



The in vitro effect of lactose on *Clostridium perfringens* alpha toxin production and the implications of lactose consumption for in vivo anti-alpha toxin antibody production

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ABSTRACT

Necro-hemorrhagic enteritis in calves, caused by *Clostridium perfringens* type A, is a fatal disease, mostly affecting calves in intensive rearing systems. The lack of development of active immunity against α toxin, an essential virulence factor in the pathogenesis, has been proposed as a main trigger. In this experimental study, the effect of a set of milk replacer components on α toxin production, and the effect of lactose on in vivo antibody production, were investigated. For the latter, Holstein-Friesian bull calves ($n = 18$) were fed an all liquid diet that contained either a milk replacer with high-lactose content (45% DM) or the same milk replacer that was lactase treated, resulting in a lactose-free equivalent. Antibody levels against α toxin were monitored from 2 to 12 wk of age. In the in vitro part of the study, a concentration-dependent inhibitory effect of lactose on in vitro *C. perfringens* α toxin activity was observed, whereas protein did not influence α toxin activity. The in vivo experiment then showed from the age of 10 wk onwards, that anti- α toxin antibody levels of high-lactose animals declined, whereas antibody levels of the animals consuming lactose-free milk replacer remained the same throughout the trial. This points to a natural decline in maternal immunity of lactose-consuming animals, that is not compensated by the development of an active immunity, resulting in inferior protection. This study suggests that dietary lactose reduces *C. perfringens* α toxin production in vivo, which may lead to a decreased antigen presenta-

tion and thus lower serum antibody levels against the toxin. Consequently, any event causing massive α toxin production puts lactose-consuming calves at higher risk of developing necro-hemorrhagic enteritis.

Key words: necro-hemorrhagic enteritis, alpha toxin, *Clostridium perfringens*, lactose, milk

INTRODUCTION

Necro-hemorrhagic enteritis (NHE) in calves (previously named enterotoxaemia) is caused by *Clostridium perfringens* type A, an anaerobic, gram-positive, spore-forming bacterium that is ubiquitously present in the environment. It is a commensal in the calf's intestine but has, under certain circumstances, pathogenic potential (Lebrun et al., 2010). Necro-hemorrhagic enteritis is characterized by sudden death with necrotic lesions and hemorrhages in the small intestine (Manteca et al., 2000). It affects different cattle breeds worldwide, but the disease is predominantly seen in suckler cow systems, and intensive rearing systems such as beef and dairy veal production (Manteca et al., 2000; Pardon et al., 2012; Goossens et al., 2017). Most cases occur during the late stage of the production period, between 4 and 8 mo of age (Valgaeren et al., 2013), resulting in high financial losses. As pre-mortal clinical signs are usually not observed, individual therapeutic interventions are not possible. Therefore, prevention is of crucial importance to lower disease incidence.

The pathogenesis of bovine NHE is not fully unraveled yet, but it is clear that *C. perfringens* α toxin plays an important role. Alpha toxin is cytotoxic for bovine endothelial cells (Verherstraeten et al., 2013; Goossens et al., 2016a,b). When it is injected in bovine intestinal loops, it causes similar lesions as the ones observed in clinical cases of NHE: epithelial cell detachment, villus tip blunting, erosion, and influx of neutrophils in the lamina propria (Morris et al., 2012). Also, it has been

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Table 1. Supplement concentrations of skim milk powder and its components (lactose, casein, and whey protein isolate) in TGY (3% tryptone, 2% yeast extract, 0.1% glucose, 0.1% L-cysteine) medium used for *Clostridium perfringens* culture¹

Item, %	In TGY medium			
	High	Intermediate	Low	Control ²
Skim milk powder	10.00	1.00	0.10	0.00
Lactose	5.00	0.50	0.05	0.00
Casein	2.80	0.28	0.03	0.00
Whey protein	0.70	0.07	0.01	0.00

¹Skim milk powder largely consists of 50% lactose, 28% casein, and 7% whey protein, on a DM basis. The lactose, casein, and whey protein concentrations correspond to their relative presence in skim milk powder.

²Unsupplemented TGY medium was used as a negative control.

shown that *C. perfringens* mutants lacking the ability to produce α toxin are barely able to cause lesions in an intestinal loop model (Verherstraeten et al., 2013). All these data thus point out that α toxin is an essential virulence factor in the pathogenesis of NHE.

The clinical observation that suckling calves and calves raised for veal production are more susceptible to NHE than conventionally reared beef calves of the same breed (Pardon et al., 2012), could be linked to α toxin as well (Niilo et al., 1974; Muylaert et al., 2010; Goossens et al., 2017). Indeed, in a study on the evolution of anti- α toxin antibodies in healthy calves of different production systems, a significant decline of anti- α toxin antibody concentration over time was demonstrated in white veal calves, whereas the titers remained unchanged for calves reared for beef (Valgaeren et al., 2015). This indicates a smooth transition from passive to active immunity in calves reared for beef. The observed antibody decline in veal calves results in an inadequate immunity, which probably explains the difference in susceptibility between both types of calves.

One of the most important differences between both types of calf production systems is the weaning management, which is typically 3–4 mo of age in beef suckler calves (Fiems et al., 2008), and 6–8 wk in dairy heifers (Eckert et al., 2015; Lynch et al., 2019). In contrast, white veal calves are not weaned at all and remain on an intensive milk diet throughout their production period, while they receive limited amounts of solid feed.

As veal calves are fed milk in larger quantities, and for a longer period of time, we hypothesized that some milk component(s) may inhibit the development of active immunity in these animals, while maternal antibody titers are gradually decreasing, because contact with α toxin should be necessary for active antibody production. Previously, we found an inhibitory effect of a commercial milk replacer on α toxin production in vitro (supplement 1; Valgaeren et al., 2015). Hitherto it is unknown which component of whole milk might inhibit the *C. perfringens* α toxin formation or presentation to the host immune system.

The main objective of the current study was to determine which element of milk replacer has an inhibitory effect on *C. perfringens* α toxin production in vitro. Thereafter, as a proof of concept, we studied the effect of the identified factor, namely lactose, on anti- α toxin antibody production in an in vivo experiment, by feeding calves either a high-lactose (**HL**) diet or a lactose-free (**LF**) equivalent.

MATERIALS AND METHODS

In Vitro Studies

Bacterial Strains and Growth Conditions. The *C. perfringens* type A strain used in this study (BCP20) was a clonal isolate from abomasal and intestinal ulcers of a calf that was diagnosed with bovine NHE (Van Immerseel et al., 2010). The strain was grown overnight in 10 mL of TGY broth (3% tryptone, 2% yeast extract, 0.1% glucose, 0.1% L-cysteine) under anaerobic conditions at 40°C. To determine the effect of different milk ingredients on the *C. perfringens* growth and α toxin production, TGY medium was supplemented with either skim milk powder (1:10, wt/vol; 1:100; 1:1000), or the skim milk powder ingredients: lactose, casein, and whey protein isolate at concentrations corresponding to their relative presence in skim milk powder on a DM basis (Table 1). Two levels of 10-fold dilutions of these concentrations were also applied, to finally gain a high, intermediate, and low level for each supplement (Table 1). For all conditions, there were at least 3 biological replicates. We also compared the ingredients at equal concentrations (5% each) in an additional experiment. Furthermore, the saccharide component (lactose and its enzymatic breakdown products glucose and galactose) was tested similarly, at concentrations as shown in Table 2. Supplemented media were inoculated with *C. perfringens* type A to verify the effect on clostridial growth and on α toxin production.

A *C. perfringens* BCP20 overnight culture was diluted (1:10,000) in each of the supplemented TGY broths,

Table 2. Concentrations of saccharides (lactose, glucose, galactose) that were supplemented to TGY (3% tryptone, 2% yeast extract, 0.1% glucose, 0.1% L-cysteine) medium¹

Item	Supplement concentration	
	mM	g/L
Lactose	146.07	50.00
Glucose	292.14	52.64
Galactose	292.14	52.64
Glucose and galactose	146.07	26.32
Control	0	0

¹Lactose was supplemented at 5% (wt/vol). Glucose, galactose, and a combination of the two, were added at equimolar quantities. Unsupplemented TGY medium was used as negative control.

followed by incubation for 24 h (anaerobically, 40°C). During this incubation, clostridial growth was determined at 0, 1, 2, 3, 4.5, 6.5, 8, and 24 h, by standard plate count of colony-forming units (cfu) on Columbia agar plates (Oxoid) supplemented with 5% defibrinated sheep blood. The plates were incubated overnight (anaerobically, 40°C), followed by a colony count. None of the test supplements affected clostridial growth. The exponential growth phase was reached after 4 h of incubation. After 5 h of growth, 2-mL aliquots were collected, and centrifuged (8,000 × g; 10 min at 4°C) to obtain cell-free supernatants. The supernatants were filtrated through a 0.22-μm filter, and stored at -20°C until further use.

Alpha Toxin Activity Assay. An egg yolk diffusion assay was performed to determine α toxin activity in the supernatants as previously described (Rigby, 1981). Briefly, Columbia agar was supplemented with 2% (vol/vol) egg yolk and round holes (∅ 7 mm) were stabbed in the agar plates, using the back of 200-μL pipette tips. Twenty microliters of the collected supernatants were added to the holes. A dilution series (1.0, 0.5, 0.25, 0.125, 0.0625, and 0.0313 U/mL) of phospholipase C (Sigma-Aldrich) was used as a standard. Plates were incubated at 37°C for 24 h and scanned with a GS-800 calibrated densitometer (Bio-Rad). Alpha toxin breaks down the egg yolk lecithin in the plate, thereby creating a dense area around the spotted supernatants. The size of the area is correlated with the α toxin activity.

The zone of opacity around each spot was measured using Quantity One software (Bio-Rad) and quantified using the α toxin standard series. The assay was performed in triplicate.

In Vivo Experiment

Diet: A High-Lactose Milk Replacer Versus a Lactose-Free Equivalent. To determine the effect of lactose on the anti-α toxin antibody development,

Table 3. Nutritional composition of milk replacer¹

Analytical component	%
DM, %	96.9
CP, % of DM	23.5
Crude fat, % of DM	18.0
Crude ash, % of DM	7.0
Crude fiber, % of DM	0.0
Calcium, % of DM	0.9
Lactose, % of DM	45.0
Phosphorus, % of DM	0.7
Sodium, % of DM	0.5
Lysin, % of DM	2.5
Methionine, % of DM	0.6
Threonine, % of DM	1.2

¹Milk replacer was prepared and fed at a concentration of 15% (wt/vol) in tap water.

calves were fed either an HL or LF milk replacer, and no solid feed. High-lactose milk replacer (23.5% CP, 18.0% fat, 45% lactose, 4.81 Mcal/kg of DM; Table 3; Nukamel), was obtained as a dry powder and prepared as instructed by the manufacturer. Gross energy was calculated using the following equation: gross energy = [(9.21 × fat) + (5.86 × CP) + (3.95 × lactose; Drackley, 2008)].

To obtain LF milk replacer without affecting any other components, the HL milk replacer was treated with lactase (EC.3.2.1.108; Disolut). To determine the minimal incubation time and lactase concentration required to break down all lactose in the HL milk replacer, the milk was treated with different lactase concentrations (ranging from 0 to 2,200 U/L) at different incubation times (ranging from 0 to 90 min) at 42°C. The residual lactose concentration present in the milk after lactase treatment was determined using a lactose assay kit (MAK017, Sigma-Aldrich). In this assay, lactose concentration is determined by an enzymatic assay, in which lactose is converted to galactose and glucose. The galactose is subsequently oxidized, resulting in a colorimetric (570 nm)/fluorometric (λ_{ex} = 535 nm/λ_{em} = 587 nm) product, proportional to the lactose present. The colorimetric product was determined every 5 min, to follow the rate of the enzymatic reaction, and verify when all lactose was hydrolyzed. This showed that a lactase concentration of 110 U/L was able to degrade all lactose within 15 min of incubation, resulting in a LF milk. To avoid the presence of any residual lactose in the LF milk during the in vivo trial, milk was treated with a 2-fold concentration of lactase (220 U/L) for at least 20 min at 42°C.

Feeding Regime and Housing Conditions.

The animal experiment was approved by the Ethical Committee of the Faculties of Veterinary Medicine and Bioscience Engineering of Ghent University (EC

2020–001). The study was conducted between March 2020 and June 2020.

For an estimation of the effect size on antibody levels, results of Valgaeren et al. (2015) were used in the `d.ind.t.t`-function of R-package MOTE. Sample size was then calculated using the `pwr`-package in R studio, which resulted in 8 animals per group for a power of 0.9. To anticipate on possible unforeseen problems, we opted for one extra animal per group. Eighteen healthy Holstein-Friesian bull calves (age 16.6 ± 3.3 d; BW 51.2 ± 4.6 kg) were transported from a single dairy farm to stables of the Veterinary Research Building at the Faculty of Veterinary Medicine, Ghent University. Using Microsoft Excel RAND function, they were randomly assigned to 2 test groups (HL, LF) by colored ear tags. Averages on BW, age, or anti- α toxin antibody levels did not differ between groups ($P = 0.89$, $P = 0.36$, and $P = 0.61$, respectively). The study was not blinded, as the researchers ought to know which diet to provide to which animals. Serum and fecal samples were given a serial number, but for ELISA and quantitative PCR (qPCR), samples of both groups were always placed on the same microtiter plates to avoid plate effects.

Animals from both groups were equally divided over 3 stables of 21.4, 21.4, and 20.4 m². During the first 3 wk after arrival, animals were kept individually, thereafter grouped per 3. The concrete floors were bedded with rubber mats, as straw could potentially be consumed, which might increase the uptake of *C. perfringens* spores. The stables were cleaned on a bidaily basis.

Animals were fed 3 times a day at 0900, 1500, and 2000 h with either the HL or LF milk replacer, following a feeding regimen in which the milk replacer volume increased during the first month. For the first 5 d, amoxicillin (70% Kela) was added twice a day to the milk (500 mg per animal), following the advice of a veterinarian specialized in veal calves. We did this (1) to avoid inter-individual variation in intestinal *C. perfringens* colonization, and (2), to emulate the preventative treatment that was commonly used in veal and dairy beef rearing. As we wanted to study the mere effect of lactose, we aimed to keep all other factors as similar as possible to what is done commercially.

The feeding amount started at 4 L per day in the first week, which then every week increased with 2 L, to reach the maximum amount of 10 L per day, from the fourth week until the end of the experiment. The daily amount was equally divided over the 3 feeding moments, to allow a longer lasting effect of lactose. Milk was prepared using a milk taxi (Holm & Laue) by dissolving the milk powder in one-third of the final amount of water (55°C), followed by 10 min of continuous mixing. Thereafter, the remaining two-thirds of water (cold) were added, again followed by 10 min

of continuous mixing. Prepared milk replacer (150 g/L) was then provided to animals of the HL group, and the remaining amount of milk in the milk taxi was lactase treated in the meantime, to obtain the LF milk replacer (220 U/L lactase, 20 min, 40–42°C, with constant mixing), after which the calves in the LF group were fed. All animals were individually fed to ensure that they all consumed the same amount of milk. The milk was offered at 40°C. Until the age of 4 wk, animals consumed only the milk replacer. Thereafter they also had ad libitum access to clean drinking water.

Serum samples and rectally collected fecal samples were taken every 2 wk, up until 12 wk after arrival, and frozen (–20°C) until further processing.

Alpha Toxin Antibody Production. *Clostridium perfringens* α toxin antibodies were quantified in the serum samples using a blocking ELISA test (K291, Bio-X Diagnostics). The test was performed according to the manufacturer's instructions. Technical replicates were performed on separate microtiter plates to avoid plate effects.

Quantitative PCR to Determine Total Bacterial Load and *C. perfringens* Load in Feces. To quantify the abundance of *C. perfringens* in the fecal samples throughout the study, DNA was extracted from 100 mg feces using the CTAB method as described (Griffiths et al., 2000), with modifications (Aguirre et al., 2019). The DNA concentration was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) and diluted to 50 ng/ μ L. Total bacteria and *C. perfringens* were quantified by performing qPCR, targeting the 16S rRNA gene and *cpa* gene, respectively. To determine the total number of bacteria, primers Uni_F (5'-ACTCCTACGGGAGGCAGCAG-3') and Uni_Rev (5'-TTACCGCGGCTGCTGG-3') were used (Mountzouris et al., 2015). The *C. perfringens* load was determined by quantifying the *cpa* gene copies using primers AlphaFW (5'-GTTGATAGCGCAGGACATGTTAAG-3') and AlphaRev (5'-CATGTAGTCATCTGTTCCAGCATC-3').

Quantitative PCR was performed using SYBR-green 2x master mix (Bioline) in a Bio-Rad CFX-96 system. For the reaction a 12- μ L total reaction mixture was used, consisting of 2 μ L of the DNA sample and 0.5 μ M final qPCR primer concentration. Each reaction was performed in triplicate. The qPCR conditions used were similar for both genes, except for the final step: one cycle at 95°C for 10 min, 40 cycles of 95 ° for 30 s, then 60°C for 1 min, and finally (for the 16s rRNA gene) a stepwise temperature increase (60 to 95°C) at 5s per 0.5°C. For the *cpa* gene, the final stepwise temperature increase was 65 to 95°C at 10s per 0.5°C.

For construction of the standard curve, the PCR product was generated using the following PCR primers:

'5-CTGGCGGCGTGCTTAACACATG-3' (forward) and '5-GCCCAGTAAATCCGGATAAC-3' (reverse) for total bacteria, and '5-AGTCTACGCTTGGGATTGAA-3' (forward) and '5-TTTCCTGGGTTGTTCAATTC-3' (reverse) for *C. perfringens* α toxin. The fragment was purified and the concentration of the linear dsDNA standard was adjusted to 10-fold dilutions from 1×10^7 to 1×10^1 copies per μL . The copy numbers of samples were determined by reading off the standard series with the Ct values of the samples.

Statistical Analysis

All analyses and figures were performed and made using GraphPad Prism 9. For the data of the in vitro experiments, a one-way ANOVA was applied for each ingredient separately, to compare α toxin activity levels after growth of *C. perfringens* in growth medium supplemented with different concentrations of the same ingredient.

For the in vivo trial, antibody titers against α toxin were compared between HL and LF animals throughout time. To do so, a mixed model with repeated measures was performed to examine the effect of both age and lactose, and possible interactions between group and sampling moment were included in the model. Post hoc analyses were performed using a Bonferroni test. Linear trends throughout time were also explored for both groups.

RESULTS

In Vitro Studies

Effect of Skim Milk Powder and Its Components on α Toxin Activity. *Clostridium perfringens* was incubated in growth medium that was supplemented either with skim milk powder, or with one of the major individual components: lactose, casein, or whey protein. None of the supplements affected bacterial growth. To investigate the effect of the supplements (Tables 1 and 2) on α toxin activity, supernatants of cultures that were incubated for 5 h in TGY containing different concentrations of the supplements, were tested in an egg yolk assay. The values (mean \pm SD) are expressed as fraction of α toxin activity relative to the control (un-supplemented TGY medium; Figure 1A). Ten percent of skim milk powder (high level) caused a significant drop in α toxin activity (0.40 ± 0.19), compared with each other level and to the control ($P < 0.0001$ for all differences). The high level of lactose (0.42 ± 0.17) differed from the low level of lactose (0.77 ± 0.30 , $P = 0.0008$) and the control ($P < 0.0001$). The intermediate level of lactose (0.59 ± 0.23) differed from

the control as well ($P = 0.0001$). At the lowest tested concentrations, none of the supplements affected the α toxin activity. Differences in α toxin activity between the different concentrations (high versus intermediate or low) within skim milk powder and lactose-supplemented medium, indicate a concentration-dependent effect. No differences were observed for any of the levels of casein and whey protein isolate. This strongly suggests that lactose is the component of skim milk powder which causes the observed effects on *C. perfringens* α toxin production.

Because casein and whey protein are present in low concentrations in skim milk powder, the effect of these components on *C. perfringens* α toxin production described above was tested at relatively low concentrations (TGY supplemented with 2.8% casein or 0.7% whey protein for the high level). To verify whether this affected the outcome of the experiment, high concentrations (5% wt/vol) of both supplements were tested as well. We observed no significant differences compared with the lower concentrations of these supplements (data not shown). Therefore, we conclude that our observations were not the result of too low test concentrations of casein and whey protein.

Effect of Saccharides on α Toxin Activity. The previous results pointed out that lactose is the predominant component responsible for concentration-dependent α toxin inhibition. In vivo, lactose is hydrolyzed in the small intestine, releasing the monosaccharides glucose and galactose. Therefore, we examined the effect of lactose as well as the monosaccharides, both individually and combined together, in equimolar concentrations as compared with lactose (Table 2). This way, we were able to compare the effect of lactose with (1) a 1:1 glucose-galactose mix, (2) glucose, and (3) galactose. All tested saccharides reduced α toxin activity, compared with the control ($P < 0.001$; Figure 1B). We observed no differences in α toxin reduction between glucose and galactose supplementation. The inhibition caused by the combination of glucose and galactose was significantly stronger than the effect of lactose alone (0.29 ± 0.08 and 0.57 ± 0.09 respectively, $P = 0.0273$). To summarize, the results confirm that both monosaccharides are interchangeable regarding the effect on α toxin production, but also that when combined, the mix of both has a larger inhibitory effect than lactose.

In Vivo Experiment

Effect of Lactose on Antibody Production Against Alpha Toxin. Considering that lactose inhibits *C. perfringens* α toxin production in vitro, we hypothesized that a diet high in lactose would inhibit

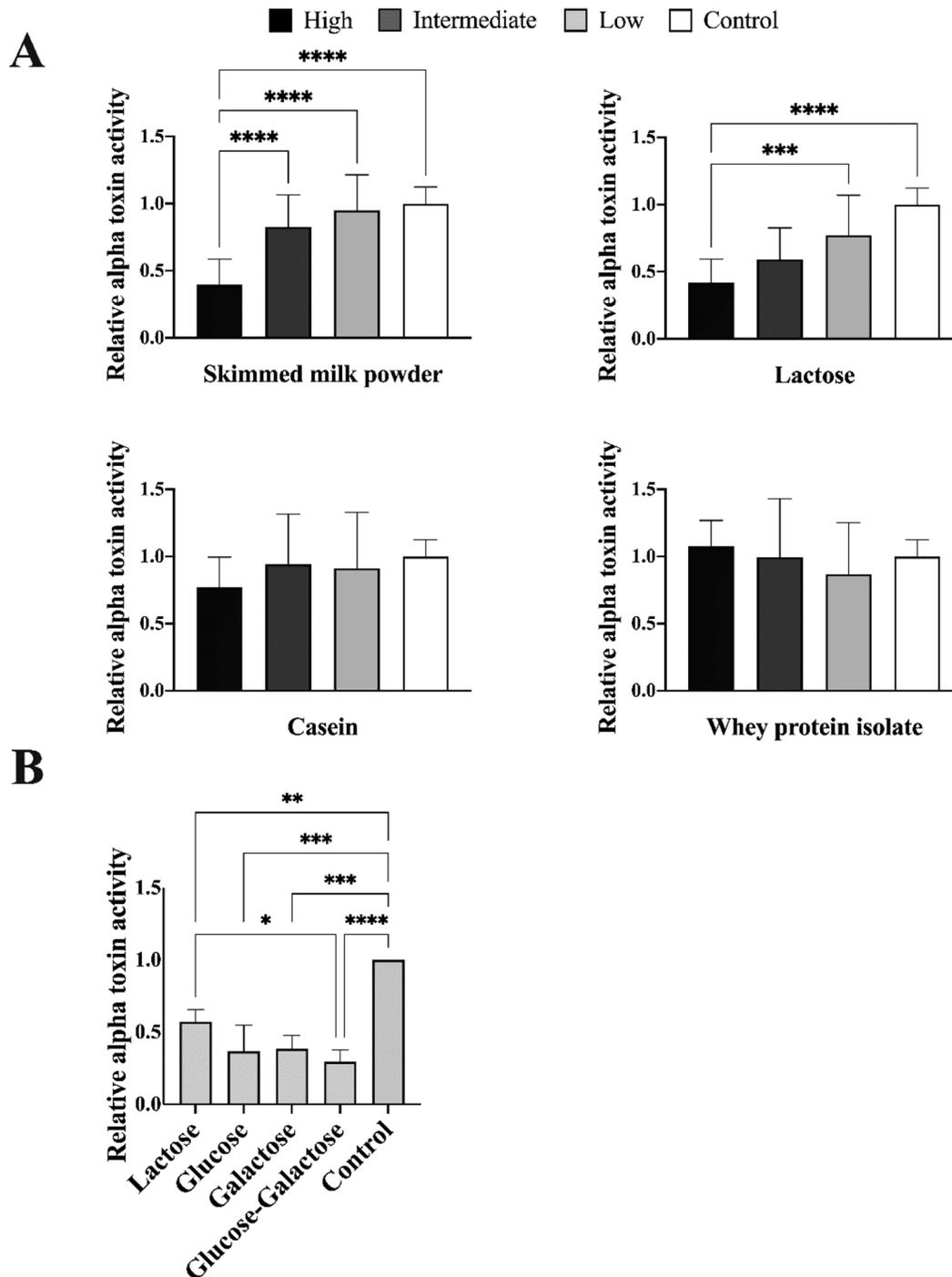


Figure 1. Relative α toxin activity of BCP20 when supplemented with (A) skim milk powder or either of its components lactose, casein, or whey protein isolate at different concentrations: high (approximately as fed), intermediate (10-fold dilution), and low (100-fold dilution), as described in Table 1, or (B) different types of carbohydrates at equimolar concentrations, as described in Table 2. Values are expressed relative to the unsupplemented TGY (3% tryptone, 2% yeast extract, 0.1% glucose, 0.1% L-cysteine) medium. Error bars represent SD. Asterisks indicate levels of significance: $*0.01 \leq P < 0.05$; $**0.001 \leq P < 0.01$; $***0.0001 \leq P < 0.001$; $****P < 0.0001$.

α toxin production in vivo as well, which consequently would interfere with the animal's development of an active immune response. This was investigated in an in

vivo feeding trial, where calves were fed a HL containing milk replacer, or a lactase-treated, LF equivalent. Presence of *C. perfringens* in feces was measured by

qPCR at biweekly intervals. Values were always below detection limit of 10^3 copies/g of feces.

Every 2 wk, the antibody production against α toxin was determined using ELISA (Figure 2). A mixed model analysis with repeated measures revealed that both age and lactose affected the antibody levels ($P < 0.0001$ and $P = 0.0117$, respectively) and that an interaction between both factors ($P = 0.0006$) was observed. A Bonferroni post hoc test showed that there was no difference between both groups from start to 8 wk. At 10 and 12 wk of age, a significant difference between the groups was observed. Whereas antibody levels in the serum of calves fed the LF milk replacer remained high (slope -0.74 ; $P = 0.23$), a significant decline with a slope of -3.25 ($P < 0.0001$) was observed in the calves fed the HL diet. The difference in anti- α toxin levels between the 2 groups was 9.4% ($P = 0.007$) at 10 wk and 15.0% ($P < 0.0004$) at 12 wk (Figure 2).

DISCUSSION

Regarding the protection of calves against *C. perfringens* α toxin, the most important virulence factor in the pathogenesis of bovine NHE, dietary interventions may be part of the solution. In the present study, we proved that lactose consumption negatively affects the antibody production against α toxin. We observed that at the start of the trial, antibody levels were indeed high, with no differences between both groups. Over time, a significant decline was observed from 10 wk of age onwards, but only in the HL animals. It is possible that these decreased levels partly cause an inadequate immunity, making animals less resilient to the activity of α toxin, and consequently, more susceptible to NHE. Antibody levels in the LF animals remained the same throughout the experimental period, which suggests that predigesting dietary lactose allowed these animals a better transition from maternal to active immunity. High antibody titers do not necessarily equal protection, but for clostridial diseases antibodies directed to the toxins were found to be very effective (Goossens et al., 2016b). This is also supported by the previously reported link between calf production system, incidence rates, and reported antibody levels (Valgaeren et al., 2015). Altogether it is suggested that the inhibiting effect of lactose, leads to a decreased antigen presentation, thereby causing an impaired immunity, which would put lactose-consuming calves at higher risk. Those calves are then more likely to develop NHE when any event that then triggers clostridial overgrowth or explosive α toxin production (e.g., poor hygiene, ileus or dysbiosis) occurs.

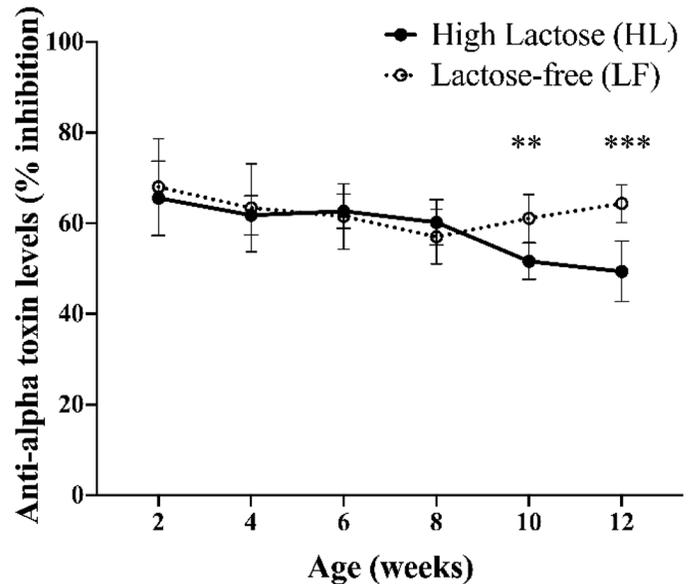


Figure 2. Antibody levels against *Clostridium perfringens* α toxin at different time points. Dots express the average percentage of inhibition, determined using a commercial antibody ELISA kit. Error bars represent the SD. The concentration of serum anti- α -toxin levels decreased in the high-lactose (HL) group, whereas the antibody levels in the lactose-free (LF) group remained at the same level. Consequently, antibody levels were significantly higher in the LF animals at wk 10 and 12. Asterisks indicate levels of significance: ** $0.001 \leq P < 0.01$; *** $0.0001 \leq P < 0.001$.

Aforementioned effect of lactose on α toxin production, is most probably a result of carbon catabolite repression, a mechanism that, among many bacterial species, also exists in *C. perfringens* (Dupuy and Sonenshein, 1998; Varga et al., 2004; Méndez et al., 2008). This mechanism allows bacteria to select which carbohydrate source to use (Brückner and Titgemeyer, 2002; Deutscher et al., 2006; Görke and Stülke, 2008), as the presence of a saccharide of preference causes an inhibition of the expression of certain genes that are often engaged in the production of virulence factors (Méndez et al., 2012). Once the “first-choice” saccharide is fully expended, the inhibition is lifted. Méndez et al. (2012) showed that glucose and sucrose suppress the production of both α and θ toxin (perfringolysin O), whereas vegetative growth, is not affected. In the in vitro experiment of the present study, we confirmed the observation of Méndez et al.: α toxin production was inhibited without compromise of bacterial growth, when glucose was present in the culture medium. We also observed this phenomenon with galactose and lactose. The general idea behind this carbon catabolite repression mechanism is that bacteria will produce certain virulence factors only when the direct environment is lacking nutrients. If nutritional availability is

sufficient, the production of virulence factors would undermine energetic profitability, hence the benefit of regulated inhibition by the means of carbon catabolite repression (Titgemeyer and Hillen, 2002).

Several studies show a decreasing lactose tolerance with age, as they are weaned and stop consuming milk, which has also been observed in calves (Huber et al., 1964). The authors found a positive link between lactose concentration in the diet, and intestinal lactase activity. This means that keeping animals on a LF diet, leads to an impaired lactose digestion. This could mean that opting for a LF diet should not be limited to the period during which active immunity against α toxin is developed, but should be continued throughout the full production period. This can be accomplished in different ways: (1) enzymatic pre-hydrolyzation of lactose, (2) substituting lactose with alternative energy sources, or (3) weaning the animal. However, it should be noted that such interventions could cause additional effects on the animal's intestinal health or its microbiota.

Nevertheless, if intestinal lactase activity is insufficient, excess lactose passes through the small intestine toward the large intestines, where it provides the commensal bacterial community with a nutrient source (Castro et al., 2016). Hindgut fermentation of this fast-degradable carbohydrate typically causes bloat and increased acidity due to d-lactic acid production (Rask Pedersen et al., 1982) which potentially leads to colic and diarrhea. In lactose intolerant calves specifically, diarrhea is the most important sign, whereas bloat is less common (Olchoway et al., 1993). In the present study, however, we observed no notable difference in fecal consistency between both groups.

The observed effects of lactose point out the indirect role that weaning management could play in the pathogenicity of NHE. Naturally, weaning occurs gradually until approximately 10 to 12 mo of age (Reinhardt and Reinhardt, 1981). During the transition process from functional monogastric to ruminant, milk is gradually replaced by solid feed. Lactose becomes a minor constituent of the diet, and thus the protective lactose block on *C. perfringens* α toxin expression is gradually lifted. At the same time, intake of *C. perfringens* spores increases due to the animal's grass consumption. It seems that this ingenious balance between a facultative pathogen and its host plays a part in creating a smooth transition from passive to active immunity in almost all individuals. That is, when the transition from milk to solid feedstuffs is respected. In the industrial veal calf sector, conversely, calves often remain on a milk diet, with sometimes limited amounts of solid feed. Consequently, lactose remains an important part of their diet, and antibody production remains suppressed. What then triggers sudden α toxin production remains

unclear, but in many cases it seems to be related to an abrupt change in dietary composition (Manteca et al., 2000).

Lactose needs to be hydrolyzed upon intestinal absorption, and its effect apparently persists longer and further down the intestinal tract than that of glucose and galactose, which are rapidly absorbed in the small intestine (Coombe and Smith, 1973). Consequently, although glucose and galactose have the ability to suppress α toxin production in vitro, in vivo they did not have this effect as measured by anti- α toxin antibodies. Enzymatic hydrolysis of the lactose immediately before ingestion of the milk, lifted this antibody response block, even though in vitro, the monosaccharides (glucose and galactose) resulting from this hydrolysis, were equally able to inhibit α toxin expression by *C. perfringens*. This may be explained by the highly efficacious and rapid receptor mediated absorption of glucose and galactose in the upper part of the small intestine, which might indicate that α toxin is naturally presented to the host immune system in the lower part of the intestinal tract. Further research in this regard should focus on the role of ileal Peyer's patches and the cecal and colonic GALT (gut-associated lymphoid tissue) in the initiation of an adaptive immune response against α toxin.

The in vivo part of this study was performed under highly experimental conditions, which did not reflect the veal calf production sector regarding housing and feed management. This way we were able to avoid variances caused by differences in feed intake, and assess the mere effect of lactose. A consequence of the latter is that our results cannot entirely be extrapolated to the field, especially considering the lack of solid feed. Due to the multifactorial character of NHE, it is not clear which combinations of predisposing factors could lead to disease. Therefore, it is difficult to correct for all possible influences. Also, a single predisposing factor may have multiple effects: it is possible that lactose, aside from the effect on *C. perfringens* and its α toxin production, also affects the host. Another aspect to consider is the fact that blocking for failure of passive transfer was not performed. However, all animals originated from the same farm (thus same colostrum management), and groups did not differ on body weight or anti- α toxin antibody levels at the start of the experiment.

CONCLUSIONS

Results of the present study show a continuing inhibitory effect of lactose on the development of antibodies against α toxin, which is a determining factor in the animal's sensitivity toward NHE. Intervening in the lactose provision at the moment the maternal

immunity starts to decline, could therefore benefit the animal's risk on disease development. This preventative approach is necessary considering the lack of sufficient therapeutic strategy due to the extremely rapid development of the disease, leading to sudden deaths. Lactose elimination can be attained by adjusting the milk replacer, or by altering weaning management. However, first, further research on this effect needs to be performed under farming conditions, to fully comprehend the practical implications of our findings.

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