Article

Appeasing pheromones against bovine respiratory complex and modulation of immune transcript expressions

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Simple Summary: Bovine respiratory complex is still a major issue in bovine feedlots. Most of the time antimicrobial molecules are used to manage these diseases with deleterious consequences for microbiota and the emergence and the shedding of resistant bacteria. To improve bovine health and to reduce the risk for cattle to develop respiratory infections, alternative molecules such as appeasing pheromones have been developed. In the current report, we tested bovine appeasing pheromones in young bulls. We treated them at the beginning of the fattening period and we measured zootechnical and health parameters over several weeks. We identified positive effects of pheromone treatment. Indeed, less clinical signs were observed in bulls who received the pheromone treatment. Regarding the potential mechanism to explain the beneficial effect of the pheromone treatment, we identified an increased expression of transcripts associated with the expression of an immune molecules involved in the recruitment of cells important to manage pathogens. Our study confirms the positive effect of appeasing pheromones and opens the doors for future studies in beef cattle.

Abstract: Bovine respiratory disease is still a major concern in feedlots and has major economic impact. Another consequence of respiratory infections is the use of antimicrobial molecules to control bacterial pathogens. This can participate to the emergence and shedding of antimicrobial resistance that can threaten animal as well as human health. Appeasing pheromones with their capacity to reduce stress and thus their ability to preserve the functions of the immune system have been proposed to reduce the use of antimicrobial substances. In the current report we assessed the effect of appeasing pheromone administration on bovine health and performance during the fattening period. Zootechnical and health parameters as well as whole blood immune transcript expressions were measured over weeks in young bulls to determine the effect of the pheromone. We observed a reduction of clinical signs at day 30 in young bulls who received the pheromone and a higher expression of interleukin 8 transcripts in this group than in the control group. Our results are in line with previous reports in bovine and other mammals and ask for further studies to shed more light on the beneficial impact of appeasing pheromones and to decipher their exact mechanisms of action.

Keywords: appeasing pheromone; bovine; respiratory infections; immune response; average daily gain

1. Introduction

Bovine respiratory disease (BRD) complex remains a major cause of morbidity and mortality in bovine feedlots with major economic consequences [1]. Multiple pathogens, including various bacteria and viruses, and several host and environment factors such as air quality are involved in the pathogenesis of BRD complex [2–5]. To control the bacterial infections, antimicrobial molecules are commonly used before – methaphylaxis – or after the detection of various clinical signs. These antibacterial treatments have major consequences not only on bovine respiratory and gut microbiota and pathogens but also on the shedding of resistant bacteria and resistance determinants into the surrounding environment [6–8]. This constitutes a serious one health issue for animals and humans. Amongst the multiple factors involved in the pathogenesis of BRD, the stress has a determinant role [5,9]. It has been shown that stress is increased during the fattening period of young bulls for several reasons including, amongst others, a recent weaning, the transportation the young bulls and their mixing in a new confined environment while they are coming from different farms [5,10–14]. Within a few days, multiple periods of stress show an impact on immune defence mechanisms of young bovine with consequences on the onset and the persistence of respiratory infectious disorders [9,15–17].

To prevent the onset of respiratory infections during the fattening period of young bulls, vaccinations targeting the main pathogens can also be used [2,18]. Currently vaccination is carried out when the young are gathering. However, this is quite late to enable a good protection at the beginning of the fattening period. New breeding management practices to limit the impact of stress on the immune system of young bovines (batches of bulls coming from a same farm and reduced transport time amongst others) could be considered. However, the implementation of these new practices could be challenging for the stakeholders. An alternative to reduce the impact of the stress could be the use in young animals of appeasing pheromones as demonstrated in several species [19–21]. The production of these hormones during nursing exist in all mammals including bovine. In cows, there is a specific area between the two mammary chains [22]. Appeasing pheromones are produced by sebaceous glands of the sulcus. Synthetic analogues of the bovine appeasing hormone have been developed, they are based on a mixture of fatty acids. They are similar to the pheromone produced by the dam at calving [23].

To the best of our knowledge, the effect of appeasing pheromone on bovine performance and disease incidence has been assessed in a limited number of studies [22,24–26]. Positive effects including increased performance and improved early detection of BRD signs were reported in dairy cattle as well as beef cattle.

Thus, in a global context of antimicrobial molecules use reduction, we decided to assess the effect of bovine appeasing pheromone over the stress, the development of respiratory disorders and whole blood immune transcript expression in young bulls over the four first weeks of fattening. Interesting observations were collected, showing a positive impact of the pheromones and contributing to a better knowledge of this alternative tool.

2. Materials and Methods

2.1. Study facilities

The study was carried out in four young bulls fattening units in western France. The basic husbandry management of these fattening units is representative of standard management used in this area. In western France, 5-10-month-old young bulls are weaned and immediately transported to a sorting facility to be sorted by breed and body weight, forming new batches that fulfill the orders of the fatteners. The newly formed batches are then transported to the fattening units for the entire fattening period. During the fattening period, young bulls are reared in barns composed of pens of 5 to 20 animals for a space allowance of 3.5 to 5.5 m²/bull. Young bulls are commonly fed with a complete diet composed of corn or grass silage and a mixture of cereals and urea.

2.2. Animals

The 265 animals utilized in the study were Charolais young bulls aged 317 days (d) (+/- 51.8 d) at arrival at the sorting facility between January and March 2018 (Table 1). The animals came from multiple suckler farms. On arrival at the sorting facility they were moved through a chute for a group of procedure. They were treated with doramectin anthelmintic (Dectomax, Elanco, Sèvres, France), vaccinated once with a vaccine containing modified-live bovine viral diarrhoea virus (BVDV) and bovine respiratory syncytial virus (BRSV) (Rispoval RS-BVD, Pfizer, Paris, France) and then weighted. The average weight of the 265 animals was 366.7 kg (+/- 30.8 kg). The farm bulls were housed and maintained in compliance with the French Ministry of Agriculture and Fishing standards for the protection of animals.

Table 1. Distribution of young bulls (YB) by batches and study Group ("pheromone" or "control")

		YB[1]	Age	Body-weight	Pheromone group					Control group				
					Batches	YB	YB per batches	Age	Body-weight	Batches	YB	YB per batches	Age	Body-weight
Fattening unit	1	60	338(+/-51)	391(+/-27)	3	36	12	343(+/-55)	391(+/-31)	2	24	12	335(+/-48)	390(+/-21)
	2	90	324(+/-43)	377(+/-22)	4	60	15	319(+/-45)	377(+/-25)	2	30	15	328(+/-37)	378(+/-14)
	3	60	338(+/-47)	371(+/-25)	2	30	15	339(+/-52)	382(+/-26)	2	30	15	337(+/-39)	361(+/-18)
	4	55	261(+/-49)	323(+/-20)	5	33	3x6 1x5 1x10	246(+/-52)	316(+/-15)	3	22	1x10 2x6	284(+/-37)	336(+/-17)
Total		265	320(+/-54)	370(+/-32)	14	159		312(+/-58)	372 (+/-36)	9	106		325(+/-45)	368 (+/-26)

[1] YB: Young Bull, age in days and body weight in kg

2.3. Batches constitution and pheromone administration

Animals were sorted by weight, conformation and farms of origin to form 23 homogeneous batches from 5 to 15 animals (Table 1). Two treatment groups were then constituted. The pheromone group consisted of 159 young bulls in 14 batches and the control group consisted of 106 young bulls in 9 batches. For a given fattening unit, batches of pheromone and control group had comparable mean weight and include young bulls with similar conformations and from same farms of origin.

2.4. Pheromone administration

Before leaving the sorting facility, animals were moved again through a chute. Five mL of SecureCattle® (Signs - Irsea Group, Apt, France) (pheromone group) or 5 mL of transcutol (Diethylene glycol monoethyl ether – Gattefossé, Saint-Priest, France) (control group), a high-purity solvent and powerful solubilizer associated with skin penetration enhancement in topical dosage forms, were deposited on coat in the middle of the forehead – at the crossing point of two imaginary lines drawn between the eyes and the center of the peer base of the opposite horns – using an Injecmaster® automatic syringe (Génia, St. Hilaire de Chaléons, France). From there, contact between young bulls from pheromone and control group was avoided.

2.5. Clinical assessments

At day 8 (D8) and D30, a veterinarian performed a visual examination for detection of BRD clinical signs (increase in respiratory rate, dyspnea, cough, nasal or ocular discharge and depression). A diagnosis of BRD was established when the animal displayed (i) nasal or ocular discharge or cough and (ii) increase in respiratory rate or depression.

2.6. Activity and behavioral observations

Two observers recorded activities, behaviors and stereotypies (Table 2) at D0, D8 and D30 during a 6 hours' observation period. Both observers looked at the pheromone and placebo young bulls. They used instantaneous sampling of individual young bulls in each group using 5 min scan sampling. Data were recorded 48 times day^{-1} each, during 6 one-hour-observation-periods from 9am to noon and 2pm to 5pm.

Table 2. Distribution of young bulls (YB) by batches and study Group ("pheromone" or "control")

	Activity, behavior and stereotypy	Description
Activity	Ruminating	Chewing regurgitated boluses of feed
	Eating feed at the feeding trough	Eating and masticating at the feeding trough
	Lying	Lying down in any resting position
	Standing idling	Standing
Behavior — Agonistic	Fighting	Engaging in headbunts
	Escaping	One young bull escape another hostile young bull
	Threatening	One young bull has an hostile behavior but no contact is made
Behavior — Non-agonistic	Chin-resting	One young bull places its chin on another young bull
	Grooming	One young bull lick another
	Sniffing	Sniffing another young bull
	Social rubbing	Rubbing another young bull
Stereotypy	Licking	Licking any equipment
	Rubbing	Rubbing repetitively own body against any equipment
	Tongue-rolling	Twisting and twirling the tongue, either inside or outside the open mouth for at least 5 seconds

2.7. Zootechnical performances

Duration of the fattening period was recorded for each young bull. Each young bull was weighted at the end of the fattening period. An individual average daily gain (ADG) (Kg/d) for the entire fattening period was calculated as the change in body weight measured at the sorting facility and at the end of the fattening period divided by the number of days elapsed.

2.8. Immune gene expression analysis using reverse-transcription quantitative polymerase chain reaction

Total RNA was extracted from the blood of 15 (control) and 18 (pheromone) bulls randomly selected in each groups from D0, after pheromone administration, to D8 using PAXgene Blood RNA Kit (Qiagen Courtaboeuf, France) as described by the manufacturer. RNA concentration was determined by measuring OD at 260nm (OD260) and the RNA quality was assessed by calculating OD260/OD280 ratio NanoDrop spectrophotometer

(NanoDrop Technologies, Wilmington, USA). cDNA was generated with iScript Reverse Transcription Supermix for reverse-transcription quantitative polymerase chain reaction (RT-qPCR) (Bio-Rad, Hercules, CA, USA) from 400 ng of RNA free of genomic DNA per reaction. Diluted cDNA (2X) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's recommendations. The qPCR conditions, for the various genes tested in the study, were 95°C for 30 seconds (s), followed by 40 cycles with denaturation at 95°C for 5 s and annealing/elongation for 30 s at selected temperature – depending of the gene assessed. Efficiencies of all the qPCR assays were comprised between 90 and 100% and we carefully followed MIQE guidelines recommendations [27]. The sequences of the primers used in the study were published previously (see Table 3) [28–33]. Real-time assays were run on a Bio-Rad CFX96 (Bio-Rad). The specificity of the qPCR reactions was assessed by analyzing the melting curves of the products and verifying the amplicon sizes. Samples were normalized internally by simultaneously using the average Cycle quantification (Cq) of two suitable reference genes (Beta actin – ACTB and Glyceraldehyde-3-phosphate dehydrogenase - GAPDH) in each sample to avoid any artefact of variation in the target gene [28]. Then, qPCR data were expressed as relative values after Genex macro analysis (Bio-Rad) [34] using the Cq from the samples for the different transcripts.

Table 3. Primer abbreviations, full names, sequences, amplicon sizes (bp), annealing temperatures (°C) and accession number or reference

Primer abbreviation and full names	Primer sequences: sense (S) and anti-sense (AS)	Amplicon sizes (bp)	Annealing temperatures (°C)	Accession number or references
REFERENCE GENES				
ACTB	S: ACGGGCAGGTCATCACCATC	166	67	28
Beta actin	AS: AGCACCGTGTTGGCGTAGAG			
GADPH	S: GGCATCGTGGAGGGACTTATG	186	62	28
Glyceraldehyde-3-phosphate dehydrogenase	AS: GCCAGTGAGCTTCCCGTTGAG			
CYTOKINES				
IL12p40	S: CACCAGCAGCTTCTTCATCA	105	60	33
Interleukin 12 subunit p40	AS: TACTCCCAGCTGACCTCCAC			
IL4	S: GCCACACGTGCTTGAACAAA	63	60	30
Interleukin 4	AS: TCTCAACAGCTTGGCAAGCA			
IL6	S: TAAGCGCATGGTCGACAAAA	150	60	32
Interleukin 6	AS: TTGAACCCAGATTGGAAGCAT			
IL8 (CXCL8)	S: AGAACTTCGATGCCAATGCAT	150	60	NM_173925
Interleukin 8	AS: GGGTTTAGGCAGACCTCGTTT			
IL17A	S: TCGTTAACCGGAGCACAAACT	120	60	32
Interleukin 17A	AS: TGGCCTCCCAGATCACAGA			
IL10	S: AGAACCACGGGCCTGACA	121	60	32
Interleukin 10	AS: ACCGCCTTGCTCTTGTTTTC			
TGFß	S: TGCTTCAGCTCCACAGAAAAGA	116	60	32
Transforming growth factor ß	AS: AGGCAGAAATTGGCGTGGT			
IFNγ	S: TTGAATGGCAGCTCTGAGAAAC	150	60	32
Interferon γ	AS: TCTCTTCCGCTTTCTGAGGTTAGA			
CHEMOKINES				
CXCL6	S: GAGAGCTGCGTTGTGTGTGT	107	60	29
Chemokine (C-X-C motif) ligand 6	AS: ACTTCCACCTTGGAGCACTG			
CCL20	S: TTCGACTGCTGTCTCCGATA	172	62	28
Chemokine (C-C motif) ligand 20	AS: GCACAACTTGTTTCACCCACT			
TRANSCRIPTION FACTORS				
FOXP3	S: TGGTGCAATCTCTGGAGCAA	116	60	30
Forkhead box P3	AS: GTCAGATGATGCCGCAGATG			
GATA-3	S: CCAGACCAGAAACCGAAAAA	234	62	31
Trans-acting T-cell-specific transcription factor GATA-3	AS: ACCATACTGGAAGGGTGGTG			
RORγ (RORC gene)	S: ACAGCCCTCGTCCTCATCAATGCC	145	60	30
RAR-related orphan receptor gamma	AS: TGGGTGGCAGCTTTGCCAGGATA			
TBX21	S: CGAGGACTATATACTGCCGC	133	61	31
	AS: CAAGACCACGTCCACATACA			

2.9. Statistical analysis

Most calculations and statistical analyses were performed in the open-source environment R version 3.5.1. (R Development Core Team, Vienna, Austria). The characteristics of young bulls assigned to the two treatment groups were compared to assess homogeneity: Student's *t*-test was used for continuous variables after checking normality using Shapiro-Wilk's test. Then, a mixed effects logistic regression model using the glm function from the package lmertest was used to characterize the association between the exposure to pheromone and BRD diagnosis (diseased or healthy). Individual bull was the experimental unit. Fattening operation and pen were considered as random factors. An interaction between the date of clinical examination and BRD diagnosis was included in the statistical model.

Proportions and count were used to describe activity and behavioral observations. Because they displayed a skewed distribution, permutation tests with 1000 replications were used to calculate *P*-values. The H0 hypothesis is that an activity or behavioral observation has a significant effect. A mixed linear model is used with the initial data set to test this effect and the Chi-square statistic is calculated for each activity or behavioral observation. Then 1000 replications with at each replication a random permutation of group (pheromone or control) for young bulls, while keeping the same proportion of pheromone groups and control groups, a random permutation of date of clinical examination (D0, D8, D30) and a random permutation of number of young bulls per pen while keeping the same number of young bulls receiving pheromone than in the initial data set. For each activity or behavioral observation, 1000 values of Chi-square statistic are then obtained. The 95% percentile of repartition of this 1000 values is calculated and compared with the value obtained with the initial data set: if superior then H0 is rejected, the activity and behavioral observation has a significant effect.

A mixed linear regression model using the lme function from the nlme package was used to characterize the association between the exposure to pheromone and ADG for the entire fattening period. Individual bull was the experimental unit. A "pen within fattening operation" nested random effect was added to the model.

Then, relative expressions of transcripts were compared using Wilcoxon signed rank test since data were paired and non-normally distributed. All the comparisons were carried out using GraphPad Prism (GraphPad Software version 7.0, San Diego, CA, USA). *P*-values less than 0.05 were considered statistically significant.

3. Results

Regarding animals included in the study, no significant difference was present between the two treatment groups regarding weight at entry (T= -0.33, df =263; *P*=0.7393). However, young bulls from the pheromone group were older than young bulls in the control group (T= -2.1647, df= 263, *P*=0.031) (see Table 1). Then, no significant difference was observed between the two treatment groups regarding number of cow-calf farms of origin per batch (T=0.29214, df=21, *P*=0.773).

3.1. Clinical signs

Clinical data were obtained for 237 young bulls (137 in the pheromone group and 100 in the control group) at D8 and 230 young bulls (139 in the pheromone group and 91 in the control group) at D30. At D8, 15% (36/237) of the young bulls were affected by BRD.

The diagnosis was established when the animal displayed (i) nasal or ocular dis-charge or cough and (ii) increase in respiratory rate or depression. The morbidity rate was 19% (26/137) in the pheromone group and 1% (10/100) in the control group. At D30, 8% (19/230) of the young bulls were sick with a morbidity rate of 4% (6/139) in the pheromone group and 14% (13/91) in the control group.

Results of the logistic regression model (Table 4) shows that interaction between exposure to pheromone and day of clinical exam was significantly (P=0.001) associated with disease showing that more clinical cases (OR=8.20) were observed in the control group compared to the pheromone group at D30.

Table 4. Association between the exposure to pheromone and BRD diagnosis (diseased or healthy). Data collected from 265 young bulls

Variables and levels		Odds ratio			*P*-value
		Estimate	95% confidence interval		
			Lower bound	Upper bound	
Exposure to pheromone	Control	*Reference*			
	Pheromone	0.48	-0.20	1.10	0.84
Day of clinical examination	D8	*Reference*			
	D30	0.19	-0.07	0.45	0.08
Pheromone x Day of clinical examination		8.20	2.32	31.90	0.001

3.2. Activities and behaviors

Activities and behaviors were observed for the 265 young bulls at D0, D8 and D30. The proportion of animals standing was significantly higher at D8 and D30 than at D0 ($P<0.001$) for all pens. The statistical model did not show any effect of the treatment group on the proportion of animals standing idling, eating food at the feeding trough or ruminating. Over all the period of observation, animals from the pheromone group moved significantly more than animals from the control group ($P<0.05$).

No effect of day of observation (D0, D8 or D30) or treatment on the number of agonistic interactions was demonstrated by the statistical model used. Analysis of the results shows that the number of agonistic interactions increases depending on the number of animals in a batch, regardless of the batch considered ($P<0.05$) (Figure 1). Too few non-agonistic interactions and stereotypies were observed to allow meaningful statistical analysis.
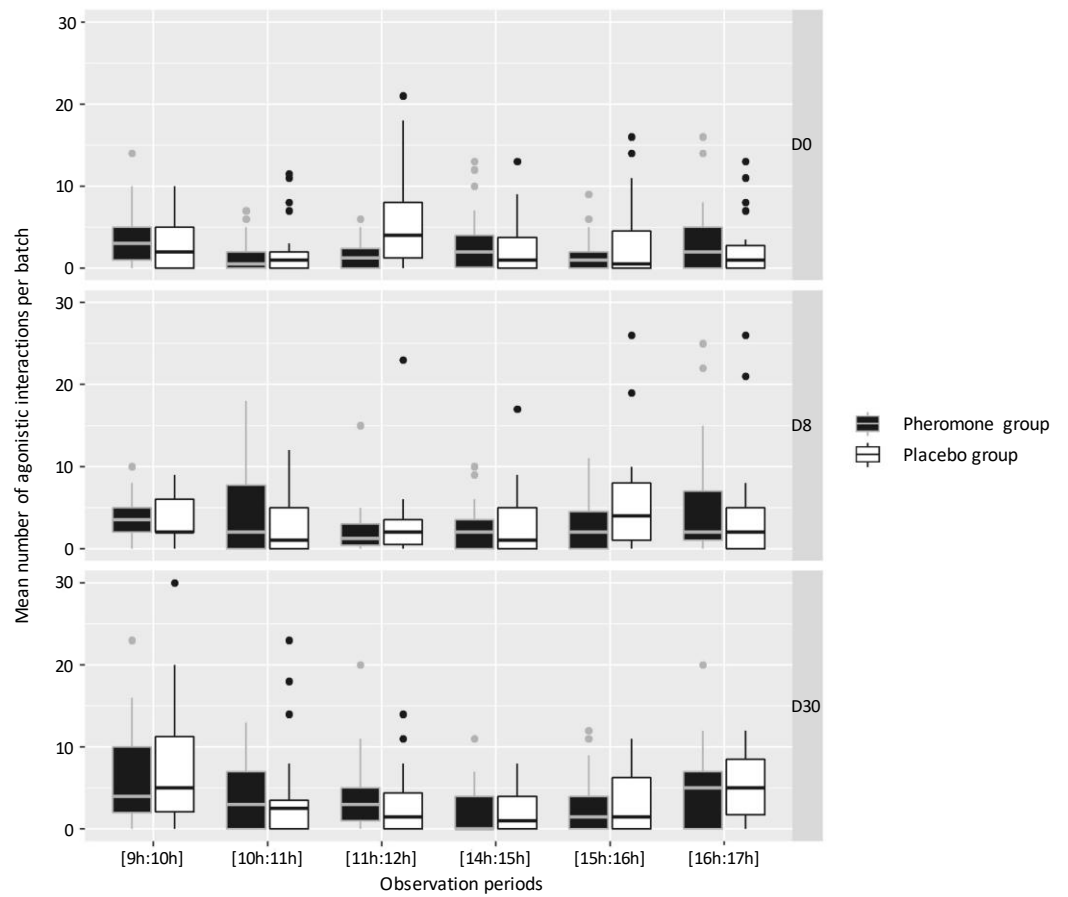
Figure 1. Activity and behavioral observations

3.3. *Average daily gain*

ADG for the entire fattening period were observed for 164 young bulls. Results of the mixed linear regression model shows that exposure to pheromone was not significantly associated with ADG (Table 5).

Table 5. Associations between exposure to pheromone, pen-size and average daily gain (kg/d) of young bulls for the entire fattening period. Data are collected from 164 young bulls.

Variables and levels		Mean ADG*			*P*-value
			95% confidence interval		
		Estimate	Lower bound	Upper bound	
Intercept		1.498			
Exposure to pheromone	Control	1.48	1.33	1.67	0.98
	Pheromone	1.48	1.33	1.67	

[1] ADG: Average Daily Gain (kg/d) calculated for the entire fattening period

3.4. Assessment of immune transcript expression by quantitative polymerase chain reaction

In an attempt to assess the impact of the pheromone treatment on the expression of various immune transcripts, we collected blood from the young bulls, extracted RNA from the cells and measured the expression of different transcripts. The selected transcripts were associated with the different common lymphocyte T helper responses (Th1, Th2, Th17 and T regulator) as well as with inflammation for the last ones (see Table 3). At D0 and at day D8, we did not observe significant differences ($P>0.05$) for the vast majority of the assessed transcripts (Table 6). However, an increasing trend for IL12p40 transcripts in the blood of control group bulls in comparison to the blood of pheromone group bulls was identified ($P=0.073$) (Figure 2 and Table 6). Similarly, an increasing trend for IL6 transcripts in control group was observed at D8 ($P=0.055$) (Figure 2 and Table 6). Interestingly, for IL8, significant differences were identified at D8 with more IL8 transcripts in the pheromone group compared to control group (see Figure 1 and Table 6). At D0, the trend was similar but the difference was not significant ($P=0.053$).

Table 6. Statistical comparisons between mRNA relative expressions in control and pheromone groups at day 0 (D0) and day 8 (D8). Levels of expression in controls are shown in the second column (High: Amplification around 17–26 cycle quantification (*Cq*), Moderate: Between 26-31 *Cq* and Low: More than 31 *Cq*). *P*-values are presented in other columns. As the data were not paired and non-normally distributed, group means were compared using the Wilcoxon Signed Rank Test (Exact). *$P<0.050$, **$P<0.010$. In bold, significant *P*-values and *P*-values nearly significant. ns: Not significant. SEM: Standard Error of the Mean

Messenger RNAs	Levels of expression (controls)	D0			D8		
		mRNA relative expressions +/- SEM		Pheromone *vs* Control	mRNA relative expressions +/- SEM		Pheromone *vs* Control
		Control (N=15)	Pheromone (N=18)		Control (N=15)	Pheromone (N=18)	
CCL20	low	13.87+/-4.13	20.72+/-4.84	ns	12.38+/-1.79	18.24+/-3.26	ns
CXCL6	moderate	5.56+/-1.64	14.77+/-6.74	ns	5.91+/-1.32	8.84+/-2.18	ns
FOXP3	moderate	6.3+/-0.9	5.85+/-0.77	ns	4.86+/-0.71	4.39+/-0.71	ns
GATA-3	high	6.32+/-0.92	8.15+/-1.26	ns	4.72+/-0.51	4.7+/-0.62	ns
IFNγ	low	18.44+/-4.49	45.86+/-16.27	ns	17.93+/-3.92	37.83+/-11.35	ns
IL10	moderate	4.15+/-0.70	4.57+/-1.11	ns	2.68+/-0.51	2.76+/-0.25	ns
IL12 p40	moderate	213.23+/-40.59	155.05+/-47.51	**0.073**	87.49+/-22.68	82.28+/-15.69	ns
IL17A	low	22.06+/-10.57	34.94+/-15.77	ns	57.53+/-30.78	43.67+/-14.51	ns
IL4	low	6.28+/-4.00	0.81+/-0.45	ns	1.13+/-0.71	3.34+/-2.13	ns
IL6	low	11.32+/-4.68	11.15+/-3.88	ns	11.33+/-2.21	5.55+/-1.78	**0.055**
IL8	moderate	231.45+/-82.35	321.85+/-62.56	**0.053**	178.25+/-95.58	330.61+/-104.61	**0.004****
RORγ	moderate	6.25+/-0.86	5.32+/-0.46	ns	4.86+/-0.64	5.03+/-0.64	ns
TBX21	moderate	8.51+/-1.47	9.33+/-1.66	ns	4.78+/-0.74	3.8+/-0.60	ns
TGFß	high	3.36+/-0.38	3.12+/-0.32	ns	2.78+/-0.37	3.32+/-0.18	ns
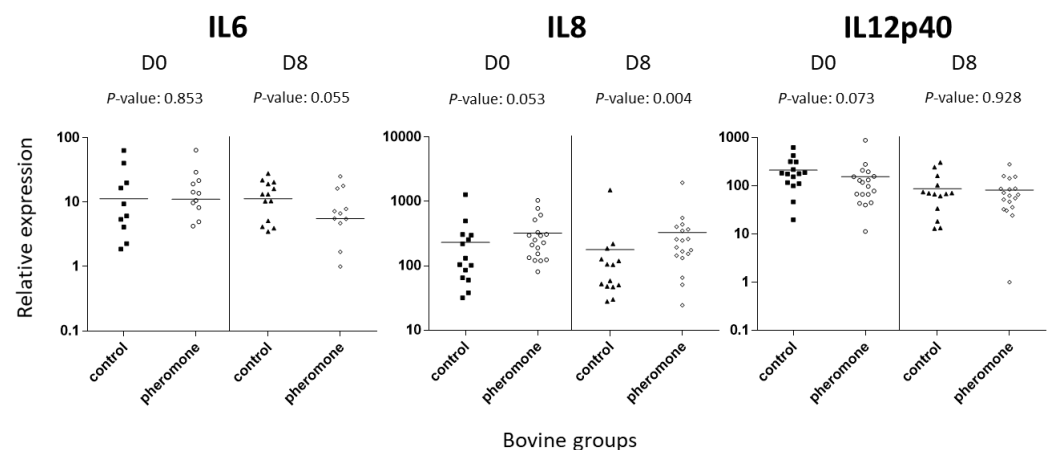
Figure 2. Blood IL6, IL8 and IL12p40 transcripts are differentially expressed between treated and untreated young bulls

4. Discussion

Fattening period in beef cattle is one of the most challenging phases within the production cycle. Indeed, young animals are exposed to multiple stresses of different natures

with major consequences in terms of welfare, health, and productivity. Stress, especially when consecutive to weaning and transportation, has a major role in the onset of various respiratory infections during fattening through its deleterious effects on the immune defenses [2,5,35]. Bacterial respiratory infection is one of the main cause of antimicrobial molecules use during metaphylaxis procedure and curative treatments [2,7,8]. The development and the spreading of antimicrobial resistance makes necessary the identification and the establishment of alternative approaches to antimicrobial molecules to reduce their use. Amongst the potential alternatives, bovine appeasing pheromone is an attractive one. As stated before, several previous reports have shown a benefit when appeasing pheromones were used in dairy and beef cattle production [22,24–26]. In our study, carried out in fattening units from western France using Charolais young bulls, we observed that significantly more clinical cases were detected at day 30 in the control group than in the group of bulls which received appeasing pheromone. This observation differs from what has been previously reported by Colombo and collaborators [26]. Indeed, in their study, incidence of BRD was greater in (P<0.05) in Angus steers who received appeasing pheromone than in the control group from days 6 to 10 and days 19 to 23. Moreover, a higher proportion (P=0.04) of appeasing pheromone group diagnosed with BRD required one antimicrobial treatment to retrieve health compared to control group [26]. There are several hypotheses to explain this discrepancy. First of all, the timing of pheromone administration and the measured parameters are different between studies. Then, in our study we just measured the presence or absence of BRD without any monitoring of pathogens while in the study of Colombo and collaborators they monitored the cumulative incidence of BRD clinical signs during a 45-days feedlot receiving period of beef steers administered an appeasing pheromone at feedlot entry and they measured the serum concentration of antibodies against various pathogens [26]. Regarding activities and behavior, we did not observe any differences between groups. Similarly, the mixed linear regression model showed that exposure to pheromone was not significantly associated with final ADG. In other studies [25,26], authors identified positive impact of appeasing pheromone over body weight gain, however, the benefit was not sustained throughout the 45-day experiment in *Bos indicus*-influenced beef cattle [25]. Moreover, in these studies authors measured intermediate ADG while we assessed final ADG.

In the last part of our study, we assessed the whole blood expression of various transcripts associated with the immune response. The analysis of blood has been demonstrated to be a valuable approach to assess various health aspects in bovine [36,37]. In our study, we selected a limited number of target genes encompassing Th1, Th2, Th17, Treg cytokines as well as associated transcription factors and chemokines. Most of the time, no significant differences were observed between control and pheromone groups of bulls both at day 0 and day 8 (after pheromone administration). However, for IL8 transcripts, we observed a clear difference between pheromone group and control group at day 8 with a higher expression of IL8 transcripts in the pheromone group than in the control group. IL8 or CXCL8 is a well-known cytokine which was first purified and molecularly cloned as a neutrophil chemotactic factor from lipopolysaccharide-stimulated human mononuclear cell supernatants [38–40]. Bovine IL8 has similar roles to its human counterpart and can massively attracts neutrophils to the respiratory system [41–43]. A positive effect of IL8 transcripts can be suggested in our conditions, especially since we observed less clinical cases of BRD in the pheromone group than in the control group. This hypothesis is strengthened by observations from multiple reports where IL8 protective effects against respiratory infections were identified [40,44]. Moreover, administration of recombinant bovine IL8 was shown to have also positive effects on uterine health and milk production [45]. Thus, a monitoring of blood IL8 transcripts in young bulls could potentially bring useful information regarding the onset of clinical respiratory signs associated with infectious diseases. Furthermore, it would be important to assess the IL8 protein concentration and respiratory infections over time in bovine treated with appeasing pheromones. Indeed, we cannot totally exclude discrepancies between transcript and protein expressions.

Additionally, IL6 transcripts, there was a clear trend for a lower expression of the transcripts in the pheromone group than in the control group. This could account for a low inflammatory status in pheromone group but asks for further research to confirm this hypothesis.

In our study, appeasing pheromones show a slight beneficial impact on bovine health and are in line with previous studies [22,24–26] also reporting an interest in using these molecules in cattle to improve animal health.

5. Conclusions

In the current study we show that appeasing pheromone can have a beneficial impact on the bovine respiratory disease complex in the context of the fattening period. Our results confirm the potential of appeasing pheromones as a complementary approach in the management of young bulls in fattening units. However, further studies are definitely warranted to determine the extent of the beneficial effects of appeasing pheromones and to precisely decipher the mechanisms underlying these effects.

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