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Short Communication

Is Raw Milk Microbiota Influenced by the use of Live Yeast (*Saccharomyces cerevisiae*) as Ruminant Feed Additive?

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Abstract

Live *Saccharomyces cerevisiae* yeast strains are widely used as feed additives for dairy cows. Although, perfectly safe and applied in several food processes, the potential contamination of milk by the strain and more generally its possible influence on the microbial profile of milk could be questioned, this concerns particularly traditional cheeses made with raw milk. In an attempt to address these questions, two experiments were performed: A trial in an experimental farm, where two groups of cows, one receiving yeast supplementation and the other one being an unsupplemented control, were compared and a field trial where herds received alternatively yeast-supplemented and control diets. In both cases, viable counts of different microbial groups, including yeasts and molds in general and *S. cerevisiae* in particular were analysed in tank milk and results from supplemented groups or periods were compared with control ones. No viable *S. cerevisiae* were detected in any of the samples analysed. Moreover, no noticeable differences in the microbial profiles were observed. This applies also to the evolution of the microbial composition during an experimental cheese processing trial. These data suggest that live yeast additive distributed to dairy cows does not influence milk microbial composition and cheese-making abilities.

Key words: Dairy cow, milk, cheese, live yeast, feed additive

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In the last present years, the use of antibiotics and chemical growth promoters in livestock production has been dramatically reduced, as a result of both regulatory constraints and consumer demand. To fulfill the need for optimizing the management of intensive animal production, more natural solutions based on plant or microbial ingredients have been proposed. Live microorganisms known as "Probiotics" or "Direct Fed Microbials" are one of the most rapidly developing categories of these natural feed supplements (Chaucheyras-Durand and Durand, 2010).

In dairy production, the most thoroughly used microbial feed supplement is live yeast, mostly of the *Saccharomyces cerevisiae* species. This species being traditionally used for centuries in food processes (baking, brewing, wine making etc.) is considered as very safe (EFSA., 2007) and is generally well accepted by both farmers and consumers. Many published studies have described the beneficial effects of daily yeast supplementation on milk yield (Desnoyers *et al.*, 2009; De Ondarza *et al.*, 2010), or milk composition, in particular on milk fat percentage (Moallem *et al.*, 2009; DeVries and Chevaux, 2014). For some strains, modes of action have been described, such as direct interactions with ruminal microbial communities or modulation of ruminal environment that change microbiota balance in a more favorable way for the animal (Chaucheyras-Durand *et al.*, 2008).

In some European countries (notably France, Italy or Spain), a significant part of cheeses available on the market is traditionally made with raw milk. The microbiological quality of such products, partly resulting from that of milk, is of paramount importance. Indeed, some microorganisms can be detrimental, i.e., pathogenic, toxinogenic or provoking flavor defects, some others are essential to the proper development of required organoleptic properties (taste, texture and colour) during cheese maturation (Desmasures and Beuvier, 2011).

Although, various yeasts and molds species, including *Saccharomyces* sp., have been identified in raw milk (Panelli *et al.*, 2013, 2014; Montel *et al.*, 2014), *S. cerevisiae sensu stricto* species has never been mentioned. Therefore, the potential presence of *S. cerevisiae* in milk when using this species as a feed supplement could be regarded as questionable, even considering its safety and inability to metabolize lactose (Chen and Chiger, 1985). On another aspect, since the traditional cheese processes, in particular the Protected Designation of Origin (PDO) cheeses, rely on microbes naturally present in raw milk (Montel *et al.*, 2014),

the potential impact on milk microbial profile when using feed additives acting on the cow gut microbiota could be questioned.

In this context, compared microbial populations in milk collected from cows supplemented with live yeast vs controls, by using two distinct approaches. First, a trial was performed in an experimental farm with well controlled conditions, where the herd was randomly split into two groups, one being supplemented and the other one being a control. Secondly, a field trial involving five farms where periods of supplementation alternated with control periods was carried out.

MATERIALS AND METHODS

Trial 1: University farm trial with contemporaneous group comparison

Trial design: The study was conducted with 40 Holstein dairy cows in the experimental farm of the University of Bologna, Italy. The lactating herd was divided into 2 groups, homogeneous for milk yield, fat and protein content, parity and body weight. Criteria to select cows undergoing the experiment were, among others, parity number <4 and 30 < days of pregnancy <160.

Animals received the same diet under the form of Total Mixed Ration (TMR) that was formulated to meet the requirements of high producing cows according to NRC (2001). The diet was based on hay (43% of the TMR) and did not include any silage in accordance to the operating instructions to produce Parmigiano Reggiano cheese. The experimental product (*Saccharomyces cerevisiae* CNCM I-1077, Lallemand Animal Nutrition, Blagnac, France) was provided at the dose of 2×10^{10} CFU of live yeast/head/day in a premixture to be included in the diet at 100 g/head/day. The control group received 100 g/head/day of a placebo which consisted in the carrier (limestone) used in the premixture.

The duration of the trial was 63 days, including a 14 days observation period with both groups receiving the same diet without yeast supplementation, followed by a 42 days period with one group receiving the yeast supplementation and the other group the placebo. At the end of the trial, the groups were kept separated during 7 days and the parameters continued to be recorded as described below.

During the whole trial, the following parameters were recorded daily: Group feed intake, individual milk production and composition (Afilab NIR technology), individual body weight. The milk was collected in two distinct tanks, one for

Table 1: Specific media and incubation conditions used to enumerate different microbial categories in Trial 1

Microbial category	Medium	Incubation temperature (°C)	Incubation time (h)	Reference
Yeasts	Sabouraud Chloramphenicol agar	25	72	Carlier (1948)
Enterobacteria	Violet Red Bile Glucose agar	37	24	ISO (2004)
Mesophilic bacteria	PCA with 1 g L ⁻¹ of skim milk	30	24	Franciosi <i>et al.</i> (2011)
Mesophilic lactobacilli	MRS agar	30	48	De Man <i>et al.</i> (1960)
Thermophilic lactobacilli	MRS agar	42	48	De Man <i>et al.</i> (1960)
Mesophilic lactococci	M17 agar	30	48	Terzaghi and Sandine (1975)
Thermophilic streptococci	M17 agar	42	48	Terzaghi and Sandine (1975)

Table 2: Specific media and incubation conditions used to enumerate different microbial categories in Trial 2

Microbial category	Medium	Incubation temperature (°C)	Incubation time (h)	Reference
Total mesophilic flora	PCA skim milk agar	30	72	ISO (2003)
Gram negative bacteria	PCA skim milk agar+inhibitors (purple cristal and vancomycin)			
		30	48	ISO (2003)
Lactic acid bacteria	MRS agar	30	72	De Man <i>et al.</i> (1960)
Enterococci	Bile Esculin agar	37	24	Swan (1954)
Enterobacteria	Violet Red Bile Glucose agar	37	24	ISO (2004)
<i>Pseudomonas</i> sp.	CFC	25	48	Mead and Adams (1977)
Yeasts and molds	Sabouraud Chloramphenicol agar	25	72	Carlier (1948)
<i>Saccharomyces cerevisiae</i>	Lysine agar (1)	25	7 days	Morris and Eddy (1957)

Lysine agar medium is usually recommended as a selective medium for "wild yeasts" as opposed to *Saccharomyces* species used for brewing or baking. On this medium, the latter grows very slowly and gives small, white colonies while the former generally grows vigorously and forms larger colonies. However *S. cerevisiae* colonies are visible even on a background of wild yeasts

each group, through separate piping circuits. Individual milk production data in the 2 groups were compared with t-test (Statistica 10.0, Vicenza, Italy).

Microbiological analysis of milk: For the purpose of evaluating the potential effect of yeast supplementation on the microbial populations playing a role in cheese organoleptic properties, it was chosen to focus on metabolically active biomass and on functional groups rather than taxonomy. That is why the viable counts determination by plating on specific media was preferred to molecular techniques. At day 49, 56 and 63, samples of tank milk from both groups were taken aseptically to be analysed for microbial content. On the same days, viable count determinations were performed on specific media as described in Table 1.

In addition, a cheese processing trial according to Parmigiano Reggiano traditional process was performed on a 300 kg batch of milk from the treated and control groups at days 49 and 56, respectively. The process involves a curdling step induced by rennet addition, a cooking step i.e., heating of the curd for 10-12 min at 56°C and a natural cooling step. At each step of the process (milk pre-curdling, curd before cooking, cooked curd) samples were taken and microbial counts were performed as described above.

Trial 2: Multi-farm trial with alternative periods of treatment

Trial design: Five dairy farms in the South-West of France (Haute-Garonne and Tarn Departments) were selected as

being geographically close to the lab where analyses were performed and comparable to each other as regards with herd size, management and feeding practices. The number of lactating cows (Holstein breed) per farm ranged from 45-77, the milk yield per cow ranged from 8000-10000 kg per year. All rations were total mixed rations based on corn silage and concentrates in comparable proportions.

The duration of the trial was 15 weeks, divided into 5 periods of 3 weeks each. During the first period, three farm managers (farms coded A, C and F) were asked to supplement their ration with live yeast (*Saccharomyces cerevisiae* CNCM I-1077, Lallemand Animal Nutrition, France) at the dose of 2×10^{10} CFU/head/day, introduced in the TMR as a premixture. The other two (B and R) did not use yeast supplementation. During the second period, the supplementation concerned the latter farms (B and R), while the former (A, C and F) interrupted it. This switch was repeated during the third and fourth periods. During the last period, none of the farms used yeast; this period was intended to evaluate potential carry-over effects of the yeast supplementation.

Microbiological analysis of milk: In each farm, samples of milk were taken aseptically from the tank every week at the same time. The No. of milkings varied from 1-4 between farms but were constant within a given farm. The samples were immediately brought to the lab in refrigerated containers to be analyzed within 6 h at maximum.

On each sample, microbial counts were performed by plating on specific agar media as described in Table 2.

Table 3: Microbial profile of tank milk samples from Trial 1 (\log_{10} CFU mL⁻¹)

Day of sampling	Group	Entero bacteria	Mesophilic bacteria	Yeasts	Mesophilic lactococci	Thermophilic streptococci	Mesophilic lactobacilli	Thermophilic lactobacilli
49	Control	2.9	4.0	2.2	3.7	3.4	2.9	2.3
	Treated	2.5	4.6	2.3	4.3	3.2	3.1	2.3
56	Control	1.1	4.2	<1	3.8	3.6	3.4	2.6
	Treated	1.9	4.8	1.6	4.5	4.5	3.3	2.9
63*	Control	2.4	3.9	2.6	3.6	3.2	3.0	2.0
	Treated	2.2	4.5	2.7	4.2	3.6	3.4	2.6
Mean±SD	Control	2.1±0.9	4.0±0.2	1.9±0.8	3.7±0.1	3.4±0.2	3.1±0.3	2.3±0.3
	Treated	2.2±0.3	4.6±0.2	2.2±0.6	4.3±0.2	3.8±0.7	3.3±0.2	2.6±0.3

*samples taken one week after the end of yeast distribution to "treated" group

Table 4: Microbial profile of tank milk samples from Trial 2

Farm	Yeast distribution	Entero bacteria	Mesophilic bacteria	Yeasts and molds	Lactic acid bacteria	Gram negative	Enterococci	Pseudomonas
A	No	2.2±0.4	4.5±0.9	2.4±0.6	2.9±0.6	3.7±0.5	3.0±0.5	3.5±0.5
	Yes	2.1±0.7	4.4±0.7	2.7±0.3	2.9±0.5	3.1±0.3	3.1±0.9	3.8±0.8
B	No	1.2±0.6	4.5±1.0	3.2±0.8	2.5±1.0	2.2±1.2	3.1±0.8	2.8±1.5
	Yes	0.9±0.7	3.9±0.5	3.0±0.4	2.3±0.6	1.9±1.7	2.6±0.5	2.3±1.4
C	No	1.1±1.0	3.6±0.6	2.6±0.6	2.0±0.2	1.4±1.2	2.6±0.4	1.3±0.9
	Yes	1.1±0.6	4.0±0.9	2.7±0.6	1.9±0.7	0.5±1.0	2.9±0.5	1.2±1.0
F	No	1.2±0.9	3.7±0.2	2.8±0.2	1.8±0.2	2.3±0.8	2.3±0.2	1.9±0.4
	Yes	1.2±0.8	4.0±0.8	2.8±0.2	1.3±0.9	2.5±0.9	2.2±0.3	2.1±1.8
R	No	1.4±0.2	4.4±0.5	3.3±0.3	1.9±0.5	2.3±1.4	2.9±0.4	2.6±0.5
	Yes	1.3±0.4	3.9±0.6	3.4±0.8	2.2±0.5	2.2±1.3	3.1±0.3	2.1±0.5
Pool	No	1.4±0.9	4.1±0.8	2.8±0.6	2.2±0.7	2.4±1.4	2.7±0.6	2.4±1.1
	Yes	1.3±0.7	4.0±0.6	3.0±0.5	2.1±0.8	2.0±1.4	2.8±0.6	2.3±1.3

(Mean±SD of 9 weekly samples taken during control periods, 6 samples during yeast distribution periods, in \log_{10} CFU mL⁻¹)

The results obtained during the yeast supplementation periods in a given farm were compared to those during the control periods in the same farm, using ANOVA with Fisher test as a statistical tool.

RESULTS AND DISCUSSION

Zoo technical parameters: For Trial 1, the group receiving yeast supplementation produced 1.3 kg more fat corrected milk (27.1 vs 25.8 kg day⁻¹) and energy corrected milk (27.4 vs 26.1 kg day⁻¹) ($p < 0.05$) as compared to the control group. A slight but significant increase in fat content was also observed (3.24 vs 3.18%, $p < 0.05$). For Trial 2, the design did not allow to accurately compare the production between yeast supplementation periods and control periods. However, no notable variations in the production parameters were observed in between periods.

Monitoring of milk contamination by live yeast supplement:

In Trial 2, the potential presence of *Saccharomyces cerevisiae* viable cells was sought for in milk samples, by plating on lysine agar medium. No colonies harbouring the characteristic shape and color of the *S. cerevisiae* CNCM I-1077 strain, plated as a control at the same time, were detected in any of the 75 milk samples analysed.

Microbial profile of raw milk: The results of the microbiological analysis of milk samples from Trial 1 are shown in Table 3. The highest viable counts were observed for total mesophilic bacteria (3.9-4.8 \log_{10} CFU mL⁻¹), closely followed by mesophilic lactococci (3.6-4.5 \log_{10} CFU mL⁻¹). This tends to indicate that lactococci are the main constituents of the mesophilic flora. These concentrations are in agreement with those described in the literature (Bouton *et al.*, 2005; Marion *et al.*, 2008). Yeasts and Enterobacteria were found at much lower concentrations (<1-2.7 and 1.1-2.9 \log_{10} CFU mL⁻¹, respectively). However, max values for these two groups appear to be higher in our samples than those reported elsewhere (Bouton *et al.*, 1998; Marion *et al.*, 2008; Desmasures and Beuvier, 2011). This may reflect the specific environmental conditions of the farm (e.g., bedding and feeding practices) and/or differences in the methods of analysis.

When comparing the microbial profiles between the two groups of cows, although the small No. of samples did not allow a proper statistical analysis, no notable differences were observed.

In Table 4, the microbial profiles of the raw milk collected during control and yeast supplementation period in each farm of Trial 2 are displayed.

Fairly consistent viable counts were found for each microbial category, with acceptable variations between

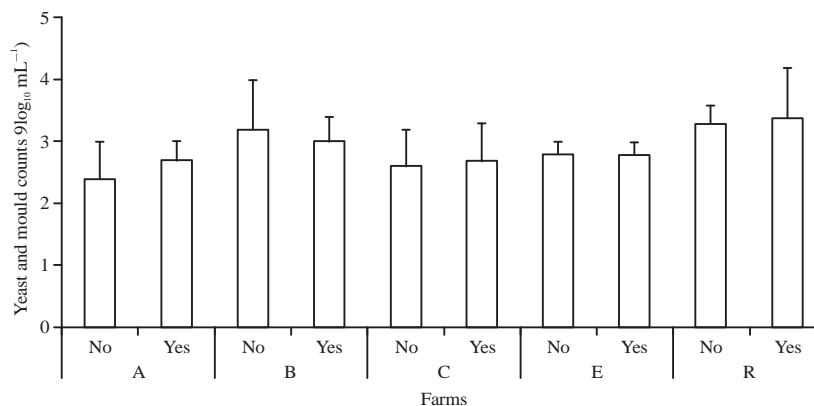


Fig. 1: Viable yeast and mould counts in tank milk collected in the different farms during non-supplemented (no) and supplemented (yes) periods of Trial 2. Means and SDs are shown

Table 5: Evolution of microbial profiles of milk and curd during cheese processing in Trial 1 (log₁₀ CFU mL⁻¹)

Matrix	Group	Entero bacteria	Mesophilic bacteria	Yeasts	Mesophilic lactococci	Thermophilic streptococci	Mesophilic lactobacilli	Thermophilic lactobacilli
Milk pre-curdling	Control	5.6	6.7	3.8	6.6	6.6	6.4	3.7
	Treated	2.9	5.1	3.4	4.7	4.3	4.3	3.0
Curd before cooking	Control	4.8	6.5	3.8	6.4	6.4	6.4	3.3
	Treated	1.9	4.7	2.6	4.3	4.2	3.8	3.4
Cooked curd	Control	0.8	5.5	<1	5.4	5.4	4.2	4.3
	Treated	<1	4.2	<1	3.5	4.6	2.3	3.1

samples within a farm on the one hand and between farms on the other hand.

As in Trial 1, mesophilic bacteria appear as the major microbial group and the levels observed are in line with published data. Gram negative bacteria, lactic acid bacteria, Enterobacteria, enterococci and pseudomonas are also found at concentrations comparable to published results (references cited above). Similarly, to Trial 1, yeast and molds counts were detected at higher levels, which might reflect methodological or environmental differences.

When comparing the results obtained during the yeast supplementation periods in a given farm to those during the control periods in the same farm, no significant differences ($p > 0.05$) were found.

No clear cut difference between supplemented and non-supplemented periods can be observed on yeast and mold counts (Fig. 1). This corroborates the findings that yeast supplementation through the ration does not appear to influence the fungal population in raw milk.

Evolution of microbial profiles during cheese processing:

The effect of processing on the evolution of the microbial profiles was similar in both groups (Table 5). The cooking step resulted in a marked decrease of mesophilic bacteria counts and a dramatic drop in Enterobacteria and yeasts, the latter was even not detected in the cooked curds. Thermophilic

streptococci were less affected by cooking and thermophilic *lactobacilli* remained essentially unchanged. These results are in accordance with those of previously published study, where a decrease in mesophilic flora and not in thermophilic bacteria after the cooking step were also described (Coppola *et al.*, 2000; De Dea Lindner *et al.*, 2008).

CONCLUSION

The present study aimed at studying the potential effect of live yeast additive daily supplementation to dairy cows on the microbiological properties of raw milk used in traditional cheese manufacturing. First of all, the question of possible passage of viable yeast cells in milk was addressed. By using a semi-selective cultural method, live *S. cerevisiae* cells were never detected in the samples analysed. It appears that although being very ubiquitous in nature, *S. cerevisiae* does not find a favorable habitat in raw milk; one of the reasons is probably linked to its lack of capacity to metabolize lactose.

The next question concerned more generally the change in milk microbial profile potentially induced by yeast supplementation. As far as the various microbial groups targeted in this study are concerned, no differences were observed between milk obtained from yeast supplemented cows vs control cows. This applies both in raw milk and in curd during cheese processing.

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