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Growth of *Lactococcus lactis* subsp *lactis* in reconstituted irradiated milk powder

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Summary — Low heat milk powder was submitted to γ-ray irradiation in order to sterilize it and then tested for growth of *L lactis* subsp *lactis* strains CNRZ 1076 (prt$^+$) and its non-proteolytic-variant, CNRZ 1075 (prt$^-$). Inhibition was observed when reconstituted milk was inoculated at a level of 10^4 but not 10^6 cfu/ml. This inhibition was partly relieved by addition of superoxide dismutase (SOD) suggesting a correlation with the production of O_2^-. The maximum stationary phase populations of CNRZ 1076 (prt$^+$) in reconstituted milk from irradiated powder and in reference milk were identical while the maximum stationary phase population of CNRZ 1075 (prt$^-$) was significantly higher in reconstituted milk made from irradiated powder. Amino acids analysis of the non-protein fraction revealed that irradiation led to a release of peptides, which likely enhanced final yields of prt$^-$. Consequently, irradiated milk acidification by *L lactis* CNRZ 1076 (prt$^+$) was delayed, while that of CNRZ 1075 (prt$^-$) was greatly stimulated.

irradiation / dried milk / *Lactococcus lactis* / growth / superoxide anion / superoxide dismutase / caseinomacropeptide

Résumé — Croissance de *Lactococcus lactis* subsp *lactis* dans du lait reconstitué à partir de poudre irradiée. *Une poudre de lait de type* low heat *a été irradiée par des rayons y. Les croissances de 2 souches de* Lactococcus lactis *subsp* lactis *— CNRZ 1076 et son variant non protéolytique - CNRZ 1075 (prt$^-$) — ont été suivies sur le lait reconstitué à partir de cette poudre. Le taux de croissance est fortement diminué lorsque l'ensemencement est réalisé à 10^4 ufc/ml. Cette inhibition est attribuée à la présence d'anion superoxyde puisqu'elle disparaît partiellement après ajout de superoxyde dismutase (SOD). Le taux de croissance devient identique à celui observé dans le lait témoin après 6 h de culture. Le niveau de population atteint en fin de croissance est le même dans les 2 laits en ce qui concerne la souche sauvage. Il est fortement augmenté en revanche dans le cas de la souche prt$^-$ (protéolyptique $^-$), ce qui est explicable par l'accroissement significatif de la teneur en peptides solubles dans le lait ionisé. En conséquence, l'acidification du lait ionisé se trouve retardée lorsqu'il s'agit de la souche prt$^+$ (protéolytique $^+$), et stimulée dans le cas de la souche prt$^-$.*

irradiation / poudre (de lait) /* Lactococcus lactis */ croissance / anion superoxyde / superoxyde dismutase / caséinomacropeptide

INTRODUCTION

Reconstituted milk powder is generally used for studying either coagulation enzyme activity (Berridge *et al*, 1943) or growth of lactic acid starter bacteria involved in cheesemaking processes (FIL, 1980). It is preferred to fresh milk which is a highly variable substrate (Vassal and Auclair, 1966).

Low heat milk powders, *ie* resulting from milk heated to ≤ 62°C for 30 min or 80°C for 15 s are usually chosen because higher heat treatments can induce formation of stimulating or inhibiting compounds (FIL, 1980) and have a detrimental effect on firmness and texture of acid and rennet gels (Dalgleish, 1990; Ferron-Baumy *et al*, 1991). However, if such low heat powders are considered as satisfactory for routine tests in cheesemaking plants – determination of activity of commercial rennet solutions or acidification rate of mesophilic starters – they are not satisfactory for the determination of acidifying activity of thermophilic lactic acid bacteria which are added to reconstituted milk at a low rate (0.05–0.2%) (Chamba and Prost, 1989). Indeed, microbiological flora of these non-sterilized milk powders is mainly composed of thermoduric species: *M lacticum, B cereus, S thermophilius*, etc (Stadhouders *et al*, 1982) which can interfere with the development of the lactic acid studied strain and thus, completely modify the results of acidifying activity tests (Chamba and Prost, 1989).

Irradiation at 20 kGy (kiloGray) of low heat milk powder was recently proposed (Chamba and Prost, 1989) in order to destroy all contaminating bacteria, viruses and bacteriophages. It was claimed that such an ionizing treatment did not induce a modification of the peptidic composition of milk and the observed growth of thermophilic lactic acid starters was similar to that determined on milk reconstituted from reference low heat powder.

However, irradiation produces water-derived compounds such as OH^-, H_2O_2 and H_3O^+(Bonet-Maury and Lefort, 1948; Dainton, 1948) which, if the concentrations are high, are known to inhibit growth of lactic acid bacteria (Condon, 1987; Piard and Desmazeaud, 1991). The water content of skimmilk powder is low (4% on average) but the characteristic 'wet dog' off-flavor (Hsu *et al*, 1972) observed in the 20 kGy irradiated powder led us to think that a small amount of water radiolysis products was likely to be present and consequently could have some detrimental effect on the development of dairy microorganisms.

The purpose of this study was to measure the effect of irradiations of different intensities of a high bacteriological quality, low heat milk powder on the growth of *Lactococcus lactis* subsp *lactis* in reconstituted milks.

MATERIALS AND METHODS

Bacteria and stock cultures

Lactococcus lactis CNRZ 1076 (prt+) and CNRZ 1075 (prt−) were obtained from INRA (CNRZ collection, Jouy-en-Josas, France). Stock cultures were maintained at −20°C in M17 broth (Biokar, Beauvais, France) with 15% w/w glycerol added. For each experiment, 0.2 ml of each culture was inoculated into 10 ml M17 broth and then incubated at 30°C overnight; the final pH was always above 6.0. The cultures were then centrifuged at room temperature (4000 *g*, 10 min) and cell pellets were washed with saline water (8.5 g/l NaCl). Optical density at 650 nm was adjusted to *ca* 0.30 with a spectrophotometer (DU 7400 Beckman Instruments, Gagny, France). This suspension was then used to inoculate milk at a level of either 10^6 or 10^4 cfu/ml milk.

Milk preparation

Skim milk powder produced from 'Bactocatch' treated raw skimmilk was obtained as described by Schuck *et al* (1994). This powder was irradiated at three levels, 5, 10 and 20 kGy with a cobalt source (Conservatome, Dagneux, France). Lait G, the commercial 20 kGy irradiated milk powder was from the Institut Technique du Gruyère (ITG, La Roche-sur-Foron, France). Reference and irradiated milk culture media were prepared by reconstituting 10 g powder in 90 g autoclaved distilled water at room temperature; after 5 min mixing, reconstituted milks were rapidly cooled down to 0°C. RISMP will be the abbreviation for reconstituted skim milk from irradiated powder. Time interval between irradiation and reconstitution was less than 6 months.

Non-clotting milk

Non-clotting milk was prepared by incubating reconstituted skim milk with 5% (w/w) cation exchange resin Chelex 100, sodium form, biotechnology grade (Bio Rad, Ivry-sur-Seine, France), for 20 min at room temperature.

Mesophilic aerophilic microflora determination

Reconstituted milk was analysed for total microflora on plate count agar (PCA, Biokar, Beauvais, France) medium and colony forming units were counted 48 h after incubation at 30°C.

Milk acidification monitoring, growth rate determination and statistics

pH development in milk culture medium was followed with a multi pH-meter (Solomat, Evry, France). Acidification kinetics were characterized by three feature points as described by Picque *et al* (1992). V_m was defined as the maximum value of |dpH/dt|, pH_m and T_m were the pH and time values corresponding to the maximum acidification rate. *L lactis* enumeration was carried out by plating sample dilutions on M17 agar with a spiral plater (Interscience, St-Nom-la-Bretèche, France). Plates were incubated 24 h at 30°C before reading. When chymosin effect on *L lactis* CNRZ 1076 growth was tested, both control milk and coagulum samples were identically homogenized with Ultra-Turrax at 20000 rpm for 30 s at room temperature.

For each experiment, growth rate was estimated according to Monod's equation (1958):

$$\mu = \frac{p}{\log_{10}{(2)}} \ (h^{-1})$$

where p is the growth slope (log (pop)•h^{-1}).

Growth rate data were statistically analysed by calculating standard deviation(s) and standard error and by the Student's *t*-test (Snedecor and Cochran, 1957).

Enzyme and peptide preparation

Crystalline chymosin and peptide [193–209] from β-casein were gifts of D Mollé (INRA, Technologie Laitière, 65, rue de St-Brieuc, 35042 Rennes Cedex, France). They were respectively purified according to Garnot and Mollé (1982) and to Coste *et al* (1992). 0.5 mg crystalline chymosin was dissolved in 1 ml sterile water (pH 6) and stocked at 4°C. Chymosin was denatured in two ways: i) heating at 100°C for 10 min; and ii) incubating at 43°C and pH 9 (adjusted with 0,1 N NaOH) for 24 h.

Caseinomacropeptide was prepared according to Brulé *et al* (1980).

Chemicals

Mannitol was from Difco Laboratories (Detroit, USA). Superoxide dismutase from bovine erythrocytes was from Sigma (St-Quentin-Fallavier, France) and catalase was from Merck (Nogent-sur-Marne, France). Amino acids and small peptides were obtained from Sigma (St-Quentin-Fallavier, France) and Bachem Fein-Chemikalien (Budendorf, Switzerland).

Milk and coagulum supernatants

500 ml reference skim milk was divided in two equal parts: to one, 60 μl/100 ml chymosin (500 mg/l concentrated solution) was added, while the other was untreated. Both batches were incu-

bated at 30°C for 1 h. Milk and coagulum were acidified to pH 4.6 with 1 N HCl, and the resulting precipitates were centrifuged at 3000 *g* for 5 min. Then, supernatants were harvested and neutralized to pH7 with 1 N NaOH.

Milk amino acid analysis

Free amino acids in skim milks were determined with an amino acid analyser Alpha plus serie 2 (Pharmacia LKB, Cambridge, UK) using the cation exchange column Ultrapac 8 resin, lithium form, (Pharmacia LKB Biochrom, Cambridge, UK). One ml sample was incubated for 1 h at 4°C with 50 mg sulfosalicylic acid, and centrifuged (3000 *g*, 15 min). Supernatants were then filtered through an 0.2 µm membrane (Gelman Sciences, Ann Arbor, USA) and diluted five times in citrate buffer (pH 2.2) before loading on the column.

Non-protein nitrogen (NPN) fraction amino acid analysis

5 ml skim milk sample was mixed with 20 ml trichloroacetic acid (TCA) 15%, held for 20 min, and filtered on Whatman paper n° 40. To remove excess TCA, 5 ml filtrate was shaken five times with 15 ml diethylic ether. About 2–3 g of filtrate were evaporated. 1 ml 6 N HCl was added before freezing in liquid nitrogen and sealing the glass tube under vacuum. Hydrolysis was performed at 110°C for 24 h. Samples were then evaporated and diluted in ultra-pure water. Evaporation was repeated twice before adding 5 ml citrate buffer (pH 2.2). After filtration through an 0.2 µm membrane, an aliquot of 50 µl was loaded on the amino acid analyser using a cation exchange column Ultrapac 7 resin, sodium form (Pharmacia LKB Biochrom, Cambridge, UK).

For each amino acid analysis, the mean of two experiments was calculated.

RESULTS

Effect of milk powder irradiation on L lactis *CNRZ 1076 growth and acidification in reconstituted skim milk (RISMP)*

Initial contamination of reconstituted skim milk was *ca* 50 cfu/ml while that from 20 kGy irradiated powder had fewer than 1 cfu/ml.

Growth of *L lactis* CNRZ 1076 in milk reconstituted from 20 kGy irradiated pow-

Table I. Effect of 20 kGy skim milk powder irradiation and level of inoculation on growth rate of *L lactis* CNRZ 1076 and acidification kinetics in reconstituted milk.
Effet de l'irradiation de la poudre de lait et du niveau d'ensemencement sur les cinétiques de croissance et d'acidification de L lactis *CNRZ 1076 dans le lait reconstitué.*

Level of inoculation		*Reference milk*	*Reconstituted skim milk from 20 kGy irradiated powder*
	μ^1 (h^{-1})	1.80	μ_1 (level of population < 10^6 cfu/ml): 1.09 μ'_1 (level of population > 10^6 cfu/ml): 1.74
10^4 cfu/ml	V_m (u pH h^{-1})	0.49	0.40
	pH_m (u pH)	5.42	5.39
	T_m	13 h	15 h 10'
	μ_1 (h^{-1})	1.79	1.78
	V_m (u pH h^{-1})	0.48	0.44
10^6 cfu/ml	pH_m (u pH)	5.48	5.40
	T_m	7 h 45'	8 h 15'

der was measured in cfu/ml and compared to that in reference milk. Two levels of inoculation were tested, 10^4 and 10^6 cfu/ml. Growth rates were calculated from at least ten observations and each experiment was repeated four times. The standard error value was *ca* 0.10.

The results are presented in table I. Statistical analysis of the growth rates by a Student's *t*-test indicated that for the 10^6 inoculation level, the growth rates were similar whether the milk powder was irradiated or not. However, when 10^4 cfu/ml was used as an inoculum, the growth rate was significantly lower in RISMP than in reference milk. Nevertheless, as soon as the population reached *ca* 10^6 cfu/ml in RISMP, growth rate suddenly rose and above this level no more differences were noted between the two media (fig 1). The growth inhibition of *L lactis* CNRZ 1076 in RISMP accounted for the delay of about 2 h which characterized the acidification patterns in milk inoculated with 10^4 cfu/ml (fig 2). Concerning maximal acidification rates, effect of skim milk powder irradiation was as negative for the 10^6 cfu/ml inoculated milk as for that inoculated at 10^4 cfu/ml (table I).

Irradiation of 5 and 10 kGy were tested with the 10^4 cfu/ml inoculation. Acidification delays compared to reference milk were respectively 20 and 55 min.

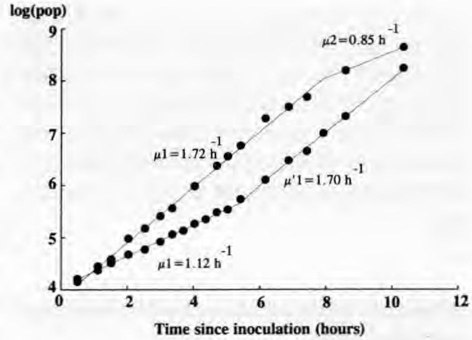

Fig 1. *L lactis* CNRZ 1076 growth in reference milk and reconstituted skim milk from 20 kGy irradiated powder (RISMP). ● RISMP; ○ reference milk.
Croissance de L lactis *CNRZ 1076 dans le lait de référence et dans le lait reconstitué à partir de poudre irradiée à 20 kGy. ● Lait reconstitué à partir de poudre irradiée; ○ lait de référence.*

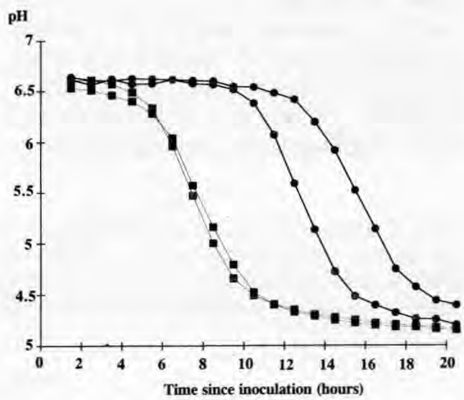

Fig 2. Milk acidification by *L lactis* CNRZ 1076. ■, reconstituted skim milk from 20 kGy irradiated powder inoculated with 10^6 cfu/ml; □, reference milk inoculated with 10^6 cfu/ml; ●, reconstituted skim milk from 20 kGy irradiated powder inoculated with 10^4 cfu/ml; ○, reference milk inoculated with 10^4 cfu/ml.
Acidification de lait reconstitué par L lactis *CNRZ 1076. ■ Lait reconstitué à partir de poudre de lait irradiée à 20 kGy inoculé à 10^6 ufc/ml; □ lait de référence inoculé à 10^6 ufc/ml; ● lait reconstitué à partir de poudre de lait irradiée à 20 kGy inoculé à 10^4 ufc/ml; ○ lait de référence inoculé à 10^4 ufc/ml.*

Effect of milk incubation prior to inoculation

20 kGy RISMP was incubated at 30°C, for 1, 2 and 2.5 h before inoculation at 10^4 cfu/ml. The incubation was static. According to the results (table II), RISMP became less and less inhibiting, as incubation prior to inoculation increased up to 2 h. However, detoxification was not observed beyond this time. There was no development of residual

mesophilic aerophilic microflora, which thus cannot account for this phenomenon. On the other hand, incubation prior to inoculation did not influence growth of *L lactis* CNRZ 1076 in reference milk. The numbers of total mesophilic aerophilic microflora varied from 48 to 69 cfu/ml after 2.5 h incubation.

Effect of catalase, superoxide dismutase and mannitol

The possible role of H_2O_2 and O_2^- in the inhibition phenomenon seen in milk from irradiated powder was tested by adding respectively 100 units of catalase, 20 and 120 units of superoxide dismutase per ml to reference milk and RISMP. Since H_2O_2 and O_2^- can react together to produce [OH•], the possible toxicity of this radical compound was also considered, by adding mannitol (0.05 and 0.1 mol/l), a scavenger of [OH•] (Fridovich, 1983). Acidification was followed in milk inoculated at 10^4 cfu/ml. The results are presented in table II.

Since catalase stimulated acidification of reference milk as well as of RISMP, it can be deduced that H_2O_2 alone is not the cause of inhibition of *L lactis* CNRZ 1076 by milk from irradiated powder. Superoxide dismutase greatly stimulated growth in RISMP, indicating that O_2^- is generated by irradiation and is strongly involved in the acidification delay. The combined activities of SOD and catalase restored totally the normal acidification pattern, compared to reference milk with both enzymes. Mannitol also slightly reduced the inhibition, indicating that hydroxyl radicals are involved in *L lactis* CNRZ 1076 inhibition too.

Effect of pH of the inoculum

The strain CNRZ 1076 was pre-cultured in RISMP. At pH 5.6 and 4.3, samples of culture were inoculated into freshly 20 kGy RISMP milk. Inoculation levels were 10^6 and 10^4 cfu/ml. Growth rates were estimated over a 4 h period ($\mu 1$). Inoculation with 10^6

Table II. Effect of incubation of reconstituted milk prior to inoculation, addition of catalase, mannitol and SOD on acidification kinetics. Inoculation level of *L lactis* CNRZ 1076 was 10^4 cfu/ml.
Effet de la pré-incubation du lait, de l'addition de catalase, mannitol et SOD sur les paramètres cinétiques d'acidification. Le taux d'inoculation de L lactis *CNRZ 1076 était de 10^4 ufc/ml.*

	Reference milk			*Reconstituted skim milk from 20 kGy irradiated powder*		
	V_m *(u pH/h)*	pH_m	T_m	V_m *(u pH/h)*	pH_m	T_m
Control	0.48	5.49	13 h	0.42	5.48	15 h 10'
1 h incubation prior to inoculation	0.49	5.45	12 h 55'	0.46	5.38	14 h 50'
2 h incubation prior to inoculation	0.52	5.43	13 h	0.46	5.44	14 h 35'
2 h 30 incubation prior to inoculation	0.50	5.40	13 h 10'	0.46	5.40	14 h 30'
Catalase	0.48	5.39	12 h 20'	0.44	5.44	14 h 30'
Mannitol 0.05 mol/l	0.52	5.43	13 h 10'	0.45	5.54	14 h 50'
Mannitol 0.1 mol/l	0.49	5.53	13 h 05'	0.44	5.38	14 h 20'
SOD 20 U/ml	0.48	5.40	12 h 55'	0.45	5.39	14 h 50'
SOD 120 U/ml	0.51	5.46	12 h 55'	0.47	5.40	13 h 45'
SOD 120 U/ml + Catalase	0.53	5.35	12 h 25'	0.49	5.45	12 h 20'

cfu/ml led to uninhibited growth rates whether the pH of the inoculum was 4.3 or 5.6 (1.72 $h^{-1} \pm 0.06$ and 1.76 $h^{-1} \pm 0.11$ respectively) but inoculation with 10^4 cfu/ml led to a $\mu 1$ value of 1.11 $h^{-1} \pm 0.09$ for pH 5.6 inoculum compared to 1.43 $h^{-1} \pm 0.07$ for pH 4.3 inoculum.

Effect of chymosin activity on L lactis *CNRZ 1076 growth in RISMP*

Inoculation level for this study was always 10^4 cfu/ml. Results are shown in table III. First of all, chymosin action (60 µl/100 ml) effectively prevented the inhibition of acidification and growth in RISMP (figs 3, 4). Secondly, neither contamination of chymosin nor milk clotting were responsible for this phenomenon since neither denaturated chymosin nor native chymosin in non-clotting milk relieved the inhibition; the effect of chymosin was due to release of peptides. Indeed, addition of supernatant of reference milk incubated with chymosin for 1 h to RISMP promoted acidification whereas addition of a supernatant of chymosin-free milk had little effect. Direct addition of the caseinomacropeptide, β casein 193–209 peptide, and other small peptides such as casein hydrolysates greatly stimulated growth of *L lactis* CNRZ 1076 in RISMP but had no significant effect on growth in reference milk. The time between addition of these molecules and the increase in growth rate of the bacteria was very short (close to 30 min) which is only half the doubling time of this strain in that medium (1 h) (fig 5).

Identical results were observed with commercial 'Lait G' which is a 20 kGy irradiated milk powder.

Table III. Effect of chymosin and peptides on *L lactis* CNRZ 1076 growth and acidification in reconstituted skim milk from 20 kGy irradiated powder. Inoculation level of *L lactis* CNRZ 1076 was 10^4 cfu/ml.
Effet de l'addition de chymosine et de peptides sur la croissance de L lactis *CNRZ 1076 dans le lait reconstitué à partir de poudre irradiée à 20 kGy et sur son acidification. Le taux d'inoculation de* L lactis *CNRZ 1076 était de 10^4 ufc/ml.*

	μ_1 (h^{-1})	s	$s.t_{0.05}$	T_m[1]
Control	1.12	0.03	0.08	15 h 10'
Native chymosin	1.59	0.03	0.08	14 h 15'
Heated chymosin 100°C, 10'	1.16	0.02	0.06	15 h
Incubated chymosin pH 9, 43°C, 24 h	1.19	0.03	0.08	15 h 15'
Native chymosin on non-clotting milk	1.56	0.04	0.11	14 h 10'
Supernatant of milk incubated with chymosin	1.65	0.03	0.08	14 h
Supernatant of chymosin-free milk	1.33	0.02	0.06	14 h 55'
Caseinomacropeptide 1 mg/ml	1.57	0.01	0.03	13 h 55' [2]
Peptide [193–209] of casein 0.8 mg/ml	1.75	0.04	0.11	13 h 50' [2]
Casein hydrolysate < 500 Da 50 µg/ml	1.81	0.05	0.14	13 h 05' [2]

[1] T_m is time corresponding to maximum acidification rate. The pH_m value is about 5.40 for every pattern.
[2] These molecules promote reference milk acidification of *ca* 15'.
[1] *Tm: temps correspondant à la vitesse maximale d'acidification. La valeur du pH_m est d'environ 5,40 pour toutes les cinétiques.*
[2] *Ces molécules n'ont qu'un faible effet sur l'acidification du lait de référence (avance de 15').*

Effect of amino acid and small peptide addition

Data presented in table IV show results obtained when individual amino acids and dipeptides were added to both reconstituted milks. They suggest that bacteria were missing essential amino acids to fight irradiated milk toxicity. The most efficient amino acids appeared to be phenylalanine, histidine, leucine and isoleucine, though no individual amino acid or peptide tried completely relieved the inhibition caused by irradiation.

Effect of milk powder irradiation on L lactis *CNRZ 1075 (prt⁻) growth*

L lactis CNRZ 1075 (prt⁻) grew more slowly in reference milk and stopped growing at lower final populations and a higher pH than CNRZ 1076 (fig 6).

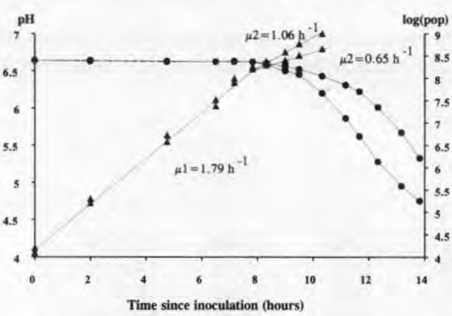

Fig 3. Chymosin addition effect on *L lactis* CNRZ 1076 growth and acidification in reference milk; ▲, log (pop) reference milk; Δ, log (pop) reference milk + chymosin; ●, pH reference milk; O, pH reference milk + chymosin.
Effet de l'addition de chymosine sur la croissance de L lactis *CNRZ 1076 et l'acidification du lait reconstitué à partir de poudre de référence.* ▲ *log (pop) lait de référence;* Δ *log (pop) lait de référence + chymosine;* ● *pH lait de référence; O pH lait de référence + chymosine.*

Early growth inhibition observed with *L lactis* CNRZ 1076 in RISMP was also observed with strain CNRZ 1075 (prt⁻) grown in this milk (fig 6). Nevertheless, the population levels achieved in late exponential phase were much higher in this milk than in reference milk. Consequently, though acidification was slower at the beginning in irradiated reconstituted milk than in reference milk, it became faster after 10 h of incubation and led to a lower final pH (fig 6).

Effect of milk powder irradiation on free amino acid fraction

No significant differences were noted when comparing the free amino acid fraction of reference and irradiated reconstituted milks

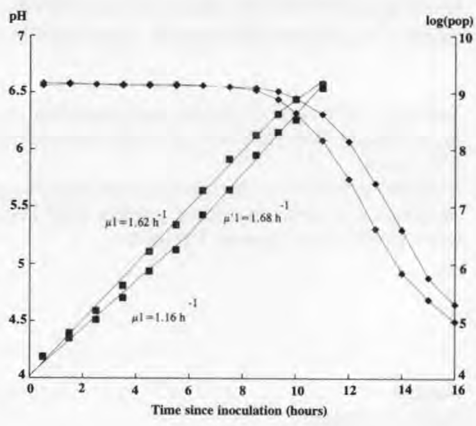

Fig 4. Chymosin addition effect on *L lactis* CNRZ 1076 growth and acidification in reconstituted skim milk from 20 kGy irradiated milk powder (RISMP). ■, log (pop) RISMP; ❑, log (pop) RISMP + chymosin; ◆, pH RISMP; ◊, pH RISMP + chymosin.
Effet de l'addition de chymosine sur la croissance de L lactis *CNRZ 1076 et l'acidification de lait reconstitué à partir de poudre irradiée à 20 kGy.* ■ *log (pop) lait reconstitué à partir de poudre irradiée;* ❑ *log (pop) lait reconstitué à partir de poudre irradiée + chymosine;* ◆ *pH lait reconstitué à partir de poudre irradiée;* ◊ *pH lait reconstitué à partir de poudre irradiée + chymosine.*

(table V). However, only 7 of the 20 amino acids were above 2 µg/ml.

Effect of milk powder irradiation on amino acids of NPN fraction

Contrary to the absence of an effect on the free amino acid fraction, the amino acid composition of the NPN fraction of milk powder was markedly increased from 347 to 447 mg/ml on irradiation (20 kGy). Most amino acids increased, phenylalanine and glycine being major exceptions (table VI).

DISCUSSION

Irradiation of milk powder at the 20 kGy level, contrary to what claimed by Chamba and Prost (1989), partly inhibited growth of a lactococcal culture. The inhibition was observed at 10^4 cfu/ml inoculation level but not at the 10^6 cfu/ml inoculation level used by Chamba and Prost (1989). A 2.5 h incubation of reconstituted milk prior to inoculation or an addition of 0.1 mol/l mannitol or of 120 U/ml of SOD partly relieved the inhibition. Addition of a mixture of SOD (120 U/ml) and catalase (100 U/ml) allowed a growth identical to that observed in unirradiated reference reconstituted milk.

Such results suggest that even in products with low water content such as milk powders, irradiation may generate water radiolysis compounds such as H_2O_2 (Bonet-Maury and Lefort, 1948; Dainton, 1948). There is also evidende that superoxide anion O_2^- may be generated by irradiation because it induces reduction of milk riboflavin (Beauchamp and Fridovich, 1971). In presence of molecular oxygen, reduced riboflavin implies the production of significant amounts of O_2^- (Massey *et al*, 1969).

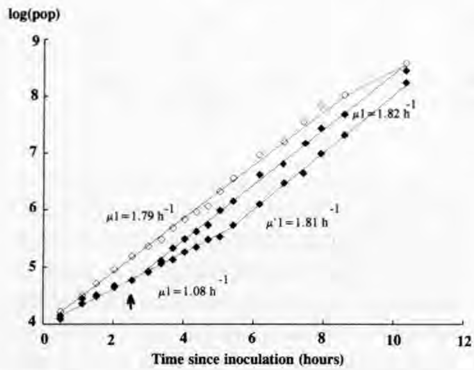

Fig 5. Effect of small peptide addition on *L lactis* CNRZ 1076 growth in reconstituted skim milk from 20 kGy irradiated powder (RISMP). ◆, RISMP; ◊, RISMP + casein hydrolysate 50 µg/ml (↑); ◊, RISMP + casein hydrolysate 50 µg/ml (t = 0 h);
Effet de l'addition de petits peptides au lait reconstitué à partir de poudre de lait irradiée à 20 kGy sur la croissance de L lactis *CNRZ 1076. ◆ Lait reconstitué à partir de poudre irradiée; ◊ lait reconstitué à partir de poudre irradiée + hydrolysat de caséine 50 µg/ml (↑); ◊ lait reconstitué à partir de poudre irradiée + hydrolysat de caséine 50 µg/ml (t= 0h).*

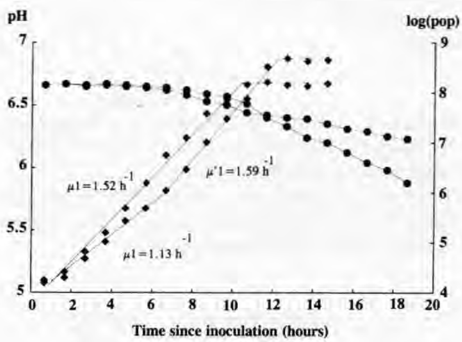

Fig 6. Effect of milk powder 20 kGy irradiation on acidification and growth of *L lactis* CNRZ 1075 (prt⁻) in reconstituted milk (RISMP). ◆, log (pop) RISMP; ◊, log (pop) reference milk; ●, pH in RISMP; O, pH in reference milk.
Effet de l'irradiation de la poudre de lait à 20 kGy sur la croissance de L lactis *CNRZ 1075 (prt⁻) et l'acidification du lait reconstitué. ◆ log (pop) lait reconstitué à partir de poudre irradiée; ◊ log (pop) lait de référence; ● pH lait reconstitué à partir de poudre irradiée; O pH lait de référence.*

Table IV. Effect of amino acids and peptides on *L lactis* CNRZ 1076 growth in reconstituted skim milk from 20 kGy irradiated powder (RISMP). The level of inoculation was 10^4 cfu/ml.
Effet de l'addition d'acides aminés et de peptides sur la croissance de L lactis *CNRZ 1076 dans le lait reconstitué à partir de poudre de lait irradiée à 20 kGy. Le taux d'inoculation était de 10^4 ufc/ml.*

	$\mu 1$ (h^{-1}) reference milk	s	$s.t_{0.05}$	$\mu 1$ (h^{-1}) RISMP	s	$s.t_{0.05}$
No supplement	1.78	0.04	0.11	1.19	0.02	0.06
Met 5 µg/ml	1.69	0.05	0.14	1.16	0.05	0.14
Pro 5 µg/ml	1.76	0.02	0.06	1.18	0.03	0.08
Ile 5 µg/ml	1.77	0.06	0.17	1.47	0.02	0.06
Val 5 µg/ml	1.66	0.03	0.08	1.15	0.04	0.11
His 5 µg/ml	1.79	0.03	0.08	1.53	0.04	0.11
Phe 5 µg/ml	1.77	0.01	0.03	1.47	0.01	0.03
Leu 5 µg/ml	1.93	0.03	0.08	1.45	0.03	0.08
Glu 5 µg/ml	1.79	0.05	0.14	1.20	0.05	0.14
Ile-Met 5 µg/ml	1.76	0.06	0.17	1.42	0.03	0.08
Ala-Pro 5 µg/ml	1.77	0.02	0.06	1.16	0.03	0.08
Ala-pro 10 µg/ml	1.76	0.02	0.06	1.15	0.04	0.11
Pro-Glu 10 µg/ml	1.79	0.05	0.14	1.18	0.02	0.06
Ile-Pro 5 µg/ml	1.78	0.04	0.11	1.36	0.03	0.08
Ile-Pro 10 µg/ml	1.76	0.01	0.03	1.46	0.01	0.03
Ile-Pro 20 µg/ml	1.80	0.04	0.11	1.44	0.06	0.17
Ile-Phe 10 µg/ml	1.77	0.03	0.08	1.45	0.03	0.08
Ala-Pro 10 µg/ml + Pro-Glu 10 µg/ml	1.79	0.02	0.06	1.26	0.02	0.06
Ile-Pro 10 µg/ml + Leu-Glu 10 µg/ml	1.99	0.05	0.14	1.49	0.04	0.11
Mean		0.034			0.031	

Table V. Effect of 20 kGy skim powder irradiation (RISMP) on the free amino-acids fraction of reconstituted milk.
Effet de l'irradiation de la poudre de lait (20 kGy) sur la composition de la fraction acides aminés libres du lait reconstitué.

	Reference milk (mg/l)	*RISMP (mg/l)*
Pser	11.5	13.3
Ser	1.5	1.8
Thr	4.7	2.2
Asp	2.3	2.5
Glu	43.4	40.4
Gly	7.2	6.5
Ala	4.2	4.7
Lys	2.3	2.4
Total	77.1	73.7

H_2O_2 and O_2^- are known to be toxic or inhibiting for lactic acid bacteria (Condon, 1987; Piard and Desmazeaud, 1991). It appears from results gathered in table II, particularly the strong restoring effect of the addition of SOD, that the main inhibiting compound present in irradiated milk powder is O_2^-. Indeed, the results suggest that dismutation of the amount of superoxide anion induced by irradiation requires either a population level of 10^6 cfu/ml of *L lactis* which possesses SOD activity (Britton *et al*, 1978; Archibald and Fridovich, 1981; Zitzelsberger *et al*, 1984; Smart and Thomas, 1987) or addition of 120 U/ml of exogenous SOD. The relief of inhibition by incubation prior to inoculation may be explained either through a spontaneous dismutation of O_2^-, possible in aqueous solutions (Bielski and

Table VI. Effect of milk powder irradiation (20 kGy) on amino acids composition of the NPN fraction of reconstituted milk (RISMP).
Effet de l'irradiation de la poudre de lait (20 kGy) sur la composition en acides aminés de la fraction NPN du lait reconstitué.

	Reference milk (mg/l)	*RISMP (mg/l)*
Ser	17.7	25.2
Asp/Asn	25.3	34.9
Thr	11.4	19.9
Glu/Gln	97.5	120.7
Gly	21.0	22.7
Ala	8.8	12.1
Lys	17.6	21.7
Pro	12.7	22.7
Val	9.5	17.7
Ile	6.1	9.4
Leu	4.5	8.9
Tyr	0.8	4.5
Phe	99.5	99.3
His	5.1	7.5
Arg	10.0	10.3
Trp	nd [1]	nd
Met	nd	nd
Cys	nd	nd
Total	347.5	437.5

[1] Not determined.
[1] *Non déterminé.*

Allen, 1977) or through the dismutation of O_2^- by milk endogenous SOD which is known as relatively thermoresistant and consequently still active in low heat milk powder (Alais, 1984). On the other hand, the relief of inhibition by using a culture with a well developed acidity is likely to be due to pH effect on O_2^- spontaneous dismutation; optimal pH for O_2^- spontaneous dismutation is around 5.0 (Bielski and Allen, 1977; Lengfelder *et al*, 1979; Götz *et al*, 1980). The internal pH of *L lactis* cells from a culture at pH 5.6 is likely to be around 6.7 to 7.0 (Otto *et al*, 1983), pH values at which spontaneous dismutation is ten times slower than at pH 5.0.

On the other hand, at pH 4.3, the intracellular pH is likely lowered to values around 5.7 (Kashket, 1987) close to optimal spontaneous dismutation pH. Dismutation of O_2^- present in irradiated powder is then accelerated and a less inhibiting effect is observed.

H_2O_2 appears to be involved in the observed inhibition only indirectly. Indeed, addition of catalase had the same promoting effect on growth of *L lactis* CNRZ 1076 in both milks (from reference and irradiated powders). Such a stimulation indicates that this strain produces H_2O_2 by itself but is lacking in endogenous catalase as are most lactic acid bacteria which are unable to synthesize hemoporphyrins (Piard and Desmazeaud, 1991). H_2O_2 can also inhibit lactic acid bacteria through the Haber-Weiss reaction (1934): $H_2O_2 + O_2^- \longrightarrow OH^- + OH^\bullet + O_2$, which allows formation of hydroxyl radical [$OH^\bullet$] known as an inhibitor lactic acid bacteria (Gregory and Fridovich, 1974). Such an [$OH^\bullet$] formation is likely in milk reconstituted from irradiated powder because of the simultaneous presence of hydrogen peroxide, superoxide anion (Piard and Desmazeaud, 1991) and lactoferrin which acts as an efficient catalyst (Ambruso and Johnston, 1981). That is suggested by the decrease in inhibition of growth of *L lactis* on the addition of 0.1 mol/l mannitol, a scavenger of [OH·] (Fridovich, 1983). However, the relatively low restoring effect of added mannitol suggests that hydroxyl radical is not the critical inhibitor for *L lactis* in the milk from irradiated powder. This weak effect of mannitol agrees with the observations of Grufferty (1981), cited by Condon (1987), who found that oxygen sensitive strains of *L lactis* were not protected by the [OH·] scavenger.

Peptides and amino acids also appear to be involved in the restoration of growth rate of *L lactis* in milk reconstituted from irradiated powder. In a previous work (Bouhallab *et al*, 1993), it was observed that growth rate of *L lactis* CNRZ 1076 in a commercial reconstituted irradiated milk powder (lait G) was slow (0.9 h^{-1}) but was enhanced by addition of tryptic digests of κ caseinomacropeptide so as to reach growth rates in reference milk seen in this study. Our results on addition of chymosin supernatant, κ caseinomacropeptide and β casein 193-209 peptide indicate that the stimulation of *L lactis* CNRZ 1076 in 'lait G' by a tryptic digest of κ caseinomacropeptide was due to a lifting of inhibition more than to a stimulation.

Possible nutritional deficiency of reconstituted skim milk from irradiated powder cannot account for the stimulation of *L lactis* CNRZ 1076 by peptides and amino acids since the non-protein fraction was increased in the latter milk compared to that in reference milk. The increase in peptidic fraction of irradiated milk, already indicated by Day *et al* (1957) was not observed by Chamba and Prost (1989) because they limited their study to a comparison of chromatographic profiles. The higher maximum stationary phase population and acidification rate of strain CNRZ 1075 (prt$^-$) in reconstituted skim milk from irradiated powder compared to that in reference milk show evidence for absence of nutritional deficiency in RISMP. The inhibition caused by O_2^-/H_2O_2 and [OH$^\bullet$] presumably affects many proteins in the cell (Lavelle *et al*, 1973; Michelson and Maral, 1983). The most probable explanation is that several essential proteins are destroyed and must be resynthesised to restore growth. The full restoration requires several hours in reconstituted milk from irradiated powder, *ie* restoration requires elimination of the toxic molecules *via* SOD and *de novo* synthesis of proteins inhibited by the toxic molecules.

The results suggest that protein synthesis requires addition of some lacking amino acids such as Leu, Ileu, His of which genes of biosynthesis are inactivated in *L lactis* dairy strains (Delorme *et al*, 1993; Godon *et al*, 1993). These amino acids would be the restricting factors for the *de novo* synthesis of proteins and especially biosynthesis of the SOD present in lactic acid bacteria, Mn-SOD (Britton *et al*, 1978) which contains many His, Ileu and Leu residues in its primary sequence (White and Scandalios, 1988; Nakayama, 1992). The irradiation causes formation of peptides which *L lactis* CNRZ 1075 (prt$^-$) could not form in unirradiated milk. However, this is insufficient for balancing inhibition of growth caused by radicals' formation in RISMP.

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