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ORIGINAL RESEARCH

Production of bacteriocin-like inhibitory substances (BLIS) by *Bifidobacterium lactis* using whey as a substrate

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The objective of this work was to evaluate the production of bacteriocin-like inhibitory substances (BLIS) by Bifidobacterium animalis *subsp.* lactis *in whey supplemented with yeast extract, inulin, Tween-80 or* L*-cysteine. Cell growth, acidification, glucose and lactose consumption as well as BLIS production were measured during fermentations carried out in shake flasks. The best additive for both cell growth and BLIS production was shown to be yeast extract, which gave the highest concentrations of biomass (9.9 log cfu/mL) and BLIS (800 AU/mL). In a bench-scale fermentor,* B. lactis *growth and BLIS production were between 6% and 25% higher than in flasks depending on the conditions assayed.*

Keywords *Bifidobacterium lactis*, Whey, Bacteriocin, Anaerobic conditions.

INTRODUCTION

Whey is a by-product of high nutritional value that contains about 4.6% lactose, 0.8% protein, 0.5% fatty acids, 0.5–0.8% ash and 0.2–0.8% lactic acid (Punidadas *et al.* 1999; Antunes 2003). Given the production of cheese, 9 kg of whey is produced for each kilogram of cheese (De Wit 2001); therefore, taking into account that the cost of whey is about 10 times lower than that of traditional cultivation semisynthetic media such as de Man, Rogosa and Sharpe (MRS) medium or *Bifidus* Selective Medium (BSM), it would be of great interest to exploit this waste as a fermentation medium for the production of high value-added biomolecules such as antimicrobial compounds.

Some probiotic species of *Lactobacillus* and *Bifidobacterium* used in the manufacture of fermented dairy products can inhibit the growth of other micro-organisms, including intestinal pathogenic and spoilage bacteria, through the production of antimicrobial compounds such as bacteriocins (Cheikhyoussef *et al.* 2010; Ristagno *et al.* 2012), which offer to the producing strains an ecological advantage in the colonisation of the gastrointestinal tract (Tamime *et al.* 2005).

Bacteriocins are usually synthesised as inactive prepeptides with an N-terminal guidance sequence (Xie and van der Donk 2004), which are transported to the cell surface during the exponential growth phase and enzymatically converted into their bioactive form. The carrier contains an N-terminal peptide moiety responsible for cleavage of the guidance peptide and a C-terminal portion responsible for the hydrolysis of ATP and energy supply (Aucher *et al.* 2005).

New bacteriocins are traditionally identified by screening bacterial isolates with antimicrobial activity, followed by purification and identification of their genetic determinants. Such screening strategies are essential for the detection and identification of potent bacteriocins of various subclasses (Balciunas *et al.* 2013; Martinez *et al.* 2013).

Bifidobacteria, which are regarded as one of the predominant genera of the gastrointestinal tract (Falk *et al.* 1998), generally inhibit a wide

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range of target micro-organisms caused by the intensive release of lactic and acetic acids as products of their metabolism (Eklund 1983). Inside this genus, at least 34 species able to produce bacteriocins had been isolated from various sources, including the intestinal tract of humans (Ventura *et al.* 2007). Unlike the current situation of *Lactobacillus* spp, only a limited number of studies have been conducted on the production of bacteriocins by bifidobacteria strains.

Yildirim and Johnson (1998) isolated the first bacteriocin produced by a *Bifidobacterium* species, known as Bifidocin B, which was proven to be very effective against foodborne pathogenic micro-organisms (Yildirim *et al.* 1999). Recently, Cheikhyoussef *et al.* (2010) discovered a novel bacteriocin produced by *Bifidobacterium infantis* in synthetic (MRS) medium supplemented with L-cysteine. This bacteriocin, called Bifidin I, showed a broad spectrum of action against both Gram-negative and Gram-positive bacteria and proved to exert a strong inhibition of the pathogen *Listeria monocytogenes*.

Several compounds have been successfully employed to stimulate either bifidobacterial growth or their production of bacteriocins. Among these, inulin is a fructose-based polysaccharide whose stimulating prebiotic effect has recently been ascribed to its ability to release fructose as a carbon source (Oliveira *et al.* 2009, 2012). Tween-80 or Polysorbate 80 is a nonionic surfactant and emulsifier derived from polyethoxylated sorbitan and oleic acid, which is often used as a viscous, liquid, water-soluble yellow solution in foods and other products (O'Neil 2006). The addition of Tween-80 to culture media was shown to be very promising in bacteriocin production (Collado *et al.* 2005), being a surfactant able to increase bacteriocin concentration by making its excretion easier. In several studies on species belonging to the *Bifidobacterium* genus and even other genera performed in different culture media, yeast extract was shown to enhance cell growth. Loquasto *et al.* (2011) observed that the addition of yeast extract to whey stimulated *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) growth compared to the non-supplemented medium. Mättö *et al.* (2006) and Kiviharju *et al.* (2007) demonstrated the beneficial effect of cysteine on the growth of *B. lactis* E2010 and *Bifidobacterium longum* ATCC 15707 in MRS medium.

Based on this background, the objective of this study was to investigate the production of bacteriocin-like inhibitory substance (BLIS) by *B. lactis* HN019 using whey as an economical culture medium and evaluating inulin, Tween-80, yeast extract or L-cysteine as additives, aiming at a future large-scale use as a cheaper method to preserve foods and as a pharmaceutical alternative to nisin.

MATERIALS AND METHODS

Micro-organisms

The commercial lyophilised strain *Bifidobacterium animalis* subsp. *lactis* HN019 (*B. lactis*) (Danisco, Sassenage, France) was used in this work for bacteriocin-like inhibitory substances (BLIS) production, while *Lactobacillus sakei* ATCC 15521, *Escherichia coli* ATCC 25922 and *Listeria monocytogenes* ATCC 13932 were used as bioindicators.

Preparation of culture media

Whey powder produced from cheese whey (Cargill Agrícola SA, Campinas, SP, Brazil) was reconstituted at a concentration of 10% (w/v) and supplemented or not with inulin (Beneo-Orafti, Mannheim, Germany), Tween-80, L-cysteine (Sigma-Aldrich, St. Louis, MO, USA) and yeast extract (Synth, Diadema, SP, Brazil) at a concentration of 1% (w/v). Whey powder was reconstituted with the aid of a magnetic stirrer for 15 min and heated at 90 °C for 5 min in a thermostatically controlled bath, model 550 A (Fisatom, São Paulo, SP, Brazil). Subsequently, the suspension was immediately cooled in an ice bath and distributed into sterile Schott vials inside a laminar flow hood.

Inoculum preparation

To perform cultivations in flasks, a preculture was prepared by adding 45 mg of lyophilised stock culture of *B. lactis* into 50 mL of *Bifidus* Selective Medium (BSM) (Sigma-Aldrich) in 250-mL Schott flasks at 37 °C with magnetic agitation at 50 rpm for 24 h in anaerobic jars (BBL GasPak System; Becton Dickinson Microbiological System, Cockeysville, MD, USA).

Cultivation in shaker flasks

An aliquot of the previous culture containing approximately 10^8 cfu/mL was transferred to 250-mL Schott flasks containing 100 mL of whey, which were then incubated in a shaker at 37 °C at 50 rpm for 30 h.

Cultivation in bench-scale bioreactor

The culture for cultivations in a bench-scale bioreactor was prepared by adding 145 mg of *B. lactis* lyophilised stock culture into 150 mL of whey in 250-mL Schott flasks at 37 °C with magnetic agitation at 50 rpm for 24 h in anaerobic jars. After reaching a concentration of approximately 10^8 cfu/mL, the preinoculum was transferred to the New Brunswick Bio-Flo 115 benchtop fermentor (Eppendorf, Enfield, USA) containing 1350 mL of whey supplemented with 1% yeast extract, which was shown to be the best additive. Fermentation in the bioreactor were performed in triplicate under anaerobic conditions ensured by continuous injection of nitrogen.

Analytical techniques

Aliquots of the culture media were collected aseptically every 3 h from cultures performed either in flasks or in bioreactor, and pH, concentrations of glucose (for synthetic media), lactose (for whey), biomass, and BLIS activity were determined. Cultures were performed in triplicate, and the results were expressed as mean values.

Samples (0.1 mL) of the fermentation broth were decimally diluted six- to ninefold with sterile peptone water. Subsequently, 1.0 mL of each dilution was transferred to Petri dishes containing BSM and agar prepared in accordance with the pour plate method (Norden and Kass 1968). The plates were placed in anaerobic jars and incubated at 37 °C for 48 h. Counts were considered acceptable when the number of colonies ranged between 30 and 300 and expressed as log cfu/mL. The maximum specific growth rate (μ_{max}) was then calculated during the exponential growth phase, according to the equation $\mu_{max} = \ln(X_2/X_1)/(t_2-t_1)$, where X_2 and X_1 are the counts (cfu/mL) at time t_2 and t_1, respectively.

The pH was measured using a pH meter, model Q-400M1 (Quimis, São Paulo, SP, Brazil).

The concentrations of lactose and lactic acid were determined by high-performance liquid chromatography (HPLC) according to the method described by Donkor *et al.* (2007). Briefly, each sample was centrifuged at 15 000 *g* for 20 min using a microcentrifuge, model U-32R (Boeckel, Hamburg, Germany). The supernatant was adequately diluted, filtered through a membrane with 0.45 μm pore diameter (Millipore, Barueri, SP, Brazil), and injected into a HPLC, model Ultimate 3000 (Dionex, Sunnyvale, CA, USA), equipped with a refractive index detector, model Shodex RI-210 (Kawasaki, Kanagawa, Japan) and a HPX-87H column (Bio-Rad, Hercules, CA, USA). Runs were carried out at 35 °C, using 5.0 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. High-purity lactose and lactic acid for use in HPLC (Sigma-Aldrich) were used at concentrations from 0.1 to 2.0 g/L as standard solutions to prepare the calibration curve.

Determination of BLIS activity

To detect the formation of BLIS, samples were centrifuged at 13 201 *g* at 10 °C for 10 min. The supernatant was used to quantify BLIS activity, and the pH was adjusted to 6.0 to avoid any interference in the activity tests. Samples were then heated at 90 °C for 10 min to avoid the possible influence of peroxides produced by *B. lactis* (Odamaki *et al.* 2011) and to inactivate its proteolytic enzymes. After agitation using a vortex mixer, a portion of supernatant was treated with 1 mg/mL trypsin (Sigma-Aldrich) to check the proteinaceous nature of BLIS (Todorov *et al.* 2004) and decimally diluted. Each dilution (40 μL) was placed into wells in BHI broth (Brain Heart Infusion), TSB (Trypticase Soy Broth) and MRS broth (Difco, Sparks, MD, USA) supplemented with 1% (w/w) agar, which, in turn, had been preinoculated with *L. monocytogenes* ATCC 13932, *E. coli* ATCC 25922 and *L. sakei* ATCC 15521 as indicator microorganisms, respectively. The antimicrobial activity was determined by the spot-on-lawn assay (Somkuti and Steinberg 2002; Apolônio *et al.* 2008) after incubating the plates at 35 °C. BLIS activity (AU/mL) was calculated as $2^n \times 1000$ μL/40 μL, where n is the final dilution showing inhibition.

Statistical analyses

All analyses were performed in triplicate, and the results were expressed as mean values. The experimental errors were expressed as standard deviation from the mean value. Values of percentage of lactose consumption, μ_{max} and lactic acid concentration were submitted to analysis of variance (ANOVA) by the Statistica 12 software (Tulsa, OK, USA).

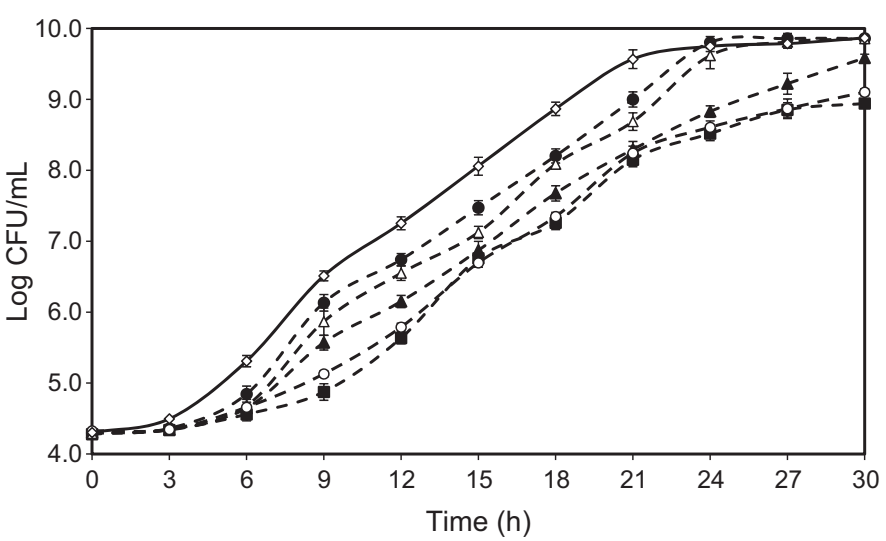

Figure 1 Biomass concentration (log cfu/mL) during *Bifidobacterium animalis* subsp. *lactis* cultivations in flasks (dashed lines). Nonsupplemented whey: (○); whey supplemented with yeast extract: (●); L-cysteine: (■); Tween-80: (▲); inulin: pH (△). Runs carried out in bench-scale fermentor (continuous lines) (◊).

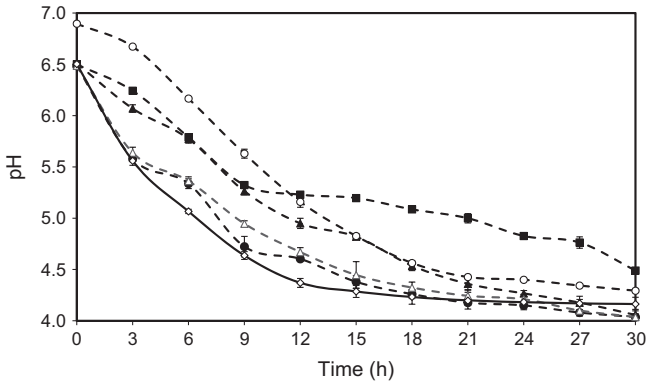

Figure 2 pH variations during *Bifidobacterium animalis* subsp. *lactis* cultivations in flasks (dashed lines). Nonsupplemented whey: (○); whey supplemented with yeast extract: (●); L-cysteine: (■); Tween-80: (▲); inulin: pH (△). Runs carried out in bench-scale fermentor (continuous lines) (◊).

RESULTS AND DISCUSSION

Cultivations in flasks

Batch cultures were performed using whey as an alternative culture medium supplemented with yeast extract (YE), L-cysteine, inulin or Tween-80, each at a concentration of 1%. These supplements were in fact shown to significantly stimulate bifidobacterial growth in previous studies (Collado *et al.* 2005; Oliveira *et al.* 2009, 2012; Loquasto *et al.* 2011). The results of these runs in terms of biomass concentration and pH along the time are illustrated in Figures 1 and 2, respectively.

At the end of the cultivation period (30 h), the pH of whey supplemented with L-cysteine was 4.5, that is 0.5 points higher than that in the presence of the other supplements. These values coincide with those reported by Loquasto *et al.* (2011) for the same micro-organism.

With respect to cell growth, the use of inulin or YE as a supplement ensured a cell concentration of *B. lactis* (approximately 9.8 log cfu/mL for both) significantly higher than those obtained with L-cysteine (8.8 log cfu/mL) or Tween-80 (9.2 log cfu/mL). Consistent with these results, when whey was supplemented with YE or inulin, the stationary phase of *B. lactis* growth started earlier (after 24 h of fermentation) compared with L-cysteine (27 h). Although whey is a natural culture medium containing nutritional substances capable of stimulating the metabolism of *B. lactis* as well as other lactic acid bacteria (Janer *et al.* 2004; Thamer and Penna 2005), it has low contents of peptides and free amino acids (Gomes *et al.* 1998) that are essential for cell growth (Antunes 2003); therefore, the strong acceleration of growth observed using YE as a supplement can possibly be attributed to its high content of these components. This stimulatory effect, already observed by Loquasto *et al.* (2011) for the same micro-organism in whey (10% v/v) supplemented with 1% YE, is consistent with the results obtained by Gomes *et al.* (1998) using hydrolysed whey supplemented with amino acids. Also, the stimulatory effect of inulin on *B. lactis* growth is in agreement with the results of a recent work, where this biopolymer was used to enhance bifidobacteria growth (Oliveira *et al.* 2009, 2012). As a result, in whey supplemented with both YE and inulin, the specific growth rate (μ_{max}) (0.60/h) was 13% higher than that obtained in nonsupplemented whey (0.53/h).

On the contrary, the addition of Tween-80 not only delayed the occurrence of the stationary phase (30 h), but also did not increase the specific growth rate of *B. lactis* when compared to whey alone.

Although *B. lactis* proved to be a bacteriocin-producing species, its BLIS activity was not very high (100 AU/mL in whey without any supplement), being *Listeria monocytogenes* the only sensitive pathogenic micro-organism among those tested. BLIS activity was 100 AU/mL with L-cysteine and Tween-80, 200 AU/mL with inulin and 400 AU/mL with yeast extract. Cheikhyoussef *et al.* (2010) and Yildirim *et al.* (1999) reported for bacteriocins produced by *Bifidobacterium infantis* BCRC 14602 and *Bifidobacterium bifidum* NCFB

Table 1 Main results of *Bifidobacterium lactis* cultivations performed in whey supplemented with different additives at a concentration of 1% (w/w). Data collected at the end of cultivations (30 h)

Supplement	*Lactose consumption (% of total)*	*Bacteriocin activity (AU/mL)**	*μ_{max} (/h)†*	*Lactic acid concentration (g/L)*
Runs in flasks				
Yeast extract	38.5 ± 0.1^{d}	400	0.60 ± 0.1^{c}	3.2 ± 0.1^{a}
L-Cysteine	36.5 ± 0.1^{ab}	100	0.55 ± 0.1^{a}	4.4 ± 0.2^{b}
Tween-80	34.8 ± 0.2^{c}	100	0.53 ± 0.1^{a}	4.6 ± 0.2^{b}
Inulin	36.2 ± 0.1^{a}	200	0.60 ± 0.2^{bc}	4.5 ± 0.1^{b}
None	36.7 ± 0.2^{b}	100	0.53 ± 0.1^{a}	4.9 ± 0.2^{bc}
Run in fermentor				
Yeast extract	40.9 ± 0.1^{f}	800	0.57 ± 0.2^{bc}	3.4 ± 0.2^{a}

*Bacteriocin activity against *Listeria monocytogenes* ATCC 13932. †Maximum specific growth rate.

Mean values (n = 3) ± standard deviations with different letters in the same column mean that they significantly differ among values of the same parameter ($P < 0.05$).

1454, both in cell-free supernatants, antimicrobial activities of 1600 and 3200 AU/mL, respectively. It is noteworthy that these researchers used MRS broth supplemented with 0.05% (w/v) L-cysteine hydrochloride, that is a medium much more expensive than whey used in this study. On the other hand, Bendali *et al.* (2008) and Bizani *et al.* (2005) reported values of antimicrobial activity similar to those obtained in this work, but the micro-organisms employed were *Lactobacillus paracasei* subsp. *paracasei* and *Bacillus cereus* 8A, both cultured in BHI medium. In all these previous studies, and in this current work, the sensitive indicator micro-organism was always *L. monocytogenes*. Regarding the heat stability, BLIS was shown to be stable in temperatures ranging from 80 to 100 °C (results not shown), confirming the findings of previous studies (Cheikhyoussef *et al.* 2009; Yildirim *et al.* 1999).

The influence of the above additives on the production of lactic acid was contrary to that on BLIS activity, in that its highest concentration at the end of cultivations was observed in nonsupplemented whey (4.90 ± 0.16 g/L) and the lowest one in whey supplemented with YE (3.23 ± 0.12 g/L), conditions under which *B. lactis* showed the minimum (100 AU/mL) and maximum (400 AU/mL) antimicrobial activities, respectively (Table 1).

Even though the percentage of lactose consumption varied slightly (from 34.8 to 38.5%) from one culture to another carried out at the same initial concentration (53.0 g/L), these results as a whole suggest that BLIS production is linked to the consumption of the carbon source. According to Gui and Li (2013), lactose consumption occurs when bacteriocin and lactic acid accumulate in the culture medium, which indicates that this sugar can be used to support the production of both. One can therefore infer that BLIS produced by *B. lactis* is a primary metabolite (Hugas *et al.* 2002) resulting from a type II fermentation according to the classification of Gaden (1959).

Cultivation in bioreactor

Fermentation was also carried out in a bench-scale fermentor using whey containing 10% (w/v) total solids supplemented with 1% (w/v) YE, because in flasks these were the conditions showing the highest biomass concentration at the end of the exponential growth phase and, at the same time, the highest BLIS activity. This experiment was performed in triplicate under conditions of temperature (37 °C) and agitation (50 rpm) that simulated at the best of those in flasks.

As can be seen in Figure 1, under these conditions, *B. lactis* showed higher and accelerated growth compared to cultivations in flasks. When grown in fermentor, the micro-organism did in fact reach the exponential growth phase, on average, 3 h faster, and the cell concentration (9.57 log cfu/mL) was significantly higher than that obtained in flasks (8.96 log cfu/mL). This result may be related to the fact that the conditions occurring in flasks are microaerophilic, while *B. lactis* is notoriously a strictly anaerobic micro-organism. Therefore, low agitation and injection of nitrogen were sufficient to homogenise the culture medium and promote an anaerobic fermentation, respectively. The percentage of lactose consumption was consequently higher in fermentor (40.9%) compared to flasks (38.5%).

As far as the acidifying capacity is concerned, it can be observed in the same figure that the pH behaviour was similar in flasks and bench-scale fermentor.

B. lactis growth in whey supplemented with YE was higher than that found after 24 h of culture (7.16 log cfu/mL) by Jalili *et al.* (2010), who cultivated the wild-type strain of the same micro-organism in the same culture medium, but with a slightly more intense agitation rate (60 rpm).

BLIS activity obtained in bench-scale fermentor using whey supplemented with YE was 100% higher (800 AU/mL) than that in flasks (Table 1).

CONCLUSIONS

Whey supplemented with yeast extract or inulin ensured the highest growth of *B. lactis* in shake flasks compared either with whey supplemented with L-cysteine or without any supplement. The addition of yeast extract led to a significant increase in the production of bacteriocin-like inhibitory substance (BLIS), thus proving to be an essential medium ingredient for cultures. In a bench-scale fermentor, *B. lactis* proved to be a promising bacteriocin producer, exhibiting a BLIS activity (800 AU/mL) under anaerobic conditions that was 100% higher than in flasks. As whey is much cheaper than traditional semisynthetic media, it may reduce the cost of BLIS production, making its use more competitive. In addition, the strain of *B. lactis* employed in this work showed antimicrobial activity against the pathogenic species *Listeria monocytogenes*, and the BLIS produced appeared to have similarity with Bifidin, even though more studies are needed to confirm this conclusion. It is however necessary to optimise BLIS production by bifidobacteria, as in the case of Bifidin I, which has a broad spectrum of action against both Gram-positive and Gram-negative bacteria.

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