

# Whey Protein-Carboxymethylcellulose Obtained by Complex Coacervation as an Ingredient in Probiotic Fermented Milk

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## Abstract

Discharge of whey proteins is still a current practice by small cheese producers. The development of low-cost alternatives for recovery of these proteins is fundamental for small producers who cannot apply expensive techniques. The present study investigated the complex coacervation technique as a cheap technology to recover proteins from sweet whey using carboxymethylcellulose, and the coacervate used as an ingredient in the formulation of probiotic fermented milk. The nutritional properties of whey-carboxymethylcellulose coacervates (WP-CMC) were evaluated in trials with animals (rats) using casein as a reference. All these parameters—the coefficient of feed efficiency (CEA), protein digestibility-corrected amino acid score (PDCAAS), and net protein ratio (NPR), as well as weight gain—were determined to evaluate protein quality. A sensory acceptance test was applied to evaluate the sensory characteristics of the product. The complex coacervation technique recovered 86% of the protein from sweet whey. No significant ( $p > 0.05$ ) differences were observed in the biological tests for both groups (WP-CMC and Casein groups) when NPR (4.98 to 5.04), digestibility (92.35 to 90.64), and CEA (0.40 to 0.42) were evaluated. Probiotic fermented milk beverage containing WP-CMC (0.78%) and guar gum (0.68%) presented good acceptability as determined by sensory evaluation. WP-CMC can be considered an ingredient with high nutritional and biological value that could be applied in probiotic fermented milk as an alternative to small producers to allocate the residual whey from cheese manufacture.

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#In memoriam.

## Keywords

**Nutritional Value, Whey Proteins, Complex Coacervation, Probiotic Fermented Milk, Environmental Impact**

## 1. Introduction

Whey is a residue from artisanal cheese production, and contains high organic load and hard biodegradability [1]. This by-product keeps almost 50% from original milk nutrients [2], and its high-quality makes it an ideal ingredient for special formulations. Whey and its derivatives present effects on body composition, energy balance, satiety, and are indicated for use as fat substitutes in some industrialized foods [3].

Beside its high biological value, sweet whey has high water content (0.9 g protein L<sup>-1</sup>). The high volume of whey from cheese processing is still a problem for small industries and producers [4], and some techniques to protein recovery (ex. ultrafiltration, microfiltration, reverse osmosis, and ion-exchange) are impractical and have a high cost. The inadequate whey disposal leads to environment impacts due to its high biochemical oxygen demand (BOD) (30.000 - 60.000 mg of O<sub>2</sub>·L<sup>-1</sup>) [5]. Thus, some simple and classic techniques can be used by small dairy producers to transform whey into an attractive product with commercial value [1] [2].

The complex coacervation technique is a simple method to recover whey proteins and to reduce the environmental impacts [6]. Complex coacervation consists of spontaneous phase separation by forming an insoluble complex between two or more polymers as a result of electrostatic interactions [7]. The electrostatic interaction results in insoluble agglomerates, which precipitate spontaneously and can be easily separated from the aqueous medium by centrifugation [8]. Whey protein-polysaccharide coacervates by complex coacervation technique have been studied to promote protein recovery and may be applied to different types of foods and biomaterials (fat replacers, viscosity agents, coating and encapsulation). This interaction should be adapted and controlled to preserve or to improve the ingredient functionality and the final quality of the products [9].

Krzeminski *et al.* [10] studied the sensory and textural effects of WP-pectin complexes added to a low-fat yogurt matrix, when compared with a full-fat control. The authors demonstrated the ability of WP-high methoxyl pectin complexes to act as fat replacers and texturizing elements in reduced-fat yogurt. Capitani *et al.* [11] reported that carboxymethylcellulose coacervates (CMC), when complexed with whey proteins (WP), protected proteins against denaturation and increased their viscosity when gel systems were studied.

A combination of probiotics and whey protein concentrate (WPC) for use in yogurt production improved the growth and survival of the probiotic microorganisms *L. acidophilus* (LAC 4, Rhodia) and *B. longum* (BL, Rhodia), especially the former [12]. Fermented milk has several health benefits and is an excellent vehicle for the inclusion of functional ingredients, such as probiotic bacteria, dietary fiber, prebiotic carbohydrates, antioxidants, and other compounds [13].

Probiotics are defined as live microorganisms that, when administrated in adequate amounts, confer health benefits on the host [14]. Several factors have been reported to affect the viability of probiotics in fermented milk, including pH, dissolved oxygen concentration, concentration of lactic acid and acetic acid, storage temperature, strains, and addition of whey protein [10].

Literature reports have demonstrated the physiological properties and technological applicability of whey proteins and whey proteins coacervated with polysaccharides. However, no reports are found on the nutritional quality and amino acids bioavailability of the polysaccharide coacervate proteins. The present study aims to evaluate both the nutritional and technological aspects of whey protein recovered by coacervation technique and its application in probiotic fermented milk.

## 2. Material and Methods

### 2.1. Materials

Sweet skimmed whey was obtained from a small-scale dairy (Espírito Santo do Pinhal, São Paulo, Brazil). Sodium carboxymethylcellulose (CMC) was obtained from Walocel CRT™ 40000 PV (Dow Chemical Company, Brazil). Commercial casein was obtained from Naarden Agro Products. L-cysteine and choline bitartrate were

purchased from Sigma (St. Louis, MO, USA). *Bifidobacterium animalis* supsp. *lactis* Bb-12 was kindly provided by Chr-Hansen/Brazil and *Streptococcus thermophilus* TA 40 by Danisco/Brazil.

## 2.2. Preparation of WP-CMC Coacervate

WP-CMC coacervate was obtained according to a previously described procedure [11] with some modifications. A flowchart with the main steps is shown in Figure 1.

An aqueous CMC solution (0.3% w/v) was stored overnight at 7°C to ensure complete hydration of the polymer to promote further interaction with whey proteins in solution. Initially, skimmed whey (WP) (adjusted to pH 3.0 using citric acid 10%) was mixed with the CMC solution (0.3% w/v), also adjusted to pH 3.0, at a WP:CMC ratio of 1:1 (v/v) and centrifuged at  $2150 \times g$  (SORVALL® RC-26 PLUS industrial scale centrifuge) at 22°C. The coacervates (WP-CMC) were resuspended at pH 6.0 with sodium carbonate  $1 \text{ mol} \cdot \text{L}^{-1}$ , and freeze-dried before use to evaluate their biological value and application as an ingredient in fermented milk.

## 2.3. Chemical Composition of WP-CMC

Protein (micro-Kjeldahl), moisture, ash and lipid contents were determined according to AOAC [15]. Residual lactose content in WP-CMC was determined by liquid chromatography, according to Burgner & Feinberg [16]. All analysis was conducted in triplicate.

The amino acid levels were determined by high-performance liquid chromatography (HPLC) using a reverse phase column on a Shimadzu HPLC (Shimadzu Corporation, Japan) and UV detector at 254 nm. The amino acids released by acid hydrolysis (110°C/22 h) were subjected to pre-column derivatization with phenyl isothiocyanate (PITC). Quantification was performed by internal calibration using amino butyric acid (AAAB) as standard [17], and based on a standard amino acid mixture (standard H/Pierce/P/N 20088). Amino acids analysis was conducted in duplicate.

## 2.4. Protein Recovery and Complex Stability under Different pH Values

After resuspension of WP-CMC in sodium carbonate to pH 6.0, protein recovery was measured by the following equation:

$$\% \text{ Protein recovery} = \frac{\text{Resuspended protein}}{\text{Initial protein}} \times 100$$

In order to evaluate the effect of pH on complex stability, protein solubility was determined in the supernatant at different pH values. The WP-CMC coacervate solution was prepared using distilled water ( $1 \text{ mg protein mL}^{-1}$ ) and the pH value was adjusted to 2.0, 3.0, 4.0, 5.0, or 6.0, and kept under stirring for 30 min. Then, samples were centrifuged at  $2150 \times g$  (SORVALL® RC-26 PLUS industrial scale centrifuge) for 30 min at 22°C. The supernatants were evaluated for protein content by Micro-Kjeldahl method [15]. The percentage of soluble protein was calculated in relation to the initial protein content ( $\text{mg protein mL}^{-1}$ ).

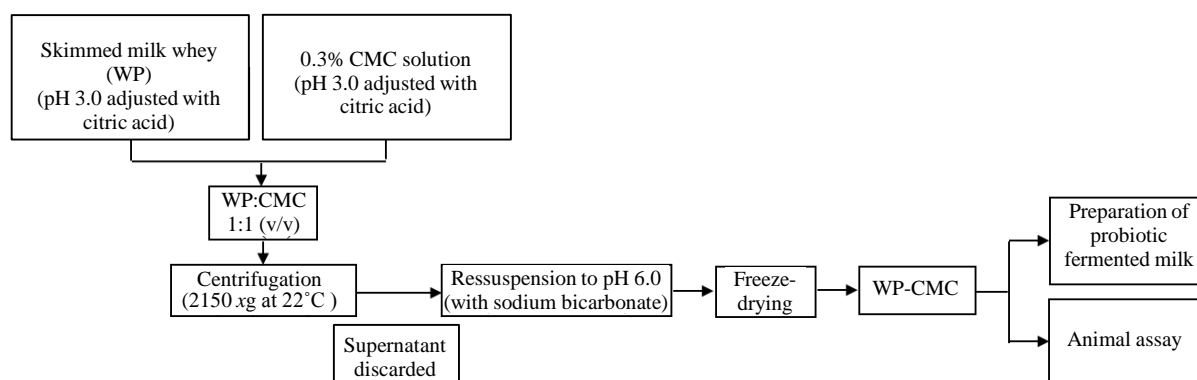


Figure 1. Preparation of whey-CMC coacervate.

## 2.5. Animals and Diets

Male wistar rats ( $n = 21$ ) with initial weight of  $56.16 \pm 3.15$  g were acclimated to standard housing conditions and fed on a 10% casein diet for 4 days. The 10% casein diet (Table 1) limits growth rate and allows differences in protein nutritional quality to be readily demonstrated [18].

Rats were then randomized according to weight into 3 groups (7 rats/group). The groups consumed *ad libitum* diets containing 10% protein consisting of Group 1—Casein (Control) or Group 2—WP-CMC coacervate (WP-CMC) for 14 days. The third group ( $n = 7$ ) was fed on a diet without protein during the same period (14 days) for determination of endogenous nitrogen loss. Apart from the protein content, nutrient levels of all diets were adequate for the growth of rats according to AING-95 [19]. Tap water, freely available for 14 days, was replaced daily during the study. Body weight and food consumption were measured every 3 days, and food consumption was corrected for spillage. Rats were placed in cages and fecal collections were made under clean-housing conditions to avoid mineral contamination. Samples were collected in acid-washed containers. All experiments were conducted according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and the Brazilian Regulations for animal experimentation (COBEA), after approval by Ethical Commission for Animal Experimentation at the University of Campinas (CEE/ UNICAMP).

## 2.6. Protein Nutritional Evaluation

The coefficient of alimentary efficiency (CAE) of the complexes was calculated according to Osborne, Mendel, & Ferry [20], by comparing the weight gain (WG) and the ingested protein (ID) in the experimental groups during 14 days. The CAE was calculated as follows:  $CAE = \text{Weight gain}/ID$ . The protein values of the WP-CMC coacervate were estimated using the net protein ratio (NPR) [18].

True protein digestibility (D%) was determined as recommended by the Food and Agriculture Organization (FAO) [21] for *in vivo* testing. Feces were collected on the 14<sup>th</sup> day of the experiment for nitrogen analysis. The amount of nitrogen consumed by the animals (I), the amount of nitrogen excreted in the feces of animals fed on a protein diet (F), and the amount of nitrogen excreted in the feces of animals fed on a protein-free diet were used to calculate de protein digestibility as follow ( $\%D = \text{endogenous nitrogen minus the amount of nitrogen excreted in the feces of animals fed on a protein-free diet} \times 100$ ).

PDCAAS was estimated by calculating the score of the most limiting essential amino acid [21]. A PDCAAS equal or greater than 1.0 indicates high-quality protein [22].

## 2.7. Preparation of Probiotic Fermented Milk

Initially, a sachet of freeze-dried probiotic culture *Bifidobacterium animalis* subsp. *lactis* Bb-12 (Chr-Hansen) was dissolved in 1 L of sterile whole milk, obtaining  $3 \times 10^9$  colony forming units·mL<sup>-1</sup> (CFU·mL<sup>-1</sup>) of viable probiotic cells. The inoculated milk was transferred to sterile tubes (10 mL) and stored at  $-18^\circ\text{C}$ . A culture of *Streptococcus thermophilus* (TA 40, kindly provided by Danisco) was previously dissolved in milk in the same way (Figure 2).

For manufacture of the fermented milk, commercial skim milk powder was dissolved in water at 10.00% (w/v), and 10.00% sugar, 0.78% WP-CMC coacervate, and 0.68% guar gum were added, according to the results obtained in a previous study [23]. Guar gum was added to minimize syneresis after fermentation.

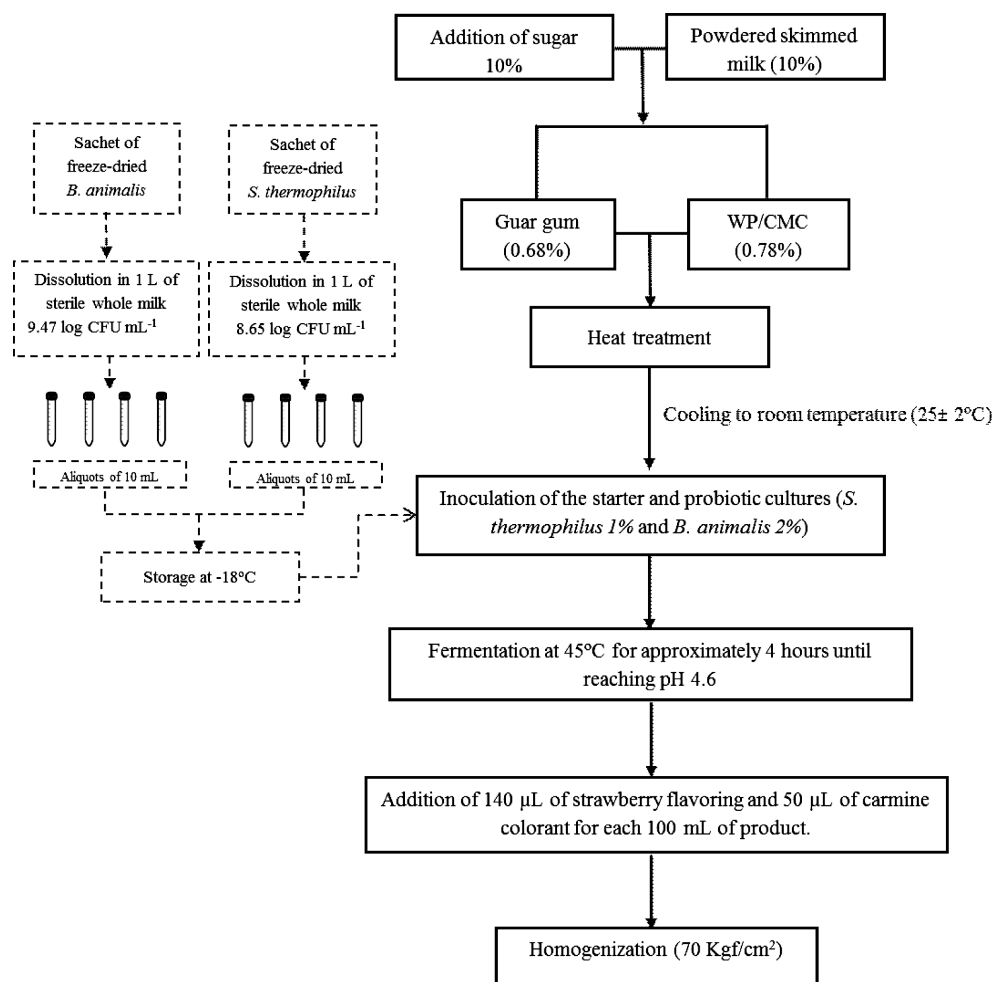
The mixture was heat-treated at  $85^\circ\text{C}$  for 30 min, and then cooled and inoculated with the probiotic bacteria and starter culture (2.00% *B. animalis* and 1.00% *S. thermophilus*), previously dissolved in sterilized milk, as described above. Fermentation was carried out at  $45^\circ\text{C}$  for approximately 4 hours until pH 4.6 was reached. At this point, 140  $\mu\text{L}$  strawberry flavoring (supplied by Symrise®) and 50  $\mu\text{L}$  carmine colorant from cochineal (provided by Corantec®) were added for each 100 mL product. The beverage was homogenized under a pressure of 70 Kg/cm<sup>2</sup> using an FT9 Armfield homogenizer, and kept under refrigeration at  $8^\circ\text{C} \pm 1^\circ\text{C}$ .

## 2.8. Microbiological Analysis of Probiotic-Fermented Milk

Microbiological analyses were carried out at the beginning and at the end of the storage period (3 and 28 days, respectively), according to the procedures recommended by the American Public Health Association [24]. The most probable number procedure (MPN) was used to determine coliforms at  $35^\circ\text{C}$  and  $45^\circ\text{C}$ , using lauryl sulfate tryptose broth (LST from Difco) and brilliant green bile lactose broth (BGBLB from Difco), incubated at  $35^\circ\text{C} \pm 1^\circ\text{C}$  for 24 - 48 h for coliforms at  $35^\circ\text{C}$  [25], and using *Escherichia coli* broth (EC from Difco) incubated at  $44^\circ\text{C}$

**Table 1.** Ingredients, chemical composition, and energy value of diets used in the biological assay with male wistar rats.

Experimental data		
Ingredients (g)	Casein	WP-CMC
Casein (84.41% <sup>a</sup> )	118.47	-
WP-CMC (55.45% <sup>a</sup> )	-	180.34
Saccharose	100.00	100.00
Soy oil	70.00	70.00
Fiber (cellulose)	50.00	50.00
Mineral mix (AIN-93G)	35.00	35.00
Vitamin mix (AIN-93G)	10.00	10.00
L-cistine	3.00	3.00
Choline bitartrate (41.1% C)	2.50	2.50
Terc butyl hydroquinone	0.014	0.014
Cornstarch	611.01	549.14
<b>Total</b>	<b>1000</b>	<b>1000</b>
Energy (kcal/g)	3.87	3.62

<sup>a</sup>Protein percentage.**Figure 2.** Preparation of probiotic fermented milk.

$\pm 1^\circ\text{C}$  for 24 h [26] for the heat-tolerant coliforms. Dichloran rose bengal chloramphenicol agar (DRBC from Difco) was used for yeast and mold counts, incubated at  $25^\circ\text{C} \pm 1^\circ\text{C}$  for 5 days [27].

The viability of the *B. animalis* culture was determined using MRS Agar (Oxoid) supplemented with 0.5% L-cysteine hydrochloride at 10.00%, 1.00% lithium chloride at 10.00% and 0.50% dicloxacillin at 0.10%.

## 2.9. Sensory Analysis

Descriptive sensory analysis was carried out after 3 days of storage at  $6^\circ\text{C}$ , by a group of 35 panelists (mean age 24 years) at the Sensory Laboratory of the Institute of Food Technology (ITAL/Campinas, Brazil). The research project was approved by the Ethics Committee for research in human beings of PUC-Campinas (n. 992/07), and all participants signed a term of consent. The overall acceptance was evaluated by the attributes appearance, consistency, taste, and overall impression, using a nine-point hedonic scale (9 = extremely like, 5 = neither like nor dislike, and 1 = extremely dislike). Sample were identified by a three random number code and served in plastic cups accompanied by natural mineral water for palate cleansing. The test was conducted in individual booths with fluorescent lighting and equipped with computerized Compusense Five version 4.8 for data collection and analysis.

## 2.10. Statistical Analysis

Analyses were performed in triplicate. Data were expressed as mean standard deviations (SD), and compared by analysis of variance (ANOVA) and Tukey's test. Statistical analysis was performed using the STATISTICA 12.0<sup>®</sup> software package for Windows (StatSoft, Inc., Tulsa, OK, USA). Differences were considered statistically significant at  $p < 0.05$ .

# 3. Results and Discussion

## 3.1. Chemical Composition

The chemical composition of the skimmed whey and the amino acid composition of the coacervate (WP-CMC) are described in **Table 2**.

As expected, proteins and lactose were the predominant components in WP-CMC and skimmed whey, respectively. As previously observed by Capitani *et al.* [11] using native PAGE-electrophoresis methodology, the complex coacervation with CMC was able to precipitate the total whey proteins (WP) in this work. A predominance of proteins (57.23%) was observed in the WP-CMC coacervate in the conditions of this study. Thus, the complex coacervation technique was efficiently to separate proteins from sweet whey, presented concentrations of the essential amino acids above the recommendations for adults [28] and maintained the branched-chained amino acids ( $\text{mg AA g protein}^{-1}$ ) from sweet whey (**Table 2**).

## 3.2. Protein Recovery and Coacervate Stability under Different pH Values

The recovery of sweet whey proteins by complex coacervation was effective, since 86% proteins were recovered as protein-binding CMC polymers (pH 3.5), when compared to the initial composition.

The coacervate proteins remained insoluble in the range of pH 3.0 to pH 6.0, as shown in **Figure 3**. Thus, the complexes did not dissociate at fermented milk pH (pH 4.6), and remained aggregated as expected, which can have a positive effect on the final viscosity of the fermented milk.

## 3.3. Body Weight Profiles and Diet Consumption

All groups fed on the protein diets, except the non-protein group, presented a positive and linear tendency towards weight gain during the experimental period (14 days). No significant differences ( $p > 0.05$ ) were observed in weight gain, diet intake, and energy ingestion between the groups (Casein and WP-CMC Groups) (**Table 3**). Thus, the complex coacervation was effective for both protein recovery and maintenance of its biological value, since no difference in weight gain was observed when compared with the control (casein group).

Although similar growth and weight gain values were found for different protein sources in the experimental groups, a lower weight gain was observed in the WP-CMC (~8.7%) during 14 days, when compared to the Casein group. The Casein group consumed  $1.43 \text{ g diet}\cdot\text{day}^{-1}$ , while the animals of the WP-CMC group consumed

**Table 2.** Chemical composition of skimmed whey and WP-CMC (dry basis) and amino acids profile when compared to the WHO/FAO/UNU requirements and commercial casein.

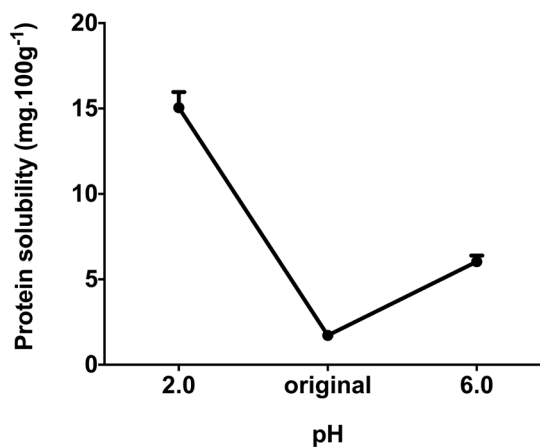
Component (g·100g <sup>-1</sup> )	Skimmed whey	WP-CMC <sup>a</sup>	
Ash	9.31 ± 0.05	1.98 ± 0.96	
Protein	13.05 ± 0.43	57.23 ± 0.32	
Lipids	0.89 ± 0.05	9.42 ± 0.00	
Lactose	76.73 ± 0.32	10.6 ± 0.30	
Amino acid (AA) (mg AA g·protein <sup>-1</sup> )	Requirement pattern <sup>b</sup>	Casein	WP-CMC
His	19.0	35.1 ± 0.4	19.5 ± 0.1
Ile	28.0	55.6 ± 0.1	51.2 ± 0.1
Leu	66.0	119.9 ± 0.5	117.1 ± 0.8
Lys	58.0	90.6 ± 0.7	82.1 ± 0.5
Met + Cys	25.0	40.3 ± 0.4	40.6 ± 0.6
Phe + Tyr	63.0	135.1 ± 1.0	70.7 ± 0.5
Thr	34.0	52.9 ± 0.1	53.7 ± 0.3
Trp	11.0	11.7 ± 0.2	13.0 ± 0.7
Val	35.0	75.5 ± 0.6	58.5 ± 0.2

<sup>a</sup>WP-CMC = whey-carboxymethylcellulose coacervate; <sup>b</sup>Reference WHO [28].

**Table 3.** Weight gain, food and protein intake, net protein ratio (NPR), true digestibility (D), and protein digestibility-corrected amino acid score (PDCAAS) of rats fed on different experimental diets.

Parameter	Diets	
	Casein	WP-CMC
Weight gain (g)	48.21 ± 4.20	43.75 ± 6.28
Total food intake (g)	120.15 ± 12.96	103.46 ± 14.70
Total protein intake (g)	12.07 ± 1.30	10.88 ± 1.53
NPR <sup>a</sup>	5.01 ± 0.26	5.04 ± 0.30
True Digestibility (% D)	92.35 ± 1.01	90.64 ± 2.68
Food Efficiency (% EF)	0.40	0.42
PDCAAS <sup>c</sup> (%)	0.98	0.93

<sup>a</sup>NPR: Net Protein Ratio; <sup>c</sup>PDCAAS: Protein Digestibility Corrected Amino Acid Score.

**Figure 3.** Solubility of protein from coacervates under different pH values.

1.23 g diet·day<sup>-1</sup>. It is well known that polysaccharides can modify food texture and thus enhance product viscosity. The dissociation of WP-CMC during the digestion process, for example, can lead to polymer release. Therefore, the digestion process (at ~pH 2.0) may have contributed to the partial separation of the polymers, and the CMC, partially soluble or in the coacervated form may have contributed to satiety, reducing slightly diet intake in this group (WP-CMC).

However, future studies on adult animals should be carried out to evaluate others parameters such as blood glucose and cholesterol. This design would provide greater support for the discussion of the benefits of using whey proteins-CMC coacervate for weight gain and satiety, in addition to developing special food formulations.

### 3.4. Nutritional Quality

High true digestibility (D) and net protein ratio (NPR) values were observed for the protein in the coacervate products and casein, with no significantly ( $p > 0.05$ ) differences between groups (Table 3). The true digestibility is an important variable affecting the nutritional value of protein food, and commercial whey proteins have a high biological quality, when compared with casein. The present results showed high and adequate digestibility ( $D > 90\%$ ) for all protein sources.

No statistical differences were observed in PDCCAS values ( $p > 0.05$ ) for all groups (Table 3). These results demonstrate the high protein quality of the WP-CMC Coacervate (PDCAAS  $\geq 1.0$ ). The PDCAAS method estimates protein quality, as it is capable of associating the essential amino acid doses with the true digestibility of the proteins. In other words, amino acids will be absorbed if proteins have high true digestibility, considering the most limiting amino acid of the chemical score. PDCAAS parameters and weight gain in experimental animals should be considered in infant formulas with high protein quality. Thus, the complex coacervation method may have efficiently maintained the biological value of the whey proteins [29].

It should be emphasized that complex coacervation is a process that principally involves electrostatic interactions, which are considered to be chemically weak [30]. The pH changes that occur during the digestion process may partially dissociate these complexes, increasing protein solubility and, consequently, its availability (Figure 3). Satisfactory weight gain and high-quality protein indicates a good availability of WP-CMC for digestion and utilization by normal metabolic pathways. In the conditions tested, the WP-CMC coacervate may be suitable for use as food ingredients with special characteristics, and can be applied in a fermented milk beverage, for example. For small dairy producers, it is a rapid and advantageous alternative, allowing protein recovery and its application in dairy products.

### 3.5. Microbiological Analysis

Microbiological analyses were carried out at the beginning and at the end of fermented milk storage (28 days). Counts lower than 3 NMP·mL<sup>-1</sup> for coliforms at 35°C and coliforms at 45°C were obtained in the beginning and at the end of the storage period. For mold and yeast counts, values lower than 10 UFC·mL<sup>-1</sup> were also observed in the fermented milk. Thus, the product presented appropriate microbiological quality according to the Brazilian legislation [31].

For a probiotic product to be marketed as presenting health-promoting benefits, it must present viable minimum probiotics count from 10<sup>8</sup> to 10<sup>9</sup> CFU for a daily-consumption sized portion of the product [32], and the fermented milk produced in the present study attained this requirement (8.44 log CFU·mL<sup>-1</sup>). The application of whey protein coacervate in probiotic fermented milk was feasible and did not interfere with the *B. animalis* viability.

### 3.6. Sensory Analysis

The sensory evaluation of the probiotic fermented milk presented scores corresponding to “moderately like” for all attributes. Among the criteria used by consumers, the highest score ( $7.3 \pm 1.1$ ) was obtained for the attribute appearance, followed by consistency ( $6.2 \pm 1.2$ ), and overall impression ( $6.0 \pm 1.6$ ) on a 9-point hedonic scale. The beverage formulation could be altered according to the target public, especially with regard to flavor, since this attribute received the lowest score ( $5.0 \pm 1.1$ ). The use of coacervate allowed the preparation of a probiotic beverage, with protein of high biological value and good acceptability.



## 4. Conclusion

Protein precipitation from sweet whey using complex coacervation with carboxymethylcellulose is an alternative to recover soluble proteins, being a rapid, efficient, low cost, and clean technique that allows small dairy producers to reuse whey from cheese manufacture. It is advantageous from the environmental point of view, since there is no discard, besides the enrichment of a fermented dairy beverage with a protein of high biological value, as observed in the experimental test. In addition, adequate probiotics viability in the probiotic fermented milk containing WP-CMC was observed. This study evidenced the potential of whey protein-carboxymethylcellulose coacervate as a low-cost strategy to recover sweet whey, which could be used to produce new ingredients for application in probiotic fermented dairy products.

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