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## A novel bioactive peptide from yoghurts modulates expression of the gel-forming MUC2 mucin as well as population of goblet cells and Paneth cells along the small intestine<sup>☆</sup>

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### Abstract

Several studies demonstrated that fermented milks may provide a large number of bioactive peptides into the gastrointestinal tract. We previously showed that beta-casomorphin-7, an opioid-like peptide produced from bovine  $\beta$ -casein, strongly stimulates intestinal mucin production in *ex vivo* and *in vitro* models, suggesting the potential benefit of milk bioactive peptides on intestinal protection. In the present study, we tested the hypothesis that the total peptide pool (TPP) from a fermented milk (yoghurt) may act on human intestinal mucus-producing cells (HT29-MTX) to induce mucin expression. Our aim was then to identify the peptide(s) carrying the biological activity and to study its impact *in vivo* on factors involved in gut protection after oral administration to rat pups (once a day, 9 consecutive days). TPP stimulated *MUC2* and *MUC4* gene expression as well as mucin secretion in HT29-MTX cells. Among the four peptide fractions that were separated by preparative reversed-phase high-performance liquid chromatography, only the C2 fraction was able to mimic the *in vitro* effect of TPP. Interestingly, the sequence [94–123] of  $\beta$ -casein, present only in C2 fraction, also regulated mucin production in HT29-MTX cells. Oral administration of this peptide to rat pups enhanced the number of goblet cells and Paneth cells along the small intestine. These effects were associated with a higher expression of intestinal mucins (*Muc2* and *Muc4*) and of antibacterial factors (*lysozyme*, *rdefa5*). We conclude that the peptide  $\beta$ -CN(94–123) present in yoghurts may maintain or restore intestinal homeostasis and could play an important role in protection against damaging agents of the intestinal lumen.  
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**Keywords:** Milk bioactive peptides;  $\beta$ -Casein; Intestinal barrier; Intestinal protection; MUC2; Mucus;  $\alpha$ -Defensin

### 1. Introduction

Milk proteins are considered as the most important sources of bioactive peptides. These remain inactive within the sequence of the parent protein until they are released by either gastrointestinal digestion or food processing. Their beneficial health effects are

classified as antimicrobial, immunomodulatory, blood-pressure-lowering (ACE inhibitory), antithrombotic, antioxidant and opioid-like, in addition to cholesterol-lowering and enhancers of mineral absorption/bioavailability [1,2].  $\beta$ -Casomorphins ( $\beta$ -CMs) are such a family of opioid peptides derived from bovine  $\beta$ -casein. They possess  $\mu$ -preferential-receptor agonist activity and consist of  $\beta$ -CM-7, -6, -5 and -4 which are obtained by successive C-terminal cleavage of the [60–66] (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) sequence in bovine  $\beta$ -casein [3]. Jinsmaa and Yoshikawa have also isolated another opioid peptide (Tyr-Pro-Val-Glu-Pro-Phe) after digestion of bovine  $\beta$ -casein by proteases [4]. It corresponds to residues [114–119] of  $\beta$ -casein and was named neocasomorphin-6. Interestingly,  $\beta$ -CMs and neocasomorphin-6 are resistant to the actions of gastrointestinal enzymes due to the presence of proline residues. They could thus reach the intestine and represent agonists for opioid receptors localized in the gut myenteric and submucosal plexus but also on epithelial cells of the human, rat, pig and guinea pig [5–7].

**Abbreviations:** CT, controls; ELLA, enzyme-linked lectin assay; PC, Paneth cell; *rdefa5*, rat defensin 5; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TP, treated with peptide; TPP, total peptide pool;  $\beta$ -CN,  $\beta$ -casein;  $\beta$ -CMs,  $\beta$ -casomorphins;  $\beta$ -CM-7,  $\beta$ -casomorphin-7.

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Intestinal goblet cells are highly polarized secretory cells that reside throughout the length of the small and large intestine. They are responsible for production of the protective mucus coat by releasing the secreted mucin MUC2 (in rodent, *Muc2*), a high-molecular-weight glycoprotein that is stored within granules in their apical compartment. Mucus lubricates the intestinal surface, limits passage of luminal molecules into the mucosa, functions as a dynamic defensive barrier against enteric pathogens and acts as a substrate and a niche which the commensal flora can colonize [8,9]. The physiological relevance of MUC2 is definitively proven by the development of spontaneous colitis and intestinal tumors in *Muc2* knockout mice [10,11]. In man, many studies also support the hypothesis that alterations in mucin synthesis, secretion and/or degradation are involved in the initiation or maintenance of a lot of intestinal disorders. For example, Pugh et al. demonstrated that active duodenal ulcerations were associated with a decreased number of goblet cells [12]. In ulcerative colitis, mucus layer and goblet cell density are also significantly reduced [13,14]. Based on these observations, the strengthening of mucus gel, in particular by nutrients, could be extremely beneficial [15]. In order to achieve this aim, we previously showed that luminal administration of  $\beta$ -CM-7 induced a strong and fast (in the first 30 min of stimulation) release of mucin in rat jejunum through the activation of the enteric nervous system and opioid receptors [16,17]. Additionally, we demonstrated that  $\beta$ -CM-7 (100  $\mu$ mol/L) can also directly act on mucus-secreting cells to induce the expression of *Muc2* and *Muc3* in intestinal rat cells (DHE) or the expression of *MUC5AC* in intestinal human cells (HT29-MTX) [7].  $\beta$ -CMs could thus regulate intestinal goblet cells via two distinct but complementary modes of action: by a direct pathway to increase the expression of intestinal mucin genes and by an indirect nervous pathway to rapidly enhance the secretory activity of intestinal goblet cells. These findings were very exciting and suggested that the presence of  $\beta$ -CMs in the intestinal lumen could improve gut health. It is necessary however to bring sufficient quantities of bioactive peptide in contact with intestinal target cells.  $\beta$ -CMs may be generated from native bovine milk  $\beta$ -casein as a result of enzymatic hydrolysis occurring in the digestive tract [18] or during technological processing of food [19]. Indeed, several studies indicated that proteolytic systems involved in fermented milks or cheese manufacture can hydrolyze  $\beta$ -casein to  $\beta$ -CM-7 or  $\beta$ -casomorphin precursors, as well as to numerous other bioactive peptides [1,20,21]. In this context, the main objective of our project was to determine if opioid peptides (or the precursors thereof, or possibly other peptides) present in yoghurts are able to stimulate the production of intestinal mucins, which would then make these dairy products a valuable way for increasing the intestinal protection. For this purpose, the total peptide pool (TPP) from a marketed yoghurt and four fractions obtained by preparative reversed-phase high-performance liquid chromatography (RP-HPLC) from this pool were tested *in vitro* in a human mucus-secreting cell line (HT29-MTX).  $\beta$ -casein f94-123 [ $\beta$ -CN(94-123)], a precursor of the sequence of neocasomorphin-6, was detected only in the active fraction and was assumed to be the active peptide. Accordingly, it was tested in the same cell line but also in rat pups to determine its *in vivo* effects and to study its impact on several actors of intestinal protection.

## 2. Materials and methods

### 2.1. Peptide fraction collection from yoghurt

#### 2.1.1. Preparation of the TPP from marketed yoghurt

Commercial yoghurt was manually mixed and centrifuged at 5000g for 30 min at room temperature. The pellet was discarded, and the peptide fraction was isolated from the supernatant by addition of 2% trichloroacetic acid (TCA) (w/v, final concentration). After 30-min equilibration, the sample was centrifuged at 5000g for 10 min. The supernatant containing TPP was filtered through Whatman no. 41 filter

paper to remove floating particles. Part of this solution was directly frozen and used for fractionation. The other part was dialyzed against osmotic water and lyophilized.

#### 2.1.2. Fractionation of the TPP by chromatography

One hundred twenty milliliters of TPP was filtered through 1.2- $\mu$ m pore filters and then separated on a preparative C18 column (LiChrospher RP18, 12  $\mu$ m, Merck, 250 $\times$ 50 mm) equilibrated in 1.06% trifluoroacetic acid (TFA; Sigma-Aldrich, St-Quentin Fallavier, France). Elution was achieved at 45 ml/min with a multistep gradient of acetonitrile in 1% TFA. The absorbance was monitored at 214 nm. Five fractions, named C1 to C5, eluted at 12%, 24%, 36%, 48% and 80% of acetonitrile, respectively, were collected and lyophilized at neutral pH. C1 fraction was found in too low quantity to be analyzed.

#### 2.1.3. Analysis of peptides by RP-HPLC

TPP and fractions were analyzed by RP-HPLC on a Waters HPLC system (Waters 2695 Separators Module, Alliance, Waters, Milford, MA, USA) with a narrow-bore symmetry C18 column (5- $\mu$ m particle size, 2.1 $\times$ 150 mm, Waters) equipped with a C18 cartridge guard. Solvent A contained 1.06% (v/v) TFA in Milli Q water, and solvent B contained 1% (v/v) TFA in acetonitrile–Milli Q water (80:20).

Separation of peptides was obtained using a linear gradient from 0% to 60% of solvent B for 60 min. The column was maintained at 40°C, the flow rate was 0.25 ml/min, and eluted peaks were detected by UV absorbance at 214 and 280 nm (Waters 2487 Dual  $\lambda$  Absorbance Detector). Before analysis, samples were first diluted in solvent A and then filtered through 0.45- $\mu$ m pore filters. For quantification of  $\beta$ -CN(94-123), the mass spectrometer was used as a detector. The surface of specific ions resulting from the ionization of different concentrations of the chemically synthesized peptide (GENOSPHERE Biotechnologies, Paris, France) was determined and used as a calibration curve.

#### 2.1.4. Identification of peptides by liquid chromatography–tandem mass spectrometry (LC-MS-MS)

Peptides were identified using nano-LC-MS-MS as described by Boutroux et al. [22], with some modifications. Samples were diluted in 1% TFA, and 5  $\mu$ l was injected into a capillary column. The separation was achieved with a linear gradient from 5% to 50% solvent B for 45 min. Solvent A contained 2% acetonitrile, 0.8% formic acid and 0.1% TFA in LC-grade water, and solvent B contained 95% acetonitrile, 0.8% formic acid and 0.1% TFA in LC-grade water. Peptides were then identified using the Mascot MS/MS ion search V.2.2. from Matrix Science (Boston, MA, USA) against a homemade database dealing with major milk proteins.

## 2.2. *In vitro* studies

HT29-MTX, a human colon carcinoma-derived mucin-secreting goblet cell line, was grown in 25-cm plastic culture flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 mg/ml penicillin–streptomycin at 37°C in a 5% CO<sub>2</sub> atmosphere in a humidified incubator as previously described [7]. To study the effect of peptide pool and fractions, cells were seeded in 12-well culture plates. Experiments were performed 21 days after cells reached confluency (see Supplemental data). All experiments were performed at least three times in triplicate.

## 2.3. *In vivo* studies

### 2.3.1. Animals

Primiparous timed-pregnant Wistar female rats were purchased (Janvier ; route des chènes secs, 53940 Le Genest Saint Isle, France) on gestational day 14. Dams were individually housed on a 12:12-h light–dark cycle with free access to food and water. The day of birth was considered to be postnatal day 1. Litters were culled to nine pups. All procedures were approved by the regional ethics committee for animal experiment (Rhône Alpes, approval no. 0195).

### 2.3.2. Experimental protocol

Peptide  $\beta$ -CN(94-123) was synthesized from GENOSPHERE Biotechnologies. The daily required amount of the drug was freshly dissolved in sterile water for the gavage procedure or in culture medium for *in vitro* studies.

Rat pups ( $n=9$  per group) were randomly assigned to receive treatment by gastric gavage (10  $\mu$ l/g body weight) with either water (control group, CT) or  $\beta$ -CN(94-123) at 0.01, 0.1 and 100  $\mu$ mol/L every day from postnatal day 10 to postnatal day 18. Treatment was delivered at the same time every day to minimize the effects of circadian rhythm. Body weight was recorded daily. Two hundred forty minutes after the last administration, rats were deeply anesthetized with pentobarbital sodium. Intestinal samples were taken for immunohistological and biochemical studies. RNA and protein were obtained from the same sample by using Nucleospin RNA/Protein kit (Macherey-Nagel GmbH & Co KG, Germany) according to the manufacturer's protocol.

### 2.4. Enzyme-linked lectin assay (ELLA) for mucins from cell culture media

An ELLA was used to measure mucin-like glycoprotein secretion as described in the Supplemental data. The amount of glycoprotein secreted in the incubation medium

Table 1  
Antibodies used for Western blot analysis

Antigen	Species	Source	Dilution
MUC4	Mouse	Abcam (ab60720)	1:1000
Lysozyme	Rabbit	Interchim (GTX72913)	1:250
Cyclophilin A	Rabbit	Cell signaling Technology (2175)	1:1000

was expressed as nanograms of mucin-like glycoprotein per  $10^6$  cells, and results are given as percent of controls.

### 2.5. Quantitative real-time polymerase chain reaction (PCR) analysis

Measures were performed with the real-time fluorescence detection method using the ep realplex Mastercycler (Eppendorf) with Maxima SYBR Green qPCR Master Mix (Fermentas) in PCR plates (see Supplemental data). Calculations were performed according to the  $2^{-\Delta\Delta Ct}$  method with correction for efficiency, and the final value was adjusted so that controls had a mean relative mRNA level of 1 [23,24].

### 2.6. Western blot analysis

Proteins (30  $\mu$ g) were separated using Nupage gel bis-Tris 10% (lysozyme, cyclophilin) or Tris acetate 3%–8% (Muc4) and then transferred onto polyvinylidene difluoride (PVDF) membrane using an XCELL II Blot Module (Invitrogen Life Technologies SAS, Villebon sur Yvette, France). The membrane was incubated with antibodies directed against a mouse monoclonal anti-MUC4 antibody, a rabbit polyclonal anti-lysozyme antibody or a rabbit polyclonal anti-cyclophilin A antibody. Primary antibodies are listed in Table 1. Bound antibodies on the PVDF membrane were detected using WesternBreeze Immunodetecting Chemiluminescent System (Invitrogen). The optical density of protein bands was visualized and pixelized with the "Image System" (ImageMaster VDS-CL, Amersham Bio-sciences) and densitometrically analyzed with Quantity one image analysis software (Biorad Laboratory, Hercules, CA, USA).

### 2.7. Histological examination of intestine for goblet and Paneth cells (PCs) determination

Intestinal tissues were removed, fixed in 90% ethanol for 24 h at  $-20^{\circ}\text{C}$  and processed into paraffin. Serial paraffin sections (4  $\mu$ m) were rehydrated and treated as described in the Supplemental data. All slides were analyzed by a single investigator who was blinded to the treatment groups. The histological features shown are representative of all tissue samples studied.

### 2.8. Statistical analysis

*In vitro* data were compared using repeated-measures analysis of variance (ANOVA) followed by the Mann–Whitney *U* test when appropriate or Mann–Whitney test alone for single comparisons. In dose–effect studies performed *in vivo*, data were analyzed by one-way ANOVA followed by Bonferroni test when appropriate. Western blot data obtained after gavage treatment were compared by Mann–Whitney test. All the data are expressed as mean  $\pm$  S.E.M. Differences with  $P < .05$  were considered significant. Statistical analyses were performed with XLSTAT, Version 2009.4.06 (Addinsoft, Paris, France).

## 3. Results

### 3.1. The peptide pool from a marketed yoghurt stimulated mucin secretion and expression in the human mucin-secreting cell line HT29-MTX

The peptidic fraction of marketed yoghurt was recovered in the supernatant after centrifugation. The TPP corresponded to the 2% TCA-soluble fraction. On the basis of their total nitrogen content as determined by a micro-Kjeldahl analysis [25], TPP represented about 10%–13% of the total protein nitrogen of yoghurt. As shown by analytical RP-HPLC analysis (Fig. 1A), the elution pattern of the TPP was very complex, with numerous peaks arising from the protease and peptidase action during milk fermentation. To assess the effect of TPP on goblet cells and mucin production, we used the human intestinal HT29-MTX cell line which secretes mucin in response to various stimulants [7]. These cells also exhibited immunopositivity for  $\mu$ -opioid receptors on their cell membranes [7]. The major mucin produced by HT29-MTX cells is the secreted mucin MUC5AC. We also studied MUC2 and MUC4 which represent, respectively, the major intestinal secreted mucin and one of the transmembrane-associated

mucins. Cell cultures were exposed to different concentrations of TPP (Y/5, Y/2 and Y) for 4 h. The Y concentration corresponds to the concentration of these peptides in the initial yoghurt. As shown in Fig. 1B, TPP induced the secretion of mucin-like glycoprotein (maximal response:  $192\% \pm 3\%$  of controls,  $P < .05$ ). The effect of TPP on the expression of MUC2, MUC5AC and MUC4 was then evaluated. Quantitative reverse transcriptase (RT)-PCR showed that TPP induced a dose-dependent increase in MUC2 and MUC4 mRNA levels compared with controls (Supplemental Fig. 1). The maximal response was obtained with Y concentration ( $220\% \pm 24\%$  and  $177\% \pm 12\%$  of controls for MUC2 and MUC4, respectively;  $P < .05$ ). In contrast, TPP did not modify the expression of MUC5AC. These results suggest that, tested at a level consistent with that found in yoghurt, the TPP is able to modulate mucin production.

### 3.2. Selective effect of a peptide fraction obtained from the peptide pool on mucin secretion and expression in HT29-MTX cells

TPP was then separated by preparative RP-HPLC. The profiles of fractions are presented in Fig. 2A. The fraction C1 corresponded to the

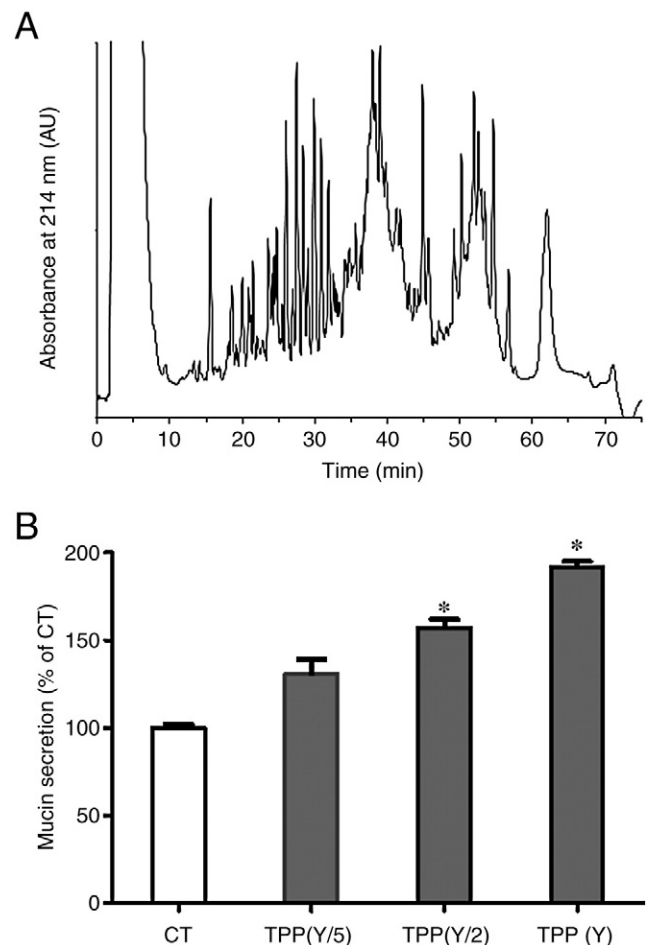


Fig. 1. TPP from yoghurt (see Methods) stimulates mucin glycoprotein secretion in HT29-MTX cells. (A) RP-HPLC elution profile of the TPP of a marketed yoghurt. Samples were analyzed by RP-HPLC on a Symmetry column (150  $\times$  2.1 mm i.d.), and elution was performed at a flow rate of 0.25 ml/min at  $40^{\circ}\text{C}$ , with a linear gradient from 0% to 60% of solvent B for 60 min. Solvent A contained 0.106% TFA (v:v) in Milli-Q water, and solvent B contained 0.1% TFA (v:v) and 80% acetonitrile (v:v) in Milli-Q water. (B) Dose response for TPP stimulation (4 h) of mucin glycoprotein release in HT29-MTX cells. The Y concentration corresponds to the content of TPP in the initial yoghurt. The amount of mucin glycoproteins in culture media was measured by ELLA. The data are expressed as mucin glycoproteins secretion in percent of control. Each point represents mean  $\pm$  S.E.M. of three to four experiments performed in triplicate. \* $P < .05$  vs. controls.

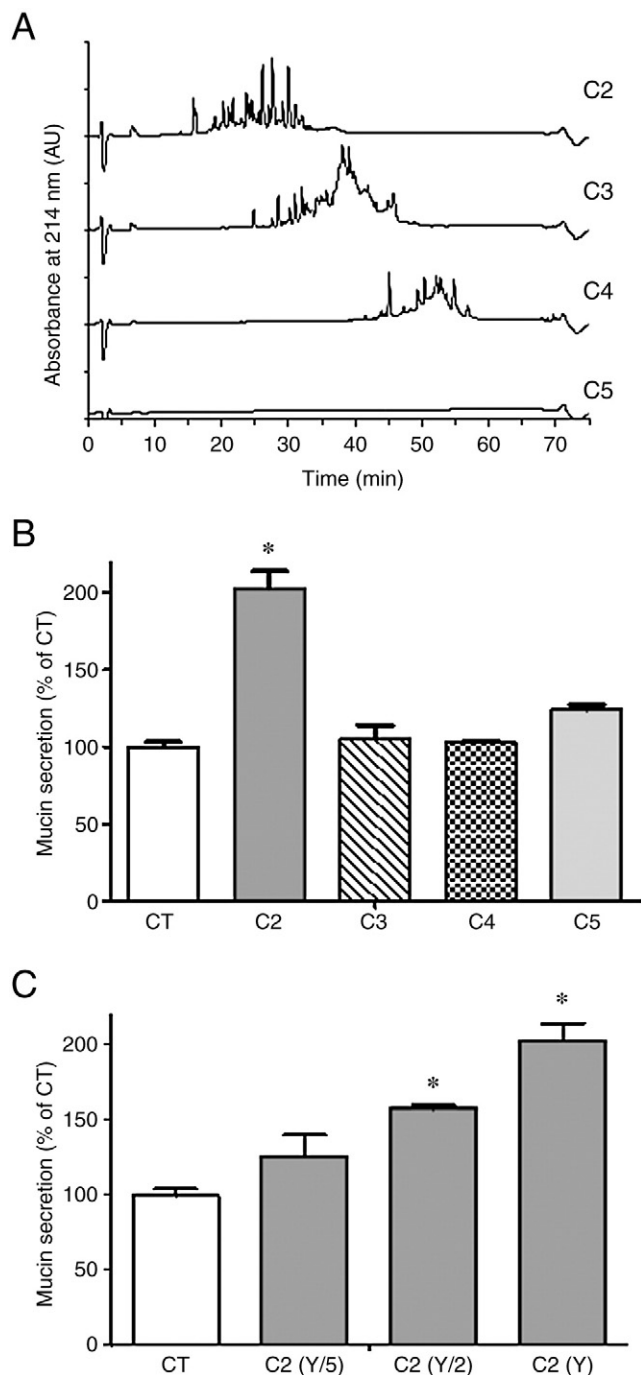


Fig. 2. C2 fraction stimulates mucin glycoprotein secretion in HT29-MTX cells. (A) RP-HPLC elution profile of peptide fractions (C2 to C5). See Fig. 1 for details. (B) Effect of different fractions (C2 to C5) of the peptide pool on mucin glycoprotein secretion (Y concentration, 4 h). The Y concentration corresponds, for each fraction, to the content of peptides in the initial yoghurt. The amount of mucin glycoproteins in culture media was measured by ELLA. The data are expressed as mucin glycoproteins secretion in percent of control. Each point represents mean $\pm$ S.E.M. of three to four experiments performed in triplicate. \* $P$ <.05 vs. controls. (C) Dose response for C2 fraction stimulation on mucin glycoprotein secretion. Each point represents mean $\pm$ S.E.M. of three to four experiments performed in triplicate. \* $P$ <.05 vs. controls.

elution of TCA. The whole peptide profile was recovered in four fractions (C2, C3, C4 and C5). The identification of peptides, by using LC-MS-MS, showed that C2, C3 and C4 contained a mixture of about 60 identified peptides, of which more than half (50%–80%) were derived from  $\beta$ -casein. In the C5 fraction, only six peptides were

identified, of which five were from  $\beta$ -casein. Table 2 is a selection of identified peptides, focused on precursors of  $\beta$ -CM-7 and neocasomorphin-6, i.e., containing the [60-66] and [114-119] region of  $\beta$ -casein, respectively. In C2 fraction, we identified three peptides ( $\beta$ -casein f94-123, f102-123 and f106-123) which may be precursors of the agonist opioid neocasomorphin-6 (sequence [114-119] of  $\beta$ -casein) [4]. The C3 fraction contained five potential precursors of neocasomorphin-6 ( $\beta$ -casein f100-123, f102-123, f106-119, f106-123 and f108-123) and three peptides ( $\beta$ -casein f57-68, f57-72 and f59-72) which are precursors of  $\beta$ -CM-7 ( $\beta$ -casein f60-66). Three peptides containing the sequence [60-66] of  $\beta$ -casein were identified in fraction C4 ( $\beta$ -casein f57-77, f57-91 and f57-93). The C5 fraction contained none in the zones of interest.

The C2–C5 fractions collected were tested for their mucin production activity in HT29-MTX cells. As shown in Fig. 2B, the overall release of mucin-like glycoprotein was raised after 4 h of exposure to C2 fraction only. This effect of C2 was dose dependent (Fig. 2C). Quantitative RT-PCR analysis also revealed that C2 fraction significantly increased *MUC2* and *MUC4* mRNA levels as compared with controls (Supplemental Fig. 2). As observed in Table 2, the peptide sequence [94-123] of  $\beta$ -casein is the only peptide present only in the active fraction C2. It may therefore be responsible for the observed biological effect. It was named peptide  $\beta$ -CN(94-123).

### 3.3. Peptide $\beta$ -CN(94-123), a peptide we detected in yoghurts, regulated the secretion and the expression of mucins in HT29-MTX cells

The presence of  $\beta$ -CN(94-123) was investigated in different marketed regular yoghurts, i.e., fermented with only *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*.  $\beta$ -CN(94-123) was detected in 80% of tested yoghurts at concentrations ranging between 0.3 and 1.7  $\mu$ mol/L. The identification failed on some yoghurts, probably because the concentration of peptide was below the detection threshold. Hence, this peptide seems to be released by the bacteria used in the manufacture of yoghurts. Accordingly, the peptide corresponding to the sequence of  $\beta$ -Casein f94-123 (GVSKVKEAMAPKHKEMPPFKYPVEPFTESQ) was chemically synthesized and tested on HT29-MTX cells.

As shown in Fig. 3A,  $\beta$ -CN(94-123) (0.01–1  $\mu$ mol/L) dose-dependently induced mucin-like glycoprotein secretion. Peptide  $\beta$ -CN(94-123) also increased the expression of *MUC2* and *MUC4* after 4 h of stimulation (Fig. 3B and 3C) but not that of *MUC5AC* (data not shown). The first significant increase in transcripts was observed with a concentration of 0.01  $\mu$ mol/L for *MUC2* and *MUC4*. These data indicate that  $\beta$ -CN(94-123), tested at concentrations close to those detected in yoghurts, was able to reproduce the effect of the TPP and of the relevant C2 fraction. In contrast,  $\beta$ -CN(94-123), tested at a concentration of 100  $\mu$ mol/L, inhibited the expression of *MUC4*.

In an attempt to determine the mechanisms involved in the effect of  $\beta$ -CN(94-123), HT29-MTX cells were pretreated for 30 min at 37°C with the mu-opioid antagonist cyprodime ( $10^{-5}$  mol/L) before addition of 0.1  $\mu$ mol/L  $\beta$ -CN(94-123) for 4 h. Interestingly, the effects of  $\beta$ -CN(94-123) on HT29-MTX cells were not prevented by pretreatment with the mu-opioid antagonist (data not shown).

### 3.4. In vivo effects of peptide $\beta$ -CN(94-123)

To determine the physiological relevance of the findings obtained from cultured goblet-like cells, pups received synthetic peptide  $\beta$ -CN(94-123) (0.01, 0.1 and 100  $\mu$ mol/L) or vehicle alone (CT rats) by oral gavage once daily for 9 consecutive days. No significant difference in body weight was observed between rats treated with the peptide (TP rats) and CT rats. The morphology of the intestine of rats subjected to  $\beta$ -CN(94-123) treatment was also normal. We assessed mucosal proliferation by determining proliferating cell nuclear

Table 2  
Identification by nano-LC-MS-MS of peptides present in fractions obtained from a marketed fermented milk

Fraction	Region	$\beta$ -casein sequence	Peptide sequence	Molecular weight
C2	Opioid region	f94-123	GVSKVKEAMAPKHKEMPPFKYPVEPFTESQ	3417.9
		f102-123	MAPKHKEMPPFKYPVEPFTESQ	2619.0
		f106-123	HKEMPPFKYPVEPFTESQ	2191.4
C3	Pro- $\beta$ -CMs	f57-68	SLVYFPGPIPN	1300.5
		f57-72	SLVYFPGPIPNSLPQ	1726.0
		f59-72	VYFPGPIPNSLPQ	1525.7
	Opioid region	f100-123	EAMAPKHKEMPPFKYPVEPFTESQ	2819.2
		f102-123	MAPKHKEMPPFKYPVEPFTESQ	2619.0
		f106-119	HKEMPPFKYPVEPF	1746.0
		f106-123	HKEMPPFKYPVEPFTESQ	2191.4
		f108-123	EMPPFKYPVEPFTESQ	1926.1
		f57-77	SLVYFPGPIPNSLPQNIPPL	2260.6
C4	Pro- $\beta$ -CMs	f57-91	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQPE	3794.4
		f57-93	SLVYFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM	4024.7

Table 2 shows a selection of peptides identified in each fraction, focused on precursors of  $\beta$ -CM-7 and neocasomorphin-6. In C5, no peptides of interest have been found.

antigen in crypt cells at the end of the oral treatment. No difference in the level of epithelial proliferation was detected between TP and CT rats (data not shown).

#### 3.4.1. Goblet cells and mucins

The histological examination of intestinal slices revealed that  $\beta$ -CN(94-123) (0.1  $\mu$ mol/L) induced an increase in the number of duodenal goblet cells per crypt-villus axis as compared with controls (Table 3). This increase in the density of goblet cells was also observed in the jejunum and ileum but not in the colon (Table 3 and Supplemental Fig. 3). Quantitative RT-PCR analysis indicated that  $\beta$ -CN(94-123) modified the expression of *Muc2* in the rat small intestine (Table 4). Peptide gavage also induced a significant increase in duodenal, jejunal and ileal *Muc4* mRNA expression (Table 5). At the protein level, Western blot analysis confirmed the increased expression of *Muc4* in intestinal epithelium of rat receiving  $\beta$ -CN(94-123) compared to controls ( $P < .05$ ) (Fig. 4).

#### 3.4.2. PCs and antimicrobial molecules

A characteristic feature of small intestinal host defense is the presence of PCs at the bottom of the crypts of Lieberkühn. Oral administration of  $\beta$ -CN(94-123) (100  $\mu$ mol/L) increased the percentage of crypts showing PCs along the intestinal tract (Supplemental Fig. 3 and Fig. 5A), which would suggest an improved state of mucosal protection. The effect of  $\beta$ -CN(94-123) on the percentage of crypts exhibiting PCs was retained when the peptide was administered at the concentration of 0.1  $\mu$ mol/L (duodenum) or 0.01  $\mu$ mol/L (jejunum, ileum). As shown in Fig. 5B, a marked increase in expression of *rat defensin 5 (rdefa5)* mRNA transcripts was also observed in the duodenum, jejunum and ileum of TP rats. Likewise, *lysozyme* mRNA transcripts were significantly augmented in the small intestine of TP rats when compared with CT intestinal tissue (data not shown). Western blot analysis confirmed the increased expression of lysozyme in intestinal epithelium of rat receiving  $\beta$ -CN(94-123) compared to controls ( $P < .05$ ) (Fig. 5C).

#### 3.4.3. Enteroendocrine cells

Because, in the small intestine, enteroendocrine cells, goblet cells and PCs arise from a common secretory progenitor [26], the distribution of enteroendocrine cells was examined with the pan-endocrine marker chromogranin A.  $\beta$ -CN(94-123) did not modify the number of enteroendocrine cells in the small intestine or in the colon (data not shown). These data indicate that  $\beta$ -CN(94-123) specifically changes the quantitative distribution of intestinal cells of secretory lineage that are directly involved in protection of the intestinal mucosa.

## 4. Discussion

The intestinal barrier, an ultimate interface maintaining the whole intestinal homeostasis, is a functional unit including the epithelial layer itself and factors such as peristalsis, gut microbiota, mucus and different secreted constituents such as defensins, lysozymes and immunoglobulins. Among these factors, intestinal mucus plays a critical role in the protection against acidic aggression, luminal proteases, mechanical insults, colonization by pathogenic bacteria and potential carcinogens [8,27,28]. The major component of the intestinal mucus is the gel-forming mucin MUC2, which is produced by goblet cells of the epithelium [29]. It should be noted that a second class of mucins, transmembrane-associated mucins, also plays a role in the protection of epithelia, in particular by providing a steric barrier that can limit direct access of pathogens [30]. They are anchored to the apical cell surface of epithelial cells and are a major component of the glycocalyx. In the intestine, MUC4 is one of such prominent transmembrane-associated mucins [31]. In the present study, we showed that proteolysis of caseins by lactic bacteria (*L. delbrueckii* ssp. *bulgaricus* and *S. salivarius* ssp. *thermophilus*) as fermented in yoghurt leads to the formation of many peptides including precursors of  $\beta$ -CMs or of neocasomorphin-6. We also established that the TPP obtained from a marketed yoghurt induces mucin secretion and the expression of *MUC2* and *MUC4*. These effects were dose-dependent, the maximum response being observed when the peptide pool was administered at a concentration corresponding to that found in yoghurts. Our results thus revealed that peptides active on mucus cells are produced in sufficient quantity in this fermented dairy product to modulate *in vitro* the secretion and expression of gastrointestinal mucins. Surprisingly, we failed to detect mucin-like glycoprotein secretion in response to C3, C4 or C5 fractions (despite the fact that C3 and C4 fractions contained peptides with the sequence of  $\beta$ -CM-7 inside). This implies that the components responsible for the biological effect are specific to the C2 fraction. We succeeded in identifying the sequence [94-123] of  $\beta$ -casein, present only in C2 fraction, as most likely the active molecule of this fraction and of the yoghurt. Indeed,  $\beta$ -CN(94-123) increased production of the intestinal secreted mucin MUC2 and of the transmembrane-associated mucin MUC4 when tested *in vitro* (0.01–1  $\mu$ mol/L) on human intestinal HT29-MTX goblet-like cells. Interestingly,  $\beta$ -CM-7, which is the reference milk bioactive peptide, enhanced intestinal mucins production only when administered at 100  $\mu$ mol/L [7]. Most importantly, we demonstrated that the effect of  $\beta$ -CN(94-123) was also observed *in vivo*. Indeed, one daily oral administration of  $\beta$ -CN(94-123) (0.1–100  $\mu$ mol/L) in rats between postnatal days 10 and 18 significantly increased the number of goblet cells along the small intestine. These effects were associated with an enhanced expression of intestinal mucins (*Muc2* and *Muc4*). This is of

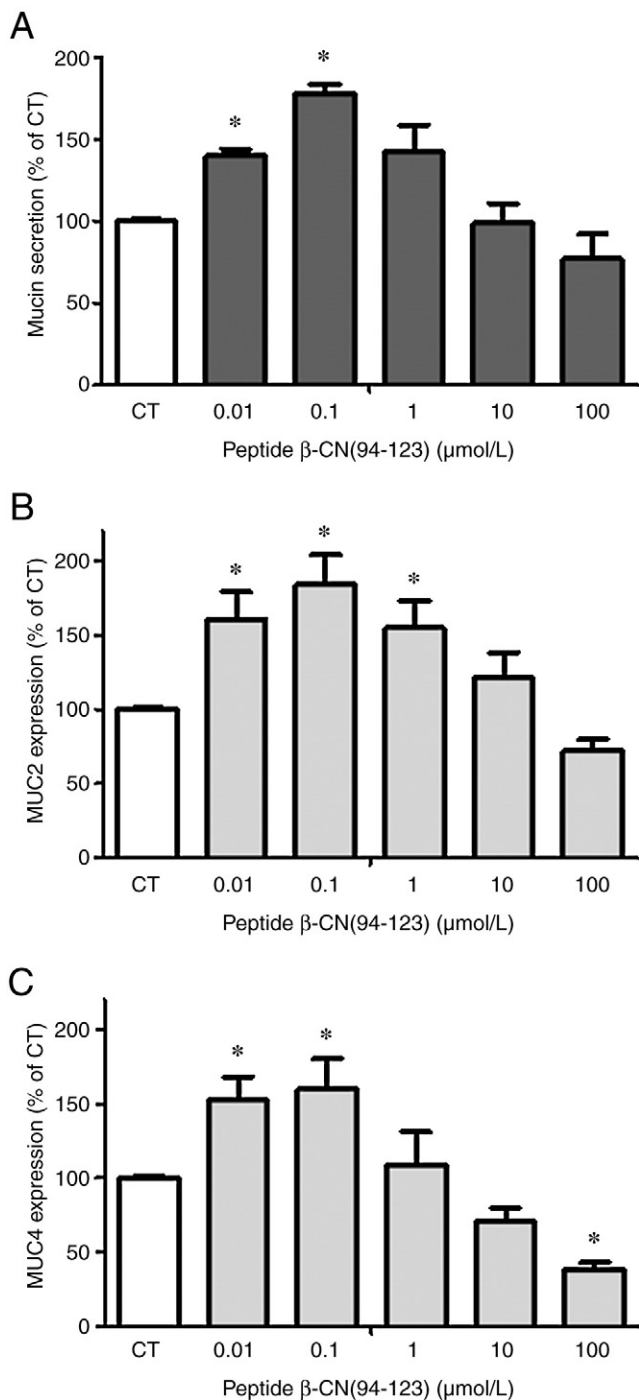


Fig. 3. Peptide  $\beta$ -CN(94-123) dose dependently increases mucin-like glycoprotein release as well as the level of *MUC2* and *MUC4* mRNA in HT29-MTX cells (4-h exposition). Each bar represents the mean percent increase vs. controls  $\pm$  S.E.M. of four experiments performed in triplicate. \* $P < 0.05$  vs. controls. (A) The amount of mucin glycoproteins in culture media was measured by ELLA. (B and C) Total RNA was isolated, and mucin mRNA levels (*MUC2* and *MUC4*) were analyzed by quantitative RT-PCR. Quantitative RT-PCR products of cyclophilin A mRNA, used as internal control, were unaffected by  $\beta$ -CN(94-123).

great consequence as, although biological activity of some food products may be demonstrated *in vitro*, activity may be lost *in vivo* after ingestion as the bioactive peptide may be degraded during digestion or may not attain active concentrations in target tissues. It should be noted however that  $\beta$ -CN(94-123) had no effect on colonic mucus cells. This could be due to the fact that dietary peptides that

Table 3  
Quantitative immunohistochemistry (number of goblet cells) after oral administration of the peptide  $\beta$ -CN(94-123)

	Duodenum (number of mucus cells/ crypt-villus axis)	Jejunum (number of mucus cells/ crypt-villus axis)	Ileum (number of mucus cells/ crypt-villus axis)	Colon (number of mucus cells/crypt)
Control group	6.65 $\pm$ 0.11	7.75 $\pm$ 0.14	7.89 $\pm$ 0.14	9.90 $\pm$ 0.31
Peptide (0.01 $\mu$ mol/L)	6.57 $\pm$ 0.23	8.01 $\pm$ 0.18	7.61 $\pm$ 0.18	9.22 $\pm$ 0.13
Peptide (0.1 $\mu$ mol/L)	8.86 $\pm$ 0.15*	9.30 $\pm$ 0.25*	9.79 $\pm$ 0.09*	10.04 $\pm$ 0.02
Peptide (100 $\mu$ mol/L)	6.61 $\pm$ 0.16	11.12 $\pm$ 0.17*	10.54 $\pm$ 0.17*	9.61 $\pm$ 0.05

Counts were performed on tissue sections ( $\times 20$  magnification) and were expressed as the mean number of goblet cells per intestinal crypt-villus axis (means  $\pm$  S.E.M.,  $n = 8$ ) or per colonic crypt (S.E.M.,  $n = 8$ ).

\*  $P < 0.05$  vs. controls.

escape digestion and enter the large intestine are rapidly broken down by colonic microbiota.

Remarkably enough,  $\beta$ -CN(94-123) (0.01–100  $\mu$ mol/L) also enhanced the number of PCs in the duodenum of rats. A significant effect on PCs was even observed in the jejunum and in the ileum as soon as the concentration of 0.01  $\mu$ mol/L. This increase in PCs was associated with a rise of *lysozyme* and *rdefa5* expression. In agreement with our results, Thoreux et al. demonstrated that the number of PCs significantly rose in the three portions of the small intestine of mice receiving a diet supplemented with yoghurt [32]. The mechanism responsible for this effect was not further investigated however. Our study is thus the first to identify a bioactive molecule that regulates this epithelial cell population. PCs are key effectors of innate immunity. These epithelial cells are located at the base of the crypts of the small intestine and are characterized by the presence of apical secretory granules containing high levels of antimicrobial factors such as lysozyme,  $\alpha$ -defensins (cryptdins in mice), phospholipase A2 and the C-type lectins RegIII $\gamma$  [33,34]. In humans, the  $\alpha$ -defensins found in PCs are  $\alpha$ -defensin-5 and -6 (HD-5 and HD-6) [35]. PCs play an essential role in controlling intestinal barrier penetration by pathogenic or commensal bacteria, in regulating the intestinal microbiota and in maintaining homeostasis of the small intestinal stem cell niche [36–39]. It should be noted that antibacterial factors secreted by PCs are retained by the mucus barrier, which allows it to regulate bacteria that are in close contact with the intestinal surface. The simultaneous stimulation of goblet cells and PCs induced by  $\beta$ -CN(94-123) should thus synergistically decrease susceptibility to microbial invasion and enhance intestinal protection. Interestingly, several studies suggested that consumption of fermented milks may reinforce mucosal integrity of the intestine. For example, dietary supplementation with milk fermented by *L. casei* DN-114 001 strongly decreased the diarrhea symptoms, infection and injuries of the intestinal epithelial cells in a model of suckling rats infected with rotavirus [40]. Vinderola et al. also demonstrated the effectiveness of the nonbacterial fraction of fermented milk in prevention against *Salmonella typhimurium* infection [41]. The mechanisms proposed to explain the fermented milk properties included improvement of the lactose digestion and of

Table 4  
RT-PCR analysis of *Muc2* after oral administration of  $\beta$ -CN(94-123) from postnatal day 10 to 18

	Duodenum	Jejunum	Ileum	Colon
Control group	100.0 $\pm$ 1.3	100.0 $\pm$ 1.2	100.0 $\pm$ 1.7	100.0 $\pm$ 2.7
Peptide (0.01 $\mu$ mol/L)	83.2 $\pm$ 0.8	102.3 $\pm$ 1.8	98.0 $\pm$ 1.4	115.1 $\pm$ 3.9
Peptide (0.1 $\mu$ mol/L)	84.0 $\pm$ 1.3	152.2 $\pm$ 1.1*	104.3 $\pm$ 1.1	107.4 $\pm$ 2.5
Peptide (100 $\mu$ mol/L)	90.1 $\pm$ 1.0	139.5 $\pm$ 1.8*	84.8 $\pm$ 1.0	121.2 $\pm$ 1.5

The expression of each gene was normalized to the reference gene level in each sample. The results are expressed as the percentage of CT rats (means  $\pm$  S.E.M.,  $n = 8$  per group).

\*  $P < 0.05$  vs. controls.

Table 5  
RT-PCR analysis of *Muc4* after oral administration of  $\beta$ -CN(94-123) from postnatal day 10 to 18

	Duodenum	Jejunum	Ileum	Colon
Control group	100.0 $\pm$ 1.7	100.0 $\pm$ 2.8	100.0 $\pm$ 2.1	100.0 $\pm$ 1.2
Peptide (0.01 $\mu$ mol/L)	156.5 $\pm$ 1.3*	160.3 $\pm$ 1.4*	157.2 $\pm$ 2.2*	102.8 $\pm$ 3.4
Peptide (0.1 $\mu$ mol/L)	120.1 $\pm$ 1.5	94.7 $\pm$ 1.2	84.1 $\pm$ 2.7	92.6 $\pm$ 5.6
Peptide (100 $\mu$ mol/L)	121.0 $\pm$ 1.4	161.4 $\pm$ 2.1*	113.1 $\pm$ 1.7	103.0 $\pm$ 4.7

The expression of each gene was normalized to the reference gene level in each sample. The results are expressed as the percentage of CT rats (means $\pm$ S.E.M., n=8 per group). \* P<.05 vs. controls.

the intestinal ecology by their antibacterial and immunostimulating effects. Our data provide an alternative explication, showing that bioactive peptides such as  $\beta$ -CN(94-123) may play a major role in this protective effect. Works are in progress in our laboratory to validate this hypothesis by analysis of the effects of  $\beta$ -CN(94-123) in several models of intestinal epithelial alterations.

How could we explain the effects of  $\beta$ -CN(94-123) on goblet cells and PCs? An exciting possibility will be that  $\beta$ -CN(94-123) could induce changes in epithelial differentiation since we found a significant rise in the density of goblet cells and PCs in the small intestine of treated rat. Furthermore, the lack of effect of  $\beta$ -CN(94-123) on enteroendocrine cells as well as on proliferation of epithelial cells in the small intestinal mucosa could suggest a specific action on the two cell lineages directly involved in intestinal protection. To confirm this hypothesis, further studies will be conducted to determine the impact of the peptide on the expression of transcription factors (Hath1, Gfi1, Klf4,...) involved in the differentiation of goblet cell and PC lineages [26]. However, we cannot exclude that  $\beta$ -CN(94-123) could increase transcription of the gel-forming mucin *MUC2* in already differentiated goblet cells of the intestine since, *in vitro*, it can enhance the expression of this mucin after direct application on differentiated intestinal goblet-like cells HT29-MTX.

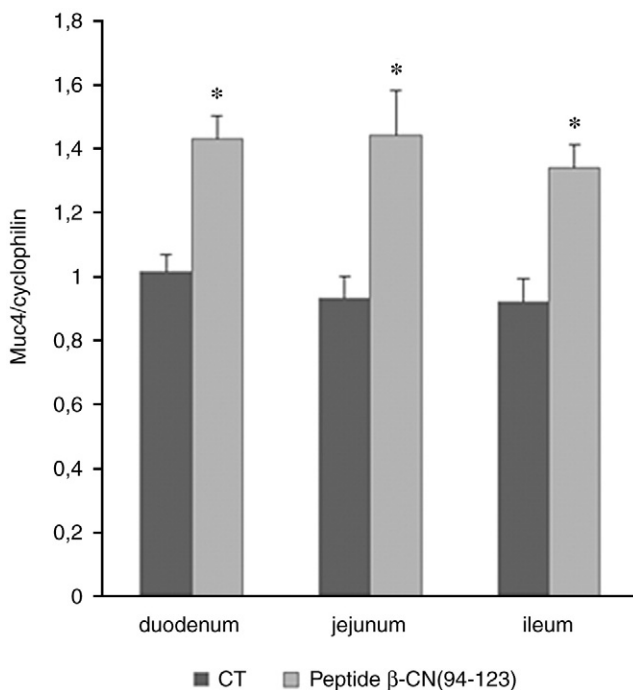


Fig. 4. Western blot analysis of *Muc4* in the small intestine after administration of  $\beta$ -CN(94-123) (0.01  $\mu$ mol/L, 9 days). The expression of *Muc4* was normalized to the reference gene level (cyclophilin A) in each sample. Data are mean $\pm$ S.E.M. \*P<.05 vs. controls (n=8 per groups). CT: control rat; TP: rat treated with the peptide.

It is interesting to note that the effect of  $\beta$ -CN(94-123) observed *in vivo* on *Muc2* and *Muc4* expression was very similar to that obtained when tested *in vitro* on HT29-MTX cells, suggesting a direct action of  $\beta$ -CN(94-123) through receptors located on the intestinal goblet cells. Consequently, it is likely that  $\beta$ -CN(94-123) or a shorter active form resulting from its partial digestion resists the action of digestive enzymes and reaches the duodenum but also the distal part of the small intestine. The length of the peptide makes the identification of the active sequence difficult. One possible candidate for the activation of goblet cells could have been the sequence  $\beta$ -CN f114-119 (neocasomorphin-6), which was previously identified as an opioid receptor agonist [26]. However, we can exclude in our study the involvement of the mu-opioid pathway. Indeed, the effect of  $\beta$ -CN(94-123) on mucin production *in vitro* was not inhibited by cyprodime, an antagonist of the mu-opioid pathway. Furthermore, we previously showed that  $\beta$ -CM-7, an opioid peptide, induces the expression of *MUC5AC* in HT29-MTX cells, while our peptide had no effect on this mucin [7]. At last, the effects of  $\beta$ -CN(94-123) on goblet cells and PCs could be explained by the amino acid composition of this peptide. Interestingly,  $\beta$ -CN(94-123) contains 30 amino acids including 5 proline residues, making it more resistant to degradation by digestive enzymes. This peptide that we identified in several marketed yoghurts at concentrations ranging between 0.3 and 1.7  $\mu$ mol/L thus seems also to be resistant to degradation by enzymes of lactic acid bacteria. Now, if we consider the activity of other preponderant amino acids (E, K) of  $\beta$ -CN(94-123) on intestinal functions, we can note that protective effects of glutamic acid have been characterized. Luminal L-glutamate (1 and 10 mmol/L) was demonstrated to enhance mucus gel thickness via the activation of afferent nerves [42]. Therefore, one can hypothesize that the peptide could be hydrolyzed to amino acids and then L-glutamate triggers the activation of mucus cells. However, as the peptide is administered at very low doses, it is unlikely that L-glutamate could be present in the intestinal lumen in sufficient quantities. Moreover, this indirect effect described by Akiba et al. involved a nervous pathway and therefore cannot explain the action of the peptide on HT29-MTX cells. Other studies are needed to determine the mechanisms by which peptide  $\beta$ -CN(94-123) exerts its activity. It thus will be possible to optimize the nutritional and health effects of this molecule. Furthermore, having identified this bioactive peptide present in yoghurts is an exciting challenge for trying to boost its synthesis and hopefully the potential health effect of these fermented milks.

Surprisingly, the effect of  $\beta$ -CN(94-123) on HT29-MTX cells was the most prominent at a concentration of 0.01  $\mu$ mol/L, and it was reversed when tested at 100  $\mu$ mol/L. One possible explanation for these results is that the interaction of  $\beta$ -CN(94-123) with a putative receptor would reach saturation, thus leading to a paradoxical inhibitory effect. Another explanation may be that our peptide potentially contains several bioactive sequences showing antihypertensive (f108-113, f114-115), opioid (f114-119) and antioxidant (f 98-105) activities [43–45]. One of these potentially active sequences could be recognized as a signal when the peptide is tested at 100  $\mu$ mol/L in HT29-MTX cells and then reduce mucin production. The effect of each bioactive sequence on the secretion and expression of mucins is to be determined *in vitro*. Special attention should be paid to the opioid sequence f114-119. Data from the literature have indeed shown that opioid peptides including  $\alpha$ -casomorphins are able to interact with somatostatin receptors [46,47], which are also present on intestinal epithelial cells as well as on the parental HT29 cells. Somatostatin, synthesized and secreted by endocrine D cells of the stomach and intestine, is known to exert an inhibitory control in the gut. On the basis of these data, we can hypothesize that interaction with somatostatin receptors could explain the reversed effect of  $\beta$ -CN(94-123). Additional experiments are required to validate this hypothesis. Anyway, it is important to note that the reverse action of



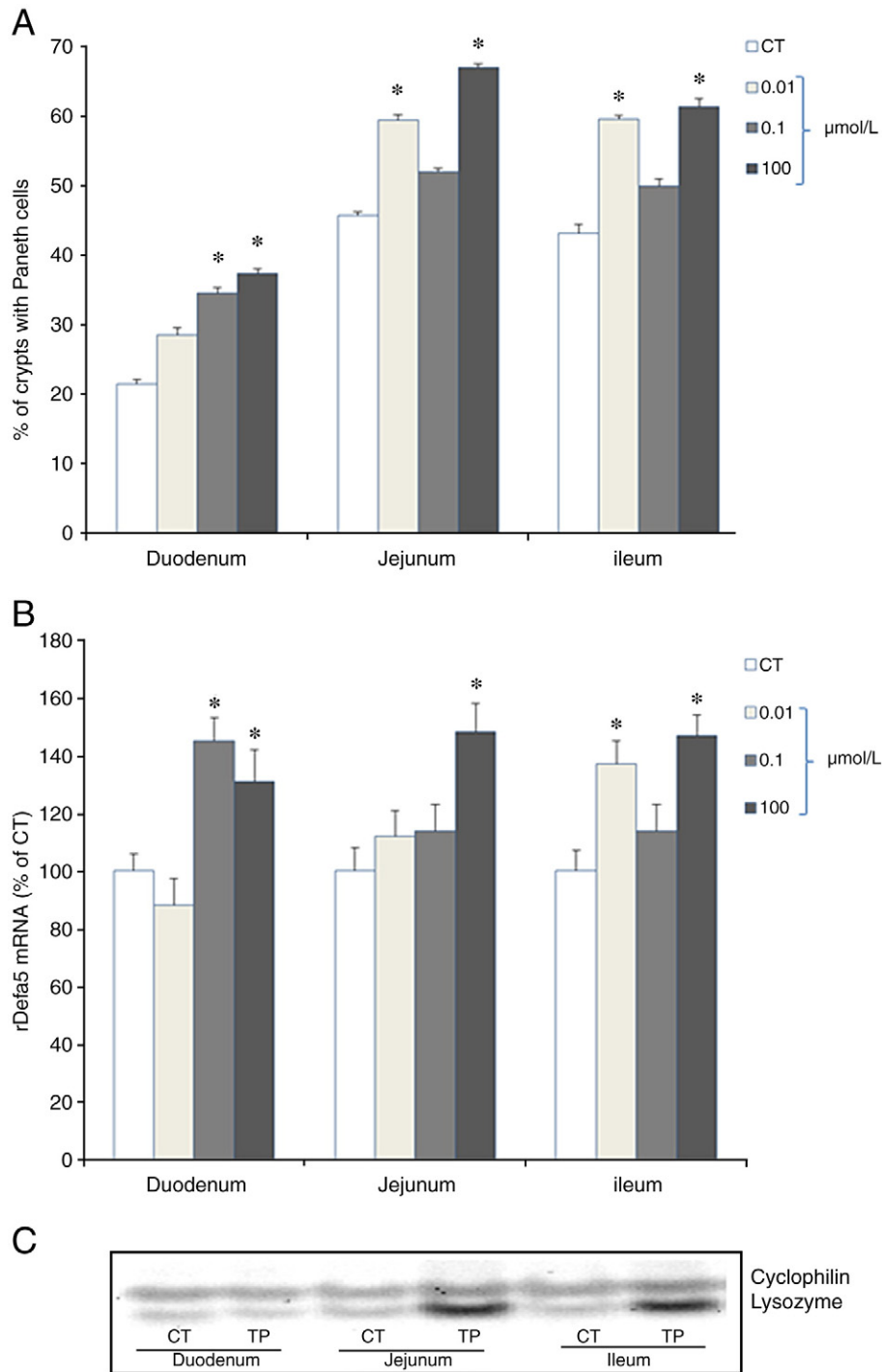


Fig. 5. Effect of peptide  $\beta$ -CN(94-123) on PCs. (A) Percent of PC-containing crypts (mean  $\pm$  S.E.M.,  $n = 8$ ) in the duodenum, jejunum and ileum of CT and TP rats (0.01, 0.1 and 100  $\mu\text{mol/L}$ ). PC counts were performed on tissue sections immunostained for lysozyme ( $\times 20$  magnification). \* $P < .05$  vs. controls. (B) Real-time PCR analysis of *rDefa5* mRNA. The expression of *rDefa5* was normalized to the reference gene level (cyclophilin A) in each sample. The results are expressed as percent of control rats (means  $\pm$  S.E.M.,  $n = 8$  per group). \* $P < .05$  vs. controls. (C) Representative Western blot picture of lysozyme and cyclophilin A in duodenum, jejunum and ileum of CT and TP rats [peptide  $\beta$ -CN(94-123), 0.01  $\mu\text{mol/L}$ ]. Lysozyme appeared as a band of 17 kDa. Eight jejunal samples were examined for both lysozyme and cyclophilin A.

$\beta$ -CN(94-123) on *MUC2* and *MUC4* expression was observed *in vitro* when the peptide was tested at a high concentration (100  $\mu\text{mol/L}$ ). We can speculate that such an inhibition could not take place after normal consumption of dairy products. Indeed, on the one hand, we have not detected such a concentration in fermented milks, and on the other hand, when the peptide was tested *in vivo* at the same high concentration, we observed no reversed effect as compared with rat receiving vehicle alone.

In conclusion, our study demonstrated that bioactive peptides that are formed in yoghurts during the hydrolysis of milk proteins by lactic acid bacteria are produced in sufficient quantities to achieve an effect on intestinal epithelial cells. Among these peptides,  $\beta$ -CN(94-123), which corresponds to the fragment (94-123) of bovine  $\beta$ -casein, modulates Paneth and mucus cells, the two intestinal epithelial cell populations that play a crucial role in intestinal protection. Because of its resistance to the digestive phase and its strong effects on two

major actors of the intestinal barrier,  $\beta$ -CN(94–123) is particularly pertinent to restore normal intestinal homeostasis and could play an important role in protection against damaging agents of the intestinal lumen.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jnutbio.2012.05.004>.

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