ABSTRACT

The objectives of this study were to determine if weaning would induce behavioral and physiological indicators of a negative affective state, and if supplementation of inactivated *Lactobacillus helveticus* (ILH) to dairy calves would reduce those indicators of negative affect during weaning. Male Holstein calves (n = 23) were enrolled in the study on d 1 of life. The calves were housed in individual pens in 1 of 4 rooms for the 42 d study. Calves began a stepdown weaning from 9 L/d of milk replacer (MR), at 150 g of MR powder/L, on d 35 and received 6 L/d on d 35 – 36, 3 L/d on d 37 – 38, and 0.4 L/d on d 39 - 42. The MR was divided between 3 meals/d until the last 0.4 L/d phase which was divided between 2 meals/d. Calves had *ad libitum* water access throughout the study and calf starter from d 28 onwards. Within room, calves were assigned to 1 of 2 treatments: 1) control (CON; n = 11) and 2) 5 g of ILH/d split over and mixed into the 0800 h and 2000 h milk feedings from d 3–42 (ILH; n = 12). Lying behavior was recorded using HOBO data loggers from d 21–41. On d 33, 37 and 41, infrared eye images were taken to determine maximum eye temperature (MET), saliva samples were collected to determine cortisol concentration, and play assessments were conducted to quantify play behavior. On d 34, 38, and 42, blood samples were collected to determine blood serotonin concentration, whereas on d 38 and 39, calves were tested with a cognitive task. A subset of calves (n = 5/treatment) were euthanized to collect gut and brain tissue samples for serotonin concentration on d 43. Weaning resulted in fewer (d 37–41, tendency: d 36), but longer (d 38–41, tendency: d 37), lying bouts and reduced play (d 41), although no changes in lying time, MET, saliva cortisol, nor blood serotonin were detected with initiation of weaning. Supplementation of ILH was associated with lower lying time throughout the study, and reduced play duration and higher salivary cortisol and MET during weaning. No differences in lying bouts, play count, blood and tissue (colon, ileum, prefrontal cortex and brain stem) serotonin concentration, and time to complete the cognitive task were detected between the treatments. Overall, weaning induced behavioral changes indicative of negative affective state, and some behavioral differences were observed with ILH supplementation both before and during weaning, with some physiological changes observed during weaning.

**Key words:** microbial-based solutions, gut-brain axis, behavior, weaning stress

INTRODUCTION

Weaning, the process of transitioning dairy calves from a diet containing milk to one entirely composed of solid feed, is a necessary step in the development of the calf. Although weaning can be a challenging experience for the calf (Jasper et al., 2008), supported by behavioral and/or physiological responses indicative of increased stress reported in many studies assessing this (Loberg et al., 2008; Miguel-Pacheco et al., 2015; Yeste et al., 2020). The feelings and emotions experienced as pleasant or unpleasant are referred to as “affective states” of animals (Fraser, 2008). There are many reasons why weaning may cause dairy calves to experience a negative affective state. These include that milk is often fed through a nipple, which facilitates sucking behavior - a behavior calves are motivated to perform (de Passille, 2001), and one that may elicit satiety (de Passille et al., 1993) and calming effects (Veissier et al., 2002). Furthermore, calves prefer to drink milk over consuming solid feeds (Webb et al., 2014) and it is their natural source of nutrition (Khan et al., 2016). Therefore, removing milk...
and the teat, a familiar sucking outlet, could inflict negative affect.

Additionally, there is high variability among calves regarding when they begin to eat solid feed and how quickly their consumption increases during weaning (de Passille and Rushen, 2016). Yet abrupt weaning methods still occur (Vasseur et al., 2010). The rapid dietary change can result in growth depressions due to challenging the lower gut resulting in insufficient energy intake (Steele et al., 2019). Perhaps at the root of the problem, on most dairy farms in North America, calves are weaned much earlier (6–8 wk of age; Vasseur et al., 2010) than they would be if raised and weaned by their dam in a natural environment (average 10 mo; Reinhardt and Reinhardt, 1981). Calves weaned early show more signs of hunger (negative affect) than calves weaned later and/or by a specified amount of voluntary starter intake (hunger inferred by more unrewarded visits to an automated milk feeder; de Passille and Rushen, 2016). Furthermore, there is great variation in when calves voluntarily wean themselves using this method, many never will wean themselves before the average weaning age on dairy farms (Benetton et al., 2019).

Another factor that may influence affective state at weaning is the gut microbiota. The gut microbiota is not only essential to gut and overall animal health and function (Malmuthuge and Guan, 2017), but also have been shown in other animal models to play a major role in mental well-being through their key involvement in the gut-brain axis (Huang and Wu, 2021). Undesirable alterations in gut microbiota composition and/or diversity, termed “dysbiosis,” and psychological stress are linked (Cryan and Dinan, 2012). Correspondingly, gut microbiota is reported to change at weaning. For example, Meale et al. (2017) reported that the most abundant ruminal phyla in dairy calves changed from Bacteroidetes to Firmicutes at weaning, the change was more rapid in calves weaned at 6 wk as opposed to 8 wk. Similarly, Li et al. (2018) reported weaning decreased some beneficial bacteria, including Alloprevotella and Oscillibacter, and increased some pathogenic bacteria, including Campylobac terales, Campylobacteraceae, and Campylobacter, in piglets. Factors likely to affect this are the change in diet and associated eating behavior (Meale et al., 2016) as well as psychological stress (de Palma et al., 2014). Therefore, methods to support the gut microbiota and the gut-brain axis may prevent or reduce negative affects associated with weaning.

Probiotics have been supplemented to support gut and overall health in dairy calves (Cangiano et al., 2020). Probiotics may also benefit mental health through their modulation of the gut-brain axis (Zhang et al., 2020), positively affecting brain physiology and behavior (Cryan and Dinan, 2012). Inactivated probiotic products (e.g., heat-killed, non-viable, microbial cells), also recently qualified as paraprobiotics (Teame et al., 2020), have gained attention since they can yield similar beneficial health affects while being safer, in that there is no risk of them causing infection, and they may be easier to transport and store (Adams, 2010; Piqué et al., 2019). Although notably, no such infections in calves from live probiotics were identified in the literature. More research is needed on paraprobiotics, as the mechanisms are not well understood (Cuevas-González et al., 2020). No studies have been identified in the literature that investigated the effects of inactivated probiotic products on cattle affective state.

One strain that has shown promise is live Lactobacillus helveticus (LH), with research in rodents supporting that LH can improve stress-related symptoms. Specifically, Liang et al. (2015) reported supplementation of LH to stressed rats improved behavioral dysfunction (anxiety, depression, and cognitive) better than an anti-depressant (selective serotonin reuptake inhibitor- citalopram). Also, LH was associated with lower plasma corticosterone and adrenocorticotropic hormone levels, higher plasma interleukin-10 levels, restored hippocampal serotonin and norepinephrine levels, and higher hippocampal brain-derived neurotrophic factor mRNA expression. Likewise, LH supplementation to mice was associated with reduced anxiety-like behavior (Ohland et al., 2013) and improved learning and memory in mice (Ohsawa et al., 2015). Considering paraprobiotic research, Maehata et al. (2019) reported that supplementation of inactivated LH (ILH) to stressed mice improved anxiety- or depressive-like behaviors and stress-induced gene expression alterations. While no differences were found on gut microbiota composition, Maehata et al. (2019) suggested the beneficial effects of ILH could be attributable to vagus nerve stimulation, altering the metabolites produced by the gut microbiota, and/or modulating the immune system (cytokines); however, they stated deeper investigation is required to identify and elucidate the mechanisms. No previous studies were identified that assessed the effect of live LH or ILH on measures of cattle (including dairy calf) affective state.

Therefore, the objectives of this study were to determine if weaning would induce physiological and behavioral indicators of negative affective state and if supplementation of ILH to dairy calves would result in reduced indicators of negative affect during weaning. We hypothesized that weaning would induce behavioral and physiological indicators of negative affective state and that those indicators would be reduced in calves receiving ILH through it positively supporting the gut-brain axis.
MATERIALS AND METHODS

Animals and Housing

This study was part of 2 larger trials (Cangiano et al., 2023 and Olmeda, 2023) focused on investigating the effects of ILH supplementation on calf immunity, gut health, intake, and growth. A randomized trial was conducted at a University of Guelph research facility (Ponsonby General Animal Facility, Ponsonby, Ontario, Canada) using 2 groups of calves (group 1 and 2 included 13 and 10 calves, respectively), for a total sample size of 23 singlet male Holstein calves. All calves were sourced from another University of Guelph research facility (Ontario Dairy Research Centre at the Elora Research Station, Elora, Ontario, Canada) located less than 10 km away. All study procedures were reviewed and approved by the University of Guelph Animal Care Committee (AUP#4470).

Within 2 h after birth, farm staff transferred calves from an individual maternity pen with their dam to a predisinfectant and washed calf crate. Calves had their navels thoroughly dipped in iodine and were fed their first colostrum meal promptly. All calves were healthy at birth based on overall appearance, rectal temperature, umbilical appearance, and nasal discharge. Following postnatal care, all calves were transported to the Ponsonby General Animal Facility (within 10 h of life) and enrolled in the study on d 1 of life. The calf crates were strapped down and transported in the back of a truck from one research facility to the other. The calf crates were disinfected with Virkon (Lanxess, Elmira, Ontario, Canada) and pressure washed between calves. For each group, calves were enrolled over approximately 1–2 mo. (calves in group 1 were enrolled between July 6, 2021 to August 17, 2021, and calves in group 2 were enrolled between September 6, 2021 and November 2, 2021). There was 1 calf (not included in the 23-calf total sample size) removed from the study within the first 21 d of age due to a leg injury.

There were 4 temperature-controlled rooms at the facility: rooms 1–3 were structurally identical while room 4 was smaller. Rooms 1–3 had 2 rows of pens (9 pens total/room), with the rows separated by a walkway, while room 4 had 1 row of 4 pens with a walkway in front. All pens were 1.20 × 1.80 × 3.60 m (height x width x length). The pens were vertical metal bar-sided (1.50 cm width, 6.00 cm apart), except 1–2 sides which were solid concrete walls depending on the pen’s location in the room. The pens had concrete floors, and were bedded with wood shavings, with a straw pack in the back half. On the inside of each pen, there were 2 bucket holders for solid feed and water buckets, as well as a hook for the milk teat bucket. After milk feedings, each calf’s milk bucket was thoroughly scrubbed using a bristled scrub brush, hot water, and Liquid Hand Soap (Agrisan, Arthur, Ontario, Canada), and then rinsed with water. The attached nipples were also cleaned by squeezing the hot soapy water through them and then squeezing clean water through them to rinse. Each calf’s bucket was then hung upside down outside of their pen to air dry.

For each group of calves, rooms were filled 1 at a time. At any given time, a maximum of 6 calves were housed in each of rooms 1–3, while a maximum of 3 calves were housed in room 4. Calves had auditory and visual contact with other calves, however, physical contact was limited due to the metal bar pen sides. Shavings were cleaned out of each pen and replaced daily, while straw was topped up throughout the wk as needed, and the whole pen was completely cleaned out and re-bedded once per wk. Each pen had a shovel (for removing dirty bedding) allocated to it, to prevent microbial spread. A few additional measures that were taken to prevent microbial spread between calves include that animal handlers put on a clean pair of gloves when moving between calves, and they also put on a new pair of plastic boot covers between calf rooms and scrubbed their boot covers in prepared disinfectant solution (Virkon, CDMV, Brampton, Ontario, Canada) between calves within rooms. Calves remained on the study until d 42. As calves in group 1 completed the trial, they were removed from their pens, which were disinfected with MS MegaDes Novo (Schippers Canada Ltd., Lacombe, Alberta, Canada), pressure washed, and prepared for the next calf (in group 2).

Feeding and Health Management

All calves were fed 2 meals of a 26% IgG standardized colostrum replacer (CR) powder (HeadStart, Saskatoon Colostrum Company Ltd., Saskatoon, Saskatchewan, Canada) on the first day of life - the first within 6 h after birth while still at the Elora Research Station and the second at approximately 12 h after birth, when they were at the Ponsonby General Animal Facility. Each colostrum meal consisted of 750 g of CR powder mixed with water between 42 and 45°C to reach a final volume of 3 L to deliver 200 g of IgG to the calf. All CR, milk replacer (MR), and electrolytes were prepared in water at this temperature. Both colostrum meals were prepared in a pail and fed in a calf bottle fitted with a nipple. The calves were encouraged to drink from the bottle, however, if they did not consume all of the colostrum meals, the refusals were fed with an esophageal tube feeder. During the second day of life, the calves were fed 2 feedings of a 50:50 mix of CR and MR (Grober Nutrition, Cambridge, Ontario, Canada; Table 1), each meal consisting of 225 g of CR powder and 225 g of MR powder mixed with water to reach a final volume of 3 L. The CR:MR meals were prepared in a pail and fed in a bucket fitted...
with a nipple. A 27% CP, and 19% fat, 47.1% lactose (DM basis) MR powder was used. It is recognized that the lactose in the MR powder was abnormally high, this was not intentional and to our knowledge did not induce scours. The MR contained no feed additives, including no antimicrobials. Animal handlers remained with the calves during these feedings and trained the calves to consume milk from the buckets fitted with nipples by allowing the calves to suckle on their gloved fingers and guiding their mouth onto the nipple. At approximately 24 h after the first colostrum meal, a blood sample was taken, which was centrifuged to collect serum. IgG levels were later determined using radial immunodiffusion, as reported in Cangiano et al. (2023). Importantly, all calves had successful transfer of passive immunity (using the categories developed by Lombard et al. [2020], all calves were in the fair category [10–17.9 g/L of IgG] or higher) and there was no difference detected between treatments.

Calves had ad libitum access to fresh water in a bucket from d 1 of life. Starting on d 3 of life, calves received 6 L/d of MR, which was increased to 9 L/d on d 7 of life. Daily MR allowance starting on d 3 was split over 3 meals (0800, 1400, and 2000 h) at 150 g of MR powder/L. The MR was prepared with water either by hand in a pail or in a milk taxi (MilkTaxi100, Holm & Laue, Westerrönfeld, Germany) depending on the number of calves at the facility at the time and fed to calves in the same buckets fitted with nipples. All colostrum, milk, and electrolyte feedings were conducted by farm staff (3 people) or researchers and volunteers (up to 10 people).

Calves were offered a high-starch texturized starter (41.3% starch, and 10.7% NDF, on a DM basis; Shur-Gain, Ontario, Canada; Table 1) on d 28 and fed ad libitum for the rest of the study. The calf starter contained no feed additives, including no antimicrobials. Orts were discarded and starter was replaced or topped up fresh as needed. Calves were weaned from MR with a stepdown method as follows: on d 35 the MR was reduced to 6 L/d (2 L /meal), on d 37 the MR was reduced to 3 L/d (1 L /meal), and on d 39 the MR was reduced to 0.4 L/d (0.2 L/meal, fed at 0800 and 2000 h meals only), which the calves received until d 42.

Samples of the MR and calf starter were collected at the beginning of each month and frozen. Samples were later thawed overnight in the refrigerator and individually placed in a drying oven at 60°C for 48 h to determine the DM content. Samples of solid feed were ground through a 1-mm sieve (Model 4 Wiley Laboratory Mill, Thomas Scientific, Swedsboro, New Jersey, USA) and were sent to A & L Canada Laboratories Inc. (London, Ontario, Canada) for chemical composition analyses. Calf starter samples were analyzed for ash (550°C; AOAC International, 2000: method 942.05), CP (N x 6.25; AOAC International, 2000: method 990.03; Leco FP-628 Nitrogen Analyzer, Leco, St. Joseph, MI), ADF (AOAC International, 2000: method 973.18), NDF with heat-stable α-amylase and sodium sulfite (AOAC International, 2000: method 2002.04), starch (heat-stable amylase and amyloglucosidase; AOAC International, 2000: method 996.11), and crude fat (AOAC International, 2000: method 920.39; Ankom XT15). Milk replacer samples were also analyzed at A & L Canada Laboratories Inc. for CP (N x 6.25; AOAC International, 2000: method 990.03; Leco FP-628 Nitrogen Analyzer, Leco, St. Joseph, MI), crude fiber (AOAC International, 2000: method Ba 6a-05; Ankom Bag Technology), and fat (total fat by acid hydrolysis using the ANKOM HCl Hydrolysis System; AOAC International, 1995: method 954.02 Section 4.5.02).

Health scoring was performed to monitor health and detect any illness, conducted daily starting at 1000 h by 1 of 3 trained researchers using the Wisconsin Calf Health Scoring App (University of Wisconsin-Madison, School of Veterinary Medicine, Madison, Wisconsin, United States), a tool used previously by other researchers, described by Cramer et al. (2019). The app allowed

Table 1. Ingredient and chemical composition (mean ± SD) of the calf starter and milk replacer fed to all calves

<table>
<thead>
<tr>
<th>Ingredient and chemical composition</th>
<th>Calf starter feed1</th>
<th>Milk replacer2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ingredient, % in ration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calf starter pellet1</td>
<td>60</td>
<td>—</td>
</tr>
<tr>
<td>Steam flaked corn</td>
<td>36</td>
<td>—</td>
</tr>
<tr>
<td>Molasses</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td><strong>Chemical Composition</strong>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM, % DM</td>
<td>88.5 ± 0.55</td>
<td>97.0 ± 0.74</td>
</tr>
<tr>
<td>CP, % DM</td>
<td>20.4 ± 1.08</td>
<td>27.0 ± 0.66</td>
</tr>
<tr>
<td>NDF, % DM</td>
<td>10.7 ± 0.61</td>
<td>—</td>
</tr>
<tr>
<td>ADF, % DM</td>
<td>5.2 ± 0.91</td>
<td>—</td>
</tr>
<tr>
<td>Crude Fiber, % DM</td>
<td>—</td>
<td>0.6 ± 0.29</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>7.2 ± 0.36</td>
<td>7.0 ± 0.22</td>
</tr>
<tr>
<td>NFC, % DM</td>
<td>57.3 ± 0.67</td>
<td>—</td>
</tr>
<tr>
<td>Starch, % DM</td>
<td>41.3 ± 0.94</td>
<td>—</td>
</tr>
<tr>
<td>Fat1, % DM</td>
<td>2.2 ± 0.27</td>
<td>19.0 ± 1.44</td>
</tr>
<tr>
<td>Lactose2, % DM</td>
<td>—</td>
<td>47.1 ± 1.44</td>
</tr>
<tr>
<td>Ca, % DM</td>
<td>1.4 ± 0.14</td>
<td>1.0 ± 0.05</td>
</tr>
<tr>
<td>P, % DM</td>
<td>0.4 ± 0.04</td>
<td>0.7 ± 0.02</td>
</tr>
<tr>
<td>Na, % DM</td>
<td>0.4 ± 0.02</td>
<td>0.6 ± 0.06</td>
</tr>
</tbody>
</table>

1Texturized calf starter feed was supplied by Shur-Gain (Ontario, Canada).
2Milk replacer was supplied by Grober Nutrition (Cambridge, Ontario, Canada).
3Calf starter pellet included ingredients: Soymeal, soft-ground wheat, barley, gluten, calcium carbonate, calf micro premix, salt, magnesium oxide, and dicalcium phosphate.
4Chemical analysis was done by A&L Laboratory Services Inc. (London, ON, Canada).
5Fat for the rations is crude fat. Fat for the milk replacer was done by an acid hydrolysis test (AOAC, 1995: Method 954.02).
6Lactose is assumed to be 100-CF-Pat-Ash.
Treatments Allocation

Within room, treatments were assigned to the pens before calves arriving to make sure that a pen was kept empty between calves on different treatments to prevent cross-contamination of microbiota and correct for any confounded treatment and room effect. Calves on the same treatment could be housed in pens next to each other. Calves were then randomly assigned by researchers to the pens within one room at a time, to keep calf ages within room as similar as possible. The treatments included: 1) control, no ILH (CON; n = 12), and 2) supplementation of inactivated Lactobacillus helveticus R0052 at 5 g/d of ILH equivalent to 2 x 10^9 cfu/d (ILH; n = 11). The sample size for the study was based on the primary outcomes of the larger studies, including IL-6 concentration (Cangiano et al., 2023, and Olmeda, 2023). Based on that sample size (minimum of 11 calves/treatment), with a CV of 7.7% for lying duration (Overvest et al., 2018), we had sufficient power to detect a 6.6% difference in lying duration (min/d) between treatments (Morris, 1999). All 23 calves underwent all behavioral and physiological measurements, except for gut and brain tissue serotonin due to a limited number of calves being dissected (5 calves/treatment). External personnel assigned letters (e.g., A and B) to the treatments, to allow the primary author and fellow researchers to be blinded to the treatments. The external personnel were not involved in any of the data collection. The CON treatment consisted of the same carrier ingredient (lactose) as the ILH treatment, which allowed both treatments to visually appear identical. Treatments were administered from d 3–42. Each day, 2.5 g of treatment/calf was fed at each of the 0800 and 2000 h feedings, mixed individually into each calf’s bucket with 1 L of milk to ensure they consumed all the product (all calves successfully did), and then the rest of the milk allowance was provided. During the last phase of weaning, the treatments were mixed into the 0.2 L of milk offered twice daily to keep the method of ILH administration consistent.

Behavioral Measurements

Electronic data loggers (HOBO Pendant G Data Logger, Onset Computer Corp., Bourne, Massachusetts, United States), validated by Bonk et al. (2013), were programmed to record leg orientation every 60 s, wrapped in veterinary bandaging tape, and secured horizontally onto each calf’s inner-rear right leg with veterinary bandaging tape (3M Vetrap Bandaging Tape, London, Ontario, Canada) on d 21. On d 28, the logger was removed and replaced with a new logger promptly secured in the same manner although to the opposite rear leg. The same procedure was repeated again on d 35 and then removed on d 42. The time of d and date each time a HOBO was placed on and/or taken off a calf was recorded for data analysis. Each time a HOBO logger was removed, data was downloaded onto a computer using HOBOware Pro Software (Onset Computer Corp.; Bourne, Massachusetts, United States) and imported into Microsoft Excel (Microsoft Corp.; Redmond, Washington, United States). The data were then analyzed to determine lying bout frequency (bouts/d), lying duration (hr/d), and daily average lying bout length (min/bout) for each calf (Overvest et al., 2018; Reedman et al., 2021).

Play behavior was induced by bedding the calves with shavings, as done by Reedman et al. (2021). Each calf was tested in this manner around 0900 h on d 33, 37, and 41, in their own pen. On these days, the bedding to induce play behavior replaced that morning’s shavings provision so that at the time of the test, there would be a greater time since the last shavings provision, further encouraging play behavior. At the time of testing, a...
A video camera (GoPro Hero 9, GoPro, California, United States) was attached to a tripod and positioned in front of the calf pen to capture the entire calf pen, and the video recording started. The researcher got the calf up if not already standing, and then using a shovel, quickly (within 1 min) bed the front half of the calf’s pen with 8 shovelfuls of clean wood shavings. All shavings were placed low to the ground to avoid getting shavings on the calf and prevent the room from getting dusty. Once the researcher finished bedding, they left the room for 3 min so there were no people in the room during the play assessment. Time spent playing and differentiation of various calf play behaviors based on an ethogram (Table 2) was later determined using the behavior coding program, Solomon Coder (Peter, 2019). For each play assessment, behavior was coded from 30 s before the gate was closed (bedding completed) to 180 s after the gate was closed (a total of 210 s). Two people coded all play videos. Interobserver reliability was calculated for each behavior by the Kappa coefficient (k), including 20% of the videos. The average Kappa (κ) was 0.86, with κ ≥ 0.76 for all behaviors assessed. Total play duration was determined by summing the total time each calf spent running and rubbing shavings (per play assessment). The total play count was determined by summing the counts of bucks, kicks, jumps, head-shake/swings, and head-butts (per play assessment). The daily bedding procedure conducted by staff (for calves that did not have a play behavior assessment that day) was similar to how researchers provided shavings for the assessment, except they did not attempt to get the calf up (if lying down).

Each calf was tested to perform a detour task on d 38 and 39 between 1000 h and 1300 h. These 2 weaning days were chosen because the goal was to test the calves’ ability to learn while in a stressed state, which may facilitate capturing any potential effect of treatment. A see-through, V-shaped apparatus was centered between the left and right sides of a 3.66 m wide by 7.32 m long pen bedded with shavings, with the point of the V 3 m away from the middle of the front of the pen, with the sides (positioned approximately 30 degrees apart) extending toward the back of the pen (Figure 1). The apparatus was made of 2, 3.5 m long, 1.1 m tall aluminum gates, attached using a metal bar and zip ties at the point where the gates met to create the V shape (detour). An empty milk bucket (identical to the ones the calves received their milk meals in) was hung using zip ties inside the point of the V matching the calves’ normal drinking height with the nipple facing outwards to where the V opens up. A small amount of MR (<0.4 L) was prepared and poured into the bucket. Figure 1 provides a visual of the detour apparatus and pen layout, which was built based off of that described by Nawroth et al. (2016) and personal communication with Dr. Heather Neave (Post-doctoral Research Fellow, Aarhus University) and Laura Whalin (PhD student, University of British Columbia). A video camera (GoPro Hero 9, GoPro, California, United States) was attached to the tripod and secured approximately 4 m above the ground of the pen, positioned near the center to capture the entire pen. The video recording was started, the calf was guided from their home pen directly to the testing pen, once all 4 hooves were on the ground inside the testing pen, the test began. The test ended when the calf found their way around the apparatus and touched the bucket/nipple or when 5 min passed, whichever came first. Later, the test time duration was determined from watching the video recordings. The pen (including the detour apparatus) was disinfected with Virkon (CDMV, Brampton, Ontario, Canada), pressure-washed, and re-bedded between calves.

**Table 2.** Description of recorded play behavior. adapted from that of previous studies (Jensen et al., 1998; Mintline et al., 2013)

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locomotor</td>
<td></td>
</tr>
<tr>
<td>Run</td>
<td>Includes:</td>
</tr>
<tr>
<td></td>
<td>• Trot: Two-beat gait, with leg movements synchronized diagonally</td>
</tr>
<tr>
<td></td>
<td>• Canter: Three-beat gait in between a trot and a gallop</td>
</tr>
<tr>
<td></td>
<td>• Gallop: Four-beat gait with a phase where all legs are off the ground</td>
</tr>
<tr>
<td>Buck</td>
<td>Both rear legs are lifted off the ground and kicked in the rear direction</td>
</tr>
<tr>
<td>Kick</td>
<td>One rear leg is lifted off the ground, sometimes tucked in under the body first, and then extended to the rear or side vigorously</td>
</tr>
<tr>
<td>Jump</td>
<td>The two forelegs are lifted from the ground, as the forepart of the body is elevated, during the last phase of the movement, the hind legs may be lifted from the ground</td>
</tr>
<tr>
<td>Turn</td>
<td>The two forelegs are lifted from the ground, as the forepart of the body is elevated and turned to one side, followed by the calf’s rear-end being elevated and moving sideways to fall into alignment with the front end</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Head-shake/swing</td>
<td>The head is shaken, swung or rotated</td>
</tr>
<tr>
<td>Rub shavings</td>
<td>Rubbing head, throat or neck in shavings, kneeling down on the two forelegs</td>
</tr>
</tbody>
</table>
Physiological Measurements

Saliva sampling was done on d 33, 37, and 41 at approximately 0930 h. A researcher entered the calf’s pen and held the calf gently, but securely, and offered the calf a swab (Salivabio Children’s Swab, Salimetrics LLC, Pennsylvania, United States). If the calf did not grab onto the swab, the researcher slowly inserted the swab into the calf’s mouth, held loosely with a sterile glove in the calf’s mouth, until the bottom 2/3 was well soaked with saliva (at least 1 mL; Loberg et al., 2008; Kovács et al., 2021) and then the swab was placed into a sterile plastic tube (Swab Storage Tube, Salimetrics LLC, Pennsylvania, United States). Two swabs were collected/calf on each sample d, 1 to be analyzed and 1 to be kept as a spare. The tubes were stored at −20°C until analysis (Meléndez et al., 2018). Samples were shipped to Salimetrics (Carlsbad, California, United States) where cortisol concentrations were determined (Pagani et al., 2017; Marti et al., 2017), assessed in duplicate. The samples were thawed to room temperature, vortexed, and then centrifuged for 15 min at 1,500 x g immediately before performing the assay. Samples were tested for salivary cortisol following manufacturer’s protocol of a high sensitivity enzyme immunoassay (Cat. No. 1–3002 Salimetrics LLC). The assay had a lower limit of sensitivity of 0.007 μg/dL, a standard curve range from 0.012 to 3.0 μg/dL, an average intra-assay coefficient of variation of 4.6%, and an average inter-assay coefficient of variation 6.0%, which meets the manufacturers’ criteria for accuracy and repeatability, and exceeds the applicable NIH guidelines for Enhancing Reproducibility through Rigor and Transparency (https://grants.nih.gov/policy/reproducibility/index.htm).

Eye pictures were taken using an infrared thermography camera (FLIR E8-XT, FLIR Systems, Wilsonville, Oregon, United States) on d 33, 37, and 41 at approximately 0930–1000 h (following saliva sampling). Two researchers entered the calf’s pen to capture the pictures. One researcher held the calf gently but securely while the other took the pictures. Pictures were taken of both eyes (Bravo et al., 2018) at a 90° angle to the animal and approximately 0.5 m distance from the eye (Stewart et al., 2007). One to 5 images were taken/eye/calf/d, with the image numbers recorded for each calf/d. Each time a calf’s photos were taken, the ambient temperature and relative humidity were recorded from a Taylor Digital Indoor Thermometer Comfort Station (Canadian Tire, Guelph, Ontario, Canada) with the device positioned at the front of each calf’s pen. These values were later used during image analysis to account for atmospheric changes (Stewart et al., 2007; Bravo et al., 2018). The pictures were downloaded onto a computer and into the software program FLIR Tools (FLIR Systems, Wilsonville, Oregon, United States) for analysis. The clearest picture of each eye/calf/d was analyzed (Lecorps et al., 2018). The maximum temperature detected within an oval area traced around the eye, including the eyeball and approximately 1 cm surrounding the outside of the eyelids, was recorded (Figure 2; Stewart et al., 2007). The Maximum Eye Temperature (MET), determined from both left and right eye pictures was averaged to obtain 1 MET value for each calf/d. The d 33 MET was assumed to reflect a baseline. The calves were frequently handled throughout the study, and especially gently during the eye images, supporting minimal induced stress that would have affected the data. Also, the saliva sampling (which occurred just prior) was considered to be a very low stress activity as it appeared to be well accepted by the calves. Due to the number of the calves, required hygiene precautions needed between calves, and therefore overall time limitation, we were unable to space out in time the measures (i.e., the saliva sampling and MET) for each calf.

Calves were blood sampled on d 34, 38, and 42 between 0830 and 1030 h. Ten mL of blood was collected from the jugular vein through a catheter (installed for sample collection as part of the larger project; d 34) or venipuncture (d 38 and 42) using a 20 gauge 1 inch needle (greiner BIO-ONE, Kremsmünster, Austria) into a 10 mL vacutainer blood collection tube with K2 EDTA.
was gently inverted approximately 8 times. Immediately following, 4 mL of blood was distributed into each of 2 6 mL glass tubes (14–961–26, Fisher Scientific, Ontario, Canada) containing 40 mg/tube of ascorbic acid (NOW Foods 100% Pure Ascorbic Acid Powder, Well.ca, Guelph, Ontario, Canada) and then mixed gently by inverting approximately 8 times. The ascorbic acid was added at 10 mg/mL, which preserves the blood by stabilizing and protecting the serotonin against oxidative loss (Connelly et al., 2020). The tubes were stored at −20°C until analysis (Marrero et al., 2019). The samples were shipped to the University of Wisconsin-Madison where they were analyzed using a serotonin enzyme immunoassay kit (IM1749; Immunotec, Beckman Coulter, Marsei le Cedex 9, France) according to the manufacturer’s instructions, assessed in duplicate (Marrero et al., 2019). The intra- and inter-assay CVs were 3.86% and 4.24%, respectively.

On d 43, a subset of calves (n = 10; 5/treatment) were euthanized using captive bolt followed by jugular exsanguination by trained personnel, and then dissected. Due to time constraints, we were required to select the 10 calves from 2 sets of calves, one set from each enrolled group, ensuring the treatments were mixed throughout each set. Calm and consistent handling by familiar animal handlers was prioritized before slaughter to try to reduce any increased stress before slaughter that may impact tissue serotonin levels. Using sterile surgical scissors and tweezers, 2, approximately 1 cm² pieces of colon were collected at 30 cm distal to the colon, gently rinsed with phosphate buffered saline (PBS), and then placed in a 1.5 mL tube, and snap frozen in liquid nitrogen. The ileum sample was collected at 30 cm proximal to the cecum, using the same procedure. The colon and ileum were selected for sampling as approximately 90% of the body’s serotonin is produced in the gut’s enterochromaffin (EC) cells (Jenkins et al., 2016). Immediately following the harvest of the digestive tract, the skull was cut into 2 halves from the poll to muzzle using a large knife and mallet. The brain halves were gently removed from the skull using gloved hands and autoclaved surgical scissors. Two, approximately 1 cm³ pieces of the prefrontal cortex (PFC) were collected from each brain half at the most anterior point of the brain (Figure 2), gently rinsed with PBS, and then placed in a small tube, and snap frozen in liquid nitrogen. Then, 2, approximately 1 cm³ pieces of the brain stem (BS) were collected from both sides if cut evenly or from whichever side was more intact and processed using the same procedure. The intent was to sample the location of the BS where the raphe nuclei are most concentrated (Figure 2). The BS (raphe nuclei) was a selected region because it is where the serotonin circuits in the brain originate (Morrisette and Stahl, 2014) and account for approximately 5% of the body’s serotonin production (Jenkins et al., 2016). The PFC was also chosen because it is a brain region greatly involved with emotion and the region most sensitive to the negative effects of stress (Arnsten, 2009). The PFC is densely innervated by serotonergic neurons (Puig and Gulledge, 2011), and stress has been reported to result in decreased PFC serotonin (Wilson et al., 2014). Following the dissection, all snap-frozen samples were transferred to and stored in a −80°C freezer until analysis. Before the analysis, 2 mL tubes were prepared: 0.1 g of 425–600 µm glass beads (G8772; Sigma-Aldrich, Ontario, Canada)

Figure 2. Left: Example of a calf eye infrared thermography image used to determine maximum eye temperature. The red triangle placement is where the software program (FLIR Tools) detected is the maximum temperature within the circle placed around the calf’s eye (lacrimal caruncle). The ambient temperature and relative humidity were measured at the time each image was taken and used during the analysis. Right: Sampled brain locations including the prefrontal cortex (A) and the brain stem (B).
were added to each and sterilized. Total protein from each sample was extracted using mortar and pestle. Then, approximately 0.2 - 0.3 g of the powder were placed into a pre-prepared 2 mL tube that was pre-cooled with liquid nitrogen. Then, 500 µL of RIPA buffer (R0278; Millipore Sigma, Ontario, Canada) and 5 µL of Halt Protease and phosphatase inhibitor cocktail (PI78441; Fisher Scientific, Ontario, Canada) was pipetted into the tube. The tube was sealed, immediately vortexed and shaken to ensure the contents were well mixed. The tubes were beat (Qiagen TissueLyser II beater, Ontario, Canada) at 30 Hz for 1 min twice and then were centrifuged at 14,000 g at 4°C for 10 min (Thermo Scientific Sorvall Legend Micro 21R Centrifuge, Mississauga, Ontario, Canada). The supernatant was collected into a new tube. Samples were diluted 50 times with PBS (P5368; Sigma-Aldrich, Ontario, Canada) to fit within the curve. The total protein concentration was determined using a Pierce BCA Protein Assay Kit (PI23227; Fisher Scientific, Ontario, Canada) according to the manufacturer’s instructions, assessed in duplicate. The aliquots were shipped to the University of Wisconsin-Madison where they were analyzed for serotonin concentration using a serotonin enzyme immunoassay kit (IM1749; Immunotec, Beckman Coulter, Marseille Cedex 9, France) according to the manufacturer’s instructions, assessed in duplicate (Marrero et al., 2019). The intra- and inter-assay CVs were 6.85% and 11.03%, respectively.

Statistical Analyses

All measurements were collected at more than one time point/calf, except tissue sample serotonin, during the study and were therefore summarized by calf and day. All statistical analyses were conducted using SAS 9.4 software (SAS Institute Inc., 2013). All data were imported into the statistical software program from Microsoft Excel (Microsoft Corp., Redmond, WA, USA). In SAS, data were assessed for normality using the UNIVARIATE procedure; all parameters were normally distributed. The GLIMMIX procedure of SAS was used for all analyses. Calf within group and room was the experimental unit in all analyses and the subject of the repeated statement for repeated measures models. Group and room were considered random effects, and treatment was used as a fixed effect. For the repeated measures models, day and the treatment by day interaction were also used as fixed effects. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. In models with multiple comparisons (e.g., with significant treatment by day interactions), a Tukey-Kramer adjustment was made for those comparisons. The fit of all repeated measure models were examined by visual evaluation of the homoscedasticity and normality of the residuals.

To address our objectives, the data for saliva cortisol, average MET, blood serotonin, total play duration and total play count were analyzed in 3 steps. First, for each outcome, the baseline (pre-weaning) values for each treatment were compared using a mixed-effect linear regression model. Next, for each outcome, the effect of treatment during the weaning period was tested using a repeated measures mixed-effect linear regression model. Finally, for each outcome, to assess how the weaning day outcomes changed from baseline, the difference from baseline for each weaning observation day was calculated and those differences were analyzed using a repeated measures mixed-effect linear regression model. The difference from baseline was calculated for each weaning value by subtracting each weaning day value from the corresponding baseline value, allowing us to test this magnitude difference from preweaning to weaning for each weaning observation day. In this model, a day effect indicated differences between weaning observation days in their magnitude change from baseline, a treatment effect indicated a difference between treatments in the magnitude difference from baseline, and finally a day by treatment interaction indicated that the change from baseline varied by both weaning day and treatment. To determine if there was a significant change from preweaning to weaning, individual mean values (for each day, treatment, or day and treatment combinations) were tested for a difference from zero.

Lying behavior (bouts, bout length, and time) data was analyzed using the same 3 steps outlined above except for the pre-weaning values, which were tested using a repeated measures mixed-effect linear regression model due to there being multiple pre-weaning data values. Also for lying behavior (bouts, bout length, and time) data, an average of the pre-weaning values was calculated and used for the models testing for a difference from baseline. The effect of treatment on the cognitive test results was tested using a repeated measures mixed-effect linear regression model. For all of the repeated measures analyses, since all parameters had equal time spacing, the covariance structures: compound symmetry (cs), heterogeneous compound symmetry (csch), heterogeneous first-order autoregressive [arh(1)], first-order autoregressive [ar(1)], and unstructured (un) were tested and the one with the lowest BIC value was used for the analysis. Lastly, the effect of treatment on the tissue sample serotonin results was tested using a mixed-effect linear regression model.

RESULTS

In the pre-weaning period, the number of lying bouts tended to vary by day ($P = 0.09$; Figure 3a) and as weaning progressed, the number of lying bouts/d decreased ($P$
< 0.001). Compared with pre-weaning, there were fewer lying bouts during weaning ($P < 0.001$). In the pre-weaning period, the daily average lying bout length tended to vary by day ($P = 0.07$; Figure 3b) and as weaning progressed, the daily average lying bout length increased ($P < 0.001$). Compared with pre-weaning, the daily average lying bout length was greater during weaning ($P < 0.001$). There were no treatment differences detected for the number of lying bouts or the daily average lying bout length throughout the study ($P \geq 0.44$). In the pre-weaning period, total lying time varied by day ($P = 0.006$; Figure 3c). Throughout the study, CON calves tended to have a greater lying time than ILH calves (pre-weaning: CON $= 18.7 \pm 0.18$ h/d, ILH $= 18.3 \pm 0.17$ h/d, $P = 0.09$; weaning: CON $= 18.5 \pm 0.14$ h/d, ILH $= 18.1 \pm 0.14$ h/d, $P = 0.06$). There was no change in lying time detected during weaning including between weaning days and when compared with pre-weaning ($P \geq 0.13$).

No treatment difference was detected in the pre-weaning total play duration ($P = 0.13$; Figure 4a) nor total play count ($P = 0.47$; Figure 4b). During weaning, calves played for a longer duration ($P < 0.001$) on d 37 (11.4 ± 2.64 s) than on d 41 (0.2 ± 0.12 s). Compared with pre-weaning, on d 41, play duration was decreased ($P < 0.001$), while there was no change on d 37 ($P = 0.30$). During weaning, there was a higher play count on d 37 (15.7 ± 2.41) compared with d 41 (1.7 ± 0.50; $P < 0.001$). Compared with pre-weaning, calves tended to have a higher play count on d 37 ($P = 0.07$), while they had a lower play count on d 41 ($P = 0.002$). Compared with pre-weaning, during weaning, supplementation of ILH was associated with a reduced play duration ($P = 0.04$), although no such treatment difference was detected for play count ($P = 0.28$).

There tended to be a treatment by day interaction ($P = 0.07$), whereby CON calves tended to complete the cognitive (detour) test faster on d 39 compared with d 38 ($P = 0.09$; Figure 5). No change was detected in ILH calves between the 2 test days ($P = 0.65$) and no differences were detected between treatments within day ($P \geq 0.15$).

No treatment difference was detected in pre-weaning salivary cortisol ($P = 0.93$; Figure 6). During weaning (d 37 and 41) calves in the ILH treatment (0.16 ± 0.01 µg/dL) tended to have a higher salivary cortisol than CON calves (0.11 ± 0.02 µg/dL). There was no difference ($P = 0.75$) detected between the weaning days (d 37 and 41). Compared with pre-weaning, on both days during weaning salivary cortisol for the ILH calves was increased ($P = 0.04$), while salivary cortisol for CON calves did not change ($P = 0.46$).

No treatment difference was detected in pre-weaning MET ($P = 0.18$; Figure 7). Calves on the ILH treatment (37.2 ± 0.26°C) tended ($P = 0.08$) to have a higher MET than CON calves (36.7 ± 0.26°C) during weaning. There was no difference ($P = 0.69$) detected between the weaning days (d 37 and 41). No changes from pre-weaning to weaning in MET were detected ($P \geq 0.15$).

No treatment difference was detected in pre-weaning blood serotonin ($P = 0.27$; Figure 8) and no treatment or day effects during weaning in blood serotonin were detected ($P \geq 0.28$). No changes from pre-weaning to
weaning in blood serotonin were detected ($P \geq 0.60$). No treatment differences were detected in tissue (colon, ileum, PFC, and BS) serotonin ($P \geq 0.25$; Figure 9).

**DISCUSSION**

As hypothesized, there were behavioral changes during weaning. Compared with pre-weaning, calves had fewer lying bouts and the length of those lying bouts was greater during weaning. Furthermore, as weaning progressed, the number of lying bouts/d further decreased and the lying bout length increased. Fewer, but longer, lying bouts may reflect a change in eating behavior. Specifically, as the calves progressed in weaning, due to reduced milk intake, the calves would have needed to consume more solid feed (Khan et al., 2007), which likely influenced their eating behavior (not measured) and, consequently, lying behavior. Despite these lying bout changes, daily lying time did not change during weaning.

Based on other weaning studies it was expected that the calves would spend more time standing and, therefore, less time lying during weaning because weaning stress is associated with more standing (and less lying) behavior in calves (Budzynska and Weary, 2008; Overvest et al., 2018; Yeste et al., 2020). During weaning, the combination of receiving less milk and not consuming enough solid feed (<800 g/d) resulted in the calves not meeting their energy and protein requirements (Olmeda, 2023). A likely reason for the calves not consuming enough feed is that the calves were only offered it for the first time (d 28) starting a week before weaning began (d 35), and therefore were likely still neophobic to that feed. It is possible that any increased standing behavior associated with stress was balanced off with increased lying behavior associated with insufficient energy and nutrient intake, resulting in no detected change in total lying time during weaning.

Play behavior can be an indicator of positive affective state or at least absence of a negative affective state in species that are normally playful (Ahloy-Dallaire et al., 2017), as animals are motivated to play when their basic needs are met (Held & Špinka, 2011). Stressors, including weaning, have been reported to result in reduced play behavior in dairy calves (Krachun et al., 2010; Miguel-Pacheco et al., 2015). As weaning progressed, calves played for a shorter duration and had a lower play count. Likely reasons for the reduced play activity on d 41 include the calves not meeting their protein and energy requirements, which would be conducive for physical activity, as well as increased psychological stress from being further along in the weaning process (Jasper et al., 2008; Krachun et al., 2010). Although, there is also a possibility that the calves habituated to the bedding events by d 41, resulting in less play. Interestingly, compared with pre-weaning, calves tended to have a higher play count on d 37. The increase in play count behaviors on d 37 may be because some behaviors, such as kicking and

**Figure 4.** Total play duration (s/d; a), and total play count (count/d; b) by treatment and d (mean ± SE). The total play duration was determined by the total time the calf spent running and/or rubbing shavings, while the total play count was determined by the total count of bucks, kicks, jumps, head-shake/swings, and head-butts the calf performed (all behaviors defined in Table 2) during a 210 s play assessment conducted around the time of bedding (0900 h). The treatments include: CON = control (calves received no Lactobacillus helveticus; n = 11) and ILH = calves received 5 g/d of inactivated Lactobacillus helveticus R0052 equivalent to $2 \times 10^9$ cfu/d, split over 2 milk feedings from d 3–42 (n = 12). The vertical dashed line indicates when weaning began (d 35). During weaning, calves played for a longer duration ($P < 0.001$) on d 37 than on d 41 (a). Compared with pre-weaning, play duration was decreased on d 41 ($P < 0.001$), and during weaning, supplementation of ILH was associated with a reduced play duration ($P = 0.04$; a). During weaning, there was a higher play count on d 37 compared with d 41 ($P < 0.001$; b). Compared with pre-weaning, calves tended to have a higher play count on d 37 ($P = 0.07$), while they had a lower play count on d 41 ($P = 0.002$; b). Significant differences ($P \leq 0.05$) detected are denoted by ‘∗’, and tendencies for differences ($0.05 < P \leq 0.1$) are denoted by ‘†’. 
head-shaking/swinging, were actually performed due to frustration associated with weaning rather than playfulness (Kiley-Worthington, 1983).

To our surprise, there were few detected changes in the physiological parameters during weaning. Saliva cortisol has been reported to increase in calves following various stressors; including in calves separated from their foster cow without any step-down weaning method (e.g., nose-flaps; Loberg et al., 2008), after 20 h of transport without a rest stop (Marti et al., 2017), and following a dystocic birth (Kovács et al., 2021). In the present study, the mean
salivary cortisol concentration in the pre-weaning period was 0.12 ± 0.01 µg/dL, which falls within the range of salivary cortisol literature values reported following the stressors in the above studies. This would potentially indicate hypothalamic-pituitary-adrenal (HPA) stressors in the above studies. This would potentially indicate hypothalamic-pituitary-adrenal (HPA) axis activation, the primary stress response system, throughout the observation period, from different stress or other factors (Mormède et al., 2007). It is possible a greater stress stimulus is needed for the cortisol levels to increase from baseline. Loberg et al. (2008) reported during the 4 d after separation from a cow, the cortisol levels of calves increased from baseline. Terminating the cow-calf bond in addition to nursing may have caused greater stress than just removing teat bucket fed milk using a step-down method in the present study (Enríquez et al., 2011). When the sympathetic nervous system is activated, blood flow to the eye may be increased, and thus, higher MET may be detected, especially in the lacrimal caruncle region (Stewart et al., 2007; Dai et al., 2015). No studies were identified that assessed MET in response to weaning stress, although MET has been reported to increase in calves following various stressors including castration (Stewart et al., 2010), following loading into a trailer (Lecorps et al., 2018), and following a 3 h transport (Bravo et al., 2018). Although the affective state of the calves appeared to be more negative during weaning based on the behavioral results, MET did not increase during weaning, perhaps because the stress was not great enough to elicit an effect (Dai et al., 2015). The calves had a lot of human contact, including being visited for the regular care practices like feeding and bedding, as well as for research purposes; it is possible this increased their stress resilience (Hulbert and Moisá, 2016), limiting physiological changes. It is possible that different weaning methods such as weaning off a cow or abrupt weaning (no step-down) could cause a greater stress stimulus that results in increased MET, although more investigation is needed. It should be further noted that the assessment of MET is demonstrating some potential use in research but its commercial use in this manor still needs to be validated.

Lower serotonin concentration in whole blood or serum has been associated with more negative affective states in humans (Cleare, 1997), dogs (Rosado et al., 2010), chickens (de Haas et al., 2013), and calves (Marrero et al., 2021). Therefore, we predicted the calves would have lower blood serotonin concentration during weaning compared with pre-weaning. Despite behavioral signs of a more negative affective state during weaning, there was no detected change in blood serotonin concentration. Likewise, Riggio et al. (2021) reported serum serotonin levels were not associated with the behavioral response of dogs to a stressful situation. Perhaps there was no detected change in blood serotonin at weaning because different stressors cause different biological responses (Moberg and Mench, 2000) and the stress elicited by the weaning conditions in the present study did not impact blood serotonin concentration, at least during the week of weaning. Again, as explained above for the MET results, it is possible the calves had good stress resilience due to the high amount of human contact they received, limiting physiological changes. Notably, the physiological functions of serotonin are still not fully understood (Bacqué-Cazenave et al., 2020). Clearly, more investigation is required to explore the effect of weaning, and associated impacts on indicators of calf affective state, on dairy calf blood serotonin levels.

Calves on the ILH treatment tended to have less lying time throughout the entire study. While more standing may be associated with stress, it is also possible that the standing time was associated with more activity. In support of this, Kelsey and Colpoys (2018) reported that beef cows supplemented with a probiotic (containing strains of Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus casei, and Lactobacillus plantarum) had higher activity. Olmeda (2023) reported the calves on the ILH treatment consumed more feed than calves on the CON treatment during weaning (on d 40), and post-weaning (on d 46, 49, 51, and 53, with tendencies for greater intake on d 43, 48, and 55). Therefore, increased standing in our ILH study calves could also be associated with greater eating time, possibly as a result of increased exploration of feed and, during the days specified, greater feed intake.

It was predicted that the calves would complete the cognitive test (detour task) faster on d 39 compared with d 38 (i.e., demonstrating learning). While this tended to be observed in the CON calves, there was no change

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**Figure 9.** Colon, ileum, prefrontal cortex (PFC), and brain stem (BS) serotonin (mean ± SE) by treatment. The treatments include: CON = control (calves received no Lactobacillus helveticus; n = 5) and ILH = calves received 5 g/d of inactivated Lactobacillus helveticus R0052 equivalent to 2x10^9 cfu/d, split over 2 milk feedings from d 3–42 (n = 5). No significant differences (P ≥ 0.25) were detected.
observed in ILH calves. Although notably, no differences were detected between the treatments on either of the test days. Stress can reduce cognitive ability (Liang et al., 2015), due to stress hormones, in particular, cortisol (Joels et al., 2018). Interestingly, during weaning, calves supplemented with ILH had higher salivary cortisol levels than CON calves, and ILH calves’ salivary cortisol levels increased from pre-weaning. Additionally, ILH calves tended to have a higher MET than CON calves during those days of weaning. These results suggest that ILH supplementation may have resulted in those calves having a greater reaction to weaning. Part of that reactivity, as noted in the increase in salivary cortisol and MET, may have also been related to the increased standing (and possibly overall activity levels; Stewart et al., 2010; Ede et al., 2019) of those ILH calves; thus, these physiological changes may reflect behavioral changes, and not necessarily stress per se. Furthermore, the treatment differences for feed intake may have impacted these results. While no such studies were identified in calves, Lemmens et al. (2011) reported a meal-induced salivary cortisol increase in men. Since the ILH calves did consume more feed during weaning compared with the CON calves, this could be another reason to explain their higher salivary cortisol during that time. Travain et al. (2016) reported increased MET in dogs during administration of treats compared with before that event, stating this could reflect a positive affective state. Since the ILH calves consumed more feed, it is possible that they similarly received satisfaction from it, which could explain the higher MET observed.

There were no treatment effects detected on blood serotonin, although numerically, blood serotonin concentration was greater in ILH calves throughout the trial. Hara et al. (2018) reported mice supplemented with heat-killed Lactobacillus casei ssp. casei 327 had higher serotonin levels in colon tissue compared with control mice. Although not reported, those mice may have had higher blood serotonin also, as the majority of the serotonin in the body is produced in the gut (Jenkins et al., 2016) and then released into the blood (Mawe and Hoffman, 2013). To our surprise, there were no detected treatment differences observed for any of the gut or brain tissues measured. This result should be considered cautiously as there was a small sample size for dissected calves (n = 5/treatment), which could have prohibited detecting differences due to variation. Other possibilities for the lack of detected effect could be attributable to there simply being no effect, a more stressful weaning model being needed to elicit an effect, or perhaps any potential increase in serotonin is less detectable in tissue compared with blood. It is also possible there is not a direct relationship between tissue and blood serotonin, and that different mechanisms are involved. Nakaita et al. (2013) reported based on a study conducted in mice, that heat-killed Lactobacillus brevis SBC8803 may induce the release of serotonin from the gut, therefore making serotonin presumably more detectable in the blood than in the gut; it is thus possible that ILH has a similar action. Regarding the brain tissues, as serotonin cannot cross the blood brain barrier (Namkung et al., 2015), blood serotonin is not necessarily related to it. It is possible that paraprobiotics lack the mechanisms of action that probiotics have to effect brain serotonin, as Wei et al. (2019) reported that supplementation of live, but not heat-killed, Lactobacillus paracasei PS23 reversed corticosterone-reduced serotonin concentrations in the brains of mice. It should be noted that no studies were identified that assessed gut or brain serotonin levels in response to the probiotic strain, LH, in live and heat-killed forms, requiring more research to be done. Overall, the detected effects of ILH supplementation are inconclusive. Olmeda (2023) and Cangiano et al. (2023) reported there were no treatment effects on gut permeability, or microbial diversity, respectively, supporting that the ILH effects were independent of those factors, and that paraprobiotics may have effects on the gut-brain axis through non-viable probiotic cells, without affecting the gut microbiota. This is further supported by the results and proposed mechanisms of Maehata et al. (2019), outlined in the introduction of the present paper. Maehata et al. (2019) was the only study identified in the literature that supplemented and investigated the effects of ILH on mental well-being in animals. These results of paraprobiotic supplementation to dairy calves do not correspond with the results of probiotic supplementation previously reported in the literature. Researchers have previously reported positive outcomes on physiological stress markers (Zhang et al., 2016; Xie et al., 2020; Lee et al., 2019) and behavioral signs of stress (Kelsey and Colpoys, 2018) from probiotic supplementation. It is possible that probiotics, as were used in these studies, exert more beneficial effects through the gut-brain axis than paraprobiotics in dairy calves. Although, as this is the first study, to our knowledge, to investigate the effect of paraprobiotics on indicators of dairy calves’ affective state, and as only one paraprobiotic was investigated, it is difficult to draw such a conclusion. As well, this was the first study, to our knowledge, that investigated the effects of the species LH on dairy calves’ indicators of stress. Thus, more research is needed to confirm LH’s, and in overall - paraprobiotic effects.

There are some notable limitations to the current study. First, as noted throughout, the sample size was small, so the findings should be considered cautiously. Further, weaning and calf age were confounded; there was no way for us to distinguish the results between them. To solve this, and to make statements of changes being a result...
of weaning specifically, in future studies, researchers could have an additional treatment of calves that are not weaned to identify if their behavior changes as it did to the calves during weaning in the present study. Only one dose of ILH was tested (5 g/d, equivalent to 2x10^8 cfu/d); it is possible that higher dosages would result in different outcomes (Zağórska et al., 2020). Future studies should also include a viable LH treatment to compare the effects of non-viable and viable LH. The last phase of weaning (0.4 L/d) was included to allow continued administration of the ILH using the same method (via milk), although administration of such small amounts of milk is not a common practice in industry. The continued administration might have changed the results, as it is possible that the small amounts of milk fed may have resulted in more stress than if they were not offered any milk following the 3 L/d weaning phase. Offering such a small amount of milk may have had that effect, followed by quick removal of the buckets, possibly resulting in increased stress from the motivation to suck without a proper outlet and frustration from not receiving a larger, more satisfying milk meal (Rushen and de Passille, 1995); as we were measuring indicators of affective state, this would be pertinent to the results. Therefore, for future studies, completing weaning and ILH supplementation after the 3 L/d weaning phase should be done to eliminate the possible increased stress from a milk ‘ tease’, better match industry practices, and therefore make the results more applicable to commercial settings. Other dietary factors that are less common in commercial settings that limit the applicability of this study to them include the MR having high lactose, and calf starter was only offered starting on d 28, a week before the early beginning weaning date of d 35. Additionally, as explained in the discussion, it is likely the calves in this study received more human contact than calves in most commercial settings, potentially improving their stress resilience and affecting the results. Finally, there is a chance the differences observed reflect random variation, although in effort to prevent this, consistent methods for all calves were prioritized and sources of variation were accounted for in the statistical model.

CONCLUSION

During weaning, there were behavioral changes in the dairy calves, including fewer but longer lying bouts and reduced play behavior, with no detected changes in physiological measures. Supplementation of ILH to calves was associated with reduced lying time throughout the study, and reduced play duration and increased salivary cortisol during weaning. More research on LH supplementation, either inactivated or in live form, to dairy calves is required to confirm and understand its effects. This study represents a preliminary investigation in these areas and lays groundwork for future calf studies, particularly in the areas of brain and gut serotonin concentrations, cognitive (detour) testing, and the impact of inactivated probiotic products on indicators of affective state.

ACKNOWLEDGMENTS

Thank you to all the staff at the Ponsonby General Animal Facility (Ponsonby, Ontario, Canada) and Ontario Dairy Research Centre (Elora, Ontario, Canada) for their part in caring for the calves. Thank you to Brooke Boon-stoppel, Claudia Jaczkowski, Katherine Perry, Sabina Czachor, and Jessica Brasier of the University of Guelph (Guelph, ON, Canada) for their assistance with sample collection and analysis. Thank you also to Veronica Fursova, Aly Hoste, Claudia Campoli, Junyu Zhang, Kehan Zhang, and Gary Cottee of the University of Guelph (Guelph, ON, Canada) for their assistance feeding and care for the calves. Thank you also to Dr. David Huyben, and Junyu Zhang of the University of Guelph (Guelph, ON, Canada) for laboratory consultation and training. Thank you to Dr. Heather Neave of Aarhus University (Aarhus, Denmark) for guidance in project design. Thank you to Lallemand SAS (France) for providing the supplement. This project received support from the Natural Sciences and Engineering Research Council of Canada (Ottawa, ON, Canada) and the Ontario Agri-Food Innovation Alliance Research Program of the University of Guelph and the Ontario Ministry of Agriculture, Food, and Rural Affairs (Guelph, ON, Canada). The authors have not stated any conflicts of interest.

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