} compared to 0.74 h^{-1} with *L. casei* encapsulated in alginate beads). This low rate can be explained by the gradual growth of *P. taetrolens* throughout the experiment, without a decrease in the number of cells,

412 whereas in fermentations with *L. casei* encapsulated in alginate the maximum growth
413 (much lower) was reached earlier (Fig. 4).

414 Lactobionic acid yields/biomass were similar for *L. casei* encapsulated in alginate/starch
415 and CMC/k-carr beads (0.19 and 0.18 g lactobionic acid/g biomass, respectively), higher
416 values than that obtained in the case of alginate beads (Table 4).

417

418 **Mixed fermentations with immobilized *P. taetrolens* and free *L. casei***

419 Mixed fermentations with entrapped *P. taetrolens* and free *L. casei* in the medium were
420 carried out to evaluate the effect of the encapsulation of the sensitive strain on its
421 competitive exclusion. Because of the low mechanical resistance of the CMC/k-carr
422 beads and the poor fermentative capacity of *L. casei* entrapped in this gelling mixture,
423 experiments were carried out employing only the alginate and the alginate/starch beads,
424 in order to establish the influence of the porosity of the encapsulating material on the
425 behaviour of *P. taetrolens*.

426

427 **Fig. 5** - Evolution of the *P. taetrolens* growth inside beads (A), lactobionic acid
428 production (B), lactose consumption (C) and lactic acid production (D) in mixed
429 fermentations with *P. taetrolens* encapsulated in alginate and alginate/starch. Pure
430 cultures of *P. taetrolens* and mixed fermentations with both microorganisms free in the
431 medium are used as controls

432

433 Fig. 5a shows the increase in the CFU number of *P. taetrolens* inside the bead during
434 fermentations, from an initial concentration of 10^8 CFUmL^{-1}. During the first 48 hours of
435 incubation, a very low increase in the biomass concentration in the two encapsulating
436 materials can be observed, the highest growth occurring from this moment onwards.

Nevertheless, the CFU number only increased 20 times with respect to the inoculation value, compared to the increase of up to 60 times found in the alginate/starch beads when *L. casei* was encapsulated (Fig. 3a). This low growth is consistent with the low lactobionic acid concentrations registered during cultures. The encapsulation of *P. taetrolens* did not lead to an improvement in lactobionic acid synthesis with respect to that obtained in mixed fermentations with both microorganisms free (Fig. 5b). Lactobionic acid productivities of 0.09 and 0.11 g L^{-1} h^{-1} were achieved with *P. taetrolens* entrapped in alginate and alginate/starch beads, respectively, not significantly higher than that achieved in free cell cultures (0.10 g L^{-1} h^{-1}). Improved lactobionic acid production was obtained with *L. casei* entrapped in alginate/starch beads, but not when *P. taetrolens* was encapsulated. The acidic micro-environment that is created inside the beads seems to be the main cause of the damage to the *P. taetrolens* cells. Entrapped cells of *P. taetrolens* are forced to suffer the acidic stress at earlier stages than free bacteria, becoming non-lactobionic-acid-producing cells and therefore leading to low productivities [1]. On the contrary, LAB have an acid tolerance response, preserving the proper physiological functions in the cells and surviving at low pH [41, 42]. This ability makes the immobilized LAB more able to survive within the acidic environment inside the bead than the *P. taetrolens* strain.

With respect to lactic acid, an improvement in its production was registered, particularly in the case of the *P. taetrolens* encapsulated in alginate, corresponding to a greater degradation of lactose (Fig. 5c and 5d).

Conclusions

This study has revealed the potential of microbial encapsulation to act as a barrier that minimizes the inhibitory effect in mixed fermentations in which antagonistic strains coexist in the same niche. The entrapment of *L. casei* in alginate/starch beads not only maintains the healthy status of the LAB, but also allows an improvement in the bioconversion performance of free *P. taetrolens*. Therefore, it can be proposed as a feasible strategy to achieve the co-production of lactic and lactobionic acids, in the context of its possible application to the production of fermented dairy products enriched in lactobionic acid. Further investigations would be necessary in order to improve the organoleptic and sensory properties of the obtained fermented product for its food application.

The protection of *P. taetrolens* when *L. casei* was encapsulated is especially significant, because it implies that encapsulation, beyond simply creating a protective environment for entrapped cells, can protect a sensitive strain in suspension against an entrapped dominant strain. The results also highlight the need to evaluate the behaviour of immobilized microorganisms, since those strains which are not able to have a tolerance response to acid stress may not be suitable for encapsulation.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure 1

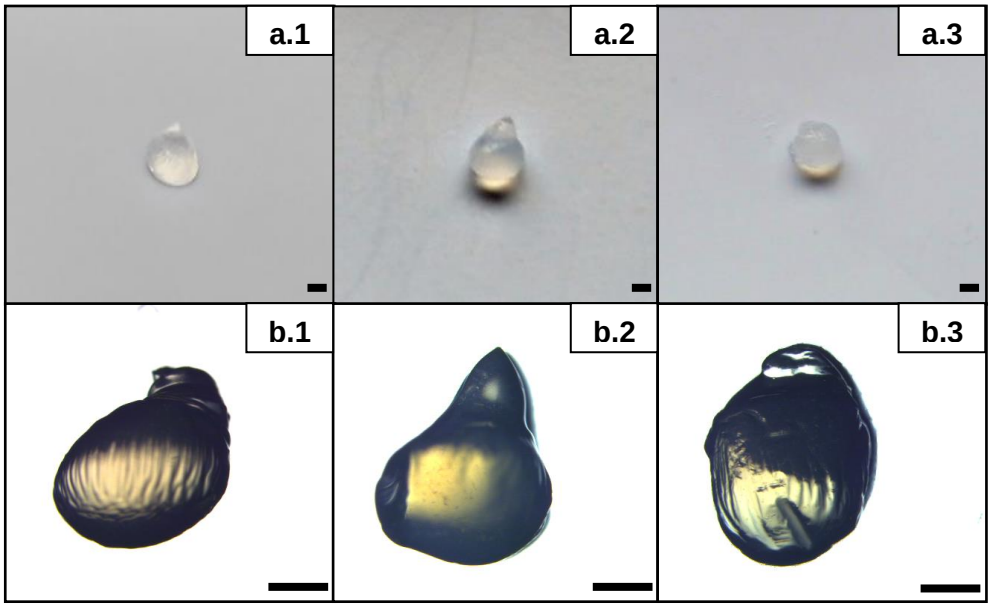


Figure 2

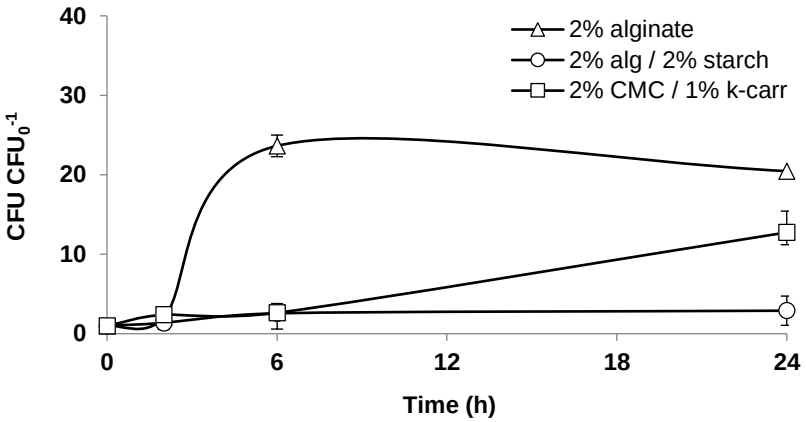
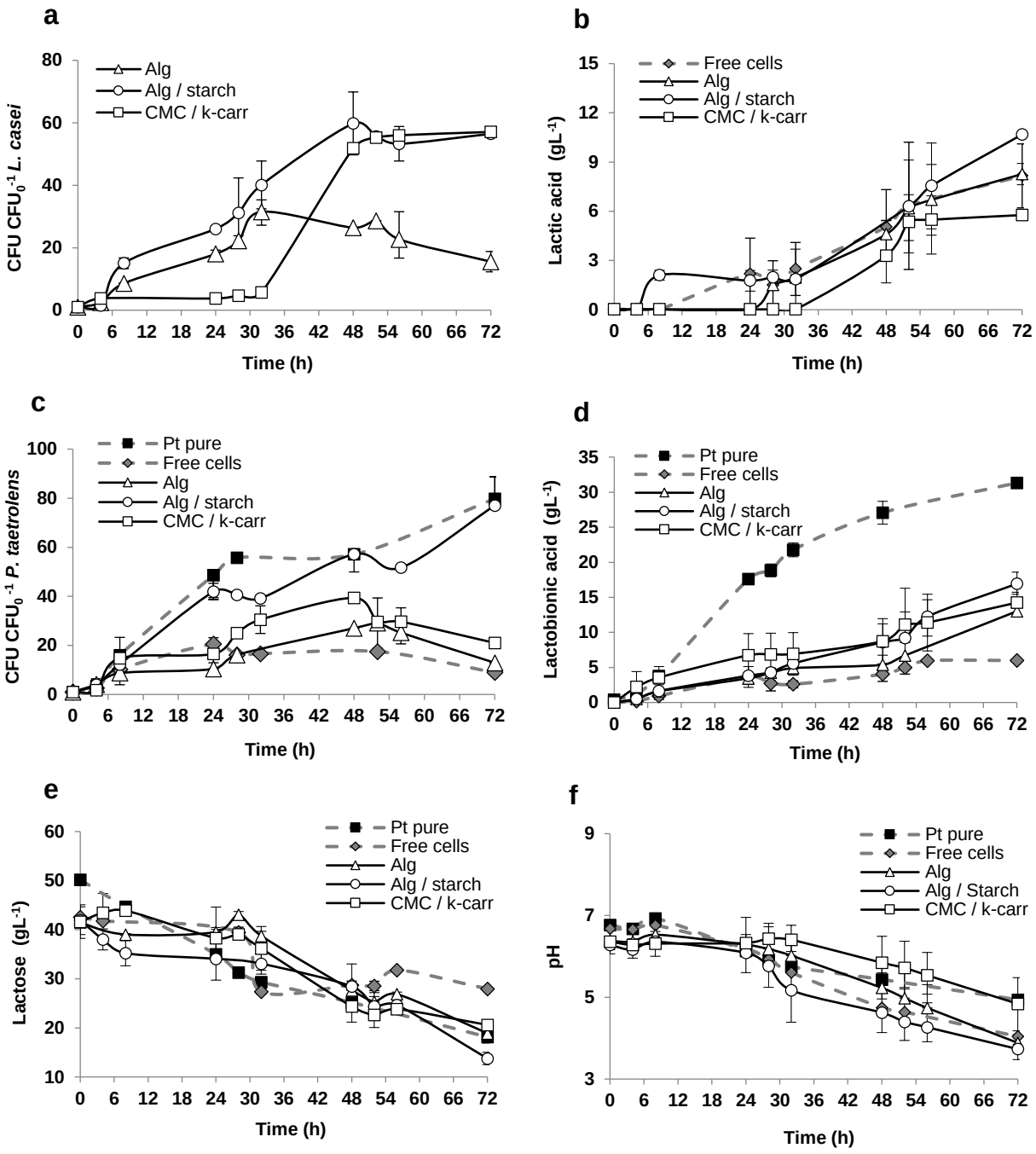

40
30
20
10
0
CFU CFU$_0^{-1}$
0
6
12
18
24
Time (h)
2% alginate
2% alg / 2% starch
2% CMC / 1% k-carr

Figure 3


a
Alg
Alg / starch
CMC / k-carr
CFU CFU$_0^{-1}$ *L. casei*
Time (h)
b
Free cells
Alg
Alg / starch
CMC / k-carr
Lactic acid (gL^{-1})
Time (h)
c
Pt pure
Free cells
Alg
Alg / starch
CMC / k-carr
CFU CFU$_0^{-1}$ *P. taetrolens*
Time (h)
d
Pt pure
Free cells
Alg
Alg / starch
CMC / k-carr
Lactobionic acid (gL^{-1})
Time (h)
e
Pt pure
Free cells
Alg
Alg / starch
CMC / k-carr
Lactose (gL^{-1})
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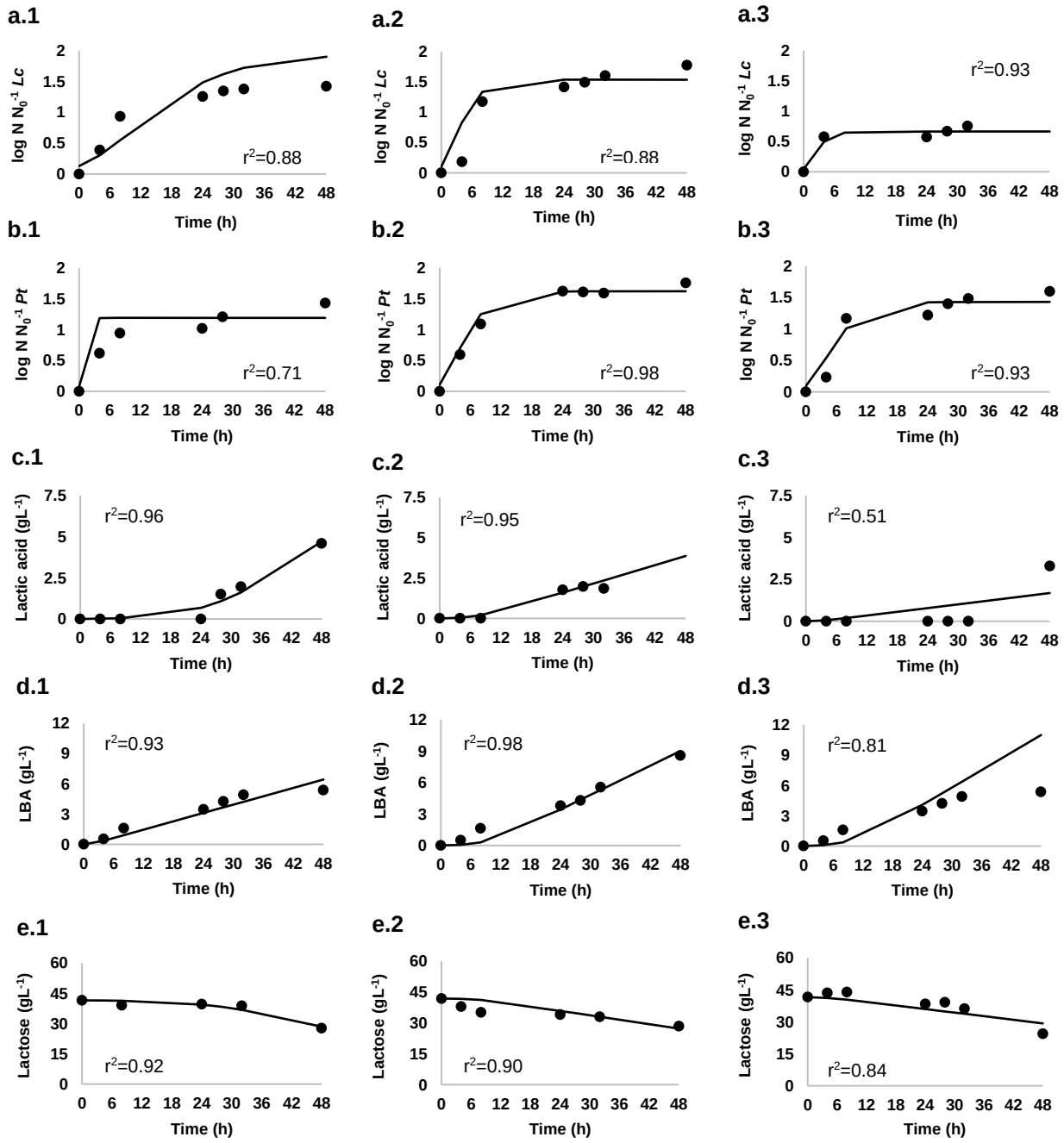
Free cells
Alg
Alg / Starch
CMC / k-carr
pH
Time (h)

Figure 4


a.1
log N N0-1 Lc
r2=0.88
Time (h)
a.2
log N N0-1 Lc
r2=0.88
Time (h)
a.3
log N N0-1 Lc
r2=0.93
Time (h)
b.1
log N N0-1 Pt
r2=0.71
Time (h)
b.2
log N N0-1 Pt
r2=0.98
Time (h)
b.3
log N N0-1 Pt
r2=0.93
Time (h)
c.1
Lactic acid (gL-1)
r2=0.96
Time (h)
c.2
Lactic acid (gL-1)
r2=0.95
Time (h)
c.3
Lactic acid (gL-1)
r2=0.51
Time (h)
d.1
LBA (gL-1)
r2=0.93
Time (h)
d.2
LBA (gL-1)
r2=0.98
Time (h)
d.3
LBA (gL-1)
r2=0.81
Time (h)
e.1
Lactose (gL-1)
r2=0.92
Time (h)
e.2
Lactose (gL-1)
r2=0.90
Time (h)
e.3
Lactose (gL-1)
r2=0.84
Time (h)

753 Figure 5

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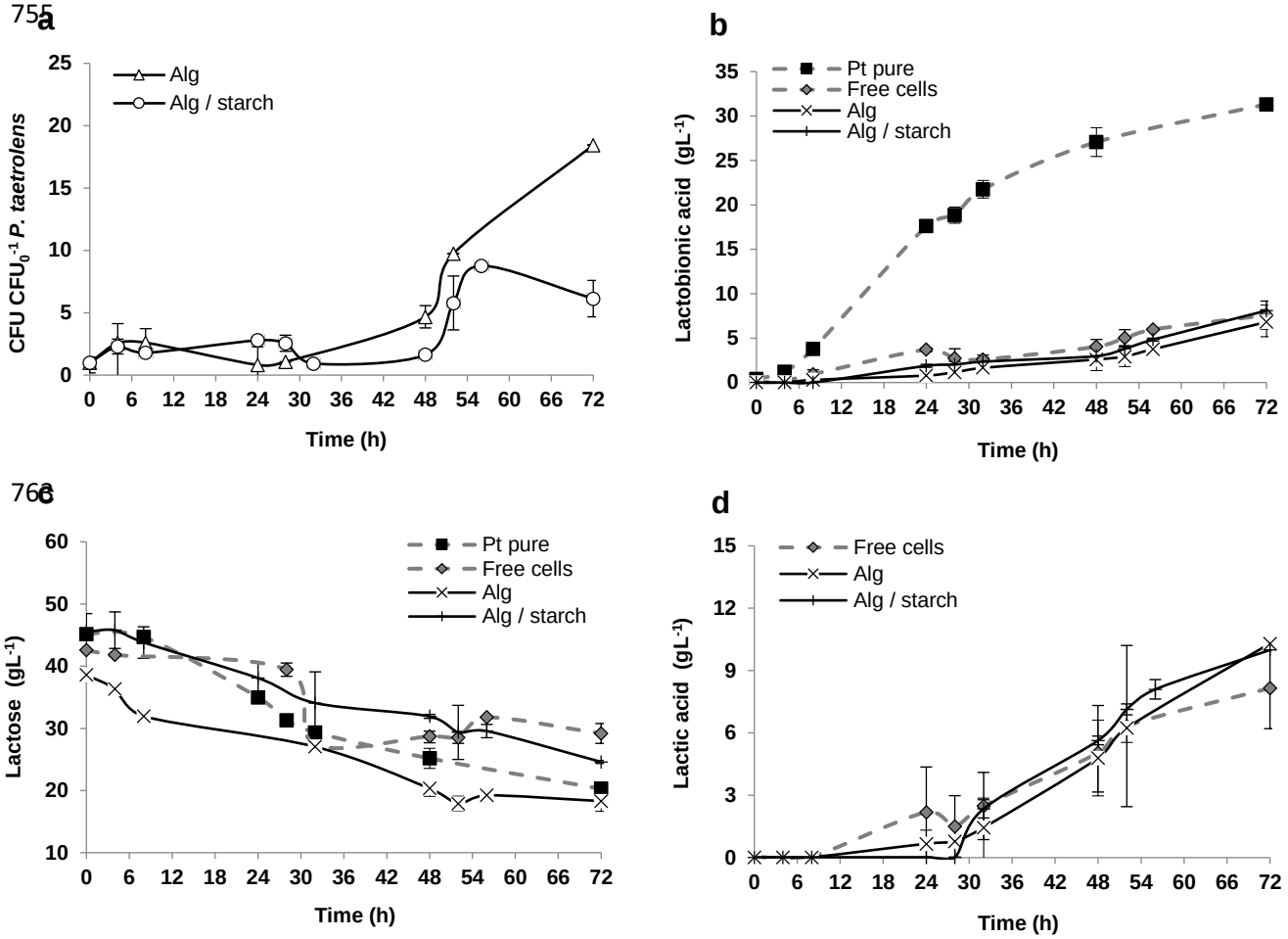

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