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# Safety Assessment of the Antimicrobial Lipopeptides Synthesized by *Bacillus subtilis* Subsp. *subtilis* CBMDC3f

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**Received:** May 10, 2017; **Published:** June 02, 2017

## Abstract

*Bacillus subtilis* subsp. *subtilis* CBMDC3f produces lipopeptides with strong antibacterial activities that were previously identified by UV-MALDI-TOF mass spectrometry as different surfactin homologues. In this work, the potential toxic effects of those lipopeptides were explored on BALB/c mice. The following samples of CBMDC3f antimicrobial metabolites were evaluated: cell-free supernatant (CFS) and purified lipopeptides fractions (LF), at different concentrations: 25, 125 and 250 mg/Kg of body weight (LF25, LF125 and LF250 groups, respectively). As a measure of hepatocellular toxicity, the serum enzymes (GOT and GPT transaminases) concentrations and liver and intestine histological analyses were performed. Values of GOT and GPT enzymes did not reveal significant changes as a result of the treatments. The histological analysis showed some signs of liver injury when CFS was administered; however, the liver injury in CFS-treated mice was significantly milder than those observed in the LF250 group. CFS, LF25 and LF125 treatments did not produce histologic changes in the small intestine. These results indicate that surfactins present in the LF of *B. subtilis* subsp. *subtilis* CBMDC3f would not produce cytotoxic effect on liver or small intestine of mice when administered at concentrations between 25 and 125 mg/Kg of body weight. Considering that the concentrations of CBMDC3f lipopeptides able to exert the anti-*Listeria monocytogenes* and anti-*Staphylococcus* activities are significantly lower than those with toxic effects; the results of this work indicate that CBMDC3f lipopeptides are promising for their application in food protection.

***Keywords:*** *Bacillus subtilis subsp. Subtilis; Lipopeptides; Surfactin; Toxicity; BALB/C Mice; Histopathologic Analyses*

## Abbreviations

CFS: Cell-Free Supernatant; LF: Purified Lipopeptides Fractions; GOT: Glutamic Oxaloacetic Transaminase; GPT: Glutamic Pyruvic Transaminase

## Introduction

The constant demand for new and effective microbicidal therapeutic agents has led to intensive research in the field of naturally occurring antimicrobials [1]. These types of compounds are synthesized by all forms of life and have important biomedical and biotechnological properties since they are considered as a possible solution to the growing problem of resistance to conventional antibiotics, fungal infections and diseases that affect human and animal health [2,3].

Bacteria of the genus *Bacillus* have acquired increasing interest and are being studied, among other applications, for their antimicrobial activities attributed to strong antimicrobial substances that are used for competition with other microorganisms [4,5]. Some of these antagonistic compounds include lipopeptides such as surfactin, fengycin, iturin and bacitracin [6-8]. However, there are few scientific reports evaluating the toxicity of these antimicrobial compounds in animal models. Those studies would be of great importance to ensure its safety and assess future biotechnological applications of these antimicrobials, for example in the food industry.

*B. subtilis* subsp. *subtilis* CBMDC3f was previously selected for its ability to synthesize lipopeptides that present a broad antimicrobial spectrum against important food pathogens [9]. Surfactin predominates among the lipopeptides synthesized by this bacterium. This biosurfactant is the main representative of the family of anionic lipopeptides and is the more powerful bio-surfactant known so far. Since its discovery and identification as a macrolide lipopeptide, this metabolite has been recognized for its high amphiphilicity and strong tendency to self-aggregation [10]. All these properties make surfactin an excellent candidate to solve a series of problems in medicine [11-13] and the food industry [14,15]. Additionally, surfactin emulsifying and surfactant activities could also be useful for applications in environmental protection [16].

*In vitro* and *in vivo* safety studies of microorganisms and/or their metabolites with potential applications as food biopreservatives is mandatory. Those studies are necessary for a microorganism to achieve its Generally Recognized As Safe (GRAS) status. For this reason, the possible cytotoxicity effects of surfactin must be determined [17]. In this regard, surfactin exhibited a milder action than other surfactants when its acute toxicity was studied in mice [18]. However, the majority of the reports have evaluated surfactin considering its potential applications in the medical and pharmaceutical area [1,3,19] but not as a food preservative or bioprotector.

The potential toxic effects of *B. subtilis* CBMDC3f lipopeptides had not been explored before. Therefore, the aim of this study was to determine whether the lipopeptides produced by *B. subtilis* subsp. *subtilis* CBMDC3f, present in the cell-free supernatant (CFS) and in the purified lipopeptide fraction (LF), exerted toxic effect in BALB/c mice.

## Materials and Methods

### Cell-Free Supernatants and Lipopeptide Fractions

Lipopeptides present in *B. subtilis* subsp *subtilis* CBMDC3f cell-free supernatant (CFS) and crude lipopeptide fractions (LF) were studied in this work. In previous studies, by using UV-MALDI-TOF mass spectrometry homologes of surfactin, fengycin, and iturin families were detected in CFS samples. In LF samples only homologes of surfactin were found [9].

The CBMDC3f strain, isolated from *Apis mellifera* L. bee gut, was previously characterized phylogenetically by rRNA analysis of the 16S subunit and the *gyrA* gene (Genbank Access Codes JX120508 and JX120516, respectively) [9]. CBMDC3f strain was grown at 37°C in MH broth (Müller Hinton, Britania, Argentina) during 24h without shaking, and its CFS was recovered by centrifugation (10,000g for 10 min at 4ºC). Subsequently, it was filter sterilized (0.22 µm pore-size cellulose acetate membranes) and maintained at 4ºC without any further conditioning until analysis.

The LF fraction was obtained from CFS. After centrifugation, CFS was acidified with HCl (c) until reaching pH 2, in order to precipitate lipopeptides. The precipitate was then recovered by centrifugation (14,000g for 25 min at 4ºC) and lipopeptides were subsequently extracted with 10 mL of methanol. The solvent was evaporated under hood and the resulting precipitate, called LF, was dissolved in sterile distilled water at pH 9 to a final concentration of 5.0 mg/mL. This solution was used for the toxicity assays. Sterile water (C1) and water at pH 9 (C2) were used as controls.

## Animals

Three-week-old female BALB/c mice with weights between 21 - 24g were provided by the Instituto de Patología Experimental, Universidad Nacional de Salta (IPE-CONICET, UNSa, Argentina). All animal experiments were carried out in strict accordance with the stan-

dards established by the "Guide for the Care and Use of Laboratory Animals" [20] and all efforts were made to minimize suffering. The Instituto de Patología Experimental Ethical Committee approved experiments (2012). Five mice per group were used and experiments were performed twice. Animals were randomly assigned into six groups and housed in stainless-steel cages with a 12h light/dark cycle (8 am - 8 pm) in a controlled environment (temperature 22ºC ± 2ºC, humidity 55% ± 2%). Mice were provided with food and water *ad libitum* during the experimental period.

### Treatment and Administration

After a week of acclimation, different groups were orally administered 0.2 mL per mouse of distilled water (control 1: C1) or distilled water at pH 9 (control 2: C2) or CFS. The remaining groups of mice were administered 25, 125 or 250 mg of LF (crude lipopeptide fraction) per Kg of body weight (LF25, LF125 and LF250 groups respectively). Two administrations of each treatment were given during the experiment, at the beginning (day 1) and on the seventh day (day 7). Animal behavior and general health features, as bristly hair and abduction or "freeze" were observed. Body weight was recorded at days 1 (2 hours before treatments with CFS or LF), 7 and 15 of experiment.

### Biochemical Analysis

Glutamic oxaloacetic (GOT) and glutamic pyruvic (GPT) hepatic transaminases were used as biochemical markers for early acute hepatic damage. Blood samples from the vessel tail were collected on days 7 and 15. Samples were incubated into glass tubes at 37ºC for 20 minutes. After coagulation, the serum was separated by centrifugation at 3500g for 10 minutes. Transaminases determination was performed following Wiener Lab. Group instruction, by a UV kinetic method (five lectures, 340 nm, 37ºC) using Clinical Autosampler Metrolab 2300 plus.

### Histopathological Examination

On day 15 of experiment, animals were sacrificed and liver and intestine samples were taken for histological studies, as previously described by [21]. Briefly, tissue samples of intestine and liver were collected and fixed in paraformaldehyde (4% v/v) and processed for paraffin embedding. The histological sections were stained with hematoxylin-eosin (HE). The slides were coded and blindly analyzed at the Laboratory of Immunobiotechnology (CERELA), who were unaware of the experimental conditions of each group. A semiquantitative scoring index was used to evaluate alterations in both organs. The presence/absence and severity of hepatic steatosis, necrosis, intralobular degeneration, hydropic degeneration, fibrosis and inflammation were considered for hepatic tissue. In addition, the presence/absence and intensity of edema, necrosis, degranulation of Paneth cells and inflammation were considered for intestine. For both tissues, each parameter was rated on a point damage scale from 1 to 4 (1, absence; 2, slight; 3, moderate; 4, severe alteration) and the final score results were expressed as the sum of the individual scores given to each parameter.

### Statistical analysis

The results of measurement of body weight and dosage of enzymes (GOT y GPT) were expressed as mean ± standard deviation of the groups and subjected to analysis with a non-parametric Kruskal-Wallis test. The differences were considered significant when $p < 0.05$. The statistical analyses of the data were performed using InfoStat statistical software.

## Results

### Clinical Signs

Clinical examination of mice was performed periodically during the experiments. The observation of clinical signs and symptoms was focused in hackles, dehydration and abduction. Normal behavior and complete absence of adverse effects were determined during testing in groups treated with *B. subtilis* subsp *subtilis* CBMDC3f CFS and LF or controls. Moreover, no death of mice occurred in any group.

**Body weight gain and food and water consumptions**

There were no differences in food and water consumption among all experimental groups. In addition, differences in mice weights between *B. subtilis* subsp *subtilis* CBMDC3f CFS and LF treatments were not significant ($p > 0.05$) during the experimental period, maintaining a range of body weight between 21 and 24 g approximately (Figure 1).

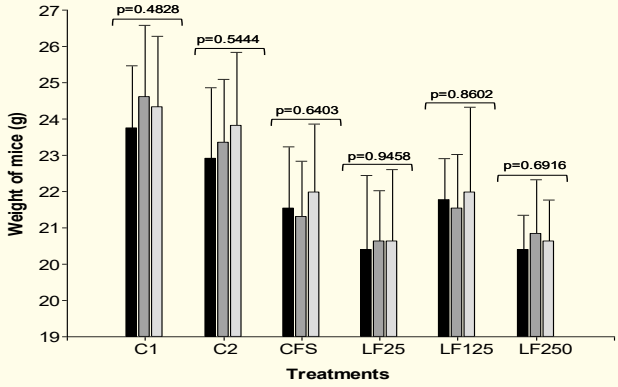


***Figure 1:*** *Effect of CBMDC3f CFS and LF administration on the weight of mice. Body weight were measured on day 1 (2 hours before the administration of treatments) (black bar), day 7 (gray bar) and day 15 (white bar). Control 1 (distilled water, C1); Control 2 (distilled water at pH 9, C2). Weight analysis was performed with a non-parametric Kruskal-Wallis test ($p < 0.05$)*

**Monitoring of liver enzymes**

No significant differences between the treatments were detected when GOT enzymatic activity was determined on day 7 of treatment (Figure 2a), since the CFS, LF25, LF125 and LF250 groups showed values of 69, 70, 70 and 79 IU/L, respectively. However, a significant difference was found ($p = 0.0361$) between the above mentioned groups compared to C1 control group (52 IU/L). In the second monitoring on day 15 of experiment (Figure 2b), GOT values decreased around 18 and 23% in CFS, FL125 and FL25 groups. These values were similar to those found for C2 (54 IU/L) and C1 (50 IU/L) control groups. In contrast, GOT activity determined for LF250 was 97 IU/L, that was considered a significant increase when compared to control groups ($p = 0.0321$).

Regarding GPT activities, the levels of this liver enzyme were similar in all the groups ($p = 0.5314$) in the first week of the experiment. GPT activity remained between 28 at 34 IU/L (Figure 2a). For the second monitoring, in general no abrupt changes were found (Figure 2b). It should be noted that no significant decrease ($p = 0.3800$) of the enzymatic activity in the FL25 (13%) and FL125 (10%) groups were observed.

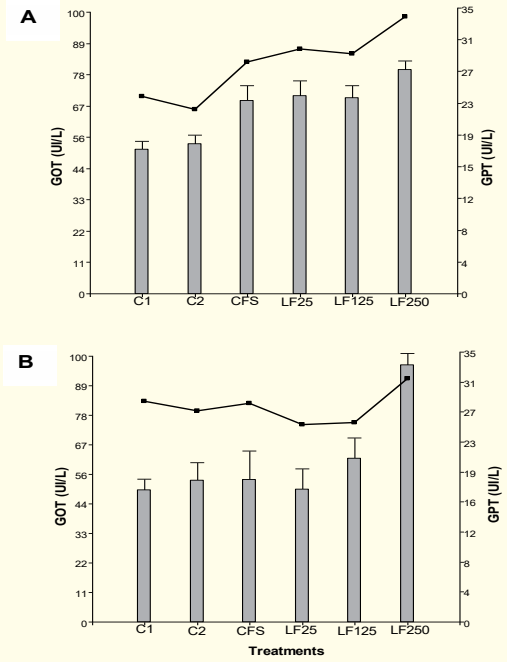


***Figure 2:*** *Effect of CBMDC3f CFS and LF administration on hepatic enzymes in serum. Values of GOT (bars) and GPT (curve) enzymes in serum (a) after the first dose (7 days) and (b) after the second dose (15 days) of each treatment. C1, Control 1 (distilled water); Control 2 (distilled water to pH 9); CFS (cell free supernatant); LF25 (lipopeptide fraction 25 mg/Kg); LF125 (lipopeptide fraction 125 mg/Kg); LF250 (lipopeptide fraction 250 mg/Kg). Analysis of GOT and GPT differences between the groups was performed with a non-parametric Kruskal-Wallis test ($p < 0.05$).*

**Histopathological analysis**

No alterations in the liver structure, sinusoidal narrowing, hepatic steatosis or inflammatory cell infiltration were observed in liver section analysis obtained from animals from control, LF25 or LF125 groups (Figure 3). Mice from these groups showed normal histologic features and there were no differences between them regarding the liver histological score (Figure 3a). On the contrary, mice that received LF250 showed moderate hydropic degeneration with mild cellular vacuolization and changes in the relationship nucleus/cytoplasm (Figure 3b). This group also showed a diffuse infiltration of inflammatory cells in the hepatic parenchyma. In addition, a mild inflammatory infiltration and hydropic degeneration was observed in the group treated with CFS (Figure 3a, b). Although histological liver lesions were detected in this group, the damage was significantly milder than observed for mice receiving FL250.

On the other hand, no structural intestinal disorders, degranulation of Paneth cells or inflammation were detected in mice treated with the CFS, LF25 or LF125, showing similar histological signs when compared to control groups (Figure 4). Mice that received CFS, LF25 or LF125 showed normal histological features in the small intestine and there were no differences for the histological score compared with controls (Figure 4a, b). However, mice that received the highest concentration of lipopeptides (LF250) presented moderate degranulation of Paneth cells and mild focal swelling (Figure 4b). The intestinal histologic score for LF250 treatment was higher than those in controls (Figure 4a).

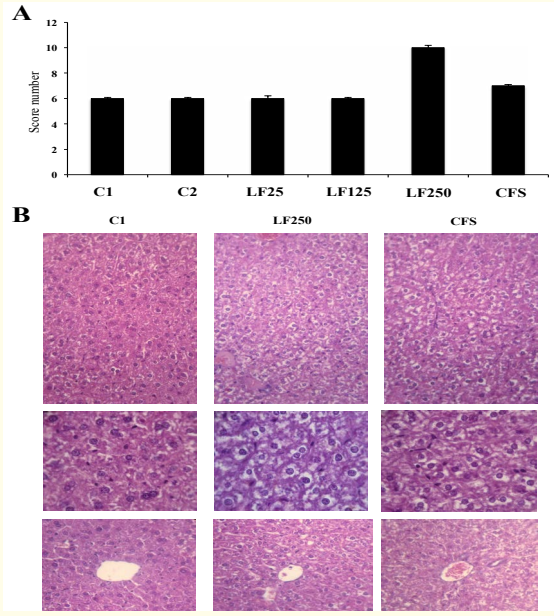


***Figure 3:*** *Effect of CBMDC3f CFS and LF administration on liver histology. (a) Score of histological lesions and (b) microscopic examination of liver tissue of mice treated with the CFS and the LF250 of CBMDC3f.*

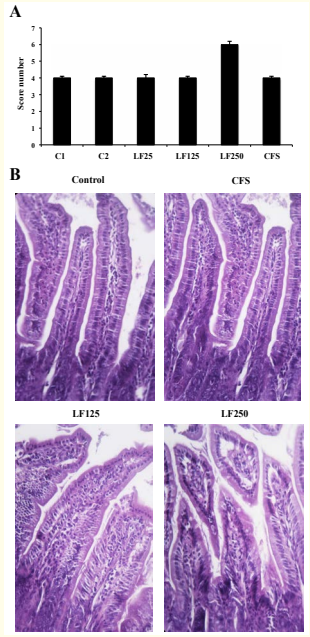


***Figure 4:*** *Effect of CBMDC3f CFS and LF administration on intestine histology. (a) Score of histological lesions and (b) microscopic examination of intestinal tissue of mice treated with the CFS, LF125 and LF250 of CBMDC3f.*

## Discussion

*B. subtilis* subsp. *subtilis* CBMDC3f was previously chosen among several *Bacillus* strains isolated in our group due to the high *in vitro* biological activity of both, its cell free supernatant (CFS) or its crude lipopeptide fraction (LF), against *Listeria monocytogenes, Staphylococcus aureus* and *Bacillus cereus* [9]. Thus, we have deepened the study of the safety of *B. subtilis* CBMDC3f CFS and LF using a mice model, considering future applications of these antimicrobials either in food or other products of industrial interest associated with the human being. It is important to remark that CFS and LF from CBMDC3f strain had been previously studied by mass spectrometry analysis, and that it was demonstrated that LF mainly contained surfactin; while surfactin, iturin and fengycin homologues were detected in the CFS [9]. Then, from a chemical of view, two different samples were tested *in vivo*. It should be noted that it was decided to study the safety of the SLC considering that it could be used as an "economic" biopreservative since purification steps of the bioactive component would be excluded. In addition, it should be considered that the concentrations of the lipopeptide samples (LF = 25, 125 y 250 mg/Kg of body weight) studied in this work were chosen according to the minimum inhibitory concentrations (CMI = 0.5 mg/mL) determined against the relevant food-borne pathogenic bacteria aforementioned [9].

Interestingly, several articles have focused their attention on the beneficial applications of lipopeptides like surfactin including their antimicrobial effects [13,22-24] and their potential as new anti-cancer drugs because of their strong activities in the inhibition of cell proliferation and induction of apoptosis in colon cancer LoVo cells [25], and human breast cancer [12].

Even though there is information about the biological activities of lipopeptides, the scientific publications strictly related to the evaluation of their toxicity are limited [3,17,26]. In this regard, Hwang., *et al*. [3] suggested that an oral intake of up to 500 mg/kg per day of surfactin C did not generate apparent toxic effects in female mice and emphasized the absence of fetotoxicity or teratogenicity. In another study of the same research group, it was reported that surfactin acted as an important anti-endotoxin agent (such as lipopolysaccharides of *Escherichia coli*) and that was more secure (i.e., less toxic) in comparison with polymyxin B [27].

In this work, the impact of the oral administration of the CFS and LF of CBMDC3f on mice body weight, clinical signs, liver enzymes and histological changes in liver and intestine was determined in order to assess the potential toxic effects of these antimicrobial samples. These parameters have been previously used by other authors to evaluate the toxicity/safety of probiotic *Lactobacillus* strains [28], the flavonol quercetin [29], the herbal preparation DAS-77 [30] or the antimicrobial peptide P34 [31]. It is noteworthy that in our work, no mice death was recorded during the assay, even with the highest concentration of the orally administered lipopeptides (250 mg/Kg). Furthermore, no adverse effect on food or water intake was observed and all mice registered weight gain similar to those observed in control animals.

It is known that the liver, the main organ for the metabolism of various substances, is the classic target of cytotoxicity studies. In this regard, the dosage of serum concentration of transaminase enzymes such as GOT and GPT is used as an indicator of acute liver damage [32-34]. When GOT enzyme was evaluated, we observed that on day 15 its levels in mice treated with CFS, LF25 and LF125 decreased between 18 and 23 %, reaching values closer to the controls. In contrast, LF250 increased GOT values between day 7 and day 15 of the study, from 80 to 97 IU/L. On the other hand, the values measured for GPT enzyme were similar to all groups during the first and second week of administration. Similarly, Almeida Vaucher., *et al*. [31] evaluated the toxicity of the antimicrobial peptide P34 synthesized by *Bacillus* spp. P34, by measuring the enzyme GPT among other biochemical parameters, and reported that after 21 days of administration, the mice did not experience an increase in the enzyme GPT with regard to the control. These results suggested these peptides could be consumed during long periods of time without adverse effects. The same authors compared the toxicity of the antimicrobial peptide P34 against 0.825 mg/Kg of nisin (food additive E234: lantibiotic internationally used as bioprotector in food) and surprisingly, noted a significant increase in GTP levels compared to controls and reported that the bacteriocin, consumed in this concentration, could lead to a significant hepatotoxic effect.

Even though the biochemical markers are relevant tools to have an overview of potential tissue injury or toxicity, the histological examination is a key analysis to have a real notion of the in vivo cytotoxicity effects of certain compounds. In this work, the histological

analysis of liver and small intestine revealed that the mice treated with lower concentrations of CBMDC3f LF (25 and 125 mg/Kg of body weight) showed normal histological features, similar to those of control animals. On the other hand, the mice that received LF 250 mg/Kg showed mild to moderate histological changes, with a mild cellular vacuolization and a non-focused infiltration on inflammatory cells from the liver parenchyma. Almeida Vaucher., *et al.* [31] conducted histological studies of the liver of mice treated with the antimicrobial peptide P34 or nisin. No cellular damage was identified for the group treated with the antimicrobial peptide, while mice receiving nisin presented histological changes that suggested liver degeneration. In addition, the presence of neutrophils indicated the induction of inflammatory response in the liver. In the present work, although liver histological lesions were detected in the group that received the CFS, they were significantly milder in comparison to the mice that received the LF250.

In addition, mice treated with the CFS, LF25 and LF125, showed normal histological features in the small intestine, similar to controls. However, as observed with the liver, intestine histological changes were detected in the mice that received LF250, with moderate degranulation of Paneth cells and mild focal swelling. Almeida Vaucher., *et al*. [31] also reported no histological changes in the intestine of mice treated with the antimicrobial peptide P34 or with nisin, on the contrary of that observed for the liver tissue.

The evaluation of the *in vitro* and *in vivo* toxicity of an antimicrobial lipopeptide is an essential step in order to be considered safe for humans [3]. From *et al.* [35] assessed the *in vitro* cytotoxicity of surfactin-like compounds produced by *Bacillus mojavensis* B31 strain. They reported cytotoxic effect on Vero cells as well as on the mobility of boar sperm; however, they pointed out that no direct evidence was found in order to infer these lipopeptides had enterotoxigenic effect. In addition, they suggested that more direct enterotoxigenicity models (such as animal models) should be used to study the role of lipopeptides in food poisoning.

Hwang., *et al.* [3] also evaluated the cytotoxicity of three different doses of surfactin C in mice (500, 1000 and 2000 mg/Kg) and observed that the compound did not exert adverse effects in concentrations superior to 1000 mg/Kg. In this work, we studied the toxicity of the LF of *B. subtilis* subsp. *subtilis* CBMDC3f in an animal model by using 250 mg/Kg as a maximal concentration. We demonstrated that the LF showed no adverse effects when two doses (LF 25 or 125 mg/Kg of body weight) were orally administered to mice. It should be noted that, the dose of LF of 250 mg/Kg of body weight is 10-fold higher than the optimal dose with antibacterial activity described previously (MIC = 0.5 mg/ml) [9]. Therefore, these results demonstrate that the concentrations of LF that are necessary to induce mild liver and small intestine toxic effects are much higher than those that would be used in practice for application in food and consumption, indicating that LF is a safe compound. This characteristic demonstrates the potential of these lipopeptides produced by the CBMDC3f strain to be applied in foods. Although the impact of the prolonged exposure of the CBMDC3f LF should be analyzed with complementary studies to evaluate a potential chronic damage, the results obtained in this work are promising since the available information regarding the evaluation of lipopeptides toxicity in animal models is scarce. This work represents a breakthrough in the scientific evidence supporting CBMDC3f LF as an alternative for food conservation.

## Acknowledgements

The authors would like to thank Celia Mora, Alejandro Uncos and Renato Uncos for their technical assistance. This work had the financial support of CIUNSa (PI 1974) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) of Argentina for the financial support (PICT2011-07/67). M.C. Audisio, P. Barroso and J. Villena are members of the Research Career of CONICET.

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