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COMPARISON OF ANTIBODY PROFILES OF BOVINE MILK REPLACERS WITHOUT & WITH EGGTEK®-C

Analysis Conducted by Arkion® Life Sciences

EXECUTIVE SUMMARY

Calves are frequently fed calf milk replacer (CMR) before weaning, and these CMR's are formulated to provide the calf optimal nutrition. The protein source in the CMR can vary, for example, spray dried plasma or whey protein concentrate, and almost all of these sources also provide IgG. However, the amount of IgG CMRs can provide and the antigen-specificity they possess is not well understood. Here, we profiled five milk components-only CMRs against nine antigens associated with different calf diseases (e.g., bovine rotavirus, *E. coli*, and different *Salmonella* strains).

We also simulated the addition of EggTek®-C to each milk replacer. EggTek-C is an egg antibody (i.e., IgY) product, manufactured by Arkion Life Sciences, designed to help a calf's immune system by providing passive immunity against an array of early calf pathogens. Addition of EggTek-C to a CMR can improve the CMR's recognition of specific disease-causing antigens and help prevent the calf from getting sick. Indeed, when EggTek-C was added to the CMRs tested here, we saw increased responses for all antigens tested. This demonstrates inclusion of EggTek-C in a CMR can help increase the amount of passive immunity the calf receives and thus improve overall health of the calf.

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CALF MILK REPLACER PROFILING AND COMPARISON

The IgG profiles of five commercial, milk components-only bovine CMRs were found against eight common enteric challenges for calves. The specific CMR brands and products used are listed in Table 1.

Table 1. CMRs used in analysis.

Brand	Product
Dairy Farmers of America (DFA)	DFA 24/20 Bova/Clarify/Baciflex
Denkavit	Denkamilk Perfect 24/20
Land O'Lakes®	Cow's Match® ColdFront®
Milk Specialties Global	Excelerate® w/Bio-Mos®
Provimi North America	Nuture® Professional 24-17 BOV CFL

In order to protect confidentiality, the five CMRs were anonymized and will be referred to as Samples A-E throughout the rest of the report.

IgG was extracted and purified from the milk replacers and then labeled with a horseradish peroxidase (see Materials & Methods for details). Direct ELISAs were then conducted against the following antigens:

- Bovine rotavirus
- Bovine coronavirus
- *Cryptosporidium parvum*
- *E. coli* (mix of K88, K99, 987P, and F41 pili)
- *Salmonella* Typhimurium
- *Salmonella* Dublin
- *Salmonella* Heidelberg
- *Clostridium perfringens* Type A
- *Clostridium perfringens* Type C/D

For the assays, the amount of IgG used in each reaction was the equivalent to a 10 oz dose of CMR, which allows a comparison of each CMR based on equivalent masses. To determine this, first the total IgG concentration of each CMR was found (Table 2) and then scaled down proportionally (see Material & Methods).

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Table 2. IgG of each CMR.

CMR Sample	IgG Conc.	Std. Dev.
Sample A	11.09 mg/g	± 1.25 mg/g
Sample B	2.01 mg/g	± 0.11 mg/g
Sample C	3.76 mg/g	± 0.07 mg/g
Sample D	5.65 mg/g	± 0.24 mg/g
Sample E	12.16 mg/g	± 1.25 mg/g

Direct ELISAs were performed on nine antigens using the five CMR samples, and the results are shown in Figure 1. The results are reported as the absorbance at 450 nm (A450). The A450 value is the raw output from the ELISA and is a measure of how much IgG is bound to the specific antigen of interest. Importantly, the A450 values cannot be compared between antigens, only between CMR samples for the same antigen. Each CMR sample was run in triplicate against each antigen, and the background absorbance was subtracted.

The differences in A450 values between the different CMRs for each antigen were statistically significant (p-value < 0.05) for all comparisons except for:

- Sample A vs Sample D for bovine rotavirus, *Salmonella* Typhimurium, *Salmonella* Dublin, *Salmonella* Heidelberg, *C. perfringens* Type A and *C. perfringens* Type C/D antigens
- Sample B vs Sample C for *C. perfringens* Type A antigen

Interestingly, though Sample A and Sample E have similar total IgG values, Sample E had significantly higher A450 values, while Sample A was more comparable to Sample D, which had about half the total IgG. Sample B, which had the lowest total IgG amount, also had the lowest A450 values for all the antigens tested.

Conclusions

There was a large degree of variance between different CMR products both in terms of total IgG and the specificity of the IgG. The differences seen in total IgG values could reflect the different protein sources used in these milk component-only CMRs (e.g., whey protein concentrate vs. dried milk components). However, the total IgG value does not tell the whole story. Though Samples A and Sample E have comparative total IgG values (p-value = 0.354), Sample E had statistically greater A450 values for all antigens tested. Thus, it is important to

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know not only the total IgG of a CMR but also the antigen specificity of the IgG, because if the IgG has no specificity for potential pathogens, it cannot help protect the calf.

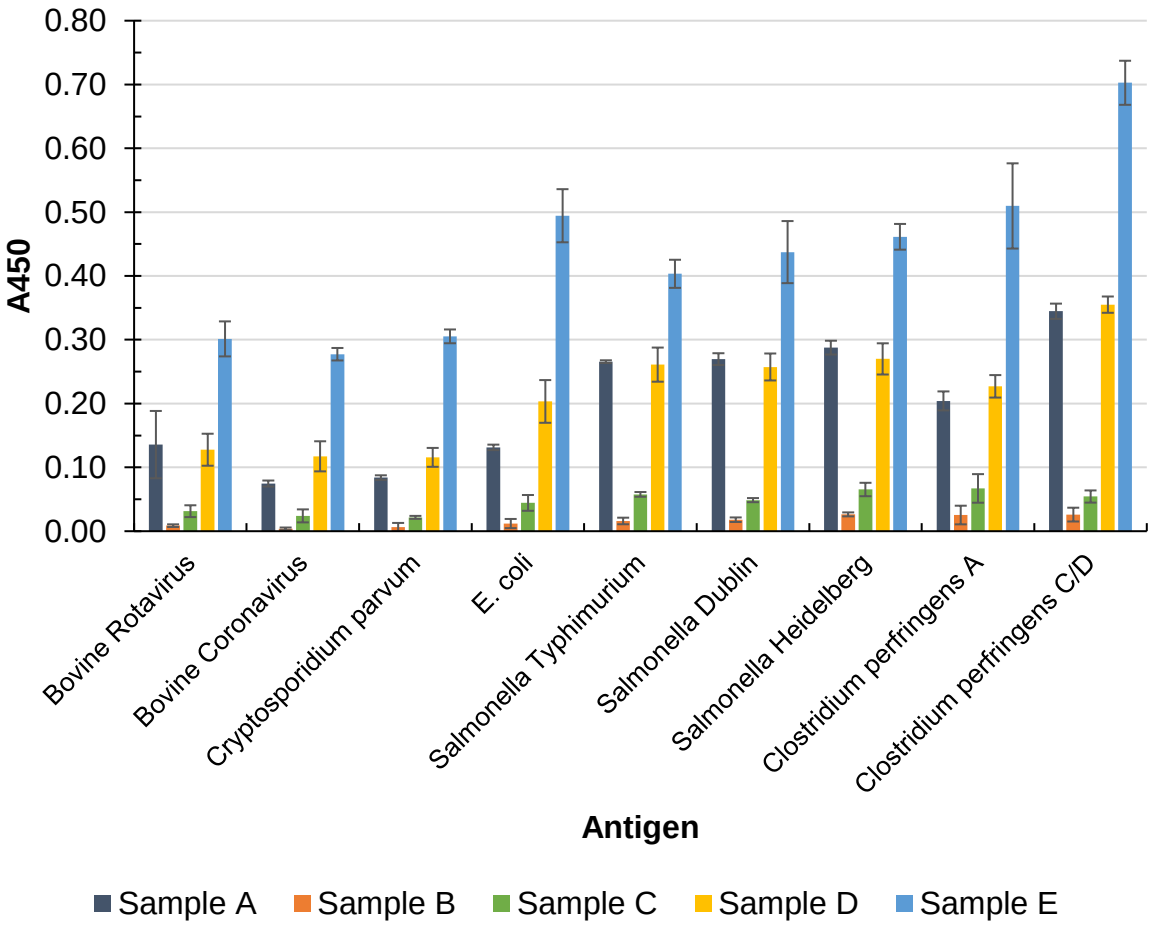

Figure 1. Specific IgG profile of each CMR. The average A450 value against each antigen is shown along with the standard deviation. Antigens are listed along the x-axis. A450 values are listed in Supplemental Table 1.

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EGGTEK-C ADDITION TO CALF MILK REPLACERS

EggTek-C is produced by vaccinating laying hens with a range of calf disease causing antigens resulting in a product to boost a calf's passive immunity, and addition of EggTek-C to CMRs can increase the antigen specific A450 value and help protect the calf from becoming ill. To simulate EggTek-C's addition to the CMRs, IgY was extracted and purified from the product and labeled with a horseradish peroxidase (see Materials & Methods for details). The same direct ELISAs were then repeated but with mixing labeled IgG and IgY together. The labeled IgY was added proportionally to simulate a 2-gram or a 4-gram addition to 10 oz of CMR. These dosages (2-gram and 4-gram) are current recommendations for daily feeding to calves.

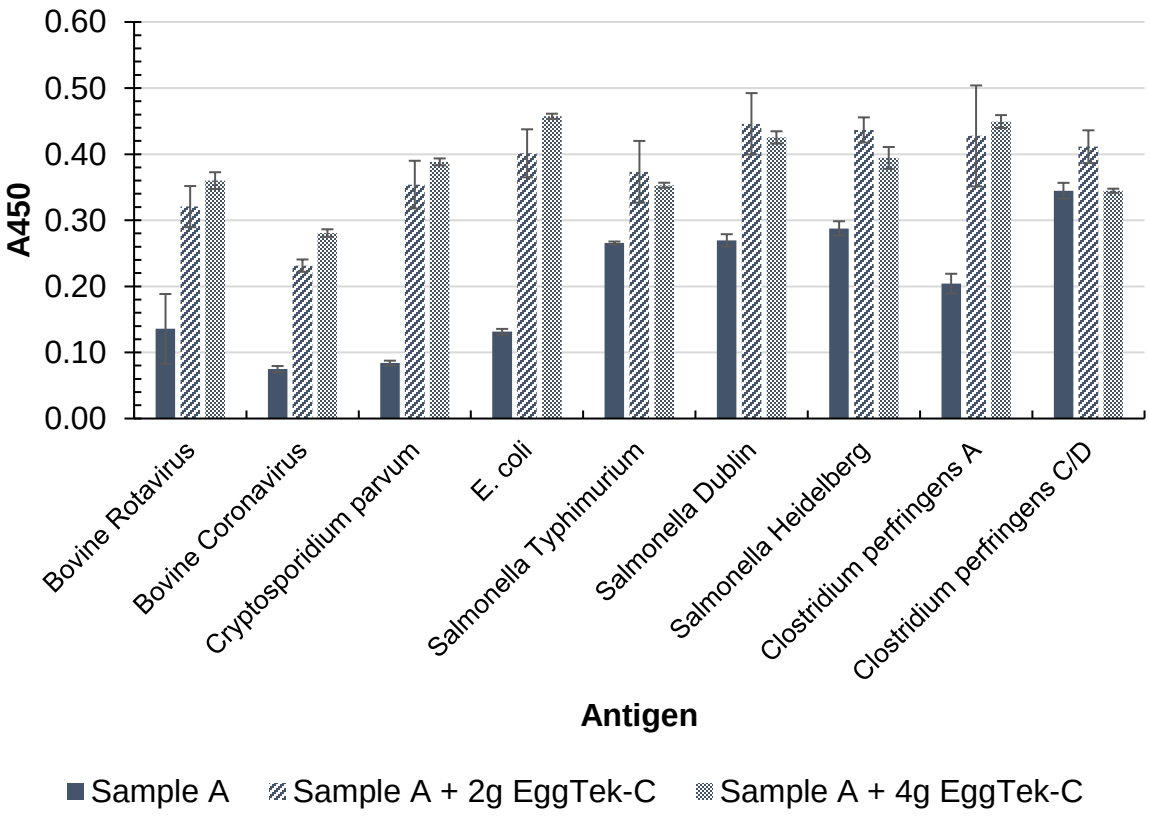

Figure 2. A450 values of Sample A with EggTek-C addition. The average A450 value against each antigen is shown along with the standard deviation. Antigens are listed along the x-axis. A450 values are listed in Supplemental Table 2.

EggTek-C addition to CMR Sample A (Figure 2) significantly improved (p-value > 0.05) the A450 values for all antigens tested. For some antigens (e.g., *C. perfringens* Type C/D), the 4g

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EggTek-C addition actually resulted in a slightly lower A450 value than the 2g addition. This is most likely due to antibody crowding and/or limited antigen on the plate. As more antibodies (both IgG and IgY) try to bind to the antigen on the plate, steric hindrance between them causes less antibody to bind to the antigen before washing, and this results in a lower A450 value. This effect was generally seen when the A450 values were already high.

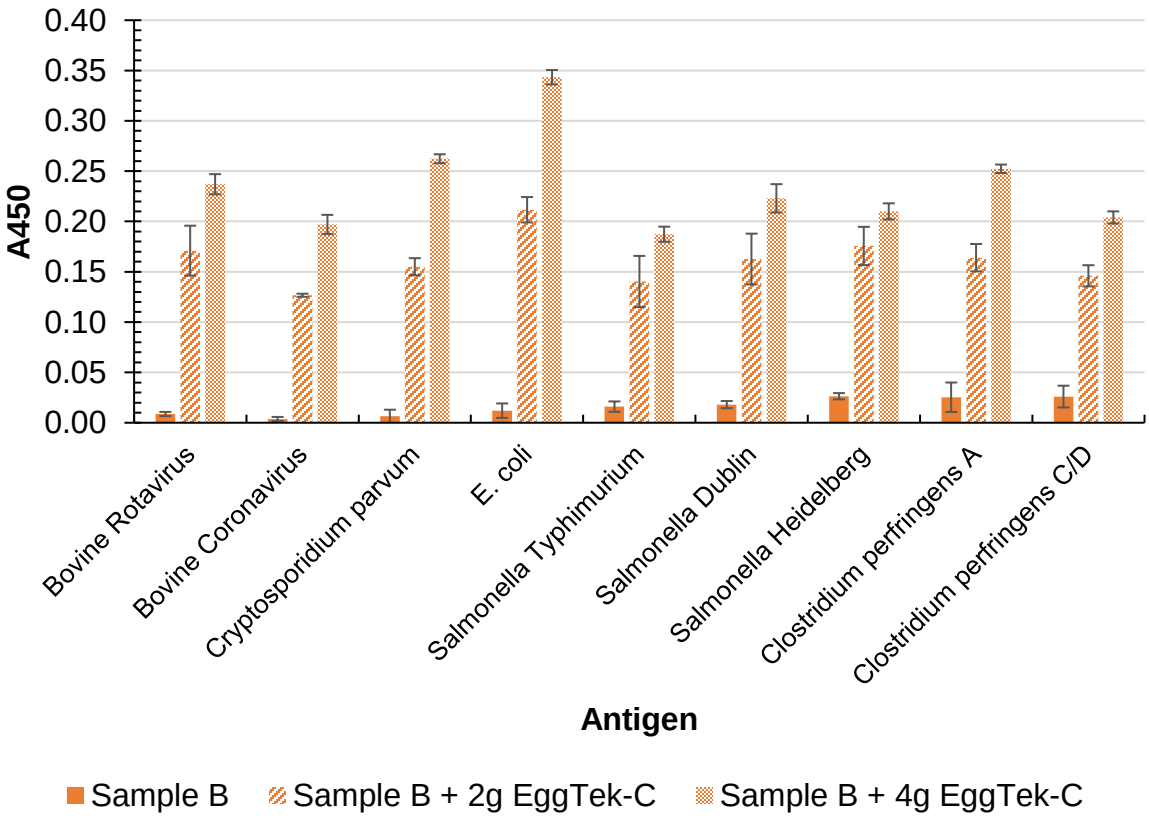

Figure 3. A450 values of Sample B with EggTek-C addition. The average A450 value against each antigen is shown along with the standard deviation. Antigens are listed along the x-axis. A450 values are listed in Supplemental Table 3.

The addition of EggTek-C to Samples B, C, and D had similar effects (Figures 3-5). In all cases, the inclusion of EggTek-C significantly improved A450 values for all antigens (p-value > 0.05).

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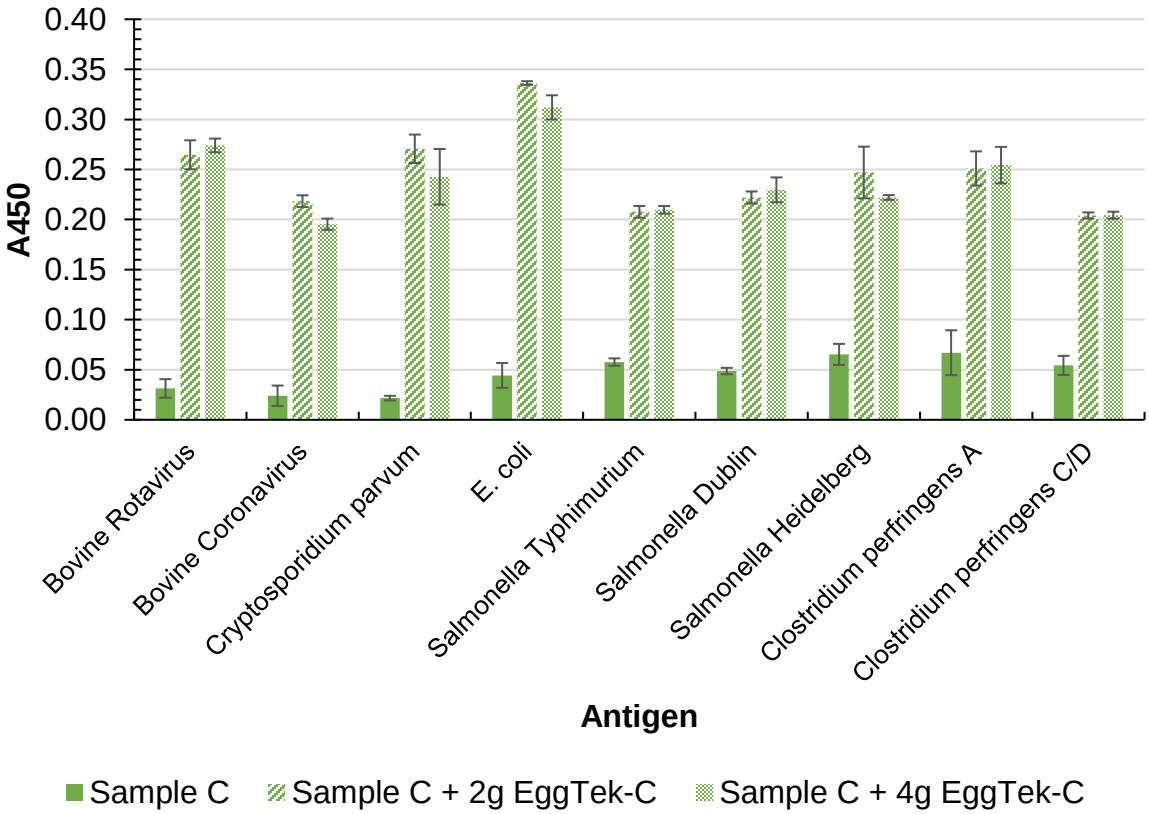

Figure 4. A450 values of Sample C with EggTek-C addition. The average A450 value against each antigen is shown along with the standard deviation. Antigens are listed along the x-axis. A450 values are listed in Supplemental Table 4.

For Sample C, the 4g addition of EggTek-C produced similar A450 values as the 2g addition. While steric hindrance may also play a role here, the A450 values are lower than those seen in Sample A, so a different phenomenon may also be causing this. Regardless, the addition of EggTek-C, whether 2 grams or 4 grams, significantly improved all A450 values.

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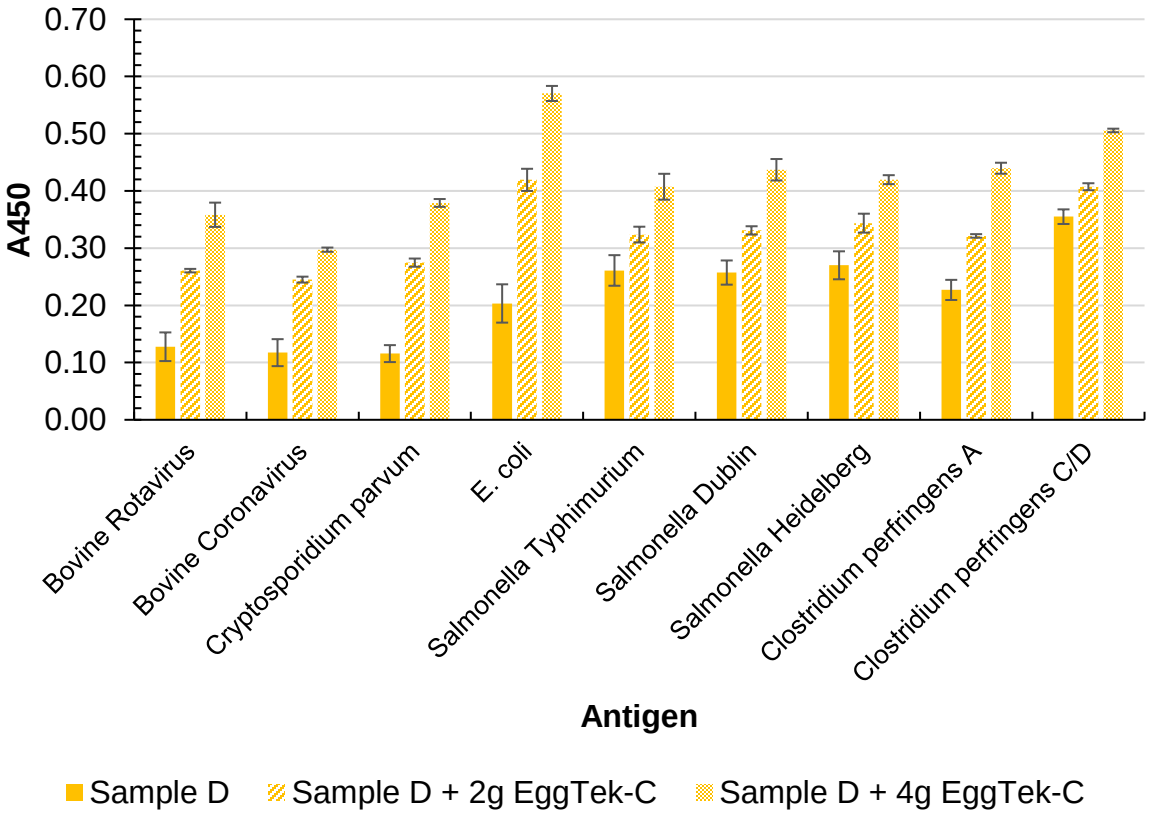

Figure 5. A450 values of Sample D with EggTek-C addition. The average A450 value against each antigen is shown along with the standard deviation. Antigens are listed along the x-axis. A450 values are listed in Supplemental Table 5.

Like the other CMRs, addition of EggTek-C to Sample E improved all A450 values. However, for *C. perfringens* antigens (both Type A and Type C/D), this increase was not statistically significant, as it was for all other antigens. Sample E already had high A450 values for both these antigens, so it is unsurprising EggTek-C did not significantly improve these titers.

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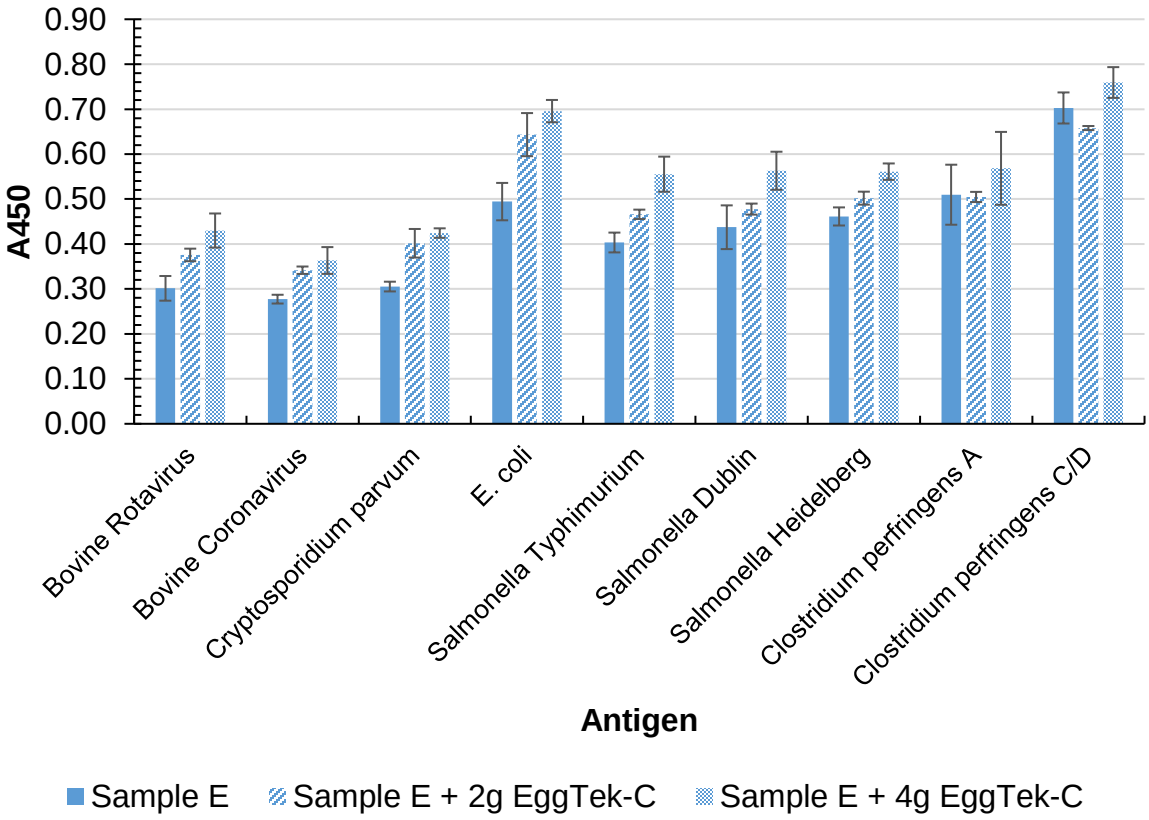

Figure 6. A450 values of Sample E with EggTek-C addition. The average A450 value against each antigen is shown along with the standard deviation. Antigens are listed along the x-axis. A450 values are listed in Supplemental Table 6.

Conclusions

The inclusion of EggTek-C in any of the CMR samples improved the A450 values for all antigens tested, and for most CMRs and antigens, this increase was statistically significant. The higher A450 values with EggTek-C imply a greater amount of binding to the antigen that should result in a healthier calf, and indeed, positive benefits are seen in the field for calves receiving 2 grams and 4 grams of EggTek-C in a feeding (corresponding to a daily dose of 4 grams and 8 grams, respectively).

In a bull calf study, conducted at Mapleview Agri Ltd. (Ontario, Canada), calves fed EggTek-C had lower mortalities in a dose-dependent manner. For the Control calves, the mortality rate was 7.5% compared to the 6.7% and 4.7% rates for 4 grams or 8 grams of EggTek-C, respectively. The decrease in mortality was more significant for calves with Failed Transfer of Passive Immunity (FTPI), defined as a total serum protein < 5.2 g/dL. These calves would not

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have received sufficient colostrum, and thus can be more prone to disease pressures. When only looking at FTPI calves (~40 calves in each treatment group), the Control mortality rate was 12.5% compared to 7.9% (p-value 0.503) and 2.4% (p-value 0.079) for the 4-gram and 8-gram dosages, respectively. These results strongly imply the higher A450 values seen in the ELISA assays with EggTek-C translates into a lower mortality rate for bull calves fed EggTek-C.

In a separate heifer calf study, conducted at a commercial dairy farm in upstate New York, the calves fed EggTek-C had few days of scours, and lower severity of scours, and had fewer intervention treatments (e.g., antibiotics) compared to the Control. Heifer calves (~50 in each group) fed either 4 grams or 8 grams of EggTek-C had on average 1.28 days (p-value 0.429) or 1.07 days (p-value 0.089) of a scours score >1 (i.e., loose or watery) compared to 1.51 days for the Control. They also had less severe scours (defined as the sum of all scour scores). The scours severity score for the Control was 6.20, while the 4-gram dosage was 5.43 (p-value 0.310) and the 8-gram dosage was 5.04 (p-value 0.088). Finally, the number of Control calves needing two or more intervention treatments was 22 compared to 12 (p-value 0.039) or 14 (p-value 0.107) for the 4-gram and 8-gram dosages, respectively. As with the bull calf study, these findings demonstrate EggTek-C can help reduce enteric disease pressure and keep calves from needing additional medical care.

In conclusion, the inclusion of either 2 grams or 4 grams of EggTek-C into a CMR leads to higher A450 values against calf disease antigens, and this can result in calves with fewer enteric health issues and lower mortality.

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MATERIALS & METHODS

Extraction and purification of IgG from CMRs. For each CMR, 15 g of CMR was reconstituted in 200 mL of warm water (43-44°C) under agitation. Once reconstituted, the pH was lowered to 4.6 with 5N HCl, incubated at 37°C for 20 minutes, and then centrifuged at 4,000 x *g* for 30 minutes at 25°C. The supernatant was retained, and the volume measured. An equal volume of cold saturated ammonium sulfate was added to the supernatant in a dropwise manner with agitation at room temperature. After the full volume of ammonium sulfate was added, the solution was stirred for an additional hour at room temperature. The solution was then centrifuged at 4,000 x *g* for 30 minutes at 4°C, and the supernatant discarded. The pellet was resuspended in 2-5 mL of PBS. The resuspended solution was next dialyzed against PBS for two rounds. The solution was further purified with the Protein A IgG Purification Kit (Thermo Scientific Rockford, IL), following the manufacturer's instructions. The elutes of IgG from each sample were finally combined and dialyzed against PBS for two rounds. The purified solution was then concentrated using a Pierce™ Concentrator, PES, 10K MWCO (Thermo Scientific Rockford, IL). The purified IgG concentrate was quantified using a Bovine IgG ELISA Kit (ICL, Inc. Portland, OR), following the manufacturer's instructions.

Extraction and purification of IgY from EggTek-C. Retains from EggTek-C production Lot 1202 were extracted and used for this analysis. The whole egg powder was first resuspended in 0.2% acetic acid at 10% (i.e., 4 g of powder into 36 g of 0.2% acetic acid), mixed with end-over-end rotation for 1 hour at room temperature, and then incubation at 4°C overnight. The next morning, the sample was centrifuged at >8,000 x *g* for 30 minutes at 4°C. The supernatant was retained, and the volume measured. An equal volume of cold saturated ammonium sulfate was added to the supernatant in a dropwise manner with agitation at room temperature. After the full volume of ammonium sulfate was added, the solution was stirred for an additional 2 minutes at room temperature. The solution was then incubated at 4°C overnight. The following morning, the solution was centrifuged at 4,000 x *g* for 15 minutes at 4°C, and the pellet was resuspended in 2-5 mL of PBS. The resuspended solution was next dialyzed against PBS for two rounds. The purified solution was then concentrated using a Pierce™ Concentrator, PES, 10K MWCO (Thermo Scientific Rockford, IL). The purified IgY concentrate was quantified using Arkion's customer total IgY ELISA protocol.

Labeling of Antibodies with Peroxidase. The purified IgG and IgY were then labeled with a horseradish peroxidase using the HRP Conjugation Kit – Lightning-Link® (abcam Waltham, MA), following the manufacturer's instructions. For each sample, 120 µg of IgG or IgY was labeled.

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Direct ELISAs. Custom direct ELISAs were performed for each antigen using the labeled antibody. Specific protocols can be requested from Arkion Life Sciences, if needed. The general outline of each ELISA was the following. Antigen was diluted in 0.1 M carbonate buffer, pH 9.6, applied to a 96-well polystyrene plate, and incubated overnight at 4°C. The next morning, the plate was washed 6 times with PBS with 0.05% Tween20, and then blocked with 3% BSA in PBS with 0.05% Tween20 at 37°C for 30 minutes. While blocking, the antibodies were mixed and diluted in the same buffer (3% BSA in PBS with 0.05% Tween20). After blocking, the plate was washed 6 times again, and then the antibody solutions were applied to the plate. Plates were incubated at 37°C for 30 or 60 minutes, depending on the antigen, and then washed 6 times again. TMB was then added to the plate and incubated at room temperature for 30 minutes in the dark. Finally, the reaction was stopped with 0.3 M sulfuric acid, and the A450 values were measured.

To determine the amount of antibody to test for the CMRs, first the total IgG concentration of the CMR was multiplied by the CMR dose (i.e., 10 oz). Next, this amount was scaled down by a factor of 10^7 for each CMR. The amount of IgY for EggTek-C was determined in a similar way (i.e., the total IgY concentration was multiplied by 2 grams or 4 grams and scaled down by a factor of 10^7).

Statistical analysis. A450 values were first corrected by subtracting the background A450 (the average A450 of the triplicate blank wells on each plate) and then averaged together. For statistical comparison, unpaired, two-tailed t-tests were performed.

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SUPPLEMENTAL MATERIAL

Supplemental Table 1. A450 values for CMR comparison. Average, corrected A450 values with their standard deviation are shown. The letters next to the A450 values indicate statistically similar groups.

	CMR Samples				
Antigen	**Sample A**	**Sample B**	**Sample C**	**Sample D**	**Sample E**
Bovine Rotavirus	0.136 ± 0.053 A	0.009 ± 0.002 B	0.031 ± 0.009 C	0.128 ± 0.025 A	0.301 ± 0.027 D
Bovine Coronavirus	0.075 ± 0.005 A	0.004 ± 0.002 B	0.024 ± 0.010 C	0.117 ± 0.024 D	0.277 ± 0.010 E
Cryptosporidium parvum	0.084 ± 0.004 A	0.006 ± 0.007 B	0.022 ± 0.002 C	0.116 ± 0.015 D	0.305 ± 0.011 E
E. coli	0.131 ± 0.004 A	0.012 ± 0.007 B	0.044 ± 0.012 C	0.203 ± 0.033 D	0.494 ± 0.042 E
Salmonella Typhimurium	0.266 ± 0.002 A	0.016 ± 0.005 B	0.058 ± 0.004 C	0.261 ± 0.027 A	0.403 ± 0.022 D
Salmonella Dublin	0.270 ± 0.009 A	0.018 ± 0.004 B	0.049 ± 0.003 C	0.257 ± 0.021 A	0.437 ± 0.049 D
Salmonella Heidelberg	0.288 ± 0.011 A	0.026 ± 0.003 B	0.065 ± 0.011 C	0.270 ± 0.024 A	0.461 ± 0.020 D
Clostridium perfringens Type A	0.204 ± 0.015 A	0.025 ± 0.015 B	0.067 ± 0.022 B	0.227 ± 0.018 A	0.510 ± 0.067 C
Clostridium perfringens Type C/D	0.345 ± 0.012 A	0.026 ± 0.011 B	0.054 ± 0.010 C	0.355 ± 0.013 A	0.703 ± 0.035 D

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Supplemental Table 2. A450 values for EggTek-C addition to CMR Sample A. Average, corrected A450 values with their standard deviation are shown. The letters next to the A450 values indicate statistically similar groups.

Antigen	CMR Samples		
	Sample A	Sample A + 2g EggTek-C	Sample A + 4g EggTek-C
Bovine Rotavirus	0.136 ± 0.053 A	0.321 ± 0.031 B	0.360 ± 0.013 B
Bovine Coronavirus	0.075 ± 0.005 A	0.231 ± 0.009 B	0.281 ± 0.006 C
Cryptosporidium parvum	0.084 ± 0.004 A	0.354 ± 0.036 B	0.388 ± 0.005 B
E. coli	0.131 ± 0.004 A	0.401 ± 0.036 B	0.457 ± 0.004 B
Salmonella Typhimurium	0.266 ± 0.002 A	0.373 ± 0.047 B	0.353 ± 0.004 B
Salmonella Dublin	0.270 ± 0.009 A	0.446 ± 0.046 B	0.425 ± 0.009 B
Salmonella Heidelberg	0.288 ± 0.011 A	0.437 ± 0.019 B	0.394 ± 0.017 C
Clostridium perfringens Type A	0.204 ± 0.015 A	0.428 ± 0.076 A	0.449 ± 0.010 B
Clostridium perfringens Type C/D	0.345 ± 0.012 A	0.411 ± 0.025 B	0.345 ± 0.003 A

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Supplemental Table 3. A450 values for EggTek-C addition to CMR Sample B. Average, corrected A450 values with their standard deviation are shown. The letters next to the A450 values indicate statistically similar groups.

Antigen	CMR Samples		
	Sample B	Sample B + 2g EggTek-C	Sample B + 4g EggTek-C
Bovine Rotavirus	0.009 ± 0.002 A	0.171 ± 0.025 B	0.237 ± 0.010 C
Bovine Coronavirus	0.004 ± 0.002 A	0.127 ± 0.002 B	0.197 ± 0.010 C
Cryptosporidium parvum	0.006 ± 0.007 A	0.155 ± 0.009 B	0.262 ± 0.004 C
E. coli	0.012 ± 0.007 A	0.212 ± 0.013 B	0.343 ± 0.007 C
Salmonella Typhimurium	0.016 ± 0.005 A	0.140 ± 0.025 B	0.187 ± 0.008 C
Salmonella Dublin	0.018 ± 0.004 A	0.163 ± 0.025 B	0.223 ± 0.014 C
Salmonella Heidelberg	0.026 ± 0.003 A	0.176 ± 0.019 B	0.210 ± 0.008 C
Clostridium perfringens Type A	0.025 ± 0.015 A	0.164 ± 0.014 B	0.252 ± 0.004 C
Clostridium perfringens Type C/D	0.026 ± 0.011 A	0.146 ± 0.011 B	0.204 ± 0.006 C

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Supplemental Table 4. A450 values for EggTek-C addition to CMR Sample C. Average, corrected A450 values with their standard deviation are shown. The letters next to the A450 values indicate statistically similar groups.

Antigen	CMR Samples		
	Sample C	Sample C + 2g EggTek-C	Sample C + 4g EggTek-C
Bovine Rotavirus	0.031 ± 0.009 A	0.265 ± 0.014 B	0.274 ± 0.007 B
Bovine Coronavirus	0.024 ± 0.010 A	0.218 ± 0.006 B	0.195 ± 0.006 C
Cryptosporidium parvum	0.022 ± 0.002 A	0.271 ± 0.014 B	0.243 ± 0.028 B
E. coli	0.044 ± 0.012 A	0.336 ± 0.002 B	0.312 ± 0.012 C
Salmonella Typhimurium	0.058 ± 0.004 A	0.208 ± 0.006 B	0.210 ± 0.004 B
Salmonella Dublin	0.049 ± 0.003 A	0.222 ± 0.006 B	0.230 ± 0.012 B
Salmonella Heidelberg	0.065 ± 0.011 A	0.247 ± 0.026 B	0.222 ± 0.002 B
Clostridium perfringens Type A	0.067 ± 0.022 A	0.251 ± 0.017 B	0.254 ± 0.018 B
Clostridium perfringens Type C/D	0.054 ± 0.010 A	0.204 ± 0.003 B	0.204 ± 0.004 B

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Supplemental Table 5. A450 values for EggTek-C addition to CMR Sample D. Average, corrected A450 values with their standard deviation are shown. The letters next to the A450 values indicate statistically similar groups.

Antigen	CMR Samples		
	Sample D	Sample D + 2g EggTek-C	Sample D + 4g EggTek-C
Bovine Rotavirus	0.128 ± 0.025 A	0.261 ± 0.003 B	0.358 ± 0.021 C
Bovine Coronavirus	0.117 ± 0.024 A	0.245 ± 0.005 B	0.297 ± 0.004 C
Cryptosporidium parvum	0.116 ± 0.015 A	0.275 ± 0.007 B	0.379 ± 0.007 C
E. coli	0.203 ± 0.033 A	0.419 ± 0.019 B	0.570 ± 0.013 C
Salmonella Typhimurium	0.261 ± 0.027 A	0.324 ± 0.014 B	0.407 ± 0.023 C
Salmonella Dublin	0.257 ± 0.021 A	0.331 ± 0.007 B	0.437 ± 0.019 C
Salmonella Heidelberg	0.270 ± 0.024 A	0.344 ± 0.017 B	0.420 ± 0.008 C
Clostridium perfringens Type A	0.227 ± 0.018 A	0.321 ± 0.003 B	0.440 ± 0.010 C
Clostridium perfringens Type C/D	0.355 ± 0.013 A	0.407 ± 0.006 B	0.506 ± 0.003 C

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Supplemental Table 6. A450 values for EggTek-C addition to CMR Sample E. Average, corrected A450 values with their standard deviation are shown. The letters next to the A450 values indicate statistically similar groups.

Antigen	CMR Samples		
	Sample E	Sample E + 2g EggTek-C	Sample E + 4g EggTek-C
Bovine Rotavirus	0.301 ± 0.027 A	0.376 ± 0.014 B	0.430 ± 0.038 B
Bovine Coronavirus	0.277 ± 0.010 A	0.342 ± 0.008 B	0.363 ± 0.030 B
Cryptosporidium parvum	0.305 ± 0.011 A	0.402 ± 0.032 B	0.424 ± 0.011 B
E. coli	0.494 ± 0.042 A	0.643 ± 0.048 B	0.696 ± 0.025 B
Salmonella Typhimurium	0.403 ± 0.022 A	0.466 ± 0.010 B	0.555 ± 0.039 C
Salmonella Dublin	0.437 ± 0.049 A	0.478 ± 0.012 A	0.563 ± 0.042 B
Salmonella Heidelberg	0.461 ± 0.020 A	0.502 ± 0.014 B	0.561 ± 0.018 C
Clostridium perfringens Type A	0.510 ± 0.067 A	0.505 ± 0.011 A	0.568 ± 0.081 A
Clostridium perfringens Type C/D	0.703 ± 0.035 A	0.658 ± 0.005 A	0.759 ± 0.034 A