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## Accelerated production of blue cheese flavors by fermentation on granular curds with lipase addition

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**Summary** — The accelerated production of blue cheese flavors was studied in the fermentation of the mold *Penicillium roqueforti* on granular curds. This substrate allows a better control of the fermentation conditions by reducing the diffusional limitations that occur in traditional cheese formats. The evolution of the fermentation is presented with lipase (E.C. 3.1.1.3) added before coagulation and when the granular curds are coated with a lipase containing slurry or with lipolyzed cream. Products containing 8–10 times higher concentrations of carbonyl compounds compared to the traditional cheese were obtained by coating the granular curds with a lipase containing slurry or with lipolyzed cream.

**blue cheese — *Penicillium roqueforti* — cheese flavors — lipase**

**Résumé** — **Production accélérée d'arôme de fromage bleu par fermentation en caillé granulaire.** La production accélérée d'arômes de fromage bleu est obtenue par la fermentation de granules de caillé par *Penicillium roqueforti*. Ce substrat a permis un meilleur contrôle des conditions de fermentation en réduisant les contraintes diffusionnelles trouvées dans les tailles des fromages traditionnels. L'évolution de la fermentation est présentée dans le cas où de la lipase (E.C. 3.1.1.3) est ajoutée avant la coagulation du lait ou par enrobage des grains de caillé et dans le cas d'addition de la crème lipolysée. Des produits ayant une concentration de carbonyles de 8 à 10 fois supérieure à celle du fromage ont été obtenus par l'enrobage des grains de caillé par de la lipase ou de la crème lipolysée sur le caillé granulaire.

**fromage bleu — *Penicillium roqueforti* — arômes fromage — lipase**

## INTRODUCTION

A large number of cheeses (Roquefort, Stilton, Gorgonzola, Bleu d'Auvergne, Cabrales, etc.) are grouped under the category of blue cheeses (Kosikowski, 1977). Despite their differences in type of milk used, size and ripening conditions, they all share the same flavor-producing agent: the mold *Penicillium roqueforti*.

The flavor of blue cheese is characterized by compounds derived from strong proteolysis and lipolysis (Adda *et al.*, 1982). The typical flavor constituents are the methyl-ketones, especially 2-heptanone, which are produced by the beta-oxidation of the free fatty acids (FFA) (Kinsella and Hwang, 1976). This transformation, which is believed to be a way to diminish the FFA inhibition, is caused by the mycelium and the spore of the mold.

Some of the methods proposed to accelerate the flavor production and to obtain flavor concentrates are: liquid fermentation (Jolly and Kosikowski, 1975; Nelson, 1970), Enzyme Modified Cheeses (EMC) (Moskowitz and Noelck, 1987; Talbot and McCord, 1981) and ripening with divided (granular) curd (Kondrup and Hedrick, 1963; Furtado *et al.*, 1984).

The technique of ripening the granular curds has also been reported in the production of Limburger, Brick and Camembert cheeses (Bryant, 1946). Recently, it has been reported that the ripening of Camembert cheese under granular conditions, and with added lipase, produces a stronger cheese in 12 days than in the traditionally ripened format (Furtado *et al.*, 1984). It is assumed that granular conditions improve the surface to volume ratio, thus reducing the diffusion effects. This allows a better control of the fermentation process.

In this work the evolution of some flavor constituents resulting from the growth of *Penicillium roqueforti* on granular curds is presented. The effect of added lipases and of lipolyzed cream was studied as a means to accelerate flavor production.

## MATERIALS AND METHODS

### Microorganisms

Three strains of *P. roqueforti* (Pr-A, Pr-C and Pr-D) were isolated from traditionally ripened cheeses. A fourth strain (Pr-B), a collection strain (CNRZ 883), was donated by the INRA, France. The lactic bacteria corresponded to a commercial starter (HM 18) Marshall-Miles, France.

*P. roqueforti* conservation and spore production was made on PD agar and lactic bacteria were kept frozen and inoculated after being propagated for 18 h in sterile non-fat milk (100 g/l) at 30°C. Spore inoculation was 10<sup>8</sup> spores per liter of milk.

### Substrate preparation

The method employed is described in Figure 1. Batches of 4 l of milk were used. The curds were cut with a mesh as proposed by Hicks *et al.* (1981). After the acidification (18 h), the matted curd was cut with a stainless steel knife or ground with a meat grinder. Salting was made directly on the granular curds (10 g salt/kg granular curds). Serum expulsion and ripening was carried in 5 l, flat bottom SS funnel covered with 8 sheets of gauze in an incubator. The ripening temperature was 25°C.

### Lipase addition

Lipase (E.C. 3.1.1.3) from *Candida cylindracea* (Sigma, St. Louis, MO., USA) was used throughout this study. The lipase was either

added directly to the milk before coagulation (LBC) (10 mg/l milk) or added to the granular curds (LGC). In the LGC experiments 5% of the lot was removed from the fermentor and diluted with sterile water (1:1) and the lipase was then added (50 mg/kg granular curds). This mixture was homogenized with an Ultra-turrax (2 min @ 20,000 rpm), and then this slurry was reincorporated into the granular curds.

### Lipolyzed cream addition (LCA)

Lipolyzed cream was prepared according to the method of Dwivedi and Kinsella (1974). UHT treated cream was diluted with sterile water to 16.5% fat and 100 mg/l of enzyme was added. This mixture was incubated for 2 days at 27°C and then heated at 75°C to inactivate the enzyme. The lipolyzed cream was coated onto the granular curds at a ratio of 1:5.

### Sampling

The analyses were performed on samples of the granular curds diluted with distilled water and homogenized with an Ultra-turrax (2 min @ 20,000 rpm).

PASTEURIZED MILK
standardized to 30 g/l fat content
addition of 0.1 g/l Ca Cl <sub>2</sub>
acidification to pH 5.9 with HCl
THERMISATION (5 min @ 63°C)
COOLING TO 35°C
INOCULATION
<i>P. roqueforti</i> spores
lactic bacteria
COAGULATION (35°C, 30 min)
rennet extract (1/10 000) 0.2 ml/l
CURD CUTTING (1 cm grains)
HEATING TO 45°C (30 min)
COOKING (30 min)
WHEY DRAINAGE (25°C, 18 h)
SALTING
RIPENING AT 25°C

**Fig. 1.** Production of granular curds for the growth and flavor production by *Penicillium roqueforti*.

*Production de caillé granulaire pour le développement et la génération d'arômes par Penicillium roqueforti.*

### Analyses

Dry weight was determined for 5 to 10 g samples with a microwave oven. The pH was recorded for diluted (1:10) and homogenized samples. The total lactic acid was measured by spectrophotometry by the Steinsholt and Calbert method, as described by Muller (1971). The soluble nitrogen (SN) was obtained by addition of 36% trichloroacetic acid (TCA) to the diluted (1:10) and homogenized samples to obtain a final TCA concentration of 12% and was measured by the Hull (1947) method, as modified by Citti *et al.* (1963), and reported as mg soluble tyrosine per g of curds. Total carbonyls (TC) were measured with the 2-4 phenyl hydrazine reagent as described by Nonus (1982), and are reported in  $\mu\text{mol}$  of methyl-ketones based on a standard curve obtained with 2-undecanone. TC analyses were also performed on different blue cheese samples bought from local stores. Free fatty acids (FFA) were measured by titration according to Deeth *et al.* (1975). Proteolytic activity (PA) was determined according to the method of Stepaniak *et al.* (1980). A unit is defined as  $\mu\text{g}$  of tyrosine liberated in 2 h at 37°C.

## RESULTS AND DISCUSSION

### Evolution of the fermentation

The evolution of the fermentation by *P. roqueforti* D on granular curds without enzyme addition is represented in Figure 2a, b and c. The behavior of the 4 strains of *P. roqueforti* without enzyme addition was very similar. In the beginning the pH decreases due to the lactic fermentation and increases after about 65 h. This pH increase is associated with the consumption of lactic acid by the mold and the production of ammonia and amines by the enzymatic action on peptides and amino acids (Adda *et al.*, 1982). There is an increase in proteolytic

activity associated with growth which solubilizes the protein. This fact, together with the increase of pH (Noomen, 1983), softens the texture of the granular support which is, by the 5th day, melted. The action of *P. roqueforti* on the milk fat produces an accumulation of FFA which increases with the rise in the pH of the system because the optimal pH for the mold lipases is found between 7.5 and 8.0 (Eitenmiller *et al.*, 1970). Total carbonyls correspond mainly to methylketones (Dwivedi and Kinsella, 1974) and are produced from the beginning due to

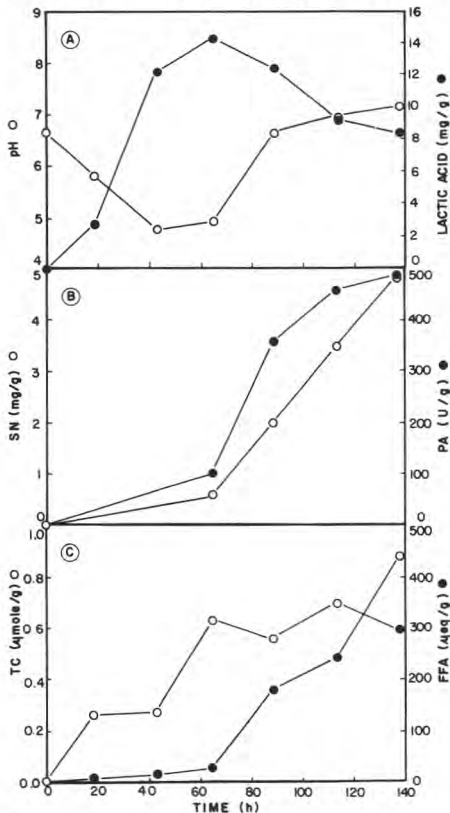


Fig. 2. A, B and C. Evolution of the fermentation of Pr-D on granular curd. *Evolution de la fermentation du Pr-D sur caillé granulaire. (Azote soluble : SN, activité protéase : PA, carbonyls totaux : TC, acides gras libres : FFA).*

the activity shown by the mold spores to make this conversion. The concentrations of the total carbonyls were similar to those present in traditionally ripened blue cheese that had an average of  $0.5 \mu\text{mol/g}$  of cheese. The stabilization of the concentration may be caused by the reduction of the mold metabolic activities due to the elevated pH. A marked reduction in the total carbonyls concentration was generally observed towards the end of the fermentation. This observation may be attributed mainly to 2 factors : the evaporation losses promoted by the high surface of the granular curds and to the transformation of the methylketones into secondary alcohols by the mold (Kinsella and Hwang, 1976). Beyond 160 h the product has a strong ammoniacal odor, a very unpleasant soapy flavor and a cream-like texture.

### Lipase addition

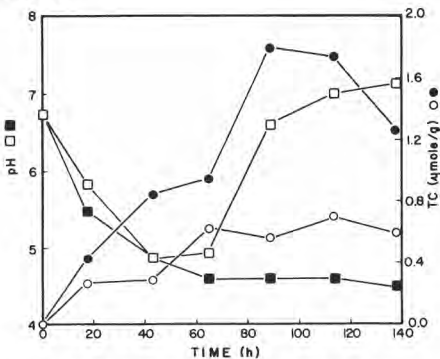
In order to increase the methylketones concentration, 3 methods for the addition of lipase were studied : enzyme addition to the milk before coagulation (LBC), coating of the curd grains with a lipase solution (LGC) and addition of lipolyzed cream (LCA).

### Addition of lipase before coagulation (LBC)

In these experiments the granular curds developed a strong characteristic blue cheese aroma associated with the methylketone production (Fig. 3). Nevertheless, mold growth did not develop until the fifth day. This inhibition might have been caused by the combined effect of pH and the FFA produced by the action of the lipase. Under low pH conditions the germination of the spores is inhibited by the high FFA concentration.

### Addition of lipase by coating the curd grains (LGC)

These experiments were made by adding the enzyme containing slurry to the granular curds after 48 h of coagulation. At this time the spore germination had already occurred and, consequently, the FFA accumulated simultaneously with the mold development. Figures 4 a, b and c show the evolution of the pH, soluble nitrogen and total carbonyl concentration for the 4 studied strains. Strain A showed a faster accumulation of soluble nitrogen which provoked the melting of the curds by the fifth day. Strains A and D produced carbonyl compounds faster than the other 2 strains. The different assays with lipase coating of the curd grains contained much higher concentrations of carbonyls than those in the traditionally ripened cheese or the granular curd fermentation without enzyme addition. The comparison of this method with and without enzyme addition is shown in Figures 5 a and b for strains B and C.



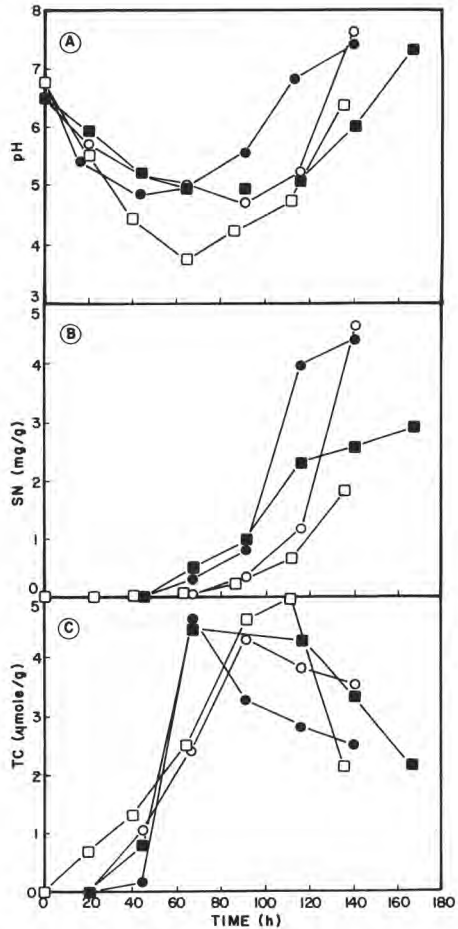
**Fig. 3.** Evolution of the fermentation of Pr-D on granular curd with addition of lipase to the milk (10 mg/l), LBC, (■ pH with lipase, □ pH control, ● total carbonyls with lipase, ○ total carbonyls in the control).

*Evolution de la fermentation du Pr-D sur caillé granulaire avec addition de lipase au lait (10 mg/l) (■ pH avec lipase, □ pH témoin, ● carbonyls totaux avec lipase, ○ carbonyls totaux du témoin).*

### Lipolyzed cream addition (LCA)

Two groups of experiments were performed with added lipolyzed cream.

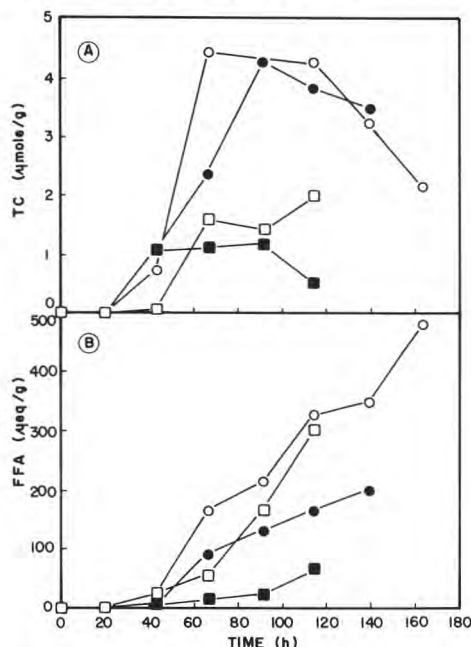
*Addition on the first and second days.* For this experiment milk with 15 g/l fat was



**Fig. 4. A, B and C.** Evolution of the fermentation of Pr-A (●), Pr-B (○), Pr-C (■) and Pr-D (□) on granular curd with addition of lipase by coverage (50 mg/kg of granular curds).

*Evolution de la fermentation du Pr-A (●), Pr-B (○), Pr-C (■) et Pr-D (□) sur caillé granulaire avec enrobage de lipase (50 mg/kg de caillé granulaire).*

used. Lipolyzed cream was added after 24 h (LCA1) or 48 h (LCA2). LCA retarded the development of mold growth (Fig. 6 a—d) compared with the control and the parallel LGC experiment. Growth was observed on the fifth day for LCA1 and on the sixth for LCA2, while for the control and the LGC control, growth started on the third day. The spores, being more resistant than the mycelium to the inhibitory effects of the FFA, transform these into methyl-ketones, thus reducing the FFA inhibition. This effect was more obvious with the experiment in which the LCA was added on the second day due to an advanced spore germination.



**Fig. 5. A and B.** Evolution of the fermentation of Pr-B and Pr-C on granular curd with addition of lipase by coverage, LGC, (50 mg/kg of granular curds). (● Pr-B with lipase; ○ Pr-C with lipase; ■ Pr-B control, □ Pr-C control). *Evolution de la fermentation du Pr-B et Pr-C sur caillé granulaire avec enrobage de lipase (50 mg/kg de caillé granulaire). (● Pr-B avec lipase, ○ Pr-C avec lipase, ■ Pr-B témoin, □ Pr-C témoin).*

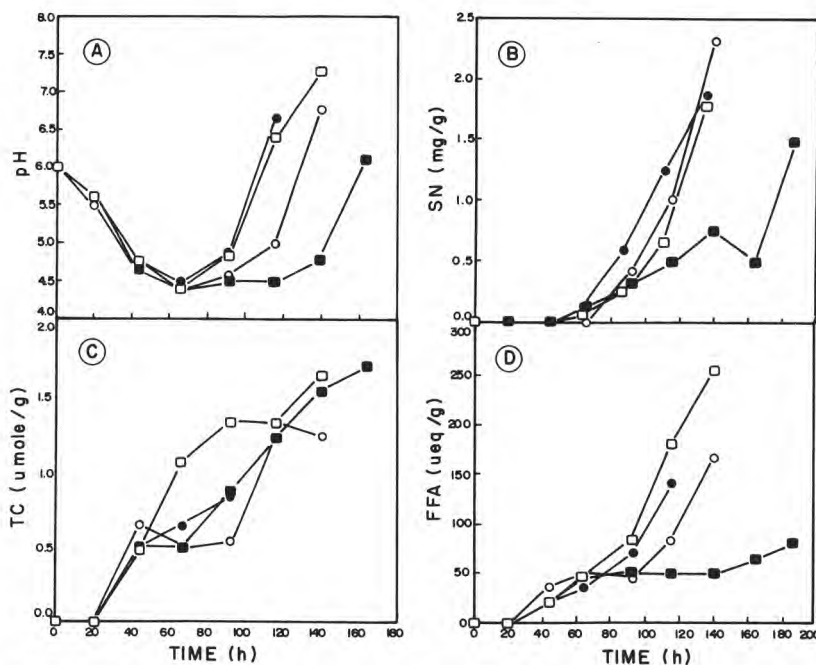
*Addition on the fourth day.* In this set of experiments (LCA4) milk with 35 g/l fat was used and the lipolyzed cream was added when there was already considerable growth and a visible sporulation. LCA on this stage should allow higher transformation rates. As can be observed in Figure 7 a and b, addition of the cream reduces the pH of the granular curds and produces a strong increase in the methyl-ketone concentration. Nitrogen solubilization and FFA, production rates were not significantly modified.

### **Effect of the fat content of the granular curds**

The fat content of the granular curds has a strong influence on the development of flavoring compounds. This is due to the fact that fat is the substrate for the production of the FFA, which are the main aroma precursors. In addition, fat acts as a solvent for the liposoluble compounds produced during the fermentation (Adda *et al.*, 1982). This last aspect is more important in granular curd fermentation than in the cheese because the increased surface area also promotes the evaporation of the volatile compounds produced by the mold. Figure 8 shows the results of a parallel experiment with milk with 15 and 35 g/l of fat using the LGC technique.

### **Effect of salting**

Salting of the granular curds was performed on the first day after milk coagulation. An extra addition of salt (20 g salt/g granular curds) on the fourth day was made as a means to slow down the ripening rates. As can be observed in Figure 9 a and b, an extra salt addition does not alter the SN, the FFA and the methyl-ketone accumulation.



**Fig. 6 A, B, C and D.** Evolution of the fermentation of Pr-D on granular curd with lipolyzed cream (16.5% fat) addition, LCA, at 24 h (○ LCA1), 48 h (■ LCA2), control (●) and control with lipase coverage (□).

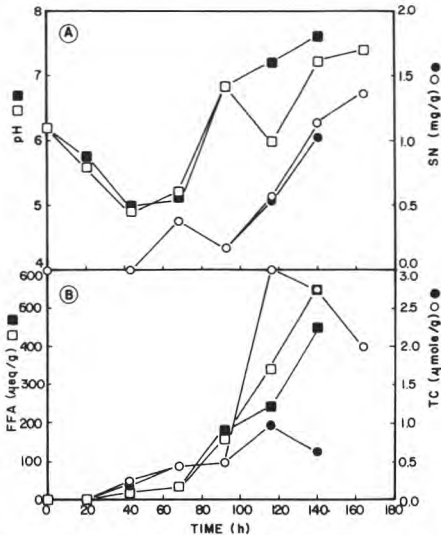
*Evolution de la fermentation du Pr-D sur caillé granulaire. Addition de la crème (16,5% M.G.) lipolisée après 24 h (○), 48 h (■), témoin (●) et témoin avec enrobage de lipase (□).*

## CONCLUSIONS

The experiments performed with granular curds were shown to be feasible methods for the accelerated production of blue cheese flavors. Fermentation times were reduced to 4 to 6 days by a combined effect of increased surface area and incubation temperature. The substrate proved to be adequate for the 4 strains studied, which follow the same evolution pattern but differ in nitrogen solubilization, FFA and methyl-ketones production rates. Under the studied conditions, the product did not develop a «moldy» flavor as was

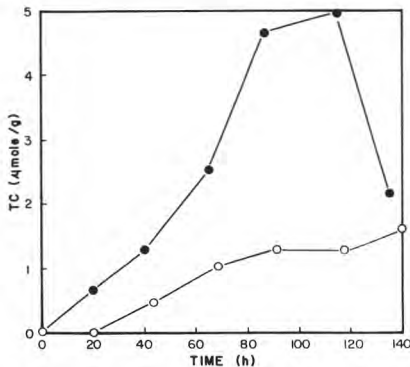
reported by Kinsella and Hwang (1976) for the assays realized by Kondrup and Hedrick (1963) with a divided curd for blue cheese flavor production.

The increased accumulation of aromatic compounds (mainly those derived from the metabolism of the FFA) has been accomplished by the LGC and LCA techniques. With LGC the carbonyl concentration may be 8–10 times higher than those found in traditionally ripened blue cheese. With LCA a strong concentration of carbonyls may be attained if a well developed mold growth already exists.



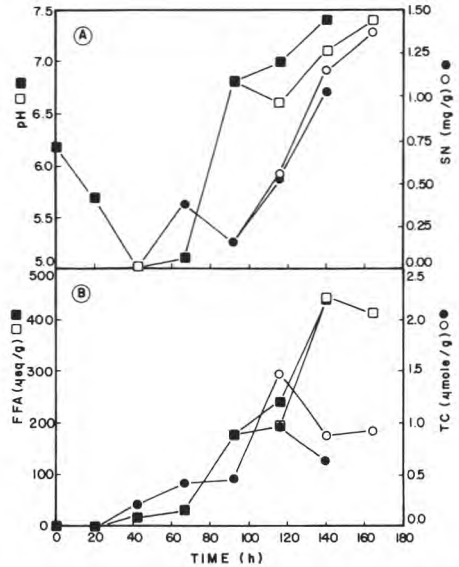
**Fig. 7. A and B.** Evolution of the fermentation of Pr-D on granular curd with lipolyzed cream (16.5 : fat) addition, LCA, at 96 h (O, □) and control (●, ■).

*Evolution de la fermentation du Pr-D sur caillé granulaire. Addition de la crème lipolysée (16,5% M.G.) après 96 h (O, □) et témoin (●, ■).*



**Fig. 8.** Evolution of the fermentation of Pr-D on granular curd with addition of lipase by coverage (50 mg/kg of granular curds). Granular curds made with milk with 35 g/l fat (●) and 15 g/l fat (○).

*Evolution de la fermentation du Pr-D sur caillé granulaire avec enrobage de lipase (50 mg/kg de caillé granulaire). Le caillé granulaire est fait avec du lait à 35 g/l M.G. (●) et 15 g/l M.G. (○).*



**Fig. 9. A and B.** Evolution of the fermentation of Pr-D on granular curd with addition of lipase by coverage (50 mg/kg of granular curds). Salting (1%) performed at 24 h (■, ●) and at 24 and 96 h (□, ○).

*Evolution de la fermentation du Pr-D sur caillé granulaire avec enrobage de lipase (50 mg/kg de caillé granulaire). Salage (1%) après 24 h (■, ●) et après 24 et 96 h (□, ○).*

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