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A multiparametric approach to assessing residual pain experienced by dairy cows undergoing digestive tract surgery under multimodal analgesia

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ABSTRACT

This study assessed residual pain responses of dairy cows undergoing fistulation surgery under multimodal analgesia using a multiparametric method combining behavioural and physiological indicators. A longitudinal study was conducted on five dairy cows, each acting as her own control. The surgery consisted of implanting a ruminal and a duodenal cannula in each cow. The multimodal drug protocol consisted of a combination of N-Methyl-D aspartic Acid antagonists, α2-agonists, and local anaesthetic during surgery, and non-steroidal anti-inflammatory drugs (**NSAIDs**) and opioid treatment postsurgery. Cow responses to surgery were monitored by direct behavioural observation, physiological assay indicators, and milk production from day (**D**) −6 days before surgery (D-6) to D13 postsurgery. From the data recorded, the variables that contributed most to the discrimination of days pre- and postsurgery were identified using factorial discriminant analysis. Components 1 and 2 of the factorial discriminant analysis explained 68.2% and 17.9%, respectively, of the total variance. Component 1 was mainly explained by haptoglobin (contribution to axis: 0.885), oxidative stress (ratio of oxidized gluthatione to reduced glutathione (**GSH/GSSG**), −0.746; vitamin E, −0.683; vitamin A, −0.597; malondialdehyde (**MDA**), 0.416), and behavioural indicators (general attitude, 0.594). On this axis, the higher the score, the higher were the apathy and haptoglobin and MDA concentrations, and the lower were the GSH/GSSG ratio and concentrations of vitamins A and E. This axis opposed cows on D-6 to cows on D3 and D5; cows on D1 and D13 were intermediate. Component 2 was mainly explained by the Hypothalamic-pituitary-adrenal axis (non-esterified fatty acid (**NEFA**), 0.686; cortisol, 0.669), milk yield (−0.725), oxidative stress (MDA, −0.584; nitric oxide (**NO**), 0.454), and behavioural indicators of pain (ear position, 0.467; leg postures, 0.431). On this axis, the higher the score, the higher the NEFA, cortisol, and nitric oxide concentrations; the more the ear and leg pain postures; and the lower the milk production and MDA concentrations. This axis opposed cows on D13 to cows on D1. These results suggest that cows may experience some pain only on D1, whereas on subsequent days, the inflammatory response and oxidative stress did not seem to be associated with pain. Our results should be considered for different surgeries to improve analgesia immediately after surgery, and to provide antioxidants along with NSAIDs to promote recovery.

Implications

We used a multiparametric method that combined behavioural and physiological indicators to assess pain in cattle before and after fistulation surgery under multimodal analgesia. We showed that the combination of anti-inflammatory drugs and morphine derivatives administered over a prolonged period effectively controlled postoperative pain. However, it was necessary to reinforce analgesia during the first 24 hours after surgery with an intravenous infusion of ketamine, lidocaine, and butorphanol and to improve the recovery of the animals by supplementing them with antioxidants. This study may allow researchers to more effectively prevent pain in animals during surgery.

Introduction

In recent years, many studies on digestion or metabolism in animal science research required long-term implantation of

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digestive cannulas and/or catheters at different levels of the digestive tract, mainly the rumen and duodenum. First, they are used to improve estimates of dietary nutrient supply using the nylon-bag technique, which remains the reference technique for many ruminant feeding systems (Hristov et al., 2019). Second, cannulated ruminants often serve as teaching tools for students in veterinary medicine, nutrition, anatomy, and physiology. Third, these techniques also enable repeated sampling or injections, and reduce the stress of sampling for the animals. Although INRAE (i.e. France's National Research Institute for Agriculture, Food and Environment) already decided that this technique should be stopped and to have no longer ruminants with permanent cannula within 5 years, we believe it would be useful to share our experience of the surgical procedures we performed over the past seven years to generate and disseminate knowledge on how to reduce pain in animals and, if possible, eliminate it. This knowledge could be used in the context of other research on surgery as diseases affecting the gastroenteric tract in ruminants sometimes require to be treated with surgeries: rumenotomy representing as much as 94% of all surgeries in goat for example (Martin et al., 2021).

Cannulation surgery includes (i) a laparotomy; (ii) an incision of the rumen, duodenum, or ileum wall to insert a silicone rubber tube; and (iii) exteriorisation of the cannula on the animal's flank. In rats and sheep, laparotomies (Roughan and Flecknell, 2001) and tension in the mesentery (Faure et al., 2017) cause pain. To our knowledge, however, only one study explored pain in dairy cows undergoing digestivetract fistulation surgery (Newby et al., 2014). Cattle are fistulated using a combination of morphine derivatives and non-steroidal anti-inflammatory drugs (**NSAIDs**) (Sams and Fubini, 1993). Multimodal analgesia is more effective than unimodal analgesia, as reported in a clinical-practice review of pain management in horses (Valverde, 2005), after bovine dehorning (Stafford and Mellor, 2005; Herskin and Nielsen, 2018), and after bovine castration (Coetzee, 2011). However, these medications may not sufficiently alleviate all impacts of surgery, particularly visceral pain caused by tension in the mesentery.

As animals cannot provide verbal reports, their pain is generally assessed through behavioural indicators (e.g. postures) and physiological indicators (e.g. activation of the hypothalamic–pituitaryadrenal (**HPA**) axis, activation of the autonomic nervous system (**ANS**), inflammation, and oxidative stress) of pain (Prunier et al., 2013). We recently developed a multiparametric method that combines behavioural and physiological indicators (Faure et al., 2017). It helped us to better detect and assess the pain experienced by ruminants under different conditions: in sheep after surgical procedures (cannulation surgeries of differing degrees of invasiveness; Faure et al., 2017), in sheep after castration (Durand et al., 2019), and in dairy cows during *Escherichia coli* mastitis (de Boyer des Roches et al., 2017). Hence, the general aim of this study was to assess residual pain responses in dairy cows before and after cannulation surgery under multimodal analgesia using a multiparametric method that combined behavioural and physiological indicators.

Material and methods

The experiment was conducted in 2014 at the experimental research Installation for Milk Production at INRAE's PEGASE research unit in France. The surgical and experimental procedures using cannulated cows followed EU Directive 2010/63/EU for animal experiments and were conducted with the approval (for request R-2012-PL-01) of the Regional Ethics Committee for Experiments on Animals (No. 7), 29 January 2013.

Animals, housing, and diet

The experiment was performed with five mid-lactation primiparous Holstein-Friesian dairy cows (537 ± 24 kg of BW; mean ± SD). They were at 219 ± 13 days in milk, 117 ± 5 days of pregnancy, and produced 17.4 ± 2.3 kg milk per day (mean ± SD). For two weeks before surgery and five weeks postsurgery, the cows were housed in individual tie stalls in an air-conditioned room with a milking system. The cows were milked twice a day, and an electronic device measured milk production. The cows received water and an *ad libitum* diet fed twice daily (50% at 0800 AM and 50% at 1800 PM) before the surgery preparation and consumed 17.2 ± 0.9 kg dry DM per day (mean ± SD). On a DM basis, the diet consisted of maize silage (64.1%), grass silage (6.2%), straw (1.5%), dehydrated alfalfa (9.3%), soybean meal (9.1%), cereal-based concentrate (7.8%), and a mineral and vitamin mixture (2.0%).

Experimental design

The experiment had a longitudinal design, with each cow acting as her own control to examine the residual pain responses (i.e. behavioural and physiological) of five lactating dairy cows undergoing ruminal and duodenal cannulation treated with a multimodal protocol of NSAID and opioid drugs.

Preparation of the animals and surgery

To prepare the cows for general anaesthesia, they received about half the usual amount of their diet on D-2 before the surgery on the morning and all the refusals were removed at 1100. In addition, percentages of cereal-based concentrate, dehydrated alfalfa, and grass silage were reduced on a DM basis from 7.8% to 3.8%, from 9.3% to 3.8%, and from 6.2% to 3.0%, respectively, while those of straw and maize silage were increased from 1.5% to 3.5% and from 64.1% to 74.8%, respectively, and the cows ate 8.3 ± 0.4 kg/d of MS (Mean ± SD) on D-2. The cows received no feed or water from one day before the surgery. During the two days of restriction, cows received IV infusion nutrients (Table 1). One day before surgery, the cows received an intramuscular injection of 0.1 mL/kg BW of antibiotic (Intramicin, Ceva Santé Animale, Libourne, France) providing 14.4 mg/kg of BW of benzylpenicillin (114 mg/mL) and 20 mg/kg of BW of dihydrostreptomycin (200 mg/mL).

Anaesthesia and analgesia protocol

Each cow was given an intramuscular injection 66.7 µg/kg of BW of Xylazine (Rompun 2%: 3.33 µL/kg of BW containing 20 mg/mL of Xylazine; Elanco, Cuxhaven, Germany) 15 minutes before leaving its stall. On arrival in the operating room, ketamine was IV injected (2.5 mg/kg of BW) to each cow (Imalgene 1000: 0.025 mL/kg BW containing 100 mg/mL of ketamine; Boehringer, Lyon, France). Anaesthesia was then maintained with an oxygen-isoflurane mixture (Oxygene: 6 l/min and air 3 l/min with during the first 30 min isoflurane represented about 5% of the gas flow and oxygen flow is reduced to represent about 2 –3%) using an endotracheal tube connected to a closed-circuit anaesthesia apparatus (Alpha 400 Veterinary Equipment, Minerve, Esternay, France). Immediately before surgery, animals were administered an IM injection of 2.083 mg/kg of BW of flunixin (Genixine: 41.66 µL/kg containing 50 mg/mL of flunixin; Ceva Santé Animale). The animals also received an antalgic IV infusion providing 7.5 µg/min per kg of BW of ketamine and 15 µg/min per kg of BW of lidocaine (during 111 min). The infusion mixture was composed of 5 mL of Imalgene 1000 and 50 mL of lurocaine (lidocaine: 20 mg/mL; Vetoquinol, Lure, France) diluted in 500 mL of physiological saline (i.e. 555 mL) at a rate of 5 mL/min. Throughout the

Table 1
Intravenous Infusion of nutrients in cows during the days of presurgery phase (D-1, D-2), of surgery (D0) and of postsurgery phases (D1–D3).

	Infused Nutrients			
Item	Ringer lactate	Glucose 30%	Rehydex	Energhepa
Laboratory	BIOLUZ, St Jean de Luz, France	Vibrac, Carros, France	DOPHARMA, Saint Herblon, France	Vibrac, Carros, France
Nutrients	Sodium chloride (6 mg/ml), Potassium chloride (0.4 mg/ml), Calcium chloride (0.2 mg/ml), Sodium lactate (3.1 mg/ml)	Glucose (300 mg/ml)	Glucose (240 mg/ml), Sorbitol (140 mg/ml)	Sorbitol (50 mg/ml), Acetylmethionine (20 mg/ml), Aspartic Acid (16 mg/ml), Arginine (8.27 mg/ml), Glutamic Acid (15.14 mg/ml)
Rate (ml/min)	50	15	15	15
Volume infused (l/d)				
D-2, D-1	5	0.500	0.500	
D0	15	0.500		
D1	15	0.500	0.500	0.500
D2	10	0.500	0.500	0.500
D3		0.500	0.500	0.500

surgery and, after the surgery, animals received IV infusions of nutrients aft (Table 1).

Surgery: Rumen and duodenal cannulation

The surgery consisted of implanting one cannula made of polyamide and polyvinyl chloride (Synthesia, Nogent-sur-Marne, France), in the rumen (123 mm of internal diameter) and one cannula in the duodenum (27 mm of internal diameter) of each cow. For rumen cannulation, each cow was placed in right lateral recumbency. First, a small circle of skin (the diameter of the cannula was 123 mm) was cut and removed in the hollow of the left flank. Second, the underlying muscles were cut respecting the direction of the muscle fibres (the least traumatic technique and limiting bleeding) and the part of the rumen just above the incision was exteriorized. Third, the cannula was introduced into the rumen and then fixed to it. Fourth, the entire cannula was placed back into the abdominal cavity so that only a small section remained outside, which was then secured to the skin. For cannulation of the duodenum, which occurred 45 min after the beginning of the surgery, the cow was placed in left lateral recumbency. First, a 20 cm incision was made through the skin and muscle on the right flank. Second, the cannula was implanted in the proximal duodenum (10 cm beyond the pylorus), exteriorized on the flank (usually 20 cm above the laparotomy) and secured to the skin. Third, the initial laparotomy was closed by peritoneal, muscular, and skin sutures.

Postoperative treatments

The isoflurane was stopped at the end of the surgery. After extubation and complete recovery of postural reflexes, the cow was given water to drink. Once the cow was standing up and walking (usually 1–2 h postsurgery), she was transferred to her individual tie stall, where she was kept for 5 weeks. From the day (**D**) of surgery (D0) to three days postsurgery (D3), 0.1 mL/kg BW of Intramicin was administered by IM injection twice daily, in the morning and in the evening. On D0, D1, D2, and D3, 2.08 mg/kg of BW per day of flunixin (as Genixine) was administered by IM injection. At 4 h and 24 h postsurgery, 16.67 μg/kg of BW of Butorphanol (Torbugesic containing 10 mg/mL of butorphanol; Zoetis, Malakoff, France) was administered by IV injection.

The cows were fed gradually increasing amount during the week after surgery to avoid acidosis and other digestive problems. On the afternoon of D0, cows received 12.6 kg DM from a diet that consisted of 62.4% maize silage, 8.0% dehydrated maize, 17.6% hay, 5.3% dehydrated alfalfa, 4.7% soybean meal, and 2% mineral and vitamin mixture on a DM basis. From D1 to D7, they received 16.10 ± 1.15 kg of DM with a gradual change in diet composition, and from D8 cows were fed ad libitum and consumed 16.2 ± 0.9 kg DM/d from D8 to D26. From D1 to D3, the cows received nutritional IV infusions (Table 1). Vigosine (50 mL/d from D1 to D3) was introduced into the rumen to regulate digestive functions (sorbitol: 250 g/L, magnesium sulphate: 250 g/L, and L-carnitine: 10 g/L; Ceva Santé Animale). From D1 to D5, cows also received 20 g Besortyl daily, introduced into the rumen (orotic acid: 45 mg/g, betaine: 50 mg/g, sorbitol: 125 mg/g, and sorbic acid: 2.5 mg/g; Novartis Santé Animale, Huningue, France) and 50 g of Rumigastryl (propionic acid salt: 424 mg/g and propionic acid calcium: 40 mg/g; Ceva Santé Animale). Cereal-based concentrate was gradually re-introduced beginning on D4. On D8, cows received an *ad libitum* diet for cannulated cows (57.7% maize silage, 9.3% dehydrated maize, 2.3% hay, 9.4% dehydrated alfalfa, 10.5% soybean meal, 9.2% cereal-based concentrate and 1.6% mineral and vitamin mixture on a DM basis).

Data collection

Behavioural observations and physiological measurements were performed from 6 days before (D-6) surgery to 13 days (D13) postsurgery (Fig. 1) by two experienced technicians.

Behavioural observations and clinical signs

To allow the cows to become accustomed to the presence of the observer for behavioural and clinical recording, and to avoid confusion between the influence of time and the consequences of surgery, the cows were subjected to blank observations five times before the challenge. Thus, cows were accustomed to human presence by the time they underwent the experimental treatment.

Behaviour was observed using focal instantaneous sampling (Martin and Bateson, 2013) on D-6 ($T - 144$ h), D0 ($T + 6$ h and $T + 12$ h), D1 ($T + 24$ h and $T + 29$ h), D3 ($T + 72$ and $T + 78$), D5 ($T + 120$ and $T + 125$ h), and D13 ($T + 312$ h). Behaviour was assessed based on pain behaviours selected from the literature (Table 2). During each observation, the observer approached the cow quietly and observed it by standing at the end of the stall (4–5 m from the animal) in order not to disturb her, and observed her for 1 min. The presence/absence of each behaviour, and for clinical signs, was recorded for each cow on a tablet computer

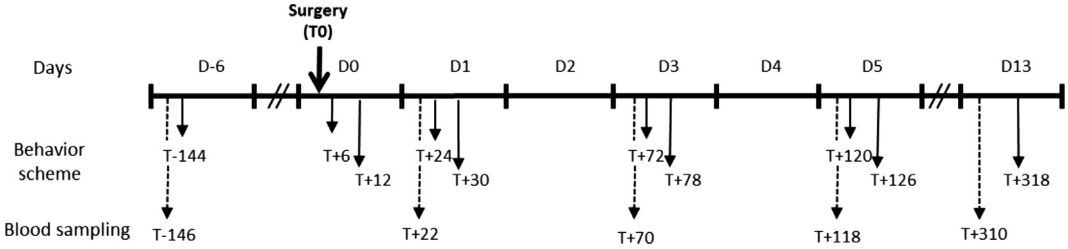

Fig. 1. Experimental design for investigating the influence of multimodal analgesic treatment of dairy cows ($n = 5$) after digestive tract surgery on physiological (from 144 h before surgery (T − 144) to 318 h postsurgery (T + 318)) and behavioural indicators (from 146 h before surgery (T − 146) to 310 h postsurgery (T + 310)).

Table 2
Description of the criteria evaluated during behavioural and clinical observations in cows.

Category	Description	Score	Composite score per category
Behaviours			
General attitude: attention to surroundings and head position (de Oliveira et al., 2014)	Attention:		Min = 0 Max = 2
	Vigilant: the cow is awake, and is attentive towards the surroundings	0	
	Sleeping: the cow is sleeping	0	
	Apathetic: the cow is awake, but does not respond to any stimulus in the environment or is orientated towards a wall	1	
	Head position:		
	Head above or at wither: Cow's head is at or above the line of spinal column	0	
	Head below withers: Cow's head is below the line of spinal column when the cow is eating, exploring, sleeping	0	
	Head below withers: Cow's head is below the line of spinal column when the cow is not eating nor exploring nor sleeping	1	
Ear position (Gleerup et al., 2015)	Ears forward: both ears are positioned forward of the perpendicular. This is often associated with the ear auricles facing forward.	0	0
	Ears plane: both ears are perpendicular to the head-rump axis. This is often associated with the ear auricles facing down.	1	1
Position of the eyelid	Opened	0	0
	Closed	1	1
Other facial expressions (Gleerup et al., 2015)	Bunching of the muzzle	1	Min = 0 Max = 1
Standing and back postures while standing (de Oliveira et al., 2014; Robertson et al., 1994)	Standing postures:		Min = 0 Max = 2
	Standing steadily: Cow is standing or resting steadily	0	
	Standing unsteadily: Cow is standing unsteadily, sometimes the body leaning against a wall; or standing with weight shifting on hind legs	1	
	Back Postures:		
	Plane back: the cow's back is plane while she is standing	0	
	Arched back: the cow's back is arched while she is standing	1	
Lying postures (Robertson et al., 1994; Molony and Kent,1997)	Sternal recumbency with the head down, either to one side or directly in front, or head up	0	0
	Sternal recumbency, and keeping the hip region off the ground	0.5	0.5
	Lateral recumbency with one shoulder on the ground	1	1
Postures of the legs while standing or lying (Robertson et al., 1994)	Normal posture of the legs	0	Min = 0 Max = 1
	One leg held in suspension	1	
	Hind legs stretched back	1	
	Crossed forelimbs or hind limbs	1	
Abnormal activities	Grinding the teeth: bruxism	1	Min = 0 Max = 3
	Lack of rumination	1	
	Trampling	1	
Clinical Signs			
Clinical signs	The cow's eyes are sunken into their orbits	1	Min = 0 Max = 3
	Hair raised: the cow's hair on its head is raised	1	
	Panting: rapid breathing	1	

(ASUS France) using the "EvaDoul" application developed by the research team.

Physiological measurements

Blood was sampled via venipuncture of the jugular vein to determine physiological and metabolic parameters at regular intervals (i.e. at 0800, before the 0830 morning meal) on D-6, D1, D3, D5, and D13. Blood samples were collected in vacutainers containing Na_2-EDTA or lithium heparin. To determine the glutathione concentration, 400 μL of the blood was deproteinised and stored at −80 °C until analysis. For the remaining blood, plasma was isolated by centrifugation at 1 600g for 10 min at 4 °C and stored at −80 °C until analysis.

Inflammation

Plasma haptoglobin concentrations were determined by immunoprecipitation as described by Auboiron et al. (1990). Tumor necrosis factor α (**TNFα**) concentrations were determined using an enzyme-linked immunosorbent assay kit (GenWay, GWB-FOF1X9).

Hypothalamic-pituitary-adrenal axis

Plasma cortisol concentrations were determined by radio-immunoassay as described by Boissy and Bouissou (1994). Non-esterified fatty acid (**NEFA**) and glucose concentrations were determined enzymatically as described by Scislowski et al. (2005).

Oxidative stress

HPLC was used to measure plasma concentrations of oxidised and reduced glutathione (**GSH** and **GSSG**, respectively; Martin and White, 1991). Plasma malondialdehyde (**MDA**) concentrations were determined by colorimetric assay (Gobert et al., 2009). Plasma nitric oxide (**NO**) concentrations were determined using a fluorometric assay kit (Item no. 780051, Cayman Chemical Company, MI, USA). Vitamins E and A were determined by HPLC as described by Gobert et al. (2009).

Production performance

Daily milk yield of each cow was recorded from D-6 to D13.

Statistical analyses

For the behavioural and clinical data, we established categories (Table 2), each of which consisted of several criteria (*e.g.* "clinical signs" included "shrunken eyes", "raised hair", and "panting"). For each cow on each day (D-6, D1, D3, D5, and D13) and for each criterion, we multiplied the frequency of observation by the initial score to obtain a weighted score. For each category, we first summed all of the weighted scores for each cow in each observation and then divided the total by the maximum weighted score possible. The final composite score for each category was thus expressed on a scale of 0–100.

For all indicators, the mean value was calculated for each time and each cow, and was then used for further analysis. Due to the small sample size and the non-normal distribution of variables, non-parametric Friedman and Nemenyi tests were used to compare the physiological, behavioural, and production responses among days.

We performed factorial discriminant analysis to statistically discriminate the five days around surgery (D-6, D1, D3, D5, and D13), as described by de Boyer des Roches et al. (2017). We performed the analysis using all behavioural, physiological (i.e. inflammation, HPA, oxidative stress), and production performance measures. To interpret the discriminant components (axes) of the factorial discriminant analysis, we focused on variables with an absolute value of correlation with an axis >0.4. We estimated the performance of the final predictive model with the current dataset by performing leave-one-out cross-validation, which calculates the probability of each animal being accurately classified for the correct day around surgery. This multiparametric analytical approach enabled us to calculate the probability of each cow belonging to each day. The number of cows correctly classified after cross-validation enabled us to calculate the performance of the final model: sensitivity (percentage of cows correctly predicted as belonging to a given day) and specificity (percentage of cows correctly predicted as not belonging to a given day). We set the significance threshold at $P = 0.05$. Statistical analyses were performed using XLSTAT 2015 (Microsoft Excel®, https://www.xlstat.com/).

Results

No animal reached the ethical limit point (see the ethics approval statement).

Dynamics of physiological, behavioural, and production performance indicators postsurgery (Table 3)

Compared to those at D-6 (median [1st–3rd quartiles]: 0.003 [0.002–0.004] mg/L), haptoglobin concentrations increased consistently from D1 and reached 0.69 [0.66–0.70] mg/L on D5, but did not increase afterwards. TNFα did not vary significantly over time ($P > 0.05$).

All oxidative stress indicators (MDA, NO, GSH/GSSG, vitamins E and A) were significantly impacted by surgery. Compared to those on D-6, the GSH/GSSG ratio decreased significantly on D2 (0.03 [0.03 –0.04]; $P < 0.05$) and remained below 0.09 until D13. NO was higher on D1, reaching 24.16 [17.86–28.24] µmol/mL. Compared to those on D-6, MDA increased consistently from D1 to D13, with peaks on D5 (0.21 [0.20–0.23] µg/dL and D13 (0.25 [0.24–0.27] µg/dL; $P < 0.05$). Compared to those on D-6, vitamins E and A decreased consistently to reach 2.72 [2.04–3.18] µg/mL on D13 and 0.16 [0.16–0.22] µg/mL on D3 ($P < 0.05$), respectively

HPA axis activity was impacted mainly on D1. Cortisol and NEFA increased to 6.43 [4.17–8.20] ng/mL and 0.317 [0.308–0.347] µg/mL on D1, respectively, but not significantly compared to those on D-6. From D3, plasma concentrations of these two indicators returned to levels similar to those on D-6. Glucose concentrations remained stable throughout the observation period, at around 0.8–0.9 g/L. Compared to those on D-6, one behavioural indicator (i.e. general attitude) varied postsurgery: the cows were more apathetic or had their head below their withers on D1 (50.0 [50.0–62.5]) and D3 (50.0 [50.0–50.0]) ($P < 0.05$), but not afterwards. Compared to that on D-6, milk yield did not decrease significantly postsurgery, except on D1.

Discriminant analysis of physiological, behavioural, and production performance indicators of cows on days around the surgery

Discrimination of the five days around surgery was tested (Tables 4 and 5, Figs. 1 and 2). Components 1 and 2 explained 68.2% and 17.9%, respectively, of the total variance of the model (for a total of 86.1%). According to correlations with each axis, component 1 was explained mainly by haptoglobin (0.885), oxidative stress indicators (GSH/GSSG, −0.746; vitamin E, −0.683; vitamin A, −0.597; MDA, 0.416), and behavioural indicators (general attitude, 0.594). This axis opposed cows on D-6 to cows on D3 and D5; cows on D1 and D13 were intermediate. Component 2 was explained mainly by the HPA axis (NEFA, 0.686, cortisol, 0.669), milk yield (−0.725), oxidative stress (MDA, −0.584; NO, 0.454), and behavioural indicators (ear position, 0.467; postures of the legs, 0.431). This axis mainly opposed cows on D13 to cows on D1. After cross-validation, all observations were correctly classified to their original days for D-6 and D13 (i.e. sensitivity of 100%). For D1, D3, and D5, four out of five observations were correctly classified (i.e. sensitivity of 80%). Specificity ranged from 95% (D3, D5, and D13) to 100% (D-6 and D1).

Discussion

Most studies that investigated pain in cattle induced by cannulation surgery often measured only one or two indices of welfare at a time (e.g. behaviour, inflammation, ANS). The current study is the first to use multiparametric evaluation of cow responses to cannulation surgery that included all aspects of the pain response (i.e. behaviour, HPA, inflammation, and oxidative stress), as well as production performances, in an integrative manner, as already performed for sheep (Faure et al., 2017; Durand et al., 2019). Despite the small sample size (five cows) and the absence of positive control groups (i.e. cows without surgery but receiving

Table 3
Median values [1st quartile–3rd quartile] of the indicators for the five Holstein-Friesian cows 6 days before surgery (D-6) and 1, 3, 5, and 13 days after surgery (D1, D3, D5, and D13, respectively). Behavioural scores are expressed on a scale of 0–100.

Indicator	D-6	D1	D3	D5	D13	Friedman *Q–P* value
Behaviour						
General attitude	0.0^{a} [0.0–0.0]	50.0^{b} [50.0–62.5]	50.0^{b} [50.0–50.0]	37.5^{ab} [25.0–50.0]	50.0^{ab} [25.0–50.0]	*Q* = 8.396 *P* = 0.078
Ear position	0.0 [0.0–0.0]	25.0 [0.0–25.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	*Q* = 12.000 *P* = 0.017
Position of the eyelid	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	*Q* = nd *P* = nd
Other facial expressions	0.0 [0.0–0.0]	0.0 [0.0-–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	*Q* = nd *P* = nd
Lying postures	0.0 [0.0–0.0]	10.0 [0.0–12.5]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	*Q* = 8.500 *P* = 0.075
Standing postures	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–16.7]	0.0 [0.0–8.3]	0.0 [0.0–0.0]	*Q* = 7.200 *P* = 0.126
Postures of the legs	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	*Q* = 9.488 *P* = 0.406
Clinical signs	0.0 [0.0–0.0]	0.0 [0.0–6.7]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	0.0 [0.0–0.0]	*Q* = 8.000 *P* = 0.092
Abnormal activities	16.7 [16.7–25.0]	33.3 [20.0–33.3]	0.0 [0.0-–16.7]	33.3 [33.3–33.3]	33.3 [16.7–33.3]	*Q* = 8.396 *P* = 0.078
Inflammation						
Haptoglobin (mg/mL)	0.00^{a} [0.00–0.00]	0.13^{ab} [0.12–0.14]	0.50^{ab} [0.43–0.50]	0.69^{b} [0.66–0.70]	0.00^{a} [0.00–0.00]	*Q* = 19.833 *P* = 0.001
TNFα (pg/mL)	0.54 [0.52–0.86]	1.38 [0.87–1.44]	0.55 [0.38–0.74]	0.45 [0.44–0.48]	0.96 [0.42–1.09]	*Q* = 6.880 *P* = 0.142
HPA axis						
Cortisol (ng/mL)	2.09^{ab} [1.75–2.56]	6.43^{a} [4.17–8.20]	2.19^{ab} [1.56–2.20]	1.80^{b} [1.80–2.08]	1.70^{ab} [1.28–1.73]	*Q* = 10.400 *P* = 0.034
Glucose (g/L)	0.78 [0.78–0.85]	0.89 [0.80–0.93]	0.89 [0.78–0.90]	0.92 [0.86–0.93]	0.80 [0.79–0.88]	*Q* = 5.920 *P* = 0.205
NEFA (mmol/L)	0.154^{ab} [0.111–0.175]	0.317^{a} [0.308–0.347]	0.101^{b} [0.088–0.186]	0.118^{ab} [0.083–0.123]	0.111^{ab} [0.097–0.119]	*Q* = 10.720 *P* = 0.03
Oxidative stress						
MDA (μg/d)	0.15^{a} [0.14–0.15]	0.15^{a} [0.13–0.18]	0.18^{ab} [0.17–0.19]	0.21^{b} [0.20–0.23]	0.25^{b} [0.24–0.27]	*Q* = 16.960 *P* = 0.002
NO (μmol/mL)	17.05^{ab} [12.31–18.90]	24.16^{a} [17.86–28.24]	13.00^{ab} [11.61–22.72]	17.25^{ab} [16.47–27.33]	12.63^{b} [8.33–17.01]	*Q* = 9.760 *P* = 0.045
GSH/GSSG	0.27^{a} [0.23–0.28]	0.03^{b} [0.03–0.04]	0.04^{ab} [0.04–0.05]	0.04^{ab} [0.03–0.05]	0.09^{ab} [0.04–0.20]	*Q* = 12.320 *P* = 0.015
Vitamin E (μg/mL)	5.24^{a} [5.13–5.84]	3.81^{ab} [3.09–4.09]	3.08^{ab} [2.74–3.79]	2.97^{ab} [2.85–3.19]	2.72^{b} [2.04–3.18]	*Q* = 13.600 *P* = 0.009
Vitamin A (μg/mL)	0.28^{a} [0.28–0.37]	0.21^{ab} [0.20–0.31]	0.16^{b} [0.16–0.22]	0.19^{ab} [0.17–0.22]	0.31^{a} [0.31–0.35]	*Q* = 16.320 *P* = 0.003
Production performance						
Milk yield (kg/d)	14.88^{ab} [14.31–15.00]	10.30^{a} [9.60–10.50]	15.20^{ab} [14.40–19.00]	16.80^{ab} [15.30–17.20]	19.30^{b} [16.10–19.50]	*Q* = 16.640 *P* = 0.002

NEFA = Non-esterified fatty acid; MDA = malondialdehyde; NO = nitric oxide; GSH/GSSG = ratio of oxidized to reduced glutathione; HPA = Hypothalamic-pituitary-adrenal; TNFα = Tumour necrosis factor α.
[a,b] Different superscripts within a row indicate a significant ($P < 0.05$) difference between days.

analgesia), the results provide new insights into how cows experience this surgery and the performance of multimodal analgesia.

During the surgery, the multimodal anaesthesia and analgesia protocol included a combination of IV infusion of lidocaine, IM injection of flunixin, IV injection of ketamine and isoflurane (gas). Analgesia consisted in IV use of lidocaine, which has already been described in multiple species. Lidocaine has both pro-motility and anti-inflammatory in addition to its analgesic effects (Hartnack, 2014). However, the effect of an IV infusion of lidocaine in combination with ketamine was more documented in horses than in cattle. In horses, it is used to prevent postoperative ileus, an important cause of increased morbidity and mortality in the early postsurgical period, particularly after small intestinal surgery (Malone et al., 2006). This property, described in horses, was the one sought by our vet for the cannulation of our cows. More recently, the analgesic efficacy of an IV constant rate infusion of a morphine-lidocaine-ketamine combination was proven in Holstein calves undergoing umbilical herniorrhaphy (Hartnack et al., 2020). Lastly, the IV infusion of lidocaine also significantly decreased the isoflurane requirement during umbilical surgery in mechanically ventilated calves (Vessal et al., 2010). It should be noted that nowadays it is procaine that is used (authorisation allowances).

Although cows received NSAIDs (flunixin) during surgery, the haptoglobin concentration increased, reaching 0.5 mg/mL on D3 and peaking at 0.69 mg/mL on D5. However, these concentrations were lower than those observed (0.88 and 0.87 mg/mL on D3 and D7 postsurgery, respectively) for a single rumen cannulation under a different NSAID (ketoprofen at 3 mg/kg BW/d; Newby et al., 2014). This difference is probably due to the type and dose of the drug. We administered four injections of flunixin, which acts on visceral pain, to cows on D0, D1, D2, and D3, while Newby et al. (2014) administered 3 mg/kg BW/d of ketoprofen, only on D0 and 24 h later. Other studies observed even higher haptoglobin concentrations than those in our cows during acute inflammation (e.g. up to 100 times higher; Conner et al., 1988). The anti-inflammatory treatment used in our study thus seems to have limited inflammation well.

In our study, glutathione oxidation, production of MDA, and loss of antioxidant reserves (vitamins A and E) clearly highlighted a situation of damaging oxidative stress from D1 to D13. The same

Table 4
Factorial discriminant analysis of behavioural, physiological, and production indicators of five Holstein-Friesian cows 6 days before surgery (D-6) and 1, 3, 5, and 13 days (D1, D3, D5, and D13, respectively) after surgery: percentage of variance explained by each component, and correlation of each variable with the component. Bold text identifies variables that contributed the most to the component (i.e. absolute value of correlation coefficient >0.4).

Factorial discriminant analysis	Component 1	Component 2
Eigenvalue		
Percentage of total variance explained by component (%)	68.2	17.9
Contribution of each variable to components:		
Behaviour		
General attitude (%)	**0.594**	0.156
Ear position (%)	−0.091	**0.467**
Position of the eyelid (%)		
Other facial expressions (%)		
Standing postures	0.381	0.107
Lying posture (%)	−0.063	0.324
Postures of the legs (%)	−0.125	**0.431**
Clinical signs (%)	−0.037	0.248
Abnormal activities (%)		
Inflammation		
Haptoglobin (mg/mL)	**0.855**	0.236
TNFα	−0.336	0.085
HPA axis		
Glucose (g/L)	0.322	0.343
NEFA (mmol/L)	−0.185	**0.686**
Cortisol (ng/mL)	−0.158	**0.669**
Oxidative stress		
GSH/GSSG ratio[1] (dimensionless)	−**0.746**	−0.331
NO (μmol/mL)	0.121	**0.454**
MDA (μg/d)	**0.416**	−**0.584**
Vitamin E (μg/mL)	−**0.683**	0.233
Vitamin A (μg/mL)	−**0.597**	−0.323
Production performances		
Milk yield (kg/d)	0.219	−**0.725**

NEFA = Non-esterified fatty acid; MDA = malondialdehyde; NO = nitric oxide; GSH/GSSG = ratio of oxidized to reduced glutathione; TNFα. = Tumour necrosis factor α.

[1] GSH and GSSG are expressed in nmol/mL but their ratio has no unit.

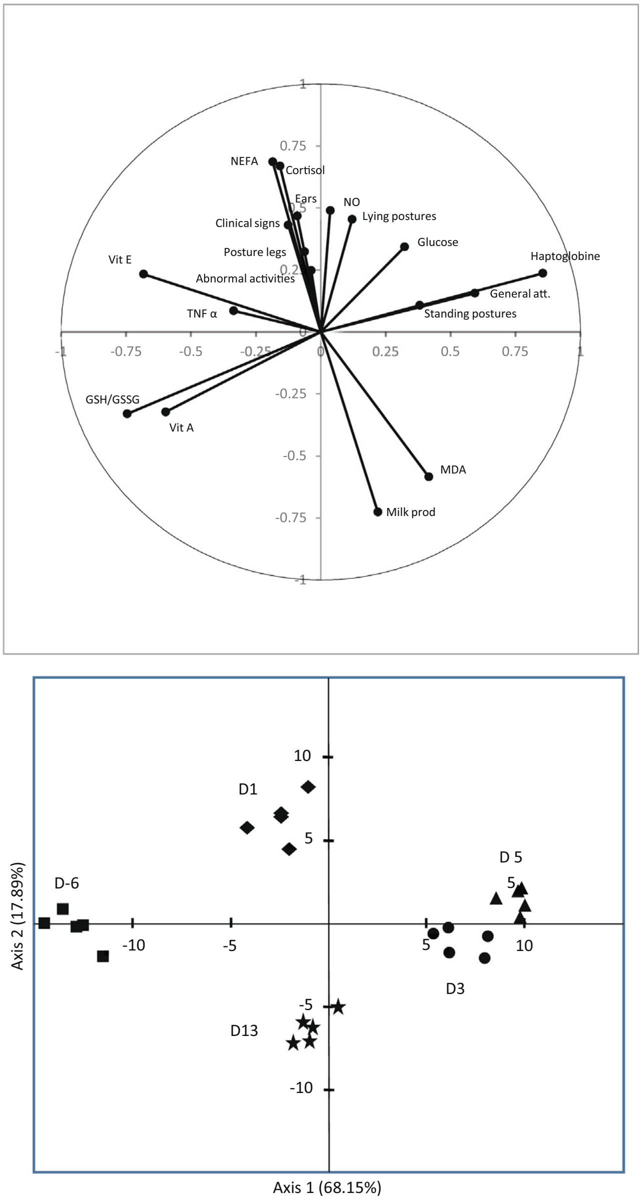

Fig. 2. Positioning of (A) variables or (B) individuals on axes 1 and 2 of factorial discriminant analysis of performance, physiological and behavioural indicators of five Holstein-Friesian cows after digestive tract surgery 6 days before surgery (D-6) and 1, 3, 5, and 13 days after surgery (D1, D3, D5, and D13, respectively). NEFA = Non-esterified fatty acid; MDA = malondialdehyde; NO = nitric oxide; GSH/GSSG = ratio of oxidized to reduced glutathione; TNFα = Tumour necrosis factor α.

results with the same intensity were observed in sheep undergoing digestive tract surgery (Faure et al., 2017). Oxidative stress is involved in many painful conditions, such as metritis, acidosis, and respiratory diseases (Celi, 2011). Moreover, it is now well accepted that reactive oxygen species generated during tissue injury have "pronociceptive" potential (Salvemini et al., 2011). An imbalance between antioxidants and oxidants was reported in several pain situations generated by inflammation (Salvemini et al., 2011). In addition to the strong inflammatory response, anaesthesia itself may have increased the production of free radicals (Naziroglu and Gunay 1999; Gadek-Michalska et al., 2005), as indicated by the high NO concentrations on D1 in our study. Recent studies highlight the anti-nociceptive valence of antioxidants due to their direct action on mediators of the inflammatory response (especially cytokines) (Pinheiro et al., 2015). In buffaloes, deleterious effects of dystocia (e.g. production of cortisol and oxidation products) can be reduced by supplementing animals with vitamin E and selenium (Sathya et al., 2007).

Table 5
Sensitivity and specificity of cross-validation of the final factorial discriminant analysis (FDA) performed on zootechnical, physiological, and behavioural indicators of five Holstein-Friesian cows 6 days before surgery (D-6) and 1, 3, 5, and 13 days after surgery (D1, D3, D5, and D13, respectively).

From\To	D-6	D1	D3	D5	D13	Sensitivity (%)	Specificity (%)
D-6	5	–	–	–	–	100	100
D1	–	4	–	–	1	80	100
D3	–	–	4	1	–	80	95
D5	–	–	1	4	–	80	95
D13	–	–	–	–	5	100	95

Surgery influenced the HPA axis slightly: cortisol and NEFA increased only on D1, but not significantly. In sheep that underwent the same surgery, but with only an NSAID treatment, the HPA axis was strongly affected, with cortisol concentrations reaching more than 60 ng/mL five days after surgery (Faure et al., 2017). The analgesic treatment applied in our study, especially morphine derivatives associated with NSAIDs, thus seems to have limited the HPA response strongly through their central analgesic action through hyperpolarisation of neurons due to agonists at κ and μ receptors, as reported by Lizarraga and Chambers (2012).

Following surgery, cows were less alert and more apathetic on D1 and D3 than they had been on D-6, but they did not significantly express other behavioural signs of pain. On D1 and even more on D3, butorphanol action already ended (half time less than

2 h; Coetzee, 2013) therefore apathy was due to pain and not to an analgesic effect. These results confirmed those of Faure et al. (2017)'s study: implanting cannulas in the rumen and the intestines increased pain-related behavioural responses, especially alertness, antalgic postures, and clinical signs.

Milk yield decreased slightly on D1, but not significantly so, due in part to the dietary restrictions that began two days before surgery. The cows recovered their pre-operative performance levels as early as D3. The contribution of energy provided through IV infusion of nutrients and the diet appeared to be well adapted to milk production by the cows, and thus limited the mobilisation of energy reserves, as indicated by plasma concentrations of NEFA. Using NSAIDs to manage postoperative pain could also have limited the decrease in milk yield, as described by Newby et al. (2014).

The analgesic regimen included a combination of an NSAID (flunixin meglumine) and morphine derivatives, which have analgesic properties on visceral pain (Heinrich et al., 2010; Coetzee, 2011; Lizarraga and Chambers, 2012) but also pain at the central level, which contributes strongly to limiting changes in behaviour. Thus, we recommend multimodal analgesia for this type of invasive surgical procedure, as already recommended for horses (Valverde, 2005). Our results confirm previous results for humans: an NSAID-morphine combination is beneficial for managing postoperative pain (Fletcher and Aubrun, 2009). In addition, morphine seems to suppress several components of the inflammatory response (i.e. IL-6, CD 11b, CD 18, and postoperative hyperthermia; Murphy et al., 2007).

The multiparametric approach showed that, compared to before surgery, the main changes in the cannulated animals were (i) an increase in inflammation (although it was partially controlled by a combination of an NSAID and morphine derivatives), (ii) an increase in oxidative stress with significant mobilisation of antioxidant reserves (i.e. vitamins A and E, and glutathione), (iii) behavioural changes (increased apathy), and (iv) a small decrease in milk yield. Although the NSAID- and morphine-mediated drug treatment strongly limited changes in the indicators of each pathway involved in pain, the multiparametric approach enabled us to differentiate cows throughout the postoperative recovery period. Thus, despite the therapeutic regimen, the cows seemed to experience residual pain (i.e. more apathy, higher cortisol concentrations) during the first few hours. On subsequent days, this response was due more to the inflammatory response and oxidative stress (i.e. sensory-discriminative component of pain), and did not seem to be associated with a negative emotional experience (i.e. emotional component of pain). These data indicate an improvement in analgesia is required immediately after surgery, especially when using morphine derivatives more consistently.

Conclusion

The multiparametric approach allowed us to assess the residual pain experienced by cows after surgery receiving a multimodal drug protocol. The NSAID- and butorphanol-mediated drug treatment strongly limited changes in the indicators of each pathway involved in pain. The cows thus experienced some pain (i.e. more analgesic postures, higher cortisol concentrations) in the first few hours after surgery, while on subsequent days, the inflammatory response and oxidative stress did not seem to be associated with a negative emotional experience. Our findings suggest the need to improve analgesia immediately after surgery and to provide antioxidants along with NSAIDs to promote cow recovery. Consequently, since 2014, we have infused (via IV) a solution of ketamine and lidocaine continuously during surgery and on D0 and D1. In addition, 1 ml of butorphanol (a morphine derivative) can be injected more frequently based on individual behavioural observations. Likewise, in order to reduce oxidative stress and its consequences (as inflammation), an appropriate complementation in vitamin E before surgery could be used.

Ethics approval

This experiment was conducted in 2014 with the approval of the Regional Ethics Committee for Experiments on Animals (CREEA No. 7: approval R-2012-PL-01, given 29 January 2013). The ethical limit point was defined as a combination of seven behaviours (i.e. no feeding, no rumination, bruxism, panting, lowered head, low ears, and closed eyes) observed for more than 12 h. If a cow reached the limit point, veterinary assessment was required to consider its welfare, including the administration of rescue analgesia, and it was excluded from the study. The criterion for euthanasia was the impossibility of reducing animal pain (e.g. posture or analgesic attitude for 48 h despite a new protocol).

Data and model availability statement

Data are available upon request from the corresponding author.

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Declaration of interest

The authors declare that they have no conflict of interest.

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