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Enhancement of growth rate and β-galactosidase activity, and variation in organic acid profile of *Bifidobacterium animalis* subsp. *lactis* Bb 12

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ABSTRACT

The behavior of *Bifidobacterium animalis* subsp. *lactis* Bb 12 under batch cultivation, after continuous culturing for up to 12 d, was monitored in skim milk-based media. Previous continuous culture for longer than 6 d affected the physiology of said microorganism. The minimum inhibitory concentrations of lactic and acetic acids increased from 18 to 26 g/l, whereas the molar ratio of acetic to lactic acid increased from 0.8 to 1.55, when the previous continuous culture increased its duration from 1 to 12 d. The specific lactose consumption rate decreased from 0.94 to 0.77 $g_{lactose}/g_{cell\ dry\ mass}/h$ within the batch culture timeframe; this was concomitant with greater amounts of acetic and formic acids, and lower amounts of lactic acid produced. The β-galactosidase activity increased as continuous culturing time increased, and reached 446 units/ml by 12 d; however, the rate of enzyme synthesis decreased concomitantly. Succinic acid was produced during the exponential growth and stationary phases of the batch culture, but the former at exponential growth phase was higher as the continuous culturing time was longer. For comparison purposes, batch cultivation of samples taken from continuous cultures by 1 and 12 d was done using a semi-synthetic medium with glucose as carbon source; a pattern similar to that observed when using skim milk-based media was observed.

1. Introduction

The current trend of supplementing various types of food products with probiotic bacteria has been driven by a rapidly increasing knowledge on the health importance of the intestinal microbiota and on their modulation factors. The predominant genus of these microflora that usually establishes in the colon is *Bifidobacterium*, which may account for up to 25% of their total viable numbers [1]. Recall that bifidobacteria are Gram-positive, non-spore forming, non-motile and (usually) catalase-negative anaerobes, which may assume various shapes [2]; since they are intrinsically heterofermentative, they can produce lactic acid, ethanol, acetic acid and formic acid through carbohydrate metabolism, as well as small amounts of carbon dioxide and succinic acid. These bacteria bring about several benefits to their human hosts, such as vitamin production, anticarcinogenic activity, immunostimulating effects, hypocholesterolemic power and pathogen inhibition; they may also shift the intestinal pH, via the aforementioned acidic metabolites [3,4]. A relatively novel industrial strain with a probiotic nature is *Bifidobacterium animalis* subsp. *lactis*, has been found in children's intestine where it promotes production of IgA (which plays a key role in their immune system); it also possesses a number of desirable technological features, viz. tolerance to oxygen, resistance to acids and ability to grow on milk-based media [5,6].

To respond with success to the demand for functional foods in the near future, technological and sensory issues are to be addressed. Nowaday, one important issue to attain optimum probiotic food manufacture is the absence of objective functions in terms of cell physiology that will assure a good functionality of the probiotic strains during food processing, and eventually in the gut during digestion. On the other hand, for a food to bear a probiotic label, survival of said probiotic bacteria during its whole shelf life is to be guaranteed [7]. In addition, a pleasant taste is required for a probiotic (as for every type of) food, besides its characteristic health beneficial effects; e.g. succinic acid conveys a proper flavor to a dairy product, but acetic acid leads to off-flavors. Finally, the

Abbreviations: d, day; CF, continuous culture; Control, cells harvested by 1 d of continuous culture, and batch cultivated afterwards, in skim milk-based medium; 6CF, cells harvested by 6 d of continuous culture, and batch cultivated afterwards, in skim milk-based medium; 8CF, cells harvested by 8 d of continuous culture, and batch cultivated afterwards, in skim milk-based medium; 10CF, cells harvested by 10 d of continuous culture, and batch cultivated afterwards, in skim milk-based medium; 12CF, cells harvested by 12 d of continuous culture, and batch cultivated afterwards, in skim milk-based medium; MIC, minimum inhibitory concentration of acetic and lactic acids; CDM, cell dry mass.

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final concentration of lactic acid should be ca. 8 g/kg and pH is to lie in the range 4.2–4.4, in order for bitterness and coagulum firmness be satisfactory [8,9]. Therefore, metabolic changes that prevail during manufacture are very important from a technological standpoint, because the amounts of organic acids are relevant toward correct development of flavor and texture in the final fermented dairy product. Note, however, that a few metabolic changes are relevant so as to allow microorganisms to obtain energy and maintain their NAD^+/NADH balance [10]; hence, a compromise should be attained between metabolic needs of the microorganism and organoleptic effects on the food product.

A few recent research studies have attained high cell densities of, and high productivities by bifidobacteria, encompassing either continuous or (repeated) batch cultures [11–16]; unfortunately, the information remains scarce on the change in physiology and metabolic activity of *Bifidobacterium* spp. during continuous culturing. Otherwise, Doleyres et al. reported that a continuous immobilized culture of *Bifidobacterium longum* exhibited improved physiological features, viz. an increase in its tolerance to various physico-chemical stresses, during long term fermentation [15].

Hence, the aim of this research effort was to ascertain the changes in the metabolic activity of *B. animalis* subsp. *lactis* Bb 12 in a batch culture, after continuous cultures had been maintained for several days in a row. The main metabolic activity indicators monitored were organic acid production (or consumption) and β-galactosidase activity, because of their impact upon the final organoleptic properties of the fermented food.

2. Materials and methods

2.1. Microorganism source

B. animalis subsp. *lactis* strain Bb 12 was purchased from Christian Hansen (Hørsholm, Denmark).

2.2. Culture medium

Preliminary tests, departing from dairy feedstocks and aimed at maximizing cell production, led to the following formulation of the growth medium: 50% rehydrated skim milk powder (10% total solids), 50% rehydrated whey permeate powder (6% total solids), 15 g/l yeast extract and 56 g/l lactose. Commercial whey permeate and skim milk powder were supplied by a local factory (Zarin Laban Pars, Karaj, Iran), and yeast extract was from Sigma–Aldrich (Dorset, UK).

2.3. Culture performance

B. animalis subsp. *lactis* Bb 12 was propagated routinely at 37 °C for 10–12 h in MRS broth (Difco, Detroit MI, USA) containing 0.5 g/l cystein hydrochloride (Merck, Darmstadt, Germany) [12]. This culture was incubated under anaerobically (in a glass jar with a gas pack). Then, 1 ml was used to inoculate 150 ml of culture medium (similar to said fermentation medium). This culture was finally incubated at 37 °C under anaerobiosis, for 10–12 h, and then further used as a pre-culture.

A 2 l-glass bioreactor (BIOFLO 2000, from New Brunswick Scientific, New Brunswick NJ, USA) was used to carry out the continuous cultures. After equipment sterilization (at 121 °C for 15 min), the bioreactor was filled with 1960 ml of medium (following previous sterilization at 121 °C for 5 min, cooling for ca. 5 h at room temperature, and reheating to 121 °C for 5 min); finally, the medium was inoculated with 40 ml of the pre-culture. The processing conditions were set according to the results of the preliminary study encompassing continuous cell production: the temperature was thus maintained at 37 ± 1 °C, and the pH at 5.5 via addition (when necessary) of 4 N NaOH. The stirring rate was set to 60 rpm, and the cultures were conducted for 12 d, under anaerobiosis provided by a CO_2 purge. The dilution (D) rate was 0.2 h^{-1}; after stabilization of the continuous culture, samples were taken every 2 d. For the sake of comparison, similar samples were also separately cultivated batchwise for 24 h, in the same bioreactor and under identical conditions (except that pH was not controlled); in this case, samples were taken every 2 h. Cells harvested on the first day of the continuous culture were taken as control.

For comparative purposes encompassing the performance of the strain selected, an alternative medium containing another carbon source was used to batch culture samples from the control and 12CF experiments. Said medium was a modified MCB (Medium for Colon Bacteria) one, which contained: 6.5 g/l peptone, 10 g/l tryptone, 10.0 g/l yeast extract (Sigma–Aldrich), 5 g/l meat extract (Sigma–Aldrich) 2.0 g/l KCl, 0.2 g/l $NaHCO_3$, 4.5 g/l NaCl, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 0.45 g/l $CaCl_2{\cdot}2H_2O$, 0.2 g/l $MnSO_4{\cdot}H_2O$, 0.005 g/l $FeSO_4{\cdot}7H_2O$, 0.005 g/l $ZnSO_4{\cdot}7H_2O$, 1.2 g/l cystein–HCl, 0.5 ml/l H_3PO_4, 0.5 g/l Tween 80, 2 g/l triammonium hydrogen citrate, 5 g/l sodium acetate trihydrate and 50 g/l glucose (pH was adjusted to 6.5). The fermentation conditions were the same as those applied to skim milk-based media [17,18]. All chemicals used were from Merk, unless otherwise indicated.

2.4. Viable cell enumeration

B. animalis viable cells were counted via plating on MRS agar (Difco, Detroit MI, USA), using the pour plate method, after appropriate dilution, followed by incubation for 48 h at 37 °C in anaerobic jars. The biomass concentration was estimated as described elsewhere [19].

2.5. Determination of minimum inhibitory concentration of acetic and lactic acids

For this purpose, diluted cultures of the probiotic strain were inoculated on MRS broth, at several levels of acetic and lactic acid concentrations (ranging from 12 to 30 g/l), but at a constant molar ratio (i.e. 1.5) of acetic to lactic acids. The tubes were incubated at 37 °C for 24 h, and cell growth was determined via optical density at 650 nm (Beckman, Fullerton CA, USA) [20].

2.6. Assay for β-galactosidase activity

β-Galactosidase activity was assayed for as reported elsewhere [21]: 1 ml of culture was added to 50 ml of 0.1 M phosphate buffer containing 0.001 M $MgSO_4$ and 0.05 M β-mercaptoethanol. Then, two drops of chloroform and one drop of 1% (w/v) sodium dodecyl sulfate were added to 1 ml of the diluted sample. After vortexing for 10 s, the mixture was placed in a water bath at 28 °C for 5 min; then, 0.2 ml of 4 mg/ml ONPG was added to the mixture and vortexed for 10 s before the reaction was started. The reaction catalyzed by β-galactosidase was quenched by 10 min via addition of 0.5 ml of 1 M Na_2CO_3. Following centrifugation at 16,266 × g for 15 min, the optical density at 420 nm was recorded (Beckman). The following formula was used to estimate the enzyme activity:

$$\text{activity of } \beta\text{-galactosidase (units/ml)} = \frac{A_{420}}{tv} \quad (1)$$

where t, v and A_{420} denote time of reaction (min), volume of sample (ml) and absorbance (420 nm), respectively. All chemicals used were from Merk.

2.7. Determination of specific rates of enzyme synthesis

The aforementioned rate, ε, was calculated using the formula:

$$\varepsilon = \frac{\Delta E}{\Delta t X} \quad (2)$$

where ΔE, Δt and X denote the enzyme activity change, the time interval and the biomass (in mg/ml), respectively [22].

2.8. Determination of organic acid profile

The concentrations of organic acids (viz. lactic, formic, succinic, citric and acetic acids) were determined by HPLC (Cecil 1100, Cambridge, UK), using a C_{18} column (Waters, Milford MA, USA) and a UV detector; the gradient of eluant was detailed elsewhere [23]. The concentrations of lactose and galactose were measured using the same HPLC system, but with a Eurokat H column kept at 65 °C (Waters) and an RI detector; the eluant was now an isochratic aqueous buffer at pH 2, and the flow rate was 1 ml/min.

2.9. Statistical analyses

The Duncan Multiple Range test was used to compare the mean values obtained in different treatments, via the SPSS v. 16 software (Chicago IL USA); the significance level selected was $P < 0.01$ or $P < 0.05$, as indicated. The bioreactor runs were replicated twice, whereas the analytical data were obtained as triplicates.

3. Results

3.1. Cell growth and β-galactosidase activity

Changes in the metabolic activity of the tested microorganisms when subject to continuous culturing, relative to the control fermentation, were visible after 6 d (data not shown). Both the viable cell numbers and the β-galactosidase activity increased significantly ($P < 0.01$) with duration of the continuous culture (Figs. 1 and 2); by 12 d, the values found were 8.9×10^7 CFU/ml and 446 units/ml, respectively, whereas the control counterparts were only 3.3×10^7 and 315, respectively. All cultures experienced a sudden drop in growth after reaching their maxima, but before

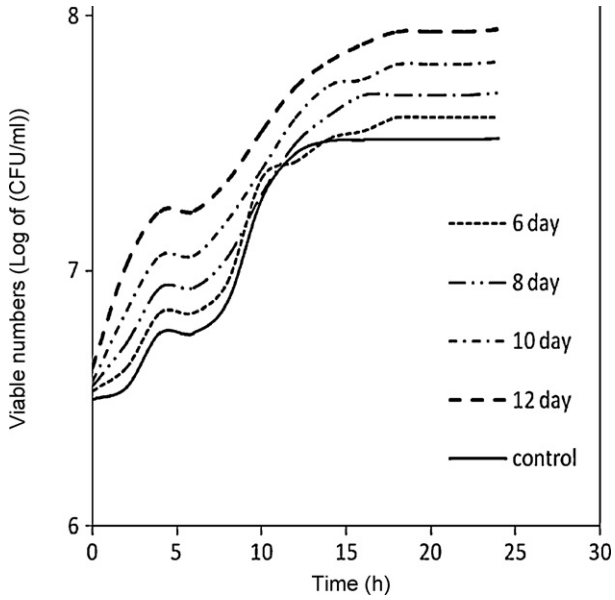

Fig. 1. Evolution with batch culture time of microbial growth, of control and experimental cultures, after continuous culturing for several times (6–12 d), in skim milk-based media.

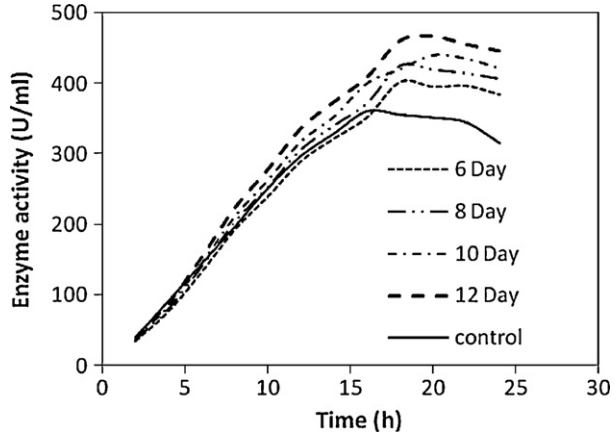

Fig. 2. Evolution with batch culture time of β-galactosidase activity, of control and experimental cultures, after continuous culturing for several times (6–12 d), in skim milk-based media.

complete lactose uptake (Table 1). The exponential growth phase in the batch cultures subject to previous continuous culturing took longer than in the control; for 12CF and 10CF data, such extension was ca. 4 h, and for 6CF and 8CF ones, it was ca. 2 h. In all media, a diauxic pattern was observed.

Inspection of Fig. 2, one finds that β-galactosidase activity increased during the exponential growth phase and at the early stationary phase of the batch culture, but eventually decreased afterwards. Said activity was higher after previous continuous

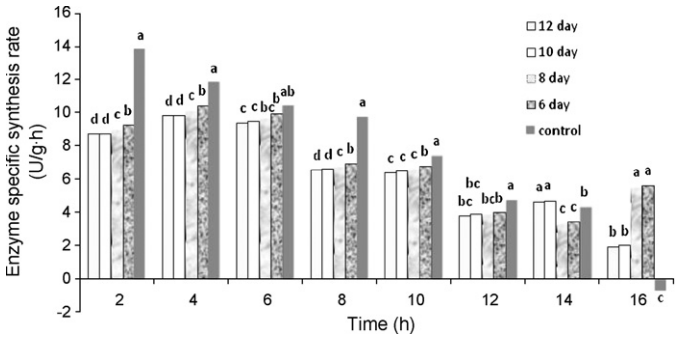

Fig. 3. Evolution with batch culture time of β-galactosidase specific synthesis rate, of control and experimental cultures, after continuous culturing for several times (6–12 d), in skim milk-based media. Means with the same letter (within the same set of columns) are not significantly different ($P > 0.05$).

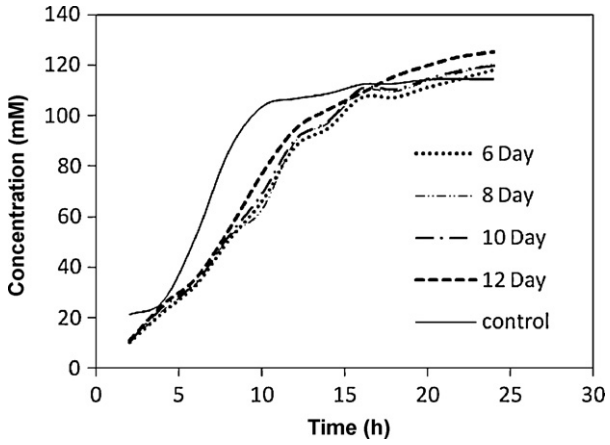

Fig. 4. Evolution with batch culture time of acetic acid concentration, of control and experimental cultures, after continuous culturing for several times (6–12 d), in skim milk-based media.

culture for a longer period; 12CF samples exhibited indeed the maximum β-galactosidase activity. When in the exponential growth phase, 12CF and 10CF samples entertained activity for an extra ca. 4 h, and 6CF and 8CF ones for an extra 2 h than the control. As apparent from inspection of Fig. 3, the specific rate of synthesis of enzyme decreased significantly ($P < 0.05$) with the previous continuous culture time; such a discrepancy was highest within the first 2 h, and decreased with batch culture time.

3.2. Kinetic parameters and organic acid profile

The results pertaining to acid production or consumption, substrate utilization and kinetic parameters are depicted in Table 1 and Figs. 4–7. The specific consumption rate of lactose was higher in the control than in the CF experiments, and decreased with time. However, the lactose consumption increased when the previous continuous culturing was longer: 12CF was thus associated with

Table 1
Selected characteristic and kinetic parameters of batch cultures, for 24 h in milk-based medium, after continuous culturing.

Previous continuous culture duration (d)	Specific sugar consumption rate ($g_{lactose}/g_{CDM}/h$)	Citric acid reduction (mM)	Lactose consumption (g/l)	MIC	Acetic/lactic acid molar ratio
6	0.87^{a}	4.13^{b}	49	19^{c}	0.9^{c}
8	0.84^{a}	2.6^{bc}	51	20^{c}	1^{c}
10	0.81^{a}	1.9^{c}	53	24^{b}	1.3^{b}
12	0.77^{a}	2.4^{bc}	55	26^{a}	1.55^{a}
^{c}ontrol	0.94^{a}	11.32^{a}	44	18a	0.8^{c}

Means with the same letter (within the same column) are not significantly different ($P > 0.05$).

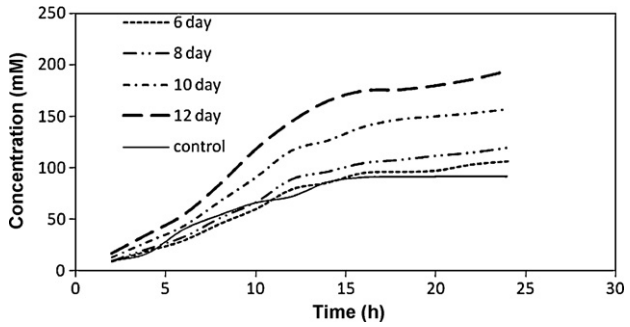

Fig. 5. Evolution with batch culture time of acetic acid concentration, of control and experimental cultures, after continuous culturing for several times (6–12 d), in skim milk-based media.

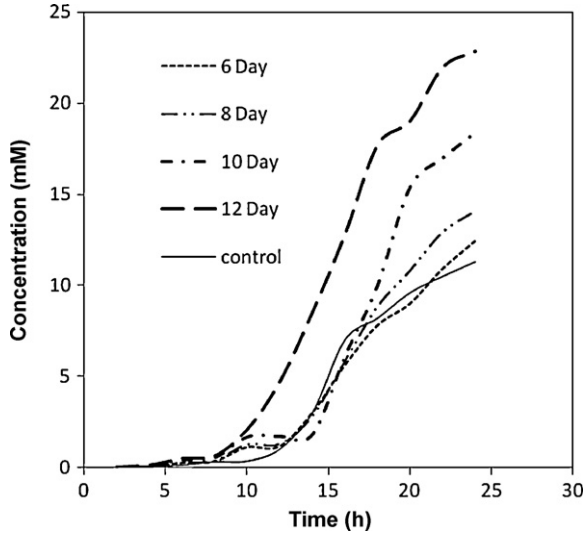

Fig. 6. Evolution with batch culture time of formic acid concentration, of control and experimental cultures, after continuous culturing for several times (6–12 d), in skim milk-based media.

the maximum absolute consumption and the minimum specific consumption rates of lactose (see Table 1).

When compared with the control, the levels of acids changed significantly with continuous culture time ($P < 0.05$). Lactic and acetic acids were produced during the exponential and stationary phases in both the control and the CF experiments, but the amount produced during the stationary phase in the CF ones rose as the period of previous continuous culturing was extended (see Figs. 4 and 5); in the 12CF case, ca. 8% of the final lactic acid concentration was produced during the stationary phase, and the corresponding amount of acetic acid was ca. 9%, whereas for the control those levels were only ca. 5% and 6%, respectively. The 12CF experiment reached maximum lactic and acetic acid concentrations of 125 and 194 mM, respectively, whereas their control counterparts attained only 114 and 91 mM, respectively. The molar ratio of acetic to lactic acid was not constant, and depended on the previous period of continuous culturing at the time of harvest; the data in Table 1 indicate that said ratio increased as this period expanded. As a result, the continuous culturing time caused a shift from lactic to acetic acid production in the batch cultures.

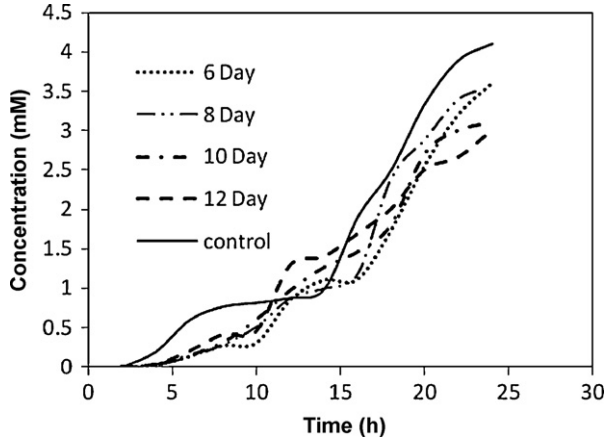

Fig. 7. Evolution with batch culture time of succinic acid concentration, of control and experimental cultures, after continuous culturing for several times (6–12 d), in skim milk-based media.

Formic acid production occurred in the stationary phase (see Fig. 6), as happened with lactic and acetic acids; 12CF had a maximum formic acid concentration of 23 mM, but the control reached only 11.3 mM. Faster rates of sugar consumption were associated with larger contents of lactic acid, and smaller contents of acetic and formic acids (see Table 1 and Figs. 4–6).

Production of succinic acid was observed in all experiments (see Fig. 7); said production took place during both the exponential growth and the stationary phases. In the control, the production of succinic acid at the stationary phase was greater than in the CF experiments, e.g. 76% vs. 33.3% in 12CF, respectively. The aforementioned production in CF at the exponential growth phase increased with fermentation time, but the total production decreased with the previous continuous culture time; therefore, maximum and minimum concentrations of succinic acid were associated with the control and 12CF data, respectively, and were 4.1 and 3.0 mM.

Variation in the citric acid level was observed only in the stationary phase (data not shown). Our microorganism consumed the aforementioned acid in the stationary phase, which was associated with succinic acid production ($R^2 = 0.9$). The control and 12CF batch cultures exhibited maximum and minimum citric acid reduction levels, respectively (see Table 1); said concentration decreased from 6CF to 10CF, but increased from 10CF to 12 CF.

3.3. Cell growth, β-galactosidase activity and organic acid profile in MCB medium

Data pertaining to growth of *B. animalis* in control and 12CF samples, using MCB medium, are shown in Table 2. It is apparent that the cell counts were much higher than in the (optimized) skim milk-based medium utilized (see Table 1 and Fig. 1); for control and 12CF samples, the cell counts were 1.9×10^9 and 5.6×10^9 CFU/ml, respectively.

Likewise to our previous results, longer preliminary continuous culturing led to enhanced viable cell counts. As happened with the lactose-containing medium, the specific sugar consumption rate in MCB decreased from 0.36 in the control to 0.27 in the 12CF sample. On the other hand, the carbohydrate concentration was not limiting: the residual glucose concentration in the control was 21 and 9 g/l in the 12CF case.

The maximum β-galactosidase activity (which was recorded by 24 h of batch fermentation – see Table 2) was greater for 12CF than for the control, but not to a significant degree ($P > 0.05$). As stated before, the biomass concentration increased when going from the control to 12CF, so the specific enzyme synthesis rate (as given by Eq. (2)) was lower in the latter than in the control (data not shown). The enzyme activity in MCB (Table 2) was indeed ca. 2.7 times lower than in milk (Fig. 2), even though the viable numbers were up to 60-fold greater (Table 2 and Fig. 1). Therefore, the specific enzyme synthesis level, for a given CFU level at the stage characterized by the maximum activity of β-galactosidase, was approximately 3 times lower in MCB. This realization might be linked to the fact that

Table 2
Selected chemical and microbiological parameters of batch cultures for 24 h in MCB medium, after continuous culturing.

Previous continuous culture duration (d)	Specific sugar consumption rate ($g_{glucose}/g_{CDM}/h$)	Glucose consumption (g/l)	Viable cell counts [Log(CFU/ml)]	Maximum β-galactosidase activity[a] (U/ml)	Acetic acid production (mM)	Lactic acid production (mM)	Succinic acid production (mM)	Formic acid production (mM)	Citric acid reduction (mM)	Acetic/lactic acid molar ratio
12	0.27[a]	41[a]	9.75[a]	166[a]	221.33[a]	127.87[a]	2[b]	70[a]	0.66[b]	1.73[a]
Control	0.36[a]	29[b]	9.28[b]	161[a]	143.82[b]	101.12[b]	3.31[a]	33[b]	3.7[a]	1.42[b]

Means with the same letter (within the same column) are not significantly different ($P > 0.05$).
[a] Attained by 24 h.

glucose was used as substrate in MCB. It thus remains to be determined whether β-galactosidase levels in MCB would have been much higher had lactose been used in the MCB medium instead. Interestingly, the levels of lactic and acetic acids were similar in the fermented milk and MCB media; this suggests that important uncoupling between growth and acid production occurred in the milk-based medium. The observation that organic acid levels were similar in the milk-based medium and MCB explains why the cells reacted similarly throughout the continuous culture period, even at very different biomass levels.

As was observed with our previous dairy medium, continuous culture caused larger acetic and formic acid production, as well as a higher acetic to lactic acid molar ratio. However, the results pertaining to the batch culture in glucose-containing medium indicated that both the control and the 12CF experiments yielded a greater acetic to lactic acid molar ratio than their skim milk-based counterparts (see Tables 1 and 2, and Figs. 4 and 5). As expected, MCB and skim milk-based media produced similar behaviors, in the case of succinic acid production and citric acid depletion, for the control and 12CF data.

4. Discussion

One major purpose of this research effort was to optimize biomass production in a continuous process, so a good growth medium should have been selected for the experiments in the first place; however, a skim milk-based medium was used rather than the MCB medium. Our choice of a skim milk-based medium for processing was indeed dictated by the intended use of a simple dairy medium that would be suitable for application in regular industrial settings, thus using milk or related feedstocks (e.g. whey and lactose) rather than a complex (and putatively more expensive) synthetic formulation. However, the patterns observed in both media tested were essentially parallel, so our major conclusions in terms of fundamental elucidation were not hampered to a significant extent by our choice.

There are two major reasons to study in depth the physiology of such probiotic bacterium as *B. animalis*. First, during growth of probiotic bacteria in the lower intestine, they compete with other local bacteria for available substrates, so lowering of the intestinal pH is a tool to assure their competitive growth; such metabolic products as acetic and lactic acids are released for this purpose, and they inhibit pathogens and/or are absorbed by the host. Second, that specific bacterium is often cultured by the food industry, either *in situ* in a food (e.g. in yoghurt manufacture) or else in a fermenter for pure culture production (e.g. for posterior inclusion later as a food supplement). Consequently, it is useful to know the kinetic parameters prevailing during growth of *B. animalis* in a particular substrate [24].

Acid resistance has been proposed as a criterion to screen for potentially probiotic *Bifidobacterium* spp. [25], and lactic and acetic acids are known inhibitors thereof [19,26]. The dilution rate experimentally found (i.e. $0.2\,h^{-1}$) is low, so this leads to development of a relatively steep variation in the concentration of those compounds under continuous culturing. Inspection of Fig. 8, one observes that lactic and acetic acids attained the levels of 208 and 185 mM, respectively, when the previous continuous culture was maintained for 12 d; the corresponding concentrations of succinic and formic acids were 2.8 and 25 mM, respectively. These organic acid concentrations can induce a stress adaptation of cells to thereto [27]; and the data in Table 1 show that the minimum inhibitory concentrations of lactic and acetic acids increased from 18 to 26 g/l, along with longer duration of the previous continuous culture. Bacteria usually respond to changes in their surrounding medium via metabolic reprogramming, which leads to a cellular state of enhanced resistance [28]; hence, variations in

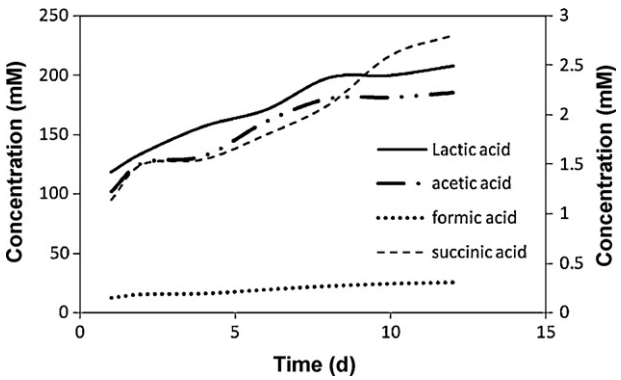

Fig. 8. Evolution with continuous culture time of organic acid concentration in the pH-controlled chemostat. The left axis entails lactic, acetic and formic acid concentrations, and the right axis succinic acid concentration.

growth, β-galactosidase activity and organic acid profile can act in combination to provide the aforementioned adaptation.

Bifidobacteria can degrade carbohydrates to acetic and lactic acids as major metabolites, through the fructose-6-phosphate shunt, with a 3:2 theoretical value for the acetic to lactic acid molar ratio [29]. However, at high intracellular sugar concentration (as happened with the processing medium used in this study), they preferentially convert pyruvic acid to lactic acid through the conventional catabolic route [30]. In general, larger amounts of acetic acid lead to larger amounts of formic acid and smaller amounts of lactic acid, and vice-versa [16]: less lactic acid available for additional ATP production (or regeneration of NAD^+) actually shifts the metabolic pathways to acetic, succinic and formic acid production (see Fig. 9A) [27]. Consequently, carbohydrate metabolism may be more efficient if the subsequent ATP handling at the substrate level is directed to higher yields of ATP [22]. According to our results, continuous culturing caused adaptation of cells to acidic conditions; hence, cells produced by this process exhibited lower lactose specific consumption rates, larger acetic to lactic acid ratios, and larger formic and succinic acid production. Therefore, the ATP yield should in principle be improved during the exponential growth and stationary phases of the batch culture, and the NAD^+ regeneration should be more efficient through the fructose 6-phosphate shunt. These variations help cells survive under such harsh condition as low pH, and thus increase their population relative to that of the control.

When bacteria are grown in a batch culture containing a mixture of two growth-limiting substrates, they typically undergo a diauxic growth [31]. Our results showed that lactose or galactose (note that the galactose concentrations by the end of culturing were not significantly different from that in unfermented medium, data not shown) were not growth-limiting in all experiments; hence, peptide components might account for this realization. In fact, it has been reported that *B. longum* 15707 also underwent a diauxic pattern when grown in skim bovine milk; this pattern could be due to the presence of different proteolytic enzymes in bifidobacteria, that provide the amino acids they required for growth [32]. Furthermore, during growth of *L. lactis* in milk-based media, regulations of the proteolytic mechanism are affected by medium peptides; heat treatment of milk has likely affected these peptides, and thus influenced the proteolytic activity of that microorganism [33]. On the other hand, recall that skim milk and whey powder from local dairies were employed in the formulation of the processing medium, so the peptide profile will be directly influenced by the intrinsic quality of the raw materials and the type of heat treatment applied afterwards [33]. Therefore, our experimental medium had three sources of peptides: whey, yeast extract and skim milk; and *B. animalis* apparently utilized all such nitrogen sources [5,34,35]. The

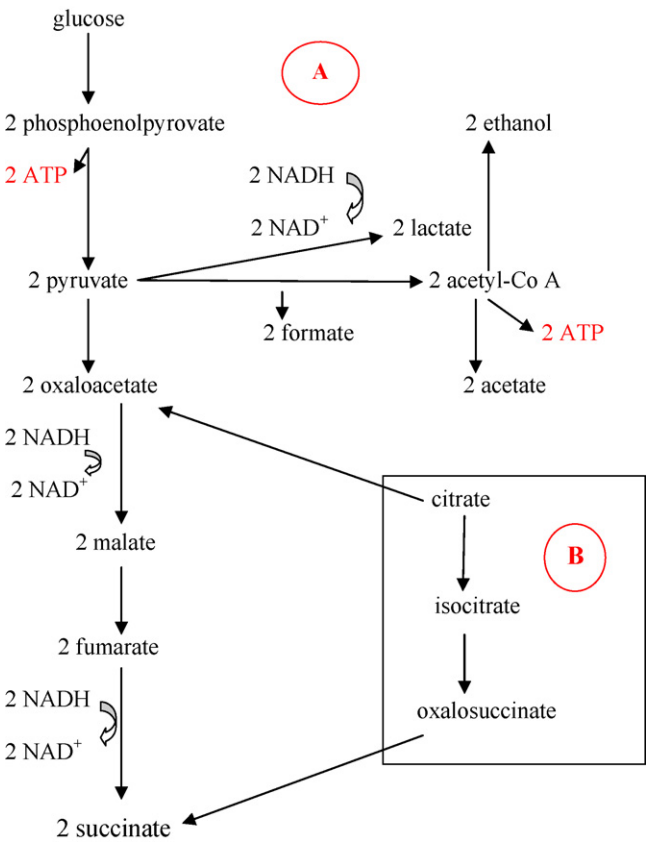

Fig. 9. Schematic representation of the fructose 6-phosphate shunt (A) and the citric acid metabolism (B) in *B. animalis* subsp. *lactis* Bb 12. Adapted from [28] and http://www.genom.jp/kegg/kegg2.html.

differential use thereof in time might justify (at least partially) the diauxic behavior observed in all batch cultures, but further studies are required to fully backup this hypothesis.

The operon encoding lactose permease is apparently activated by the presence, within the same transcriptional unit, of the β-galactosidase gene [35,36]. When the lactose specific consumption rate increases, the expression of this gene raises as well. Therefore, a relationship between the specific rate of enzyme synthesis and the specific rate of lactose consumption was observed in our experiments. The increase in β-galactosidase activity with fermentation time could not, however, be fully rationalized by existing knowledge: accumulation of hydrogen ions is indeed a factor that reduces β-galactosidase activity in the stationary phase [22].

It has been reported that *B. animalis* subsp. *lactis* Bb 12 cannot metabolize citric acid in milk at 37 °C, but the reverse holds at 45 °C [9]; our results showed that this microorganism can metabolize citric acid in milk enriched with whey protein and yeast extract. Furthermore, a strong relationship between succinic acid production and citric acid consumption has been found. Succinic acid is a growth-associated compound, but citric acid level decrease is associated with the stationary phase [9,17,24]. However, according to our data, the production of succinic acid continues until the stationary phase is reached. The proposed metabolic mechanism for *B. animalis* subsp. *lactis* Ad011 entails production of succinic from citric acid, as evidenced in Fig. 9. Carbohydrates are accordingly degraded through the fructose-6-phosphate shunt, oxaloacetate is converted to fumarate, and succinic acid is eventually produced therefrom (Fig. 9A); alternatively, citrate is converted to isocitrate, and oxalosuccinate is produced from it and later converted to succinic acid, or citrate is converted to oxaloacetate directly, and succinic acid is produced from it (Fig. 9B). Therefore, it could

be hypothesized that *B. animalis* subsp. *lactis* Bb 12 would generate succinic acid following such pathways. However, increases in succinic acid production and citric acid depletion were not likely associated with continuous culture time; production of succinic acid may be a tool to assure the redox balance [17]. Cells in 12CF do indeed handle larger amounts of lactose and organic acids; hence, in order for the organism to equilibrate its redox performance, a shift to synthesis of succinic acid and consumption of citric acid prevailed relative that applying to 10CF. More experimental work is, nevertheless, required before one can establish which metabolic pathway holds in our bifidobacterium strain, encompassing production of succinic acid and interaction with citric acid.

Growth of *B. bifidum* in samples of control and 12CF in the glucose-containing medium showed essentially the same behavior as in the skim milk-based medium, except for the end product concentration, i.e. β-galactosidase activity. Wide differences in the levels of end products brought about by bifidobacteria have been reported in the literature as depending on the strain at stake, the carbon source of the culturing medium and the growth conditions applied [37]. A previous study has claimed that bifidobacteria grown on glucose produce more lactic acid and less acetic acid than in lactose-containing media [37]. Our results showed that the control and 12CF samples produce more acetic and formic acids, and (obviously) less lactic acid in glucose-containing medium than in its skim milk-based counterpart; they are consistent with results reported elsewhere [12,17], indicating that growth on glucose causes a greater acetic to lactic acid molar ratio than on lactose. Since lactose is an inducer of β-galactosidase activity, growth in the presence of glucose constrains only the observed activity of enzyme [37], as perceived with MCB medium (for both control and 12CF samples).

Preconditioning treatments (which are conventional methods to promote cellular resistance) lead to reduced cell activity and yield [27]. Hence, there is a considerable advantage of resorting to continuous culturing for biomass production of *B. animalis* [14]: cells produced in this way, which are in the exponential growth (or, at most, in the early stationary) phase, are prone to both a high viability and a good metabolic activity relative to their batch cultured counterparts [15]. Our results have actually shown that continuous culturing can increase the acid resistance of *B. animalis*, and that such a resistance may improve the functionality of probiotic bacteria in the food, and eventually in the gut [38]. These increases in acid resistance are simultaneous with variation in β-galactosidase activity and organic acid production – especially in terms of acetic to lactic acid molar ratio, which plays an important role upon organoleptic acceptance of the final fermented food.

Except for β-galactosidase synthesis, the physiological phenomena occurring in milk at low biomass levels (i.e. $<10^8$ CFU/ml) were similar to those in MCB, where viable counts were well above 10^9 CFU/ml. Hence – presumably due to uncoupling between growth and acidification, the biomass level did not seem to affect the evolution of cells during continuous culture. Nevertheless, an industrial application would require a milk-based medium, so further work is needed encompassing supplementation of the milk medium with food-grade ingredients, aiming at an increase of the viable counts by (at least) a factor of 50.

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