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Comparison of bifidobacterial growth-promoting activity of ultrafiltered casein hydrolyzate fractions

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Summary — Growth rates and acid production of the most common dairy-related bifidobacteria (*Bifidobacterium infantis, B breve* and *B longum*) were determined in the presence of casein hydrolyzates produced by the action of three proteolytic enzymes (alcalase, chymotrypsin and trypsin). Casein hydrolyzates were fractionated with a two-step ultrafiltration process to study the effect of molecular mass of peptides on bifidobacterial growth. The retentate of the second ultrafiltration (nominal molecular cut-off of membranes was 1000 Da) was called mixture of polypeptides (MP) and the permeate fraction which was mainly composed of free amino acids and small peptides was called AA. These hydrolyzate fractions were characterized by a very different concentration of some amino acids (glu, tyr, phe). Among MP and AA casein fractions, trypsin MP fraction at a final concentration of 2% in synthetic medium (Garches medium) exhibited a higher growth-promoting activity on the three bifidobacterial species tested. However, addition of alcalase AA fraction at a final concentration of 1 or 2% repressed the growth and acid production of *B breve* and *B longum*.

bifidobacteria / casein hydrolyzate fraction / amino acid content / stimulation

Résumé — Comparaison de l'activité stimulante sur la croissance des bifidobactéries de fractions ultrafiltrées d'hydrolysats caséiques. *Les taux de croissance et l'activité acidifiante de souches de bifidobactéries communément utilisées en industrie laitière* (Bifidobacterium infantis, B breve *et* B longum) *ont été estimés en présence d'hydrolysats enzymatiques de caséine obtenus en utilisant l'alcalase, la chymotrypsine et la trypsine. Les hydrolysats de caséine ont été séparés en 2 fractions distinctes grâce à un procédé d'ultrafiltration en 2 étapes, afin d'étudier l'influence de la masse moléculaire des peptides sur la croissance des bifidobactéries. La fraction rétentat de la seconde étape d'ultrafiltration (seuil de coupure des membranes de 1 000 Da) correspondait au mélange polypeptidique (MP) et le filtrat fut identifié comme un mélange de peptides de faible masse moléculaire et d'acides aminés libres (AA). Ces fractions d'hydrolysat sont caractérisées par*

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une concentration très différente de certains acides aminés (glu, tyr, phe). Parmi les fractions MP et AA des différents hydrolysats caséiques, l'hydrolysat trypsique MP ajouté à un milieu synthétique (milieu Garches) à une concentration finale de 2% a un effet stimulant supérieur sur la croissance des 3 espèces de bifidobactéries testées. La croissance et la production d'acide de B breve *et* B longum *ont été inhibées par l'ajout de la fraction AA de l'hydrolysat alcalasique à une concentration finale de 1 ou 2%.*

bifidobactérie / hydrolysat de caséine fractionné / acide aminé / stimulation

INTRODUCTION

Bifidobacteria have gained importance in dairy industry as their positive physiological influences are better known (Marteau *et al,* 1990). *Bifidobacterium longum, B breve, B bifidum* and *B infantis* may be used in dairy products including cultured milks, ice cream, yoghurt and cheeses (Modler *et al,* 1990; Reuter, 1990; El-Soda *et al,* 1992). However, bifidobacteria require undefined biological products to grow in complex culture media such as milk (Poch and Bezkorovainy, 1988, 1991; Desjardins *et al,* 1990a,b).

Kehagias *et al* (1977) demonstrated that a soluble peptide fraction from acid hydrolyzate of bovine casein stimulated the growth of *Bifidobacterium bifidum* var *pennsylvanicus.* This strain is a fastidious microorganism used as model in numerous studies on bifidobacteria growth promoters present in human milk (Bezkorovainy and Topouzian, 1981; Petschow and Talbott, 1990, 1991). Recent studies have shown that both protein nitrogen and non-protein nitrogen in human milk and cow milk also promote the growth of other bifidobacterial species from human origin (Poch and Bezkorovainy, 1988, 1991; Petschow and Talbott, 1991). Poch and Bezkorovainy (1988) indicated that the best growth promoters for bifidobacteria were bovine casein digest and yeast extract rather than human milk whey. More recently, Poch and Bezkorovainy (1991) reported that κ-casein digested by trypsin showed the best growth-promoting activity among bovine milk casein components. Proulx *et al* (1992) noted that peptides obtained from casein hydrolyzates might be a more preferable source of nitrogen than free amino acids. Commercial casein hydrolyzate (N-Z case: enzymatic hydrolyzate) allows better growth of bifidobacteria than ultrafiltered casein hydrolyzate due to a higher content in small peptides (< 2000 Da).

The purpose of this study was to determine the effect of casein hydrolyzate fractions on growth rates and activities of the most common dairy-related bifidobacteria cultivated in synthetic Garches medium. Casein hydrolyzates produced by the action of three proteolytic enzymes (alcalase, chymotrypsin and trypsin) have been separated with a two-step ultrafiltration (UF) process to study the influence of molecular mass of peptides and amino acid composition on bifidobacterial growth.

MATERIALS AND METHODS

Production of casein hydrolyzates

Sodium caseinate (ICN Pharmaceuticals Inc, Irvine, CA, USA) was hydrolyzed with chymotrypsin, trypsin (Sigma, St Louis, MO, USA) or alcalase (Novo Industria a/s Enzymes Division, Bagsvaerd, Denmark), and separated by UF according to Turgeon and Gauthier (1990). The hydrolysis of 60 l of sodium caseinate solution was carried out for 45 min using the following conditions: substrate, 3.5% final concentration of

protein; enzyme to substrate ratio, 1/200 (pH 8.0); temperature, 40 ± 1°C. The pH was maintained at 8.0 by addition of 4 N NaOH. The proteolytic products were removed continuously by UF using two hollow fiber membranes with a nominal molecular cut-off of 30 000 Da (HF1-43-PM30, Romicon Inc, Woburn, MA, USA). The permeate was further separated by a second UF using two membranes with a nominal molecular cut-off of 1000 Da (HF1-43-PM1, Romicon Inc, Woburn, MA, USA). The retentate of the second UF was called mixture of polypeptides (MP). The permeate fraction was composed mainly of amino acids and small peptides (AA). Each fraction was lyophilized and stored at room temperature.

Chemical analysis

The degree of hydrolysis (DH) of each fraction was evaluated in triplicate by measuring the amount of α-amino nitrogen with the o-phthaldialdehyde spectrophotometric method (OPA) using L-leucine as standard (Church *et al*, 1983). Total content of peptide bonds used for calculation of DH was 8.2 meqv g^{-1} protein (Adler-Nissen, 1977). Molecular mass distribution profiles of casein hydrolyzates were determined by high performance size-exclusion chromatography (HPSEC, LKB Instrument Inc, Rockville, MD, USA) using a TSK-2000SW column (Vijayalakshmi *et al*, 1986). The amino acid composition of the casein fractions was determined with a high performance amino acid analyser system (6300 Beckman, Palo Alto, CA, USA), following acid hydrolysis (HCl 6 N, 100°C, 24 h) (Turgeon and Gauthier, 1990). Total protein concentration of different casein hydrolyzates was measured using the macro-Kjeldahl method (AOAC, 1984). A nitrogen to protein conversion factor of 6.38 was applied.

Strains and cultivation

Bifidobacterium infantis (ATCC 15697), *Bifidobacterium breve* (ATCC 15700), *Bifidobacterium longum* (ATCC 15707) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Working cultures were propagated by transfer in lactobacilli MRS medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.05% L-cysteine (Sigma, St Louis, MO, USA). Active cultures used as inoculum were freshly prepared by growing the organisms in supplemented MRS medium. After 18 h of growth at 37°C in an anaerobic chamber with 5% CO_2, 10% H_2 and 85% N_2 (anaerobic glove box; Forma Scientific, Marietta, OH, USA), cells were centrifuged at 7649 g for 15 min in a Sorval RC-5B centrifuge (Dupont Col, Newtown, CT, USA). Cell pellets were washed, resuspended in Mc Ilvaine buffer 100 mol/l pH 6.0 and standardized to obtain an inoculum of 5 x 10^7 colony forming units per ml (cfu/ml).

Growth experiments

Garches medium (Raynaud and Bizzini, 1971) without bacto casamino acids was used to test the casein hydrolyzate fractions. It contained per liter the following: 5 g asparagine, 0.2 g cystine, 5 g sodium acetate anhydrous, 10 g lactose, 0.9 g potassium phosphate diabasic, 2.33 g sodium phosphate diabasic, 0.5 g magnesium sulfate, 1.0 mg p-aminobenzoic acid, 10 µg biotin and 1.0 mg calcium pantothenate. The casamino acids usually present in Garches medium were substituted by MP and AA casein fractions from each enzyme in concentrations of 0.5, 1.0 or 2.0% (w/v). Garches medium containing 1% of casamino acids was used as control. Modified Garches medium was distributed in 15-ml aliquots in 30-ml assay tubes, equilibrated under anaerobic atmosphere for 48 h and finally inoculated at 5% with the standardized inoculum. Each fermentation condition was performed in triplicate.

Viable cell counts per ml (cfu/ml) were done in duplicate by spread-plating on supplemented MRS lactobacilli agar plates which were incubated anaerobically at 37°C for 48 h. Cell density was determined by spectrophotometry at 635 nm (Spectrophotometer model 390, Sequoia-Turner Corporation, Mountain View, CA, USA). Uninoculated modified Garches medium was used for blank.

For the determination of titratable acidity, samples of 5 ml were withdrawn, mixed with 5 ml of distilled water and titrated with N/9 NaOH to the endpoint of pH 8.6 using a pH meter (Radiometer model PHM84 and titrator model TTT80, Copenhagen, Denmark). The results are expressed in mmol of NaOH of which the value at time 0 has been subtracted. Growth and acidity were measured after 0, 4, 8, 12 and 24 h of incubation.

Statistical analysis

Analysis of variance was used to determine the effect of casein hydrolyzate fraction combinations (fraction x concentration) and time (combination x time) on unit of optical density (UOD) and titratable acidity of the bifidobacteria tested. To stabilize the variance, a logarithmic transformation was applied on UOD values. A significant interaction between casein hydrolyzate combinations and time was found, indicating the effect of time varies depending upon the combination studied ($P = 0.0001$).

The time effect was partitioned into linear, quadratic and cubic components using orthogonal polynomials. Since the time effect was linear for each combination ($P = 0.0001$), the slope corresponding to specific growth rate (μ) was used for further casein hydrolyzate combination comparisons. When comparison was made at given concentration, the parallelism of slopes was tested using the combination x time interaction. Finally, statistical analyses for testing effects of casein hydrolyzate fractions on acid production (titratable acidity) at 24 h for concentration 0.5% and 2.0% were performed using the general linear models procedure of SAS (SAS Institute Inc, 1989). The comparison between casein hydrolyzate fractions for a specific concentration or between concentrations for a specific fraction was done by using multiple comparisons on least square means and by controlling the confident level (α).

RESULTS AND DISCUSSION

Figures 1, 2 and 3 show the growth of *Bifidobacterium infantis, B breve* and *B longum* in the presence of 1% of casein hydrolyzate fractions produced by the action of alcalase, chymotrypsin and trypsin, respectively. Growth curves of each species of bifidobacteria tested were different. Figures 1A and D show that *B infantis* exhibited similar growth in Garches medium containing either 1% of MP or AA casein fractions. However, growth curves expressed in terms of log cfu/ml of *B breve* and *B longum* sharply decreased in the presence of alcalase AA fractions (fig 1E, F). Specific growth rates estimated from growth curves expressed in UOD of *B breve* and *B longum* with this latter fraction were low as compared to the presence of alcalase MP fractions (fig 1B, C).

Figure 2 shows that *B infantis* exhibited the highest growth performance (fig 2A, D) and *B longum* the lowest (fig 2C, F). No significant difference was observed between specific growth rates obtained on chymotryptic MP and AA fractions for *B infantis* (fig 2A, D), *B breve* (fig 2B, E) and *B longum* (fig 2C, F). No decrease of the population of *B breve* and *B longum* was observed in the presence of chymotryptic AA fraction (fig 2E, F).

Finally, growth curves and specific growth rates of *B breve* and *B longum* were higher in the presence of tryptic MP fraction (fig 3B, C) as compared to those with tryptic AA fraction (fig 3E, F). Comparison of the three types of casein hydrolyzates indicates that tryptic MP fraction allowed better growth performance of *B breve* and *B longum* in Garches medium (fig 3) than the other fractions (figs 1, 2). However, no difference was observed between casein fractions for *B infantis*.

In a second set of experiments, comparison of specific growth rates and acid production of *B infantis, B breve* and *B longum* obtained with casein fractions at a final concentration of 0.5 and 2% was done with the original Garches medium containing 1% of casamino acids instead of hydrolyzates (tables I, II). Differences of growth and acid production between casein fractions were observed according to the concentration of 0.5 and 2% of ultrafiltered hydrolyzates fractions. In addition, growth of bifidobacteria was stimulated in the presence of casein fraction as compared to the addition of amino acids.

Table I shows that, for *B infantis*, no significant difference ($P > 0.05$) was observed between AA or MP casein fraction from alcalase, chymotryptic or tryptic hydrolyzates

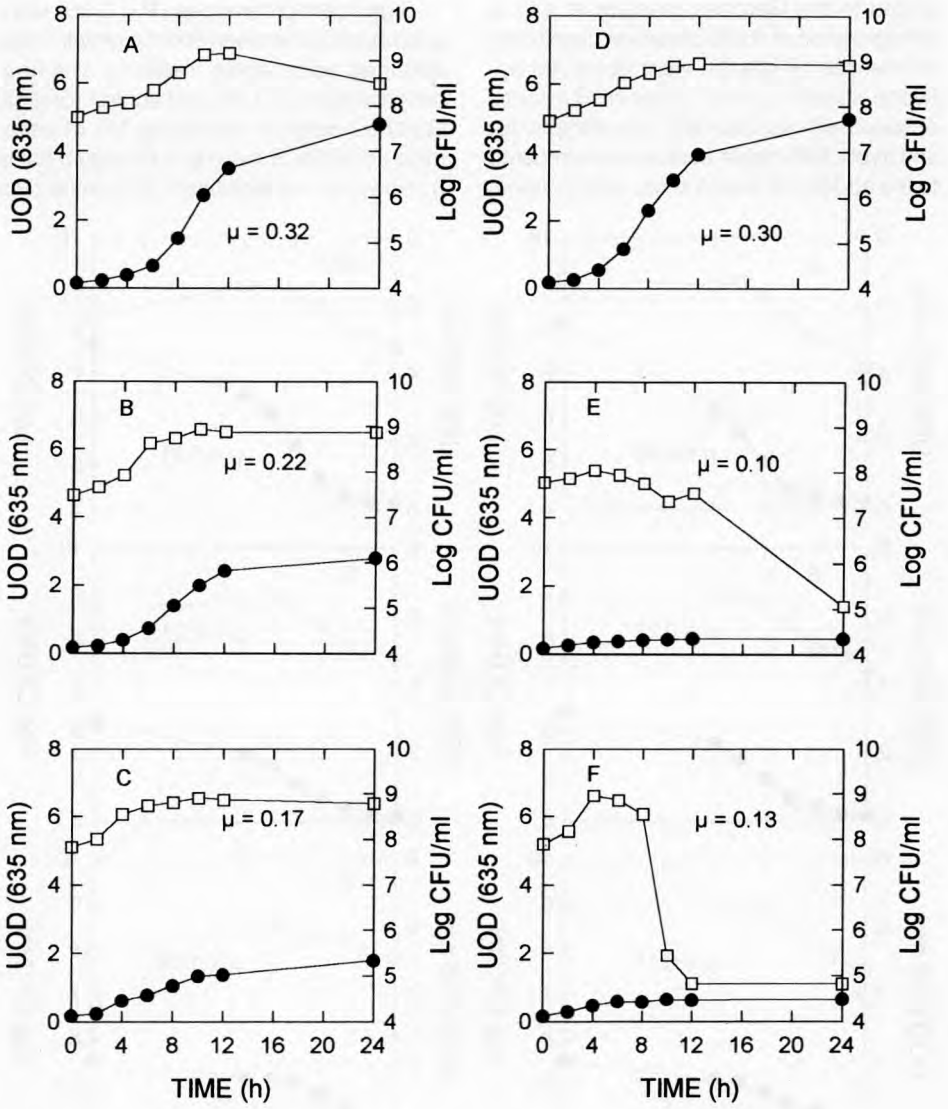

Fig 1. Growth curves in Garches medium with MP (left) and AA (right) fractions from alcalase hydrolyzate at final concentration of 1% for *B infantis* (**A, D**), *B breve* (**B, E**), and *B longum* (**C, F**). Symbols: ● unit of optical density (UOD); ❑ log cfu/ml. Legend: MP, mixture of polypeptides; AA, amino acids and small peptides mixture.

Cinétiques de croissance de B infantis *(A, D),* B breve *(B, E), et* B longum *(C, F) dans le milieu Garches contenant les fractions MP (gauche) et AA (droite) de l'hydrolysat alcalase à une concentration finale de 1%. Symboles :* ● *unité de densité optique (UOD);* ❑ *log cfu/ml. Légende : MP : mélange polypeptidique; AA: mélange de peptides de faible masse moléculaire et d'acides aminés libres.*

added to the Garches medium at a final concentration of 0.5%. However, significant differences ($P < 0.05$) were observed between specific growth rates of *B infantis* obtained with alcalase MP, chymotryptic AA and tryptic MP casein fractions as compared to the addition of amino acids only (control).

Significant differences ($P < 0.05$) were also observed between specific growth rates obtained with casein fractions at a final concentration of 0.5% and control (original Garches medium containing 1% of casamino acids) for *B breve* and *B longum* (table I). However, no significant difference ($P >$

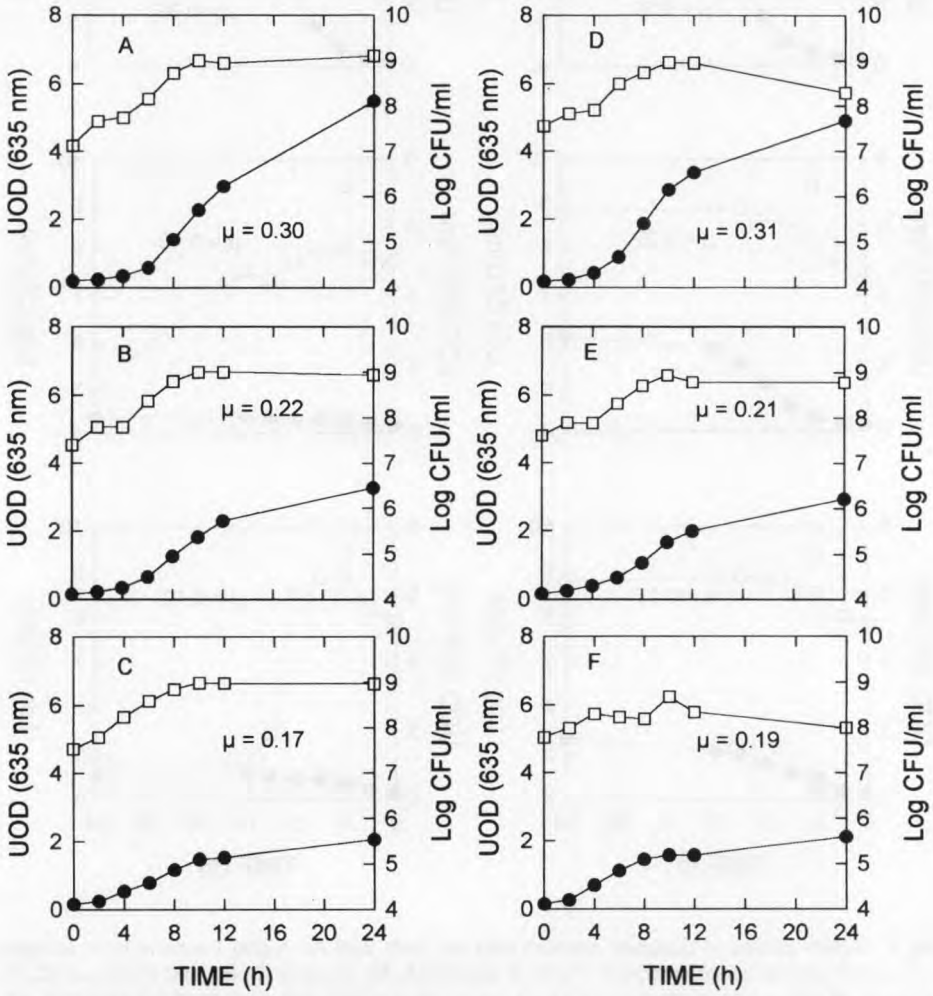

Fig 2. Growth curves in Garches medium with MP (left) and AA (right) fractions from chymotryptic hydrolyzate at final concentration of 1% for *B infantis* (**A, D**), *B breve* (**B, E**) and *B longum* (**C, F**). Symbols: ● unit of optical density (UOD); ❑ log cfu/ml. See fig 1 for legend.

Cinétiques de croissance de B infantis *(A, D),* B breve *(B, E) et* B longum *(C, F) dans le milieu Garches contenant les fractions MP (gauche) et AA (droite) de l'hydrolysat chymotrypsique à une concentration finale de 1%. Symboles :* ● *unité de densité optique (UOD);* ❑ *log cfu/ml. Voir la fig 1 pour la légende.*

0.05) was observed between AA or MP casein fractions of the three types of hydrolyzates tested. For *B infantis*, significant differences ($P < 0.05$) were observed in terms of specific growth rates between casein fraction at a final concentration of 2% and casamino acids, except for alcalase AA fraction (table I). The specific growth rates of *B infantis* with ultrafiltered fractions at a concentration of 2% were not different from rates observed with a concentration of 0.5%, except for tryptic MP fraction. The growth of *B breve* and *B longum* was stimulated ($P < 0.05$) by an increase of chymotryptic and

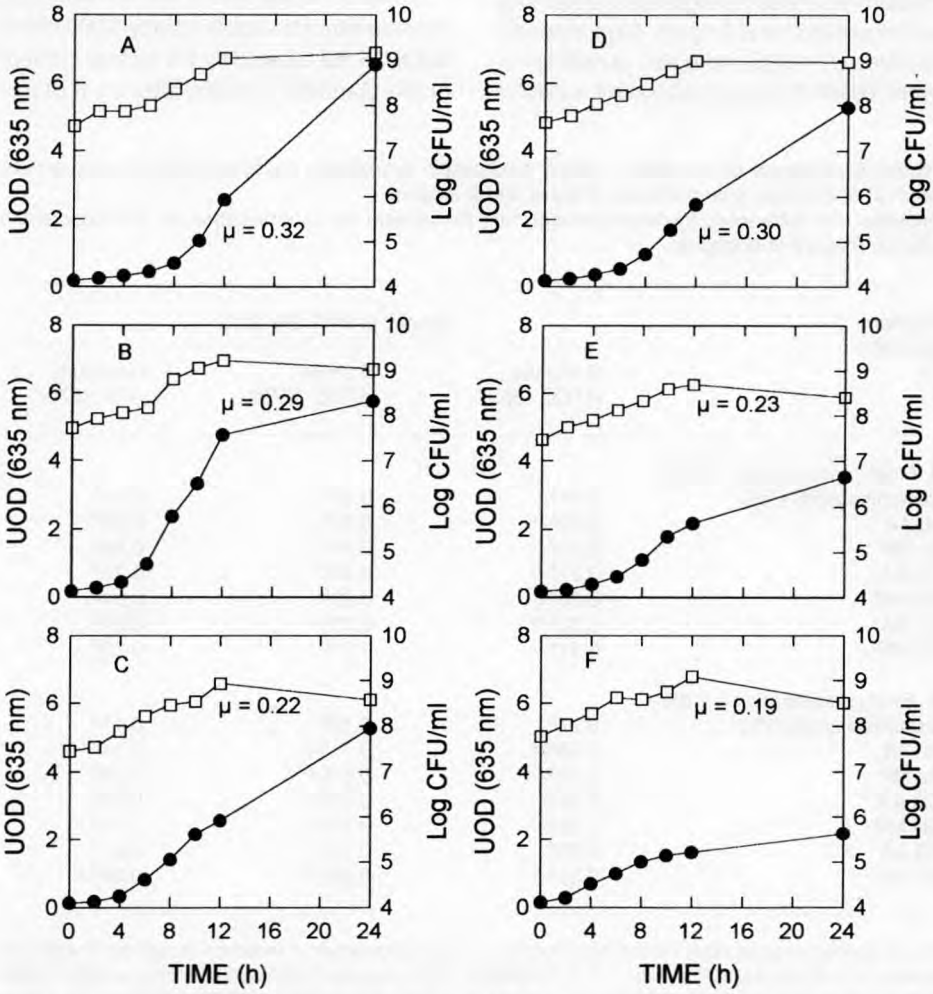

Fig 3. Growth curves in Garches medium with MP (left) and AA (right) fractions from tryptic hydrolyzate at final concentration of 1% for *B infantis* **(A, D)**, *B breve* **(B, E)** and *B longum* **(C, F).** Symbols: ● unit of optical density (UOD); ❑ log cfu/ml. See fig 1 for legend.

Cinétiques de croissance de B infantis *(A, D),* B breve *(B, E), et* B longum *(C, F) dans le milieu Garches contenant les fractions MP (gauche) et AA (droite) de l'hydrolysat trypsique à une concentration finale de 1%. Symboles :* ● *unité de densité optique (UOD);* ❑ *log cfu/ml. Voir la figure 1 pour la légende.*

tryptic MP casein hydrolyzate fractions from 0.5 to 2% whereas no additional stimulation occurred ($P > 0.05$) with the three AA casein fractions (table I). Addition of alcalase MP at a final concentration of 2% stimulated *B breve* as compared to the addition of 0.5% of this fraction to the Garches medium whereas no significant difference was observed for *B longum*. Significant differences ($P < 0.05$) were also observed between casein fractions and control, except in the presence of alcalase AA fraction at a final concentration of 2% for *B breve*. *B longum* exhibited low specific growth rate ($P < 0.05$) in the presence of 2% of alcalase AA fraction as compared to the addition of chymotryptic and tryptic fractions.

Table II shows that *B infantis* exhibited the maximum titratable acidity (0.60 mmol NaOH/ 5 ml) allowed by the lactose content of the Garches medium after 24 h of fer-

Table I. Comparison of the effect of ultrafiltered casein hydrolyzate fractions on the specific growth rates (μ) of *Bifidobacterium infantis, B breve* and *B longum*.
Influence des différentes fractions d'hydrolysats de caséine sur la croissance de *Bifidobacterium infantis, B breve* et *B longum*.

Fraction content	*Specific growth rate (h^{-1})*		
	B infantis *ATCC 15697*	B breve *ATCC 15700*	B longum *ATC 15707*
A. Final concentration = 0.5%			
Casamino acids (1%)	0.27^{A}	0.13^{A}	0.11^{A}
AL AA	$0.30^{A,B}$	0.27^{B}	0.25^{B}
AL MP	$0.32^{B,C}$	0.24^{B}	0.25^{B}
CH AA	$0.31^{B,C}$	0.24^{B}	0.25^{B}
CH MP	$0.30^{A,B}$	0.20^{B}	0.24^{B}
TR AA	$0.30^{A,B}$	0.25^{B}	0.27^{B}
TR MP	$0.31^{B,C}$	0.21^{B}	0.28^{B}
B. Final concentration = 2.0% [a]			
Casamino acids (1%)	0.27^{A}	0.13^{A}	0.11^{A}
AL AA	$0.28^{A,B}$	$0.11^{A,b}$	$0.14^{B,b}$
AL MP	$0.32^{B,C}$	$0.31^{C,b}$	0.28^{C}
CH AA	0.34^{C}	$0.24^{B,C}$	0.28^{C}
CH MP	$0.32^{B,C}$	$0.31^{C,b}$	$0.31^{C,b}$
TR AA	0.30^{B}	0.23^{B}	0.31^{C}
TR MP	$0.37^{D,b}$	$0.35^{D,b}$	$0.38^{D,b}$

[a] Final concentration of casein hydrolyzate fractions in Garches medium. [b] Presence of significant difference between concentrations at $P < 0.05$. [A, B, C, D] Different letters represent significant difference between casein hydrolyzate fractions and control at $P < 0.05$. Legend: AL, casein hydrolyzate fraction from alcalase; CH, casein hydrolyzate fraction from chymotrypsin; TR, casein hydrolyzate fraction from trypsin; AA, amino acids and small peptides mixture; MP, mixture of polypeptides.

[a] *Concentration finale des fractions d'hydrolysat de caséine dans le milieu Garches.* [b] *Présence d'une différence significative entre les concentrations à* $P < 0,05$. [A, B, C, D] *Des lettres différentes représentent une différence significative entre les fractions d'hydrolysat de caséine et la culture témoin à* $P < 0,05$. *Légende : AL, fractions de l'hydrolysat alcalase de la caséine; CH, fractions de l'hydrolysat chymotrypsique de la caséine; TR, fractions de l'hydrolysat trypsique de la caséine; AA, mélange de peptides de faible masse moléculaire et d'acides aminés libres; MP, mélange polypeptidique.*

mentation with 1% of casamino acids (control) and 0.5% of ultrafiltered fractions, except with 0.5% of alcalase AA fraction ($P < 0.05$). The presence of AA and MP casein fractions stimulated the acid production of *B breve* and *B longum* as compared to the addition of 1% of casamino acids ($P < 0.05$) although the increase of acid production was lower with 0.5% of alcalase AA fraction ($P < 0.05$). In the presence of AA and MP casein fractions at a final concentration of 2%, acid production of *B infantis* was lower ($P < 0.05$) with alcalase and tryptic AA fractions as compared to the addition of

Table II. Comparison of the effect of ultrafiltered casein hydrolyzate fractions on acid production by Bifidobacterium infantis, *B breve* and *B longum*.
Influence des différentes fractions d'hydrolysats de caséine sur la production d'acide par Bifidobacterium infantis, B breve et B longum.

Fraction content	*Titratable acidity (mmol NaOH/5ml)* [a]		
	B infantis *ATCC 15697*	B breve *ATCC 15700*	B longum *ATCC 15707*
A. Final concentration = 0.5%[b]			
Casamino acids (1%)	0.61^{A}	0.22^{A}	0.17^{A}
AL AA	0.46^{B}	0.44^{B}	0.46^{B}
AL MP	0.63^{A}	0.56^{C}	0.61^{C}
CH AA	0.59^{A}	0.53^{C}	0.59^{C}
CH MP	0.60^{A}	0.54^{C}	0.61^{C}
TR AA	0.60^{A}	0.58^{C}	0.61^{C}
TR MP	0.61^{A}	0.53^{C}	0.60^{C}
B. Final concentration = 2.0%[b]			
Casamino acids (1%)	0.61^{A}	0.22^{A}	0.17^{A}
AL AA	0.47^{C}	$0.14^{B,c}$	$0.12^{B,c}$
AL MP	0.60^{A}	0.60^{D}	0.58^{D}
CH AA	0.57^{B}	0.56^{D}	0.57^{B}
CH MP	$0.58^{A,B}$	0.61^{D}	0.59^{D}
TR AA	$0.46^{C,c}$	$0.45^{C,c}$	$0.47^{C,c}$
TR MP	$0.58^{A,B}$	0.55^{D}	0.59^{D}

[a] Titratable acidity determined after 24 h of fermentation.
[b] Final concentration of casein,hydrolyzate fractions in Garches medium.
[c] Presence of significant difference between concentrations at $P < 0.05$.
[A] Different letters represent significant difference between casein hydrolyzate fractions at $P < 0.05$
Legend: AL, casein hydrolyzate fraction from alcalase; CH, casein hydrolyzate fraction from chymotrypsin; TR, casein hydrolyzate fraction from trypsin; AA, amino acids and small peptides mixture; MP, mixture of polypeptides.
[a] *Acidité titrable déterminée après 24 h de fermentation.*
[b] *Concentration finale des fractions d'hydrolysat de caséine dans le milieu Garches.*
[c] *Présence d'une différence significative entre les concentrations à* $P < 0,05$.
[A] *Des lettres différentes représentent une différence significative entre les fractions d'hydrolysat de caséine et la culture témoin à* $P < 0,05$.
Légende : AL, fractions de l'hydrolysat alcalase de la caséine; CH, fractions de l'hydrolysat chymotrypsique de la caséine; TR, fractions de l'hydrolysat trypsique de la caséine; AA, mélange de peptides de faible masse moléculaire et d'acides aminés libres; MP, mélange polypeptidique.

0.5% of casein fractions and 1% of casamino acids (table II). For *B breve* and *B longum,* acid production drastically decreased with a concentration of 2% of alcalase AA fraction as compared to the other casein fractions ($P < 0.05$). These results are in agreement with the decrease of population of *B breve* and *B longum* observed in the presence of 1% of alcalase AA fraction (fig 1E, F). Decrease of acid production by the three species tested was also observed with tryptic AA fraction.

The results indicate that the presence of alcalase, chymotryptic and tryptic casein fractions in Garches medium stimulated growth and acid production of *B breve* and *B longum* as compared to the addition of 1% of amino acids, except with alcalase AA casein fraction at a final concentration of 2%. Addition of AA and MP casein fractions (except alcalase and tryptic AA fractions) resulted in acid production activities (0.50–0.60 mmol NaOH/5 ml) similar to those observed with the first-step ultrafiltered casein hydrolyzates of alcalase, chymotrypsin and trypsin after 48 h of fermentation (Proulx *et al,* 1992). These results indicate that the second UF step did not eliminate essential elements for growth of bifidobacteria. In the presence of the three types of casein fractions, growth rates of *B infantis* were comparable to the addition of amino acids whereas acid production was lower in the presence of alcalase AA fraction at final concentrations of 0.5 and 2%. Specific growth rates and acid production of *B breve* and *B longum* were also repressed in the presence of alcalase AA fraction at concentrations higher than 1%. In some cases, growth promoters such as bovine serum albumin digest may be an inhibitor for growth of bifidobacteria (Poch and Bezkorovainy, 1988). Proulx *et al* (1992) previously noted that growth and acid production of bifidobacteria were higher in the presence of peptides than of free amino acids.

The length of peptides is a well-known factor important for the growth and acid production of lactic acid bacteria (St-Gelais *et al,* 1993). Table III shows that chymotryptic MP fraction contained high amounts of large peptides over 2000 Da (30%) than tryptic (11%) and alcalase (1.5%) MP fraction. Alcalase AA and MP fractions were both essentially composed of protein components smaller than 2000 Da (>98.5%). The tryptic MP fraction exhibited the highest growth-promoting activity at a concentration of 2% among the casein fractions for the three bifidobacteria tested. This fraction was characterized by low levels (< 2%) of peptides higher than 5000 Da as compared to the chymotryptic MP fraction (table III). These results suggest that peptides higher than 5000 Da did not have the most stimulating effect on growth of bifidobacteria.

It was found that κ-casein digested by trypsin enhances the growth of bifidobacteria (Poch and Bezkorovainy, 1991). These authors suggested that disulfide/sulphydryl residues of κ-casein in combination with another tryptic peptide are required for growth of some species of bifidobacteria. Recently, Bouhallab *et al* (1993) noted that tryptic digest of caseinomacropeptide separated into two fractions by UF stimulated the growth and acid production of *Lactococcus lactis* subsp *lactis* in skim milk.

The results also indicate that growth of bifidobacteria was stimulated by an increase of concentration from 0.5 to 2% of MP casein fraction in Garches medium. The types of peptides found in MP fraction from tryptic hydrolyzates stimulated the growth of bifidobacteria in a dose-response dependent manner (Bouhallab *et al,* 1993). However, the length of peptides and amino acid content of casein fractions could lead to growth inhibition. Moreover, the addition of 2% of tryptic AA fraction did not stimulate growth of bifidobacteria tested and inhibited acid production of *B infantis*. This result suggests that the repressive effect was rela-

Table III. Molecular mass distribution of protein components of mixture of polypeptides (MP) and amino acids and small peptides fractions (AA) obtained by alcalase, chymotrypsin or trypsin hydrolysis of sodium caseinate.
Distribution de la masse moléculaire des composés protéiques du mélange de polypeptides (MP) et des fractions de peptides de faible masse moléculaire et d'acides aminés libres (AA) obtenus à partir de l'hydrolyse alcalase, chymotrypsique ou trypsique de caséinate de sodium.

Hydrolyzate	*Fraction*	*Molecular mass distribution[a] (%)*		
		> 5 000 Da	*5 000- 2 000 Da*	*< 2 000 Da*
Alcalase	MP	0.00	1.54	98.46
	AA	0.00	0.98	99.02
Chymotrypsin	MP	18.49	10.77	70.74
	AA	4.65	6.43	88.92
Trypsin	MP	1.40	9.38	89.22
	AA	1.77	5.48	92.75

[a] Molecular mass distribution is calculated from the integration of the total surface of the chromatogram. The total surface is separated in three ranges of molecular mass and expressed in percentage of the total surface.
[a] *La distribution de la masse moléculaire est estimée à partir de l'intégration de la surface totale du chromatogramme. La surface totale est divisée en 3 zones de masse moléculaire et exprimée en pourcentage de la surface totale.*

ted to the degree of proteolysis which affectsthe balance in low molecular mass peptides and amino acid content in AA fractions.

Table III shows that the alcalase AA fraction had a higher content in small peptides (< 2000 Da) than the other fractions tested. Proulx *et al* (1992) found that commercial casein hydrolyzate which was the best for stimulating growth of bifidobacteria also contained a high content (99.6%) in small peptides (< 2000 Da). Moreover, Cheng and Nagasawa (1984) found that peptides ranging from 1100 to 2300 Da stimulated acid production of *B breve* and *B infantis*. Although the alcalase AA fraction had a peptide content lower than 2000 Da comparable to that of commercial casein hydrolyzate and alcalase MP fraction, specific growth rates and acid production of *B breve* and *B longum* were lower than results observed in the presence of alcalase MP fraction.

The enzyme specificity and nature of UF membranes might be involved in the differences observed between MP and AA fractions in terms of molecular mass distribution profiles and amino acid content. The net charge and hydrophobicity seemed to be the predominant factors determining the passage of amino acids or peptides through polysulfone membrane (Bard *et al,* 1992). So, we observed that amino acid content has not been equally dealt out by UF separation.

The amino acid content of peptide fractions was modified after UF separation as shown in table IV. The amount of glutamic acid in both alcalase AA and MP casein hydrolyzates was lower than chymotryptic and tryptic AA and MP casein hydrolyzates (AA fraction: 6.17, 17.58, 17.30 g/16 g N

Table IV. Amino acids content (expressed in g/16 g N) of casein hydrolyzate fractions obtained by alcalase, chymotrypsin and trypsin.
Contenu en acides aminés (exprimé en g/16 g N) des fractions de l'hydrolysat de caséine obtenu à l'aide de l'alcalase, de la chymotrypsine et de la trypsine.

Amino acids	*Casamino acids*	*Alcalase*		*Chymotrypsin*		*Trypsin*	
		MP	*AA*	*MP*	*AA*	*MP*	*AA*
ASP	8.42	7.40	8.74	7.43	5.83	7.54	5.14
THR	5.10	5.43	5.68	3.99	3.02	4.71	3.04
SER	5.99	5.43	6.04	5.59	3.81	5.44	4.34
CYS	ND	ND	ND	ND	ND	ND	ND
GLU	6.36	5.87	6.17	20.82	17.58	19.63	17.30
PRO	15.41	13.65	15.82	10.33	10.47	10.89	10.90
GLY	2.67	2.80	2.91	1.80	1.87	1.76	2.10
ALA	6.35	4.26	5.24	2.77	3.49	2.79	3.46
VAL	7.94	9.51	9.31	6.51	6.31	6.24	6.37
MET	2.03	1.20	2.75	2.76	3.49	2.79	3.46
ILE	5.97	5.13	5.98	4.22	4.28	4.40	3.07
LEU	9.97	6.23	8.64	9.72	9.11	9.34	12.32
TYR	2.02	8.12	1.03	5.06	6.32	4.32	9.47
PHE	5.72	7.96	4.45	5.06	6.32	5.17	7.03
TRP	ND	ND	ND	ND	ND	ND	ND
HIS	3.34	4.11	4.08	2.52	3.27	2.88	2.57
LYS	7.37	6.92	7.80	6.47	9.46	7.08	6.11
ARG	5.32	3.67	3.95	3.61	4.33	3.33	2.59

ND: not determined. Tryptophan was destroyed during hydrolysis.
ND: non déterminé. Le tryptophane a été détruit au cours de l'hydrolyse.

and MP fraction: 5.87, 20.82 and 19.63 g/16 g N). The content in aromatic acids tyrosine and phenylalanine was lower in the alcalase AA fraction than MP fraction (AA fraction: 1.03 and 4.45 g/16 g N *versus* MP fraction: 8.12 and 7.96 g/16 g N, respectively). The content in leucine and tyrosine was higher in AA fraction from tryptic casein hydrolyzate than MP fraction (AA fraction: 12.32 and 9.47 g/16 g N *versus* MP fraction: 9.34 and 4.32 g/16 g N, respectively). Moreover, the highest content in leucine was found in tryptic AA fraction. This fraction also contained the lowest concentrations in isoleucine and arginine (3.07 and 2.59 g/16 g N, respectively).

The alcalase AA had an amino acid content very similar to that of casamino acids (table IV). The differences observed between alcalase MP and AA fractions on activity of *B breve* and *B longum* might be explained by the high percentage of free amino acids *versus* small peptides (table III). This level was also very similar to that observed for casamino acids (100%; data not shown). In addition, the increase of the concentration of alcalase AA fraction from 0.5 to 2.0% resulted in growth and acid production inhibition for some species. Tyrosine and phenylalanine concentrations were much lower in alcalase AA fraction than in MP fraction and could explain the growth difference with *B breve* and *B longum*.

In conclusion, tryptic digest of casein can be used to supplement milk media for growth of bifidobacteria, especially *B breve* and *B longum*. Growth-promoting activity of the tryptic MP fraction can be explained by the presence of an optimal content in small peptides (< 2000 Da). Medium supplementation with alcalase AA fraction and casamino acids were not adequate for bifidobacterial species with higher nitrogen requirements than *B infantis*. Further studies on growth-promoting activity of peptides from casein hydrolyzate fraction should include determination of the balance supply of essential amino acids and concentration of total amino acid content which might have a repressive effect.

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