Description of innate immunity and hematological changes in Holstein calves during the gradual weaning process

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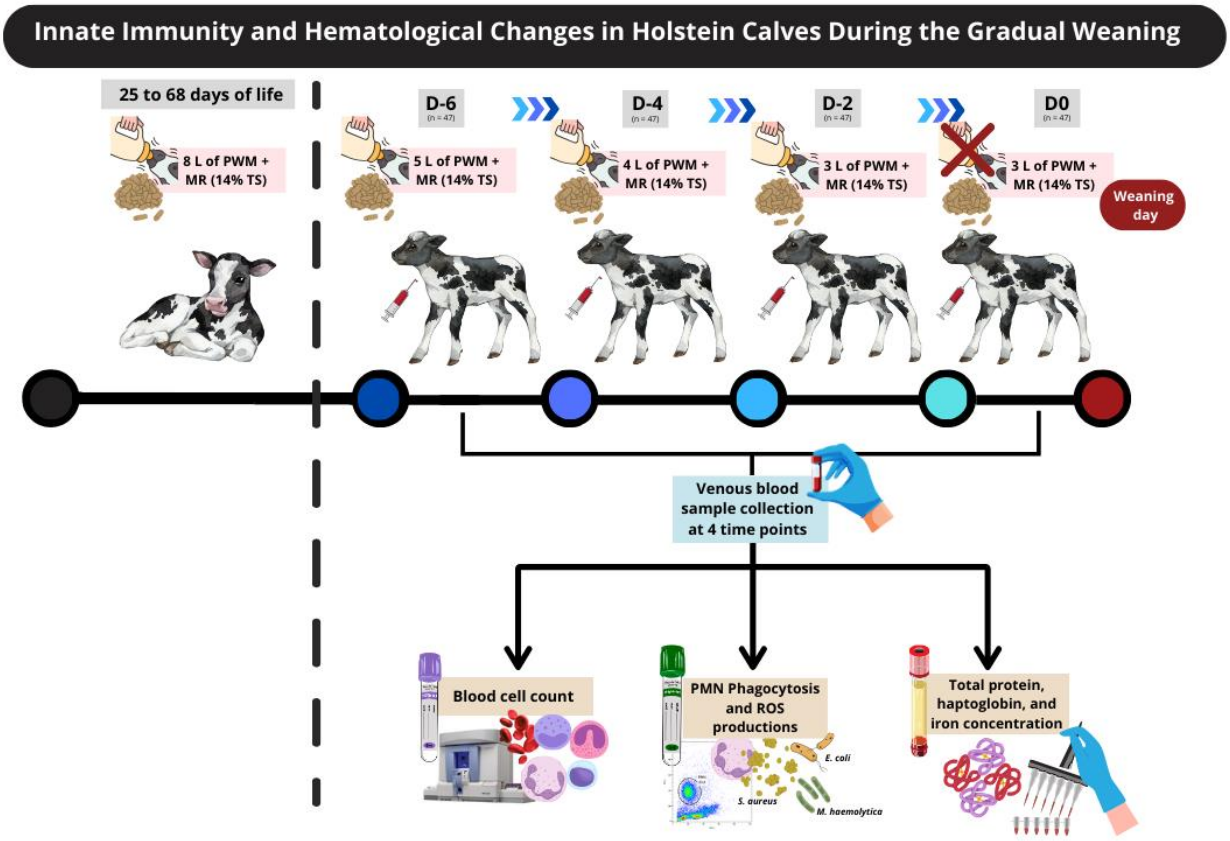
SUPPLEMENTARY FILE



PWM: pasteurized waste milk; MR: milk replacer**;** TS: total solids.

Supplementary Figure S1.

SUPPLEMENTARY MATERIAL & METHODS

Feed and Weaning management

All calves were fed 7 L of slow-pasteurized (60 °C for 30 min) waste milk (PMW) per day to 24 days of age. This was divided into two meals per day. Starting at 25 days of age, the calves were started on a milk replacer (MR; Nattimilk Emax®, Auster Animal Nutrition Ltd., Brazil; Supplemental Table 1). This was continued until the day of weaning (D0), which occurred between 69 and 85 days of age, depending on the day of enrolment. The total solids (ST) in the liquid diet were adjusted to 14% by adding a milk replacer to the pasteurized waste milk, totaling 8 L of liquid diet. Therefore, the calves fed with 8 L of total

liquid diet received 1,154 g of total solids per day (considering a milk density of 1,040). During the reduced liquid feeding phase, water and pellet feed (Rumileite LS® Guabi Nutrition and Animal Health, Brazil; Supplemental Table 2) were provided *ad libitum*.

When the calves entered the weaning period, they met the weekly selection criteria. They were sorted according to their size and weight. This was blocked using standardization in the group that was weaned during the same week. The calves included in the study received 8 L of milk replacer mixture per day and were split into two feeds. They were fed 5 L/day starting on D-6 before full weaning. Subsequently, each calf received 1 L of milk in a stepwise ratio after three days of the protocol. The last liquid feed was 3 L per day, followed by the next day with a total interruption of the liquid feed on D0.

Animals' inclusion criteria

To be included in the study, each calf was evaluated for passive immune transfer (PIT) by measuring serum total solids (≥ 8.4%) and total serum protein (≥ 5.5% - Godden et al., 2019). Additionally, a clinical evaluation of the vital parameters of each cow and calf was performed (Feitosa and Benesi, 2014). Calf rectal temperature was measured using a digital thermometer and fecal scores were assessed according to the Calf Health Scoring Criteria of the University of Wisconsin (McGuirk, 2008). The Wisconsin respiratory score adapted by Decaris et al. (2023) was used to monitor the bovine respiratory disease (BRD). This system evaluates the presence of ocular discharge, nasal discharge, cough, head and ear positions, and rectal temperature. A calf was considered positive for BRD if it scored 2 or 3 (on a scale of 0–3) on at least two parameters of the scoring system. None of the calves showed umbilical inflammation.

Health calves' management

The calves were dehorned using hot iron one week before weaning. After dehorning, an employee applied 10% iodine tincture and tetracycline powder to the lesions on each calf. In addition, the calves were intranasally administered a commercial vaccine containing attenuated live infectious bovine rhinotracheitis (IBR), parainfluenza type 3 (PI3), and bovine respiratory syncytial virus (BRSV) (Inforce 3®, Zoetis, Brazil) at seven days of age.

Thirty days later, a second intranasal dose of the same vaccine and an initial dose of an injectable commercially killed vaccine (Cattle Master Gold FP5/L5®; Zoetis, Brazil) containing IBR, bovine viral diarrhea (BVD), PI3, BRSV, and leptospirosis was administered. 21 days later, a second dose of vaccine was administered. The animals also received a prophylactic dose of toltrazuril (Baycox®, Elanco, Brazil) to block the development of eimeriosis 10 d after weaning. The calves were also subjected to weight estimation using a chest circumference tape.

Selected Biochemical parameters

Serum aliquots were thawed overnight at 4 °C in a refrigerator. The samples were then homogenized using an automatic homogenizer for further testing using an automated biochemical analyzer (Daytona Model, Randox Laboratories Ltd., Co. Antrim, UK). To measure iron concentration, a commercial kit (UIBC Iron, Randox, Cat. 357599), according to the manufacturer's instructions.

Inflammation was monitored by determining the total serum protein, total serum solids, and haptoglobin (Hp) concentrations. Serum total protein (g/dL) was estimated using an optical refractometer in the range of 0–12 g/dL. The percentage of solids in the serum was measured using a Brix refractometer. Hp was assessed using a turbidimetric assay based on the properties of haptoglobin and meta-hemoglobin differential binding[25].

Phagocytosis assessment

Phagocytosis of bacterial particles killed *S. aureus* and *E. coli* labeled with pHrodo™ Red, using a previously established flow cytometry technique[18,26]. The assays were conducted in polypropylene tubes suitable for flow cytometry measurements. To perform the phagocytosis assay, 100µL of heparinized blood was incubated with 12µL *of Staphylococcus* aureus (pHrodo™ Red *S. aureus* BioParticles™ Conjugate for Phagocytosis ref. A10010) and 12µL *Escherichia coli* (pHrodo™ Red *E. coli* BioParticles™ Conjugate for Phagocytosis ref. P35361) for 30 minutes at 37ºC[18]. Data were collected using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego, CA, USA). For each sample, 20,000 events were acquired based on a pre-calibrated forward

angle and 90-light scatter gate defining granulocytes. Data were collected using the CellQuest® software (Becton Dickinson Immunocytometry Systems, San Diego, CA, USA). The data were recorded and analyzed using FlowJo® software (version 7.6.1 for Windows, Tree Star Inc., Ashland, OR, USA). Polymorphonuclear cells fell into established gates, which were first documented using samples containing cells only. These gates were directly applied to all other samples from the same animal on each test day. A similar process was established by setting the threshold to determine the frequency (as % of events) of positive cells for fluorescence in the FL2 channel (orange-red). The quantity of bacteria taken up was estimated using the median fluorescence intensity (MFI) on a logarithmic scale.

Reactive Oxygen Species (ROS) assessment

Production of ROS was assessed using the conversion of the fluorescent dye dihydrorhodamine 123 (Thermo Fisher Scientific, Massachusetts, USA, Cat. D632) to Rhodamine-123 in the cells as previously described[18]. A fraction of leukocytes was suspended in RPMI-1640 medium without phenol red (Sigma-Aldrich, St. Louis, MO, USA, cat. 7509) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT cat. G6392, inactivated at 56°C for 30 min), and 2 mM l-glutamine (Cat: 21051–024, Gibco, Brazil) at 3×10^6 viable cells/mL. Samples were plated in triplicate wells of a flat bottom 96 well plate (100μL per well) for assessment of endogenous production (medium only) or induced production of ROS by *Escherichia coli* (1:10), *Staphylococcus aureus* (1:5), and *Mannheimia haemolytica* (1:5). Stock bacterial strains were cultured in a liter of Brain Heart Infusion medium overnight, washed, and centrifuged three times to achieve a final volume of 10mL of a concentrated bacterial solution. The bacterial strains were inactivated by incubation with the solution for an hour at 60 °C. Serial dilutions of bacterial strains were performed to determine the ideal titration to achieve maximum production of induced ROS. Phorbol 12-myristate-13-acetate (PMA Sigma-Aldrich) Louis, USA, Ref 8139)10^{-6} M was used to induce maximal enzyme activity from the cells and allow comparison of NADPH oxidase activity between assays. RPMI-1640 and RPMI-1640 supplemented with DH-R123 were used as negative and spontaneous fluorescence controls, respectively. The plates were then placed in a CO_2 incubator (Sanyo CO_2 Incubator; Sanyo, Osaka, Japan) for 2 h at 37°C. Finally, the conversion of DH-R123 to R-123 yielding a fluorescent signal was captured using a plate reader (excitation wavelength 485 nm and emission wavelength 538 nm;

SpectraMax® M5, Molecular Devices, Sunnyvale, CA USA) The ROS for each sample was presented as a response ratio (RR): induced ROS (AFU) divided by endogenous ROS (AFU).

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SUPPLEMENTAL TABLES

Supplemental table 1. Guarantee levels of the milk replacer (Nattimilk E Max®, Auster Animal Nutrition, Brazil) were provided to the pre-weaned calves during the experiment.

Item	Quantities
Calcium	6 g/kg
Crude protein	220 g/kg
Cupper	8,98 mg/kg
Fat	170 g/kg
Flavomycin	20 mg/kg
Iron	70 mg/kg
Lactose	450 g/kg
Lysine	15,3 g/kg
Methionine	5,94 g/kg
Phosphorus	6,1 g /kg
Sodium	5,5 g/kg
Threonine	10 g/kg
Tryptophan	2,8 g/kg
Vitamin A	55.000 IU/kg
Vitamin D3	10.000 IU/kg
Vitamin E	200 IU/kg
Vitamin K3	5,5 mg/kg
Zinc	140 mg/kg

Supplemental table 2. Guarantee levels of pelleted feed (Rumileite LS Guabi®, Brazil) provided to the pre-weaned calves and during the weaning period *ad libitum*.

Item	Quantities (g/kg)
Humidity (max)	130g
Crude protein (min)	200g
Ethereal extract (min)	35g
Crude fiber (max)	80g
Mineral mix (max)	85g
Calcium (max)	15g
Phosphorus (min)	6g

Supplemental table 3. Hematological (ADVIA 2120i®) and Biochemical parameters (mean ± SEM) of pre-weaned Holstein calves (PWC) during the weaning period: 6 days (D -6), 4 days (D -4), 2 days before (D -2), and in the weaning day (D0).

Parameters	D -6	D-4	D -2	D0 (WEANING)	P-value
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	
RBC(×10^6/dL)	10.00 ± 0.24^a	9.92 ± 0.23^a	9.81 ± 0.23^a	9.64 ± 0.22^a	0.7105
HGB (g/dL)	11.29 ± 0.13 ^{a}	11.17 ± 0.13^a	11.19 ± 0.13^a	10.74 ± 0.23^b	0.8360
MCV (fL)	37.85 ± 0.76^a	37.53 ± 0.74^a	37.93 ± 0.69^a	37.48 ± 0.62^a	0.9582
MCH (pg)	11.58 ± 0.29^a	11.52 ± 0.27^a	11.81 ± 0.31^a	11.60 ± 0.25^a	0.8942
MCHC (g/dL)	30.50 ± 0.15^a	30.62 ± 0.14^a	30.44 ± 0.23^a	30.89 ± 0.16^a	0.2625
CHCM (g/dL)	31.38 ± 0.12^b	32.01 ± 0.10^a	31.36 ± 0.13^b	31.71 ± 0.19^{ab}	0.0029
CH (pg)	11.82 ± 0.24^a	11.91 ± 0.24^a	11.80 ± 0.21^a	11.86 ± 0.20^a	0.9856
RDW (%)	23.50 ± 0.37^a	23.70 ± 0.38^a	23.01 ± 0.36^a	23.09 ± 0.35^a	0.4907
HDW(g/dL)	3.71 ± 0.08^b	4.13 ± 0.07^a	3.95 ± 0.06^a	4.12 ± 0.07^a	0.0002
PLT(×10^3/µL)	537.85 ± 22.99^a	554.87 ± 8.45^a	548.52 ± 19.89^a	535.06 ± 17.05^a	0.8836
Ret (×10^9/L)	11.58 ± 1.43^b	10.74 ± 1.35^b	16.43 ± 7.80^{ab}	20.44 ± 2.01^a	0.0106
CHr (pg)	18.78 ± 0.27^b	18.50 ± 0.59^b	22.33 ± 2.58^a	17.69 ± 0.36^b	0.0026
CHm	12.44 ± 0.12^{ab}	12.44 ± 0.13^{ab}	13.43 ± 1.28^a	12.06 ± 0.32^b	0.2423
MPV (fL)	6.73 ± 0.10^b	6.23 ± 0.06^c	7.64 ± 0.16^a	7.05 ± 0.15^b	<0.0001
WBC (×10^3/µL)	11.56 ± 0.49^{ab}	10.48 ± 0.39^b	12.16 ± 0.65^a	11.37 ± 0.36^{ab}	0.1096
NEUTR (×10^3/µL)	4.05 ± 0.31^{ab}	3.46 ± 0.23^b	4.96 ± 0.59^a	4.25 ± 0.22^{ab}	0.0443
NEUTR (%)	33.08 ± 1.52^{ab}	31.77 ± 1.28^b	36.45 ± 1.89^a	36.06 ± 0.99^a	0.0668
LYMPH (×10^3/µL)	6.48 ± 0.20^a	6.05 ± 0.19^a	6.43 ± 0.21^a	6.29 ± 0.18^a	0.3947
LYMPH (%)	56.90 ± 1.85^a	58.82 ± 1.24^a	55.59 ± 1.91^a	55.63 ± 1.00^a	0.4180
N:L ratio	0.62 ± 0.04^a	0.60 ± 0.03^a	0.81 ± 0.11^a	0.67 ± 0.02^a	0.1169
MONO (×10^3/µL)	0.68 ± 0.04^a	0.71 ± 0.04^a	0.52 ± 0.03^b	0.62 ± 0.04^{ab}	0.0021

MONO (%)	5.82 ± 0.20^{b}	6.67 ± 0.27^{a}	4.54 ± 0.24^{c}	5.45 ± 0.25^{b}	<0.0001
EOS (×10^{3}/µL)	0.23 ± 0.03^{a}	0.15 ± 0.02^{a}	0.20 ± 0.03^{a}	0.20 ± 0.03^{a}	0.2471
EOS (%)	2.03 ± 0.25 ^{a}	1.45 ± 0.13^{a}	1.71 ± 0.28^{a}	1.89 ± 0.26^{a}	0.3398
BASO (×10^{3}/µL)	0.09 ± 0.01^{a}	0.07 ± 0.00^{b}	0.07 ± 0.01^{b}	0.07 ± 0.00^{b}	0.0058
BASO (%)	0.78 ± 0.03^{a}	0.71 ± 0.03^{ab}	0.58 ± 0.05^{c}	0.64 ± 0.03^{bc}	0.0013
LUC (×10^{3}/µL)	0.06 ± 0.00 ^{ab}	0.08 ± 0.02^{a}	0.04 ± 0.00^{b}	0.05 ± 0.00^{ab}	0.0714
LUC (%)	0.49 ± 0.03 ^{b}	0.59 ± 0.03^{a}	0.33 ± 0.02^{c}	0.43 ± 0.03^{b}	<0.0001
LI	2.14 ± 0.05^{b}	2.60 ± 0.03^{a}	1.87 ± 0.03^{c}	2.17 ± 0.06^{b}	<0.0001
MPXI	4.05 ± 0.46	4.70 ± 0.36	3.32 ± 0.45	5.48 ± 1.15	0.1439
WBCP (×10^{3}/µL)	10.13 ± 0.46^{a}	9.83 ± 0.37^{a}	10.45 ± 0.67^{a}	10.09 ± 0.33^{a}	0.8360
TP (g/dL)	7.28 ± 0.13^{a}	6.84 ± 0.10^{b}	6.91 ± 0.08^{b}	6.79 ± 0.08^{b}	0.0018
Hp (g/dL)	4.10 ± 1.08^{a}	2.24 ± 0.37^{b}	1.82 ± 0.19^{b}	2.16 ± 0.28^{b}	0.0223
Iron (µMol/L)	30.61 ± 1.45^{bc}	34.39 ± 1.63^{ab}	35.48 ± 1.53^{a}	28.11 ± 1.41^{c}	0.0020

RBC = Red Blood Cells; HGB = Hemoglobin; PCV = Packed Cell Volume; MCV = Mean Corpuscular Volume; MCH = Mean Corpuscular Hemoglobin; MCHC = Mean Corpuscular Hemoglobin Concentration; CHCM = Cell Hemoglobin Concentration Mean; CH = Cellular Hemoglobin Content; RDW = Red Cell Distribution Width; HDW = Hemoglobin Distribution Width; PLT = Platelets; Ret = Reticulocytes; CHr = Mean hemoglobin content of reticulocytes; CHm = Cellular hemoglobin content of mature red blood cells(pg); MPV = Mean Platelet Volume; WBC = White Blood Cells count; NEUTR = Neutrophils; LYMPH = Lymphocytes; MONO = Monocytes; EOS = Eosinophils; BASO = Basophils; LUC = Large Unstained Cells; LI = Lobularity Index; MPXI = Myeloperoxidase Intracellular Index; WBPC = WBC count from the Peroxidase channel; TP = Total Protein; Hp = Haptoglobin.

Significant differences were considered when $P<0.05$ by the PROC GLM test.

Supplemental table 4. Polymorphonuclear cell phagocytosis rate (%) and mean fluorescence intensities (MFI) consistent values challenged by *S. aureus*, *E. coli*, and *M. haemolytica* (mean ± SEM) of pre-weaned Holstein calves (PWC) during the weaning period: 6 days (D -6), 4 days (D -4), 2 days before (D -2), and in the weaning day (D0).

Parameters	D -6	D -4	D -2	D0 (WEANING)	P-value
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	
Phagocytosis rate (%) of *E. coli*	63.37 ± 2.01[a]	58.2±1.68[a]	49.93±2.56[b]	58±1.65[a]	<0.0001
Relative uptake of *E. coli* (MFI)	69.82±7.14[a]	59.84±6.04[ab]	50.64±5.9[b]	52.6±4.57[ab]	0.1006
Phagocytosis rate (%) of *S. aureus*	89.66±1.02[a]	91.9±0.76[a]	89.46±0.99[a]	86.28±1.34[b]	0.0027
Relative uptake of *S. aureus* (MFI)	94.7±1.29[a]	96.8±0.75[a]	96.13±0.92[a]	94.16±1.22[a]	0.2669

Significant differences were considered when $P < 0.05$ by the PROC GLM test.

Supplemental file 5. Reactive oxygen species (ROS) production in arbitrary fluorescence unit (AFU) of cells challenged by PMA, *S. aureus*, *E. coli*, and *M. haemolytica* (mean ± SEM) of pre-weaned Holstein calves (PWC) during the weaning period: 6 days (D -6), 4 days (D -4), 2 days before (D -2), and in the weaning day (D0).

Parameters	D -6	D -4	D -2	D0 (WEANING)	P-value
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	
Unstimulated Cells	42.29 ± 3.2[a]	48.61 ± 3.68[a]	40.85 ± 3.27 [b]	31.85 ± 1.89[b]	0.003
PMA + stimulated cells	5.76 ± 0.26 [ab]	5.06 ± 0.20 [b]	5.61 ± 0.24 [b]	6.63 ± 0.26 [a]	0.2532
***S. aureus* + stimulated cells**	216.97 ± 11.92 [a]	223.26 ± 10.41 [a]	202.63 ± 9.38 [a]	197.59 ± 8.7[b]	0.1131
***E. coli* + stimulated cells**	146.51 ± 7.19[a]	147.16 ± 6.74[a]	137.76 ± 5.53[a]	143.09 ± 5.87[a]	0.7095
***Pure M. haemolytica*+ stimulated cells**	102.91 ± 5.46[a]	111.98 ± 4.76[a]	106.11 ± 5.22[a]	105.96 ± 4.75[a]	0.6369
***M. haemolytica* 1:5 + stimulated cells**	78.39 ± 4.42[a]	85.33 ± 4.54 [a]	79.27 ± 4.95 [a]	77.17 ± 3.99 [a]	0.5828

Significant differences were considered when $P < 0.05$ by the PROC GLM test.

Supplemental file 6. Response ratio (RR) of reactive oxygen species (ROS) production of cells challenged by PMA, *S. aureus*, *E. coli*, and *M. haemolytica* (mean ± SEM) of pre-weaned Holstein calves (PWC) during the weaning period: 6 days (D -6), 4 days (D -4), 2 days before (D -2), and in the weaning day (D0),

Parameters	D -6	D -4	D -2	D0 (WEANING)	P-value
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	
PMA stimulation index	5.76 ± 0.26 [ab]	5.06 ± 0.20 [b]	5.61 ± 0.24 [b]	6.63 ± 0.26 [a]	0.0002
***S. aureus* stimulation index**	4.02 ± 0.20 [a]	3.44 ± 0.17 [a]	3.88 ± 0.17 [a]	2.54 ± 0.12 [b]	<0.0001
***E. coli* stimulation index**	2.08 ± 0.07 [ab]	1.87 ± 0.06 [b]	1.93 ± 0.07 [b]	2.30 ± 0.08 [a]	0.0003
***Pure M. haemolytica* stimulation index**	2.83 ± 0.16 [b]	2.56 ± 0.10 [b]	2.92 ± 0.13 [b]	3.54 ± 0.15 [a]	<0.0001
M. haemolytica* stimulation index *1:5	2.08 ± 0.10 [b]	1.92 ± 0.08 [b]	2.10 ± 0.08 [b]	2.53 ± 0.10 [a]	<0.0001

Significant differences were considered when $P < 0.05$ by the PROC GLM test.