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Original article

Effect of some technological treatments of milk on *in vivo* gastric emptying of immunoreactive whey proteins

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Summary — The effects of technological treatments of milk on the *in vivo* immunoreactive β-lactoglobulin and lactoferrin gastric emptying were studied in calves given 3 different diets: raw skim milk (RSM), pasteurized skim milk (PSM) and pasteurized and acidified skim milk (Y). The emptying of these proteins was evaluated in collected effluents leaving the stomach over a 12-h period by a competitive enzyme linked immunoassay (ELISA). The sensitivity of developed ELISA was 1.5 µg/ml for β-lactoglobulin and 50 ng/ml for lactoferrin. Immunoreactive β-lactoglobulin was detected throughout the digestion period and was emptied, as was the fresh matter with RSM and Y, whereas it was strongly retained in the stomach with PSM. Amounts equivalent to 60% of ingested β-lactoglobulin were emptied 7 h after the meal for RSM and Y and 40% for PSM. Immunoreactive lactoferrin was detectable only for 1.5 h after the meal and only in digesta from calves fed RSM. An amount equivalent to 22% of ingested lactoferrin was emptied in an immunoreactive form.

β-lactoglobulin / lactoferrin / gastric emptying / calf

Résumé — Effets de traitements technologiques du lait sur la vidange gastrique *in vivo* de la β-lactoglobuline et de la lactoferrine immunoréactives. *Afin de comparer les effets de traitements technologiques du lait sur la vidange gastrique* in vivo *de la β-lactoglobuline et de la lactoferrine immunoréactives, trois régimes ont été étudiés chez le veau préruminant : lait écrémé cru (RSM), lait écrémé pasteurisé (PSM) et lait écrémé pasteurisé et acidifié (Y). La vidange de ces protéines a été quantifiée par collecte des effluents gastriques pendant 12 h et dosage par ELISA. Deux ELISA ont été développés et leurs sensibilités respectives étaient de 1,5 µg/ml pour la β-lactoglobuline et de 50 ng/ml pour la lactoferrine. La β-lactoglobuline immunoréactive a été détectée dans tous les effluents. Sa vidange suit celle de la matière fraîche dans le cas du RSM et du Y alors qu'avec PSM sa vidange est nettement ralentie par rapport à la matière fraîche. Après 7 h de digestion, l'équivalent de 60% de la β-lactoglobuline ingérée a été collecté dans le cas de RSM et de Y et seulement 40% sont collectés avec PSM. La lactoferrine immunoréactive n'a pu être détectée que dans les digesta provenant du régime RSM et seulement pendant 1 h 30 après le repas. L'équivalent de 22% de la lactoferrine ingérée était vidangée 7 h après le repas.*

β-lactoglobuline / lactoferrine / vidange gastrique / veau

INTRODUCTION

A number of diverse biological roles have been suggested for several whey proteins, especially lactoferrin and β-lactoglobulin. Lactoferrin (Lf), the major iron binding protein in human whey, has been involved in the high bioavailability of iron in human milk because of its high affinity for iron (Cox *et al,* 1979). It may function in the process of iron absorption through interaction with intestinal receptors because specific receptors has been evidenced on the small intestine brush border of several animal species such as rabbits, monkeys and mice (Mazurier *et al,* 1985; Davidson and Lönnerdal, 1988; Hu *et al,* 1988, 1990). A bacteriostatic effect of lactoferrin in the gastrointestinal tract has also been proposed based on *in vitro* experiments demonstrating the ability of the apoprotein to inhibit the growth of a variety of potentially pathogenic bacteria presumably by depriving the organism of iron required for growth (Bullen *et al,* 1972; Law and Reiter, 1977). β-lactoglobulin (BLg) binds retinol and may function as a physiological retinoid carrier protein since specific receptors for BLg exist in the intestine of neonatal calves (Papiz *et al,* 1986). Moreover, BLg is generally considered as the main factor responsible for milk protein immunoreactivity and intolerance (Huang *et al,* 1985; Koritz *et al,* 1987).

For Lf or BLg to play any of these roles in the gastrointestinal tract, resistance to gastric and intestinal digestion would be required. Human Lf undergoes limited digestion in the presence of pepsin, trypsin or chymotrypsin, whereas bovine Lf was found to be less resistant to proteolysis even in its iron saturated form (Line *et al,* 1976; Brock *et al,* 1976; Brines and Brock, 1983; Legrand *et al,* 1984). There is no information about the effect of heat on Lf structure or on its resistance to proteolysis. Britton and Koldovsky (1987) demonstrated *in vitro* with gastric or luminal fluids of suckling or weanling rats that human Lf digestion may vary with respect to postnatal age of the rat, segment of the gastrointestinal tract and dietary state. Native BLg is resistant *in vitro* to peptic and chymotryptic digestion because of its stable conformation and heating at 50, 60 or 70 °C for 15 min does not affect resistance of BLg, while heating at 80 and 90 °C significantly decreases its resistance to proteolysis (Reddy *et al,* 1988). *In vivo,* in experimental animals, native Blg is resistant to gastric digestion and apparently remains undegraded and immunoreactive after it passes through the stomach (Miranda and Pélissier, 1983; Yvon *et al,* 1984). It was found undegraded with heat treated milk and acidified milk (Scanff *et al,* 1990) but no quantitative information about the emptying kinetics and the immunoreactivity of this protein is available. No information exists on the *in vivo* gastric proteolysis and emptying of Lf.

In order to determine the effect of technological treatments of milk on *in vivo* quantitative gastric emptying of immunoreactive BLg and Lf, 3 diets were studied: raw skim milk, pasteurized skim milk and pasteurized and acidified skim milk. Studies were made with the preruminant calf, a monogastric animal model.

MATERIALS AND METHODS

Diets

Three types of test meals were prepared from the same skim milk: (RSM) raw skim milk, (PSM) pasteurized skim milk (Y) pasteurized and acidified skim milk. To obtain diets PSM and Y, milk was heated at 95 °C for 45 s. For diet Y, the milk was then inoculated (3%) with a

preculture of mixed strains, *Streptococcus thermophilus* (CNRZ TJ160) and *Lactobacillus bulgaricus* (CNRZ 1B369), in milk, and then incubated at 42 °C for 4 h. The samples were brought to 4 °C and kept at that temperature for 15 days. Polyethylene glycol (PEG) 4 000 was added to each diet to a final concentration of 1% just before animal feeding in order to estimate the ratio of endogenous secretions in the emptied matter.

Animals

Two preruminant Friesian calves were used. At about 1 month of age, each animal was fitted with a reentrant duodenal cannula and an abomasal cannula. Proximal and distal parts of the duodenal cannula were located around 8 and 15 cm after the pylorus, respectively (Ash, 1962). Animals were allowed to recover from surgery for 7 days before digesta collection was started. Their normal diet was a milk substitute based on skim milk powder, whey powder and tallow.

Experimental procedure

This has been previously described (Scanff *et al*, 1990). For each experiment, 2 animals were used simultaneously. The day before the experiments in the morning the animals received their normal diet to which sodium citrate had been added in order to accelerate gastric emptying (Frantzen *et al*, 1973); in the evening they only received water. Under these conditions, the abomasum was empty at the moment of the first meal in the morning of the experimental days. Each fasted calf received once a week the 3 experimental diets (190 g/kg live weight$^{0.75}$) successively. The whole effluent from the calf stomach was collected over 7 h, fractions being taken every 10 min for the first 30 min, every 15 min for the second 30 min, and every 30 min until to the end of the 7 h period. A second identical test meal was then given, and samples were collected with the same periodicity up to 5 h after this meal.

Aliquots were used to measure pH and then returned to samples. Each sample was immediately precipitated with trichloroacetic acid (TCA) to a final concentration of 12%. Samples were centrifuged at 2 000 *g* for 20 min. The pellet was resuspended in water. Supernatants and sediments were kept at –20 °C until analysis.

Sample analysis

A competitive enzyme linked immunoassay (ELISA) was used to quantify BLg and Lf in each digesta. Anti-BLg and anti-Lf sera were obtained by immunizing rabbits with BLg or Lf previously purified in our laboratory. Peroxidase-coupled anti-rabbit IgG was purchased from Pasteur Diagnostic.

Microtiter plates (Nunc, Denmark) were coated either with BLg (5 µg/ml) in 0.1 mol/l EDTA, NaOH pH 9.3 buffer or with Lf (0.25 µg/ml) in a carbonate–bicarbonate pH 9.6 buffer for 2 h at 37 °C or 18 h at room temperature. Then the plates were washed (washing buffer: NaCl 9 g/l, Tween 0.05% in 10 mmol/l, pH 7.4 phosphate buffer; each wash step consisted of 5 fill/empty cycles with this buffer), pretreated with EIA buffer (10 mmol/l pH 7.4 phosphate buffer containing 0.15 mol/l NaCl and 0.05% human serum albumin as blocking agent) and stored at 4 °C until use.

For analysis, samples were prepared from the TCA insoluble fraction of digesta which were first solubilized by increasing the pH to 7.5 and then diluted in EIA buffer. Antisera, BLg or Lf for standard curves were also diluted in EIA buffer. For assays, antisera and samples (diluted digesta or diluted protein) were distributed in washed plates without preincubation for BLg or with a preincubation step (30 min, 37 °C) for Lf. Incubation was performed for 2 h at 37 °C, plates were then washed and the correctly diluted peroxidase coupled anti-rabbit IgG was added. Plates were incubated for 30 min at 37 °C and washed again. Enzymatic reaction was realized by addition of orthophenylendiamin dihydrochloride (Sigma) and H_2O_2. The appropriate enzymatic reaction time had been determined previously and absorbance of the content of each well was measured in a Titertec Multiskan MC (Flow Laboratories) after this time. The results were expressed in terms of $B/B_0 \cdot 100$ where B and B_0 represent the absorbance values measured on the bound fractions in the presence (B) or absence (B_0) of competitors (BLg or Lf for cal-

ibration curves and samples). Standard curve and calculation of sample concentrations were performed using a linear log-logit transformation. Sample concentrations were determined from dilutions giving a B/B_0 close to 50%. All measurements were made in duplicate.

In vitro Lf hydrolysis and electrophoresis

Lf was purified from whey according to Law's and Reiter's ion exchange method (1977) modified using a SP-Zetaprep 100 cation exchanger cartridge (LKB, Bromma, Sweden). Solubilized (either in pH 4.7 or pH 2.7 citrate phosphate buffer) Lf was submitted to rennet hydrolysis (E/S = 1/2 500 or 1/250 000 w/w). After 15, 30, 60, 120 or 180 min incubation time, the reaction was stopped by increasing the pH to 9 with ammonia and samples were dried. Samples were subsequently subjected to electrophoresis in a 10–20% linear gradient polyacrylamide gel containing 0.1% SDS (Laemmli, 1970).

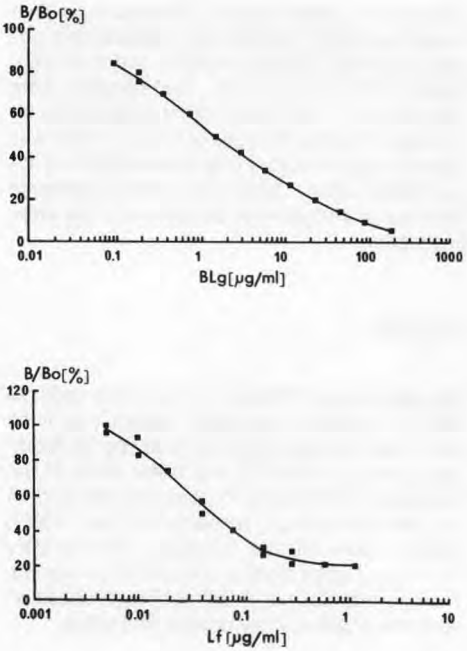

Fig 1. Standard inhibition curves for β-lactoglobulin and lactoferrin in ELISA. B/B_0*100 = F([BLg or Lf]).
Courbes étalon des dosages ELISA. B/B_0*100 = F([BLg ou Lf]).*

RESULTS

ELISA conditions

Standard curves for BLg and Lf obtained with the described conditions are shown in figure 1. The sensitivity of the assays was characterized by the concentration of standard BLg or Lf inducing 50% inhibition of B_0. The B/B_0 = 50% values for BLg were between 1.5–2 µg/ml. For Lf 50% inhibition values were between 50–70 ng/ml. Concentrations as low as 8 ng/ml for Lf and 0.2 µg/ml for BLg were detectable, but could not be quantified.

Cross-reactions between anti BLg and α-lactalbumin, bovine serum albumin, α_{s1}-casein, β-casein or κ-casein were investigated. Cross reactions between anti Lf and α-lactalbumin, BLg, bovine serum albumin, αs1-casein, β-casein or κ-casein were also tested. The total cross-reactivity coefficients were found to be < 0.1%. Considering the proportions of these proteins in milk, the error due to this cross-reactivity was always < 5%. The intra-assay coefficients of variation were always < 5%.

BLg gastric emptying

Immunoreactive BLg was measured in diets and in gastric effluents collected throughout the digestion time. 3.25 g/l, 2.50 g/l and 2.25 g/l were present in RSM, PSM and Y diets respectively. Seven h after the first meal 74, 59 and 40% of ingest-

ed BLg was emptied in an immunoreactive form for RSM, Y and PSM respectively. With the diets RSM and Y around 50% of the BLg ingested during the 2 meals was emptied 12 h after the first meal, whereas 30% was emptied with diet PSM (fig 2). Fresh matter emptying was previously measured and represented the ingested volume after 7 digestion hours for the 3 diets (Scanff *et al,* 1990). Therefore the ratio of emptied BLg *versus* emptied fresh matter after 7 digestion hours was 96, 95 and 54% for RSM, Y and PSM respectively (fig 3).

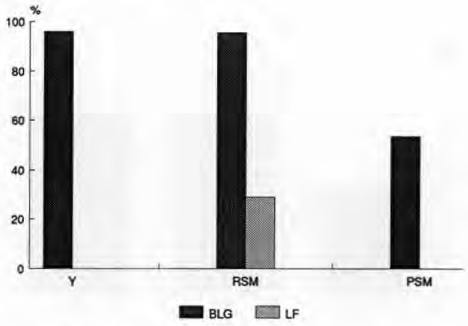

Fig 3. Cumulative amount of immunoreactive β-lactoglobulin or lactoferrin emptied during the first 7 h in relation to the emptied fresh matter.
Quantités cumulées de β-lactoglobuline ou de lactoferrine immunoréactives vidangées durant les 7 premières h de digestion et rapportées à la vidange de matière fraîche.

Lf gastric emptying

74 mg/l of immunoreactive Lf was determined in RSM. Because a 10-fold lower quantity was found in PSM and Y, immunoreactive Lf was not measured in digesta of calves fed these diets. In order to check if technological treatments of milk modify the structure of Lf and consequently the Lf-anti Lf reaction, a 0.1% Lf aqueous solution was heated to 95 °C for 45 s as for PSM and Y. A 90% decrease in Lf immunoreactivity was induced by this treatment. In digesta from calves fed RSM, immunoreactive Lf was detectable only during 1.5 h after the meal. The same pattern was observed after the second meal. Thus, at 7 h as at 1.5 h after the meal, 22% of the ingested Lf was emptied in an immunoreactive form (fig 4). Lf hydrolysis by rennet was one of the possible explanations for such a low immunoreactive Lf emptying and this was tested *in vitro*. The electrophoretic pattern of Lf submitted to rennet hydrolysis (E/S = 1/2 500) at pH 4.7 or pH 2.7 showed that Lf was not hydrolyzed at pH 4.7, whereas it was degraded at pH 2.7 (fig 5a). Electrophoresis of hydrolysates obtained at pH 2.7 with a E/S ratio close to that observed in the stomach of calf for Lf (fig 5b) showed that after 15-min incubation, Lf began to be hydrolyzed and after a 120-min incubation Lf was totally degraded.

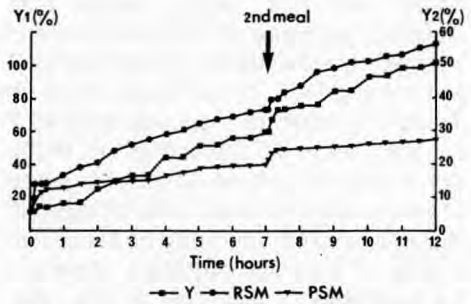

Fig 2. Cumulative amount of immunoreactive β-lactoglobulin leaving the abomasum for the three diets in percentage of ingested β-lactoglobulin with the first meal (Y1) or in percentage of ingested β-lactoglobulin with the 2 meals (Y2).
Quantités cumulées de β-lactoglobuline immunoréactive vidangées de l'estomac au cours de la digestion, pour les 3 régimes. Ces quantités sont exprimées en pourcentage de β-lactoglobuline ingérée lors du premier repas (Y1) ou lors des 2 repas (Y2).

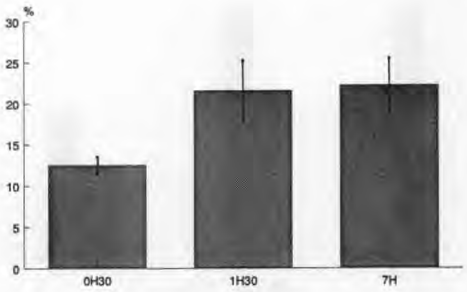

Fig 4. Emptied immunoreactive lactoferrin 30 min, 1.5 h and 7 h after the first meal in percentage of ingested lactoferrin with the first meal.
Quantités de lactoferrine immunoréactive vidangées à 30 min, 1,5 h et 7 h de digestion, exprimée en % de la lactoferrine ingérée lors du premier repas.

DISCUSSION

The ELISA described for BLg was not highly sensitive but it was sufficient for our study as BLg was present in a relatively high quantity. The interest of such an assay was to minimize the serum quantity required compared to methods such as immunoelectrophoresis. In contrast, the ELISA described for Lf was highly sensitive since only a few ng per ml could be detected. The same sensitivity was previously obtained by Le Magnen *et al* (1989) with a competitive ELISA, whereas a more highly sensitive test (0.4 ng/ml) was described by Henghoj and Schaffalitzky de Muckadell (1985) with a single sandwich ELISA.

Gastric emptying of immunoreactive BLg in RSM or Y fed calves was the same as the fresh matter, whereas it was slowed down compared to the fresh matter emptying after PSM meal (fig 3). This was probably due to a retention of BLg in the stomach since BLg is known to interact with caseins by heating. In fact, heating at 95 °C for 0.5 or 1 min results in the association of 58 or 66% of the BLg with the casein fraction (Noh and Richardson, 1989). These interaction have no consequences with Y since casein and whey proteins are emptied at the same rate throughout digestion (Scanff *et al,* 1990). With PSM, casein coagulated and whey proteins were also retained in the stomach because of these interactions. At the time of the second meal of PSM coagulation occurred faster than with the first meal (Scanff *et al,* 1990). At the time of the second meal, the non-emptied products of the first meal contributed to minimize the increase of pH in the stomach and consequently accelerated the coagulation. This could explain the difference of BLg emptying between the 2 meals.

With RSM immunoreactive Lf was no longer detected in digesta 1.5 h after the meal and the proportion of emptied Lf represented only 1/3 of the proportion of emptied Blg after 7 h (fig 3). This could be consistent with the pepsic rather than chymosic hydrolysis of the protein since Lf was hydrolyzed *in vitro* by rennet at pH 2.7 and not at pH 4.7. *In vivo* the pH of the effluents 1.5 h after the meal was still not 2.7 but was below 4.7 (Scanff *et al,* 1990). One h after the first meal 20% of ingested Lf was emptied whereas 34% of ingested BLg was emptied. This could be the consequence of a starting hydrolysis. However, the hydrolysis of lactoferrin at the postprandial pH may be minimal as was previously observed (Spik *et al,* 1982; Britton and Koldovsky, 1989). Thus the retention of Lf in the coagulum is likely responsible since an *in vitro* coagulation test on raw milk showed that only 50% of Lf was found in the whey fraction after coagulation. Moreover, some authors have demonstrated that Lf interacts with casein in milk (McEnroe and Donnelly, 1987).

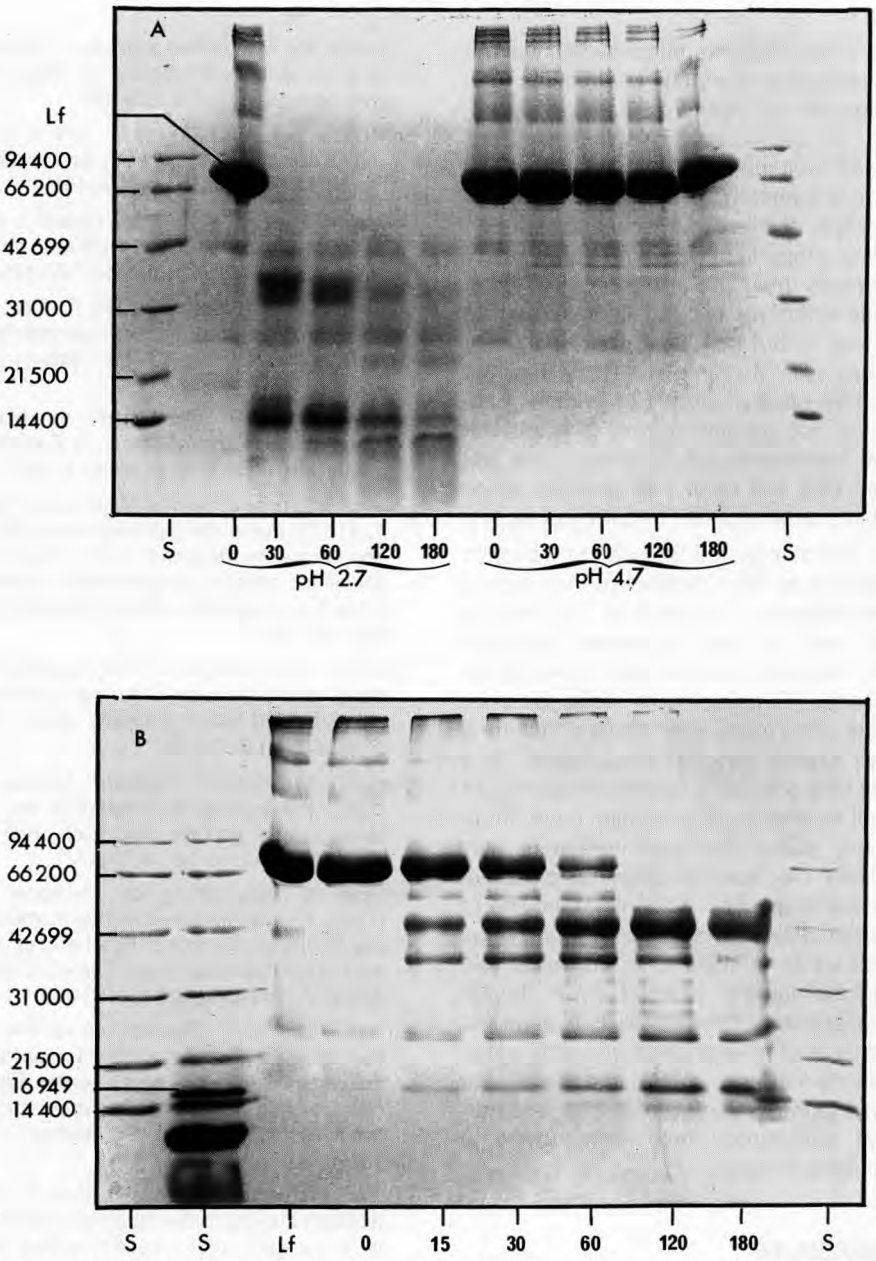

Fig 5. SDS-PAGE of lactoferrin hydrolyzed with rennet for indicated time (min). S = molecular weight standard. Lf = lactoferrin; 4A: pH 2.7 or 4.7, E/S = 1/2500, T = 37 °C. 4B: pH 2.7, E/S = 1/250000, T = 37 °C.

SDS–PAGE de l'hydrolysat obtenu après action de la chymosine sur la lactoferrine pendant les temps indiqués. S = étalon de masse moléculaire. Lf : lactoferrine. 4A : pH 2,7 ou 4.7, E/S = 1/2500, T = 37 °C. 4B : pH 2,7, E/S = 1/250000, T = 37 °C.

In Y and PSM the heat treatment probably induced a change in Lf structure. This change did not modify its *in vitro* susceptibility to pepsic hydrolysis (result not shown) but it modified its immunoreactivity and consequently could also affect the properties of this protein such as the iron binding properties. Therefore the dietary treatments (heat and acidification) alter the gastric emptying of both immunoreactive BLg and Lf but not in the same way. For Lf, they alter the immunoreactivity of the specific protein whereas BLg immunoreactivity is not or only slightly altered. But these treatments induce interactions between BLg and other milk proteins which lead to a slower gastric emptying of BLg.

A part of ingested BLg and Lf was thus emptied in an immunoreactive form mainly at the beginning of digestion. For them to fulfull any of their supposed biological roles, they also have to resist other digestion steps. BLg seems to resist the pancreatic step even after heating but to a lower degree (Reddy *et al,* 1988). BLg could thus ensure its function of binding or retinol transport but could also have an allergenic effect. The heat treatment only modified the quantity of immunoreactive BLg that is emptied. In contrast, bovine Lf was not resistant to pancreatic enzymes (Brock *et al,* 1976) and its biological role could be largely affected even though some authors demonstrate that a pepsic product of Lf always binds iron with a lower affinity (Line *et al,* 1976) and that bovine Lf was found in the feces of infants fed artificial milk supplemented with bovine Lf (Spik *et al,* 1982).

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