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The relationship between ruminal mat consistency of calves and mortality or immunity

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Two studies were conducted on the relationship between ruminal mat consistency (RMC) and (1) calf mortality or (2) immunity. Assessment of RMC was by ten repeated manual compressions of the middle rumen; good-RMC showed no fluid bounce-back (FBB), while poor-RMC showed FBB. The first study examined whether RMC affected calf mortality. Monthly feeding management guidance (FMG) was implemented for 5 years on a commercial farm with Holstein–Friesian × Japanese black calves, directing increased forage consumption for poor-RMC calves aged 50–180 days. Compared to 35 control farms, the FMG period showed significantly lower total mortality (3.8% vs. 0.8%, $P < 0.001$, OR = 4.7) and lower respiratory mortality (2.3% vs. 0.1%, $P < 0.001$, OR = 18.3). The second study tested the hypothesis that RMC affects calf immunity. Peripheral blood of good-RMC (n = 6) and poor-RMC (n = 6) calves was compared. Good-RMC showed higher phagocytosis in monocytes ($P = 0.05$, r = 0.63) and granulocytes ($P = 0.02$, r = 0.78), and higher numbers of CD4 + ($P = 0.07$, r = 0.58), CD8 + ($P = 0.07$, r = 0.60), and B-cells ($P = 0.03$, r = 0.62). In mRNA expression, good-RMC showed lower *TNF-α* ($P = 0.02$, r = 0.70), *IL-1* ($P = 0.09$, r = 0.49), *IL-12* ($P = 0.04$, r = 0.59) and *IL-10* ($P = 0.13$, r = 0.44), and higher *IL-16* ($P = 0.07$, r = 0.53) compared to poor-RMC. These results suggest that RMC affects calf mortality and immunity, emphasizing the importance of feeding management in improving sustainability.

Keywords Bovine respiratory disease, Cytokine mRNA, Flow cytometry, Mortality, Phagocytic ability, Ruminal mat

Nutrition strategies are paramount in addressing key issues in the livestock industry, encompassing environmental impact, economic sustainability, animal health and welfare, and antibiotic resistance. Failures in nutritional management increase morbidity and mortality[1,2], resulting in economic losses[3], increased risk of antimicrobial resistance[4], and worsened animal welfare[5,6]. In particular, mortality indicates animal welfare and one of the potential reputational risk to the bovine industry, necessitating more effective control schemes[7].

The key points of nutritional management include: "*(1) nutrient components of the ingredients used to construct the diet, (2) the amount of each ingredient in the diet, and (3) the amount of the diet consumed.*" (NASEM, 2021[8]). Therefore, evaluating feed quality, fiber source and non-fiber source ratio, and dry matter intake (DMI) is essential in bovine nutrition, as these factors affect rumination and milk production[9], growth and rumen bacterial composition[10], health and immunity[11], and morbidity and mortality[1]. Ideally, all animals should consume feed equitably. However, in intensively managed commercial herds, discrepancies between designed and actual consumption and inter-individual variation in forage-to-concentrate ratio (F:C ratio) are rather prevalent[12]. Factors such as feed sorting[13], housing density and feed bunk competition[14], heat stress[15], and social hierarchy[16] contribute to this practical issue of nutritional management. Equitable feed consumption would stabilize herd productivity and health by mitigating under- and over-nutrition and reducing the incidence of subacute ruminal acidosis (SARA). These are the basic concepts of precision nutrition, currently implemented in dairy and feedlot cattle[17], but slow to be adopted for calves[18]. This is presumably because longstanding recommendations against feeding forage prior to weaning, with only limited amounts suggested after weaning[19].

Ingested feed by ruminants is initially passed into the rumen, where it is gradually micronized through rumination and mastication before being transferred to the lower digestive tract. The rumen digesta forms a layered structure consisting of a gas, a solid, and a liquid layer from the dorsal to the ventral sac of the rumen[20]. Rumen digesta with low specific gravity and relatively large particle size (e.g., fiber) floats solidly above the liquid layer and is called the ruminal mat (RM)[20]. RM has two characteristics–thickness and consistency–

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both of which vary with feed composition[21]. The persistent decrease in ruminal pH from overfeeding highly fermentable carbohydrates or underfeeding fiber is referred to as SARA[22–24]. Under these feeding conditions, the RM becomes thin and soft, leading to reduced rumination time[21,25,26]. SARA induces peripheral translocation of rumen lipopolysaccharide (LPS) and systemic inflammation[27]. However, clinical diagnosis of SARA is difficult because of the absence of overt symptoms[28].

Feed intake or inter-individual differences can be assessed through several ways. Simple approaches include evaluating the depression of the left para-lumbar fossa as the rumen fill score (RFS)[29] and evaluating the ratio of abdominal circumference to chest circumference (A/C ratio)[30]. These are relatively robust measures of DMI[31,32], but they do not assess the F:C ratio[33]. Sensor-based approaches dominate the realm of precision feeding but require initial investments and dedicated application. Feeding status is estimated indirectly[17], via ruminal parameters such as pH and temperature[34], or via behavioral parameters such as triaxial acceleration, pressure, and chewing sounds[35,36]. The concept of quantifying ruminal mat consistency (RMC) is a more direct assessment of feeding that has been studied since the 1970's[21,37,38], but has yet to be applied commercially due to the need for surgery. However, Izumi et al. assessed RM using a simplified method, the ruminal mat score (RMS), by detecting fluid bounce-back (FBB) when administering repeated manual compression to the middle rumen of heifers with low forage intake[39]. RMS has been reported to have diurnal variation, with higher scores in post-feeding and lower scores in pre-feeding, due to fiber digestion and its subsequent transfer to the lower digestive tract. Furthermore, pre-feeding RMS positively correlates with DMI and rumination time[39]. This RMS assessment requires no initial costs and could potentially be applied globally, even in low-income regions or small-scale farms, thereby contributing to the development of sustainable livestock production.

This report is proof-of-concept for assessing inter-individual differences in feed consumption via RMS and providing feedback to improve feeding management in calf herds. In Study 1, routine RMC evaluations were conducted on a model commercial farm to determine whether nutritional interventions toward more calves with a thick and firm RM would impact their health. In Study 2, we tested the hypothesis that the RMC in calves affects their immune status.

Results

Study 1: whether RMC affects calf mortality

Annual aggregation of poor RMC population and forage proportion of the total mixed rations (TMR)

As shown in Table 1, the forage proportion (DM (%)) of TMR starting from 90 days of age on model farms was 39.9 ± 3.1% from 2013 to 2016. The poor RMC population by age cohort of calves evaluated through the period with FMG was 86.6% for under 90 days old, 51.0% for 91–120 days old, 34.1% for 121–150 days old, and 27.8% for 151–180 days old.

Mortality and the causes of death

During the period 2006–2011, FMG was not implemented in either the control farms or the model farm. In contrast, during the period 2012–2016, FMG was introduced in the model farm, whereas the control farms did not adopt it. As shown in Fig. 1, there was no significant difference in the survival rate of calves between the model farm and the control farms during 2006–2011 ($P = 1.0$). However, during 2012–2016, the survival rate in the model farm with FMG was significantly higher than that in the control farms without FMG ($P < 0.001$). Table 2 shows comparison for the mortality rates of calves younger than 365 days old between the control farms and the model farm. During the period 2006–2011, FMG was not implemented in either the control farms or the model farm. The total mortality was 4.7% (1,625/34,624) in the control farms and 4.1% (108/2,625) in the model farm ($P = 0.19$, odds ratio[OR] = 1.1, 95% confidence interval[CI] of OR as 95% CI = 0.9–1.4), with no significant difference. For cause-specific breakdown, respiratory mortality was 2.9% (997/34,624) in the control farms and 1.9% (51/2,625) in the model farm ($P = 0.005$, OR = 1.5, 95% CI = 1.1–2.0), which was small-sized effect although P value was below the significance level. Gastrointestinal mortality was 0.9% (328/34,624) in the control farms

	Year				
	2013	2014	2015	2016	Total
Annual average of forage proportion of TMR (DM %, Mean ± SD)	42.2 ± 0.6	41.1 ± 2.2	36.3 ± 3.4	41.0 ± 0.6	39.9 ± 3.1
Age cohort (days old)	**Number of poor RMC calves/evaluated calves (head) (proportion of poor RMC (%))**				
Under 90	32/36 (88.9)	82/98 (83.7)	93/101 (92.1)	71/86 (82.6)	278/321 (86.6)
91–120	8/21 (38.1)	44/74 (59.5)	37/73 (50.7)	33/71 (46.5)	122/239 (51.0)
121–150	8/19 (42.1)	24/72 (33.3)	23/67 (34.3)	24/74 (32.4)	79/232 (34.1)
151–180	4/18 (22.2)	17/43 (28.3)	14/52 (26.9)	19/64 (29.7)	54/194 (27.8)

Table 1. The annual variation of forage proportion of the TMR and distribution of poor RMC calves in each age cohort on the model farm (Study 1). Forage proportions were presented as an average of monthly value ± SD. These data are a compilation of monthly RMC evaluation for FMG, so the proportion of poor RMC after FMG is lower than that shown in this table.

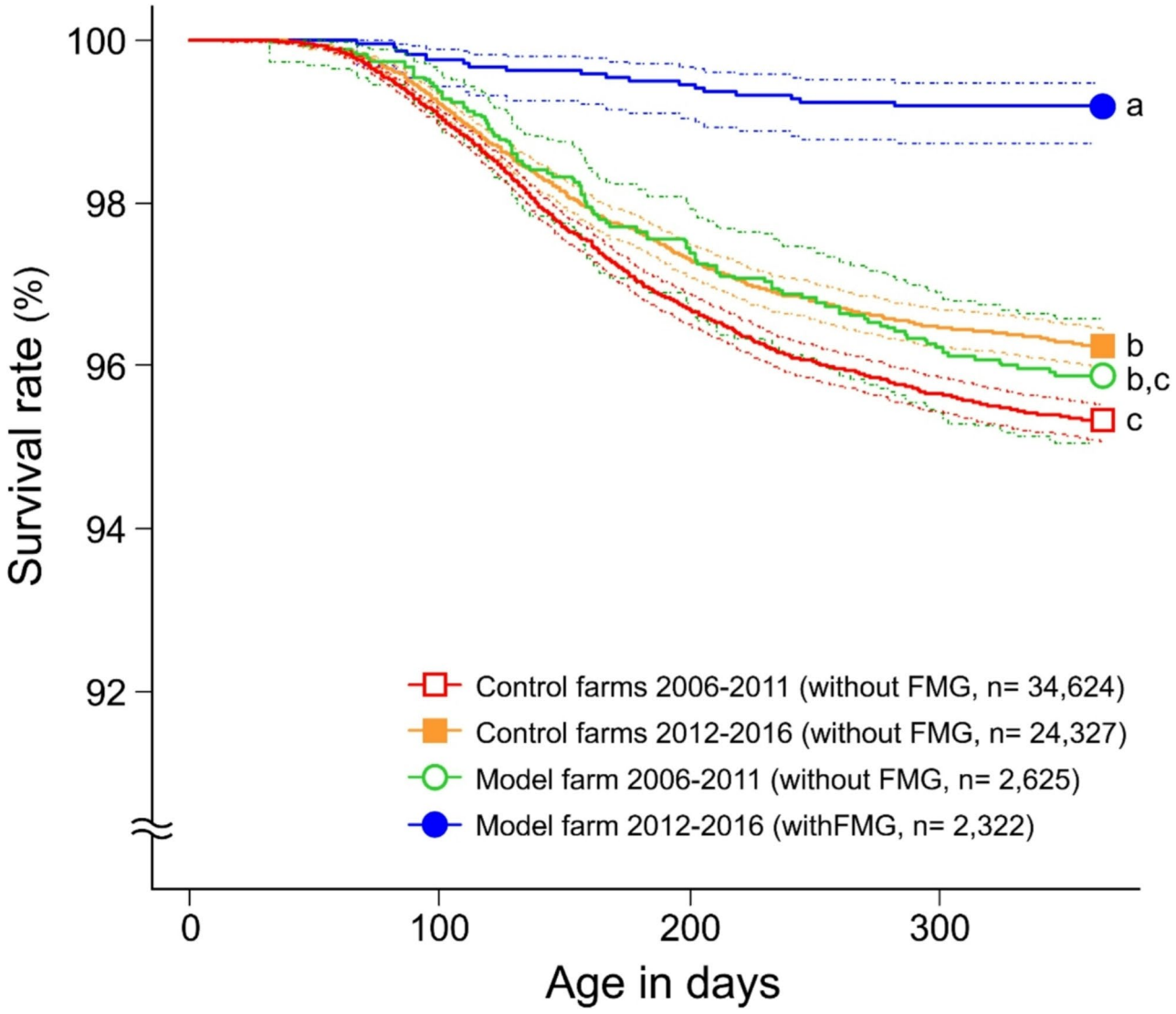

Fig. 1. Kaplan–Meier survival curves for calves younger than 365 days-old in groups without feeding management guidance (FMG), with FMG, and the control farms. (Study 1) Log rank test (two-sided). Solid line: survival rate curve, Dashed line: 95% confidence interval. Statistical significance between different codes: a, b, c for $P \leq 0.001$.

and 1.2% (32/2,625) in the model farm ($P = 0.18$, OR = 0.8, 95% CI = 0.5–1.2), with no significant difference. Cardiovascular mortality was 0.6% (209/34,624) in the control farms and 0.6% (15/2,625) in the model farm (P = 1.00, OR = 1.1, 95% CI = 0.6–1.9), with no significant difference. Other mortality was 0.3% (91/34,624) in the control farms and 0.4% (10/2,625) in the model farm (P = 0.25, OR = 0.7, 95% CI = 0.4–1.5), with no significant difference. During the period 2012–2016, FMG was implemented in the model farm, whereas the control farms did not adopt it. The total mortality in the model farm was significantly lower at 0.8% (19/2,322) compared with 3.8% (919/24,327) in the control farms (P < 0.001, OR = 4.8, 95% CI = 3.0–8.0), with a large-sized effect. For cause-specific breakdown, respiratory mortality in the model farm was significantly lower at 0.1% (3/2,322) compared with 2.3% (564/24,327) in the control farms (P < 0.001, OR = 18.5, 95% CI = 6.3–89.9), with a large-sized effect. Gastrointestinal mortality of the model farm was significantly lower at 0.3% (6/2,322) compared with 0.7% (177/24,327) in the control farms (P = 0.004, OR = 2.9, 95% CI = 1.3–8.0), with a medium-sized effect. Cardiovascular mortality was 0.2% (15/2,322) in the model farm and 0.5% (121/24,327) in the control farms (P = 0.06, OR = 2.4, 95% CI = 1.0–7.5), although the p-value met the tendency level, the result was determined no practical effect according to the predefined effect size criterion (i.e., no effect if the 95% CI of OR included 1.0). Other mortality was 0.2% (5/2,322) in the model farm and 0.2% (57/24,327) in the control farms (P = 1.00, OR = 1.1, 95% CI = 0.5–3.6), with no significant difference.

Item	Control farms		Model farm		Statistics		
	Number (head)	Rate (%)	Number (head)	Rate (%)	P-value	OR	95%CIof OR
From 2006 to 2011	Without FMG		Without FMG				
Purchased calves	34,624		2,625				
Dead (Total mortality)	1,625	4.7	108	4.1	0.19	1.1	0.9–1.4
Respiratory diseases	997	2.9	51	1.9	0.005*	1.5	1.1–2.0
Gastrointestinal diseases	328	0.9	32	1.2	0.18	0.8	0.5–1.2
Cardiovascular diseases	209	0.6	15	0.6	1.00	1.1	0.6–1.9
Other diseases	91	0.3	10	0.4	0.25	0.7	0.4–1.5
From 2012 to 2016	Without FMG		With FMG				
Purchased calves	24,327		2,322				
Dead (Total mortality)	919	3.8	19	0.8	< 0.001**	4.8	3.0–8.0
Respiratory diseases	564	2.3	3	0.1	< 0.001**	18.5	6.2–89.9
Gastrointestinal diseases	177	0.7	6	0.3	0.004*	2.9	1.3–8.0
Cardiovascular diseases	121	0.5	5	0.2	0.06†	2.4	1.0–7.5
Other diseases	57	0.2	5	0.2	1.00	1.1	0.5–3.6

Table 2. Comparison of the total and the disease-specific mortality in calves younger than 365 days old of the control farms and periods without FMG or with FMG (Study 1). Fisher's exact test (two-sided). FMG: Feeding management guidance. Purchased: Calves purchased under 120 days old. Dead: Among calves purchased, those who died younger than 365 days old. Control farms: 35 farms that manage calves in a style similar to model farm except for the presence of the FMG. Without FMG: the period between 2006 and 2011 on the model farm. With FMG: the period between 2012 and 2016 on the model farm. OR: Odds ratio. CI: Confidence interval. †, * and ** for $P \leq 0.1$, $P \leq 0.05$ and $P \leq 0.001$ compared to the control farms respectively.

Item	Group		P-value	Effect (*r*)	power
	Poor RMC (n = 6)	Good RMC (n = 6)			
Age in days	80.3 ± 3.4	81.7 ± 6.5	0.67	0.16	0.07
Chest girth (cm)	100.3 ± 2.1	103.5 ± 4.6	0.17	0.50	0.31
Abdominal girth (cm)	110.0 ± 4.0	114.0 ± 7.0	0.26	0.40	0.22
A/C ratio	1.10 ± 0.04	1.10 ± 0.04	0.84	0.06	0.04

Table 3. Comparison of mean age and body measurement of calves tested (Study 2). Welch's *t*-test (two-sided). Values for each item are presented as mean ± SD. RMC: Ruminal Mat Consistency. Poor RMC: group in which fluid bounce back was detected. Good RMC: group in which FBB was not detected. A/C ratio: Abdominal girth/ Chest girth.

Study 2: whether RMC affects calf immunity

Age and body measurement of calves tested

As shown in Table 3, calf age ($P = 0.67$, r = 0.16), chest girth ($P = 0.17$, r = 0.50), abdominal girth ($P = 0.26$, r = 0.40), and A/ C ratio ($P = 0.84$, r = 0.06) did not show significant difference between poor RMC calves and good RMC calves. The mean age, chest girth, abdominal girth, and A/C ratio of these calves were approximately 80 days old, 100 cm, 110 cm, and 1.10, respectively.

Cell fraction, number, and phagocytosis of peripheral blood

As shown in Table 4, cell fraction did not show statistical significance between poor RMC calves and good RMC calves. The numbers of $CD4^+$ T cells ($P = 0.07$, $r = 0.58$), $CD8^+$ T cells ($P = 0.07$, $r = 0.60$), and MHC class II^+ cells ($P = 0.07$, $r = 0.54$) in good RMC calves tended to be higher and had a large-sized effect than those in poor RMC calves. The numbers of MHC class II^+ IgM^+ (B) cells ($P = 0.03$, $r = 0.62$) in good RMC calves were significantly higher and had a large-sized effect than that in poor RMC calves. The phagocytic index of granulocytes ($P = 0.02$, r = 0.78) and monocytes ($P = 0.05$, r = 0.63) in good RMC calves were significantly higher and had a large-sized effect than those in poor RMC calves. Other items were neither significant nor did they exhibit any tendencies.

Cytokine mRNA expression

As shown in Fig. 2, *TNF-α* ($P = 0.02$, $r = 0.70$) and *IL-12* ($P = 0.04$, $r = 0.59$) mRNA expression in good RMC calves was significantly lower and had a large-sized effect than those in poor RMC calves. *IL-1β* ($P = 0.09$, $r = 0.49$) mRNA expression in good RMC calves tended to be lower and had a medium-sized effect than that in poor RMC calves. *IL-16* ($P = 0.07$, $r = 0.53$) mRNA expression in good RMC calves tended to be higher and had a large-sized effect than that in poor RMC calves. *IL-10* ($P = 0.13$, $r = 0.44$) and *IFN-γ* ($P = 0.13$, $r = 0.44$) had a medium-sized effect and the P-value subtly exceeded the tendency level, but the effect size and the boxplot of *IL-10* mRNA

Item	Group		P-value	Effect (*r*)	Power
	Poor RMC (n = 6)	Good RMC (n = 6)			
Cell fraction (%)					
Granulocyte	33.8 ± 17.0	31.2 ± 6.6	0.74	0.14	0.06
Monocyte	2.4 ± 1.0	2.0 ± 0.6	0.40	0.29	0.14
Lymphocyte	63.9 ± 16.7	66.8 ± 6.8	0.71	0.15	0.06
Cell number (cells/μL)					
WBC	8,716.7 ± 4,333.8	9,433.3 ± 1,007.3	0.71	0.17	0.06
Granulocyte	3,434.1 ± 3611.1	2,921.8 ± 544.7	0.75	0.15	0.05
Monocyte	183.4 ± 43.6	184.3 ± 42.0	0.97	0.01	0.03
Lymphocyte	5,104.1 ± 1234.4	6,327.3 ± 1,066.5	0.10	0.51	0.38
$CD4^+$ cells	1,097.4 ± 195.7	1,407.7 ± 307.5	0.07 †	0.58	0.45
$CD8^+$ cells	551.9 ± 83.3	698.0 ± 151.2	0.07 †	0.60	0.44
$CD4^+CD8^+$ cells	20.0 ± 8.7	31.7 ± 17.3	0.18	0.48	0.29
γδ T cells	1,395.3 ± 567.2	1,474.0 ± 458.9	0.80	0.09	0.05
$CD335^+$ cells	315.6 ± 203.1	287.7 ± 111.7	0.78	0.11	0.05
MHC class II^+ cells	1,673.4 ± 504.4	2,297.4 ± 555.3	0.07 †	0.54	0.44
MHC class II^+ IgM^+ cells	547.5 ± 343.8	1,017.6 ± 315.4	0.03*	0.62	0.53
Phagocytic index (%)					
Granulocyte	93.6 ± 3.1	97.9 ± 1.3	0.02*	0.78	0.65
Monocyte	57.5 ± 6.5	69.5 ± 11.2	0.05*	0.63	0.49

Table 4. Comparison of fraction, number, and phagocytic index of cells in peripheral blood (Study 2). Welch's *t*-test (two-sided). Values for each item are presented as mean ± SD. RMC: Ruminal Mat Consistency. Poor RMC: group in which fluid bounce back was detected. Good RMC: group in which FBB was not detected. CD: Cluster Differentiation. MHC: Major Histocompatibility Complex. † and * for $P \leq 0.1$ and $P \leq 0.05$, respectively.

expression suggests a type II error, indicating that it was lower in good RMC calves than that in poor RMC calves. Other items were neither significant nor did they exhibit any tendencies.

Discussion

This is the first report to assess the feasibility of estimating the actual consumption of calf herds via RMC on a commercial farm, as well as the relationship between RMC and immunity. Both good- and poor- RMC were identified in all age cohorts examined in the commercial farm, indicating that inter-individual differences in feed consumption exist even in calf herd. The most surprising finding was that nutritional intervention toward increase the number of good RMC calves based on RMC assessment decreased total mortality, especially by reducing the respiratory disease mortality. In addition, the results of immunological assays suggested that poor RMC calves, although seemingly healthy, had a decreased phagocytic ability, a decreased number of acquired immune cells, and a raised mRNA expression of several pro- and anti- inflammatory cytokine. The RMS or the RMC presented in this study may possibly be confused with the RFS[29]. However, this study demonstrated that a calf herd could be segmented into those in which FBB could not be detected and those in which FBB could be detected, despite no significant difference in the A/ C ratio, which is analogous to the RFS. Therefore, the RFS and the RMC were considered distinct indicators, and assessing both rather than one may help the better feed consumption estimation.

Study 1: whether RMC affects calf mortality

From 2013 to 2016, the forage proportion of TMR at the model farm fluctuated around 40%. The poor RMC population was highest (86.6%) in the under 90 cohort and decreased into 27.8% in the 151–180 cohort, indicating that they had individual differences and age related variation in consumed forage proportion or DMI. The feeding practice that increases calf starter rapidly from 1 kg at 55 days to 2.5 kg at 90 days was believed to cause poor RMC of the under 90 days cohort. The mortality of calves aged 50 to 365 days in this study were 4.7% (95%CI = 4.5–4.9) in 2006–2011 period of the control farms, 3.8% (95%CI = 3.5–4.0) in 2012–2016 period of the control farms, 4.1% (3.4–4.9) in the 2006–2011 period of the model farm without FMG, and 0.8% (0.5–1.3) in the 2012–2016 period of the model farm with FMG. Calf mortality have been reported as follows: Santman-Berends et al. (2019) found a 3.1% mortality rate for weaned dairy calves aged 56 days to 1 year in the Netherlands[40]. Raboisson et al. (2013) reported 3.1% for calves aged 1 to 6 months and 4.1% from 6 months to first calving in France[41]. Hyde et al. (2020) reported 4.15% for beef calves and 8.31% for dairy calves aged 0 to 6 months in Great Britain[42]. The total mortality of this study was generally consistent with previous reports, but the mortality of 0.8% in the 2012–2016 period of the model farm with FMG was remarkably low and no previous reports have maintained a mortality at 0.8% over a long term of five years.

One of the limitations of this study is that mortality rates could not be compared on the same farm over the same period. In general, direct comparison of mortality rates over short and different periods (e.g., control group in the previous year and treatment group in the current year) is not recommended because mortality

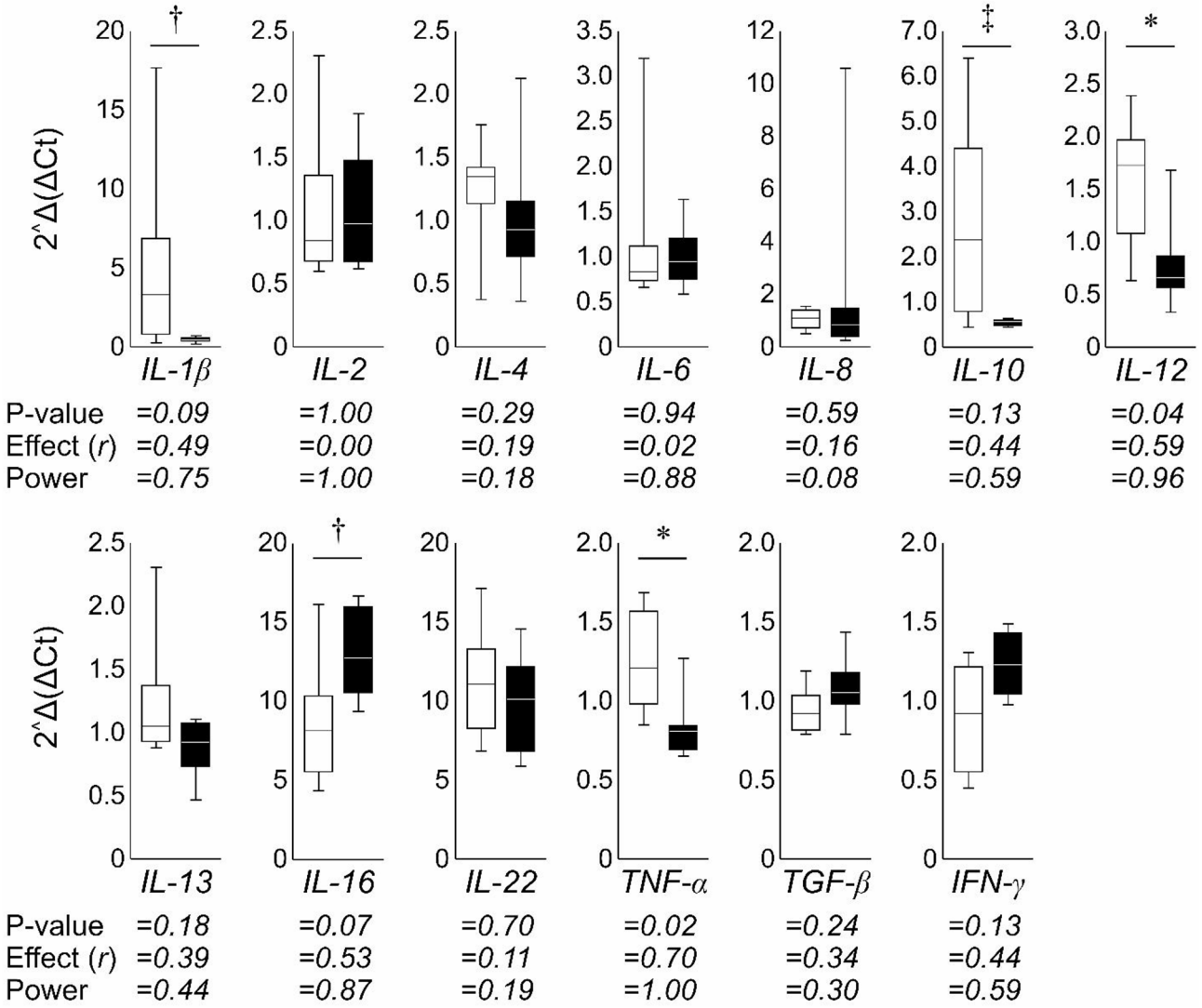

Fig. 2. Comparison of relative expression levels of cytokine mRNA in peripheral blood mononuclear cells in poor RMC and good RMC calves. (Study 2). Mann-Whitney *U* test (two-sided). Box plots are shown with maximum, third quartile, median, first quartile, and minimum. White box plot: poor-RMC group (n = 6), Black box plot: good-RMC group (n = 6). † and * for $P \leq 0.1$ and $P \leq 0.05$ respectively, ‡: the difference could not be judged only by P-value.

rates vary with factors such as year[7], environmental temperature[42], and nutrition[43]. On the other hand, it was not feasible to apply two different feeding procedures in parallel to the model commercial farm. Therefore, this study conducted a relatively long term nutritional intervention for five years, and compared the mortality of the model farm using regional benchmarking approach, regarding the full prefecture-wide mortality over the same period as control. The mortality comparison in this study negates the possibility that the model farm were low-mortality even without FMG, and suggests that the unique FMG to increase good RMC calves lowered total mortality rates.

As in many previous studies[44,45], respiratory and gastrointestinal diseases were the most common causes of calf death as in this report. In particular, bovine respiratory diseases (BRD) are typically understood to be a multifactorial opportunistic infection that require infectious agents and stressors[46]. Also, BRD are referred as the most expensive diseases, due to direct losses from death, depreciated carcass weight and quality, and increased medical treatment and labor costs[45,47]. But some consider it to be one of the clinical phenotypes of the SARA[11]. In this study, the FMG, which increases good RMC calves aged 50 to 180 days by increasing forage intake, resulted in a remarkably lower respiratory mortality rate of 0.1% compared to 2.6% in the control farms. These findings demonstrated the feasibility of the concept that nutritional intervention based on RMC assessment affects calf health and suggests that it may expanded into a new prophylaxis for BRD without antibiotics. Further research is needed to determine if FMG application on other farms will achieve similar mortality and how the RMC affects production outcomes.

While monthly RMC assessments in this study proved effective in reducing calf mortality, the optimal interval remains open to discussion. To enable shorter FMG intervals, automating data analysis and visualization through a dedicated application, or integrating RMC assessment with sensor-based or image processing technologies, could help reduce the need for manual data collection. Nonetheless, the observed reduction in mortality even with monthly FMG suggests that our predefined procedures to improve poor RMC also played an important role.

Study 2: whether RMC affects calf immunity

Our immune assay results suggests that good-RMC group possessed enhanced innate immune function, a greater abundance of acquired immune cells, and lower expression of both pro- and anti- inflammatory cytokine mRNA. This study was carried out limiting the age and the body size of the calves, which was approximately 80 days old, 100 cm of chest girth, and 110 cm of abdominal girth. This age and size limitation was employed mainly because immune parameters in calves under 6 months old are known to vary with age[48,49] and nutrition[50]. This limitation enabled us to compare the immunity excluding age and body size or condition dependent variations. Nevertheless, further research is needed to determine whether these findings can be extrapolated to other age groups.

The result of flow cytometry suggested that phagocytosis of both monocytes and granulocytes, and the number of lymphocytes that include $CD4^+$ cells, $CD8^+$ cells, B cells, and MHC class II^+ cells were higher in good RMC calves than that of poor RMC calves. Phagocytosis is one of the fundamental functions of monocytes and granulocytes and recognized as the first line of innate defense against invading pathogens[51,52]. The elevated phagocytic index of both monocytes and neutrophils in the good-RMC group suggests superior innate immune function and increased resistance to infection. Additionally, $CD4^+$, $CD8^+$, B cells, and MHC class II^+ cells, all of which are known to be essential for acquired immunity[53,54]. The elevated number of these cells in the good-RMC group suggests the advantages in acquired immunity. There was no observed difference in the numbers of granulocytes, monocytes, $CD335^+$ cells and γδ T cells between the good- and poor-RMC groups. The result of peripheral blood mononuclear cells (PBMC) cytokine mRNA assays suggested that good-RMC group exhibited lower expression in both pro- and anti-inflammatory cytokine mRNA such as *TNF-α*, *IL-1β*, *IL-12*, and *IL-10* than that of poor-RMC group. Macrophages or monocytes are known to secrete TNF-α, IL-1, IL-6, IL-8, and IL-12 when exposed to inflammatory stimuli[55]. These cytokines facilitate the recruitment of inflammatory cells by vasodilation and increased permeability, but their overproduction causes autoimmune diseases, with unregulated excess production leads to acute systemic inflammation[55,56]. IL-10, produced by activated macrophages, T cells, and B cells, functions to suppress macrophage activation and the production of TNF-α, IL-1, IL-6, IL-8, and IL-12[57]. Our result suggested that the good-RMC group reduced need for inhibitory regulation due to lower stimulatory inputs, whereas the poor-RMC group had elevated both pro- and anti-inflammatory reaction. Although poor RMC calves looked clinically healthy at the sampling, cytokines seemed to be competing with each other internally. Levels of *IL-16*, a chemoattractant for $CD4^+$ cells[58], tended to be higher in the good-RMC group, potentially contributing to the elevated numbers of peripheral $CD4^+$ cells.

Kim et al. (2019)[59] reported a positive correlation between ruminal pH and the numbers of $CD4^+$ and $CD8^+$ cells in SARA-challenged weaning transition calves. Similarly, Schnabel et al. (2020)[60] observed that a high grain diet reduced the proportion of peripheral $CD4^+$ cells. Kim et al. also noted a positive correlation between ruminal LPS and peripheral blood TNF-α concentrations[59]. SARA induces peripheral translocation of ruminal LPS, a typical inflammatory stimulus, leading to systemic inflammation[27,61]. Thus, although it would not be conclusive, our immunoassay results consistently providing circumstantial evidence of a new hypothesis that poor RMC was closer to SARA. Currently, clinical diagnosis of SARA is difficult because of the absence of overt symptoms[28] but RMC and RMS by repeated rumen compression method is simple enough to assess in 15 s at most. Further research on the relationship between RMC and SARA may lead to the development of a simplified clinical diagnosis for SARA.

Lastly, this study did not address the relationship between RMC and growth. Terler et al. (2022) and Poier et al. (2022) reported in a series of studies[62,63] that when part or all of the calf starter was replaced with medium or high-quality hay, rumen development was comparable across all nutritional management, and the growth of calves fed exclusively high-quality hay was similar to that of calves receiving conventional nutritional management. This suggests that high-quality hay is essential for our proposed nutritional strategy aimed at increasing calves with good RMC.

Conclusion

This study demonstrated the preliminary concept that assessing inter-individual differences in actual consumption via RMC evaluation in the commercial farm. Nutritional intervention aimed at improving RMC, which implies optimizing F: C ratio or forage consumption, was suggested to reduce mortality and may have possibility to support a smoother early rumen development or transition to functional ruminant. In addition, the remarkable finding that improving RMC particularly reduced BRD-related deaths may lead to the development of a novel preventive measure for BRD. This validated concept could be applied globally, not only in the development of sensor-based precision feeding, but also on farms lacking the latest IoT devises. Calves with good RMC were found to have superior innate and acquired immunity, along with lower levels of both pro- and anti-inflammatory cytokines compared to poor RMC calves. Further research into the relationship between RMC and SARA may lead to a simplified clinical diagnose for SARA. These findings have the potential to contribute resolving key issues and advancing sustainability in the bovine industry by improving calf health.

Materials and methods

Evaluation of RMC and RMS (repeated rumen compression method)

The evaluation of RMC by the repeated rumen compression method was performed with the animal's head restrained using a halter and rope. The area targeted for administering repeated compression was on the left side of the animal, along a horizontal line between the knee joint and the last rib, slightly anterior (head side) to the midpoint of the line, as illustrated in Fig. 3. The designated area was strongly pressed 10 times consecutively, either with the fist or the base of the palm. The force applied was sufficient to shift the animal slightly to the right, but maximum force was exerted if the animal's weight prevented shifting. The frequency of the compressions was 10 to 15 s per 10 times. In calves regarded to have poor RMC, the ruminal mat gradually softens and liquefies in response to repeated compressions, allowing for the detection of FBB. Conversely, FBB would not be detectable in calves with good RMC after a set of 10 compressions. Additionally, the RMC can be quantified into a 5-point RMS scale according to the number of compressions required to detect FBB and the consistency of the RM after 10 compressions: RMS 1 indicates the thinnest and the softest RM, where FBB is detected at the first compression; RMS 2 detects FBB within 2 to 5 compressions; RMS 3 detects FBB within 6 to 10 compressions; RMS 4 does not detect FBB even after 10 compressions, but the RM in the designated area is softer than paper clay; and RMS 5 indicates the thickest and firmest RM that does not detect FBB even after 10 compressions, and the RM in the designated area is as hard as paper clay. If it is unconfident, 0.5 point increments (e.g., RMS 2.5 if unconfident whether the RMS is 2 or 3) are acceptable. These procedures are demonstrated in Supplementary materials S1 in the video.

Model farm overview and procedures used to raise calves

Holstein Friesian × Japanese Black calves were used in this study. Calves were raised on a model commercial farm located in Miyazaki prefecture, Japan. Briefly, they were purchased at approximately 55 days of age at a calf auction and then fattened until about 27 months old. The average number of calves purchased by the farm was approximately 450 head a year. After arrival, the calves were given 6 L/day milk replacer (DM600g, CP24%, Fat25%) using a calf feeder or bucket, and this was reduced to 3 L/day at 3 days before weaning at about 90 days old. The forage comprised a mixture of timothy hay, oats hay and paddy rice straw mixed at 7:2:1, and forage was fed ad libitum. Pelleted calf starter (CP18%, TDN76%) was gradually increased from 1 kg/head on arrival at the farm to a maximum of 2.5 kg/head at weaning. After weaning, the calf starter and the mixed hay were gradually reduced and replaced with a TMR within a month. These calves were group fed and the time of

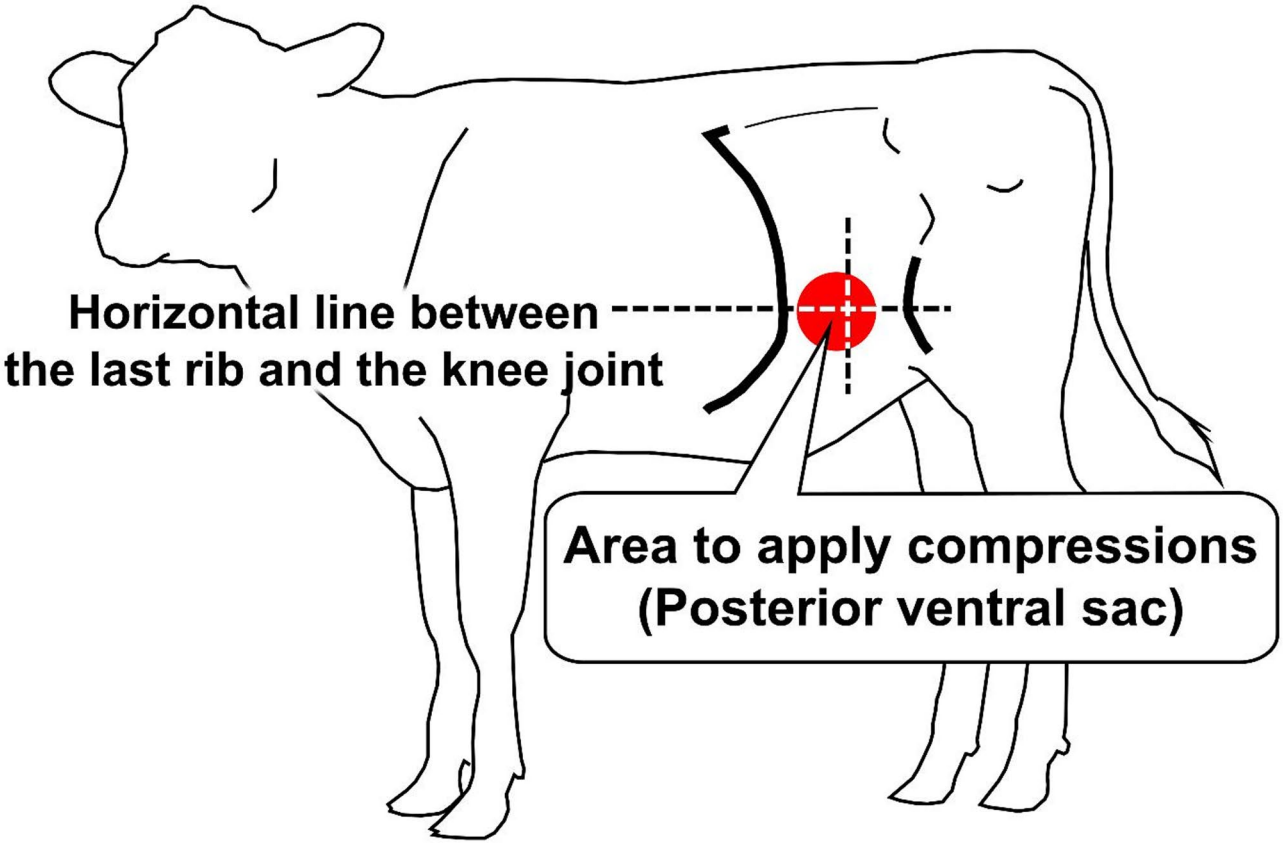

Fig. 3. The area targeted for administering repeated compression to detect fluid bounce back of the rumen (red circle). The center point of the targeted area was on the left side of the animal, along a horizontal line between the knee joint and the last rib, slightly anterior (head side) to the midpoint of the line.

feeding was 0800 h to 0900 h and 1600 h to 1700 h. As prophylactics, ivermectin pour-on (IvermecPO, Sasaeah pharmaceutical Co., Ltd.), viral respiratory disease pentavalent vaccine (Cattlewin 6, Kyoto Biken Laboratories, Inc.), *Mannheimia haemolytica* inactivated vaccine (Rispoval, Zoetis), toltrazuril (Baycox bovine, Bayer), and long-acting amoxicillin injection (Amostuck LA inj., Meiji Seika Pharma Co., Ltd.) were administered the day after arrival. Calves were dehorned at about 100 days old. The calves' bedding was coarse sawdust.

Study 1: whether RMC affects calf mortality

The protocol of the study 1 was approved by the Institutional Animal Care and Use Committee of the University of Miyazaki, Japan (Approval No.: 2007–002). All procedures of the study 1 were conducted in full compliance with the ARRIVE guidelines. All methods of study 1 were carried out in accordance with relevant guidelines and regulations. The model farm received a comprehensive explanation of the study's objectives, methods, and potential risks and benefits, and provided informed consent to participate. Feeding management proposals were only implemented with the farm's consent, which included the right to withdraw at any time, and personal information was strictly protected.

Feeding management guidance (FMG)

Feeding management guidance was implemented on a model farm from August 2012 to December 2016. FMG was conducted once a month on calves aged between 50 and 180 days with the aim of increasing the population of calves with good RMC. A total of 2,322 calves, representing all calves purchased between 2012 and 2016, were managed under FMG with no exclusions. The FMG consisted of evaluation of calf herd RMC and treatment of calves with poor RMC. In addition, from 2013 to 2016, interviews and records about the TMR composition were included. RMC evaluation was performed between 1300 and 1500 h. To evaluate calf herd RMC, at least 10% of the calves, with a minimum of four animals, were randomly selected as pilot calves to represent the herd. These calves were confirmed by a veterinarian to be free of any clinical symptoms such as respiratory disease or diarrhea. Once selected, the pilot calves were not changed for any reason and were subjected to monthly RMC evaluations up to approximately 180 days old. After weaning, they were randomly assigned to pens containing 8–10 animals. This procedure allowed for the estimation of RMC distribution by age group or pen, and the model farm was instructed to adjust the forage-to-concentrate ratio for herds with poor RMC until improvement in RMC was observed. The aim of the more detailed FMG was to increase the forage intake. To achieve this, we instructed not to empty forage, to reduce unconsumed grass like late-cutting or poor storage quality silage, to provide at least three types of forage, with additional amounts of the fastest consumed type. If increasing forage intake provided ineffective and the number of poor RMC calves expanded, we also recommended reducing or temporarily discontinuing the concentrate feeding. We often organized pens for poor RMC calves and merged calves from several pens. To summarize the FMG recordings, the annual average and standard deviation of forage proportion of the TMR, and the annual prevalence of poor RMC calves within each age cohort were aggregated.

Extent and causes of mortality

Data from Livestock insurance operated by the Miyazaki Agricultural Mutual Aid Association were used to aggregate the mortality and causes of death. The insurance policy mandates owners report all national ear tag IDs, along with purchase and sale dates, to calculate premiums. Also, a veterinarian-certified death certificate specifying the disease and date of death must be submitted to claim payments. The survey period was set into the period without FMG, from 2006 to 2011, and the period with FMG, from 2012 to 2016. The survey period was divided into two phases: without FMG (2006–2011) and with FMG (2012–2016), based on the presence of FMG in the model farm. The mortality rate of 35 commercial farms in Miyazaki Prefecture, which raised calves in a style similar to the model farm but without FMG, was calculated for each period. This rate was regarded as the full prefecture-wide mortality rate and used as a benchmark for comparison with that of the model farm during the corresponding period. Calves aged 120 days or less at the time of purchase were extracted from the database for mortality calculations. From 2006 to 2016, a total of 64,708 calves were purchased by the 36 farms, including the model farm. No randomization or sample size calculations were performed as this survey represented a full prefecture-wide tabulation. However, data on 810 calves legally culled due to the foot-and-mouth disease outbreak in 2010 were excluded. The model farm was not subjected to this culling. Of the total 63,898 calves after exclusion, 34,624 were from control farms (2006–2011, without FMG), 24,327 from control farms (2012–2016, without FMG), 2,625 from model farm (2006–2011, without FMG), and 2,322 from model farm (2012–2016, with FMG). The Kaplan–Meier survival curves, mortality rates, and causes of death were compared between the control farms and periods without FMG, and between the control farms and periods with FMG. The follow-up period for survival analysis was set at 365 days form the birth, with the endpoint defined as the death age in days. Calves that survived the follow-up period were censored at 365 days. The mortality, including causes of death, was calculated as the percentage of calves in each group that died within 365 days old. The causes of death listed on the death certificates were classified into respiratory, gastrointestinal, cardiovascular, and other diseases.

Study 2: whether RMC affects calf immunity

The protocol of the study 2 was approved by the Institutional Animal Care and Use Committee of the University of Miyazaki, Japan (Approval No.: 2019-001). All procedures of the study 2 were conducted in full compliance with the ARRIVE guidelines. All methods of the study 2 were carried out in accordance with relevant guidelines and regulations. The model farm received a comprehensive explanation of the study's objectives and methods, and provided informed consent to participate. Sample collection was conducted exclusively with the farm's consent, which included the right to withdraw at any time, and personal information was strictly protected.

Experimental design, exclusion criteria, and sample collection and selection

In Study 2, we aimed to compare two levels of blood samples from the calves of the model farm, poor-RMC and good-RMC groups, to test the hypothesis that differences in RMC affect immunity. The sample size was initially set to n = 6 for each group, which is the minimum required for nonparametric test. As no prior studies were available to estimate an appropriate sample size, post-hoc power analyses were conducted to assess detection power. Also it was a simple two-group comparison without a control group as there were no deliberate diet changes. Blood samples were planned to collect from the pilot calves selected in the same procedure as in Study 1 during the monthly RMC evaluation at the model farm, with collection ceasing once the number of samples in both the poor- and good-RMC groups reached six or more. Samples eligible for statistical analysis tested were planned to limit those collected from calves aged 70 days or older and before weaning, i.e., around 80 days old. Sample selection was further refined to ensure that the average of age and body size of the two groups were nearly equal. These were because of the following reasons: immune status was known to vary with age up to 6 months old and nutritional status; transportation and environmental acclimation could be potential confounders; poor-RMC calves were rarely observed in the post-weaning herd (90 days or older) of the model farm in 2019; farm consent was not obtained to collect samples from calves other than the pilot calves; and ethical concerns precluded intentionally inducing poor-RMC in the post-weaned herd for research purposes. In Study 2, RMC was classified as good if RMS 3.5 or higher, and poor if RMS 3 or lower. Sampling was carried out between 1400 and 1500 h before the evening feeding in conjunction with the monthly RMC evaluation for FMG from October 2019 to January 2020. All pilot calves were confirmed to be healthy by a veterinarian, and information including national ear tag IDs, RMS, chest girth, abdominal girth, and A/C ratio was recorded before blood samples were collected from the jugular vein into tubes containing disodium EDTA (EDTA-2Na, VP-NA070K, TERUMO). Samples were transported in the dark at room temperature, and subjected to white blood cell count, lymphocyte subset, and phagocytosis tests on the day of collection, and the remaining samples were stored at -80 °C until evaluation of cytokine mRNA expression. Of a total of 53 samples from 25 pilot calves, 26 samples from post-weaned calves and 8 samples from younger than 70 days old were excluded. From the remaining 19 samples, 6 from the 7 good-RMC samples and 6 from the 12 poor-RMC samples were selected to ensure that the average age and body size of the two groups were nearly equal.

White blood cell (WBC) number and fraction

WBC number was measured using a cell counter (Celltac-α, MEK-6550, Nihon-Koden). Leukocyte fractions were assessed via microscopic examination of May-Grünwald-Giemsa-stained blood smears. The blood smear preparation and staining procedures were as follows. Blood samples were smeared by wedge method onto glass slides, immediately forced cold air-dry, and stained by the overlay method. The Giemsa's stain solution (GSS) + phosphate buffered saline (PBS) stain solution (GSS + PBS) was prepared by mixing 250 µl of GSS (Muto Pure Chemicals Co., Ltd) with 3 ml of PBS (M/15 PBS, pH 6.4, Muto Pure Chemicals Co., Ltd). May–Grünwald's stain solution (Muto Pure Chemicals Co., Ltd) was applied to the slides for 2 min, followed by an equal volume of PBS for an additional 3 min. The slides were then stained with GSS + PBS for 15 min.

Lymphocyte subsets

Two ml of blood with EDTA-2Na was diluted with an equal volume of PBS (0.01 mol/L, FUJIFILM Wako Pure Chemical Corporation) and layered on Ficoll-Paque™PLUS medium (GE Healthcare Bio-Sciences). After samples were centrifuged at 400 × *g* for 30 min at room temperature, the layer of PBMC was collected and washed with PBS. The part of isolated PBMC were stored at -80 °C until cytokine mRNA measurement. Erythrocytes were lysed with lysis buffer and the isolated PBMC were resuspended in PBS supplemented with 0.5% bovine serum albumin and 0.05% sodium azide (BSA-PBS). Resuspended PBMC were placed in a round-bottomed 96-well plate and following fluorescent monoclonal antibodies (mAb) were added before incubation at 4 °C for 60 min: anti-CD4 (200 × dilution, ILA11A, Monoclonal Antibody Center at Washington State University), anti-CD8 (200 × dilution, CC63, Bio Rad), γδ TCR (200 × dilution, GB21A, Monoclonal Antibody Center at Washington State University), anti-CD335 (100 × dilution, AKS1, Bio Rad), anti-MHC class II (200 × dilution, TH14B, Monoclonal Antibody Center at Washington State University), anti-IgM (100 × dilution, P-PIG45A2, Monoclonal Antibody Center at Washington State University). For fluorescence labeling of mAb, a fluorescein isothiocyanate (FITC) labeling kit-NH_2, HiLyte™ Fluor 555 (F555) labeling kit-NH2, and HiLyte™ Fluor 647 labeling (F647) kit-NH2 (Dojindo Laboratories) were used according to the manufacturer's instructions. After staining, cells were washed twice with BSA-PBS, and the labeled cells were counted using a flow cytometer (BD FACS Canto II Flow Cytometer, BD Biosciences). Analyses were conducted on gated lymphocyte cell populations based on their forward scatter (FSC) and side scatter (SSC). The number of lymphocytes in each subset was calculated as follows:

Phagocytosis

Peripheral blood collected in EDTA-2Na tubes was centrifuged at 780 × g for 10 min at room temperature. The buffy coat was collected, washed with PBS, and the erythrocytes were lysed with lysis buffer. The cells were washed with PBS and resuspended in RPMI1640 (FUJIFILM Wako Pure Chemical Corporation, containing 10% heat-inactivated fetal calf serum and antibiotics). Cell viability was assessed using a trypan blue-dye exclusion test, and the number of cells was adjusted to 4.0×10^6 cells/ml with RPMI1640. One ml of the cell suspension was dispensed into a 24 microwell plate, and 1 µl of a 2.5% FITC-labeled latex beads suspension (1 µm, No. 1030, Sigma-Aldrich Chemicals) was added. The cells were incubated for 60 min at 37°C in a 5% CO_2 atmosphere. The supernatant containing the free beads was discarded by washing three times with BSA-PBA. The cells were placed in a round-bottomed 96-well plate and stained at 4°C for 30 min using the following mAbs: F555-labelled anti-granulocyte mAbs (100 × dilution, CH138A, Monoclonal Antibody Center at Washington State University)

Gene	Accession number	bp	Direction	Sequence (5' to 3')
IL -1β	NM_174093	111	For (232)	GCCTACGCACATGTCTTCCA
			Rev (322)	TGCGTCACACAGAAACTCGTC
IL-2	NM_180997	111	For (113)	TGCTGGATTTACAGTTGCTT
			Rev (203)	TCAATTCTGTAGCGTTAACCT
IL-4	NM_173921	142	For (103)	ATCAAAACGCTGAACATCCTC
			Rev (223)	TCCTGTAGATACGCCTAAGCTC
IL-6	NM_173923	168	For (162)	AGCTCTCATTAAGCGCATGG
			Rev (311)	ATCGCCTGATTGAACCCAG
IL-8	NM_173925	175	For (86)	GTACAGAACTTCGATGCCAA
			Rev (240)	TTCTGCACCCACTTTTCCTTG
IL-10	NM_174088	103	For (305)	GGCCTGACATCAAGGAGCAC
			Rev (387)	CTCTTGTTTTCGCAGGGCAGA
IL-12	NM_174356	178	For (796)	CCGCATTCCTACTTCTCCCT
			Rev(955)	ACACAGATGCCCATTCACT
IL-13	NM_174089	178	For (19)	GCGGTCATTGTTCTTATCTGCT
			Rev (178)	CTGCACAGTACATGCTGCT
IL-16	NM_001075253	176	For (363)	CTGCACCTCTCAGCCTGGTC
			Rev (518)	CTCCTGCCAAGCTGAACCCAA
IL-17A	NM_001008412	196	For (221)	TCCACCGCAATGAGGACCC
			Rev (397)	GCCACCAGCATCTTCTCCGA
IL-22	EF560596	100	For (107)	TCACGTCGCACTGTAGGCTC
			Rev (184)	ATGTTATCTGCCAAACTAGCCTT
TNF-α	NM_173966	128	For (206)	CCATCAACAGCCCTCTGGTTC
			Rev (312)	ATTGGCATACGAGTCCCACCAC
TGF-β	NM_001166068	196	For (526)	AACAATTCCTGGCGCTACCTC
			Rev (700)	AACTGAACCCGTTAATGTCCAC
IFN-γ	NM_174086	108	For (353)	TGATTCAAATTCCGGTGGAT
			Rev (442)	TCTTCCGCTTTCTGAGGTT
GAPDH	NM_001034034	130	For (159)	GTTCAACGGCACAGTCAAGGCAGAG
			Rev (289)	ACCACATACTCAGCACCAGCATCAC

Table 5. Primer sequences used for real-time quantitative PCR analysis (Study 2). *IL*: Interleukin, *TNF-α*: Tumor Necrosis Factor-α, *TGF-β*: Transforming Growth Factor-β, *IFN-γ*: Interferon-γ, *GAPDH*: Glyceraldehyde 3-phosphate dehydrogenase, bp: base pairs, For: Forward, Rev: Reverse.

and F647-labelled anti-MHC class II mAbs (200 × dilution, TH14B, Monoclonal Antibody Center at Washington State University). The fluorescence of the cells was analyzed under a flow cytometer. Granulocytes and monocytes were gated according to their FSC and SSC; the gated cells were sorted on the basis of fluorochromes. Phagocytic indexes of granulocytes and monocytes was reported as follows:

RT-qPCR

Total RNA was extracted from PBMCs stored at − 80 °C using an RNeasy PLUS Mini Kit (Qiagen) according to the manufacturer's instructions. RT-qPCR was performed using One Step TB Green™ PrimeScript™ PLUS RT-PCR Kit (Takara Bio.) according to the manufacturer's instructions. RT-qPCR primer pairs were designed using Oligo7 software (Molecular Biology Insights, https://www.oligo.net/) (Table 5). RT-qPCR conditions consisted of reverse transcription at 42 °C for 5 min, initial PCR activation at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, 57 °C for 30 s, and 72 °C for 30 s. The RT-qPCR assay was performed using a Real-time PCR_Quant Studio 3 System (Thermo Fisher Scientific). For quantification of target mRNA, a comparative Ct method ($2^{-\Delta\Delta Ct}$ method/Livak method) using GAPDH as an internal control was used. Data were analyzed using Quant Studio Design and Analysis software v1.5.1 (Thermo Fisher Scientific, https://www.thermofisher.com/jp/ja/home/global/forms/life-science/quantstudio-3-5-software.html).

Statistical analysis

For the survival rate of Study 1, a log rank test (two-sided) was performed to determine the survival rate curve by the Kaplan–Meier method. For the mortality and the disease classification, Fisher's exact test (two-sided) was used. These results were shown as percentages together with the 95% confidence interval, P-value, and OR. For Study 2, we examined the difference between poor RMC and good RMC by referring to the proper statistical techniques with small samples[64]. For body measurements, phagocytosis, and peripheral blood leukocytes were tested using Welch's *t*-test (two-sided), which is said to be robust against unequal variances, assuming the population is normally distributed and not homoscedastic. These data were presented as the mean,

standard deviation (SD), P-value, effect (*r*), and post-hoc detection power. For cytokine mRNA expression, the Mann–Whitney *U* test (two-sided) was selected since cytokines often do not meet the requirements for a normal distribution[65]. These data were presented as the boxplot of maximum, median, minimum, first and third quartiles, P-value, effect (*r*), and post-hoc detection power. The results of the normality test were not adopted because the power of the normality test was low in all the items of study 2. All statistical analyses were performed using EZR ver1.42 (Saitama Medical Center, Jichi Medical University, https://www.jichi.ac.jp/usr/hema/EZR/statmed.html)[66], a graphical user interface for R, using R ver. 4.0.0 (R Foundation for Statistical Computing, https://www.r-project.org/)[67]. For the post-hoc detection power analysis of cytokine data assuming a non-normal distribution, the wmwpow package ver0.1.3 (https://www.rdocumentation.org/packages/wmwpow/versions/0.1.3)[68] was used. The P-value, effect size (*r*) and power were calculated with the α level taken as 5% and the β level was taken as 20%[69]. We considered $P \leq 0.05$ as significant, $P \leq 0.1$ as a tendency, $OR \geq 3.0$ as a large-sized effect, $OR \geq 1.5$ as a medium-sized effect, $1.0 < OR \leq 1.5$ as a small-sized effect, $r \geq 0.5$ as a large-sized effect, and $r \geq 0.3$ as a medium-sized effect. In the case of Welch's *t*-test, the effect (*r*) was calculated from the *t*-value (*t*) and the degree of freedom (*df*). In the case of the Mann–Whitney *U* test, it was calculated from the Z-value (*Z*) and the number of samples (*n*) as follows.

$$r = \sqrt{\frac{t^2}{t^2 + df}}$$

$$r = \frac{Z}{\sqrt{n}}$$

Data availability

All data are available at https://github.com/ShinskeAbe/Bovine-RMC-mortality-immunity/.

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Author contributions

S.A., Y.S., and M.Y. conceived and planned the research. S.A. and K.S. carried out the FMG, and contributed to sample collection and mortality calculation. S.A. and M.Y. carried out the immunological assays. S.A. took the lead in writing the manuscript and created all illustrations and a supplementary video. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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