**Report: Deliverable 2.2.2 Passive transfer of immunity from cows to calves and antibodies in nursing cows' milk**

**Authors: Bieber, A[1]., Nicolao, A.[2], Pomies, D. [2], Mathieu, A.[2], Koczura, M.[2], Bouchon, M.[2], Constant, I.[2], Walkenhorst, M. [1], Eppenstein, R.[1], Probst, J.K.[1], Thüer, S.[1], Baki, C.[1], Spengler Neff, A.[1], Martin, B.[2]**

**[1] Research Institute of Organic Agriculture, FiBL, Department of Livestock Sciences, Ackerstrasse 119, CH-5070 Frick, Switzerland**

**[2] Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genès-Champanelle, France**

## 1. Research questions

A common belief is that separating the calf at birth limits the risk of transmission of diseases which is better for both calf and cow health. The efficiency of calf cow contact (CCC) systems in achieving passive immune transfer is partly controversially discussed: on one hand, sucking of calves by their dam is thought to be less efficient in controlling colostrum ingestion (Beaver et al., 2019). On the other hand, some studies report that calves left with the dam have higher levels of IgG absorption and serum IgG concentrations than calves bottle-fed colostrum (Selman et al., 1971; Stott et al., 1979), while others did not identify differences in IgG concentration in rearing systems with longer cow contact (Hillmann et al. 2019). Colostrum-derived passive immunity is vital to health, performance, and welfare of neonatal calves (Mcgee & Earley, 2019). Calves are born agammaglobulinemic because the cow's placenta prevents immunoglobulin (Ig) transmission in utero (Godden, 2008). Consequently, the transfer of sufficient immunoglobulin G (IgG) to the neonatal calf through colostrum is essential to provide the calf with immunological protection and resistance against disease (Conneely et al., 2013). Transfer of immunoglobulin G, which represents 85% to 95% of total immunoglobulins (Larson et al., 1980), occurs from the blood through the mammary epithelium and accumulates in the mammary gland before parturition (Conneely et al., 2013). IgG levels of < 10 g/L at 24–48 h of age in calves' serum indicate an inadequate transfer of IgG (Besser et al., 1991). An optimum colostrum management is required within the few hours after birth to allow calves receiving a sufficient amount of clean and high-quality colostrum (Johnsen et al., 2019). In any case, the main factors to obtain an optimal transfer of immunity are the timing of colostrum ingestion, the quantity and the quality of colostrum and, when it is possible, the presence of the dam (Johnsen et al., 2019). When calves are allowed to suckle colostrum directly from the dam, factors such as rapidity to stand, to walk and to find teats or good maternal bond, are parts of the success of rapid colostrum ingestion (Mcgee & Earley, 2019).

Suckling is also thought to improve udder health (Fröberg et al., 2005; Margerison et al., 2002). Several studies reported that suckling decreases the milk somatic cell counts (SCC) during the suckling period and this can be related to a lower mastitis incidence (Boden & Leaver, 1994; Krohn, 2001; Margerison et al., 2002). In addition to SCC, another factor in determining udder health could be lactoferrin (LF). LF is a bioactive multifunctional protein of the transferrin family. LF is present mainly in the secretions of all mammals, especially in milk (Wang et al., 2021). In milk of lactating animals, LF plays a key role in

mammary gland defense mechanisms (Cheng et al., 2008). LF participates in bacterial infections because bacteria require iron for growth, and under certain conditions, LF can inhibit bacteria by chelating iron (Weinberg, 1978). Some studies found a correlation between SCC and LF (Cheng et al., 2008; Harmon et al., 1975). This finding suggests that milk LF may be helpful as an indicator for intra-mammary infection in dairy cows (Cheng et al., 2008). For neonatal calves, milk LF, thanks to its broad spectrum of anti-microbial properties and to its regulatory functions in the immune system may also play a role for the preservation of calves' health. It was demonstrated that claves receiving LF supplemented milk had less days of disease with less serious cases of diarrhoea as well as higher weight gains (Prenner et al., 2007). In humans, Mondal et al. (1998) demonstrated that significantly greater concentrations of LF in breast milk reduced episodes of diarrhoea in infants. We hypothesized that similar mechanisms could exist also in bovine.

Finally, suckling seems to improve the health of animals, or at least this is the perception of farmers and one of the main motivations that leads them to implement this practice (Michaud et al., 2018). On-farm trials were conducted in Switzerland (CH) and France (FR) to test 1- passive transfer of immunity from cows to calves and 2- whether immune (IgG) and antimicrobial (LF) parameters in dairy cows' milk would change by nursing. Calves' plasma and milk from cows being milked and additionally suckled twice daily for 30 minutes after milking (CH) or between morning and evening milking (FR) was compared to milk from dairy cows in similar lactations stages, but only milked.

## 2. Material and Methods

### 2.1 Animals and study design

**CH**: Directly after birth, calves were randomly assigned by sex and parity status of the mother (primi- or mulitparous) to become either a bucket fed (BF) or a mother suckling (MS) calf. Cow- calf couples involved in this study stayed together for a few days post partum (CH Farm1: approx. 7 days, CH Farm2: approx. 2 days). Colostrum ingestions occurred directly from the mother in all calves. Colostrum intake was checked by means of a blood sample taken within a maximum of 48 hours post partum. In the blood serum the proportion of total protein was determined by means of a refractometer (a minimum of 50 g/L required). Afterwards calves from both feeding regimes were moved to the group pen and housed together in deep straw littered barns with outdoor runs. Cows were kept in a loose housing barn on both farms involved in CH trials. Cows grazed in summer, in winter they were fed hay, grass-silage and grass-clover silage, but no concentrates. On farm 1 the old German Friesian cows (DNS) had an annual milk yield of 6000 kg, while the Swis Fleckvieh (SF) cows on farm 2 achieved an average annual milk yield of 5500 kg. In CH trials, cows with calf contact (CC) met their respective MS calf for approx. 30 minutes in the cow waiting area after having been milked. Milk intake was regularly assessed by weighing the MS calves before and after milking and was controlled by a feeding scheme for BF calves. BF calves were fed with teat buckets during the same time slots as MS calves. We aimed at achieving comparable milk intakes, as we wanted to eliminate the effect of higher milk intake usually associated with cow-calf contact rearing systems. In both groups each calf consumed around 900 kg milk during four months (farm 1: BF= 1000 kg, MS= 940 kg, farm2: BF=888 kg, MS=977 kg) and achieved comparable weight gains during this time (daily weight gain: BF= 807 ± 42 g/d, MS= 815 ± 46 g/d)**.**

**FR:** At INRAE experimental farm 'Herbipole', we tested two different suckling strategies on animal performance and behavior as described in D2.1 (Trial 3 "Volame 3" experiment). Briefly, a 14-cow 'Control'

rearing system (C group) was compared during 14 weeks to two suckling systems. In the C group, calves were separated from dams immediately after birth and fed with an automatic milk feeder until weaning. new-born Control-calves received at least 2.0 L of fresh colostrum from a feeding bottle. If there was no good-quality fresh colostrum available (< 24% Brix, measured by refractometer), then good-quality thawed and reheated colostrum was provided. In the 'Dam' group (D), dam-calf contact was allowed from birth to weaning, between morning and evening milking. In the 'Mixed' group (M), calves were kept with dams until three weeks (as in D group) before being separated and reared as in C group. Calves spent the first three days after birth with their dams in specific pens and during these days, animal caretakers checked at least twice a day whether the calves properly suckled their dam. All calves were weaned at about 11 weeks. In all groups, five male calves were sold at the age of 3-4 weeks. On average, over 14 weeks, Dam and Mixed cows produced 25.1% less milk at parlour than C cows; milk fat content was 3.6 g/kg lower in the Dam group compared to Control and Mixed cows, and milk protein content was intermediate between Control and Mixed. After 11 weeks, Dam calves weighed 20.5 kg more than Control and Mixed calves.

**2.2 Milk sampling and analysis**

**CH:** Milk samples were taken directly after birth (colostrum sample) and at week 3, 8, 12, 16 post partum. In total 44 cow-calf couples from two farms were involved: 21 with restricted cow-calf contact of 0.5 h twice a day and 23 cows without calf contact (calves were teat bucket fed twice daily).
Milk samples were frozen after collection and stored at -18°C. After all samples from the Swiss trials had been obtained, the frozen milk samples were sent from Switzerland to France in insulated Styropor boxes, 1/3 filled with dry ice. They were transported within 48 hours without any interruption of the cold chain.

**FR**: Milk samples were taken in 30 mL dry vials/tubes at the first milking after calving, at the evening milking three days after calving (colostrum), at week 3 when all calves were still suckling, at week 7 and 9 when calves (9 per group) were still sucking in the Dam group and finally at week 13 when all calves were weaned. In total 42 cow-calf couples were involved. Milk samples, collected separately one morning and the following evening, were frozen at -20°C until the analysis of immunoglobulin G (IgG) and lactoferrin (LF).

All milk samples were analysed in the Agrolabs' laboratory (Aurillac, France). An enzyme-linked immunosorbent assay (ELISA) was used to determine lactoferrin content (mg/L) and a radial immunodiffusion method (Bovine IgG1 Test from IDBiotech, Issoire, France) to determine immunoglobulin G content (mg/L) in cows' milk.

**2.3 Blood serum sampling and analysis**

**CH:** Within 48 hours post partum a blood sample of 10ml was taken from the Vena jugularis externa after shaving and disinfection with 70 % alcoholic solution using a sterile V2A cannula (Braun-Melsungen, Sterican 1,3 mm). Thereafter, whole blood samples without anticoagulant were taken in weeks 3, 8, 12 and 16. The blood samples were stored at room temperature for at least 1 hour until the blood coagulated and then spun in a centrifuge (Heareus Multifuge 1S Centrifuge) at 1300 G for 15 minutes to obtain serum. Serum was pipetted into approximately 2 mL cups and frozen at −20°C for later IgG analysis.

**FR**: Individual blood samples were taken at the jugular vein of all the 42 calves at 48 hours after birth into 10 mL vacutainer tubes, then just before the separation (Week 3). Finally, samples were taken on week

10, before weaning in 27 female calves. Blood samples were immediately centrifuged for 20 min at 3000 × g and 4°C. Serum was pipetted approximately 1 mL into 3 x 1.5 mL cups for each tube (= 9 cups) and frozen at −20°C for later IgG analysis.

All blood serum samples were analysed in the VetAgro Sup laboratory (Marcy l'Etoile, France), applying a radial immunodiffusion method (Bovine IgG1 Test from IDBiotech, Issoire, France) to determine the immunoglobulin G content (IgG, in mg/dL).

**2.4 Statistical analysis**

**CH**: In order to test the effect of calf contact versus calf absence on immunoglobulin G and lactoferrin content in cows' milk we applied the following model:

$$\mathbf{y_{ijkl} = \mu + T_i + Time_j + (T \times D)_{ij} + Sex_k + Parity_l + Farm_m + CY_n + CS_o + Cow_p + ColostrumValue + e_{ijklmnop}}$$

where: $y_{ijkl}$ is the dependent trait (immunoglobulin content in serum, immunoglobulin content in milk or lactoferrin content in milk); μ is the overall mean; $T_i$ = treatment ( for serum samples: I = bucket fed calves; mother suckling calves; for milk samples: i= with calf contact; without calf contact), $Time_j$= week of observation (j: 2, 8, 12 or 16 weeks after calving), $(T \times D)_{ij=}$ is the interaction between treatment and week of observation, $Sex_k$ = sex of the calf (k=male, female), $Parity_l$ = parity of the cow (l=primiparous, multiparous), $Farm_m$ (m= farm 1, farm 2), $CY_n$= calving year (n= 2018, 2019 or 2020), and $CS_o$= calving season (o= season 1 from August to November or season 2 from December to March) are fixed effects. $Cow_p$ is the random effect of the cow. ColostrumValue is the covariate of immunoglobulin G in the colostrum sample, only used in the models with immunoglobulin G in serum and milk as dependent traits, and $e_{ijkl}$ is the residual error

Models were estimated in R version 3.6.3 (2020-02-29, R Core Team 2020).The differences between least square means of the fixed effects were verified by Tukey tests using the «emmeans» package (version 1.4.5, Lenth, 2020). We checked normal distribution of the residuals of all models by visually inspecting residual plots. Statistical significance was assumed at $P<0.05$, with tendency between $P>0.05$ and $P<0.10$.

**FR**: All data were analysed using the SAS 9.4 software package (SAS Institute Inc., Cary, NC). Colostrum sampled on day 1 and 3 were analysed using the GLM procedure with animal group (Control, Dam and Mixed) and breed (Holstein and Montbéliarde) included in the model. Data related to milk were analysed using the MIXED procedure, with animal as random factor, group (Control, Dam and Mixed), breed (Holstein and Montbéliarde), milking (morning, evening) and interaction Week x Group as fixed factors and date (week 3, 7, 9 and 13) as repeated factor. Data related to blood plasma were analysed using the MIXED procedure, with animal as random factor, group (Control, Dam and Mixed), breed (Holstein and Montbéliarde), sex (male, female) and interaction Week x Group as fixed factors and date (day 2, week 3 and 10) as repeated factor. For all data, normality of residuals was checked using the Shapiro-Wilk test. Frequency of health disorders around weaning was compared between groups using a Chi-squared test. Significance was set at $P < 0.05$.

## 3. Results and conclusions:

### 3.1 Passive immunity transfer

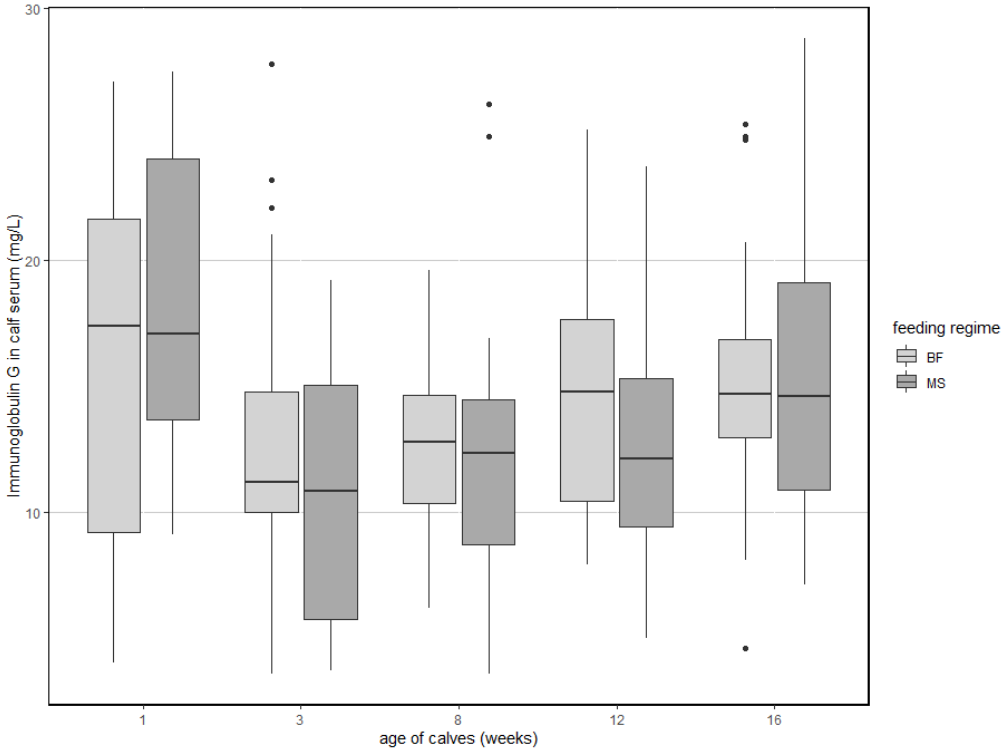
**CH:** In the Swiss trials the colostrum period did not differ between BF and MS calves- both stayed with their mother and ingested the colostrum naturally. Therefore, we expected no difference in the first point of observation. Immunoglobulin content in calf serum did not differ between feeding groups at any observation point (Table 1 and Figure 1), but showed a clear change over time in form of a marked drop followed by an increase again (Figure 1).

***Table 1. Average content of immunoglobulin G (IgG in mg/L) in serum of calves fed by teat bucket twice per day (BF) or suckling the mother twice per day (MS) as least square means (LSM), standard error (SE) and confidence intervals (CI) in Swiss trials***

| Variable | Feeding regime | | | | | | P |
|---|---|---|---|---|---|---|---|
| | BF ($n_{animals}$= 25) | | | MS ($n_{animals}$= 22) | | | |
| | LSM ± SE | CI | $n_{obs}$ | LSM ± SE | CI | $n_{obs}$ | |
| IgG Serum (mg/L) | 13.0 ± 0.7 | 11.6-14.3 | 91 | 13.2±0.7 | 11.8-14.6 | 84 | 0.801 |

$n_{animals}$= number of animals, nobs= number of observations, P-value of the feeding effect

***Figure 1. Content of immunoglobulin G (IgG in mg/L) in serum of calves reared with teat bucket feeding (BF) or mother suckling (MS) twice a day at week 1, 3,8,12 and 16 of life***



Immunoglobulin in calf serum did not differ between feeding groups at any time of measurement, but showed a clear change over time in the form of a marked drop followed by a rise again

**FR**: In the French trial, Control-cows tended to have a higher concentration of IgG in colostrum at first milking after calving (Table 2). No group effect was found for IgG concentration of colostrum at Day3, and no breed effects were observed at Day1 and Day3 after calving. Suckling cows (Dam- and Mixed-) had lower IgG concentrations of colostrum at Day1 probably because the IgG-richest colostrum was already suckled by calves before the first milking while it was not the case in Control-cows. The IgG concentration in colostrum is known indeed to very rapidly decrease (Johnsen et al., 2019) during the first days post-partum, as we observed in this experiment (Table 2).

***Table 2. IgG concentration (g/L) of colostrum of the three groups of cows at the first milking after calving (Day1) and three days after calving (Day3). Values reported are adjusted- and P-values per Group (Control, Mixed, Dam) and per Breed (Holstein [Ho], Montbeliarde [Mo]).***

| | Group | | | Breed | | *P-Value* | |
|---|---|---|---|---|---|---|---|
| | Control | Mixed | Dam | Ho | Mo | Group | Breed |
| IgG colostrum Day1 (g/L) | 61.0 | 39.2 | 44.6 | 51.3 | 45.2 | 0.07 | 0.43 |
| IgG colostrum Day3 (g/L) | 1.61 | 1.53 | 1.71 | 1.67 | 1.57 | 0.81 | 0.69 |

The IgG concentration in calves' serum (Table 3) was similar in the different groups and no interaction was observed with the week. At day 2, in all groups, the average IgG levels were high (Figure 2), which indicates that the passive immunity transfer was adequate for most calves, except for 3, 2 and 4 calves (out of 14) in groups Control, Dam and Mixed respectively whose plasma IgG concentration was below the threshold of 10 g/L (Besser et al., 1991). The plasma IgG concentration decreased significantly from week 3 to 10 (Figure 2) and was significantly higher in female than in male calves (18.2 ± 1.2 vs 14.1 ± 1.3 g/L, P= 0.03).

***Table 3. IgG serum of calves concentration (mg/L) of the three groups of calves (Control, Mixed, Dam). Values reported are adjusted- and P-values of IgG serum per Group (Control, Mixed, Dam) and, per Breed (Holstein [Ho], Montbeliarde [Mo]), Group × Breed interactions, Sex of calves and Week of age in the French trial.***

| | Group | | | *P-Value* | | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Mixed | Dam | Group | Breed | Group × Breed | Sex | Week |
| IgG Serum (mg/L) | 18.5 | 14.5 | 15.4 | 0.18 | 0.37 | 0.61 | 0.03 | 0.0002 |

We conclude from these measurements that in our experimental conditions, the passive immunity transfer from cows to calves was adequate in all groups and that CCC practices implemented had no adverse effect on neonatal calves' immunity and no effect on the further build-up of the active immune defense during the pre-weaning period.

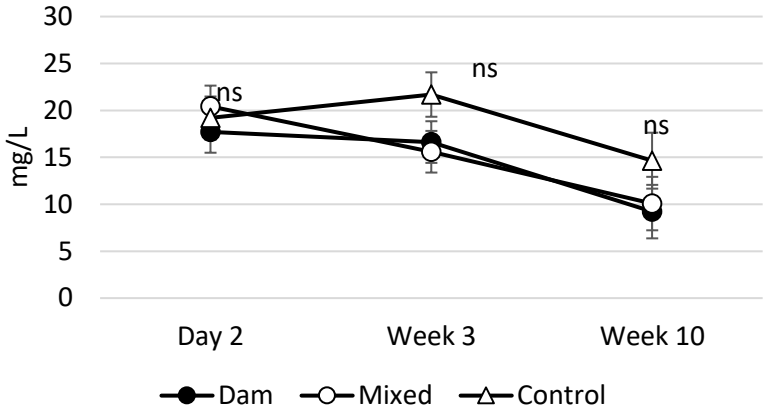



***Figure 2. IgG serum of calves concentration (average and standard error) of the three groups of calves at Day 2 after birth, at Week 3 (before the separation of Mixed-calves) and at Week 10 (before weaning of calves).***

As conditions of colostrum feeding did not differ between BF- and MS calves in Switzerland no conclusion on passive immune transfer via colostrum for differing feeding regimes can be derived from these trials. However, since the observation period extended into the week 12 and 16 of life, an increase of IgG in calf serum could be observed in Swiss trials for both groups, reflecting the build-up of active immune defense.

### 3.2 Immunoglobulin and lactoferrin in cows' milk

#### 3.2.1 Swiss experiments:

Immunoglobulin G content (mg/L) in cows' milk did not differ between feeding regimes (Table 4) and considerably decreased after 3 weeks (Table 5, Figure 4). Lactoferrin content (mg/L) in milk did not differ between treatment groups (Table 4) either, and in CH did not vary with time (Table 5, Figure 4). The variability of lactoferrin content was much higher in cows with calf contact (Figure 4).

***Table 4: Average content of immunoglobulin G (IgG in mg/L) and lactoferrin (mg/L) in cow milk from cows with or without calf contact as least square means (LSM), standard error (SE) and confidence intervals (CI) in Swiss trials***

| Variable | Milk of cows | | | | | | P |
|---|---|---|---|---|---|---|---|
| | without calf contact ($n_{animals}$= 23) | | | with calf contact ($n_{animals}$= 21) | | | |
| | LSM ± SE | CI | $n_{obs}$ | LSM ± SE | CI | $n_{obs}$ | |
| IgG (mg/L) | 397 ± 32 | 118-657 | 82 | 366 ± 32 | 159-573 | 83 | 0.605 |
| Lactoferrin (mg/L) | 227 ± 46 | 69-386 | 85 | 260 ± 46 | 102-418 | 83 | 0.330 |

$n_{animals}$= number of animals

***Table 5. Time course of immunoglobulin G (IgG in mg/L) and lactoferrin (mg/L) content in cow's milk with (n=21) and without calf contact (n=23) as least square means in Swiss trials***

| Variable | Treatment | Time (weeks) | | | | P treatment |
|---|---|---|---|---|---|---|
| IgG (mg/L) | | 3[a] | 8[b] | 12[b] | 16[b] | |
| | With calf contact | 399 | 312 | 306 | 270 | 0.114 |
| | Without calf contact | 500 | 350 | 350 | 345 | |
| Lactoferrin | | 3 | 8 | 12 | 16 | |
| (mg/L) | With calf contact | 277 | 211 | 277 | 237 | 0.104 |
| | Without calf contact | 204 | 233 | 201 | 146 | |

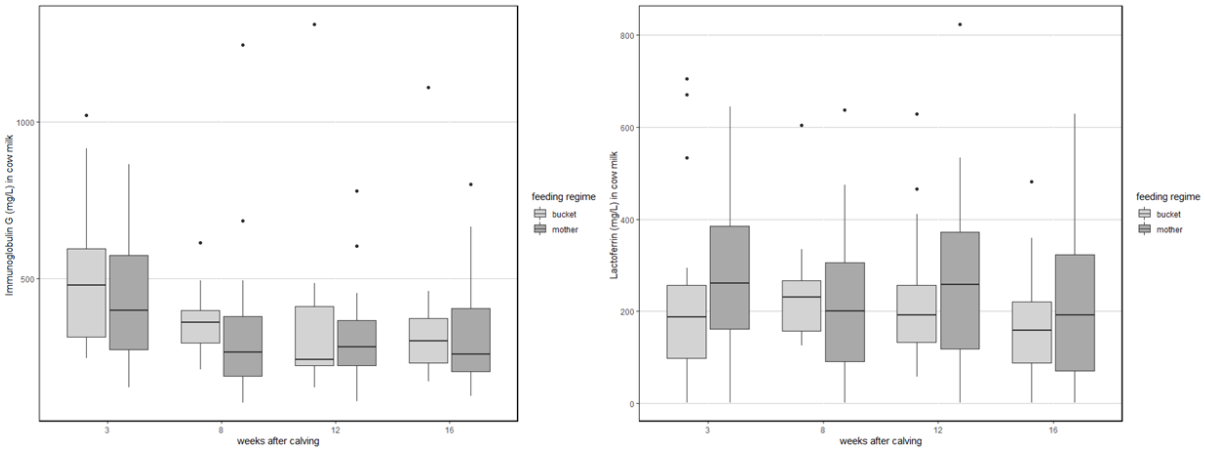



***Figure 4. Content of immunoglobulin G (IgG in mg/L) and lactoferrin (mg/L) in milk of cows with (mother) and without calf contact (bucket) at week 3,8,12 and 16 post partum***

From Swiss trials we conclude that prolonged contact twice a day to the calf beyond the colostrum period did not modulate the content of immunoglobulin G and antimicrobial compounds (lactoferrin) in dairy milk.

**3.2.2 French experiment:**

By contrast, in the French experiment, milk IgG concentration was significantly higher in Mixed-cows in week 3, in comparison to Control and Dam-cows (Table 6 and Figure 4). Milk IgG concentration clearly decreased after week 3 where group-differences observed in week 3 disappeared. The time related decrease observed in both French and Swiss experiments is in line with the well described immune development in calves. The higher IgG concentration found in the French trial only in Mixed cows is very surprising considering that in week 3, all Mixed and Dam cows were suckling their calves. Therefore, differences do not seem to be due to calves' suckling. Further investigations need to be done in order to try to understand this surprising result. Nevertheless, as in the Swiss trials, we conclude that cows' sucking does not seem to have any effects on the IgG concentration of milk.

***Table 6. Milk lactoferrin and IgG concentration (mg/L) of the three groups of calves (Control, Mixed, Dam). Adjusted values and P-values per Group (Control, Mixed, Dam), per Breed (Holstein [Ho], Montbeliarde [Mo]), Group × Breed interactions, and Week of lactation (3, 7, 9 and 13).***

| | Group | | | SEM | *P-Value* | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Mixed | Dam | | Group | Breed | Group × Breed | Week |
| Milk Lactoferrin (mg/L) | 196 | 138 | 149 | 29.2 | 0.13 | 0.13 | <0.04 | <0.001 |
| IgG Milk (mg/L) | 343 | 433 | 395 | 28.6 | 0.03 | 0.93 | <0.001 | <0.001 |

Contrary to the Swiss results, milk LF concentration increased steadily from week 3 to 13 and in Week 13, milk LF was significantly higher in Control cows compared to Dam and Mixed-cows (Table 4, Figure 2B). Again, this result is very surprising and does not seem to be related to calves' suckling as in week 13, all calves were weaned in all groups. Here again, further investigation needs to be done in order to try to understand this surprising result that could be linked to milk SCC, slightly higher in average in Control cows in week 13.

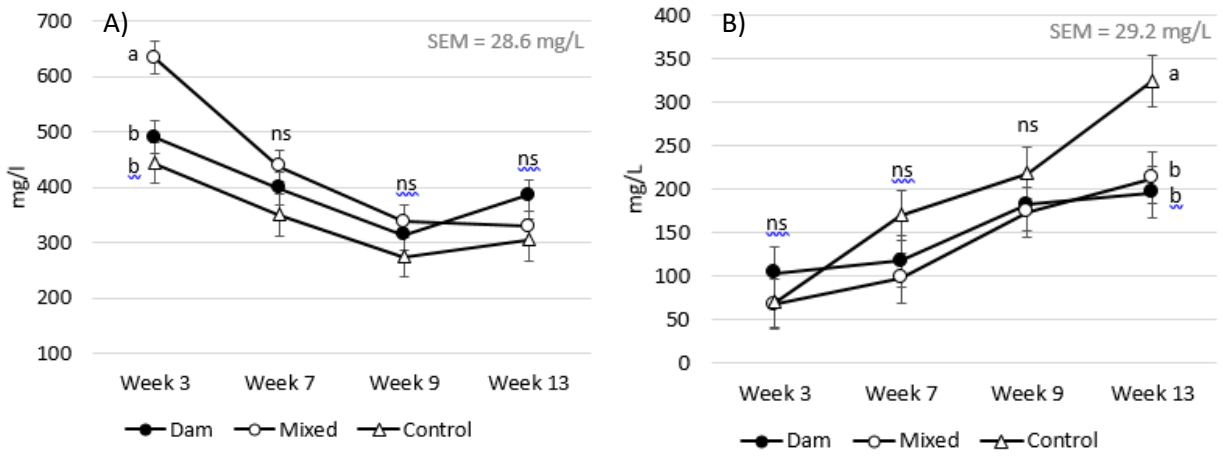



***Figure 4. Time course of IgG (A) and Lactoferrin (B) concentration in milk (LSM from the model and standard error) of the three groups of cows by weeks of lactation.***

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