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# Preliminary researches to standardize a method of quantitative analysis on *Lactobacillus acidophilus* in poultry feed

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## ABSTRACT

The study focuses on the method and the problems about quantitative analyses in the research on *Lactobacillus acidophilus* after its addition to commercial poultry-feed, whose rough grinding is not suitable for the "IDF Standard quantitative method for lactic acid bacteria count at 37°C" employed in dairy products. Poultry-feed was prepared every month. A sample was collected before and after adding *Lactobacillus acidophilus*, while analyses were carried out respectively at T 0, 15 and 28 days after the food storage at 4-6°C.

The best outcomes (more 30% of recovered cells compared to the standard method) resulted from samples subjected to the homogenization and the addition of Skim Milk Powder.

*Key words:* *Lactobacillus acidophilus*, Probiotics, Quantitative analyses, Poultry-feed.

## RIASSUNTO

RICERCHE PRELIMINARI PER STANDARDIZZARE UN METODO DI ANALISI  
QUANTITATIVA PER *LACTOBACILLUS ACIDOPHILUS* NEL MANGIME AVICOLO.

*Il lavoro riporta la metodica e i problemi relativi alle analisi quantitative per la ricerca di Lactobacillus acidophilus in seguito all'integrazione in mangime commerciale per galline ovaiole, la cui grossolana macinazione non consente di adottare la metodica "IDF Standard quantitative method for lactic acid bacteria count at 37°C" approntata per i derivati del latte. Il mangime veniva preparato ogni mese. Prima e dopo l'integrazione con Lactobacillus acidophilus si prelevava un campione. Le analisi si svolgevano rispettivamente al T 0, dopo 15 e 28 giorni di stoccaggio dell'alimento a 4-6°C.*

*I risultati migliori (30% di cellule recuperate in più rispetto alla metodica standard) sono stati ottenuti con i campioni sottoposti a omogeneizzazione ed aggiunta di Skim Milk Powder.*

Parole chiave: *Lactobacillus acidophilus*, Probiotici, Analisi quantitative, Mangime avicolo.

## Introduction

Probiotics are defined as living microorganisms producing health effects on the host upon ingestion, provided that they are ingested in “adequate amounts” (Guarner and Schaafsma, 1998; Araya *et al.*, 2001). The physiological status of the flock, the probiotic strain(s) involved (including its vitality and liveability within the gastrointestinal tract), the administered dose (CFU) and the treatment’s length are the main factors influencing “on field” results. Today, the European Food Safety Authority authorizes only safe and effective microbial strains in target species. According to EC Regulation No. 2003/1831, probiotics are feed additives of the functional group 4 (b) “stabilizers of the intestinal flora”.

The aim of our research was to adapt the “IDF Standard quantitative method for lactic acid bacteria count at 37°C” to count the probiotics added to poultry feed, evaluating their stability at 15 and 28 days after the storage. These series of tests were performed to investigate the process’ influence, at different storage periods, on the physiological states of lactobacilli. The reason for modifying the standard method is due to the growth of *Lactobacillus acidophilus* in the milk and its dairy products, physically different from the mash.

## Material and methods

### *Sampling methods*

25 kg of complete poultry feed (mash) and 25 g of additive, composed of lactose and freeze-dried *Lactobacillus acidophilus* D2/CSL cells ( $1 \times 10^9$  CFU/g), were mixed together in a pharmaceutical mixer for 4 minutes. Then, it was used the sampling method ISO 2170 (for cereals and ground legumes) to collect six definitive feed sam-

ples (500 g each) in sterile bags, which were submitted to microbiological counting, in order to ascertain both the actual number and the homogeneous distribution of LAB cells in feed. Since the poultry-feed could be naturally contaminated with lactobacilli, the sampling and counting were carried out to check the feed without additive. In total, six different feed samples, produced at a month one from another, were checked to verify the method’s reliability. Each lot was analysed at 0 time, 15 and 28 days after the food storage at 4-6°C. The *Lactobacillus acidophilus* D2/CSL strain was characterized before mixing it with the feed to be used as negative control.

The sample batches submitted to counting were: A=feed without probiotic (“control”); B=feed added with *L. acidophilus* D2/CSL; C=feed added with *L. acidophilus* D2/CSL and homogenized with Ultraturrax® at 10000 rpm; D=feed sample treated as “C” and diluted with reconstituted skim milk immediately before the analyses.

As seen, Ultraturrax reduces the feed’s granulometry, enabling the homogenization of the sample.

The addition of Skim Milk Powder is fundamental for the survival of *Lactobacillus acidophilus*.

### *Counting of lactobacilli in feed*

Method: IDF Standard n. 117A/1988 “Yogurt-enumeration of characteristic microorganisms - colony count technique at 37°C”:

- weigh aseptically 10 g of feed sample in a stomacher bag.

- Add 90 mL of sterile physiologic solution (0.9%) to the 10 g sample (1<sup>st</sup> dilution). The samples belonging to D group were diluted into 90 mL of reconstituted skim milk (10% skim milk powder in water), Oxoid, England.

- Submit the first dilution into stomacher for 90 seconds.

- Continue with the following decimal dilutions transferring 10 mL from the first dilution (-1) in 90 mL (concludes 2<sup>nd</sup> dilution), Repeat the same procedure until the 9<sup>th</sup> dilution.

- Inoculate aseptically 1mL per each plate according to the dilution to carry out (two plates are prepared for each dilution).

- Pour 13-15 mL of MRS agar medium (Biolife, Italia) in each plate containing the diluted sample (the temperature of medium is usually between 45-48°C maximum and minimum).

- Blend the added medium in a rotatory way (anti-clock and clockwise).

- Let the plate dry aseptically.

- Incubate in anaerobic conditions for 3 days at 37°C.

- Count the colonies.

#### Statistical analysis

Statistical analyses were performed using the General Linear Model procedure of SAS® statistic package (SAS, 1997), with

treatment and age employed as sources to analyse the variation (ANOVA). Student's t-test was applied to the calculations of the least square means difference.

#### Results and discussion

We studied a suitable homogenization method to count the probiotic freeze-dried lactobacilli added to poultry-feed. From the first count, it was noticed the existence of some tough mash particles (ground limestone, milled cereals, and so on) in the feed, which caused, during the stomacher homogenization with physiologic solution, bag's ruptures, leakage of the liquid and losses of probiotic bacteria. We carried out preliminary researches to find out that the type of bag is fundamental for the success of the analysis. In particular, those bags with a filter must be rejected, as they retain too much feed particles which alter the dilution.

Three days after the incubation at 37°C, we counted the bacterial colonies in MRS agar.

Table 1 shows the standard deviation, the survival average and percentage of

Table 1. Average and SD of *L. acidophilus* verified in the 4 groups (CFU/g feed).

Groups	A		B			C			D		
	Aver.	± SD	Aver.	% on theoretic	± SD	Aver.	% on theoretic	± SD	Aver.	% on theoretic	± SD
1 day	450	100	9 x 10 <sup>5</sup>	90	1.15x10 <sup>5</sup>	9.37x10 <sup>5</sup>	93.75	75x10 <sup>3</sup>	9.75x10 <sup>5</sup>	97.5	70x10 <sup>3</sup>
			a			a,b			b		
15 days	450	150	5.7x10 <sup>5</sup>	57.5	1.5x10 <sup>4</sup>	7.52x10 <sup>5</sup>	75.20	30x10 <sup>3</sup>	9.62x10 <sup>5</sup>	96.2	74x10 <sup>3</sup>
			a			b			C		
28 "	250	50	4x10 <sup>5</sup>	40	70x10 <sup>3</sup>	5.3x10 <sup>5</sup>	53.0	30x10 <sup>3</sup>	6.97x10 <sup>5</sup>	69.7	22x10 <sup>3</sup>
			a			b			c		

Small letters:  $P < 0.05$ ; capital letters:  $P < 0.01$ ; no difference for same letters.

*Lactobacillus acidophilus* D2/CSL, compared to the initial integration.

Despite a good mixing of the probiotic in the feed, we found that only in some samples the counting was consistent with the expected one ( $1 \times 10^6$  CFU  $g^{-1}$ ). The preliminary grinding of the feed sample, added with Ultraturrax for two minutes at 10000 rpm (paying particular attention to avoid the feed overheating), was fundamental to recover the probiotic colonies actually integrated to the feed (Table 1). Probably the *L. acidophilus* cells adhere to the roughly ground feed materials (e.g. corn, ground limestone, etc.) and they free themselves when further separated. C and D samples also showed a better homogeneity in the analytical quantitative results, as indicated by lower standard deviations (Table 1).

Compared to UFC, the group is statistically relevant, above all, group A shows the lowest UFC/g ( $P < 0.01$ ) values, while groups C and D present the highest ones. As regards the samples analyzed at different days of storage, they don't show any significant differences ( $P > 0.05$ ).

The dilution with reconstituted Skim Milk Powder (D sample) increased the recovered number of lactobacilli, with results of  $1 \times 10^6$  CFU  $g^{-1}$ , near to the expected ones.

The differences between D samples and the other types increased at 2 and 4 weeks of food storage, in accordance with the results of Bernardeau *et al.* (2001). The efficacy of the standard method was considerably more sensible if applied to grinding samples (C and D). In fact, 15 and 28 days after the storage, the rate of recovered CFU was higher than 18% and 10% respectively in C vs. B samples. Furthermore, D sam-

ples showed, absolutely, the best recovery of CFUs, due to the grinding and the addition of skim milk, which led to differences of 30% with the B ones at 28 day.

Maybe, substituting the physiologic solution with the skim milk as diluent, a positive effect on the viability of the cell is achieved and the beneficial effect appears, especially after the storage.

## Conclusions

Due to the number of involved variables, our research, aiming to ameliorate the standard method to count lactobacilli in poultry feed, is to be considered as a preliminary work.

Certainly, the samples'granulometry is an important aspect to consider. In particular we found that reducing the feed particle size the CFUs recovery improved and the addition of Skim Milk contributes to dilute the samples better.

The decrease of probiotic freeze-dried cells during the feed storage is a well known phenomenon influenced by a number of environmental factors (Abbiss, 1983). The storage at low temperatures is surely favourable to a better bacterial viability. Generally, poultry-feed is stored in silo at temperatures which vary from winter to summer, according to the location. That is why, further analyses on feeds stored at different temperatures are necessary.

In addition, researches concerning the stability of freeze-dried lactobacilli in different feed typologies (e.g. pellets, crumbled) must be carried out in order to optimize the zootechnical performances expected from the probiotic administration.

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